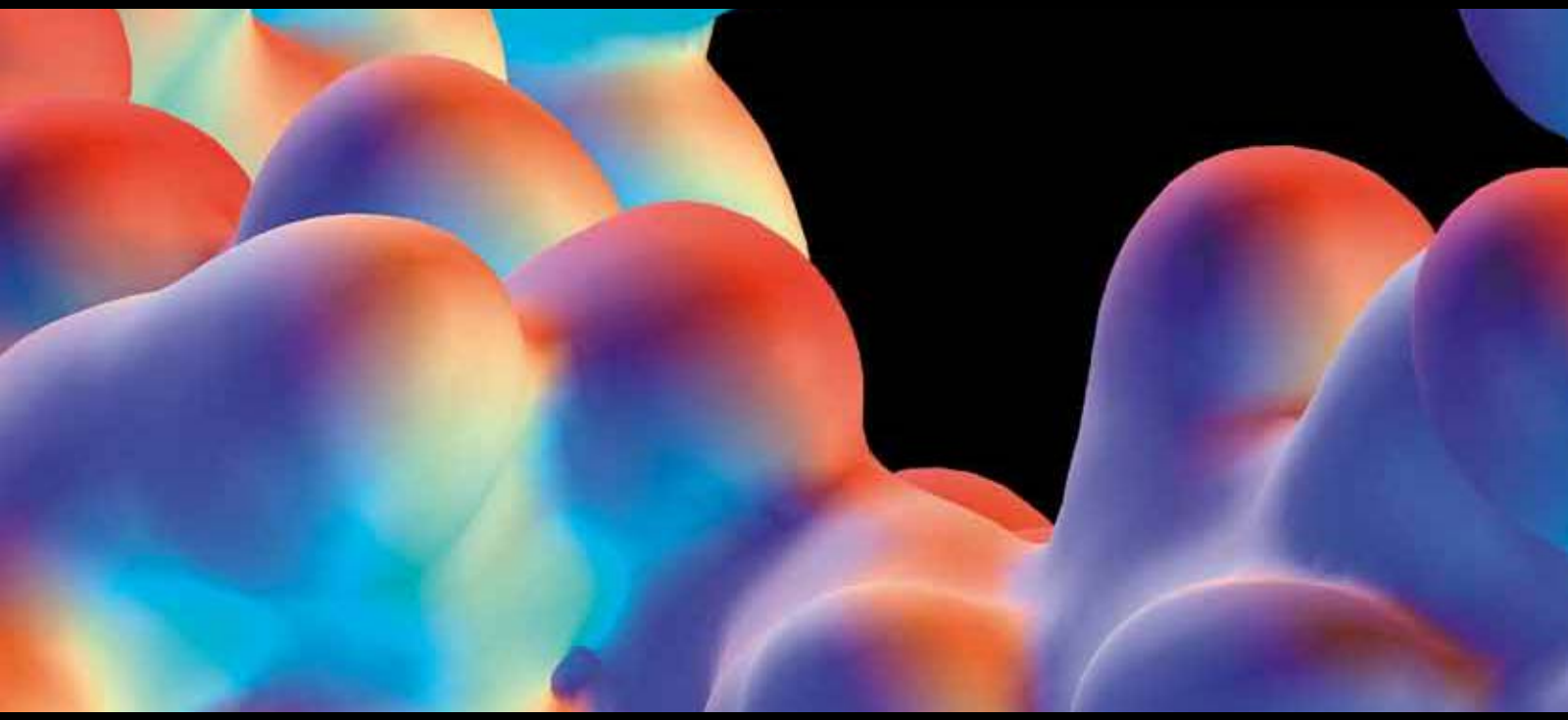
Enzyme Research

Regulation and Function of Protein Kinases and Phosphatases

Guest Editors: Heung-Chin Cheng, Robert Z. Qi, Hemant Paudel, and Hong-Jian Zhu



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Editorial

Regulation and Function of Protein Kinases and Phosphatases

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Protein kinases and phosphatases are enzymes catalysing the transfer of phosphate between their substrates. A protein kinase catalyses the transfer of γ-phosphate from ATP (or GTP) to its protein substrates while a protein phosphatase catalyses the transfer of the phosphate from a phosphoprotein to a water molecule. Even though both groups of enzymes are phosphotransferases, they catalyse opposing reactions to modulate the structures and functions of many cellular proteins in prokaryotic and eukaryotic cells. Among the various types of posttranslational modifications, protein phosphorylation and dephosphorylation are the most prevalent modifications regulating the structures and functions of cellular proteins in a wide spectrum of cellular processes, ranging from cell fate control to regulation of metabolism. For example, even though protein kinase genes constitute only 2% of the genomes in most eukaryotes, protein kinases phosphorylate more than 30% of the cellular proteins [1]. Owing to the significant roles of protein kinases and phosphatases in cellular regulation, this special issue focuses on their regulation, and functions. In this issue, there are two research articles and seven reviews on various topics related to the structure, regulation and functions of protein kinases and phosphatases. Together, they give the readers a glimpse of the roles played by protein kinases and phosphatases in regulating many physiological processes in both prokaryotic and eukaryotic cells. They also highlight the complexity of the regulation of protein kinases and phosphatases.

Phosphorylation regulates protein functions by inducing conformational changes or by disruption and creation of protein-protein interaction surfaces [2, 3]. Conformational changes induced by phosphorylation are highly dependent on the structural context of the phosphorylated protein. Upon phosphorylation, the phosphate group regulates the activity of the protein by creating a network of hydrogen bonds among specific amino acid residues nearby. This network of hydrogen bonds is governed by the three-dimensional structure of the phosphorylated protein and therefore is unique to each protein. The most notable example of regulation of protein function by phosphorylation-induced conformational changes is glycogen phosphorylase [4]. Glycogen phosphorylase, made up of two identical subunits, is activated upon phosphorylation of Ser-14 of each subunit by phosphorylase kinase [4]. Phosphorylation of Ser-14 in one monomer creates a network of hydrogen bonds between the phosphate group and the side chains of Arg-43 of the same monomer as well as Arg-69 of the other monomeric subunit [5]. This network induces significant intra- and intersubunit configurational changes, allowing access of the substrates to the active sites and appropriately aligning the catalytically critical residues in the active sites for catalysis of the phosphorolysis reaction.

Phosphorylation can also modulate the function of a protein by disrupting the surfaces for protein-ligand interactions without inducing any conformational changes. For example, phosphorylation of Ser-113 of the bacterial isocitrate dehydrogenase almost completely inactivates the enzyme without inducing any significant conformational changes [6, 7]. The phosphate group attached to Ser-113 simply blocks binding of the enzyme to isocitrate. Likewise, phosphorylation can also create ligand-binding surface

without inducing conformational changes. For example, tyrosine phosphorylation of some cellular proteins creates the binding sites for SH2 domains and PTB domains [8, 9].

The functions of protein kinases and phosphatases are mediated by their target substrates. Understanding how protein kinases and protein phosphatases recognise their respective substrates is one of the methods used by various investigators to elucidate the physiological functions of these important enzymes. Before completion of the human genome project, most protein kinases were discovered after the discoveries of their physiological protein substrates. The most notable example is phosphorylase kinase which was discovered after glycogen phosphorylase was discovered to be regulated by phosphorylation. However, in the postgenomic era, the genes encoding protein kinases and phosphatases of an organism are known upon completion of the genome project. The challenge now is to identify their physiological protein substrates.

Protein kinases employ two types of interactions to recognize their physiological substrates in cells: (i) recognition of the consensus phosphorylation sequence in the protein substrate by the active site of the protein kinase and (ii) distal interactions between the kinase and the substrate mediated by binding of docking motif spatially separated from the phosphorylation site in the substrate and interaction motif or domain located distally from the active site of the kinase [1, 10]. These interactions contribute to the ability of protein kinases to recognize their protein substrates with exquisite specificity. Defining the structural basis of these interactions is expected to benefit identification of potential physiological substrates of protein kinases. Relevant to this, the orientated combinatorial peptide library approach developed in the 1990s and the more recently developed positional scanning peptide library approach allow rapid determination of the optimal phosphorylation sequence of many protein kinases [11, 12]. Notably, Mok et al. reported using this approach to define the optimal phosphorylation sequences of 61 out of 122 protein kinases encoded by the *Saccharomyces cerevisiae* genome [13]. Scanning the proteomes for proteins that contain motifs similar to the optimal phosphorylation sequence of a protein kinase will assist the identification of potential physiological substrates of the kinase [10]. Armed with the knowledge of many known three-dimensional structures of protein kinases with the peptide substrate bound to the active site, Brinkworth et al. designed the PREDIKIN program capable of predicting the optimal phosphorylation sequence from the primary structure of a protein serine/threonine kinase [14, 15]. Besides the peptide library approaches, researchers can also search for cellular proteins in crude cell or tissue lysates that are preferentially phosphorylated by a protein kinase *in vitro*. This method, referred to as "kinase substrate tracking and elucidation (KESTREL)" has led to the identification of potential physiological protein substrates of a number of protein kinases [16]. Finally, using specific synthetic small-molecule protein kinase inhibitors, researchers were able to perform large-scale phosphoproteomics analysis to identify physiological protein substrates of a specific protein kinase in cultured cells [2].

Substrate specificity of protein phosphatases is governed by interactions between interaction motifs or domains located distally from the phosphatase active site and distal docking motifs spatially separated from the target phosphorylation sites in protein substrates [17, 18]. Little is known about the role of the active site-phosphorylation site interactions in directing a protein phosphatase to specifically dephosphorylate its protein substrates. Using the oriented phosphopeptide library approach, several groups of researchers were able to define the optimal dephosphorylation sequences of several protein tyrosine phosphatases [19, 20], suggesting the active site-phosphorylation site interactions also play a role in dictating the substrate specificity of protein tyrosine phosphatases. Finally, the substrate-trapping mutant approach pioneered by Flint et al. in the last decade has allowed identification of physiological protein substrates of many phosphatases [21].

In this special issue, the two research articles focus on how pyruvate dehydrogenase kinase and Akt recognise their physiological substrates. The article by T. A. Hirani et al. explores how pyruvate dehydrogenase directs its recognition and phosphorylation by pyruvate dehydrogenase kinase. The article by R. S. Lee et al. reported results of their investigation that aims to decipher the regulatory mechanism governing substrate specificity of the various isoforms of Akt. The review article by A. M. Slupe et al. focuses on the structural basis governing how protein phosphatase 2A recognises its physiological substrates in cells.

It is well documented that aberrant regulation of protein kinases and phosphatases contributes to the development of diseases. For example, constitutive activation of many protein tyrosine phosphatases is known to cause cancer and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Protein kinases and phosphatases are regulated by protein-protein interactions, binding of ligands, and reversible or irreversible covalent modifications such as phosphorylation and limited proteolysis. In this special issue, the article by I. Nakashima et al. summarizes how protein tyrosine kinases are regulated by redox reactions. C. F. Dick et al. reviewed how the activity of protein and acid phosphatases in yeast, plants, and other microorganisms is regulated by inorganic phosphate.

Among the cellular processes in which protein kinases and phosphatases are involved, this issue contains review articles detailing how protein kinases and phosphatases regulate cell cycle, mediate toll-like receptor signaling, and control of cell fate and potassium channel and intracellular calcium concentration in renal tubule epithelial cells.

In addition to protein phosphatases, acid phosphatases are involved in regulation of many biological processes such as an organism's adaptation to stress and hydrolysis of phosphorylcholine. This issue contains three review articles on the function, catalytic mechanism, and regulation of this important group of phosphatases.

Heung-Chin Cheng
Robert Z. Qi
Hemant Paudel
Hong-Jian Zhu

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Research Article

Asp295 Stabilizes the Active-Site Loop Structure of Pyruvate Dehydrogenase, Facilitating Phosphorylation of Ser292 by Pyruvate Dehydrogenase-Kinase

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We have developed an *in vitro* system for detailed analysis of reversible phosphorylation of the plant mitochondrial pyruvate dehydrogenase complex, comprising recombinant *Arabidopsis thaliana* $\alpha 2\beta 2$-heterotetrameric pyruvate dehydrogenase (E1) plus *A. thaliana* E1-kinase (AtPDK). Upon addition of MgATP, Ser292, which is located within the active-site loop structure of E1α, is phosphorylated. In addition to Ser292, Asp295 and Gly297 are highly conserved in the E1α active-site loop sequences. Mutation of Asp295 to Ala, Asn, or Leu greatly reduced phosphorylation of Ser292, while mutation of Gly297 had relatively little effect. Quantitative two-hybrid analysis was used to show that mutation of Asp295 did not substantially affect binding of AtPDK to E1α. When using pyruvate as a variable substrate, the Asp295 mutant proteins had modest changes in k_{cat}, K_m, and k_{cat}/K_m values. Therefore, we propose that Asp295 plays an important role in stabilizing the active-site loop structure, facilitating transfer of the γ-phosphate from ATP to the Ser residue at regulatory site one of E1α.

1. Introduction

The α-ketoacid dehydrogenase complexes comprise a unique family of large multienzyme structures, including the pyruvate-, α-ketoglutarate-, and branched-chain α-ketoacid dehydrogenase complexes and the related glycine decarboxylase complex [1–5]. These complexes have molecular mass values from 4 to 9 MDa and are among the largest non-viral protein assemblies. All family members have multiple copies of three central enzymes: E1, an α-ketoacid decarboxylase/dehydrogenase; E2, a dihydrolipoyl acyltransferase; E3, a dihydrolipoyl dehydrogenase. The overall reaction converts the α-ketoacid, NAD^+, and reduced coenzyme A to CO_2, NADH, and an acyl-CoA. The complexes are defined by their α-ketoacid specificity

In prokaryotic cells the α-ketoacid dehydrogenase complexes are localized in the cytoplasm, while in eukaryotic cells they are within the mitochondrial matrix. Plant cells have a second form of the pyruvate dehydrogenase complex (PDC) which is localized within the plastid stroma [1, 2]. In addition to subcellular compartmentation, the activities of all of the complexes are regulated by product inhibition. The branched-chain α-ketoacid dehydrogenase complexes (BCKDCs) and mitochondrial PDCs are additionally regulated by reversible phosphorylation, mediated by intrinsic E1-kinases [1–3, 6] and P-E1-phosphatases [6, 7].

The PDC E1α active-site loop includes two (sites 1 and 2) of the three phosphorylation sites present in mammalian PDC (Figure 1). Plant PDC E1α sequences include a conserved Ser residue corresponding to mammalian phosphorylation site 1 and a Ser residue one position upstream of site 2. The Asp295 residue, conserved in both PDC and BCKDC E1α subunits, is located three residues from phosphorylation site 1. The results from studies using peptide substrates

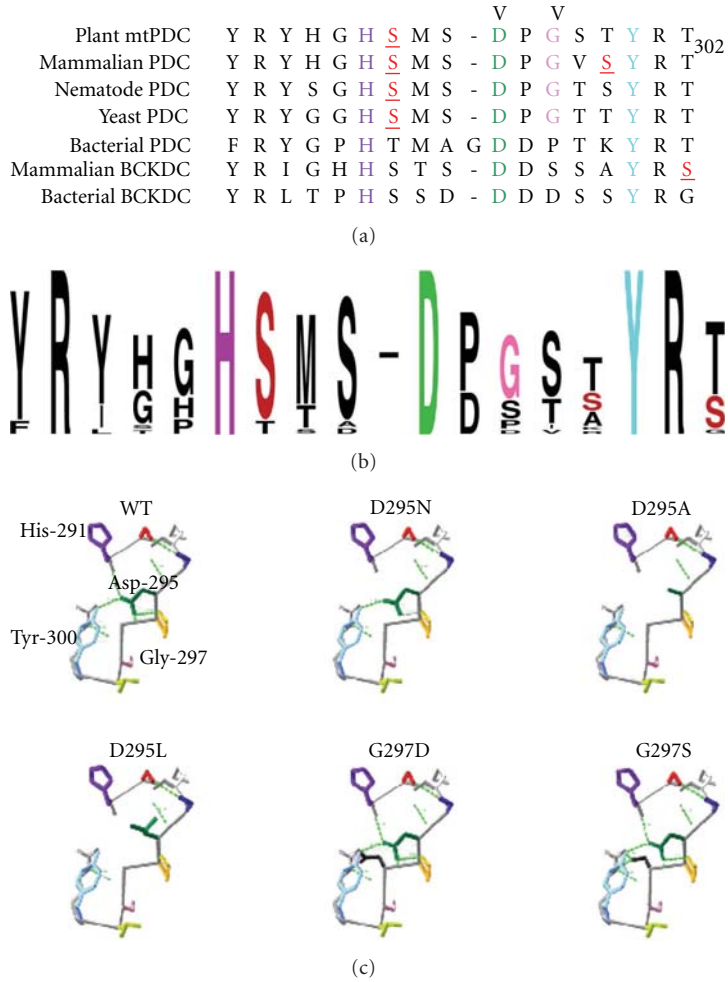

Figure 1: Molecular modeling and in silico mutagenesis of the active-site loop of AtPDC E1α. (a) Alignment of residues in the active-site loop of α-ketoacid dehydrogenase E1α sequences. Numbering is based upon the *A. thaliana* PDC E1α sequence. The plant mtPDC sequence is identical in *Pisum sativum*, *A. thaliana*, *Glycine max*, *Lycopersicon esculentum*, *Solanum tuberosum*, *Beta vulgaris*, *Nicotiana tabacum*, *Populus hybrida*, *Lotus corniculatus*, *Medicago truncatula*, *Zea mays*, and *Oryza sativum*. The mammalian PDC sequence is identical in *Rattus norvegicus*, *Mus musculus*, and *Homo sapiens*. The nematode PDC sequence is identical in *Ascaris suum* and *Caenorhabditis elegans*. The yeast PDC sequence is identical in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Kluyveromyces lactis*. Bacterial PDC sequences are identical from *Bacillus subtilis* and *Bacillus stearothermophilus*. The mammalian BCKDC sequence is identical in *H. sapiens*, *M. musculus*, and *Ovis aries*. The bacterial BCKDC sequence is from B. subtilis. The carats denote the Asp and Gly residues targeted in this study. The red underlined Ser residues correspond to mammalian pyruvate dehydrogenase phosphorylation sites 1 and 2 and mammalian BCKDC phosphorylation site 2. (b) The consensus active-site loop primary sequence presented as output from the WebLogo program [9]. (c) Carbon-alpha trace from modeling of residues His-291 to Arg-301 in the active site loop of AtE1α. The same colors have been used for each residue in all panels. Hydrogen bonds are indicated with green dashed lines. Traces were built using Swiss-PDB viewer (version 3.7).

indicate that this residue is important for phosphorylation of E1 at site 1, and it has been proposed to be involved with PDK binding [8]. The Gly297 residue is conserved only in forms of PDC E1 that are phosphorylated, and there is a corresponding Ser residue in the BCKDC E1α sequences. This suggests that a small residue in this position is required for an appropriate conformation of the active-site loop for E1 phosphorylation.

Limited proteolysis has been used to probe the reaction mechanism of the dehydrogenase/decarboxylase (E1) component of mammalian α-ketoacid dehydrogenase complexes [10–13]. In all cases, loss of catalytic activity resulted from cleavage of the E1α subunit, while E1β remained unaffected. Limited proteolysis of *Bacillus stearothermophilus* PDC revealed that it is the surface loop of the E1α subunit structure that is susceptible to cleavage [14]. In the crystal structures of the BCKDC E1 from *Pseudomonas putida* [15] and *Homo sapiens* [16] and of the PDC E1 from *H. sapiens* [17], it is clear that this loop lies in the funnel-shaped active site formed by the E1α and E1β subunits and is 20–25 Å from the binding site of the thiamine pyrophosphate cofactor. The primary sequence of the active-site loop is conserved among plant and mammalian PDC E1 proteins, and, while sequence conservation with BCKDC is less,

both loops have a similar structure [16, 17] suggesting that the individual residues (conserved or variable) perform the same biochemical function.

Site-directed mutagenesis of the active-site loop from mammalian PDC E1α highlighted the roles of Arg267 in binding the carboxyl group of pyruvate and Asp276, Tyr281, and Arg282 in both decarboxylation of pyruvate and the subsequent reductive acetylation of the lipoyl domains of E2 [18]. In addition, Ala scanning mutagenesis of the active-site loop of RnBCKDC E1α provided evidence that Arg288, His292, and Asp296 are also involved in catalysis [19, 20]. Moreover, the clinically deleterious H263L and R273C mutations of HsPDC E1α result in greatly reduced PDC activity [21, 22]. These residues correspond to Arg287, His291, Asp295, Tyr300, and Arg301 of the *Arabidopsis thaliana* PDC E1α (AtPDC E1α) sequence (Figure 1).

To better understand the catalytic mechanisms and regulatory properties of the plant mitochondrial PDC, it was necessary to develop a method to produce the recombinant enzyme that is both soluble and correctly assembled into the $\alpha 2\beta 2$ heterotetramer. Previous attempts to use *Bacillus subtilis* [23] and *Pichia pastoris* [24] expression systems were plagued by problems with protein solubility and stability and codon usage bias. Herein, we describe a two-plasmid approach for expression of catalytically active heterotetrameric AtPDC E1 in *Escherichia coli*. Using a minimum *in vitro* assay system of recombinant AtPDC E1 and AtPDK, we have begun detailed analyses of the reversible phosphorylation of E1. We verified that the Ser residue corresponding to mammalian site 1 is a site of regulatory phosphorylation. Additionally, we targeted Asp295 and Gly297, residues conserved in the active-site loop but not previously tested for their contribution(s) to the catalytic activity of E1, and role(s) in phosphorylation-site recognition by PDK. Our results suggest that Asp295, but not Gly297, is involved in both E1 catalysis and phosphorylation.

2. Experimental Procedures

2.1. Plasmid Construction. A two-plasmid system was used for expression of AtPDC E1. Primers DDR321 and 322 (see Table S1 in Supplementary Material available online at doi:10.4061/2011/939068) were used to amplify the AtPDC E1α reading frame [25]. The DDR321 primer includes a NdeI site, a H_6-affinity tag, and starts with S18 of E1α, the first residue after the N-terminal mitochondrial targeting sequence. The PCR product was cut with NdeI and XhoI, then ligated with similarly digested pT7-7 [26], yielding pDDR-E1α.

Similarly, primers DDR323 and 324 (Table S1) were used to amplify the AtPDC E1β reading frame [27]. The DDR323 primer includes a NcoI site, the FLAG epitope (DYKD-DDDK) [28], and starts with A30 of E1β, the first residue after the N-terminal mitochondrial targeting sequence. The PCR product was digested with NcoI and HinDIII, then ligated with similarly digested pET28 (Novagen, Madison, WI, USA), yielding pDDR-E1β.

2.2. Protein Expression and Purification. The *E. coli* BL21 (DE3) host cells (Novagen, Madison, WI, USA) were sequentially transformed to express the GroE chaperonins [29], AtPDC E1β-pET28, and finally AtPDC E1α-pT7-7. Protein expression was induced by adding IPTG to a final concentration of 0.1 mM. Individual transformants were screened for high-level expression of soluble AtPDC E1. Cell growth, lysis, and affinity purification of recombinant AtPDC E1 and AtPDK are described elsewhere [30].

2.3. Size-Exclusion Chromatography. Assembly of heterotetrameric AtPDC E1 was determined by size-exclusion chromatography. A Superose 12 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was equilibrated with 50 mM TES-NaOH, pH 7.4, containing 50 mM NaCl, 0.1 mM TPP, and 0.2 mM $MgCl_2$. Column fractions were analyzed by SDS-PAGE, and the AtPDC E1 subunits were detected by immunoblotting [31]. The E1α monoclonal antibodies have been previously described [32]. Anti-FLAG antibodies were from the Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Site-Directed Mutagenesis. Site-directed mutagenesis of AtPDC E1α was accomplished using the QuikChange kit from Stratagene (La Jolla, CA, USA). The S292D/S298A double mutant was constructed by preparing the S298A mutant first, then using it as the template for a second round of mutagenesis. The mutagenic primer pairs were DDR162 and DDR163 (S292A), DDR431 and DDR432 (S298A), DDR425 and DDR426 (S292D), DDR444 and DDR445 (G297S), DDR446 and DDR447 (G297D), DDR448 and DDR449 (D295N), DDR450 and DDR451 (D295A), and DDR452 and DDR453 (D295L) (Table S1). All mutations were verified by DNA sequencing.

2.5. Far-UV Circular Dichroism Spectroscopy. The various expressed E1 proteins were examined by far-UV CD spectroscopy using an AVIV Model 202 spectrometer (Piscataway, NJ, USA) to determine if the site mutations introduced any significant perturbation in AtPDC E1 structure. Samples were analyzed at room temperature using a 0.1 cm path-length cuvette, and spectra were recorded from 190 to 300 nm at 1 nm intervals with a bandwidth of 1 nm. For each analysis, 200 μL of purified protein in 20 mM KH_2PO_4/K_2HPO_4 (pH 7.4) at a concentration of 100–200 μg/mL was used.

2.6. Phosphorylation of AtPDCE1. Phosphorylation was determined by measuring the incorporation of [^{32}P] from [γ-^{32}P]ATP (6000 Ci/mmole, Perkin-Elmer, Boston, MA, USA) into AtPDC E1. Recombinant E1 (1 μg) was mixed with recombinant AtPDK (0.25 μg) and kept for 30 min on ice. Reactions were conducted at 30°C in 20 mM TES-NaOH, pH 7.4, containing 5 mM $MgCl_2$, 50 mM KCl, and 0.2 mM total ATP. Reactions were terminated after 90 min by adding 1/5 volume of 5x SDS-PAGE sample buffer (15% (w/v) SDS, 15% (v/v) 2-mercaptoethanol, 50% (v/v) glycerol). Proteins were resolved by SDS-PAGE, electroblotted to nitrocellulose, and examined by autoradiography. The incorporation of

radiolabel into AtPDC E1 was quantified by liquid-scintillation spectrometry of the bands excised from Ponceau S stained membranes.

2.7. Peptide Mass Mapping of Phosphorylated E1α by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. The AtPDC E1α S298A mutant was phosphorylated by AtPDK using nonradioactive ATP. After SDS-PAGE, the AtPDC E1α band was excised and subjected to in-gel tryptic digestion. Desalted tryptic peptides in acetonitrile/water/88% formic acid (700/290/10, V/V/V) were applied to a 96x2 hydrophobic stainless steel target and topped with an equal volume of a 10-mg/mL solution of alpha-cyano-4-hydroxycinnamic acid (Fluka, St. Louis, MO, USA) in acetonitrile/water/10% trifluoroacetic acid (600/370/30, V/V/V). The sample and matrix were allowed to cocrystallize under ambient conditions. Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry was performed with an Applied Biosystems Voyager DEPro instrument equipped with a 20-Hz 337-nm nitrogen laser (Applied Biosystems, Inc., Foster City, CA, USA). Negative ion spectra were acquired in the linear delayed extraction mode under optimized conditions using close-external calibration based on a commercial mixture of peptides (Applied Biosystems).

2.8. Tandem MS Analysis of Tryptic Peptides. The desalted tryptic peptides from the phosphorylated AtPDCE1α S298A mutant protein were analyzed by nanospray quadrupole time-of-flight mass spectrometry (Q-TOF MS) using an Applied Biosystems/MDS Sciex (Foster City, CA, USA) QStar/Pulsar instrument fitted with a nanospray source (Proxeon Biosystems, Odense, Denmark). A stable spray was achieved at 750 V in the presence of nitrogen curtain gas for sample dissolved in acetonitrile/water/88% formic acid (500/490/10, V/V/V). Positive ion spectra were acquired in the profile MCA mode at a pulser frequency of 6.99 kHz. Collision-induced dissociation spectra (MS/MS) were acquired for peptides selected by the first quadrupole at low resolution (3–5 amu window) and fragmented in the collision quadrupole at appropriate nitrogen collision gas pressures and collision energy settings. Fragmented ions were assigned nomenclature according to [33].

2.9. Measurement of Catalytic Activity. The catalytic activity of WT AtPDC E1 and site-directed mutants was determined by assaying the decarboxylation of [1-^{14}C] pyruvate (20 mCi/mmol, Perkin-Elmer, Boston, MA, USA) in the presence of 1.0 mM $K_3Fe(CN)_6$ as the electron acceptor [34]. Reactions were preincubated for 2 min at 30°C before adding 1 mM [1-^{14}C] pyruvate and 1 μg AtPDC E1. One unit of enzyme activity is 1 μmol of CO_2 released per min at 30°C. All of the data presented comprise a minimum of two biological replicates plus three technical replicates.

Kinetic constants were determined by measuring decarboxylation of pyruvate in the presence of 1.8 μM $K_3Fe(CN)_6$. The lower concentration of $K_3Fe(CN)_6$ reduced the otherwise high and variable background values to near zero. A fixed variable concentration of pyruvate ranging from 50 to 900 μM was used with 1 μg AtPDC E1. Data were fitted to the appropriate equations using the program Origin 7 (OriginLab Corporation, Northampton, MA, USA). The K_m and k_{cat} values were determined from initial-rate measurements and fit to the equation $v = (k_{cat} \times S)/(K_m + S)$, where S = substrate concentration.

2.10. Bacterial Two-Hybrid Analysis. Interactions between AtPDK and AtPDC E1α were analyzed using the bacterial two-hybrid (B2H) system (BacterioMatch I, Stratagene, La Jolla, CA, USA). Primers DDR471 and DDR472 were used to amplify WT AtPDK [30], while primers DDR473 and DDR474 were used to amplify both WT and mutant forms of E1α. The AtPDK sequence was cloned into pBT, and the AtE1α constructs were cloned into pTRG. Pairs of constructs were used to cotransform the *E. coli* BacterioMatch I reporter strain. Selection was on Luria-Bertani agar containing 250 μg/mL carbenicillin, 12.5 μg/mL tetracycline, 34 μg/mL chloramphenicol, and 50 μg/mL kanamycin. Cells selected by antibiotic screening were additionally screened on LB plates containing 80 μg/mL X-gal plus 0.2 mM β-galactosidase inhibitor (to inhibit endogenous β-galactosidase). Interactions between PDK and the E1α mutants were quantified by measuring β-galactosidase activity [35, 36]. The microplate spectrophotometric assay uses o-nitrophenyl-β-D-galactoside as the substrate.

3. Results

3.1. In Silico Analysis of the E1α Active-Site Loop. The active-site loop is a structural feature critical for both catalysis by E1 and phosphorylation of E1 by PDK. Overall, there is 50% sequence identity among the amino acid residues that comprise the active-site loop of diverse members of the α-ketoacid dehydrogenase family (Figure 1(a)). Two of the conserved residues were targeted in this study, Asp295 and Gly297. Located three residues C-terminal of phosphorylation site 1 (Ser292), Asp295 is the only acidic residue in proximity to this phosphorylation site. It is entirely conserved among α-ketoacid dehydrogenase sequences.

The primary sequence of AtPDC E1α was threaded through the crystal structure of HsE1α [17]. The resulting model indicates that Asp295 protrudes into the loop and can interact with His291 and Tyr300 (Figure 1(b)). The results from *in silico* mutagenesis of Asp295 indicate that these interactions would be disrupted if the hydrophilic side chain was replaced with an uncharged polar residue, that is, D295N. In the D295A and D295L mutants, interactions with His291 and Tyr300 would be completely abolished. The active-site loop is a highly charged region. If Asp295 plays a structural role in maintaining the structure of the loop, the mutations would disrupt substrate binding and/or interaction with AtPDK.

Of the five residues proximal to Ser292, Gly297 is conserved in eukaryotic PDC sequences, but not in those of the BCKDC. Since Gly297 is not in the immediate vicinity of Ser292, mutation would not be expected to directly affect

phosphorylation of E1 but might still be involved with either presentation of the phosphorylation site to the kinase-active site or catalysis. The corresponding position in BCKDC contains a Ser residue suggesting that a small residue at this position may be sufficient for the structural conformation of the active-site loop. *In silico*, the G297D and G297S mutants do not affect the N-terminal interactions of Asp295 but induce an additional interaction with Arg301 (Figure 1(c)).

3.2. Expression of Recombinant AtPDCE1. A two-plasmid approach was employed for expressing high yields of correctly assembled and fully active $\alpha_2\beta_2$ heterotetrameric AtPDC E1 in *E. coli*. Expression of only the AtPDC E1α or E1β subunits individually invariably yielded only insoluble subunits (Figure 2(a)), while coexpression of the α- and β-subunits resulted in a substantial amount of soluble protein (Figure 2(b)). Immobilized metal affinity chromatography was used to purify recombinant AtPDC E1; analysis of the purification steps by SDS-PAGE is shown in Figures 2(b) and 2(c). The co-occurrence of α- and β-subunits was verified by immunoblotting (Figure 2(d)). Based upon visual examination of Coomassie Blue-stained SDS gels, it was estimated that final preparations of AtPDC E1 were ~95% pure.

To confirm the assembly of heterotetrameric AtPDC E1, the purified protein was subject to size-exclusion chromatography. Most recombinant protein eluted from the Sepharose 12 column at a position corresponding to an M_r value of 160,000, which is what would be expected for an $\alpha_2\beta_2$ heterotetramer. The far-UV CD spectra of the E1 proteins indicated no significant changes in secondary structure between WT AtPDC E1 and any of the site-directed mutants (data not presented).

3.3. Catalytic Activity of Recombinant AtPDC E1. The specific activity values for the WT and mutant forms of AtPDC E1 are shown in Table 1. The activity of the S292A mutant was reduced by 25%, while there was no change in the activity of the S298A mutant. In contrast, the activities of the S292D and S292D/S298A mutants were reduced by >90%. The activities of the G297D and G297S mutants were reduced by 25 to 30%, while those of the D295A and D295N mutants were reduced by 60%. The D295L mutant protein was essentially inactive (1% residual activity).

The kinetic parameters of WT and the mutants, using pyruvate as the variable substrate, are shown in Table 2. The k_{cat} values for the D295A and D295N mutants were reduced ~70%. The K_m pyruvate values for the D295A and D295N mutants were increased 4- to 6-fold compared with WT AtE1. The k_{cat} value for the G297D mutant was reduced by 50% without any change in K_m pyruvate. In these mutants, the catalytic efficiency (k_{cat}/K_m) was reduced by 3.5 to 35-fold, reduction that, although significant, is modest for the changes expected on mutation of residues directly involved in substrate binding and/or catalysis.

3.4. Phosphorylation of AtPDC E1 In Vitro. Incubation of WT AtPDC E1 with recombinant AtPDK using 200 μM [γ-^{32}P] ATP resulted in the phosphorylation of a single polypeptide species (Figure 3(a), lane 1). To analyze regulatory phosphorylation site 1 of AtPDC E1, three different mutants were constructed. The S292A mutant targets the conserved phosphorylation site 1, while the S298A mutant targets potential phosphorylation of the putative site 2. The S292D/S298A double mutant was constructed to mimic phosphorylation of AtPDC E1α Ser292 in the absence of a residue that could be phosphorylated at site 2.

Incubation of the mutant proteins with recombinant AtPDK plus 200 μM [γ-^{32}P] ATP resulted in phosphorylation of a single polypeptide species for the S298A mutant (Figure 3, lane 3). There were only trace amounts of phosphate incorporated into the S292A mutant (2% of WT) and into the S292D/S298A double mutant (1% of WT).

3.5. Analysis of the Phosphorylated AtPDC E1α S298A Mutant by Mass Spectrometry. To verify that Ser292 was the site phosphorylated *in vitro*, the S298A mutant was phosphorylated with recombinant AtPDK plus unlabeled ATP, then subjected to in-gel tryptic digestion. The MALDI-TOF MS analysis of the tryptic peptides revealed an ion at M-H$^-$ 1656.9 Da, which corresponds to the phosphopeptide Tyr286-Arg301 (Figure 4(a)). This was the only peptide detected by negative ion nanospray QqTOF precursor ion analysis that yielded the diagnostic fragment ion for

Table 1: The specific activities of WT and mutant AtPDC E1 proteins were determined by assaying the decarboxylation of [1-^{14}C] pyruvate in the presence of 1.0 mM $K_3Fe(CN)_6$ as the electron acceptor.

Enzyme	Specific activity (units/mg)	%
WT AtPDCE1	0.32 ± 0.01	100
S292A	0.24 ± 0.06	74
S298A	0.31 ± 0.02	97
S292D	0.02 ± 0.03	6
S292D/S298A	0.03 ± 0.03	9
D295A	0.12 ± 0.01	37
D295N	0.12 ± 0.01	37
D295L	0.003	1
G297D	0.24 ± 0.03	75
G297S	0.22 ± 0.04	68

Table 2: Kinetic parameters of WT AtPDC E1 and site-directed mutants. Activities were determined by assaying decarboxylation of [1-^{14}C] pyruvate in the presence of 1.8 μM $K_3Fe(CN)_6$.

Enzyme	K_m pyruvate (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)
WTAtE1	45 ± 18	159 ± 14	3.5
D295A	178 ± 108	52 ± 12	0.3
D295N	257 ± 144	38 ± 10	0.1
G297D	66 ± 24	70 ± 7	1.0

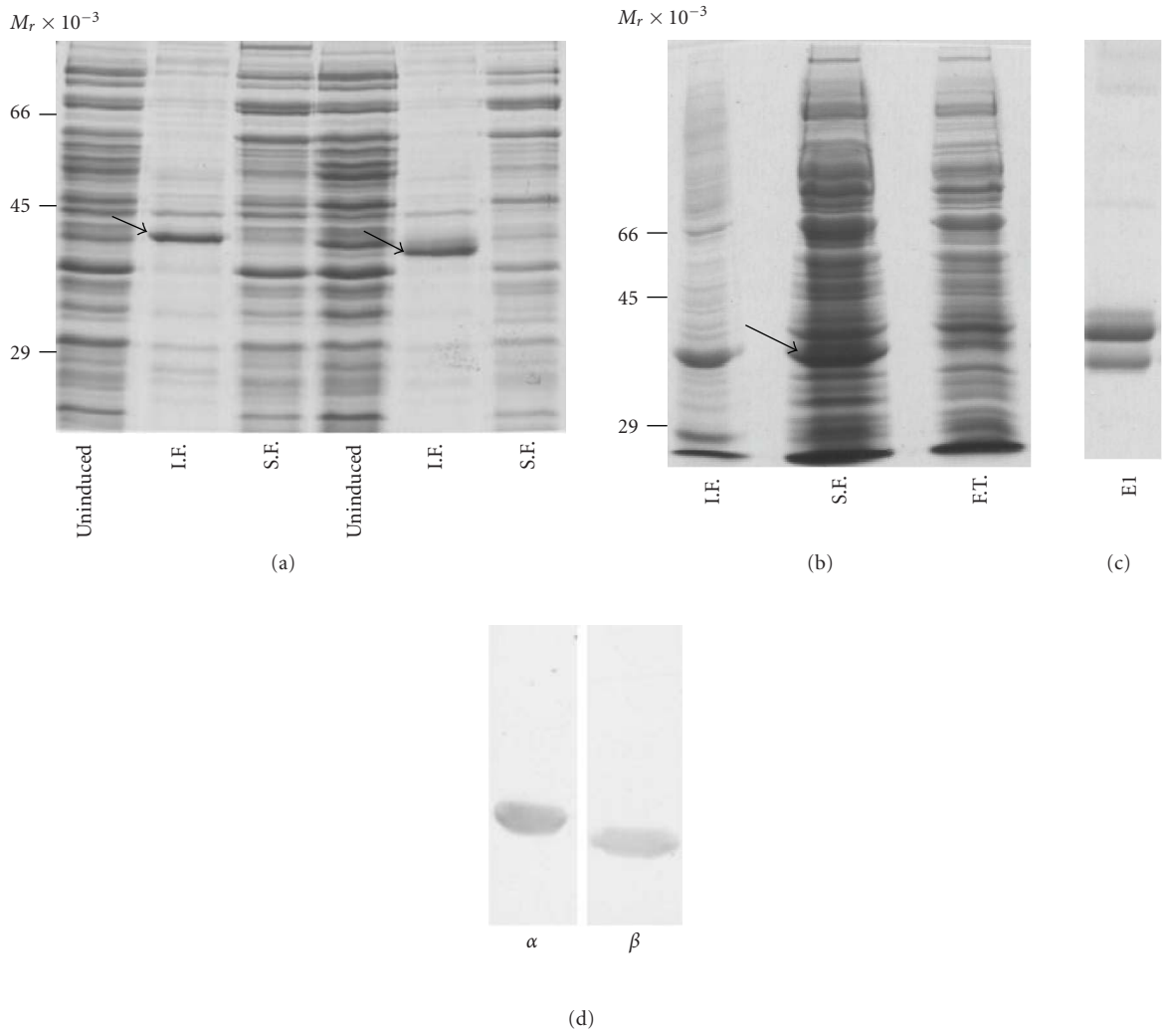

Figure 2: Expression and purification of *A. thaliana* pyruvate dehydrogenase. (a) Bacterial expression of AtPDC E1α and E1β. An SDS gel of total proteins from uninduced *E. coli* BL21 (DE3) cells containing pT7-7:AtE1α; insoluble (I.F.) and soluble (S.F.) fractions from induced cells; total proteins from uninduced cells containing pET28:AtE1β; insoluble and soluble fractions from induced cells. The positions of AtPDC E1α and E1β are indicated by arrows. (b) An SDS gel of a typical 2-plasmid AtPDC E1 α plus β coexpression and purification profile using the HiTrap chelating column charged with 0.1 M $NiSO_4$; insoluble fraction, soluble fraction, flow through (F.T.). The position of AtPDC E1β in the soluble fraction is indicated by an arrow. (c) A Coomassie-stained SDS gel of AtPDC E1 after immobilized metal affinity chromatography. (d) Immunoblot analysis of AtPDC E1. The E1α subunit was detected with monoclonal anti-E1α antibodies, and the E1β subunit was detected with monoclonal anti-FLAG antibodies. The positions of molecular mass markers are indicated on the left for (a) and (b). All gels (except the Immunoblot) were stained with Coomassie Blue.

phosphopeptides, 78.96 Da (the PO_3^- ion). The sequence of this peptide was confirmed by positive ion nanospray QqTOF tandem MS analysis of the triple-charge ion at 553.55 Da in the peptide mixture (Figure 4(b)). The assignment of phosphate to Ser292 is supported by the presence of fragment ions $b5^+$ (662.25 Da), $b6^+$ (793.23 Da), and a complete y-ion series including $y10^+$ (1164.41 Da). The most plausible interpretation of these data is that the Tyr286-Arg301 tryptic peptide is phosphorylated at Ser292. Tandem MS analyses of the double-charge ion at 829.8 Da and the triple-charge ion for the Met-oxidised form of the peptide at 558.9 Da provided additional evidence for phosphorylation of Ser292 (data not shown).

3.6. Phosphorylation of the Asp295 and Gly297 Mutants. Comparison of WT and mutant AtPDC E1 proteins incubated with AtPDK for 90 min showed substantial differences in phosphorylation (Figures 5(a) and 5(b)). In comparison with WT E1, phosphorylation of the D295A and D295N mutants was reduced by 92 and 83%, respectively, while phosphorylation of the D295L mutant was abolished (~3% residual phosphorylation) (Figure 5(a)). In contrast, phosphorylation of the G297D and G297S mutants was little affected, being reduced 9 and 13%, respectively.

The data in Figure 5(c) show a time-dependent increase in phosphorylation of the G297D mutant, similar to that seen

with the WT protein. In contrast, the low-level phosphorylation in the D295A and D295N mutant proteins did not increase with an increase in time.

3.7. B2H Analysis of Protein Interactions. Interaction between PDK and E1 in our reconstituted *in vitro* system comprises a multistep sequence including recognition, binding, and phosphotransfer. We used the B2H synthetic genetic system to test the possibility that reduced phosphorylation of the AtPDC E1α D295A and D295N mutants was due to impaired binding of PDK. In these assays, protein interaction is quantified using the β-galactosidase reporter. Interactions between AtPDK and the mutant forms of AtPDC E1α were slightly reduced relative to WT E1α (Figure 6). It does not appear, however, that these small decreases in protein interaction are adequate to explain the large decreases in phosphorylation (Figure 5).

4. Discussion

Two approaches were used previously to express recombinant plant mitochondrial PDC E1. Staphylococcal protein A-based vectors were constructed to direct secretion of the E1α and E1β subunits of *P. sativum* pyruvate dehydrogenase from separate strains of *B. subtilis* [23]. A bipartite vector was designed for simultaneous expression of E1α and E1β subunits in the cytoplasm of *P. pastoris*, but very low yields were the result of an unfavorable codon-usage bias [24]. While useful for analytical studies of E1 assembly, these systems yielded too little protein for detailed biochemical characterizations.

The envisioned analyses necessitated development of a method for heterologous expression and rapid purification of active, correctly folded and assembled recombinant AtPDC E1. The *E. coli* coexpression strategy routinely yielded ~8 mg of purified E1 per 500 mL, and more than 90% of this was assembled into heterotetramers. Catalytic activity was seemingly not influenced by the N-terminal His_6-tag. When individually expressed at high levels neither subunit was soluble, suggesting that correct folding and assembly of large amounts of the heteromeric enzyme require the simultaneous presence of both subunits. Complexity in the folding and assembly pathway was not unexpected based upon previous reports [23, 24, 37, 38] and the reported participation of the GroE chaperonins [39–41].

Recombinant tetrameric AtPDC E1 catalyzed decarboxylation of pyruvate in the presence of high (1.0 mM) and low (1.8 μM) concentrations of the artificial electron acceptor $K_3Fe(CN)_6$ (Tables 1 and 2). The specific activity of the recombinant enzyme was 0.32 μmol min^{-1} mg^{-1} protein in assays containing 1 mM $K_3Fe(CN)_6$. This is ~2-fold higher than the values reported for recombinant HsPDC E1 [42] or native *E. coli* E1 [43] under similar assay conditions. The K_m pyruvate value for recombinant AtPDC E1 is similar to that of native pea mitochondrial PDC [44] but cannot be compared directly because of differences in assay procedures.

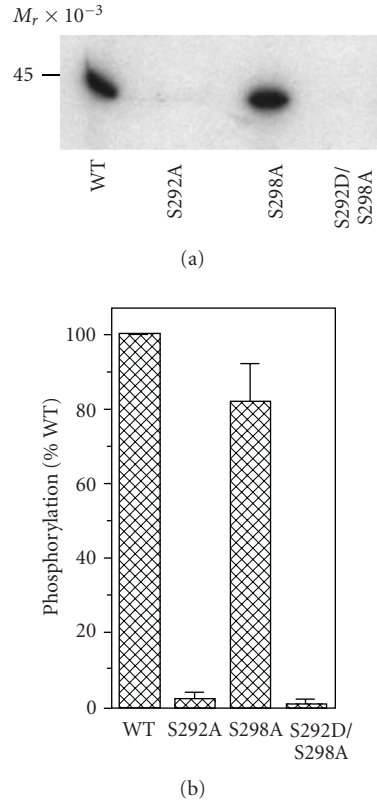

Figure 3: Phosphorylation of recombinant AtPDC E1 by recombinant AtPDK. (a) Autoradiograph of the *in vitro* phosphorylation of wild-type (WT) AtE1α, and the S292A and S298A mutants, by recombinant AtPDK. One μg of E1 was incubated with 0.25 μg PDK and 200 μM [γ-^{32}P]ATP, at 30°C for 90 min. When the film was developed after 3 h, phosphorylation of the WT and the S298A proteins was visible, while no phosphorylation was observed for the S292A and the double mutants. Furthermore, no phosphorylation of the S292A or double mutants was observed when the same film was developed after 16 h. (b) Phosphorylation of the mutant proteins relative to WT. The 100% phosphorylation value of WT was 1.0 ± 0.04 μmol ^{32}P mol AtPDC E1^{-1}. Data are the mean values from at least three separate determinations ± SEM.

There are currently no literature k_{cat} values for recombinant E1 proteins.

The catalytic activity of the AtPDC E1 S292A mutant was reduced by 26% (Table 1), which is essentially identical to the effect previously reported for HsPDC E1 [42]. The activity of the AtPDC E1 S292D/S298A double mutant was reduced by 90% (Table 1). Replacement of the HsPDC E1 site 1 Ser residue with the P-Ser mimic Glu reduced decarboxylase activity but abolished PDC activity. This result agrees with the previous observations that phosphorylation has a more pronounced effect on reductive acetylation than on substrate binding [43, 45].

Recombinant AtPDC E1 was efficiently phosphorylated *in vitro* by AtPDK. One mole of phosphate was incorporated per mol of subunit dimer. (Figure 3). The slight ^{32}P incorporation on the S292A mutant and the unaffected phosphorylation level of the S298A mutant catalyzed by

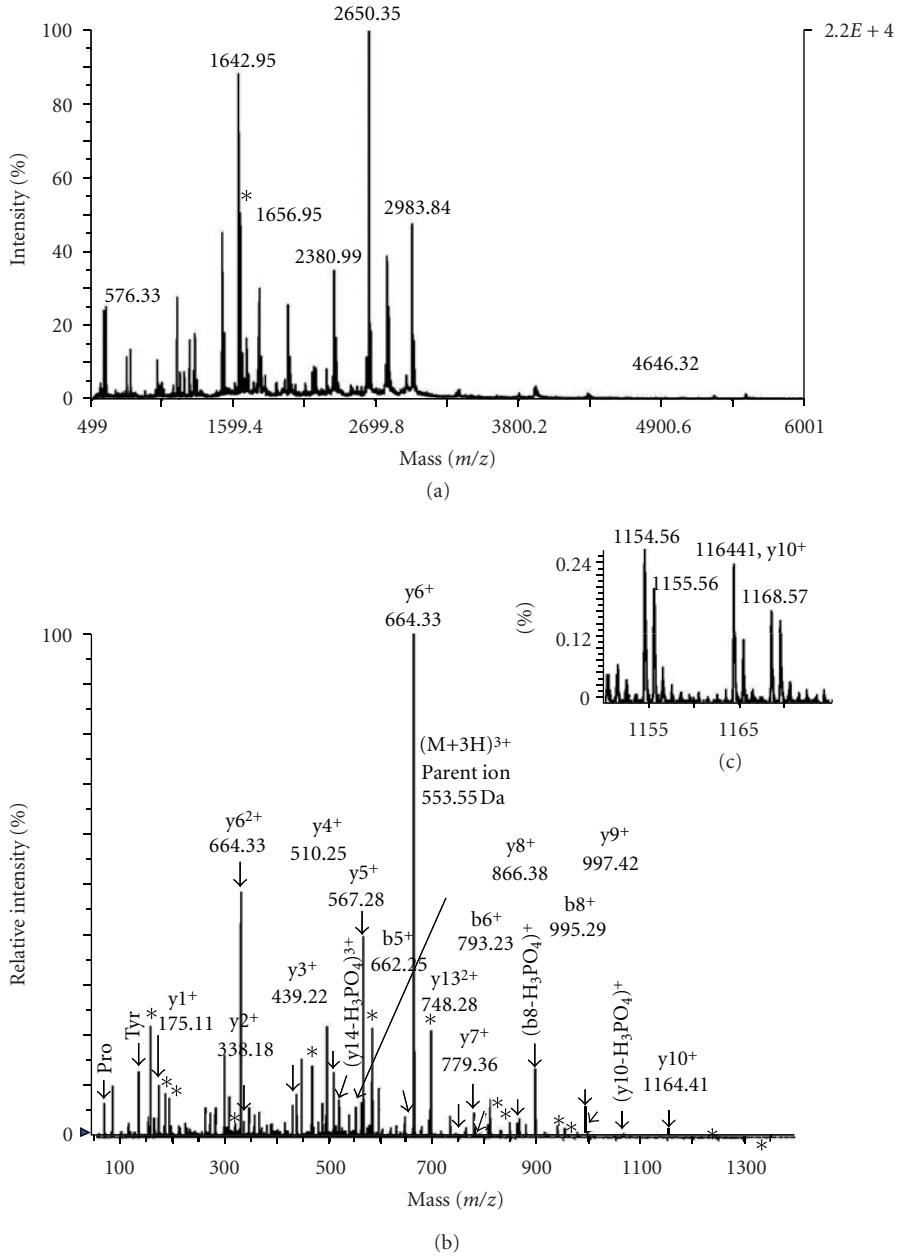

Figure 4: Analysis of the AtPDC E1α S298A mutant phosphorylation site by mass spectrometry. (a) MALDI-TOF MS analysis of tryptic peptides from the AtPDC E1α S298A mutant. The 1656.9 *m/z* ion corresponds to the phosphopeptide Tyr286 to Arg301. (b) Fragmentation of the *m/z* 1656.9 peptide by nanoelectrospray tandem MS. Fragment ion spectrum from the triple-charge molecular ion at *m/z* 553.55. The ions $b5^+$ (662.25), $b6^+$ (793.23), and $y10^+$ (1164.41) indicate P-Ser292. The asterisks mark the fragment ions from the S298A-methylated peptide that was also present in the parent ion envelope at $(M + 3H)^{3+}$ of 553.95 Da. (c) Expanded spectrum of the $y10^+$ ion.

AtPDK suggested that only Ser292 is phosphorylated in our assay condition (Figure 3). Analysis of the S298A mutant by QqTOF tandem MS verified phosphorylation of only Ser292 (Figure 4). Our finding that only site 1 is phosphorylated with the reconstituted minimum *A. thaliana* system is in agreement with results obtained using the recombinant human proteins [46] and validate use of the plant system for further mechanistic studies.

There is no reason to believe that the enzymatic mechanism is somehow different in the absence/presence of E2. While the rate of E1 phosphorylation by PDK is higher in the presence of E2 (and higher yet with PDC) [47], this stimulation is due to a direct kinetic effect; that is, ADP disassociates faster from PDK when it is bound to E2 [48]. Our results are consistent with a previous study, using two-dimensional separation of phosphoproteins

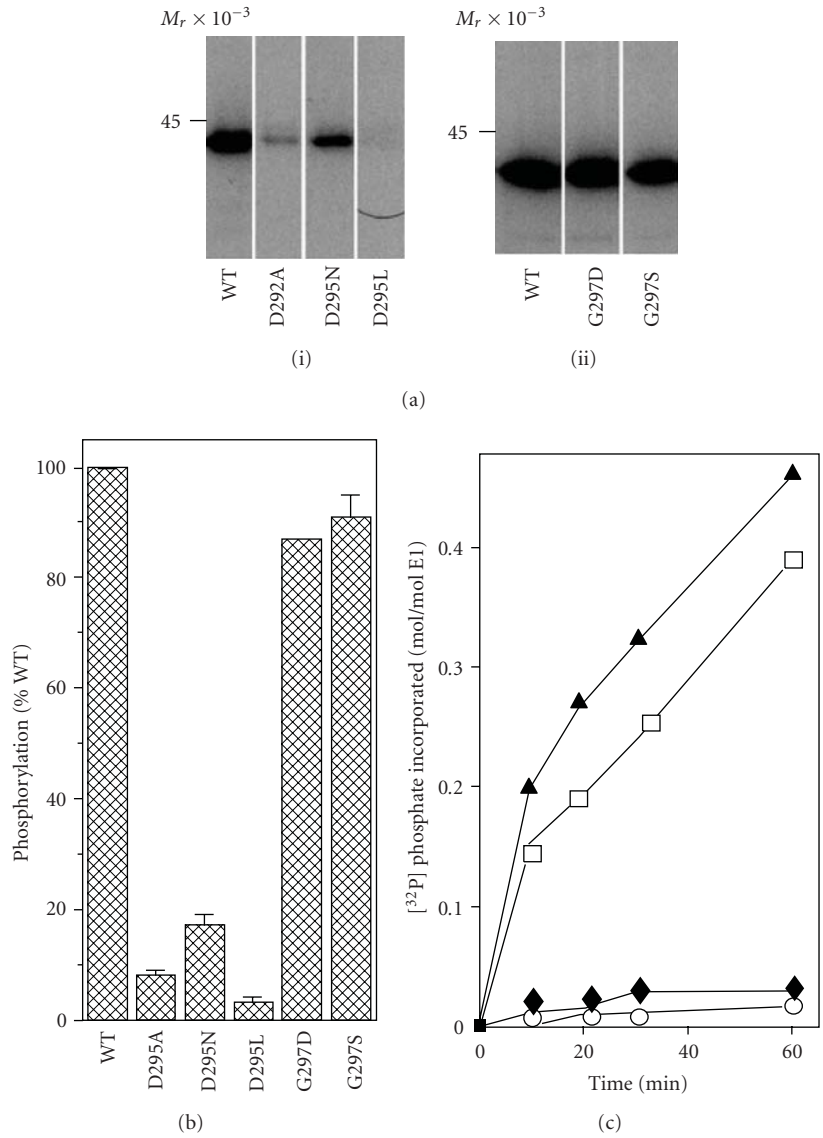

Figure 5: Phosphorylation of AtPDC E1α active-site loop mutants by AtPDK. (a) (i) Autoradiograph comparing phosphorylation of WT AtPDC E1 and the Asp295 mutant. (a) (ii) Autoradiograph comparing phosphorylation of WT AtPDC E1 and the Gly297 mutant. One μg each of the WT and mutant proteins was incubated with 0.25 μg AtPDK and 200 μM [γ-^{32}P]ATP at 30°C for 90 min. In each case the entire reaction mixture was loaded onto the gels. After electrophoresis, all gels were exposed to film for 3 h. (b) Relative (to WT) phosphorylation of the Asp295 and Gly297 mutants is indicated. Data are mean values from at least three separate determinations ± SEM. The value for 100% of WT phosphorylation was 0.7 ± 0.02 μmol ^{32}P μmol AtPDC E1^{-1}. (c) Time-course of phosphorylation. The WT AtPDC E1 (▲), and the G297D (□), D295A (◯), and D295N (◆) mutant proteins, were phosphorylated as described in (a). Reactions volumes were 100 μL. Samples of 20 μL were removed at each time point, mixed with SDS-sample buffer, heated, and then resolved by SDS-PAGE.

from potato mitochondria followed by MS analysis, which identified Ser292 as the only residue phosphorylated [49]. While phosphorylation of only site 1 was expected with the minimum *in vitro* system, multi-site phosphorylation remains a possibility upon addition of E2 or more likely as PDC.

Phosphorylation of Ser292 was affected considerably by changes to Asp295, being reduced in the order D295N < D295A < D295L (Figure 5(a)). Interaction between E1α and PDK is a multistep process that involves recognition of the phosphorylation-site region, docking of the PDK with this region, and subsequent transfer of the phosphoryl group. It is unlikely that the major reduction in phosphorylation of these mutants was due to inhibition of PDK binding to E1, because there was no significant reduction of interaction between AtPDK and the D295N or D295A mutants (Figure 6). It is logical that the reduction in phosphorylation of the active-site loop mutants is due to disruption of the transfer of the γ-phosphate from ATP to E1α Ser292, possibly the result in ordering or positioning of the active-site loop.

Some protein Ser/Thr kinases have an affinity for amino acids with acidic, basic, or hydrophobic functional groups located at defined positions N- or C-terminal to the phosphorylation site [50]. Our results are similar to an earlier study employing synthetic peptides as substrates for PDK. In those studies, peptides with the D295N substitution were poorer substrates for bovine PDK [8]. The Asp295 residue is one of a group of charged residues that form an H-bonding network that stabilizes the active-site loop [51], making it accessible to the lipoyl domain of E2. Perturbations in this network affect substrate channeling, and phosphorylation is believed to promote this disorder [45, 51]. Based on the results from *in silico* mutagenesis, we hypothesize that interactions with His291 and Tyr300 would be disrupted if Asp295 were substituted (Figure 1(b)). Even substitution with Glu would be predicted to abolish the H-bond with His291, although we did not test this experimentally. The reduced phosphorylation seen with all changes to Asp295 is consistent with the proposal that altering the acidic residue C-terminal to Ser292 destabilizes the structure of the active-site loop. Alternatively, it is possible that conservation of Asp295 is part of a mechanism that maintains the active-site loop disordered and provides access to Ser292.

Changes to Asp295 have been reported to have pleiotropic effects on phosphorylation of E1. Phosphorylation of RnBCKDC E1 was not affected by mutagenesis of Asp295 [19], but was inhibited by mutagenesis of Arg288, which corresponds to Arg285 of AtPDC E1α. In contrast, the same mutations of HsBCKDC E1 did not inhibit phosphorylation [51]. The D296E mutant in HsPDC E1α was still phosphorylated, although in these analyses the effect of phosphorylation was determined indirectly by measuring the decrease in activity of an already catalytically compromised mutant protein [52].

The catalytic response to the D295A change has been studied in other systems but using a similar assay method, which simplifies comparisons. In the case of AtPDC E1, the catalytic activities of the D295A and D295N mutants were reduced considerably (Table 1). The K_m pyruvate values of these mutant proteins were increased 5-fold, while the k_{cat} decreased by >70%. The response of the HsBCKDC E1 to the D295A mutation was essentially the same as with AtPDC E1 in terms of catalytic activity and k_{cat} values; however, in that instance, the K_m pyruvate was unaffected [51]. In a case of extreme contrasts, this D > A mutation of RnBCKDC E1 yielded a catalytically dead protein [19], while the activity of the *B. stearothermophilus* PDC E1α actually increased 3-fold (and the K_m pyruvate value by 10-fold) [18]. The modest but consistent reduction in k_{cat} and k_{cat}/K_m values seen with the AtPDC E1 mutants are in agreement with the proposal that Asp295 stabilizes the active-site loop conformation. The differences seen with ostensibly closely related enzymes suggest small or subtle differences in loop structure or stability. In the case of the more structurally distant *E. coli* PDC E1, the H-bonding loop-stabilizing pattern also involves nonconserved residues [53].

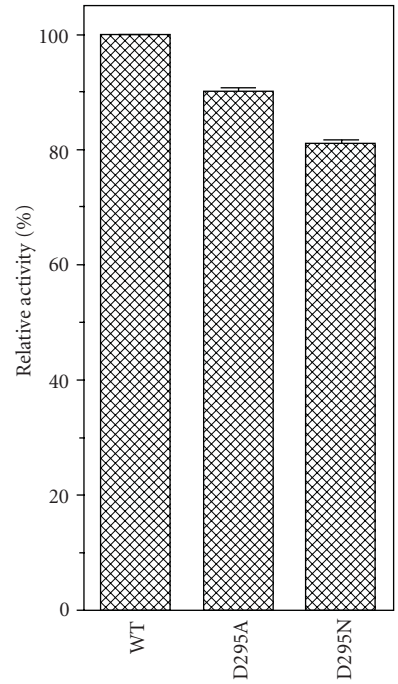

Figure 6: Quantitative analysis of interactions between AtPDC E1 and AtPDK. Quantitative B2H analysis was used to study interaction between WT AtPDC E1α, the D295A, and D295N mutants and AtPDK. The AtPDK sequence was cloned into the Stratagene B2H vector pBT, while the E1α sequences were cloned into pTRG. After selection, protein interactions were quantified using the β-galactosidase reporter. The value for 100% of WT activity was 3 μmol min^{-1} 1.0 A600^{-1}. Data are the mean ± SEM from two separate experiments, each assay conducted in triplicate.

Binding of the TPP cofactor confers an ordering to the E1α active-site loop. When the site 1 Ser residue is phosphorylated, the presence of the phosphoryl group prevents this ordering [53]. The mechanism involved has been referred to as a "steric clash" between the P-Ser at site 1 and a nearby Ser residue which then nullifies a hydrogen-bonding network [45]. Some of the effects that we have seen with the AtPDC E1α Asp295 mutants could be explained by this same mechanism. The disordered phosphorylation loops impede the binding of lipoyl domains of the PDC E2-core to E1 [45]. The importance of structural order/disorder on protein phosphorylation is the subject of much current attention [54, 55].

The conserved Gly297 residue does not have an obvious role in the phosphorylation of the site 1 Ser residue. Phosphorylation was not affected in either the G297S mutant, where residue size is similar, or the G297D mutant which represents a drastic change (Figure 5(b)). These results seem to indicate that the residue present in this position is not directly involved with either formation or stabilization of the active-site loop structure. The reduction in k_{cat}, without any change in K_m pyruvate, suggests that replacing Gly297 with a polar residue, such as Asp, might be interfering with interactions between Tyr-300

and the carboxyl group of pyruvate, either directly or via steric hindrance.

Since all of the mutant subunits assembled into heterotetramers, neither Asp295 nor Gly297 is critical for subunit association. Moreover, the far-UV CD spectra of the mutant proteins were not distinguishable from the spectra obtained for the WT protein (data not presented), indicating that no gross structural perturbations occurred as a result of the mutations.

The defined *in vitro* system that we have developed is suitable for additional detailed analyses of the mechanism of regulatory phosphorylation of the plant mitochondrial PDC. Future studies will include the recombinant plant E2 proteins and domains and will address the differences in behavior between the mono- and dilipoyl forms of this core component of the PDC [56].

Conflict of Interests

The authors declare no conflict of interests.

Abbreviations

At:	*Arabidopsis thaliana*
BCKDC:	Branched-chain α-ketoacid dehydrogenase complex
B2H:	Bacterial 2-hybrid
E1:	α-ketoacid dehydrogenase/decarboxylase
E1α:	α-subunit of E1
Hs:	*Homo sapiens*
PDC:	Pyruvate dehydrogenase complex
PDK:	Pyruvate dehydrogenase-kinase
Rn:	*Rattus norvegicus*
WT:	Wild type.

Acknowledgments

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Research Article

Relative Expression Levels Rather Than Specific Activity Plays the Major Role in Determining *In Vivo* AKT Isoform Substrate Specificity

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The AKT protooncogene mediates many cellular processes involved in normal development and disease states such as cancer. The three structurally similar isoforms: AKT1, AKT2, and AKT3 exhibit both functional redundancy and isoform-specific functions; however the basis for their differential signalling remains unclear. Here we show that *in vitro*, purified AKT3 is ~47-fold more active than AKT1 at phosphorylating peptide and protein substrates. Despite these marked variations in specific activity between the individual isoforms, a comprehensive analysis of phosphorylation of validated AKT substrates indicated only subtle differences in signalling via individual isoforms *in vivo*. Therefore, we hypothesise, at least in this model system, that relative tissue/cellular abundance, rather than specific activity, plays the dominant role in determining AKT substrate specificity *in situ*.

1. Introduction

The AKT protooncogene comprises a family of three highly homologous serine/threonine kinases (AKT1, 2 and 3) [1], that play major regulatory roles in a wide range of cellular processes including cell survival, growth, proliferation, angiogenesis, and metabolism [2–4]. These processes are key to normal development and often dysregulated in disease. Consistent with these wide-ranging roles, to date, more than 100 AKT substrates have been identified that are integral in the regulation of one or more of these cellular processes [3].

Each AKT isoform consists of a pleckstrin homology domain (>75% identity), a linker region (>17% identity), a kinase domain (>87% identity), and a carboxyl tail with a hydrophobic motif (>66% identity) [1]. The two key regulatory phosphorylation sites on AKT are also conserved, Ser473 and Thr308 in AKT1, Ser474 and Thr308 in AKT2, and Ser472 and Thr305 in AKT3 [5]. Thr308 is phosphorylated by 3-Phosphoinositide-dependent kinase 1 (PDK1) and Ser473 by mammalian target of rapamycin (mTOR) complex 2 (mTORC2) [6–9]. Recent studies have suggested that phosphorylation at Ser473 dictates AKT signaling to a specific set of substrates, and phosphorylation at Thr308 determines the kinase activity [8, 10].

Whilst all three AKT isoforms share a high sequence identity and exhibit functional redundancy, there is also genetic evidence that they can function distinctly. This is illustrated in the distinct phenotypes exhibited by the single isoform knockout (KO) mice and the severe phenotypes exhibited by double KO (dKO) mice. AKT1$^{-/-}$ mice are not 100% viable, with surviving mice that reach adulthood being 15–20% smaller than their wild type (wt) and heterozygous counterparts [11]. Furthermore, AKT1$^{-/-}$ mice exhibit a smaller brain and liver as a result of a reduction in cell number when compared to wt mice [12] indicating that AKT1 is important in regulating proliferation. Heart size

was also reduced; however it was caused by a decrease in cell size [12], suggesting that AKT1 predominantly affects cell growth in this tissue. The AKT2$^{-/-}$ mice exhibited a very different phenotype, reminiscent of mice with Type 2 diabetes mellitus, suggesting a specific role for this isoform in glucose metabolism [13] although more recent studies in AKT1$^{-/-}$ mice have indicated that both AKT1 and 2 are required for the regulation of glucose homeostasis in the periphery [14]. AKT3$^{-/-}$ mice are not growth retarded but show a 25% reduction in brain size due to decreased cell number and a smaller cell size [12]. The phenotypes of AKT dKO mice were more severe than that observed in single KO mice. AKT1/2$^{-/-}$ mice die shortly after birth and suffer from severe dwarfism, impaired skin development, skeletal muscle atrophy, impaired adipogenesis, and bone development [15], none of which was observed for the individual KO mice. The additive effect of AKT1 and AKT2 suggests a functional redundancy between these two isoforms. AKT1/3$^{-/-}$ mice die between E11-E12, indicating the importance of AKT1 and AKT3 in postnatal survival [16]. AKT2/3$^{-/-}$ mice are viable but smaller than their wt counterparts [17]. This less severe phenotype than the other compound mutants indicates a dominant role for AKT1, at least in development.

Recent studies suggest that the different AKT isoforms can signal via distinct subsets of downstream pathways and that these subsets can vary depending on cellular and tissue context [12], reinforcing the need to investigate isoform-specific signaling in different cell types. For example, AKT3 but not AKT1 is crucial for activation of the mTORC1/S6K1 signaling pathway in brain [12]. Furthermore, cellular context has been seen to be important for AKT1 signaling where AKT1$^{-/-}$ livers were smaller due to reduced cell number while AKT1$^{-/-}$ hearts were smaller entirely because of reduced cell size [12]. In addition, evidence for distinct signaling pathways to and from individual AKT isoforms is beginning to accumulate. For example, reduction of PTEN expression in melanocytes activates AKT3 apparently without affecting the activity of AKT1 or 2 [18]. While the mechanism for this selective activation remains to be elucidated it provides an intriguing precedent.

Defining the mechanism(s) of differential signaling via these highly homologous AKT isoforms will be critical for understanding the role played by AKT in many essential cellular functions. It is clear that differential expression patterns can explain some of these functional roles. AKT1 is widely expressed in tissues including the brain, heart, lung, skeletal muscle, thymus, and skin while AKT2 is predominantly expressed in brown fat and the heart, and AKT3 was most abundantly expressed in the brain [16]. Moreover, AKT isoforms have been shown to localize to different subcellular compartments in a cell-line-specific manner [19, 20]. In unstimulated cells, AKT1 localised in the cytoplasm, AKT2 at the mitochondria, and AKT3 in the nucleus of breast, prostate, and liver cancer cell lines [20]. However, insulin-stimulated adipocytes demonstrated a higher level of AKT2, than AKT1, localization at the plasma membrane, thus linking AKT2 to the isoform-specific regulation of GLUT4 translocation and glucose metabolism [19].

In addition to variation in expression and localization patterns, differences in the intrinsic activity of the isoforms in phosphorylating synthetic peptide substrates have been reported [21–24]. Indeed, we have shown previously that purified AKT isoforms vary with their specific activity in phosphorylating model peptide substrates, with AKT3 being ~15-fold more active than AKT1 which was ~10-fold more active than AKT2 [25]. These findings were consistent with the findings of Walker et al. [24] who purified each isoform from HEK293 cells and further activated them by incubation with PDK1. In these experiments, a truncated form of AKT3 was 3-fold more active than AKT1 that was 5-fold more active than AKT2 [24]. Here we have examined whether these fundamental differences in enzyme kinetics contribute to substrate specificity *in vivo*.

Given the cell-type-specific regulation of isoform-dependent signaling described above, we chose to directly compare the intrinsic activity of individual AKT isoforms purified from HEK293 cells with their ability to phosphorylate *in vivo* substrates in the same cell type. HEK293 were chosen as they enable high levels of expression of exogenous AKT to allow purification of sufficient kinase to enable detailed kinetic analysis of enzyme activity. We focused our study on AKT 1 and 3 predominantly given the low activity of AKT2 after confirming this using isoforms immunopurified from HEK293 cells stimulated with serum, insulin, and pervanadate. We first confirmed that AKT3 was indeed more active than AKT1 in peptide kinase assays and against a newly identified protein substrate, ribosomal protein S7 (rpS7) (see supplementary Figure 1 in Supplementary Material available online at dois 10.4061/2011/720985; [26]). We have used gain- and loss-of-function approaches employing constitutively active forms of the isoforms and specific siRNAs to examine *in vivo* AKT isoform-specific signaling towards established direct AKT substrates involved in cell proliferation, survival, and growth including GSK3α/β, FoxO1/3a, MDM2, and PRAS40, and via the activation of the mTORC1 pathway leading to cell growth. In contrast to the marked differences in intrinsic enzyme activity between the AKT isoforms, only subtle differences in *in vivo* substrate specificity were observed. This thus suggests that AKT isoform-specific regulation of cellular function is, in a large part, dictated by the relative expression levels in the relevant cell type.

2. Material and Methods

2.1. Cell Culture and Treatments. Human embryonic kidney (HEK293) cells were cultured in Dulbecco's Modified Essential Medium (DMEM) plus 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antimycotic/antibiotic (Am/Ab, Invitrogen) and maintained at 37°C in 5% CO_2. Cells were serum-starved (DMEM no FBS) for 24 hours then stimulated with 1 μM insulin, 0.1 μM pervanadate or 10% FBS for 20 minutes or pretreated with 5 μM AKT inhibitor (AKTi, Calbiochem, # 124017), 20 nM rapamycin (Calbiochem, #553210), or both for 30 minutes then stimulated with 10% FBS for 20 minutes.

2.2. Harvesting Cells for Protein Lysates. Proteins were harvested as described in [25]. Briefly, cells were lysed in Rac Lysis Buffer (RLB: 50 mM Tris-hydrochloride (Tris-HCl) pH7.5, 1% (v/v) NP-40, 120 mM sodium chloride (NaCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium fluoride, 40 mM β-glycerophosphate, 0.1 mM sodium vanadate, 1 mM benzamidine, complete EDTA-free protease inhibitor cocktail (Roche), and phosSToP phosphatase inhibitor cocktail (Roche)) and cleared by centrifuging (16000 $\times$g, 15 minutes, 4°C). Samples were snap frozen and stored at −80°C. The protein concentration was determined using the DC protein assay kit (BioRad).

2.3. Expression and Purification of Recombinant AKT Isoforms. HEK293 cells were transfected via the calcium phosphate method [27] with pCDNA3 HA-tagged wtAKT1, wtAKT2, or pCMV5 HA-tagged wtAKT3 (Pearson Laboratory), pCDNA3 GST-wtAKT1 [28], pCMV5 GST-wtAKT3 [25], or pCDNA3 constitutively active myristoylated (myr) HA-tagged AKT1, AKT2, or AKT3 (Pearson Laboratory). Cells were treated and harvested into RLB and lysates cleared by centrifugation (14500 $\times$g, 15 minutes, 4°C). HA-tagged AKT isoforms were immunoprecipitated from cleared lysates (100 μg) using Protein A Sepharose 4B beads (Zymed). GST-tagged AKT isoforms were purified by affinity chromatography as described in [28, 29]. Eluates were combined, buffer exchanged by overnight dialysis against 0.27 M sucrose in RLB, and purified GST-tagged wtAKT concentrated using Centricon 50s (Millipore), snapped frozen, and stored at −80°C. Samples were resolved by SDS-PAGE alongside known concentrations of bovine serum albumin (BSA), Coomassie R-250 stained, and the concentration determined by densitometry using Scion Image software (Scion Incorporation).

2.4. Expression and Purification of HIS-rpS7. Human ribosomal protein S7 (rpS7, gift from Dirk Görlich, Heidelberg [30]) was cloned into the pQE-60 vector using restriction sites NcoI and BamHI and transformed into XL-Blue E.coli cells. Expression of HIS-rpS7 was induced with 1 mM isopropyl-β-D-thio-galactoside (IPTG) for 5 hours, cells pelleted (6000 $\times$g, 4°C, 10 minutes), resuspended in buffer A (300 mM NaCl, 50 mM sodium phosphate buffer, pH 7.0), and sonicated. The insoluble fraction was pelleted as above, resuspended in 20 mL of buffer B (8 M urea in buffer A), and sonicated. The clarified sample was tumbled with talon resin (Clontech) for 2 hours at room temperature (RT), washed 3 times with buffer B, and HIS-rpS7 eluted with 150 mM imidazole. HIS-rpS7 was dialysed sequentially for 4 hours at 4°C against: 4 M urea in $PBS^{-/-}$; 2 M urea in $PBS^{-/-}$, 20% glycerol and 0.5 mM phenylmethylsulphonyl fluoride (PMSF); 0.5 M urea in $PBS^{-/-}$, 20% glycerol and 0.5 M PMSF; and twice in $PBS^{-/-}$, 20% glycerol and 0.5 mM PMSF. Purified HIS-rpS7 was clarified by centrifugation (20000 $\times$g, 2 minutes, 4°C) and stored at −80°C.

2.5. SDS-PAGE and Immunoblotting. Protein lysates (20–50 μg) were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) alongside Benchmark Prestained Protein Ladder (Invitrogen) or PageRuler Prestained Protein Ladder Plus (Fermentas). Proteins were then transferred onto immobilon-P membrane (Millipore) and immunoblotted with primary antibodies listed in Supplementary Table 1. Antimouse and antirabbit horseradish peroxidase-conjugated secondary antibodies (BioRad) were used at 1:2000 dilution. Signals were detected by enhanced chemiluminescence (Perkin Elmer) onto film and quantified by densitometry using ImageJ 1.42q (National Institutes of Health, USA). Paired t-test statistical analysis was performed using GraphPad Prism version 5.00, GraphPad Software, San Diego, Calif, USA, http://www.graphpad.com/.

2.6. Coomassie R-250 Staining. After SDS-PAGE, gels were stained with Coomassie R-250 (0.2% Coomassie R-250, 50% ethanol, 10% acetic acid) for 1 hour then destained with 25% ethanol plus 7% acetic acid.

2.7. Knockdown of Endogenous AKT Isoforms. Individual AKT isoforms were silenced using On-Target plus SMARTpool small interfering RNAs (siRNAs) purchased from Dharmacon (AKT1: L-003000-00, AKT2: L-003001-00, AKT3: L-003002-00). Control siRNAs targeting enhanced green fluorescent protein (EGFP) were purchased from Sigma-Proligo (siEGFP 5′ sequence: 5′-GCAGCACGACUUCUUCAAGTT-3′, siEGFP 3′ sequence: 5′-CUUGAAGAAGUCGUGCUGCTT-3′). HEK293 cells at 50–60% confluency were transfected with 25 nM of siAKT1, siAKT2, or siAKT3 either individually or simultaneously using lipofectamine reagent (Invitrogen) following the manufacturer's instructions. After 4 hours, the media was replaced with DMEM plus 10% FBS and 1% Am/Ab then incubated overnight.

2.8. Direct Kinase Assay. Direct kinase assays were performed as described in [31]. Briefly, purified or immunoprecipitated AKT were incubated with [γ-^{32}P]ATP and varying concentrations of RPRAATF peptide or HIS-rpS7 at 30°C for 20 minutes. The assay was terminated by spotting onto p81 paper, washed, dried, and [γ-^{32}P] counted using the Tri-Carb 2100TR liquid scintillation analyzer (Skudtek Scientific, Pty Ltd). For whole cell lysates, the reaction was terminated by adding 10 μL of 10% trichloroacetic acid. The sample was then cleared, spotted onto p81 paper, and counted as above. In the case of HIS-rpS7, samples were resolved by SDS-PAGE, the gel stained with Coomassie R-250, dried, the appropriate bands excised and counted as above.

2.9. Two Dimensional Gel Electrophoresis (2DGE). All chemicals were purchased from GE Healthcare or Sigma. Protein samples lysed in RLB were precipitated using the Ettan 2D Clean up Kit (GE Healthcare), resuspended in labelling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris) and the protein concentration determined using the Ettan 2D Quant Kit (GE Healthcare). Protein samples (50 μg) were adjusted to pH 8.5 with 100 mM sodium hydroxide, labelled in the dark with 400 pmol of CyDye Fluor minimal dye Cy2 (GE Healthcare) for 30 minutes on ice and then quenched

with 10 mM lysine. Labelled samples were combined with unlabelled proteins to a total of 250 μg, adjusted to a final volume of 340 μL (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.004% (w/v) bromophenol blue (BPB), 1% (w/v) DTT, and 1% (v/v) IPG buffer) and passively rehydrated onto an 18 cm, nonlinear pH 3–11 Immobiline DryStrip gel (GE Healthcare). Proteins were focused using the Ettan IPGphor3 (GE Healthcare) at 20°C with 50 μA per strip using the following protocol: step and hold at 150 V for 3 hours, step and hold at 300 V for 3 hours, gradient to 1000 V for 6 hours, gradient to 10000 V for 1 hour, step and hold at 10000 V for 3 hours. Immobiline DryStrip gels were incubated in equilibration buffer (6 M Urea, 2% (w/v) SDS, 50 mM Tris·HCl pH 8.8, 0.002% BPB, 30% (v/v) glycerol) containing 1% (w/v) DTT for 15 minutes, and then incubated in equilibration buffer containing 2.5% (w/v) iodoacetamide for 15 minutes. Proteins were resolved in a 12.5% gel at 0.5 W/gel for 1 hour, then 17 W/gel for 3 hours using the Ettan DALT*six* gel running tank (GE Healthcare). Gels were either stained with Coomassie G-250 (10% (w/v) ammonium sulphate, 0.1% Coomassie (w/v) G-250, 3% (v/v) orthophosphoric acid, 20% (v/v) ethanol) or transferred onto Hybond-LFP (GE Healthcare) and immunoblotted with the Phospho-AKT Substrate (PAS) antibody (Supplementary Table 1) and Cy5 conjugated secondary antibody (GE Healthcare). Signals were detected by scanning with the Typhoon tri9100 (GE Healthcare). Total protein (Cy2) and PAS (Cy5) signals were overlayed using ImageQuant (GE Healthcare). Protein spots of interest were manually excised from the Coomassie G-250 stained gel and identified by mass spectrometry.

2.10. Mass Spectrometry. Protein samples are resolved by SDS-PAGE, Coomassie R-250 stained and proteins of interest excised, in-gel tryptic digested and identified using LC-ESI-MS/MS using an Agilent 1100 Series HPLC coupled to an Agilent LC/MSD Trap XCT Plus Mass Spectrometer fitted with an HPLC Chip cube (Agilent, Palo Alto, Calif). Mass spectrometry was performed by the Core Facility at Peter MacCallum Cancer Centre, utilising facilities at Bio21 RTF (Parkville, VIC, Australia). The data acquired were analysed by correlating the peptide masses obtained to predict peptide masses from proteins in the NCBI nonredundant database using the Mascot search engine (Matrix Science).

3. Results

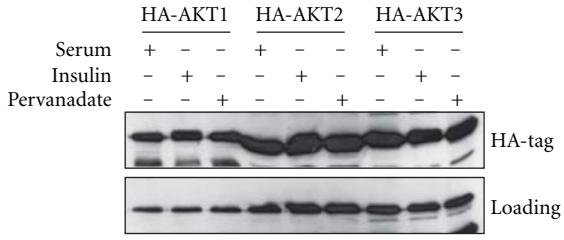
3.1. Relative Specific Activity of AKT Isoforms. Previously data from our laboratory using model peptide substrates revealed that AKT3 was ~15-fold more active than AKT1 which was ~10-fold more active than AKT2 [25]. To confirm that AKT2 was intrinsically considerably less active compared with AKT1 and 3 when expressed in HEK293 cells, HA-tagged versions of each isoform were immunopreciptated from HEK293 cells stimulated with serum, insulin, or pervanadate (Figures 1(a) and 1(b)). Under all conditions, AKT2 activity was minimal, despite robust expression. Thus, we have focused the present studies largely on AKT1 and 3.

To confirm that this difference in relative enzyme activity extended to the ability of the isoforms to phosphorylate a protein substrate, expressed GST-tagged AKT1 and 3 isoforms were purified from pervanadate-treated HEK293 cells and the kinetics of phosphorylation of the RPRAATF peptide [6, 31] compared to that with rpS7 protein. rpS7 has recently been identified as an *in vivo* substrate of the AKT signaling network [26], and we have confirmed this as a direct *in vitro* AKT substrate (Supplementary Figure 1).

GST-AKT3 was ~47-fold more active than GST-AKT1 towards the RPRAATF peptide substrate, with their V_{max} values calculated as 315.90 ± 88.95 nmol/min/mg and 6.73 ± 0.69 nmol/min/mg, respectively (Figures 1(c) and 1(d)). GST-AKT3 was ~5-fold more active than GST-AKT1 in phosphorylating rpS7 with a V_{max} value of 12.67 ± 0.47 nmol/min/mg and 2.27 ± 0.20 nmol/min/mg, respectively (Figure 1(e)). While the differences in specific activity were marked, the fold change was considerably less with the protein compared to the synthetic peptide substrate. Furthermore, GST-AKT3 had a lower affinity for the rpS7 protein than GST-AKT1, with K_m values of 6.43 ± 0.62 μM and 1.24 ± 0.63 μM, respectively. Thus, despite the markedly higher V_{max} for AKT3, its elevated activity will be dependent on higher substrate concentrations than AKT1 meaning that the local concentrations of specific substrates may be critical in determining isoform-specific signaling.

3.2. Gain of Function Approach to Determine AKT Substrate-Specific Phosphorylation. In order to examine the potential outcomes of these different intrinsic enzyme properties on *in vivo* substrate phosphorylation, HA epitope tagged forms of constitutively activated N-terminal myristoylated (myr) AKT1, 2, and 3 were expressed in serum-starved or stimulated HEK293 cells (panAKT western blot, Figures 2(a) and 2(b)). Transfection conditions were optimised to achieve similar expression levels for each isoform as indicated by the anti-HA western blot (Figures 2(a) and 2(c)). Analysis of AKT regulatory phosphorylation sites revealed that enforced expression of each isoform resulted in constitutive phosphorylation at Ser473 (Figures 2(a) and 2(d)). Thr308 phosphorylation was also markedly increased in myrAKT1 and myrAKT3 transfected cells (Figures 2(a) and 2(e)), consistent with elevated total AKT activity compared with cells expressing myrAKT2. Direct kinase assays confirmed that myrAKT1 and 3 exhibit constitutive activity (Figure 2(f)). To examine the *in vivo* effects of enforced expression of these specific isoforms, we took a targeted substrate approach, focusing on known direct AKT substrates involved in key cellular processes: proliferation (GSK3α and GSK3β); survival (FoxO1, FoxO3a and MDM2); growth (PRAS40) (Figure 3).

3.2.1. Effect of Enforced AKT Isoform Expression on Proliferation: GSK3α and GSK3β. Glycogen synthase kinase 3 (GSK3) is a negative regulator of cell proliferation [4, 32, 33] and has been implicated in various diseases including diabetes, alzheimer's, and cancer [34]. GSK3 is expressed as two isoforms, GSK3α and GSK3β, in similar tissues [35], and their activity is inhibited by AKT which phosphorylates the regulatory site Ser21 and Ser9, respectively [32, 36]. Overexpression of myrAKT1 and 3, but not myrAKT2, significantly



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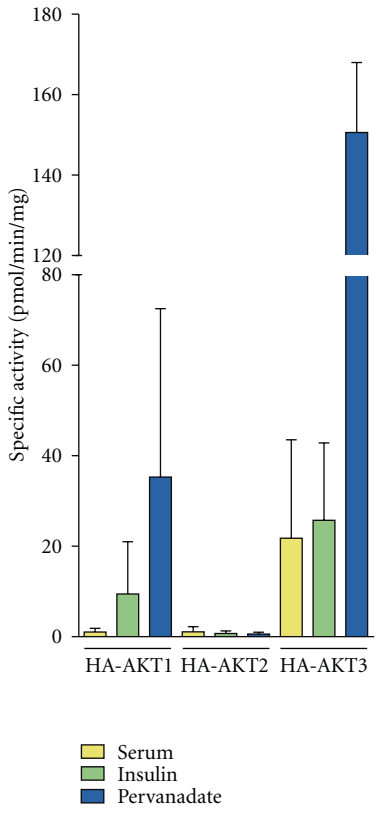

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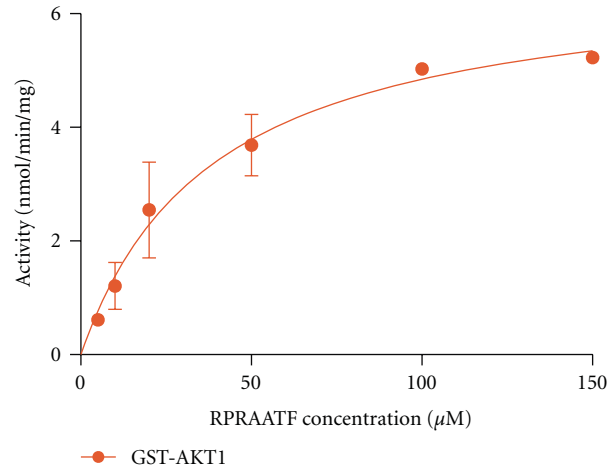

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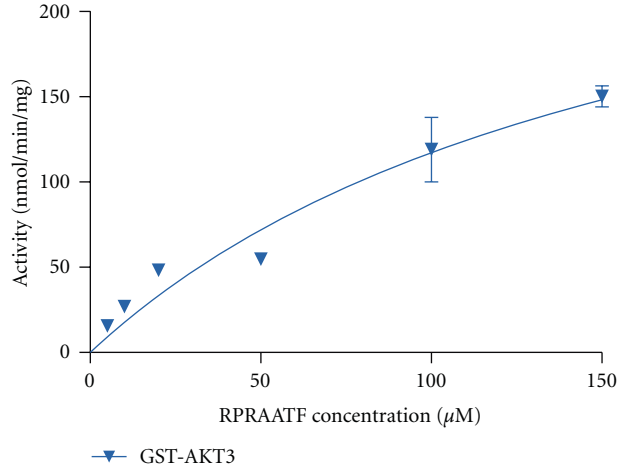

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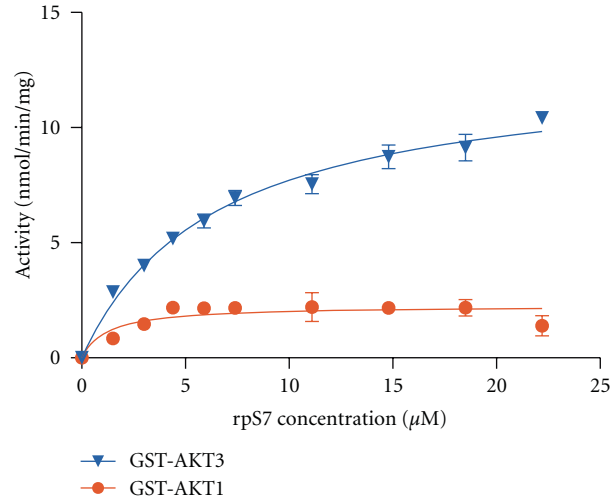
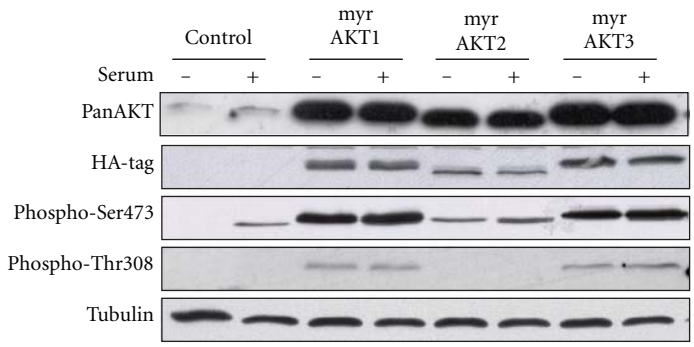


(e)

Figure 1: AKT3 is more active than AKT1 towards both the RPRAATF peptide and rpS7 protein substrate. HA-AKT isoforms were expressed in HEK293 cells, stimulated with 10% serum, 1 μM insulin, or 0.1 μM pervanadate and harvested into RLB. (a) expression of HA-AKT isoforms was detected by immunoblotting with the anti-HA antibody. (b) HA-AKT isoforms were immunoprecipitated from cleared protein lysates (100 μg) and assayed towards the RPRAATF peptide for activity. Samples were assayed in duplicate. n = 1–3. Error bars: mean ± SD. GST-AKT1 and GST-AKT3 were expressed in HEK293 cells, stimulated with 0.1 μM pervanadate, and harvested into RLB, then purified by GST-pull down and (c and d) assayed against increasing concentrations of the RPRAATF peptide or (e) rpS7. Data points were fitted to the Michaelis-Menten equation using GraphPad Prism version 5.00, GraphPad Software, San Diego, Calif, USA, http://www.graphpad.com/. n = 1, where samples were assayed in duplicate. Graph shows mean of duplicates.



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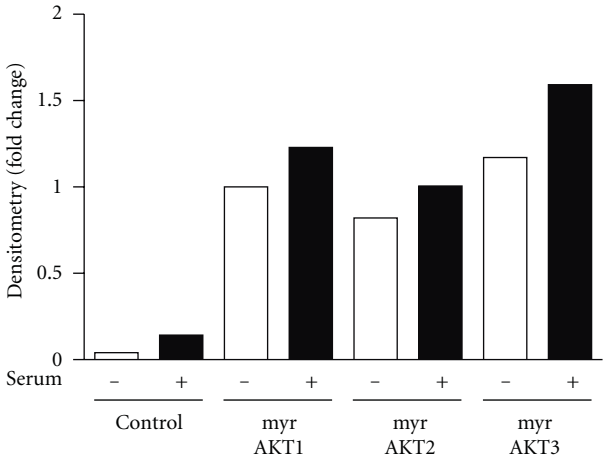

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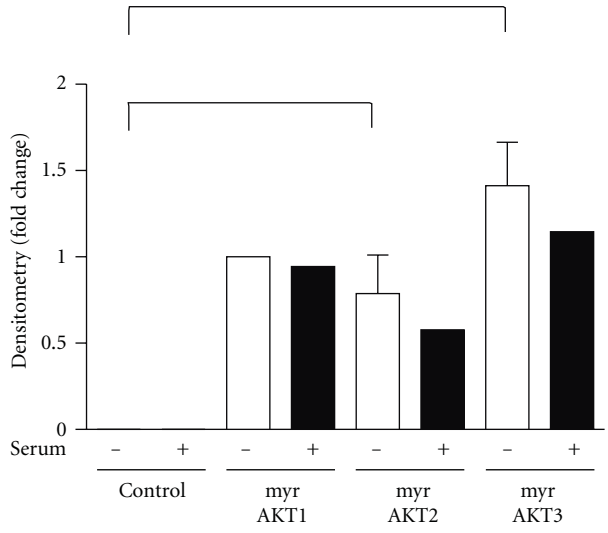

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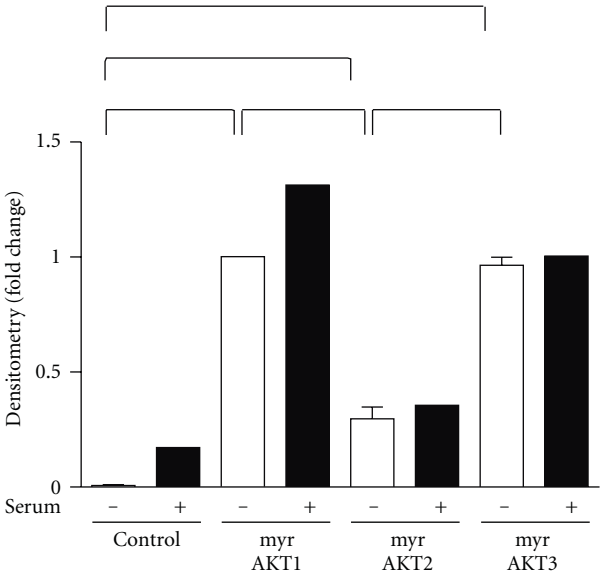

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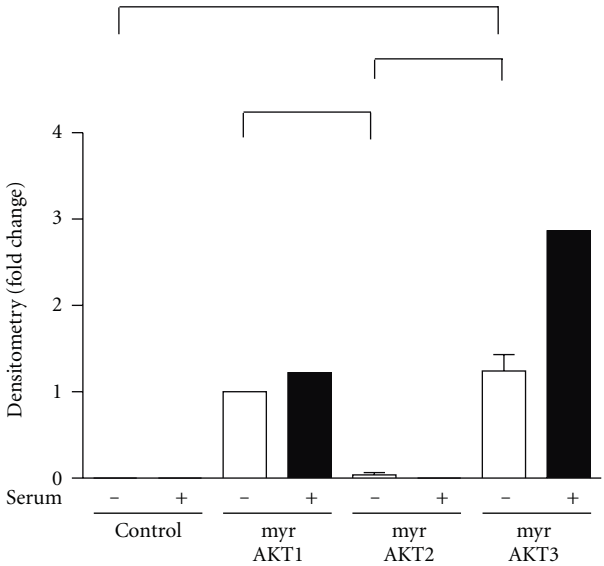

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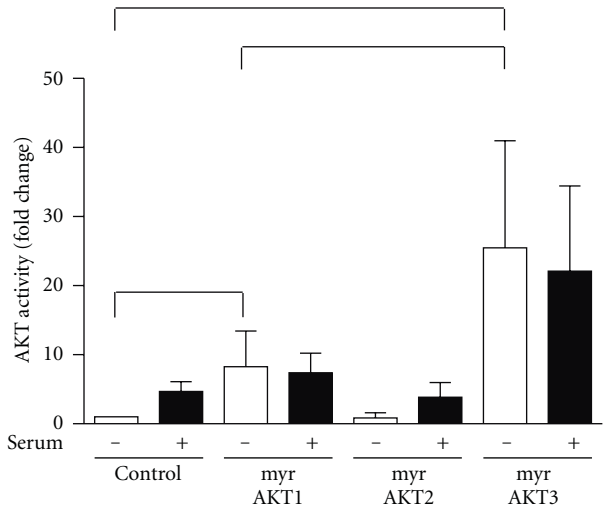


(f)

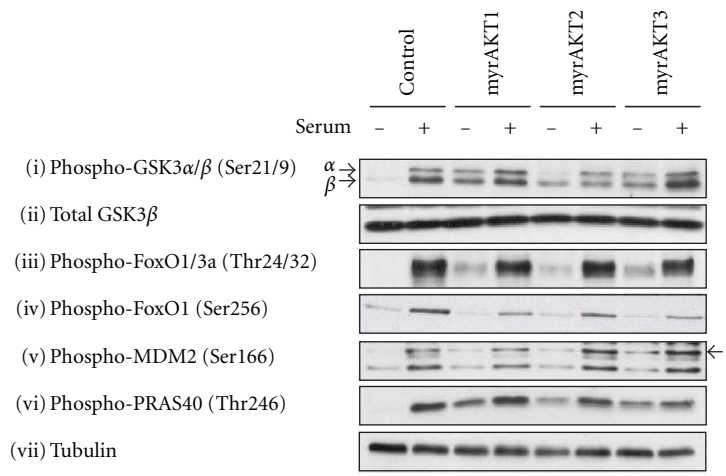
Figure 2: Comparison of AKT isoform-specific activation *in vivo* and activity *in vitro*. HEK293 cells were transfected with the pCDNA3 vector (control) or myrAKT isoforms, serum-starved for 24 hours, then stimulated with 10% serum for 20 minutes. (a) protein lysates (20–50 μg) were separated by SDS-PAGE, transferred onto PVDF membrane, and immunoblotted. Western blots are representative of $n = 1$–5 experiments. Signals were quantified by densitometry using ImageJ 1.42 q (National Institutes of Health, USA), normalised to loading and expressed as fold change over myrAKT1 serum-starved samples. (b) panAKT. $n = 1$ (c) HA-tag. (d) phospho-Ser473. (e) phospho-Thr308. (c–e) Serum-starved samples: $n = 4$, stimulated samples: $n = 1$. Error bars: mean ± SD. (f) protein lysates (20 μg) were incubated with the RPRAATF peptide substrate in the presence of [γ-^{32}P]ATP at 30°C for 20 minutes to determine total AKT activity. Each sample was assayed in duplicate. Levels of AKT activity are represented as fold change over the serum-starved control sample. $n = 2$–6, Graph shows mean ± S.D. Statistical analysis was performed using the paired t-test (GraphPad Prism version 5.0, GraphPad Software, San Diego, Calif, USA). Paired t-test was not calculated between serum-starved control and myrAKT1 for (c and e) as the fold difference was the same for all blots quantified. P values >0.05 are not significant, P values 0.01 to 0.05 (*), P values 0.001 to 0.01 (**), and P values <0.001 (***).

increased GSK3α (Ser21) phosphorylation under serum-starved conditions (Figures 3(a)(i), and 3(b)). However, all three myrAKT isoforms significantly increased GSK3 (Ser9) phosphorylation under serum-starved conditions (Figures 3(a)(i), and 3(c)), suggesting that AKT2 specifically targeted GSK3β over GSK3α despite its apparent extremely low peptide phosphorylating activity. The data presented here suggests that constitutive AKT1 and 3 but not AKT2 activity are sufficient to phosphorylate GSK3α, while all three isoforms are sufficient in phosphorylating GSK3β.

3.2.2. Effect of Enforced AKT Isoform Expression on Survival: FoxO1, FoxO3, and MDM2. One mechanism by which AKT modulates cell survival involves phosphorylation and thus inactivation of the FoxO family of transcription factors which regulate the expression of various genes associated with apoptosis, the cell cycle, and DNA repair [37]. AKT directly phosphorylates FoxO1 at residues Thr24 and Ser256, and FoxO3a at Thr32 [37–40], however, other kinases are also capable of FoxO protein phosphorylation [37–40].

In this study, FoxO1 (Thr24) and FoxO3a (Thr32) were robustly phosphorylated in response to serum (Figure 3(a)(iii)). In serum-starved conditions, the overexpression of all three constitutively active AKT isoforms (Figures 3(a)(iii), and 3(d)) modestly increased FoxO1/3a (Thr24/32) phosphorylation with myrAKT1 and 3 being more potent than myrAKT2. This suggests that all isoforms are sufficient to phosphorylate FoxO1/3a at residues Thr24 and 32, respectively. However, in all three cases this was not additive with serum treatment (Figure 3(a)(iii)) indicating that serum stimulation causes maximal phosphorylation at these sites. Interestingly, none of the constitutively active myrAKT isoforms was sufficient to induce FoxO1 (Ser256) phosphorylation in serum-starved conditions (Figures 3(a)(iv), and 3(e)). Indeed, serum induced phosphorylation of FoxO1 at Ser256 trended lower upon expression of constitutively active AKT isoforms.

AKT also regulates cell survival by phosphorylating murine double minute 2 (MDM2) at residue Ser166, which results in its translocation into the nucleus and binding to p53, a tumour suppressor that acts as a transcription factor to express proapoptotic genes. The p53/MDM2 complex is then sequestered to the cytoplasm for proteasomal degradation [4, 41, 42]. Overexpression of all myrAKT isoforms was sufficient to induce phosphorylation under serum-starved conditions (Figure 3(a)(v), top band indicated by arrow), thus suggesting that all isoforms are sufficient to phosphorylate MDM2 at Ser166. However, this phosphorylation was not additive with serum when compared to the control. The differences in the signal to noise between individual western blots made statistical analysis of MDM2 phosphorylation from multiple gels impractical. However,



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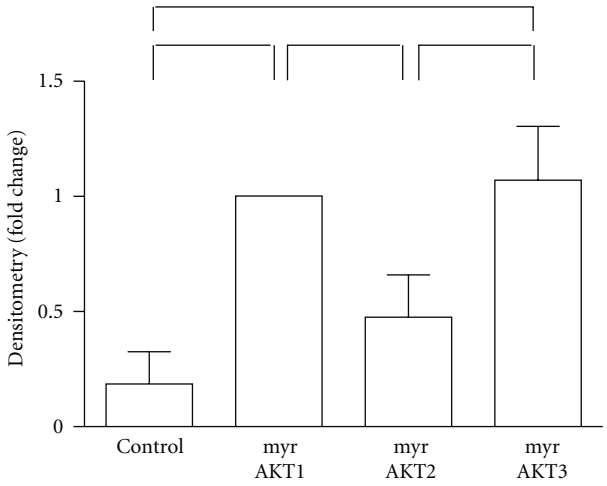

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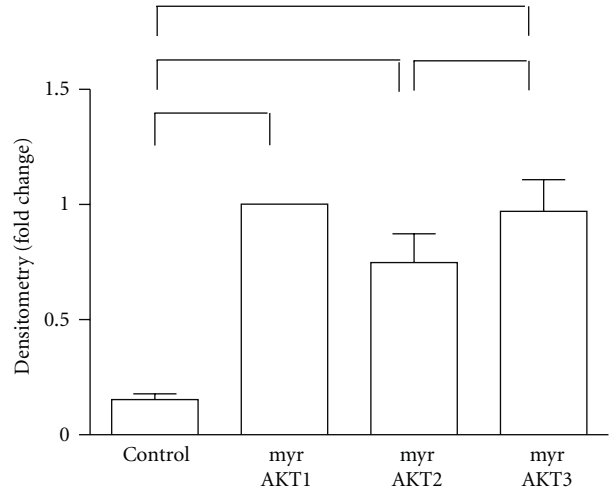

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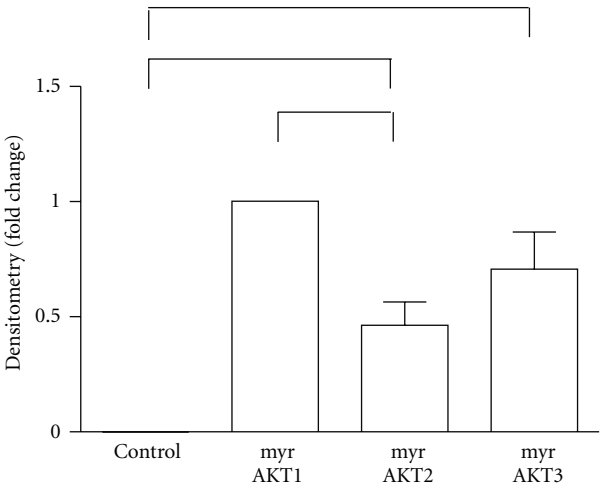

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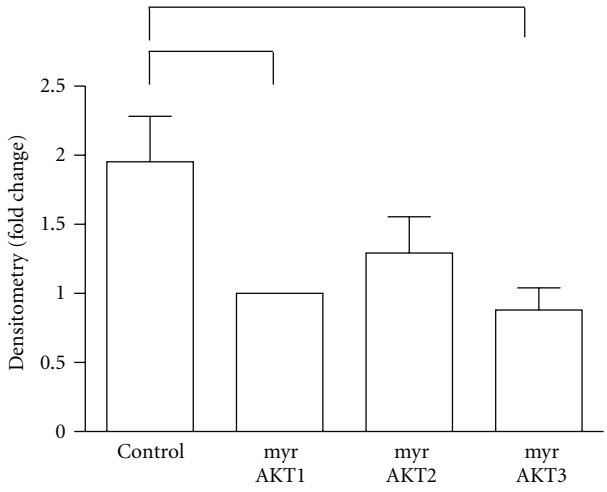

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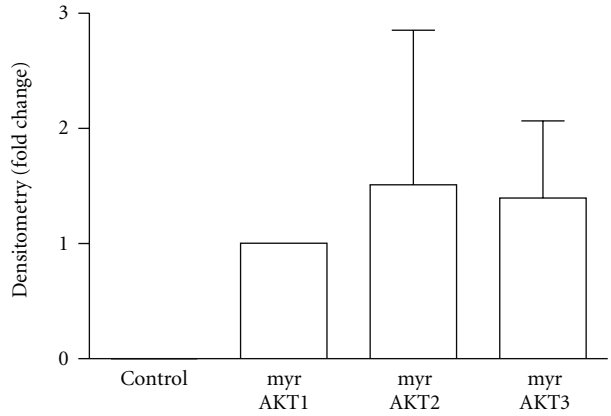

Figure 3: Differential isoform-specific signaling to direct AKT substrates *in vivo*. (a) Protein lysates (20–50 μg) generated from HEK293 cells transfected with the pCDNA3 vector (control), or overexpressing HA-tagged myrAKT isoforms were resolved by SDS-PAGE, transferred onto membrane, and immunoblotted. Western blots are representative of $n = 2$–5 experiments. Signals from serum-starved samples were quantified by densitometry using ImageJ 1.42q (National Institutes of Health, USA), normalised to loading, and expressed as fold change over myrAKT1 serum-starved samples. (b) phospho-GSK3α (Ser21). $n = 5$. Error bars: mean ± SEM. (c) phospho-GSK3β(Ser9). $n = 5$. Error bars: mean ± SEM. (d) phospho-FoxO1/3a (Thr24/32). $n = 5$. Error bars: mean ± SEM. (e) phospho-FoxO1 (Ser256). $N = 5$. Error bars: mean ± SEM. (f) phosph-PRAS40 (Thr246). $n = 2$. Error bars: mean ± SD. Statistical analysis was performed using the paired t-test (GraphPad Prism version 5.0, GraphPad Software, San Diego, Calif, USA). Paired t-test was not calculated between serum-starved control and myrAKT1 for (d) as the fold difference was the same for all blots quantified. P values >0.05 are not significant, P values 0.01 to 0.05 (*), P values 0.001 to 0.01 (**), and P values < 0.001 (***).

as consistent trends were observed, a representative blot is presented.

3.2.3. Effect of Enforced AKT Isoform Expression on Growth: PRAS40. AKT signals to mTORC1 via two converging pathways to regulate cell growth. One pathway involves the proline rich AKT substrate of 40 kDa (PRAS40) and the other the tuberous sclerosis complex 2 (TSC2) [43–45]. Specifically, PRAS40 inhibits Ras homolog enriched brain-(Rheb)-dependent mTORC1 activity by binding to the raptor subunit of mTORC1, and this is relieved upon its phosphorylation at Thr246 by AKT [46]. Overexpression of each of the constitutively active AKT isoforms resulted in robust PRAS40 (Thr246) phosphorylation in serum-starved conditions (Figures 3(a)(vi), and 3(f)). As observed for FoxO1/3a, this phosphorylation was not additive upon serum stimulation, nor was any differential isoform specificity observed.

3.3. Effect of Enforced AKT Signaling down the mTORC1 Pathway. AKT is known to mediate cell growth by signaling through the mTORC1 pathway primarily due to mTORC1 phosphorylation of initiation factor 4E-Binding Protein 1 (4E-BP1) and activation of S6 Kinase 1 (S6K1) that phosphorylates ribosomal protein S6 (rpS6) [47]. mTORC1 also activates rDNA transcription [48]. These signals converge to promote ribosomal biogenesis and function [49]. Thus in this study 4E-BP1 and rpS6 were used as readouts of AKT signaling down the mTORC1-growth signaling pathway (Figure 4).

3.3.1. Effect of Enforced AKT Isoform Expression on 4E-BP1. The function and activity of 4E-BP1 is modulated by phosphorylation at multiple residues [50]. The 4E-BP1 residues Thr37 and Thr46 are priming sites that require phosphorylation by mTORC1 before other key residues are available to kinases such as ERK2 and JNK [50, 51]. In serum-starved conditions overexpression of all myrAKT isoforms modestly increased 4E-BP1 (Thr37/46) phosphorylation when compared to control (Figure 4(a)(i)). Again, this phosphorylation was not additive upon serum stimulation, nor was any differential isoform specificity observed. As for MDM2, the differences in the signal to noise between individual western blots made statistical analysis of 4E-BP1 (Thr37/46) phosphorylation from multiple gels impractical. However, as consistent trends were observed, a representative blot is presented.

3.3.2. Effect of Enforced AKT Isoform Expression on Ribosomal Protein S6. Activation of the mTORC1/S6K1 pathway results in the phosphorylation of rpS6 at Ser235, Ser236, Ser240, and Ser244 [52, 53]. In serum-starved cells, overexpression of all three myrAKT isoforms increased rpS6 Ser235/236 and Ser240/244 phosphorylation when compared to the control (Figures 4(a)(ii)(iii), 4(b) and 4(c)), although this was considerably less than the phosphorylation observed in response to serum stimulation. Like 4E-BP1, no further elevation in rpS6 Ser235/236 and Ser240/244 phosphorylation was observed upon serum stimulation.

In conclusion, these experiments reveal that despite the enforced expression of AKT isoforms with very different

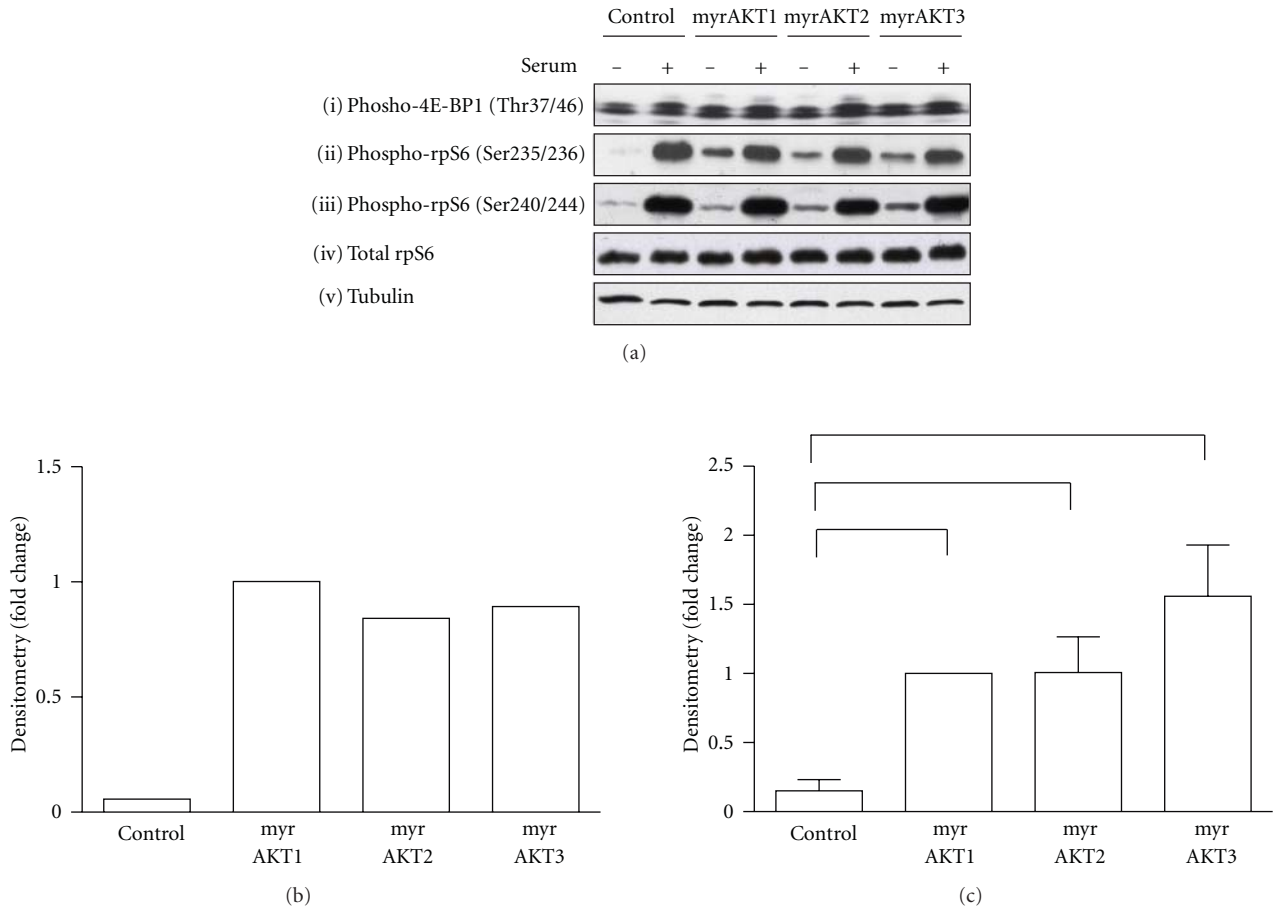

Figure 4: Expression of all three AKT isoforms is sufficient for signaling down the mTORC1 pathway. (a) Protein lysates (20–25 μg) generated from HEK293 cells transfected with the pCDNA3 vector (control), or overexpressing HA-tagged myrAKT isoforms were resolved by SDS-PAGE, transferred onto membrane, and immunoblotted. Western blots are representative of $n = 1 - 5$ experiments. Signals from serum-starved samples were quantified by densitometry and normalised to loading and expressed as fold change over myrAKT1 serum-starved samples. (b) phospho-rpS6 (Ser235/236). $n = 1$. (c) phospho-rpS6 (240/244). $n = 5$. Error bars: mean ± SEM. Statistical analysis was performed using the paired t-test (GraphPad Prism version 5.0, GraphPad Software, San Diego, Calif, USA). P values >0.05 are not significant, P values 0.01 to 0.05 (*), P values 0.001 to 0.01 (**), and P values < 0.001 (***).

intrinsic kinase activities towards defined peptide and protein substrate, the resulting *in vivo* phosphorylation patterns of known substrates is largely isoform independent. Some subtle differences were identified including the lack of phosphorylation of GSK3α by myrAKT2 and its reduced phosphorylation of FoxO1/3a (Thr24/32).

3.4. Loss of Function Approaches to Determine AKT Substrate Specific Phosphorylation. To further assess potential differences in the requirement for specific isoforms for substrate phosphorylation we used siRNA approaches to knockdown the expression of each isoform individually and in combination (Figures 5 and 6). Western blot analysis using isoform-specific antibodies showed that the siRNAs used specifically targeted their respective AKT isoform (Figure 5(a)). Moreover, panAKT western blot analysis indicated that of the three isoforms, the individual knockdown of AKT1 and AKT3 most robustly reduced total AKT expression (Figures 5(a) and 5(b)), thus suggesting that these two isoforms may be more abundantly expressed in HEK293 cells. Moreover, when both AKT1 and AKT3 expression were reduced, an additive reduction in endogenous AKT expression was demonstrated (Figures 5(a) and 5(b)).

The contribution of each AKT isoform to total AKT activity *in vivo* was also analysed by examining the levels of phosphorylation at the two regulatory residues Ser473 and Thr308 (Figures 5(c)–5(e)). It should be noted that combined knockdown of all isoforms did not fully ablate AKT expression or phosphorylation but did reduce the levels of active kinase to a similar level to the AKT inhibitor (AKTi), which we have shown to inhibit all AKT isoforms in HEK293 cells when used at 5 μM (Chan et al. [54]).

As with the overexpression studies we took a targeted approach focusing on AKT substrates involved in key cellular processes: proliferation (GSK3α and GSK3β); survival (FoxO1, FoxO3a and MDM2); growth (PRAS40). However, many direct AKT substrates are also phosphorylated by other kinases; for example, GSK3β and MDM2 are also substrates

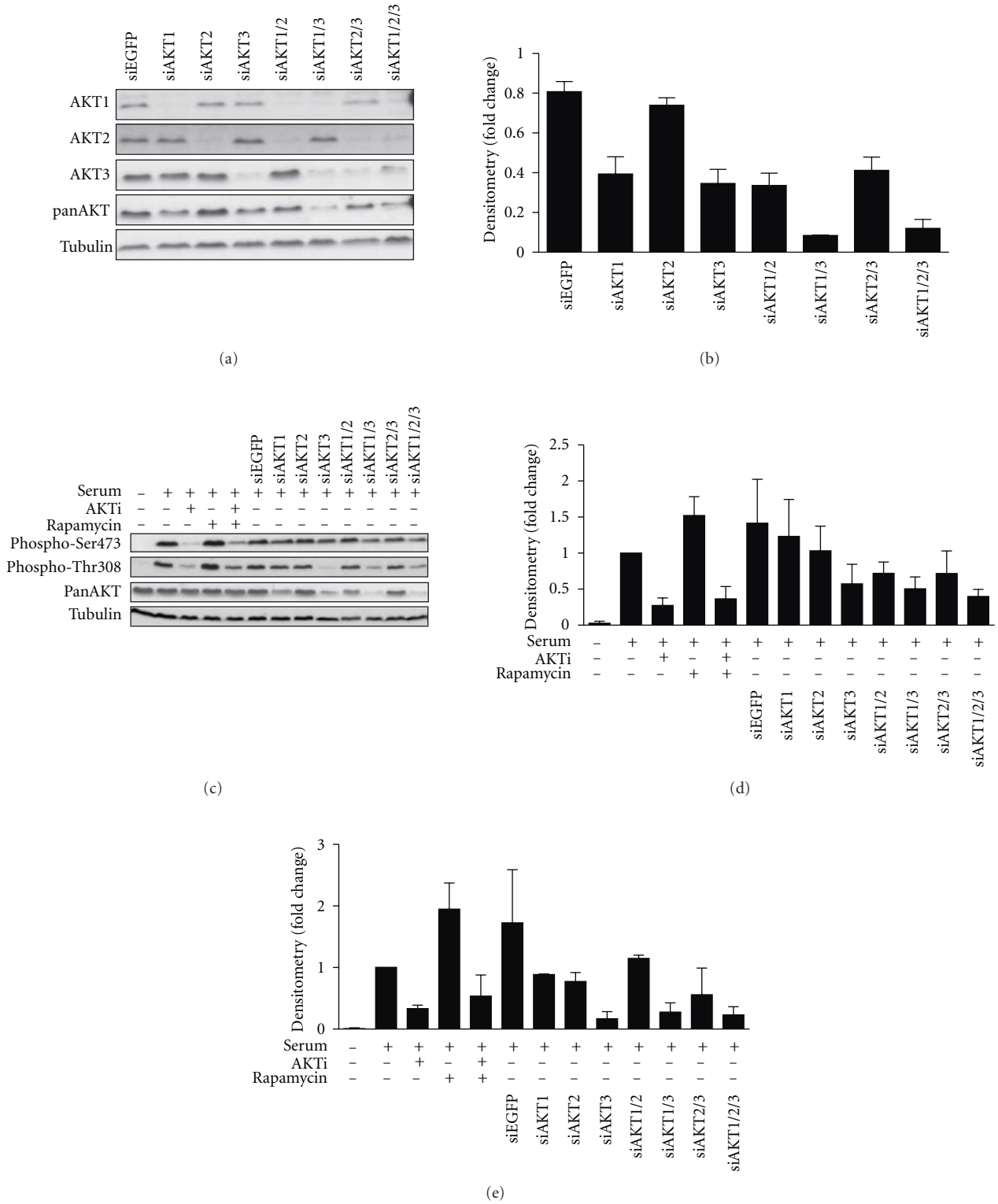

Figure 5: Specific knockdown of endogenous AKT isoforms. HEK293 cells were serum-starved for 24 hours and pretreated with either 5 μM AKTi, 20 nM rapamycin, or both for 30 minutes prior to stimulation with 10% serum for 20 minutes and harvesting into RLB. Endogenous AKT expression was knocked down, either individually or simultaneously, with 25 nM of siRNAs towards specific AKT isoforms and harvested into RLB. siEGFP was used as the control. Protein lysates (20–25 μg) were separated by SDS-PAGE, transferred onto PVDF membrane, and immunoblotted. Western blots are representative of $n = 3$ experiments. Western blot signals were quantified by densitometry, normalised to loading and expressed as fold change over the serum-stimulated control. (a) Specificity of isoform-specific knockdown and their effects on total AKT expression were analysed by immunoblotting with isoform-specific and panAKT antibodies. (b) panAKT. $n = 3$. Error bars: mean ± SEM. (c) Total AKT activation levels were analysed by immunoblotting with phospho-Ser473 and phospho-Thr308 antibodies. (d) Phospho-Ser473. $n = 3$. Error bars: mean ± SEM. (e) Phospho-Thr308. $n = 2$, Error bars: mean ± SD.

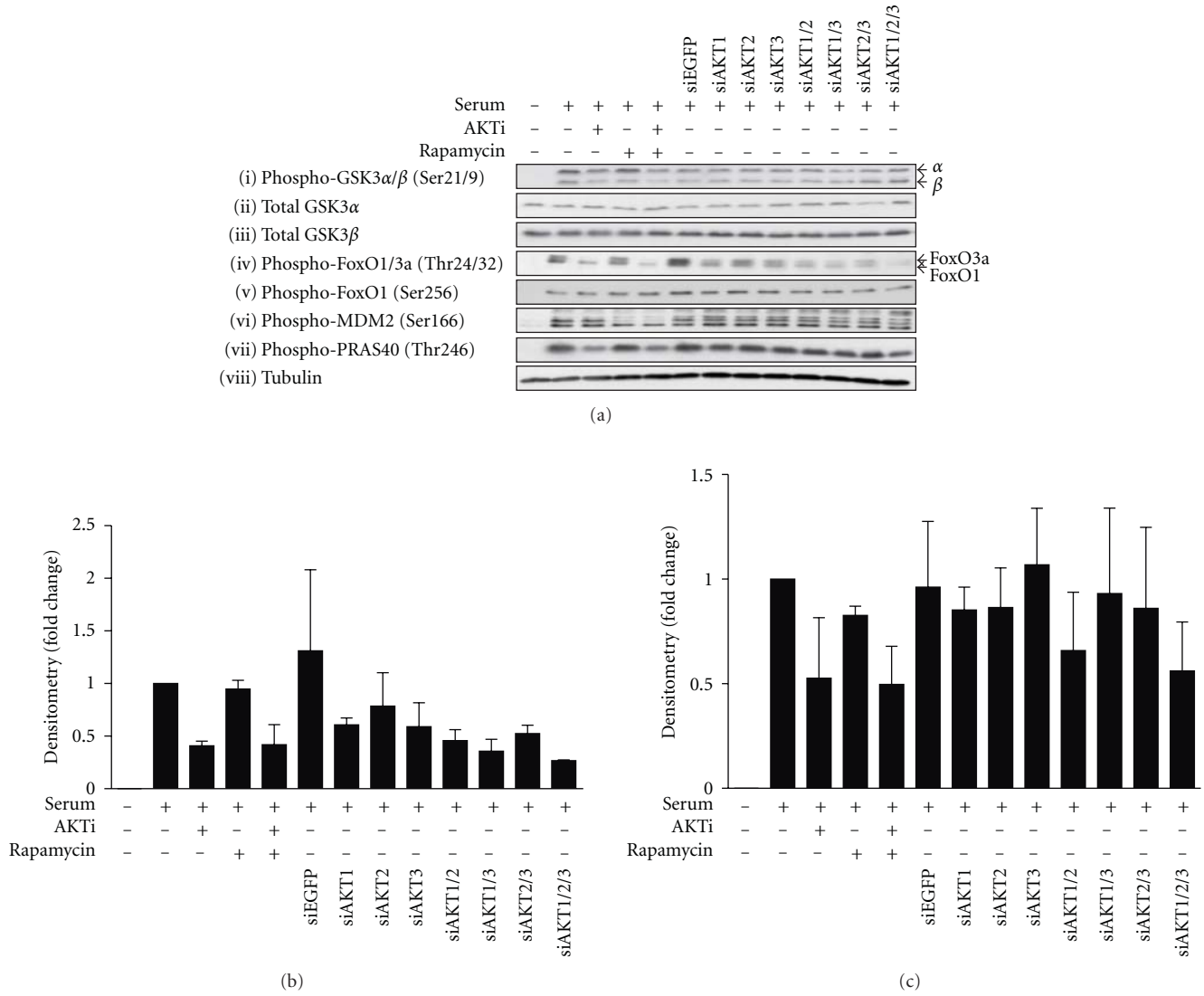

Figure 6: Dependence of AKT isoform-specific expression for signaling down cell proliferation, survival, and growth pathways. (a) HEK293 cells were serum-starved for 24 hours before pretreatment with 5 μM AKTi, 20 nM rapamycin or both for 30 minutes prior to stimulation with 10% serum and harvested into RLB. Expression of endogenous AKT isoforms were knocked down in HEK293 cells, either individually or simultaneously, with 25 nM of siRNAs towards specific AKT isoforms and harvested into RLB. siEGFP was used as the control. Protein lysates (20–25 μg) were separated by SDS-PAGE, transferred onto PVDF membrane, and immunoblotted. Western blots are representative of $n = 3$ experiments. Intensity of western blot signals were quantified by densitometry, normalised to loading, and expressed as fold change over the serum-stimulated control. (b) phospho-FoxO1/3a (Thr24/32). $n = 2$. Error bars: mean ± SD. (c) phospho-PRAS40 (Thr246). $n = 2$. Error bars: mean ± SD.

of S6K1, a downstream kinase of mTORC1 [55, 56]. Thus, to delineate direct AKT substrates from mTORC1-mediated signaling, cells were treated with specific inhibitors of AKT (AKTi) and/or mTORC1 (rapamycin) (Figure 6).

3.4.1. Effect of AKT Isoform Knockdown on Proliferation: GSK3α and GSK3β. Specific reduction in AKT isoform expression individually or in combination did not modulate the level of phosphorylated or total GSK3α (Ser21) or GSK3β (Ser9) (Figure 6(a)(i)–(iii)). In fact, complete inhibition of AKT activity with AKTi only marginally reduced GSK3 phosphorylation (Figure 6(a)(i)) despite the results showing their dependence on AKT expression in the overexpression studies (Figure 3(a)(i)). This may be due to other kinases such as PKA and S6K1 that have been shown to also play an active role in GSK3α (Ser21) and GSK3 (Ser9) phosphorylation [57]. In summary, under the conditions tested, AKT is sufficient but not necessary for phosphorylation of GSK3α (Ser21) and GSK3 (Ser9).

3.4.2. Effect of AKT Isoform Knockdown on Survival: FoxO1, FoxO3, and MDM2. Phosphorylation of FoxO1/3a (Thr24/32) was markedly reduced upon knockdown of each of the three AKT isoforms with AKT1 and 3 being more

potent than AKT2 (Figures 6(a)(iv), and 6(b)). This is consistent with overexpression studies where myrAKT1 and 3 were shown to phosphorylate FoxO1/3a (Thr24/32) more intensely (Figures 3(a)(iii), and 3(d)). Moreover, an additive reduction in FoxO1/3a (Thr24/32) phosphorylation was demonstrated upon the knockdown in expression of all three AKT isoforms (Figures 6(a)(iv), and 6(b)). When combined with the AKT overexpression studies we conclude that all AKT isoforms are both sufficient and necessary for the phosphorylation of FoxO1/3a at Thr24 and Thr32, respectively. In contrast knockdown of all AKT isoforms together had no effect on FoxO1 at Ser256 (Figure 6(a)(v)). This is consistent with the data of Brognard et al. [58] who also failed to observe altered FoxO1 (Ser256) phosphorylation upon the knockdown of AKT isoform-specific expression in H157 cells, a non-small-cell lung cancer cell line. Additionally, treatment with AKTi did not alter FoxO1 phosphorylation at Ser256 (Figure 6(a)(v)). This is consistent with the lack of effect of overexpressing AKT on this substrate (Figures 3(a)(iv), and 3(e)). Taken together, these results suggest that in HEK293 cells, AKT may mediate cell survival by phosphorylating FoxO1/3a at Thr24/32 but not Ser256.

Phosphorylation of MDM2 (Ser166) was not altered upon the individual or compound knockdown of the AKT isoforms (Figure 6(a)(vi)). This suggests that AKT expression is not necessary for MDM2 Ser166 phosphorylation, despite overexpression of AKT being sufficient to increase its phosphorylation (Figure 3(a)(v)). Moreover, MDM2 phosphorylation at Ser166 was not reduced upon treatment with AKTi (Figure 6(a)(vi)). This is in contrast to treatment with the mTORC1 inhibitor rapamycin, which had a dramatic effect on MDM2 (Ser166) phosphorylation (Figure 6(a)(vi)). This suggests that under these conditions, AKT does not regulate MDM2 (Ser166) phosphorylation while mTORC1 does and that mTORC1 must be activated in an AKT-independent manner [59–61].

3.4.3. Effect of AKT Isoform Knockdown on Growth: PRAS40. Phosphorylation of PRAS40 (Thr246) was not significantly modulated by knockdown of any individual AKT isoform (Figures 6(a)(vii), and 6(c)) although there was a subtle reduction when RNAi to all three AKT isoforms were combined. Moreover, AKTi treatment, but not rapamycin, reduced PRAS40 (Thr246) phosphorylation (Figures 6(a)(vii), and 6(c)). In combination with the above data this would suggest that AKT is both sufficient and necessary for PRAS40 (Thr246) phosphorylation; however the AKT isoforms are functionally complementary with respect to this substrate, with knockdown of all giving additive inhibition.

3.4.4. Effect of AKT Isoform Knockdown on mTORC1 Signaling Pathway. Consistent with the modest increase in 4E-BP1 (Thr37/46) phosphorylation induced by all constitutively active AKT isoforms (Figure 4(a)), knockdown of each individual AKT isoform reduced 4E-BP1 phosphorylation as evidenced by disappearance of the lowest mobility species (Figure 7(a)(i), indicated by an arrow). Furthermore, phosphorylation of the Ser235/236 site on rpS6 (Figures 7(a)(ii), and 7(b)) was also reduced upon knockdown of each individual AKT isoform. In contrast, reduction of phosphorylation of Ser240/244 in rpS6 required knockdown of AKT1 and was optimal with all three (Figures 7(a)(iii), and 7(c)) raising the possibility that AKT1 exhibits some nonredundant signaling to rpS6 phosphorylation. Interestingly for both of these substrates all three of these phosphorylation sites were robustly reduced with either AKTi treatment or rapamycin (Figure 7(a)(i–iii)), consistent with the importance of AKT in the regulation of mTORC1 function which functions upstream of both 4E-BP1 and rpS6.

3.5. Differences in Signaling between AKT Isoforms Are Subtle In Vivo. These data demonstrate dramatic differences in peptide kinetics for the three AKT isoforms, which were less marked when using a protein substrate *in vitro* (Figures 1(c)–1(e)). However regulation of the level of phosphorylation for some known *in vivo* substrates, whether by overexpression or specific knockdown of individual AKT isoforms, was extremely subtle. Only GSK3α (Ser21) and FoxO1/3a (Thr24/32) exhibited clear differential regulation by the AKT isoforms. Thus we took a third approach, to identify potential AKT isoform-specific substrates by performing an unbiased screen on cells overexpressing myrAKT1 or myrAKT3 and utilizing 2DGE combined with western blot analysis with the phospho-AKT substrate (PAS) antibody that detects phosphorylation at AKT-specific motifs [62]. Consistent with our studies of validated substrates, the patterns of phosphorylation of more than 30 AKT regulated PAS signals were similar (Figure 8). However, some differences were also observed. A group of proteins were found to be more efficiently phosphorylated by myrAKT1 compared to myrAKT3 (Figures 8(c) and 8(d), white circle) while 5 phosphoproteins 4 of which were seemingly related, were more efficiently phosphorylated by myrAKT3 than myrAKT1 (Figures 8(c) and 8(d), black circle). Of the 5 spots more efficiently phosphorylated by myrAKT3, four overlapped with Coomassie G-250 stained spots (Supplementary Figure 2(c)), and these were excised, digested, and subjected to mass spectrometry analysis. All four spots were identified as eukaryotic translation elongation factor 2 (eEF2, Supplementary Figure 3). The potential and significance of AKT mediated regulation of eEF2 remains to be elucidated. Thus, in general the patterns of phosphorylation observed using the 2DGE approach were similar for the two AKT isoforms, although some subtle isoform-specific differences were observed, consistent with the findings obtained above for GSK3 and FoxO1/3a phosphorylation (Figure 3). Thus, while relative expression levels are likely to underlie the major isoform-specific signaling observed at least in HEK293 cells, differences in intrinsic enzyme activity may contribute to subtle modifications of the differential effects of AKT on the regulation of a range of cellular processes.

4. Discussion

AKT consists of a family of three homologous isoforms, AKT1, 2, and 3, whose role in maintaining cellular homeostasis is thought to be pivotal in regulating processes

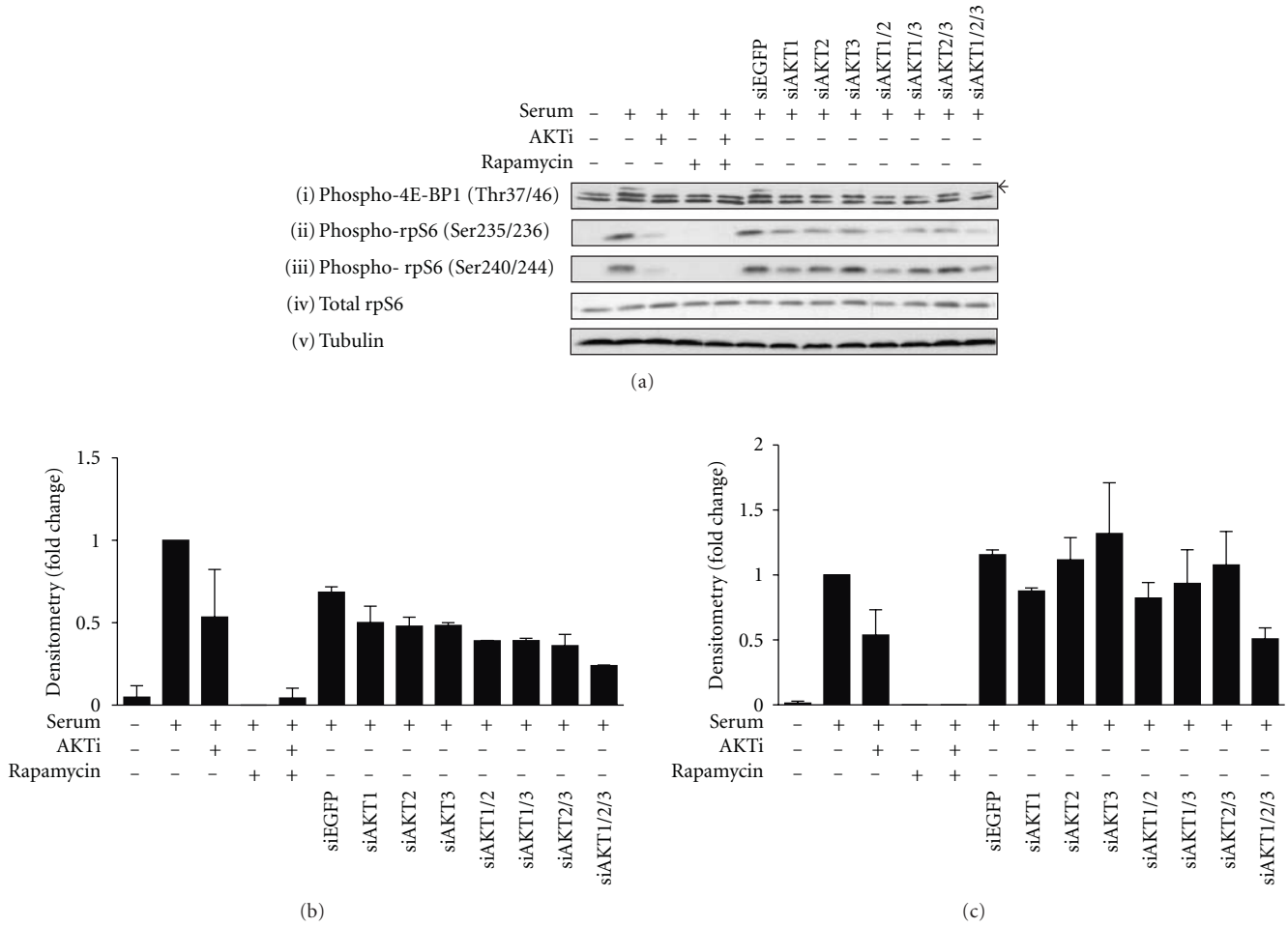

Figure 7: Effect of AKT expression on activation of the mTORC1 pathway. (a) HEK293 cells were serum-starved for 24 hours before treatment with 5 μM AKTi, 20 nM rapamycin or both for 30 minutes prior to stimulation with 10% FBS and harvested into RLB. Expression of endogenous AKT isoforms were knocked down in HEK293 cells, either individually or simultaneously, with 25 nM of siRNAs towards specific AKT isoforms, and harvested into RLB. siEGFP was used as the control. Protein lysates (20–25 μg) were separated by SDS-PAGE, transferred onto PVDF membrane, and immunoblotted. The arrow indicates the hyperphosphorylated band of phospho-4E-BP1. Western blots are representative of $n = 3$ experiments. Intensity of western blot signals was quantified by densitometry, normalised to loading and expressed as fold change over the serum-stimulated control. (b) Phospho-rpS6 (Ser235/236). $n = 2$. Error bars: mean ± SD. (c) Phospho-rpS6 (Ser240/244). $n = 3$. Error bars: mean ± SEM.

such as cell survival, growth, proliferation, angiogenesis, and metabolism [3]. While much is known about the complex regulation of this pathway, many questions remain unanswered. Though the substrates that mediate some of its pleiotropic effects have been characterised, many critical substrates remain to be identified [3]. Furthermore, studies involving single and double knockout mice suggest that the AKT isoforms may differentially signal to produce distinct phenotypes [11–17, 63–65]. The basis of this differential signaling and its impact on determining the roles of the individual isoforms in regulating specific cellular functions remain unclear.

Recently, potential AKT isoform-specific substrates have come to light, providing one clear mechanism for differential signaling; using loss-of-function studies, Brognard et al. [58] showed that TSC2 (Ser939 and Thr1462) phosphorylation was dependent on AKT1 and AKT2, but not AKT3, expression. Moreover, they showed that p27 (Thr157) phosphorylation was specifically dependent on AKT3 expression. Additionally GSK3α (Ser21) and MDM2 (Ser166) phosphorylation was shown to be exclusively dependent on AKT2 expression.

Here we have addressed the question of whether isoform-specific signaling was due to differences in the intrinsic activity of the isoforms. Previous reports have shown that AKT3 is the most active isoform towards a range of peptide substrates [21, 24, 25]. We confirmed that AKT3 had the highest specific activity compared to AKT1 and AKT2, phosphorylating the RPRAATF synthetic peptide with~47-times the specific activity of AKT1. Importantly, we showed that this hierarchy of activity was conserved for a protein substrate (rpS7), but the difference in activity was reduced to 5-fold. These data indicated that the differences in intrinsic kinase activity towards protein substrates may be more

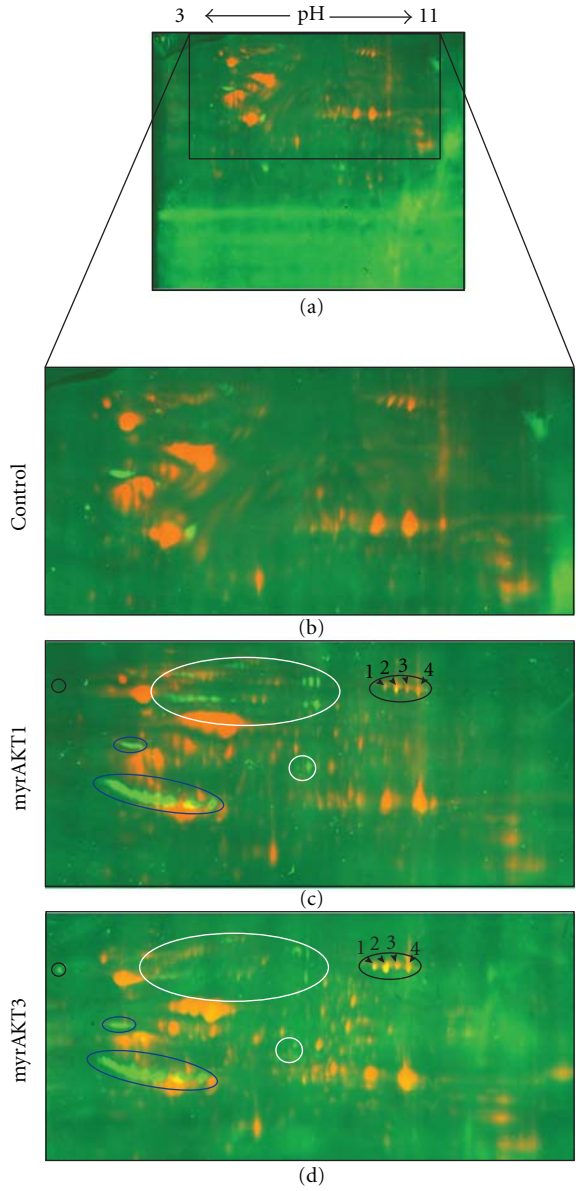

Figure 8: Identification of eEF2 as a potential AKT substrate. HEK293 cells transfected with the pCDNA3 vector (control) or expressing similar levels of myrAKT1 or myrAKT3 were serum-starved for 24 hours prior to harvesting into RLB. Samples were processed in duplicate. Cy2 labelled protein samples (250 μg) were loaded onto 18 cm broad range IPG strips with a nonlinear pH range of 3–11, focused and resolved by SDS-PAGE. After 2DGE, gels were transferred onto Hybond-LFP membrane and then immunoblotted with the PAS antibody and Cy5-conjugated secondary antibody. Membranes were scanned using the Typhoon trio9100 for both Cy2 and Cy5 signals. Cy2 and Cy5 signals were overlayed using ImageQuant (GE Healthcare). Cy2 (total protein) signals are represented in red. Cy5 (PAS) signals are represented in green. Overlayed signals are represented in yellow. (a) control. (b–d) enlarged region of membrane containing control, myrAKT1 or myrAKT3 samples, respectively. Proteins more efficiently phosphorylated by myrAKT1 are circled in white, by myrAKT3 are circled in black, and with equal efficiencies for both are circled in blue. The four protein spots (spots 1–4) were excised from the myrAKT1 Coomassie R-250 stained gel (Supplementary Figure 2) and identified as eEF2 by mass spectrometry analysis (Supplementary Figure 3). $n = 1$.

modest than first thought and potentially of less impact *in vivo*. Indeed, this concept is reinforced by the observation that AKT3 had a 5-fold lower affinity than AKT1 towards the rpS7 protein meaning at limiting substrate concentrations, the activity of the isoforms may be very similar. Similar differences in the K_m for peptide phosphorylation were observed previously [25]. Alternatively, it is possible that colocalization of AKT3 with important substrates will facilitate local concentrations required for maximum AKT3 activity and provide a measure for isoform-specific signaling not evident in the cytoplasm. Such sites may include the translational apparatus or the nucleolus where ribosomal proteins, such as rpS7 and ribosomal RNA are assembled into ribosomes. Supporting this hypothesis is the recent finding that AKT3 localises to the nucleus in a range of cell lines including HEK293 [20].

To further examine the potential for isoform-specific phosphorylation of substrates *in vivo* we determined the effect of enforced expression of constitutively active isoforms on the phosphorylation of key validated substrates, GSK3α and GSK3β, FoxO1/3a, MDM2, and PRAS40. These observations were complemented by loss-of-function studies using isoform-specific siRNA reagents. Regulation of the level of phosphorylation of these *in vivo* substrates, whether by overexpression or specific knockdown of individual AKT isoforms, was extremely subtle, indicating that relative expression levels are likely to underlie the major isoform-specific signaling observed at least in HEK293 cells. Expression of each AKT isoform contributed to the phosphorylation of FoxO1 at residue Thr24, but not at Ser256 (Figure 6(a)(iv)(v), and 3(d) and 3(e)) consistent with previous findings in H157, a non-small-cell lung cancer cell line [58]. In addition, we showed isoform-specific differences in the phosphorylation of FoxO1/3a (Thr24/32). Overexpression of myrAKT1 and 3 resulted in a greater level of FoxO1/3a (Thr24/32) phosphorylation when compared to myrAKT2 (Figure 3(a)(iii), and 3(d)). Consistent with these findings, the knockdown of AKT1 and AKT3 expression individually reduced the level of FoxO1/3a (Thr24/32) to a greater extent than AKT2 (Figure 6(a)(iv), and 6(b)). This indicates that the isoforms might vary in the strength or efficiency to phosphorylate FoxO1/3a (Thr24/32), thus resulting in different abilities to regulate cell survival and proliferation.

Taken together, these data indicate that despite exhibiting very different intrinsic enzyme activity, *in vivo* phosphorylation patterns induced by the activation of specific AKT isoforms are very similar when expressed at similar levels in a given cell type. These findings imply that differential expression, activation, or localization of the isoforms may play more dominant roles in determining isoform-specific functions. However, differences in intrinsic enzyme activity may contribute to subtle modifications of the differential effects of AKT on the regulation of a range of cellular processes, including glucose metabolism and cell proliferation (via GSK3) and cell survival (via FoxO1/3a). It is important to note that the basis of isoform-specific signaling is likely to vary depending on the type of cell or tissue. Certainly, recent studies suggest that the substrates targeted by different AKT

isoforms can vary depending on cellular and tissue context [12].

AKT isoforms have been shown to localise to different subcellular compartments in a cell-line-specific manner as described above [19, 20]. It remains possible that overexpression of individual isoforms may subvert signal specificity by changing/overwhelming the specificity of AKT subcellular localization. Moreover, the AKT isoforms are expressed in various tissues at different levels. AKT1 is ubiquitously expressed, while AKT2 is predominantly expressed in brown fat and the heart [16], correlating with the role of AKT2 in diabetes and glucose metabolism. AKT3 was most abundantly expressed in the brain [16] correlating with its role in attaining normal brain size. Furthermore, there is growing evidence that the differential expression of specific AKT isoforms is associated with individual tumour types. AKT1 activity is frequently elevated in breast and prostate cancers [66] while AKT2 has been shown to be upregulated in pancreatic and ovarian carcinomas [67, 68]. We have shown that AKT3 is upregulated in 20% of ovarian cancers [25]. Thus, it is also possible that, while our results reveal only subtle differences in isoform-specific signaling in epithelial cells, this may vary in other cell types. Further experiments will be required to explore this possibility using a panel of primary human cell types.

Conflict of Interests

The authors declare that there is no conflict of interests.

Abbreviations

4E-BP1:	4E-binding protein 1
FoxO:	Forkhead box O
eEF2:	Eukaryotic translation elongation factor 2
HEK293:	Human embryonic kidney cell line
MDM2:	Murine double minute 2
MS:	Mass spectrometry
mTOR:	Mammalian target of rapamycin
mTORC1:	mTOR Complex 1
mTORC2:	mTOR Complex 2
myr:	Myristoylated
PAS:	Phospho-AKT Substrate
PDK1:	Phosphoinositide dependent kinase 1
PRAS40:	Proline-rich AKT substrate of 40 kDa
rpS6:	Ribosomal protein S6
rpS7:	Ribosomal protein S7
S6K1:	S6 Kinase 1
siRNAs:	Small interfering ribonucleic acids.

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Review Article

Determinants for Substrate Specificity of Protein Phosphatase 2A

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Protein phosphatase 2A- (PP2A-) catalyzed dephosphorylation of target substrate proteins is widespread and critical for cellular function. PP2A is predominantly found as a heterotrimeric complex of a catalytic subunit (C), a scaffolding subunit (A), and one member of 4 families of regulatory subunits (B). Substrate specificity of the holoenzyme complex is determined by the subcellular locale the complex is confined to, selective incorporation of the B subunit, interactions with endogenous inhibitory proteins, and specific intermolecular interactions between PP2A and target substrates. Here, we discuss recent studies that have advanced our understanding of the molecular determinants for PP2A substrate specificity.

1. Introduction

Cellular adaptation requires biochemical processes including post-translational mechanisms to modify existing proteins. Catalyzed by opposing kinases and phosphatases, reversible phosphorylation of serine, threonine, and tyrosine residues is now appreciated as a fundamental regulatory mechanism with the majority of phosphorylation (>99%) occurring on serine and threonine residues [1, 2]. Due to their untapped therapeutic potential, protein phosphatases have been identified as promising targets for xenobiotic manipulation through rational drug design (reviewed in [3–6]). In particular, the ubiquitously expressed protein phosphatase 2A (PP2A) has been proposed as a target for the treatment of a number of pathologies ranging from neurodegenerative diseases such as Alzheimer disease to a variety of neoplasias [7–9].

Compared to other members of the phosphoprotein phosphatase (PPP) superfamily of serine/threonine phosphatases, a detailed understanding of the mechanism by which PP2A recognizes substrates and mediates site-specific dephosphorylation remains to be developed. Sequence and structural homology of the catalytic subunits of PPP family members has revealed a conserved catalytic mechanism in which a divalent metal cation activates a water molecule to hydrolyze phospho-serine/threonine without the formation of a phosphoenzyme intermediate (reviewed in [10–12]). Despite a shared catalytic mechanism, substrate specificity within the PPP family is mediated by distinct mechanisms of substrate recognition. For example, the PPP calcineurin (also known as protein phosphatase 2B) has been shown to interact with two consensus sequences, PxIxIT and LxVP, found on nonsubstrate-interacting proteins and target substrates (reviewed in [11, 13]). For protein phosphatase-1 (PP1), substrate specificity is conferred by incorporation of PP1-interacting proteins via a conserved docking motif with a general consensus sequence of RVxF (reviewed in [11, 12]). At present, consensus sequences in PP2A substrates have not been identified. This review will focus on our emerging understanding of PP2A substrate specificity, which appears to involve additive effects of multiple discrete interactions.

PP2A is a highly conserved serine/threonine phosphatase which, depending on the tissue of origin and cell type, may account for up to 1% of cellular protein and the majority of serine/threonine phosphatase activity [14]. The physiological functions of PP2A have been implicated in all facets of cellular existence (reviewed in [15]). Further, PP2A functions as a critical tumor suppressor whose interruption leads to proliferative diseases [14]. The heterotrimeric holoenzyme is composed of a catalytic subunit (C) a scaffold subunit (A) and one member of four families of regulatory subunits (B) (Figure 1). The diversity of PP2A heterotrimers is achieved through expression of two C subunits, two A subunits and approximately fifteen B subunits in vertebrates. The B

subunits are derived from four diverse gene families (B, B′, B″, and B‴) that have little sequence similarity between families but maintain high sequence similarity within families. The B family (B55, PR55, PPP2R2) of regulatory subunits consists of four genes (α, β, γ, δ), the B′-family (B56, PR61, PPP2R5) is comprised of five isoforms (α, β, γ, δ, ε), the B″ family (PR72, PPP2R3) includes three isoforms (α/PR72/130, β/PR59, γ/PR48), and the B‴ family (PR93/PR110) is made up of three proteins (SG2NA, striatin, and mMOB1). There is some controversy as to whether the B‴ family members, most notably SG2NA, are bona fide PP2A regulatory subunits that always associate with the AC dimer or whether they are merely regulated by association with the PP2A dimer. Given the large number of PP2A subunits, it is thought that each cell expresses a dozen or more distinct holoenzyme complexes which act on a diverse array of substrates. PP2A holoenzyme diversity has been the subject of several excellent papers [14, 15, 18].

Like PP1, regulatory subunit incorporation is thought to dictate the substrate specificity of the PP2A complex, however, only recently have molecular studies begun to develop insight into the mechanism by which the regulatory subunit acts [15]. The results from recent studies suggests a multitiered mechanism wherein PP2A substrate specificity arises from (1) subcellular localization of PP2A defined by the B subunit, (2) selective holoenzyme assembly by posttranslational modification, (3) interaction with specific endogenous inhibitors, (4) interactions between the B subunit and phosphosubstrates at sites distant from the active site, and (5) B-subunit residues which infiltrate the catalytic cleft of the C subunit. This paper will provide a summary of these studies and how the understanding of the determinants of PP2A substrate specificity has advanced.

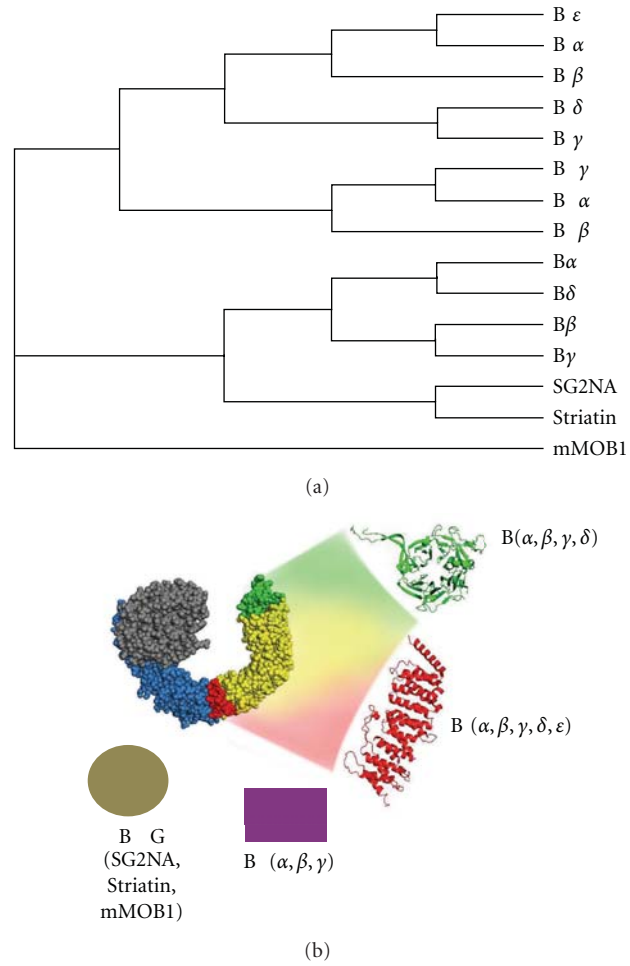

Figure 1: Diversity of the B-regulatory subunits and the structure of the PP2A holoenzyme complex. (a) Cladogram of the human PP2A B-regulatory subunits generated using ClustalW [16] and shown with Dendroscope [17]. (b) Structure of the dimeric A scaffold subunit and C catalytic subunit (gray). The shaded portions of the A scaffold subunit indicate the known docking sites for the B regulatory subunits; green (HEAT repeat 1) for the B family of regulatory subunits, red (HEAT repeat 8) for the B′ family of regulatory subunits, and yellow (HEAT repeat 2–7) for the docking site area common for the B and B′ families. Cartoon structure of the B (green ribbon), B′ (red ribbon), B″ (purple rectangle), and B‴ (tan circle) families of regulatory subunits. Cartoon structures were generated using the Protein Data Bank accession codes 3DW8 and 2NPP.

2. Mechanisms of Substrate Specificity

2.1. Subcellular Localization of the Holoenzyme Complex. The heterotrimeric holoenzyme is targeted to discrete subcellular locales dictated in part by which B-regulatory subunit is incorporated. The localization imparted by the B-regulatory subunit dictates the spatial sphere of influence of the holoenzyme complex for potential substrates. This mechanism of targeting PP2A activity is highlighted by extensive studies of the B family of regulatory subunits. For instance, the B family regulatory subunits target the holoenzyme to different cellular compartments in the brain [19]. Specifically, Bα and Bβ are primarily cytosolic where as the Bγ-regulatory subunit associates with a detergent-resistant protein fraction consistent with an interaction at the cytoskeleton [19].

Similar diversity has been observed in the B′ family of regulatory subunits. A C-terminal nuclear export signal common to B′α, B′β, and B′ε which, when these regulatory subunits are incorporated into the PP2A holoenzyme, results in cytoplasmic localization of the heterotrimer[20]. B′δ and B′γ isoforms lack a similar sequence and are found primarily in the nucleus [21]. In cardiomyocytes, B′α interacts with the protein ankyrin-B through its C-terminus which leads to localization at the cardiac M-line [22]. B′γ on the other hand has been shown to target the holoenzyme complex to subnuclear structures in cardiomyocytes where PP2A/B′γ may regulate gene expression [23]. Loss of proper subcellular targeting of PP2A has been implicated in the biogenesis and aggressive phenotype of neoplastic growths. Specifically, a truncated form of B′γ (ΔB′γ), has been isolated from a melanoma cell line wherein the PP2A/ΔB′γ complex is targeted to the trans-golgi network, blunts p53 responsiveness, contributes to genetic instability and increases metastatic motility [24–27].

Further complexity arises from differing localization imparted by alternative splicing of regulatory subunit genes.

This was first observed in a member of the B family, Bβ. Two neuron-specific isoforms of Bβ, Bβ1, and Bβ2, are generated by the use of an alternative 5′ exon which results in the production of a divergent N-terminal extension on Bβ2 [28, 29]. The N-terminal extension of Bβ2 directs the holoenzyme complex to the outer mitochondrial membrane (OMM) by targeting the mitochondrial translocase complex and forming an abortive complex resistant to import into the mitochondrial matrix [28, 30]. The OMM-directed PP2A/Bβ2 complex promotes fragmentation of the mitochondria reticulum and increases cell susceptibility to proapoptotic insults through an unknown mechanism [31]. Alternative splicing of B-regulatory subunits directing subcellular localization of the PP2A holoenzyme has also been observed in both the B′ and B″ families. Two B′ε isoforms differ by the inclusion or exclusion an N-terminal nuclear localization signal leading to isoform-specific nuclear or cytoplasmic localization [32]. The murine-specific B″ family member, B″δ, is also subject to alternative splicing leading to isoform-specific nuclear or cytoplasmic localization through an as yet unidentified mechanism [33].

Localization is also imparted by interactions between the PP2A holoenzyme complex and other effectors. For example, the interaction between PP2A/B′ and shugoshin during meiosis is crucial for spatial and temporal regulation of sister chromatid disjunction. During meiosis, the cohesin complexes, which link the arms of bivalent chromosomes and the centromeres of sister chromatids, must be released in a stepwise fashion by the protease separase; the cohesin complex is firstly hydrolyzed along the arms of the bivalent chromosomes for completion of anaphase I and secondly at the centromeres of sister chromatids for completion of anaphase II. During anaphase I, the centromeric cohesin complex is protected from separase-dependent proteolysis by shugoshin [34, 35]. Shugoshin recruits PP2A/B′ to the centromere which likely results in dephosphorylation of the cohesin complex leading to protection of the cohesin complex from separase-dependent proteolysis [36, 37]. Through cocrystallization, Xu and colleagues, have revealed that dimeric human shugoshin 1 interacts with PP2A/B′γ through a coiled-coil region across a broad composite surface of the C and B′γ subunits [38]. Other PP2A-effector interactions have recently been reviewed elsewhere [14].

2.2. Selective Holoenzyme Assembly and Activation. Incorporation of specific regulatory subunits is influenced by reversible posttranslational modification of the C subunit. Many groups have shown that the C-terminus of the C subunit is modified through phosphorylation and methylation on Y307 and L309, respectively [39–46]. Phosphorylation of Y307 is catalyzed by src kinase and is likely opposed by PP2A-catalyzed autodephosphorylation of this phosphotyrosine [46]. Phosphorylation of Y307 selectively inhibits recruitment of the B family and some B′ family members to the dimeric AC complex whereas B″ recruitment is not effected [44].

Methylation of the C subunit at the C-terminal L309 is catalyzed by the protein phosphatase methyltransferase (PPM1) and is opposed by the phosphatase methylesterase (PME-1) [47–51]. Reversible methylation of PP2A is absolutely critical as knocking-out PME-1 in mice changes the phosphoproteome and results in early perinatal lethality [52]. Further, methylation of the C subunit is a dynamic process which plays a role in cellular response to acute stimuli [53]. The recruitment of the B subunit to the AC dimer has been postulated to require methylation of the C subunit for some of the B-subunit families [39]. However, conflicting results have been reported that may reflect differences in experimental design and will be discussed further. Studies wherein PP2A/B is isolated from intact cells have revealed that methylation of the C subunit at L309 is required for incorporation of the B family of regulatory subunits into the holoenzyme complex [39, 40, 42, 44, 54, 55]. Conversely, in vitro assembly of the PP2A/B holoenzyme complex does not require methylation of the C subunit for incorporation of the B family of regulatory subunits [56, 57]. Similarly controversial, the requirement for C subunit L309 methylation was observed to be study-specific in in vitro PP2A/B′ timer formation [58, 59]. Methylation was dispensable for isolation of the PP2A/B′ holoenzyme complex from intact cells [44]. The role of methylation of the C subunit in recruitment of B″ and B‴ families of regulatory subunits is less controversial with methylation of the C subunit being dispensable [44, 54]. For more information, the reader is directed to an excellent recent paper [60].

Posttranslational modifications which influence formation of the PP2A holoenzyme complex also occur on the B subunit. PP2A/B′ negatively regulates the ERK MAP kinase signal transduction pathway [61]. Through formation of a ternary complex of the early response gene product *IEX-1*, B′γ1, and ERK, ERK mediates its own disinhibition by phosphorylation of B′γ1 on S327 leading to B′γ1 disassociation from the PP2A holoenzyme [62]. Since S327 is conserved among B′-subunit family members, it is likely that other B′ subunits are regulated similarly. Additionally, Bα is likely phosphorylated on S167 to disrupt the Bα subunit from the AC dimer in early mitotic stages. However, PP2A/Bα activity is necessary to resolve the mitotic spindles and conclude mitosis; therefore, autodephosphorylation may occur on Bα to allow efficient PP2A/Bα heterotrimer formation and cell cycle progression [63]. Thus, phosphorylation of the B-regulatory subunits also influences holoenzyme assembly and, therefore, substrate specificity.

Phosphorylation of the B subunit of heterotrimeric PP2A also potentiates the catalytic activity of the holoenzyme complex. In response to activation of D1 dopamine receptors on striatal neurons, cAMP-dependent protein kinase A (PKA) phosphorylates B′δ at S566 increasing activity of the PP2A/ B′δ holoenzyme towards dopamine- and cAMP-regulated neuronal phosphoprotein (*DARPP-32*) [64, 65]. Dephosphorylation of T75 on *DARPP-32* by PP2A/B′δ disinhibits PKA-mediated phosphorylation of *DARPP-32* at T34 which converts *DARPP-32* into a potent PP1 inhibitor leading to changes in neuronal signaling. This circuit acts to attenuate phospho-T75 inhibition of T34 phosphorylation of *DARPP-32*. This circuit has been shown to be differentially regulated by psychomotor stimulants and antipsychotics acting on different striatal neuron subpopulations [66].

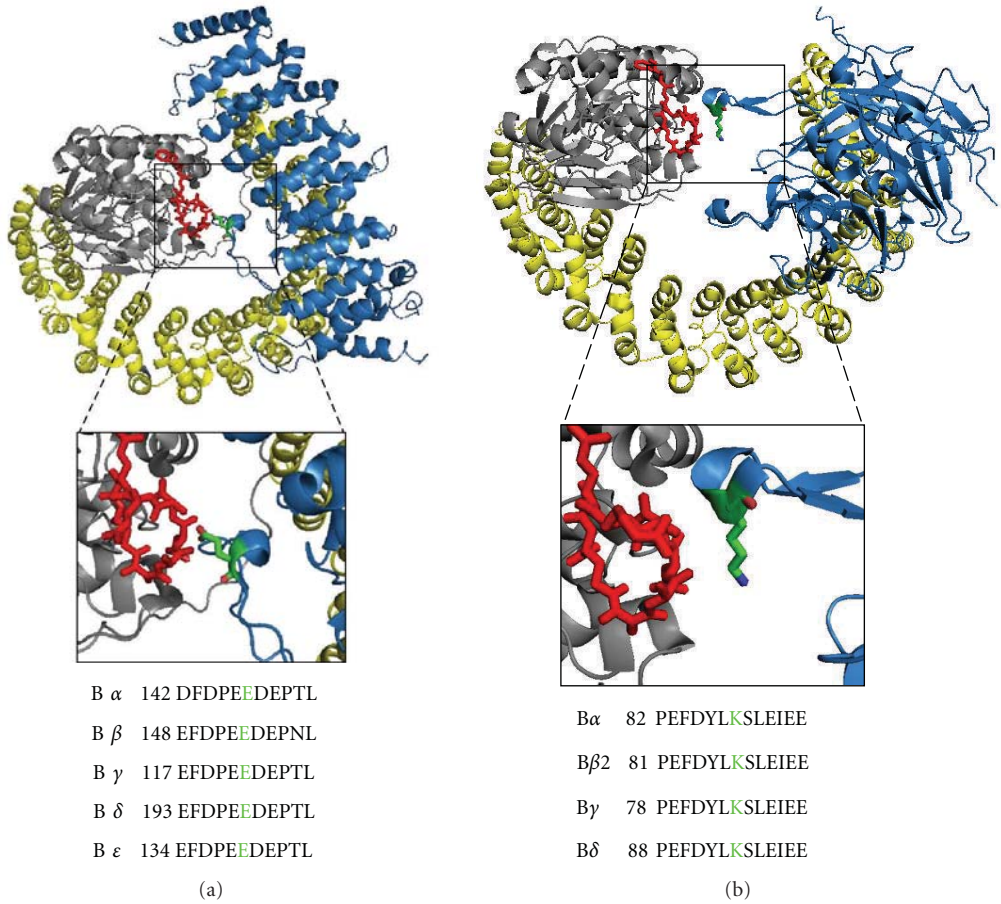

Figure 2: A portion of the B′-and B-regulatory subunits contacts the active site of the C-catalytic subunit. Ribbon diagrams showing a "top-down" view of two heterotrimeric holoenzyme PP2A complexes. The subunits of the holoenzyme complexes are color-coded with the catalytic C subunit in gray, the scaffold A subunit in yellow, and B subunits in blue. The PP2A inhibitor microcystin-LR is shown near the active site as a red stick figure. (a), Structure of the PP2A/B′ γ holoenzyme (PDB 2NPP); inset highlights the infiltration of the B′ γ-subunit residue, E122 (green), into the catalytic core *above*. *Below*, sequence alignment of the B′ family of regulatory subunits with the conserved glutamate residue (green). (b), Structure of the PP2A/Bα holoenzyme (PDB 3DW8); inset shows infiltration of the Bα-subunit residues, K88 (green), into the catalytic core *above*. *Below*, sequence alignment of the B family of regulatory subunits with the conserved lysine residue (green).

2.3. Regulating PP2A through Protein Inhibitors. While assembly of the many trimeric PP2A holoenzymes directs cellular localization and substrate specificity, further regulation is afforded through binding of specific protein inhibitors of PP2A. One such inhibitor SET (I_2/TAF-1) is upregulated during the progression of chronic myelogenous leukemia through BCR/ABL activity and results in decreased PP2A activity. Importantly, restoring PP2A activity prevents disease progression in an animal model of leukemogenesis [67]. An additional PP2A inhibitor is the protein CIP2A (cancerous inhibitor of PP2A), overexpression of which is associated with several human malignancies. CIP2A associates with c-myc to protect its phosphorylated S62 from PP2A-directed activity stabilizing the c-myc protein and allowing it to promote oncogenesis [68].

Recently an interplay between the *Drosophila* serine/threonine kinase Greatwall (gwl) and PP2A/Bδ was observed during mitotic entry in two separate studies [69, 70]. PP2A/Bδ activity prevents mitotic entry by maintaining Cdc25 in a dephosphorylated and inactive state. Gwl reverses this inhibition through the phosphorylation of S67 of both α-endosulfine (Ensa) and cyclic adenosine monophosphate-(cAMP-) regulated phosphoprotein-19 (Arpp-19). Phosphorylation converts Ensa and Arpp-19 into very specific inhibitors of PP2A/Bδ activity and produces activation of Cdc25 leading to cell cycle progression. Similar cell cycle regulatory activity has been observed with the mammalian ortholog of gwl, MASTL; however, the MASTL-PP2A interaction has yet to be characterized [71].

2.4. Substrate Recruitment by Regulatory Subunit Interactions Distant from the Active Site. Once targeted to specific subcellular locales, the PP2A holoenzyme must recruit and dephosphorylate target substrates. Recent structural studies have begun to suggest the mechanism by which the regulatory subunit of PP2A mediates initial binding to target substrates.

The B family of regulatory subunits adopts a seven-bladed β-propeller structure [72]. Other β-propeller proteins have been shown to bind ligands in the central depression on the top surface of the toroid [73]. Crystallization of PP2A/Bα revealed a cluster of acidic residues in this depression that is available to recruit potential substrates containing a basic motif [56]. In this same study, the acidic central depression of Bα was experimentally confirmed to bind the microtubule-associated protein tau, an established PP2A/Bα substrate. Several conserved aspartate and glutamate residues in Bα engage in weak, electrostatic interactions across a large basic portion of tau and support dephosphorylation of tau at multiple sites through cycles of binding and unbinding [56].

Structurally divergent B′ family members may recruit substrates in a similar fashion as the B family. The crystal structure of PP2A/B′γ shows that the B′ subunit contains 18 stacked α-helices which adopt 8 huntingtin elongation A subunit Tor- (HEAT-) like repeats [58, 59]. A portion of these HEAT-like repeats interact with the A subunit of the holoenzyme to mediate regulatory subunit incorporation into the holoenzyme complex. Like the Bα subunit, an acidic patch is exposed in the B′ family of regulatory subunits and may mediate protein-protein interactions and substrate recruitment [59].

2.5. Substrate Recruitment by Regulatory Subunit Interactions Near the Active Site. Structural studies of PP2A have revealed a conserved loop in the B′ family of regulatory subunits which infiltrates the catalytic core of the holoenzyme [58, 59] (Figure 2(a)). At the tip of this loop is a conserved glutamate residue, E153 (B′β numbering), which contacts through its carbonyl oxygen the catalytic subunit and through its carboxyl group a cocrystallized microcystin molecule in the active site. Mutational analysis revealed that E153 is an absolute requirement for efficient dephosphorylation of tyrosine hydroxylase (TH), a known PP2A/B′β substrate, as well as other as yet unidentified cellular substrates of this PP2A holoenzyme [74]. Further, it was determined that E153 of B′β interacts with R37 and R38 of TH to mediate dephosphorylation of both S31 and S40 on TH. Positively charged residues in the vicinity of target phospho-serine/threonine residues could represent a consensus sequence for B′-subunit-mediated dephosphorylation. Further, the infiltrating loop is likely a conformationally dynamic structure which is not sterically hindered by surrounding portions of the A, C, or B subunits. Since R37/38 are important for dephosphorylation of both upstream (S40) and downstream (S31) sites, it appears that the orientation of phosphopeptides relative to the catalytic cleft is not constrained by additional interactions. Collectively, the above observations support a model in which the sites of interaction between the substrate and the B regulatory subunit that are distant and near the active site together control substrate specificity. First, the interaction occurring at sites distant from the active site increases the local substrate concentration. Following this initial substrate recruitment, the interactions near the active site mediate site-specific dephosphorylation.

Although divergent in its sequence, an analogous structure from the β-propeller fold of the B family of regulatory subunits extends to the catalytic core of the holoenzyme [56] (Figure 2(b)). This loop places conserved residues of the B-family subunits very near the holoenzyme active site. Unpublished observations generated in our lab suggests that of these loop residues K87 of Bβ2 may play a similar role as E153 of B′β in site-specific dephosphorylation of target substrates; however, further characterization of this substrate specificity loop is required.

3. Conclusion

PP2A is a ubiquitous protein phosphatase responsible for the dephosphorylation of many different intracellular targets. The diverse repertoire of potential substrates for PP2A is imparted by the incorporation of one of fifteen unique B-regulatory subunits. Recent studies have increased our understanding of the mechanisms by which the B subunit imparts specificity to the holoenzyme complex. Through selective incorporation of the B-regulatory subunit, the holoenzyme complex is recruited to discrete subcellular locales which define the sphere of influence for the phosphatase. Secondly, interactions between endogenous inhibitors and specific PP2A heterotrimers further restrict phosphatase activity. As shown for the B family, regulatory subunits mediate low-affinity interactions with substrates to increase the local concentration of substrates. Through a flexible substrate selectivity loop which contacts the catalytic subunit, interactions between the regulatory subunit and phosphosubstrate may mediate multiple nearby dephosphorylation events. With the current structural information available for the PP2A complexes, future high-resolution studies will further define the molecular mechanism of PP2A substrate specificity. As general, inhibitors of PP2A are either clinically irrelevant or toxic, as in the case of the small molecule inhibitor microcystin, novel methods to increase the specificity of PP2A inhibition or activation must be developed. A clearer understanding of the PP2A substrate specificity mechanisms will serve as the foundation for rational drug design of selective inhibitors and activators of specific PP2A holoenzyme complexes.

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Review Article

Control of Genetically Prescribed Protein Tyrosine Kinase Activities by Environment-Linked Redox Reactions

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Recent observations on environment-linked control of genetically prescribed signaling systems for either cell activation or cell death have been reviewed with a focus on the regulation of activities of protein tyrosine kinases (PTKs). The environment-linked redox reactions seem to primarily affect cell surface receptors and cell membrane lipid rafts, and they induce generation of reactive oxygen species (ROS) in cells. ROS thus generated might upregulate the catalytic activities of PTKs through inactivating protein tyrosine phosphatases that dephosphorylate and inactivate autophosphorylated PTKs. Recent evidence has, however, demonstrated that ROS could also directly oxidize SH groups of genetically conserved specific cysteines on PTKs, sometimes producing disulfide-bonded dimers of PTK proteins, either for upregulation or downregulation of their catalytic activities. The basic role of the redox reaction/covalent bond-mediated modification of protein tertiary structure-linked noncovalent bond-oriented signaling systems in living organisms is discussed.

1. Introduction

Enzymes, of which structures and functions are prescribed in genes, basically mediate all metabolisms needed for the development and functions of living organisms. Among the many enzymes, protein tyrosine kinases (PTKs) are known to play key roles in intracellular signaling for the development and functions of cells [1]. The mechanisms for regulation of the catalytic activities of PTKs seem to be principally prescribed in genes. These mechanisms include phosphorylation and dephosphorylation of specific tyrosine residues on the kinase protein by other PTKs or protein tyrosine phosphatases (PTPs), the structures and functions of which are all under genetic control [2–8]. For example, the catalytic activity of Src, a representative PTK, is known to be downregulated through phosphorylation of Tyr527 located in the tail of Src protein by CSK, another PTK, which leads to binding of the tail to the specific structure on the SH2 domain of Src to make the catalytic domain of Src closed [2, 3]. Additionally, it is known to be upregulated by dephosphorylation of phosphorylated Tyr527, which allows the catalytic domain to open, inducing phosphorylation of Tyr416 as the major autophosphorylation site and upregulation of the kinetic activity to phosphorylate exogenous specific substrates [2–4, 7, 8]. On the other hand, dephosphorylation of phosphorylated Tyr416 by PTPs downregulates the kinase activity [5, 6, 8].

Results of several early studies have, however, suggested that the catalytic activities of PTKs are modulated by exposure of cells to environmental chemicals or oxidative agents [9–13]. It could be that the catalytic activities of PTKs are upregulated through redox mechanism-based inactivation of PTPs which otherwise dephosphorylate phosphorylated tyrosines at the major autophosphorylation sites of PTKs [11–14]. Recent evidence has, however, also suggested that environment-linked chemical or redox reactions directly attack PTKs for modification of their catalytic activities and subsequent signaling for cell activation [15–19]. This may occur through oxidization of SH groups of specific cysteines on PTK molecules by ROS that are generated intracellularly after clustering of cell surface receptors in association with membrane lipid rafts by environment-linked chemical or

redox reactions [15, 20–24]. Chemical or redox reaction-mediated clustering of cell surface receptors in association with membrane lipid rafts has further been shown to deliver signals for either cell activation or cell death depending on the conditions [20, 24–30].

Recent observations on how environment-linked chemical or redox reactions control genetically prescribed signaling for cell activation or cell death are reviewed here, following several related review articles on this matter [9, 10, 28, 31–38]. Additionally, significance of these observations is briefly discussed from the viewpoint of crosstalk between environment-linked chemical or redox reaction- (covalent bond)-mediated and genetically prescribed (protein tertiary structure-linked noncovalent bond-oriented) regulation of intracellular signaling for either cell activation or cell death.

2. Environment-Linked Chemical or Redox Reaction-Mediated Bypass of Genetically Prescribed Receptor-Ligand Recognition

Initial activation of PTKs is usually linked to recognition by cell surface receptors of specific ligands. Thus, receptor type PTKs such as EGFR first bind specific ligands such as EGF to be dimerized on cell membranes for activation. On the other hand, Src family PTKs as nonreceptor type PTKs with a hydrophobic structure at the N-terminus are activated following specific ligand-induced clustering of cell surface receptors in association with membrane lipid rafts. Lipid rafts are known to work as a station of molecules for signal transduction to which the nonreceptor type PTKs are attached under the cell membrane [39–43]. Earlier experiments showed that this receptor-ligand bond-mediated step for activation of either receptor type [26] or Src family nonreceptor type [17, 20–23, 44, 45] PTKs in cells can be bypassed by environment-linked chemical or redox reactions. These reactions induce clustering of receptors and membrane lipid rafts, which somewhat mimics ligand-mediated receptor clustering. Environmental agents for such chemical or redox reactions include thiol-reactive Hg^{2+} ions [20–22, 44–46], arsenite [24, 29] and methane sulfonate compounds (1,4-butanediyl-bismethanethiosulfonate (BMTS) [45, 47], protein amino group-reactive carbonyl compounds (glyoxal, methylglyoxal) [23, 48, 49], and protein sulfhydryl, amino, and imidazole groups-reactive 4-hydroxynonenal (HNE) [26, 27, 50].

This pathway of activation of PTKs has been most extensively studied in cells carrying Src family PTKs such as c-Src (primary and tertiary structures of human c-SRC are illustrated in comparison with those of human c-RET in Figure 1) and Lck, which direct other PTKs such as ZAP70 and Syk and a large family of protein serine/threonine kinases downstream of the signal transduction cascade. Exposure of cells carrying c-Src or Lck to Hg^{2+}, arsenite, glyoxal, or 1,4-butanediyl-bismethanethiosulfonate (BMTS) induced chemical or redox reaction-mediated clustering of membrane rafts. This occurred in close association with clustering of glycosylphosphatidyl inositol- (GPI-) anchored proteins in the rafts as the potential direct targets of the redox reactions and Src family PTKs that are bound to lipid rafts with a myristoylated membrane-targeting structure at the N terminus (20, 53). This signaling event was followed by activation of Src family PTKs as well as generation of ROS [23, 46]. Generation of ROS possibly underlay the mechanism of activation of PTKs and ultimately led to cell activation or cell death. All of these signal transduction pathways were inhibited by methyl beta cyclodextrin, a compound that disrupts lipid rafts by removing cholesterol from membranes [24]. This suggested a crucial requirement of the integrity of membrane lipid rafts in the redox reaction-linked signal pathway that apparently mimics the genetically prescribed receptor-ligand bond-mediated signal transduction. HNE, which is able to pass through the plasma membrane, also triggered another signal transduction pathway for cell death, possibly through scavenging intracellular glutathione [27]. These results suggest that genetically prescribed signal transduction pathways for cell activation or cell death, including the step of PTK activation, can be bypassed or modified at different steps by environment-linked chemical or redox reaction-mediated pathways.

As briefly mentioned in the introduction section, the receptor-ligand bond-mediated signaling pathway for activation of Src kinase is originally prescribed by genes. This pathway includes phosphorylation of Tyr527 in the tail of Src protein by CSK to be bound to the SH2 domain of Src for stabilizing and inactivating the kinase molecule [2–4, 7]. Binding between cell surface receptors attached to membrane lipid rafts, including GPI-anchored proteins, and their ligands may trigger the signal pathway for dephosphorylation of phosphorylated Tyr527 of Src kinase (or its equivalent tyrosine residues of other Src family PTKs) by specific PTPs. This should result in disruption of the bond between phosphorylated Tyr527 and SH2 domain of Src and induction of autophosphorylation of Tyr416 for activation of the catalytic activities of Src or other Src family PTKs [5, 6, 8]. Recently, the question of whether this is the only mechanism for triggering Tyr416 autophosphorylation and activation of Src or other Src family PTKs has been repetitiously discussed [10, 31–37].

Pu et al. [44] investigated whether the environment-linked chemical or redox reaction-oriented signaling pathway that bypasses the genetically prescribed cell surface receptor-ligand interaction-mediated pathway includes the above-described Tyr527-mediated regulatory mechanism of Src kinase. They revealed that cells carrying Tyr527-missing v-Src or cells missing CSK for phosphorylation of Tyr527, which are thereby already active, were still reactive to Hg^{2+} for superactivation of the kinases. It was concluded from these results that the environment-linked chemical or redox reaction-mediated pathway triggers a new mechanism for Tyr416 autophosphorylation and activation of Src kinase independent of dephosphorylation of previously phosphorylated Tyr527.

Oxidative agents are known to inactivate PTPs of which cysteine residues in the catalytic domain [51] are sensitive to the agents. It has long been suggested that this is the central or even the only mechanism of upregulation of PTKs by oxidative agents, maintaining the autophosphorylation level

of major tyrosine residues in the catalytic domain of PTKs [11, 12, 52]. ROS produced in cells through the mechanism triggered by environment-linked chemical or redox reaction-mediated clustering of cell surface receptors/membrane rafts might actually inactivate PTPs, which in turn upregulate the catalytic activity of PTKs [14].

3. Redox Reaction-Mediated Direct Modification of Molecular Structures of PTKs for Regulation of Their Catalytic Activities *In Vitro*

In earlier experiments, Pu et al. [15] and Akhand et al. [16] exposed Src kinase proteins that had been immunoprecipitated from cell lysates in a medium with detergents to sulfhydryl-reactive Hg^{2+} [53] or nitric oxide- (NO-) generating S-nitroso-N-acetyl penicillamine (SNAP) [16]. They then measured the catalytic activities of Src proteins for Tyr416 autophosphorylation and tyrosine phosphorylation of specific substrates.

Pu et al. [15] showed that the catalytic activity of immunoprecipitated c-Src from NIH3T3 cells was elevated severalfold by exposure to low to moderate concentrations (0.5–50 μM) of Hg^{2+}, whereas it was decreased by exposure to a high concentration (500 μM) of Hg^{2+}. N-acetylcysteine neutralized this Hg^{2+} effect, suggesting the involvement of a redox reaction in the mechanism. Addition of the protein tyrosine phosphatase inhibitor Na_3VO_4 to the reaction mixture did not inhibit the Hg^{2+}-mediated activation. This result provisionally eliminates the possibility that Hg^{2+}-mediated inactivation of PTPs was the central mechanism for upregulation of the c-Src activity. Hg^{2+} was further found to be capable of activating Tyr527-defective v-Src kinase and c-Src kinase from mutant cells defective in Tyr527-phosphorylating Csk kinase. Cyanogen bromide cleavage maps revealed that Hg^{2+} selectively promoted the autophosphorylation at Tyr416 and that the previously *in vivo* radiolabeled phosphorus on Tyr527 was not deleted. These observations suggested that Hg^{2+} triggered the redox reaction-linked mechanism for promotion of both autophosphorylation and general catalytic activity of Src kinase *in vitro* independent of Tyr527-linked or PTP-mediated regulation. The exact target structure of the redox reaction for promotion of autophosphorylation of c-Src was not, however, identified in these experiments.

Exposure of Src kinase to SNAP was also shown to promote Tyr416 autophosphorylation independent of Tyr527-linked or PTP-mediated regulation [16]. Interestingly, promotion of Tyr416 autophosphorylation, which accompanied increase in overall kinase activity of c-Src, was found to be closely linked to S–S bond-mediated aggregation of Src molecules. This observation supported a hypothetical view that the environment-linked oxidative agent-triggered signal transduction pathway for upregulation of c-Src activity includes sulfhydryl-oxidation-mediated structural modification of Src proteins.

Further experiments demonstrated that exposure of cells carrying RET kinases to ultraviolet (UV) [17, 54] or osmotic pressure [18] intracellularly induced activation of these kinases. Either c-RET proto-oncogene as a receptor type PTK or its consistently active oncogene mutants, including an extracellular domain-deleted (nonreceptor type) mutant RET-PTC-1 (primary and tertiary structures of c-RET in comparison with those of c-SRC are illustrated in Figure 1), were activated. Interestingly, UV- or osmotic pressure-mediated activation of RET-PTC-1 accompanied an increase in the amount of S–S bond-mediated dimers of the kinase proteins. Exposure of cells to UV or osmotic stress increased S–S bond-mediated dimerization from 1% as the background level to 4% of total RET-PTC-1 proteins, and the fraction of dimerized RET-PTC-1 selectively displayed a high level of autophosphorylation [17, 18]. This was demonstrated by an *in vitro* kinase assay under the condition with or without a reducing agent (2-mercaptoethanol) for the kinase proteins that had been immunoprecipitated from the UV- or osmotic stress-exposed cells. It was speculated that the UV-induced or osmotic-stress-generated ROS in cells attacked intracellular domains of the RET kinase to oxidize the domains for S–S bonded dimerization, ultimately resulting in promotion of autophosphorylation and activation of the kinase.

More recently, direct evidence has been provided by Kemble and Sun [19, 38] for oxidative modification of purified Src proteins accompanying changes in the levels of their catalytic activities. They isolated Src proteins from bacteria lysates containing recombinant Src proteins by adding iminodiacetic acid-agarose beads charged with $NiCl_2$ to the lysates. The isolated Src proteins were active when reduced by adding dithiothreitol (DTT) to a medium containing Src proteins and retained 8–25% of their full activity when oxidized by adding H_2O_2 to the medium. This downregulation of the kinase activity was shown to be caused by oxidation of specific cysteine residues on Src, resulting in an S–S bridge for dimerization of Src molecules. Their study results formally proved that some cysteine residues on Src proteins are capable of being oxidized for dimer formation with downregulation of the kinase activity. This effect of dimer formation is, however, apparently opposite to the above-described effect on immunoprecipitated RET-PTC-1 kinase, in which the kinase activity was upregulated in linkage to S–S bond-mediated dimerization of kinase proteins [17, 18].

The reason why dimer formation of Src or RET-PTC-1 due to S-S bonds between specific cysteine residues induced apparently opposite effects on their kinase activities in the experiments performed by two different study groups [17–19] is not clear. However, in the model of RET-PTC-1, kinase molecules must have been exposed to possibly mildly oxidative environments in cells. Intracellular environments are normally maintained for reducing because of the presence of large amounts of reducing agents such as glutathione and thioredoxin, and the oxidizing/reducing balance in cells therefore does not change drastically even after production of ROS in cells (57). The mildly oxidative environment possibly selectively affected SH groups of specific cysteines for fine structural modification of kinase proteins, which was needed for maintenance and upregulation of the kinase activity.

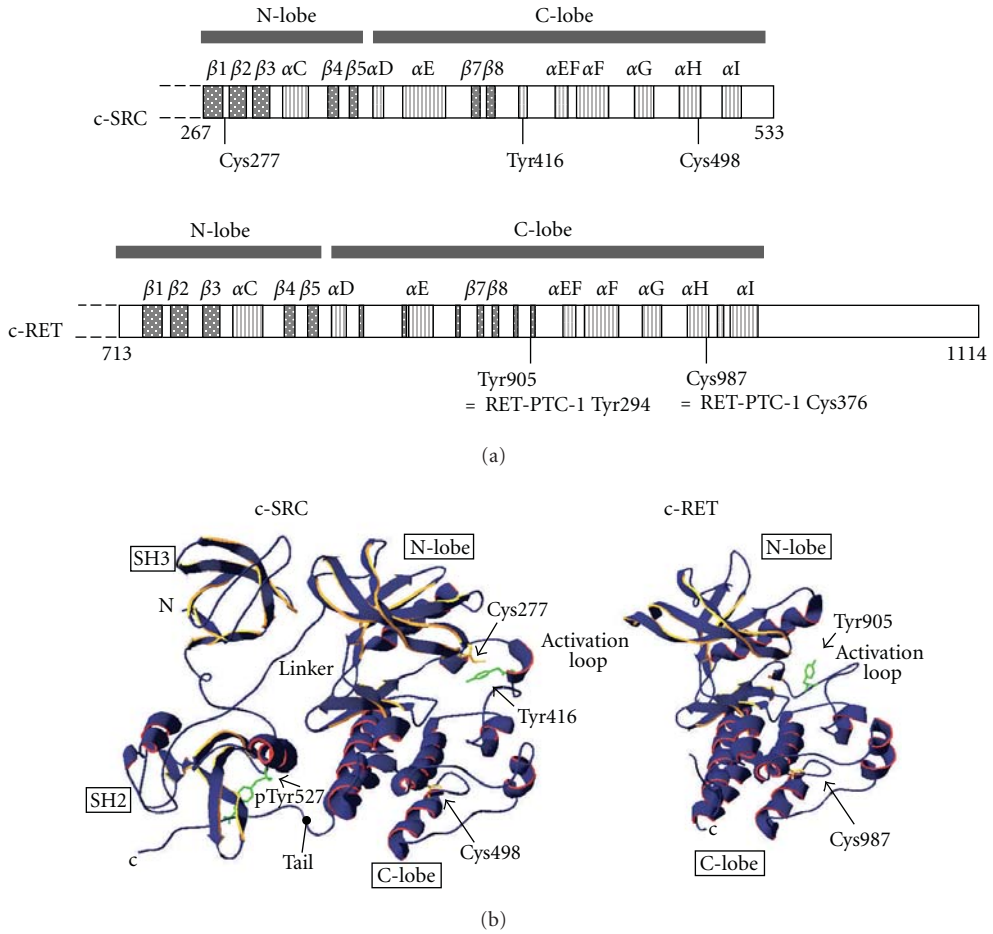

Figure 1: Shematic illustration of structures of human c-SRC and human c-RET kinase proteins. Primary and tertiary structures of c-SRC (PDB 2SRC) and c-RET (PDB 2IVS) are deduced from the data in Protein Data Bank (PDB). (a) Primary structures of c-SRC (top) and c-RET (bottom) kinase domains. α: α-helix; β : β-sheet. (b) Tertiary structures of SH3, SH2, and kinase (N-lobe and C-lobe) domains of c-SRC (left) and kinase domain of c-RET (right). Positions of Tyr416 of c-SRC and Tyr905 of c-RET as major autophosphorylation sites and those of Cys277/Cys498 of c-SRC and Cys987 of c-RET (equivalent to Cys376 of RET-PTC-1) as well as positions of N-terminal (N) and C-terminal (C) amino acids are shown.

RET-PTC-1 molecules were possibly located in a row under the cell membrane, which should make two neighboring kinase molecules easily accessible to each other for S–S bonded dimerization at specific cysteines. S–S bonded dimerization in close linkage to upregulation of the kinase activity [17, 18] is direct evidence of the involvement of oxidative structural modification in redox-mediated upregulation of PTK activities. S–S bonded dimerization may not, however, be an absolute requirement for the oxidative effects for upregulation of PTKs as discussed in the next section.

In contrast, isolated recombinant Src proteins prepared by Kemble and Sun [19], which were probably arranged in a random manner *in vitro*, were affected by rather heavily oxidative agents such as oxygen in air and H_2O_2, which possibly oxidized free SH groups on multiple cysteines on Src but selectively induced dimerization through S–S bonds between specific cysteines, probably inducing a major structural change of Src for downregulation of the kinase activity.

4. Identification of Submolecular Structures in PTK Molecules as the Targets of Redox Reaction for Regulating the Catalytic Activities of PTKs

Kemble and Sun [19] studied the target cysteine residues responsible for S–S bonded dimer formation of Src proteins by preparing mutant proteins in which each of totally ten cysteine residues of Src was replaced with alanine. They showed that dimer formation occurred only through oxidation of Cys277, which is located in the catalytic domain of Src.

On the other hand, Kato et al. [17, 54] and Takeda et al. [18] examined the sensitivity to ultraviolet light

irradiation or osmotic stress of RET-PTC-1 (a nonreceptor type PKC) mutants. Each of two cysteine residues (Cys365 and Cys376) on the alpha-helix H region downstream of the catalytic domain of RET-PTC-1 was replaced with alanine. They found that the mutants in which Cys376 of RET-PTC-1 is replaced with alanine almost totally lost their background ability for inducing autophosphorylation of Tyr294 as the major autophosphorylation site [55]. The mutants at the same time lost their abilities to form S–S bonded dimers of kinase proteins in cells to which a high level of autophosphorylation was associated (*in vivo*), even after exposure to ultraviolet irradiation or osmotic stress. These results are in contrast to another observation that replacement of Cys365 of RET-PTC-1, a cysteine located near Cys376 on alpha-helix H, with alanine did not detectably alter either background ability for autophosphorylation of Tyr294 or the ability to form S–S bonded dimer formation in cells. Correspondingly, a single mutation in Cys498 of v-Src, an equivalent of Cys376 of RET-PTC-1, but not in other cysteines yielded clear suppression of kinase activity and temperature sensitivity in cell transformation [56]. Takeda et al. [57] further confirmed that all RET-PTC-1 mutants in which C376 of RET-PTC-1 was replaced with glycine, lysine, threonine or serine lost their background level of activity for autophosphorylation of Tyr294 in cells, even though comparable amounts of RET proteins were obtained from each of these mutants, suggesting that possibly accelerated degradation of the mutant proteins is not the reason for the above-mentioned loss of abilities. All of these results suggested that Cys376 of RET-PTC-1, an equivalent of Cys498 of Src or Cys987 of c-RET, is crucially involved in the maintenance and upregulation of catalytic activities of these kinase proteins in cells. Moreover, because both S–S bonded dimer formation and Tyr294 autophosphorylation were simultaneously lost in the Cys376-missing RET-PTC-1 mutant, it is likely that Cys376 plays a crucial role in the redox regulation of the kinase activity of this PTK through oxidative structural modification.

Cys277 of Src is located in the sequence context of GQGCFG on the small N-terminal lobe of the catalytic domain and is conserved in 8 of more than 90 human PTKs examined [19]. On the other hand, Cys376 of RET-PTC-1 or Cys498 of Src is located in the MXXCW motif on the C-terminus of alpha-helix H and is conserved in all but one (FGR: threonine in place of cysteine) of 82 human PTKs examined in the database Swiss-Prot (18/11/2004), suggesting a crucial role in regulation of the kinase activity of PTKs in general [32, 33, 55].

Structural modification of Cys376 of RET-PTC-1, Cys498 of Src or its equivalent cysteine residues on almost all PTKs in a mildly oxidative environment in the cell could thus work to basically maintain or to upregulate the kinase activity. In contrast, modification of Src proteins at Cys277 or its equivalent cysteine residues on 8 out of more than 90 human PTKs in possibly heavily oxidative environments evidently acted to downregulate it (see Figure 2). The reason of the different effects of S–S linked dimerization on RET-PTC-1 and Src might simply be that RET-PTC-1 is missing the cysteine equivalent to Cys 277 of Src. Pu et al. [15] have, however, shown that exposure of immunoprecipitated Src proteins to low to moderate levels of sulfhydryl-reactive Hg^{2+} *in vitro* caused upregulation of the kinase activity, whereas exposure of these proteins to a high level of Hg^{2+} downregulated the activity. The latter observation might have involved oxidization of Cys277, although S–S bonded dimerization was not detected in their experiment. It is possible that long-term exposure to oxygen in air (*in vitro*) during preparation of recombinant Src proteins [19] caused the proteins to be oxidized at a number of free SH groups of cysteines possibly including both Cys277 and Cys498. S–S bonded dimerization, however, occurred only between Cys277, of which the downregulatory effect mostly overcame the potential upregulatory effect by oxidization of Cys498.

Taken together, the results indicate that oxidative structural modification of PKTs might bidirectionally control their kinase activities in the following manner. Fine structural modification by oxidization of Cys376 of RET-PTC-1 or its equivalent cysteines in mildly oxidative environments works for maintenance and upregulation of kinase activities of almost all PTKs carrying equivalent cysteine residues. In contrast, more extensive structural modification of PTKs through oxidization of Cys277 of Src or its functionally equivalent cysteine residues on other PTKs in heavily oxidative environments downregulates their kinase activities. The fine structural modification through oxidization of Cys376 of RET-PTC-1, Cys498 of Src or its equivalent cysteine residues on other PTKs for kinase activation may not necessarily require S–S bonded dimerization of kinase proteins. Oxidization of the SH group to S–X seems still effective, because exposure of immunoprecipitated Src proteins to sulfhydryl-reactive Hg^{2+} successfully induced upregulation of the kinase activity without S–S bonded dimerization of the kinase proteins *in vitro*.

5. Further Crosstalk between Genetically Prescribed and Environment-Linked Signaling Systems for Regulating Kinase Activities of PTKs

FGR, an exceptional PTK that does not carry Cys376 of RET-PTC-1-equivalent, carries threonine in place of cysteine in the MXXCW motif. Takeda et al. [57] therefore investigated the mechanism for this exceptional PTK to keep its kinase activity. They prepared a number of RET-PTC-1 mutants in which Cys376 was replaced with glycine, lysine, threonine, or serine and examined the kinase activities of imunoprecipitated mutant RET-PTC-1 from cell lysates *in vitro*. As described in the previous section, all of these mutant proteins almost totally lost their basic kinase activities *in vitro*. Surprisingly, however, cells carrying Cys376Ser or Cys376Thr mutant RET-PTC-1, but not those carrying Cys376Gly or Cys376Lys mutant RET-PTC-1, displayed almost normal levels of autophosphorylation at Tyr294 *in vivo* (in cells). Correspondingly, Cys376Thr/Ser, but not Cys376Gly/Lys, was capable of inducing cell transformation almost as effectively as parental RET-PTC-1 as an active oncogene.

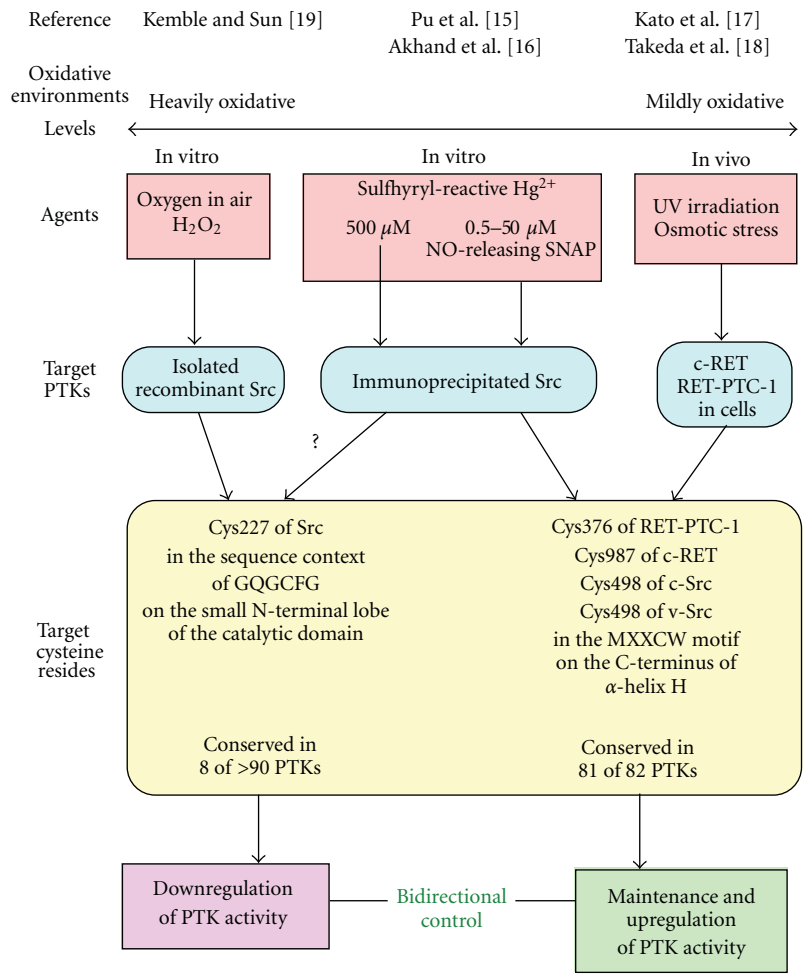

Figure 2: Summary of observations for bi-directional control of PTKs through their potential structural modification by redox reactions. Kemble and Sun demonstrated a pathway for definitive structural modification-mediated downregulation of the kinase activity of Src in heavily oxidative environments *in vitro* through disulfide-bonded dimerization of Src proteins at Cys277. On the other hand, Kato et al. and Takeda et al. revealed another pathway for possibly fine structural modification-mediated upregulation of kinase activities of RET-PTC-1 in a mildly oxidative environment *in vivo* through disulfide-bonded dimerization of RET proteins at Cys376. Pu et al. and Akhand et al. further demonstrated that exposure of immunoprecipitated Src proteins from cell lysates to low to moderate levels of sulfhydryl-reactive Hg^{2+} or NO-releasing SNAP induced Tyr527/PTP-independent activation of Src kinase, whereas their exposure to a high level of Hg^{2+} downregulated it.

Further study demonstrated that the essential role of the cysteine at the MXXCW motif in initiating the kinase activity of RET-PTC-1 could be partially replaced by the activity of protein kinase C as a serine/threonine kinase *in vivo* (but not *in vitro*). This result suggested that the PTK missing the cysteine at the MXXCW motif uses an alternate PKC-mediated pathway as a rescue system for maintaining the kinase activity. PKC itself in the rescue pathway should, however, be activated by another PTK that possesses the cysteine at the MXXCW motif. It is therefore speculated that there is a new pathway of crosstalk between genetically prescribed and environment-linked mechanisms for regulation of PTKs. Crosstalk between Src and RET kinases has also been reported as Src-mediated repair of function-impaired RET-MEN2A (another oncogenic RET mutant) with substitutions of tyrosines in the COOH-terminal kinase domain for phenylalanine [58].

6. Crosstalk between Environment-Linked and Genetically Prescribed Signals for Either Cell Activation Or Cell Death

Environment-linked oxidative signals have also been shown to induce apoptotic cell death, basically sharing a common initial step for cell activation, by inducing clustering of cell surface receptors in association with membrane lipid rafts [24]. This signaling pathway was shown to induce generation of reactive oxygen species (ROS) and activation of apoptosis signal-regulating kinase 1 (ASK-1), c-Jun amino-terminal

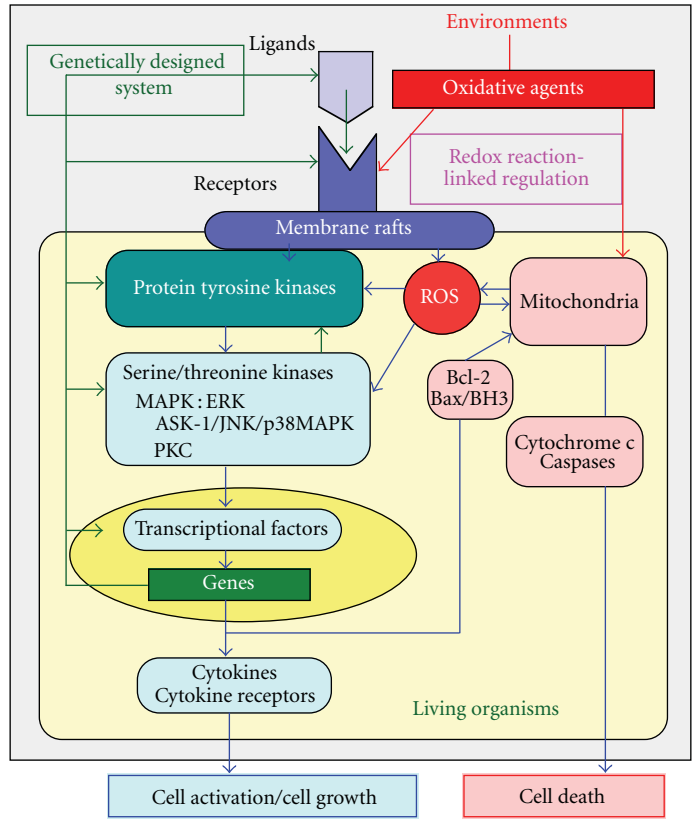

Figure 3: Suggested crosstalk between the genetically prescribed system in living organisms and environments through chemical or redox reactions in the signal transduction for either cell activation or cell death. The genetically prescribed signaling system includes noncovalent bond-oriented interaction between receptors and specific ligands and subsequent activation of PTKs, serine/threonine kinases, and transcriptional factors. This system is regulated by environment-linked covalent bond-mediated redox reactions, which primarily attack cell surface receptors in association with cell membrane lipid rafts, resulting in intracellular production of ROS. ROS might directly or indirectly affect PTKs, ASK-1, or mitochondria for promotion of subsequent signal transduction, which ultimately leads to either cell activation/growth or cell death.

kinase (JNK), and p38 MAPK and reciprocal regulation of Bcl-2/Bax [24, 48, 50, 59, 60] or downregulation of ERK [49]. This pathway was further shown to cause mitochondria to release cytochrome c, leading to activation of caspases for DNA fragmentation and apoptotic cell death, or to release of a high level of ROS for necrotic cell death [25]. Environment-linked oxidative agents might also more directly affect mitochondria [30] or induce an intracellular reduced glutathione level [27], ultimately leading to apoptotic or necrotic cell death.

These observations suggest extensive crosstalk between microenvironment-linked and genetically prescribed signals for inducing either cell activation or cell death (see Figure 3). Switching for either cell activation or cell death seems to depend on the nature and levels of environment-linked chemical or oxidative signals and on the time of involvement of environment-linked signals in relation to the stage of genetically prescribed development and functions of the cells.

A number of environment-linked chemical or oxidative signals seem to be changed to intracellular oxidative signals through generation of ROS as potential second messengers in signal transduction in cells [34, 35]. ROS may lead to either cell activation or cell death depending largely on the level of ROS generated [20, 25]. Thus, a low level of environment-linked oxidative stress probably induces a low grade of ROS production for upregulation of PTKs. This causes downstream activation of MAP family kinases such as ERK, serine/threonine kinases, and transcriptional factors for production of specific cytokines and cytokine receptors, ultimately leading to cell activation and proliferation. In contrast, a slightly increased level of oxidative stress induces apoptotic cell death through a cascade of signaling such as a higher grade of ROS production and downstream activation of the ASK-1-mediated signal pathway including JNK/p35MAPK, Bax/BH3, cytochrome c, and caspases. Higher levels of stress induce necrosis accompanying a high-grade ROS production possibly through serious damage of mitochondria [47].

A low level of ROS is always produced by mitochondria and may be used for controlling the activation levels of PTKs physiologically [32]. A higher level of ROS can be produced in cells after direct attack of mitochondria by environment-linked oxidative agents. Evidence has been provided for a

mechanism to localize ROS signal at a specific subcellular compartment, which is essential for promoting redox-linked signaling pathways after cell surface receptor activation [61]. ROS production for inducing either cell activation or cell death has been suggested to be membrane raft linked [24, 62, 63] and might be localized through interactions of NADPH oxidase with signaling platforms associated with lipid rafts [61]. Favoring in part this speculation, Kawamoto et al. recently obtained experimental results suggesting that cells exposed to sulfhydryl-reactive arsenite generate ROS outside mitochondria (unpublished observation).

7. A Potentially Basic Role of Environment-Linked Control of Genetically Prescribed Signaling Systems in Living Organisms

Both the development and functions of living organisms are basically self-regulated by information natively included in genes. Thus, genes code for tertiary structures of proteins, including receptors and their ligands, which provide noncovalent bond-oriented molecular switches in signal transduction for either cell activation or cell death. However, the environment conditionally gives some covalent bond-mediated nongenetical information to living organisms [9], particularly through environment-linked oxidizing or reducing (redox) reactions. The redox reactions are principally reversible and might therefore replace some of the genetically prescribed noncovalent bond-mediated molecular switches for signal transduction.

Recent evidence introduced here suggests that the environment-linked covalent bond (chemical or redox reaction-) mediated information plays a crucial role in controlling the genetically prescribed noncovalent bond-oriented signaling systems for cell activation or cell death. The key target molecules for the covalent bond-mediated information are membrane lipid raft-associating cell surface receptors and intracellular PTKs. PTKs as crucial targets of redox regulation are capable of triggering subsequent cascade reactions of signaling elements such as protein serine/threonine kinases and transcription factors. The idea for general importance of redox regulation of PTKs in overall signal transduction in living organisms corresponds well to the fact that the specific cysteine residue as the target of redox regulation is highly conserved in a number of human PTKs [33].

8. Conclusion

Environment-linked chemical or redox reactions control the genetically prescribed signal delivery system for either cell activation or cell death in living organisms. Accumulated evidence indicates that this includes environment-mediated control of PTKs as a master key of intracellular signal transduction for cell activation and of several elements of signal transduction for cell death. The pathway for environment-linked control of PTKs include (1) clustering of cell surface receptors in association with membrane lipid rafts, potentially bypassing receptor-ligand interaction, and (2) membrane lipid raft-linked production of ROS beneath the membrane. This leads to (3) ROS-mediated inactivation of PTPs, which in turn upregulate PTKs, and to (4) ROS-mediated oxidative structural modification of PTKs for bi-directional direct control of PTKs through oxidization of SH groups of specific cysteines on PTKs to either S–S or S–X. Upregulated PTKs promote the subsequent signal transduction, ultimately resulting in either cell activation or cell death. Intracellular ROS produced might also promote other pathways of signal transduction for cell death. It is concluded that environment-linked chemical or redox reactions, which are mediated by covalent bonds, bidirectionally control the genetically prescribed and thereby protein tertiary structure-linked noncovalent bond-oriented signaling systems for either cell activation or cell death in living organisms.

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Review Article

Inorganic Phosphate as an Important Regulator of Phosphatases

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Cellular metabolism depends on the appropriate concentration of intracellular inorganic phosphate (Pi). Pi starvation-responsive genes appear to be involved in multiple metabolic pathways, implying a complex Pi regulation system in microorganisms and plants. A group of enzymes is required for absorption and maintenance of adequate phosphate levels, which is released from phosphate esters and anhydrides. The phosphatase system is particularly suited for the study of regulatory mechanisms because phosphatase activity is easily measured using specific methods and the difference between the repressed and derepressed levels of phosphatase activity is easily detected. This paper analyzes the protein phosphatase system induced during phosphate starvation in different organisms.

1. Introduction

Regulation of cellular processes, such as cellular differentiation, proliferation, cell death, mobility, metabolism, survival, and organization of the cytoskeleton, in response to some stimuli is fundamental to all aspects of cell life [1–4]. Protein phosphorylation is one of the most common mechanisms used to regulate these processes. Processes that are reversibly controlled by protein phosphorylation require both a protein kinase and a protein phosphatase [5–7].

Traditionally, protein kinases have been studied more intensely than protein phosphatases due to the earlier view that protein kinases confer fine regulation to protein phosphorylation, whereas protein phosphatases merely act to remove phosphate groups. Only in the last decade was it realized that protein phosphatases are also regulated by a variety of mechanisms and are of no less importance to cellular physiology than protein kinases [8]. Protein phosphatases are able to hydrolyze phosphomonoester metabolites, releasing inorganic phosphate (Pi) from these substrates [5, 9].

Phosphorus, in the form of inorganic phosphate (Pi), is one of the most important macronutrients for all organisms [7, 10–13]. It is not only used in the biosynthesis of cellular components, such as ATP, nucleic acids, phospholipids, and proteins, but it is also involved in many metabolic pathways, including energy transfer, protein activation, and carbon and amino acid metabolic processes [14]. Large amounts of phosphate are required for cell survival. In plants, Pi is essential for growth and development [15]. In fungi, the Pi signal transduction pathway regulates the expression of many phosphate responsive genes that are involved in scavenging and Pi uptake from extracellular sources [16]. In trypanosomatid parasites, Pi influences their ability to correctly grow and colonize in the invertebrate host [17, 18].

In summary, protein phosphatases and kinases are necessary for Pi homeostasis during the acquisition, storage, release, and metabolic integration of Pi [19–23]. The purpose of this paper is to summarize the regulation of phosphatases by inorganic phosphate, with an emphasis on the role of these enzymes in cell biology.

2. Feedback Control of Phosphatases by Inorganic Phosphate: The *PHO* Pathway

Saccharomyces cerevisiae has several phosphatases with different specificities, cellular location, and permeases used in Pi uptake. The set of genes responsible for these activities are coordinately repressed by the Pi concentration in the growth medium [24]. The cell acquisition, storage, release, and metabolic integration of Pi requires the participation of many essential enzymes such as extracellular acid phosphatases (APases), phosphodiesterases, Pi transporters, polyphosphate kinases, alkaline phosphatases (ALPases), and endopolyphosphatases [19–21]. The activities of these enzymes are intrinsically linked to Pi homeostasis, and they are subjected to regulation via the Pi signal transduction pathway (PHO) in response to varying Pi levels [21, 24].

In one current model for *PHO* regulation, the positive regulator (or positive factor) Pho4p, encoded by the *PH04* gene, is indispensable for transcriptional activation of *PHO* genes by its activity and location. In high Pi medium, a cyclin-dependent kinase (CDK) complex consisting of Pho80p and Pho85p inhibits Pho4p function by hyperphosphorylation. Hyperphosphorylated Pho4p remains in the cytoplasm and is unable to activate the transcription of the *PHO* genes [25, 26]. When the Pi concentration in the medium is sufficiently low, Pho81p inhibits the function of the Pho80p-Pho85p complex [26, 27], allowing Pho4p to relocate to the nucleus and activate transcription of the *PHO* genes [20, 25]. These genes encode for the high-affinity transporters Pho84p and Pho89p; secreted acid phosphatases Pho5p, Pho11p, and Pho12p; other related proteins that increase Pi recovery from extracellular sources [24, 28].

This *PHO* pathway has been described in different organisms such as plants [29–34], bacteria [35–37], and fungi [16, 24, 38, 39].

3. The Phosphatase System in Yeast

Initially, it was observed that a several phosphatase genes are modulated by the Pi concentration in the culture medium; thus, the PHO pathway was initially characterized by differentially expressed phosphatases [24].

In *S. cerevisiae*, the transcription of genes encoding acid and alkaline phosphatases and the Pi transporter is coordinately repressed and derepressed depending on the Pi concentration in the culture medium [20]. Most of the phosphatases synthesized under Pi limiting conditions are extracellularly located or are associated with the plasma membrane or cell wall [40].

The Pi-regulated phosphatase genes include *PHO5* [41], which encodes for the major fraction of repressible acid phosphatases (rAPase; optimum pH 3-4; EC 3.1.3.2), and its isozymes *PHO10* and *PHO11* [42]. These three rAPases are glycoproteins that are found in the cell wall or the periplasmic space. They are responsible for phosphate scavenging and work in conjunction with high-affinity transporters to acquire phosphate when the Pi concentration in the environment is low [24, 43]. The rAPase encoded by the *PHO5* gene is glycosylated during secretion across the membrane and is localized in the periplasmic space [41]. Pho5p is responsible for >90% of APase activity [43].

Because the *PHO5* gene product constitutes the bulk of the acid phosphatases, *PHO5* regulation is key to cellular phosphate homeostasis. Transcriptional activators, Pho4p and Pho2p, are required to generate the active chromatin structure in the *PHO5* promoter and stimulate transcription. Pho80p-Pho85p is a cyclin/cyclin-dependent kinase complex that phosphorylates Pho4p at multiple sites to negatively regulate Pho4p function [44]. Huang and O'Shea [45] carried out a high-throughput, quantitative, enzymatic screen of a yeast deletion collection, searching for novel mutants defective in *PHO5* expression. Among the constitutive mutants, the *pho80* and *pho85* strains showed the most elevated levels of Pho5 phosphatase activity and *PHO5* mRNA under high-phosphate conditions, consistent with their central role in the *PHO* pathway. Complete loss of the high kinase activity (Pho80p-Pho85) results in full activation of the Pho4 transcription factor, leading to full *PHO5* expression.

Another important class of phosphatases in *S. cerevisiae* is the alkaline phosphatases (ALPase; optimum pH 8; EC 3.1.3.1). *PHO8* [46] encodes for a nonspecific repressible alkaline phosphatase (rALPase). It is a vacuole-localized glycoprotein that cleaves diverse substrates to retrieve phosphate from intracellular products [24]. Pho8p is an Mg^{2+}/Zn^{2+}-dependent dimeric protein, similar to the ALPase in *Escherichia coli* and in mammalian cells [47]. The enzyme product of *PHO13* is a monomeric protein and is specific for p-nitrophenyl phosphate (pNPP) and histidinyl phosphate. This enzyme was strongly activated by Mg^{2+} ions, with a pH optimum of 8.2 and a high specific activity for pNPP, with a mean value of 3.6×10^{-5} M [4].

4. Phosphorus Stress Modulates Acid Phosphatases in Plants

Acid phosphatases (APases) may be active against a wide array of organic phosphates present in soil. These enzymes are nonspecific orthophosphoric monoester phosphohydrolases (EC 3.1.3.2) that cleave Pi from ester linkage sites. Secreted plant phosphatases sustain 50% activity over a broad pH range (4.0–7.6), maintain 80% activity over a broad temperature range (22°C–48°C), and are stable at temperatures as high as 60°C, making them ideal candidates for active soil enzymes [48–50].

APases are abundant in Arabidopsis and are represented by at least four gene families. A recent survey of the annotated Arabidopsis genome identified sequences for 1 His APase, 4 phosphatidic APases, 10 vegetative storage protein APases, and 29 purple APases [50, 51].

In recent years, there has been considerable interest in purple APases (PAPs). Comparative analysis of the structure of PAPs from higher plants and mammals has allowed for the identification of conserved sequence and structural motifs in this type of enzyme from many eukaryotic species [51, 52].

Biochemically, plant PAPs function as homodimeric proteins with a molecular mass of ~55 kDa per monomer,

whereas mammalian PAPs are typically monomeric proteins with a molecular mass of ~35 kDa [51–53]. Many PAPs are glycoproteins that are targeted to the secretory pathway [52]. One PAP from *Spirodela oligorrhiza* has been found to be glycosylphosphatidylinositol anchored in the cell [54]. Structurally, plant PAPs have two domains. The NH2-domain does not have catalytic function. The COOH-domain has the metal center and is the catalytic domain of the enzyme. Another PAP from *Lupinus albus* may contain a third domain, with a structure resembling that of sterol desaturases, at its carboxyl terminus [55]. It is not known how common the latter two forms of posttranslational modification are in PAPs from other species. PAPs are metalloenzymes that have a binuclear metal ion complex in its active site. Their characteristic pink to purple color is due to a charge transfer transition by a tyrosine residue coordinating a ferric ion [52, 56]. This enzyme can hydrolyze phosphoric acid esters and anhydrides [57].

PAPs have been isolated from *Phaseolus vulgaris* (common bean) [58], *Glycine max* (soybean) [57], *Lupinus albus* (white lupin) [59], *Lycopersicon esculentum* (tomato) [60], *Triticum aestivum* (wheat) [61], *Hordeum vulgare* (barley) [61], *Zea maize* (maize) [61], and *Oryza sativa* (rice) [61].

The plant response to Pi starvation can be divided into two categories: the specific response and the general response. The specific responses promote efficient mobilization and acquisition of Pi from growth medium and intracellular stores. The general responses allow for long-term survival by coordinating cell metabolism with nutrient availability and growth potential [14, 62]. The implementation of these strategies requires changes in the expression profiles of hundreds of genes, as demonstrated by the transcriptome analyses of *Arabidopsis thaliana* (Arabidopsis) [14, 63].

During Pi starvation, plants enhance phosphatase expression as a general response [50, 63]. Phosphatase production is linked to Pi deficiency, and a positive correlation between acid phosphatase production and Pi nutrition has been proposed [64–66]. For example, plants such as lupins, which are more efficient in acquiring Pi from the soil, produce significantly more phosphatase in comparison to cereals [66].

Wu et al. [14] analyzed the regulation of protein phosphatases in Arabidopsis and found that three genes for PAP were induced by Pi starvation. In addition, the gene At1g25230 was induced more than 2-fold, showing that this gene is responsive to Pi starvation.

In the rice genome, a total of 26 putative PAP genes were identified, and Pi starvation induced the expression of 10 rice PAP genes, suggesting that these play important roles in the acclimation of rice to low Pi conditions [67].

In *Lycopersicon esculentum* (tomato), LePS2, is induced by Pi starvation [66]. It is noted that LePS2 phosphatases represent the first cytoplasmatic phosphatases that are components of the Pi starvation response [31]. Suspending tomato cells in Pi-starved medium led to a PAP-specific activity increase of approximately 4-fold, but the PAP-specific activity remained low and constant in cells maintained in high Pi medium. The increase of PAP activity in cells growth in Pi-starved medium demonstrates that PAP could have a role in enhancing the availability and utilization of Pi and may be pivotal for mobilizing intracellular Pi by sensing a lack of Pi in tomato [60, 66].

5. Ectophosphatases as Pi Sensors

The plasma membrane of cells may contain enzymes whose active sites face the external medium rather than the cytoplasm. The activities of these enzymes, referred to as ectoenzymes, can be measured using intact cells [17, 68–73]. Ectophosphatases and ectokinases have been detected in several microorganisms, including protozoa [69, 71, 74–78], bacteria [79–82], and fungi [83–88].

Many studies have demonstrated a role of ectophosphatases in acquiring Pi for use in the growth of various cell types [73, 74, 86, 89]. In fungi cells (*Fonsecae pedrosoi*), the depletion of Pi from the culture medium apparently induces the expression of different ectophosphatase activities [86]. The cultivation of these fungi in the absence of exogenous Pi has been shown to result in the generation of fungal cells expressing an ectophosphatase activity 130-fold higher than that expressed by fungi cultivated in the presence of Pi [85]. Trypanosomatid cells have ectophosphatases that provide Pi by hydrolyzing phosphomonoester metabolites [17, 18, 51, 60, 73, 81]. For example, in *T. rangeli*, a low Pi concentration in the growth medium induces the expression of a different ectophosphatase activity [17, 73], suggesting that this enzyme leads to hydrolyzes phosphorylated compounds present in the extracellular medium. This hydrolysis could contribute to the acquisition of Pi during the development of *T. rangeli* epimastigotes [18].

Under conditions of Pi limitation, fluorescent bacteria *Pseudomona* express a set of phosphate starvation genes [90, 91]. For example, at least 56 Pi starvation proteins are induced in the *P. putida* strain KT2442 [90], and, in the *P. fluorescens* strain DF57, induction of several phosphate starvation genes has been reported [91].

In many eukaryotes, the nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family of proteins is directly responsible for phosphate hydrolysis from extracellular nucleotides. NPP1 to −3 are found in nearly all human tissue types, and these enzymes contain an alkaline ectonucleotide pyrophosphatase/phosphodiesterase type 1 domain. In *S. cerevisiae*, *NPP1* and *NPP2* are upregulated via Pi-regulated transcription [43].

6. Concluding Remarks

Pi is a compound that is growth limiting in various organisms when its availability is low in many ecosystems [38]. The induction of phosphatase activity in response to Pi starvation is a common phenomenon among organisms acquiring Pi from the environment. These enzymes are able to hydrolyze phosphorylated substrates to supply a source of Pi during a shortage of nutrients. In *Saccharomyces cerevisiae*, the Pi starvation signal triggers increased production of at least four types of phosphatases: (1) the acid phosphatases Pho5, Pho11, and Pho12, which are localized in periplasmic space;

(2) the alkaline phosphatase Pho8, which is localized in the vacuole; (3) the glycerol phosphatase Hor2; (4) the putative polyphosphatase Phm5, which is localized in the vacuole. All of these enzymes can contribute to increased levels of free Pi [92]. However, other functions could be attributed to these enzymes.

Del Pozo et al. [93] purified an acid phosphatase, AtACP5, that is induced by Pi starvation in *Arabidopsis thaliana*. This enzyme presents two activities, hydrolysis of phosphorylated substrates and peroxide formation. The phosphatase activity probably reflects a role in Pi mobilization; the peroxidation activity suggests that AtACP5 could also play a role in the metabolism of reactive oxygen species [34].

Taken together, Pi starvation-induced phosphatases play a role in an organism's adaptation to stress, though other roles can be found.

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Review Article

Interaction between Calcineurin and Ca^{2+}/Calmodulin Kinase-II in Modulating Cellular Functions

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Roles of calcineurin (CaN), a Ca^{2+}/calmodulin- (CaM-) dependent protein phosphatase, and Ca^{2+}/CaM-dependent protein kinase-II (CaMKII) in modulating K^+ channel activity and the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) have been investigated in renal tubule epithelial cells. The channel current through the cell membrane was recorded with the patch-clamp technique, and $[Ca^{2+}]_i$ was monitored using fura-2 imaging. We found that a CaN-inhibitor, cyclosporin A (CyA), lowered the K^+ channel activity and elevated $[Ca^{2+}]_i$, suggesting that CyA closes K^+ channels and opens Ca^{2+}-release channels of the cytosolic Ca^{2+}-store. Moreover, both of these responses were blocked by KN-62, an inhibitor of CaMKII. It is suggested that the CyA-mediated response results from the activation of CaMKII. Indeed, Western blot analysis revealed that CyA increased phospho-CaMKII, an active form of CaMKII. These findings suggest that CaN-dependent dephosphorylation inhibits CaMKII-mediated phosphorylation, and the inhibition of CaN increases phospho-CaMKII, which results in the stimulation of CaMKII-dependent cellular actions.

1. Introduction

Protein kinases and phosphatases regulate major parts of functional proteins through the phosphorylation and dephosphorylation of individual proteins. The characterization and roles of many kinds of protein kinases and phosphatases have been widely investigated in a variety of cells.

Ion channels are well-known functional proteins and their activity can be estimated by monitoring ion currents using the patch-clamp technique. Since the activity of several kinds of channel protein is regulated by protein kinase-mediated phosphorylation and phosphatase-mediated dephosphorylation [1, 2], the investigation of regulatory mechanisms for ion channels often provides important findings regarding the functional significance of protein kinases and phosphatases. To date, it has been demonstrated that some serine/threonine protein kinases, such as protein kinase A (PKA), protein kinase G (PKG), and protein kinase C (PKC), affect ion channel activity. In renal tubule cells, PKA [3–5] and PKG stimulates, [6, 7] and PKC inhibits [3, 5] the activity of inwardly rectifying K^+ channels. In such cases, it was suggested that the PKA- or PKG-mediated phosphorylation site was different from the PKC-mediated phosphorylation site, since the effects of PKA and PKG on the channel functions were different from those of PKC. Although the PKG-mediated site is still unknown, molecular analyses have revealed that PKA-mediated and PKC-mediated sites exist in channel proteins [8, 9]. As for the effect of protein phosphatase on the activity of K^+ channels, protein phosphatase-1 (PP-1) [10] and protein phosphatase-2A (PP-2A) [10, 11] were reported to inhibit channel activity in renal tubule cells, suggesting that PKA-mediated phosphorylation was dephosphorylated by PP-1 and/or PP-2A.

It was also reported that the inhibitory effect on channel activity of PKC was mimicked by Ca^{2+}/CaM kinase II (CaMKII) in renal tubule cells [12]. However, the phosphatase which induces dephosphorylation of PKC or the CaMKII site remains unknown. Recently, we discovered that calcineurin (CaN), a Ca^{2+}/CaM-dependent protein phosphatase, had an opposite effect to CaMKII on channel activity in human renal tubule cells [12].

Based on the recent reports including our data [13], we have reviewed the functional significance and mutual effects of CaN and CaMKII on channel activity, as well as on

modulation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We will further discuss the regulatory mechanisms controlling channel activity and $[Ca^{2+}]_i$ by phosphorylation and dephosphorylation in cultured renal tubule cells, which we have used in recent studies [7, 10, 13].

2. Mutual Effects of Calcineurin (CaN) and CaMKII on K^+ Channel Activity in Human Tubule Cells

CaN is a Ca^{2+}-dependent protein phosphatase [14], often called protein phosphatase-2B (PP-2B) [15], and is known to be involved in some functions of various cells [15–17]. Since CaN induces the dephosphorylation of several phosphorylated protein serine/threonine residues [14, 17, 18], it is likely that phosphorylation induced by Ca^{2+}-dependent protein kinase, such as PKC or CaMKII, is dephosphorylated by CaN.

It was demonstrated that cyclosporin A (CyA), an inhibitor of CaN, suppressed the activity of the inwardly rectifying K^+ channels in renal tubule cells [19, 20]. We also found that inhibitors of CaN, CyA and FK520, both of which are well-known immunosuppressive agents [21], suppress K^+ channel activity in cell-attached patches of tubule cells under normal conditions [19, 20]. Since inhibitors of CaN are effective under normal conditions, CaN would be functionally activated even in a normal $[Ca^{2+}]_i$. However, the mechanism of CyA-induced channel inhibition is still unknown. To examine the involvement of Ca^{2+}-dependent protein kinase in CyA-induced K^+ channel suppression, we applied a PKC inhibitor, GF109203X [22], and a CaMKII inhibitor, KN-62 [23], and observed their effects on CyA-induced channel suppression. As the results, the CyA-induced channel suppression was not affected by GF109203X, but significantly attenuated by KN-62 [13], suggesting that the CyA-induced channel inhibition is mediated mainly by CaMKII. Although PKC may have an inhibitory effect on the inwardly rectifying K^+ channels in renal tubule cells [3, 5], our results suggest that the major candidate evoking CyA-induced channel inhibition is CaMKII [13]. Indeed, Western blot analysis revealed that CyA increased phospho-CaMKII, an active form of CaMKII [13].

The direct effects of CaN and CaMKII on channel activity were also examined in inside-out patches. Before investigation of the effects of CaN and CaMKII, we analyzed the direct effects of cytoplasmic Ca^{2+} and CaM on channel activity, since it was reported that the activity of some channels was directly affected by Ca^{2+} [24] or CaM [16]. We confirmed that Ca^{2+} (1 μM) or CaM (0.6 μM) barely affected the K^+ channels from the inside of the cell membrane, at least under our experimental conditions [13]. Moreover, the application of CaN in the presence of Ca^{2+} and CaM also induced no appreciable change in channel activity [13]. These findings suggest that CaN has no inhibitory effect on channel activity. In contrast, CaMKII markedly suppressed the channel activity. Then, we tested the direct effect of CaN on channel activity in inside-out patches in the presence of Ca^{2+}, CaM, and CaMKII. The suppressed channel activity due to CaMKII was restored following the application of CaN [13]. These results suggest that CaMKII-mediated phosphorylation has an inhibitory effect on channel activity, and CaN reactivates channels by the dephosphorylation of CaMKII-mediated phosphorylation sites. However, the phosphorylation and dephosphorylation sites of kinases and phosphatases are still unclear. It is also possible that CaN induces the dephosphorylation of phospho-CaMKII, resulting in the inhibition of CaMKII.

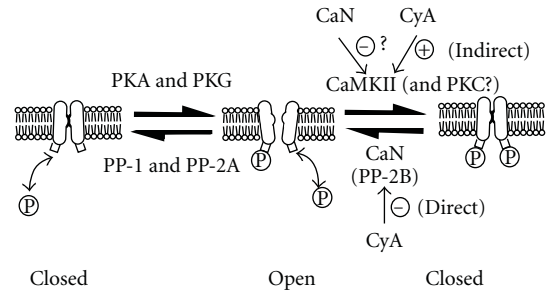

Figure 1: A schematic representation of the model for the regulation of the inwardly rectifying K^+ channels in renal tubule cells by phosphorylation and dephosphorylation processes. CaN is often called protein phosphatase-2B (PP-2B). The circled "P" indicates phosphate. Circled "+" and "–" indicate stimulation and inhibition, respectively. Small squares attached to the lower part of the channel are putative phosphorylation sites. See text for details.

A schematic representation of the mechanism of K^+ channel regulation by phosphorylation and dephosphorylation is shown in Figure 1. Previously, it was reported that PKA- and PKG-mediated phosphorylation induced channel opening (an active state) in proximal tubule cells [4–6] and that the open channels were closed by PP-1 and PP-2A [10], suggesting that PKA- or PKG-mediated phosphorylation was dephosphorylated by PP-1 or PP-2A. In addition to the above data, our findings strongly suggest that CaMKII phosphorylates other sites, resulting in the closed state of channels. On the other hand, CaN induces channel opening by the dephosphorylation of the CaMKII-mediated phosphorylation site or may inhibit CaMKII by the dephosphorylation of phospho-CaMKII. CyA directly inhibits CaN and indirectly increases the active type of CaMKII, phospho-CaMKII, through inhibition of the dephosphorylation process by CyA.

3. Role of CaN and CaMKII in Modulation of $[Ca^{2+}]_i$ in Human Tubule Cells

Our data on $[Ca^{2+}]_i$ measurement showed that both CyA and FK520, inhibitors of CaN, elevated $[Ca^{2+}]_i$ [13], similar to the data reported previously [25, 26]. Thus, it is suggested that CaN has an inhibitory effect on Ca^{2+}-release from the intracellular Ca^{2+}-store. As mentioned above, CyA-induced K^+channel suppression results from stimulation of CaMKII-dependent processes. Thus, CaMKII may be a key factor for CaN inhibitor-mediated $[Ca^{2+}]_i$ elevation in the tubule cells. The following experiments with inhibitors of both CaMKII and CaN revealed that CyA-induced $[Ca^{2+}]_i$ elevation was blocked by KN-62 [13], suggesting the importance

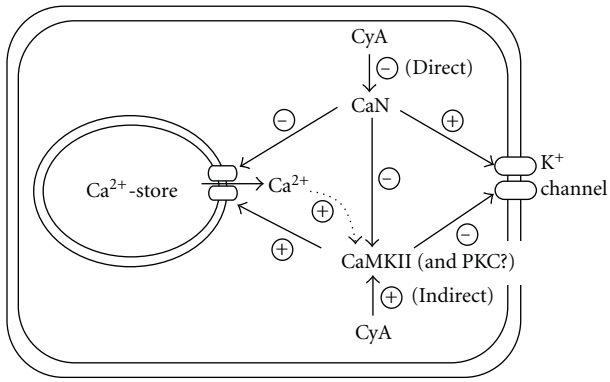

Figure 2: A simplified model of the involvement of CaN and CaMKII in the modulation of K^+ channel activity and Ca^{2+}-release channels in intracellular Ca^{2+}-stores in renal tubule cells. Circled "+" and "–" indicate stimulation and inhibition, respectively. Actions of CyA on Ca^{2+}-release channel and CaMKII are also shown. Ca^{2+} released from the Ca^{2+}-store may further enhance the activity of CaMKII, as shown by the dotted line.

of CaMKII in the elevation of $[Ca^{2+}]_i$. Similar data were demonstrated in a previous report showing that CaMKII stimulates Ca^{2+} release from Ca^{2+}-stores in skeletal muscle [27].

Figure 2 shows a simplified putative model of the mechanisms regarding Ca^{2+}-release channels in intracellular Ca^{2+}-stores and K^+ channels in the cell membrane by CaN and CaMKII. CaN activates the K^+ channel in the cell membrane and inhibits the Ca^{2+}-release channels of intracellular Ca^{2+}-stores. However, CaMKII inhibits K^+ channels and activates Ca^{2+}-release channels. Although there is a report of the role of CaN in the stimulation of Ca^{2+} flux via the 1,4,5-triphosphate receptor [28], our data strongly suggest that the inhibition of CaN stimulates Ca^{2+}-release channels [13]. Moreover as mentioned above, the inhibition of CaN-mediated dephosphorylation by CyA increases phospho-CaMKII, an active form of CaMKII. Thus, it is suggested that $[Ca^{2+}]_i$ elevation by CyA is induced mainly by increased phospho-CaMKII through activation of the Ca^{2+}-release channels of the intracellular Ca^{2+}-store. It is also conceivable that CaN exhibits an inhibitory effect on CaMKII, probably by the dephosphorylation of the phospho-CaMKII. The elevated $[Ca^{2+}]_i$ may further stimulate the activity of CaMKII, which would enhance the suppression of K^+ channel activity, as shown by the dotted curve in Figure 2. Moreover, the elevated $[Ca^{2+}]_i$ may stimulate not only CaMKII but also PKC.

4. Functional Relationship between CaN and CaMKII

Previous reports have shown that CaN plays several key roles in cellular functions. As for Ca^{2+}-release from intracellular stores, CaN is directly involved in its regulation [26], or CaN inhibitors lead to a higher probability of the ryanodine receptor (RyR)/Ca^{2+}-release channels being open [25]. On the other hand, CaMKII reportedly stimulates the Ca^{2+}-release in skeletal muscle cells [27]. Thus, it is likely that the CaMKII-mediated stimulation of Ca^{2+}-release was similar to the enhanced Ca^{2+}-release induced by the inhibition of CaN, as observed in our study [13]. These reports support our experimental data that CaN and CaMKII have opposite effects on channel activity. However, it has been reported that target phosphorylation sites dephosphorylated by CaN are mainly mediated by PKA [29]. Indeed, the functional coupling of CaN and PKA was shown to modulate Ca^{2+}-release in ventricular myocytes [29]. It has also been demonstrated that Na^+/K^+ ATPase at the basolateral membrane of kidney tubular epithelia was inhibited by CaN [30] and stimulated by PKA [31]. On the other hand, a cardiac Na^+/Ca^{2+} exchanger was reported to be regulated by CaN and PKC [32]. A cellular process dependent on mitogen-activated protein kinase was reported to be negatively regulated by CaN [33]. Thus, protein kinases opposed to CaN-mediated processes would act not in unity. Only a few reports suggested that CaMKII-mediated processes were abolished by CaN [34, 35]. Our data indicate the possibility that CaMKII-mediated phosphorylation is blocked by CaN [13], although it is still unclear whether the CaMKII-mediated phosphorylation site is identical to the CaN-mediated dephosphorylation site. It is also conceivable that the target dephosphorylation site for CaN is the phosphorylation site of CaMKII. In such a case, CaN would directly inhibit CaMKII activity by the dephosphorylation of phospho-CaMKII.

Our data suggest that CaN-mediated protein dephosphorylation would be dominant compared to CaMKII-mediated phosphorylation under normal conditions, since CaN inhibitors were found to significantly suppress K^+ channel activity, but an inhibitor of CaMKII alone did not [13]. Although the involvement of CaN in the modulation of channel activity is variable [36–39], our data suggest that the maintenance of channel opening under normal conditions would require the CaN-mediated action to exceed the CaMKII-mediated action in the tubule cells used in our study. This notion was supported by a previous report that the CaN content in proximal tubule cells is high among the several nephron segments [40]. However, it is still unknown whether the site of CaN-mediated dephosphorylation is identical to that of CaMKII-mediated phosphorylation in the modulation of channel activity. Moreover, there remains a possibility that the elevation of $[Ca^{2+}]_i$ may be dependent on the entry of extracellular Ca^{2+} across the cell membrane [41]. Recently we also demonstrated Ca^{2+} entry across the cell membrane in renal tubule cells [42], although a CyA-induced $[Ca^{2+}]_i$ elevation is considered to mainly be mediated by Ca^{2+}-release channels in the membranes of intracellular Ca^{2+}-stores. Further studies are necessary to clarify the precise mechanisms for the molecular regulation of channel activity as well as $[Ca^{2+}]_i$ by protein kinases and phosphatase, such as CaMKII and CaN.

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Review Article

Canonical and Alternative Pathways in Cyclin-Dependent Kinase 1/Cyclin B Inactivation upon M-Phase Exit in *Xenopus laevis* Cell-Free Extracts

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Cyclin-Dependent Kinase 1 (CDK1) is the major M-phase kinase known also as the M-phase Promoting Factor or MPF. Studies performed during the last decade have shown many details of how CDK1 is regulated and also how it regulates the cell cycle progression. *Xenopus laevis* cell-free extracts were widely used to elucidate the details and to obtain a global view of the role of CDK1 in M-phase control. CDK1 inactivation upon M-phase exit is a primordial process leading to the M-phase/interphase transition during the cell cycle. Here we discuss two closely related aspects of CDK1 regulation in *Xenopus laevis* cell-free extracts: firstly, how CDK1 becomes inactivated and secondly, how other actors, like kinases and phosphatases network and/or specific inhibitors, cooperate with CDK1 inactivation to assure timely exit from the M-phase.

1. Introduction

The cell cycle regulation comprises a network of numerous kinases and phosphatases. CDK1 is a major kinase necessary both for the S-phase and M-phase progression. Identification at the end of XX century of the CDK1 as a major regulator of the cell cycle made the understanding of its own regulation a fascinating topic. The use of *Xenopus laevis* cell-free extracts has had a large impact on these studies.

CDK1 belongs to the family of Cyclin-Dependent Kinases (CDKs). It has been the first CDK described in yeast (as a product of *cdc2* or *cdc28* gene depending on species) and human (called $p34^{cdc2}$). The name of CDKs comes from the association of these kinases with the regulatory subunits called cyclins. Similarly to all other kinases CDK1 has in its amino-terminal domain an xGxPxxxxREx sequence (where x represents any amino acid). This conserved region corresponds to a cyclin-binding domain [1]. There are twenty-one CDK-coding genes in the human genome [2]. However, only a few of them are involved in cell cycle regulation. In *Xenopus laevis* oocytes and early embryos a handful of CDKs are expressed (CDK1, CDK2, CDK5, CDK9; our unpublished data). Two major CDKs taking part in cell cycle regulation are CDK1 and CDK2. Only CDK1 seems to be involved in M-phase regulation, while CDK2 is a major player in S-phase progression. The similarity in three-dimensional structure of these two kinases helps to understand some aspects of their regulation.

CDK1 activity picks only for a very short period of time upon G2/M transition and falls down rapidly at the M-phase exit. The structure of CDK1 is bilobate, similar to the cyclic AMP-dependent protein kinase, but contains a unique helix-loop segment that interferes with ATP and protein substrate binding. In its monomeric inactive form, CDK1 binds to the ATP in a conformation, which prevents a nucleophilic attack by hydroxyl substrate on the β-γ phosphate bridge of ATP [3].

Cyclins, the regulatory subunits of CDKs, are encoded by at least 15 different genes in human genome [2]. Only some of them are expressed in oocytes and early embryos of *Xenopus laevis*. B-type cyclins are main regulators of CDK1. In *Xenopus laevis* early developmental stages five of B-type

cyclins (B1–B5) were identified [4]. B1 and B2 cyclins play a major role in M-phase regulation in *Xenopus laevis* since they are associated with the majority of CDK1s. Upon M-phase exit cyclins are degraded sequentially beginning from cyclin B1 and ending with cyclin B5. This sequential degradation of cyclins reflects inactivation of successive parts of CDK1. Thus just a brief look at the composition and metabolism of CDK1/cyclin B complexes illustrates the complexity of the system, and this is just the tip of the iceberg.

2. M-Phase Control in *Xenopus laevis* Oocytes via MPF, CSF, and Calcium Signalling

Amphibian oocytes have been excellent model system allowing the discovery of the basic principles of M-phase regulation. Most of the molecules regulating M-phase entry, maintaining, and exit were identified with the help of the amphibian experimental model. The enzymatic complex of CDK1 and cyclin B is the universal regulator of the M-phase. It was first discovered as an activity called Maturation Promoting Factor (MPF) by Masui and Markert in 1971 [5]. MPF drives both meiotic and mitotic cell cycle via M-phase entry induction. Masui and Markert discovered the MPF activity in experiments involving a cytoplasmic transfer between mature and immature oocytes of *Rana pipiens* and *Xenopus laevis*. Such a transfer invariably induced resumption of meiotic maturation, that is, M-phase entry in G2-arrested immature oocytes. Initially, the MPF activity was called a Maturation Promoting Factor because of its ability to induce maturation upon injection into immature oocytes. However further studies have shown that the very same factor induces also the mitotic M-phase. Thus the name of Maturation Promoting Factor was changed to M-phase Promoting Factor. Soon after, the MPF was shown to have an M-phase-inducing activity regardless of the species. This suggested the key role of this molecule in the induction of the M-phase of cell cycle in all eukaryotic cells. After numerous efforts, MPF was identified as a complex of Cyclin-Dependent Kinase 1 (CDK1) and its regulatory subunit cyclin B [6–9].

Masui and Markert [5] demonstrated the presence of another factor that stabilized MPF in MII-arrested oocytes. They called it the CSF for CytoStatic Factor. Identification of the molecular identity of CSF took more time and energy than identification of MPF. The key player in the CSF pathway was discovered recently as a protein called Early mitotic inhibitor 2 (Emi2) [10–13]. Emi2 arrests the Ubiquitin/Proteasome System (UPS) by an inhibitory association with APC/C^{Cdc20} ubiquitin ligase necessary for cyclin B ubiquitination and targeting the CDK1/cyclin B to proteasomes where cyclin B becomes degraded. Thus, CSF holds APC/C in an inactive state assuring MII arrest in oocytes, and Emi2 is the most downstream effector.

Amphibian oocytes, similarly to the majority of other vertebrate oocytes, are ovulated in MII-arrested state. Mos/. . ./ERK2 MAP kinase pathway stabilizes Emi2 during this period. The stabilization of Emi2 is achieved via phosphorylation on Thr 336, Ser 342, and Ser 344 by the most downstream enzyme of ERK2 MAP kinase pathway, the Rsk90 kinase. These phosphorylations promote Emi2-PP2A interaction and thus antagonize Emi2 phosphorylation by CDK1/cyclin B [14]. Other residues, namely T545 and T551, are phosphorylated by CDK1/cyclin B [15]. These phosphorylations are removed by protein phosphatase 2A (PP2A) assuring the turnover of Emi2 phosphorylation during the oocyte MII arrest (Figure 1).

Upon fertilization, the spermatozoon entering the oocyte induces a burst of free calcium in the ooplasm. The calcium signaling plays a major role in triggering the developmental program of the embryo. The rapid increase in calcium concentration inactivates CSF. The first step is activation of calcium-dependent kinase 2 (CaMK2), which phosphorylates Emi2. This turns Emi2 into a substrate for Plk1, the kinase which is already active in MII oocytes before fertilization, but unable to phosphorylate Emi2 before it becomes modified by CaMK2 (Figure 1). The double CaMK2- and Plk1-mediated modification of Emi2 following fertilization makes this protein recognizable by $SCF^{\beta\text{-TRCP}}$ ubiquitin ligase. $SCF^{\beta\text{-TRCP}}$ triggers Emi2 polyubiquitination followed by its proteasome-dependent degradation and disappearance from the ooplasm. This complex process is necessary to remove the CSF activity and to release the MII arrest of oocytes upon fertilization via APC/C activation and triggering cyclin B polyubiquitination [10–12].

Despite the major role of the Mos/. . ./ERK2 MAP kinase pathway resulting in Emi2 stability other alternative CSF pathways seem also involved in MII arrest induction and maintenance. For example, CDK2/cyclin E pathway was shown to induce a CSF-like arrest in Xenopus laevis cell-free extracts [16]. A checkpoint kinase Mps1 (Monopolar spindle 1) is necessary for this action of CDK2/cyclin E [16]. Further details of the role of this alternative CSF pathway and especially its elimination upon oocyte activation remain unknown.

The CSF inactivation is not the only effect of the rise in calcium concentration upon fertilization. The calcium burst also triggers a transient activation of a phosphatase called calcineurin [17, 18], which is absolutely necessary for oocyte activation. However, it is unknown whether calcineurin acts on CDK1 or on some of its substrates. Nevertheless, these results pinpoint the network of kinases and phosphatases involved in phosphorylation and dephosphorylation events of numerous proteins as major mean to regulate the cell cycle transition necessary to initiate the embryo development.

3. CDK1 Inactivation without Cyclin B Degradation

Proteolytic degradation of cyclin B via UPS plays a major role in cell cycle regulation. Its perturbation disorganizes the cell cycle progression [19–21]. Inhibition of the proteolytic activity of proteasome with inhibitors like MG115, MG132, or ALLN arrests cells in M-phase with high cyclin B content and equally high CDK1 activity. The same inhibitors block also cyclin B degradation in *Xenopus laevis* cell-free extracts; however, they neither arrest CDK1 inactivation nor provoke the M-phase arrest [22–24]. This important difference between the reaction of intact cells and cell-free extracts

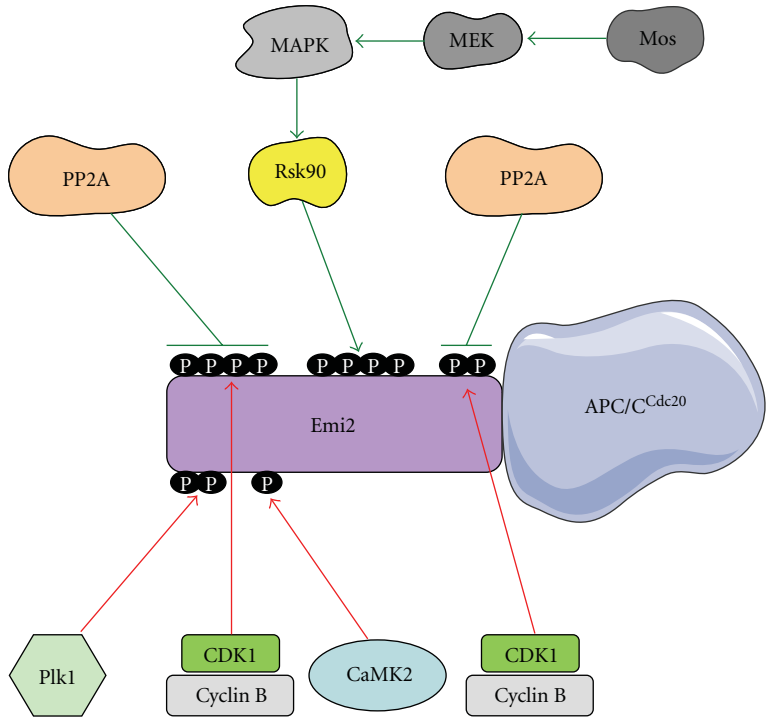

Figure 1: Regulation of Emi2 association with APC/C^{Cdc20}. Phosphorylation sites in the upper part of Emi2 are inhibitory for the association and are protected during MII arrest (green arrows and symbols of inhibition), while the sites in the bottom part of Emi2 are activatory for the association and the CSF-arrest exit (red arrows).

strongly suggests that CDK1 inactivation proceeds without cyclin B degradation at least in *Xenopus laevis* oocytes and embryo extracts. The reason for such a different reaction of intact cells and cell-free extract to proteasome inhibition remains unclear. It seems reasonable to speculate that in the case of somatic cells some upstream substrate of proteasome pathway must be degraded before cyclin B could be targeted for degradation. In this case the proteosome inhibition would inhibit cyclin B degradation indirectly, via action of remaining upstream substrate of UPS. However, the identity of a potential UPS substrate conditioning cyclin B degradation in intact cells remains unknown.

The first step in cyclin B degradation is its polyubiquitination by APC/C ubiquitin ligase (Figure 2). This process takes place when cyclin B is still associated with CDK1. Thus, APC/C-mediated polyubiquitination targets the proteasome not only cyclin B but the whole complex, which is still active when cyclin B is in the polyubiquitinated state. The proteasome induces or catalyses the dissociation of cyclin B from its CDK1 partner. Nishiyama and colleagues [22] have shown that the activity of the 26S proteasome involved in cyclin B dissociation from CDK1 is associated with its 19S regulatory subunit. It seems that the lid of the proteasome could be involved in this process. The 19S subunits of proteasome may also deubiquitinate and concomitantly denature cyclin B before it becomes loaded into the 20S proteasome catalytic chamber. This hypothetical modification of cyclin B may, as a side effect, trigger its dissociation from CDK1. Takeo Kishimoto's group named the dissociating activity of the proteasome a "nonproteolytic activity" to distinguish it from the classical proteolytic activity [22].

The APC/C activity is itself positively regulated in large part by CDK1/cyclin B-dependent phosphorylations of its numerous subunits [25, 26]. These phosphorylations trigger cyclin B polyubiquitination, dissociation from CDK1, and degradation resulting in CDK1 inactivation. In addition, the substrate specificity of APC/C changes during mitosis, due to the switch in its regulatory subunit, from Cdc20 (Fizzy) to Cdh1 (Fizzy related). This switch is possible after Cdc20 polyubiquitination by APC/C and its subsequent degradation. Thus, CDK1/cyclin B indirectly regulates itself via controlling APC/C activity at least at two different levels, which in turn determines the stability of cyclin B (reviewed in [27, 28]).

4. CDK1 Inactivation Is Inhibited by Interference with Cyclin B Polyubiquitination

Ubiquitin is a highly evolutionary conserved small (76 amino acids) polypeptide. Its COOH terminus is covalently linked to lysine residues of a substrate (e.g., cyclin B) *via* an isopeptide bond (for a review see [29]). Polyubiquitination proceeds *via* multiple rounds of ubiquitination during which the COOH terminus of a new ubiquitin molecule forms an isopeptide bond with the lysine residue of ubiquitin

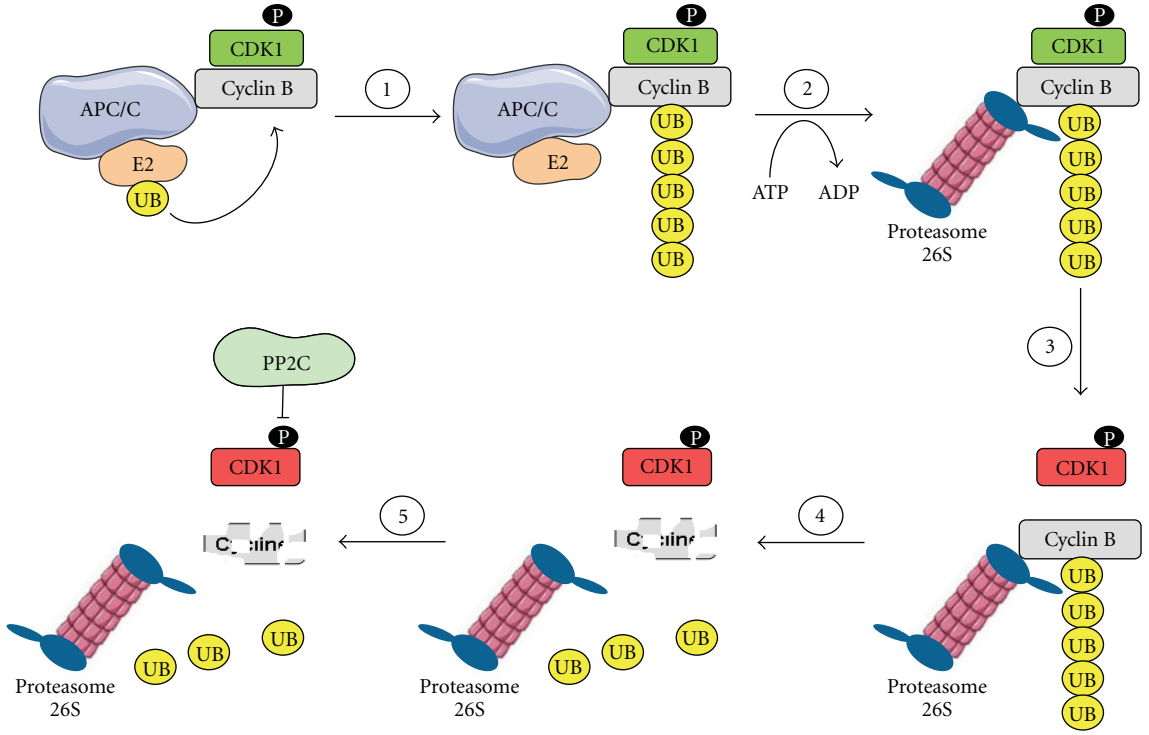

Figure 2: Canonical pathway of CDK1/cyclin B inhibition.

previously attached to the substrate. This process is mediated by three sequentially acting enzymes (E1, E2 and E3, the last being an ubiquitin ligase). Lysine 48 of ubiquitin molecule is one of the major residues involved in polyubiquitination, mediating subsequent targeting of substrates to the proteasome. However, all seven lysine residues present within the molecule of ubiquitin are able to form isopeptide bonds [29–31]. A mutation of lysine 48 (K48) to arginine (R) severely affects the process of polyubiquitination [32, 33]. Such ubiquitin mutant (UbiK48R) was used to perturb ubiquitination of proteins during the M-phase in the cell-free mitotic extracts upstream from the inhibition of the proteolytic activity of the proteasome. The interference with the polyubiquitination pathway via UbiK48R arrests cyclin B dissociation from CDK1 and its degradation [34]. Therefore, the polyubiquitination pathway appears to be necessary for the targeting of cyclin B complexed with CDK1 to the proteasome. This in turn results in the maintenance of high activity of CDK1 and keeping the extract in the M-phase. Thus UPS inhibition at the level of polyubiquitination and not at the level of the proteasome proteolytic activity inhibits effectively CDK1 inactivation. This points to the importance of cyclin B dissociation from CDK1 and not the degradation of cyclin B.

5. Alternative Pathways to Inactivate CDK1

5.1. Dephosphorylation of CDK1 Threonine 161. The phosphorylation of threonine 161 residue of CDK1 is necessary for activation of CDK1. Thus, the dephosphorylation of this site may inactivate CDK1 independently of cyclin B dissociation and proteolysis [35, 36]. The dephosphorylation is catalyzed by the okadaic-acid-(OA-)sensitive type 2C protein phosphatases (PP2Cs) [36]. The comparison of the dynamics of CDK1 inactivation with the dynamics of CDK1 Thr161 dephosphorylation upon M-phase exit has shown that the latter follows CDK1 inactivation [24]. The detailed analysis of the interaction between CDK1 and cyclin B2 (one of five cyclins B potentially present in *Xenopus laevis* early embryos) upon M-phase exit revealed that the dissociation of cyclin B2 from CDK1 perfectly correlates with the dynamics of CDK1 inactivation and not with CDK1 threonine 161 dephosphorylation [24]. Thus, CDK1 threonine 161 residue dephosphorylation is a relatively late step in CDK1 inactivation and perhaps plays a role in the ultimate switching off of the CDK1, thus protecting the cell against unscheduled and premature reactivation of the kinase after the M-phase exit.

5.2. Transient Inhibitory Phosphorylation of CDK1 on Threonine 14 and Tyrosine 15. A study in *Xenopus laevis* cell-free extracts has shown that a transient phosphorylation of CDK1 on tyrosine 15 (and most probably on threonine 14) could also participate in CDK1 inactivation. D'Angiolella and colleagues have shown that in cycling cell-free extracts the cyclin B1-associated histone H1 kinase activity diminishes more rapidly than the level of cyclin B1 protein [37]. This was not the case for cyclin A-associated activity and the diminution of the level of cyclin A protein. Detailed analysis of M-phase exiting extracts both during mitosis and

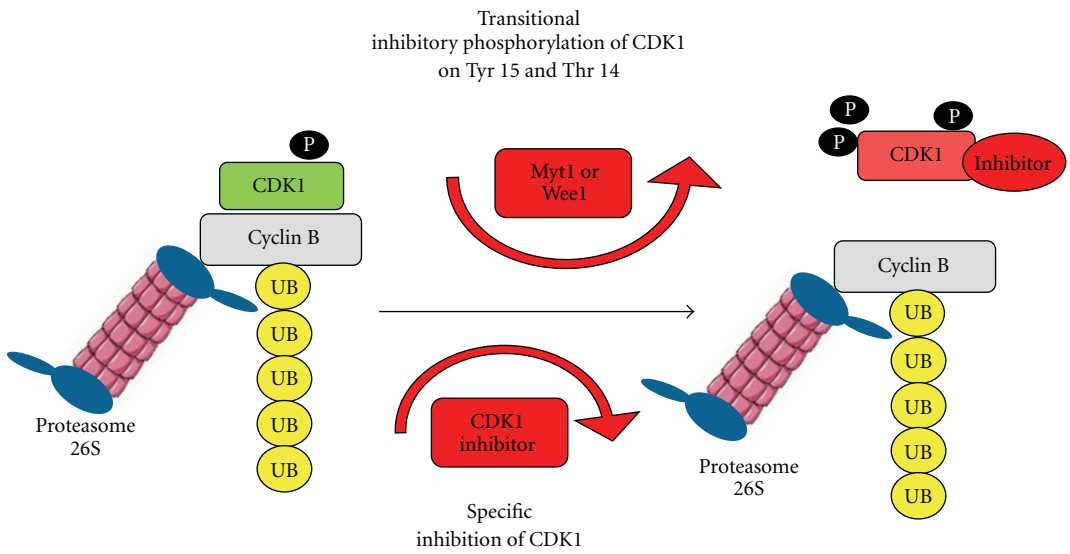

Figure 3: Hypothesis of alternative pathways involvement during CDK1/cyclin B dissociation.

meiosis (CSF extract treated with calcium) has revealed a transient rephosphorylation of cyclin B-associated CDK1 on tyrosine 15 following the initial drop in CDK1 inactivation. This short-lasting increase in tyrosine 15 phosphorylation could be mediated by a transient increase in association of CDK1 with Wee1 kinase catalysing the reaction of phosphorylation at this site. However, another study has shown that CDK1 dephosphorylation at tyrosine 15 precedes the kinase inactivation [38] (Figure 3). Thus, it is not entirely clear to what extend this kind of regulation influences the dynamics of CDK1 inactivation.

5.3. Specific Inhibitors May Participate in CDK1 Inactivation. Data from yeast and HeLa cells suggest that the specific inhibitors could also be involved in CDK1 inactivation upon mitotic exit. Cdc6 protein, well known as a key S-phase regulator, could play this role [39, 40] (Figure 3). There is no data supporting such a role of Cdc6 in *Xenopus laevis* cells. However, since Cdc6 was shown to inhibit CDK1 both in yeast and in human cells it seems possible that it also plays the same role in amphibians. CDK1 inhibition specifically during the M-phase exit may lead, together with the transient CDK1 phosphorylation on tyrosine 15, to more rapid and more effective inhibition of CDK1.

5.4. Cooperation of CDK2 and PKA in CDK1 Inactivation. The next alternative pathway of CDK1/cyclin B inactivation implies the participation of CDK2. It was shown that CDK2/cyclin E enables to maintain the high CDK1/cyclin B activity during mitosis in *Xenopus laevis* cell-free extract [41]. Premature inactivation of CDK2 during the mitotic M-phase induces an increase in protein kinase A (PKA) activity and speeds up CDK1/cyclin B inactivation and cyclin B degradation. Thus, CDK2/cyclin E seems to be coupled with PKA activity in assuring a correct timing of CDK1/cyclin B inactivation and the M-phase exit. However, it remains unknown how CDK2 and PKA act in concert on CDK1 and cyclin B degradation pathway.

Crystallographic studies of another CDK2 enzymatic complex, namely, CDK2/cyclin A that has fundamental role in S-phase regulation, have shown details of the mechanism underlying inactivation of this kinase [2, 42]. CDK1/cyclin B complex has never been studied with such accuracy. As the two complexes are closely related the mechanism proposed for CDK2 may also apply to CDK1. The active site of CDK1 could undergo conformational changes in its PSTAIRE helix and T-loop upon the dissociation of cyclin B, as it happens with CDK2 upon dissociation of cyclin A. Such a conformational change prevents proper interaction of the enzyme with ATP and inactivates the kinase [2, 42]. The cyclin subunit determines substrate specificity of CDKs (reviewed in [43]). Thus, the loss of cyclin subunit should be immediately followed by efficient inactivation of CDK1 to protect the cell against possible phosphorylation of undesired substrates. A part of this job may be performed by the transient inhibitory phosphorylation of tyrosine 15. Another possibility to eliminate unspecific activity of cyclin-free CDK1 would be a direct inhibition via its association with a specific inhibitor, for instance, Cdc6.

6. Kinase/Phosphatase Network upon M-Phase Exit

In HeLa cells CDK1 inactivation is not sufficient to assure the successful transition to interphase. The efficient transition to interphase requires a phosphatase activity (or activities) dephosphorylating CDK1 mitotic substrates [44]. The activation of these phosphatases is clearly proteasome-dependent, but independent of cyclin B degradation. PP2A was shown to be the major phosphatase dephosphorylating CDK1 substrates in interphase *Xenopus laevis* eggs extract [45]. PP2A is a heterotrimer composed of the catalytic C-,

scaffolding A-, and regulatory B-type subunits represented by different isoforms. The B-type subunits are responsible for the substrate specificity of the whole complex [46]. Mochida and colleagues [45] have shown that PP2A containing the B55δ subunit is the major phosphatase controlling the exit from the M-phase via dephosphorylation of CDK1 phospho-substrates. It was proposed that the newly discovered Greatwall kinase could play a role of a phosphatase suppressor [47]. Greatwall kinase was discovered in a screen for *Drosophila* mutants defective in chromosome condensation [48]. Greatwall is a ubiquitous evolutionarily conserved protein kinase, known in humans as MAST-L kinase, belonging to the AGC family of Ser/Thr kinases [49]. Depletion of this kinase from M-phase extracts induced activation of an okadaic-sensitive phosphatase that acts on CDK1 substrates and on the mitotic exit. Addition of this kinase to interphase extracts inhibited dephosphorylation of CDK1 substrates [50, 51]. Moreover, the inability of Greatwall-depleted cell-free extracts to enter M-phase was reverted by removal of PP2A-B55 delta [50]. It was shown recently by two independent laboratories that a small protein called cAMP-regulated phosphoprotein-19 (ARPP-19), a close relative of another small protein alpha-endosulfine (ENSA), was an ideal substrate for Greatwall kinase in *Xenopus laevis* cell-free extracts [52, 53]. Thus, the network of kinases and phosphatases governing CDK1 substrates dephosphorylation, and thus, the transition to interphase following the M-phase was discovered.

7. Conclusions

Several pathways control CDK1 inhibition upon M-phase exit. The major pathway called here canonical involves dissociation of cyclin B from CDK1 and is followed by cyclin B degradation and disappearance from the cell. Other probably minor pathways, including phosphorylation and dephosphorylation of CDK1 at different sites, and active inhibition of CDK1 kinase activity, may play supplementary role in shortening the process of CDK1 inactivation. This hypothetical role of CDK1 accelerator could be of particular importance for very fast cleaving embryos such as amphibian embryos. CDK2/cyclin E and PKA seem also to exercise an important control over the timing of CDK1/cyclin B inactivation. The alternative pathways in CDK1/cyclin B inactivation may be important in certain unique conditions when the canonical pathway becomes ineffective. For example, in rat one-cell embryos treated with MG132 the M-phase exit probably occurs without cyclin B degradation [54]. Also in mouse oocytes undergoing maturation and fertilized in vitro by numerous spermatozoids CDK1/cyclin B is inactivated rather via threonine 161 dephosphorylation than full cyclin B degradation [55]. Surprisingly, recent studies of the minimal control of CDK network in fission yeast suggest that modulation of CDK1 activity and not its proteolytic or phosphorylation-dependent regulation could play an ancestral role during evolution [56]. This discovery will certainly stimulate further studies on noncanonical pathways regulating M-phase exit, and it is possible that the canonical pathway will become noncanonical and vice versa.

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Review Article

Protein Kinase C and Toll-Like Receptor Signaling

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Protein kinase C (PKC) is a family of kinases that are implicated in a plethora of diseases, including cancer and cardiovascular disease. PKC isoforms can have different, and sometimes opposing, effects in these disease states. Toll-like receptors (TLRs) are a family of pattern recognition receptors that bind pathogens and stimulate the secretion of cytokines. It has long been known that PKC inhibitors reduce LPS-stimulated cytokine secretion by macrophages, linking PKC activation to TLR signaling. Recent studies have shown that PKC-α, -δ, -ε, and -ζ are directly involved in multiple steps in TLR pathways. They associate with the TLR or proximal components of the receptor complex. These isoforms are also involved in the downstream activation of MAPK, RhoA, TAK1, and NF-κB. Thus, PKC activation is intimately involved in TLR signaling and the innate immune response.

1. Introduction

Protein kinase C (PKC) is a family of protein serine /threonine kinases centrally involved in intracellular signal transduction. The PKC isoforms are divided into 3 subfamilies based on their activation requirements: the conventional isoforms, PKC-α, -βI, -βII, and -γ, require calcium, diacylglycerol, and phosphatidylserine; the novel isoforms, PKC-δ, -ε, -η, and –θ, require diacylglycerol and phosphatidylserine but are calcium independent; the atypical isoforms, PKC-ζ and λ/ι, require only phosphatidylserine [1]. Different isoforms of PKC are involved in such pivotal functions as cell growth, differentiation, apoptosis, motility, and secretion. Accordingly, these enzymes have been implicated in many disease states including cancer and cardiovascular disease [2–6]. The role of PKC in cancer is complicated by the tissue-specific, and often opposing, effects of the different isoforms on cell cycle and apoptosis. Similarly, the role of PKC in heart disease is complex because components of the disease (myocyte hypertrophy, cardiac function, fibrosis, and inflammation) are influenced in different ways by the different isoforms.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are critical for the effective innate immune response to infection [7–9]. Signaling from different TLRs varies but is initiated by the recruitment of TIR-containing adaptor proteins (e.g., TIRAP recruits MyD88, TRAM recruits TRIF). MyD88 recruits IRAK4, IRAK1, IRAK2, and TRAF6. Phosphorylation and degradation of IRAK1 releases this complex into the cytoplasm where it binds and activates TAK1 downstream. Through an as yet unknown, and possibly indirect, mechanism, TAK1 activates the IKKβ → IkB-α → NF-κB pathway for induction of proinflammatory genes. TAK1 also activates the MAPK cascades that influence gene expression. TLR binding of TRIF recruits TRAF6, βRIP1, and TAK1 for activation of MAPK, IRF3, NF-κB, and transcription of interferon-β. The TRIF pathway also stimulates the secretion of proinflammatory cytokines although to a lesser degree than the MyD88 pathway.

Initial evidence for the involvement of PKC in TLR signaling came from observations that altering PKC activity in cells of the innate immune system affected cytokine secretion. Subsequently, LPS and other TLR ligands were shown to activate most of the PKC isoforms expressed in monocytes, macrophages, dendritic cells, and neutrophils [10–14]. A large number of studies have shown that pharmacological inhibition of PKC, or its depletion by long-term treatment with phorbol esters, decreases LPS-stimulated

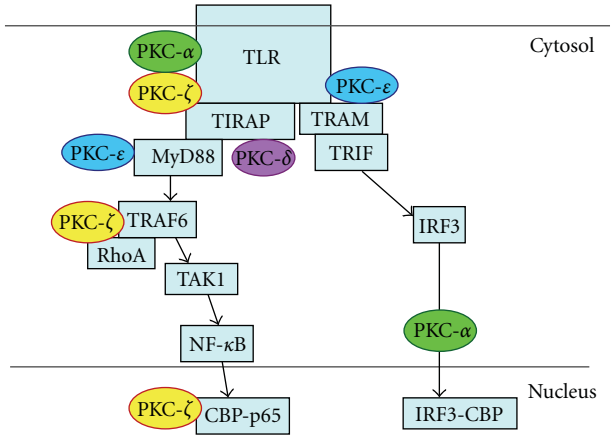

Figure 1: PKC isoforms act at many levels in TLR signaling. PKC-α associates with the TLR2 signaling complex in a MyD88-dependent manner. PKC-α is also required for TLR3-mediated IRF-3 binding to CBP and IFN-β gene induction. Interaction of PKC-δ with TIRAP is required for the downstream activation of NF-κB. PKC-ε associates with complexes of MyD88 and TLR2 or TLR4 and is necessary for downstream signaling. PKC-ε is also required for the phosphorylation of TRAM. PKC-ζ is activated upon ligation of TLR2 or TLR4. During signaling by these receptors, active PKC-ζ binds to TLR2, associates with TRAF6 and RhoA, and is required for full transcriptional activation of p65.

cytokine secretion [10, 15–17]. Accordingly, acute activation of PKC with phorbol esters increases cytokine secretion [11, 15, 17, 18].

Results from studies performed over the last decade have identified four PKC isoforms that impact different steps in TLR signaling. This paper will summarize recent advances detailing the role of PKC-α, -δ, -ε, and -ζ in activation of the initial TLR signaling complex, activation of RhoA, and transcription factors (Figure 1).

2. PKC Isoforms Involved in TLR Signaling and Host Defense

2.1. PKC-α. Conventional PKC isoforms have been implicated in cytokine secretion by several studies showing that Gö6976, which inhibits PKC-α and β, blocks TLR-stimulated cytokine secretion by macrophages [14, 19–22]. A series of studies found that 264.7 RAW macrophages expressing a dominant negative (DN) PKC-α have reduced LPS-stimulated TNF-α, IL-1β, iNOS, and NF-IL6 (CAAT/enhancer-binding protein β) induction [23, 24]. These cells also had defects in phagocytosis, phagosome maturation, and killing of intracellular pathogens, suggesting that PKC-α is also involved in other, non-TLR-mediated aspects of innate immunity [25–28]. A further link between PKC-α and TLR-mediated responses is the finding that PPARγ modulates phorbol ester-induced NF-κB activation and TNF-α secretion by preventing the activation of PKC-α [18].

Findings by Langlet et al. [29] revealed that, in human DC, PKC-α inhibition blocked IL-12p40 secretion induced by TLR2/6, TLR 2/1, TLR5, and IL-1R, but not TLR3. The role of PKC-α was dissected using Gö6976, expression of DN PKC-α, and DC from PKC-$\alpha^{-/-}$ mice. Using these approaches, it was determined that activation of PKC-α is required for TLR2/1-mediated activation of MAPK, NF-κB, and AP-1 as well as secretion of TNF-α, IL-6, and IL-10 by DC. That immunoprecipitation of PKC-α from TLR2/1-activated DC captured TLR2 provides additional evidence that PKC-α is linked to TLR2 signaling. As TLR2 was not found in PKC-α immunoprecipitates of cells from MyD88$^{-/-}$ mice suggests that MyD88 links TLR2 with PKC-α.

Johnson et al. [30] found that poly (I : C), a TLR3 ligand, activated PKC-α. Downregulation of PKC-α with siRNA, or expression of DN PKC-α, blocked TLR3-stimulated IFN-β production in dendritic cells (DCs). Interfering with PKC-α activity did not change the activation of IRF3 in terms of phosphorylation, dimerization, nuclear translocation, or DNA binding but did inhibit IRF-3 transcriptional activity induced by TRIF and TBK1 overexpression. This latter effect is due to decreased IRF-3 binding to the coactivator, CREB binding protein (CBP) which requires PKC-α activation.

2.2. PKC-δ. Several studies have implicated PKC-δ in TLR-mediated cytokine secretion [12, 31–34]. Inhibition of PKC-δ with Rottlerin, or its downregulation, consistently decreases activation of NF-κB, secretion of inflammatory cytokines, and production of nitric oxide by cells of the innate immune system. Results from Kubo-Murai et al. [35] suggest that PKC-δ is involved in TLR signaling through its interaction with TIRAP. Specifically, PKC-δ in macrophage lysates bound to immobilized TIRAP via the TIR domain of TIRAP. Additionally, PKC-δ depletion results in the loss of kinase activity in immobilized TIRAP implicating PKC-δ as the relevant kinase for propagating signaling from the TIRAP complex. That downregulation of PKC-δ severely depresses the activation of p38 MAPK and NF-κB by ligands for TLR4, and TLR2 underscores its importance in signaling via these receptors. To date, this is the only report demonstrating a direct role for PKC-δ in TLR signaling.

Cecal ligation and puncture (CLP) is an established animal model for sepsis and has been used to study TLR signaling *in vivo*. Recently, it was shown that, when administered intratracheally, a PKC-δ-inhibiting peptide reduced the lung injury associated with CLP-induced sepsis in rats [36]. This inhibitor blocked sepsis-induced phosphorylation of PKC-δ. Animals given the inhibitor prior to CLP had reduced levels of the chemokines CINC-1 and MIP-2 in lung lavage and blood samples. At the same time, there was reduced infiltration of inflammatory cells into the lungs and less pulmonary edema. The authors linked the protective effects of the inhibitor to reduced NF-κB activation and chemokine production by macrophages, endothelial cells, and epithelial cells [37–39]. This is consistent with a previous study showing that PKC-$\delta^{-/-}$ mice had reduced cytokine production, neutrophil infiltration, and lung injury due to asbestos [40].

Sphingosine kinase 1 (SphK1) has been implicated in inflammation through the formation of sphingosine 1 phosphate [41–44]. SphK1 is induced and activated by several immune stimuli including LPS. In LPS-treated macrophages, PKC-δ lies downstream of SphK1 activation and is required for the activation of NF-κB [37]. Furthermore, the inhibition of SphK1 *in vivo* reduced LPS- and sepsis-stimulated cytokine secretion and mortality. These studies support a TLR4 → SphK1 → PKC-δ → NF-κB → cytokine pathway. It remains to be determined if the same pathway links SphK1 to TNFR1 and IL-1R [43, 44].

Beyond the scope of this paper are the effects of this PKC isoform in several TLR-independent aspects of inflammation including signaling via TNFR1, neutrophil activation, and endothelial cell function [43–48].

2.3. PKC-ε. A role for PKC-ε in host defense was apparent when Castrillo et al. [49] observed that PKC-$\varepsilon^{-/-}$ mice were difficult to breed due to infections of the uterus. Additionally, these animals had a 2- to 3-fold reduction in survival time after infection with Gram-negative or Gram-positive bacteria. Several aspects of the immune system in these animals were normal although macrophages were defective in the production of LPS-stimulated TNF-α, IL-1β, PGE_2, and nitric oxide. There were also deficits in LPS-stimulated MAPK and NF-κB activation. Other studies, using PKC-ε-specific inhibitors, depletion with antisense oligonucleotides, and macrophages from PKC-$\varepsilon^{-/-}$ mice, verified that PKC-ε is critical for LPS-stimulated TNF-α and IL-12 secretion by DC and macrophages [10, 50, 51]. *In vivo* administration of a PKC-ε inhibitor reduced the inflammation associated with a murine model of cardiac transplantation [52]. Thus, PKC-ε has a likely role in inflammation and host defense [53, 54].

More recent studies have sought to clarify the role of PKC-ε in TLR signaling. PKC-ε is phosphorylated by all TLRs that signal through MyD88, that is, TLR1 through 9 except TLR3 in macrophages [55]. Upon TLR4 ligation with LPS, PKC-ε is phosphorylated on Ser-346 and 368 and binds to 14-3-3β. Association with 14-3-3β requires the presence of MyD88. Phosphorylation of these serines is critical for PKC-ε signaling as cells expressing PKC-ε S346A/S368A fail to activate an NK-κB reporter in response to ligands for TLR4 and TLR2. These findings suggest that the complex of TLR, MyD88, 14-3-3β, and PKC-ε is required for gene induction. Of note, since PKC-ε can be phosphorylated by PKC-α, it is possible that the effects of PKC-α on cytokine secretion are shared by PKC-ε [56, 57].

In addition to its role in MyD88-dependent signaling, PKC-ε is also involved in TLR4 activation via the TRAM pathway. The TRAM pathway primarily stimulates the production of IFN-β and RANTES. Phosphorylation of TRAM in LPS-stimulated macrophages allows it to dissociate from the membrane and bridge TLR4 with TRIF. McGettrick et al. [11] found that recombinant PKC-ε, but not PKC-ζ, phosphorylates TRAM on Ser-16, and $TRAM^{-/-}$ macrophages reconstituted with TRAM S16A do not signal. That this phosphorylation of TRAM did not occur in PKC-$\varepsilon^{-/-}$ cells and that macrophages from PKC-$\varepsilon^{-/-}$ mice have reduced production of IFN-β places PKC-ε in the TLR4 → PKC-ε → Ser-16 TRAM → IFN-β pathway.

2.4. PKC-ζ. This atypical PKC isoform is a component of the signaling pathways for IL-1R and TNFR [58–60]. More recently, PKC-ζ has been shown to be involved in the activation of TLR, IRAK, RhoA, and NF-κB [61–64].

PKC-ζ is downstream of TLR2 in human macrophages stimulated with *Mycobacterium tuberculosis* (MTB) [64]. Specific inhibitors of PKC-ζ or its downregulation blocked ERK activation and TNF-α secretion stimulated by MTB. PKC-ζ was also found to associate with immunoprecipitated TLR2 but not TLR4 after stimulation with MTB or peptidoglycan. Finally, PKC-ζ did not associate with TLR2 in THP-1 cells expressing DN PKC-ζ. These results suggest a TLR2 → PKC-ζ → ERK pathway for production of TNF-α in response to MTB.

IRAK phosphorylation is an early event in TLR signaling. Phosphorylated IRAK is subsequently degraded which acts as a negative feedback control on the signaling pathway. Using a panel of protein kinase inhibitors, Hu et al. [61] demonstrated that the phosphorylation of IRAK by TLR4 is mediated by PKC in THP-1 cells. PKC-ζ was found in IRAK immunoprecipitates from LPS-stimulated cells. Furthermore, macrophages from wild-type mice responded to LPS with activation of PKC-ζ and phosphorylation of IRAK, but macrophages from C3H/HeJ mice that have a nonfunctional TLR4 did not. Although not yet directly tested, these results suggest that IRAK is a PKC-ζ substrate.

Several studies have highlighted a role for RhoA in TLR signaling [65–69]. Two papers have directly implicated PKC-ζ in TLR2 and TLR4 signaling through the activation of RhoA and subsequent activation of NF-κB [62, 63]. Teusch et al. [63] found that full transcriptional activation of p65 by TLR2 ligands in monocytic cells requires the activity of both RhoA and PKC-ζ. Inhibition of PKC-ζ, or expression of its dominant negative mutant, reduces the phosphorylation of p65 on serine 311 and its transcriptional activity. PKC-ζ transiently associates with RhoA with a time course similar to that of RhoA activation. The authors concluded that PKC-ζ mediates at least part of the effects of RhoA on gene transcription.

Further refinement of the position of PKC-ζ in the TLR → RhoA → NF-κB pathway has been elucidated by the studies of Huang et al. [62]. They reported that treatment of macrophages with LPS activates PKC-ζ and that interfering with PKC-ζ activation blocks LPS-stimulated activation of NF-κB and cytokine production. As in the TLR2 studies above, PKC-ζ was present in anti-RhoA or anti-TRAF6 immunoprecipitates of cell lysates from LPS-stimulated macrophages. Inhibition of RhoA or TRAF6 blocked PKC-ζ activation, placing PKC-ζ downstream of RhoA and TRAF6. Similar experiments place TAK1 between PKC-ζ and NF-κB. That is, inhibition of PKC-ζ blocked TAK1 phosphorylation, and constitutively active PKC-ζ fails to activate NF-κB in TAK1-depleted cells. Together, these studies are consistent with the following model: TLR2/4 → RhoA/TRAF6 → PKC-ζ → TAK1 → s311 p65 → cytokine induction.

Cigarette smoke is a major cause of lung inflammation which is exacerbated by its common contamination with LPS. Yao et al. [70] recently demonstrated that PKC-$\zeta^{-/-}$ mice exposed to smoke/LPS had less lung inflammation than wild-type mice. Bronchoalveolar cells from mice exposed to smoke/LPS had activated PKC-ζ that translocated to the nucleus in association with p65 and CBP. This was associated with the phosphorylation (Ser311) of p65 and induction of cytokines. All of these events were reduced in PKC-$\zeta^{-/-}$ mice.

Surfactant protein A (SP-A) limits several aspects of inflammation in the lung, partly due to stabilization of IkB-α [71]. Moulakakis et al. [72] found that PKC-ζ is required for this effect. SP-A activates PKC-ζ, limits its translocation to the nucleus, and stabilizes IkB-α. In alveolar macrophages from PKC-$\zeta^{-/-}$ mice, these protective effects were lost. That is, in PKC-$\zeta^{-/-}$ macrophages, SP-A failed to inhibit LPS-stimulated IkB-α activation, and NF-κB DNA binding was nearly normal. It was concluded that the effect of SP-A $\rightarrow$ PKC-ζ axis may function as a brake on the inflammatory response of alveolar macrophages.

3. Perspective

The establishment of PKC in TLR signaling as well as in other inflammatory processes may provide a means to treat inflammatory diseases. Inhibition of a kinase required for so many important biological functions may be expected to result in substantial side effects. However, several inhibitors of PKC have proven to be well tolerated in clinical trials [73–75].

The studies detailed above provide convincing evidence that PKC is directly involved in TLR signaling. In most cases, further studies are needed to determine the nature of the interaction between PKC isoforms and other proteins in the signaling cascades and then to identify the specific PKC substrates. Another important area of investigation is the integration of the TLR $\rightarrow$ PKC axis with the involvement of these same isoforms (PKC-α, -δ, and -ζ) and possible interaction of the isoforms in non-TLR-dependent aspects of innate immunity. PKC-α may contribute to phagocytosis and pathogen killing [25–28], PKC-δ is involved in signaling via TNFR1, neutrophil activation, and endothelial cell function [43–48], and PKC-ζ participates in signaling through TNFR and IL-1R [58–60]. Thus, PKC isoforms likely sit at different nodes in the signaling web. Understanding their contribution to individual pathways as well as their position in the web is necessary to avoid misinterpreting results of experiments such as one in which a PKC inhibitor appears to reduce the TNF-α response to a TLR ligand, but, in fact, the inhibitor blocked the secondary augmentation of TNF-α secretion mediated through the TNFR. Finally, understanding the role of TLR-mediated activation of specific PKC isoforms will immediately provide insights into the mechanisms of the disease.

One of the more important advances during the 20th century was the alleviation of a great deal of the suffering from infectious diseases. We may hope for a similar advance with regard to inflammatory diseases in the 21st century.

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Review Article

Protein Kinases and Phosphatases in the Control of Cell Fate

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Protein phosphorylation controls many aspects of cell fate and is often deregulated in pathological conditions. Several recent findings have provided an intriguing insight into the spatial regulation of protein phosphorylation across different subcellular compartments and how this can be finely orchestrated by specific kinases and phosphatases. In this review, the focus will be placed on (i) the phosphoinositide 3-kinase (PI3K) pathway, specifically on the kinases Akt and mTOR and on the phosphatases PP2a and PTEN, and on (ii) the PKC family of serine/threonine kinases. We will look at general aspects of cell physiology controlled by these kinases and phosphatases, highlighting the signalling pathways that drive cell division, proliferation, and apoptosis.

1. Introduction

Cells are exposed to several extracellular signals simultaneously. Maintaining the fidelity of intracellular transduction systems, resulting in the amplification of specific biological responses, is crucial in eliciting the appropriate physiological response. The accurate selection of effector molecules is required, and these molecules must be finely regulated in their activation and deactivation, often by phosphorylation and dephosphorylation events. Protein phosphorylation is a reversible posttranslational modification that plays key roles in several physiological processes and is often deregulated in pathological conditions. The balance between activation and deactivation of signalling pathways is a delicate one, which is regulated not only through phosphorylation by kinases, but also through dephosphorylation events induced by a diverse range of phosphatases. The majority of oncogenes identified thus far encode protein kinases, and dysregulation in their activity is required for cancer initiation and maintenance. Intuitively, by counterbalancing the activity of kinases, phosphatases should primarily act as tumour suppressors [1, 2]. Approximately one-third of proteins encoded by the human genome are presumed to be phosphorylated during their life cycle, accounting for an estimated 100,000 different phosphorylation sites in a cellular proteome [3]. The structural changes imparted by the phosphorylation of specific residues afford exquisite mechanisms for the regulation of protein functions by modulating protein folding, substrate affinity, stability, and activity. In many cases, phosphorylation results in switch-like changes in protein function, which can also bear to major modifications, that is, in the catalytic function of other enzymes, including kinases. Moreover, protein phosphorylation often leads to a structural change of the protein that can induce changes in interaction partners or subcellular localization.

Phosphorylation acts as a molecular switch for many regulatory events in signalling pathways that drive cell division, proliferation, differentiation, and apoptosis. One of the main strategies for achieving the proper outcome is the compartmentalization of both protein kinases and phosphatases, to ensure an appropriate balance of protein phosphorylation [4]. The spatial distribution of kinases and phosphatases implies that a gradient of phosphorylated substrates exists across different subcellular compartments. This spatial separation not only regulates protein phosphorylation but can also control the activity of other proteins, enzymes, and the transfer of other posttranslational

modifications. The most important routes showing the fundamental importance of subcellular localization of kinases and phosphatases are perhaps the mitogen-activated protein-kinase- (MAPK-) mediated pathways, the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR)-dependent signalling, as well as those involving protein kinase A (PKA) and protein kinase C (PKC), both of which act as key transducers in many signalling cascades. These signalling pathways (MAPK, PI3K/Akt/mTOR, PKA, PKC) interact at numerous levels and at multiple intracellular sites to regulate many fundamental cellular processes. Crosstalk between signalling pathways is a common theme in cell regulation, which usually depends on cell context and plays an important role in fine-tuning the biological responses. It is now well established that MAPK and PI3K are two of the most predominant oncogenic routes, and they are intimately linked together [5, 6]. The signal transduction ensuing from these pathways is complicated by a remarkable number of interconnections [7] (Ras-MAPK pathway modulates PI3K pathway at multiple levels: MAPK—c-Jun—PTEN, Ras—PI3K, ERK—TSC2, RSK—TSC2, RSK—S6, RSK—eIF4B, and so does MKK4—JNK pathway through the activation of NF-κB; for a recent review see [8]). Both MAPK and PI3K pathways may result in the phosphorylation of many downstream targets and impose a role in the regulation of cell survival and proliferation. Overexpression of these pathways in acute myeloid leukemias (AML) has been associated with a worse prognosis than overexpression of a single pathway [9], while activation of the MAPK (Raf/MEK/ERK) cascade is suppressed in some prostate cancer cell lines which express high levels of activated Akt [10, 11]. Hyperactivation of Akt has been found in cellular models of prostate cancer, such as LNCaP cells, as well as in prostate cancer specimens, particularly in advanced stages of the disease [12]. It has been demonstrated that in prostate cancer, PI3K-Akt survival pathway could be affected also by PKC activation. Indeed, PKC promotes apoptosis in LNCaP cells through activation of p38 MAPK, and inhibition of the Akt survival pathway [13]. Also PKA can act as a central hub that interacts with a variety of other signaling pathways, not only mediating but also communicating cAMP effects to Akt, PKC, MAPK and other pathways [14]. Recent studies indicated that cAMP-dependent signalling is closely interwoven with the PI3K/Akt pathway [15]. Akt is of tremendous importance for several neuronal key signalling events, including cell differentiation, proliferation, and survival [16]. Neuronal survival and axonal regeneration mediated by PI3K-dependent Akt signalling were shown to be induced by elevated cAMP levels [15, 17]. Finally, it is important to state that several of these major protein kinases in the cell, in particular Akt, PKC, and ERK MAP kinases, are substrates for the protein phosphatase 2a (PP2a), which appears to be the major phosphatase in eukaryotic cells that downregulates activated protein kinases. Thus, PP2a is likely to play an important role in determining the activation of protein kinase cascades [18].

In the first part of this paper we will focus on the PI3K/Akt/mTOR pathway, specifically on the kinases Akt and mTOR, along with the phosphatases PP2a and PTEN (phosphatase and tensin homolog deleted on chromosome 10). The PI3K/Akt/mTOR signalling cascade is crucial to many widely divergent physiological processes which include cell cycle progression, transcription, translation, differentiation, apoptosis, motility, and metabolism [19]. After the p53 pathway, the PI3K/Akt/mTOR signalling pathway is one of the most mutated pathways associated with human tumour and contributes to both cancer pathogenesis and therapy resistance [20]. The binding of insulin, insulin-like growth factor-1 (IGF-1), and other growth factors to its related receptors can activate/phosphorylate PI3K, which catalyses the synthesis of the lipid phosphatidylinositol 3,4,5-trisphosphate (PIP3). These lipid products in turn interact with proteins via their pleckstrin homology (PH) domain, which allows the recruitment of other signalling molecules to the cell membrane [21]. Akt and phosphoinositide-dependent kinase-1 (PDK1) are PI3K's main downstream effectors: they contain a C-terminal PH domain, which binds the membrane-bound PIP3. Here, Akt is activated through phosphorylation mediated by PDK1 [22] and by the mammalian target of rapamycin complex 2 (mTORC2) [23]. In turn, activated Akt phosphorylates many target proteins to regulate a broad range of cellular processes which include cell survival, growth, proliferation, angiogenesis, metabolism, and migration. This pathway is activated in many tumours as a result of amplification/overexpression of PI3K (either PI3CA or the p85 subunit) [24], or of the amplification/overexpression/mutation of Akt [25]. Moreover, a tight counterregulation by phosphatases has emerged as a crucial process to control PI3K/Akt/mTOR-dependent signalling. Elevated Akt activation in human cancers can result from its enhanced phosphorylation due to loss of the PTEN tumour suppressor [26] or PP2a, which is now considered to be a tumour suppressor as well since it can also dephosphorylate Akt and thus downregulate its activity [27]. Evidence accumulated over the past years has highlighted the presence of different players of this intricate pathway in almost all the different subcellular compartments (namely, cytosol, plasma membrane, nucleus, mitochondria, endoplasmic reticulum (ER) and mitochondrial-associated membranes (MAMs)), where they can both modulate each other and act on a plethora of different substrates (Figure 1). In the following sections, we will discuss these events and how they are implicated in the control of cell fate.

In the final part, we will address more in depth the PKC family of serine/threonine kinases that, when activated, can be translocated from one intracellular compartment to another, thus being able to affect a wide variety of cellular processes. PKC is one of the most extensively studied kinase families and has been implicated in cell proliferation, differentiation, apoptosis, tumour promotion, and neuronal activity [28]. There are 12 isoforms of PKC termed conventional (PKCα, PKCβ and PKCγ), novel (PKCδ, PKCε, PKCη and PKCθ), atypical (PKCζ and PKCλ), and PKN and PKC-related (PKN1, PKN2 and PKN3) forms. There is strong evidence that this family of kinases can be related to the PI3K/Akt/mTOR signalling pathway at some levels.

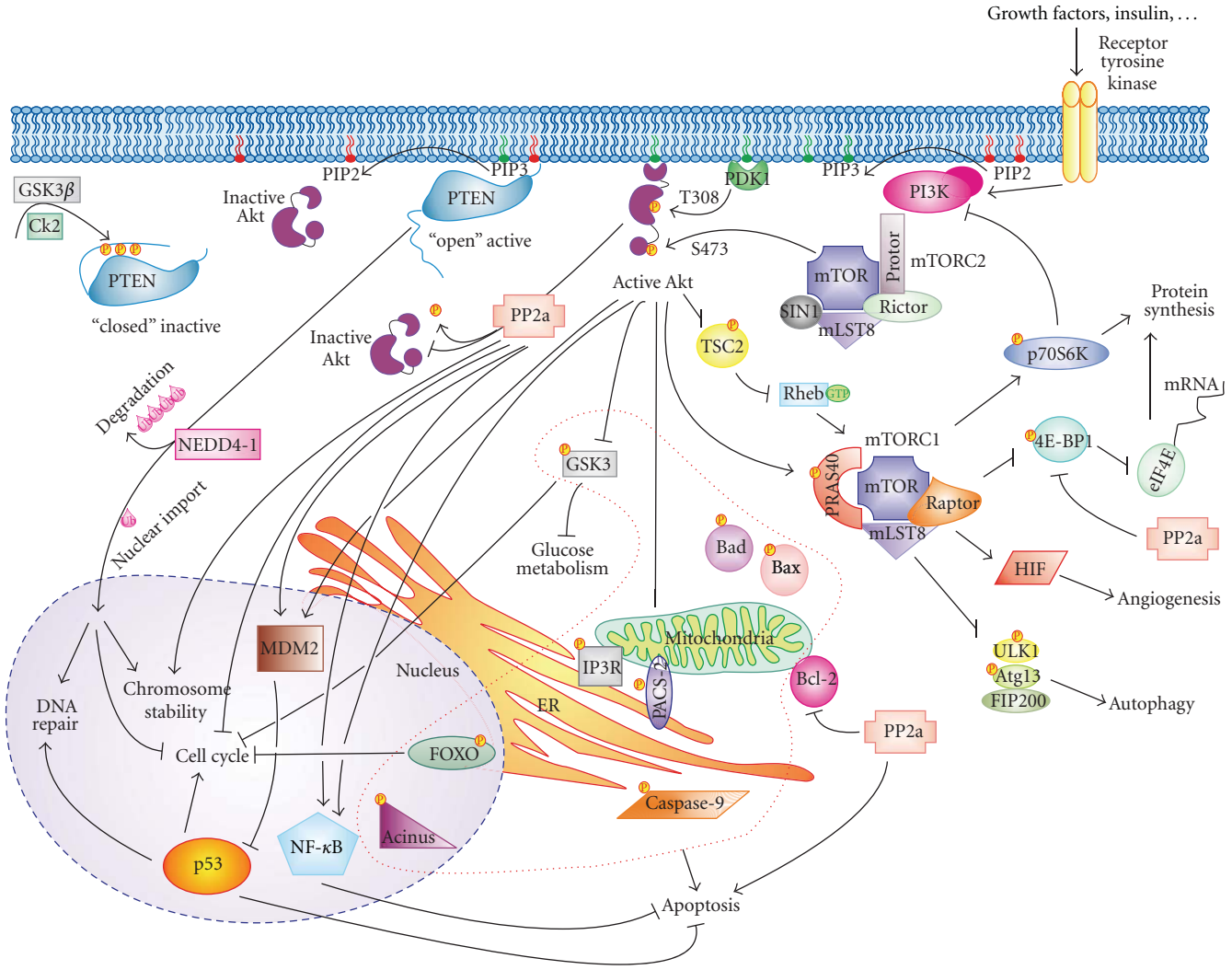


Figure 1: PI3K signalling pathway: The phosphatidylinositol 3-kinase (PI3K) signalling pathway begins with PI3K activation by receptor tyrosine kinases after growth factors or insulin stimulation. PI3K activity phosphorylates and converts the lipid second messenger phosphatidylinositol 4,5-bisphosphate (PIP2—indicated by red phosphoinositide) into phosphatidylinositol 3,4,5-triphosphate (PIP3—indicated by green phosphoinositide), with consequent double phosphorylation/activation of Akt kinase. Akt promotes cell proliferation and survival by phosphorylation/inhibition of several proapoptotic targets (hold by the red dotted line), or by activation of mTOR complex 1, an important regulator of several processes, such as autophagy, angiogenesis and protein synthesis. Protein phosphatase 2a (PP2a) family members are able to dephosphorylate/inhibit Akt, favouring apoptosis also by direct dephosphorylation of Bcl-2. The tumour suppressor phosphatase PTEN negatively regulates PI3K signalling by dephosphorylating PIP3, converting it back to PIP2. A mono-uniquitinated/active form of PTEN is able to translocate into the nucleus, promoting DNA repair, cell cycle arrest and chromosome stability.

As such, a deeper understanding could yield improvements not only in how their mutations and dysregulation play causal roles in human diseases, but may also provide insights to develop agonists and antagonists for use in therapy. In this respect, it is well known that PKC belongs to the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) family which includes Akt [29]. PKC and Akt share some common kinases (i.e., PDK1 and mTORC2) that perform important regulatory functions [30–33]. Moreover, some PKC isoforms interact with Akt although their specific effect on Akt activity is isoform dependent [34, 35]; it has also been demonstrated that high glucose induced Akt activation, in a PKCβ-dependent manner [36]. In addition, it has been found that activation of PKCη leads to the activation of Akt and mTOR signalling pathway, promoting glioblastoma cells proliferation [37]. PKC activity seems to be related also to PP2a and PTEN. PKCδ not only physically associates with the PP2a catalytic subunit (PP2a/C) but also phosphorylates the phosphatase to increase its activity [38]; this is the case for PKCα too, which seems to be the primary mediator of the ~2-fold increase in PP2a activity observed in intestinal cells after PKC-signalling activation [39]. Regarding PTEN, suppression of its activity by transforming growth factor-β (TGF-β) has been shown to be specifically mediated by

PKCα. On the topic of PKCs, we will summarise their general characteristics and role in different cell signalling pathways, as well as the result of their deregulation in cell fate.

For reasons of brevity, we will not discuss much further the MAPK- and PKA-mediated pathways; interested readers should refer to [40–44].

2. Akt Kinase

The serine/threonine kinase Akt constitutes an important node in diverse signalling cascades downstream of growth factor receptor tyrosine kinases. Akt plays an essential role in cell survival, growth, migration, proliferation, polarity, metabolism (lipid and glucose), cell cycle progression, muscle and cardiomyocyte contractility, angiogenesis, and self-renewal of stem cells. Altered Akt activity has been associated with cancer and other disease conditions, such as diabetes mellitus, neurodegenerative diseases, and muscle hypotrophy [45].

Among the downstream effectors of PI3Ks, Akt is the most important and best studied [46]. Three Akt isoforms have been identified in mice and humans [47]. These three Akt proteins, although encoded by distinct genes localized on different chromosomes, have approximately 80% amino acid identity and similar domain structures. Each isoform possesses an N-terminal PH domain of approximately 100 amino acids, with a high similarity to PH domains found in other signalling molecules that bind 3-phosphoinositides [48]. Biochemical analysis revealed that the PH domain of Akt binds to both PIP3 and phosphatidylinositol 4,5-bisphosphate (PIP2) with similar affinity [49]. Moreover, in contrast to PH domains of other proteins, the head group of PIP3 is localized in a significantly different orientation in the PH domain of Akt, and it lacks a specific tyrosine that is conserved in PH domains of other proteins (e.g., DAPP1, GRP1, BTK) [50]. The kinase catalytic domain, located in the central region of the protein, shows a high degree of similarity to those found in PKA and PKC [51]: the relation with PKA and PKC explains the other name of Akt, PKB. Also present in this region is a threonine residue (T308 in Akt1) whose phosphorylation is necessary for the activation of Akt. Following the kinase domain is a C-terminal tail of around 40 amino acids, containing a second regulatory phosphorylation site (S473 in Akt1). This region possesses the F-X-X-F/Y-S/T-Y/F hydrophobic motif (where X is any amino acid) that is characteristic of the AGC kinase family. In mammalian Akt isoforms, this motif is identical (FPQFSY) [52]. Phosphorylation at T308 and S473 occurs in response to growth factors and other extracellular stimuli and is essential for maximal Akt activation [53].

The crystal structure of activated Akt was determined in 2002 [54] and underlines the fundamental role of the hydrophobic motif as an allosteric regulator of the kinase activity: in fact, with Akt phosphorylated on threonine in the catalytic loop but lacking its carboxyl-terminal hydrophobic motif, the αB and C-helices in the aminoterminal lobe of the kinase domain and the activation loop are disordered [55]. Replacing the hydrophobic motif with a similar sequence from the PKC-related kinase 2 (PRK2), containing an acidic residue instead of the Ser (called the PDK1-interacting fragment (PIF) sequence) [56], or mimicking the phosphorylation in S473 through a substitution with aspartate leads to a stabilization of the kinase domain in active state. In other words, the molecular interaction between the phosphorylated hydrophobic motif and the groove, formed by the αB and C-helices, stabilizes the whole catalytic domain: this association is critical for the complete activation of Akt.

As mentioned above, the activation of the PI3K signalling pathway by growth factor stimulation leads to recruitment of Akt to the plasma membrane through binding to PIP3. At the plasma membrane, Akt can be phosphorylated on two residues, T308 and S473, by two different kinases, PDK1 [22, 57] and the mTORC2 [23], respectively. Once activated, Akt translocates to various subcellular compartments, including the Golgi, ER, mitochondria, and nucleus [58], where it phosphorylates substrates or interacts with other molecules (Figures 1 and 3). Consensus motif analysis indicates that there are potentially thousands of cellular substrates for Akt; about 50 of these have been characterized so far. Through phosphorylation, Akt may either positively or negatively affect the function of these substrates, alter their subcellular localization, or modify their protein stabilities [45]. As a protooncoprotein and the primary target of PI3K, Akt was first characterized for its function in regulating cell survival and cell proliferation, and its antiapoptotic activity is solved through the inactivation of many proapoptotic factors. Constitutive activation of Akt leads to uncontrolled cell proliferation, inhibited apoptotic pathways, and strong cell cycle dysregulation, typical hallmarks of many human cancers. Akt is able to directly or indirectly modulate apoptosis [59]. The direct effects are linked to phosphorylation events or interactions with cell death actors, whereas the indirect regulation of apoptosis is mediated through transcriptional responses to apoptotic stimuli.

Active Akt migrates to both the cytosol and the nucleus. Nevertheless, the relative contribution of Akt signalling at the plasma membrane, the cytosol, and the nucleus remains to be elucidated. Nuclear Akt may fulfil important antiapoptotic roles [60] (Figure 1). Indeed, Akt plays a crucial role in determining cell fate by regulating fundamental transcriptional factors for the expression of pro- or anti-apoptotic molecules, for example, YAP, CREB, FOXO proteins, and NF-κB, as well as the E3 ubiquitin ligase MDM2, a known negative regulator of the tumour suppressor p53 [61]. Phosphorylation of CREB and NF-κB induce upregulation of antiapoptotic Bcl-2 family members, whereas phosphorylation of FOXO family members leads to their nuclear exclusion and inactivation [62, 63], with a consequent decreased transcriptional activity that is required for promoting apoptosis. Interestingly, Trotman et al. have shown that the promyelocytic leukemia protein (PML) tumour suppressor prevents cancer by inactivating phosphorylated Akt (pAkt) inside the nucleus. PML specifically recruits the Akt phosphatase PP2a as well as pAkt into PML nuclear bodies. PML-null cells are impaired in PP2a phosphatase activity towards Akt and thus, accumulate nuclear pAkt. As

a consequence, the progressive reduction in PML dose leads to the inactivation of FOXO3a-mediated transcription of the proapoptotic Bim and the cell cycle inhibitor $p27^{kip1}$ [64].

At the same time, Akt deliver antiapoptotic signals via different proteins directly modulated by Akt phosphorylation. Bad is one of the first discovered targets of Akt phosphorylation [65]. Bad is a proapoptotic member of the Bcl-2 family of proteins, able to bind Bcl-2 or Bcl-XL, blocking their antiapoptotic activities. Phosphorylation of Bad on S136 by Akt disrupts its interaction with Bcl-2/Bcl-XL, localized on the outer mitochondrial membrane, sequestering Bad in the cytosol, through the interaction with 14-3-3 protein. In an analogous way, phosphorylation by Akt of proapoptotic Bax protein on S184 suppresses its translocation to mitochondria, preventing Bax conformational change, a typical event that occurs after apoptotic induction [66]. In addition, the caspase cascade is further inhibited by Akt phosphorylation of procaspase 9 [67], inactivated through phosphorylation in S196, a residue that, however, is not conserved in other mammalian species [68]. Also Acinus, one of the most relevant proapoptotic factors, responsible for chromatin condensation, is phosphorylated by Akt in S422 and S573, with a consequent resistance of the protein to the activator cleavage by caspase 3 [69]. Moreover, Akt phosphorylates the two isoforms, α and β, of the glycogen synthase kinase 3 (GSK-3) in S21 and S9, respectively, promoting their inactivation [70] and blocking its ability to induce apoptosis in response to a wide range of stimuli (reviewed in [71]). Interestingly, GSK-3 β is a target of Akt phosphorylation/inactivation also inside mitochondria [72], but the role of this mitochondrial interaction remains to be clarified.

Akt is at the crossroads of several mitochondria-mediated cell death pathways and exerts a major role in apoptosis due to its networking with mitochondria in the regulation, and interconnection of metabolic pathways and cell survival. Notably, the mitochondrial import and export mechanisms for Akt have yet to be investigated. Also linked to mitochondrial metabolism is the Akt activity on hexokinase. Hexokinase catalyzes the first step of glycolysis: in particular, hexokinases I and II are found to directly interact with mitochondria, and their expression is increased in tumours [73], providing a putative explanation of the Warburg effect. In cancer cells, hypoxic conditions induce the activation of the PI3K/Akt cell survival pathway [74] and the association of Akt with mitochondria [75]. Akt promotes binding of hexokinase II to the mitochondrial voltage-dependent anion channel (VDAC) [76]. The high affinity of hexokinase II to VDAC allows it to selectively utilize intramitochondrial ATP to phosphorylate glucose and directly couple glycolysis to oxidative phosphorylation [77]. By promoting the interaction hexokinase-VDAC, Akt seems to maintain the "opening state" of the channel, protecting cells from apoptotic events through conservation of the mitochondrial integrity [76]. Despite this, other studies show how the hexokinase-VDAC binding mediates channel closure rather than VDAC opening, still maintaining the ability of hexokinases to inhibit cell death [78, 79], especially reducing the mitochondrial calcium (Ca^{2+}) overload, a primary event that triggers cytochrome *c* release.

Connected to Ca^{2+}-induced apoptosis, the inositol 1,4,5-trisphosphate receptor (IP3R), the main ER Ca^{2+}-release channel, is phosphorylated by Akt due to the presence of a consensus substrate motif that is conserved in all the three IP3R isoforms. The consensus substrate motif (R-X-R-X-X-S/T) for Akt kinase is located in the C-terminal portion of the IP3R, and it is also conserved in IP3Rs cloned from several different species, with the exception of *Caenorhabditis elegans* [80]. The IP3R is involved in Ca^{2+} mobilization from intracellular stores, where channel activity is largely under the control of IP3 binding. This receptor is also involved in fundamental processes such as fertilization, mitosis, and apoptosis. In fact, there is general agreement in the literature that Ca^{2+} efflux from the ER and Ca^{2+} accumulation into mitochondria are linked to the effects of various apoptotic stimuli [81]. Akt, probably through its kinase activity, reduces Ca^{2+} release from the IP3R and protects the cells from apoptosis induced by several Ca^{2+}-mediated apoptotic stimuli [82, 83]. Moreover, a recent paper by our group explains how the protein PML functions at the MAMs and ER levels to suppress Ca^{2+} transfer to the mitochondria. Knock-out cells for PML show a strong reduction of IP3R Ca^{2+} release, due to a hyperactivation of Akt. PML is a negative regulator of Akt, forming a macrocomplex composed by PML, Akt, the phosphatase PP2a, and IP3R type 3 at ER/MAMs. In the absence of PML, PP2a cannot inhibit Akt, with a consequent increase in IP3R phosphorylation, limited Ca^{2+} release, and protection from apoptosis [84] (see also below, PP2a section). Another protein regulated by Akt that mediates interorganelle signalling and transmits apoptotic signals from the ER to mitochondria is phosphofurin acidic cluster-sorting protein-2 (PACS-2). Akt phosphorylates this protein in S473, favouring the binding with 14-3-3, which represses PACS-2 apoptotic activity [85].

One of the best-conserved functions of Akt is its role in promoting cell growth. The predominant mechanism appears to be through activation of the mammalian target of rapamycin complex 1 (mTORC1), which is regulated by both nutrients and growth factor signalling. Akt has been suggested to directly phosphorylate mTOR on S2448 [86], but the role of this phosphorylation remains still unclear. The mTORC1 activation by Akt seems due to the inhibitory phosphorylation of the tuberous sclerosis complex 2 (TSC2, also known as tuberin), a tumour suppressor, critical negative regulator of mTORC1 signalling [87]. This complex functions as a GTPase-activating protein (GAP) for the small G protein Ras homologue enriched in brain (RHEB). The decreased GAP activity of the complex leads to accumulation of RHEB-GTP and activation of mTORC1. However, it remains poorly understood how TSC2 phosphorylation by Akt leads to decreased GAP activity [88]. Moreover, a second Akt substrate has been found to be involved in mTORC1 regulation, the proline-rich Akt substrate of 40 kDa (PRAS40, also known as Akt1 substrate 1). Akt was shown to directly phosphorylate PRAS40 on T246 [89], and this phosphorylation was fundamental for

14-3-3 binding. PRAS40 associates with mTORC1, negatively regulates it; therefore, phosphorylation by Akt and association with 14-3-3 are crucial for insulin to stimulate mTOR [90]. Akt regulation of mTORC1 through TSC2 and PRAS40 phosphorylation is also important for cell proliferation, controlling the translation of proteins important for cell-cycle progression. Akt-mediated cell proliferation and oncogenic transformation has been shown to be dependent on mTORC1 activation [91], raising the possibility that mTORC1 is the dominant Akt target in cells transformed with mutant Ras [92].

Considering the strong connection between Akt and mTORC1 and the role of this signalling, frequently dysregulated in cancers, in the next section, we will focus our attention on the major component of the mTOR complex, the kinase mTOR.

3. mTOR Kinase

The PI3K pathway includes a number of critical effectors that are involved in basic cellular functions, such as cell growth control, the cell cycle and DNA damage checkpoints, and recombination or maintenance of telomere length. One of this family members is mTOR, now recognized as a central regulator in a diverse array of vital cellular processes, including proliferation, growth, differentiation, and survival [93]. The physiological importance of mTOR is undoubtedly demonstrated by the fact that the knockout of mTOR in mice is primordially embryonic lethal [94], and the dysregulation of the mTOR pathway is associated with increased transformation and oncogenesis [20].

Structurally, mTOR possesses in the N-terminus up to 20 tandem HEAT motifs, including a protein-protein interaction structure of two tandem antiparallel α-helices found in huntingtin, elongation factor 3, the A subunit of protein PP2a, and TOR [95]. The C-terminus consists of mutated FRAP-ataxia-telangiectasia (FAT), a transformation/transcription-domain-associated protein domain, an FRB domain (FKPB12 (FK506-binding protein 12 kDa)-rapamycin binding), a catalytic kinase domain containing also an ATP-binding site, a probable autoinhibitory or repressor domain, and an FATC (FAT carboxy-terminal) domain. The kinase domain is between the FRB domain (which is C-terminal to the FAT domain) and the FATC domain, located at the C-terminus of the protein [96]. It is speculated that the HEAT repeats serve to mediate protein-protein interactions, the FRB domain is responsible to provide a docking site for the FKBP12/rapamycin complex, and FAT and FATC domains modulate mTOR kinase activity via unknown mechanisms [97]. The catalytic kinase domain in the C-terminus has a high similarity to the catalytic domain of PI3K, so mTOR is considered a member of the PIKK (PI3K-related kinase) family, but there is no experimental evidence that it displays lipid kinase activity [98]. In mammalian cells, mTOR exists in two distinct complexes called complex 1 (mTORC1) and complex 2 (mTORC2).

mTORC1 is rapamycin-sensitive and consists of the mTOR catalytic subunit, Raptor (regulatory associated protein of mTOR), mLST8 (also known as GβL), and PRAS40 [99]. Whereas the function of mLST8 is not really clarified, Raptor regulates mTORC1 functioning as a scaffold for recruiting mTORC1 substrates. PRAS40 is phosphorylated by Akt at T246 releasing its inhibitory effects on mTORC1. mTORC1 is a master controller of protein synthesis, integrating signals from growth factors within the context of the energy, and nutritional conditions of the cell. Activated mTORC1 regulates protein synthesis by directly phosphorylating 4E-BP1 (eukaryotic initiation factor 4E binding protein-1) and p70S6K (ribosomal p70S6 kinase), translation initiation factors that are important to cap-dependent mRNA translation, which increases the level of many proteins that are needed for cell cycle progression, proliferation, angiogenesis, and survival pathways [100]. Many diverse signals (such as growth factors, amino acids, glucose, energy status, and different forms of stress) and pharmacological agents (such as rapamycin) regulate the mTORC1 pathway.

In the other complex, mTORC2, mTOR also contains the mLST8 protein and additionally interacts with a protein called Sin1 (stress-activated protein kinase-interacting protein), a second protein termed Protor (protein observed with Rictor), and, instead of Raptor, Rictor (rapamycin-insensitive companion of mTOR) is present [101] (Figure 1). The interaction between Rictor and mTOR is not blocked by the drug rapamycin nor affected by nutrient levels, which are conditions known to regulate mTORC1. mTORC2 modulates cell survival in response to growth factors by phosphorylating Akt on Ser473 which enhances subsequent Akt phosphorylation on Thr308 by PDK1 [23]. Another downstream target of mTORC2 is serum/glucocorticoid-regulated kinase 1 (SGK1) [102]. Also the hydrophobic motif phosphorylation of PKCα has been shown to be mediated by mTORC2 [103]. Moreover, mTORC2-mediated phosphorylation of Akt and conventional PKC (cPKC) in their C-terminal turn motif (TM) is crucial for their proper carboxyl-terminal folding, stability, and signalling [32, 33].

The mTOR signalling pathway is activated during a wide variety of cellular responses: it regulates growth by maintaining the appropriate balance between anabolic processes, such as macromolecular synthesis and nutrient storage, and catabolic processes, like autophagy and the utilization of energy stores. Active mTOR enhances cell growth promoting protein translation and increasing cell mass by controlling a subset of mRNAs that are thought to promote cell growth and proliferation.

Most of protein translation is modulated at the level of initiation, through the positioning of the ribosome at the AUG codon. Cellular mRNAs contain a cap structure at their 5′ terminus [104], and it has been demonstrated that a strong repressor of cap-mediated translation is 4E-BP1 [105]. mTOR phosphorylates and inactivate the translation inhibitor 4E-BP1, inducing its dissociation from the translation initiation factor eIF4E, which can bind the cap structure at the 5′ termini of mRNAs, thereby allowing cap-dependent translation (in fact, dephosphorylated 4E-BP1 binds and

inhibits eIF4E) of proteins involved in cell proliferation and survival [106]. Moreover, mTOR is able to activate the p70S6K (through T389 phosphorylation) which activates the 40S ribosomal protein S6 via phosphorylation at S240/244 [107], a required process for translation and cell growth. mTOR is also able to monitor the regulation of ribosome biogenesis, an aspect that fundamentally occurs during the translation of mRNAs of the ribosomal proteins and the synthesis of ribosomal RNA (rRNA). Transcription of ribosomal DNA (rDNA) and transfer RNA (tRNA) genes by RNA polymerase I (Pol I) and III (Pol III) is a major rate-limiting step in the biogenesis of ribosomes. Nuclear localized mTOR is involved in Pol-I- and Pol-III-mediated transcription of rDNA and tRNA genes. It has been demonstrated that mTOR is associated with the promoters of 45S rDNA and genes of 5S rDNA and tRNAs [108]. mTOR also regulates Pol-I and Pol-III-mediated transcription through phosphorylation of TIF-IA and upstream binding factor (UBF), two transcription initiation factors of Pol I [109, 110]. The phosphorylation of TIF-IA in Ser44 is indispensable for its activity, while phosphorylation in Ser199 inhibits TIF-IA. Indeed, it has been demonstrated that inhibition of mTOR signalling inactivates TIF-IA by decreasing phosphorylation at Ser44 and enhancing phosphorylation at Ser199 [111]. Additionally, this effect not only regulates the activity of TIF-IA, but also controls its intracellular localization. Treatment of cells with mTOR inhibitors causes translocation of a significant part of TIF-IA into the cytosol (from the nucleus) inhibiting the formation of the transcription-initiation complex [111].

The signalling components upstream and downstream of mTOR are frequently altered in a wide variety of human tumours. Mutations in several tumour-suppressor genes (such as TSC1, TSC2, LKB1, *PTEN*, *VHL*, NF1, and PKD1) trigger the development of different diseases [100]. To confirm a primary role of mTOR in cancer development, it was shown that the inhibition of this kinase is fundamental for blocking cell growth and motility in a number of tumour cell lines [112]. Different data provide evidence that the mTOR pathways receive stimulatory signals from Ras, and, ultimately, these pathways drive tumorigenesis through the coordinated phosphorylation of proteins that directly regulate protein synthesis, cell-cycle progression, and cancer development. It has been demonstrated that Ras is able to determine an ERK-dependent phosphorylation of TSC2. This results in the suppression of its biochemical and biological tumour-suppressive functions (the loss of TSC2 is the typical aspect of the tumour syndrome tuberous sclerosis) and in the activation of S6K through the mTOR-dependent site T389. This shows a direct control of translation through a mechanism that involves the TSC1/2-mTOR pathway and Ras-MAPK (mitogen-activated protein kinase) signalling [113].

mTOR is also able to regulate the expression of the hypoxia-inducible factor-1 alpha (HIF-1α). Hypoxia occurs in the majority of tumours, promoting angiogenesis, metastasis, and resistance to therapy. Responses to hypoxia are orchestrated in part through the activation of the hypoxia-inducible factor family of transcription factors (HIFs) [114]. HIFs are heterodimeric transcription factors composed of an α and a β subunit (whose levels are controlled by oxygen tension [115]) and activates the transcription of 100–200 genes involved in cellular metabolism and the adaptation of cells to hypoxic conditions. A number of interesting potential connections have emerged between HIFs and mTOR, and there are clear examples of these pathways regulating each other and common downstream pathways. Perhaps the most firmly established is the ability of mTOR to influence HIF1A translation: mTORC1 signalling increases HIF-1α protein levels by promoting mRNA translation from its 5′UTR [116–118]. At the same time, hypoxia and low oxygen levels inhibit mTOR signalling through multiple mechanisms. One of these determines a decrease in cellular ATP levels with consequent activation of AMPK (AMP-activated protein kinase), which inhibits mTOR via TSC2 and raptor phosphorylation [119].

Moreover, the mTOR pathway is the most studied pathway regulating mammalian autophagy. Autophagy, which is highly conserved from yeast to humans, is a bulk degradation process involved in the clearance of long-lived proteins and organelles. Dysfunction in the autophagy pathway has been implicated in an increasing number of human diseases, from infectious diseases to cancer and neurodegeneration. mTOR negatively regulates autophagy, and, downstream of this kinase, there are more than 30 different genes regulating autophagy in yeast (known as the *ATG* genes), and many of these have mammalian orthologs [120]. mTORC1 regulates numerous proteins that are required for the execution of the autophagic program, including a macromolecular complex (FIP200,ULK1/Atg1) implicated in the initiation step of autophagosome formation. mTORC1 is able to phosphorylate ULK1 (unc-51-like kinase 1), moderately reducing ULK1 kinase activity and, consequently, blocking autophagy [121] (Figure 1).

Despite the central role of mTOR in cell physiology, relatively little is known about its precise subcellular distribution and the underlying functional significance. Essentially, mTOR is localized in the cytosol where it plays the important function of maintaining an appropriate balance between anabolic and catabolic processes. However, following different stimuli, mTOR can be driven into other compartments where it performs its activity (Figure 3). Previous studies indicate that mTOR is present in both the cytoplasm [122] and nucleus [123, 124]. Biochemical characterization in association with confocal microscopy identified a new localization of mTOR in the ER and the Golgi apparatus; specifically, mTOR is a peripheral ER membrane protein, tightly anchored to the ER/Golgi membranes [125]. ER and Golgi localization sequences of mTOR have also been identified [126], suggesting that anchoring to the ER/Golgi is important for mTOR signal transduction. ER and the Golgi apparatus are internal membrane structures constituting more than one-half of the total membranes in a cell. These compartments have an important role in processing and packaging macromolecules and sequestration of Ca^{2+}, which has been involved in mechanisms capable of activating both cell suicide programs as well as prosurvival mechanisms [127]. It is, therefore, possible to hypothesize that mTOR could play its role of amino acid sensor in the ER/Golgi

side. Moreover, the Rag GTPase family has been shown to be an amino acid-specific regulator of mTOR [128], a condition that promotes the translocation of the kinase to a surface of endomembrane compartments, identified recently as the lysosomal surface. In fact, a trimeric complex, named Ragulator, (encoded by the genes *MAPKSP1*, ROBLD3, and c11orf59) interacts with the Rag GTPase, localizing these and mTOR to lysosomes, a translocation necessary for mTOR activity [129].

Recent data suggest that mTOR signalling might also regulate mitochondrial function [130]. Cunningham et al. have shown that mTOR is necessary for the maintenance of mitochondrial oxidative function. mTOR inhibition determines lower oxygen consumption, mitochondrial membrane potential, and cellular ATP levels. Conversely, hyperactivation of mTORC1 increases mitochondrial DNA copy number, as well as the expression of many genes encoding proteins involved in oxidative metabolism [131]. Interestingly, starting from the observation that mTOR regulates cell growth by sensing nutrients, ATP levels, and osmotic stress, and since energy metabolism is a critical aspect of mitochondria, Desai and coworkers demonstrated a direct interaction of a large portion of mTOR with mitochondria, exactly at the level of the outer mitochondrial membrane. As validation of this direct control of mitochondrial function by mTOR, induced mitochondrial dysfunction results in mTOR-mediated growth regulation, suggesting the presence of a critical crosstalk between mitochondrial activity and growth regulation mediated by mTOR [122]. Even if the mechanism remains still unclear, mTOR might be localized to mitochondria via FKBP38 (a member of the FK506-binding family of proteins), a mitochondrial protein that should bind to the FRB domain of mTOR [132]. Moreover, a recent study has identified mTOR in a complex with the mitochondrial outermembrane proteins Bcl-XL and VDAC1 and demonstrated that Bcl-XL, but not VDAC1, is a kinase substrate for mTOR in vitro; the employment of mTOR inhibitors caused the dissociation of mTOR from Bcl-XL, modulating the mitochondria metabolism [133].

As reported above, mTOR is strongly involved in growth, survival, metabolism, and cancer development. A variety of transformed or tumour cells with deregulated mTOR signalling have shown higher susceptibility to inhibitors of mTOR than normal cells. The identification of mTOR as a potential target for anticancer therapeutics occurred after the discovery of the antineoplastic properties of the natural product rapamycin. This lipophilic macrolide was isolated from a strain of *Streptomyces hygroscopicus* indigenous to Easter Island (known as Rapa Nui) more than 20 years ago [134]. Rapamycin was initially developed as an antifungal agent. However, its major application quickly changed after rapamycin was proven to have immunosuppressive and antiproliferative properties. Upon entering the cells, rapamycin binds the small protein receptor called FKBP12. The rapamycin/FKBP12 complex specifically binds to mTOR and potently interferes with its function, inhibiting signals required for cell cycle progression, cell growth, and proliferation, leading to cell-cycle arrest in the G1 [97]. This inhibition blocks the activation of two fundamental downstream signalling effectors: p70S6K and 4E-BP1. By inhibiting cell cycle progression, growth, and division, rapamycin (and its analogues, termed rapalogs) produce several immunosuppressive and antiproliferative effects. These molecules were expected to become a breakthrough for the treatment of different types of cancer. However, rapamycin and rapalogs demonstrated only a limited clinical efficacy. This is explained since the complex rapamycin/FKBP12 is able to inhibit mTORC1 only partially, and in addition mTORC2 is resistant to rapamycin. Moreover, there is another possible outcome: feedback inhibition of PI3K signalling may be relieved by rapamycin treatment, presumably by inhibiting the downstream activation of S6K, which can serve as a negative regulator of PI3K signalling [135]. In this situation, inhibition of mTORC1 relieves a feedback inhibition of PI3K and results in enhanced PI3K signalling to Akt. To bypass these issues, a new molecule directed at both mTOR complexes simultaneously was reported recently. This inhibitor binds to the ATP-binding site in the mTOR catalytic domain, inhibiting mTOR completely (both mTORC-1 and -2) and minimizing the feedback activation of PI3K-Akt signalling [136].

Despite its humble beginning two decades ago, the protein kinase mTOR is now recognized as a critical mediator of the cellular response to many types of stress, including DNA damage as well as drops in the levels of energy, glucose, amino acids, and oxygen. The physiological functions of mTOR continue to expand, and it has been demonstrated that mTOR is involved in a wide variety of human diseases. Recent data suggest that use of mTOR inhibitors result in antitumor activity against several types of refractory malignancies. It is hoped that a more detailed understanding of the mTOR signalling cascade will lead to new therapies for many different human disorders.

4. PP2a Phosphatases

PP2a is a family of serine-threonine phosphatases highly conserved and ubiquitously expressed, implicated in the control of diverse cellular processes through the negative regulation of signalling pathways initiated by protein kinases. PP2a phosphatase activity has been linked to the regulation of the cell cycle, signal transduction, DNA replication, transcription, and translation [137, 138].

The structure of PP2a has been extensively studied. PP2a is a heterotrimer consisting of a catalytic subunit (the 36 kDa C subunit, PP2a/C), a structural subunit (the 65 kDa A subunit, PP2a/A), and a regulatory subunit (the B subunit, PP2a/B, which can vary in size from 50 to 130 kDa). The A and C subunits are ubiquitously expressed and form a catalytic complex (PP2a/AC) that interacts with at least three families of regulatory subunits (B55, B56, and PR72/130) and tumour antigens (e.g., SV40 small T antigen). The generation of a great diversity of PP2a holoenzymes is due to the existence of several isoforms for each subunit and their combination. Numerous studies implicate specific roles for PP2a subunits in regulating physiological functions. The variable B-type subunits are expressed differentially by tissue

and temporally during differentiation and/or development. They exert a crucial role in the control of PP2a activity by regulating substrate selectivity and regulating the catalytic activity in a wide range of biological processes [139]. In addition, there is evidence that the regulatory B subunits may target the catalytic complex to intracellular sites such as microtubules, the nucleus, and the mitochondria (Figures 1 and 3) [140, 141]. Some PP2a hetero-trimers display a quite restricted subcellular distribution [142]. Nuclear localization signals have been identified experimentally in B56α, and B56ε, while nuclear export signals are present in B56α, B56β and B56ε [143, 144]: as a consequence, B56 proteins can be detected at centromeres (B56α colocalized with pericentrin, a centrosomal marker) from prophase to metaphase during mitosis [145], while during interphase, B56α, B56β, and B56ε undergo nuclear-cytoplasmic shuttling, with a prevalent localization in the cytoplasm [139]. Nuclear PP2a proteins containing different B56 isoforms exert a further function in maintaining the stability of cohesin at centromeres. The accurate chromosome segregation during mitosis and meiosis depends on shugoshin proteins that associate with PP2a/B56, which is thought to dephosphorylate cohesin and thereby, protecting cohesin degradation and prophase chromosome dissociation [146]. Although most regulatory subunits do not contain obvious targeting motifs, their presence certainly influences the compartmentalization of PP2a, such as focal adhesions [147] and the microtubule cytoskeleton [142]. Moreover, ceramide, the central molecule of sphingolipid metabolism which generally mediates antiproliferative responses, such as cell growth inhibition, apoptosis induction, senescence modulation, ER stress responses, and/or autophagy, was found to promote the upregulation of a regulatory subunit (B56α) and thus promotes translocation of PP2a to the mitochondria. C2-ceramide, but not inactive C2-dihydroceramide, was found to specifically activate a mitochondrial PP2a, which rapidly and completely induced Bcl-2 dephosphorylation and correlated closely with ceramide-induced cell death [148, 149].

This explains partially the variety of specific physiological functions carried out by the enzyme and underlines the potential complexity of PP2a's role and its regulation [150] in several signalling pathways by regulating the activity of protein kinases, protein phosphatases, or their substrates [151]. Hence PP2a activity needs to be tightly regulated, and this is achievable through enzyme modulation at different levels. Therefore, in addition to the heterooligomeric composition of the PP2a complexes, which is the most important one, PP2a can also undergo posttranslational modifications and be controlled by its association with inhibitory proteins [150]. Specifically, the highly conserved C-terminal sequence of the PP2a catalytic subunit contains tyrosine residues, including Y307, which can be phosphorylated, resulting in inactivation of the enzyme [152], and PP2a can reactivate itself by autodephosphorylation. At least two endogenous protein inhibitors, designated I1PP2a (also known as pp32 or PHAP-1) and I2PP2a (also known as SET), were identified as noncompetitive inhibitors of PP2a activity [153]. The biological significance of I1PP2a is still mostly unknown. Conversely, I2PP2a is known to be phosphorylated on two serine residues (most likely by PKC) and is required in chromatin remodelling. The importance of its potent inhibitory activity towards PP2a is underlined in its involvement in a form of acute non-lymphocytic myeloid leukemia with t(6,9) and in BCR/ABL-driven leukemias (CML and Ph1-ALL), suggesting that disruption of normal PP2a regulation may also play a role in the pathogenesis of these leukemias [150]. Moreover, protein-protein interactions between PP2a and other intracellular components contribute to the specificity of PP2a signalling: the selective association of PP2a with scaffolding proteins directs the phosphatase to specific signalling modules [154].

Interaction of PP2a with other proteins might also recruit PP2a activity towards specific substrates in apoptotic signalling. PP2a promotes cell survival by negatively regulating the PI3K/Akt pathway, by associating to and directly dephosphorylating Akt, with consequent inactivation of the kinase [155] (Figure 1). Trotman et al. have shown that Akt and PP2a are corecruited into PML nuclear bodies and that the inability to recruit PP2a to Akt in *PML* deficiency resulted in an accumulation of nuclear phosphorylated Akt, which drives tumorigenesis [64]. Moreover, PML, Akt, and PP2a colocalize with the IP3R3 in high molecular weight complexes at the ER and MAMs, supporting a model in which dephosphorylation of Akt at the MAMs might occur through PML-mediated recruitment of PP2a. Reduced cellular sensitivity to apoptotic stimuli was observed in cells with high Akt activity, as a result of diminished Ca^{2+} flux from the ER through the IP3R. This effect is highly specific to Ca^{2+}-mediated apoptotic stimuli and explains a more direct role of PP2a in regulating cell survival through changes in Ca^{2+} signalling in the ER, cytosol, and mitochondria [84]. Also in the sarcoplasmic reticulum of cardiac muscle cells, PP2a has been shown to generate a macromolecular complex with the ryanodine receptor Ca^{2+}-release channel, the FK506-binding protein FKBP12.6, PKA, PP-1, and the muscle A-kinase anchoring protein (AKAP), where it is probably involved in the regulation of channel activity [156, 157].

The PP2a proapoptotic function is also mediated by direct dephosphorylation of specific proteins: PP2a both inactivates the antiapoptotic Bcl-2 and activates the proapoptotic factor Bad (Figure 1). Dependent on the survival stimuli and cell type, Bad becomes phosphorylated at S112, S136 and/or S155 by different prosurvival kinases such as mitochondrial cAMP-dependent protein kinase (PKA) and cytosolic Akt, which cooperatively mediate binding of Bad to 14-3-3 proteins, causing cytoplasmic retention of Bad. This interferes with the ability of Bad to translocate to the mitochondrial membrane and to inhibit the antiapoptotic Bcl-2 protein. In the absence of survival stimuli, Bad is dephosphorylated and colocalises and binds with prosurvival Bcl-2 members at the mitochondrial membrane, leading to apoptotic cell death. Finally, the A/PR65 subunit of PP2a is a substrate of caspase-3. Hence, partial or total loss (by further degradation) of the A/PR65 subunit resulted in an increase in PP2a activity towards yet unidentified substrates, which leads to the increase of the apoptotic response [138].

A direct involvement of PP2a in the regulation of the cell cycle has been extensively demonstrated. The distribution of the protein differs with cell-cycle phase, is markedly accumulated in the nucleus at the G1/S border and in S phase, regulating the G1- to S-transition [158]. PP2a activity is also modulated by cell cycle-related events: indeed, the methylation levels of nuclear and cytoplasmic PP2a vary during the G0/G1 and G1/S phases [154]. PP2a is required in the control of the Cdk1/cyclin B complex (also known as maturation-promoting factor, MPF) [142]. In early G2, PP2a, containing a B56δ/B55α-targeting subunit [159], keeps MPF in the inactive precursor state by inhibiting the activities of both CAK (Cdk-activating kinase) and Wee1 kinase [156, 160]. Together, CAK, Wee1, and the dual-specificity kinase Myt1 switch off Cdk1/cyclin B [142]. PP2a also inhibits complete phosphorylation of Cdc25 [156], which is a critical upstream regulator of Cdk1 [160]. Indeed, final activation of MPF occurs when the inhibitory Thr14 and Tyr15 are dephosphorylated by the dual-specificity phosphatase Cdc25. PP2a is also positively implicated in the exit from mitosis, mediating cyclin B destruction [156]. Lastly, full mitotic exit requires the inhibition of both PP2a/B55α and PP2a/B55δ complexes, which is obtained by the action of the kinase Mast-I (Greatwall), a target of Cdk1/cyclin B. Once this system is active, the cell switches to complete mitosis, implying that inhibition of protein phosphatases is critically important for this process [156, 159, 161].

Moreover, PP2a can dephosphorylate and inactivate MEK1 and ERK-family kinases, therefore, inhibiting mitogenic signals; this effect is obtained directly and indirectly, through the inactivation of PKCζ enzyme [162], which in turn stimulates the MAPK cascade through Raf-1 independent activation of MEK [150, 154]. Accordingly, PP2a exerts its anti-mitogenic function through the negative regulation of the eukaryotic initiation factor 4E (eIF4E) and eIF4F assembly, which requires the MAPK Mnk1/2 signalling mediating eIF4E phosphorylation at S209. Inhibition of PP2a causes increased Mnks and eIF4E phosphorylation, resulting in the dissociation of eIF4E from 4E-BP1 and the subsequent increase in eIF4F assembly, potentiating the expression of certain oncogenic proteins such as Mcl-1 and c-Myc [163] (Figure 1). The SV40 small t-antigen too, a well-known PP2a inhibitor and a cell-transforming viral protein, is believed to transform cells in part by hyperactivation of the MAPK pathway as a consequence of PP2a inactivation [150].

PP2a can also dephosphorylate MDM2 at T216, and this is thought to activate MDM2 resulting in the degradation of p53, a proapoptotic transcription factor [164]. PP2a inhibits the mTOR pathway too, through direct inactivation of the S6 kinase, which is responsible for promoting cell growth [150]. Other PP2a substrates involved in prosurvival signals are proteins integrated in the signal-dependent activation of the NF-κB pathway, which indeed regulates the expression of multiple genes involved in the control of cell growth, division, and survival. Specifically, PP2a dephosphorylates the NF-κB protein family members RelA [150]. PP2a interacts with IKK in the multicomponent protein kinase termed the IKK signalosome, facilitating the induction of IkB kinase activity, targeted degradation of IkB, and release of NF-κB to its nuclear site of action following the stimulation with the proinflammatory cytokine TNF. The precise mechanism of PP2a action on IKK remains unclear. However, given the key role that IKK plays in inflammation, the kinase/phosphatase interaction described represents an attractive therapeutic target for small molecule inhibitors [165].

By altering the functions of proteins involved in mitogenic and apoptotic signalling pathways, PP2a is considered to be a tumour suppressor, and many observations support a role for PP2a in tumorigenesis. Indeed, the dysfunction of particular PP2a complexes regulates specific phosphorylation events necessary for cancer initiation. Moreover, the disruption of functional PP2a complexes also induces transformation. Mutations of both structural subunits contribute with low frequency to different subsets of human cancers by distinct mechanisms [166]. In conclusion, characterization of the specific physiological substrates and the regulation of PP2a holoenzyme assembly will be one of the main requirements to identify the in vivo function and regulation of PP2a in different signal transduction pathways.

5. PTEN Phosphatase

In 1997, two groups independently searching for tumour suppressors on chromosome 10q23, a locus that is frequently deleted in a variety of human cancers [167], identified a new phosphatase termed PTEN (phosphatase and tensin homolog deleted on chromosome 10), also known as MMAC (mutated in multiple advanced cancers) [168] or TEP1 (transforming growth factor-β-regulated and epithelial-cell enriched phosphatase 1) [169]. Subsequently, *PTEN* somatic mutations were identified in almost all human tumour types, especially those of the brain (above all glioblastoma), prostate, and endometrium; moreover, mutation of at least one allele occurs in one-third or more of breast, colon, and lung tumours [170]. Therefore, nowadays the PTEN gene appears to be the second most frequently mutated tumour-suppressor gene in human cancers after TP53 [171]. Germline mutations in PTEN are associated with a wide and diverse clinical spectrum of inherited autosomal dominant disorders characterized by developmental disorders, neurological deficits, multiple hamartomas, and an increased risk of breast, thyroid, and endometrial cancers. Collectively, these are referred to as the PTEN hamartoma tumour syndromes (PHTS), which include Cowden syndrome (CS), Lhermitte-Duclos disease, Bannayan-Riley-Ruvalcaba syndrome, and Proteus and Proteus-like syndromes [172]. Some of the patients with these disorders also show macrocephaly, mental retardation, ataxia, and seizures. Recently, a subset of autistic patients with macrocephaly were found to bear PTEN mutations [173]. Various mouse models in which the *PTEN* gene is deleted further demonstrate its crucial role as a tumour suppressor in multiple tumour types, and the consequences of its loss in human health and disease (for recent reviews, see [174, 175]). Notably, these studies

revealed the requirement of PTEN for embryonic development and provide evidence for its haploinsufficient tumour-suppressive activity (one functional allele is not enough to sustain a wild-type condition) [176–180]. Today, it is well documented that PTEN function affects diverse cellular processes such as cell-cycle progression, cell proliferation, apoptosis, ageing, DNA damage response, angiogenesis, muscle contractility, chemotaxis, cell polarity, and stem cell maintenance.

As its name suggests, PTEN has been shown to be a non-redundant, evolutionarily conserved dual-specificity phosphatase that is capable of removing phosphates from protein and lipid substrates [181, 182]. PTEN harbours the Cys-X_5-Arg-Thr/Ser (CX_5RT/S) phosphatase catalytic signature and shares primary sequence similarity with members of the protein tyrosine phosphatase superfamily [183]. This initially suggested that PTEN is a protein phosphatase and prompted the expectation that its function would be to suppress directly oncogenic tyrosine kinase signalling [184]. Indeed, early studies showed PTEN-dephosphorylated serine, threonine, and tyrosine residues in peptide substrates in vitro [185] and focal adhesion kinase (FAK) in vivo [186]. Studies carried out so far have firmly established that the protein phosphatase activity of PTEN plays a role in the regulation of cellular processes, especially cell migration [187, 188]. In addition, there are reports that PTEN can dephosphorylate itself [189], the platelet-derived growth factor receptor [190], and various studies have demonstrated that the protein phosphatase activity of PTEN regulates MAPK phosphorylation and cyclin D1 expression [191, 192].

Ironically, although PTEN represents the only protein tyrosine phosphatase that can unambiguously be termed a tumour suppressor, it is its lipid phosphatase function that has been shown to be crucial for maintaining tissue homeostasis [181]. Specifically, the primary target of PTEN in cancer is the lipid second messenger intermediate PIP3 [182]. PTEN removes the phosphate from the three-position of the inositol ring to generate PIP2, thereby, directly antagonizing signalling through the PI3K/Akt pathway [8, 26, 193, 194]. The loss of *PTEN* leads to constitutively high levels of PIP3, which promotes the recruitment of a subset of proteins that contain a pleckstrin homology domain to cellular membranes, including PDK1 and Akt kinase. As discussed before, Akt is the major downstream effector of PI3K signalling that can phosphorylate a wide array of substrates and, thus, stimulates cell growth, proliferation, and survival (Figure 1) [45].

The obvious biological effect caused by a modest change of PTEN expression level in mouse models also emphasizes that PTEN function needs to be precisely controlled in a temporally and spatially specific manner. Yet, the function and regulation of PTEN has turned out to be amazingly complex. PTEN can be regulated through multiple mechanisms as well as epigenetic effects: transcriptional modulation, posttranscriptional mechanisms (also through coding-independent function of pseudogene PTENP1 mRNA, as recently discovered [195]), posttranslational modifications (including oxidation, acetylation, phosphorylation, and ubiquitination), binding partners, and subcellular localization—all these potentially impact PTEN levels and/or function. Every one of these mechanisms has been extensively reviewed elsewhere [196–198]; here, we will only provide a brief overview, with particular emphasis on new perspectives that have emerged about the importance of PTEN's subcellular localization (Figures 1 and 3).

Human PTEN encompasses 403 amino acids and is characterized by five functional domains: a short N-terminal PIP2-binding domain, a phosphatase domain, a C2 domain, a C-terminal tail containing PEST (proline, glutamic acid, serine, threonine) sequences, and a PDZ (postsynaptic density protein—*Drosophila* disc large tumour suppressor—zonula occludens 1 protein) interaction motif [26]. The majority of the missense mutations found in human tumours and CS occur in the phosphatase domain and affect the catalytic activity of PTEN [199]. Nevertheless, mutations also occur in all other domains of PTEN, strongly suggesting that these different domains are physiologically relevant to PTEN-related tumorigenesis.

PTEN's amino acid sequence and tertiary structure explain a great deal about PTEN's unique enzymatic properties. Detailed analysis of the PTEN crystal structure [200] revealed that its phosphatase domain contains all the highly conserved residues of the signature motif CX_5RT/S present in the active sites of protein tyrosine phosphatases and dual-specificity phosphatases, required for protein phosphatase action but, in addition, has two unique Lys residues that might interact with negatively charged PIPs. Moreover, the PTEN active site pocket is wider and slightly deeper than other protein phosphatases, allowing both bulky PIP3 and smaller phosphoaminoacids to be substrates. However, the shape of the active site pocket, electrostatic interactions with positively charged sidechains, and hydrogen bonds with polar sidechains make PIP3 binding more favourable [201]. Interestingly, PTEN protein- and phosphoinositide-phosphatase activities can be uncoupled. For example, the C124S mutation (which is the catalytic site residue) inactivates both lipid and protein phosphatase activity [181] but the G129E mutation disrupts the lipid but not the protein phosphatase activity of PTEN [182]. The presence of this mutation in patients with PHTS and sporadic human tumours clearly indicates that loss of the lipid phosphatase activity is sufficient to cause these clinical phenotypes. The unimpressive activity of PTEN towards phosphopeptide substrates, while consistent with the idea that it is primarily a lipid phosphatase, does not exclude the possibility that PTEN is also a protein phosphatase, but one with an exquisite substrate specificity, as recently demonstrated [183].

Even though PTEN has multiple domains for membrane association, in most mammalian cell types it does not show an obvious association with the plasma membrane. Instead, PTEN usually appears diffusely in the cytosol, with a somewhat variable nuclear component. The crystal structure revealed that PTEN has a C2 domain that harbours basic residues essential for phospholipid membrane binding in vitro [202]. In addition, the C-terminal, apparently unstructured and flexible tail of PTEN, contains a cluster of serine and threonine phosphorylation sites (S370, S380, T382, T383, and S385) which may regulate its stability, activity,

and recruitment to the membrane [203]. Multiple kinases including casein kinase 2 (CK2) [204], GSK3β [205], PICT-1 [206], and Rock [207] are capable of phosphorylating the PTEN C-terminal tail. Vazquez et al. have proposed that when phosphorylated PTEN assumes a closed conformation (due to interaction between the phosphorylation cluster of the C-terminal tail and the positively charged surface on the C2 domain), this enhances its stability but opposes membrane binding, thus keeping PTEN inactive in the cytoplasm (in terms of its lipid phosphatase activity) [208, 209]. Dephosphorylation leads to a more active phosphatase, but also more unstable and subject to ubiquitin-mediated proteasomal degradation (Figure 1). The identity of PTEN's phosphatase is still unclear (and also how it might be activated), but it has been proposed that PTEN could be its own phosphatase, dephosphorylating itself [189].

More recently, infrared spectroscopy experiments have also indicated that the PTEN N-terminal domain can bind specifically to PIP2; this is involved in PTEN membrane targeting and, in addition, causes a conformational change within the enzyme and its allosteric activation [210, 211]. Single-molecule studies using total internal reflection fluorescence microscopy (TIRF) have shown that PTEN associates stably but transiently (about 150 ms) with the plasma membrane [212], and localization of PTEN to the plasma membrane is presumably influenced by the local PIP2 and PIP3 concentrations [213]. As such, PTEN is an "interfacial enzyme", which exists in a high-activity state when bound transiently at membrane surfaces containing its substrate and other acidic lipids, such as PIP3 and phosphatidylserine. This mechanism ensures that PTEN functions in a spatially restricted manner and may explain its involvement in forming the gradients of PIP3, which are necessary for generating and/or sustaining cell polarity during motility, in developing neurons and in epithelial tissues [214]. Moreover, other potential functional consequences of this are suggested by experiments that identified pools of PIP3 in the nuclear matrix and on the ER and other endomembrane compartments [215, 216].

The identification of nuclear PIP2 and PIP3 [217, 218], as well as other key components of the PI3K pathway including PI3Ks, PDK1, and Akt, indicate that PTEN might also function as a PIP3 lipid phosphatase in the nucleus [219]. It is now well documented that a pool of PTEN protein is located and functional within the nucleus; accumulating evidence also suggests that the role of nuclear PTEN is not the same as that of cytoplasmic PTEN. Importantly, loss of this nuclear PTEN population correlates with increased tumorigenicity [220–222]. PTEN nuclear shuttling is mediated by ubiquitination; as described above, PTEN contains the two so-called PEST motifs characteristic of short-lived proteins that are subject to ubiquitin-mediated degradation by the proteasome. In support of this, Wang et al. [223] have identified NEDD4-1 as an E3-ligase for PTEN. In addition to promoting polyubiquitination and, therefore degradation of PTEN, NEDD4-1 can also catalyse monoubiquitination of PTEN, which was shown by Pandolfi's laboratory to promote PTEN nuclear import (Figure 1) [224]. The same group have also identified HAUSP as a critical and essential enzyme for PTEN deubiquitination and nuclear exclusion, a mechanism which involved also the tumour-suppressor PML [225]. Nuclear PTEN affects a variety of biological functions: chromosomal stability through an interaction with the centromeric-binding protein CENP-C, which enhances centromere stability specifically and overall genomic stability [226]; DNA-damage responses through its ability to upregulate the transcription of Rad51, which leads to double-strand break repair [226]; cell-cycle regulation by inducing G0-G1 arrest, probably as a result of cyclin D1 downregulation [227]. In addition, injection of PTEN into isolated nuclei enhances apoptosis-induced DNA fragmentation [228]. Recently, Song et al. have also revealed that nuclear PTEN interacts with the anaphase-promoting complex (APC) and enhances its stability and tumour-suppressive activity, in a phosphatase-independent manner [229].

Notably, even if in cells the vast majority of PTEN appears to exist as a monomer and usually only a small percentage of cellular PTEN stably binds to a particular associating factor, PTEN can also be recruited to special plasma membrane locations through binding of its PDZ-interaction motif with PDZ-containing membrane-anchored proteins [230] including MAGI-2 [231], PAR-3 [232], the microtubule-associated serine/threonine kinases MAST-1 and MAST-3 [233], and NHERF [234]. A recent proteomics study suggests that there are additional PTEN-interacting proteins [235], remarkably PP2a, which attracted our attention and is also addressed in this paper. Some PTEN-binding proteins might simply act as an adaptor protein to guide PTEN to specific cellular compartments and subsequently to function there. Although the physiological relevance of many of these interactions needs to be validated, it is possible that some of these associating proteins can regulate PTEN function, including its enzymatic activity; conversely, it is also likely that PTEN regulates the function of its binding partners [236].

Other interesting studies carried out by Liu et al., using fluorescence recovery after photobleaching (FRAP) of GFP-PTEN, revealed that nuclear PTEN had a very rapid diffusion and appeared not to be tethered. Conversely, cytoplasmic PTEN diffused more slowly, suggesting that there are transient interactions with immobile cytoplasmic structures [237]. The apparent tethering of PTEN to cytoplasmic structures raises an intriguing question. Does tethering enhance PTEN activity by placing PTEN close to substrate or does it prevent PTEN from acting at the plasma membrane? [201]. Data regarding PTEN's subcellular localization could lead to new insights, especially considering the recent work of Zhu et al., demonstrating a mitochondrial location of PTEN and its crucial role as mediator of apoptosis [238].

It is possible that a subpopulation of cellular PTEN is dedicated to (and sufficient for) a specific biological function and that there are specific regulatory mechanisms defining this subpopulation. Research on these regulatory mechanisms should provide exciting avenues of investigation and may identify additional functions of PTEN that depend on its subcellular localization. These should help us further elucidate the importance of this protein in human health

and disease and—given the distinct localization of PTEN in normal and cancerous cells—might identify potential therapeutic targets.

6. Protein Kinase C

PKC comprises a multigene family of related serine/ threonine kinases that involve a lot of signal transduction pathways, for example, cell proliferation, differentiation, apoptosis, and autophagy [239]. PKCs exhibit a high molecular heterogeneity, occurring in at least 10 different isoforms differing in biochemical properties and sensitivity to activators. PKCs are lipid-sensitive enzymes that are activated by growth factor receptors that stimulate phospholipase C (PLC), the enzyme that generates diacylglycerol (DAG) and inositol trisphosphate (IP3), with the latter being involved in the mobilization of intracellular Ca^{2+}. There are also pharmacological activators of PKC, such as phorbol 12-myristate 13-acetate (PMA). PMA exerts its function by anchoring PKCs in their irreversibly active conformations to the plasma membrane [240]. PKC isozymes are classified as Ca^{2+}-dependent, Ca^{2+}-independent, and atypical, according to their sensitivity to Ca^{2+} and DAG. For their activation, the conventional PKC (cPKCs: α, βI, βII, γ) require additional free Ca^{2+} and DAG. The novel PKCs (nPKCs: δ, ε, η, θ) are activated by DAG; the atypical PKCs (aPKCs: ζ, λ/ι) are independent of Ca^{2+} and DAG [241].

The PKC polypeptide consists of a C-terminal catalytic domain and an N-terminal regulatory domain that are separated by a flexible hinge region [242]. cPKCs contain four homologous domains (C1, C2, C3, and C4) interspaced by isozyme-unique variable domains (V1, V2, V3, V4, and V5). The C1 region is a putative membrane-binding domain, the C2 region appears to be related to the Ca^{2+} sensitivity of the enzyme, the C3 region contains the catalytic site and the ATP-binding site, and the C4 region appears to be necessary for recognition of the substrate to be phosphorylated. In nPKCs, there is no C2 homologous domain (and thus, they are insensitive to Ca^{2+}); aPKCs lack both the C2 and one-half of the C1 homologous domain (and thus, they are insensitive to both DAG and Ca^{2+}) (Figure 2(a)) [243, 244]. In PKCs there are three constitutively phosphorylated sites in the kinase domain. These phosphorylations occur on the activation loop site and two C-terminal sites (TM) [245]. The maturation of PKCs occurs through ordered and constitutive phosphorylations that are very important for their stability and catalytic activity [246]. The first phosphorylation event (by PDK1) is controlled by the conformation of PKC, which is in an open conformation; the pseudosubstrate is removed from the substrate-binding cavity, and the activation loop site is free to be phosphorylated by PDK1 [247]. The phosphorylation of the TM, which depends on mTORC2 [245], stabilizes the structure of mature PKC by anchoring the C-terminal tail on the upper lobe of the kinase [245, 248].

PKC isoforms are unique, not only with respect to primary structure but also on the basis of expression patterns, subcellular localization, activation in vitro, and responsiveness to extracellular signals. PKC isozymes are homologous enzymes in which their subcellular localization determines the functional specificity. The localization facilitates crosstalk between different signalling intermediates, targeted substrate phosphorylation, and regulation of catalytic activity [239]. PKCs are thought to reside in the cytoplasm in an inactive conformation and, after cell activation, these proteins translocate to the plasma membrane, cytoplasmic organelles, or nucleus (Figure 3) [249, 250]. Translocation of PKCα, β, δ, ε, and ζ to mitochondria, Golgi, nuclear, or perinuclear regions results in regulation of mitosis, cell survival pathways, and apoptosis [251]. In the cytosol, PKCs interact with several different proteins, including receptors for activated C kinase (RACKS), the product of the par-4 gene, zeta-interacting protein, lambda-interacting protein, and AKAPs. PKCs are also involved in remodelling the actin cytoskeleton, partly by phosphorylating specific PKC substrates such as the myristoylated alanine-rich PKC substrate (MARCKS) protein and pleckstrin. As such, PKCs influence many distinct aspects of cell function [252]. PKC translocation to the plasma membrane generally has been considered the hallmark of activation (and frequently has been used as a measure of PKC isoform activation in cells). PKC activation in the plasma membrane results in serine phosphorylation and endocytosis of transmembrane proteins and receptors [251]. PKC isoforms also translocate to specialized membrane compartments such as lipid rafts or caveolae [253]. A sizeable body of evidence collected over the last 20 years has also shown PKC to be capable of translocating to the nucleus. Furthermore, PKC isoforms are resident within the nucleus. Studies from independent laboratories have led to the identification of quite few nuclear proteins which are PKC substrates and to the characterization of nuclear PKC-binding proteins which may be critical for fine-tuning PKC function in this cell microenvironment. Several lines of evidence suggest that nuclear PKC isozymes are involved in the regulation of biological processes as important as cell proliferation and differentiation, gene expression, neoplastic transformation, and apoptosis (interested readers should refer to [249, 254]). As mentioned above, certain PKCs also accumulate in mitochondria. Nearly 15 years ago, the α and β isoforms of PKC were detected in a subset of mitochondria in carp retinal Müller cells [255]. These immunoelectron microscopy studies showed that the kinase was localized in the inner membrane. Since then, several data have confirmed that PKC isoforms play a direct role in regulating mitochondrial function. In particular, two isoforms of nPKCs, PKCδ, and PKCε show opposite effects on apoptosis: activation of PKCδ induces and/or enhances the apoptotic events that occur during ischemia-reperfusion and malignant progression of cancer cells, whereas activation of PKCε inhibits and/or reduces these events (recently reviewed in [244]).

Mitochondria mediate diverse cellular functions including energy generation and intracellular signalling through the generation of ROS (reactive oxygen species) and regulation of intracellular Ca^{2+} signalling [244, 256]. Changes in the spatial distribution and concentration of Ca^{2+} in the cytoplasm constitute a very important intracellular signalling

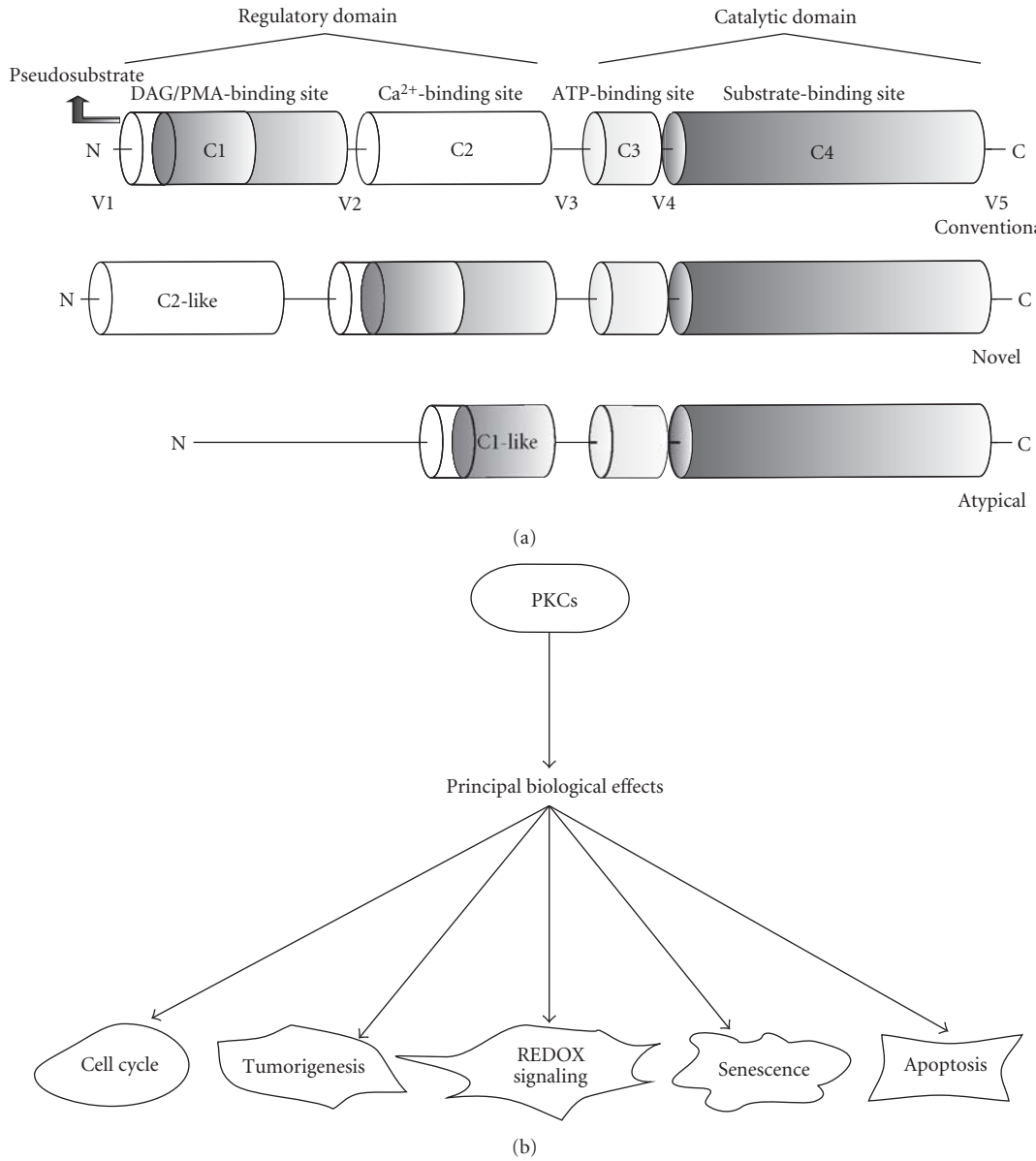


Figure 2: (a) Domain structure of protein kinase C (PKC) isoforms. All PKC family members comprise four conserved domains (C1–4) and five variable (V) domains. All isoforms contain a pseudosubstrate domain (PS) that maintains PKC in a catalytically inactive form. The C1 domains are the molecular sensors of phorbol 12-myristate 13-acetate (PMA)/diacylglycerol (DAG) in cPKC and nPKC isoforms. Atypical PKCs have only one C1 domain and are unable to bind DAG. The C2 domains function as calcium-dependent phospholipid binding modules in cPKCs, whereas nPKC C2 domains do not bind calcium, and aPKCs are lacking these domains. (b) PKCs signalling. The diagram summarizes current knowledge of the PKCs signalling pathways. Full and further details are provided in the text.

pathway in cellular physiology, including cell proliferation [257]. Ca^{2+} is an activator of some PKCs, and at the same time PKC-dependent phosphorylation reactions change the spatiotemporal pattern of cellular Ca^{2+} responses. PKC isoforms are involved in decoding high- and low-frequency Ca^{2+} spiking [258] and also in shaping the Ca^{2+} signals and Ca^{2+} release from the ER after agonist stimulation [259, 260]. In particular, PKCα is responsible for reduction of ER Ca^{2+} release, PKCζ is involved in the increase of mitochondrial Ca^{2+} uptake, while PKCβ and PKCδ are involved in the reduction of mitochondrial Ca^{2+} uptake in HeLa cells [260]. In addition, signalling by PKCs has been shown to encompass a remarkable complexity during redox stress, with differences among PKC isoforms also belonging to the same subgroup [250]. Intracellular redox state is routinely subjected to modifications during cell life, and redox stress may be involved in the pathogenesis of several diseases [244]. PKCs contain, both in the N-terminal regulatory domain and in the C-terminal catalytic domain, regions that are susceptible to redox modifications. Redox stresses change the phosphorylation state of PKC and in turn may regulate their activity; hence, PKCs are proposed to be activated in

Cytosol

Protein	Function	Ref.
Akt	Antiapoptotic roles: Phosphorylation by Akt of proapoptotic Bax protein on S184 suppresses its translocation to mitochondria. Caspase cascade is further inhibited by Akt phosphorylation of procaspase 9. Anti-apoptotic roles, cell cycle, and glucose metabolism regulation: Akt phosphorylates the two isoforms, α and β, of the glycogen synthase kinase 3 (GSK-3) in S21 and S9 respectively, promoting their inactivation. Cell growth, translation, autophagy, and cell cycle: Activation of mTORC1.	[66, 67, 70]
mTOR	Autophagy, protein synthesis: mTORC1 directly phosphorylates and inactivates 4E-BP1 whereas activates p70S6K. mTORC1 regulates numerous proteins that are required for the execution of the autophagic program. Cell growth and survival: mTORC2 phosphorylates SGK1 and cPKC. mTORC1 increases HIF-1-α protein levels by promoting mRNA translation.	[100, 102, 103, 106, 107, 118, 121]
PP2a	Cell growth and survival: Inactivates Akt through directly association and dephosphorylation. Dephosphorylates and inactivates MEK1 and ERK-family kinases, therefore inhibiting mitogenic signals, through the inactivation of PKCζ enzyme. Negative regulation of eIF4E. Inhibits the mTOR pathway, through direct inactivation of the S6 kinase Apoptosis induction: Dephosphorylates and activates the proapoptotic factor Bad.	[162, 163]
PTEN	PTEN is phosphorylated and assumes a closed conformation; this enhances its stability but opposes membrane binding.	208, 209]
PKCs	PKCs reside in the cytoplasm in an inactive conformation. Remodelling the actin cytoskeleton: Partly by phosphorylating MARCKS and pleckstrin Life-span determination: PKCβ, activated by oxidative conditions in the cell, induces phosphorylation of $p66^{Shc}$, and triggers its mitochondrial accumulation.	[252, 262]

Plasma membrane

Protein	Function	Ref.
Akt	Activation: Akt is recruited to the plasma membrane through the specific binding of PIP3 to the PH domain and is activated through phosphorylation on T308 and S473, by two different kinases, PDK-1 and the mTORC2, respectively.	[22, 23, 57]
mTOR	Cell survival: mTORC2 phosphorylates Akt on Ser473 in response to growth factors.	
PTEN	Directly antagonizes the PI3K/Akt pathway: through its lipid phosphatase activity converts PIP3 to PIP2. Cell migration: Directly associates with FAK and can reduce its tyrosine phosphorylation.	[182, 186]
PKCs	Activation: PKC translocation to the plasma membrane generally has been considered the hallmark of activation. Involved in serine phosphorylation and endocytosis of transmembrane proteins and receptors.	[251, 253]

MAMs

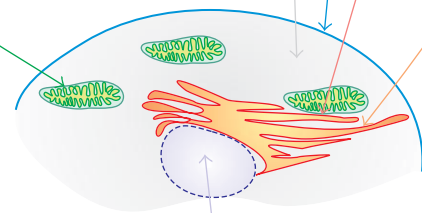
Protein	Function	Ref.
Akt	Antiapoptotic roles: Akt is localized in a specific multi-protein complex with IP3R-3, PP2a, and PML, that is essential for Akt- and PP2a-dependent modulation of IP3R phosphorylation and in turn for IP3R-mediated Ca^{2+} release from ER. Akt phosphorylates PACS-2 and represses its apoptotic activity.	[84, 85]
PP2a	Apoptosis induction: Dephosphorylation of Akt at the MAMs occurs through PML-mediated recruitment of PP2a.	[84]

Endoplasmic reticulum

Protein	Function	Ref.
Akt	Antiapoptotic roles: Akt phosphorylates IP3Rs, significantly reducing their Ca^{2+} release activity.	[80, 82, 83]
mTOR	ER and Golgi localization sequences of mTOR have been identified	[126]
PP2a	In the sarcoplasmic reticulum of cardiac muscle cells, PP2a has been shown to generate a macromolecular complex with the ryanodine receptor Ca^{2+}-release channel, the FK506-binding protein FKBP12.6, PKA, PP-1, and AKAP.	[156, 157]

Mitochondria

Protein	Function	Ref.
Akt	Antiapoptotic roles: Phosphorylates Bad allowing its sequestration in the cytoplasm Regulation of apoptosis and glucose metabolism: Phosphorylates/inactivates GSK-3 β Promotes the interaction between hexokinase and VDAC.	[65, 72, 76]
mTOR	Cell growth and survival: mTOR might be localized to mitochondria via FKBP38 or through complex with Bcl-XL and VDAC1. mTOR inhibition determines lower: oxygen consumption, mitochondrial membrane potential, and cellular ATP levels. Conversely, hyperactivation of mTORC1 increases mitochondrial DNA copy number, as well as the expression of many genes encoding proteins involved in oxidative metabolism.	[130, 131, 132, 133]
PP2a	Apoptosis induction: Dephosphorylates and inactivates the antiapoptotic Bcl-2.	[148, 149]
PTEN	Apoptosis regulation: Mitochondrial PTEN has a crucial role as mediator of apoptosis.	[238]
PKCs	Regulation of intracellularCa^{2+} signalling: PKCα is responsible for reduction of ER Ca^{2+} release, PKCζ is involved in the increase of mitochondrial Ca^{2+} uptake, while PKCβ and PKCδ are involved in the reduction of mitochondrial Ca^{2+}. Apoptosis regulation: PKCδ induces apoptosis whereas activation of PKCε inhibits it.	[244, 260]



Nucleus

Protein	Function	Ref.
Akt	Antiapoptotic roles, cell cycle regulation: Regulates fundamental transcriptional factors for the expression of pro- or antiapoptotic molecules, for examples, YAP, CREB, FOXO proteins, and NF-κB. Regulates the E3 ubiquitin ligase MDM2, a known negative regulator of the tumour suppressor p53 Phosphorylation of acinus results in its resistance to caspase cleavage in the nucleus and the inhibition of acinus-dependent chromatin condensation.	[61, 62, 63, 64]
mTOR	Regulation of ribosome biogenesis: Phosphorylation of TIF-IA and UBF, two transcription initiation factors of Pol I	[109, 110, 111]
PP2a	Chromosome stability and accurate chromosome segregation: Protecting cohesin degradation Cell survival and growth: Akt and PP2a are corecruited into PML nuclear bodies. The inability to recruit PP2a to Akt in *PML* deficiency resulted in an accumulation of nuclear pAkt, which drives tumorigenesis. Dephosphorylates and activates MDM2 resulting in the degradation of p53 Dephosphorylates the NF-κB protein family members RelA Cell cycle regulation: Required in the control of the Cdk1/cyclin B complex	[64, 146, 150, 154, 156, 164]
PTEN	Directly antagonizes the PI3K-Akt pathway: PTEN might also acts as a PIP3 lipid phosphatase in the nucleus. Loss of nuclear PTEN correlates with increased tumorigenicity. Chromosomal stability: Docking onto the centromeric-binding protein CENP-C. DNA repair: Upregulates the transcription of Rad51. Cell-cycle regulation: Induces G0-G1 arrestia result of cyclin D1 downregulation. Interacts with the anaphase-promoting complex (APC) and enhances its stability and tumour-suppressive activity.	[219, 220, 221, 222, 226, 227, 229]
PKCs	Cell proliferation and cell cycle: PKCα is antiproliferative and causes G1 cell-cycle arrest associated with ERK activation and inhibition of p21 and p27. PKCδ is involved in proliferation defects affecting G1 and G2 phase and is capable of downregulating the expression of cyclins causing cell cycle arrest. PKC/PKCα can also induce cell cycle withdrawal and growth suppression through downregulation of cyclin D, mediated by activation of 4E-BP1. PKC-mediated 4E-BP1 dephosphorylation is dependent on the activity of PP2a.	[263, 264, 265, 266, 267, 268, 269]

Figure 3: Summary of Akt, mTOR, PP2a, PTEN, and PKC intracellular localization and functions: each table summarized the various physiological processes regulated in the different subcellular compartments. Full and further details are provided in the text.

oxidative stress conditions [261]. Recently, we demonstrated that PKCβ, activated by oxidative conditions in the cell, induced phosphorylation of $p66^{Shc}$ (a protein that translates oxidative damage into cell death, since it acts as producer of ROS within mitochondria) and triggered mitochondrial accumulation of the protein after it is recognized by the prolyl isomerase Pin1 [262].

As mentioned previously, PKCs are also involved in cellular proliferation, in particular during the G1 and the G2/M phases of cell cycle. PKCα is antiproliferative and causes G1 cell-cycle arrest associated with ERK activation and inhibition of p21 and p27 in the intestinal epithelial cells [263]. PKCδ is involved in proliferation defects affecting G1 and G2 phases [264], and is capable of downregulating the expression of cyclins causing cell-cycle arrest in lung adenocarcinoma cells [265, 266]. PKC/PKCα can also induce cell cycle withdrawal and growth suppression in intestinal epithelial cells, an event associated with rapid downregulation of cyclin D1 [267, 268], mediated by activation of 4E-BP1 and inhibition of cap-dependent translation initiation [269]. PKC signalling has been shown to promote dephosphorylation of Thr45 and Ser64 on 4E-BP1, residues directly involved in its association with eIF4E, and further studies have indicated that PKC-mediated 4E-BP1 hypophosphorylation is dependent on the activity of PP2a [39].

The PKC family has been implicated in several pathologies as neoplastic transformation, ageing, diabetes, and angiogenesis. Several studies indicate that increased PKC levels can be found in malignant transformation in breast [270], lung, and gastric carcinomas [271] and in disease progression. PKCs have distinct and opposing roles in cancer development. For example, PKCε overexpression has proliferative properties in rat fibroblasts [272] and has been shown to be upregulated in various types of cancer [273], while PKCα and PKCδ are downregulated [274, 275]. In fact, PKCδ is antiproliferative, and these effects are tissue-dependent [266, 276]. The levels of PKCβII are relevant in colonic epithelial cell proliferation and progression to colon carcinogenesis. Increased expression of PKCβII was observed in both aberrant crypt foci (ACF; preneoplastic lesions of the colon) and colon tumours, as compared to normal colonic epithelium [244]. There is also a correlation between changes in PKC expression and disease progression.

In conclusion, PKCs are involved in a wide array of cellular pathways (Figure 2(b)) and potentially represent a good biological target in the treatment of disease. For example, assuming that redox stresses change the phosphorylation state and thus the activity of PKC, a potential therapeutic strategy could be the modulation of PKC activity using antioxidant agents to prevent ROS concentrations in cancer, since the formation of free radicals appears to have an important role in tumour progression. There are several cellular nonenzymatic antioxidants, such as glutathione, thiols, vitamins and metals, isoflavones, polyphenols and flavanoids. Antioxidants alone, however, are not specific and must necessarily be associated with drugs specific for different isoforms [244].

7. Conclusions and Perspectives

This paper describes the functional relationship between protein kinases and protein phosphatases in regulating signalling transduction pathways in eukaryotic cells, with a particular focus on cell cycle and apoptosis. The ability to balance a complex network of signal transduction pathways is a key element in homeostasis, enabling a cell to react accurately to external stimuli by proliferating, differentiating, or even dying. The balance between prosurvival and proapoptotic signalling is very sophisticated and the integration of the complex web of incoming signals will ultimately determine cell proliferation or cell death. One of the main mechanisms by which a normal cell appropriately and promptly transduces signals is the reversible and dynamic process of protein phosphorylation [277]. The multiple positive and negative interactions, and the subsequent cellular responses are further modulated by regulated localization of different signalling molecules to key intracellular locations, to facilitate exquisite control of function. Disturbing of this complex equilibrium results in the deregulation of a plethora of cellular processes and subsequent development of diseases. Indeed, the majority of oncogenes identified thus far encode protein kinases, and dysregulation in their activity is required for cancer initiation and maintenance. Although abundant evidence supports the role of kinase oncogenes in cancer development, the study of protein phosphatases and their regulation has only recently become an expanding field of research aimed at shedding light on the importance of these proteins in cancer. Specific protein phosphatases may also play a key role in cancer progression by acting as important regulators of kinase activation and the phosphorylation status of proteins involved in signal transduction [166, 196].

Both protein phosphatases and protein kinases play a role in modulating cell responses, yielding a wide array of potential pharmaceutical targets in cancer research. Towards these proteins, it is possible to develop small molecules that specifically inhibit or activate any abnormal cell responses that lead to cell proliferation or cell death [266, 278]. Indeed, several targeted therapies focused on inhibiting particular kinases have now been approved for clinical use, while an increasing number of studies are aimed at identifying new targets and strategies based on the counterbalancing activation of phosphatases.

Abbreviations

4E-BP1: Eukaryotic initiation factor 4E binding protein-1
ACF: Aberrant crypt foci
Acinus: Apoptotic chromatin condensation inducer in the nucleus
AGC: cAMP-dependent, cGMP-dependent, and protein kinase C
AKAP: A-kinase anchoring protein

AMPK: 5′ AMP-activated protein kinase
APC: Anaphase-promoting complex
Asp: Aspartate
ATG: Autophagy-related
ATP: Adenosine triphosphate
Bad: Bcl-2-associated death promoter
Bax: Bcl-2-associated X protein
BTK: Bruton's tyrosine kinase
Bcl-2: B-cell lymphoma 2
Ca^{2+}: Calcium ions
CaMKIV: CaM-dependent kinase IV
Cdk: Cyclin-dependent kinases
CK2: Casein kinase 2
CREB: cAMP response element-binding
DAG: Diacylglycerol
DAPP1: Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide
eIF4: Eukaryotic initiation factor 4
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated kinase
FAK: Focal adhesion kinase
FAT: FRAP-ataxia-telangiectasia
FATC: FAT carboxy-terminal
FIP200: Focal adhesion kinase family interacting protein of 200 kD
FKBP12: FK506-binding protein 12 kDa
FOXO: Forkhead box O
FRAP: Fluorescence recovery after photobleaching
FRB: FKPB12-rapamycin binding
GAP: GTPase-activating protein
GRP1: General receptor for phosphoinositides-1
GSK-3: Glycogen synthase kinase 3
HIF: Hypoxia-inducible factor
HK: Hexokinase
IGF-1: Insulin-like growth factor 1
IP3: Inositol 1,4,5-trisphosphate
IP3R: Inositol 1,4,5-trisphosphate receptor
LCMT1: Leucine carboxyl methyltransferase 1
MAMs: Mitochondrial-associated membranes
MAPK: Mitogen-activated protein kinase
MARCKS: Myristoylated alanine-rich PKC substrate
MAST 1/3: Microtubule-associated serine/threonine kinases MAST-1 and MAST-3
MDM2: Murine double minute 2
MMAC: Mutated in multiple advanced cancers
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
p70S6K: Ribosomal p70S6 kinase
PACS-2: Phosphofurin acidic cluster sorting protein-2
pAkt: Phosphorylated Akt
PDK1: Phosphoinositide-dependent kinase-1
PDZ: Postsynaptic density protein—*Drosophila* disc large tumour suppressor—zonula occludens 1 protein
PH: Pleckstrin homology
PI3K: Phosphoinositide 3-kinase
Pin1: Prolyl isomerase
PIP2: Phosphatidylinositol 4,5-bisphosphate
PIP3: Phosphatidylinositol 3,4,5-trisphosphate
PKA: Protein kinase A
PKB: Protein kinase B
PKC: Protein kinase C
aPKC: Atypical PKC
cPKC: Conventional PKC
nPKC: Novel PKC
PLC: Phospholipase C
PMA: Phorbol 12-myristate 13-acetate
PML: Promyelocytic leukemia protein
PP1: Protein phosphatase 1
PP2a: Protein phosphatase 2a
PRAS40: Proline-rich Akt substrate of 40 kDa
PRK2: PKC-related kinase 2
PTEN: Phosphatase and tensin homolog deleted on chromosome 10
RACKS: Receptors for activated C kinase
Raptor: Regulatory associated protein of mTOR
RHEB: Ras homologue enriched in brain
Rictor: Rapamycin-insensitive companion of mTOR
ROS: Reactive oxygen species
Ser: Serine
SGK1: Serum/glucocorticoid regulated kinase 1
Sin1: Stress-activated protein kinase-interacting protein
STAT: Signal transducer and activator of transcription
TEP1: Transforming growth factor-β-regulated and epithelial-cell-enriched phosphatase 1
Thr: Threonine
TM: Turn motif
mTOR: Mammalian target of rapamycin
mTORC1/2: Mammalian target of rapamycin complex 1/2
TSC1/2: Tuberous sclerosis complex 1/2
VDAC: Voltage-dependent anion channel
YAP: Yes-associated protein.

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Review Article

Acid Phosphatases of Budding Yeast as a Model of Choice for Transcription Regulation Research

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Acid phosphatases of budding yeast have been studied for more than forty years. This paper covers biochemical characteristics of acid phosphatases and different aspects in expression regulation of eukaryotic genes, which were researched using acid phosphatases model. A special focus is devoted to cyclin-dependent kinase Pho85p, a negative transcriptional regulator, and its role in maintaining mitochondrial genome stability and to pleiotropic effects of *pho85* mutations.

1. Introduction

Reactions of protein phosphorylation and dephosphorylation play a significant regulatory role in cell processes. Activity of many proteins, such as regulatory proteins, histones, permeases of various compounds, and plenty of enzymes, depends on working of protein kinases and phosphatases.

Phosphorus is known to be one of the most necessary macroelements, used in biosynthesis of the most important cell macromolecules, such as nucleic acids, proteins, and lipids. Reducing the level of inorganic phosphate (P_i) in medium leads to changes of cell physiology, influences on ATP synthesis, DNA replication, and other key processes in the cell [1]. In case of phosphate shortage, the cell can replace it by different phosphate-containing organic compounds, which undergo the cleavage of ether bonds by the phosphatases that results in release of P_i and its subsequent importation into the cell. Most of the genes encoding phosphatases are activated and repressed concordantly depending on P_i concentration in the medium. The special positive and negative regulators in and out of cell transduce the signals about that [2].

The budding yeast *Saccharomyces cerevisiae* appeared to be the most convenient object for researching structure and functions of the phosphatases. Family of nonspecific acid phosphatases is one of the most popular molecular models in yeast genetics that gives unique opportunities for investigating different functions of phosphatases and protein kinases.

2. Phosphate Metabolism

In living organisms, the phosphate is present basically in the form of orthophosphate (HPO_4^{2-}). In the yeast, P_i can be found as free ion, but the most part of it is bound in phospholipids, nucleotides, phosphoproteins, and phosphorylated hydrocarbons. An excess of P_i is accumulated in the form of polyphosphates, which represent linear polymers of orthophosphoric acid. The polyphosphate comprises phosphate atoms linked by anhydride bonds that results in its capability of storing energy and releasing it by the bonds' hydrolysis [3]. Also P_i has an important role in the intracellular pH maintenance. Beside this, P_i acts as a substrate and an effector for many enzymes (for example, phosphofructokinase) and regulates many metabolic pathways. Reaction speed alterations of any process leading to P_i release or its consumption affect the level of intracellular P_i. Decrease of P_i quantity in the medium and in the cell can be compensated by using the intracellular phosphate resources as ATP, phosphoenolpyruvate, sugar phosphates, and polyphosphates [4]. Analysis of almost 6200 yeast genes revealed 22 genes whose expression is sharply increased for the lack of phosphate. This gene group was designated

as *PHO*-regulon. Also P_i concentration influences on the expression of many other genes, which may not participate directly in phosphate metabolism, but their transcription is activated or repressed for the lack of phosphate in the medium [5].

The enzymes of phosphate metabolism include the following: isozymes of the nonspecific acid phosphatase (AP), which provide detachment of phosphate group from the phosphate-containing organic compounds in medium; transport proteins, that is, permeases with different phosphate affinity; alkaline phosphatases; polyphosphatases; polyphosphate kinases; also enzymes with phytase activity [6]. APs of *S. cerevisiae* are of special interest because they being extracellular enzymes are localized in cell wall and periplasmic space that significantly simplifies qualitative and quantitative methods of enzyme activity testing and also procedure of their extraction. Studying AP isozymes of yeast strain GRF18 (Dr. Hinnen, USA) and the strains from Peterhoff Genetic Collection (PGK), St. Petersburg, Russia revealed that yeast *S. cerevisiae* synthesize three isozymes of acid phosphatases designated as AP1, AP2, and AP3 [7]. AP1 is synthesized constitutively, while others are repressed by high phosphate concentration. Fractionating on Sephadex G-200 columns showed that all three AP isozymes (AP1, AP2, and AP3) represent glycoproteins with molecular masses more than 200 kDa (Figure 1) [8, 9]. Molecular masses of the polypeptides after deglycosylation were shown to be 57 kDa for AP1, 58 kDa for AP2, and 55 kDa for AP3 [9]. These results agree with the data of Bostian and coworkers obtained in translation product analysis of the total yeast polyA-RNA in cell-free system [10]. Studying properties of the APs showed that all of them have optimum pH value in acid range and that AP1 has lower optimum pH value near 3,7–3,8 than AP2 and AP3 (pH 4,6) (Figure 2). This feature provides the ability of hydrolyzing extracellular substrates in periplasmic space.

Constitutive AP1 is more thermostable than repressible APs (Figure 3). AP1 shows full activity at 40°C, while AP2 maintains 40% of its activity during 15 minutes, and AP3 is almost inactivated at 40°C [8, 9].

Exploration of the conditions for repressible APs accumulation in cultural medium revealed that AP3 activity inhibition occurs at the lower concentration of KH_2PO_4 than that for AP2 [9] (Figure 4).

The constitutive AP1 is encoded by gene *PHO3* of *S. Cerevisiae*; it hydrolyses different phosphate-containing substrates in periplasmic space (thiamine pyrophosphates, in particular). The increase of thiamine concentration in medium leads to *PHO3* transcription blockage [11].

Repressible AP2 and AP3, encoded by genes *PHO5* and *PHO10,11,* respectively, are synthesized in conditions of low phosphate only. Therefore, the regulation system of repressible APs is a convenient model for comparable genetic analysis of enzymes biosynthesis mechanisms [12]; In derepression conditions, the APs' fraction is mainly composed of AP2 (Pho5p), and the minor fraction consists of Pho10p and Pho11p, which have 87% amino acid sequence identity with Pho5p. Genes *PHO3* and *PHO5* are linked in II chromosome of *S. cerevisiae* [13]. The coding regions of

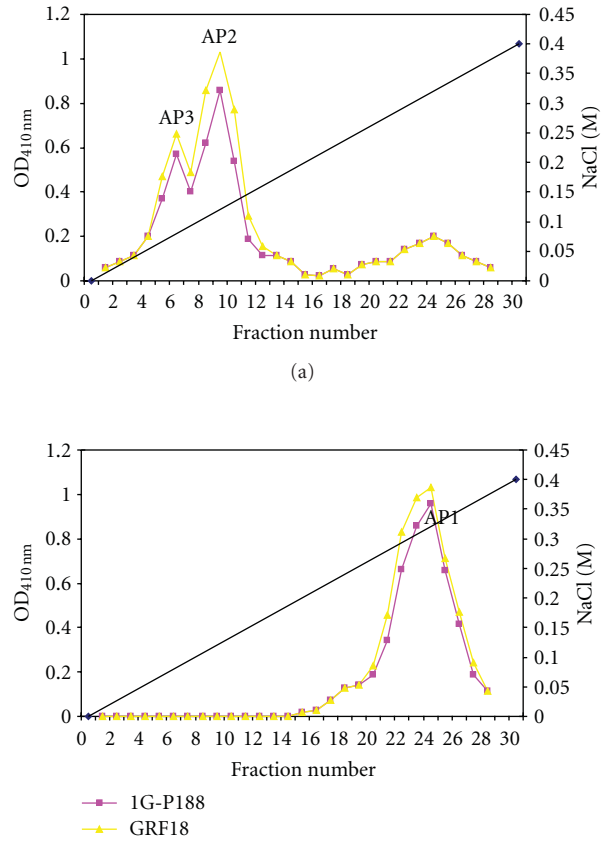

Figure 1: Gel-filtration of APs: (a) yeast strains 1G-P188 (PGK) and GRF18 (USA), cultivated in low-P_i medium; (b) strains 1G-P188 (PGK) and GRF18 (USA), cultivated in high-P_i medium.

both genes have high rate of homology in nucleotide (82%) and amino acid (87%) sequences. This rate decreases to 65% towards flanking noncoding regions and becomes very low in promoter regions of these genes [14]. Genes of acid phosphatases family *PHO3*, *PHO5*, *PHO10,* and *PHO11* are possibly the result of ancestral gene duplication [15].

2.1. Phosphate Transport. Transmembrane transportation of P_i is the first and obligatory step of its assimilation by cell. Uncombined phosphate is transported into cell by special permeases. There are three transport systems, which depend on concentrations of P_i and also H^+ and Na^+ ions. The presence of different transport systems with high and low phosphate affinity provides flexibility for yeast phosphate metabolism in continuously changing environmental conditions. Mutations affecting P_i transport occur in a set of genes: *PHO84, PHO86, PHO87, PHO88, PHO89,* and *GTR1* and still unknown gene encoding low-affinity transporter. Such mutants are characterized by constitutive *PHO5* expression. High-affinity transport system includes two phosphate permeases Pho84p and Pho89p with dissociation constant K_m ~8 μM [16, 17]. Expression of genes *PHO84* and *PHO89* is repressed by phosphate abundance [18].

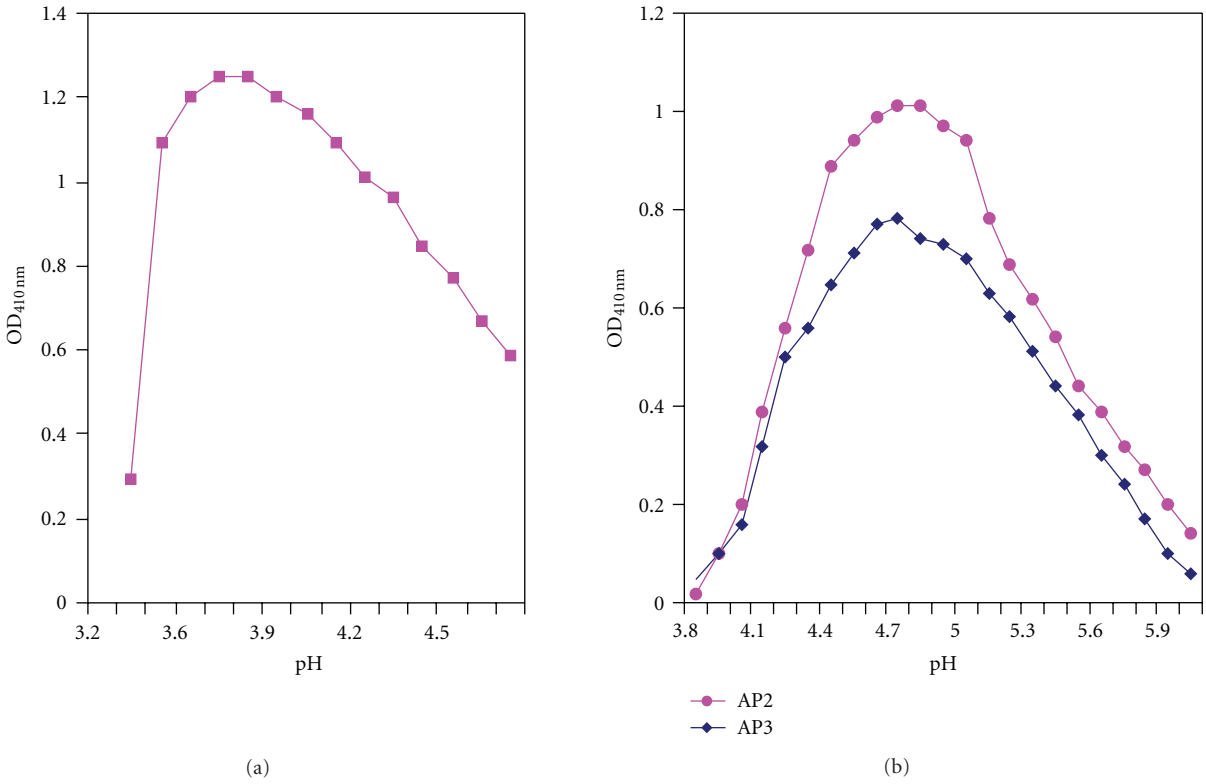

Figure 2: The pH dependence of enzyme activity: (a) AP1; (b) AP2 and AP3.

The main phosphate permease of this system is Pho84p [16]. It has K_m value ranging from 1 to 15 μM and works at pH 4,5. Pho84p is a symporter of H^+ and symports 2-3 ions with one phosphate. Furthermore, Pho84p was shown to transport selenium [19] and manganese [20]. Pho84p activity is inhibited by arsenate that allows to get selectively mutations *pho84* by picking yeast clones resistant to arsenate [21]. The quantity of Pho84p decreases in case of phosphate concentration increase or carbon source depletion in the medium [22]. Pho84p combines into complexes with some other proteins participating in P_i transport: Pho86p, Pho87p, Pho88p, and Gtr1p. The last one plays a special role: N-terminal domain of Gtrp1p (35,8 kDa) is similar to yeast protein Ypt1p and comprises a region for binding GTP. Gtr1p is a member of *ras* family of GTP binding proteins. Gene *GTR1* is localized in yeast chromosome XIII near gene *PHO84*. The disruption *pho84* leads to constitutive synthesis of APs and affects P_i assimilation by the cell. The complex Gtr1p-Pho84p is supposed to have a regulatory function and to be a receptor of P_i concentration signals [23]. Permease Pho84p is packed to COP II-coated vesicles and transported to the cell surface with the help of Pho86p [24, 25].

Another P_i transporter with high affinity is encoded by gene *PHO89*. It has K_m value near 0,5 μM and performs Na^+-associated P_i cotransportation and works at pH 9,5 [26].

Low-affinity phosphate transport system includes three permeases: Pho87p, Pho90p, and Pho91p with dissociation constant $K_m \sim 770\,\mu$M [16].

Expression of genes *PHO84*, *PHO89*, *PHO87*, *PHO90,* and *PHO91* is regulated according to the models of positive and negative feedback loops [18]. Low-affinity permeases' activity is regulated with the participation of protein Spl2p [27]. The expression of genes *PHO84* and *SPL2* is controlled by transcription factor Pho4p whose activity depends on intracellular P_i concentration.

2.2. Genetic Control of AP1 Synthesis. Transcription rate of gene *PHO3* is regulated by thiamine (vitamin B1), which is a necessary component for enzymatic reactions in Krebs cycle and pentose-phosphate pathway of carbohydrate oxidation. The yeast is able to receive extracellular thiamine from a medium using the special membrane transporter, encoded by gene *THI7*, and also to create its own intracellular thiamine using the enzyme, encoded by gene *THI4* [28]. There are some recessive mutations revealed in *PHO3*, which lead to blocking AP1 activity and changing its properties [7, 29, 30]. Unlike wild-type yeast cells, *pho3* mutants are characterized by significantly reduced intensity of thiamine pyro- and monophosphates transport. It means that AP1 is responsible for hydrolyzing thiamine phosphates in periplasmic space and is specialized on this class of phosphate-containing compounds [31]. Thiamine addition results in repressing Pho3p activity and thiamine biosynthesis enzymes [32]. *PHO3* expression was shown also to depend on the type of nitrogen source in cultural medium [33]. It was proved that *PHO3* expression level is

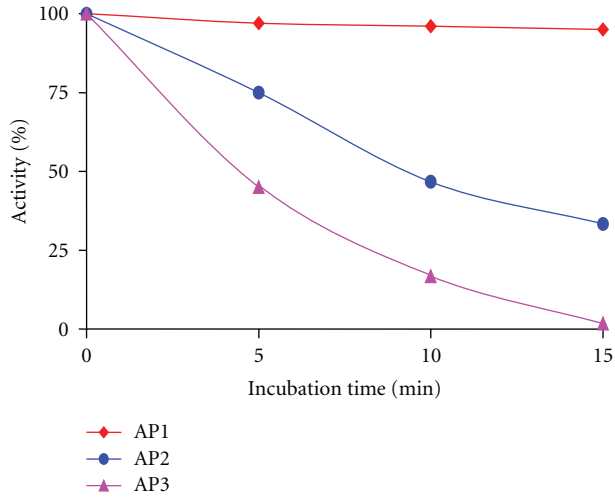

Figure 3: Thermostability of AP's at 40°C.

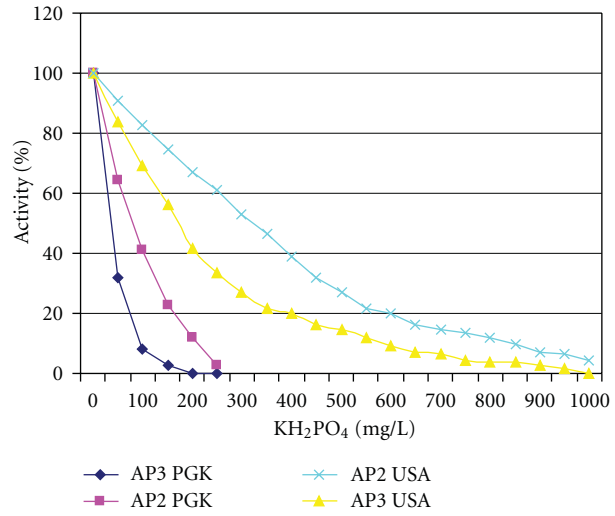

Figure 4: AP2 and AP3 enzyme activity at different KH_2PO_4 medium concentration. APs from yeast strains of PGK (St. Petersburg, Russia) and GRF18 (USA) were analyzed.

decreased while yeast cells use poor nitrogen sources like urea [34].

2.3. Genetic Control of Repressible APs Synthesis. The regulation of repressible APs genes' expression is one of the most well-studied genetic systems in yeast *S. cerevisiae* [2]. The regulation of *PHO5* gene, encoding major repressible AP, differs from *PHO10* and *PHO11* by higher rate of expression during phosphate absence and stringent repression during phosphate abundance [14]. Genes *PHO10* and *PHO11* are the result of duplication [35]. Promotor regions of nonlinked genes *PHO5* and *PHO11* have a strong homology and are regulated in coordination [36]. Because of its accurate regulating mechanism, gene *PHO5* promotor is widely used in biotechnology for industrial production of heterologous proteins in the yeast [37].

There are some recessive mutations revealed in gene *PHO5*, which affect AP2 activity; its deletion leads to growth speed decrease in complete and mineral media [12, 38]. Mutations *pho5* occurred in signal peptide damage a secretion level of the protein that was synthesized in cells successfully [39]. Intracellular AP2 represents a dimer [40]; in periplasmic space, APs form an oligomerous enzyme comprising Pho5p, Pho11p, and Pho12p [41].

Nowadays there are multiple levels of gene *PHO5* expression regulation discovered that displays an importance of this gene for the yeast cell [42–44].

The expression of the genes encoding phosphate metabolism enzymes (*PHO*-regulon) is regulated by transcriptional activators Pho4p and Pho2p, cyclin-CDK complex Pho85p-Pho80p, and inhibitor of cyclin-CDK complex Pho81p [25, 45].

Transcription induction of gene *PHO5* is associated with chromatin reorganization in promotor region [46, 47]. While in repression conditions (when phosphate is abundant), promotor of *PHO5* gene is packaged into four nucleosomes and only 70 bp region remains unoccupied and nuclease sensitive. Two nucleosomes flank the UAS1 region (5′-AATTAGCACGTTTTCGCCATA-3′) that is unoccupied, while UAS2 (5′-GCACTCACACGTGGGA-3′) and TATA-box are packed into the nucleosomes-2 and -1, respectively [48]. When phosphate concentration is low, activator Pho4p is imported in the nucleus and docks UAS1 and UAS2 regions of promotor. This leads to changing the chromatin structure and nucleosome removal [49]. It was shown that mutations in UAS1 or UAS2 lead to tenfold promotor activity decrease, and double mutations in both regulatory sequences are followed by lack of *PHO5* induction [50].

When extracellular phosphate concentration is high, cyclin-CDK complex Pho85p-Pho80p hyperphosphorylates the transcriptional activator Pho4p that leads to its localization in cytoplasm and inactivation [51]. Free of TFs gene *PHO5* promotor is a target for nucleosomal chaperon Spt6p that restores nucleosome structures in the promotor. Spt6p inhibition results in that *PHO5* promotor stays free of nucleosomes and transcription occurs *in vivo* even in the absence of the activator. Therefore, Pho4p function may be to maintain promotor in nucleosome-depleted state [52]. It was proposed earlier that chromatin remodeling takes place in the absence of DNA replication [53] and requires the presence of Pho4p activating domain [54]. This domain can interact with the transcription apparatus and assist in forming the initiation complex on the promoter only after nucleosomes' removal [55]. It is remarkable that Spt6p absence and transcription-prone chromatin can be followed by DNA replication [56].

Also SAGA protein complex contributes to *PHO5* expression activation. The attraction of this complex depends on Pho4p activity. Histone acetyltransferase Gcn5p is one of the complex subunits. Deletion *gcn5* leads to nucleosome rearrangements in *PHO5* promotor and drastic reduction of transcription rate [57]. Another member of the SAGA complex is Spt3p. Its functions differ from Gcn5p: deletion *spt3* leads to the reduction of *PHO5* transcription affecting TBP interaction with TATA-box but not touching the

chromatin structures. Double mutant *gcn5spt3* displays the same phenotype as mutants *spt7* or *spt20* [58]. They are not able to form the SAGA complex at all. Another subunit is Ada2p, whose absence results in chromatin remodeling delay like in case of mutation *gcn5*. Factor Ada2p controls Gcn5p activity via SANT domain. Thus, the SAGA complex appears to be coactivator of *PHO5* promotor along with Pho4p [59].

2.4. Phosphate Concentration Signals Transduction Pathways. The AP2 and AP3 synthesis is repressed in response to high concentration of P_i. To research transduction pathways for this signal, the mutants were selected characterized by constitutive synthesis of APs. As a result, some more genes were identified in addition to known *PHO2, PHO4, PHO80, PHO85,* and *PHO81*. Gene *PMA1* encodes ATPase of plasma membrane. Gene *ACC1* is involved in fatty acid synthesis. Also a novel, nonessential gene *PHO23* was identified. The mutants *pho84*, *pho86,* and *pma1* are defective in high-affinity phosphate uptake, whereas *acc1* and *pho23* are not. Hence, the products of these genes control different steps of signal transduction about high phosphate concentration to the regulatory factors of *PHO*-regulon expression [60].

Pho84p was shown to be not only high-affinity permease but also a signal transmitter of phosphate and glycerol-3-phosphate quantities. A novel term was proposed for the molecules like, "transceptor" [61].

2.5. Cyclin-Dependent Protein Kinase Pho85p. Yeast *S. cerevisiae* is the most well-studied eukaryotic model object for signal transmission and adaptive response molecular mechanisms studies. The main components of signalling networks are well conserved from yeast to higher eukaryotes. The central role in the regulation of signalling cascades in yeast is executed by nutrients, while in higher eukaryotic cells, the same role is played by hormones and growth factors. In yeast *S. Cerevisiae,* several nutrient-controlled pathways which adapt cell growth and proliferation, metabolism, and stress resistance, have been estimated. These pathways form general signalling network which guarantees that yeast cells enter resting phase G_0 in order to pass through the period of nutrient depletion and, on the other hand, to be capable of cell proliferation fast resumption, when nutrient conditions become favorable. Key roles in this signalling network play nutrient-sensory protein kinases PKA, Snf1p, Tor1p, Tor2p, Sch9p, and Pho85p. Pho85 is a cyclin-dependent protein kinase with a wide range of substrates, which is a functional homologue of Cdk5p [62]. Protein kinase Pho85p takes part in phosphate and glycogen metabolism regulation, autophagy regulation, stress adaptation, proline utilization, regulates proteolysis, and cell polarity.

Cyclin-dependent protein kinases (CDKs) are the group of serine-threonine kinases and have multiple regulatory functions of great importance in such global processes as cell cycle, cell differentiation, and cell response to environmental changes. *S. cerevisiae* CDK family comprises Cdc28p, Ssn3p, Kin28p, Pho85p, and Cak1p [63]. Binding CDK with cyclin protein followed by phosphorylation leads to the activation of catalytic CDK subunits [64]. Cyclins define CDK substrate specificity. Nowadays, ten cyclins Pcl1–10 and at least nineteen substrates (Pho4p, Sic1p, Rvs167p, etc.,) of Pho85p are known [63, 65].

Gene analysis indicated that *PHO85* encodes protein kinase highly homologous to Cdc28p (51% amino acid identity), the key cell cycle regulator in the yeast [62, 66]. But still the overexpression of one CDK does not compensate mutations in the other one. In view of the inviability of strains with *cdc28* mutations, Cdc28p is thought to be the main cell cycle kinase [67]. The presence of Pho85p is not necessary for viability, but this protein kinase assures normal metabolic and cell cycle dynamics reversion after stress [63].

Mutations *pho85* were firstly identified as recessive mutations of repressible APs' constitutive synthesis in the sake yeast strains (original gene name—*PHO U*) and in the strains of Peterhoff Genetic Collection (originally—*ACP82*). Studying epistatic interactions between different *pho* mutations and mutations in gene *PHO85* revealed a negative role of CDK in *PHO5* expression regulation [12, 68, 69].

The role of protein kinase is not restricted by transcription regulation. A great number of *pho85* phenotypic effects were described [70, 71]. Yeast mutants *pho85* generally do not grow at 37°C, have abnormal cell morphology [72], are incapable of growing on nonfermentable carbon substrates (ethanol and glycerol), accumulate glycogen [73], are sensitive to aminoglycoside antibiotics [74], and cannot use proline as a sole nitrogen source [75]. Disruption of gene *PHO85* leads to forming phenotype of nuclear thermosensitivity (*ts*) and respiratory incompetence *[rho-]* affecting mitochondrial DNA [70]. The diploids heterozygous for *pho85* are characterized by defects of sporulation [76], that makes it difficult to analyze these features of the mutants. The *pho85* phenotypes' research is of special interest because of homology between the yeast kinase Pho85p and the main mammalian neurogenesis protein kinase Cdk5p [77]. Mitochondria dysfunction is typical for many neurodegenerative diseases.

PHO85 transcription does not depend on cell cycle stage, phosphate concentration, and α-factor. *PHO85* expression increases during sporulation, nitrogen depletion, and after heat shock. It is remarkable that the quantity of kinase monomers is constantly high in the cell.

Ogawa and coworkers showed that CDK Pho85p has a significant role in gene expression regulation [5]. They used flavopiridol, a specific inhibitor of kinases Cdc28p and Pho85p, to analyze its influence on genome expression. This confirmed Pho85p participation in transcription control of *PHO*-regulon, stress response genes (*CTT1, HSP12,* and *UBI4*) and other genes working in stationary phase [78].

2.6. CDK Structure. Cyclin-dependent protein kinase structure is extremely conservative. Take space structure of human Cdk2p as an example (http://pdbwww1.sdsc.edu/pdb/explore.do?structureId=1B38). Cdk2 consists of 11 subdomains, which are packed in two lobes with an active site cleft among. The N-terminus of Cdk2p is important for ATP binding and correct orientation of the molecule to the catalytic and protein-binding sites. The C-terminus determines binding of protein substrates. Amino acid residues which are localized in three short β-sheet sites between two lobes are other important players for Cdk2p catalysis [79]. The conservative

PSTAIRE motive is necessary for cyclin binding in all CDKs, and Cdk2p is not a singularity [80].

The functional analysis of *PHO85* gene mutations was done in two laboratories [70, 81]. It is rather interesting to mention that CDK Pho85p needs no phosphorylation by other kinase in order to be active [82].

2.7. Pho85p Regulators

2.7.1. Cyclins. The expression of cyclin genes is coordinated according to cell cycle phases. Cyclins play a double role in kinase activity regulation. On the one hand, programmed cyclin synthesis and degradation through the cell cycle leads to the well-timed kinase activation; on the other, cyclins determine substrate specific binding of kinase [83]. Cyclins are identified in many species, and it is shown that despite the specificity of cyclin-kinase interaction, CDK can be activated by different cyclins, and cyclins in its turn can activate different kinases. An exception to this rule applies yeast *S. cerevisiae*, and the principle of "one cyclin activates one kinase" was formed above them. All cyclins have conservative domain—cyclin box, which consists of 5 α-helixes [84]. It should be mentioned that the role of cyclins is not limited by cell cycle control.

The fact, gene PHO85 encodes CDK, and its mutations lead to different pleiotropic effects, engaged Pho85p cyclins, and substrates searches. Due to two-hybrid screening it is known that ten cyclins Pcl1p-Pcl10p can interact with Pho85p. PCL1–PCL10-deleted strains were constructed and characterized, on which ground Pcl1–Pcl10 cyclins were divided into two subfamilies: Pho80 subfamily (Pho80p, Pcl6p, Pcl7p, Pcl8p, and Pcl10p) and Pcl1, 2 subfamily (Pcl1p, Pcl2p, Pcl5p, Pcl9p, and Clg1) [85].

The Subfamily of Pho80p Cyclins. Cyclins of this family take part in metabolism control, such as stress response, temperature shock, nitrogen starvation, and stationary stage of growth.

Pho80p. Pho80p was one of the first identified Pho85 cyclins. Unlike the other cyclins, *PHO80* expression is not changed through the cell cycle progression [86]. *PHO80* mutations have recessive inheritance and lead to constitutive synthesis of repressible acid phosphatases [87]. In *pho80* PGC yeast strains, two types of mutations were identified: recessive with constitutive synthesis of repressible acid phosphatases and semidominant mutations, which lead to the lack of repressible acid phosphatase expression phenotype [88]. *PHO80* expression is not governed by phosphate concentration, but still it does depend on Pho85p functionality. There are some contradictory data concerning Pho80p self-transcription regulation [88, 89]. *PHO80* transcription level is decreased during heat shock or nitrogen starvation, but during the stationary stage of growth, *PHO80* mRNA level is increased several times [33]. *PHO80* disruption has no lethal effect. There are some pleiotropic effects of *pho80* mutation, just to name few: permeability deoxythymidine monophosphate increase [90], vacuoles segregation defects [29, 30, 91, 92], decreased growth on nonfermentable carbon source containing medium [93], and methyl-methane sulphonate sensibility [94]. *PHO80* mutations lead to aminoglycosidic antibiotics resistance [33, 74]; *pho80* strains are characterized by ions K+, Mg2+, Zn2+, Ca2+, and Mn2+ sensibility increase [34, 95]. One of the most possible Pho80p partners is kinase Pho85p in all the above-mentioned processes and phenotypes. *PHO85* gene overdose compensates the effects of *pho80* deletion partially and shows allele-specific effect [93].

Pho80p-Pho85p complex phosphorylates transcriptional factor bHLH-Pho4p [96, 97], as a result Pho4p stays in cytoplasm and is not capable of *PHO*-regulon genes activation on the medium with phosphate abundance [98]. *PHO80* overexpression leads to *PHO5* transcription inhibition restoration in *pho85* strains, which means that cyclin abundance can lead to nonspecific interactions with other kinases [98].

Pcl6p and Pcl7p. Pcl6p and Pcl7p cyclins have 46% identity. *PCL6* mRNA level is constant during all stages of cell cycle, but is increased during heat shock and nitrogen starvation and decreased in stationary phase of growth significantly, while *PCL7* mRNA level is increased by sixfold. *PCL6* insertional mutants have complete medium growth defects [33]. Pcl6p-Pho85p complex is able to phosphorylate Pho4p *in vitro*, but *in vivo* it is still unknown whether Pho4p is Pcl6p-Pho85p complex's substrate or not. Pcl6p and Pcl7p cyclin's role in metabolism control is rather contradictory. Some researchers have shown that Pcl6p-Pho85p complex regulates transcription of genes *PUT1* and *PUT2*, which encode proline utilization components [5, 75]. *PCL6* and *PCL7* mRNA increase during nitrogen starvation suits this hypothesis. But in another research work, it has been shown that Pcl6p-Pho85p and Pcl7p-Pho85p complexes control glycogen accumulation [99]. *PCL7* expression varies during cell cycle progression unlike other cyclin's genes of Pho80p subfamily. *PCL7* mRNA level is increased in the middle of S-phase of growth. Pcl7p-Pho85p complex activity is inhibited by Pho81p during phosphate starvation. Authors have considered a role of Pcl7p-Pho85p complex in cell cycle regulation [100]. Since cyclins Pcl7 and Pcl6 interact with common substrate, it is fair to assume that this substrate is Pho4p as in *PUT1,2* gene expression regulation and so in glycogen accumulation process.

Pcl8p and Pcl10p. Other members of Pho80p cyclin subfamily are Pcl8p and Pcl10p, which also form a complex with kinase Pho85p and phosphorylate glycogen synthase Gsy2p as *in vitro* so *in vivo*. Gsy2p phosphorylation prevents glycogen accumulation on phosphate-abundant medium [101]. As a conclusion of Pho80p subfamily cyclins description, it should be mentioned that protein interaction analysis by HMS-PCI (high-throughput mass spectrometric protein complex identification) method showed Pho85p interaction only with cyclins Pcl6p, Pcl7p, Pcl8p, and Pcl10p [102].

Pcl1/2p Subfamily Cyclins. Pcl1/2p subfamily cyclins are characterized by expression fluctuation during cell cycle progression. *PCL1/PCL2* expression pattern research has shown

mRNA concentration decrease during heat shock, nitrogen starvation, and stationary stage of growth. Therefore, cyclins, that provide metabolic processes, are of key importance at stress conditions, rather than cyclins, which are necessary for cell cycle progression.

PCL1, *PCL2*, and *PCL9* have maximum expression at early G_1 phase *PCL5* mRNA level is constant during cell cycle progression but increases during nitrogen starvation, thermo shock, or stationary phase of growth [33]. *Pcl1/pcl1, pcl2/pcl2/, clg1/clg1, pcl5/pcl5, pcl9/pcl9* diploids, and *pho85*-deleted strains have common phenotype of abnormal cell morphology. Haploid cells of Δ*pho85* strains are characterized by large round cells with giant vacuoles. Homozygous *pho85/pho85* diploids also have some morphological defects: wide bud neck, granular cytoplasm, and elongated cells, but this phenotype is more noticeable on complete medium rather than on mineral [103]. One of the most interesting Pcl2p-Pho85p and Pcl9p-Pho85p substrates is protein Rvs167, which is homologous to mammalian amphiphysin-1. Rvs167p takes part in actin cytoskeleton regulation and cell survival after starvation [104]. *PCL9* transcription is activated by transcriptional factor Swi5p. Pcl9p-Pho85p complex is able to phosphorylate Pho4p *in vitro* and plays an important role in M/G_1 checkpoint transition [105]. *PCL2* transcription activation also takes place after α-factor treatment and *PCL1*; *PCL9* mRNA level is decreased on the contrary. α-factor treatment leads to G1 stage arrest as far as *CLN1*, *CLN2* genes are low expressed and Cdc28p is inactive. Cell cycle progression is blocked, and Pcl2p-Pho85p is still functionally active.

Clg1p is another member of Pcl1/2p subfamily with 27% homology to Pcl1p. *CLG1*-mutated strains have no visual phenotypic effects, and *CLG1* expression is constant during cell cycle progression [65]. *CLG1* mRNA level fluctuations take place during environment changes: it is decreased during amino acid and nitrogen starvation and is increased at stationary phase four times [33]. It is possible that Clg1p is the cyclin, which is responsible for the regulation of stationary phase genes by protein kinase Pho85p.

Cyclins compete with each other for Pho85p kinase binding, so that cyclins expression fluctuations make the delicate regulation different depending on Pho85p processes possible.

Pho85p-Pho80p Substrates. Cyclins determine the kinase substrate specificity. 19 Pho85p substrates were identified [62, 63]. Bound with different cyclins, protein kinase Pho85p takes part in the regulation of G1/S progression, cell polarity, *PHO*-regulon genes expression, and signal transduction [106]. Strains with *pho85* deletion are characterized by different phenotypic effects, such as constitutive synthesis of repressible acid phosphatases, slow growth with G_1 arrest, glycogen accumulation, cell morphology changes, sporulation and cell polarization defects, irregular cell divisions, actin depolarization, endocytosis defects, and high frequency of thermosensitive and respiratory-deficient cells genesis [107].

Respiratory-deficient phenotype is associated with mitochondrion functions defects. Mitochondria participation in different vital cellular processes dictates the necessity of delicate organelle function regulation existence according to the environmental changes. In cells of higher eukaryotic organisms, kea role in numerous stress response signals is played by transcription, factor p53 which blocks cell cycle progression and triggers apoptosis in many cell types, including neurons. Phosphorylated by Cdk5 transcription factor p53 is accumulated in nucleus, where it induces pro-apoptotic genes and mitochondria-mediated apoptosis in response to genotoxic or oxidative stress. Cdk5p prevents p53 ubiquitylation and downregulation [108]. Cdk5 is implicated in both development and disease of the mammalian central nervous system. Protein kinase Pho85p is a functional homologue of Cdk5p. The loss of Cdk5 in mice is perinatal lethal, and overactive Cdk5 induces apoptosis, meanwhile *pho85* mutations have no lethality effect. The existence of high conservativity in mitochondria functions regulation mechanisms permits to project revealed in yeast mechanisms to higher eukaryotes.

Respiratory, carbon, and amino acid metabolisms take part in the regulation of mitochondrial functions in yeast [109]. An important role in mitochondria functions plays phosphate, but the consequences of phosphate metabolism defects on mitochondria functions in yeast are deficiently studied. Respiratory-deficient phenotype of *pho85* strains was thought to be a pleiotropic effect of the mutation [81]. Genetic analysis has shown that respiratory deficiency of *pho85* strains is estimated by nonnuclear determinant [110]. Respiratory deficiency of *pho85* strains can be the consequence of different reasons:

(i) point mutations or deletions of mitochondria DNA (mtDNA), as it was shown for *abf2*, *msh1*, *mip1*, and *rim1* mutants [111];

(ii) mtDNA synthesis defects in the consequence of nucleotide pool changes (*adk1*, *sml1*) [112, 113];

(iii) changes of protein quantity, which take part in mtDNA synthesis and compactization, because of nuclear genes expression level changes (*ILV5*, *ACO1*) or organelle protein transport defects (*tom20, pam17,* and *tom6*) [114, 115];

(iv) mitochondria transport defects, which are determined by changes in mitochondria morphology (*mje1, pam18, ssc1, tim17, tim23, tim44, tom20, tom40, tom7,* and *tom70*) or by actin cytoskeleton structure or regulation changes (*act1*, *trp1*, *bni4*, *rvs167*, *sac6*, *sla1*, *prk1*, and *myo3*) [115, 116];

(v) mtDNA destabilization by mitochondrial mitochore structure changes or by mtDNA transport defects (*puf3*, *mdm31*, *mdm34*, *mmm1*, *mdm10*, and *mdm12*) [117];

(vi) mitochondria membrane potential changes, as a consequence of membrane permeability defects (*crd1*, *atp10*, *atp11*, *atp12*, *atp25*, *fmc1*, *oxa1*, and *mir1*) [118].

Respiratory-deficient cells of *pho85* strains appear on the medium with glucose when mitochondria functions

are repressed. Mitochondria GFP marking has shown that respiratory-deficient phenotype of *pho85* strains is not a consequence of mitochondria morphology or transport defects [119, 120]. It was shown that *pho85* strains are characterized by high nuclear DNA mutation rate [121].

mtDNA mutations frequency of active dividing cells depends neither on the moment of mutation appearance during cell divisions nor on replication regulation factors, mtDNA recombination, and segregation. These processes determine mutation fixation rate in multicopy pool of mtDNA. It was shown that *pho85* mutations lead to changes in expression pattern of genes *ABF2*, *ACO1*, *CCE1*, *MSH1-6*, *PIF1*, *MDM12*, *MMM1*, *MMM2*, *KGD1,* and *RPO41*, which encode mtDNA replication, reparation, and segregation proteins [5]. We have shown that *[rho^0]* appearance in *pho85* strains is not a consequence of mutation accumulation, but is a result of mtDNA loss. Nucleoids loss of *pho85* strains is taking place on the medium with glucose and high concentration of phosphate. It was shown that in such environment respiratory-deficient cells have selective advantage [120].

The loss of mtDNA is a dynamic process, and respiratory-deficient cell population consists of cells with full mtDNA loss *[rho^0]* so of respiratory-deficient cells with low mtDNA copy number, which is not enough for respiration [122]. *[rho^0]* colonies would not be able to revert on glycerol medium, but cells with low mtDNA copy number could reproduce normal mtDNA quantity, respiration, and growth on glycerol. We selected revertants, which had restored *pho85* strains respiration capability. Phenotype analysis and test for allelism revealed that respiration restoration of *pho85* strains is a consequence of *pho4*, *pho81*, *pho84,* and *pho87* mutations.

In *pho85* strains, transcription factor Pho4p is always localized in nucleus, where it activates the expression of *PHO*-regulon genes, which encode phosphate permeases Pho84p and Pho87p, for example. As a result, an intracellular phosphate level is uncontrollably growing. Under such conditions, *pho85* cells with secondary mutations in genes encoding permeases Pho84p or Pho87p, in regulatory genes *PHO4* or *PHO81* or unidentified genes, get a selective advantage [120].

Increase of intracellular phosphate concentration can lead to some negative effects, like plasmalemma or mitochondria membrane potential changes [123], PKA signal pathway activation [124], and so forth. Mutations preventing uncontrolled phosphate transport apparently normalize its cytoplasm concentration. Therefore, in the course of *Saccharomyces* evolution, regulatory networks which modify mitochondrion functions according to the changes not only of carbon or nitrogen source but also of changes in phosphate concentration has formed [120].

Pho85p and Cyclin's Chaperones. Chaperones take part in protein kinases space structure organization. Yeast protein kinase Cdc28p form complexes with cyclins through some extra factors. Hsp90p's cochaperone Cdc37p is one of them. It was shown that Cdc37p is an important factor for Cdc28p-Cln2p and Cdc28p-Clb2p activation *in vitro*. Cdc37p has genetic interactions with 4 protein kinases: Cdc5p, Cdc7, Cdc15p, and Cak1p [125]. It is assumed that protein kinases stabilize inactive Cdc28p preparing it for cyclin binding [126]. Cdc37p and Cdc28p also have a genetic interaction, which was shown in synthetic lethality and suppressions analysis, but there are no evidences of Cdc37p and Cdc28p physical interactions. Two-hybrid analysis has shown physical interaction of N-terminus Cdc28p with C-terminus of Cdc37p, but still full-size Cdc28p protein did not interact with Cdc37p [125].

Pho85p's chaperones have not been identified yet. We have selected different *pho85* missense mutants, which led to molecule conformation changes. One of these mutants had F82L substitution, which had led to Pho85p folding defects and constitutive synthesis of repressible acid phosphatases. Extragenic suppressions search of strains, which restore normal repressible acid phosphatases synthesis, have revealed a few genes. One of these suppressor genes is localized to a 2 cM interval from *PHO85*, like what *EGD1* gene does. *EGD1* encodes chaperone of Gal4p and of some ribosomal proteins [70]. It is fair enough to assume that Egd1p can regulate Pho85p conformation, but extra experiments are necessary for an exact conclusion.

2.7.2. Pho85p Inhibitors. Inhibitors and cyclins extend opposite effects. The majority of CDK's activity is controlled by cyclin binding. CDK inhibitors play a role of tumor suppressors and development regulators in mammalian cells so search of mechanisms it acts with is of great importance. Nowadays, three CDK inhibitors in yeast *S. Cerevisiae* are known:

(1) Far1p, which is activated by phosphorylation during alpha factor treatment and leads to cell cycle arrest at G_1 stage by Cdc28p-Cln1p and Cdc28p-Cln2 inhibition [127];

(2) Sic1p, which inhibits Clb5p-Cdc28p and Clb6p-Cdc28p during G_1 and Pcl2p-Pho85p; it also prevents DNA synthesis as long as cell passes through G_1/S boundary [128];

(3) Pho81p is the only one identified Pho85p cyclin. *PHO81* mutations are characterized by two types of phenotype: one of them is recessive and leads to the lack of *PHO5* derepression, and other one is dominant and is characterized by constitutive synthesis of *PHO5* [5]. *PHO81* gene has been cloned [129].

PHO81 promotor has Pho4p, Pho2p binding sites and negative regulatory sequence (NRS). NRS-localized deletions lead to *PHO81* expression increase at fourfold [130]. Main *PHO*-regulon regulatory proteins take part in *PHO81* transcription regulation. Pho81p contains 6 ankyrin repeats, that in mammalian homologues are used for cyclin-kinase complex attaching [131]. In case of Pho81p, it was shown that, ankyrin repeats do not influence the inhibitor-cyclin binding so inhibition itself. However, neither N-terminus nor C-terminus of the protein has an influence on interaction of cyclin-kinase complex with Pho81p. The 80 amino acid

(645–724 aa) sequence was identified which is sufficient for cyclin-kinase complex interaction.

Activity regulation of a great number of proteins is determined by its localization. There are conformable proteins activators and Cln1p, Cln2p cyclins. Pho81p is predominantly localized in nucleus, but still it is can be situated in cytoplasm and endoplasmic membranes. Pho81p-Pho80p-Pho85p complex is constitutive, but kinase inhibition by inhibitor takes place only under phosphate starvation conditions. It is supposed that Pho81p inhibits kinase by means of cyclin binding, which leads to changes in 3(R121), 5(E154) helixes of cyclin, and kinase's PSTAIRE domain interaction [132].

Pho81p is also able to inhibit Pcl7p-Pho85p complex functioning during phosphate starvation. *PCL7* mRNA level is maximally increased during S-phase, so it is fair to assume that replication arrest is taking part according to the phosphate level [100].

So that Pho81p receives signals of phosphate concentration changes and transmits it to other proteins of signal network. Pho81p also interacts with adenylate cyclase Cdc35p. Adenylate cyclase controls PKA producing cAMP and is a part of RAS signal pathway, which in its turn takes part in glucose starvation signal pathway. Pho81p-Cdc37p interaction can lead to cAMP concentration changes in response to phosphate availability and transmit signal to PKA taking part in control of growth, proliferation, and other vital processes.

Pho81p-Cdc35p complex is localized in cytoplasmic membrane. It was shown that Pho81 can interact with a great number of other proteins, like mitotic growth component Emg1p or cortical cytoskeleton component Cof1p, but these complexes have cytoplasm localization [102]. It is possible that Pho81p influences Pho85p-cyclin complex functions according to phosphate concentration changes. Another Pho80p-Pho85p complex inhibitor is Spl2p. Spl2p and Pho81p have 27% identity [133].

Spl2p controls phosphorylation of other substrates except Pho4p, but *SPL2* transcription is regulated by Pho4p. *SPL2* mutations were identified as supressors of *plc1* mutants. *PLC1* gene encodes phosphoinositide-specific phospholipase C involved in regulating many cellular processes.

Proteolytic degradation also plays a significant role in kinase inhibitors regulation. Far1 and Sic1 target Cdc28p for degradation. Cdc28p inactivation leads to cell accumulation of inhibitors.

2.8. Transcription Regulation of PHO and ADE Genes by Metabolic Intermediates. Recent studies have revealed that several key metabolic intermediates take part in phosphate and purine metabolism [134, 135]. A 5-phosphoribosyl-5-amino-4-imidazolecarboxamide (AICAR) and succinyl-AICAR belong to these regulatory molecules [136]. TF Pho2p is involved in the regulation of AICAR-dependent genes. During AICAR accumulation, the affinity of Pho2p to promotors of phosphate and purine metabolism genes also raises. It was shown that AICAR *in vitro* interacts with TF Pho2p and Pho4p in purine starvation conditions. *In vivo* Pinson and coauthors have revealed that AICAR can stimulate the formation of Pho2p-Pho4p and Pho2p-Bas1p complexes [44]. Thus, under the purine starvation conditions, TF Bas1p and Pho4p compete for Pho2p binding that provides the coordinated regulation of nucleotides biosynthesis and P_i uptake in cell.

It was shown that SAICAR affects only the formation of Pho2p-Bas1p complex and correspondingly the regulation of purine metabolism. Supposably the physiological reason of different cell response on the accumulation of AICAR and SAICAR appears to be the result of the adenylosuccinate lyase (ASL) function which metabolizes SAICAR in the inosin-5′-monophospate (IMP) and AMP biosynthesis *de novo* [137].

Another interesting example that illustrates cross-regulation of purine biosynthesis and phosphate metabolism is the constitutive transcription of *PHO5* on the background of *ado1* and *adk1*, encoding adenosine and adenylate kinases. In both cases, Pho4p is localized in nucleus even if the phosphate concentration is high [138].

Gauthier and coauthors have shown that Ado1p and Adk1p work upstream Pho81p, independently of phosphate concentration, but in response to concentration changes of ATP as the target product and AICAR as intermediate product [139].

Beside the regulation of *PHO*-genes by P_i and purine metabolism intermediates, some other mechanisms were also identified. There are models illustrating that *PHO*-genes expression also depends on the concentration of another P_i sources in the cell such as polyphosphates and inositol pyrophosphates.

2.9. Polyphosphates Metabolism. Polyphosphates are commonly used throughout almost all organisms as the reserve pool of P_i [3]. Polyphosphates are the linear polymers of phosphoric acid where the phosphate residues are linked by phosphoanhydride bonds as in ATP. Thus, polyphosphates are not only the reserve pool of phosphates but also play role as high-energy molecules. The number of phosphate residues can range from 3 to 1000. In yeast, 37% of all phosphate is accumulated as polyphosphate and 90% of polyphosphates, are localized in vacuoles.

It is known that the polymerization of phosphates is catalyzed by polyphosphates kinase in *Escherichia coli* (*E. coli*). The reverse reaction is the result of exopolyphosphatase function [1]. The major exopolyphosphatase in yeast *S. cerevisiae* is encoded by gene *PPX1* [140]. Ppx1p is localized in mitochondrial matrix, cytosol, and cell membrane. Ppx1p has restricted substrate specificity and can not cleave pyrophosphates, ATP, and cyclic forms of tripolyphosphates. Other enzymes were also discovered: exopolyphosphatases similar to Ppx1p localized in cell membrane and vacuoles (Ppx2p, Ppx3p) and the vacuolar endopolyphosphatase Ppn1p [140–143]. Many of the genes encoding enzymes of polyphosphates metabolism were discovered by the DNA Chip technology while searching the genes regulated by *PHO*-system.

By the analysis of genes expression levels on the medium with, high- and low-P_i concentrations, three genes were revealed in the wild-type and *PHO*-genes mutants: *PHM2*, *PHM3*, and *PHM4*, which had the expression levels similar

to *PHO5* and *PHO84*. The homology of *PHM2*, *PHM3*, *PHM4,* and *PHM1* was also identified; *PHM1* showed the expression level similar to *PHO8* and *PHO86*. These genes encode integral proteins of vacuolar membrane, which form the heterotetrameric complex and regulate the activity of vacuolar H+-ATPase [144, 145]. Besides, genes encoding glycerol phosphatase Hor2p and ATP-phosphoribosyl transferase His1p are also regulated by the P_i concentration, and under starvation conditions their expression also increases along with *PHO5*, *PHO11*, *PHO12*, *PHO8,* and *PHM5*.

The AA sequences of Phm1p and Phm2p are identical for 58%. The N-end of the Phm1p, Phm2p, and Phm3p has a domain, which shows 33% homology with the N-end of Pho81p and Pho87p [18]. Strains Δ*phm1*, Δ*phm2*, Δ*phm3*, Δ*phm4*, and Δ*phm1*Δ*phm2* are viable and have the same growth dynamics as the wild-type strain. Meanwhile, such mutant cells demonstrate different levels of polyphosphate accumulation: Δ*phm1* has low level of polyphosphate, and Δ*phm2* leads to the significant decrease of polyphosphate level and to the reduction of polyphosphate chain length. In strains with double deletion Δ*phm1*Δ*phm2,* none of the polyphosphates are detected, which indicates the dubbing functions of Phm1p and Phm2p. Strains Δ*phm3* and Δ*phm4* do not contain polyphosphate also. Thus, Phm1p, 2p, 3p, and 4p play an important role in polyphosphate accumulation.

In yeast, polyphosphates serve as emergency stores of phosphate. When polyphosphate synthesis critically decreases, unassociated phosphates are accumulated in cell. Accordingly to this situation, the phosphate transport by phosphate permeases is going to be locked by the gene expression decrease. Thus, metabolisms of phosphates and polyphosphates have mutual mechanisms of regulation.

Such a scheme of mutual regulation also exists in bacteria *E. coli* and *Vibrio cholera*. Bacterial promotors of *ppk-ppx* operons have so named *pho*-box [5, 146], while phosphate starvation regulatory factor *phoB-phoR* docks *pho*-box and activates the polyphosphatases transcription [146].

Thus, the regulation of polyphosphate metabolism genes by phosphate concentration is a conservative feature of yeast and bacteria.

2.10. Inositol pyrophosphates: The New Form of Intracellular Signaling. Inositol pyrophosphates are high-energy compounds involved in many biological functions of a cell. Inositol pyrophosphates take part in the regulation of such processes as vesicular transport, apoptosis, DNA reparation, telomere stability, and stress-motivated signaling, but exact mechanisms of this influence are still not clear [147]. Inositol pyrophosphates IP_7 and IP_8 are the most characterized molecules of this signaling system, which were found in all eukaryotic cells from amoeba to neurons of higher mammals. Pyrophosphorylation reactions constantly occur, so the concentration of IP_7 and IP_8 normally is high. A lot of substrates which are phosphorylated by IP_7 were found in yeast and mammals [148]. The main advantage of phosphorylation by IP_7 is the absence of necessity to special kinase functioning, like in ATP-dependent phosphorylation. IP_7 phosphorylates substrates in direct way. The phosphorylated IP_7 peptides are more resistant to phosphatases cleaving than peptides, which have been phosphorylated by means of ATP, because of another type of phosphate bond. By energy content, pyrophosphate bond of IP_7 is equal to or even exceeds pyrophosphate bond of ATP, what indicates a great potential of these molecules in cell [149]. Pyrophosphorylation can be a new signaling system of eukaryotic cells [150].

The main players in inositol pyrophosphates biosynthesis are inositol hexakisphosphate kinases (IP6K) [151]. In yeast, *S. cerevisiae* IP6K are encoded by *KCS1* and *VIP1* genes. Strains Δ*kcs1* almost do not contain IP_7 and IP_8, but deletion of Ddp1p phosphatase gene (*DDP1*) on the Δ*kcs1* background suppresses this phenotype due to IP6K Vip1 [152]. IP6Ks Vip1 and Kcs1 synthesize different isomers of pyrophosphates. The existence of such differences in IP_7 enantiomer biosynthesis might have a great physiological value. Thus, Lee with coauthors showed that IP_7 which has been synthesized by kinase Vip1p represses Pho85p-Pho80p complex [153]. Meanwhile, the products of Kcs1p do not repress Pho85p-Pho80p. But the Kcs1p influence on *PHO*-regulon also exists as far as mutants *kcs1* are characterized by constitutive expression of *PHO5* and phosphate transport efficiency decrease [43]. Thus, the mechanisms of *PHO*-system regulation differ in the case of two IP_7 enantiomers.

Despite the complicated phosphate cellular regulation, which includes the mechanisms of mutual control of orthophosphates, polyphosphates, and inositol pyrophosphates pathways, in order to regulate the cytoplasm phosphate concentration, Pho85p functions are of critical importance.

3. Conclusion

Recent researches have revealed a significant role that nonprotein factors play in gene expression regulation in eukaryotic and prokaryotic cell. Especially, lots of data were obtained about regulatory function of different RNA molecules in response to environmental signals. The yeast *S. cerevisiae* have some well-studied samples of gene regulation by noncoding RNAs [154–156].

Three well-known asRNAs in yeast relate to phosphate metabolism. First, there is RNA molecule transcribed in antisense orientation from gene *PHO84* of the high-affinity permease of phosphates. This asRNA participates in the repression of its own gene in senescent cells [154]. Second, short asRNA is transcribed from gene *KCS1* in the presence of activator Pho4p and low phosphate concentration in medium. *KCS1* encodes structure of kinase involved in signal transduction about phosphate depletion. This asRNA blocks normal synthesis of Kcs1p that leads to the increase of Vip1p kinase activity. Vip1p competes with Kcs1p and intensifies derepression effect from phosphate depletion [157]. The third case is the one known sample of positive regulation by asRNA in the yeast. This asRNA (2400 n.) is transcribed in *PHO5* locus in repressive conditions of high phosphate, exceeds the *PHO5* ORF length (1400 n.), and binds the upstream region of gene *PHO5*. This binding leads to chromatin remodeling, histone removal from promotor DNA, and therefore *PHO5* transcription activation as soon as phosphate concentration goes down in the medium [158].

The latest analysis of cDNA libraries and RNA polymerase II binding sites mapping in *S. cerevisiae* genome revealed much more antisense RNAs with unknown functions. The future of expression control researches in eukaryotes is connected with studying the RNA role in yeast gene regulation, and *PHO*-regulon genes stand first in a queue.

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Review Article

Phosphorylcholine Phosphatase: A Peculiar Enzyme of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa synthesizes phosphorylcholine phosphatase (PchP) when grown on choline, betaine, dimethylglycine or carnitine. In the presence of Mg^{2+} or Zn^{2+}, PchP catalyzes the hydrolysis of *p*-nitrophenylphosphate (*p*-NPP) or phosphorylcholine (Pcho). The regulation of *pchP* gene expression is under the control of GbdR and NtrC; dimethylglycine is likely the metabolite directly involved in the induction of PchP. Therefore, the regulation of choline metabolism and consequently PchP synthesis may reflect an adaptive response of *P. aeruginosa* to environmental conditions. Bioinformatic and biochemistry studies shown that PchP contains two sites for alkylammonium compounds (AACs): one in the catalytic site near the metal ion-phosphoester pocket, and another in an inhibitory site responsible for the binding of the alkylammonium moiety. Both sites could be close to each other and interact through the residues ^{42}E, ^{43}E and ^{82}YYY^{84}. Zn^{2+} is better activator than Mg^{2+} at pH 5.0 and it is more effective at alleviating the inhibition produced by the entry of Pcho or different AACs in the inhibitory site. We postulate that Zn^{2+} induces at pH 5.0 a conformational change in the active center that is communicated to the inhibitory site, producing a compact or closed structure. However, at pH 7.4, this effect is not observed because to the hydrolysis of the $[Zn^{2+}L_2^{-1}L_2^0(H_2O)_2]$ complex, which causes a change from octahedral to tetrahedral in the metal coordination geometry. This enzyme is also present in *P. fluorescens*, *P. putida*, *P. syringae*, and other organisms. We have recently crystallized PchP and solved its structure.

1. Introduction

Bacteria possess multiple proteins with the capacity to catalyze the hydrolysis of phosphoric esters in either acidic or alkaline media. In addition to having different optimal pH values for their activity, these enzymes differ in their dependence on metal ions, which are generally divalent cations found in the second or third period of the periodic table. In our previous review, we summarized work showing that the synthesis of *Pseudomonas aeruginosa* phosphorylcholine phosphatase (PchP) in the presence of low or high orthophosphate concentration depends on choline, betaine, dimethylglycine, or carnitine added to the culture medium as the carbon and/or nitrogen source. In addition, the gene for PchP was located, and the most current information on the kinetic, biochemical, biophysical, and molecular characteristics of PchP was summarized [1]. Phosphorylcholine (Pcho), phospho-rylethanolamine, and *p*-nitrophenylphosphate (*p*-NPP), substrates of PchP, are hydrolyzed to orthophosphate plus choline, ethanolamine, and *p*-nitrophenol, respectively [2, 3]. Because *P. aeruginosa* is an opportunistic Gram-negative bacterium capable of producing infections at different levels in higher organisms, we dedicated our effort toward in-depth characterization of PchP. Therefore, this paper focuses on fundamental studies at the molecular, biochemical, bioinformatic, and biophysical levels, with the goal of compiling all current information on PchP.

2. Molecular Studies

PA5292 was identified as the locus encoding PchP in the *P. aeruginosa* PAO1 genome using various microbiological

and molecular experiments [4]. After this identification our interest was focused on the regulation of *pchP* gene expression. Wargo et al. reported that the induction of *pchP* transcription by glycine betaine, a product of choline oxidation, via betaine aldehyde, is mediated by GbdR, an AraC family transcription factor [5]. The same authors also described GbdR as a specific regulator of genes involved in choline metabolism [6]. The construction of a Δ*gbd*R strain allowed us to confirm the direct impact of GbdR on choline catabolic genes because no growth was detected with this mutant in media with choline, choline/NH_4^+, or choline/succinate [7]. In addition, with wild-type *P. aeruginosa*, we have also observed that choline, betaine, dimethylglycine, or carnitine (which is metabolized to betaine) utilization was absolutely necessary for the synthesis of PchP [8–10]. In addition, it is known that in the absence of preferred sources, a two-component system comprising the global regulators NtrBC and CbrAB is activated by the depletion of nitrogen and carbon in many bacteria including *P. aeruginosa* [11–13]. As choline and the various amino acids are not typically preferred carbon or nitrogen sources, we hypothesized that besides GbdR, enzymes whose synthesis is dependent on choline catabolism might be also under the control of NtrBC and/or CbrAB. In control experiments with histidine where NtrBC and CbrAB were activated [11, 12], no β-galactosidase activity (a measure of *pchP* expression) was detected [7]. The specific induction produced by choline led us to focus other experiments in this direction, utilizing Δ*cbr*B, Δ*ntr*C, and Δ*ntr*C/Δ*cbr*B mutants of *P. aeruginosa* PAO1, containing the construction P1::*lacZ* (7) inserted into the bacterial chromosome. These experiments showed that CbrB is crucial when choline is the only nutrient for growth because it was observed that the Δ*cbr*B strain did not grow in media containing choline as a carbon or carbon/nitrogen source [7]. With choline as nitrogen source and succinate as carbon source, growth of the Δ*cbr*B mutant was evident, and the increase of β-galactosidase activity was similar to the observed in the wild-type strain [7]. Contrary to experiments with the Δ*cbr*B mutant, the Δ*ntr*C strain did not grow in a choline/succinate medium but did grow in media containing choline as the carbon or carbon/nitrogen source. In these two last culture media experiments, the β-galactosidase activity was reduced by only 65% with respect to the wild-type strain [7]. The 35% of remnant activity suggested that other regulators besides NtrC might be participating in the synthesis of PchP, but this point has not yet been resolved.

However, experiments with choline or dimethylglycine as carbon and nitrogen sources by *P. aeruginosa* PAO1 P1::*lacZ* showed the rapid response of the promoter during the beginning of the lag phase of growth; the production of β-galactosidase activity, at the maximum point, was approximately 50% higher in dimethylglycine medium than in choline medium [16]. These results together with findings from other laboratories [5, 6] demonstrated that GbdR and CbrB are indispensable for choline metabolism when it was the carbon source. In addition, GbdR and NtrC are necessary for the expression of PchP; dimethylglycine is likely the metabolite directly involved in the induction of PchP. Therefore, the regulation of choline metabolism and consequently PchP synthesis may reflect an adaptive response of *P. aeruginosa* to environmental conditions. This is in line with some results described in *P. fluorescens*, where a similar pattern of regulation for the catabolism of certain amino acids (histidine, proline, leucine, isoleucine, and valine) is also controlled by CbrAB and NtrBC. The specific induction of enzymes involved in their metabolism is indispensable in the presence of the specific substrate [13].

In order to explain the participation of specific regulator GbdR and the global regulators NtrBC and CbrAB in the synthesis of PchP, we first focused our attention on the transcriptional organization of the *pchP* gene [7]. Bioinformatic predictions, confirmed experimentally by site-directed mutagenesis and transcriptional fusion analyses, led to the conclusion that full *pchP* expression depends on an upstream region located −188 to −68 bp from the ATG start codon. Although this sequence has a score below the best prediction for a σ^{54}-dependent promoter, it contains (i) the conserved −24 GG and −12 GC elements characteristic of putative σ^{54}-dependent promoters, (ii) a region that resembles an IHF binding site, and (iii) a potential EBP binding site resembling the palindromic NtrC-binding consensus site [17, 18]. The dependence of *pchP* expression on the σ^{54} factor was confirmed using a Δ*rpo*N mutant strain, which showed a strong reduction in expression (≅70%). On this point, we assumed that the residual expression may result from a second promoter that could be activated by GbdR. Because the upstream region of PA5380 (*gbdR*), as analyzed by PromScan software, has a high similarity score (86 out of 100) with σ^{54}-dependent promoters, we suggest that a cascade of events happen when choline is the alternative source of carbon, nitrogen, or carbon and nitrogen; (i) NtrB and CbrA respond to the absence or deficiency of the preferred carbon or nitrogen sources. (ii) These sensors activate, through phosphorylation, the respective response regulators NtrC and CbrB, which are enhancers of σ^{54}-dependent promoters. (iii) As choline is an alternative carbon and nitrogen sources for growth, all genes involved in its catabolism or related to choline catabolism, for example, *gbdR, pchP* (and some other) are first activated. (iv) Once the intracellular concentration of GbdR increased, the enzymes of choline catabolism are directly activated [6] and specifically interact with the *pchP* promoter near the −12 box [5]. This interaction may move the σ^{54}- polymerase from the promoter, so transcription from other promoter sequences can occur. In conclusion, we are only beginning to understand this activity because *pchP* expression now appears much more complex than previously anticipated, suggesting that more than one form of RNA polymerase and multiple transcriptional regulators could be involved.

3. Bioinformatic Studies

Initial bioinformatics studies indicated that PchP belongs to the haloacid dehalogenase hydrolase (HAD) superfamily. According to the following reference [[24], (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi)],"*The haloacid*

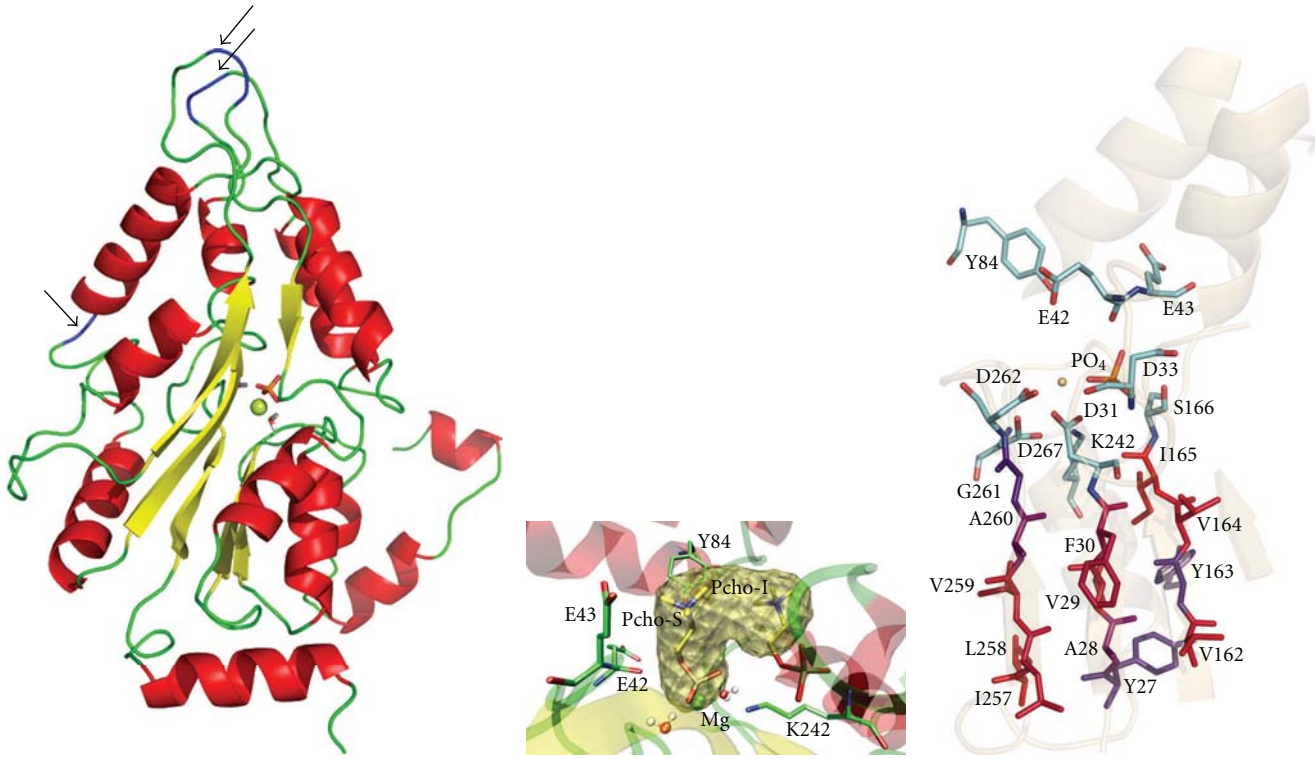


Figure 1: Model of PchP. (a) Cartoon representation. The points where loops were eliminated for modeling are indicated by an arrow. (b) A model of PchP representing the pocket detected by ICM-Pro and two conformations of Pcho. Cartoon representation of the secondary structure, stick representation of certain residues and Pcho, and msms (solvent excluded surface) representation of the pocket. Pcho-S: substrate conformation, Pcho-I: inhibitor conformation. (c) Stick representation of residues from the active site of members of the HAD superfamily, plus the hydrophobic pocket surrounding the active site. The residues of the active site are colored by element, and the rest are colored by hydrophobicity according to the Kyte and Doolittle scale [14], in which the most hydrophobic residues are red, and the most hydrophilic are blue.

dehalogenase-like superfamily includes L-2-haloacid dehalo genase, epoxide hydrolase, phosphoserine phosphatase, phosphomannomutase, phosphoglycolate phosphatase, P-type ATPase, and many others, all of which use a nucleophilic aspartate in their phosphoryl transfer reaction. All members possess a highly conserved alpha/beta core domain, and many also possess a small cap domain, the fold and function of which is variable. Members of this superfamily are sometimes referred to as belonging to the DDDD super-family of phosphohydrolases." The *pchP* gene published in the *Pseudomonas* genome database $_{V2}$ indicates that it codes for a protein containing 349 amino acids. However, because PchP is exported to the periplasmic space, it produces a mature protein containing 327 amino acids. Therefore, motifs I, II, and III, which are characteristic of enzymes belonging to the HAD superfamily, are found at ^{31}**D**M**D**NT^{35}, ^{166}S, and ^{242}K/^{261}G**D**TPDS**D**^{267} (the aspartyl residues involved in the catalysis of PchP are denoted in bold and underlined) [25, 26].

3.1. Molecular Modeling. As previously indicated [24], all members of the HAD superfamily share a similar catalytic mechanism that uses a nucleophilic aspartate, but the overall homology among these enzymes is small. Their sequence identity is less than 15% and is focused on three short motifs that form the active site [27]. In previous modeling studies of PchP, threading techniques were employed using the *Methanococcus jannaschii* phosphoserine phosphatase atomic coordinates as a template (*mj*PSP, PDB code 1F5S) [28]. This model was very useful for determining the catalytic relevance of the residues in the active site and for the description of phosphate and Mg^{2+} binding [25, 26]. However, the template was much shorter than PchP, and 3d-pssm did not model several regions; consequently the final PchP model was segmented. These problems required major improvements to the model to allow the use of a docking approach to make advances in determining the quaternary ammonium-binding site. Therefore, a strategy was implemented combining protein fold recognition and comparative modeling by satisfaction of spatial restraints. First, the PchP sequence (lacking the signal peptide) was submitted to the PHYRE server [29], which is a more accurate and updated version of 3d-pssm. This server performs alignments with proteins of known structure according to sequence identity, secondary structure as predicted by PSI-PRED, and solvent accessibility. The best-ranked template was phosphoserine phosphatase from *M. jannaschii,* with

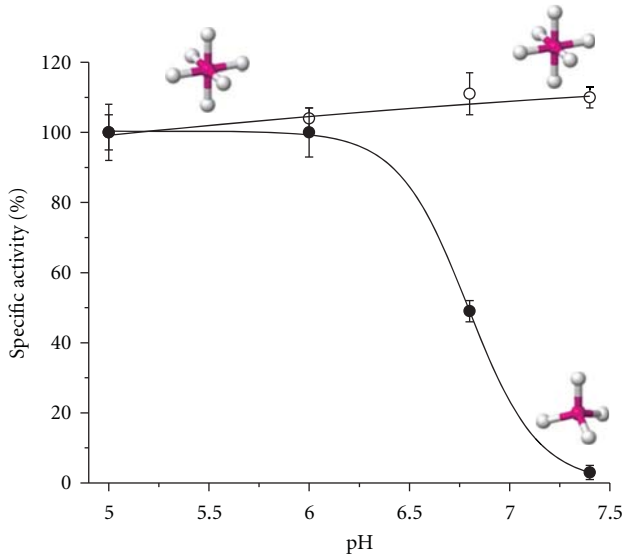


Figure 2: Effect of pH on PchP activity in the presence of 10 mM Mg^{2+} (open circle) or 0.06 mM Zn^{2+} (filled circle). The buffers used were 100 mM HAc/NaAc, pH 5.0, 100 mM HAc/KAc, pH 6.0, 50 mM Hepes/NaOH, pH 6.8, and 50 mM Hepes/NaOH, pH 7.4. Insert shows the coordination sphere for each metal.

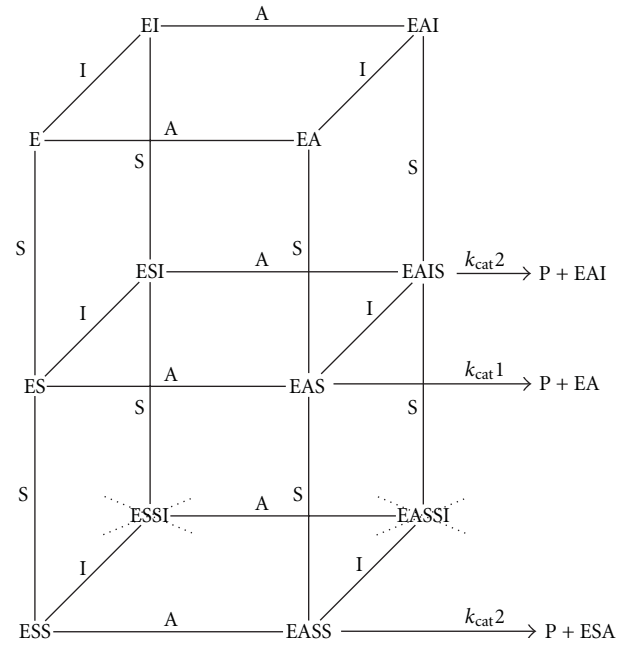


Figure 3: Scheme of the catalytic mechanism of PchP in the presence of metal ion (A), Pcho (S), and AAC (I). All reactions that occur in the cubes are reversible. Different true K_M, K_A, K_I, K_{SI}, and k_{cat} values are published elsewhere [15]. Kinetic data does not indicate that ESSI and EASSI complexes reformed.

100% confidence, but once again, template size was a problem. While *mj*PSP has 222 residues, PchP has 327; this results in regions without a template for PchP modeling. In addition, the secondary structure predicted for those regions was coiled and formed large loops. Therefore, it was not possible to model those loops with an acceptable level of accuracy, and those regions were discarded for the next step (residues 56–67, 120–136, and 206–224). This edited alignment was the input alignment for MODELLER. The final model was minimized and equilibrated using molecular dynamics, and the regions containing the three mega-loops that were eliminated (indicated by arrows) are shown in Figure 1(a). Like all members of the HAD superfamily, the model of PchP possesses an α/β core domain that consists of a central parallel β-sheet flanked by α-helices on both sides. The catalytic scaffold is located in this central core, which is composed of four loops. Loop 1 contains the nucleophilic aspartate, loops 2 and 3 contain the serine from motif II and the lysine from motif III, respectively, and loop 4 contains the two aspartate residues of motif III involved in metal coordination. Despite the absence of the three mega-loops, the new model was significantly improved compared to the previous one [25] and allowed docking studies to be performed in the active site region.

3.2. Docking Assays. Docking studies with Pcho performed with wild-type PchP (wtPchP) only showed one major conformation for the substrate regardless of whether the entire protein or only the HAD pocket was mapped (Figure 1(b)). The alternative conformations were very similar, with only slightly modified rotation angles. The presence of two interaction sites for Pcho in PchP was measured in crude extracts [30] and in the purified enzyme [3], and attention was focused on finding an explanation for this observation. A second conformation of Pcho was detected when the docking study was performed with the wtPchP-Pcho complex obtained after the first docking. A second molecule of Pcho was docked into the pocket at its alkylammonium moiety in an empty zone of the pocket (Figure 1(b)). The binding energy of the second conformation was found to be much higher (-75.5 Kcal mol^{-1} versus -4.9 Kcal mol^{-1}), indicating that the substrate first binds in the catalytic position with high affinity, and only when this site is occupied is a second molecule of substrate able to bind into the pocket in an inhibitory conformation. The inhibitory conformation of the substrate may block the entry and/or exit of the substrate and products; the enzyme-substrate-substrate complex can be productive, but with much lower efficiency. This result explains the inhibition caused by high concentrations of substrate. Considering the pocket shape detected by ICM-Pro, as well as the kinetic and docking results, we hypothesized that the binding pocket is composed of a catalytic site (with a subsite for the phosphate moiety and a subsite for the alkylammonium moiety) together with an inhibitory site capable of recognizing the alkylammonium moiety of Pcho (or another NR_4^+ group) (Figure 1(b)). Therefore, it appeared likely that different alkylammonium compounds (AACs) could bind to both sites. Kinetic experiments seemed to confirm this possibility (the data are discussed at the end of the next section).

4. Biochemical Studies

PchP was discovered as an acid phosphatase (AcPase) whose activity, measured with *p*-nitrophenylphosphate (*p*-NPP) in

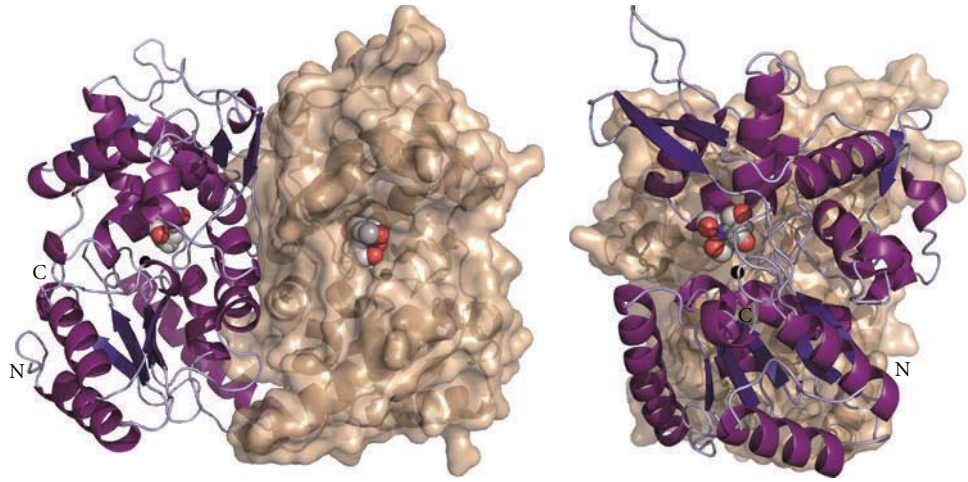
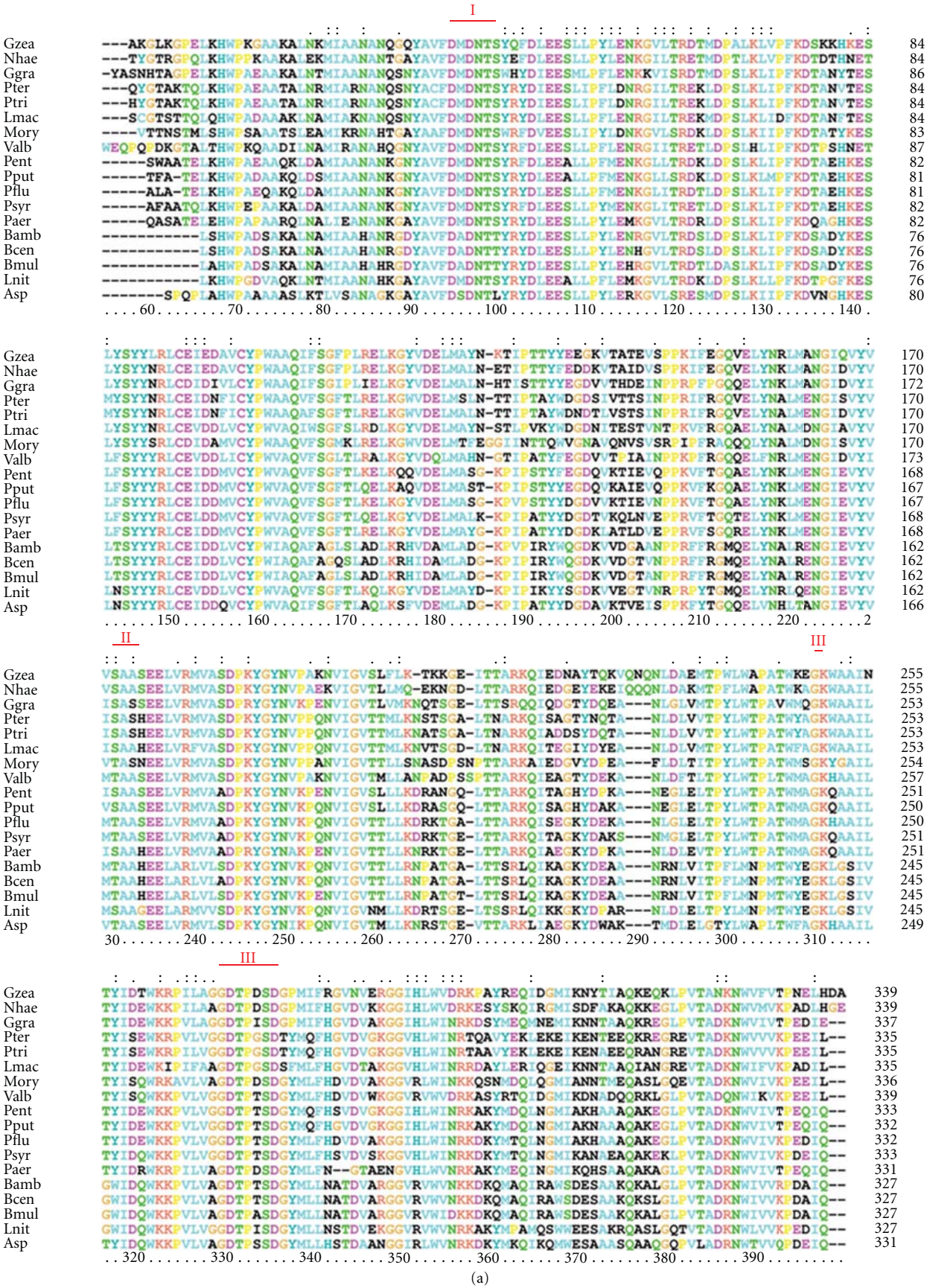


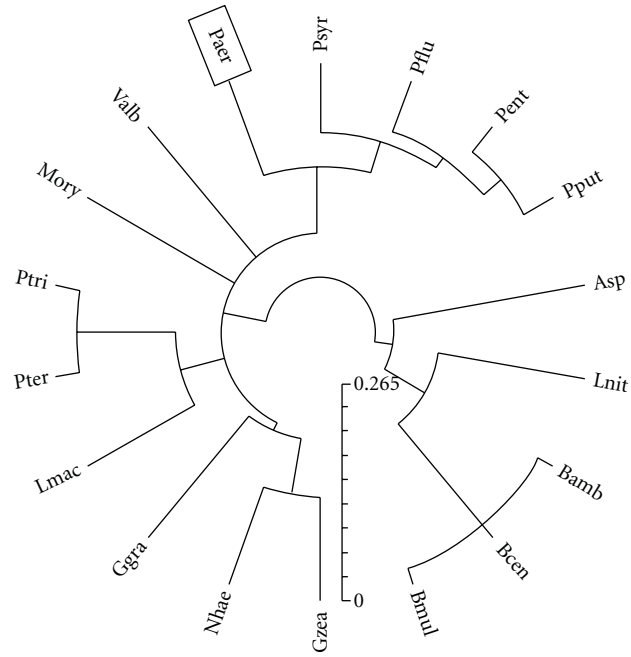
Figure 4: Two perpendicular views of a ribbon representation of the dimeric PchP crystallographic structure. The atoms at the active site are displayed in a spherical mode. The preparation of the figure from crystallographic data was performed by Lourdes Infantes and Armando Albert from IQFR-CSIC, Madrid, Spain.

the presence of Mg^{2+} at pH 5.0, is inhibited by choline, betaine, acetylcholine, Pcho, and many AACs [8, 9, 15]. These unusual acid phosphatase characteristics, measured in the laboratory [8, 31] or described by other authors [32–36], led to the proposal that the AcPase produced by *P. aeruginosa* grown in the presence of choline or derivative metabolites is capable of catalyzing the hydrolysis of *p*-NPP, Pcho, and phosphoethanolamine [3]. Remarkably, the optimum pH (measured in the presence of Mg^{2+}) depends on the substrate used; it is approximately 5.0 and 6.0 for *p*-NPP and phosphoethanolamine, respectively. The optimal pH range for Pcho was found to have a plateau between 5.0 and 8.0 [3]. Considering these data and the fact that PchP acts in the periplasmic space (a compartment where the pH may vary according to environmental conditions) [37], kinetic properties, including saturation by substrate, activation by divalent cations, and inhibition by AACs, were studied with Pcho as the substrate at pH 5.0 and 7.4, and with *p*-NPP as the substrate at pH 5.0 (with this latter substrate, there is no enzymatic activity at pH 7.4). The first studies were performed with a recombinant enzyme, initially as an intein fusion [4, 25, 26] and later with the enzyme expressed with a histidine tag [38]. The catalytic importance of the aminoacyl residues on motifs, I, II, and III was shown through site-directed mutagenesis of the aspartyl (^{31}D, ^{33}D) and threonyl (^{35}T) residues of motif I, of the seryl (^{166}S) residue of motif II, and of the lysyl (^{242}K), glycyl (^{261}G), and aspartyl residues (^{262}D, ^{265}D, and ^{267}D) of motif III (Figure 1(c)) [25]. By comparison with the phosphoserine phosphatase of *M. jannaschii* [28], ^{31}D is phosphorylated during phosphoester hydrolysis, and the oxygen atom of the carboxyl group of ^{31}D may be involved in nucleophilic attack on the phosphorus atom of the substrate (either *p*-NPP or Pcho). The ^{33}D residue is important for catalysis because it participates in the phosphorylation of ^{31}D. Considering the motif III residues ^{262}D, ^{265}D, and ^{267}D, it has been shown that ^{262}D and ^{267}D are the aspartyl residues involved in catalysis. Additionally, it has been shown that the positively charged group of lysyl residue ^{242}K is necessary to stabilize the negative charge of the phosphate [25]. After confirming the residues that interact with phosphate and magnesium, we focused our interest on the interaction with the choline moiety of the substrate. This approach was based on comparison to the human enzyme PHOSPHO1 (a phosphocholine/phosphoetanolamine phosphatase also belonging to the HAD superfamily) [39], PHOSPHO2 [40], and choline binding proteins of Gram (+) bacteria [41]. We selected residues ^{42}E, ^{43}E, and the aromatic triplet ^{82}YYY^{84} as possible candidates to interact with Pcho and *p*-NPP. Using a comprehensive approach including homology modeling, site-directed mutagenesis, and kinetic and docking studies, we show that the residues mentioned above interact differently with Pcho and *p*-NPP. We found that ^{43}E of PchP is critical for the distinctive inhibition produced by high concentrations of Pcho and that the absence of the side chain in that position makes PchP a more efficient acid phosphatase. ^{42}E has relevance in substrate recognition because its substitution with alanine decreased activity with *p*-NPP but increased activity with Pcho. Because mutations in residues ^{43}E and the three tyrosines affect both the affinity for substrate and the inhibitory effect for high levels of Pcho, we believe that both a catalytic and regulatory site are present in PchP and that they are near each other in space and even share residues [42]. After identifying the catalytic pocket of PchP through kinetic experiments and developing a single structural model [25, 26], the interactions of the phosphate moiety with the activating metal ions Mg^{2+}, Zn^{2+}, and Cu^{2+} were investigated [26]. The first results obtained with the recombinant enzyme confirmed previous findings [3, 43]. The new results indicated that at pH 5.0, with *p*-NPP plus suitable concentrations of different metal ions, the activity of PchP measured in the presence of Zn^{2+} or Cu^{2+} was higher compared to that with Mg^{2+}. Classic kinetic experiments such as saturation curves with substrate or



(a)

Figure 5: Continued.



(b)

Figure 5: (a) Amino acid sequence alignment of PchP with proteins from different organisms. The sequences analyzed were as follows: Paer, *Pseudomonas aeruginosa* PAO1; Psyr, *Pseudomonas syringae pv. tomato str.* DC3000; Pflu, *Pseudomonas fluorescens* Pf-5; Pent, *Pseudomonas entomophila* L48; Pput, *Pseudomonas putida* KT2440; Asp, *Azospirillum sp.* B510; Lnit, *Lutiella nitroferrum* 2002; Bamb, *Burkholderia ambifaria* MC40-6; Bcen, *Burkholderia cenocepacia* MC0-3; Bmul, *Burkholderia multivorans* ATCC 17616; Gzea, *Gibberella zeae* PH-1; Nhae, *Nectria haematococca* mpVI 77-13-4; Ggra, *Glomerella graminicola* M1.001; Lmac, *Leptosphaeria maculans*; Pter, *Pyrenophora teres* f. teres 0-1; Ptri, *Pyrenophora tritici-repentis* Pt-1C-BFP; Mory, *Magnaporthe oryzae* 70-15; Valb, *Verticillium albo-atrum* VaMs.102. The alignment was constructed using the program CLUSTAL-X [19]; (∗) indicates identical residues, (:) indicates conserved residues, and (.) indicates semiconserved residues. The three catalytic motifs of the HAD superfamily are marked in red. (b) Evolutionary relationships of 18 taxa (linearized). The evolutionary history was inferred using the neighbor-joining method [20]. The optimal tree with the sum of branch length = 2.68745267 is shown. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages [21]. The clock calibration to convert distance to time was 0.002 (time/node height). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [22] and are in units of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 338 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [23].

metal ions led to the conclusion that with the *p*-NPP substrate, the catalytic efficiency of PchP activated by Zn^{2+} is approximately 2.5- or 3.2-fold more efficient than with Cu^{2+} or Mg^{2+}, respectively. The K_M value of *p*-NPP was found to be identical when the enzyme was measured with Mg^{2+} or Zn^{2+} but increased approximately 3.5-fold in the presence of Cu^{2+}. However, the K_A value indicated that Zn^{2+} and Cu^{2+} have higher affinity than Mg^{2+} for the metal site of PchP (0.01 mM, 0.02 mM, and 0.6 mM for Zn^{2+}, Cu^{2+}, and Mg^{2+}, resp.) [26]. These results with wtPchP led to experiments designed to obtain kinetic parameters with different mutants. Although different catalytic constants were obtained, no appreciable difference with respect to the activation produced by different divalent cations was detected. Other divalent cations such as Mn^{2+}, Co^{2+}, and Cr^{2+} (experiments were performed in strict conditions to avoid the oxidation of Cr^{2+} to Cr^{3+}) also activated acid phosphatase activity. The results obtained by replacing Zn^{2+} with Co^{2+} will be useful for the study of different physicochemical properties through the utilization of electronic spectroscopy of Co^{2+}-PchP derivatives. The catalytic site of PchP lacks both histidine and cysteine, two amino acids that contribute nitrogen or sulfur atoms to coordination complexes in many Zn^{2+}- or Cu^{2+}-dependent enzymes that generally form tetrahedral or square planar complexes, respectively [44–47]. The utilization of the METALDETECTOR program [48] also indicated that the probability that either histidine or cysteine bound to the metal ion was very low (less than 10% [49]). After this information our focus was placed on the idea that Zn^{2+}, Cu^{2+}, and Mg^{2+} may form octahedral coordination complexes with the oxygen atom of the carboxylic group of aspartic acid and the main chain carbonyl oxygen. Therefore, we concluded that the three metal ions form an octahedral complex with electron pairs from six oxygen atoms from the two –COO–, one ($O\delta_1$) (^{31}D), one ($O\delta_2$) (^{262}D), one carbonyl (^{33}D), one phosphate (O_1–P), and two water molecules [25, 26, 38]. All of these donor groups are Lewis bases, and the metal ions are Lewis acids. Pearson [50, 51] introduced the concept of "hardness," (η) "*The nonchemical meaning of the word "hardness" is resistance to deformation or*

change;" this concept is useful for explaining the reasons why Cu^{2+} and Zn^{2+} are better activators than Mg^{2+}. Chemically speaking, "*hardness is resistance of the chemical potential to change in the number of electrons*" [52]. This author also introduced the concept of chemical hardness and softness in connection with the behavior of Lewis acids and bases, adding, "*hard acids prefer to coordinate to hard bases and soft acids to soft bases.*" Therefore, an acid with hardness η_A would prefer to bind to a base with similar hardness, η_B. In our case [26], Mg^{2+} is a hard acid ($\eta_A = 32.5$), whereas Zn^{2+} and Cu^{2+} have intermediate values (with η_A values of 8.3 and 10.8, resp.) [52]. The hardness parameters (η_B) for Lewis bases participating in octahedral complexes are approximately 6 to 7. Because these values are closer to the hardness values of Zn^{2+} and Cu^{2+} and are supported by kinetic data, we conclude that it is easier for these metal ions to form coordination complexes in the active site of PchP than for Mg^{2+}. Next, the catalytic mechanism of PchP was studied; Cu^{2+} was discarded for simplicity, and the following experiments were performed with Mg^{2+} and Zn^{2+}. The catalytic constant (k_{cat}) values with *p*-NPP [53] or Pcho [15] as substrates were found to be 1.7- and 1.2-fold higher for Zn^{2+} than for Mg^{2+}, respectively. The experiments were performed at pH 5.0 (in acetate buffer) and pH 7.4 (in Hepes buffer) because with Pcho as the substrate and Mg^{2+} as the activator, the optimal pH is a plateau between 5.0 and 8.0 [3]. The first experiments, which were needed to set experimental conditions for the following kinetic studies, were surprising. With Mg^{2+}, the same enzymatic activity was measured at pH 5.0 and 7.4. With Zn^{2+}, pronounced activity was found at pH 5.0, but no activity was detected at pH 7.4 (Figure 2). We next focused on this lack of PchP activity with Zn^{2+} at neutral or alkaline pH values. The model of the enzyme, including amino acids in the vicinity of motifs I, II, and III, was considered in addition to the ionization of different PchP active site groups involved in the ionization of Pcho and the possible hydrolysis of the metal ion [38]. The model presented in Figure 1(c) shows the hydrophobic amino acid residues located in the β-sheets motifs I (^{27}YAVF^{30}) and III (^{257}ILVA^{260}). These residues are responsible for creating an environment with a low dielectric constant (ε) which according to Dudev and Lim [54] is necessary for the coordination of the metal ion. Kinetic results, obtained at pH 7.4 with saturated Mg^{2+} and low Zn^{2+} concentrations and analyzed with the simulator DYNAFIT [55], indicated that Zn^{2+} might bind to free PchP or PchP-Mg^{2+} complexes with the same affinity to form the nonproductive complex E-Zn^{2+}. In addition to this experiment, it has also been shown that inhibition produced by Zn^{2+} in the presence or absence of Mg^{2+} is reversible and dependent on the pH of the reaction. To explain the changes in activity with Zn^{2+} and without Mg^{2+}, the ionization state of the enzyme was ruled out as a factor. However, we considered in detail the change of Pcho ionization due to the inactivation of PchP at pH 7.4. We considered the ionization of O-Pcho using the soft- ware [ACD, Inc. (http://www.acdlabs.com)] according to the equations

$$\begin{aligned} &O{=}P(O{-}\text{choline})(OH)_2 \\ &\quad \rightleftarrows O{=}P(O{-}\text{choline})(OH)O^- + H^+, \quad pK_2 \cong 6.0 \end{aligned} \tag{a}$$

$$\begin{aligned} &O{=}P(O{-}\text{choline})(OH)O^- \\ &\quad \rightleftarrows O{=}P(O{-}\text{choline})(O)_2{}^{=} + H^+, \quad pK_3 \gg 8.0 \end{aligned} \tag{b}$$

Equation (b) was discarded, and (a) was used. The Henderson-Hasselbalch equation ($pH = pKa + \log[A^-]/[HA]$) was used for pH 5.0, 7.4, and pH was set to the pK_2 value. According to (a):

at pH 5.0; $5.0 = 6.0 + \log[A^-]/[HA]$ and results in the following: $[A^-]/[HA] = 0.1$,

at pH 6.0; $6.0 = 6.0 + \log[A^-]/[HA]$ and results in the following: $[A^-]/[HA] = 1$,

at pH 7.4; $7.4 = 6.0 + \log[A^-]/[HA]$ and results in the following: $[A^-]/[HA] = 25$,

at pH 7.4, the main ionic species $[A^-] = [O{=}P(O{-}\text{choline})(OH)O^-]$ is approximately 250 times more concentrated than at pH 5.0. Under the assay condi-tions, this anion (free in solution or as a substrate bound to the enzyme) is neutralized by monovalent cations present in the reaction buffer (Na^+ or K^+ at pH 5.0 or 7.4). This is true when enzyme's activity is measured in the presence of Mg^{2+} or Zn^{2+}. The protonation/deprotonation of O-phosphocholine at different pH values does not change in the presence of Mg^{2+} or Zn^{2+}. Therefore, it was assumed that the inhibition produced by Zn^{2+} is caused by an intrinsic property of this ion. The catalysis produced by PchP in the metal ion binding site to form either an octahedral complex $[Mg^{2+}L_2^{-1}L_2^0(H_2O)_2]$ or $[Zn^{2+}L_2^{-1}L_2^0(H_2O)_2]$ is formed by Me^{2+}-two COO^- (provided by ^{31}D and ^{262}D residues), -one C=O (provided by ^{33}D), -one O=P (from $O{=}P(O{-}\text{choline})(OH)O^-$ or $O{=}P(O\text{-choline})OH_2$), and the oxygen atoms from two molecules of water. The charge on $O{=}P(O{-}\text{choline})(OH)O^-$ was ignored because it is not part of the coordination sphere of the metal ion. After these considerations, the results were explained considering the molecular model of the PchP active site [25, 26, 38] in addition to previously described data and theoretical calculations [44, 45, 56–64] related to the coordination of the metal ion with water and their hydrolysis at different pH values, the interaction of carboxylic and carbonyl groups, the substitution of metal ions in the active site, and the hydrophobic cavity with a low ε proximal to the active site of PchP. The inhibition produced by Zn^{2+} at pH 7.4 may be interpreted as the change from octahedral to tetrahedral coordination geometry, which is produced by the hydrolysis of the $[Zn^{2+}L_2^{-1}L_2^0(H_2O)_2]$ complex. Zn^{2+}, which has an octahedral coordination at pH 5.0 and forms a complex with a charge of zero $[Zn^{2+}L_2^{-1}L_2^0(H_2O)_2]$, may change to a negatively charged complex of either $[Zn^{2+}L_2^{-1}L_2^0(OH)^{-1}(H_2O)]^{-1}$ or $[Zn^{2+}L_2^{-1}L_2^0\ (OH)_2^{-1}]^{-2}$ at pH 7.4. Therefore, the loss of catalytic activity at pH 7.4 may produce changes in the coordination geometry at the metal binding site of PchP from an octahedral (active enzyme) to a tetrahedral (inactive enzyme) arrangement. Contrary to what occurs with Zn^{2+}, the neutral complex of $[Mg^{2+}L_2^{-1}L_2^0(H_2O)_2]$ that forms an octahedral complex does not change between pH 5.0 and 7.4. The consequence is that at both pH values, the PchP activity is similar when Mg^{2+} is utilized as an activator ion [38] (Figure 2). An

interesting point emergent of our results is to emphasize that the presence or absence of activity in enzymes of the HAD superfamily may be caused by the hydrolysis of the metal produced by variation of the pH in the reaction mixture. For example, an enzymatic activity measured at neutral or alkaline pH can be active when Mg^{2+} is present but can be inactivated by Zn^{2+} or other ions belonging to the transition metals. As above indicated, the another point of interest was to understand the catalytic mechanism of PchP with Pcho as the substrate, Mg^{2+} or Zn^{2+} as activators, and the effect produced by AACs. Saturation curves with different concentrations of Pcho analyzed in the DYNAFYT software have led to the conclusion that the catalytic mechanism follows a random sequential mechanism for the binding of Pcho, Mg^{2+}, or Zn^{2+} (Figure 3) [15]. However, these ions do not change the K_M value of Pcho. The noteworthy difference between these ions is that the K_A value for Zn^{2+} is approximately one thousand times lower than the values obtained with Mg^{2+}. Another difference is that Zn^{2+} is more effective than Mg^{2+} at avoiding the inhibition produced by high Pcho concentrations. A random mechanism also occurs for the interaction of a second substrate molecule at the second site of the enzyme, which apparently has an affinity for the choline moiety of the substrate or another AAC. These mechanisms are independent of the characteristics of the central metal ion; however, Zn^{2+} is more effective than Mg^{2+} at alleviating the inhibition produced by the entry of the second Pcho molecule or different AACs [15]. This result suggests that Zn^{2+} induces a conformational change in the active center that is communicated to the peripheral anionic site and produces a compact or closed structure. In contrast, a relaxed or open conformation occurs when Mg^{2+} acts as the metal ion activator of the enzyme. To confirm the presence of a second site for the alkylammonium moiety in the PchP molecule, the following compounds were utilized: trimethylamine, tetramethylammonium, choline, chlorocholine, betaine, hexamethonium, decamethonium, tubocurarine, and neostigmine. All these compounds produce inhibition with both competitive and partially uncompetitive components. The presence of two K_i values also indicates that these compounds, independent of the degree of inhibition produced (the changes produced for the nonalkylammonium moiety present in the inhibitor), might bind to two sites of PchP (e.g., for tetramethylammonium, the K_i1 and K_i2 values are 0.17 and 0.035 mM, respectively) [15]. The concordance between these results and the above-men-tioned bioinformatic studies led to the proposal that PchP contains an active site with subsites where the metal ion, the phosphate, and the trimethylammonium moieties are bound. The inhibitory site responsible for the inhibition by high substrate concentrations or different AACs could be a peripheral site located in the vicinity of the active site (Figures 1(b) and 1(c)).

5. Biophysical Properties and Crystallization of PchP

Simultaneously, attention was focused on studying the physicochemical properties of PchP to facilitate crystallizing the enzyme. The first approach to obtaining enough enzyme to achieve its crystallization and to study its physicochemical properties was performed as previously described [53]. The enzyme was purified from urea, and the inclusion bodies were solubilized and refolded by dialysis. The refolded PchP consisted of a mixture of native PchP and an alternatively folded enzyme aggregate that was slowly converted to the native state. It was proposed that the active enzyme is a dimer, and the catalytic parameters of native PchP for the hydrolysis of *p*-NPP were found to be in excellent agreement with the values reported previously for PchP expressed in *Escherichia coli* as an N-terminal fusion to intein or a histidine tag and purified in the folded state [25, 26, 53]. Although some data were collected, the preparation was not useful for obtaining crystals capable of diffracting. Therefore, efforts were focused on obtaining the enzyme as previously described [38]. Using this preparation technique, crystals were obtained [65], and the crystallization of PchP in the presence of different ligands will be used to solve its structure and to reveal the catalytic mechanism and the possible conformations produced by the different ligands. A structure of the enzyme has recently been obtained and is shown in Figure 4.

6. Other Microorganisms Containing PchP

Before the PA5292 gene in the *P. aeruginosa* PAO1 genome was found to be responsible for the synthesis of PchP [4], this protein was known as a hypothetical protein. This enzyme is also present in *P. fluorescens, P. putida, and P. syringae* [49, 66]. The PchPs found in these bacteria were also activated by Mg^{2+}, Zn^{2+}, and Cu^{2+}; Zn^{2+} was the best metal activator of all these enzymes. The kinetic data and the K_A values for the metal ions defined two groups; one of the groups is formed by *P. aeruginosa* and *P. putida* and has a low affinity for Mg^{2+} with apparent K_A values of 130 μM and 190 μM, respectively, and the other group is formed by *P. fluorescens* and *P. syringae,* which have a high affinity for Mg^{2+} with apparent K_A values of 38 μM and 30 μM, respectively. Catalytic efficiency is defined as the relationship V_{max}/K_M; in this respect, the enzyme isolated from *P. fluorescens* is better at catalyzing the hydrolysis of *p*-NPP in the presence of Mg^{2+}, Zn^{2+}, or Cu^{2+} with V_{max}/K_M values of 2.3, 2.5, and 3.4 for Mg^{2+}, Zn^{2+}, and Cu^{2+}, respectively, followed by those of *P. aeruginosa* and *P. putida* ($\cong$0.3–0.4 $Umg^{-1}mM^{-1}$ for the three metals) and finally by *P. syringae* ($\cong$0.03–0.04 $Umg^{-1}mM^{-1}$, for the three metals) [49]. Finally, information from many genomes utilizing the precomputed BLAST results for "http://www.ncbi.nlm.nih.gov/protein/15600485 phosphorylcholine phosphatase [*Pseudomonas aeruginosa* PAO1]" matched 351 proteins in 173 species (331 bacteria, represented principally by *Pseudomonas* and *Burkholderia* species such as *P. aeruginosa*, *P. fluorescens*, *P. syringae*, *P. savastanoi*, *P. entomophila*, *P. putida*, *B. ambifaria*, *B. cenocepacia*, *B. multivorans*, and other bacteria such as *Azospirillum sp*, and *Lutiella nitrofe*). PchP activity has also been found in plant pathogenic fungi such as *Glomerella graminicola*, *Leptosphaeria maculans*, *Pyrenophora teres, P. tritici*, *Gibberella*

zeae, *Nectria haematococca*, *Verticillium albo-atrum*, *Magnaporthe oryzae*, and *Phaeosphaeria nodorum* [67]. A multiple alignment of PchP found in different organisms utilizing Clustal X [19] is shown in Figure 5(a); evolutionary relationships and a phylogenetic tree produced by the software [20–23] are shown in Figure 5(b). Because all of these organisms might contain PchP with similar properties to the enzyme found in *P. aeruginosa,* and the crystallized enzyme in the presence of different ligands will soon be solved, the global approach used by our group to study PchP will culminate with the determination of its structure. We expect that further studies might be applied to the development of new and very effective inhibitors of PchP through metal binding, phosphate binding, or alkylammonium binding levels.

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