[Frontiers in Bioscience 4, d372-385, March 15, 1999]

LIFE AMONG THE PRIMITIVES: PROTEIN O-PHOSPHATASES IN PROKARYOTES

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1. ABSTRACT

Prokaryotes contain at least five distinct families of protein O-phosphatases, including AceK, the chimeric isocitrate dehydrogenase kinase/phosphatase, and four protein phosphatase families first identified and characterized in Eukaryotes. The latter consist of the PPP and PPM families of protein-serine/threonine phosphatases, and the low molecular weight and conventional families of protein-tyrosine phosphatases. Prokaryotic protein O-phosphatases participate in the regulation of metabolic processes and the transduction of environmental signals. Certain pathogenic bacteria employ protein-tyrosine phosphatases as virulence factors, injecting them into host cells where they enzymatically perturb the phosphorylation state of proteins therein. While our understanding of protein O-phosphorylation events in Prokaryotes only now is emerging from its infancy, their phylogenetic diversity and malleability to genetic manipulation render these "simple'" organisms powerful vehicles for answering fundamental questions concerning the origins and evolution of this key biological regulatory mechanism.

2. INTRODUCTION

2.1. Protein phosphorylation in prokaryotes

The regulation of cellular processes via the interconversion of proteins between functionally distinct phosphorylated and dephosphorylated forms has been the subject of intensive scrutiny for nearly five decades (1). Studies of eukaryotic organisms have revealed protein phosphorylation to be a remarkably versatile and sophisticated mechanism for exerting regulatory control. In each mammalian cell a thousand or more phosphoprotein "bits" are woven together to form an integrated information processing network – a bioorganic computer – capable of coordinating a myriad of cellular processes in response to a wide spectrum of internal and external cues (2-4).

By contrast, our understanding of protein phosphorylation processes in prokaryotic organisms is only now emerging from its infancy. Protein phosphorylation events have been implicated in the regulation of a number of processes in prokaryotic organisms (Reviewed in 5-13). These include:

- Chemotaxis and phototaxis
- Osmoregulation
- Sporulation
- Expression of stress response genes
- Catabolite repression
- Coordination of nitrogen and carbon metabolism
- Photosystem biosynthesis and function
- Synthesis of secondary metabolites
- Differentiation [e.g., heterocyst formation in cyanobacteria]
- Infective mechanisms of pathogens
- Regulation of the PTS sugar transferase system

However, only in relatively few instances has the architecture of the protein phosphorylation cascades in prokaryotic organisms been elucidated in full molecular detail. This disparity reflects both the long lag period preceding the recognition that prokaryotes were the sites of protein phosphorylation (14-16), and the lingering consensus that "primitive" organisms have little to contribute to our understanding of the signal transduction processes taking place in medically-relevant eukaryotes (7, 17). The tendency of bacterial signal transduction research to focus upon the two-component paradigm, in which histidine protein kinases phosphorylate aspartate residues on response regulator proteins/domains (5, 9), further reinforced the impression that phylogenetically diverse organisms shared little in common with the "higher"

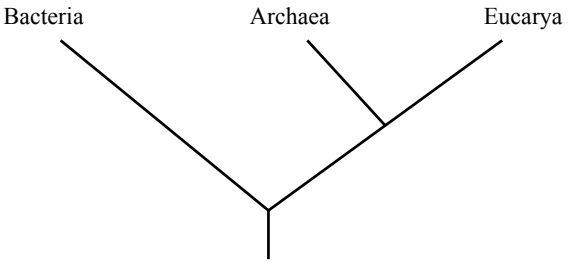

Figure 1. A schematic representation of the rooted phylogenetic tree.

organisms whose protein phosphorylation networks predominantly targeted the side hydroxyl chains of serine, threonine, and tyrosine [O-phosphorylation].

The current decade has witnessed the discovery of surprising and unexpectedly pervasive parallels between the protein kinases and protein phosphatases of eukaryotic and prokaryotic organisms. Biochemical, molecular genetic, and genomic analyses indicate that prokaryotic organisms harbor homologs of the predominant family of protein O-kinases from Eukaryotes, those sharing the catalytic domain features of the cAMP-dependent protein kinase, as well as the four major families of protein O-phosphatases from Eukaryotes (6, 11, 12, 18-20). Conversely, homologs of "bacterial" histidine protein kinases and phosphoaspartyl response regulator proteins have been discovered in several Eukaryotes (9, 21, 22). These findings have raised fundamental questions concerning the origins and evolution of protein phosphorylation as a global biological regulatory mechanism, and have revealed the potential of hitherto neglected prokaryotic organisms as important resources for addressing them.

2.2. A brief overview of phylogeny

For many years scientists believed that the natural world was bipartite in nature, that the organisms within it fell within two distinct groups: the Prokaryotes and the Eukaryotes. However, while these terms often are assumed to represent phylogenetic classifications, they are in fact morphological in origin. The distinguishing feature by which the Eukaryotes were defined was the possession of an internal, nuclear membrane that segregated their genomic material away from the bulk of the cytoplasm (23). By contrast, the definition of the term Prokaryote was essentially negative in nature. Quite literally, a prokaryote is any cellular organism that is not a Eukaryote – the leftovers so to speak. Subsequent attempts were made to refine this scheme into a molecularly-based taxonomy using comparisons of metabolic architecture, protein and tRNA sequence information, etc. (24-27). However, it required the emergence of molecular biological techniques for the isolation and sequencing of genetic material on a mass scale before scientists were able to analyze phylogenetic relationships from a truly genetic perspective (28). Surprisingly, these examinations have revealed the living world to be tripartite in nature (29) (figure 1). The Eukaryotes first identified on the basis of their intracellular compartmentation proved to comprise a single, coherent phylogenetic domain – the Eucarya [The still valid and much more commonly used term Eukaryote will be used in throughout the remainder of this article.]. However, the same cannot be said of the Prokaryotes. Rather, the organisms gathered under the Prokaryote umbrella were revealed to be members of two different, and quite distinct, phylogenetic domains – the Bacteria and the Archaea, sometimes referred to as the Eubacteria and Archaebacteria, respectively (Reviewed in 28, 30).

The Bacteria include those types of the organisms traditionally encountered in a typical survey course in microbiology: *Escherichia coli, Pseudomonas aeruginosa, Paracoccus denitrificans, Salmonella typhimurium, Staphylococcus aureus, Haemophilus influenzae,* etc. At first glance, the Archaea appeared to be specialized Bacteria adapted to life in extreme environments; those characterized by high temperatures, extreme salinity, low oxygen tension, acidic pH, or some combination thereof. However, such superficial comparisons have proved quite deceptive. *A priori,* one might predict that, since the Bacteria and Archaea share many common morphological features, they would reside on the same "branch" of a rooted phylogenetic tree, i.e. a tree that assumes the existence of a single common [i.e. "universal"] ancestor. Such a tree would leave the superficially more diverse Eucarya alone on their own unique branch. In fact, the opposite now appears to be the case. The Archaea and Eucarya sprout from the same branch of the tree, evolutionary "first cousins" so to speak. It is the Bacteria that stand alone (figure 1). While the Archaea represent a distinct phylogenetic domain, numerous archaeal genes and gene products resemble their eukaryotic counterparts more closely than their bacterial ones. These include proteins as diverse as 3-hydroxy-3-methylglutaryl-coenzyme A reductase, DNA-dependent RNA polymerase, initiation factor 5A, elongation factor 2, F_0,F_1ATPase, and numerous ribosomal proteins. In addition, the Archaea use methionyl tRNA, rather than N-formyl-methionyl tRNA, to initiate translation. The frequency with which such similarities have been encountered has triggered speculation that the Archaea number among their ancestors the proto-Eukaryote that gave rise to the nuclear/cytoplasmic portions of present day Eucarya (31).

2.3. Prokaryotic protein O-kinases

In Prokaryotes, the phosphoproteins containing the phosphomonoesters of serine, threonine, and/or tyrosine that serve as the physiological substrates of the prokaryotic protein O-phosphatases are generated by the action of at least four distinct types of protein O-kinases. The first are protein kinases homologous to the predominant family of such enzymes in Eukaryotes whose prototype is the catalytic subunit of the cAMP-dependent protein kinase (11, 32). The second are derivatives of the protein histidine kinases that target serine residues. The best known of these is SpoIIAB from *Bacillus subtilis* (33) and its homologs from the *B. subtilis* Rsb operon (34, 35). Third is the newly discovered HPr kinase (36, 37) and the fourth is AceK, the isocitrate dehydrogenase kinase/phosphatase (38). The existence of a second, distinct type of protein

Table 1. The protein O-phosphatases of prokaryotes

Family	Aliases	In Bacteria?	Archaea?	Eukaryotes?
AceK		Yes		
PPP	PP1/2A/2B superfamily	Yes	Yes	Yes
PPM	PP2C	Yes		Yes
Low MW PTP	Small, acid PTP	Yes	ORFs identified	Yes
Conv. PTP		Yes	ORFs identified	Yes

kinase from *B. subtilis* that phosphorylates the Hpr protein *in vitro,* PrkA, also has been reported (39). In Eukaryotes, only the first named of these families are known to contain members that phosphorylate the phenolic hydroxyl group of tyrosine. Representatives of all four protein kinase families have been identified and characterized in the Bacteria, while open reading frames potentially encoding both cAMP-dependent protein kinase-like proteins and histidine protein kinases have thus far been identified in the Archaea (18, 20, 40, 41). However, it has yet to be determined whether any of the latter include serine/threonine-specific variants. No data have yet come to light indicating whether or not any Prokaryote contains a homolog of the other known family of eukaryotic protein kinases that includes myosin heavy chain kinase A and elongation factor-2 kinase (42-44).

2.4. Prokaryotic protein O-phosphatases

Five distinct families of prokaryotic O-phosphatases have been recognized in prokaryotic organisms. The first to be discovered was AceK, the isocitrate dehydrogenase kinase/phosphatase, which acts upon a serine residue that guards the active site of isocitrate dehydrogenase [IDH]. To date, AceK has been encountered only in members of the Bacteria. The remaining protein phosphatase families were first identified and characterized in Eukaryotes. They include the PPP and PPM families of protein-serine/threonine phosphatases and the low molecular weight [low MW] and conventional families of protein-tyrosine phosphatases [PTPs]. Included among the conventional PTPs are so-called dual-specific protein phosphatases that hydrolyze the aryl phosphomonoester phosphotyrosine and the alkyl phosphomonoesters phosphoserine and phosphothreonine. These families and our current appreciation of their distribution are summarized in table 1.

Most prokaryotic protein O-phosphatases mimic their better-known eukaryotic counterparts, serving in the traditional role of regulators of protein function. However, some pathogenic Bacteria have adapted protein O-phosphatases, specifically conventional protein-tyrosine phosphatases, to serve as molecular missiles (45, 46). These enzymes are secreted into the infected host, where they assist the initiation or progression of infection via enzymatically perturbing the phosphorylation state, and hence functional status, of host proteins. Certain of the cyanobacteria attack the phosphorylation networks of other organisms using an alternative strategy. They elaborate polypeptide inhibitors of PPP family protein phosphatases such as microcystin LR whose potency renders them highly toxic, and oftentimes fatal, to man and other animals (47).

Another noticeable difference between prokaryotic protein O-phosphatases and their eukaryotic counterparts is the higher frequency with which multifunctional variants have been encountered among the former. The eukaryotic branches of the various protein phosphatase families display a high degree of specialization in the nature of the phosphoamino acids that they target. The eukaryotic members of the PPP and PPM families are thought to be almost exclusively serine and threonine specific (48). The low MW PTPs are tyrosine-specific (49). While the conventional PTP family includes some enzymes classified as dual-specific, only the VHR family of "PTPs" and Cdc14 from yeast exhibits the ability to dephosphorylate serine, threonine, and tyrosine on a broad range of substrates (50, 51). The other dual-specific PTPs, those of the MAP kinase phosphatase (52) and Cdc25 subfamilies (53, 54), are highly specialized enzymes that target closely juxtaposed phosphotyrosine and phosphoserine or phosphothreonine residues forming an extended phosphorylation site on a single protein target, either MAP kinase or Cdc2, respectively. Thus, these enzymes are characterized by an extremely narrow substrate specificity rather than "dual-specificity". Studies of the catalytic capabilities of those few prokaryotic protein O-phosphatases characterized to date indicate a much higher frequency of dual-specificity and perhaps even multifunctionality, such as dephosphorylation of the phosphoramides of histidine and lysine, *in vitro*.

Little evidence exists for determining the extent to which the greater apparent catalytic versatility of prokaryotic protein O-phosphatases becomes realized *in vivo*. The apparent failure of prokaryotic protein phosphatase families to fall into the serine/threonine- and tyrosine-specific niches so characteristic of their eukaryotic cousins may be attributable to their smaller numbers and heterogeneous distribution. While virtually every eukaryotic organism studied to date possesses PPPs, PPMs, and multiple families of PTPs to service their large and diverse population of phosphoproteins, most prokaryotes contain only handful, sometimes as few as one or two, protein O-phosphatases (18). Consequently, it is rare for these organisms to contain ORFs for all of the protein O-phosphatase archetypes listed in table 1. Intriguingly, genome analysis suggests that the distribution of protein O-phosphatase(s) archetypes among the Prokaryotes has been surprisingly random. No single protein phosphatase archetype is common to all Prokaryotes, nor to all Bacteria or all Archaea. Instead, it appears that these prototypes have been treated as relatively interchangeable units that can be tailored to meet the needs of each individual organism. Presumably, this reflects the original broadly-specific, multifunctional nature of ancestral protein phosphohydrolases. It also suggests that the amino acid specialization exhibited by eukaryotic protein O-phosphatases may have developed after their divergence

from the bacterial and perhaps even the archaeal line. Reconstructing the selection and adaptation processes for these proteins should reveal much about the development of protein phosphorylation-dephosphorylation as a regulatory mechanism of fundamental importance.

3. AceK, THE ISOCITRATE DEHYDROGENASE KINASE/PHOSPHATASE

The first prokaryotic phosphoprotein to be identified was isocitrate dehydrogenase [IDH] from *E. coli* (15). IDH is inactivated by phosphorylation of a serine, residue 113 in the protein from *E. coli* (55, 56). The covalently bound phosphate serves as an electrosteric blocking group that prevents the binding of the polyanionic substrate isocitrate (57, 58). Phosphorylation and concomitant inactivation of IDH occurs when *E. coli* was grown on acetate as sole carbon source (59). IDH controls the partitioning of catabolic carbon flow between the Krebs cycle and the glyoxylate bypass. The latter pathway must be employed for the accumulation of carbon building block material when acetate is the sole carbon source.

Attempts to purify the protein kinase and protein phosphatase responsible for phosphorylating and dephosphorylating IDH revealed the two activities to be inseparably linked (38, 60). Genetic analyses confirmed that these activities were the product of a single gene (61). This gene, *aceK,* encodes a polypeptide of 67 kDa whose predicted product is unique, bearing little resemblance to other known protein kinases and none to known protein phosphatases (62, 63). *In vitro,* several metabolites including isocitrate, 3-phosphoglycerate, AMP ADP, oxaloacetate pyruvate, and alpha-ketoglutarate stimulate the protein phosphatase activity and concomitantly inhibit the protein kinase activity of the IDH kinase/phosphatase; while NADP, citrate, fructose-6-phosphate, and glyoxylate inhibit protein kinase activity with no effect on protein phosphatase activity (64, 65). It is presumed that physiological control of the opposing catalytic functions of the IDH kinase/phosphatase is effected via integration of the combined allosteric inputs of some or all of these compounds in a manner somewhat analogous to that by which key metabolic enzymes such as phosphofructokinase and glycogen phosphorylase are modulated (66).

Studies utilizing enzyme kinetics and site-directed mutagenesis indicate that the phosphorylation and dephosphorylation of IDH by AceK both take place at the same active site (67, 68), suggesting that the enzyme represents a modified kinase in its basic nature. It is postulated that the active site can be conformationally configured through the binding of allosteric modulators to favor one or the other catalytic process (68). The precise nature of the proposed conformational configuration event remains to be elucidated.

In the laboratory, the AceK-catalyzed dephosphorylation of IDH requires the presence of an adenine nucleotide, either MgATP or MgADP (38). Since non-hydrolyzable analogs of MgATP do not support phosphoester hydrolysis, MgADP appears to be the relevant factor. Presumably, MgATP is converted to MgADP by the relatively high intrinsic ATPase activity of AceK prior to protein dephosphorylation *in vitro.* The product of the protein phosphatase reaction is P_i and not MgATP, however, indicating that dephosphorylation does not proceed via a simple reversal of the phosphotransfer reaction. It is postulated that phosphate is first transferred from the phosphoprotein to MgADP, forming MgATP as a transient intermediate that is quickly hydrolyzed to ADP and P_i (68). The resulting divergence in their reaction stoichiometries permits each reaction to proceed in a thermodynamically-independent manner, as would be the case if the activities resided on distinct polypeptides resembling conventional protein kinases and phosphatases. AceK remains unique in biology. While bifunctional enzymes have been encountered that carry out metabolic reactions, no other bifunctional protein phosphotransferases/ phosphohydrolases have been reported.

4. THE PPP FAMILY

The PPP family of protein phosphatases represents the most quantitatively significant source of protein-serine/threonine phosphatase activity in higher Eukaryotes (69). Its most prominent members include PP1, PP2A, and PP2B – also known as calcineurin (70) – that share a common catalytic core domain approximately 35 kDa in size (71). In PP1 and PP2A, this core domain is contained on a catalytic subunit whose location and activity are modulated by the binding of a spectrum of auxiliary subunits (48, 72-74). The high degree of sequence commonality among the eukaryotic members of the PPP family ranks them among the most highly conserved protein catalysts yet encountered (75). Examination of more phylogenetically diverse organisms has led to the realization that the PPPs are a subfamily of a larger group of phosphohydrolases such as diadenosine tetraphosphatase that act upon low molecular weight organophosphate compounds (71, 76-78). The essential phosphohydrolase signature/consensus sequence for this superfamily is DXH-X_{25}-GDXXD-X_{25}-GNHD/E (71, 78).

The first Prokaryote-associated member of the PPP family to be identified was the product of open reading frame [ORF] 221 of bacteriophage lambda (79). This discovery marked the first encounter with a "eukaryotic" protein kinase or protein phosphatase that had escaped what was then presumed to be its normal eukaryotic orbit. ORF221, while only about two-thirds the length of the catalytic subunits of PP1 and PP2A from Eukaryotes, nevertheless encoded a completely functional protein O-phosphatase (80). It differed from its eukaryotic counterparts in several other ways. First, it required the addition of exogenous metal ions, Mn^{2+}, for activity. While eukaryotic PPPs are also metalloenzymes (81), they bind metal ions with sufficient tenacity to render such supplementation unnecessary. The bacteriophage protein phosphatase was resistant to the classic inhibitor of PP1 and PP2A from eukaryotes, okadaic acid, and displayed multifunctional potential. *In vitro,* recombinant ORF221 will dephosphorylate traditional PPP substrates containing phosphoserine or phosphothreonine, as well as proteins

containing phosphotyrosine or the phosphoramide phosphohistidine (82). The physiological significance of these activities is unknown, as well as the precise role of ORF221 in the lambda phage life cycle. *E. coli* infected with lambda gt10, which contains the complete ORF221 sequence, exhibit a dramatic rise in protein O-phosphatase activity (80). However, lambda gt11, which contains a truncated version of ORF221 (79), does not show increased protein O-phosphatase activity inside of infected bacteria (80), yet still is able to propagate itself within the host.

More recently, several examples of PPP family members encoded by the genomes of members of the Archaea and Bacteria have been characterized in some detail and their genes cloned and sequenced. They include PP1-arch1 from the thermophilic archaeon *Sulfolobus solfataricus* (83, 84), PP1-arch2 from the methanogenic archaeon *Methanosarcina thermophila* TM-1 (85, 86), Py-PP1 from *Pyrodictium abyssi* TAG11 (87), PP1-cyano1 and PP1-cyano2 from the cyanobacteria *Microcystis aeruginosa* PCC7820 and *M. aeruginosa* UTEX2063, respectively (88, 89), and PrpA and PrpB from *E. coli* (90). These prokaryotic representatives of the PPP family appear to share several properties in common. All can be expressed as active monomers of approximately 25 - 35 kDa, behavior reminiscent of the catalytic subunit of PP1 from eukaryotes, which also can exist as an autonomous functional unit. By contrast to eukaryotic PPPs, no evidence of regulatory subunits was uncovered when PP1-arch1, PP1-cyano1, or PP1-cyano2 were isolated from their native organisms (84, 89). In each instance, however, the phosphatase-containing fractions were detected using assays that measured catalytic activity. Hence, the existence of inactive heteromeric forms of the enzymes, such as the complex of the catalytic subunit of mammalian PP1 with its regulatory subunit, I-2, cannot be ruled out at this time. The archaeal and cyanobacterial enzymes all required exogenous metals for expression of full activity, the most effective of which was Mn^{2+} [PrpA and PrpB reportedly were assayed in the presence of Mn^{2+}, but no information regarding activity in the absence of metal ions was cited (90).]. PP1-arch1, PP1-cyano-1, and PP1-cyano2 all proved resistant to classic PPP inhibitors such as microcystin LR and okadaic acid (84, 89). PP1-arch2 (85, 86) and Py-PP1 (87), however, displayed moderate sensitivity to these compounds. Particularly in the case of the cyanobacterial enzymes, such resistance may be essential for survival in the presence of their endogenous toxins or those produced by neighboring microbes.

PP1-arch1 and PP1-arch2 exhibit high, approximately 30%, identity to the catalytic subunits of eukaryotic PP1 and PP2A and were active toward the phosphoseryl, but not the phosphotyrosyl, substrates against which they were tested (83-86). By contrast, the four bacterial PPPs exhibited dual-specific protein phosphatase activity *in vitro,* hydrolyzing protein-bound phosphotyrosine, phosphoserine, and phosphothreonine (89, 90). In addition, PP1-cyano1 and PP1-cyano2 hydrolyzed the phosphoramide bonds of both phosphohistidine- and phospholysine- containing macromolecular substrates (89). It remains to be seen whether the full spectrum of catalytic capabilities exhibited by the bacteriophage and bacterial PPPs is utilized *in vivo.* However, it may be noteworthy that no protein phosphatase has yet been identified that acts on the multiple phosphohistidyl proteins present in a typical bacterium.

Few data are available concerning the physiological role of archaeal PPPs. Fractionation of extracts from laboratory cultures grown on rich media indicate that the archaeons *S. solfataricus* (83), *M.thermophila* TM-1 (85), and *Haloferax volcanii* (91) all contain a single dominant source of protein-serine/threonine phosphatase activity that has all the characteristics of a PPP family enzyme. The gene for Py-PP1 from *P. abyssi* is cotranscribed with a gene, *canB,* encoding a protein subunit of the unique, tubular extracellular network found in this archaeon – implying a role in the regulation or modification of network proteins (87).

We know significantly more concerning cellular functions of established and potential PPPs from bacterial organisms. Genetic experiments indicate PrpA and PrpB of *E. coli* form part of a signal transduction pathway that senses protein misfolding caused by heat shock or other stresses (90). This pathway turns on expression of the sigma E regulon that encodes a periplasmic protease responsible for degrading misfolded proteins. Intriguingly, this pathway also includes an apparent two-component module consisting of a potential histidine protein kinase, CpxA, and response regulator, CpxR. Details of how these units interact with one another, such as their relative positions within the proposed pathway, are presently unknown. PrpA itself was induced by heat shock, and overexpression of the enzyme led to the accumulation of several known heat shock proteins (90).

When the gene for a potential PPP from *Anabaena* PCC7120, *prpA,* was subjected to insertional inactivation, cells grew normally on media containing fixed nitrogen (92). However, growth stagnated within a few days following a switch to nitrogen-free media. This inability to sustain diazotrophic growth correlated with aberrations in the differentiation of heterocysts. While heterocyst-like structures appeared and were accompanied by the concomitant biosynthesis of heterocyst-specific glycolipids, many of the structures appeared to be empty and others exhibited a noticeable degree of transparency relative to wild-type. The activity of the dinitrogen-fixing enzyme nitrogenase was 4 to 40 fold lower than wild-type as well. Intriguingly, *prpA* is closely [301 bp] juxtaposed with the gene for a potential eukaryote-like protein kinase, *pknE.* While these two genes are transcribed separately, insertional inactivation of *pknE* produced cells whose phenotype exhibited many similarities to those in which *prpA* had been inactivated, suggesting that these two enzymes work together to modulate portions of the heterocyst differentiation program (92).

5. THE PPM FAMILY

The PPM family of protein phosphatases in Eukaryotes are protein-serine/threonine phosphatases that

require the presence of an exogenous divalent metal ion, usually Mg^{2+}, for activity. The most prominently known members of this family are PP2C (93) and pyruvate dehydrogenase phosphatase (94). In Prokaryotes, three distinct PPM family protein phosphatases have been characterized at the genetic and biochemical level in the bacterium *Bacillus subtilis* (95, 96). Analysis of the *B. subtilis* genome indicates the presence of at least two more open reading frames that may encode PPM protein phosphatases. In fact, PPM-like ORFs can be identified in almost every bacterium for which such data is available (18, 19, 97). Surprisingly, no biochemical evidence for the existence of PPM-like enzymes in members of the Archaea has been reported, and analysis of the three archaeal genomes currently completed failed to reveal any ORFs potentially encoding a PPM homolog (18). However, given the limited nature of the sample population, it would be premature to conclude that the Archaea are devoid of protein O-phosphatases of the PPM family.

The most intensively studied of the PPMs in *B. subtilis* is SpoIIE, member of a complex signal transduction network responsible for modulating sporulation (95). SpoIIE is a membrane protein whose C-terminal portion harbors its catalytic domain. Like other PPMs, SpoIIE exhibits Mg^{2+}-dependent protein-serine phosphatase activity (95, 98). The target of this activity is SpoIIAA, which regulates the activity of the sigma factor – sigma F – that controls expression of sporulation genes (33). During sporulation, *B. subtilis* divides into two compartments of unequal size, each containing a copy of the genome. These compartments remain physically associated, separated by a septum. Phosphorylation of SpoIIAA on serine 58 by a protein-serine/threonine kinase, SpoIIAB, resembling a protein-histidine kinase maintains this anti-sigma factor in an active state until compartmentation is complete. Subsequent dephosphorylation of SpoIIAA by SpoIIE releases sigma F from the grip of the anti-sigma factor, triggering a program of gene expression within the smaller compartment that commits it to differentiation as an endospore. Specificity and timing in SpoIIE action appears to be confirmed by recruiting the enzyme to the appropriate face of the septum separating prespore from mother cell (99, 100). However, the role of SpoIIE may be more complex than simply inactivating SpoIIAA, since deletion of the gene for SpoIIE also results in impaired septation (100, 101). The protein phosphatase activity of SpoIIE appears to be essential for sporulation, since genetic constructs harboring mutations designed to alter conserved amino acid residues within its protein phosphatase domain failed to complement the sporulation defect in *spoIIE* null strains (98). Intriguingly, while the SpoIIAB protein kinase would phosphorylate a mutationally-altered form of SpoIIAA in which the phosphoacceptor serine has been replaced by a threonine, the resulting phosphothreonyl SpoIIAA was not dephosphorylated by SpoIIE (95).

Like SpoIIE, two other PPMs within *B. subtilis,* RsbX and RsbU, are involved in a signal transduction cascade that targets a sigma factor involved in activating the transcription of stress response genes. In the case of RsbX and RsbU, they constitute portions of a partner-switch pathway that regulates the activity of sigma B (96). In slowly growing or stationary phase cells, activated sigma B binds to RNA polymerase, altering its promoter recognition properties such that a set of 40+ genes become transcribed (102, 103). The products of these genes help confer resistance to a variety of environmental stresses. Both RsbX and RsbU display Mn^{2+}-dependent protein-serine phosphatase activity *in vitro* (96). The physiological substrate of RsbU is RsbV, whose dephospho form binds the protein kinase RsbW, dissociating an RsbW-sigma B complex and releasing active sigma B. RsbW is also a SpoIIAB-like protein kinase that phosphorylates RsbV. The substrate for RsbX is RsbS, a phosphoprotein that modulates the interaction of RsbT with the PPM RsbU. Binding of RsbT to RsbU stimulates the latter's protein phosphatase activity. Dephosphorylation of RsbS by RsbU causes the former to bind RsbT and attenuate its stimulatory activity toward RsbU. RsbT is also a SpoIIAB-like protein kinase that targets RsbS. This intricately coupled regulatory ballet provides an elegant means for producing a regulatory cascade responsive to both external [RsbX-RsbS-RsbT partner-switch module] and internal [RsbU-RsbV-RsbW module] factors (96).

The genome sequence of the cyanobacterium *Synechocystis* PCC6803 contains several ORFs whose predicted products exhibit resemblance to PPM-family PPs (18, 19). One of these ORFs, known as slr1860 or *icfG,* has been studied at the genetic level (104). The results of these experiments suggested that *icfG* coordinately regulates the metabolism of glucose and inorganic carbon since *icfG*$^-$ cells did not survive when asked to switch from heterotrophic growth on glucose to autotrophic growth on inorganic carbon, or vice-versa. The key role of glucose or a derivative thereof as mediator of this switch was indicated by a) the requirement of glucose for expression of *icfG,* and b) the ability of many TCA cycle intermediates or their derivatives to overcome the growth inhibitory effects of glucose on carbon-source switching. It should be noted, however, that it has yet to be determined whether the protein product of *icfG* possesses protein phosphatase activity.

6. THE CONVENTIONAL PROTEIN-TYROSINE PHOSPHATASES

What have for many years been referred to as the protein-tyrosine phosphatases, or PTPs, are in fact a set of distinct enzyme families that have converged upon a common catalytic mechanism (49, 105). This mechanism involves the participation of a Cys-Xaa_5-Arg active site motif in which the thiol group acts as the nucleophile to form a phosphocysteinyl intermediate (106, 107). The active site signature sequence is flanked at some distance by an aspartate that serves as a general acid/base (108, 109). The position of the active site signature sequence within the catalytic domain and its position relative to the key aspartate provide the most striking points of difference between the low molecular weight [LMW] PTPs and the other families of PTPs, a collection that includes

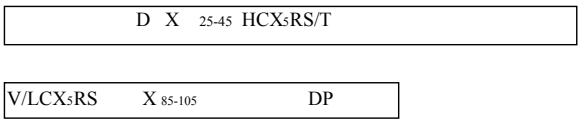

Figure 2. Distinguishing features of catalytic domains of conventional [TOP] and low molecular weight [BOTTOM] PTPs.

conventional PTPs and the Cdc25 family of dual-specific protein phosphatases [DSPs] (105). The DSPs hydrolyze protein-bound phosphoserine and/or phosphothreonine residues in addition to phosphotyrosine. In the conventional PTPs, the Cys-Xaa_5-Arg motif is located in the central portion of the catalytic domain, which is roughly 230 amino acids in length, and usually is flanked by a histidine residue immediately N-terminal to the nucleophilic cysteine and by a serine or threonine residue immediately C-terminal to the arginine (figure 2). The aspartate that serves as the general acid/base is located 30-50 residues to the N-terminal side of the catalytic cysteine. The Cdc25 family is distinguished by the presence of a glycine-proline sequence following the conserved arginine. Its members frequently lack the active site histidine almost always found in conventional PTPs as well. Although they display a superficial resemblance to the conventional PTPs in the orientation of their catalytically essential amino acids, X-ray crystallography has revealed the molecular architecture of Cdc25 to be significantly more similar to that of the sulfur transfer protein rhodanese (110). No prokaryotic enzymes or ORFs have yet been discovered whose sequence features suggest they are members of the Cdc25 family.

The first conventional PTP to be discovered from a Prokaryote was YopH [sometimes referred to as Yop51 or Yop2b] from the bacterium *Yersinia pseudotuberculosis* (111). The members of the genus *Yersinia* are human pathogens which include among their number *Y. pestis,* the causative agent of bubonic plague (112). YopH from *Y. pestis* exhibits near complete [99%] sequence identity with its counterpart in *Y. pseudotuberculosis* (111). The gene for YopH had previously been demonstrated to be essential for virulence of *Y. pseudotuberculosis* (113). Inactivation of YopH by mutational alteration of the active site cysteine residue to alanine significantly reduced virulence, indicating that the catalytic activity of the PTP was essential for pathogenesis (45). Inactivation of YopH had no effect on growth of *Y. pseudotuberculosis* in culture, however (45). Coupled with the observation that the PTPase becomes secreted/translocated into mammalian target cells, this indicated that the principal and probably exclusive target of the enzyme was the tyrosine phosphorylated proteins of the infected host.

Murine tissue culture cells infected with *Y. pseudotuberculosis* exhibited decreased phosphorylation of polypeptides with M_r's of 200, 120, and 60 kDa (45). Using a substrate trapping mutant of YopH, the second of these proteins has been identified as $p130^{Cas}$ present in focal adhesions (114). The stabilizing effect of the substrate trapping mutant of YopH on focal adhesions strongly suggests that dephosphorylation of $p130^{Cas}$ by the PTP destabilizes these cytoskeletal anchors, rendering them impotent as centers for the assembly of the signaling complexes that help trigger host infection responses (114). YopH is encoded by a large virulence plasmid in *Yersinia* that carries a host of other genes involved in pathogenesis, including a eukaryote-like protein kinase (115). The extrachromosomal location of the YopH gene and the extracellular/eukaryotic site of action of its protein product have been interpreted as signs that the bacterium acquired it from a mammalian host organism via a horizontal gene transfer event (111).

The practice of using PTPs as molecular missiles for the conquest of animal cells by bacterial pathogens does not appear to be restricted to *Yersinia*. Two strains of the pathogenic bacterium *Salmonella, S. typhimurium* (46) and *S. typhi* (116), contain similar, multidomain PTPs. Each resembles a fusion product of the YopE cytotoxin and the YopH PTP of *Yersinia* . In contrast to these Yop virulence determinants, both SptP of *S. typhimurium* and StpA of *S. typhi* are encoded within the bacterial chromosome. Like YopH, SptP is secreted (46) and disrupts the actin cytoskeleton of the infected host (117). Using mutationally altered variants of SptP, it was determined that its PTP activity is both necessary and, once it has entered cells, sufficient for cytoskeletal perturbation (46). Paradoxically, given its homology with both YopH and SptP, deletion of StpA from *S. typhi* did not affect either the entry or survival of the latter pathogen in tissue culture cells (116).

Not all bacterial PTPs function as virulence factors targeted against eukaryotic proteins. The first chromosomally-encoded member of the PTP family to be discovered among the prokaryotes was IphP from the cyanobacterium *Nostoc commune* UTEX584 (118). IphP exhibits relatively faint homology to eukaryotic PTPs (105). This fact, coupled with the free living nature of the host organism and the anchoring of the gene which encodes it within the core genome, suggests that IphP likely is of bacterial ancestry and that it acts upon phosphoproteins within *N. commune*. The widespread distribution of ORFs encoding putative PTPs among the Archaea and Bacteria (table 1), including several other non-pathogenic species, further supports the notion that indigenous prokaryotic PTPs exist (18).

IphP is a protein of 294 amino acids that contains a 24 amino acid leader sequence (118). When IphP was produced by expression in *E. coli,* it was observed that the protein was secreted into the surrounding culture media with concomitant cleavage of the leader sequence. The recombinant protein exhibited dual-specific protein phosphatase activity toward a wide range of exogenous proteins, including the ERK2 MAP kinase (119). While the subcellular location of IphP in its native milieu has yet to be determined, the leader sequence presumably targets the enzyme to an extracytoplasmic region such as the thylakoid lumen or the periplasmic space. A dual-specific protein phosphatase activity with an electrophoretic mobility similar to that of IphP was recently detected in an

Table 2. Summary. Protein tyrosine phosphorylation in Prokaryotes

ORGANISM	FACTORS THAT INFLUENCE PHOSPHORYLATION	REFERENCES
Archaea		
Haloferax volcanii		130
Methanosarcina thermophila TM-1		130
Sulfolobus solfataricus		130
Bacteria		
Acinetobacter calcoaceticus		131
Acinetobacter johnsonii		132
Anabaena PCC7120	Light	133
Clostridium thermohydrosulfuricum		134
Escherichia coli		126
Myxococcus xanthus	Developmental cycle	135
Nostoc commune UTEX584	Fixed nitrogen	118
Prochlorothrix hollandica	Light	136
Pseudomonas aeruginosa		125
Pseudomonas solanacearum		137
Streptomyces hygroscopicus	Growth phase, Rich vs. minimal culture media	138
Streptomyces griseus	Growth phase, Rich vs. minimal culture media	138
Streptomyces lividans	Growth phase, Rich vs. minimal culture media	138
Synechococcus PCC7942	Light	134

extracytoplasmic fraction from the cyanobacterium *Anabaena* PCC7120 (120).

7. THE LOW MOLECULAR WEIGHT PROTEIN-TYROSINE PHOSPHATASES

The LMW PTPs are distinguished by an active site signature sequence lying at or very near the extreme N-terminus of the catalytic domain, which spans a region of approximately 140 amino acids (figure 2). The residue preceding the catalytic cysteine is generally a valine, leucine, or isoleucine while the conserved arginine is generally followed by a serine or threonine as in the conventional PTPs. The aspartate that serves as the general acid/base is found 85 -105 residues to the C-terminal side of the cysteine, and often is followed by a proline.

Genome and other database searches indicate that both archaeal and bacterial organisms contain LMW PTPs (18, 19, 121). Only two of these have been characterized, both from members of the Bacteria: PtpA from *Streptomyces coelicolor* A3(2) (121) and Ptp from *Acinetobacter johnsonii* (122). Both displayed activity toward free phosphotyrosine and phosphotyrosine-containing peptides *in vitro.* Tests for DSP activity were performed with free phosphoamino acids, which generally are non-substrates even for bona fide DSPs (119). Hence it remains to be determined whether either of these proteins has such potential. Overexpression of the *ptpA* gene in *Streptomyces lividans* led to an increase in the production of secondary metabolites such as the antibiotic undecylprodigiodsin, A-factor, and actinorhodin (123). This effect was copy number dependent. Stimulation of secondary metabolite production was observed in cultures of *S. lividans* harboring 3-4 copies of *ptpA* per cell, but not in those containing only 1-2. The catalytic activity of PtpA apparently was required for stimulation, since expression of mutationally-altered forms of the *ptpA* in which the codon for the active site cysteine residue was changed to that for alanine had no effect. Paradoxically, disruption of the endogenous *ptpA* gene in *S. coelicolor* A3(2) had no detectable effect on the production of pigmented antibiotics or other cellular processes such as mycelium or spore formation (121).

8. PHOSPHOTYROSINE IN PROKARYOTES.

Presuming that IphP and other potential prokaryotic PTPs target proteins within the bacterium or archaeon from which they originate, it would be natural to ask whether Prokaryotes contain phosphotyrosyl proteins. While the existence of phosphotyrosyl proteins in Prokaryotes has long been a subject of debate (17, 124), recent years have witnessed the accumulation of credible evidence for their presence in numerous members of the Archaea and Bacteria (table 2) Relatively few of these proteins have been identified at the molecular level, however. Recent examples include flagellins a and b in *Pseudomonas aeruginosa* (125) and TypA from *E. coli,* a protein that displays intriguing similarities to GTP-binding elongation factors (126). In numerous Bacteria, the degree of phosphorylation and/or presence of phosphotyrosyl proteins displayed dynamic variations as cells progressed through their growth and/or differentiation cycle, as well as in response to the availability of nutrients and, in photosynthetic organisms, light (table 2). While correlative in nature, these changes suggest that at least some of the tyrosine phosphorylation events reported in the literature ultimately will prove to possess regulatory significance.

9. OTHER PROKARYOTIC PHOSPHATASES.

9.1. HprP, the HPr Protein Serine Phosphatase

During carbon catabolite repression the histidine-containing protein, HPr, of the phosphoenol pyruvate:sugar phosphotransferase [PTS] system becomes phosphorylated

on a serine residue (127) by a novel protein kinase (36, 37). Phosphorylation of HPr dramatically decreases the activity of the PTS system. The protein phosphatase that dephosphorylates HPr, HprP, is a divalent metal ion-dependent enzyme that is stimulated by inorganic phosphate and inhibited by ATP (128). Recently, the gene for this phosphatase reportedly was cloned from *B. subtilis* (37). The DNA-derived amino acid sequence predicted a protein product whose sequence resembled bacterial phosphoglycolate phosphatases. HprP thus becomes the first representative of a novel class of protein O-phosphatases.

9.2. Archaeal Alkaline p-Nitrophenyl Phosphatase

The archaeon *Halobacterium halobium* contains a low molecular weight, Mn^{2+}-dependent phosphomonoesterase that displays optimum catalytic activity at alkaline pH (129). The enzyme displayed activity toward free phosphotyrosine, p-nitrophenyl phosphate, and the phosphorylated serine and/or threonine residues of casein. However, numerous other low molecular weight phosphomonoesters and the phosphoprotein phosvitin were not dephosphorylated at detectable rates, behavior more consistent with that of a protein phosphatase than a non-specific phosphomonoesterase. Primary sequence information on this phosphohydrolase has yet to be forthcoming.

10. PERSPECTIVES

Just one decade ago, interest in the protein O-phosphatases of prokaryotic organisms was virtually nil. Only one protein O-phosphatase, AceK, had been characterized in molecular detail. Work on signal transduction in Prokaryotes was confined almost exclusively to the "bacterial" two-component system that targeted aspartyl residues for phosphorylation. The short half-life of this acyl phosphate moiety begged the question as to whether these organisms required protein phosphatases at all. Following the pioneering studies of Cohen and colleagues (79, 80) and Guan and Dixon (111), interest in prokaryotic protein O-phosphatases has steadily grown, helping catalyze a literal paradigm shift in our view of protein phosphorylation events in Prokaryotes. In the past few years we have come to realize:

- Many prokaryotes are the sites of extensive, vigorously active signal transduction networks.
- Prokaryotes make extensive use of protein phosphomonoesters of serine, threonine, and tyrosine for signal transduction purposes.
- Prokaryotes contain protein O-kinases that are homologs of the dominant superfamily of protein O-kinases in eukaryotes.
- Prokaryotes contain protein O-phosphatases drawn from nearly every major class of protein O-phosphatases from eukaryotes: PPP, PPM, conventional PTP, and LMW PTP.

Much remains to be accomplished, however. While we have made remarkable progress in the past few years in identifying the protein kinases and protein phosphatases that are the agents of prokaryotic signal transduction, our knowledge of the specific signals transduced by each and the phosphoproteins that form their ultimate targets remains fragmentary.

The discovery of what were once considered to be "eukaryotic" protein O-phosphatases and protein O-kinases in Prokaroytes, coupled with the discovery of what were once considered "prokaryotic" two-component signalling modules in Eukaryotes, raises provocative questions concerning the origins and evolution of protein phosphorylation:

- When did proteins first become modified by phosphorylation?
- When was phosphorylation adapted to modulate the functional properties of proteins, i.e. when did it develop its regulatory role?
- When did nature first employ protein phosphatases to modulate protein structure and function in a reversible manner?
- What were the original functions of the enzymes that eventually evolved to form today's signal transduction enzymes?
- How did this multiplicity of signal transduction enzymes become distributed throughout phylogeny?

At first glance, the discoveries outlined herein may be viewed as hopelessly complicating our picture of the origins and evolution of protein phosphorylation networks. However, in the long run, the spectrum of information and tools offered by the members of the prokaryotic world will more than compensate for our momentary discomfiture. The age and diversity of the Prokaryotes add depth and breadth to the archaeological record of protein phosphorylation events contained within their genomes. The malleability of numerous Prokaryotes to genetic manipulation, coupled with their robustness in the face of a wide range of nutritional and environmental stresses, renders the analysis of their protein phosphorylation networks by molecular genetic means relatively facile. Their greater quantitative simplicity renders the elucidation of the complete molecular architecture of their signal transduction networks and the determination of how these networks function as integrated information processing systems possible in the near term. By tracing the evolutionary history of signal transduction through the full range of the phylogenetic spectrum, the day that we reach the ultimate goal of understanding the signal transduction networks of the human animal will be advanced greatly.

11. ACKNOWLEDGEMENTS

We gratefully acknowledge the support of the National Institutes of Health (R01 GM55067 to P.J.K.).

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Keywords: Protein Phosphorylation, Protein Phosphatases, Protein-Serine/Threonine Phosphatases, Protein-Tyrosine Phosphatases, Dual-Specific Protein Phosphatases, Bacteria, Archaea

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Received 9/15/98 Accepted 2/10/99