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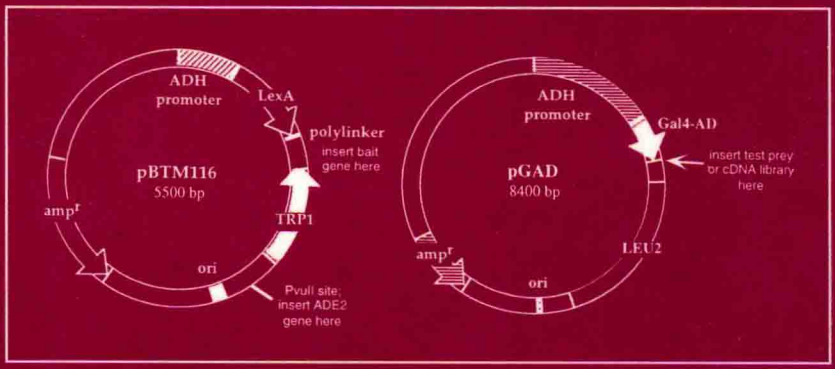
Edited by

John W. Ludlow



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METHODS IN MOLECULAR BIOLOGY™

Protein Phosphatase Protocols

Edited by

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Protein Phosphatase Protocols

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Preface

A major mechanism by which cells regulate protein function is to place phosphate groups on serine and threonine residues. Though the steady-state level of protein phosphorylation depends on the relative activities of both kinases and phosphatases, a much greater effort has previously gone into the study of the former that the latter. Today, however, there is an increasing appreciation for the role that protein phosphatases play in the dynamic process of protein phosphorylation. To date, there are four major types of protein serine/threonine phosphatase catalytic subunits, designated protein phosphatase type 1, 2A, 2B, and 2C. Each has been identified by the techniques of protein chemistry and enzymology and can be distinguished from one another by their preference for specific substrates as well as their sensitivity to certain activators and inhibitors.

Protein Phosphatase Protocols has been assembled in response to the growing interest these enzymes are receiving. The goal of this compilation is to provide a "how-to" experimental guide to aid newcomers as well as seasoned veterans in their research endeavors, thus further contributing towards our ever increasing knowledge of serine/threonine phosphatases.

What you have before you contains contributions by many of the current and emerging leaders in the field. To highlight just a few, these chapters contain step-by-step information on how to isolate novel phosphatases and regulatory subunits, assay for activity, and generate immunological reagents for both biochemical and biological characterization of these enzymes. Though it is obviously not possible to include contributions by each and every researcher in this field, every effort was made to be inclusive, and avoid being exclusive, regarding the methods used to investigate these phosphatases. We hope that you find our work both informative and thought provoking.

John W. Ludlow

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Contents

1

Prokaryotic Protein-Serine/Threonine Phosphatases

Peter J. Kennelly

1. Introduction

1.1. Prokaryotic Protein-Serine/Threonine Phosphatases: A Brief Review

1.1.1. Why Study Protein Phosphorylation Events in Prokaryotes?

As this chapter deals with the protein-serine/threonine phosphatases of prokaryotic organisms, some comments on the role of prokaryotes in the study of these important enzymes would appear to be in order. Prokaryotic organisms dominate the living world. They represent by the largest source of biomass on the planet, forming the indispensable foundation of the food chain upon which all other living organisms depend. They are the exclusive agents for carrying out biological nitrogen fixation, and are responsible for the majority of the photosynthetic activity that generates the oxygen we breath. In absolute numbers, in number of species, in range of habitat, and in the spectrum of their metabolic activities, the prokaryotes far outpace their eukaryotic brethren. More immediately, in humans prokaryotes perform essential functions in the digestion and assimilation of nutrients, whereas infection by bacterial pathogens can lead to illness or death.

The intrinsic biological importance of prokaryotic organisms in the biosphere renders them important and interesting objects of study ***(1)***. Be that as it may, the question remains as to why protein phosphorylation in prokaryotes should be of interest to "mainstream" signal transduction researchers whose attention has long been fixed on humans and other higher eukaryotes. At least part of the answer lies in the recent realization that prokaryotes and eukaryotes employ many of the same molecular themes for the construction and operation of their protein phosphorylation networks ***(2,3)***. Virtually every major family

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of protein kinases or protein phosphatases identified in eukaryotic organisms possesses a prokaryotic homolog(s), and vice versa. Consequently, the prokaryotes represent a voluminous library of fundamentally important, universally applicable information concerning the structure, function, origins, and evolution of protein kinases, protein phosphatases, and their target phosphoproteins. In addition, prokaryotes offer significant advantages as venues for the study of protein kinases and protein phosphatases, particularly with regard to dissecting their physiological functions and the factors that influence them. Prokaryotes carry out their life functions and the regulation thereof utilizing a many-fold smaller suite of genes and gene products than does the typical eukaryote. Although they employ molecular mechanisms as subtle and sophisticated as any found in "higher" organisms, the fewer "moving parts" in the prokaryotes materially facilitates the design, execution, and analysis of molecular genetic experiments. In addition, their robustness in the face of a wide range of nutritional and environmental challenges greatly facilitates the identification and analysis of resulting phenotypes. The prokaryotes thus represent a rich and presently underutilized tool for understanding the fundamental principles governing the form and function of protein phosphorylation networks.

1.1.2. Not All Prokaryotes Are Created Equal: A Brief Outline of Phylogeny

Most readers of this chapter were taught that all living organisms could be grouped into two phylogenetic domains whose names were often given as the eukaryotes and the prokaryotes ***(4)***. However, these latter terms actually refer to a morphological classification, not a genetic/hereditary one ***(5)***. The term eukaryote describes those organisms whose cells manifest internal compartmentation, more precisely the presence of a nuclear membrane. The prokaryotes include all organisms lacking such intracellular organization, in other words everything that is not a eukaryote. Early studies of phylogeny based on the first protein sequences, the gross structural and functional characteristics of key macromolecules, the architecture of common metabolic pathways, and so forth, suggested that this morphological classification of living organisms paralleled their hereditary relationships. However, as researchers gained facility with the isolation, sequencing, and analysis of DNA, a truly genetic outline of phylogeny has emerged, one that groups living organisms into three distinct phylogenetic domains—the *Eucarya*, *Bacteria,* and *Archaea (Archaebacteria)* ***(6)***.

Whereas the prior supposition that the eukaryote morphological phenotype characterized members of a coherent phylogenetic domain—the *Eucarya*—proved correct, molecular genetic analysis revealed that the prokaryotes segregated into two distinct and very different domains: the *Bacteria* and the

Archaea. The domain *Bacteria* includes essentially all of the prokaryotic organisms one encounters in a typical microbiology course: *E. coli, Salmonella, Pseudomonas, Rhizobium, Clostridia, Staphylococcus, Bacillus, Anabaena,* and so on. The domain *Archaea,* on the other hand, is populated largely by extremophiles that occupy habitats whose heat, acidity, salinity, or oxygen tension render them hostile, if not deadly to other living organisms. However, it would be wrong to suppose that the *Archaea* are simply a set of unusual bacteria. Examination of the genes encoding their most fundamentally important macromolecules, ranging from DNA polymerase to ribosomal RNAs, make it clear that the *Archaea* have much more in common with the *Eucarya* than they do with the superficially-similar *Bacteria* ***(6,7)***. The earliest detectable branch point in the evolutionary time line resulted in the segregation of the *Bacteria* away from the organism that eventually gave rise to both the *Eucarya* and the *Archaea.* The common progenitor of these latter domains then evolved for a considerable period following this first bifurcation. As a consequence, many investigators believe that present day archaeons still possess numerous features reflective of ancient proto-eukaryotes ***(7)***. This combination of prokaryotic "simplicity" with high relatedness to medically relevant eukaryotes render the *Archaea* a particularly intriguing target for the study of protein phosphorylation phenomena.

1.1.3. Prokaryotic Protein-Serine/Threonine Phosphatases Identified to Date

When one considers that the modification of prokaryotic proteins by phosphorylation-dephosphorylation first was reported nearly 20 yr ago ***(8–10)***, surprisingly little is known about the enzymes responsible for the hydrolysis of phosphoserine and phosphothreonine residues in these organisms. The first prokaryotic protein-serine/threonine phosphatase to be identified and characterized was the product of the *aceK* gene in *E. coli* ***(11).*** This gene encodes a polypeptide that contains both the protein kinase and protein phosphatase activities responsible for the phosphorylation-dephosphorylation of isocitrate dehydrogenase. Today, AceK remains an anachronism by virtue of its hermaphroditic structure, and because the sequences of its protein kinase and protein phosphatase domains are unique, exhibiting no significant resemblance to other protein kinases or protein phosphatases ***(12)***.

The next prokaryote-associated protein-serine/threonine phosphatase to be discovered was ORF 221 encoded by bacteriophage λ ***(13,14)***. This enzyme, and a potential protein encoded by an open reading frame in bacteriophage ϕ80, exhibit significant sequence homology with the members of the PP1/2A/2B superfamily, one of the two major families of eukaryotic protein-serine/threonine phosphatases ***(15)***. Whereas this represented the first discovery of a eukaryote-like protein phosphorylation network component having any asso-

ciation with a prokaryotic organism, the mobility and malleability of viral vectors begged the question of whether the genes for these protein phosphatases were bacterial in origin. Moreover, it remains unclear to what degree a protein phosphatase from a pathogen can shed light on how bacterial proteins are dephosphorylated under normal physiological circumstances.

More recently, two unambiguously bacterial enzymes have been described that possess protein-serine/threonine phosphatase activity. The first, IphP from the cyanobacterium *Nostoc commune* ***(16)***, is a dual-specificity protein phosphatase that acts on phosphoseryl, phosphothreonyl, and phosphotyrosyl proteins in vitro ***(17)***. Like other dual-specific protein phosphatases, IphP contains the characteristic HAT (His-Cys-Xaa_5-Arg, or His-Arg-Thiolate) active site signature motif characteristic of protein phosphatases capable of hydrolyzing phosphotyrosine ***(18)***. The second is SpoIIE from *Bacillus subtilis*, a bacterial homolog of the second major family of "eukaryotic" protein-serine/threonine phosphatases, the PP2C family ***(19,20)***.

"Eukaryotic" protein-serine/threonine phosphatases have been uncovered in the *Archaea* as well. In the author's laboratory a protein-serine/threonine phosphatase, PP1-arch, has been purified, characterized, cloned, and expressed from the extreme acidothermophilic archaeon *Sulfolobus solfataricus* ***(21,22)***. This protein is a member of the PP1/2A/2B superfamily, with whose eukaryotic members it shares nearly 30% sequence identity ***(22)***. Surveys of two other archaeons, which are phylogenetically and phenotypically distinct from *S. solfataricus,* the halophile *Haloferax volcanii* and the methanogen *Methanosarcina thermophila* TM-1, indicate that PP1-arch from *S. solfataricus* is the first representative of what may prove to be a widely distributed family of archaeal protein-serine/threonine phosphatases ***(23,24)***. This recently has been confirmed at the sequence level through the cloning of a second form of PP1-arch from *M. thermophila* via the polymerase chain reaction (PCR).

1.1.4. Limited Applicability of Cohen's Scheme to the Classification Prokaryotic Protein-Serine/Threonine Phosphatases

Recent experience with prokaryotic protein phosphatases has revealed that Cohen's criteria for classifying the protein-serine/threonine phosphatases cannot be extrapolated with confidence to prokaryotic enzymes. To briefly review, in the early 1980s, Cohen and coworkers compiled a set of functional attributes characteristic of each of the major protein-serine/threonine phosphatases found in eukaryotes ***(25)***. These attributes included their preference for dephosphorylating the α- vs the β-subunit of phosphorylase kinase, their sensitivity to the heat-stable inhibitor proteins I-1 and I-2, and the (in)dependence of their catalytic activity on the presence of divalent metal ions such as Mg^{2+}, Mn^{2+}, or Ca^{2+}. In later years sensitivity to potent microbial toxins—such as microcystin-

LR, okadaic acid, and tautomycin—that inhibited the activity of PP1 and PP2A were added to the list ***(26)***. While this scheme soon was adopted as standard for the classification of eukaryotic protein-serine/threonine phosphatases, attempts to apply it to prokaryotic enzymes have met with mixed success. For example, PP1-arch from *S. solfataricus* is okadaic acid-insensitive and requires exogenous divalent metal ions for activity ***(21)***. Under Cohen's scheme, this would classify it as a member of the PP2C family. However, the amino acid sequence of PP1-arch clearly places it in the PP1/2A/2B superfamily ***(22)***. The same holds true for another divalent metal ion-dependent, okadaic acid-insensitive PP1/2A homolog, ORF 221 from bacteriophage λ ***(14)***.

1.2. An Overview of Methods for Assaying, Purifying, and Identifying Clones of a Prokaryotic Protein-Serine/Threonine Phosphatase, PP1-Arch

We use [^{32}P]phosphocasein that has been phosphorylated using the catalytic subunit of the cAMP-dependent protein kinase ***(27)*** as a general-purpose substrate for the assay of protein-serine/threonine phosphatase activity in prokaryotic organisms. Although it is a eukaryotic phosphoprotein, all of the prokaryotic protein-serine/threonine phosphatases that have been studied ***(16,17,21–24)***, as well as the ORF 221 protein-serine/threonine phosphatase from bacteriophage λ ***(14)***, hydrolyze phosphocasein at a usefully high rate in vitro. Its major virtue resides in the fact that it is readily prepared in quantity by procedures that are simple and economical with regard to both effort and expense. The major drawback of phosphocasein is the very high quantity of unlabeled phosphate that is already bound to it, which renders it unsuitable for determining kinetic parameters. However, for routine applications—those requiring knowledge of the relative protein phosphatase activity present in a sample such as surveying cell homogenates or column fractions, screening potential activators or inhibitors, and so on—phosphocasein is entirely suitable.

For the assay of PP1-arch, a sample of protein phosphatase is incubated with [^{32}P]phosphocasein in the presence of a divalent metal ion cofactor and a protein carrier, bovine serum albumin (BSA). Inclusion of the divalent metal ion cofactor is very important. Every PP1/2A homolog characterized to date in both the *Archaea* ***(21,23,24)*** and bacteriophage λ ***(14)*** requires divalent metal ions for activity, as does the bacterial PP2C homolog SpoIIE ***(20)***. (Eukaryotic PP1 is a metalloenzyme ***(28)***, but it normally binds divalent metal ions in a sufficiently tenacious manner to render the addition of exogenous cofactors unnecessary.) In the author's experience, Mn^{2+} has proven the most efficacious and general cofactor. However, activation by Co^{2+}, Ni^{2+}, or Mg^{2+} has been observed on occasion ***(21,23,24)***. The assay is terminated by adding trichloroacetic acid (TCA) and centrifuging. With the assistance of the BSA carrier, the