Protein Phosphatases: Properties and Role in Cellular Regulation

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Summary. Protein phosphorylation is a principal regulatory mechanism in the control of almost all cellular processes. The nature of the protein phosphatases that participate in these reactions has been a subject of controversy. Four enzymes, termed protein phosphatases 1, 2A, 2B, and 2C, account for virtually all of the phosphatase activity toward phosphoproteins involved in controlling glycogen metabolism, glycolysis, gluconeogenesis, fatty acid synthesis, cholesterol synthesis, and protein synthesis. The properties, physiological roles, and mechanisms for regulating the four protein phosphatases are reviewed.

Over the last 10 years it has become clear that protein phosphorylation is a major mechanism for controlling the activities of enzymes and other proteins, and that it is involved in regulating many cellular processes. An important generality that has emerged is that enzymes in biodegradative pathways are activated by phosphorylation, whereas enzymes in biosynthetic pathways are inactivated (*1–5*). This concept suggested that different metabolic pathways might be regulated by the same protein kinases and protein phosphatases, or by different protein kinases and protein phosphatases that respond to identical effector molecules. These ideas are already established for protein kinases. For example, for many hormones cyclic adenosine monophosphate (cyclic AMP) is a second messenger, which transmits information from plasma membrane receptors to the interior of the cell. The effects of this second messenger on biosynthetic and biodegradative pathways are a consequence of the phosphorylation, by cyclic AMP–dependent protein kinase (*6*), of various regulatory enzymes in these pathways. Similarly, a number of different protein kinases are activated by the same calcium-ion (Ca^{2+}) binding protein, calmodulin, which mediates many of the actions of physiological stimuli where calcium ions act as a second messenger.

The steady-state level of phosphorylation of any cellular protein is dependent on the balance of the activities of the protein kinases and protein phosphatases that act on the protein; it is becoming increasingly clear that protein phosphatases, as well as protein kinases, are important targets for cellular regulation. Although considerable progress has been made in characterizing the protein kinases in cellular regulation, the nature of the protein phosphatases involved in these phosphorylation systems has been a subject of controversy. A large number of protein phosphatase preparations have been isolated with the use of various phosphorylated protein substrates, but little attempt has been made to establish the relation between these preparations. In this article, we review work from our laboratory and that of others indicating that relatively few protein phosphatases are involved in cellular regulation.

Initial Studies on Protein Phosphatases

Initial work in our laboratory dealt with the nature of the protein phosphatases in rabbit skeletal muscle that act on phosphorylase kinase (Table 1). At physiological Mg^{2+} concentrations, this enzyme is phosphorylated on two serine residues by cyclic AMP–dependent protein kinase. Phosphorylation of one of these residues (on the β subunit) markedly enhances activity. Phosphorylation of the second serine (on the α subunit) has little effect on activity, and the role of this reaction is unclear although phosphorylation of both serine residues occurs in vivo in response to epinephrine (*1, 4*).

Two enzymes, protein phosphatase 1 and protein phosphatase 2, were separated which specifically dephosphorylated the β subunit and the α subunit of phosphorylase kinase, respectively (*4, 7*). Subsequently, protein phosphatase 1 was also shown to dephosphorylate glycogen phosphorylase and glycogen synthase (Table 1). In contrast, protein phosphatase 2 had little activity toward these substrates (*4, 7*). Protein phosphatase 1 was found to be inhibited by low concentrations of two thermostable inhibitor proteins, termed inhibitor 1 and inhibitor 2, while protein phosphatase 2 was unaffected by these proteins (*8, 9*).

While these studies were in progress, Lee and co-workers (*10*) purified to apparent homogeneity a phosphorylase phosphatase with an approximate molecular weight of 35,000 (35K) from rat liver. They used an unusual procedure involving precipitation with 80 percent ethanol at room temperature, which not only denatured many contaminating proteins but also appeared to dissociate the 35K catalytic subunit from larger molecular weight complexes containing other subunits (*10*). This procedure was widely adopted and used to purify apparently similar 35K catalytic subunits from rat liver, heart muscle, and rabbit skeletal muscle (*11*). The catalytic subunit was also found to be active on phosphorylase kinase and glycogen synthase (*10*), and for several years we thought that this enzyme was identical to a 35K form of protein phosphatase 1 (*4, 7*). Subsequently, however, the 35K protein phosphatase, prepared from mammalian liver (*11*) or skeletal muscle (*12*) by the method of Brandt and co-workers (*10*), was found to be composed of two distinct enzymes. One was identical to protein phosphatase 1, while the second shared several properties with protein phosphatase 2. However, the high phosphorylase phosphatase activity of the second enzyme distinguished it from protein phosphatase 2 (*11*), and this observation prompted us to reinvestigate the nature of the protein phosphatases in liver, skeletal muscle, and other tissues.

Classification of Protein Phosphatases

Seven protein kinases were used to phosphorylate 13 substrate proteins on a minimum of 19 different serine and threonine residues (Table 1). These substrates, which correspond to phosphorylated proteins involved in the regulation

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of glycogen metabolism, glycolysis, gluconeogenesis, fatty acid synthesis, cholesterol synthesis, protein synthesis, and muscle contraction, were used to investigate the nature of the protein phosphatases involved in the control of these metabolic pathways (*1, 13–19*).

Four enzymes have been identified that appear to account for virtually all of the protein phosphatase activities in tissue extracts acting on these phosphorylated proteins (*13–15*). In addition, these enzymes explain most, if not all, of the protein phosphatase activities that have been described (*14–17*). The four enzymes can be grouped into two classes (Table 2). The type 1 protein phosphatase (that is, protein phosphatase 1) selectively dephosphorylates the β subunit of phosphorylase kinase and is inhibited by nanomolar concentrations of inhibitor 1 and inhibitor 2, whereas the type 2 protein phosphatases selectively dephosphorylate the α subunit of phosphorylase kinase and are insensitive to the inhibitor proteins (*13*).

A catalytic subunit of protein phosphatase 1 has been purified to homogeneity from skeletal muscle (*12*) and shown, on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, to have a molecular weight of 35K (*11, 12, 20*). An inactive form of protein phosphatase 1, termed the MgATP-dependent protein phosphatase (MgATP, magnesium adenosine triphosphate), can also be isolated (*20, 21*), and this species consists of a 1:1 complex between the catalytic subunit of protein phosphatase 1 and inhibitor 2 (*20*). Activation of this species requires prior incubation with MgATP and a protein kinase (see below)

Table 1. Phosphoprotein substrates used in characterizing protein phosphatases. All substrates are from skeletal muscle unless otherwise stated. References to the sequence determinations are given in (*13*). The phosphorylation of phosphorylase and phosphorylase kinase (β subunit) explain the enhanced rates of glycogenolysis in skeletal muscle during muscle contraction and in response to epinephrine (*1–5*). Phosphorylated histones are commonly used as in vitro substrates for protein phosphatases. Abbreviations are: cyclic AMP-PK, cyclic AMP–dependent protein kinase; PhK, phosphorylase kinase; GSK, glycogen synthase kinase; CaM, calmodulin; RK, HMG-CoA reductase kinase; RKK, HMG-CoA reductase kinase kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; eIF, eukaryotic initiation factor (protein synthesis); MLCK, myosin light chain kinase; Ala, alanine; Arg, arginine; Asn, asparagine; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Met, methionine; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

Substrate	Sequence	Protein kinase	Effect on activity
	Glycogen metabolism		
Phosphorylase kinase (β subunit)	—Ala-Arg-Thr-Lys-Arg-Ser(P)-Gly-Ser-Val-Tyr-Glu-Pro-Leu	Cyclic AMP-PK	Increase
Phosphorylase kinase (α subunit)	Phe-Arg-Arg-Leu-Ser(P)-Ile-Ser-Thr-Glu-Ser	Cyclic AMP-PK	None
Phosphorylase a	Glu-Lys-Arg-Lys-Gln-Ile-Ser(P)-Val-Arg-Gly-Leu-Ala	PhK	Increase
Glycogen synthase (site 1a)	Pro-Gln-Trp-Pro-Arg-Arg-Ala-Ser(P)-Cys-Thr-Ser-Ser-Ser	Cyclic AMP-PK	Decrease
Glycogen synthase (site 2)	Pro-Leu-Ser(P)-Arg-Thr-Leu-Ser(P)-Val-Ser-Ser-Leu-Pro	PhK, cyclic AMP-PK, GSK-4, CaM-dependent GSK (*53*)	Decrease
Glycogen synthase (sites 3a, 3b, 3c)	Arg-Pro-Ala-Ser(P)-Val-Pro-Pro-Ser(P)-Pro-Ser(P)-Leu-Ser-Arg	GSK-3	Decrease
Inhibitor 1	Arg-Arg-Arg-Arg-Pro-Thr(P)-Pro-Ala-Thr	Cyclic AMP-PK	Increase
	Glycolysis and gluconeogenesis		
Pyruvate kinase (rat liver)	Ala-Gly-Tyr-Leu-Arg-Arg-Ala-Ser(P)-Leu-Ala-Gln-Leu-Thr	Cyclic AMP-PK	Decrease
	Fatty acid synthesis		
ATP-citrate lyase (rat mammary gland, rat liver)	Thr-Ala-Ser(P)-Phe-Ser-Glu-Ser-Arg	Cyclic AMP-PK	None
Acetyl CoA carboxylase (rat mammary gland)	Unknown	Cyclic AMP-PK	Decrease
	Cholesterol synthesis		
HMG-CoA reductase (rat liver)	Unknown	RK	Decrease
HMG-CoA reductase kinase (rat liver)	Unknown	RKK	Increase
	Protein synthesis		
Initiation factor eIF-2 (α subunit) (rabbit reticulocytes)	Unknown	eIF-2α kinase	Decrease
	Muscle contraction		
Myosin (P light chain)	Ala-Ala-Ala-Glu-Gly-Gly-Ser(P)-Ser-Asn-Val-Phe-Ser-Met	MLCK	None/increase
	Nuclear proteins		
Histone H1 (calf thymus)	Gly-Ala-Ala-Arg-Arg-Lys-Ala-Ser(P)-Gly-Pro-Pro-Val-Ser	Cyclic AMP-PK	
Histone H2B (calf thymus)	Arg-Lys-Arg-Ser(P)-Arg-Lys-Glu-Ser(P)-Tyr-Ser-Val-Tyr-Val	Cyclic AMP-PK	

and involves the phosphorylation of inhibitor 2 on a threonine residue (*20*). It should, however, be mentioned that, in freshly prepared extracts, protein phosphatase 1 has been reported to have a molecular weight of 250K (*14, 22*). It is not yet clear whether this higher molecular weight is due to the presence of additional subunits or whether the molecular weight of the native form of the catalytic subunit is greater than 35K. Nevertheless, the substrate specificity and susceptibility to inhibitors 1 and 2 of protein phosphatase 1 in freshly prepared extracts is similar to that of the 35K catalytic subunit (*8, 18*).

There are three type 2 enzymes, termed protein phosphatases 2A, 2B, and 2C, and these appear to be distinct from one another (*13, 14, 16, 17*). Three forms of protein phosphatase 2A have been resolved by anion exchange chromatography on DEAE-cellulose (*14*). These species, termed protein phosphatases $2A_0$, $2A_1$, and $2A_2$, have apparent molecular weights on gel filtration of 210K, 210K, and 150K, respectively; and each contains the same 38K catalytic subunit. This catalytic subunit appears to be identical to the second protein phosphatase (approximate molecular weight of 35K) (*11, 12*) observed in preparations purified by the method of Lee and co-workers (*10*). Protein phosphatases $2A_1$ and $2A_2$ have been purified to homogeneity by several groups of investigators (*16, 23–25*). Protein phosphatase $2A_1$ contains two subunits (with apparent molecular weights of 60K and 55K) in addition to the catalytic subunit, and protein phosphatase $2A_2$ contains only the 60K subunit complexed with the catalytic subunit. Imaoka *et al.* (*25*) separated the 60K, 55K, and 38K subunits of protein phosphatase $2A_1$ by gel filtration on Sephacryl S-200, followed by chromatography on DEAE-Sephadex in the presence of 6*M* urea. In the absence of urea, the 38K catalytic subunit reassociated with the 60K subunit or with the 60K plus 55K subunits to form species similar to protein phosphatases $2A_2$ and $2A_1$, respectively. However, the catalytic subunit did not form a complex with the 55K subunit alone, suggesting that, in protein phosphatase $2A_1$, this subunit interacts with the 60K subunit rather than the catalytic subunit. Protein phosphatase $2A_0$ has not yet been obtained in homogeneous form, and its subunit structure is at present unknown.

Protein phosphatase 2B is a Ca^{2+}-dependent enzyme (activation constant, $A_{0.5}$ = 0.5 to 1.0 μM) whose activity is stimulated tenfold by calmodulin ($A_{0.5}$ = 6.0 n*M*) (*17, 26*). This enzyme corresponds to the α-phosphorylase kinase phosphatase that we originally isolated from rabbit skeletal muscle (*4, 7*). Although initially reported to require Mn^{2+} for activity, it is now clear that this divalent cation was only substituting for Ca^{2+} in the activation of this enzyme. Protein phosphatase 2B has been purified to homogeneity from rabbit skeletal muscle (*17, 26*). After SDS-polyacrylamide gel electrophoresis, three components termed A, A′, and B with apparent molecular weights of 61K, 58K, and 15K, respectively, were observed (*26*). The molar ratio (A + A′):B was found to be 1:1. This subunit structure was strikingly similar to that reported for a major calmodulin-binding protein of neural tissue termed modulator-binding protein (*27*), calcineurin (*28*), or CaM-BP_{80} (*29*), which is composed of an A subunit (61K) and a B subunit (15K). In addition, calcineurin from bovine brain was found to contain a Ca^{2+}- and calmodulin-dependent protein phosphatase activity that was indistinguishable from protein phosphatase 2B in terms of its specific activity and substrate specificity (*26*).

These results indicate that calcineurin and protein phosphatase 2B are the same protein. The exact relation between the A and A′ components of muscle protein phosphatase 2B remains to be established, but several possibilities have been discussed (*26*). Component A of calcineurin is the subunit that binds to calmodulin (*27, 28*), and the B component binds four Ca^{2+} moieties with affinities in the micromolar range (*28*). Since protein phosphatase 2B is a Ca^{2+}-dependent enzyme whose activity increases by a factor of 10 in the presence of calmodulin, it is likely that component A is the catalytic subunit, and component B may confer Ca^{2+} sensitivity to the enzyme in the absence of calmodulin. Therefore, protein phosphatase 2B resembles phosphorylase kinase (*30*) in being regulated by two different calcium-binding subunits, one of which is an integral subunit, the second only interacting in the presence of Ca^{2+}.

Table 2. Classification of protein phosphatases (*13*).

Protein phosphatase	Inhibition by inhibitor 1 and inhibitor 2	Specificity		Phosphorylase phosphatase activity	Regulators
		Phosphorylase kinase	Substrate		
1	Yes	β Subunit	Broad	High	Inhibitor 1, inhibitor 2 Glycogen synthase kinase 3
2A	No	α Subunit	Broad	High	Unknown
2B	No	α Subunit	Narrow	Very low	Ca^{2+} and calmodulin
2C	No	α Subunit	Broad	Very low	Mg^{2+}

Protein phosphatase 2C (*13, 14, 16*) is a Mg^{2+}-dependent enzyme ($A_{0.5}$ = 1.0 m*M*), originally identified in liver and cardiac muscle as a "specific" glycogen synthase phosphatase separable from the major phosphorylase phosphatase activities in these tissues (*31*). Subsequent studies have demonstrated that, while this enzyme has little activity on phosphorylase, it is *not* specific for glycogen synthase but has a very broad substrate specificity (see below). Purification of protein phosphatase 2C from rat liver (*32*) and turkey gizzard (*23*) yielded a homogeneous preparation consisting of a single 43K subunit.

A study of the substrate specificities of the type 1 and type 2 protein phosphatases (Table 3) demonstrated that protein phosphatases 1, 2A, and 2C each have broad but distinct substrate specificities. In contrast, protein phosphatase 2B had significant activity on only three substrates, namely inhibitor 1, the α subunit of phosphorylase kinase and the P light chain of myosin (*13*).

Mechanisms for Regulating Type 1 and Type 2 Protein Phosphatases

The activity of protein phosphatase 1 is regulated by three proteins, namely inhibitor 1 (*33*), inhibitor 2 (*34*), and the protein kinase that activates the MgATP-dependent protein phosphatase (*35*). Each has been purified to homogeneity and characterized extensively. An important role for inhibitor 1 is indicated by the finding that this protein is only an active inhibitor after phosphorylation on a specific threonine residue by cyclic AMP–dependent protein kinase (*33*). In skeletal muscle, the extent of phosphorylation of inhibitor 1 is increased by epinephrine (*36*) and decreased by insulin, which antagonizes the effects of low concentrations of β-adrenergic agonists

(*37*). This antagonism appears to result from the ability of insulin to suppress the small rise in cyclic AMP produced by low concentrations of β agonists.

Since protein phosphatase 1 is active on many proteins that are phosphorylated by cyclic AMP–dependent protein kinase (see Tables 1 and 3), the activation of inhibitor 1 may prevent the dephosphorylation of these proteins and thus amplify the effects of cyclic AMP on their phosphorylation. Protein phosphatase 1 is also active on a number of proteins that are phosphorylated by protein kinases distinct from cyclic AMP–dependent protein kinase (Tables 1 and 3). The inhibition of protein phosphatase 1 by phosphoinhibitor 1 may therefore allow cyclic AMP to modulate the phosphorylation of these substrates. This idea is supported by studies of the effects of epinephrine on the phosphorylation (inactivation) of glycogen synthase in rabbit skeletal muscle. In normally fed animals, glycogen synthase contains approximately 3 moles of phosphate bound covalently to each subunit. After administration of epinephrine this value increases to 5 moles per mole of subunit with most of the increase occurring on serine residues, termed site 2 and sites (3a + 3b + 3c) (*38*). The increased phosphorylation of site 2 was not unexpected since it is phosphorylated by cyclic AMP–dependent protein kinase and phosphorylase kinase. The increased phosphorylation of sites (3a + 3b + 3c), however, was surprising, since these sites are phosphorylated by a different protein kinase, termed glycogen synthase kinase 3 (*35*). Since protein phosphatase 1 is the major protein phosphatase in rabbit skeletal muscle acting on glycogen synthase (see below), inhibition of this enzyme by phosphoinhibitor 1 may explain the enhanced phosphorylation of sites (3a + 3b + 3c) in response to epinephrine. Similar mechanisms have been proposed for regulating 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (*39*) and the aminoacyl transfer RNA synthetase complex (*40*) in rat liver in response to glucagon.

Protein phosphatase 2B accounts for about 70 percent of the total potential activity on inhibitor 1 in skeletal muscle extracts when assays are performed in the presence of micromolar concentrations of Ca^{2+} (*18*). In skeletal muscle this could be an important adaptive mechanism for synchronizing the rate of glycogenolysis with the strength and duration of contraction. Koshland (*41*) has defined adaptation as the "desensitization of a repeated external signal (for example, epinephrine) in order to deemphasize that signal in relation to others (for example, electrical excitation)." The dephosphorylation of inhibitor 1 by protein phosphatase 2B (if it occurred in vivo) would provide a mechanism for activating protein phosphatase 1 during contraction. This should decrease the steady level of phosphorylase a and so deemphasize the effect of epinephrine during contraction. This may be important for preventing too rapid a depletion of glycogen during low frequency stimulation of muscle. Such an action of protein phosphatase 1 should also stimulate the rate at which glycogen is resynthesized when contraction ceases. In order to test this hypothesis, it will be necessary to investigate whether low frequency electrical stimulation of muscle decreases the phosphorylation state of inhibitor 1 and the level of phosphorylase a that has previously been elevated by epinephrine. However, should these ideas be correct, the state of phosphorylation of inhibitor 1 (and hence the activity of protein phosphatase 1) would be regulated by both cyclic AMP and Ca^{2+} (Fig. 1). The activation of protein phosphatase 1 by protein phosphatase 2B could represent the first example of a protein phosphatase cascade system.

The activation of the MgATP-dependent protein phosphatase may be another important mechanism for regulating protein phosphatase 1. The protein kinase required for this activation has now

Table 3. Substrate specificities of type 1 and type 2 protein phosphatases (PrP). Activity is expressed as the percent of phosphate released in the assays relative to the dephosphorylation of either the α or β subunit of phosphorylase kinase for all substrates except HMG-CoA reductase and HMG-CoA reductase kinase. With reductase and reductase kinase, activity is expressed as the percent of conversion to the active or inactive form, respectively. The concentration of HMG-CoA reductase is expressed as the enzyme activity after complete activation by protein phosphatase (*13*). Values are an indication of the substrates that each protein phosphatase is capable of acting on, but should not necessarily be interpreted as being representative of rates that occur in vivo [Table 1 and (*13*)].

Substrate	Concentration		Phosphate per mole of protein	Relative activities			
	mg/ml	μ*M*		PrP 1	PrP 2A	PrP 2B	PrP 2C
			Glycogen metabolism				
Phosphorylase kinase	0.8	2.4	1.8	100 (β)	100 (α)	100 (α)	100 (α)
Phosphorylase a	1.0	10	1.0	27	17	1	2
Glycogen synthase							
Site 1a	0.11	1.2	0.4	57	92	3	21
Site 2	0.11	1.2	0.6	103	92	1	86
Sites (3a + 3b + 3c)	0.11	1.2	0.9	28	63	2	8
Inhibitor 1	0.04	2	0.9	54	100	192	19
			Glycolysis and gluconeogenesis				
Pyruvate kinase	0.06	1.0	0.7	20	39	3	58
			Fatty acid synthesis				
ATP-citrate lyase	0.12	1.0	0.8	18	79	2	15
Acetyl CoA carboxylase	0.24	1.0	0.7	21	28	1	18
			Cholesterol synthesis				
HMG-CoA reductase	3.0 U/ml			21	17	1	186
HMG-CoA reductase kinase	1.0 U/ml			80	124	4	1814
			Protein synthesis				
Initiation factor eIF-2	0.014	0.1	0.9	16	44	1	5
			Muscle contraction				
Myosin light chains	0.07	4	0.5	40	449	74	19
			Nuclear proteins				
Histone H1	0.04	2	0.6	523	231	2	9
Histone H2B	0.030	2	0.8	228	340	8	224

been purified to a state approaching homogeneity and is identical to glycogen synthase kinase 3 (*35*). Thus, this protein kinase catalyzes two apparently antagonistic reactions, namely the phosphorylation of glycogen synthase and the activation of the protein phosphatase that dephosphorylates a number of proteins including glycogen synthase. It has been shown (*38, 42*) that glycogen synthase is phosphorylated at sites (3a + 3b + 3c) in vivo, but the physiological importance of the activation of the MgATP-dependent protein phosphatase remains to be established. It is possible that the activation of this enzyme does not occur at the same time or in the same cellular compartment as the phosphorylation of glycogen synthase. For example, while the catalytic subunit of protein phosphatase 1 is specifically associated with the protein-glycogen complex where glycogen synthase is located (see below), inhibitor 2 is not. However, sites (3a + 3b + 3c) are the serine residues that become dephosphorylated in response to insulin, and this underlies the activation of glycogen synthase by this hormone (*42*). This implies that glycogen synthase kinase 3 activity is decreased by insulin. It is therefore of interest to examine the effects of insulin on the phosphorylation state of inhibitor 2 in vivo.

Inhibitor 2 combines with the catalytic subunit of protein phosphatase 1 to form the MgATP-dependent protein phosphatase (see above) (*20, 43*). At higher concentrations (1 to 10 n*M*), this protein inhibits protein phosphatase 1 (*8, 20*) by a different mechanism, which appears to result from the binding of inhibitor 2 to a distinct site on the enzyme. However, it is unclear whether this second mechanism of inhibition occurs in vivo.

The mechanisms for regulating protein phosphatases 2A and 2C are not understood at present. However, one of the three forms of protein phosphatase 2A, namely protein phosphatase $2A_0$, is catalytically inactive (*14*) and its activity is only expressed after dissociation of the 38K catalytic subunit. In contrast, protein phosphatases $2A_1$ and $2A_2$ are spontaneously active, although dissociation of the catalytic subunits from partially purified preparations of these enzymes results in a severalfold activation of protein phosphatase $2A_1$ and a much smaller increase in protein phosphatase $2A_2$ (*14, 18*). The activation of protein phosphatase $2A_1$ under these conditions appears to result from the dissociation of the 55K subunit (see before), which suppresses the phosphorylase phosphatase and glycogen synthase phosphatase activities of this species (*25*).

Role of Type 1 and Type 2 Protein Phosphatases in Cellular Regulation

Glycogen metabolism. Several observations indicate that protein phosphatase 1 is the major protein phosphatase involved in the activation of glycogen synthesis and the inactivation of glycogenolysis in skeletal muscle (Fig. 2, left). In this tissue about 50 percent of the protein phosphatase 1 is specifically bound to the protein-glycogen complex, the functional particles on which glycogen synthase, phosphorylase, and phosphorylase kinase are located (*4*), whereas significant amounts of the type 2 protein phosphatases are not associated with this fraction (*18*). When skeletal muscle extracts were assayed at near physiological *p*H and Mg^{2+} concentration (Table 4), protein phosphatase 1 accounted for 90 percent, > 95 percent, and 60 to 75 percent of the protein phosphatase activities toward phosphorylase, phosphorylase kinase (β subunit), and glycogen synthase, respectively. Protein phosphatase 2A accounted for the remainder of the activity toward these substrates, since the contribution of protein phosphatase 2C was negligible.

The regulation of glycogen metabolism in the liver by protein phosphatases appears to be more complex than in skeletal muscle (Fig. 2, right). The specific activities of protein phosphatases 2A and 2C in liver extracts are two times higher than in skeletal muscle extracts, while that of protein phosphatase 1 is three to four times lower (*18*). Consequently, protein phosphatase 2A accounts for 50 percent or more of the phosphorylase phosphatase and glycogen synthase phosphatase activities in liver extracts, while protein phosphatase 2C accounts for a small, but significant, proportion toward site 2 of glycogen synthase (Ta-

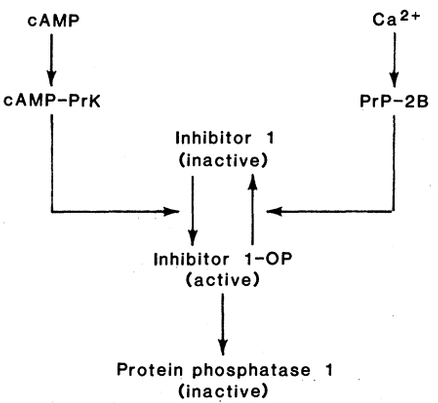

Fig. 1. Hypothesis for the regulation of the activity of protein phosphatase 1 by cyclic AMP and calcium ions via phosphorylation and dephosphorylation of inhibitor 1. Protein phosphatase 2B is not the only inhibitor 1 phosphatase in mammalian cells. Protein phosphatase 2A is also active on this substrate in vitro (Table 3) and this enzyme may be the most important inhibitor 1 phosphatase in the absence of Ca^{2+} (for example, resting muscle) (Table 4). Protein phosphatase 1 can also act on inhibitor 1 in vitro since this protein does not prevent its own dephosphorylation (*4*). However, the reaction is unusual because it has an absolute requirement for Mn^{2+} (Mg^{2+} and Ca^{2+} cannot substitute for this divalent cation). The physiological significance of this reaction is, therefore, questionable since high concentrations of Mn^{2+} do not exist in vivo. On the other hand, a protein, termed the deinhibitor, has been identified in liver that not only prevents the inhibition of protein phosphatase 1 by inhibitors 1 and 2, but is reported to enable the dephosphorylation of inhibitor 1 by protein phosphatase 1 to occur in the absence of Mn^{2+} (*54*). The abbreviations are: *cAMP-PrK*, cyclic AMP–dependent protein kinases; *PrP-2B*, protein phosphatase 2B; *Inhibitor 1-OP*, phosphoinhibitor 1.

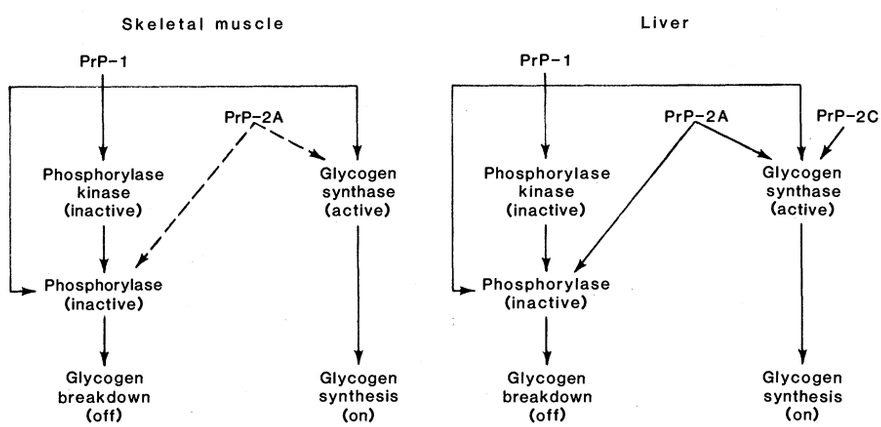

Fig. 2. The involvement of protein phosphatases (*PrP*) 1, 2A, and 2C in the control of glycogen synthesis and breakdown in skeletal muscle (left) and liver (right). The broken lines indicate that, in skeletal muscle, protein phosphatase 2A accounts for only a minor proportion of the activities on phosphorylase (12 percent) and on site 1a, site 2, or sites (3a + 3b + 3c) of glycogen synthase (24 to 34 percent) (see Table 4).

ble 5). Nevertheless, protein phosphatase 1 is still likely to play an important role in this process since it accounts for an appreciable portion of the phosphorylase phosphatase and glycogen synthase phosphatase activities and for about 80 percent of the phosphorylase kinase (β subunit) phosphatase activity (Table 5). Furthermore, it is the only protein phosphatase specifically associated with the protein-glycogen complex in liver, although the proportion bound to this fraction (10 percent) is less than in skeletal muscle (see above) (*18*).

A complication in assessing the contribution of protein phosphatases toward particular substrates is the finding that protein phosphatases 1 and 2A are strongly inhibited in concentrated liver extracts, whereas protein phosphatase 2C is not (*18*). Consequently, protein phosphatase 2C accounts for a much higher proportion of the activity toward site 2 of glycogen synthase (25 percent) when assays are carried out at low dilution. Since the protein concentration in liver cells is 40 times greater than in the "low dilution" assays, it is possible that the activities of protein phosphatases 1 and 2A are even lower in vivo and that the contribution of protein phosphatase 2C is greater. The macromolecule (or macromolecules) responsible for inhibition of protein phosphatases 1 and 2A in concentrated liver extracts is unknown, although it appears to be distinct from inhibitors 1 and 2 (*18*). Because of this, it is unclear whether such inhibition occurs in vivo or whether it is an in vitro artifact. This phenomenon is not observed in skeletal muscle extracts (*18*).

Other metabolic processes. Protein phosphatases 1, 2A, and 2C are each present in all tissues so far examined (*18*). The high concentrations of these enzymes in tissues such as brain and adipose tissue, where glycogen metabolism is of little importance, and their broad substrate specificities suggest that they may be important in regulating additional metabolic processes. Pursuing this idea, we have examined the role of these protein phosphatases in dephosphorylating several phosphoenzymes involved in regulating glycolysis, gluconeogenesis, fatty acid synthesis, and cholesterol synthesis. The enzymes ATP-citrate lyase, acetyl CoA carboxylase, and pyruvate kinase are phosphorylated by cyclic AMP–dependent protein kinase both in vitro and in vivo (*44*). In the case of the last two enzymes, the decrease in activity following phosphorylation results in the inhibition of fatty acid synthesis and of glycolysis, respectively, in response to hormones which elevate cyclic AMP. The role of the ATP–citrate lyase phosphorylation is unclear, however, since no changes in its kinetic parameters have been observed as a result of its phosphorylation (*44*). In cholesterol synthesis HMG-CoA reductase is the rate limiting enzyme. The phosphorylation of HMG-CoA reductase and HMG-CoA reductase kinase appear to underlie the short-term regulation of sterol synthesis in liver in response to insulin, glucagon, and cholesterol (*39*).

Protein phosphatases 1, 2A, and 2C are active on each of these five substrates in vitro (Table 3). However, when liver extracts were assayed at high dilution, and at near physiological *p*H and Mg^{2+} concentration, protein phosphatase 2A accounted for 80 to 95 percent of the activity toward pyruvate kinase, ATP-citrate lyase, and acetyl CoA carboxylase, while protein phosphatase 2C accounted for 60 to 70 percent of the activity toward HMG-CoA reductase and HMG-CoA reductase kinase. The remaining activity toward the last two substrates was due to protein phosphatases 1 and 2A (Table 5). When extracts

Table 4. Contribution of type 1 and type 2 protein phosphatases (PrP) to the total protein phosphatase activity in rabbit skeletal muscle acting on the phosphorylated proteins of glycogen metabolism. Protein phosphatases 1, 2A, and 2C were assayed in the presence of 1.0 m*M* EGTA and 1.0 m*M* $MgCl_2$ ("resting muscle"). Protein phosphatase 2B was measured in the presence of 1.0 m*M* EGTA and 0.85 m*M* $CaCl_2$ ("contracting muscle," 3 μ*M* free Ca^{2+}). Other conditions are described in (*18*) and Table 1.

Substrate	Relative contribution (percent) of PrP			
	1	2A	2B	2C
Resting muscle				
Phosphorylase	88	12	0	0
Glycogen synthase				
Site 1a	62	36	0	2
Site 2	74	24	0	2
Sites (3a + 3b + 3c)	75	24	0	1
Phosphorylase kinase (β subunit)	94	6	0	0
Phosphorylase kinase (α subunit)	17	79	0	4
Inhibitor 1	0*	100	0	0
Contracting muscle				
Phosphorylase kinase (α subunit)	6	29	63	2
Inhibitor 1	0*	35	65	0

*But see the legend to Fig. 1.

Table 5. Contribution of type 1 and type 2 protein phosphatases (PrP) to the total protein phosphatase activities in liver acting on the phosphorylated proteins involved in glycogen metabolism, glycolysis, gluconeogenesis, fatty acid synthesis, and cholesterol synthesis. The phosphatases were assayed in a solution containing 1.0 m*M* EGTA and 1.0 m*M* magnesium acetate at either a final dilution of 1 to 6 (low dilution) or of 1 to 600 (high dilution) as described (*18*). Assays with phosphorylase, glycogen synthase, and phosphorylase kinase as substrates were performed with rabbit liver extracts, while assays with other substrates were done with rat liver extracts. The concentrations of protein phosphatases 1, 2A, and 2C in rat and rabbit liver extracts are very similar (*18*) (Table 1).

Substrate	Percent of total activity					
	Low dilution			High dilution		
	PrP 1	PrP 2A	PrP 2C	PrP 1	PrP 2A	PrP 2C
Glycogen metabolism						
Phosphorylase	38	62	0	47	53	0
Glycogen synthase						
Site 1a	23	69	8	17	80	3
Site 2	36	39	25	22	66	11
Sites (3a + 3b + 3c)	40	51	9	32	66	2
Phosphorylase kinase (β subunit)	79	21	0	74	26	0
Glycolysis and gluconeogenesis						
Pyruvate kinase	3	48	49	8	82	10
Fatty acid synthesis						
Acetyl CoA carboxylase	2	60	38	12	84	4
ATP-citrate lyase	9	73	18	3	94	3
Cholesterol synthesis						
HMG-CoA reductase	< 1	< 1	100	15	23	62
HMG-CoA reductase kinase	< 5	< 5	100	7	20	73

were assayed at low dilution, protein phosphatase 2C accounted for virtually all of the measurable activity toward HMG-CoA reductase and HMG-CoA reductase kinase and it also became a significant activity toward pyruvate kinase and acetyl CoA carboxylase, accounting for 40 to 50 percent of the total activity in each case (Table 5). It is of interest, however, that a significant proportion of the protein phosphatase 1 in liver (20 percent) is associated with the microsomal fraction. Since HMG-CoA reductase (but not HMG-CoA reductase kinase) is tightly bound to this fraction (*39*), it remains possible that protein phosphatase 1 is an HMG-CoA reductase phosphatase in vivo.

Protein phosphatases 1 and 2A may be important in regulating protein synthesis. Protein synthesis initiation factor eIF-2 (eukaryotic initiation factor), which is involved in the formation of the preinitiation complex containing methionine transfer RNA (Met-tRNA$_f$), guanosine triphosphate (GTP), and the 40*S* ribosomal subunit, is phosphorylated on its α subunit by two different protein kinases. One termed eIF-2α kinase is inhibited by heme, while the second is dependent on double-stranded RNA for activity. These phosphorylation reactions, which occur on the same peptide, lead to the inhibition of protein synthesis initiation (*45*). Protein phosphatases 1 and 2A account for virtually all of the activity toward eIF-2 in mammalian tissues (*13, 16*). Furthermore, protein phosphatase 1 is the only protein phosphatase associated with ribosomes, and the addition of inhibitor 2 to reticulocyte lysates increases the phosphorylation of eIF-2 and inhibits protein synthesis (*46*). In addition, protein phosphatases 1 and 2A are both very active in dephosphorylating (activating) the aminoacyl transfer RNA synthetase complex (*40*).

In smooth muscle and in nonmuscle cells, phosphorylation of myosin P light chain appears to be required for the actomyosin adenosine triphosphatase (ATPase) activity. In skeletal muscle, myosin P light chain is also phosphorylated, but the role of this phosphorylation is unclear since it has no effect on actomyosin ATPase in this tissue (*47*). Protein phosphatases 1, 2A, 2B, and 2C are very active on the isolated P light chain of myosin (*13*), but it is not yet clear whether these enzymes are also active on this substrate when it is associated with the myosin heavy chains in the myofibril.

The observation that protein phosphatase 2B is apparently identical to calcineurin, coupled with the high level of this protein phosphatase in brain (particularly in the neostriatum), and its immunohistochemical localization at postsynaptic densities and microtubules of dendrites (*29*), suggests a role in neurotransmitter action and microtubular function. In conjunction with the calmodulin-dependent cyclic AMP–phosphodiesterase that is present in brain, it is possible that protein phosphatase 2B has an important adaptive function in enhancing the Ca^{2+}-signal in neuronal tissue at the expense of the cyclic AMP signal. The identification of physiological substrates for protein phosphatase 2B in the brain is of considerable interest. In this regard, Greengard and coworkers (*48*) have noted that, after incubation of isolated synaptosomes from rat brain cortex with ^{32}P-labeled inorganic phosphate, depolarization increased the incorporation of ^{32}P into several proteins but decreased the incorporation into two others with molecular weights of about 90K. The decreased phosphorylation might be due to the activation of a Ca^{2+}-dependent protein phosphatase (for example, protein phosphatase 2B).

Recently, it has been reported that microinjection of either inhibitor 1 or inhibitor 2 into *Xenopus* oocytes delayed the progesterone-induced division of these cells (*49*). This result suggests that protein phosphatase 1 may catalyze one or more dephosphorylation reactions that trigger meiotic maturation of these cells.

It is important to emphasize, however, that the nature of the protein phosphatases acting in newly discovered protein phosphorylation systems will have to be carefully defined in order to determine whether the type 1 and type 2 protein phosphatases, or additional protein phosphatases, are involved. It is already evident that distinct protein phosphatases participate in at least two other protein phosphorylation systems. In mitochondria, pyruvate dehydrogenase is regulated by phosphorylation-dephosphorylation. The protein phosphatase that acts on this enzyme is Ca^{2+}-dependent but clearly differs from protein phosphatase 2B in its subunit structure, and by the fact that it only binds a single mole of Ca^{2+} per mole (*50*). Recently, a novel class of protein kinase has been identified that phosphorylates its target proteins on tyrosine residues, rather than the more common serine or threonine residues. The transforming proteins of several RNA tumor viruses as well as the receptors for three growth-promoting peptides (insulin, epidermal growth factor, and platelet-derived growth factor) also possess this novel protein kinase activity (*51, 52*). Recent studies (*52*) suggest that the major protein phosphatases that act on phosphotyrosyl proteins are distinct from the protein phosphatases described in this article.

Conclusions

Considerable progress has now been made toward understanding the nature of the protein phosphatases involved in cellular regulation and, with the exception of protein phosphatases 1 and $2A_0$, the molecular structure of these enzymes has been elucidated. In addition, several mechanisms for regulating protein phosphatases 1 and 2B have been identified, although further work is needed to determine whether mechanisms also exist for regulating protein phosphatases 2A and 2C. Because of the broad and overlapping substrate specificities of protein phosphatases 1, 2A, and 2C, the physiological roles of these enzymes in tissues other than skeletal muscle need to be more clearly defined.

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Funding More NIH Research Grants

Proposals of a multidisciplinary group of biomedical scientists

H. George Mandel

Biomedical scientists are acutely aware of the growing inadequacy of financial support for research. Proposals to the National Institutes of Health (NIH) that are highly rated by peer review and that a few years ago would have been funded are now without support. In constant dollars, NIH appropriations for competing research projects (*1*) have actually gone down since 1979. The number of eligible applications submitted and recommended for payment by study sections has been growing steadily, but the number of awards has remained unchanged or has declined (Fig. 1).

The country's capacity for biomedical research, which has been built during many years of encouragement and support from the federal government, and which has been dramatically effective in improving our understanding of the basis of many human diseases and the design of rational treatment, is rapidly deteriorating. At the present time we lack the program stability needed to continue to attract and retain capable young scientists in biomedical research, and on-again-off-again funding will dissuade many scientists from research pursuits.

To maintain our capacity in biomedical research, which has become one of the country's greatest resources, and to provide the stability and diversity essential to it, federal appropriations for biomedical research must be increased. For fiscal 1982 it would have required an additional $300 million (the total NIH appropriation was about $3.6 billion) to fully fund, for 1 year, 50 percent of the approved competing grant applications (*2*). There could be no better investment in the health of our nation.

Until this fiscal goal can be attained, some measures are required to avoid serious damage to our hopes of progress. A letter to *Science* (*3*) expressed the concern of the Association for Medical School Pharmacology about the future of our biomedical research capacity. Under the present procedure for awarding the available funds, many excellent research projects are being terminated or cannot

Members of the group are Irwin Fridovich, president of the American Society of Biological Biochemists (ASBC); Lowell M. Greenbaum, secretary of the Association for Medical School Pharmacology (AMSP); Harold F. Hardman, president of the American Society for Pharmacology and Experimental Therapeutics (ASPET); H. George Mandel, chairman of a subcommittee of ASPET on NIH funding procedures and policies; Alan H. Mehler, chairman of ASBC's ad hoc Committee on Research Support; Gerald C. Mueller, president of the American Association for Cancer Research; Walter C. Randall, president of the American Physiological Society; Dante G. Scarpelli, president of the American Association of Pathologists; Frank G. Standaert, president of AMSP; William J. Whelan, president of the Association of Medical School Departments of Biochemistry; and Julius S. Youngner, president of the Association of Medical School Microbiology Chairmen. The opinions expressed do not necessarily reflect those of the membership of these organizations. John F. Sherman, vice-president of the Association of American Medical Colleges and formerly deputy director of NIH, attended the preliminary meeting as a resource person.

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