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The Structure, Role, and Regulation of Type 1 Protein Phosphatases

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ABSTRACT: Type 1 protein phosphatases (PP-1) comprise a group of widely distributed enzymes that specifically dephosphorylate serine and threonine residues of certain phosphoproteins. They all contain an isoform of the same catalytic subunit, which has an extremely conserved primary structure. One of the properties of PP-1 that allows one to distinguish them from other serine/threonine protein phosphatases is their sensitivity to inhibition by two proteins, termed *inhibitor 1* and *inhibitor 2*, or *modulator*. The latter protein can also form a 1:1 complex with the catalytic subunit that slowly inactivates upon incubation. This complex is reactivated *in vitro* by incubation with MgATP and protein kinase F_A/GSK-3. In the cell the type 1 catalytic subunit is associated with noncatalytic subunits that determine the activity, the substrate specificity, and the subcellular location of the phosphatase. PP-1 plays an essential role in glycogen metabolism, calcium transport, muscle contraction, intracellular transport, protein synthesis, and cell division. The activity of PP-1 is regulated by hormones like insulin, glucagon, α- and β-adrenergic agonists, glucocorticoids, and thyroid hormones.

KEY WORDS: protein phosphatases, type 1, dephosphorylation, serine/threonine.

I. INTRODUCTION

A large number of cellular processes are regulated by conformational changes of proteins that result from the reversible phosphorylation of specific serine, threonine, or tyrosine residues. The phosphorylation state of a protein is determined by a dynamic equilibrium between the activities of the protein kinase(s) and protein phosphatase(s) that catalyze the phosphorylation and dephosphorylation reactions, respectively. A considerable number of protein kinases[1–3] and protein phosphatases[4–7] have already been identified and characterized, and for some of them the physiological role and regulation have been elucidated. The importance of these enzymes for cellular activity is strikingly illustrated by the extreme phylogenetic conservation of their primary structure and by the tight hormonal and metabolic control of their activity.

This review deals specifically with a class of serine/threonine protein phosphatases that are best known as the type 1 protein phosphatases. They comprise a group of enzymes that contain an isoform of the same catalytic subunit, but differ in the noncatalytic subunits that determine the activity, the substrate specificity, and the intracellular location of the phosphatase. The interest in these phosphatases is growing rapidly as evidence is accumulating that they are present in all eukaryotic cells, where they play an essential role in such diverse processes as glycogen metabolism, intracellular transport, muscle contraction, protein synthesis, and cell division. It has been our aim to provide an up-to-date and in-depth review of the enzymology and the physiological role and regulation of type 1 protein phosphatases. For historical aspects in the development of the field, the reader is referred to other reviews.[8–10]

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II. CLASSIFICATION AND NOMENCLATURE OF SERINE/THREONINE PROTEIN PHOSPHATASES

The most widely adopted classification is that proposed by Ingebritsen and Cohen.[11,12] They distinguish two groups of serine/threonine protein phosphatases. The protein phosphatases of type 1 (PP-1) are inhibited by two proteins, termed *inhibitor 1* and *inhibitor 2*, or *modulator*, and preferentially dephosphorylate the β-subunit of phosphorylase kinase. In contrast, protein phosphatases of type 2 (PP-2) are insensitive to inhibitor 1 and modulator and preferentially dephosphorylate the α-subunit of phosphorylase kinase. Type 2 protein phosphatases are further subdivided according to their requirement for divalent cations; PP-2A do not require cations, while PP-2B and PP-2C show an absolute dependence on Ca^{2+} (plus calmodulin) and Mg^{2+}, respectively. Some investigators are rather reluctant to accept a nomenclature that is based on numbers and prefer more descriptive names.[4,10] Thus, PP-1 are also known as ATP, Mg-dependent (AMD) protein phosphatases or as phosphorylase phosphatases, PP-2A as the polycation-stimulated (PCS) protein phosphatases, PP-2B as calcineurins or Ca^{2+}-dependent protein phosphatases, and PP-2C as the Mg^{2+}-dependent protein phosphatases. The catalytic subunit of type 1 protein phosphatases is also referred to as protein phosphatase C-I[13,14] or F_C.[15]

The classification system introduced by Cohen's group applies to all examined eukaryotic cells[11,12,16–18] and provides a framework for the initial characterization of a phosphatase. Its major drawback is that it cannot accommodate all known serine/threonine protein phosphatases. It would still be feasible to extend the classification system to include such completely different enzymes as the mitochondrial protein phosphatases.[19] However, it is more difficult to classify those phosphatases that on one account belong to type 1 or type 2, but on another account clearly differ from these enzymes. Thus, a protein phosphatase has been described that is inhibited by inhibitor 1 and modulator but does not dephosphorylate phosphorylase kinase at all.[20] Other reports deal with enzymes that preferentially dephosphorylate the β-subunit of phosphorylase kinase, but are either not affected[21] or even stimulated[21a] by modulator. A further complexity arises from the recent discovery of protein phosphatases that catalyze the dephosphorylation of both phosphotyrosine and phosphoserine/phosphothreonine residues.[21b,21c]

It is clear from the previous discussion that the specificity in the dephosphorylation of the α- and β-subunits of phosphorylase kinase, and the sensitivity to inhibitor 1 and modulator, are not sufficient as criteria for the classification of serine/threonine protein phosphatases. Type 1 protein phosphatases possess, however, some additional characteristics that allow one to differentiate them from other protein phosphatases (Table 1). The most profoundly tested properties are the ability of type 1 protein phosphatases to be converted by modulator into an inactive, MgATP-dependent form and the resistance of the phosphorylase phosphatase activity of PP-1 to trypsin and chymotrypsin. The ultimate identification as a type 1 protein phosphatase lies, of course, in the determination of the primary structure of the catalytic subunit.

TABLE 1
Specific Characteristics of Type 1 Protein Phosphatases

1. Inhibition by inhibitor 1 and modulator (IV.A and B)
2. Preferential dephosphorylation of the β-subunit of phosphorylase kinase (III.C)
3. Conversion by modulator into a MgATP-dependent form (V.A)
4. Phosphorylase phosphatase activity either unaffected or increased by trypsin or chymotrypsin (III.A)
5. Phosphorylase phosphatase activity inhibited by heparin and basic proteins, and stimulated by *p*-nitrophenyl phosphate (III.A)
6. Rather low sensitivity to inhibition by okadaic acid, as compared to the sensitivity to microcystin-LR, calyculin A, and tautomycin (III.A)

Note: Section numbers in parentheses.

It has become customary to name the different species of PP-1 according to their subcellular localization. However, a uniform system to denote the enzymes and their subunits is not yet in use. We propose a capital letter to identify the intracellular location of the enzyme (Table 2). This leaves the possibility of using small letters to differentiate between several species in the same cellular compartment (e.g., PP-1Sa and PP-1Sb). By analogy, a subscript capital letter could be used to denote the catalytic subunit (e.g., PP-$1G_C$ for the catalytic subunit of the PP-1G) and a subscript small letter to differentiate between isoforms of the type 1 catalytic subunit (e.g., PP-1_{Ca} and PP-1_{Cb}).

TABLE 2
Proposed Nomenclature of Type 1 Protein Phosphatases

Cellular location	Proposed name
Cytosol (soluble fraction)	PP-1S
Glycogen	PP-1G
Endoplasmic reticulum	PP-1E
Myosin	PP-1M
Nuclei	PP-1N

III. THE CATALYTIC SUBUNIT OF PP-1

A. Enzymic Properties

1. Purification and Resistance to Proteolysis

An essential step in the purification of PP-1_C is its dissociation from noncatalytic subunits. Classically, this is attained by precipitation with 80% ethanol[22–25] or 50% acetone[26,27] at room temperature. The catalytic subunit of PP-2A also resists a treatment with ethanol,[28] but can subsequently be separated from PP-1_C by chromatography on covalently bound polylysine[25] or heparin.[24] PP-1_C migrates on SDS/PAGE with an apparent molecular mass between 33 and 38 kDa. The lower-molecular-weight species originate from the intact 37 to 38-kDa polypeptide by proteolysis during purification.[24,25,29] Proteolysis of PP-1_C from skeletal muscle can be largely overcome by the use of proteinase inhibitors,[24,25] but it has not yet been possible to obtain homogeneous, intact PP-1_C from liver.[30–33]

PP-1_C is destroyed by pronase, proteinase K, papain, and *Staphylococcus aureus* V8 proteinase.[26,33,34] However, the enzyme remains active during incubation with trypsin or chymotrypsin, in spite of its degradation from 37 to 38 kDa to 33 to 34 kDa.[29,35,36] Because the amino terminus of the phosphatase is still blocked after proteolysis by trypsin or chymotrypsin, this "nicked" phosphatase differs only from intact PP-1_C by the absence of 30 to 40 carboxy-terminal amino acids.[23,37] A treatment of PP-1_C with (chymo)trypsin decreases the activity toward some substrates (e.g., glycogen synthase, myosin light chain) severalfold, which can be explained by an increase of the K_m.[38,38a] In contrast, chymotrypsin not only increases the K_m for phosphorylase by about 50%, but also increases the V_{max} of the phosphorylase phosphatase reaction to the same extent.[38] In standard assay conditions the phosphorylase phosphatase activity of PP-1_C is either unaffected[27,39,40] or up to twofold increased[25,29] by incubation with (chymo)trypsin, which indicates that the V_{max} effect at least compensates for the lower substrate affinity. The role of the C-terminal region of PP-1_C in determining the substrate affinity is further substantiated by recent observations that the K_m of the nicked catalytic subunit for phosphorylase is decreased about 20% by addition of micromolar concentrations of a synthetic pentadecapeptide corresponding to the C terminus of PP-1_C.[38a] Incubation with this peptide did, however, not affect the V_{max} of the phosphorylase phosphatase reaction, nor did it have any effect on intact PP-1_C.

The resistance of PP-1_C to trypsin has been used to estimate the "total" activity of type 1 protein phosphatases in tissue homogenates and in subcellular fractions.[41,42] Trypsin will indeed generate free PP-1_C by destruction of the noncatalytic subunits that often inhibit type 1 protein phosphatases. On the other hand, the activity of PP-2A holoenzymes, as well as their catalytic subunit, is considerably decreased by trypsin.[43,44] In the absence of divalent cations, the phosphorylase phosphatase activity in trypsinized fractions from skeletal muscle or liver thus stems for at least 90% from PP-1_C.[39,41] This assay of total

activity of PP-1 does, however, not include the inactive, MgATP-dependent form of PP-1, unless Mn^{2+} was present during proteolysis (Section V.B).

2. Effect of Phosphorylation by a Tyrosine Kinase

PP-1_C is inactivated through phosphorylation by tyrosine protein kinase $pp60^{v\text{-}src}$, the product of the *src* oncogene.[38,40,45] At the maximal attainable stoichiometry of 0.34 mol phosphate per mole PP-1_C, the phosphorylase phosphatase activity was decreased by 39%.[40] Kinetic analysis indicates that 20% phosphorylation of PP-1_C by $pp60^{v\text{-}src}$ increases the K_m by 50% but does not affect the V_{max} of the phosphatase.[38] This K_m effect can explain why Tyr-phosphorylated PP-1_C was found to be completely inactive,[40] because the latter assays were performed at a substrate concentration that is at least fivefold below the K_m.[38,46] The phosphorylated tyrosine residue(s) has been localized in the C-terminal domain of PP-1_C that is removed by chymotrypsin. PP-1_C is no longer a substrate for $pp60^{v\text{-}src}$ after complexation with the modulator protein.[38] It is not known yet whether phosphorylation of PP-1_C on a tyrosine residue also occurs *in vivo*.

3. Inhibition by Polypeptides and Polyamines

The interaction of PP-1_C with inhibitor 1 and modulator is discussed in Sections IV.A and B. The free regulatory subunits of cAMP-dependent protein kinase (both type 1 and type 2) inhibit the phosphorylase phosphatase activity of PP-1_C with a maximum between 20 and 70%.[47–50] Such an inhibition has also been observed when PP-1_C is complexed with noncatalytic subunits.[47,51] Half-maximal and maximal inhibitions of the phosphorylase phosphatase activity have been obtained at concentrations of regulatory subunits varying between 50 and 250 n*M* and 50 and 2000 n*M*, respectively.[47,49,51] In comparison, the intracellular concentration of cAMP-dependent protein kinase in most tissues is 200 to 800 n*M*[52] and that of PP-1S in skeletal muscle has been estimated at about 500 n*M*.[29] The inhibitory potency of the regulatory subunit is cancelled by complexation with the catalytic subunit of cAMP-dependent protein kinase.[47,49,51] At variance with a report by Gergely and Bot,[53] more recent investigations indicate that the inhibition is noncompetitive with respect to the substrate.[47,49,51] However, the fivefold increase in the inhibitory potency of the type 2 regulatory subunit after phosphorylation by the catalytic subunit of cAMP-dependent protein kinase seems to be explained by the generation of a competitive inhibitor.[48,50,51] No evidence has been presented yet for a control of PP-1 by the regulatory subunits of cAMP-dependent protein kinase *in vivo*.

Basic polypeptides (protamine, histones, polylysine) and polyamines like spermine potently inhibit the dephosphorylation of phosphorylase by PP-1_C, but they do not affect, or even stimulate, the dephosphorylation of many other substrates.[34,54–58] Polyamines have also been reported to bind to phosphorylase.[59] Further, the inhibition of phosphorylase phosphatase by spermine is lost with a peptide substrate, obtained by proteolysis of phosphorylase *a*.[54] Taken together, these observations indicate that the effects of basic polypeptides are substrate-directed. However, a direct interaction of polyamines with PP-1_C cannot be excluded either, because the phosphorylase phosphatase activity is already half-maximally inhibited by a concentration of polylysine that is more than 30-fold lower than the substrate concentration,[55] and because PP-1_C is retained by polylysine-Sepharose.[25]

4. Inhibition by Cytotoxins

In recent years a number of cytotoxins have been identified as potent and rather specific inhibitors of PP-1 and PP-2A. The best known is okadaic acid, a polyether fatty acid that causes a half-complete inhibition of PP-2A at 0.1 to 1 n*M*, and of PP-1 at 10 to 500 n*M*.[60–65] Tautomycin, which is a structural analogue of okadaic acid,[66] calyculin A,[63] and the cyclic heptapeptide microcystin-LR[67,68] have recently been reported to inhibit PP-1 and PP-2A with an IC_{50} between

0.1 and 2 n*M*. From the available data, it is not clear whether[66,67] or not[68] PP-2A is much more sensitive to microcystin-LR than PP-1. Actually, these inhibitors are so potent that the IC_{50} depends on the concentration of the phosphatases during the assays.[62,66,68] Interestingly, the native type 1 protein phosphatases and PP-1_C appear to be about equally sensitive to these cytotoxins.[62,66,68]

Okadaic acid does not inhibit a protein phosphatase, encoded by the genome of bacteriophage lambda, that is homologus to PP-1_C and PP-$2A_C$ but has a truncated carboxyl-terminus that makes the protein about 100 residues shorter.[69,70] This indicates that okadaic acid binds to a noncatalytic region in the C-terminal third of PP-1_C and PP-$2A_C$. On the other hand, okadaic acid prevents the binding of microcystin-LR and tautomycin to PP-2A, whereas inhibitor 1 and modulator prevent the binding of microcystin-LR to PP-1.[66,68] Taken together, these data indicate that all these inhibitors have the same or overlapping binding sites.

The differential sensitivity of PP-1 and PP-2A to okadaic acid makes this inhibitor a useful device for the identification of these enzymes (Table 1). In addition, this toxin can enter the cell freely[64] and is therefore being used for the identification of physiological substrates of PP-1 and PP-2A (Section VII).

5. Other Non-Protein Effectors

The sulfated polysaccharide heparin inhibits the dephosphorylation of phosphorylase and phosphorylase kinase by PP-1_C but stimulates the dephosphorylation of glycogen synthase and pyruvate kinase by this phosphatase.[55,57,71,72] The retention of PP-1_C by covalently bound heparin[72] indicates that these effects may at least in part be due to binding of heparin to the phosphatase.

Glucose, glucose-6-phosphate, and caffeine increase the phosphorylase phosphatase activity of PP-1_C, whereas agents like AMP, ADP, glucose-1-P, and fructose-1-P have the opposite effect.[73–75*–77] These effects are probably substrate-directed because they are absent with a peptide substrate that contains the phosphorylation site of phosphorylase.[73,74,76] In addition, the existence of binding sites for these substances on phosphorylase has been clearly established.[78,79]

Oxidized glutathione and other disulfides fully inhibit PP-1_C, probably by reaction with a single sulfhydryl residue with formation of a mixed disulfide.[80,81–83(1)*–85] Likewise, oxalyl thioesters are inhibitory to PP-1_C, possibly by oxalylation of one or more enzymic thiol groups.[86*] The inhibition of PP-1_C by disulfides and oxalyl thioesters can be reversed by addition of sulfhydryl groups like reduced glutathione, 2-mercaptoethanol, or dithiothreitol.[81,82,85,86]

The activity of PP-1_C becomes irreversibly blocked during incubation with ATP, pyrophosphate, inorganic phosphate, or fluoride.[22,28,87–90] However, after removal of these inhibitors, the activity can be partially or completely restored in a time-dependent way by Mn^{2+} or Co^{2+}.[22,88,91] Although Mn^{2+} as such either has no effect or is slightly inhibitory of the phosphorylase phosphatase activity of PP-1_C[22,23,56,92] certain preparations of PP-1_C gradually convert to a Mn^{2+}-dependent form during storage.[92] Mn^{2+} is also an essential component in the renaturation of PP-1_C that is expressed in insect cells using a baculovirus vector containing cDNA of PP-1_C.[93] Finally, Mn^{2+} broadens the substrate specificity of PP-1_C (Section III.C) and is able to reactivate free, modulator-inactivated PP-1_C (Section V.B). In spite of all these effects of Mn^{2+}, PP-1_C does not appear to be a real metallo-enzyme because it cannot bind stoichiometric amounts of Mn^{2+}[94,94a] and because extremely low levels of Mn^{2+} and Co^{2+} have been found in a phosphatase preparation that was probably a mixture of PP-1_C and PP-$2A_C$.[95]

p-Nitrophenyl phosphate (*p*NPP) has been described as a specific stimulator of PP-1_C.[96,97] This effect results from an increase of the V_{max} and is unrelated to the hydrolysis of *p*NPP. A half-maximal stimulation has been obtained at 0.1 m*M* *p*NPP. At 1 m*M* *p*NPP the phosphorylase phosphatase activity of PP-1_C is stimulated about twofold.

* It is possible that the protein phosphatases that have been used in these studies were a mixture of PP-1_C and PP-$2A_C$.

B. Structure

1. Isoforms

Two isoforms of PP-1_C have been identified in rabbit skeletal muscle by analysis of cDNA clones.[37,98,99] Their calculated molecular weights are 37.5 kDa (PP-1α) and 35.4 kDa (PP-1β). The N-terminal 33 amino acids of PP-1α show no homology with the N-terminal region of the PP-1β, which is 19 residues shorter. However, beyond amino acid 33 of PP-1α, the nucleotide sequence for both isoforms is identical, including the 3′-untranslated region (Table 3). This has been taken as evidence that PP-1α and PP-1β are generated from the same gene by differential transcription and/or splicing of the mRNA.[99] In a more recent report, the same group[93] pointed out that PP-1β might be a cloning artifact, because the distinct 5′-region of its cDNA was also joined to an unrelated sequence in another clone.

cDNA clones encoding rabbit liver and skeletal muscle PP-1α have an identical nucleotide sequence, indicating that they are derived from the same gene.[33] The amino acid sequences of PP-1α from human liver (residues 23–330),[100]

TABLE 3
Structural Homology of Rabbit PP-1α with the Catalytic Subunit of Other Protein Phosphatases

Name	Source	Number of amino acids	Percentage of identity with rabbit PP-1α		Ref.
			Overall[a]	Residues 60–130[b]	
Homologues of PP-1_C					
PP-1α	Rabbit skeletal muscle	330	100	100	37, 99
	Rabbit liver	330	100	100	33
	Rat liver	330	100	100	99a
	Rat kidney	330	100	100	99b
	Human liver	—	—	100	100
	Drosophila head	302	92	99	101
PP-1β	Rabbit skeletal muscle	311	95	100	98, 99
dis2m1/PP-1γ2	Mouse brain	337	90	100	102
	Rat testis	337	90	100	99a
PP-1γ1	Rat liver	323	93	100	99a
dis2m2/PP-1δ	Mouse brain	327	89	97	102
	Rat liver	327	89	97	99a
bimG	*Aspergillus nidulans*	323	86	97	103
dis2/*bws1*	*Schizosaccharomyces pombe* (fission yeast)	327	82	96	102, 104
sds21	*Schizosaccharomyces pombe*	322	74	92	102
DIS2S1	*Saccharomyces cerevisiae* (budding yeast)	312	83	99	102
Other (putative) protein phosphatases					
PP-$2B_C$[c]	Human brain	524	38	60	105
PP-2Aα	Rabbit skeletal muscle	309	41	82	106
PP-X	Rabbit liver	307	39	55	107
PP-Y	*Drosophila* head	314	59	82	107, 108
PP-Z1	*Saccharomyces cerevisiae*	348	63	82	107, 108a
*orf*221	Bacteriophage lambda	221	—	41	69
	Bacteriophage Φ80	—	—	41	69

[a] In the overlapping region, according to the alignment illustrated in the indicated references.
[b] According to the alignment shown in the indicated references.
[c] The calculations are based on the sequence of the isoform that was termed *calcineurin A-2*.

rat liver,[99a] and rat kidney[99b] are identical to the rabbit enzyme, but the nucleotide sequence shows about 10% differences spread throughout the coding region. The gene of human PP-1α has been assigned to a region of chromosome 11 (band 11q13) that is important in the pathogenesis of certain cancers.[100]

Screening of a mouse-brain cDNA library with fragments of the *sds21*$^+$ gene of fission yeast, which encodes a homologue of the type 1 catalytic subunit (Table 3), has allowed the identification of two other mammalian isoforms of PP-1_C.[102] These isoforms, termed "*dis2m1*" and "*dis2m2*" are about 90% identical to rabbit PP-1α (Table 3). The differences are spread all over the polypeptide, which shows that they are encoded by different genes. Sasaki et al. used fragments of *dis2m1* and *dis2m2* as probes for the identification of isoforms of PP-1_C in cDNA libraries from rat liver and testis.[99a] Three of the isolated clones encoded proteins that are identical to PP-1α, *dis2m1* (PP-1γ2), and *dis2m2* (PP-1δ). A fourth clone, encoding a protein designated PP-1γ1, has an identical nucleotide sequence as the clone encoding PP-1γ2, except for the 3'-terminal coding region.[99a] This suggests that PP-1γ1 and PP-1γ2 are produced from the same gene by alternative splicing. The *dis2m1* (PP-1γ2) and *dis2m2* (PP-1δ) isoforms of PP-1_C are expressed in two different mRNA sizes in most rat tissues.[109] The larger sized mRNA appears to be the more abundant species, except in the testis, where the smaller mRNA of *dis2m1* is present at much higher levels. Comparison between the primary structure determined by peptide sequencing and cDNA analysis indicates that the isoform of the catalytic subunit present in PP-1G from rabbit skeletal muscle represents PP-1δ.[110]

2. Evolutionary Conservation and Homology with Other Phosphatases

The primary structure of PP-1_C shows an extreme phylogenetic conservation (Table 3). PP-1α from *Drosophila* and rabbit are 92% identical.[101] This agrees with the remarkable similarity in enzymatic and regulatory properties of PP-1_C in both groups.[57] Homologues of PP-1_C in yeast and *Aspergillus* are more than 70% identical to rabbit PP-1α (Table 3).

The primary structure of PP-1_C is strikingly homologous with that of PP-$2A_C$ and PP-$2B_C$ (Table 3) but is not related to that of PP-2C.[111] The high degree of homology between PP-1_C and PP-$2A_C$ explains the earlier finding that they have common antigenic determinants.[112,113] PP-1_C is also homologous to putative protein phosphatases, termed *PP-X*, *PP-Y*, and *PP-Z*, that are known only from sequence analysis of cDNA libraries (Table 3). The homology with PP-1_C can also be extended to a protein phosphatase encoded by the genome of bacteriophage lambda (*orf*221) and Φ80,[69,70] and even to regions of mammalian alkaline phosphatases[98] and purple acid phosphatases.[114]

The homology between different protein phosphatases is especially pronounced in the region corresponding to residues 60 to 130 of PP-1α (Table 3). This domain is therefore likely to contain the catalytic site and is probably not involved in the interaction with specific effectors of PP-1_C, like inhibitor 1 or modulator. However, some other regions of PP-1_C are also extremely conserved and may be essential for activity. Thus, a particular mutation in yeast is lethal due to the substitution of a single amino acid (Arg 245 is replaced by Gln) in a homologue of PP-1_C.[115] This Arg residue is conserved in all species of PP-1_C, PP-$2A_C$, and PP-$2B_C$ that have been investigated so far.

3. The Occurrence of a Dimer

cDNA sequencing and Northern hybridization have shown that PP-1_C is synthesized as a protein of 35 to 38 kDa.[37,98,99] Yet, Brautigan's group has reported that affinity-purified polyclonal antibodies against PP-1_C recognize polypeptides of both 35 to 38 kDa and 70 kDa following SDS/PAGE of partially or extensively purified PP-1_C from rabbit skeletal muscle.[26,116] Two lines of evidence suggest that the 70-kDa polypeptide is a dimer of the 35 to 38-kDa phosphatase.[116] First, both immunoreactive species have the same amino acid composition and give identical peptide maps after cleavage with CNBr. Second, at a slightly basic pH and after removal of reducing agents, the pure 38-kDa PP-1_C can be converted into a 70-kDa polypeptide; this "dimer" is redissociated into a polypeptide of

about 35 kDa by incubation with one of several proteases. Brautigan and co-workers have also reported that the antibodies against PP-1_C from skeletal muscle bind to polypeptides of 35 and 60 kDa in liver extracts from fetal and adult rats, but no evidence has been presented indicating that the 60-kDa protein is a dimer of PP-1_C.[117] The finding that a dramatic increase in the concentration of the 60-kDa polypeptide during the last few days of fetal development is not associated with any increase in the total activity of PP-1 rather indicates that this 60-kDa protein is not a type 1 protein phosphatase.

Mainly on the basis of results of cross-linking experiments, Khatra has concluded that the catalytic activity of the glycogen-bound protein phosphatase from rabbit skeletal muscle resides in a dimer.[36] This dimer (83–85 kDa) is monomerized by limited trypsinization but, contrary to the findings of Brautigan's group, it also dissociates during SDS/PAGE (38 kDa). More recently, Pesi and Villa-Moruzzi[118] reported that a 70-kDa complex is formed by exposure of PP-1_C to 33 m*M* NaF. This dimerization is not observed after removal of the C-terminal domain of PP-1_C by trypsin. Removal of fluoride reportedly causes little dissociation of the dimer, but the complex is dissociated by boiling in SDS.

More data is needed to establish unequivocally whether or not PP-1_C exists as a dimer in the cell. In the light of reports that serum albumin (67 kDa) is strongly antigenic and copurifies with PP-1_c,[14,119] results on immunoblotting with polyclonal antibodies against PP-1_C should be interpreted very cautiously.

C. Substrate Specificity

PP-1_C has a very broad substrate specificity (Section VII), which overlaps with that of other protein phosphatases.[11] However, PP-1 can be differentiated from other phosphatases because it dephosphorylates the β-subunit of phosphorylase kinase about 10 to 100 times faster than the α-subunit.[29,57,120,121] With phosphorylase, which is one of the best and most commonly used substrates of PP-1_C, specific activities of 10 to 50 μmol/min/mg at 30°C have been noted.[24,27,29,32] This corresponds to a molecular activity of roughly 400 to 2000 (moles of phosphate released per minute per mole of phosphatase).

The substrate specificity of protein kinases is to a large extent determined by the primary structure in the immediate vicinity of the phosphorylated residue.[122,123] The same may be true for PP-2A.[122,124–126] This explains why peptide substrates can readily be used with these enzymes. In contrast, PP-1_C fails to dephosphorylate peptides with a sequence that corresponds to the phosphorylation site of model substrates of the phosphatase.[124,125,127] This indicates that the determinants for substrate recognition of PP-1_C do reside not only in the primary structure but also in the higher order structure of the substrate.

The substrate specificity of PP-1_C is narrowed or enlarged quite dramatically by interaction with noncatalytic subunits or with Mn^{2+}. The role of the noncatalytic subunits in the determination of the substrate specificity of PP-1 is strikingly illustrated by hepatic PP-1G, which has a severalfold lower phosphorylase phosphatase activity but a much higher glycogen-synthase phosphatase activity, when compared with the free catalytic subunit (Section VI.B.2). On the other hand, the inability of PP-1_C to dephosphorylate peptide substrates is partially or completely overcome by preincubation of the phosphatase with Mn^{2+}.[124,127] The rate of dephosphorylation of inhibitor 1,[24,46,128,129] modulator phosphorylated on Thr 72[130–133] and thiophosphorylated phosphorylase[92] by PP-1_C is also dramatically improved by Mn^{2+}. Finally, free PP-1_C has been reported to dephosphorylate the nonprotein substrate *p*NPP in the presence of Mn^{2+} or Mg^{2+},[23,134,134a] but this is not a general finding.[97,135]

IV. HEAT-STABLE REGULATORY PROTEINS OF PP-1

A. Inhibitor 1 and DARPP-32

The related polypeptides inhibitor 1 and DARPP-32 are acid- and heat-stable proteins that turn into strong and specific inhibitors of type 1 protein phosphatases after phosphorylation by cAMP-dependent protein kinase. Inhibitor 1 was discovered together with another inhibitor of PP-

1, which was termed *inhibitor 2* and is now also known as modulator.[128,136–138] DARPP-32 was initially described as a phosphoprotein of 32 kDa (SDS/PAGE) in dopamine-innervated brain regions.[139] Addition of dopamine or cAMP to slices of these brain regions increased the phosphorylation of DARPP-32, hence its name — *dopamine and cAMP-regulated phosphoprotein, M_r = 32 kDa.*

1. Structure and Physicochemical Characteristics

The primary structure of inhibitor 1 from rabbit and rat skeletal muscle has been determined by protein sequencing and cDNA cloning, respectively.[140–142] Rat inhibitor 1 (171 amino acids) is five residues longer than the rabbit protein. The overall identity between rat and rabbit inhibitor 1 is 80%, but this value increases to nearly 100% in the N-terminal domain. Unexpectedly, the latter region is homologous to a part of soluble guanylate cyclase.[143] The primary structure of DARPP-32 from bovine brain has been elucidated by protein sequencing[144] and cDNA cloning.[145] It consists of 202 residues, and an optimized alignment of rabbit inhibitor 1 and bovine DARPP-32 yields an overall identity of 27%, but the homology is again higher in the N-terminal domain. The primary structure of a heptapeptide that contains the phosphorylation site of inhibitor 1 as well as DARPP-32 is also homologous to a region of G substrate, a neuron-specific protein that is phosphorylated by cGMP-dependent protein kinase.[146] Northern hybridization analysis has indicated the presence of a major mRNA of 0.7 kb and a minor mRNA of 1.8 kb for inhibitor 1 in rat and rabbit tissues.[141] For bovine DARPP-32, only mRNA of 1.65 to 1.8 kb has been detected.[145]

The calculated molecular masses of inhibitor 1 from rabbit skeletal muscle and of DARPP-32 from bovine brain are 18.7 and 22.6 kDa, respectively.[140,144] This is much lower than the estimates of 26 and 32 kDa obtained from SDS/PAGE.[128,136,137,147] Inhibitor 1 and DARPP-32 from the rat have an even lower mobility on SDS/PAGE,[142,148] but at least in the case of inhibitor 1 this is partially due to a slightly higher molecular mass.[141] The low electrophoretic mobility of inhibitor 1 and DARPP-32 may be related to the very hydrophilic nature of these proteins, resulting in an abnormally low binding of SDS.[144,149] Both proteins are also very asymmetric, as judged from the high apparent molecular mass (approximately 60 kDa) that is deduced from gel filtration.[147,150,151]

The N-terminal part of inhibitor 1 and DARPP-32, which is the most conserved region, also includes the inhibitory domain. Indeed, a fragment containing residues 9 through 54 of rabbit inhibitor 1 retains the full inhibitory power.[152] Likewise, a synthetic peptide comprising residues 9 through 38 of DARPP-32 has only a five-fold higher IC_{50} than that of the intact protein.[153] A comparison of the inhibitory potency of synthetic peptides has shown that the presence of an isoleucine at position 9 and a phosphorylated threonine at position 34 are essential features for the interaction of DARPP-32 with PP-1_C. Substitution of phosphothreonine 34 for a phosphoserine results in a dramatic increase of the half-maximally effective inhibitor concentration.

2. Control by Phosphorylation

After phosphorylation by cAMP-dependent protein kinase, inhibitor 1 and DARPP-32 act as instantaneous inhibitors of PP-1_C with a K_i between 0.5 and 10 n*M*.[46,154,155] Inhibition of the native holoenzymes of PP-1 requires much more inhibitor 1 and is time dependent (Section VI).[92,135,156] At phosphatase concentrations that are considerably lower than the K_i values,* inhibitor 1 and DARPP-32 behave as mixed-type inhibitors of PP-1_C.[46,153,155]

Thr 35 of inhibitor 1 and Thr 34 of DARPP-32 are extremely well phosphorylated by cAMP-dependent protein kinase.[147,157–159] Yet, small peptides (six to eight residues) modeled on the sequence surrounding the phosphorylation sites of inhibitor 1 and DARPP-32 are relatively poor substrates for this kinase.[153,160] Other factors, residing in domains further from the phosphor-

* At these phosphatase concentrations, Michaelis–Menten kinetics apply because the concentration of free inhibitor is virtually the same as the total inhibitor concentration.

ylation site, must therefore potentiate the rate of phosphorylation in the intact proteins. A recent comparison of synthetic peptides indicates indeed that residues 38 through 48 of DARPP-32 are essential for an efficient phosphorylation of Thr 34 by cAMP-dependent protein kinase.[153]

The phosphorylation of inhibitor 1 and DARPP-32 by cAMP-dependent protein kinase in intact cells has been clearly established (Section VIII.B). In addition, Ser 67 of inhibitor 1 appears to be phosphorylated in skeletal muscle *in vivo*, but the significance of this phosphorylation and the identity of the underlying kinase are still unknown.[140] DARPP-32 is phosphorylated at Ser 45 and Ser 102 by casein kinase 2.[157] Ser 102 is also phosphorylated under basal conditions in striatal slices of rat brain. Phosphorylation by casein kinase 2 does not affect the inhibitory potency of DARPP-32 but facilitates phosphorylation by cAMP-dependent protein kinase. Inhibitor 1 is not substrate for casein kinase 2.

Inhibitor 1 and DARPP-32 are no longer inhibitory after dephosphorylation of the site that is controlled by cAMP-dependent protein kinase. Dephosphorylation of this phosphothreonine is catalyzed by PP-2A and particularly well by PP-2B.[153,155,161] PP-1_C is effective only in the presence of unphysiological concentrations of Mn^{2+}[24,46,128,129,153] or after preincubation with the deinhibitor protein (Section IV.C). Inhibitor 1 is unable to block its own dephosphorylation by PP-1_C.[129] One explanation is that binding of inhibitor 1 as a substrate prevents its binding as an inhibitor, possibly due to the presence of two overlapping binding sites. The existence of more than one binding site for inhibitor 1 is also indicated by observations that the deinhibitor prevents the inhibition of PP-1_C by inhibitor 1 but at the same time stimulates the dephosphorylation of inhibitor 1 by this phosphatase (Section IV.C). Furthermore, the large difference in the affinity of PP-1_C for inhibitor 1 as a substrate (K_m of 200–700 n*M*) and as an inhibitor (K_i of 1–10 n*M*)[46,129] is also consistent with the presence of two binding sites.

3. Concentration and Distribution

The average concentration of inhibitor 1 in rabbit liver, skeletal muscle, and rat brain neostriatum is 1 to 2 μ*M*, which is severalfold higher than the cytosolic concentration of type 1 protein phosphatases.[142,150,154,162] The level of DARPP-32 in certain brain areas is even higher and has been estimated at 10 to 50 μ*M*.[148,162]

Inhibitor 1 and DARPP-32 are cytosolic proteins[150,163,164] having a rather peculiar tissue distribution. Inhibitor 1 is present in mammalian skeletal muscle, heart, kidney, uterus, and adipose tissue.[136–138,142] DARPP-32 has been identified in brain regions with dopaminergic innervation, but also in the adrenal medulla, pineal gland, choroid plexus, and brown adipose tissue.[148,165] Both inhibitors are present in the striatonigral neurons of rat brain[162] and in renal tubule cells,[164] but rabbit reticulocytes do not contain any phosphorylatable inhibitor of type 1 protein phosphatases.[166,167] Remarkably, DARPP-32 appears to be present in bovine adipose tissue,[168] but inhibitor 1 is the isoform present in rat adipose tissue.[151] Northern blotting and Western blotting, as well as activity assays, have shown that inhibitor 1 is present in the liver of rabbits, guinea pigs, and sheep, but not in mouse and rat liver.[137,141,142]

B. Modulator (Inhibitor 2)

1. Structure and Physicochemical Properties

Modulator has been obtained in a homogeneous form from rabbit skeletal muscle and rat adipose tissue.[130,149,154,169,170] This protein resembles inhibitor 1 and DARPP-32 in that it is resistant to denaturation by heat and acid and that it has a highly asymmetric structure and a low content of hydrophobic amino acids. The calculated molecular weight of modulator from rabbit skeletal muscle (22.9 kDa for 204 residues) is also considerably lower than the values obtained from gel filtration (50–100 kDa) and SDS/PAGE (31 kDa).[171–173] Similar to what has been found for inhibitor 1 and DARPP-32, rat modulator also has a lower electrophoretic mobility than the rabbit protein.[142,170] In spite of all these similarities, the determination of the primary structure of modulator from rabbit skeletal muscle by protein sequencing and by cDNA cloning has not revealed any homology with inhibitor 1 or DARPP-32.[172,174] Northern blotting has led to

the identification of two mRNA species (1.7 and 2.7 kb) for modulator.[174] Both species seem to be present in several rabbit tissues, except for the liver, which contains only the larger mRNA.

Western blotting of the heat-stable protein fraction of several tissue extracts with polyclonal antibodies against modulator shows a major immunoreactive polypeptide of 31 to 33 kDa.[175–177] However, several additional and larger polypeptides are recognized in unboiled tissue extracts.[142,175–177] These larger polypeptides cannot be precursors of modulator because cDNA cloning has proven that it is synthetized as a protein of 22.9 kDa.[174] It has also been shown that one of these polypeptides (60 kDa) is not a constituent of PP-1.[178] The recognition of multiple polypeptides by anti-modulator antibodies is therefore best explained by common antigenic determinants in unrelated proteins.

2. Interaction with Protein Phosphatases

Modulator blocks the activity of PP-1 in two distinct ways, namely, by impeding the substrate binding (*inhibition*) and by inducing a conformational change of the catalytic subunit (*inactivation*). As will be discussed in more detail in Section V, modulator is also required for the enzymatic reactivation of inactive PP-1_C. Due to these multiple effects, it was proposed to replace the original name *inhibitor 2* by *modulator*.[179]

The inhibition of PP-1 by modulator is of a competitive nature[46] and is thus probably accounted for by binding of modulator and substrate at the same or overlapping binding sites. Modulator inhibits PP-1_C virtually instantaneously (Figure 1A) with a K_i between 3 and 10 nM.[46] In contrast, the inhibition of type 1 holoenzymes is time dependent and requires much more modulator (Section VI).[39,41] The inhibition of PP-1_C can be completely cancelled by the specific destruction of modulator by trypsin (Figure 1B, 0 min).

Inactivation of PP-1_C differs from inhibition in that it is time dependent ($t_{1/2}$ = 10–30 min in various incubation conditions) and that it cannot be reversed by simple removal of modulator by, for example, proteolysis (Figure 1B). Thus, the assay of phosphorylase phosphatase before and after trypsin allows one to differentiate between inhibition and inactivation of PP-1_C. Furthermore, inactivation of PP-1_C is already observed at equimolar concentrations of phosphatase and modulator, whereas about 10 times more modulator is required for inhibition.[22,180] This indicates that PP-1_C possesses two separate binding sites for modulator, i.e., a low-affinity "inhibition site" and a high-affinity "inactivation site."

It has been reported that modulator, at concentrations that are 100 to 300 times higher than those required for the inhibition of PP-1_C, is also inhibitory to PP-$2A_C$.[113,181] This contrasts, however, with more recent findings showing that the activity of PP-$2A_C$ or a PP-2A holoenzyme is not significantly affected by up to 5 μM of modulator.[41] The reason for this discrepancy remains unclear, but it does not seem to be related to differences in the preparations of modulator.[182] Modulator has also been reported to inhibit protein phosphatases from brain capillaries, reticulocytes, and amphibian oocytes that can be classified neither as type 1 nor as type 2 enzymes (Sections VI.A.3 and VII.D).

3. Effects of Phosphorylation

Modulator can be phosphorylated on Thr 72 by kinase F_A/GSK-3,* and the dephosphorylation of this site is catalyzed by PP-1 and PP-2A as well as PP-2B.[130] The phosphorylation of modulator Thr 72 represents a transient step in the reactivation of the MgATP-dependent form of PP-1 (Section V.C). Due to rapid dephosphorylation of PP-1_C, the maximally observed stoichiometry of modulator phosphorylation is very low. This makes it difficult to obtain conclusive data on the physiological occurrence of this modification. On one hand, fast-atom-bombardment mass spectrometry on peptides, obtained by proteolysis of modulator that was prepared in the presence of protein phosphatase inhibitors, did not reveal any phosphate in Thr 72.[173] On the other hand, modulator that was immunoprecipi-

* This kinase was originally described as an "activating factor" (F_A) of the MgATP-dependent form of PP-1.[183,184] Later it was found to be identical to glycogen synthase kinase-3 (GSK-3).[184,185] Hence the name F_A/GSK-3.

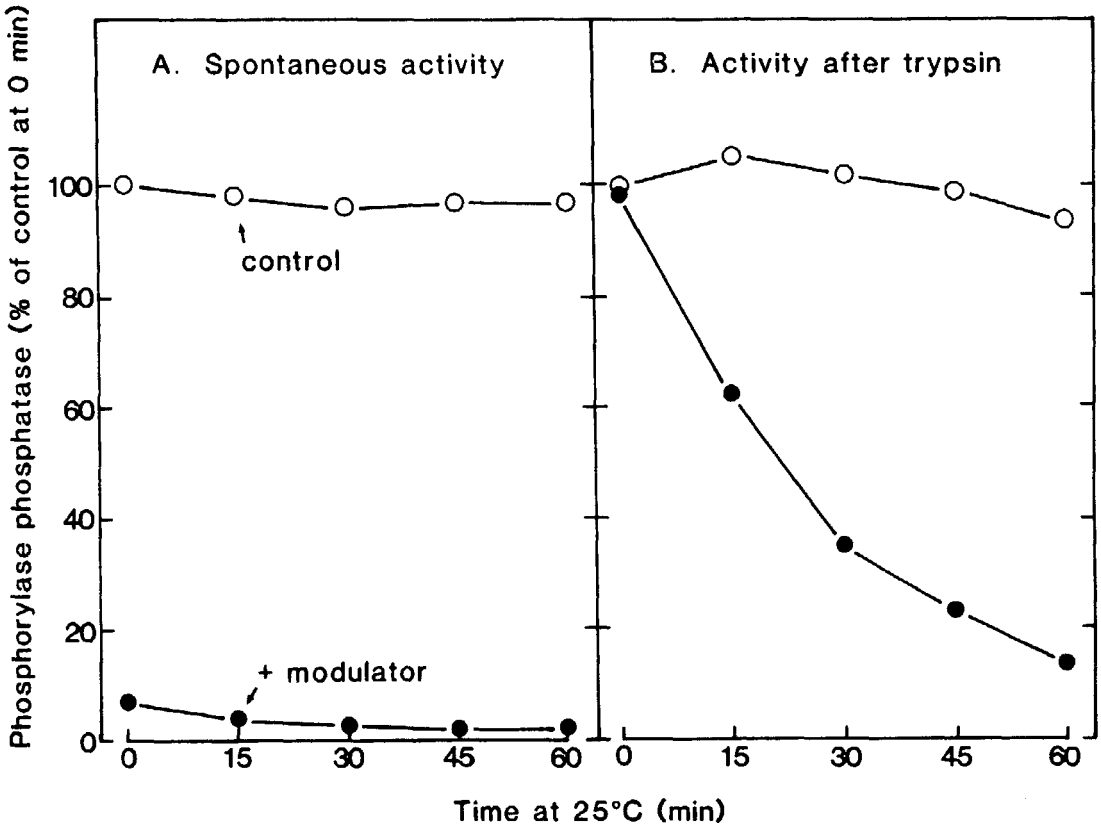

FIGURE 1. (A) inhibition and (B) inactivation of PP-1_C by modulator. The type 1 catalytic subunit (5 n*M*) was incubated at 25°C in the absence or presence of modulator (200 n*M*). Every 15 min samples were diluted sixfold for the assay of phosphorylase phosphatase before (A) or after (B) incubation with trypsin. The activities are expressed as a percentage of the spontaneous activity of the control at time zero. (Adapted from Bollen and Stalmans.[90])

tated from extracts of ^{32}P-labeled mouse diaphragms[186] or rat fat cells[187] contained not only phosphoserine but also a minor fraction of phosphothreonine. In the latter instance, it remains to be seen whether indeed Thr 72 was labeled.

Casein kinase 2 phosphorylates *in vitro* Ser 86, Ser 120, and Ser 121 of modulator,[32,188] which is probably physiologically relevant because these are also the sites that are phosphorylated in modulator that has been prepared in the presence of protein phosphatase inhibitors.[173] The extent of phosphorylation by casein kinase 2 is increased by a preincubation of modulator with PP-1_C and PP-2A.[189] Phosphorylation of modulator Ser 86 by casein kinase 2 increases the phosphorylation of Thr 72 by F_A/GSK-3 and thereby stimulates the reactivation of the MgATP-dependent form of PP-1 at limiting concentrations of F_A/GSK-3.[32,188]

Modulator is phosphorylated until 1 mol/mol by casein kinase 1.[190] However, the phosphorylation level is almost twofold higher after preincubation of modulator with PP-2A, but about 10 times lower when modulator is prepared in the presence of the phosphatase inhibitor fluoride. This indicates that the casein kinase 1 sites are phosphorylated *in vivo*. The residues that are phosphorylated by casein kinase 1 have not yet been localized, but peptide mapping indicates a partial overlapping with the phosphorylation sites of casein kinase 2.[190] Phosphorylation by casein kinase 1 stimulates phosphorylation of free modulator by F_A/GSK-3, but, contrary to the observations with casein kinase 2, this modification blocks the F_A/GSK-3-mediated activation of the MgATP-dependent form of PP-1.

Finally, modulator can be phosphorylated on undefined residues by cAMP-dependent protein kinase and on tyrosine residue(s) by the insulin receptor, the EGF receptor, and pp60, but these phosphorylations have no known effect and do not appear to occur *in vivo*.[32,94,173,191]

4. Subcellular Localization and Tissue Distribution

Upon fractionation of tissue extracts, significant amounts of modulator are recovered only in the cytosolic fraction.[150,154] However, immunofluorescence studies indicate that modulator may also be present in the nucleus.[191a,342] In freshly prepared tissue fractions, modulator is not free but appears to be associated with other polypeptide(s) (Section VI.A.1).[192,193]

Modulator has been identified in all investigated mammalian tissues, including rat liver and rabbit reticulocytes, which lack inhibitor 1 and DARPP-32.[142,166,167,175,176] *Drosophila* heads[16] and starfish oocytes[194] also contain modulator. The concentration of modulator in skeletal muscle[154] and fat cells[170] has been estimated at about 0.3 μM.

C. Deinhibitor

Merlevede's group has isolated from the glycogen fraction of dog liver a polypeptide (5.5–17.7 kDa, according to various estimates) that was termed *deinhibitor* because it antagonizes the inhibition of PP-1_C by inhibitor 1 and modulator.[195,196] The inhibition of PP-1_C by inhibitor 1 is reversed by deinhibitor in a time-dependent way, but this effect is unrelated to the stimulatory effect of deinhibitor on the dephosphorylation of inhibitor 1 by PP-1_C.[197–199] Deinhibitor also prevents the modulator-induced conversion of PP-1_C to the MgATP-dependent form and increases the final extent of activation of the MgATP-dependent form at limiting concentrations of F_A/GSK-3.[196,200] The effective concentrations of deinhibitor are in the nanomolar range but increase with higher levels of inhibitor 1 or modulator.[196] Deinhibitor is completely inactivated during phosphorylation by cAMP-dependent protein kinase.[199,201] However, for unknown reasons, the maximal stoichiometry of phosphorylation that has been obtained amounts to only 0.02 mol/mol. Deinhibitor is best reactivated by dephosphorylation with PP-2A.[202,203]

Interestingly, deinhibitor also seems to reverse the effects of "inhibitory" polypeptides other than inhibitor 1 and modulator. The evidence is that the spontaneous phosphorylase phosphatase activity of cytosolic and microsomal type 1 protein phosphatases that do not contain inhibitor 1 or modulator is increased severalfold by deinhibitor.[41,97,204] On the other hand, the phosphorylase phosphatase activity of the glycogen-bound type 1 protein phosphatase from rat liver is barely affected by deinhibitor, possibly because it is already "deinhibited" by endogenous deinhibitor.[41] However, deinhibitor has a much larger stimulatory effect on partially purified PP-1G,[41] which may indicate that the endogenous deinhibitor is lost during purification.

The glycogen-binding subunit of PP-1G from rabbit skeletal muscle resembles deinhibitor in that it also decreases the sensitivity to inhibitor 1 and modulator.[205] Furthermore, this "deinhibitor" effect is also lost after phosphorylation of the glycogen-binding subunit by cAMP-dependent protein kinase (Section VI.B.1.b). On this basis it has been suggested that deinhibitor represents a proteolytically derived fragment of the glycogen-binding subunit that has retained the phosphorylation site and the ability to interact with PP-1_C.[205] Our failure to detect heat-stable deinhibitor activity in a freshly prepared hepatic glycogen fraction as well as in purified PP-1G[206] is in agreement with this hypothesis.

V. THE MgATP-DEPENDENT FORM OF PP-1

A. The Inactivation of PP-1_C by Modulator

1. Kinetics and Mechanism of Inactivation

A 1:1 complex between PP-1_C and modulator (CM) is formed by mixing equimolar amounts of both components.[22,35,180,207] At high dilution, modulator binds specifically to the inactivation site of PP-1_C (Section IV.B.2). This binding does not immediately affect the activity of the phosphatase, but during prolonged incubation the active complex (C_aM) is converted to a completely inactive form (C_iM). The inactivation is probably associated with a tighter interaction between modulator and PP-1_C because dissociation of C_iM on anion-exchange chromatography is obtained only at pH 6, whereas a neutral pH suffices to

dissociate C_aM.[94] C_iM is identical to the native complex between PP-1_C and modulator that has been isolated from several tissues and that is also known as the MgATP-dependent form of PP-1,[208] as F_CM,[15]* or still as PP-1I.[5]

The modulator-induced inactivation of PP-1_C is kinetically explained by a decrease in the V_{max}.[211] It is an intramolecular process, because the rate of inactivation is independent of dilution.[131] The phosphatase is not reactivated by the chromatographic[94,212] or proteolytic[90,180] removal of modulator, indicating that the inactivation is associated with a lasting conformational change in PP-1_C. On the other hand, the same modulator can be used repeatedly for inactivation of PP-1_C without any difference in the rate of inactivation.[211] This suggests that the slowness of inactivation is not due to a modification (e.g., dephosphorylation) of modulator. Because the inactivation process follows first-order kinetics,[90,131] it can also be excluded that the binding of modulator to PP-1_C is the rate-limiting step for inactivation.

PP-1_C can thus be viewed as a hysteretic enzyme because it responds slowly to the binding of modulator.[213] With a $t_{1/2}$ for inactivation of about 10 to 30 min,[27,90,131,180,207,214] PP-1_C belongs to the slowest responding of all known hysteretic enzymes.[213,215] The structural basis for this hysteretic behavior of PP-1_C is not known. In general, slow transitions can result from the requirement for several small conformational changes to occur simultaneously.[216] The probability for the occurrence of such a configuration decreases dramatically with the number of involved changes. Alternatively, rotation or sliding of large domains with respect to each other, or sulfhydryl–disulfide interconversions, may also be at the basis of slow isomerization processes.[215] The ability of disulfides to block the activity of PP-1_C in a reversible and time-dependent way (Section III.A.5) indicates that SH-SS rearrangements may play a role in the modulator-induced inactivation of PP-1_C. However, this effect of disulfides cannot be identical to the inactivation caused by modulator because it cannot be reversed by Mn^{2+} or by kinase F_A/GSK-3.[211]

2. Inhibitors of the Inactivation

The inactivation rate of PP-1_C is not affected by high concentrations of modulator that cause a complete inhibition of the enzyme (Figure 1). On the other hand, the inactivation process is impaired by phosphoinhibitor 1[217,218] and by deinhibitor,[196] but their effects can be overcome by increasing the concentration of modulator. Myelin basic protein has also been reported to prevent the modulator-dependent inactivation of PP-1_C.[219]

The inactivation of PP-1_C by modulator at pH 7.4 is virtually completely blocked by addition of 5 m*M* Mg^{2+},[217] or 0.3 m*M* fluoride,[90] or 0.15 *M* KCl,[211] or by raising the pH to 8.5.[211] Fluoride is effective at concentrations that are at least 20-fold lower than those required for inhibition of PP-1_C. That the protection against inactivation is unrelated to inhibition of the phosphatase activity is also indicated by observations that other well-known phosphatase inhibitors like pyrophosphate and ATP, if anything, accelerate the modulator-induced inactivation of PP-1_C.[131,211] Further investigations have shown that the effect of fluoride on the inactivation of PP-1_C is instantaneous and reversible, and does not result from dissociation of the phosphatase-modulator complex.[90] The similar effect of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF)[90] has been traced to rapid production of F^- in aqueous medium at neutral pH.[219a] Finally, the effect of fluoride is critically dependent on the pH and is lost by decreasing the pH to 6.5.[211]

B. Reactivation by Divalent Metal Ions

C_iM can be reactivated by incubation with (chymo)trypsin in the presence of Mn^{2+} (1 m*M*), but a treatment with proteinases or Mn^{2+} alone is ineffective.[35,134,208] Proteolysis by trypsin[220] or chymotrypsin[22] decreases the apparent molecular mass of the phosphatase–modulator complex on gel filtration from 70 to 53 kDa. However, a combined proteinase–metal treatment decreases the molecular mass to 35 kDa, which is similar to that of free PP-1_C.[35,220] This indicates that frag-

* The term F_C stands for *catalytic factor* and was originally used to describe the MgATP-dependent form of PP-1.[183,209] When it was later realized that this form consists of both the catalytic subunit and the modulator, F_C was replaced by F_CM.[179,210]

ments of modulator that are still attached to PP-1_C after proteolysis are dissociated when Mn^{2+} is also present. Yet, the complete removal of modulator does not explain the recovery of the protein phosphatase activity. Indeed, free PP-1_C that is obtained by the chromatographic dissociation of C_iM is inactive unless Mn^{2+} is added.[94,212] The latter data indicate that it is Mn^{2+} that ultimately causes the reactivation of PP-1_C.* The role of proteolysis could then consist in making the metal-binding site accessible by destruction of modulator. Such a mechanism can also explain early reports that some preparations of native C_iM are activated by Mn^{2+} alone,[179,221,222] if it is supposed that the metal-binding site has become accessible due to partial proteolysis of modulator during purification. It has also been reported that C_iM is activated by Mn^{2+} plus 150 m*M* KCl to the same extent as by a combined treatment with trypsin-Mn^{2+}.[223] It is again possible that the role of salt lies in the uncovering of the binding site for Mn^{2+}.

The reactivation of free, inactive PP-1_C by Mn^{2+} is instantaneous but appears to proceed in two steps.[83,94,224] Initially, the reactivation can be reversed by metal chelation. After incubation for about 1 h this reversibility is lost, yet no incorporation of labeled Mn^{2+} can be demonstrated at this time. The reactivation of PP-1_C by Mn^{2+} shows an absolute requirement for reducing agents, indicating that sulfhydryl groups are involved in the reactivation process.[83] In this respect, it is worthy of note that cysteine sulfhydryl groups can chelate Mn^{2+}.[255]

Besides Mn^{2+}, other metals like Co^{2+} and Mg^{2+} can also activate free inactive PP-1_C and, in the presence of proteinases, C_iM.[94,133,208,222] The effectiveness of Mg^{2+} is interesting because it is of potential physiological significance and because this metal also stabilizes PP-1_C in another way, namely, by preventing the modulator-induced inactivation.[217] The failure of some investigators[94,134,222] to obtain reactivation of PP-1_C with Mg^{2+} may be related to its high K_a (>3 m*M*) when compared with the K_a for Mn^{2+} or Co^{2+} (<0.1 m*M*), to specific requirements like the presence of detergents, and to the origin of the phosphatase (skeletal muscle vs. heart).[133,208]

C. Reactivation by F_A/GSK-3

C_iM can be reconverted to C_aM by incubation with MgATP and F_A/GSK-3. A few other protein kinases have been identified that can catalyze the same reaction.[226–228] Due to the complexity of the system, the mechanism for the conversion of C_iM to C_aM will be discussed here on the basis of a model that is illustrated in Figure 2. This model accounts for most of the experimental data and represents an updated version of previous proposals.[4,15,131,229]

1. Phosphorylation of Modulator by F_A/GSK-3

Thr 72 of modulator is the only residue that is phosphorylated during incubation of C_iM with MgATP and F_A/GSK-3.[134,207,210] This phosphorylation does not by itself explain the conversion of C_iM into C_aM, because the (thio)phosphorylated intermediate is inactive[131,132] and because free inactive PP-1_C is not reactivated by addition of modulator that is phosphorylated on Thr 72.[94,230] Furthermore, much higher phosphorylation stoichiometries can be obtained with ATPγS as the phosphate donor,** but this results in less or even no activation.[131,207,208,230]

That phosphorylation of modulator merely initiates the reactivation process is also indicated by repeated observations that there is, at most, a superficial correlation between phosphorylation and activation.[94,131,207,210] During incubation of C_iM with F_A/GSK-3 and MgATP, the phosphorylation level of modulator rapidly reaches a plateau, but the activation continues for a while. Actually, full activation of the phosphatase has been obtained with phosphorylation levels that never exceeded 0.15 mol/mol modulator.[132,207,232] The continuous hydrolysis of ATP during the reactivation of C_iM[210] indicates that

* Interestingly, the time-dependent inactivation of PP-1_C by fluoride and pyrophosphate is also reversed by metals.[22]

** Thiophosphorylated proteins are more resistant to the action of protein phosphatases.[231]

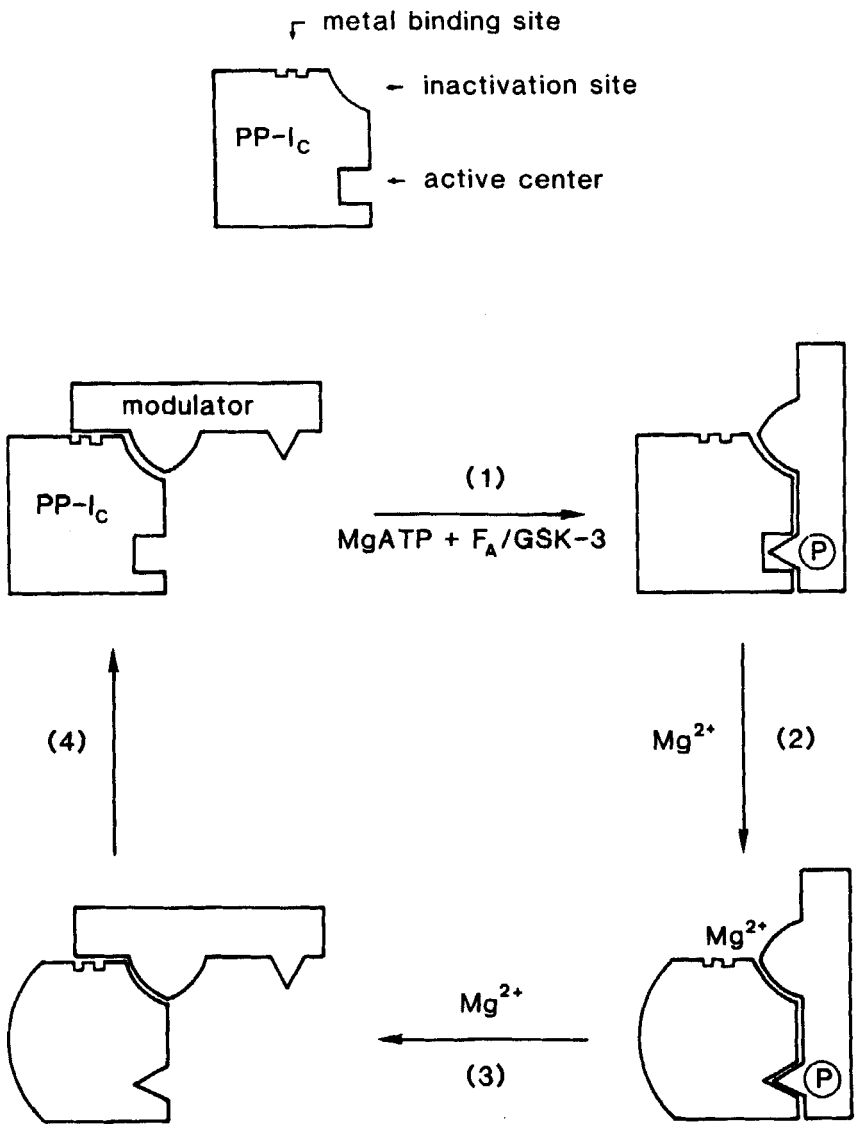

FIGURE 2. Model for the interconversion between C_iM and C_aM. The proposed model distinguishes four steps in the cyclic activation and inactivation of PP-1_C: (1) Phosphorylation of modulator in C_iM by F_A/GSK-3 induces conformational changes that uncover a metal-binding site on the catalytic subunit (Section V.C.1). (2) Upon binding of Mg^{2+}, the catalytic subunit is converted into the active conformation (Section V.C.2). (3) The dephosphorylation of modulator allows the access of exogenous substrates to the catalytic site (Section V.C.2). (4) The activated phosphatase slowly reverts to the inactive MgATP-dependent form (Section V.A.1).

the substoichiometric phosphorylation of modulator is accounted for by rapid dephosphorylation. This conclusion is also supported by the higher phosphorylation stoichiometry that is obtained when the phosphatase inhibitor pyrophosphate is present[134] or when ATPγS is used instead of ATP.[131,207,208,230]

2. Binding of Mg^{2+} and Dephosphorylation of Modulator

The role of phosphorylation of modulator Thr 72 probably consists in uncovering a binding site for Mg^{2+} on the catalytic subunit (Figure 2, steps 1 and 2). Direct evidence for this view is that

C_iM cannot be activated by Mg^{2+} unless it has been phosphorylated.[131,132,208] Remarkably, free inactive PP-1_C is instantaneously activated by Mn^{2+},[83,94,224] but the activation of phosphorylated C_iM by Mn^{2+} is time dependent.[131,132] This indicates either that metal binding to the phosphointermediate is slow or that modulator has to be dephosphorylated before exogenous substrates can be acted upon. The latter possibility is more likely because the activation of thiophosphorylated C_iM by Mg^{2+} is much slower than that of the phosphorylated complex and because the rates of dephosphorylation and activation are closely correlated.[131,132]

The dephosphorylation of (thio)phosphorylated modulator shows an absolute dependence on the presence of Mn^{2+} or Mg^{2+}.[130–133] Furthermore, the dephosphorylation reaction follows first-order kinetics and is independent of the dilution of CM, indicating that it represents an intramolecular process.[131] Because modulator is a competitive inhibitor of PP-1_C,[46] the site that dephosphorylates modulator (active center) is probably identical to the inhibitory site. It has been proposed that the dephosphorylation of modulator involves its transfer from the inactivation site to the active center.[5,15,130] However, the observation that no free modulator appears during the activation process[233] argues against a dissociation and reassociation of modulator. Unless phosphorylation by F_A/GSK-3 reverses the affinity of modulator for both binding sites, a transfer of modulator to the active site is also unlikely because it would involve a shift from a high-affinity to a low-affinity binding site (Section IV.B.2). The model in Figure 2 obviates these objections by suggesting that modulator remains bound to the inactivation site during the conversion of C_iM to C_aM, but that phosphorylation by F_A/GSK-3 allows modulator to interact also with the active center.

3. Activity Toward Exogenous Substrates

After the dephosphorylation of modulator and its release from the active center, C_aM becomes active toward exogenous substrates (Figure 2, step 3). At this stage, however, the phosphorylase phosphatase activity can be further increased (up to 10-fold) by a treatment with trypsin.[94,134,180,230] In part, this effect of trypsin can be explained by the proteolytic degradation of the C-terminal region of PP-1_C, which increases its phosphorylase phosphatase activity up to two-fold (Section III.A.1). However, the increased phosphatase activity may to a certain extent also be accounted for by the proteolytic destruction of modulator that remains bound to the inhibition site of PP-1_C, especially at low dilutions of C_aM. The observation that the specific activity of C_aM is independent of the dilution only at low concentrations[217] indeed indicates that modulator is inhibitory not only when present in a large excess over PP-1_C (see Section IV.B.2), but also when both components are present in a 1:1 ratio at high concentrations. That the inhibition by modulator depends on the dilution of C_aM may account for the large variability (between 5 and 1000%) in the reported stimulation of C_aM by trypsin.[94,134,180,230]

4. Effectors of the Reactivation by F_A/GSK-3

The rate and extent of reactivation of C_iM is determined by the concentration of F_A/GSK-3.[184,234] Removal of the kinase or disruption of the MgATP complex not only blocks further activation, but also allows the C_aM that has been formed to reconvert slowly to C_iM.[94,131,221] The regulatory subunit of cAMP-dependent protein kinase and myelin basic protein block the activation of C_iM by F_A/GSK-3.[47,219] These proteins probably act by preventing the phosphorylation by F_A/GSK-3 because their effect is overcome by increasing the concentration of the kinase. High concentrations of modulator and of inhibitor 1 that cause a complete inhibition of the phosphatase do not block the autocatalytic dephosphorylation of modulator,[130,217,232] nor do they prevent the conversion of C_iM to C_aM.[210,217,230] Deinhibitor and low concentrations of fluoride (0.5 m*M*) do not interfere with the rate of activation by F_A/GSK-3 but enhance the final level of reactivation at limiting concentrations of the kinase.[90,196]

5. Comparison between the Activation by F_A/GSK-3 and Trypsin-Mn^{2+}

The phosphorylation of modulator by F_A/GSK-3 and the degradation of modulator by trypsin may represent equivalent processes, in that they allow the binding of Mg^{2+} or Mn^{2+} to the catalytic subunit. In both cases the activation is eventually brought about by metal binding to the catalytic subunit and should yield an enzyme with the same properties. Yet, some differences have been noted between the phosphatases activated in both ways. Thus, the activation by kinase F_A/GSK-3 generally brings out more activity than that obtained with trypsin-Mn^{2+},[27,35,133,208] in spite of the fact that trypsinolyzed PP-1_C usually displays a higher phosphorylase phosphatase activity than the intact catalytic subunit (Section III.A.1). The metal-activated enzyme also has a broader substrate specificity.[127] One may wonder, however, whether such differences are not due to the difference in metal (Mn^{2+} vs. Mg^{2+}), rather than to the generation of enzymes with divergent properties. In this respect, it is significant that the degree of activation of C_iM by F_A/GSK-3 is more prominent with MgATP than it is with MnATP.[134,208] Also, PP-1_C has a broader substrate specificity in the presence of Mn^{2+} than it has in the presence of Mg^{2+} (Section III.C).

VI. CHARACTERISTICS AND STRUCTURE OF THE HOLOENZYMES

A. Cytosolic Type 1 Protein Phosphatases

1. Complexes with Modulator

A well-characterized cytosolic species of PP-1 is the complex between inactive PP-1_C and modulator (C_iM) that has been described in the previous section. This form has been isolated from rabbit skeletal muscle,[35,134,210] bovine heart,[208] and pig brain[235] and has also been identified in invertebrates like fruit flies[16] and starfish oocytes.[194] The concentration of C_iM has been determined from the increase in the phosphorylase phosphatase activity during incubation with MgATP and F_A/GSK-3. This method is not very straightforward for crude tissue fractions;[35,236,237] it is hampered, for example, by the large background caused by spontaneously active protein phosphatases, by the interference of other protein kinases (e.g., casein kinases), and by the inhibition of PP-1 by ATP (Section III.A.5). In spite of these difficulties, some groups have reported that C_iM represents a major fraction (30–100%) of the potential cytosolic phosphorylase phosphatase activity in several tissues.[134,174,208,235,238] C_iM has also been detected in immunoprecipitates that were obtained by incubation of mouse-diaphragm extracts with antibodies against modulator.[186]

A major problem in the assessment of the physiological relevance of C_iM is that its total recovery increases during purification.[35,130,208,221] To account for these observations, one might assume that C_iM exists in the cell in a dynamic equilibrium with C_aM and that, in the absence of MgATP after tissue homogenization, C_aM is gradually converted to C_iM. However, neither C_iM nor C_aM may be present in the initial purification steps, when an inhibitor of the modulator-induced inactivation (5 m*M* Mg^{2+} [217]) is included in the homogenization and purification buffer.[237] Instead, modulator appears to be associated with a largely latent type 1 protein phosphatase. Even at high dilutions, the activity of this enzyme is increased 5- to 10-fold by a preincubation with trypsin, or after complexation of modulator by free inactive PP-1_C or by modulator-specific antibodies. This enzyme cannot be further activated by F_A/GSK-3, but it is gradually converted into an F_A/GSK-3-dependent form during incubation at 30°C. It furthermore exhibits the same apparent molecular weight as C_iM when measured by sucrose density-gradient centrifugation or by gel filtration. Based on these and other results, it has been suggested that this latent phosphatase contains active PP-1_C that is inhibited by one or possibly two modulator subunits.[237] Further characterization of this phosphatase will require its complete purification. This has not yet been possible because the enzyme gradually converts into a form that cannot be distinguished from C_iM, in spite of the continuous presence of Mg^{2+}.

In the absence of Mg^{2+} or fluoride in the purification buffer, only about 60% of the mod-

ulator in rabbit skeletal muscle is recovered as C_iM.[193] The remainder is present in another complex, termed *MX*, that can be separated from C_iM by chromatography on blue Sepharose. Both modulator complexes show the same composition on SDS/PAGE, and they also behave identically during gel filtration and sucrose density-gradient centrifugation. However, it has not been possible to generate protein phosphatase activity from the MX complex by incubation with F_A/GSK-3 or metal ions, with or without an additional trypsin treatment. Further investigations are therefore needed to establish whether MX, like C_iM, represents modulator that is complexed to an inactive form of PP-1_C.

2. High-Molecular-Weight Protein Phosphatases

The major spontaneously active phosphorylase phosphatase in a freshly prepared cytosol from cardiac/skeletal muscle and liver migrates during gel filtration with an apparent molecular weight of 260 kDa.[118,239–242] It has not been unequivocally shown that this activity stems from a type 1 protein phosphatase, but it is not unlikely because PP-1 accounts for 40 to 90% of the basal cytosolic phosphorylase phosphatase activity in these tissues[41,92,243] and because cytosolic type 1 protein phosphatases of high molecular weight have indeed been identified.[97,244]

Goris and Merlevede[97] have found in dog liver a 260-kDa PP-1 that is mainly cytosolic. The apparent molecular weight of this enzyme on gel filtration decreased during purification to 160 kDa, indicating the loss or proteolytic degradation of a polypeptide. Silver staining after denaturing electrophoresis revealed a putative catalytic subunit of 37 kDa and a subunit of 75 kDa. The most remarkable characteristics of this phosphorylase phosphatase are a two- to three-fold increase of its catalytic activity by limited trypsinization and a 100-fold-lower sensitivity to inhibition by inhibitor 1 and modulator, when compared with free PP-1_C.

The major protein phosphatase in human erythrocyte cytosol is a Mn^{2+}-dependent protein phosphatase 1 with an apparent molecular weight of 180.000 on gel filtration.[244] SDS/PAGE of the homogeneous enzyme shows a putative 36-kDa catalytic subunit and a noncatalytic subunit of 62 kDa. This enzyme can be reversibly bound to the plasma membrane, but the membrane-associated form is inactive.[245] The cytosol of immature red blood cells (reticulocytes) also contains a Mn^{2+}-dependent PP-1, but, contrary to the Mn^{2+}-dependent protein phosphatase from erythrocytes, it represents only a minor fraction of PP-1S.[246,247]

3. Protein Phosphatase-1-like Enzymes

A protein phosphatase from reticulocytes[248] and from the soluble fraction of brain capillaries[249] behaves like PP-1 in that it is resistant to trypsin and is inhibited by modulator. However, its molecular weight on SDS/PAGE (56 kDa) does not correspond to that of PP-1_C. Moreover, the N-terminal 24 amino acids of the brain enzyme have been determined and show no homology with any known isoform of mammalian PP-1_C.[99,99a,100,249] Another remarkable characteristic of this 56-kDa enzyme is that it is inhibited by micromolar concentrations of Zn^{2+}.[248,249] The enzyme from reticulocytes requires Mn^{2+} for activity, but Mn^{2+} also blocks the binding of modulator.[248] In intact cells the reticulocyte enzyme may be associated with the cytoskeleton, because it can be bound to a spectrin-associated protein, termed *regulin*, that results in an increase of the phosphatase activity.

B. Glycogen-Bound PP-1

1. PP-1G from Rabbit Skeletal Muscle

a. Structure and Characteristics

With phosphorylase as substrate, 30 to 60% of the PP-1 activity in a skeletal muscle extract is associated with glycogen.[92,250] The concentration of PP-1G has been estimated at 200 n*M*.[205,251] PP-1G has been purified as a 1:1 complex between PP-1_C and a glycogen-binding subunit (G subunit) of 103 kDa on SDS/PAGE.[205] More recent studies indicate that the 103-kDa polypeptide originates from a 161-kDa subunit by proteolysis during purification.[252] The complex

between the G subunit and PP-1_C can be dissociated by incubation with 2 *M* NaCl, but a spontaneous recombination occurs after the removal of salt.

The full primary structure of the G subunit has been obtained from analysis of cDNA clones and genomic DNA.[254] The calculated molecular mass (124 kDa) is significantly lower than the values obtained from SDS/PAGE (161 kDa). Tissue distribution analysis has shown that this G subunit is expressed in skeletal muscle, heart, and diaphragm but not in kidney, liver, lung, and brain. The N-terminal part of the muscle-type G subunit, which contains the phosphorylation sites and the binding sites for glycogen and PP-1_C,[253,254] shares significant homology with the *Saccharomyces cerevisiae GAC1* gene product.[254] The *GAC1* product is also involved in glycogen accumulation, which is consistent with this protein being the yeast homologue of the G subunit.

PP-1G binds with high affinity to glycogen. At physiological concentrations of the phosphatase, a half-complete binding is obtained at about 0.1 mg/ml of glycogen and complete binding at less than 5 mg/ml.[252] This indicates that, even after exhaustive exercise, skeletal muscle contains enough glycogen (about 4 mg/ml cytosol[255]) to bind all G subunits. At physiological ionic strength and in the presence of glycogen, the catalytic efficiency of PP-1G is up to sixfold higher than that of free PP-1_C.[251] However, in the absence of salt, the release of the catalytic subunit from PP-1G does not affect its phosphorylase phosphatase activity, and even increases the rate of dephosphorylation of glycogen synthase and phosphorylase kinase by two- to threefold.[251] This indicates that the interaction of the G subunit with PP-1_C at low ionic strength inhibits the activity toward glycogen synthase and phosphorylase kinase. On the other hand, a preincubation with trypsin or chymotrypsin increases the activity of PP-1G toward phosphorylase and phosphorylase kinase by 35 to 100% but decreases the synthase phosphatase activity down to 50%.[39,135,205] The difference from the previous data may be explained by the proteolytic removal of the C-terminal domain of PP-1_C, which affects the kinetic parameters of the phosphatase (Section III.A.1).

The inhibition of PP-1G by inhibitor 1 and modulator is not instantaneous, a faster inhibition occurring at higher temperature and ionic strength.[135,205] In addition, the inhibition of the protein phosphatase activity in a crude protein–glycogen complex requires about 10 times more inhibitor 1 and modulator than does the inhibition of PP-1_C.[39] This lower sensitivity to inhibitor 1 and modulator has, however, not been noted with purified PP-1G,[205] which indicates that a deinhibitor-like activity is lost during purification of the phosphatase. This deinhibitor activity may be localized in the fragment of the G subunit that is proteolytically removed during purification. Prolonged incubation of purified PP-1G with modulator causes inactivation, with formation of an inactive ternary complex (PP-1_C, G subunit, and modulator) that can be reactivated by kinase F_A/GSK-3.[205]

b. Phosphorylation of the G Subunit

As illustrated in Figure 3, the G subunit possesses within a stretch of 28 amino acids five serine residues, organized into four sites that can be phosphorylated *in vitro* by cAMP-dependent

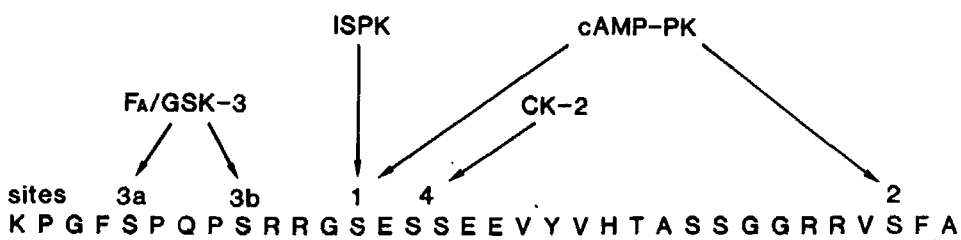

FIGURE 3. Phosphorylation sites of the G subunit of PP-1G from skeletal muscle. cAMP-PK: cAMP-dependent protein kinase; ISPK: insulin-stimulated protein kinase; CK-2: casein kinase-2. (Based on published data.[256–258])

protein kinase,[252,257] F_A/GSK-3,[259,260] casein kinase 2,[256] and a recently described insulin-stimulated protein kinase (ISPK) that is related to the 90-kDa S6 kinase II.[258,261] Heretofore, only sites 1, 2, and 3a have been shown to be phosphorylated *in vivo*.[261]

Sites 1 and 2 are phosphorylated by cAMP-dependent protein kinase.[252,257] These sites are preceded by the Arg-Arg-X- sequence, which is the basic determinant for substrate recognition by this kinase. Phosphorylation by cAMP-dependent protein kinase results in a dissociation of the catalytic subunit from the G subunit, which remains bound to glycogen.[257] The phosphorylation of site 2 (not site 1) is rate-limiting for the release of the catalytic subunit. The insulin-stimulated protein kinase phosphorylates specifically site 1. This modification increases the activity of PP-1G toward glycogen synthase and phosphorylase kinase two- to threefold, but it does not affect the phosphorylase phosphatase activity.[258] Studies on peptides have shown that the prior phosphorylation of site 1 is required for the phosphorylation of sites 3b and 3a by F_A/GSK-3 and of one of the two serine residues of site 4 by casein kinase 2.[256,259,260] The phosphorylation by F_A/GSK-3 also occurs in an ordered manner, site 3b being phosphorylated before site 3a.[256]

PP-1G with the G subunit phosphorylated on site 2 is an excellent substrate for intermolecular autodephosphorylation by PP-1G, as well as for dephosphorylation by PP-2A, PP-2B, and PP-2C.[252,257] On the other hand, the dephosphorylation of site 1 by any of these protein phosphatases is 4 to 100 times slower. A study with synthetic peptides has shown that the dephosphorylation of site 1 by PP-$2A_C$ requires prior phosphorylation of site 3a.[256]

2. Hepatic PP-1G

a. Regulatory Properties

The glycogen-bound protein phosphatase accounts for nearly 40% of the spontaneous phosphorylase phosphatase activity in a liver extract.[41] Based on the trypsin-revealed phosphorylase phosphatase activity that is associated with the glycogen particles,[39,263] the concentration of hepatic PP-1G is about 5 to 10 times than that of skeletal muscle. This corresponds to a concentration of 20 to 40 n*M* for the liver enzyme. PP-1G is nearly completely bound to glycogen at a concentration of 1 mg/ml, less than that in the liver of an overnight-fasted rat.[264,265] In contrast to glycogen synthase and phosphorylase, which have a tenfold lower affinity for glycogen, PP-1G should thus always be completely associated with glycogen in the liver of animals on a normal feeding schedule.

The inhibition of PP-1G by inhibitor 1 and modulator is time-dependent.[39,265] Furthermore, the inhibition by modulator involves dissociation of the enzyme with formation of a complex between modulator and catalytic subunit.[265] Compared to PP-1_C, the glycogen-bound enzyme from rat liver is also about 1000 times less sensitive to inhibition by modulator.[41] It remains to be seen whether this extreme resistance to modulator is accounted for by a heat-stable deinhibitor protein, as no such protein can be detected in preparations of PP-1G (Section IV.C).

The dephosphorylation of glycogen synthase by hepatic PP-1G is inhibited by physiological concentrations of Ca^{2+}.[266,267] At the optimal free Ca^{2+} concentration of 0.3 μ*M*, the inhibition is synergistically increased from 45 to 85% by 20 mg/ml of glycogen. This Ca^{2+} effect is not observed with phosphorylase as substrate and is insensitive to calmodulin and calmodulin antagonists.

Phosphorylase-*a* does not affect its own dephosphorylation by hepatic PP-1G, but it blocks the dephosphorylation of other substrates like glycogen synthase, ribosomal protein S6, HMG-CoA reductase, and phosphorylase kinase.[263,268,269] This inhibition accounts for the lag in the activation of glycogen synthase during incubation of a liver extract.[269–271] The basic evidence is that the latency corresponds to the time required to convert phosphorylase-*a* to the non-inhibitory *b* form and that the latency can be abolished or prolonged by removal or addition of phosphorylase-*a*, respectively. The concentration of phosphorylase-*a* that causes 50% inhibition of the activity of PP-1G is 2 to 20 n*M* with glycogen synthase as substrate and 50 to 100 n*M*

with other substrates.[268,272] These values are up to 100-fold lower than the K_m for dephosphorylation of phosphorylase, which is clear evidence that the inhibition is of an allosteric nature. The inhibitory potency of phosphorylase-*a* is also about 1000-fold decreased after trypsinolysis of the phosphatase, which indicates that this effect of phosphorylase is mediated by a noncatalytic subunit of the phosphatase. The allosteric inhibition by phosphorylase is specific for the phosphorylated *a* form and requires some salt (e.g., 5 m*M* phosphate or sulfate, or 100 m*M* KCl) and glycogen.[263,264,268,269,271] AMP cancels the effect of phosphorylase-*a* and is therefore used to assay the synthase phosphatase activity of PP-1G quantitatively, irrespective of the presence of phosphorylase-*a*.[270] It has been reported that fructose-1-P also cancels the inhibition of synthase phosphatase by phosphorylase-*a*.[273] However, in a more recent study it was shown that this effect is an artifact that results from the use of the *bis*-cyclohexylammonium salt of fructose-1-P.[274] The control of PP-1G by phosphorylase-*a* is absent in skeletal muscle[268,269] but may also be operative in leukocytes[275] and adipose tissue.[276]

Glucose controls the activity of PP-1G indirectly by decreasing the concentration of phosphorylase-*a* (Section VIII.C.2). However, glucose has also been reported to increase the synthase phosphatase activity in a protein–glycogen complex independently of the level of phosphorylase-*a*.[277–279] In the latter case a half-maximal effect has been obtained at 27 m*M* glucose in the absence, and at 10 m*M* glucose in the presence of caffeine, adenosine, or ADP. Glucose increases the synthase phosphatase activity by up to twofold, but the increase appears to be relatively larger in the presence of physiological concentrations of MgATP and glucose-6-P.[280] The effect of the latter additions is explained by a 70% inhibition of the basal synthase phosphatase activity, without any influence on the final extent of activation by glucose.[280]

b. Substrate Specificity

Hepatic glycogen synthase *b* is well activated by PP-1G from rat liver but not at all by PP-1_C.[41,272] *In vitro* phosphorylated glycogen synthase from skeletal muscle is also dephosphorylated twofold faster by hepatic PP-1G.[263] Furthermore, it has been reported that the ratio between the activities of synthase phosphatase and phosphorylase phosphatase is about 50 times higher in liver than in skeletal muscle.[263] All these observations agree with the proposal that hepatic PP-1G contains a "specifying subunit" that is required for recognition of glycogen synthase as substrate.[41] On the other hand, the holoenzyme must also have structural features that restrain the activity of the catalytic subunit toward phosphorylase by 70 to 90%.[39,41,263,272]

At high dilutions (2% of the physiological concentration), PP-1G is barely able to activate hepatic glycogen synthase, but the activation is synergistically increased by addition of a similarly diluted cytosolic type 1 protein phosphatase.[41,211,281] This synergism is gradually lost at lower dilutions of the phosphatases. The degree of synergism also varies with the type of synthase *b*[281] and can be enhanced by additional phosphorylation of the substrate with a cocktail of protein kinases.[282] A possible explanation for the synergism may be that the activation of hepatic glycogen synthase requires the dephosphorylation of both PP-1G-specific and PP-1S-specific sites.

c. Subunit Composition

Extensive trials to purify hepatic PP-1G by classic chromatographic techniques have failed because the enzyme displays a marked tendency to dissociate.[41,272] This problem has recently been circumvented by the separate purification of the noncatalytic subunit(s) and their subsequent recombination with PP-1_C.[272] For this purpose PP-1G is transferred from glycogen to covalently bound β-cyclodextrin (cycloheptaamylose). After the removal of PP-1_C and contaminating proteins with 2 *M* NaCl, elution with β-cyclodextrin yields a single protein on native electrophoresis and two polypeptides (161 and 54 kDa) on SDS/PAGE. Several lines of evidence indicate that one or both of these polypeptides represent the noncatalytic subunit(s) of PP-1G. First, while bound to β-cyclodextrin-Sepharose, these polypeptides recombine with PP-1_C but not with PP-$2A_C$. Second, this reconstitution is prevented by prein-

cubation of the column with antibodies against these polypeptides, and these antibodies can also be used for immunoprecipitation of PP-1G. Third, the reconstituted enzyme displays nearly the same characteristics as nonpurified PP-1G, i.e., it binds to glycogen, its phosphorylase phosphatase activity is stimulated by trypsin, it is resistant to inhibition by modulator, and its synthase phosphatase activity is inhibited by phosphorylase-*a* and by Ca^{2+}.

Unexpectedly, the sequence of a tryptic peptide from the 54-kDa polypeptide turned out to be identical to the cDNA-derived sequence 286 to 297 of the α-amylase precursor from rat pancreas (note added in proof to Reference 272). The identity has been confirmed by Western blotting with antibodies against α-amylase and by activity assays.[206] Purified PP-1G from dog liver displays essentially the same characteristics as the rat liver enzyme but lacks the 54-kDa polypeptide. These data provide strong evidence that α-amylase is not an essential subunit of PP-1G; presumably it becomes artificially bound to PP-1G during purification.

C. Protein Phosphatase 1 Associated with the Endoplasmic Reticulum

A major part of the endoplasmic reticulum in homogenates from skeletal and cardiac muscle sediments during low-speed centrifugation with the myofibrillar fraction. Proteins in this membrane fraction can be separated from the myofibrils by solubilization with Triton-X 100.[243,250,283,284] This myofibrillar "detergent extract" contains about as much PP-1 as is present in a low-speed muscle extract. The remainder of the endoplasmic reticulum is recovered in the "microsomal" fraction that is obtained by high-speed centrifugation of the postmitochondrial supernatant. The microsomal fraction from skeletal muscle[92,250] and liver[41] contains 15 to 25% of the phosphorylase phosphatase activity in a crude extract. At least 80% of this activity belongs to PP-1 and is probably associated with the endoplasmic reticulum, although a binding to contaminating fractions like lysosomal or plasma-membrane vesicles cannot be excluded. Moreover, when no extra precautions are taken, the microsomal fraction contains PP-1G associated with contaminating glycogen. A glycogen-free microsomal fraction can be prepared by α-amylolysis *in vitro*.[263,264] Specifically for the liver, residual glycogen can be removed by treatment of fasted animals with glucagon.[264] Without such precautions, the endoplasmic reticulum that is prepared by discontinuous sucrose-gradient centrifugation still contains small quantities of glycogen[285,286] that are probably sufficient to bind significant amounts of PP-1G.[271,287]

Conflicting reports as to whether PP-1 in the microsomal fraction of skeletal muscle is solubilized either by Triton-X 100[250] or by dilution or salt extraction[212] may indicate the presence of multiple species. PP-1 released by detergent from membranes in the myofibrillar and microsomal fraction of skeletal/cardiac muscle is similar and possibly identical to PP-1G.[250,284] A first argument is that the solubilized enzyme binds with high affinity to glycogen. Second, antibodies against the G subunit of muscle PP-1G recognize a polypeptide of the same size in the myofibrillar and microsomal membrane fraction. Further, this polypeptide is phosphorylated by cAMP-dependent protein kinase on the same tryptic peptides that are obtained from the G subunit. Finally, phosphorylation by cAMP-dependent protein kinase releases $PP\text{-}1_c$ from the microsomal fraction. Further, this polypeptide is phosphorylated by cAMP-dependent protein kinase on the same tryptic peptides that are obtained from the G subunit. Finally, phosphorylation by cAMP-dependent protein kinase releases $PP\text{-}1_C$ from the microsomal fraction. The C-terminal domain of the G subunit contains a hydrophobic region that potentially serves to anchor the protein to membranes.[254] This provides additional evidence for the involvement of the same G subunit in targeting $PP\text{-}1_C$ to the endoplasmic reticulum and to glycogen.

In contrast to the above findings for cardiac and skeletal muscle, PP-1E and PP-1G from the liver do not have a common G subunit. Indeed, PP-1E from rat liver has no affinity for glycogen,[263] and, unlike PP-1G, it is not retained by β-cyclodextrin-Sepharose.[206] Another difference between hepatic PP-1G and PP-1E is the 10-fold-higher sensitivity of the latter to inhibition by inhibitor-1 and modulator.[41,263] PP-1E also dis-

plays a rather weak synthase phosphatase activity,[41,263] and the allosteric inhibition by phosphorylase-*a* is restricted to the substrate glycogen synthase.[263,268] Nearly all the synthase phosphatase activity associated with microsomes can be extracted with salt, in contrast to what has been found for the glycogen-bound activity.[263]

D. Myosin-Bound PP-1

Detergent-purified myofibrils from *skeletal muscle* contain at least as much PP-1 as is present in a postmitochondrial supernatant.[243,283] This phosphatase remains associated with the actomyosin complex during repeated precipitation (at 55 m*M* NaCl) and solubilization (at 600 m*M* NaCl) of the complex.[243] However, PP-1M is solubilized from the actomyosin complex at 300 m*M* NaCl. This soluble PP-1M migrates during gel filtration with an apparent molecular weight of 110 kDa. It displays no affinity for glycogen, but it can be sedimented with added phosphatase-free actomyosin or with pure myosin. Inhibitor 1 and modulator inhibit PP-1M in a time-dependent way, but, when measured after a preincubation period, the sensitivity of PP-1M and PP-1_C to these inhibitory proteins is the same. With phosphorylase-*a* as the reference substrate, PP-1M is about three to four times more effective than PP-1G in dephosphorylating myosin.[283] A preincubation with chymotrypsin decreases the activity of PP-1M toward myosin by half, but increases the phosphorylase phosphatase activity to a similar extent.

Cardiac muscle contains much less spontaneoulsy active PP-1 than does skeletal muscle, and most of the latter is present in the myofibrillar fraction.[283,288] PP-1 in detergent-washed myofibrils from cardiac muscle can be released by freeze-thawing in the presence of 0.5 *M* salt, and the phosphorylase phosphatase activity of the released enzyme is increased about 25-fold by the proteolytic release of the catalytic subunit.[288] As noted for the enzyme from skeletal muscle, cardiac PP-1M dephosphorylates myosin about three times faster than phosphorylase.[283]

Mammalian and avian *smooth muscle* contains a protein phosphatase that dephosphorylates myosin about 40 times faster than phosphorylase at nearly physiological ionic strength.[21,289–292] This enzyme, termed *SMP-IV* (smooth muscle phosphatase IV), binds with high affinity to myosin, especially thiophosphorylated myosin, but it is not clear which part of SMP-IV is associated with the myofibrillar fraction after subcellular fractionation of smooth muscle homogenates. Although SMP-IV preferentially dephosphorylates the β-subunit of phosphorylase kinase, it does not qualify as a true type 1 protein phosphatase because it is not affected by inhibitor 1 or modulator. The M_r of the catalytic subunit of SMP-IV on SDS/PAGE (40 kDa) is also larger than that of PP-1_C (37–38 kDa). Another unusual property is the extreme resistance of SMP-IV to inhibition by ATP, pyrophosphatase, and NaF.

E. Nuclear PP-1

The nuclei of liver cells,[293–295] HeLa cells,[296] and *Xenopus* oocytes[297] contain high levels of PP-1. The average concentration of PP-1 in liver nuclei exceeds even five times the extranuclear activity of PP-1.[294,295] In nuclei from rat liver and *Xenopus* oocytes that are broken by sonication or by a hypotonic shock, 40 to 80% of PP-1 is particulate.[293–295,297] Incubation of this insoluble fraction with salt or DNAase results in the release of free PP-1_c, indicating that these treatments dissociate particulate PP-1N. In the nucleoplasm of hepatic nuclei, a single species of PP-1 has been detected (60 kDa in sucrose density-gradient centrifugation) whose phosphorylase phosphatase activity is stimulated about 10-fold by trypsin.[293] Two species of PP-1 have been identified in the nucleoplasm of *Xenopus* oocytes, with apparent molecular weights of 90 and 185 kDa, as deduced from gel filtration.[297]

F. Other Cellular Locations

Purified rat liver plasma membranes contain a phosphorylase phosphatase activity that may belong to type 1 because the activity is increased about 10-fold by trypsin.[298] In reticulocytes small amounts of PP-1 appear to be associated with ribosomes.[166] High levels of PP-1 have been detected in synaptic particulate fractions and in the

synaptosol.[299] In bovine retina, a species of PP-1 is associated with rod outer segments.[300] Finally, in *Paramecium* at least 75% of PP-1 is bound to the ciliary membrane.[301]

VII. ROLE OF TYPE 1 PROTEIN PHOSPHATASES

Table 4 provides a list of the major phosphoproteins that have been shown to be dephosphorylated *in vitro* by type 1 protein phosphatases. As will be discussed further, there is increasing evidence that a number of these proteins are also physiological substrates of PP-1.

A. Glycogen Metabolism

Glycogen metabolism is regulated mainly by changes in the activities of glycogen synthase, phosphorylase, and phosphorylase kinase that result from alterations in the phosphorylation state of serine residues.[5,271] Protein phosphatases promote glycogen synthesis and inhibit glycogenolysis by activation of glycogen synthase and inactivation of phosphorylase and phosphorylase kinase.

With the use of specific phosphatase inhibitors like modulator, okadaic acid, and antibodies against PP-$2A_C$, it has been established that in crude tissue extracts, PP-1 and PP-2A account for nearly all the phosphatase activity toward the enzymes of glycogen metabolism.[41,62,92,306,307] In dilute extracts of skeletal muscle, PP-1 contributes for a major part to the dephosphorylation of exogenous glycogen synthase (60–75%), phosphorylase (85–90%), and the β-subunit of phosphorylase kinase (>95%). In general, these numbers are somewhat lower for extracts from liver, brain, heart, kidney, and adipose tissue.

Although the assay of protein phosphatases in tissue extracts can give an idea of the potential importance of PP-1 in the regulation of glycogen metabolism, the data have to be interpreted cautiously. First, such assays do not take into account the possible role of protein phosphatases (mainly type 1) in the particulate fraction that is sedimented at low speed. Second, the contribution of different protein phosphatases varies with dilution of the tissue fractions, which is at least partially due to dissociation of some enzymes.[41,92] Third, the activity of protein phosphatases may depend on the origin and the phosphorylation state of the substrate.[41,308,309] For example, at least 90% of the glycogen synthase

TABLE 4
***In vitro* Substrates of Type 1 Protein Phosphatases**

Carbohydrate metabolism	Lipid metabolism
Glycogen synthase	Acetyl-CoA carboxylase
Phosphorylase	Hormone-sensitive lipase
Phosphorylase kinase (β-subunit)	ATP-citrate lyase
Pyruvate kinase	HMG-CoA reductase
Calcium transport	Muscle contraction
Phospholamban	Myosin light chain
Voltage-operated Ca^{2+}-channel	Myofibril C protein
Nuclear proteins	Protein synthesis
Histones H2B and H1	Initiation factor eIF-2
Protein N-60	Double-stranded-RNA-dependent protein kinase
	Heat-shock protein 90
Phosphatase regulatory proteins	Ribosomal protein S6
Inhibitor-1	S6 kinase
Modulator	
G subunit of muscle PP-1G	
Deinhibitor	

Note: Based on data from References 11, 58, and 302 through 305; see also Section VII.

phosphatase activity in a liver extract stems from PP-1 when derived from the activation rate of "natural" hepatic glycogen synthase, but this value decreases to about 50% when the activity is determined from the rate of dephosphorylation of *in vitro* phosphorylated muscle glycogen synthase.[41] A final reason that it is risky to transpose the results obtained from assays in crude tissue fractions to the intact cell is that such assays do not allow one to take into account the role of the intracellular environment. Of particular importance in this respect are selective effectors of one type of protein phosphatase (e.g., phosphorylase-*a* and Ca^{2+} for hepatic PP-1G) and the limits imposed *in vivo* by the specific subcellular location of substrate and protein phosphatase.

Evidence for a decisive role of PP-1 in the dephosphorylation of glycogen synthase comes from studies on cells having an altered activity of a species of PP-1. One example is the liver of insulin-dependent diabetic rats and of adrenalectomized, starved rats, which shows a rather specific loss of the glycogen-associated synthase phosphatase activity (Section VIII.E). This deficiency is associated with a failure of glucose to activate glycogen synthase in isolated hepatocytes. Moreover, the restoration of the phosphatase activity, after, for example, a treatment of diabetic animals with insulin, corresponds closely to an improved capacity for activation of glycogen synthase. The reverse situation applies to the liver of fasted obese *fa/fa* rats, in which a selective increase in the glycogen-associated synthase phosphatase activity is associated with a glucose-induced "superactivation" of glycogen synthase in isolated cells.[309a] It has also been demonstrated that PP-1 plays a role in the activation of glycogen synthase in yeast *in vivo*. A yeast mutant that is defective in a gene that encodes for a particular isoform of PP-1_C has less active glycogen synthase.[310] Transformation studies have shown that the level of active glycogen synthase increases considerably in the presence of the intact gene.[311]

B. Calcium Transport

A Ca^{2+} pump in the sarcoplasmic reticulum uses the energy of ATP hydrolysis to transport Ca^{2+} into its lumen.[312,313] In heart and possibly also in slow-contracting skeletal muscle and smooth muscle, this Ca^{2+}-ATPase is regulated through phosphorylation of an associated protein, termed *phospholamban*. Phospholamban inhibits the Ca^{2+}-transporting enzyme, but this inhibition is relieved by phosphorylation of phospholamban at Ser 16 by cAMP-dependent protein kinase or at Thr 17 by a Ca^{2+}/calmodulin-dependent protein kinase. Cardiac low-speed extracts and myofibril-associated membranes each contain similar activities of protein phosphatases acting on phospholamban (both Ser 16 and Thr 17).[284] Nearly three quarters of this activity is accounted for by PP-1. A major part of the membrane-associated phospholamban phosphatase activity stems from an enzyme that is similar and possibly identical to PP-1G.[284] However, the sarcoplasmic reticulum has also been reported to contain a phospholamban phosphatase (presumably of type 1) that seems to differ from PP-1G because it migrates during sucrose density-gradient ultracentrifugation with an apparent M_r of only 46 kDa.[314]

Phosphorylation by cAMP-dependent protein kinase of the cardiac L-type Ca^{2+}-channel, or a closely associated protein in the plasmalemma, is accompanied by an enhancement of its "open" probability during depolarization.[315,316] This means that a larger number of Ca^{2+} channels are opened during depolarization, which results in an increased uptake of calcium from the extracellular space. There is strong evidence that PP-1 plays an essential role in the dephosphorylation of this voltage-dependent calcium channel *in vivo*. Thus, intracellular perfusion of single voltage-clamped ventricular myocytes with PP-1_C abolishes the increased inward calcium current that is induced by the β-agonist isoprenaline.[317] Furthermore, perfusion of the cells with modulator increases the Ca^{2+} uptake before and after stimulation with isoprenaline by 20 and 70%, respectively; after the washout of isoprenaline it also increases two- to threefold the time required for the return of the Ca^{2+} uptake to the basal value.

Depolarizing stimuli also open Ca^{2+} channels in the excitable ciliary membrane of *Paramecium*.[301] The resulting influx of calcium causes a reversal of the ciliary beat and triggers backward swimming. Okadiac acid, which is an ex-

clusive inhibitor of PP-1 in *Paramecium*, prolongs the duration of backward swimming in response to depolarization. This indicates that the voltage-operated Ca^{2+} channel is closed by a dephosphorylation event that is mediated by PP-1. Recently, a 42-kDa protein in the ciliary membrane of *Paramecium* has been identified as a specific substrate for PP-1, but it is not yet known whether this substrate is a component of the voltage-operated Ca^{2+} channel.[317a]

C. Muscle Contraction and Intracellular Transport

Myosin is phosphorylated by a specific Ca^{2+}/calmodulin-dependent myosin light-chain kinase. The role of this phosphorylation in cardiac and skeletal muscle is not well understood, but, among other things, it increases *in vitro* the actin-activated ATPase activity of myosin.[318] In extracts of these cells, nearly all the phosphatase activity toward native myosin is accounted for by PP-1, and a major fraction of this activity is associated with myosin (PP-1M).[283] Earlier findings that PP-2A is the dominant cardiac myosin phosphatase[319] can probably be explained by the rapid postmortem loss of PP-1 in this tissue.[284]

In smooth muscle, the phosphorylation of myosin light chain allows myosin to interact with actin and thereby initiates contraction.[289] Smooth muscle contains a type-1-like protein phosphatase (SMP-IV) that has a high affinity for myosin and dephosphorylates this substrate with high specificity (Section VI.D). When added to chemically skinned chicken gizzard cell bundles, SMP-IV also dephosphorylates the endogenous myosin.[320]

In non-muscle cells myosin is freely soluble in the nonphosphorylated state.[321] Phosphorylation enables myosin to assemble into filaments and to interact with actin. These contractile bundles or microfilaments play a role in such diverse processes as phagocytosis, cytokinesis, and organelle movements. Microinjection of $PP\text{-}1_C$ into mammalian fibroblasts results in an extensive dephosphorylation of myosin light chain and a disassembly of the microfilament network.[322] The injection of a similar amount of $PP\text{-}2A_C$ does not produce these effects, indicating that this phosphatase does not act as a myosin phosphatase *in vivo*. Additional evidence for a role of PP-1 in the dephosphorylation of myosin in non-muscle cells comes from immunofluorescence studies that have demonstrated a close association of $PP\text{-}1_C$ with a fibrillar network of microfilaments.[322]

D. Protein Synthesis

The overall rate of protein synthesis is enhanced by the dephosphorylation of eukaryotic initiation factor 2 (eIF-2) and by the phosphorylation of ribosomal protein S6. eIF-2 is required for positioning initiator-tRNA on the small ribosomal 40S subunit, and this function is blocked by phosphorylation of two serine residues in the α-subunit of eIF-2.[246,323] The phosphorylation of S6 on five carboxy-terminal serine residues allows the 40S ribosomal subunit to form initiation complexes more efficiently.[324–326]

eIF-2 is well dephosphorylated by $PP\text{-}1_C$ *in vitro*, and PP-1 accounts for 30 to 70% of the eIF-2 phosphatase activity in extracts of reticulocytes, 3T3 cells, and hepatocytes.[11,307] The essential role of PP-1 in the dephosphorylation of eIF-2 is illustrated by the finding that modulator increases the phosphorylation level of eIF-2 and inhibits protein chain initiation in reticulocyte lysates.[307,327] This agrees with the report that microcystin, which is a more potent inhibitor of PP-1 than okadaic acid, increases the phosphorylation of eIF-2 in reticulocyte lysates at lower concentrations than does okadaic acid.[327a]

PP-1 may also control the phosphorylation level of eIF-2 indirectly, by affecting the activity of eIF-2 kinases (Figure 4). In reticulocytes eIF-2 can be phosphorylated by two highly specific protein kinases, termed *heme-controlled eIF-2α protein kinase* and *double-stranded-RNA-dependent protein kinase*.[246,323] The latter kinase is activated by autophosphorylation in the presence of double-stranded RNA and is efficiently dephosphorylated by a cytosolic PP-1 from reticulocytes.[247] However, due to its tight association with ribosomes, double-stranded-RNA-dependent protein kinase may be dephosphorylated *in vivo* by a ribosome-associated PP-1.[166] The activity of the heme-sensitive kinase is increased severalfold by phosphorylation of an associated

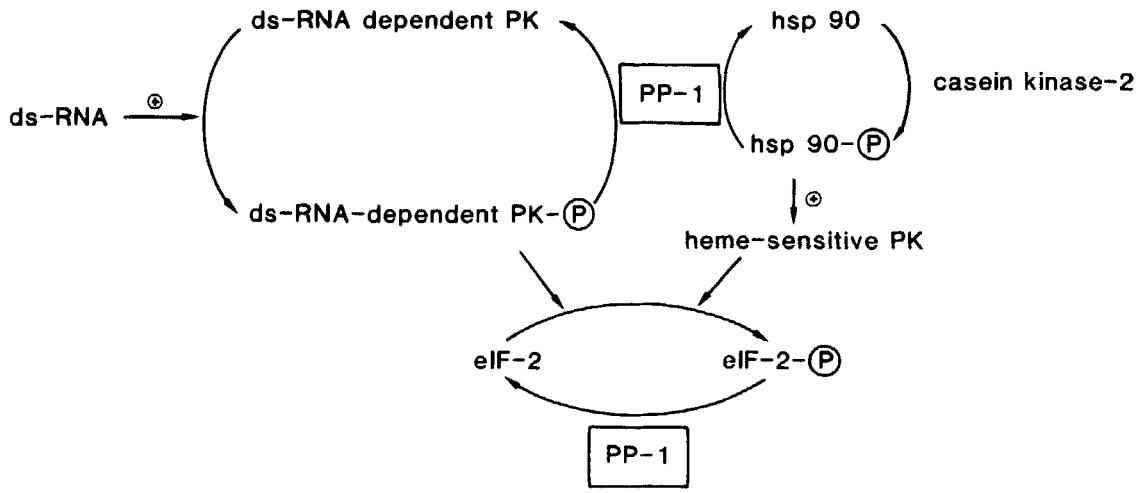

FIGURE 4. PP-1 promotes the dephosphorylation of eIF-2. ds-RNA: double-stranded RNA; PK: protein kinase; hsp 90: heat-shock protein of 90 kDa. (Based on published data.[11,246,247,307,323,327,328])

heat shock protein (hsp 90), and this protein is well dephosphorylated by a Mn^{2+}-dependent PP-1 that is also complexed to the heme-controlled protein kinase[328]

In dilute fractions from liver,[263] 3T3 cells,[329] and oocytes,[20,45] the majority of S6-phosphatase activity stems from PP-1. Moreover, PP-1 dephosphorylates S6 and the model substrate phosphorylase at a similar rate.[45,263] More direct evidence for a role of PP-1 in the dephosphorylation of S6 is that microinjection of modulator into oocytes increases the phosphate content of S6.[45] Two cytosolic protein phosphatases have been identified that account for most of the S6 phosphatase activity in *Xenopus* oocytes and eggs.[20] One enzyme has an apparent M_r of 200 kDa on gel filtration and behaves as a typical PP-1 with respect to substrate specificity and inhibition by inhibitor 1 and modulator. The second enzyme migrates as a 55-kDa protein during glycerol density-gradient centrifugation, is inhibited by nanomolar concentrations of inhibitor 1 and modulator, but is unable to dephosphorylate phosphorylase or phosphorylase kinase. PP-1 can also control the phosphorylation level of S6 indirectly, i.e., by inactivation, through dephosphorylation of S6 kinase(s). However, this may not be a physiological role of PP-1 because PP-2A appears to be more efficient in dephosphorylating S6 kinase.[329,330]

That PP-1 dephosphorylates both eIF-2 and S6 is at first glance paradoxical because the dephosphorylation of these substrates has opposite effects on protein synthesis. It can, however, be envisaged that two independently controlled species of PP-1 are involved in the dephosphorylation of these proteins. It is also possible that the control of the phosphorylation state of eIF-2 and S6 is exerted at the level of the protein kinases.

E. Cell Division

1. Regulatory Proteins of the Cell Cycle

Biochemical analysis of amphibian oocyte maturation has originally led to the identification of a protein, now known as M-phase promoting factor (MPF), that can trigger mitotic and meiotic division in eukaryotic cells without requiring protein synthesis.[331,332] On the other hand, the study of conditional yeast mutants has resulted in the description of a number of so-called cdc genes (cell division cycle) that are required for completion of the cell cycle. The product (p34) of one of these genes ($cdc2^+$) is a protein kinase that is essential for entry into mitosis. In recent years both approaches for investigation of the cell cycle have merged as it has become clear that MPF is the uniform trigger for cell division in eukaryotes and that $p34^{cdc2}$ represents the catalytic subunit of MPF.

The level of $p34^{cdc2}$ is fairly constant throughout the cell cycle, but it associates with a protein, termed *cyclin*, that accumulates beyond a threshold level at the end of the interphase. This "pre-MPF" then undergoes modifications, including the dephosphorylation of $p34^{cdc2}$ and the phosphorylation of cyclin, that result in the activation of the complex. MPF catalyzes the phosphorylation of specific proteins (e.g., lamins, histone H1, and microtubuli-associated proteins), and this results in structural changes such as nuclear envelope breakdown, chromosome condensation, and spindle formation. Once activated, MPF promotes its own inactivation and the return to interphase by inducing the proteolytic degradation of cyclin.[333]

2. Role of PP-1 in the Generation of MPF in Oocytes

$p34^{cdc2}$ does not appear to be a substrate for PP-1 *in vivo* because its dephosphorylation is not blocked by okadaic acid.[334] However, several investigations connect PP-1 with MPF, although it is not clear whether PP-1 promotes or blocks the formation of MPF. On one hand, injection of oocytes with inhibitor 1[335] or modulator[336] prevents the progesterone-induced but not the MPF-induced maturation, indicating that PP-1 is required for the generation of MPF. This agrees with the finding that inhibitor 1 can slow down the posttranslational activation of a precursor of MPF in a cell-free system of *Xenopus* oocytes, presumably by preventing PP-1 from inactivating a protein that inhibits MPF.[337] On the other hand, some studies indicate that PP-1 prevents the production of MPF. Thus injection of PP-1 inhibits the maturation of starfish oocytes induced by 1-methyladenine.[338] Also, the injection of anti-PP-1_C antibodies induces maturation in sea cucumber oocytes (but not starfish oocytes).[334] It has also been reported that okadaic acid induces MPF activation in oocytes[334,339] and fibroblasts,[340] but these results do not necessarily point to a role of PP-1 because okadaic acid also inhibits PP-2A.

3. PP-1 Modulates the Entry into Mitosis

Microinjection of fibroblasts in early interphase with $p34^{cdc2}$ induces a pseudomitotic response.[341] However, $p34^{cdc2}$ does not produce this effect when injected during the phase of chromosome replication (S phase), unless it is administered together with modulator-specific antibodies.[342] One obvious interpretation of these data is that a response to $p34^{cdc2}$ during the S phase requires the activity of a type 1 protein phosphatase that is blocked by modulator. In this respect, it is also significant that the level of modulator in fibroblasts oscillate during the cell cycle, with sharp increases during the S phase and during mitosis.[342]

The fission yeast gene $cdc25^+$ encodes a putative serine/tyrosine protein phosphatase that stimulates the activity of $p34^{cdc2}$.[21c,104] The action of this protein is antagonized by the product of $wee1^+$, a serine/tyrosine protein kinase.[104,342a] Genetic disruption of $cdc25^+$ results in an arrest in late interphase, whereas disruption of $wee1^+$ causes cells to enter mitosis precociously. The requirement for $cdc25^+$ can be relieved by mutations that eliminate the function of $wee1^+$, so that the $cdc25^{ts}$-$wee1^-$ double mutant* does not show a mitotic block. Screening for wild-type sequences that prevent this double mutant from entering mitosis at restrictive temperatures did not result in the expected isolation of the $wee1^+$ gene but led to the identification of a gene, termed "$bws1^+$" (bypass of the wee suppression), which encodes for a protein that is 82% identical to an isoform of mammalian $PP\text{-}1_C$ (PP-1α in Table 3)[104] These data indicate that PP-1 can influence the entry into mitosis by somehow opposing the function of $cdc25^+$.

4. PP-1 is Required for Completion of Mitosis

The *dis* (defective in sister-chromatid disjoining) mutants of fission yeast enter mitosis with normal timing but show a block in chro-

* $cdc25^{ts}$ is a temperature-sensitive mutant and shows an arrest only in late innterphase in the so-called restrictive temperature range.

mosome disjoining at a restrictive temperature.[102] One of these mutants, termed "*dis2-11*", could be complemented with the authentic *dis2*$^+$ gene or with one of three other suppressor genes, including *sds21*$^+$. The products of *dis2*$^+$ and *sds21*$^+$ show 82 and 74% identity, respectively, with the α-isoform of PP-1_C (Table 3). The *dis2*$^+$ gene is identical to the *bws1*$^+$ gene (see above), which interferes with the timing for entry into mitosis, indicating that this isoform of PP-1_C acts pleiotropically in the cell cycle. Comparison of the phosphorylase phosphatase activities in wild types and mutants has shown that the products of *sds21*$^+$ and especially *dis2*$^+$ account for nearly all the cellular activity of PP-1.[115] Both isoforms of PP-1_C have overlapping functions *in vivo*, because mutants are lethal only when both the *dis2*$^+$ and the *sds21*$^+$ genes are disrupted.[102] The lethality of this double mutant can be avoided by multicopy plasmids carrying either the *dis2*$^+$ or the *sds21*$^+$ gene but not by plasmids carrying either of two genes encoding fission yeast homologues of PP-$2A_C$.[102,115] Another suppressor gene that complements *dis2-11*, designated "*sds22*$^+$", encodes a 30-kDa protein that consists almost entirely of leucine-rich amino acid repeats and is enriched in the particulate nuclear fraction.[343] *sds22*$^+$ is essential for completion of mitosis but becomes dispensable upon high dosage of the type 1 catalytic subunit that is encoded by *sds21*$^+$. These data have been taken as evidence that the product of *sds22*$^+$ facilitates PP-1-mediated dephosphorylation reactions by interacting with a type 1 protein phosphatase or its substrate(s).

A conditional mutant of the fungus *Aspergillus nidulans*, termed "*bimG11*" (blocked in mitosis), phenotypically resembles the *dis* mutants of fission yeast in that its chromosomes fail to separate during the anaphase at restrictive temperatures.[103] The *bimG*$^+$ gene encodes a protein that is 86% identical to the α-isoform of mammalian of PP-1_C. This putative phosphatase is likely to be localized in the nucleus because the *bimG11* mutant has an abnormally high level of nuclear phosphoproteins.

Contrary to what has been found for fission yeast and *Aspergillus*,[102,103,115] the loss of only one of several existing isoforms of PP-1_C is lethal to *Drosophila*.[344] This isoform, which is encoded by locus 87B on chromosome 3, accounts for about 80% of the cellular activity of PP-1. Mutants of *Drosophila* that lack the 87B-isoenzyme die at a larval stage. These mutants show defects in mitosis, as illustrated by a deficient mitotic spindle organization, an abnormal sister-chromatid segregation, hyperploidy, and an excessive degree of chromosome condensation.[345] Interestingly, a mutation that still allows the expression of about 20% of the normal activity of a 87B-isoenzyme is also lethal but shows little evidence of abnormal mitosis. This indicates that the 87B-isoform of PP-1_C has a second vital function, in addition to its role in mitosis, and that completion of mitosis requires only a threshold level of this phosphatase.

VIII. PHYSIOLOGICAL REGULATION OF TYPE 1 PROTEIN PHOSPHATASES

A. Regulation of the MgATP-Dependent Form of PP-1

1. Phosphorylation and Turnover of Modulator

The phosphorylation state of modulator in skeletal muscle is not affected by an *in vivo* administration of adrenalin or insulin.[173] However, the addition of insulin to fat cells or 3T3 cells results in an increase of the phosphate content of modulator by 40 to 60%.[174,187] At least 90% of this increase stems from phosphorylation of serine residue(s). It has been proposed that this insulin effect is mediated by casein kinase 2,[174] which indeed displays an increased activity in insulin-treated 3T3 cells.[346,346a] The phosphorylation of modulator by casein kinase 2 promotes the conversion of C_iM to C_aM by facilitating the phosphorylation by kinase F_A/GSK-3 (see Section IV.B.3) and possibly explains the 20 to 40% increase of active PP-1_C (measured as phosphorylase phosphatase after trypsin) within a few minutes after addition of insulin to 3T3 cells.[347,348]

The concentration of modulator shows large variations during the cell cycle of fibroblasts, with sharp (up to eightfold) increases during mitosis and during the S phase.[191a,342] This agrees with the high content of PEST sequences in modulator[172] that are common to proteins with a

high turnover.[349] On the other hand, the overall level of PP-1_C does not appear to fluctuate during the cell cycle of fission yeast.[102,115] These data suggest that the concentration of complexes between modulator and PP-1_C is not constant at different stages of the cell cycle.

2. Hormonal Regulation of Kinase F_A/GSK-3

A major part of kinase F_A/GSK-3 is bound as an inactive form to membranes but can be activated by solubilization of the membranes with Triton-X 100.[238,350–354] The remaining 10 to 50% of the kinase is recovered as an active cytosolic enzyme. Exposure of platelets[351] or adipocytes[238] to insulin, or of epidermoid carcinoma A431 cells to EGF,[353] reportedly results within a few minutes in a manifold increase of the cytosolic activity of kinase F_A/GSK-3, with a corresponding decrease in the activity that can be extracted from membranes. Insulin has also been found to increase the cytosolic activity of kinase F_A/GSK-3 in 3T3 cells without, however, affecting this kinase activity in the particulate fraction.[346a,348] Consistent with a regulatory role of insulin, the cytosolic activity of F_A/GSK-3 is decreased by 25 to 35% in skeletal muscle of streptozotocin-diabetic rats.[355,355a]

In spite of all these reports on hormonally induced activity changes of F_A/GSK-3, the data should be interpreted cautiously because the employed assay is not necessarily specific for this kinase. Indeed, other kinases can also catalyze the conversion of C_iM to C_aM,[191a,226–228] and the phosphorylation of C_iM by kinase F_A/GSK-3 is modulated by the activities of casein kinases 1 and 2 (Section IV.B.3).

It remains an open question whether the hormonal activation of kinase F_A/GSK-3 causes a conversion of C_iM to C_aM *in vivo*. Evidence in favor of this hypothesis comes from the report that the activation of F_A/GSK-3 by insulin in 3T3 cells is associated with a 20 to 40% increase in the cytosolic trypsin-revealed phosphorylase phosphatase activity.[348] However, it can also be argued that the increased phosphatase activity is mediated by casein kinase 2 (Section VIII.A.1). Claims by Yang and co-workers[238,353] that the activation of kinase F_A/GSK-3 in adipocytes and A431 cells is associated with an increased cytosolic activity of PP-1 do not seem to be justified by the illustrated data. In the latter studies the phosphatase activities were measured only after preincubation of the cytosol with MgATP. Therefore, it is possible that the increased phosphatase activity was not induced by insulin in the cell, but was generated artifactually during the subsequent incubation of the cytosol. Indeed, the increased activity of F_A/GSK-3 after hormone addition would be expected to result in a more complete activation of endogenous C_iM during subsequent incubation of the cytosol with MgATP. This explanation is all the more likely because the activity of F_A/GSK-3 before the hormone treatment was too low to convert all endogenous C_iM to C_aM.[238,353]

B. Phosphorylation of Inhibitor 1 and DARPP-32

The ratio between the activity of inhibitor 1 before and after maximal phosphorylation by cAMP-dependent protein kinase can be taken as an index of phosphorylation of Thr 35.[356] Under basal conditions this ratio is only 0.1 to 0.3 in skeletal muscle,[356–358] heart,[359] and rabbit liver.[142] Higher values have been found in cardiac and skeletal muscle when no precautions were taken to prevent the release of catecholamines from the adrenal glands following anesthesia.[163,358] However, in spite of a very low activity ratio of cAMP-dependent protein kinase, inhibitor 1 is nearly 50% active in freshly isolated epididymal fat pads.[151]

Administration of β-adrenergic agonists increases the activity ratio of inhibitor 1 in skeletal muscle,[356,357,360] heart,[163,359] and adipose[151] tissue to 0.6 to 0.8. In rabbit liver the activity ratio of inhibitor 1 has been found to increase from 0.14 to 0.42 following an intravenous injection of glucagon.[142] At high basal activities of inhibitor 1, insulin decreases its phosphorylation level in skeletal muscle and in adipose tissue.[151,361] Insulin also antagonizes the effect of low concentrations of β-adrenergic agonists on inhibitor 1, and this is associated with a decreased concentration of cAMP.[358] In cardiac muscle the effect

of β-adrenergic agonists on inhibitor 1 is antagonistically affected by the neurotransmitter acetylcholine.[359] However, acetylcholine does not affect the level of cAMP and may therefore act by decreasing the sensitivity of cAMP-dependent protein kinase to cAMP or by increasing the inhibitor 1 phosphatase activity.

The activity of DARPP-32 is controlled in a similar way. In brain striatal slices the inhibitory potency of DARPP-32 is increased by agents (e.g., the neurotransmitter dopamine) that act through cAMP.[139,362] The cAMP-mediated phosphorylation of DARPP-32 is reversed by *N*-methyl-D-aspartate, which activates a subclass of glutamate receptors.[362] The latter effect is due neither to a change in the activity of cAMP-dependent protein kinase nor to a dephosphorylation of DARPP-32 on serine residues, which may disfavor the phosphorylation by cAMP-dependent protein kinase (see Section IV.A.2). *N*-Methyl-D-aspartate may therefore act by increasing the DARPP-32 phosphatase activity. A good candidate for this function is PP-2B, which efficiently dephosphorylates DARPP-32 *in vitro*. *N*-Methyl-D-aspartate also has the potential to increase the activity of PP-2B because it raises the intracellular Ca^{2+}-concentration.[362] Further, PP-2B and DARPP-32 have a similar cellular distribution in the brain.[155]

C. Short-Term Regulation of PP-1G

1. Control of Muscle PP-1G by Phosphorylation

The injection of adrenalin into rabbits increases the phosphorylation of site 2 on the G subunit of PP-1G in skeletal muscle.[262,363] This phosphorylation is expected to translocate the catalytic subunit to the cytosolic compartment (see Section VI.B.1). At physiological ionic strength, the released $PP\text{-}1_C$ is much less active than the holoenzyme and may, in addition, be blocked by inhibitor 1, which is also phosphorylated after the administration of adrenalin. Inhibitor 1/DARPP-32 and site 2 of the G subunit belong to the best-known substrates for PP-2B *in vitro*.[153,257] However, the processes that lead to the reassociation of the holoenzyme with glycogen particles have not yet been examined.

Site 1 of the G subunit becomes phosphorylated after injection of insulin.[258] Phosphorylation of this site increases the phosphatase activity toward glycogen synthase and phosphorylase kinase and can thus explain both the antiglycogenolytic and glycogenic effect of insulin on skeletal muscle.[258] The phosphorylation of site 1 plus the decreased phosphorylation level of inhibitor 1 can account for the acute 25 to 30% stimulation of the synthase phosphatase activity in skeletal muscle extracts after *in vivo* administration of insulin.[364,365] Site 1 is also phosphorylated upon injection of adrenalin.[262] However, the phosphorylation of site 1 by cAMP-dependent protein kinase is probably not immediately effective because this kinase at the same time dissociates the enzyme by phosphorylating site 2. It has been proposed that the role of the phosphorylation of site 1 by cAMP-dependent protein kinase exists in priming PP-1G for rapid glycogen systhesis following the reassociation of the catalytic subunit.[258]

The phosphorylation of inhibitor 1 and of the G subunit by cAMP-dependent protein kinase allows this kinase to change the phosphorylation state of residues that are not its direct substrates. A likely example is glycogen synthase, which, after an injection of adrenalin, becomes phosphorylated on serine residues that are not readily phosphorylated by cAMP-dependent protein kinase.[366,366a]

2. Control of Hepatic PP-1G by Phosphorylase-a

The mechanism for the acute regulation of hepatic PP-1G seems to differ from that of the skeletal muscle enzyme. It has, indeed, not been possible to phosphorylate hepatic PP-1G,[272] nor to dissociate the catalytic subunit of hepatic PP-1G, by incubation with cAMP-dependent protein kinase in phosphorylation conditions[265] or by injection of glucagon into anesthetized rats.[211] Neither do rat and mouse liver contain inhibitor 1 or DARPP-32.[137,141,142] Instead, the acute regulation of hepatic PP-1G may be mediated by changes in the activity of phosphorylase kinase (Section VIII.D.1) and of phosphorylase (Figure 5).

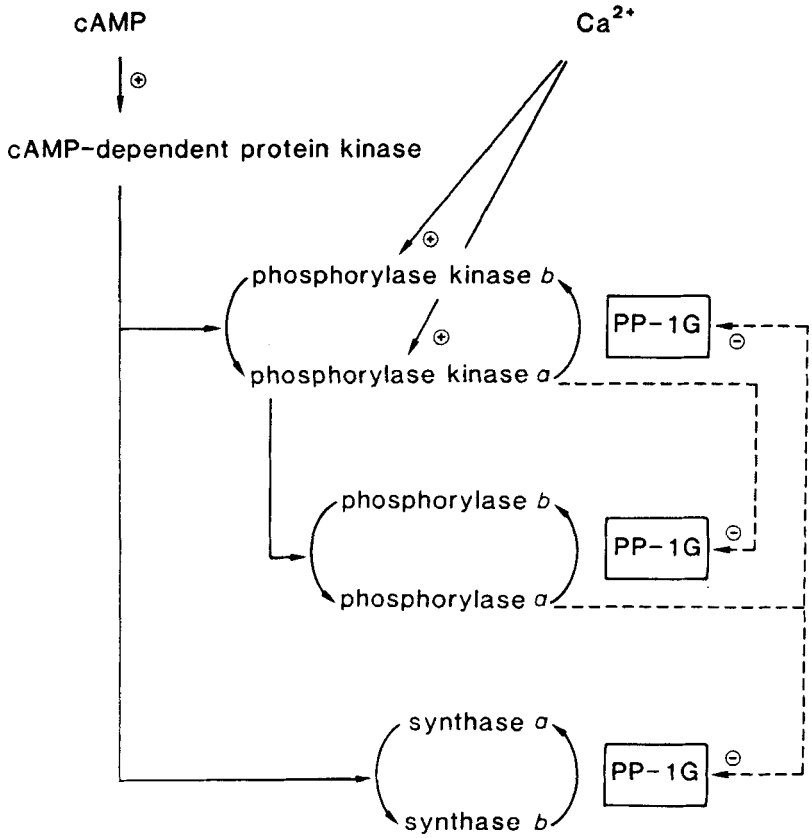

FIGURE 5. Regulation of hepatic PP-1G by cAMP- and Ca^{2+}-agonists. The activity of phosphorylase kinase and phosphorylase is increased by cAMP- and Ca^{2+}-agonists. Phosphorylase-*a* acts as an allosteric inhibitor in the dephosphorylation of glycogen synthase and phosphorylase kinase by PP-1G (Section VI.B.2). It is proposed that active phosphorylase kinase also inhibits directly or indirectly the dephosphorylation of phosphorylase by PP-1G (Section VIII.D.1).

a. PP-1G Is Inhibited by Phosphorylase-a

Glycogenolytic agents enhance the concentration of hepatic phosphorylase-*a* by increasing the activity of phosphorylase kinase (Figure 5), either through phosphorylation by cAMP-dependent protein kinase (e.g., glucagon) or through direct stimulation by Ca^{2+} (e.g., vasopressin). Phosphorylase-*a* not only catalyzes the rate-limiting step of glycogenolysis, but also sustains glycogenolysis and blocks glycogen synthesis by inhibiting the action of PP-1G on phosphorylase kinase and on glycogen synthase, respectively (Figure 5). Part of the evidence is that phosphorylase-*a* acts as an allosteric inhibitor of PP-1G *in vitro* (Section VI.B.2.a). Also, perfusion of livers with a medium containing either glucagon or vasopressin results within a few minutes in a strong inhibition (up to 75%) of the glycogen-synthase phosphatase activity, as measured in liver homogenates.[367] This effect is likely to be mediated by phosphorylase-*a* because it is absent when synthase phosphatase is assayed in the presence of AMP[368] or of antibodies against phosphorylase.[367] The inhibition of the glycogen-associated synthase phosphatase activity by phosphorylase-*a* may thus explain, at least in part, the inactivation of hepatic glycogen synthase by Ca^{2+}-agonists. Additional evidence is that this inactivation is preceded by a latency of 30 s, which corresponds to the time required for activation of phosphorylase.[369] The stimulatory effect of Ca^{2+} on the inactivation of glycogen synthase during incubation of liver extracts with MgATP is also dependent on the presence of phosphorylase-*a*.[370]

The control of PP-1G by phosphorylase-*a* also operates after the administration of glucose and insulin.[271,371] These agents may therefore activate hepatic glycogen synthase by decreasing the concentration of phosphorylase-*a* below the threshold level that is inhibitory to PP-1G. This threshold corresponds *in vivo* to about 10% of the total hepatic phosphorylase concentration. The mechanism by which insulin provokes an inactivation of phosphorylase is not yet fully understood, but, besides a decreased activity of cAMP-dependent protein kinase, PP-1 may be involved (Section VIII.D). Glucose promotes the inactivation of phosphorylase by binding directly to the *a*-form, which thereby becomes a better substrate for inactivation by phosphorylase phosphatase.

b. Regulation of the Sensitivity of PP-1G to Phosphorylase-a

The activation of glycogen synthase by partially purified PP-1G is completely inhibited by less than 50 n*M* phosphorylase-*a*,[264,269] whereas the threshold to phosphorylase-*a* is 20 to 60 times higher *in vivo*[372] and in isolated hepatocytes.[373,374] This indicates that the sensitivity of PP-1G to phosphorylase-*a* is subject to regulation. Further evidence for this view comes from the identification of molecules that interfere with the inhibition of PP-1G by phosphorylase-*a*. One such effector is a glycogen-bound protein that is induced by glucocorticoids and that renders phosphorylase-*a* noninhibitory (Section VIII.E.1). The inhibition of PP-1G by phosphorylase-*a* is also canceled *in vitro* by AMP. If this uncoupling effect of AMP also applies to intact cells, then an increased concentration of AMP would be expected to cause an activation of glycogen synthase, irrespective of the concentration of phosphorylase-*a*. This mechanism possibly accounts for the activation of hepatic glycogen synthase by fructose, adenosine, glutamine, and inhibitors of mitochondrial ATP synthesis;[375–378] these conditions are, indeed, associated with an increased concentration of AMP.[376,378]

The glycogen concentration may be another factor that affects the inhibition of PP-1 by phosphorylase-*a*. The normal diurnal variations in the glycogen concentration do not appear to have an effect on the sensitivity of PP-1G to phosphorylase-*a*, because the activation of glycogen synthase by glucose in isolated hepatocytes from fed and fasted rats occurs at the same threshold level as that of phosphorylase-*a*.[373] However, the liver of fasted animals contains significant amounts of both phosphorylase and glycogen synthase in the active form.[264,271] This indicates that the inhibition of synthase phosphatase by phosphorylase-*a* is incomplete, possibly because glycogen synthase and/or phosphorylase are not bound to glycogen particles in the fasted state.[263,264] Compared to a condition without glycogen, the addition of 5 mg/ml of glycogen has been reported to increase the sensitivity of PP-1G to phosphorylase-*a* 20-fold.[263] On the other hand, the glucose-induced activation of glycogen synthase in hepatocytes from *gsd/gsd* rats, which contain very high levels of glycogen (100–150 mg/g liver), requires a concentration of phosphorylase-*a* that is at least fivefold lower than the threshold concentration for phosphorylase-*a* in hepatocytes from normal rats.[379] In addition to the direct inhibitory effect of high glycogen concentrations on the synthase phosphatase activity,[375,379] this could be a mechanism for glycogen to limit its own upper concentration.

D. Other Mechanisms for the Acute Regulation of PP-1

1. Hepatic Type 1 Protein Phosphatases

When administered *in vivo* or added to isolated liver preparations, insulin and glucose induce within a few minutes an increase in the hepatic activities of phosphorylase phosphatase and glycogen-synthase phosphatase (assayed in the presence of AMP).[368,380,381] The effects of glucose and insulin are additive, and their combined administration *in vivo* increases the phosphatase activities in a liver extract by about 35%.[368] The stimulatory effect of insulin plus glucose results specifically from increases in the V_{max} of cytosolic and glycogen/microsomal PP-1. By using perfused livers, it has also been shown that vanadate mimics the acute effect of insulin on synthase phosphatase and phosphorylase phosphatase.[382]

The administration of either vasopressin or glucagon to anesthetized rats or to isolated perfused livers results in an acute decrease of the phosphorylase phosphatase activity in a liver extract by some 25%, but is without effect on the hepatic synthase phosphatase activity, when measured in the presence of AMP.[368,381] The effects of vasopressin and glucagon are not additive and stem at least in part from decreases in the V_{max} of cytosolic and particulate PP-1.[368]

The mechanism of the above-mentioned acute regulation of hepatic protein phosphatases is not yet understood, but the available data suggest that the control is mediated by transferable cytosolic effectors of PP-1.[368] However, it is clear that the effects on synthase phosphatase and phosphorylase phosphatase are unrelated to a change in the activity of a heat-stable protein phosphatase inhibitor.[368,381] Furthermore, the effects have typically been obtained with rat liver,[368,381] which does not contain inhibitor 1.[137,141,142]

Glucagon and vasopressin do not affect the phosphorylase phosphatase activity in the liver of the *gsd/gsd* rat, which lacks phosphorylase kinase activity.[383] Furthermore, the addition of a physiological concentration of partially purified and activated hepatic phosphorylase kinase to a cytosol or glycogen/microsomal fraction decreases the V_{max} of the phosphorylase phosphatase reaction by half, whereas the nonactivated kinase has no effect. The activated phosphorylase kinase does not affect the activity of glycogen-synthase phosphatase, which does not respond to glucagon or vasopressin. These data indicate that the phosphorylase kinase plays an essential role in the transduction of the effect of glucagon and vasopressin to phosphorylase phosphatase (Figure 5). It is unclear at present whether activated phosphorylase kinase inhibits phosphorylase phosphatase directly, or whether the effect is mediated by a contaminating protein that becomes inhibitory upon phosphorylation by phosphorylase kinase.

2. Protein Phosphatase 1 in 3T3 Cells

Insulin rapidly increases the phosphorylase phosphatase activity in 3T3 cells by 20 to 50%.[347,348,384] Contrary to the hepatic effects of insulin,[368] the response of phosphorylase phosphatase in 3T3 cells is not lost at high dilutions or after preincubation of the fractions with trypsin.[347,348] This indicates that the increased phosphatase activity in 3T3 cells stems from a higher concentration of active PP-1_C. Fifteen minutes after withdrawal of serum from cultured 3T3 cells, the cytosolic S6-phosphatase activity is increased about twofold.[385] Western blotting with antibodies against PP-1_C suggests that this increased S6-phosphatase activity is also associated with a higher amount of the type 1 catalytic subunit. The level of PP-1_C in insulin-stimulated or serum-deprived 3T3 cells increases too fast to be accounted for by a *de novo* synthesis of the phosphatase. However, the increased concentration of PP-1_C may be caused by a translocation of PP-1_C from a particulate fraction. As discussed above (Section VIII.A), the effects of insulin can also be explained by a conversion of C_iM to C_aM.

E. Long-Term Hormonal Control

1. Glucocorticoids

a. Effects of Glucocorticoids on Hepatic Glycogen Metabolism

An administration of glucocorticoids causes an inactivation of phosphorylase and an activation of glycogen synthase in the liver within 3 h.[386–388] These activity changes can be explained by a 50 to 100% increase of the glycogen-bound phosphorylase phosphatase activity and by a lesser inhibition of PP-1G by phosphorylase-*a*.[388,389] The mechanism by which glucocorticoids increase the phosphorylase phosphatase activity is not yet understood, but it involves protein synthesis.[388] On the other hand, it has been shown that glucocorticoids induce the synthesis of a glycogen-associated protein that relieves synthase phosphatase from inhibition by phosphorylase-*a*. It is likely that this ''deinhibiting'' protein also cancels the allosteric inhibition of the dephosphorylation of phosphorylase kinase by PP-1G. If so, this glucocoriticoid-induced protein is expected to cause both an activation of glycogen synthase and an inactivation of phosphorylase.

The glycogenic action of glucocorticoids is not mediated by an increased level of circulating insulin or glucose.[387] However, studies on cultured hepatocytes have shown that glucocorticoids increase the sensitivity to these glycogenic agents.[386,390–393] An increased sensitivity to glucose and insulin may contribute to the glucocorticoid-induced activation of glycogen synthase and inactivation of phosphorylase *in vivo* but cannot explain the effects of glucocorticoids on PP-1G.

b. PP-1G in Adrenalectomized Animals

Rats that have been either starved for 48 h or adrenalectomized maintain a 30 to 40% lower hepatic glycogen-synthase phosphatase activity than normal animals, when measured with the natural substrate, which is exclusively activated by type 1 protein phosphatases.[394,395] This decrease does not appreciably affect the glucose-induced activation of glycogen synthase in intact cells. However, the combination of adrenalectomy and starvation for 48 h results in an almost complete loss of the synthase phosphatase activity.[394–396] This is associated with a complete failure of glucose to activate glycogen synthase in isolated hepatocytes and *in vivo*.[395] The low synthase phosphatase activity in the liver of adrenalectomized starved rats can almost entirely be accounted for by a loss of functional PP-1G.[204] With hepatic glycogen synthase as substrate, the glycogen-associated synthase phosphatase activity in adrenalectomized, starved rats amounts to only 10% of the control value (Figure 6). The

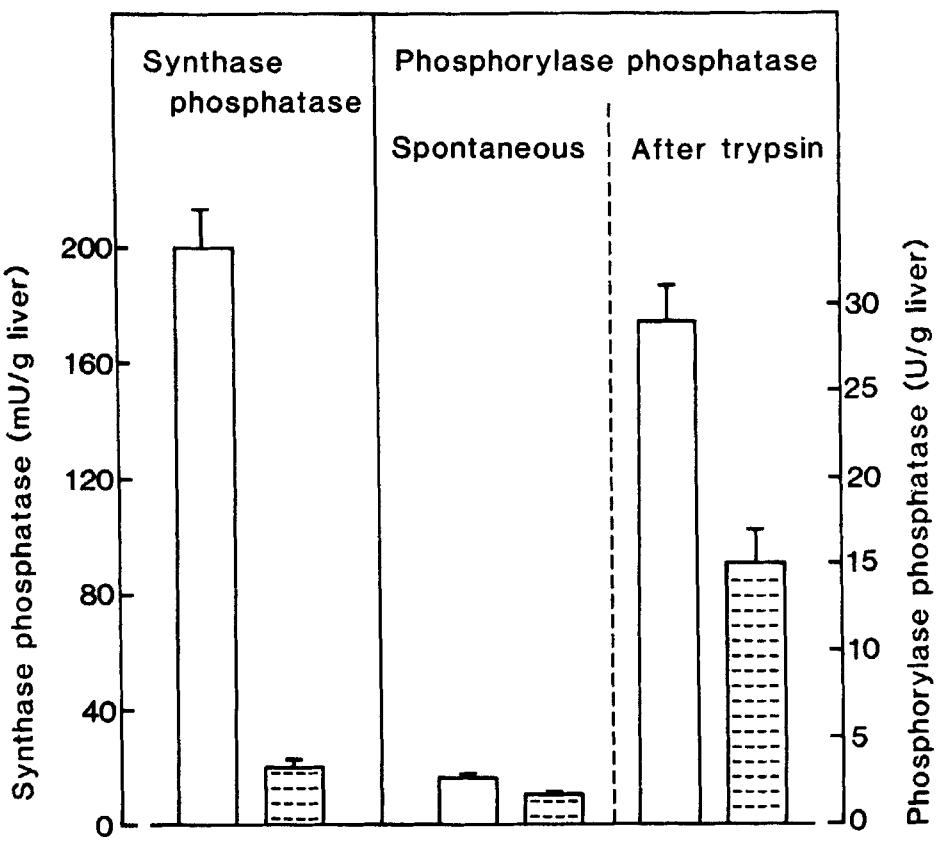

FIGURE 6. The glycogen-associated activities of synthase phosphatase and phosphorylase phosphatase in livers of adrenalectomized starved rats. Cytosol was prepared from glycogen-depleted livers from either glucagon-treated normal, fasted rats (open bars) or adrenalectomized, 48-h-starved rats (hatched bars). Glycogen was then added, and protein phosphatase activities were measured in the reisolated protein-glycogen complex. Bars represent SEM (n = 3–4). (Unpublished data.[211])

nature of this drastic decrease in synthase phosphatase activity is not yet fully understood. It can at least in part be explained by a loss of glycogen-associated PP-1_C, as indicated by the 50% decrease of both the spontaneous and the trypsin-revealed phosphorylase phosphatase activity of PP-1G (Figure 6).

The hepatic synthase phosphatase activity in adrenalectomized starved rats is partially restored by refeeding standard laboratory chow or glucose, or by an administration of glucocorticoids.[286,395,396] This recovery is paralleled by an improved capacity for activation of glycogen synthase in intact hepatocytes. Adrenalectomized starved rats have a very low circulating insulin concentration, and this is corrected by refeeding.[286,387] In contrast, the action of glucocorticoids in adrenalectomized 48 h-starved rats is, at least during the first 4 h, not associated with a significant increase of circulating insulin.[387]

In the fed rat the hepatic phosphorylase phosphatase activity is either unaffected[397] or somewhat decreased[394] by adrenalectomy. In contrast, skeletal muscle extracts from adrenalectomized rats have a 20% higher phosphorylase phosphatase activity than extracts from normal rats. Also, after adrenalectomy this phosphatase activity no longer decreases in response to an injection of adrenalin.[398] Furthermore, muscle extracts from adrenalectomized rats contain 40 to 50% less heat-stable inhibitor than that present in control rats. These data could be rationalized by a specific loss of inhibitor 1 in skeletal muscle after adrenalectomy and could also explain why rat liver, which lacks inhibitor 1,[137,141,142] does not show the same alterations after adrenalectomy.

c. The Ontogeny of PP-1G in the Fetal Liver

Glucocorticoids act as the physiological trigger for the gradual accumulation of hepatic glycogen at the end of fetal development, by inducing the synthesis of both glycogen synthase[386] and synthase phosphatase.[399] The synthesis of these enzymes can be induced prematurely by administration of dexamethasone and is delayed by glucocorticoid deficiency.[386,399] The increase of the hepatic synthase phosphatase activity in the terminal quarter of fetal development stems almost exclusively from an increase of PP-1G, with little or no contribution of the cytosolic synthase phosphatase activity, which is already present before the onset of glycogen synthesis.[399] Margolis[400] reported a considerable increase of the microsomal synthase phosphatase activity, but it cannot be excluded that this result was caused by an increased activity of glycogen-associated PP-1G that contaminated the microsomal fraction (Section VI.C). For unknown reasons, other investigators have not detected any change at all in the hepatic activity of synthase phosphatase during late fetal growth.[117]

2. Insulin

a. PP-1 in Insulin-Dependent Diabetes

The basal activity of glycogen synthase is very low in cardiac and skeletal muscle of insulin-dependent diabetic rats, and insulin does not increase this activity acutely as is observed in normal animals.[401–403] These deficiencies can at least in part be explained by a 40 to 60% lower synthase phosphatase activity.[401–404] The nature of this decreased synthase phosphatase activity has not yet been investigated, but, by analogy with the lesion in the liver (see below), it may be due to a lack of functional PP-1G. This view is supported by the recent report that the glycogen fraction from skeletal muscle of diabetic rats contains subnormal levels of immunoprecipitable G subunit.[355a] The amount of glycogen-associated PP-1_C may also be decreased, as indicated by a 40% lower trypsin-revealed phosphorylase phosphatase activity.[355,355a] It is also tempting to speculate that the lower synthase phosphatase activity in cardiac/skeletal muscle of diabetic rats is partially caused by a decreased phosphorylation of site 1 of the G subunit, as a result of a lesser activity of the insulin-dependent protein kinase that phosphorylates this site (Section VI.B.1). Because phosophorylation of site 1 does not affect the phosphorylase phosphatase activity of PP-1G,[258] this hypothesis may explain why the latter activity, in contrast to the synthase phosphatase activity,[401–404] is not significantly altered in muscle extracts of diabetic animals.[355,403,405] It

will be important to investigate whether the synthase phosphatase activity of PP-1G in the diabetic muscle is decreased to a larger extent than its phosphorylase phosphatase activity.

The liver of insulin-dependent diabetic rats also contains less than normal levels of active glycogen synthase and fails to respond to a glucose load with an activation of glycogen synthase.[271,374,406,407] In addition to an extrahepatic change, probably involving an altered insulin/glucagon ratio, this lack of activation of glycogen synthase is caused by an intrahepatic defect that develops between 2 and 3 d after the administration of the diabetogenic agent alloxan.[271,374] The only intrahepatic defect that has been found in this respect is a decreased synthase phosphatase activity.[285,404,405,407,408] The magnitude of the decrease depends on the assay conditions (e.g., type of substrate) and on the severity of the diabetic state.[406,409] With the "natural" substrate, the synthase phosphatase activity in the liver of severely diabetic rats amounts to only about 10% of the control activity. The synthase phosphatase activity is also decreased by 50 to 80% in the liver of spontaneously insulin-dependent diabetic BB rats.[407,410] Similar to what has been found for adrenalectomized starved rats, the decreased activity of hepatic synthase phosphatase in diabetic animals can almost entirely be accounted for by the loss of functional PP-1G, with little or no loss of the cytosolic synthase phosphatase activity.[409] It should also be noted that the phosphorylase phosphatase activity in the liver of diabetic animals is nearly normal.[285,408,409,411]

The restoration of the synthase phosphatase activity in cardiac muscle and in the liver of diabetic rats requires protein synthesis[402,412] and can be achieved by a treatment with insulin.[402,406,409] The hepatic deficiency is also normalized by the addition of vanadate to the drinking water for about 2 weeks.[413] With either treatment, the restoration of the phosphatase activity is strictly correlated with an improved capacity for activation of glycogen synthase in intact hepatocytes.[409,413] Remarkably, the synthase phosphatase activity in primary cultures of hepatocytes from diabetic rats is not restored by insulin.[414,415] However, the activity is recovered during long-term incubation of the cells with cortisol and triiodothyronine, and the restoration is at most somewhat more complete when insulin is also added. This may indicate that the long-term effects of insulin of PP-1G are indirect.

b. PP-1 in Non-Insulin-Dependent Diabetes

The synthase phosphatase activity in skeletal muscle extracts of insulin-resistant humans has been reported to be 20 to 25% lower than that of normal subjects.[365] However, several studies have shown that the livers of non-insulin-dependent diabetic animals display an either normal or increased activity of PP-1. Thus, the livers of spontaneously diabetic Chinese hamsters with hyperglycemia but normal insulinemia contain 25% more type 1 phosphorylase phosphatase activity than do control livers.[416] The obese *fa/fa* rat, with elevated blood glucose and insulin concentrations, has also been reported to contain nearly twofold higher activities of synthase phosphatase and phosphorylase phosphatase[417] in a liver fraction comprising probably both PP-1E and PP-1G.[287] Others found only a selective increase (two- to threefold) in the glycogen-associated synthase phosphatase activity in the livers of fasted obese rats, and this increase was associated with a glucose-induced "superactivation" of glycogen synthase in isolated hepatocytes.[309a] Hyperinsulinemic and hyperglycemic *db/db*[407] and *ob/ob* mice[211,418] maintain an essentially normal (type 1) synthase phosphatase activity in their livers. Taken together, these data indicate that a chronic hyperglycemia does certainly not result in a decrease of the hepatic activity of synthase phosphatase. This suggests that the decreased hepatic synthase phosphatase activity in insulin-dependent diabetes is due to the lack of insulin, rather than to the increased blood glucose concentration.

c. Long-Term Effect of Insulin in 3T3 Cells

In addition to the acute effects of insulin on PP-1 in 3T3 cells (Section VIII.D.2), insulin has also been reported to increase the activity of a type 1 S6 phosphatase by twofold within 2 h.[329]

3. Thyroid Hormones

Liver homogenates from hyperthyroid rats contain a more than twofold higher phosphorylase phosphatase activity[419] and a 40 to 60% higher synthase phosphatase activity[420] than control homogenates. These changes are associated with a lower basal level of phosphorylase-*a* and an increased basal activity of glycogen synthase.[419–421] On the other hand, the induction of hypothyroidism does not affect the hepatic levels of phosphorylase-*a* and phosphorylase phosphatase,[419] but decreases the synthase phosphatase activity by 40%, and this is associated with a lower basal activity of glycogen synthase and a diminished activation of glycogen synthase by glucose in isolated hepatocytes.[420] The effects of an altered thyroid status on the hepatic synthase phosphatase activity involve both cytosolic and glycogen-bound type 1 protein phosphatases.[420]

4. Hepatic PP-1G in gsd/gsd Rats

It has been reported recently that the liver of the *gsd/gsd* rat, which lacks phosphorylase kinase, also contains fewer type 1 protein phosphatases.[383] In comparison with normal Wistar rats, the glycogen/microsomal fraction of *gsd/gsd* livers contains 75% less synthase phosphatase and 60% less phosphorylase phosphatase activity. This deficiency is at least to a large extent due to a lower amount of PP-1_C in the particulate fraction. The cytosolic (type 1) synthase phosphatase activity is also 50% lower than that in control livers. It is not evident why the level of PP-1 is lower in *gsd/gsd* livers, but, in addition to the lack of phosphorylase kinase, the continuously high level of glycogen could play a role.

IX. FUTURE DIRECTIONS

The availability of the primary structure of PP-1_C (Section III.B) has opened the way for the localization of the active center and of the domains that interact with noncatalytic subunits, inhibitor 1, modulator, and nonprotein effectors. The recent description of a technique[390] for obtaining large amounts of active recombinant PP-1_C may also initiate studies on the tertiary structure of the enzyme.

A further understanding of the mechanism whereby certain cytotoxins inhibit PP-2A and PP-1 may lead to the development of specific inhibitors of PP-1. As these inhibitors can penetrate intact cells, they may become of paramount importance in the identification of physiological substrates of PP-1. Another approach to establish the physiological role of PP-1 comes from the study of mutants of yeast and *Drosophila* that lack a subunit of PP-1. The latter approach is more time consuming, but it has the advantage that it can differentiate between different species of PP-1. It can also be expected that the investigation of mutants will result in the identification of novel regulatory polypeptides of PP-1.

With the exception of inhibitor 1, modulator, and the glycogen-binding subunit of PP-1G, our knowledge of the identity, structure, and regulation of the noncatalytic subunits of PP-1 is still rather limited. The main reason is the difficulty to obtain pure type 1 holoenzymes, and this cannot be entirely circumvented by techniques of molecular biology. Another major question that still needs to be addressed is the physiological significance of the interconversion between C_iM and C_aM. The discovery of inhibitors that block the spontaneous conversion of C_aM to C_iM after tissue homogenization (Section V.A.2) may help to solve this problem.

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