Regulation of protein phosphatase-1

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Reversible protein phosphorylation is a major regulatory mechanism of intracellular signal transduction. Protein phosphatase 1 (PP1) is one of four major types of serine-threonine phosphatases mediating signaling pathways, but the means by which its activity is modulated has only recently begun to come into focus.

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Chemistry & Biology 2000, **7**:R13–R23

1074-5521/00/$ – see front matter

Introduction

The pathways and regulatory mechanisms by which cells process extracellular signals is a central issue in biology [1]. Reversible protein serine/threonine phosphorylation is a significant component of the intracellular signaling machinery, directing such diverse functions as neurotransmission, muscle contraction, glycogen synthesis, T-cell activation, neuronal plasticity and cell proliferation. Although the regulation of the large family of protein kinases has been a major focus for several decades, it is only recently that the regulation of protein phosphatases has been studied in the same detail [2].

In eukaryotic cells, eight types of serine/threonine phosphatases have been identified, and they are classified according to their requirements for metals, substrate specificities, and sensitivities to various peptide and natural product inhibitors [3]. Four of these types of serine/threonine phosphatases, PP1, PP2A, PP2B (calcineurin) and PP2C, have been studied in detail. PP1, PP2A and PP2B are structurally related to each other, whereas PP2C appears to have a distinct evolutionary background. PP1 is a major eukaryotic protein serine/threonine phosphatase that regulates diverse cellular processes such as cell-cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription and neuronal signaling [4]. This review focuses on how the activity and compartmentalization of PP1 are controlled by association with regulatory subunits, and on some of the resultant downstream cellular effects of these associations.

Association of PP1 with regulatory subunits

In contrast to the protein serine/threonine kinases [5], PP1 does not display obvious consensus sequence selectivity, dephosphorylating multiple substrates *in vivo* and *in vitro* [6]. Its action is modulated, however, through the formation of heteromeric complexes with a variety of regulatory subunits [7]. These include the heat-stable inhibitors, inhibitor-1 (I-1), its homolog DARPP-32 (dopamine- and cAMP-regulated phosphoprotein; molecular weight 32 kDa), and inhibitor-2 (I-2) [8].

PP1 is also regulated by its interaction with a variety of protein subunits that act in a manner distinct from the inhibitor proteins and that appear to target the catalytic subunit (PP1c) to specific subcellular compartments. These targeting subunits serve to localize PP1c in proximity to particular substrates, and also to reduce its activity towards other potential substrates [9,10]. In this respect, many of the targeting proteins appear inhibitory towards phosphorylase a, the substrate that is commonly used in

Table 1

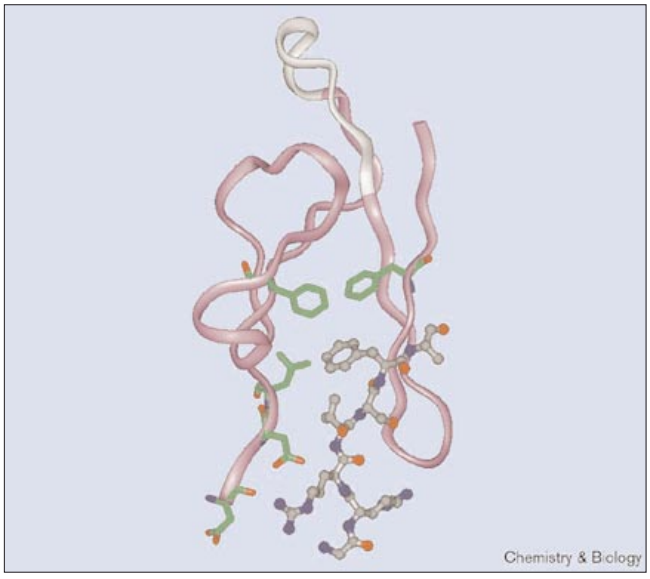
Characterized PP1-interacting proteins and putative PP1c-binding sequences.

Protein/gene	Residues	Sequence
Non-mammalian		
Gac1p/GAC1 (Yeast G_M homolog) *S67070*	65–76	TSPE**KNVRF**AIE
mel-11/MEL-11 (*C. elegans* M_{110} homolog) *U86640*	12–23	LQAK**QQITF**NVV
GIP1 (Yeast homolog of G_M) *P38229*	441–452	QKKK**RCVNF**RNK
	486–497	DRST**SSVRF**DEN
GIP2 (Yeast homolog of G_M) *6320895*	216–227	LIRS**KSVHF**DQA
GIP2h/YIL045W (Yeast homolog of G_M) *S49933*	191–202	LQRS**KSVHF**DRV
	411–422	KVFV**KNIYF**DKK
SCD5 (Yeast/role in secretory function) *S62061*	26–37	GLGP**PSVSF**DFG
	268–279	NFKS**KKVRF**SEH
YFL023W (Yeast) *6321086*	31–42	DIRS**RLVRF**IND
YAL014 *L05146*	95–106	KESL**KKVRF**KND
YFR003c (Yeast) *S56258*	45–56	MPTR**HNVRW**EEN
SHP1 (Yeast) *6319413*	272–283	PLKL**LDVQF**GQE
	365–376	CNST**DTVKF**LYE
REG1 (Yeast/glucose metabolism) *6320231*	460–471	PTKN**RHIHF**NDR
REG2 (Yeast homolog of REG1) *6319525*	163–174	KPRE**RHIKF**NDN
sds22 [Egp1] (*Saccharomyces cerivisiae*) *6322655*	295–306	LSRL**ETIYL**EGN
sds22 (*Schizosaccharomyces pombe*) *CAB11482*	284–295	LKKL**ETVYF**EGN
cet09a5.9 (*C. elegans* sds22 homolog) *Z36753*	281–292	LKGL**QTVYL**ERN
Mammalian		
Human G_M *AAB94596*	57–68	SSGT**RRVSF**ADS
Rabbit G_M *A40801*	60–71	SSGG**RRVSF**ADN
PTG *AAB49689*	56–67	NQAK**KRVVF**ADS
PTG	149–160	TVKV**KNVSF**EKK
G_L *CAA77083*	56–67	KKVK**KRVSF**ADN
Human R5 *4885559*	79–90	NQAK**KRVVF**ADS
U5 *AAC60216*	61–72	SQKK**KRVVF**ADM
$M_{110[}$ (MYPT1) *4505317*	30–41	KRQK**TKVKF**DDG
Ryanodine-sensitive calcium release channel protein *AAB32056*	Unknown	
Splicing factor PSF *P23246*	358–369	RGRQ**LRVRF**ATH
Ribosomal Protein L5 *P22451*	12–23	YFKR**YQVKF**RRR
p53BP2 *Q13625*	793–804	IAHG**MRVKF**NPL
NIPP-1 *AAD22486*	195–206	KRKN**SRVTF**SED
Spinophilin *AAB72005*	443–454	PAPS**RKIHF**STA
PNUTS *AAB96775*	393–404	GRKR**KTVTW**PEE
GRP-78 *P20029*	61–72	RITP**SYVAF**TPE
Human Muscle PFK *P08237*	42–53	IFTG**ARVFF**VHE
Human sds22 homolog *CAA90626*	143–154	ELEI**LDISF**NLL
	352–360	QIDA**TFVRF**
$HSV_{\gamma 1}$34.5 protein *g585297*	187–198	PATP**ARVRF**SPH
Hamster Gadd34 *A56535*	500–511	PLRA**RKVHF**SEN
Mouse MyD116 (Gadd34 homolog) *S10001*	543–554	PLKA**RKVHF**AEK
Mouse 110pRB *P13405*	279–290	IDEV**KNVYF**KNF
	184–195	VLKI**SWITF**LLA
Human 110pRB *P06400*	285–296	IDEV**KNVYF**KNF
	190–201	VLKV**SWITF**LLA
Rat Neurabin I *AAC53454*	452–463	IPAN**RKIKF**SCA
Human HOX11 *P31314*	11–22	PGHA**EPISF**GID
Human DARPP-32 *AAB30129*	3–14	PKDR**KKIQF**SVP
Human MYPT2 *4505319*	48–59	RRGS**PRVRF**EDG
Porcine aorta inhibitory protein *BAA22995*	Unknown	-RH**ARVTV**KYD
Human I-1 *Q13522*	4–15	DNSP**RKIQF**TVP
Human I-2 *CAA55475*	Unknown	
Human I-3 [87]	35–46	RKPE**KKVEW**TSD

The Genbank accession numbers are written in italics, and the putative consensus binding motifs are indicated in bold.

phosphatase assays [11]. It is likely, however, that each targeting protein recruits active PP1c to dephosphorylate a specific phosphoserine or phosphothreonine residue in the target substrate. In this way PP1c can act on diverse cellular targets with considerable selectivity. This manner of controlling phosphatase activity is apparently not unique

Figure 1



X-ray closeup of the $G_{M[63–75]}$ peptide bound to PP1c. The ribbon represents PP1c residues 240–297, and the grey portion is the β12–13 loop corresponding to Try272–Gly280. The PP1c sidechains shown in green are (counterclockwise from top right) Phe293, Phe257, Leu243, Asp242 and Asp240. The bound peptide has the sequence Gly–Arg–Arg–Val–Ser–Phe–Ala, and is shown as: grey, carbon; red, oxygen; and blue, nitrogen atoms. Not shown are the parallel hydrogen-bonding interactions between the peptide and the edge of the β14 strand. The graphics for Figures 1–3 were generated using Insight, and the coordinates for the complex [20] were obtained online (www.pdb.bnl.gov; PDB code 1FJM).

to PP1. For example, the catalytic subunit of PP2A is typically isolated as a heterotrimer [8], and is further regulated by interaction with specific targeting subunits [12].

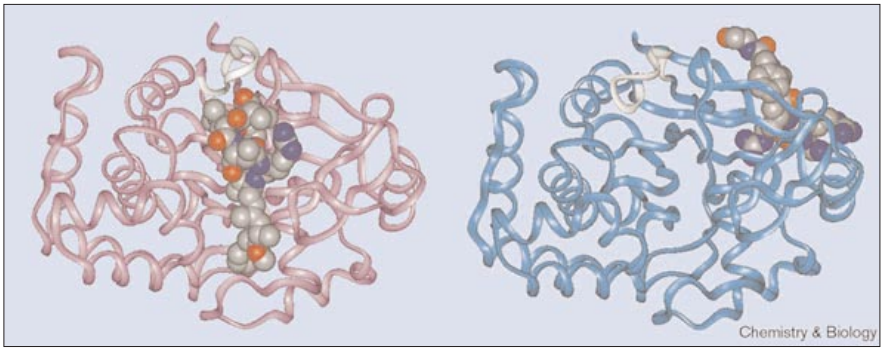
The number of known PP1c targeting subunits is rapidly increasing. To date, nearly 30 unique mammalian proteins have been identified (Table 1). In addition, homologs of several of these mammalian proteins have been identified in yeast. The observation that PP1 associates with only one inhibitor or targeting subunit at a time has suggested that the interaction of the different subunits with PP1 is mutually exclusive, and that the subunits bind to the same or overlapping sites on PP1.

Structural basis for the interaction of PP1c and regulatory subunits

Many of the important studies of PP1 targeting subunits have involved the G_M protein that targets PP1c to glycogen and the M_{110} protein that targets PP1 to myosin. Initial studies of G_M indicated that short peptides retained PP1-binding affinity [13]. Other studies of the inhibitor proteins DARPP-32 and I-1 indicated that short peptides, including the threonine residue phosphorylated by protein kinase A (PKA), also were able to bind to and inhibit PP1c [14]. More recently, studies of G_M and M_{110} [15,16], DARPP-32 [17,18] and I-1 [14], and peptide display library analysis [19] have indicated that PP1 interacts with the various targeting and inhibitor proteins through a short amino acid motif. The exact sequence of the motif is not identical, but one or more basic amino acids are followed by two hydrophobic residues separated by a variable amino acid (Table 1). The identification of the basic/hydrophobic motif in PP1c binding subunits provides a structural basis for their interaction with PP1 in a mutually exclusive manner.

To elucidate the detailed structural basis for the binding of PP1 by the regulatory proteins, a peptide corresponding to the relevant region of the G_M subunit ($G_{M[63–75]}$), was co-crystallized with PP1c, and the structure was determined using X-ray crystallography [20]. The structure shows that residues 64–69 of the $G_{M[63–75]}$ peptide bind in an extended conformation to a hydrophobic groove (Figure 1), near the carboxy-terminal region of PP1c, located on the opposite side of PP1c from the active site (Figures 2,3) [21,22]. Selective deletions of residues from this sequence have shown that the valine and phenylalanine residues are the most important, as removal

Figure 2



Comparison of the X-ray crystal structures of PP1c complexed with the inhibitor microcystin-LR [21] and the $G_{M[63–75]}$ peptide [20]. On the left is the PP1c–microcystin complex; PP1c is shown as a purple ribbon, and microcystin bound to the enzyme active site is shown in space-filling representation. On the right is the PP1c–$G_{M[63–75]}$ peptide complex; PP1c (blue ribbon) is bound to the peptide. In all structures the β12–13 loop is shown in grey. Note the conformational homology between the two PP1 structures, with the exception of the β12–13 loop. The coordinates for the PP1c–$G_{M[63–75]}$ peptide were kindly provided by David Barford.

Figure 3

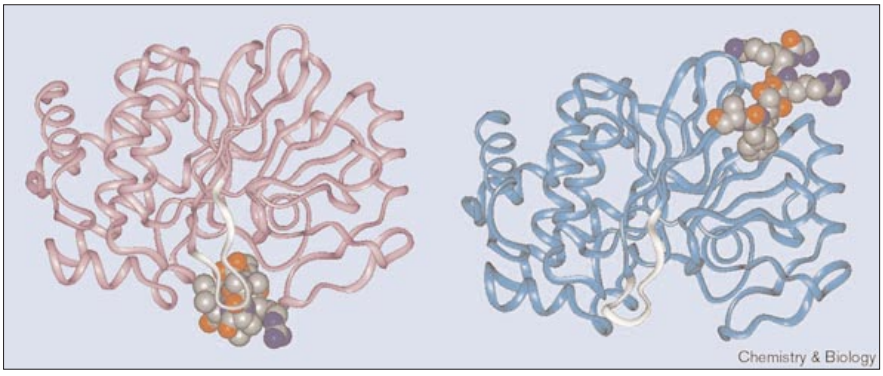


Figure resulting from rotation of the structures in Figure 2 by 90° about the X axis.

of either abolishes binding to PP1c [20]. Similar conclusions have been made from mutation studies of DARPP-32 [17,18]. In addition, the peptide display studies indicated that tryptophan can substitute for phenylalanine [19], and one example of this sequence has been found in the PNUTS protein, a nuclear PP1 targeting subunit (Table 1). The Arg/Lys–Arg/Lys–Val/Ile–Xaa–Phe/Trp motif (where Xaa is any amino acid) therefore represents a consensus sequence for the recognition and binding of regulatory subunits with PP1c.

Role of PP1 regulation by targeting subunits

The glycogen-binding subunits

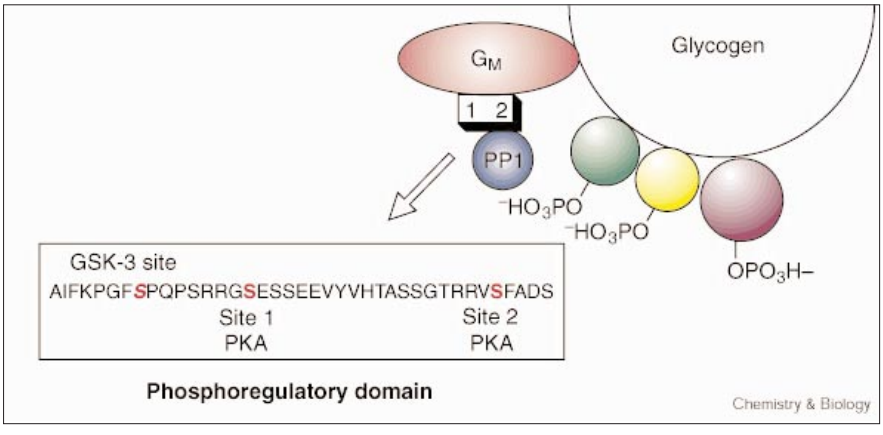
The skeletal muscle glycogen-bound PP1 subunit (G_M)

One of the primary sites of PP1c localization is skeletal muscle glycogen stores, where PP1c is bound to the G_M targeting subunit, forming the PP1c–G_M phosphatase holoenzyme (PP1-G). G_M is a 124 kDa glycogen-binding protein found in mammalian skeletal and cardiac muscle [23], where it accounts for up to 60% of the phosphorylase phosphatase activity in muscle extracts [24]. G_M binds to glycogen with high affinity (K_{app} ~ 6 nM), and also tightly associates with the sarcoplasmic reticulum in striated muscle [25].

In vivo, G_M localizes PP1c to three glycogen-binding enzymes involved in glycogen metabolism: glycogen phosphorylase (phosphorylase), phosphorylase kinase and glycogen synthase (Figure 4). The phosphorylated form of each is a good substrate for PP1c *in vitro* [8], but the activity of PP1c towards these enzymes is increased as much as eightfold [26] when associated with G_M and glycogen. This increased activity does not extend to other PP1c substrates, such as myosin light chain, that do not bind to glycogen. In addition, as a result of the mutually exclusive interaction of inhibitor and targeting subunits, PP1c is less sensitive to cytosolic inhibitors when it is bound to the G_M subunit [27].

In response to adrenalin, a series of regulatory phosphorylation events occur within the region of G_M that includes the PP1c-binding domain, resulting in the inhibition of PP1-G (Figures 4,5) [28]. Activation of PKA leads to rapid phosphorylation of site 1 (Ser48), and this is followed by

Figure 4



Representation of the functional PP1-G holoenzyme bound to glycogen and juxtaposed with potential phosphorylated substrates. The boxed portion of G_M is the region containing the phosphoregulatory sites 1 and 2, and the open arrow indicates an expansion of that region. The expansion shows the amino acid sequence of the phosphoregulatory domain and the PP1c-binding domain (site 2). Serine residues regulated by cAMP-dependent protein kinase or protein kinase A (PKA) are red and bold, and the serine residue phosphorylated by GSK-3 kinase is red, bold and italics. The green, yellow and purple balls are phosphorylated PP1 substrates, such as phosphorylase, glycogen synthase and phosphorylase kinase.

phosphorylation of site 2 (Ser67) [29]. Site 2 is within the PP1c targeting motif of G_M and its phosphorylation results in an estimated four orders of magnitude decrease in binding affinity of G_M for PP1c [30]; PP1c released from the G_M subunit possibly becomes associated with cytosolic I-1 (see below) [29,30]. Site 1 phosphorylation is thought to trigger the release process, perhaps by inducing a conformational change that increases the rate of site 2 phosphorylation [30]. Whatever the mechanism, release of PP1c results in rapid inhibition of phosphatase activity towards the glycogen-bound substrates. The release of PP1c into the cytosol is readily reversed by inhibition of kinase activity, and occurs following the dephosphorylation of site 2, perhaps by PP2B (Figure 5) [30]. The response to an adrenalin signal is therefore a double phosphorylation of G_M, which in turn causes the PP1c subunit to migrate from the glycogen stores to the cytosol with concurrent deactivation. The overall cellular effect is to promote glycogenolysis and mobilization of glucose energy stores in response to the release of adrenalin.

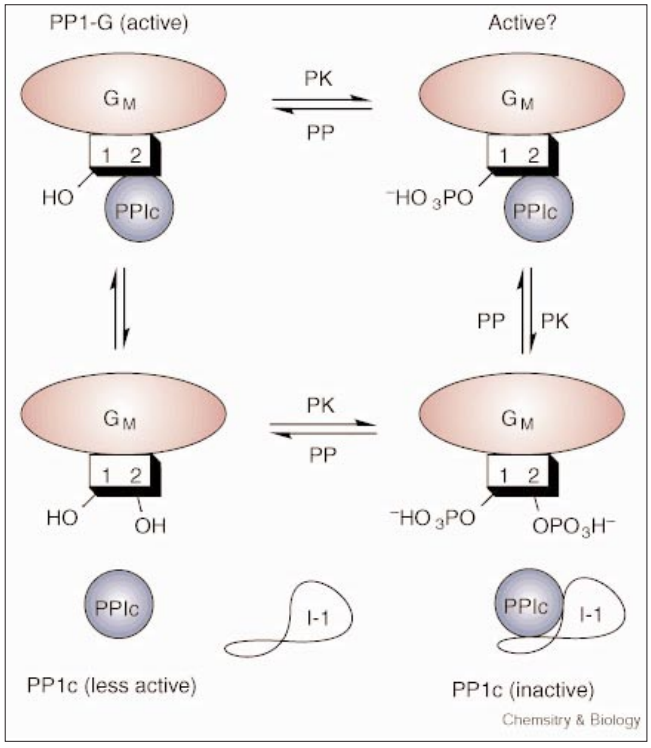
The activity of PP1 bound to glycogen can be stimulated by insulin. Although details of the early events in this pathway remain to be elucidated, the penultimate event in skeletal muscle appears to be the activation of PP1-G [31,32]. This insulin-stimulated activation of PP1-G in skeletal muscle has been attributed to phosphorylation of the G_M subunit at Ser48 (site 1 in Figure 4) [31] by a mammalian homolog of S6 kinase II [33], termed insulin-stimulated protein kinase (ISPK) [31,32]. This proposed role for ISPK is in question [32], however, and the observed increase in PP1-G activity may be due to the absence of glycogen and low salt concentration [30,34].

Regulation of PP1 by PTG

The PP1 regulatory subunit PTG is another glycogen-binding protein highly expressed in adipocytes and other insulin-sensitive tissues, such as skeletal muscle, heart and liver [35,36]. It is most closely related to the hepatic form of glycogen-binding subunit (G_L; 42% identity), but is less homologous to the G_M subunit (26% identity) and does not contain the phosphoregulatory sites found in G_M. In the PP1c targeting motif, PTG has a valine residue in place of the serine equivalent to residue 67 in G_M, indicating that it is not regulated by the phosphorylation/PP1c release mechanism that modulates PP1-G activity. PTG binds not only to PP1c and glycogen, but also to the major enzymatic regulators of glycogen synthesis, namely phosphorylase, glycogen synthase and phosphorylase kinase [36], participating in adrenalin/insulin-initiated signalling events in glycogen stores.

On the basis of studies of insulin-stimulated glycogen synthesis in fibroblasts and adipocytes [36,37], PTG is thought to act as a molecular scaffold upon which PP1c and its substrates are juxtaposed with glycogen, thereby increasing the specific activity of PP1c by localization. In addition, these studies have raised the possibility that the PP1 inhibitor DARPP-32 (see below) also plays a role in the regulation of glycogen synthesis [37]. Insulin may stimulate the dephosphorylation, or prevent the phosphorylation, of DARPP-32, possibly through the reduction of PKA activity. Conversely, activation of PKA would result in phosphorylation of DARPP-32 and inhibition of PP1, and consequently glycogen synthase activity.

Figure 5



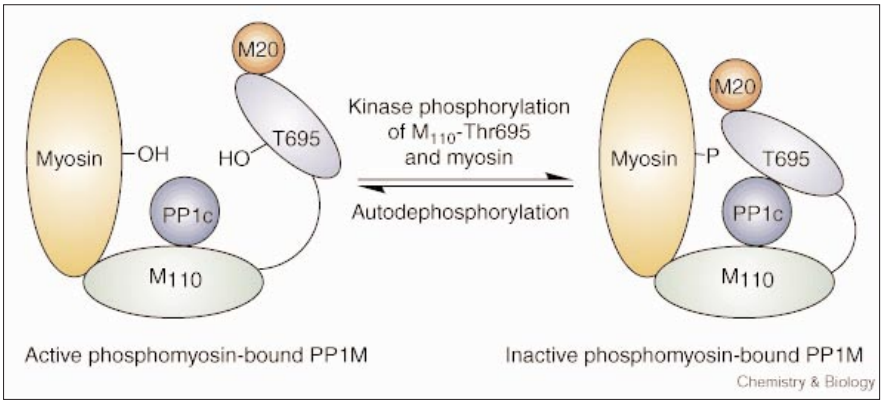
Model for the phosphoregulation of PP1-G. The boxed region of G_M is the phosphoregulatory domain (see Figure 4), and 1 and 2 refer to sites 1 and 2, respectively. I-1 is the cytosolic PP1c inhibitor-1. PK indicates protein kinase activity (PKA), and PP indicates protein phosphatase activity (PP2B, PP2A or autodephosphorylation by PP1c). The fully active PP1-G holoenzyme is shown in the upper left. The species in the lower left represents the population of unassociated PP1c and G_M subunits from the dynamic equilibrium of their association. The species in the upper right is the site 1 phosphorylated holoenzyme, the activity of which is not well established. The species in the lower right is the inactive phosphatase resulting from site 2 phosphorylation which promotes release of the catalytic subunit; the released catalytic subunit is shown complexed with I-1. Adrenalin signaling drives the equilibrium towards the lower right, whereas insulin drives the equilibrium towards the upper left.

The myosin-binding subunits

The smooth-muscle myosin-binding PP1c regulatory subunit (M_{110})

A different PP1-regulatory protein complex, smooth muscle myosin phosphatase (PP1-M), is involved in regulating

Figure 6



Proposed mechanism of M_{110} regulation of PP1. PP1c is shown associated with the amino terminus of the phospho-myosin-bound M_{110} subunit. The phosphoregulatory Thr695 hydroxyl, located near the carboxyl terminus of M_{110}, is shown. The carboxy- and amino-terminal portions of M_{110} are shown linked by the central portion. The small M20 subunit is shown bound near the carboxyl terminus of M_{110}, and the phosphorylated myosin residue is represented by P. The active myosin phosphatase is on the left, whereas the inhibited myosin phosphatase is on the right. In the proposed mechanism, the equilibrium is shifted to the right (inhibition) by Ca^{2+}-stimulated kinase activity, resulting in muscle contraction, whereas the equilibrium is shifted to the left (active) following the depletion of mobilized Ca^{2+}, resulting in muscle relaxation.

muscle contraction, cell motility and cell shape. The PP1-M holoenzyme consists of a heterotrimer containing PP1c (perhaps a specific isoform) [38], a large (110–130 kDa) subunit termed M_{110} (also termed MYPT1) [39], and a small (21 kDa) subunit termed M20; a multienzyme form of PP1-M associated with myosin light chain kinase (MLCK) and calmodulin has also been observed [40]. The small M20 subunit does not play any known role in the regulation or activation of the holoenzyme, so the M_{110} subunit has been the focus of efforts to elucidate the mechanism controlling PP1-M activity. The holoenzyme binds to myosin and myofibrils, accounting for ~80% of the phosphatase activity towards phospho-myosin. PP1-M is also found at cell–cell contact sites [41]. In smooth muscle, activated PP1-M dephosphorylates the 20 kDa regulatory myosin light chain, resulting in muscle relaxation. The smooth-muscle myosin-binding subunits have been recently reviewed [42]; the subject will therefore only be summarized here, with the addition of recent reports.

The amino-terminal 38 amino acids of M_{110} comprise the PP1c-binding region [16], and the myosin- and M20-binding regions are located towards the carboxyl terminus [15,20,43]. The M_{110} subunit binds more strongly to phospho-myosin than to dephospho-myosin, a characteristic that prevents product inhibition.

PP1-M is negatively regulated by phosphorylation of the M_{110} subunit. Smooth-muscle contraction is stimulated by Ca^{2+} or/and G-protein-coupled kinase activation, resulting in phosphorylation of myosin and the M_{110} subunit of PP1-M by a member of the Rho-kinase family [44]. This phosphorylation of the M_{110} subunit results in inhibition of PP1-M, but in contrast to the G_M subunit, the catalytic subunit is not released into the cytosol. Instead, autodephosphorylation of the phosphorylated PP1-M results in full recovery of phosphatase activity.

In M_{110}, the targeting motif at the amino terminus is responsible for binding to PP1 and juxtaposing the active site close to phospho-myosin substrate, whereas the carboxy-terminal portion is responsible for regulating the holoenzyme (Figure 6) [42]. In the proposed mechanism, phosphorylation of the M_{110} subunit on Thr695 (numbering from chicken gizzard M_{110}) by an activated kinase is followed by docking of the phosphorylated region into the active site, resulting in inhibition of myosin phosphatase activity. Autodephosphorylation reverses the active site occupation/inhibition, leading to active myosin phosphatase and muscle relaxation. Net inhibition or activation is dependent upon the level of kinase activity, which is stimulated by Ca^{2+} and ATP [40]. This mechanism of regulation is analogous in some respects to interaction of phosphorylated I-1 and DARPP-32 with PP1 (see above), but in I-1 and DARPP-32 the phosphothreonine residue is not autodephosphorylated.

Skeletal muscle myosin phosphatase

In skeletal muscle, the major myosin phosphatase is a complex between the β isoform of PP1 and the MYPT2 gene product (61% identity to MYPT1), but the mechanism of its regulation is not well characterized [45].

Nuclear regulatory subunits

Nuclear PP1 targeting subunits

PP1 plays an important role in the nucleus, where it interacts with at least two targeting proteins, NIPP-1 [46] and PNUTS [47]. NIPP-1 is a heat- and acid-labile protein that migrates on an SDS–PAGE gel with a molecular weight of ~45 kDa [46]. The heterodimeric complex of PP1c and NIPP-1, denoted $PP1N_{NIPP\text{-}1}$, bind strongly to RNA, and the binding preference is for U-rich regions [48]. It is believed, therefore, that NIPP-1 is a PP1 regulatory subunit that targets PP1 activity to as yet unknown RNA-bound substrates. Although less well

characterized, PNUTS has also been shown to contain several carboxy-terminal domains that are likely to interact with RNA or DNA [47].

$PP1N_{NIPP-1}$ is regulated by reversible phosphorylation. NIPP-1 is an excellent substrate for PKA and casein kinase-2 (CK-2) *in vitro* [49]. Each kinase phosphorylates two unique sites; PKA phosphorylates Ser199 and Ser178, whereas CK-2 phosphorylates Ser204 (adjacent to the PP1c binding motif) and Thr161 [50]. Phosphorylation of NIPP-1 by either kinase results in increased activity of PP1c towards phosphorylase, and the action of both kinases results in an additive effect. In addition, phosphorylation of $PP1N_{NIPP-1}$ by PKA reduced the affinity of NIPP-1 for PP1c, although treatment of $PP1N_{NIPP-1}$ with CK-2 did not [51]. Phosphorylation of NIPP-1 in a heterodimeric complex with PP1c results in an activation of the holoenzyme without release of NIPP-1 [50], indicating a mechanism of regulation distinct from PP1-G. The activation resulting from PKA and CK-2 phosphorylation of NIPP-1 is dependent on salt concentration, being greatest at physiological salt concentrations [50].

The cytosolic PP1 inhibitors

Inhibitor-1 (I-1) and DARPP-32

I-1 and DARPP-32 are homologous inhibitors of PP1c that are regulated by phosphorylation by PKA. The heat- and acid-stable inhibitor I-1 was first purified from rabbit skeletal muscle [52], although the protein has a relatively wide tissue distribution [53]. In contrast, DARPP-32 is highly expressed in neurons in the basal ganglia, where it is regulated by the neurotransmitter dopamine [4], although lower levels of the protein are found in other brain regions and non-neuronal tissues. Both I-1 and DARPP-32 are typically cytosolic, although an isoprenylated, membrane-bound form of DARPP-32 has been found in some cell types [37,54].

I-1 and DARPP-32 are both elongated monomers with no obvious tertiary structure [52,55]. Within the amino-terminal 40 amino acids, I-1 and DARPP-32 are 60% identical [56,57], and in contrast to the targeting subunits discussed above, both are clearly inhibitors of PP1c. In their unphosphorylated forms, however, the two proteins are poor inhibitors of PP1c [52,58]. Phosphorylation of either protein at a conserved threonine residue (Thr35 in I-1, Thr34 in DARPP-32) converts either protein into potent PP1c inhibitors (IC_{50} ~1 nM) [59,60]. Both I-1 and DARPP-32 show mixed competitive/noncompetitive kinetics for inhibition of PP1, suggesting they interact with PP1 at different sites on the enzyme [61,62].

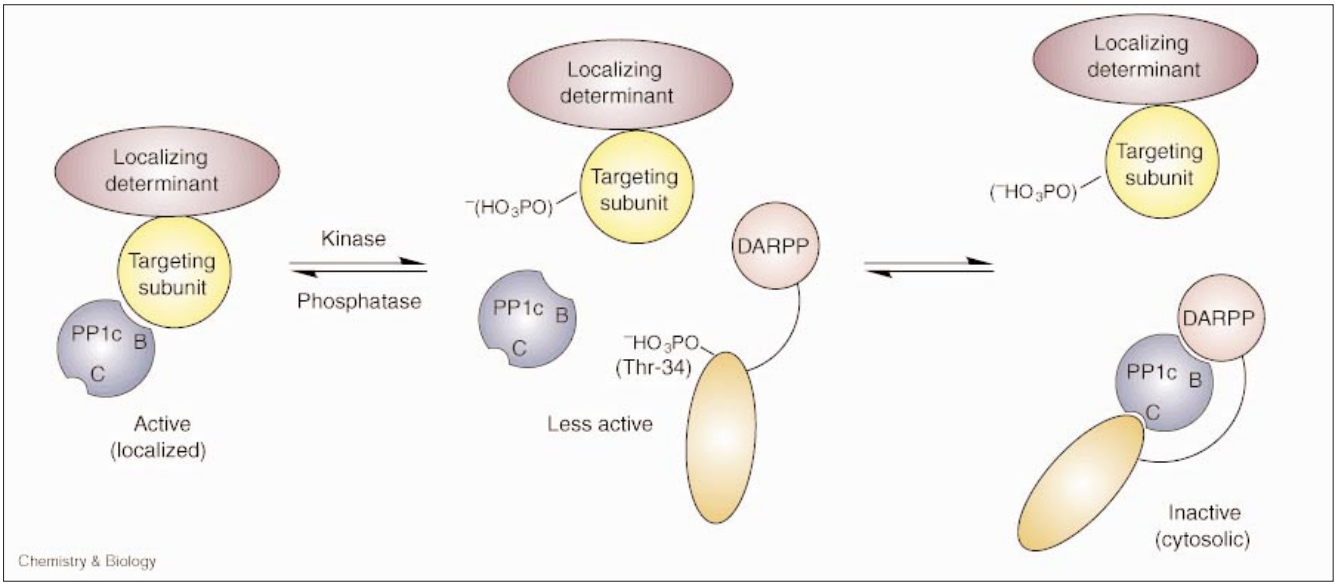
Recent structure–function studies have provided strong support for the two-site model for regulation of PP1c by I-1 and DARPP-32 [14,18]. Early proteolysis studies determined that the amino-terminal 54 residues of I-1 retained activity similar to the full-length protein, and that a region containing residues 9–22 was essential [63]. Subsequent studies using synthetic amino-terminal DARPP-32 fragments implicated two specific subdomains that are required for binding and PP1c inhibition [18,58,62]. One subdomain contains the Arg/Lys–Arg/Lys–Val/Ile–Xaa–Phe/Trp targeting motif discussed above [18]. The second subdomain corresponds to the residues surrounding the phosphorylated Thr35 in I-1 or Thr34 in DARPP-32 (Arg–Arg–Pro–Thr(P)–Ala–Met–Leu). The targeting motif interacts with PP1c at a site removed from the active site, but inhibition results because the phosphothreonine occupies the active site in such a way that catalysis (autodephosphorylation) cannot take place [17,18].

On the basis of these and additional observations, a model for PP1c regulation by I-1 or DARPP-32 can be proposed (Figure 7) [30,64]. In the dephosphorylated state, I-1 or DARPP-32 is inactive, and PP1 can associate with various targeting subunits, allowing dephosphorylation of selective substrates. Following an increase in PKA activity, I-1 or DARPP-32 are phosphorylated and activated. In parallel, PKA may also phosphorylate specific targeting proteins close to or within the targeting motif, thereby releasing PP1c, which is then inhibited by the activated I-1 or DARPP-32. Inhibition of PP1 by this mechanism works in a synergistic fashion with activated PKA, or other active protein kinases, to increase phosphorylation of downstream substrates.

Much evidence indicates that DARPP-32 plays a central role in mediating the actions of dopamine in the central nervous system [65]. Thr34 of DARPP-32 is phosphorylated by PKA in response to activation of the D1 subclass of dopamine receptors [66]. Inhibition of PP1 by phospho-DARPP-32 in turn regulates the phosphorylation status of many downstream targets, including various neurotransmitter receptors and ion channels. The reverse process, in which DARPP-32 is dephosphorylated, results in disinhibition (activation) of PP1. In neurons, glutamate signaling at the N-methyl-D-aspartate (NMDA) receptor results in activation of PP2B (calcineurin), which dephosphorylates Thr34 in DARPP-32 [67]. *In vitro*, DARPP-32 is also efficiently dephosphorylated by PP2A [60,68], and recent studies suggest that PP2B and PP2A may act synergistically in neurons to dephosphorylate Thr34 [69].

Although DARPP-32 and I-1 are phosphorylated by PKA and regulate PP1 by the same mechanism, there are significant differences in the phosphorylation of the two proteins by other protein kinases. A recent study has shown that DARPP-32 is phosphorylated at Thr75 by cyclin-dependent kinase 5 (cdk5) in intact neurons [70]. Phosphorylation of Thr75 converts DARPP-32 into an inhibitor of PKA, and prevents phosphorylation of Thr34. Reduction in phospho-Thr75 in neurons results in increased

Figure 7



Model for PP1c interaction with DARPP-32. The regulatory subunit binding site and the active site on PP1c are labeled B and C, respectively. A targeting subunit is shown bound to a localizing determinant. The two subdomains of DARPP-32 are represented as a pink ball (subdomain 1 containing the targeting motif) and an orange oval (subdomain 2 containing the phosphoregulatory domain), connected by a linker. The species shown at the far left is the active PP1 phosphatase holoenzyme. DARPP-32 is activated by Thr34 phosphorylation, possibly accompanied by phosphorylation on the targeting subunit that reduces its affinity for PP1c, and the released PP1c is fully inhibited by two-point binding with phospho-DARPP-32 (shown as the species on the far right).

phosphorylation of PKA substrates. Thus, depending on whether Thr34 or Thr75 are phosphorylated, DARPP-32 appears to control PP1 or PKA, respectively. DARPP-32 is also readily phosphorylated *in vitro* on Ser137 and Ser189 by CK-1 [71], and Ser45 and Ser102 by CK-2 [72]. CK-2 phosphorylation of Ser102 increases the rate and extent of phosphorylation at Thr34 by PKA (but not by PKG). CK-1 phosphorylation of Ser137 inhibits dephosphorylation of Thr34 by PP2B, without affecting the inhibition of PP1c [73]. Significant *in vivo* phosphorylation of Ser102 and Ser137 have been observed [71], and it has been proposed that CK-1 and/or CK-2 phosphorylation function to reinforce PP1 inhibition *in vivo*. Additionally, PP2C appears to be the primary phosphatase responsible for Ser137 dephosphorylation *in vivo* [74], therefore indicating that PP2C may indirectly regulate PP2B, and PP1, through dephosphorylation of DARPP-32.

Inhibitor-2 (I-2)

A third PP1 inhibitor, I-2, was first discovered in rabbit skeletal muscle [75]. In its unphosphorylated form, I-2 potently inhibits PP1c (IC_{50} ~1 nM) [76]. In contrast to the other inhibitors, however, inhibition of PP1c by I-2 exhibits competitive kinetics, implying that I-2 does not interact with PP1c in the same way as do I-1 and DARPP-32 [61]. The inhibition of PP1c by I-2 is competitive with respect to inhibition by okadaic acid [77], suggesting that a primary binding site of I-2 interacts with the active site of PP1. I-2 does not share any amino acid sequence with I-1 and DARPP-32, and no obvious conserved Arg/Lys–Arg/Lys–Val/Ile–Xaa–Phe/Trp targeting motif is present. A recent study has elucidated some of the details of the interaction of I-2 with PP1c, however [17]. The results obtained indicate that I-2 interacts with PP1c via a unique amino-terminal motif that is distinct from that found in I-1, DARPP-32, and the other targeting proteins discussed above. In addition, a central region of I-2 is also required for binding and to PP1c and inhibition of enzyme activity. This central region appears to interact with the region of PP1 that contains the binding site for the conserved Arg/Lys–Arg/Lys–Val/Ile–Xaa–Phe/Trp motif, but the actual motif is not present.

The physiological role and mechanism of regulation of I-2 remain unclear, despite much research. PP1 isolated as a soluble complex with I-2 is historically termed the ATP/Mg-dependent phosphatase, and *in vitro* GSK-3 phosphorylation of I-2 in this complex activates the phosphatase catalytic subunit [78]. The physiological relevance of this has been questioned, however [79]. I-2 is also phosphorylated at the Ser82, Ser120 and Ser121 residues by CK-2 *in vitro* [80,81] and *in vivo* [82,83], but the CK-2 phosphorylations are reported not to affect the phosphatase activity of the complex [80,84]. Possibly phosphorylation may influence the subcellular distribution of I-2 [83]. There is evidence that I-2 is involved in modulating

cell-cycle events, because its expression levels and nuclear localization peak during the S and M phases [83,85]. Interestingly, PP1 also localizes to the nucleus during the G_2/M transition of the cell cycle [86].

Conclusions

The localization, activity and substrate selectivity of PP1 are intimately intertwined and are controlled by interaction with a large number of regulatory proteins. Interaction of these regulatory proteins with PP1 appears in most, if not all, cases to be dependent on binding of a conserved Arg/Lys–Arg/Lys–Val/Ile–Xaa–Phe/Trp motif to a site on the catalytic subunit of PP1 that is removed from the active site. Interaction via this targeting motif ensures mutually exclusive binding, and increases the activity of PP1 towards specific substrates, dependent upon which regulatory subunit is bound. Further positive or negative regulation of activity is achieved by reversible phosphorylation of the regulatory subunit. Cytosolic inhibitors also contribute to the regulation of phosphatase activity and appear to interact with the same PP1 regulatory site, as well as the active site itself. The elucidation of a putative PP1c binding consensus sequence opens the door for developing nonpeptide ligands to selectively disrupt the interaction of the regulatory subunits with PP1c, thereby inhibiting or activating specific PP1 functions. In addition, PP1c-regulatory subunit heterodimers can participate in the formation of much larger complexes, suggesting further complexity in PP1 regulation.

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