Protein phosphatase 1 – targeted in many directions

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Summary

Protein phosphatase 1 (PP1) is a major eukaryotic protein serine/threonine phosphatase that regulates an enormous variety of cellular functions through the interaction of its catalytic subunit (PP1c) with over fifty different established or putative regulatory subunits. Most of these target PP1c to specific subcellular locations and interact with a small hydrophobic groove on the surface of PP1c through a short conserved binding motif – the RVxF motif – which is often preceded by further basic residues. Weaker interactions may subsequently enhance binding and modulate PP1 activity/specificity in a variety of ways. Several putative targeting subunits do not possess an RVxF motif but nevertheless interact with the same region of PP1c. In addition, several 'modulator' proteins bind to PP1c but do not possess a domain targeting them to a specific location. Most are potent inhibitors of PP1c and possess at least two sites for interaction with PP1c, one of which is identical or similar to the RVxF motif.

Regulation of PP1c in response to extracellular and intracellular signals occurs mostly through changes in the levels, conformation or phosphorylation status of targeting subunits. Understanding of the mode of action of PP1c complexes may facilitate development of drugs that target particular PP1c complexes and thereby modulate the phosphorylation state of a very limited subset of proteins.

Key words: PP1, Protein targeting, Insulin action, Smooth muscle contraction, Pre-mRNA splicing, AKAP, Neurabin, 53BP2

Introduction

The human genome encodes ~500 protein kinases, approximately two thirds of which are serine/threonine kinases. The four distinct families of protein phosphatases (Barford 1996; Kobor et al., 1999), however, comprise only ~150 members, of which fewer than 40 are serine/threonine phosphatases (International human genome sequencing consortium). The ratio of serine/threonine-specific kinases to serine/threonine-specific phosphatases is also high (6:1) in *Drosophila* (Morrison et al., 2000). How do such a small number of protein phosphatases dephosphorylate thousands of proteins while allowing the level of phosphorylation of each of these proteins to be regulated independently? Over the past decade, an understanding of the molecular mechanisms that underlie this versatility has emerged in the case of one of the major eukaryotic protein phosphatases: protein phosphatase 1 (PP1). The PP1 catalytic subunit (PP1c) can form complexes with >50 regulatory (bona fide or putative) subunits in a mutually exclusive manner. The formation of these complexes converts PP1c into many different forms, which have distinct substrate specificities, restricted subcellular locations and diverse regulation. This allows numerous cellular functions that rely on PP1 to be controlled by independent mechanisms (Table 1). Here, I discuss the plethora of PP1 regulatory subunits recently discovered in higher eukaryotes, their interactions with PP1c and their functions in signalling cascades. Earlier reviews on PP1 can be found elsewhere (Cohen, 1989; Shenolikar and Nairn, 1991; Bollen and Stalmans, 1992; Shenolikar, 1994; Wera and Hemmings, 1995; Brautigan, 1997; Barford, 1998; Aggen et al., 2000; Bollen, 2001).

The protein phosphatase 1 catalytic subunit

PP1c is a member of the PPP family of protein serine/threonine phosphatases, which in humans includes, PP1, PP2A, PP4, PP6, PP2B/calcineurin, PP5 and PP7 (Cohen, 1997; Barton et al., 1994). Multiple genes encode PP1c isoforms in most eukaryotes, the exception being in *Saccharomyces cerevisiae*, which possesses only one gene (*Glc7*) encoding PP1c (Stark, 1996; Dombrádi, 1997). The four mammalian *PP1c* gene products initially identified were designated PP1α, PP1β (also termed PP1δ), PP1γ_1 and PP1γ_2, the latter two arising through alternative splicing (Cohen, 1988; Dombrádi et al., 1990; Sasaki et al., 1990). An additional human PP1α variant, PP1α_2 (Durfee et al., 1993; Yoshida et al., 1999), is similar to the PP1α isoform that is predicted from the human genome sequence (Ensembl, ENSP00000176139), indicating that alternative splicing also gives rise to at least two α isoforms.

Mammalian PP1c isoforms possess distinct tissue distributions and subcellular localisations (Shima et al., 1993; da Cruz e Silva et al., 1995; Andreassen et al., 1998). Mutants of different PP1 isoforms in *Drosophila* (Axton et al., 1990; Raghavan et al., 2000) give rise to very different phenotypes, and inhibition of expression of a single isoform in a mammalian cell line blocks cytokinesis (Cheng et al., 2000). However, the small number of PP1c isoforms, their near 90% amino-acid sequence identity and their broad and similar substrate specificities in vitro support the tenet that it is predominantly the regulatory subunits with which PP1c interacts that control the specificity and enormous diversity of PP1 function. Although a small fraction of PP1c molecules may be inhibited by phosphorylation during the cell cycle (Berndt et al., 1997; Kwon et al., 1997), most forms of regulation are also achieved through the regulatory subunits.

Regulatory subunits of PP1c

A classification of 45 established or putative mammalian regulatory subunits of PP1c is presented in Table 1. An

Table 1(A). Protein phosphatase 1 regulatory subunits in mammals

Regulatory subunit	RVxF motif	Physiological function regulated	Tissue/subcellular location	Reference
Glycogen targeting				
G_M (R_{GL}, R3) *PPP1R3A*	+	Glycogen metabolism	Skeletal muscle, heart, glycogen particles	Stralfors et al., 1985; Tang et al., 1991
G_L (R4) *PPP1R3B*	+	Glycogen metabolism	Liver, glycogen particles	Moorhead et al., 1995; Doherty et al., 1995
R5 (protein targeting to glycogen, PTG) *PPP1R3C*	+	Glycogen metabolism	Widely distributed, high in liver and muscle, glycogen particles	Doherty et al., 1996; Printen et al., 1997
R6 *PPP1R3D*	+	Glycogen metabolism?	Widely distributed glycogen particles	Armstrong et al., 1997
Myosin/actin targeting				
M_{110} (myosin phosphatase targeting subunit, MYPT1, myosin binding subunit, MBS, M130) *PPP1R12A*	+	Smooth muscle relaxation	Smooth muscle and non-muscle cells myofibrils–myosin	Alessi et al., 1992; Chen et al., 1994a; Shimizu et al., 1994; Shirazi et al., 1994
MYPT2 (PP1bp55, M_{20}-spliced form) *PPP1R12B*	+	Skeletal muscle contraction	Skeletal muscle, heart, brain, myofibrils	Fujioka et al., 1998; Damer et al., 1998; Moorhead et al., 1998
p85 *PPP1R12C*	+	Actin cytoskeleton?	Widely distributed	Tan et al., 2001
Spliceosomal/RNA targeting				
NIPP1 (nuclear inhibitor of PP1, Ard1-fragment) *PPP1R8*	+	Pre-mRNA splicing	Widely distributed, nucleus	Van Eynde et al., 1995
PSF1 (polypyrimidine tract-binding protein associated splicing factor)	+	Pre-mRNA splicing?	Widely distributed, nucleus	Hirano et al., 1996
p99 (R111, phosphatase 1 nuclear targeting subunit, PNUTS) *PPP1R10*	+	RNA processing or transport?	Widely distributed, nucleus	Kreivi et al., 1997; Allen et al.,1998
Hox11 (homeodomain transcription factor)		Cell cycle checkpoint	Hematopoietic cells nucleus	Kawabe et al., 1997
HCF (human factor C1, host cell factor)		Transcription, cell cycle	Widely distributed	Ajuh et al., 2000
Endoplasmic reticulum targeting				
L5 ribosomal protein		Protein synthesis?	Widely distributed, ribosome	Hirano et al., 1995
RIPP1 (ribosomal inhibitor of PP1)		Protein synthesis?	Ribosome	Buellens et al., 1996
GADD34 (growth arrest and DNA damage protein) *PPP1R15A*	+	Protein synthesis	Widely distributed, stress inducible, endoplasmis reticulum	Novoa et al., 2001; Conner et al., 2001
Proteasome targeting?				
Sds22 *PPP1R7*		Exit from mitosis	Widely distributed, nucleus	Stone et al., 1993; Dinischiotu et al., 1997
Nuclear membrane targeting				
AKAP 149 (A-kinase anchoring protein 149)	+	Nuclear envelope reassembly (dephosphorylation of B-type lamins)	Widely distributed, nuclear envelope	Steen et al., 2000; Steen and Collas, 2001
Plasma membrane and cytoskeleton targeting				
Neurabin I *PPP1R9A*	+	Neurite outgrowth Synapse morphology	Neuronal, plasma membrane and actin cytoskeleton	McAvoy et al., 1999; MacMillan et al., 1999
Spinophilin (neurabin II) *PPP1R9B*	+	Glutamatergic synaptic transmission and dendritic morphology	Ubiquitous, plasma membrane and actin cytoskeleton	Allen et al., 1997; Satoh et al., 1998
NF-L (neurofilament-L)		Synaptic transmission?	Neuronal, plasma membrane and cytoskeleton	Terry-Lorenzo et al., 2000
AKAP220 (A-kinase anchoring protein 220)	+	Co-ordination of PKA and PP1 signalling	Brain, testis, peroxisomes/cytoskeleton	Schillace and Scott, 1999
Yotiao (A-kinase anchoring protein)		Synaptic transmission (NMDA receptor ion channel activity)	Neuronal post-synaptic density	Westphal et al., 1999
BH-protocadherin-c	+	Neuronal cell-cell interactions?	Neuronal membrane	Yoshida et al., 1999
Ryanodyne receptor		Calcium ion channel activity?	Skeletal and cardiac muscle sarcoplasmic reticulum	Zhao et al., 1998
NKCC1 (Na-K-Cl cotransporter)	+	Transcellular chloride ion transport (dephos-phorylation of NKCC1)	Epithelia, plasma membrane	Darman et al., 2001
Centrosome targeting				
AKAP 350 (CG-NAP, centrosomal and protein, AKAP450	+	Centrosomal functions?	Widely distributed, centrosome	Takahashi et al., 1999
Nek2 (NIMA related protein kinase 2)	+	Centrosome separation	Widely distributed, centrosome and cytoplasm	Helps et al., 2000

Table 1(A). *Continued*

Regulatory subunit	RVxF motif	Physiological function regulated	Tissue/subcellular location	Reference
Microtubule targeting				
Tau		Microtubule stability and function?	Neuronal microtubules	Liao et al., 1998
Mitochondrial targeting				
Bcl2	+	Apoptosis? (dephosphorylation of Bad)	Widely distributed mitochondrial membrane	Ayllón et al., 2000; Ayllón et al., 2001
Targeted to specific substrates?				
53BP2 (TP53BP2, p53-binding protein 2) *PPP1R13A*	+	Cell cycle checkpoint? (dephosphorylation of p53?)	Widely distributed cytosol	Helps et al., 1995
Rb (retinoblastoma protein)		Cell cycle progression (dephosphorylation of Rb)	Widely distributed nucleus	Durfee et al., 1993
PRIP-1 (phospho-lipase C-related inactive protein, p130, PLC-L_1)	+	Ins(1,4,5)P_3-mediated Ca^{2+} signalling?	Brain, cytosol	Yoshimura et al., 2001
PFK (phosphofructokinase)		Glycolysis? (dephos-phorylation of PFK?)	Skeletal muscle, F-actin?	Zhao and Lee, 1997b
Unclassified				
PP1bp80		Regulation of chaperones?	Skeletal muscle	Damer et al., 1998
MYPT3 *PPP1R16A*	+	?	Widely distributed microsomes and cytosol	Skinner and Saltiel, 2001

Table 1(B). Protein phosphatase 1 regulatory subunits – inhibitor proteins, chaperones and activity modulators

Regulatory subunit	RVxF motif	Physiological function and/or effect on PP1c	Tissue/subcellular location	Reference
I-1 (inhibitor 1) *PPP1R1A*	+	Inhibition of PP1c	Widely distributed cytosol	Huang and Glinsmann 1976; Cohen, 1989
DARPP-32 (dopamine and cAMP-regulated phosphoprotein M_r 32000) *PPP1R1B*	+	Inhibition of PP1c Integration of neurotransmitter signals in the neostriatum	Brain, kidney, cytosol	Hemmings et al., 1984; Shenolikar and Nairn, 1991
I-2 (inhibitor 2) *PPP1R2*	(+)	Molecular chaperone, inhibition of PP1c	Widely distributed, cytosol and nucleus	Huang and Glinsmann 1976; Cohen 1989
Inhibitor-3 (HCG V) *PPP1R11*	+	Inhibition of PP1c?	Widely distributed	Zhang et al., 1998
CPI-17 (PKC potentiated inhibitor) *PPP1R14A*		Inhibition of PP1c	Smooth muscle	Eto et al., 1997
PHI-2 (phosphatase holoenzyme inhibitor) *PP1R14B*	+	Inhibition of PP1 holoenzymes	Widely distributed	Eto et al., 1999
I_1^{PP2A} (PHAP1)		Stimulation of PP1c and inhibition of PP2A?	Widely distributed	Katayose et al., 2000
I_2^{PP2A} (SET, PHAPII, TAF1β)		Stimulation of PP1c and inhibition of PP2A?	Widely distributed	Katayose et al., 2000
G-substrate (cGMP-dependent protein kinase substrate)		Inhibition of PP1c	Brain	Aitken et al., 1981; Hall et al., 1999
Grp78 (glucose-regulated protein, member of HSP-70 family)		Molecular chaperone?	Widely distributed, stress inducible	Chun et al., 1994; Chun et al., 1999

The table includes mammalian proteins that are reported to interact with PP1c. An additional nine related proteins have been identified from the human genome sequence (Ceulemans et al., 2001). A testis-specific isoform of I-2 encoded by a separate gene has been reported in the rat (Osawa et al., 1996). *Drosphila* PP1c-interacting proteins, which have no reported mammalian homologue, are Bifocal (Helps et al., 2001), Kinesin-like protein 38 (KLP38) (Alphey et al., 1997) and testis-specific protein related to I-2 (I-t; Helps et al., 1998). Yeast PP1c regulatory subunits are described in Stark (Stark, 1996). Synonyms for the mammalian proteins are placed in brackets. The human genome nomenclature is included (in italics) for those PP1c regulatory subunits that are classified solely as PP1c regulators. PPP1R4, PPP1R5 and PPP1R6 have recently been reclassified as PPP1R3B, PPP1R3C and PPP1R3D as they are all glycogen-binding subunits of PP1c and therefore isoforms. Novel glycogen-binding subunits are PPP1R3E, PPP1R3F and PPP1R3G (see text). If the RVxF motif has been mapped this is indicated by a + sign. (+) indicates that the proposed motif is not a classical RVxF motif. For other proteins, an RVxF motif has not been mapped or may not exist. A question mark indicates that there is no experimental evidence for the proposed physiological function of the PP1c complex. References for the identification of the regulatory subunit as a PP1c-interacting protein are given. References to other properties and functions of these proteins are given in the text.

additional nine related proteins have been identified from the human genome sequence (Ceulemans et al., 2001), and there are likely to be more PP1-interacting proteins (Campos et al., 1996; Ajuh et al., 2000). For >50% of the proteins in Table 1 the interaction with PP1c is well documented in that the complex has been purified and the interaction site mapped. The physiological functions of several PP1 complexes are now well defined, whereas in other cases the site of PP1c binding has not been identified and the in vivo function of the complex has yet to be examined. Surprisingly, the majority of PP1 regulatory subunits (unless they are isoforms) share no significant sequence similarities that can be identified by

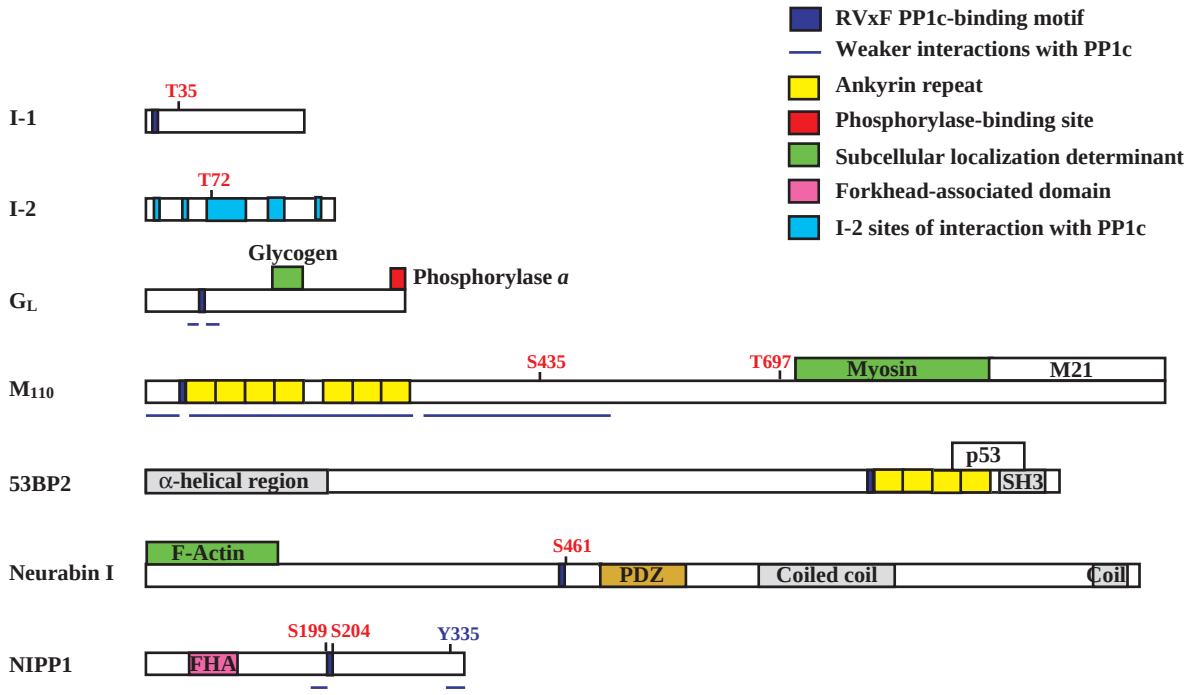

Fig. 1. Schematic representation of several regulatory subunits of PP1 in higher eukaryotes. The RVxF PP1c-binding motif (dark blue) is immediately N-terminal to the ankyrin repeats in M_{110} and 53BP2. The RVxF motif in neurabin I and II precedes the PDZ domain but is not adjacent to it. Regions of weaker interactions with PP1c are indicated by a dark blue-line in G_L, M_{110} and NIPP1 but may turn out to be smaller than indicated when fully documented. Weaker interaction sites have not been mapped for 53BP2 and neurabin I and may not have been fully mapped in other proteins. The Y335 site in NIPP1 may interact with PP1c independently of the RVxF motif. Some binding sites for I-2 (shown in turquoise) act independently of each other. Serine and threonine residues that alter the interaction with PP1c when phosphorylated are indicated (see Table 2). The allosteric binding site for phosphorylase *a* on G_L is indicated. The binding regions for the protein or molecule that determines subcellular localization are shown in green. The binding site for myosin shown on M_{110} is that determined by Johnson et al. (Johnson et al., 1997) but the ankyrin repeat region might also bind to myosin (Hirano et al., 1997). The M_{21} subunit binds to the C-terminal region of M_{110}. In the case of NIPP1, although RNA binds near the C-terminus, the region interacting with the splicing machinery is the FHA domain. All regulatory subunits are drawn to the same scale, M_{110} and I-1 being 1122 and 170 residues in length, respectively.

computer analysis (Fig. 1) and thus cannot be uncovered by database searching. Methods used to identify them include biochemical purification of the PP1 complexes, yeast two-hybrid analyses and screening of expression libraries with labelled PP1.

Nearly ten years ago, it was proposed that PP1 regulatory subunits target PP1c to distinct subcellular locations (Hubbard and Cohen, 1993), and current data support this targeting hypothesis. Table 1A classifies most regulatory subunits into distinct targeting categories and separates them from the 'modulator' proteins in Table 1B; the latter include inhibitor proteins, chaperones and activator proteins, which do not target PP1c to specific subcellular locations. Below I focus on a few of the subunits, before discussing the interactions between PP1c and its regulatory subunits in detail.

A variety of PP1c-targeting subunits can target the enzyme to glycogen. These include G_M, G_L, R5 (also known as PTG) and R6, for which a glycogen-targeting domain has been mapped (Doherty et al., 1996; Armstrong et al., 1997; Armstrong et al., 1998; Wu et al., 1996; Wu et al., 1998). A similar domain is present in three novel forms encoded in the human genome (Ceulemans et al., 2001). Low skeletal muscle glycogen levels in mice carrying a targeted disruption of the gene that encodes G_M confirm a role for G_M in the regulation of glycogen metabolism (Suzuki et al., 2001). G_M can also associate with the sarco(endo)plasmic reticulum in striated muscle through a C-terminal hydrophobic domain (Hubbard et al., 1990; Tang et al., 1991; Berrebi-Bertrand et al., 1998). Two targeting subunits, M_{110} (also known as MYPT1, MBS and M130) and MYPT2, also localise PP1c to myosin, although there is controversy about the location of the myosin-binding site (Hirano et al., 1997; Johnson et al., 1997).

PP1c dephosphorylates a wide range of substrates in vitro. Targeting enhances specificity, permitting PP1c to dephosphorylate only those substrates in the vicinity of the targeted complex. However, interaction of targeting subunits with PP1c also modulates its substrate specificity, most probably by an allosteric mechanism. Binding of G_M enhances the activity of PP1c towards the glycogen-bound substrates glycogen phosphorylase, glycogen synthase and phosphorylase kinase, whereas interaction with the myosin-targeting subunit M_{110} enhances the activity of PP1c towards myosin P-light chains and suppresses its activity towards glycogen phosphorylase (Cohen 1989; Alessi et al., 1992). Most other targeting subunits inhibit phosphorylase phosphatase activity but are not known to enhance activity against other substrates probably because their in vivo substrate is not known.

Several A-kinase-anchoring proteins (AKAPs) keep PKA and PP1c in close proximity, as well as targeting PP1c to particular subcellular locations (Schillace and Scott, 1999).

For example, Yotiao, an AKAP that binds to both PKA and PP1c, is localised in the postsynaptic structure of the neuromuscular junctions in skeletal muscle, interacts with the NMDA receptor and enhances the activity of PP1c towards the NMDA receptor (Feliciello et al., 1999; Westphal et al., 1999). AKAP350 (also known as AKAP450 and CG-NAP) is a scaffolding protein that assembles several protein kinases and phosphatases, including PP1c, at the centrosome throughout the cell cycle and at the Golgi apparatus during interphase (Takahashi et al., 1999).

The protein kinase Nek2, which has been implicated in the regulation of centrosome separation (Fry et al., 1998), interacts directly with PP1c and targets it to centrosomes (Helps et al., 2000). PP1c might keep both Nek2 and the Nek2 substrate C-Nap1 dephosphorylated prior to mitosis. The kinase-phosphatase complex might then act as a molecular switch to rapidly initiate centrosome separation.

NIPP1 was initially identified as a nuclear inhibitor of PP1c that binds to RNA (Van Eynde et al., 1995; Jagiello et al., 1997). It colocalises with pre-mRNA splicing factors in a 'speckled' nuclear distribution and has been implicated in pre-mRNA splicing (Trinkle-Mulcahy et al., 1999). Interaction of NIPP1 forkhead-associated (FHA) domain with pre-mRNA splicing factors targets NIPP1-PP1c to the splicing machinery (Boudrez et al., 2000; Jagiello et al., 2001).

Regulatory subunits of PP1c that do not appear to target it to a particular subcellular location may instead target PP1c to specific substrates. In some cases, such as that of Rb, the regulatory subunit itself is the PP1c substrate. Rb is dephosphorylated during the cell cycle by PP1c, a process that may underlie its growth suppressing properties. Other such regulatory subunits might efficiently prevent PP1c from dephosphorylating neighbouring proteins; for example, 53BP2 potently inhibits the phosphorylase phosphatase activity of PP1c (Helps et al., 2000). 53BP2 interacts with the tumour suppressor p53 (Gorina and Pavletich, 1996) and enhances p53-mediated activation of transcription (Iwabuchi et al., 1998), possibly by facilitating the dephosphorylation of one or more sites on p53. The cytosolic inositol 1,4,5-trisphosphate-binding protein PRIP-1 binds to PP1c (Yoshimura et al., 2001), but the presence of a pleckstrin homology domain raises the question of whether it is targeted to membranes in response to signals that produce particular inositol phospholipid second messengers.

Most modulator proteins are low molecular mass thermostable inhibitor proteins (Table 1B). The existence of many inhibitor proteins that bind to PP1c suggests that the activity of the untargeted free catalytic subunit must be kept under strict control. Some inhibitor proteins are regulated by signalling pathways. DARPP-32 and inhibitor 1 (I-1), for example, are converted to PP1 inhibitors by PKA (Shenolikar and Nairn 1991; Wang et al., 1995), and CPI-17 is converted to a PP1 inhibitor by Rho-associated kinase and/or PKC (Eto et al., 1997).

Interaction of regulatory subunits with PP1c

The RVxF motif

The RVxF motif is a short, conserved PP1c-binding motif initially identified in studies of the glycogen-targeting subunits, G_M and G_L, and the myosin-targeting subunit M_{110}. Although G_M and G_L are only 23% identical, highly conserved sections exist (Doherty et al., 1995), and a 31-residue G_M peptide corresponding to one of these can dissociate the M_{110}-PP1c complex in vitro (Johnson et al., 1996). The same peptide specifically slows the relaxation of permeabilized smooth muscle fibres (Gailly et al., 1996). Crystallisation of a shorter version of this peptide (13 residues) as a complex with PP1c identified the RVxF motif as the main sequence that mediates the interaction with a hydrophobic groove on the surface of PP1c, which is remote from the active site (Egloff et al., 1997). A negatively charged region of PP1c accommodates basic residues preceding the RVxF motif.

Several lines of evidence, not least the mutually exclusive nature of regulatory subunit binding (see above), indicate that highly conserved variations of the consensus (R/K)(V/I)xF mediate binding of regulatory subunits other than G_M and G_L to PP1c. Alignment of the ankyrin-repeat regions in M_{110} and 53BP2 showed that the sole common sequence (apart from the ankyrin repeats) in the PP1c-binding fragments from these proteins is (R/K)VxF, and only peptides encompassing these residues can block the interaction of regulatory subunits with PP1c (Egloff et al., 1997). Regulatory subunit peptides containing the RVxF motif block the interaction of other targeting subunits with PP1, including p99 (Kreivi et al., 1997) (also termed PNUTS) and Nek2 (Helps et al., 2000). Mutation of the valine or phenylalanine residue within this motif can destroy or severely weaken interaction with PP1c, clearly demonstrating that this is a major site of interaction for NIPP1 (Trinkle-Mulcahy, 1999; Beullens et al., 1999), spinophilin (also named neurabin II) (Hsieh-Wilson et al., 1999), Nek2 (Helps et al., 2000) and AKAP 220 (Schillace et al., 2001).

An alternative approach, employing screening of a peptide library with PP1c, identified peptides containing VxF and VxW sequences often preceded by basic residues as PP1c-binding motifs (Zhao and Lee, 1997b). Variants of the (K/R)(V/I)x(F/W) motif in which the basic residue is not adjacent to the valine residue have been identified in mammalian proteins – for example, the sequence RKSVTW in p99 (Kreivi et al., 1997; Allen et al., 1998). Most yeast PP1c regulatory or putative regulatory subunits also possess this type of motif (Egloff et al., 1997). Thus a general consensus for the PP1c-binding motif is (R/K)x_1(V/I/)x_2(F/W), where x_1 may be absent or any residue except large hydrophobic residues, and x_2 is any amino acid except large hydrophobic residues, phosphoserine and probably aspartic acid.

Several studies provide evidence that this motif is critical in vivo. Delivery of the G_M PP1c-binding peptide through a patch pipette into the Hek293 cells transfected with the AKAP yotiao modulates the NMDA receptor currents of cells expressing the NR1A subunit (with which yotiao interacts) but not those of cells expressing the control NR1C subunit (Westphal et al., 1999). Disruption of the interaction between spinophilin and PP1c by introduction of a peptide containing the RVxF motif into neostriatal neurons curtails the regulation of AMPA-glutamate channels by dopamine, which indicates the critical importance of PP1c targeting in the function of spinophilin (Yan et al., 1999). In *Drosophila* rescue of the null *bifocal* mutant with wild-type bifocal protein (a PP1c-targeting protein), but not with bifocal mutant lacking the phenylalanine residue in the PP1c- binding motif,

demonstrates that PP1c targeting is the essential in vivo feature of bifocal that determines morphological changes in photoreceptor cells during development (Helps et al., 2001). In *S. cerevisiae*, Reg1p targets PP1c to hexokinase II (Hxk2p), which it subsequently dephosphorylates. Restoration of Hxk2p dephosphorylation in a *reg1* deletion mutant with wild-type Reg1p but not a mutant lacking a phenylalanine residue in the PP1c binding motif demonstrates that the motif is critical for targeting of PP1c to Hxk2p in vivo (Alms et al., 1999).

The degenerate RVxF motif occurs in >10% of all proteins encoded in the human genome, the majority of which are unlikely to bind to PP1c. So how are particular RVxF motifs selected? Some may be inaccessible, buried in a hydrophobic core, but many must be on the surface. The only regulatory subunit of PP1c for which there is a 3D structure is 53BP2, which has been cocrystallised with p53 (Gorina and Pavletich, 1996). Although no ternary complex of p53, 53BP2 and PP1c has been demonstrated, 53BP2 binds more tightly to PP1c than to p53 (Helps et al., 1995). The C-terminal region of 53BP2 (residues 796-1005) contains the RVxF motif (residues 798-801), four ankyrin repeats and an SH3 domain. The exact structure of the RVxF motif cannot be seen, since this part of the chain is mobile in the crystal, but Fig. 2 shows that the ankyrin repeats form an arm that appears to present the RVxF motif at its tip like a hand. In M_{110}, the RVxF motif is also positioned immediately N-terminal to the ankyrin repeats. It will be interesting to determine whether regulatory subunits that do not possess ankyrin repeats present the RVxF motif in a similar exposed 'hand' ready to grip PP1c.

Targeting subunits also interact with PP1c through additional contacts

Despite the importance of the RVxF motif for the interaction of targeting subunits with PP1c, additional interactions might not only stabilise binding but also provide contacts crucial for modulating the activity and/or specificity of PP1c. Several studies indicate that binding and regulation of activity/specificity can be separated. In M_{110}, the region responsible for enhancing myosin phosphatase activity precedes the KVxF motif and is located within the first 35 residues, whereas the region involved in suppressing phosphorylase phosphatase activity requires the ankyrin repeats that follow the RVxF motif (Johnson et al., 1996; Tanaka et al., 1998). The RVxF motif determines binding of M_{110} to PP1c (Egloff et al., 1997) and, although this region does not affect activity/specificity, it is a prerequisite for three additional interactions (involving the N-terminal region, ankyrin repeats and central region) that modulate activity/specificity (Tóth et al., 2000) (Fig. 1). The basic region preceding the RVxF motif appears to strengthen the binding of G_L to PP1c (Armstrong et al., 1998), whereas a similar region in NIPP1 modulates PP1c activity, the RVxF motif having no effect on activity (Beullens et al., 1999). In spinophilin, a domain preceding the PP1c-binding motif modulates the activity of PP1c, whereas the RvxF motif determines binding (Hsieh-Wilson et al., 1999). Similarly, secondary interactions at sites other than the RVxF motif act synergistically in the AKAP220-PP1c complex to inhibit PP1c (Schillace et al., 2001). Thus the emerging picture for several targeting subunits is that the strongest association with PP1c is through the RVxF motif and that this enables several weaker interactions, which can enhance binding or modulate activity and specificity.

Although most of these secondary interactions are not sufficiently strong to enable the formation of complexes in the absence of the RVxF motif, a second independent interaction of NIPP1 with PP1c, through Tyr 335 near its C-terminus, has been reported (Beullens et al., 2000; Bollen, 2001). This second site remains bound to PP1c in buffers containing 0.05 M NaCl after disruption of the interaction at the RVxF motif and binds to a region of PP1c that is distinct from the RVxF-binding site. However, other studies performed at 0.5 M NaCl concentrations do not detect this secondary interaction (Trinkle-Mulcahy, 1999), indicating that it is much weaker than the interaction with the RVxF motif, which can persist at this salt concentration. AKAP220 also possesses a second such binding site near its C-terminus, and mutation of the RVxF motif significantly decreases but does not completely abrogate binding of an AKAP220 C-terminal fragment to PP1c in an overlay assay (Schillace and Scott, 2001).

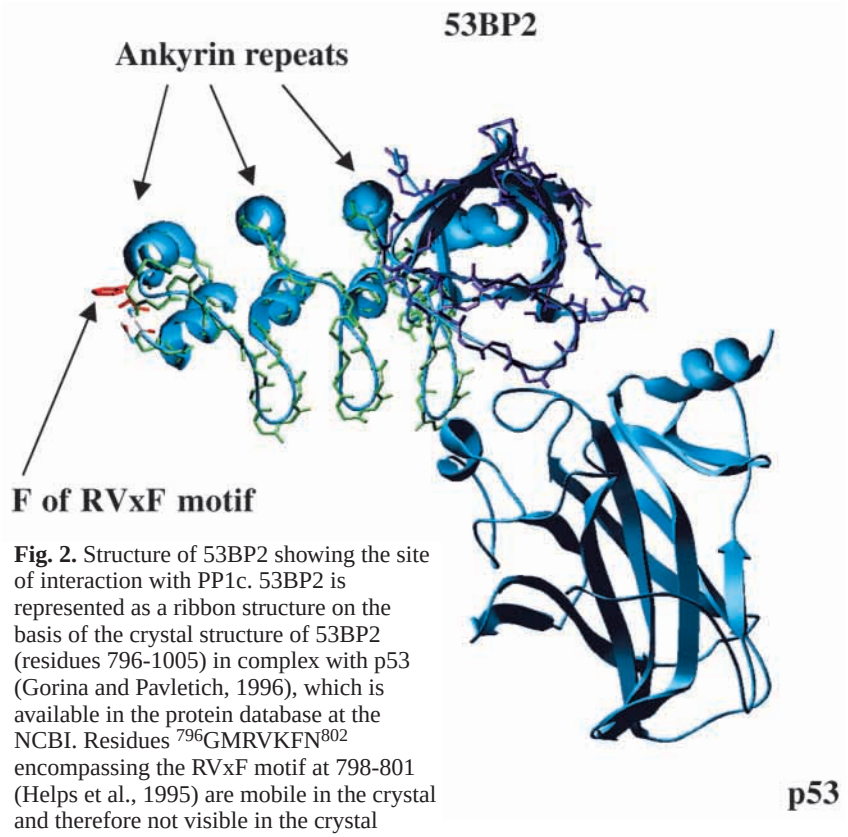

Fig. 2. Structure of 53BP2 showing the site of interaction with PP1c. 53BP2 is represented as a ribbon structure on the basis of the crystal structure of 53BP2 (residues 796-1005) in complex with p53 (Gorina and Pavletich, 1996), which is available in the protein database at the NCBI. Residues ^{796}GMRVKFN^{802} encompassing the RVxF motif at 798-801 (Helps et al., 1995) are mobile in the crystal and therefore not visible in the crystal structure. In the figure, Phe801 (depicted in red) and Asn802 have been added to the structure and illustrate that the position of the RVxF motif is highly exposed at the end of the ankyrin repeats (backbone indicated in green). The backbone of the SH3 domain of 53BP2 is indicated in purple.

Regulatory subunits that do not possess an RVxF motif

Various regulatory subunits that bind to PP1c do not seem to possess RVxF motifs (Table 1). In many cases more data are required to demonstrate specific binding and in vivo interactions; nevertheless, some of these proteins appear to interact with the site in PP1c that binds to the RVxF motif. Human factor C1 (HCF, also known as host cell factor) contains no RVxF motif, but peptides containing the RVxF motif partially block the association of HCF with PP1c, indicating that HCF interacts with the RVxF binding site on PP1c (Ajuh et al., 2000).

Rb interacts with PP1c through a region comprising residues 301-773 (Durfee et al., 1993; Putoni and VillarMoruzzi, 1997; Tamraker et al., 1999). However, there is no discernible RVxF motif in this region of Rb. Binding does not appear to be through interaction of the phosphorylated protein with the catalytic site of PP1c, since it occurs in the presence of toxins that block catalytic activity (Tamraker et al., 1999). It would be interesting to know whether RVxF-containing peptides block the Rb-PP1c interaction.

Interaction of inhibitor proteins with PP1c

Association of inhibitor proteins with PP1c was recognised, nearly two decades ago, to involve at least two sites for binding to PP1c. I-1 and its isoform DARPP-32 are low molecular mass, thermostable inhibitor proteins that potently inhibit PP1c when they are phosphorylated on a threonine residue. The N-terminal sequence of I-1 is required for binding and inhibition, together with pThr, which is presumed to bind at the active site (Aitken et al., 1982; Endo et al., 1996). Synthetic peptides and mutation of DARPP-32 identified an N-terminal KIQF motif (similar to a RVxF motif) preceded by basic residues that is required for inhibition and conserved in I-1 (Kwon et al., 1997a; Huang et al., 1999). Modelling studies also indicated that ^{8}KIQF^{12} in I-1 and DARPP-32 can readily be accommodated at the RVxF-binding site in PP1c (Egloff et al., 1997; Barford et al., 1998).

Inhibitor 2 (I-2) – unphosphorylated – inhibits PP1c. Conversion of PP1c to an active form in the complex can be induced by phosphorylation of I-2 at Thr72 (Cohen, 1989; Bollen and Stalmans, 1992) and suggests a role for I-2 as a molecular chaperone (Alessi et al., 1993; MacKintosh et al., 1996). Deletion and mutagenesis studies have led to the conclusion that several sites in I-2 interact with PP1c: the N-terminal domain is involved in inhibition, whereas other regions control inactivation and reactivation (Park and DePaoli-Roach, 1994). An N-terminal sequence, ^{12}IKGI^{15}, is essential for inhibition (Huang et al., 1999). Another interaction appears to involve the RVxF-binding site on PP1c, as peptides containing the motif effectively attenuate inhibition (Buellens et al., 1999; Helps and Cohen, 1999; Huang et al., 1999; Yang et al., 2000). Alignment of I-2 sequences from lower and higher eukaryotes identified a conserved phenylalanine/tryptophan residue (Trp46 in human I-2), in a sequence similar to the RVxF binding motif, that was essential for full inhibition (Helps and Cohen, 1999). Other mutagenesis studies have indicated that the sequence ^{144}KLHY^{147} might be equivalent to the RVxF motif, and a molecular model involving five interaction sites has been proposed (Yang et al., 2000) (Fig. 1).

Interaction sites on PP1c

The hydrophobic RVxF-binding groove near the C-terminus of PP1c includes residues Ile169, Leu243, Phe257, Leu289-Cys291 and Phe293, which interact mainly with valine and phenylalanine residues in the extended G_M peptide containing the RVxF motif (Egloff et al., 1997) (Fig. 3). The G_M peptide thus runs parallel to the β-strand, β14 (Leu289-Leu296), which forms the edge of this groove. The channel is flanked by a negatively charged region, which can accommodate several basic residues commonly found N-terminal to the VxF sequence in many targeting subunits. Mutation of residues in the hydrophobic groove of yeast PP1c (Glc7p) also implicate this region in binding to regulatory subunits, as several such mutants cannot substitute for wild-type PP1c despite retaining catalytic activity in vitro (Wu and Tatchell, 2001). The hydrophobic groove is remote from the active site, which binds to the substrate and the phosphorylated residues of inhibitor proteins (Fig. 3). Thus modulation of PP1c activity by targeting subunits is likely to involve allosteric transitions, although targeting subunits might also correctly position specific substrates close to the catalytic site. The β12-13 loop, which partially guards the entrance to the active site, is believed to undergo conformational changes readily and is crucially important for binding of tumour promoters and toxins,

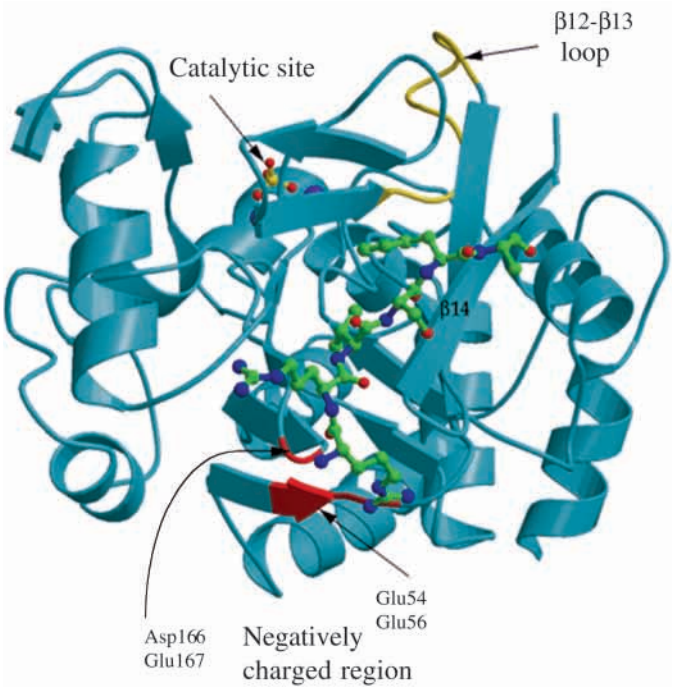

Fig. 3. Structure of the PP1c–RVxF-containing-peptide complex. PP1c is shown as a ribbon structure, and residues RRVSFA of the peptide are shown as green sticks with positively charged groups in blue and negatively charged groups in red. The G_M peptide lies in a groove running parallel to the β-strand β14 (Leu289 –Leu296). The N-terminal arginine residue of the peptide is close to a negatively charged region that is likely to bind to other basic residues that are often found N-terminal to RRVxF. This negatively charged region on PP1c encompasses Glu54, Glu56, Asp166, and Glu67, which are involved in binding to the ^{12}IKGI^{15} motif of I-2. The phosphate analogue tungstate is shown at the catalytic site. The β12-β13 loop containing Cys273 that covalently binds to microcystin is indicated in yellow. The figure was kindly produced by David Barford from the crystal structure of the PP1c-$G_{M[63-75]}$ peptide complex presented at the 1996 FASEB conference on protein phosphatases (Egloff et al., 1997).

including microcystin and okadaic acid (Egloff et al., 1995; Goldberg et al., 1995; MacKintosh et al., 1995; Zhang et al., 1996). Changes in this region can also affect the binding of I-1 and I-2 (Conner et al., 1998; Conner et al., 1999). Consequently, toxins can partially interfere with the binding of inhibitor proteins but do not prevent the binding of targeting subunits to PP1c. The ^{12}IKGI^{15} motif of I-2 binds to a negatively charged region close to the RVxF-binding groove (Conner et al., 2000). This region is in the same location as that proposed to bind basic residues preceding the RVxF motif in many targeting subunits (Egloff et al., 1997). Thus, the major area of PP1c that interacts with regulatory subunits appears to be the hydrophobic groove (to which the RVxF motif binds) and the neighbouring negatively charged region (Fig. 3).

Regulation of PP1c complexes

Much of the current interest in PP1c complexes is centred upon how they are regulated by extracellular and intracellular signals. The best studied of these is reversible phosphorylation, but mechanisms such as allosteric regulation and inducible expression of targeting subunits are now also being shown to be important.

Inducible expression of PP1c targeting subunits

A subject that has intrigued scientists for more than two decades is how insulin activates glycogen synthesis and whether activation is mediated, at least in part, by PP1. Insulin signalling involves a PI-3-kinase-dependent pathway that leads to inhibition of GSK3, which phosphorylates and inhibits glycogen synthase (Cohen, 1999). Thus insulin, by suppressing GSK3 activity, promotes the dephosphorylation and activation of glycogen synthase. The sites phosphorylated by GSK3 are believed to be dephosphorylated by glycogen-targeted forms of PP1c, and insulin might not only inhibit GSK3 but also activate glycogen-targeted form(s) of PP1. In the liver, the glycogen-targeting subunits of PP1c identified are G_L, which is the most abundant, R5 (also known as PTG) and R6. In diabetic rats, in which the insulin-producing pancreatic β-cells have been destroyed by streptozotocin, hepatic glycogen synthase phosphatase activity is substantially decreased but restored by insulin treatment (Bollen and Stalmans, 1984; Bollen and Stalmans, 1992). Examination of the livers of these animals revealed that the levels of G_L and R5 proteins, their associated PP1c activity and mRNAs are substantially decreased in the diabetic state and restored by insulin treatment (Doherty et al., 1998; Browne et al., 2001). Starvation of control animals also reduces the level of G_L and R5 proteins and mRNAs, whereas refeeding restores them (Doherty et al., 1998; O'Doherty et al., 2000; Browne et al., 2001). In contrast, the level of R6 and its associated phosphatase activity was not influenced by these conditions, which indicates that it is not regulated by insulin (Doherty et al., 1998; Browne et al., 2001).

Allosteric regulation

G_L-PP1c provides the best example of allosteric regulation of targeted PP1c complexes by a protein modulator. Studies initiated three decades ago indicated that the level of the activated form of phosphorylase (phosphorylase *a*) modulates hepatic glycogen synthesis by inhibiting glycogen synthase phosphatase activity. Thus, there is a lag period before glycogen synthesis resumes following glycogen breakdown; this lag corresponds to the time taken for phosphorylase *a* to be converted to its inactive form (Stalmans et al., 1971; Miller et al., 1981; Mvumbi et al., 1983; Bollen and Stalmans, 1992). Phosphorylase *a* (at nanomolar concentrations) potently inhibits the glycogen synthase phosphatase activity of a glycogen-targeted form of PP1 by an allosteric mechanism, without affecting the ability of PP1 to dephosphorylate phosphorylase *a* at micromolar concentrations (Alemany et al., 1986; Mvumbi and Stalmans, 1987). More recently, phosphorylase *a* was found to bind to a short section (16 residues) at the C-terminus of G_L, a sequence that is absent from the other glycogen-targeting subunits, G_M, R5 and R6

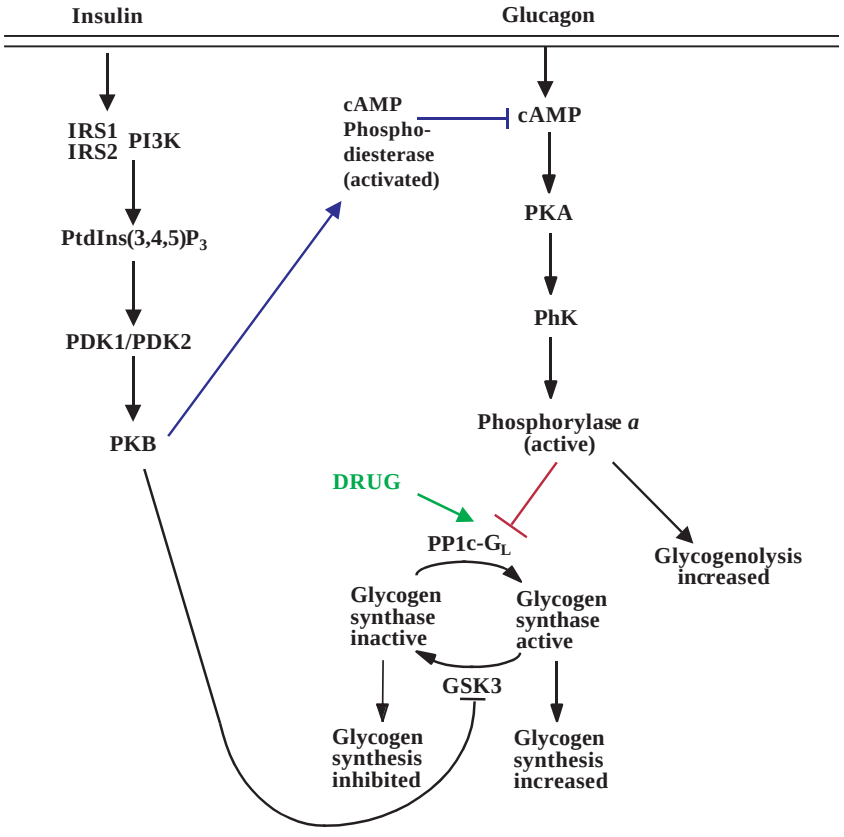

Fig. 4. Pathway by which insulin might stimulate glycogen synthesis through PP1 in the liver. Insulin is thought to activate cyclic-AMP phosphodiesterase, thereby lowering cyclic-AMP and leading to decreased levels of active phosphorylase (phosphorylase *a*). This relieves the phosphorylase *a* allosteric inhibition of G_L-PP1c, increasing glycogen synthase phosphatase activity. Insulin also increases the levels of G_L protein and mRNA.

Table 2. Serine and threonine residues phosphorylated in some targeting subunits with modulation of PP1c interaction

Regulatory subunit	Species	Sequence encompassing RVxF motif	Sites phosphorylated in other regions	Effect of phosphorylation
G_M	Rabbit	60 SSGGRRVSFADN 71	S48	Dissociation
G_L	Rat	56 KKVKKRVSFADN 67	None	
NIPP1	Cow	195 KRKNSRVTFSED 206		Dissociation (central region)
Neurabin I	Rat	452 IPANRKIKFSCA 463		Decreased binding
M_{110}	Rat	30 KRQKTKVKFDDG 41	S435/T697	Activation/inhibition
I-1	Rat/rabbit	4 DNSPRKIQFTVP 15	T35	Inhibition

Amino-acid sequences surrounding the RVxF PP1c-binding motif of several PP1c regulatory subunits are shown. Blue indicates conserved residues in the RVxF motif. Highlighted in red are serine and threonine residues that, when phosphorylated, modulate interaction with PP1c. Sites of phosphorylation that have no known effect on interaction with PP1c are not shown. The effect of the modulation is indicated in the last column. G_M, I-1 and neurabin I are phosphorylated by PKA. NIPP1 is phosphorylated on Ser199 by PKA and on Ser204 by CKII. For the M_{110}-PP1c complex, phosphorylation at Ser 435 by a mitotic kinase leads to stimulation of activity, whereas phosphorylation at Thr697 by Rho-associated kinase (or a related kinase) leads to inhibition.

(Armstrong et al., 1998). Since insulin can lower hepatic cyclic AMP levels, thereby decreasing the level of phosphorylase *a*, one short term action of insulin may be mediated through a decrease in the level of phosphorylase *a*, which alleviates the allosteric inhibition of G_L-PP1c (Armstrong et al., 1998; Bollen et al., 1998) (Fig. 4).

Reversible phosphorylation

Various cellular signalling pathways are believed to regulate phosphorylation of targeting subunits and inhibitor proteins to control PP1c activity in particular PP1c complexes. Probably the majority of these are reversibly phosphorylated, but the effects of phosphorylation on the complexes have been studied in only a few cases. Three unrelated targeting subunits (G_M, neurabin I and NIPP1) are phosphorylated within or close to the RVxF motif, which decreases PP1c binding (Table 2). Phosphorylation can also occur at other sites and may either increase or decrease phosphatase activity. Likewise, phosphorylation of several inhibitor proteins (I-1, DARPP-32, CPI-17 and PHI) strengthens their inhibitory properties, whereas phosphorylation of other inhibitors (I-2) activates PP1c.

The role of G_M and inhibitor 1 phosphorylation in the regulation of glycogen metabolism

Phosphorylation of G_M by PKA in response to adrenaline on Ser48 and Ser67 (Table 2) occurs in vivo, but insulin does not increase phosphorylation at either site (Walker et al., 2000). Both phosphorylation sites are conserved between rabbit and human G_M sequences (Chen et al., 1994b). Since Ser67 lies within the RVxF motif (Egloff et al., 1997), phosphorylation triggers dissociation of PP1c from G_M and thus inactivation of PP1c towards the glycogen-bound substrates, phosphorylase, phosphorylase kinase and glycogen synthase (Hubbard and Cohen, 1993). Coincident phosphorylation of I-1 is believed to lead to inhibition of free PP1c by insertion of pThr 34 at the active site (Cohen, 1989). Targeted disruption of the *I-1* and *G_M* genes in mice indicates that neither is essential for insulin activation of glycogen synthesis in skeletal muscle (Scrimgeour et al., 1999; Suzuki et al., 2001). The activation of glycogen phosphorylase by 10 μM adrenaline is unaffected in I-1-knockout mice, although submaximal doses of hormones were not investigated (Scrimgeour et al., 1999). However, these results are perhaps not surprising since PKA phosphorylates phosphorylase kinase and glycogen synthase directly, as well as G_M and I-1, and the effect of the former phosphorylations may override the more subtle effects of G_M and I-1 phosphorylation.

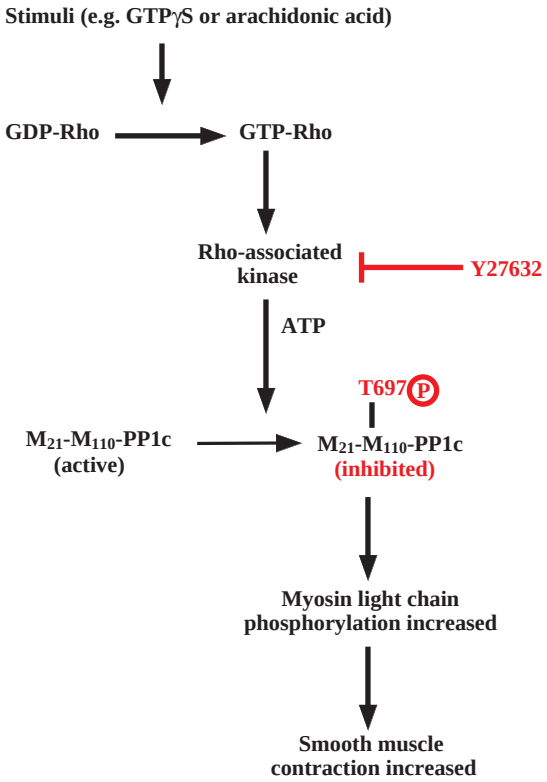

Fig. 5. Pathway by which smooth muscle contractility can be stimulated at constant Ca^{2+} levels. Agonists activating excitatory serpentine receptors coupled to heterotrimeric G-proteins, GTPγS or arachidonic acid convert GDP-Rho-A to its GTP-bound form, which activates Rho-associated kinase. Phosphorylation of M_{110} on Thr697 by Rho-associated kinase leads to inhibition of M_{20}-M_{110}-PP1c activity, thereby increasing the phosphorylation of Ser19 on the regulatory myosin light chain in the absence of alterations in the Ca^{2+}-dependent myosin light chain kinase activity. The pathway is blocked by Rho-associated kinase inhibitor Y27632. Contractility in non-muscle cells can be activated by lysophosphatidic acid through a similar pathway.

The G_L-PP1c complex does not appear to be regulated by phosphorylation. Although G_L possesses a serine residue in its PP1c-binding region (KRVSF), the preceding lysine residue means that the sequence differs from the PKA phosphorylation consensus sequence (RRxS/T) present in G_M (RRVSF). Other glycogen-targeting subunits do not possess a serine residue within the PP1c-binding motif. Perhaps surprisingly, mutation of Ser67 to threonine in G_M results in a G_M-PP1c complex that cannot be phosphorylated and dissociated by PKA (Liu et al., 2000).

Regulation of M_{110} phosphorylation in smooth muscle and non-muscle cells

The Ca^{2+}/calmodulin-regulated myosin light chain kinase (MLCK) mediates smooth muscle contraction in response to a rise in intracellular Ca^{2+} levels by phosphorylating the myosin P-light chains at Ser19. Dephosphorylation is catalysed by a trimeric complex containing PP1c with M_{110} and M_{21}, causing smooth muscle relaxation. However, GTPγS and some agonists can initiate contractility in smooth muscle and non-muscle cells through a different pathway, which can operate at constant Ca^{2+} levels and appears to involve RhoA and Rho-associated kinase (Kimura et al., 1996; Fu et al., 1998; Hartshorne et al., 1998) (Fig. 5). The sites phosphorylated in M_{110} are Thr697 and Thr855 (rat M_{110} isoform 2 sequence), which are distant from the PP1c-binding motif (Feng et al., 1999; Kawano et al., 1999) (Fig. 1; Table 2). Thiophosphorylation of Thr697, but not Thr855, inhibits PP1c activity in vitro, and Thr697 is phosphorylated in 3T3 cells that have been stimulated by lysophosphatidic acid. This effect is blocked by the Rho-associated kinase inhibitor Y27632 (Feng et al., 1999; Uehata et al., 1997).

The smooth muscle inhibitor protein CPI-17 is a potent inhibitor of the M_{110}-PP1c complex, as well as PP1c, when phosphorylated in vitro on Thr38 by PKC (Eto et al., 1997), Rho-associated kinase (Koyama et al., 2000) or a MYPT-associated kinase (MacDonald et al., 2001). Stimulation of smooth muscle by agonists leads to phosphorylation of Thr38 (Kitazawa et al., 2000) and thus would be expected to represent an additional mechanism for inhibition of the M_{110}-PP1c complex.

Totsukawa et al. have detected a mitosis-specific phosphorylation of M_{110} in cultured rat embryo cells by immunoblotting with an antibody that recognises only the dephosphorylated epitope of M_{110}, which is present during interphase (Totsukawa et al., 1999). Using mitotic and interphase *Xenopus* egg extracts as the source of kinase, they mapped the phosphorylation site to Ser435 in rat M_{110}. Phosphorylation of Ser435, in contrast to Thr697 phosphorylation, increases the myosin light chain phosphatase activity of the M_{110}-PP1c complex and also enhances the binding of M_{110} to myosin II. This cell-cycle-dependent phosphorylation suggested the presence of an additional signalling pathway in non-muscle cells, which has been proposed to operate at the onset of mitosis and involve a mitotic kinase. Activation of myosin phosphatase during mitosis and dephosphorylation of myosin light chains would be expected to lead to disassembly of microfilaments during prophase, whereas reversal at cytokinesis should lead to reassembly.

Regulation of NIPP1 by phosphorylation

In nuclear extracts, NIPP1 is present as an inactive complex with PP1c. This heterodimeric complex can be activated by phosphorylation of the central domain of NIPP1 by PKA and CKII at sites within and close to the RVxF motif (Vulsteke et al., 1998) (Table 2). Native hepatic NIPP1 has a reduced affinity for PP1c after phosphorylation by PKA in vitro and after glucagon-induced phosphorylation in vivo (Jagiello et al., 1995). Further regulation of NIPP1 may occur through its N-terminal region, which consists of a forkhead-associated (FHA) domain, a known phosphopeptide-interaction module (Li et al., 2000). The FHA domain of NIPP1 interacts with CDC5L, a human homologue of *S. pombe* Cdc5p, which regulates pre-mRNA splicing, and this interaction depends on the phosphorylation of CDC5L by kinases such as cyclin-E-Cdk2 (Boudrez et al., 2000).

Regulation of neurabin I by phosphorylation

The actin-binding, PDZ-containing protein neurabin I may link the actin cytoskeleton to the plasma membrane and is required for neurite outgrowth and synapse formation (Nakanishi et al., 1997). Neurabin I binds to and inhibits PP1c (MacMillan et al., 1999; McAvoy et al., 1999). In vitro phosphorylation of Ser461 by PKA, which is located immediately C-terminal to the RVxF motif, decreases binding of neurabin I to PP1c, although it does not cause complete dissociation. The Ser461-Glu mutation, which mimics phosphorylation by PKA, reduces the inhibitory activity of neurabin I towards PP1c, raising the possibility that the complex participates in a cyclic AMP-PKA signalling mechanism (McAvoy et al., 1999).

Role of DARPP-32 and inhibitor-1 phosphorylation in brain signalling cascades

The signalling pathways involving the dopamine- and cAMP-regulated phosphoprotein DARPP-32 and PP1c in the brain have been studied extensively (Shenolikar and Nairn, 1991; Greengard et al., 1998; Price and Mumby, 1999). Indeed, Paul Greengard was awarded a Nobel Prize in 2000 for his contributions to the understanding of dopamine-regulated signalling cascades, particularly the important role played by DARPP-32 in areas of the brain (neostriatum) receiving high dopaminergic input. PKA-catalysed phosphorylation of DARPP-32, an isoform of I-1 highly expressed in brain, is critical for its inhibition of PP1c and for crosstalk between different signalling pathways in postsynaptic regions of neurons. The process is implicated in linking dopamine receptor activation to changes in membrane potential and/or receptor modulation. The neurotransmitter dopamine, acting on D1-like receptors, causes activation of PKA and phosphorylation of DARPP-32 on Thr34, leading to inhibition of PP1c (Hemmings et al., 1984; Hemmings et al., 1989). Conversely, glutamate (another neurotransmitter) acting on NMDA receptors, increases Ca^{2+} entry, stimulating PP2B/calcineurin, which causes the dephosphorylation of DARPP-32 and thus activation of PP1c (Halpain et al., 1990). Activation of D2-like receptors may also stimulate PP1 through dephosphorylation of DARPP-32 catalysed by the Ca^{2+}/calmodulin-dependent PP2B or inhibition of PKA (Nishi et al., 1999). By contrast, activation of adenosine A2 receptors leads

to the phosphorylation of DARPP-32 and inhibition of PP1 (Svenningsson et al., 2000). Targets of PP1 activity in dopaminergic neurons include neurotransmitter receptors and ion channels such as the NR1 subunit of the NMDA glutamate receptor (Snyder et al., 1998), the AMPA-type glutamate receptor (Yan et al., 1999), the $GABA_A$ receptor β1 subunit (Flores-Hernandez et al., 2000) and the Na^+/K^+ATPase ion pump (Aperia et al., 1991; Fiscone et al., 1998). Targeted disruption of the *DARPP-32* gene produces mice that lack or have greatly diminished responses to dopamine, psychostimulant and antipsychotic drugs, decreased dopamine-induced phosphorylation of the NR1 subunit of the NMDA receptor and altered dopamine-induced activities of several ion channels (Fienberg et al., 1998). These studies confirm the important role played by DARPP-32 in integrating neuronal signalling cascades that modulate responses to dopamine (Fienberg and Greengard, 2000).

The activity of DARPP-32 is regulated by phosphorylation at several sites other than Thr34. Surprisingly, phosphorylation of DARPP-32 by Cdk5 at Thr75 prevents the phosphorylation of DARPP-32 by PKA at Thr34 and hence inhibition of PP1 (Bibb et al., 1999). Since a Cdk5 inhibitor increases dopamine-induced phosphorylation of other PKA substrates, this regulation might operate in vivo.

PP1 has been implicated in the process of long-term depression (LTD), a stimulation-dependent decrease in synaptic efficacy observable in postsynaptic neurons (Mulkey et al., 1994). Addition of a fragment of I-1 thiophosphorylated on Thr35 by PKA blocks LTD and requires active PP2B for this effect. It has been suggested that a protein phosphatase cascade involving the dephosphorylation of I-1 by the Ca^{2+}/calmodulin-dependent PP2B and the subsequent activation of PP1 is required for the generation of LTD (Mulkey et al., 1994). More recently, long-term potentiation (LTP), a synaptic mechanism thought to be involved in learning and memory and mediated by an increase in synaptic efficacy, was shown to be deficient in I-1-knockout mice at perforant path-dentate cell synapses but not at other synapses (Allen et al., 2000). However, the performance of the mice in spatial learning tests was unaffected. Like DARPP-32, I-1 has recently been reported to be phosphorylated by Cdk5 (at Ser65), which converts it to a less efficient substrate for PKA (Bibb et al., 2001).

Deregulation of PP1 by viruses

Mammals respond to infections by dsRNA viruses by producing interferons that induce the synthesis of the dsRNA-activated protein kinase (PKR). This kinase then phosphorylates eIF2α to shut down host protein synthesis, which destroys the cell and thereby prevents viral replication. New, virus-free, cells are then produced by the body. To circumvent this, herpes simplex virus produces a protein, $\gamma^{34.5}$, which is homologous to GADD34 and binds to PP1c through an RVxF motif. The multicomponent 340 kDa complex formed dephosphorylates eIF2α to prevent the shut off of host protein synthesis (He et al., 1997).

HCF is required for progression through the G1 phase of the cell cycle (Goto et al., 1997) and comprises a family of 110-300 kDa human proteins generated by processing of a precursor protein encoded by a single gene (Wilson et al., 1993). HCF binds to and potently inhibits the phosphorylase phosphatase activity of PP1c (Ajuh et al., 2000). During infections with herpes simplex virus, HCF plays a vital role in the transcription of the intermediate early genes of the virus by forming a complex with the viral protein VP16 and the host cell protein OCT1. As interaction of PP1c and VP16 with HCF is mutually exclusive, it appears that HCF dissociates from PP1c to interact with VP16. Thus, dissociation of host proteins from PP1c and binding of viral proteins such as $\gamma^{34.5}$ to PP1c are newly discovered mechanisms that appear to be crucial for viral replication in mammalian cells.

Conclusions

Recent research has uncovered a large number of bona fide and putative regulatory subunits of PP1. In vivo evidence for the crucial functional importance of PP1-targeting subunits has come from gene disruption, peptide inhibitor studies and mutational analyses. The interaction of different targeting subunits with the same region of PP1c (which encompasses the RVxF-binding groove and adjacent negatively charged region) explains the mutually exclusive nature of the binding of targeting subunits. However, further experiments are needed if we are to determine whether a second binding site, in proteins such as NIPP1 and AKAP220, can initiate interactions with a completely different region of PP1c. Although I-2 can form a ternary complex with G_M-PPIc (Strålfors et al., 1985), and PHI (an isoform of CPI-17) has recently been observed to form a ternary complex with M_{110}-PP1c (Eto et al., 1999), ternary complexes between PP1c and two different targeting subunits have not been observed.

Targeting to a specific subcellular location is important for other members of the PPP family. PP2A has a dimeric core, containing a catalytic subunit and a regulatory subunit. The latter binds to a large number of variable subunits in a mutually exclusive manner. Many of these are targeted to specific subcellular locations (Wera and Hemmings, 1995; Janssens and Goris, 2001). It seems likely that this mode of regulation will also be important for PP4 and PP6 (Cohen, 1997).

The observed alterations in the levels of PP1c-targeting subunits, as in the case of G_L (Doherty et al., 1998), raise some interesting questions: for example, upon the disappearance of the targeting subunit, is PP1c distributed among the other targeting subunits or, as would appear more likely, is it inhibited by inhibitor proteins and/or rapidly degraded? Although the first scenario may present some problems, glycogen-targeted PP1c is only ~10% of total hepatic PP1c, and therefore redistribution among about 50 other complexes might not affect these processes significantly. The timing of induction of PP1c-targeting subunits may be particularly important during development and is probably coordinated with synthesis of PP1c.

A current aim of Ser/Thr phosphatase research is to develop drugs that interfere with these enzymes in a manner that would be useful for the treatment of human disorders. Although the widely used immunosuppressants cyclosporin and FK506 target the catalytic site of PP2B/calcineurin, this approach does not seem promising for PP1, given its interaction with numerous targeting subunits. In order to obtain drugs that modulate a specific PP1 function, it would be preferable to interfere with the interaction between the targeting subunit and

the catalytic subunit, the binding of the targeting subunit to the target or the interaction of the targeting subunit with a regulator. At first sight, it might seem difficult to block the interaction of one targeting subunit with PP1c without affecting other targeting subunits that bind to the same region. However, this was the argument initially used to suggest that it would be impossible to target specific kinases, since all ~500 belong to the same superfamily and bind ATP at the active site. Yet many specific protein kinase inhibitors that compete with ATP have recently been developed and are effective because each drug makes some contacts with residues outside the conserved ATP-binding pocket (Cohen, 1999). Targeting of specific protein-protein interactions with a small-molecule drug requires only that the site of interaction is not too large, as is the case for the PP1c-binding region. Sites of interaction of targeting subunits with the target have not been precisely mapped, except in the case of the glycogen-targeting subunits. The discovery that only a small region at the C-terminus of G_L interacts with the allosteric regulator, phosphorylase *a*, provides a rationale for searching for small molecules that block this interaction and relieve the inhibition of glycogen synthase phosphatase and thus increase glycogen synthase activity (Armstrong et al., 1998) (Fig. 4). Raising hepatic glycogen synthesis by this mechanism should lower blood glucose levels and be beneficial in disorders such as diabetes, in which hyperglycaemia is a severe problem (Armstrong et al., 1998).

Drugs that target the regulation of PP1c complexes are also promising. A relatively specific inhibitor of Rho-associated kinase that decreases the phosphorylation of M_{110} at Thr697 has been developed and shown to activate smooth muscle myosin light chain phosphatase and decrease phosphorylation of myosin light chains. This should cause relaxation of smooth muscle such as that in the arterial wall, which explains why the drug can normalise blood pressure in animal models of hypertension (Uehata et al., 1997). An inhibitor that decreases the interaction of M_{110} with PP1c may also be useful. Similarly, modulation of levels or activities of DARPP-32 and the PP1c complexes targeted to receptors and ion channels offer promise for intervention in neurological disorders. Finally, drugs that bind to viral proteins, such as herpes simplex virus $\gamma^{34.5}$ and interfere with their interaction with PP1c might be beneficial to combat certain viral infections.

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