Purification and characterisation of p99, a nuclear modulator of protein phosphatase 1 activity

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Abstract We have purified a form of protein phosphatase 1 (PP1) from HeLa cell nuclei, in which the phosphatase is complexed to a regulatory subunit termed p99. We report here the cloning and characterisation of the p99 component. p99 mRNA is widely expressed in human tissues and immunofluorescence analysis with anti-p99 antibodies showed a punctate nucleoplasmic staining with additional accumulations within the nucleolus. The C-terminus of p99 contains seven RGG RNA-binding motifs, followed by eleven decapeptide repeats containing six or more of the following conserved residues (GHRPHEGPGG), and finally a putative zinc finger domain. Recombinant p99 suppresses the phosphorylase phosphatase activity of PP1 by >90% and the canonical PP1-binding motif on p99 (residues 396–401) is unusual in that the phenylalanine residue is replaced by tryptophan.

Key words: Protein phosphatase 1; Nucleus; Immunocytochemistry; Cell regulation

1. Introduction

Protein phosphatase 1 (PP1), one of the major serine/threonine-specific protein phosphatases of eukaryotic cells, is controlled by accessory proteins termed targetting subunits, that regulate its substrate specificity, determine its subcellular localisation and confer the ability to be regulated by extracellular signals [1]. A number of cytosolic targetting subunits have been identified, of which the best characterised are those that direct PP1 to glycogen and to myosin. A small peptide from the glycogen-targetting subunit has been cocrystallised with PP1 and the structure solved to 3 Å resolution [2]. This analysis revealed that the hexapeptide sequence Arg-Arg-Val-Ser-Phe-Ala interacts with a small hydrophobic channel on the surface of PP1 [2]. Similar Arg/Lys-Arg/Lys-Val/Ile-Xaa-Phe sequences are present in many other PP1-targetting proteins and, in the case of the myosin-targetting subunit [3] and p53BP2 [2,4], there is direct evidence that this motif is likely to be important in binding to PP1.

PP1 activity is not only present in the cytosol, but is also located and even enriched in the nucleus [5,6] where it is likely to regulate nuclear processes including pre-mRNA splicing [7,8] and the cell division cycle [9–11]. Two major forms of nuclear PP1 have been resolved by anion exchange chromatography of rat nuclear extracts [12]. The species eluting at a lower concentration of NaCl comprised PP1 complexed to a 41 kDa RNA-binding protein [13], which has been cloned, characterised and termed NIPP1 (nuclear inhibitor of PP1) because it prevents the dephosphorylation of a number of substrates by PP1 [14,15]. The second major species of PP1 that eluted at a higher concentration of NaCl than the PP1-NIPP1 complex was found to comprise PP1 complexed to a PP1-binding protein of apparent molecular mass 111 kDa [12].

We have previously identified a role for PP1 in the regulation of pre-mRNA splicing [7,8]. To extend these analyses, we have investigated proteins present in HeLa nuclear extracts which interact strongly with PP1. Here we report the purification, cloning and characterisation of a novel human nuclear PP1-binding protein that we term p99.

2. Materials and methods

2.1. Materials

HeLa cell nuclear extracts were purchased from Computer Cell Culture Center (Mons, Belgium), DEAE-Sepharose from Pharmacia (Milton Keynes, UK) and endoproteinase Glu-C from Boehringer Mannheim (East Sussex, UK). The EST clone (Genbank accession: R11639) encoding a fragment of p99 (residues 45–310) was provided by U.K. HGMP Resource Centre (Cambridge, UK), and antibodies against the protein encoded by R11639 were generated by the Scottish Antibody Production Unit (Carluke, Ayrshire, UK). Microcystin-Sepharose was provided by Dr. Carol MacKintosh (MRC Protein Phosphorylation Unit, Dundee) and peptides were synthesised by Mr. F.B. Caudwell (MRC Protein Phosphorylation Unit, Dundee) or by Dr. Grahame Bloomberg (Department of Biochemistry, University of Bristol, UK).

2.2. Purification of the p99-PP1 complex

HeLa cell nuclear extract was fractionated on an 8×5 cm column of DEAE-Sepharose equilibrated with 20 mM HEPES pH 7.9, 0.1 M KCl and 0.05% (by mass) Triton X-100 (Buffer A). The flow-through containing the NIPP1-PP1 complex was collected and the p99-PP1 complex was eluted with Buffer A containing 0.25 M KCl. The 0.25 M KCl eluate was then chromatographed on a 1.4×0.8 cm column of microcystin-Sepharose [16]. The flow-through was reapplied to the column a second time to ensure that all of the p99-PP1 complex was bound and, after washing with Buffer A, bound proteins were eluted with Buffer A containing 3 M NaSCN.

2.3. Protein blotting analysis

Human PP1 (the γ1 isoform) [4] was labelled with digoxygenin (DIG-PP1) and used in Far Western assays essentially as described [17]. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using a semi-dry transfer apparatus. After transfer, the filters were blocked in 20 mM Tris/HCl pH 7.5, 150 mM NaCl and 2% non-fat dry milk (w/v). The anti-R11639 serum was used at a 1:4000 dilution in the same buffer, while DIG-PP1 was used at 1 μg/ml of this buffer. The secondary antibodies from Pierce (Chester, UK) were used according to the manufacturer's recommendations.

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2.4. Construction of vectors for expression of proteins in E. coli

The DNA was cut out from the EST-R11639 clone using restriction enzymes *Xho*I and *Hin*dIII, and subcloned into the same sites in pRSET B (Invitrogen, NV Leek, The Netherlands). Complementary oligonucleotides (5′-GGGATCCATGGGTTCGGGTCCCATAGACCCCAAAGAACT-3′) and (5′-CAAGCTTAGAAAACCCCCAGGAACCCAAGCAAGTG-3′) to the clone Fb19 were synthesised which incorporated *Bam*HI and *Hin*dIII restriction sites. A p99 cDNA was obtained using these oligonucleotides in a PCR amplification with HeLa cell poly(A)+cDNA. A DNA fragment of the expected size was isolated, cleaved with *Bam*HI and *Hin*dIII and cloned into the same restriction sites in expression vector pRSET A (Invitrogen).

2.5. Expression of proteins in E. coli and their purification

p99 and its fragments expressed from pRSET vectors contain an additional 36 residues at their N-termini which include a hexahistidine tag for affinity purification on nickel-nitrilotriacetate and an enterokinase recognition site. All p99 constructs were transformed into *E. coli* AD494 (DE3) pLys S cells. Single colonies were grown at 37°C in LB medium (1l) containing 0.1 mg/ml ampicillin (Sigma, Poole, UK) to an A 595 nm of 0.5. Protein expression was induced by the addition of IPTG (3 mM) for 3 h. Cells were harvested and resuspended in 15 ml of 20 mM HEPES pH 7.9, 0.5 M NaCl containing 'complete' proteinase inhibitor cocktail (Boehringer, one tablet per 50 ml) (Buffer B) and 6 M guanidinium-HCl. The suspension was sonicated, centrifuged for 25 min at $20\,000 \times g$, and the supernatant decanted and added to 2 ml of nickel-nitrilotriacetate agarose (QIAGEN, Sussex, UK) pre-equilibrated in Buffer B plus 6 M guanidinium-HCl. After mixing end over end for 1 h, the suspension was poured into a column, and the beads washed with the same buffer containing 20 mM imidazole until no protein could be detected in the eluate. The bound proteins were eluted with Buffer B plus 0.6 M imidazole, dialysed against 20 mM HEPES pH 7.9, 50 mM KCl, 0.1% (by vol) 2-mercaptoethanol and stored in aliquots at −20°C. Human PP1α [18] and human PP1γ [4] were expressed and purified as described.

2.6. Confocal microscopy

HeLa cells were grown on coverslips in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and 100 U/ml penicillin and streptomycin (Gibco BRL, Berlin, Germany). Cells were washed in PBS and fixed for 10 min with 3.7% paraformaldehyde in CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 2 mM EDTA) at room temperature. Permeabilisation was performed with 1% (by mass) Triton X-100 in CSK buffer for 15 min at room temperature. Immunofluorescence staining using affinity purified polyclonal sheep antibodies to the p99 fragment (residues 45–310) and Texas Red or FITC conjugated anti-sheep secondary antibodies (Jackson Immunoresearch Laboratories, Stratech Scientific Ltd., UK) was performed as described [19]. Fluorescence microscopy was carried out using the Zeiss LSM 410 Confocal Laser Scanning Microscope with excitation wavelengths of 488 nm (FITC) and 543 nm (Texas Red).

2.7. Assay of PP1

^{32}P-labelled rabbit skeletal muscle phosphorylase (containing 1.0 mol phosphate per mol subunit) was prepared by phosphorylation with phosphorylase kinase. PP1 was assayed by the dephosphorylation of phosphorylase (10 μM) as described [20].

3. Results and discussion

3.1. Purification of the PP1-interacting protein p99 from HeLa cell nuclear extracts

Proteins in nuclear extracts from HeLa cells were separated by SDS-PAGE, blotted on to a nitrocellulose membrane and probed with digoxigenin-labelled PP1Cγ (DIG-PP1) to identify putative PP1-binding proteins [17]. Using this technique, we detected two major PP1-binding proteins with apparent molecular masses of 41 and 114 kDa (Fig. 1), similar to the results obtained previously for rat liver nuclear extracts [12]. We infer that the 41 kDa protein was NIPP1 since it was recognised by anti-NIPP1 antibodies (data not shown).

In order to purify the form of PP1 that was complexed to the larger protein, we first chromatographed HeLa cell nuclear extracts on DEAE-Sepharose to resolve this species from the PP1-NIPP1 complex (Fig. 1). The eluate containing the larger interacting protein but not NIPP1 was then subjected to affinity chromatography on microcystin-Sepharose (Section 2.2) which binds PP1 and PP2A (and hence the proteins with which they interact) [16]. The microcystin-Sepharose eluate contained six major protein staining bands, two of which corresponded to the 114 kDa PP1-binding protein and the catalytic subunit of PP1 (Fig. 1). A number of smaller bands were not characterised further. These may either be the result of proteolysis or alternatively could be subunits of other phosphatases, such as PP2A.

The protein band of interest was cut out from the gel, digested with endoproteinase Glu-C and, after chromatography on a Vydac C_{18} column (Hesperia, CA, USA), five peptides were sequenced. These sequences were then used to interrogate protein databases which identified several expressed sequence tags (ESTs) containing DNA sequences that could potentially code for this protein. The longest of these (R11639) had an open reading frame (ORF) of 266 residues and included two of the peptides identified from the purified protein sample. This fragment was expressed in *E. coli*, purified as a 38 kDa protein (Fig. 2A), and used to raise antibodies in sheep. These antibodies specifically recognised a band in nuclear extracts that comigrated with the 114 kDa band detected by DIG-PP1 labelling (Fig. 2A and B), establishing that EST-R11639 encodes a fragment of p99.

At this juncture, a cDNA sequence termed Fb19 was de-

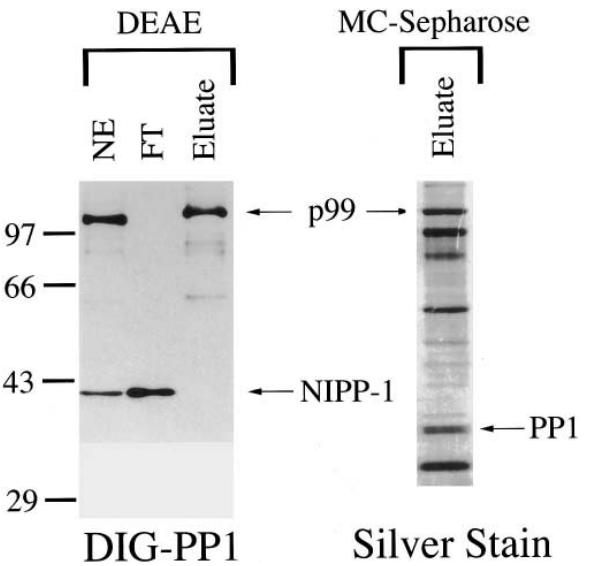

Fig. 1. Purification of p99 from HeLa cell nuclear extracts. Proteins from the nuclear extract (NE), the DEAE flow-through (FT) and the 0.25 M KCl eluate (Eluate) were separated by SDS-PAGE and transferred to nitrocellulose membranes. PP1-interacting proteins were detected by probing the filters with DIG-PP1. The eluate was further purified using microcystin (MC)-Sepharose and bound material eluted with 3 M NaSCN (Eluate), subjected to SDS-PAGE and silver stained. The positions of p99, NIPP-1, PP1 and the marker proteins glycogen phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa) are indicated.

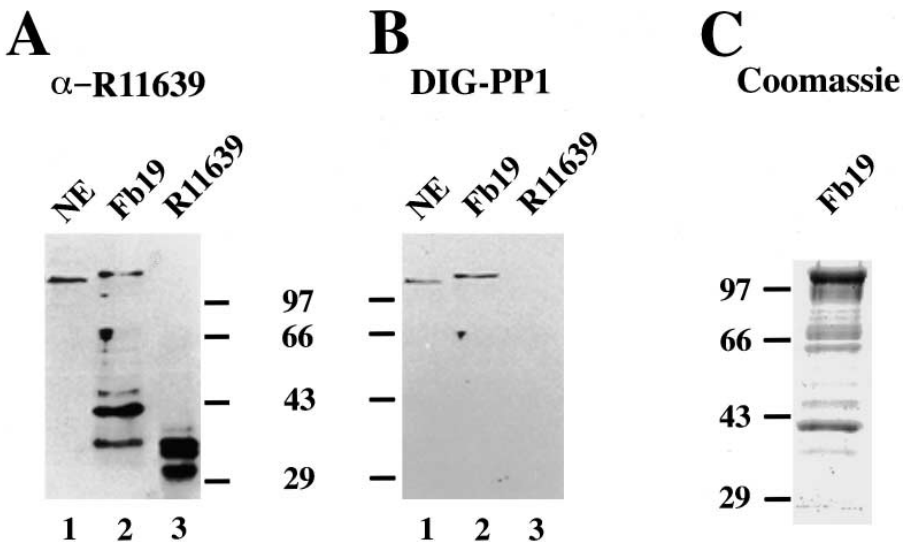

Fig. 2. Identification of clone Fb19 as full-length p99. A: Nuclear extract (NE), Fb19 protein expressed in *E. coli* and purified on nickel-nitrilotriacetate agarose (Fb19) and the fragment of p99 encoded by EST-R11639 (R11639) were separated by SDS-PAGE, transferred to nitrocellulose and probed with the anti-R11639 antibody. B: The filter from A was stripped and reprobed with DIG-PP1. C: Coomassie blue-stained gel showing the Fb19 protein after purification from *E. coli* extracts.

posited into Genbank which not only contained EST-R11639, but also the remaining three peptides identified by protein sequencing (Fig. 3). A cDNA corresponding to the ORF of Fb19 was obtained by PCR amplification of HeLa cell poly-(A)+cDNA and expressed in *E. coli* as a fusion protein with a hexahistidine tag at the N-terminus. After purification, this protein migrated on SDS-PAGE as a major protein staining band (Fig. 2C) that was recognised by DIG-PP1, and which had a slightly slower mobility than 114 kDa (Fig. 2B); this is consistent with the 36 residue N-terminal extension of the expressed protein (Section 2.5). The preparation also contained a number of minor bands of lower molecular mass (Fig. 2A and C) that were not recognised by DIG-PP1 (Fig. 2B), and which are probably proteolytic fragments. Taken together, these results show that the Fb19 cDNA encodes a protein that is either very similar or identical to the 114 kDa PP1-interacting protein.

The Fb19 protein, hereafter termed p99, comprises 940 residues with a predicted molecular mass of 99 kDa, and no obvious homologies with other protein sequences have been detected. The initiating methionine is followed by a glycine residue, suggesting that during translation the methionine is removed and that the glycine is likely to become myristylated. The presence of myristyl groups and other potential post-translational modifications, such as phosphorylation, could explain why endogenous p99 migrates with an apparent molecular mass of 114 kDa. Sequence analysis also shows five consecutive basic amino acids between residues 157–161 and 306–310 and six basic amino acids over the sequence between residues 391–397, some of which may play a role in nuclear import by acting as nuclear localisation signals [23]. The C-terminal half of the molecule is extremely rich in glycine and proline residues and there are seven Arg-Gly-Gly boxes be-

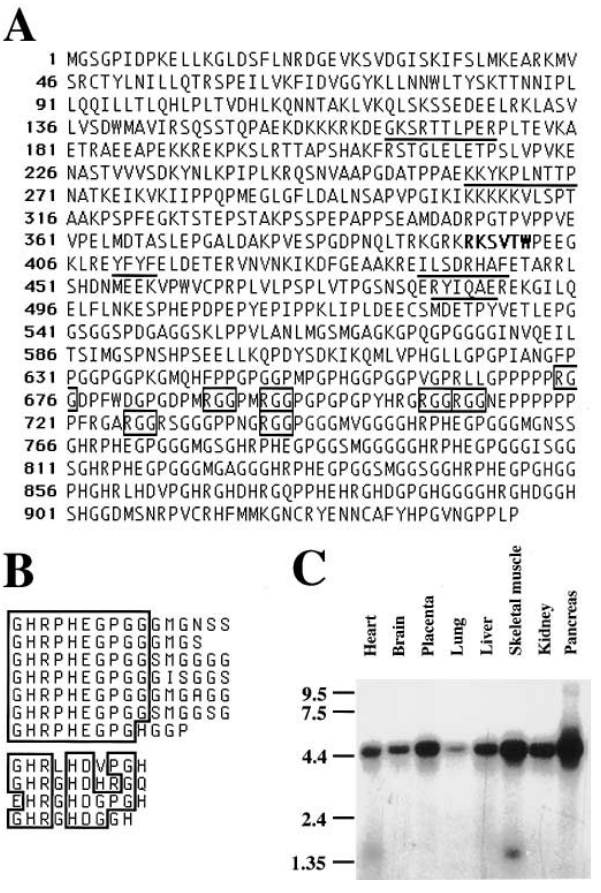

Fig. 3. The amino acid sequence of p99 and its expression pattern in human tissues. A: The protein sequence of Fb19 is shown using the one-letter code for amino acids. The sequences of the endoproteinase Glu-C generated proteolytic fragments of p99 are underlined and the PP1-binding motif is shown in boldface type. The RGG repeats at the C-terminus are boxed. B: Alignment of the seven highly conserved decapeptide repeats and the four imperfect repeats between residues 750 and 898 of p99. C: Northern blot analysis of various human tissues. Each lane contains 2 μg of poly(A)+RNA isolated from the indicated tissues. The blot was hybridised with a *Pst*I DNA fragment from EST R11639 as probe (corresponding to nucleotides 833–1316 of Fb19 cDNA).

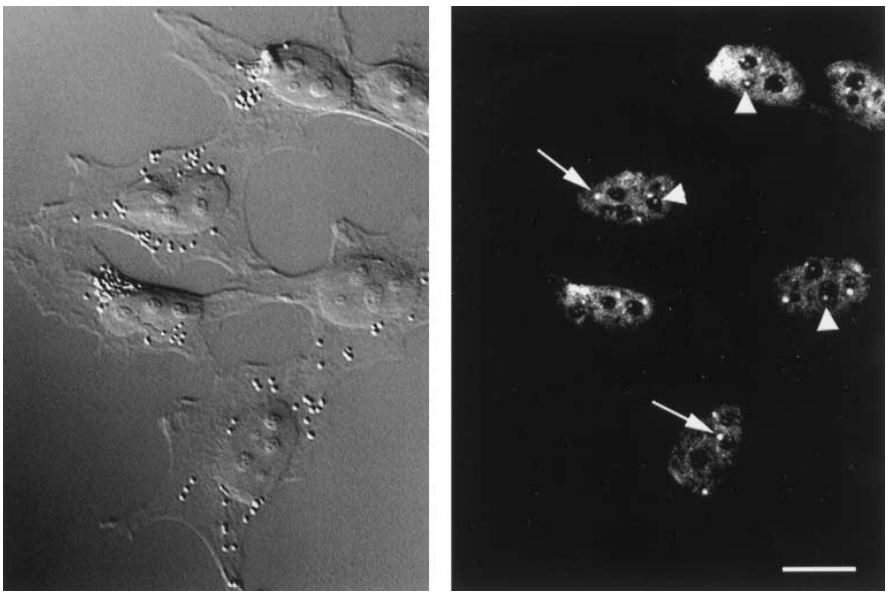

Fig. 4. Confocal image of paraformaldehyde fixed, Triton X-100 permeabilised HeLa cells stained with affinity purified sheep polyclonal antibody to the EST-R11639 fragment of p99 and Texas Red conjugated anti-sheep IgG secondary antibody. As shown, the nuclear staining pattern is punctate, with increased labelling at discrete nucleoplasmic bodies (arrows) and additional accumulations within the nucleoli (arrowheads).

tween residues 674 and 740 (boxed in Fig. 3A). Such repeats have been shown to mediate RNA binding in other proteins [21]. These are followed almost immediately by seven decapeptide repeats between residues 750 and 850 and then by four further variants of the decapeptide repeat. The repeat motif is Gly-His-Arg-Pro-His-Glu-Gly-Pro-Gly-Gly, as shown in the alignment in Fig. 3B. The extreme C-terminus (residues 912–931) contains a putative zinc finger of the Cys-$(Xaa)_8$-Cys-$(Xaa)_5$-Cys-$(Xaa)_3$-His type. This motif has been shown to bind zinc in vitro and is frequently repeated in such zinc-binding proteins [22].

3.2. Tissue distribution of p99 mRNA

We next investigated the expression pattern of p99 mRNA in different cell types by Northern blot hybridisation. A major 4.5 kb mRNA species was detected in all tissues examined (Fig. 3C), consistent with the size of the Fb19 cDNA deposited to Genbank. In heart and skeletal muscle, a minor 1.3 kb band also hybridised with the p99 cDNA probe (Fig. 3C, lanes 1 and 6). This smaller mRNA could result from alternative RNA processing and/or usage of another transcription initiation site.

3.3. p99 is predominantly localised to the cell nucleus and expressed in a wide range of tissues

Although p99 was purified from HeLa cell nuclear extracts, the possibility that this protein is present in the cytoplasm as well as the nucleus had not been excluded. To determine the subcellular localisation of p99, HeLa cells were labelled for indirect immunofluorescence with the affinity purified anti-R11639 antibody and Texas Red conjugated anti-sheep secondary antibodies (Fig. 4). This showed a punctate nucleoplasmic staining pattern including several bright nucleoplasmic bodies per nucleus. Additional labelling was detected within the nucleoli of these cells. Preincubation of the antibody with the p99 fragment encoded by EST-R11639 resulted in the loss of staining (data not shown). The labelling pattern confirmed that p99 is a nuclear protein and further suggests that its function may be localised to discrete areas of the nucleus.

3.4. Interaction of expressed p99 with PP1 in vitro

p99 was found to inhibit the phosphorylase phosphatase activity of PP1γ (Fig. 5) and PP1α (data not shown). The concentration required for 50% inhibition was similar to the concentration of PP1 in the assays, demonstrating a stoichiometric interaction between the two proteins (Fig. 5). p99 suppressed PP1 activity by $>90\%$, similar to the effect of NIPP1 under the same conditions (data not shown). The fragment of p99 (residues 45–310) encoded by EST-R11639, which did not interact with DIG-PP1 (Fig. 2B), had no effect on PP1 activity (Fig. 5).

The observation that residues 45–310 of p99 do not interact with PP1 raised the question of where the PP1-binding site was located. Many PP1 modulators from all eukaryotic cells share a common motif with a consensus sequence comprising two or more basic residues lying just N-terminal to a Val/Ile-Xaa-Phe sequence [2]. In the case of the glycogen-targetting subunit, this motif has been shown to interact with a small hydrophobic channel on the surface of PP1 [2]. However, inspection of the amino acid sequence of p99 did not initially reveal such a motif, suggesting that p99 might interact with a different region of PP1. In order to investigate this question, we incubated the p99-PP1 complex with a 32 residue peptide from the PP1-binding protein p53BP2 which contains a 'canonical' PP1-binding motif. This peptide has been shown previously to disrupt the interaction between PP1 and its glycogen-targetting and myosin-targetting subunits [2]. The peptide from p53BP2 also disrupted the interaction between p99 and PP1, as shown by a dose-dependent increase in the phosphorylase phosphatase activity of the p99-PP1 complex (Fig. 6A). In contrast, a 16 residue peptide from NIPP1 that lies outwith the putative PP1-binding site (residues 175–190) did not reactivate the p99-PP1 complex (Fig. 6A). These observations

suggested that p99 interacts with the same (or at least an overlapping) region of PP1 as other targetting subunits.

In order to narrow down the site(s) of interaction between p99 and PP1, we expressed a fragment of p99 encompassing residues 1–675 and found that it bound to DIG-PP1 (data not shown). Taken together with lack of binding of the fragment encoded by EST-R11639 (residues 45–310), this suggested that the binding site was either located in the first 45 residues from the N-terminus or between residues 310 and 675.

While we could find no match to the consensus within the first 45 residues, the region between residues 310 and 675 contains a sequence Arg-Lys-Ser-Val-Thr-Trp (residues 396–401) which is similar to the PP1-binding motif of other targetting subunits. The major difference is the substitution of a tryptophan for the phenylalanine that is conserved in this motif in all other PP1-targetting subunits analysed to date. At this juncture, we learned at the EMBO/FEBS workshop on Protein Phosphatases (September 28th 1997) that Angus Nairn and coworkers had cloned a rat protein that was highly homologous to human p99. These investigators had found a similar variation of the PP1-binding motif and established by mutagenesis that the tryptophan was important for binding of their protein to PP1 (Y.-G. Kwon, P. Allen, P. Greengard and A.C. Nairn, manuscript in preparation). We therefore synthesised a 16 residue peptide corresponding to residues 393–408 of human p99 and showed that it could disrupt the p99-PP1 complex, as indicated by an increase in phosphorylase phosphatase activity (Fig. 6B). Changing Trp401 to Ala abolished the ability of this peptide to disrupt the p99-PP1 complex, whereas changing Trp401 to Phe had no effect (Fig. 6B). Taken together, these data indicate that residues 396–401 are indeed critical for the interaction of p99 with PP1. p99 is the first PP1-binding protein in which the apparently invariant Phe residue is replaced by another amino acid. Whether other PP1-binding proteins contain Trp at this position, and the significance of this variation, remain to be evaluated.

In the glycogen-targetting subunit of PP1 from skeletal muscle, cyclic AMP-dependent protein kinase phosphorylates the serine residue located between the Val and Phe residues of the Val-Xaa-Phe motif. This disrupts the interaction with PP1

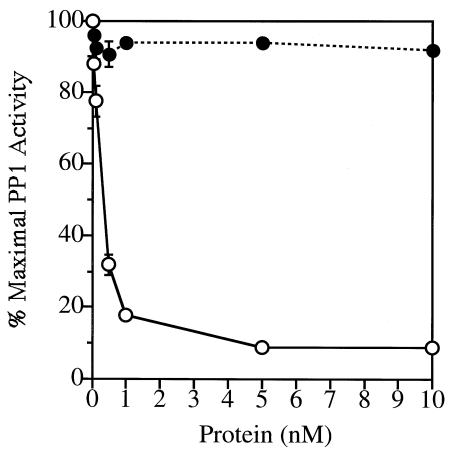

Fig. 5. Effect of full-length p99 (open circles, full line) and the fragment encoded by EST-R11639 comprising residues 45–310 (closed circles, broken line) on the phosphorylase phosphatase activity of PP1γ. PP1 activity is plotted as a percentage of the activity observed in the absence of both proteins. Data are shown as mean ± S.E. for 3–6 experiments.

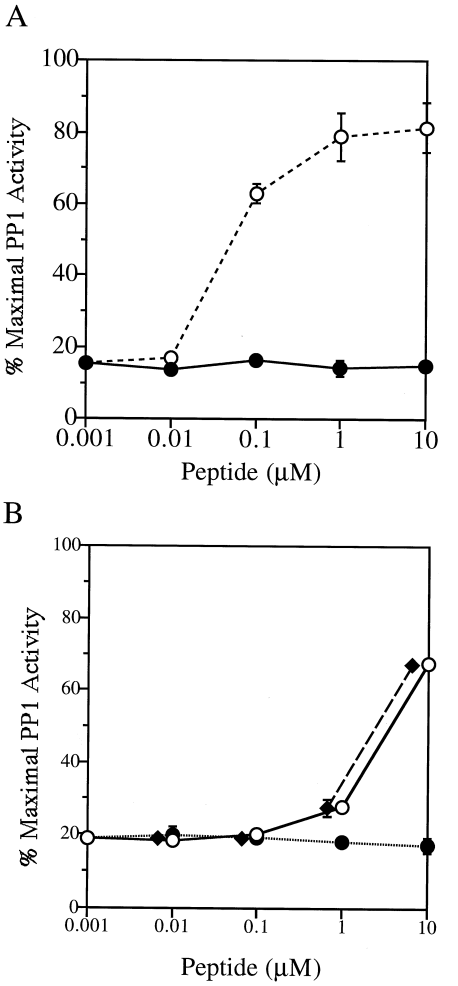

Fig. 6. Disruption of the interaction between PP1 and p99 by synthetic peptides containing PP1-binding motifs. A: PP1 activity was measured in the presence of 5 nM p99 and indicated concentrations of the peptide GKRTNLRKTGSERIAMGMRVKFNPLALLLDSC (open circles, broken line) containing the putative PP1-binding site from p53BP2 (underlined) or a control peptide REKPQTLPSAVKGD from a region of NIPP1 that does not interact with PP1 (closed circles, full line). B: Same as A, except that the peptides were from the putative PP1-binding region of p99 (residues 393–408). The peptide corresponding to the wild-type sequence (GRKRKSVTWPEEGKLR) is shown by the open circles and full line, a peptide in which Trp401 was changed to Phe by the closed diamonds and broken line, and a peptide in which Trp401 was changed to Ala by the closed circles and dotted line. Data are shown as mean ± S.E. for 3–6 experiments.

and is believed to be one of the mechanisms by which adrenalin stimulates glycogenolysis and inhibits glycogen synthesis (reviewed in [1]). NIPP1 also contains phosphorylatable residues within the putative PP1-binding domain, and its ability to suppress the activity of PP1 can be relieved by phosphorylation in vitro [12,15]. It is therefore intriguing that the residue between the Val and Trp residues in p99 is Thr, while a Ser residue lies immediately N-terminal to the Val. This raises the possibility that the interaction of p99 and PP1 is modulated by reversible phosphorylation of the p99 protein.

Preliminary data show that p99 is an RNA-binding protein, which is consistent with the presence of multiple RGG boxes. An ability to bind RNA, combined with the nuclear localisation pattern shown here, could be consistent with a role for

the p99-PP1 complex in regulating either RNA processing or transport within the nucleus. Future experiments will focus on identifying functional domains in the p99 protein and defining its role in regulating nuclear processes.

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