PPX, a novel protein serine/threonine phosphatase localized to centrosomes

Neil D. Brewis, Alasdair J. Street, Alan R. Prescott and Patricia T. W. Cohen

The amino acid sequence of a novel mammalian protein phosphatase, termed PPX (and designated PPP4 in the human genome nomenclature), has been deduced from the cDNA and shown to be 65% identical to PP2Aα and PP2Aβ and 45% identical to PP1 isoforms, the predicted molecular mass being 35 kDa. PPX was expressed in the baculovirus system. Its substrate specificity and sensitivity to the inhibitors, okadaic acid and microcystin, were similar (but not identical) to the catalytic subunit of PP2A. However, PPX did not bind the 65 kDa regulatory subunit of PP2A. The intracellular localization of PPX was investigated by immunofluorescence using two different antibodies raised against bacterially expressed PPX and a PPX-specific peptide. These showed that although PPX was distributed throughout the cytoplasm and the nucleus, intense staining occurred at centrosomes. The centrosomal staining was apparent in interphase and at all stages of mitosis, except telophase. In contrast, antibodies directed against bacterially expressed PP2A were not specifically localized to centrosomes. The human autoantibody #5051, which stains the pericentriolar material, colocalizes with PPX antibodies, suggesting that PPX may play a role in microtubule nucleation.

Key words: cDNA/centrosome/microtubule nucleation/ okadaic acid/protein phosphatase

Introduction

The reversible phosphorylation of proteins on serine and threonine residues, catalysed by protein kinases and protein phosphatases, is a ubiquitous mechanism for the regulation of cellular processes including metabolism, the cell cycle, gene expression and neuronal function. Most protein serine/threonine phosphatases have broad substrate specificities in vitro, which necessitated a classification based largely on specific inhibitors and activators. Type 1 phosphatases (PP1) dephosphorylate the β subunit of phosphorylase kinase specifically and are inhibited by nanomolar concentrations of the thermostable proteins inhibitor-1 and inhibitor-2, whereas type 2 protein phosphatases preferentially dephosphorylate the α subunit of phosphorylase kinase and are unaffected by the inhibitor proteins. The type 2 protein phosphatases comprise three enzymes (PP2A, PP2B and PP2C) which can be distinguished by their requirement for divalent cations and their sensitivity to naturally occurring inhibitors. The activity of PP2A, like PP1, has no absolute requirement for divalent cations and these enzymes are potently inhibited by the tumour promoter okadaic acid and the algal toxin microcystin. In contrast PP2B is dependent on Ca²⁺ and calmodulin and almost insensitive to okadaic acid and microcystin, while PP2C requires Mg²⁺ for activity and is completely resistant to the inhibitors (reviewed in Cohen, 1989; Cohen and Cohen, 1989; Shenolikar and Nairn, 1991; see also MacKintosh et al., 1990).

The protein serine/threonine phosphatases comprise two distinct gene families, PP1, PP2A and PP2B being related in sequence, while PP2C is structurally distinct. Mammalian PP1 and PP2A show ~45% overall amino acid sequence identity. PP2B is more distantly related, with an N-terminal catalytic domain showing ~40% identity to either PP1 or PP2A and a C-terminal domain which binds Ca²⁺ and calmodulin (reviewed in Cohen and Cohen, 1989; Cohen, 1990). Multiple isoforms of all four types of protein phosphatase have been cloned. In mammals, three isoforms have been identified for PP1 which show >90% identity, two for PP2A with >97% identity and three for PP2B with ~80% identity (Cohen, 1990; Sasaki et al., 1990; Muramatsu et al., 1992).

There has been considerable pressure during evolution to conserve the structures of protein phosphatases; for example the sequences of PP1 and PP2A from Drosophila are >90% identical to their mammalian homologues (Cohen, 1990). This implies that these protein phosphatases perform essential cellular functions and/or interact with many proteins. Mutations in PP1 and PP2A genes are lethal and cause defects in cell division in several organisms. In Drosophila (Axton et al., 1990; Dombrádi et al., 1990), Aspergillus (Doonan and Morris, 1989) and Schizosaccharomyces pombe (Ohkura et al., 1989), mutations in PP1 prevent the separation of chromosomes at anaphase, whereas mutants in PP2A in S. pombe undergo premature mitosis (Kinoshita et al., 1990).

Screening of cDNA libraries with PP1, PP2A and PP2B clones at reduced stringencies has identified several novel protein phosphatases, not so far identified as enzymes or proteins. These commonly display <70% identity to PP1, PP2A and PP2B and therefore cannot be regarded as isoforms of these enzymes. They include PPX (da Cruz e Silva et al., 1988; Cohen et al., 1990) in mammals, PPV (Cohen et al., 1990) and PYP (Dombrádi et al., 1989) in Drosophila and PPZ1 and PPZ2 (da Cruz e Silva et al., 1991) in Saccharomyces cerevisiae. Investigation of mutants of transcription regulation (SIT4) in S. cerevisiae (Arndt et al., 1989) and retinal degeneration (rdgC) in Drosophila (Steele et al., 1992) has identified two more novel protein phosphatases, which are functionally distinct from PP1, PP2A and PP2B. Polymerase chain reactions using oligonucleotides constructed to conserved regions of the protein serine/threonine phosphatases have shown the existence of two more novel protein phosphatases in S. cerevisiae, at least four more in Drosophila and predict

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many further unidentified phosphatases in higher eukaryotes (Chen et al., 1992).

PPX was originally identified by analysis of a partial cDNA (da Cruz e. Silva et al., 1988). Here we present the complete cDNA sequence of rabbit PPX, the first mammalian novel phosphatase to be reported, examine the properties of expressed PPX and demonstrate that native PPX is localized at centrosomes, implicating this phosphatase in the control of microtubule nucleation.

Results

PPX cDNA and the encoded protein

The rabbit PPX cDNA sequence has a 182 nucleotide (nt) 5\' non-coding region followed by an open reading frame of 921 nt and a 3\' non-coding region with a putative polyadenylation signal at 1191 (Figure 1). However, if this signal is functional, 400 nt of poly(A) tail or additional nucleotides in the 5\' non-coding region are required to account for the 1.8 kb PPX mRNA shown in Figure 2. The context of the ATG, with a G situated at position +4 and a purine situated at position -3 compares fairly favourably with the consensus sequence for translation initiation CC[A/G]CCATGG (Kozak, 1991).

The predicted translation product is a 307 amino acid protein with a molecular mass of 35 kDa. This amino acid sequence shows high identity with protein serine/threonine phosphatases, being most closely related to rabbit PP2A\(\alpha\) and PP2A\(\beta\) (65–66% identity). It is less similar to mammalian PP1 isofoms (45–46% identity) and the catalytic domain of mammalian PP2B isofoms (39–41% identity).

**PPX mRNA in mammalian tissues**

Analysis of PPX mRNA by Northern blotting shows that a 1.8 kb transcript is present in all tissues examined, which is clearly distinct from the 2 kb transcript of PP2A\(\alpha\) (Figure 2) and PP2A\(\beta\) (data not shown). The same results were obtained with two different PPX probes. The PPX mRNA was 3-fold higher in testis than in most other tissues (Table I). Control studies using the same RNA preparations, demonstrated that the level of PP2A\(\alpha\) mRNA was not elevated in testis (Figure 2).

**Expression of rabbit PPX in insect cells from a baculovirus vector**

In order to obtain active PPX, the baculovirus expression system was employed, since expression in *Escherichia coli* produced inactive, insoluble enzyme (see Materials and methods and Discussion). Cotransfection of the PPX cDNA in the transfer vector pVL.SG (Graber et al., 1992) and the baculovirus AcRP23.lacZ DNA, digested with Bsu36I, produced high levels of recombination as described in Kitts et al. (1990). After incubation with 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside (X-gal), 70% of the viral plaques were colourless showing replacement of the *lacZ* sequence with that of PPX. This facilitates identification of recombinants and compares very favourably with the use of wild type nuclear polyhedrosis virus DNA (without restriction enzyme digestion) where the recombination frequency is found to be only ~0.1% (Summers and Smith, 1987).

Expression of PPX by the recombinant baculovirus was detected in extracts by immunoblotting with anti-PPX antibodies. The level of expression was estimated to be ~0.5 mg PPX per litre of cell culture reaching a steady level at

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**Fig. 1.** cDNA and predicted amino acid sequence of protein phosphatase X from rabbit liver. The putative polyadenylation signal is underlined.
30 h post infection. Although most of the expressed PPX was found in the 14,000 g pellet, ~10% was present in the cytosol (Figure 3).

**Purification of expressed rabbit PPX from insect cell extracts**

Cytosolic extracts of *Spodoptera frugiperda* 9 (Sf9) cells infected with recombinant PPX virus were chromatographed on Mono Q to separate the expressed PPX from endogenous protein phosphatases. Four major peaks of phosphatase activity were observed using glycogen phosphorylase as substrate (Figure 4). Most of the activity in the first and second peaks was inhibited by inhibitor-2 and could therefore be identified as Sf9 cell PP1. The phosphatase activity of the third and fourth peaks was unaffected by inhibitor-2, but completely inhibited by 2 nM okadaic acid (data not shown), indicating that these peaks were likely to be forms of Sf9 cell PP2A (Cohen *et al.*, 1988a). Immunoblotting of the column fractions with anti-PPX antibodies showed that PPX eluted at ~300 mM NaCl, between Sf9 cell PP1 and PP2A (Figure 4, lower panel). No PPX immunoreactivity was found in the flowthrough fractions that did not bind to the column. The PPX peak fraction (number 49, Figure 4) was concentrated from 0.5 ml to <0.2 ml. The phosphatase activity in the concentrated fraction was found to be insensitive to inhibitor-2 and completely inhibited by 2 nM okadaic acid, showing that it contained little if any PP1 activity.

The PPX in the concentrated fraction 49 from Mono Q was further purified by gel filtration on Superose 12. The major peak of phosphatase activity contained all the anti-PPX immunoreactivity and eluted at the position expected for a protein of 35 kDa (Figure 5). This peak was absent in the corresponding Superose 12 profile of Sf9 cells infected with wild type virus (Figure 5). The small amount of phosphatase activity eluting earlier than PPX contained no anti-PPX immunoreactivity, was insensitive to inhibitor-2 and was inhibited completely by 2 nM okadaic acid. It is therefore likely to be a high molecular weight form(s) of PP2A.

**Properties of soluble rabbit PPX expressed from baculovirus**

In order to test whether PPX could bind the same regulatory subunit as PP2A, the PPX-containing fractions from the Superose 12 column in Figure 5 were mixed with a molar excess of the 65 kDa regulatory A subunit of PP2A, also designated PR65 (Hemmings *et al.*, 1990) and again gel filtered on Superose 12. All the PPX immunoreactivity and the majority of the activity eluted in the position expected for the free catalytic subunit (Figure 6). A control experiment showed that the free PP2A catalytic subunit when mixed with the A subunit eluted as expected as a high molecular mass complex earlier than the 67 kDa marker (data not shown).

![Fig. 2. Tissue distribution of rabbit PPX mRNA compared with PP2A α mRNA. 25 µg of total RNA were loaded per lane and fractionated on a denaturing 1% agarose gel. The membrane was hybridized with a Smal – EcoRI fragment of the rabbit PPX cDNA. M, smooth muscle; T, testicle; K, kidney; Lu, lung.](image)

![Table 1. Relative levels of PPX expression in various rabbit tissues determined by phosphorimaging analysis of PPX mRNA on Northern blots](table)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PPX signal/amount total RNA^a</th>
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<tbody>
<tr>
<td>Liver</td>
<td>2.2</td>
</tr>
<tr>
<td>Heart</td>
<td>1.4</td>
</tr>
<tr>
<td>Whole brain</td>
<td>1.3</td>
</tr>
<tr>
<td>Skeletal muscle</td>
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<tr>
<td>Smooth muscle</td>
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<td>1.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.0</td>
</tr>
<tr>
<td>Lung</td>
<td>1.0</td>
</tr>
</tbody>
</table>

^aValues estimated relative to the levels in kidney.

![Fig. 3. Expression of PPX from recombinant baculovirus in Sf9 cells. Detection is by immunoblot analysis, using anti-PPX antibodies. The samples are uninfected cells, wild type (AcRP23 lac Z) virus and PPX recombinant virus (Ac.PPX) at 42 h post infection. P, 14,000 g pellet; S, 14,000 g supernatant. PPX expressed in bacteria was used as a standard (Con). The equivalent of 0.1 ml culture was loaded per lane. Sizes of molecular weight markers (Pharmacia) are given in kDa.](image)

989
Fig. 4. Chromatography on Mono Q of an extract from Sf9 cells infected with the PPX recombinant baculovirus. Phosphatase activity was measured using phosphorylase as substrate and PPX detected by immunoblotting with anti-PPX antibodies. 50 mg protein was applied to the column equilibrated in 20 mM triethanolamine–HCl pH 7.5, 0.1 mM EGTA, 5% glycerol, 1 mM DTT (buffer A). After washing until the absorbance at 280 nm was <0.02, the column was developed with a linear gradient of NaCl to 0.5 M in buffer A. The closed and open symbols show the phosphatase activity with (●) and without (○) inhibitor-2. The broken line (---) shows the NaCl gradient and the bottom panel shows an immunoblot of the fractions using the anti-PPX antibodies.

Fig. 5. Gel filtration of the PPX on Superose 12. PPX-containing fractions from Mono Q (Figure 4) were concentrated using a Centricon-10 microconcentrator and applied to the column. The upper panel compares the elution profiles of PPX-baculovirus (○) and wild type-baculovirus (□) infected cell extracts. Phosphatase activity was measured using glycogen phosphorylase as substrate. The lower panel shows an immunoblot of the fractions analysed with anti-PPX antibodies. The column buffer was 50 mM triethanolamine–HCl pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM DTT, 0.03% Brij-35. The flow rate was 0.4 ml/min and 0.2 ml fractions were collected. The elution positions of the molecular mass markers, catalase (240 kDa), BSA (67 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa) are shown.

These results indicate that PPX does not bind the A subunit with high affinity.

The phosphatase activity eluting earlier than PPX (Figure 6) is presumably explained by the presence of some contaminating PP2A catalytic subunit which interacts with the A subunit to form a higher molecular mass complex. The PPX-containing fractions after this second gel filtration (Figure 6) appeared to be free of the PP2A catalytic subunit.
since they did not cross react with antibodies raised against
PP2A (data not shown).

The PPX preparation, free of PP2A (Figure 6), was
assayed using a variety of substrates (Table II). PPX
deprophosphorylated the α subunit 2-fold faster than the β
subunit of phosphorylase kinase, similar to experiments with
PP2A carried out in parallel (data not shown). PPX was
active on all substrates tested in the absence of divergent
cations, but like PP2A, its activity was stimulated by 1 mM
Mn²⁺, particularly in assays using the synthetic peptide
stimulation by Mn²⁺ has been noted previously for PP2A
(e.g. Agostini et al., 1987). When matched for phosphor-
lyase activity, the relative activity of PPX towards
most substrates was similar to PP2A. However, it was
13-fold lower than PP2A towards cdc2 kinase-labelled
HMG-I protein (described in Nissen et al., 1991).

Both PPX and PP2A were most active against the synthetic
peptide RRAT[^32P]VA when assayed in the presence of 1
mM Mn²⁺. This substrate was therefore used to examine
the effect of phosphatase inhibitors. In these assays PPX and
PP2A were diluted to give the same phosphatase activity
towards this substrate. The apparent IC₅₀ values for
inhibition of PP2A and PPX by okadaic acid were 0.07 and
0.2 nM respectively, while those for inhibition of PP2A and
PPX by microcystin were 2 and 8 pM respectively (Figure 7).

Cytological localization of PPX and PP2A

The subcellular localization of PPX in human fetal lung cells
(MRC-5) and epidermal carcinoma cells (A431) was
examined by immunofluorescence using polyclonal affinity
purified anti-PPX protein and anti-PPX peptide antibodies.

Although PPX staining was observed throughout the
cytoplasm and more strongly in the nucleus of cells in
interphase, very intense staining with PPX antibodies was
observed at the centrosomes (Figure 8a and i). Staining of
the same cells with anti-tubulin antibodies shows the position
of the microtubules organized from the centrosome

Protein phosphatase X, a centrosomal enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PP2A</th>
<th>PPX</th>
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<tbody>
<tr>
<td>Phosphorylase a (phosphorylase kinase)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Phosphorylase kinase α (cAMP dependent protein kinase)</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>Casein (cAMP dependent protein kinase)</td>
<td>58</td>
<td>43</td>
</tr>
<tr>
<td>Histone H1 (cdc2 kinase)</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Caldesmon (cdc2 kinase)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>HMG-I (cdc2 kinase)</td>
<td>18</td>
<td>1.4</td>
</tr>
<tr>
<td>RRAT[^32P]VA + 1 mM Mn²⁺ (PKA)</td>
<td>200</td>
<td>225</td>
</tr>
</tbody>
</table>

The enzymes were diluted to the same phosphorylase phosphatase activity
before assay (taken as 100%). The kinase used to phosphorylate the
substrate is in parenthesis.

(Figure 8b). The same distribution of anti-PPX stain was
observed using methanol or p-formaldehyde as fixatives and
in all cell types examined, including not only MRC-5
(Figure 8a) and A431 (Figure 8e—h) cells, but also human
foreskin fibroblasts (data not shown). The staining was
judged to be specific, since it was abolished by preincubation
of the PPX antibodies with excess PPX protein or excess
PPX peptide (Figure 8d).

The anti-PPX antibodies stained the duplicated
centrosomes intensely in prophase (Figure 8e), and both
spindle poles at metaphase (Figure 8f) and anaphase
(Figure 8g). However, during telophase the intensity of
staining decreased and could not be detected in most cells
at this stage of cell division (Figure 8h).

In Figure 8i—l, cells are double stained with anti-PPX
antibodies and the human autoantibody #5051 from a
sclerodema patient, which is known to detect the centrosome
by binding to the pericentriolar material surrounding the
centrioles (Calarco-Gillam et al., 1983). Closer inspection
(inset) of the centrosomal region showed that PPX staining
was also localized to the pericentriolar region. The stain
appears to radiate from the centrosome, being absent from
the centrioles.

To test whether PPX localization at the centrosomes
requires the presence of microtubules, cells (A431) were
preincubated with 20 μM nocodazole for 1 h prior to fixation
and staining. Staining with anti-tubulin and anti-PPX
antibody showed that while nocodazole had depolymerized
the microtubules, it did not affect the pericentriolar localization
of PPX (data not shown).

Staining of cells with anti-PP2A antibodies showed
cytoplasmic and nuclear locations, with increased nucleolar

![Figure 6. Gel filtration of a mixture of PPX and the A subunit of PP2A on Superose 12. PPX-containing fractions in Figure 5 were concentrated, mixed with 6 μg of the A subunit of PP2A and applied to the column. Other details are given in Figure 5.](image-url)
Fig. 8. Immunofluorescent localization of PPX and PP2A in the human MRC-5 fixed with methanol (panels a–d and i–l) and A431 fixed with p-formaldehyde (panels e–h) cells. Biotinylated anti-PPX protein antibodies, anti-PPX peptide antibodies and anti-PP2A protein antibodies were detected with an avidin–fluorescein conjugate. Anti-tubulin and anti-#5051 antibodies were detected with anti-rat and anti-human antibodies respectively coupled to Texas Red. DNA was visualized with propidium iodide. Fluorescent micrographs were taken with a confocal microscope. (a) Staining of the nucleus, cytoplasm and centrosome with anti-PPX protein antibodies (green); (b) the same cell stained with anti-tubulin antibodies (red); (c) cell stained with anti-PP2A antibodies (green); (d) cell stained anti-PPX peptide antibodies which had been preincubated with excess PPX peptide for 3 h. Panels (e)–(h) show cells in different stages of mitosis double stained with anti-PPX peptide antibodies (green) and propidium iodide (red): (e) prophase, (f) metaphase, (g) anaphase and (h) telophase. Panel (i) cell stained with anti-PPX peptide antibodies (green). Panel (j) the same cell stained with #5051 antibodies (red). Inset are enlargements of the centrosome showing staining of the pericentriolar material. Panels (k) and (l), pericentriolar matrix stained with anti-PPX peptide antibodies (green) and #5051 antibodies (red), respectively, showing separation of centrosomes after duplication. The scales shown by the bars are: panels (a)–(d), (i) and (j) 50 μm; panels (e)–(h) 10 μm; insets (i) and (j) and panels (k) and (l) 2 μm.

staining (Figure 8c). In contrast to the situation with PPX, staining of the centrosome with anti-PP2A antibodies was not observed in any cells.

Discussion

**PPX is a novel mammalian protein phosphatase**

The complete amino acid sequence of PPX, deduced from the cDNA, demonstrates that PPX is a novel mammalian protein phosphatase more closely related to PP2A than PP1. While rabbit PP2Aα and PP2Aβ isoforms show 97% amino acid identity, PPX is only 65% identical to both PP2Aα and β. This amount of sequence divergence indicates that PPX cannot simply be regarded as an isoform of PP2A, but is more likely to be functionally distinct. The mRNA transcript for PPX is present in all mammalian tissues examined, demonstrating that PPX is not a tissue-specific form of PP2A. The level of PPX mRNA transcript is 3-fold higher in testis than in other tissues. Assuming that this reflects the level of protein, PPX may be important in some aspects of spermatogenesis (see below).

In lower eukaryotes, none of the published sequences show
strikingly high identity to rabbit PPX. Of the *S.cerevisiae* phosphatases, rabbit PPX is most closely related to PP1H (Ronne *et al.*, 1991), but the level of identity is insufficient to conclude whether or not rabbit PPX and *S.cerevisiae* PP1H are homologues. It will be interesting to see whether PP1H is also localized to the pericentriolar material.

**Expression of rabbit PPX in heterologous systems**

Expression of rabbit PPX cDNA in bacteria as a fusion or non-fusion protein using the T7 polymerase system, or as a glutathione-S-transferase fusion protein (data not shown) produced high level expression, but all three expressed forms of the protein were insoluble. Insolubility has been reported for bacterial expression of PP1 from rabbit skeletal muscle (Berndt and Cohen, 1990) and maize (Smith and Walker, 1991). More recently a bacterial system capable of expressing active phosphorylase, which uses a weaker *trp−lac* hybrid promoter in conjunction with lower growing temperatures and lower IPTG levels (Browner *et al.*, 1991), has yielded soluble active PP1 (Zhang *et al.*, 1992).

However, rabbit PP2Aα could not be expressed in a soluble form in this vector (D.Barford and P.T.W.Cohen, unpublished data). Aggregation of PPX expressed in the T7 polymerase or glutathione-S-transferase systems was not prevented by using either lower temperatures or induction with non-saturating IPTG levels.

The insoluble PPX from the T7 expression system proved useful as a source of immunogen for the production of anti-PPX antibodies and the affinity purified antibodies showed no cross reactivity against several phosphatase catalytic subunits tested (see Materials and methods), despite the similarities in sequence.

The baculovirus/insect cell system (reviewed in Miller, 1989) has been used for the expression of PP1 to produce both soluble active enzyme as well as large amounts of insoluble protein (Berndt and Cohen, 1990). In order to obtain an active form of PPX we therefore used this system. Although PPX was largely expressed as a particulate form, 10% was soluble and active, providing sufficient material for purification and examination of the properties of PPX.

**PPX is similar to PP2A in many but not all biochemical properties**

PPX expressed from baculovirus was separated from endogenous insect cell phosphatases and judged to be free of PP1 by its lack of inhibition by inhibitor-2 and free of PP2A since it did not cross react with anti-PP2A antibodies or bind the regulatory A subunit of PP2A. The substrate specificity of PPX and its response to inhibitors were similar to those of PP2A. The inhibition of PPX by okadaic acid and microcystin coupled with insensitivity to inhibitor-2 and preferential dephosphorylation of the phosphorylase kinase α subunit places PPX in the ‘PP2A-like’ class and distinguishes it from PP1, PP2B and PP2C.

It is important to note that PPX and PP2A are inhibited by similar concentrations of okadaic acid and microcystin-LR. It is therefore essential that when ascribing physiological roles to PP2A by the use of these inhibitors, additional criteria are used in conjunction with the inhibitors to differentiate PP2A from ‘PP2A-like’ enzymes such as PPX and PPV.

The specific activity of PPX towards phosphorylase was 150 mU/mg (with the PPX concentration being estimated by immunoblotting). This value is ~20-fold lower than that for PP2A, and all other substrates tested (Table II) were as low or even lower. There are several explanations for these values. First, the substrate specificity of PPX may differ from PP2A, and in particular PPX may have much higher activity towards its physiological substrate(s) that have still to be identified. Secondly, expressed PPX may not be folded or modified correctly to produce a fully active enzyme. Thirdly, purified PPX may contain some catalytically inactive enzyme.

The 65 kDa regulatory A subunit of PP2A binds tightly to the PP2A catalytic subunit. Although the amino acid sequence similarity of PPX and PP2A catalytic subunits suggests that they might bind a common regulatory subunit, gel filtration of PPX mixed with the A subunit of PP2A showed that there was no high affinity interaction between these two subunits. Presumably PPX either exists in vivo as a free catalytic polypeptide or interacts with its own distinct regulatory subunit(s). The latter is more likely because we have recently observed that the native forms of PPX in liver cytosol are larger than those of the free catalytic unit (N.D.Brewis and P.T.W.Cohen, unpublished data).

PP1 (Axton *et al.*, 1990; Kinoshita *et al.*, 1990), PP2A (Sneddon *et al.*, 1990) and S14 (Sutton *et al.*, 1991) play crucial roles in cell division. A PP2A-like activity, termed INH, has been described (Lee *et al.*, 1991) which can inhibit activation of pre-maturation promoting factor in crude extracts of frog oocytes. INH from bovine heart was shown to be a protein phosphatase of molecular mass ~150 kDa. The catalytic subunit was sensitive to okadaic acid, insensitive to inhibitor-2 and possessed immunoreactivity with a monoclonal anti-PP2A antibody. However, some properties of INH did not appear to match precisely those of any previously isolated forms of PP2A. Since PPX and PP2A display considerable amino acid sequence identity, it is possible that the monoclonal antibodies employed in these experiments recognize an epitope common to the two enzymes. We therefore tested INH (kindly provided by Dr T.Lee and Professor M.Kirschner) with our antibodies, known to differentiate between PPX and PP2A. In immunoblotting experiments INH interacted with PP2A and not with PPX-specific antibodies, proving that PPX is not the catalytic subunit of INH and indicating that INH is likely to contain the catalytic subunit of PP2A.

**PPX localizes to the centrosomes**

Although anti-PPX antibodies detected PPX in the cytoplasm and in the nucleus, the pericentriolar material showed the strongest interaction with both anti-PPX protein and anti-PPX peptide antibodies, suggesting that PPX may have a role in centrosome function. Centrosomes are microtubule organizing centres, the pericentriolar material serving as a focal point for microtubule nucleation at the onset of mitosis, and the preferred site of microtubule nucleation in interphase (reviewed in Fuller *et al.*, 1992). The microtubule nucleating activity of centrosomes is regulated by phosphorylation and is probably under the control of cyclin A-cdc2 kinase (Buendia *et al.*, 1992). Cdc2 kinase has been shown to be associated with the centrosome (Bailly *et al.*, 1989). Many studies suggest a role for an okadaic acid sensitive phosphatase in this function, although the phosphatase involved was generally regarded as being PP2A (Picard *et al.*, 1989; Rime *et al.*, 1990). Incubation of mouse
ooocytes with 1 μM okadaic acid overcame arrest by isobutyl methyl xanthine. Okadaic acid also inhibited spindle formation, without interfering with the capacity of tubulin to polymerize (Alexandre et al., 1991). LLC-PK cells were blocked in a metaphase-like state within 6–8 h by 8–40 nM okadaic acid, with microtubules being present but not stabilized. Longer incubations with okadaic acid led to disruption of the metaphase plate and development of multilobar spindles (Vandré and Wills, 1992). While PP2A has been shown to be a negative regulator ofcdc2 kinase in S.pombe, by use of a dominant mutation in the gene for the catalytic subunit for PP2A (Kinoshita et al., 1990), the nature of the okadaic acid sensitive phosphatase involved in microtubule nucleation is less clear. From the immunological localizations presented here, which show that PPX is concentrated at the centrosomes and that PP2A is widely distributed throughout the cell, PPX is a more likely candidate for a phosphatase involved in microtubule organization.

Studies on the ability of centrosomes to nucleate microtubule asters from purified tubulin show that a phosphorylated epitope, recognized by the monoclonal antibody MPM-2, is important for microtubule nucleation. Preincubation of centrosomes with MPM-2 antibody or treatment with alkaline phosphatase which removes the phosphorylated epitope blocks all microtubule nucleation (Centonze and Boris, 1990). Since PPX is inhibited by okadaic acid and microtubule nucleation is blocked or altered by this inhibitor, PPX is unlikely to be the phosphatase that directly dephosphorylates the MPM-2 epitope. The observation that anti-PPX antibodies detect PPX at the centrosomes at all stages of the cell cycle but do not readily detect them at telophase may be important. It suggests either that PPX dissociates from the centrosomes at telophase or that the PPX epitope(s) may become masked. It is relevant that the microtubule network undergoes rearrangement at this time in the cell cycle.

Although an okadaic acid sensitive phosphatase has been recognized to be involved in microtubule nucleation at centrosomes, PPX is the first protein phosphatase to be localized to centrosomes. The existence of PPX mRNA in all tissues examined implicates PPX in function(s) that are common to all cells. Higher levels of PPX mRNA in testis may reflect the fact that the basal body of the sperm flagellum is related to the centrosome of other cell types and has the capacity to nucleate radial microtubules following fertilization (Kuriyama and Kanatani, 1981). It is interesting to note that we have recently cloned the PPX cDNA from Dro sophila and shown that its mRNA is highly expressed in the embryo (N.D.Brewis and P.T.W.Cohen, unpublished data), a tissue undergoing rapid mitotic divisions. Finally, sequence analysis of PPX cDNA from man (Brewis and Cohen, 1992), rabbit and Drosophila demonstrates that PPX protein structure has been highly conserved during the course of evolution, implying that PPX performs an essential cellular function.

### Materials and methods

#### Materials

- Oligonucleotides were synthesized by Alastair I.Murchie on an Applied Biosystems 394 DNA synthesizer (Foster City, CA, USA).
- Nitrocellulose filters (Hybond C), nylon membranes (Hybond N*) and the Enhanced Chemiluminescence system were obtained from Amersham International plc., Bucks, UK. The baculovirus AcRP23 lacZ and the transfer vector pHVLS were gifts from Dr R.D.Possee, Institute of Virology, Oxford, UK and Dr S.G.Graber and Dr J.C.Garrison, University of Virginia, Charlottesville, VA, USA respectively. TC-100 medium and lipofectin were from Gibco BRL, Paisley, Scotland. Fetal calf serum (PCS) was purchased from Northumbria Biologicals Ltd, Cramlington, Northumberland, UK. Okadaic acid was a gift from Dr Y.Takakutani, Fujisawa Pharmaceutical Company, Tokyo. Tauromycin was provided by Dr K.Isôna, Antibiotics Laboratory, Institute of Physical and Chemical Research, Saitama, Japan. Microcystin-LR was purified by Dr C.Mackintosh as described in Mackintosh et al. (1990). The catalytic subunit of protein phosphatase 2A (Cowling et al., 1988a) and inhibitor-2 (Cowling et al., 1988b) were purified to homogeneity from rabbit skeletal muscle by Dr D.L.Schelling, Casein, partially hydrolysed and dephosphorylated, was obtained from Sigma Chemical Co. Ltd, Poole, Dorset, UK. The A subunit (PR 65) of PP2A, expressed by baculovirus and purified to homogeneity, was a generous gift from Dr B.Favre and Dr B.A.Hemmings, Friedrich Miescher-Institut, Basal. Mono Q columns (0.5 x 5 cm) and Superose 12 columns (1.2 x 30 cm) were obtained from Pharmacia Ltd, Milton Keynes, UK. A431 cells (Giard et al., 1973), MRC-5 cells (Jacobs et al., 1970) and human foreskin fibroblasts, passed 6–10 times, were kindly provided by Dr E.Smythe, Dr S.M.Pickles (Department of Biochemistry, Dundee University) and Dr I.Kill (Department of Biology, Dundee University), respectively. Biotin N-hydroxysuccinimide ester was obtained from ICN Immunobiologicals, High Wycombe, Bucks, UK. Human #503 antibodies were from Professor M.Kirschner. Rat anti-tubulin antibodies and Texas Red-labelled donkey anti-rabbit antibodies were obtained from Jackson Immunoresearch, West Baltimore Pike, PA and from Sera-Lab Ltd, Sussex, UK, respectively. All other immunological reagents were purchased from Vector Laboratories, Burlingame, USA. Nocodazole was obtained from Sigma Chemical Co. Ltd, Poole, Dorset, UK.

#### Screening of the cDNA library

A rabbit liver cDNA Agt10 library (Clontech) was screened on duplicate nitrocellulose filters. One set of filters was hybridized at 60°C with the EcoRI–Smal fragment (nt 315–880 in Figure 1) of the partial rabbit PPX cDNA (da Cruz e. Silva et al., 1988). The other set was hybridized at 60°C with an oligonucleotide, GACCCAGTCCCCGATTAGC, made at the 5’ end of the partial cDNA sequence (nt 522–542 in Figure 1) as described in Cohen (1991). The cDNA probe was labelled by random hexanucleotide priming with [α-32P]dATP (Feinberg and Vogelstein, 1984) and the oligonucleotide was labelled at the 5’ end with [γ-32P]dATP (Sambrook et al., 1989). Screening of 400 000 plaques yielded one clone with a 1.4 kb insert and two with 1.1 kb inserts. The positive clones were subcloned into Bluescript pKS+ (Stratagene, La Jolla, USA) and DNA sequencing was performed using dideoxy chain termination (Sanger et al., 1977) with Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH, USA) and oligonucleotide primers. The three clones contained identical sequence, with the two shorter inserts starting at nt 265 of PPX cDNA.

#### Northern blot analysis of the tissue distribution of rabbit PPX mRNA

Total RNA was purified from various rabbit tissues using a guanidinium thiocyanate/phenoI extraction method (Chomczynski and Sacchi, 1987). Electrophoresis and blotting were carried out as described in Kroczek and Siebert (1990) except Hybrid N* membranes were used. Membranes were hybridized overnight in 0.25 M Na2HPO4 (titrated to pH 6.5 with H3PO4), 7% SDS, 1 mMEDTA (Church and Gilbert, 1984) at 65°C with the cDNA probe described above and an EcoRI–Smal fragment nt 183 to 880 containing the 5’-non-coding region and part of the coding region. The membranes were washed at 65°C in 0.1 M NaCl, 1.5 mM sodium citrate, 0.1% SDS. The hybridization signals were quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). The control probe was a 524 bp Sph1–EcoRI fragment of the 3’-non coding region of rabbit PP2Aα (da Cruz e. Silva and Cohen, 1987).

#### Expression of PPX in bacteria and production of antibodies

The PPX full length cDNA (Figure 1) was cleaved at the initiating ATG codon with Ncol, and the overhanging end was filled in by the Klenow fragment of DNA polymerase I (Sambrook et al., 1989). The product was further digested with HindIII and the PPX coding region fragment was gel purified and ligated into the Smal and HindIII sites of the pT7.7 vector (Tabor and Richardson, 1985) for production of a protein with the eight N-terminal amino acids of the gene 10 protein fused to PPX. The construct was checked by DNA sequencing. The pl17-7-PPX plasmid was transformed into E.coli BL21 (DE3) containing the lysP plasmid (Studier et al., 1990) and grown in Luria Broth containing 25 μg/ml chloramphenicol and 50 μg/ml ampicillin.
Concentrations of peptide were determined using NcoI-EcoRI digestion followed by fluorography (Laemmli, 1970). The proteins were stained with Coomassie Blue R-250. This expression system produced rabbit PPx as an insoluble 35 kDa protein at a concentration of ~30 mg/l culture.

The washed pellet from 6 ml bacteria expressing PPX was applied to a 18 x 16 x 0.2 cm column in a similar buffer (20 mM Tris pH 7.5, 100 mM NaCl, 15 mM MgCl₂, 0.02% Tween 20, 2.5 mM CaCl₂, 0.5 mM KCl, 1.76 mM KH₂PO₄, 10.1 mM Na₂HPO₄ pH 7.4 (PBS), 0.015% SDS). The protein was quantified by the method of Bradford (1976), with bovine serum albumin (BSA) as a standard. This material was used as a standard on immunobots and for immunization of hens as described in Harlow and Lane (1988). Rabbit PPx2-aa was expressed as a non-fusion protein in the T7 polymerase system and processed as for PPx for the production of antibodies.

Antibodies were also raised in hens against a synthetic peptide EAAPQETGRIPSKPKVADY corresponding to the C-terminal amino acids 287–305 of PPX. The peptide was synthesized on a resin using standard tertiary butoxy carbonyl (tBoc) chemistry (reviewed in Atherton and Sheppard, 1989). After cleavage from the resin, the peptide was covalently bonded to BSA as follows. 5 μmol (10 mg) of peptide were dried to remove traces of acetic acid, redissolved in 0.75 ml water and mixed with 10 mg BSA in 0.25 ml 0.4 M K₂HPO₄ pH 7.5. 0.5 ml 20 mM glutaraldehyde was added dropwise over a 5 min period. The reaction was allowed to go to completion (30 min) and then 150 μl 1 M glycine was added. The mixture was then dialysed against 150 vol PBS at 4°C with six buffer changes. The peptide was injected into hens under the regime used for the PPx protein.

Egg yolks (1 vol) were emulsified with 1 vol of PBS and then shaken vigorously with 1 vol of chloroform, until the mixture thickened. After 24 h at 4°C, the mixture was reshenk briefly and then centrifuged at 10 000 g for 15 min. The supernatant was assayed for the presence of antibodies. Anti-PPx and anti-PPA2 antibodies were purified by affinity chromatography using bacterially expressed PPX and PPX2A respectively linked to cyanogen bromide activated Sepharose (Pharmacia, Uppsala, Sweden). The antibodies, diluted in 5 vol of 20 mM Tris–HCl pH 7.5, were applied to the column, eluted with 100 mM triethanolamine–HCl pH 2.5 and immediately neutralized with 1 M Tris–HCl pH 8. They were concentrated and the buffer was exchanged with PBS, 0.02% sodium azide using a Centricron-30 microconcentrator (Amicon, Beverly, MA, USA). Anti-peptide antibodies were affinity purified in a similar manner except that the affinity column comprised PPX peptide covalently linked to Affigel 15 according to the manufacturer’s instructions (Bio-Rad, Hemel Hempstead, UK).

Immunoblotting on to nitrocellulose (BA 85, Schleicher and Schuell, Dassel, Germany) was carried out essentially as described in MacDougall et al. (1989) using a Transblot apparatus (Bio-Rad). Gels were transferred to nitrocellulose for 50 V h/cm. Non-specific binding was reduced by incubation in 4% Marval dried milk powder (Premier Brands, Birmingham, UK) in 20 mM Tris–HCl (pH 7.5), 500 mM NaCl for 1 h. The blot was then incubated with anti-PPX antibodies (1 μg/ml) and 1 mg/mL BSA for at least 5 h. PPX antibody binding was detected using biotin labelled goat anti-hen IgG antibodies and a mixture of biotinylated horse radish peroxidase and phenol red avidin according to the manufacturer’s instructions. Peroxidase activity was detected by fluorography with 3,3’-diaminobenzidine (DAB) as substrate.

Hen antibodies raised against rabbit PPX expressed in E.coli and anti-peptide antibodies were affinity purified and tested over a range of concentrations for specificity using various protein phosphatases by immunoblotting. The concentration of 1 mg/ml was used to detect the anti-PPX and anti-anti-PPX peptide antibodies exhibited any cross reactivity against 1 μg of the catalytic subunits of PP2A, PPV, PPV or PPI (data not shown). The anti-protein antibodies could detect as little as 5 ng of PPX. The anti-PPX peptide antibodies were less sensitive detecting only >20 ng of PPX on a blot. The anti-PP2A antibodies showed no cross reactivity against PPX, PPV, PPV or PPI.

Expression of PPX from baculovirus

The NcoI–EcoRI fragment of the rabbit PPX cDNA (at 1–1225) was subcloned in the same two restriction sites of the pvLSVG transfer vector (Graber et al., 1992). S99 cells were cultured as in Summers and Smith (1987). 10⁶ S99 cells were washed with serum-free TC-100 medium and cotransfected with 0.5 μg of PPX transfer vector and 100 ng baculovirus DNA (AcRP23.lacZ) using 10 μg lipofectin according to the manufacturer’s instructions. The AcRP23.lacZ viral DNA was used because the replacement of the β-galactosidase gene by cDNA in the pVL SO vector can be detected in the presence of X-gal. In the first selection, the baculovirus DNA was digested at a unique Bosu′ site in the lacZ sequence to select against non-recombinant plaques (Kitts et al., 1990). 1.5 ml serum-free TC-100 medium was added and the cells were incubated at 28°C. After 8 h the medium was changed and supplemented with 10% FCS. Three days post-transfection, the virus particles were harvested by spinning the cells at 1000 g for 5 min. The supernatant was assayed for binding of the antibody by the addition of 0.55 ml supernatant and overlaying them with agarose and then TC-100 medium/10% FCS. After 3–5 days incubation, the medium was replaced by 0.25% X-gal in TC-100 medium supplemented with 5% FCS. Recombinant viruses were detected by clear plaques after overnight incubation. The plaques were picked and the recombinant virus purified by one more round of screening in the presence of X-gal and grown to high titre stocks by serial amplification in two monolayer and one suspension culture. The presence of PPX cDNA insertion into the viral DNA was verified by dot blot hybridization (Summers and Smith, 1987) with a PPX cDNA probe (data not shown).

PPX was expressed by infecting a suspension culture of S99 cells (at 10⁶ cells/ml) with recombinant baculovirus at a multiplicity of infection of 1. At 42 h post infection, the cells were harvested, washed and lysed by freezing and thawing four times in 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.025% DTT, 0.1 mM EGTA. Particular material was removed by centrifugation at 14 000 g for 10 min at 4°C and the supernatant filtered through a 0.2 μm disposable filter (Millipore, Molsheim, France).

Protein phosphatase assays

32P-labelled rabbit skeletal muscle glycogen phosphatase was prepared by phosphorylation by phosphorylase kinase to a stoichiometry of 1 mol phosphate per mol subunit as described (Cohen et al., 1988a). The specific activity of the ATP used for all phosphorylations was ~10⁶ c.p.m./μmol. cAMP-dependent protein kinase was used to phosphorylate phosphorylase phosphorylase kinase (1.7 mol phosphate/mol) (Stewart et al., 1981), bovine casein (3 mol phosphate/mg) (McGowan and Cohen, 1987) and the synthetic peptide RRATV (0.7 mol phosphate/mol) (Deana et al., 1990). 32P-labelled calf thymus histone H1 (2.15 mol phosphate/mol), chromatin high mobility group I (HMGI) (2.4 mol phosphate/mol) and caldesmon (1.3 mol phosphate/mol) were prepared by phosphorylation with mammalian p34cdc2 purified through the DEAE-cellulose step and were all kindly provided by Professor T.A. Langan, University of Colorado, Denver.

Protein phosphatase assays were performed in the absence of divalent cations as described in Cohen et al. (1988a) using the following phosphorylated substrates: glycogen phosphorylase at 10 μM, casein at 65 μM, RRATV, histone H1, HMGI and caldesmon each at 2 μM. Assays were terminated with 20% TCA in the case of histone H1 when 12.5 μg TCA was added followed by 30 min on ice to ensure complete precipitation. Dephosphorylation of the α and β subunits of phosphorylase kinase was quantified by the method described in Stewart et al. (1981). RRAT(32)PVA dephosphorylation was quantified using acid molybdate and extraction with organic solvents (Deana et al., 1990). One unit of activity is that amount of enzyme which catalyses the release of 1 μmol [32]Piphosphate/min.

Immunofluorescence staining

Affinity purified antibodies were biotinylated using a succinimidyl ester of biotin. Antibody, 1 mg/ml in 0.1 M NaHCO₃ pH 8, was mixed with N-hydroxysuccinimide-activated avidin to a ratio of 10:1 (w/w). After 4 h at ambient temperature, the reaction was terminated by the addition of 1 vol of 1 M NH₄Cl. Uncoupled biotin was removed by extensive dialysis.

Cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, plated on to 12 mm glass coverslips and allowed to grow for a further 24–48 h. After a brief wash at 37°C in PBS, the cells were fixed in either 90% methanol/10% 100 mM MES pH 6.9, 1 mM EGTA, 1 mM MgCl₂ at ~20°C for 30 min in 4% paraformaldehyde in PBS at ambient temperature for 20 min. They were then washed in PBS, permeabilized for 10 min in 1% Nonidet P-40 in PBS, washed further in PBS and incubated for a few minutes at ambient temperature first in 3% BSA fraction V (crystalline grade) in PBS, and then in avidin/biotin blocking solution. The cells were covered with a solution of biotinylated primary antibody (diluted 10–50 fold in PBS) and placed in a humidified chamber at 6°C overnight. The cells were washed in PBS, incubated with a 10 μg/ml solution of the avidin–fluorescein conjugate for 20 min, washed in PBS, washed in 0.15 M NaCl, 0.1 M NaHCO₃ pH 8.4, incubated with either rat anti-tubulin antibodies, human # 5051 antibodies (Calarco-Gillam et al., 1983), or propidium iodide (0.1
ag/ml) for 1 h. The anti-tubulin antibodies and #5051 antibodies were detected using anti-rat antibodies and anti-human antibodies conjugated to Texas Red respectively. The coverslips were washed, mounted and the fluorescence examined using a Bio-Rad MRC 600 laser scanning confocal microscope.

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