# Carboxymethylation of nuclear protein serine/threonine phosphatase X

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Specific rabbit polyclonal antibodies against peptides corresponding to the highly homologous protein serine/threonine phosphatase 2A and X catalytic subunits (PP2A/C and PPX/C respectively) were used to investigate the cellular and subcellular distribution of PP2A/C and PPX/C, as well as their methylation state. Immunoblots of rat tissue extracts revealed a widespread distribution of these enzymes but particularly high levels of PP2A/C and PPX/C in brain and testes respectively. In addition, immunoblots of subcellular fractions and immunocytochemical analyses of rat brain sections demonstrated that PPX/C is predominantly localized to the nucleus, whereas PP2A/C is largely cytoplasmic. Treatment of nuclear extracts with alkali resulted in increased PPX/C immunoreactivity to a polyclonal antibody directed against the C-terminus; no change in PPX immunoreactivity was observed using an antibody against an internal peptide. Alkali treatment of brain and liver cytosolic and nuclear extracts did not change the molecular mass or the isoelectric point of PPX/C. Furthermore, tritiated PPX/C was immunoprecipitated from COS cell extracts incubated with the methyl donor *S*-adenosyl-L-[*methyl*-$^3$H]methionine. Thus the increase in immunoreactivity probably results from removal of a carboxymethyl group from PPX/C, as has been shown previously for PP2A/C [Favre, Zolnierowicz, Turowski and Hemmings (1994) J. Biol. Chem. **269**, 16311–16317]. Together, our results indicate that the PPX catalytic subunit is a predominantly nuclear phosphatase and is methylated at its C-terminus.

## INTRODUCTION

The reversible phosphorylation of proteins on serine and threonine residues is an important mechanism for the regulation of diverse pathways controlling cellular physiology [1]. Although less attention has been given to the protein serine/threonine phosphatases than to the serine/threonine kinases, it is nonetheless clear that this group of proteins plays an integral role in the control of cell growth and differentiation. The protein serine/threonine phosphatase 2A (PP2A) family of enzymes has been implicated in the regulation of many cellular signalling molecules including regulation of metabolic enzymes, cytoskeletal proteins, cell-surface receptors, ion channels, protein kinases and transcription factors [2–8].

PP2A is a multimeric enzyme composed of a catalytic subunit associated with two regulatory subunits; one is the A regulatory subunit and the other is the B or variable regulatory subunit [2]. The regulatory mechanisms that have been described for PP2A include post-translational modifications of the catalytic subunit, which directly alter phosphatase activity [9–11], and association of the catalytic subunit with variable regulatory subunits, which may direct subcellular localization and/or dictate substrate selectivity (reviewed in [2–7]). One post-translational modification, the methylation of the C-terminal leucine residue of the catalytic subunit of PP2A by a novel methyltransferase, has been demonstrated using both biochemical and immunological techniques [11–14]. Recent data have revealed that this carboxymethylation increases PP2A activity *in vitro* [11] and may be important for regulation of this enzyme during the cell cycle [13]. (The term carboxymethylation is used as a descriptor for O-methylation of the C-terminal $\alpha$-carboxy group, also referred to as methyl esterification of the C-terminal amino acid.)

Protein serine/threonine phosphatase X (PPX) is a novel phosphatase that is highly homologous in amino acid sequence to PP2A/C [15,16]. In fact, the catalytic subunit of PPX and PP2A (PPX/C and PP2A/C respectively) are identical at the extreme C-terminus where methylation of PP2A/C has been shown to occur [11]. The recombinant PPX catalytic subunit exhibits substrate specificity and sensitivity to phosphatase inhibitors that is similar to, but not identical with, that of PP2A/C [16]. Immunofluorescence studies in human fetal lung cells (MRC-5) and epidermal carcinoma cells (A431) have demonstrated that PPX/C is distributed throughout the cytoplasm and nucleus, with intense staining at centrosomes, implicating PPX in the control of microtubule nucleation [16]. This is in contrast with PP2A/C, which is dispersed throughout the cell, with predominant localization in the cytoplasm [17]. Although the molecular basis for this differential subcellular distribution of PPX/C and PP2A/C is not known, a reasonable hypothesis is that regulatory subunits and/or post-translational modification of the catalytic subunit play an important part in directing the catalytic subunits of these enzymes to specific regions within the cell.

In this report, we utilized subcellular fractionation and immunocytochemistry to examine the cellular and subcellular distribution of PPX in rat tissues. In addition, alkali-enhanced immunoreactivity and *in vitro* labelling with *S*-adenosyl-L-[*methyl*-$^3$H]methionine ([$^3$H]SAM) have been used to investigate whether PPX/C is carboxymethylated. The data reveal that PPX is predominantly nuclear and that PPX/C like PP2A/C, is methylated on its C-terminus.

## MATERIALS AND METHODS

### Materials

Goat anti-rabbit antibody–alkaline phosphatase conjugate, amplified alkaline phosphatase immunoblot assay kit, prestained SDS/PAGE molecular-mass standards, urea and 1-ethyl-3-(3-

Abbreviations used: PP2A, protein serine/threonine phosphatase 2A; PPX, protein serine/threonine phosphatase X; PP2A/A, A regulatory subunit of PP2A; PP2A/C, PP2A catalytic subunit; PPX/C, PPX catalytic subunit; RLNE, rat liver nuclear extract; RLCE, rat liver cytosolic extract; RBNE, rat brain nuclear extract; RBCE, rat brain cytosolic extract; [$^3$H]SAM, *S*-adenosyl-L-[*methyl*-$^3$H]methionine; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide; HA, haemagglutinin.

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dimethylaminopropyl)carbodi-imide (EDAC) were obtained from Bio-Rad (Richmond, CA, U.S.A.). *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester was from Pierce (Rockford, IL, U.S.A.). The 12CA5 anti-haemagglutinin (HA) monoclonal antibody was purchased from Berkeley Antibody Co. (Richmond, CA, U.S.A.). Ampholines (pH 3.5–10 and pH 5–8), epoxyaminohexyl-Sepharose and GammaBind Plus Sepharose were from Pharmacia LKB Biotech Inc. (Uppsala, Sweden). All tissues were obtained from Harlan Bioproducts for Science (Indianapolis, IN, U.S.A.). [$^3$H]SAM and ENTENSIFY fluorography reagents were purchased from DuPont–NEN (Boston, MA, U.S.A.). CentriPrep-30 concentrators were from Amicon (Beverly, MA, U.S.A.).

### Generation of phosphatase antisera

Synthetic peptides corresponding to amino acid residues 298–309 of human PP2A/C (PHVTRRTPDYFL), 294–307 of human PPX/C (RGIPSKKPVADYFL) and 7–19 of the human PP2A/A subunit (DDSLYPIAVLIDE) were coupled to keyhole limpet haemocyanin at pH 7.4 using EDAC as previously described [18]. An internal peptide corresponding to amino acid residues 289–302 of human PPX/C (CGAPQETRGIPSKKPV) was synthesized with N-terminal glycine and cysteine residues. This peptide was coupled to keyhole limpet haemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as previously described [18]. The peptide–keyhole limpet haemocyanin (250 $\mu$g) conjugates were injected subcutaneously into rabbits, first in complete Freund's adjuvant followed by two boosts in incomplete adjuvant. Blood was collected 1 week after the second boost, and the antisera were characterized by immunoblotting selected rat tissues and subcellular fractions in the absence and presence of the appropriate peptides.

### Immunoaffinity purification of antibodies

Anti-peptide antibodies were purified from rabbit antisera by affinity chromatography [18]. The appropriate synthetic peptide was attached covalently to epoxyaminohexyl-Sepharose with EDAC according to the manufacturer's instructions. The slurry was transferred to a column and washed extensively with Tris buffer (10 mM Tris/HCl, pH 7.5). Rabbit antiserum diluted 1:5 with Tris buffer was passed over the peptide–Sepharose conjugate three times. The resin was washed with Tris buffer and then with Tris buffer containing 500 mM NaCl. Bound antibodies were eluted by sequential addition of 100 mM glycine, pH 2.5, followed by 100 mM triethylamine, pH 11.5. The eluates were pooled, and the antibodies concentrated and dialysed against Tris-buffered saline containing 0.02 % $NaN_3$ using CentriPrep-30 concentrators.

### Preparation of tissue homogenates

Rat tissues were homogenized (two 15 s bursts) using a Polytron in 5 ml of buffer (10 mM Tris/HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 5 $\mu$g of leupeptin/ml, 20 $\mu$g of soya bean trypsin inhibitor/ml and 0.1 mM PMSF) per g of tissue. Tissue homogenates were stored at −80 °C.

### Preparation of rat liver and rat brain cytosolic and nuclear extracts

Rat liver nuclear extract (RLNE) and rat brain nuclear extract (RBNE) were prepared by a modification of the method of Gorski et al. [19]. Extracted nuclear proteins were precipitated with solid $(NH_4)_2SO_4$ (0.36 g/ml) and collected by centrifugation at 90000 ***g*** for 45 min. The pelleted proteins were either stored at −20 °C or resuspended and dialysed in 25 mM Hepes, pH 7.6, containing 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 10 % glycerol. Rat liver cytosolic extract (RLCE) and rat brain cytosolic extract (RBCE) were prepared using a modification of a method previously described [20]. Briefly, rat livers or brains were homogenized in 2.5 vol. (by weight) of a buffer consisting of 50 mM Tris/HCl, pH 7.5, 5 mM magnesium acetate, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM PMSF and 20 % glycerol. The homogenate was centrifuged at 23000 ***g*** for 30 min. The resulting supernatant was carefully removed, leaving approx. 20 % behind, and centrifuged at 180000 ***g*** for 60 min. The top 80 % of the supernatant was carefully removed, divided into aliquots in appropriate containers, quickly frozen in an ethanol/solid $CO_2$ bath, and stored at −80 °C.

### Alkali treatment and analysis of cellular extracts

The indicated amounts of RBCE, RBNE, RLCE and RLNE were incubated with an equal volume of either control buffer (50 mM Tris/HCl, pH 7.5) or 0.2 M NaOH for 30 min at 37 °C [11]. The alkali-treated extracts were then neutralized with the appropriate amount of 2 M HCl. Both control and alkali-treated samples were solubilized in electrophoresis sample buffer, boiled for 10 min and analysed by SDS/PAGE and immunoblotting. For two-dimensional gel electrophoresis, samples were solubilized in isoelectric focusing sample buffer [21] and solid urea was added to saturation. The proteins were separated by isoelectric focusing followed by SDS/PAGE as previously described [21]. Gradients were obtained by mixing 2.80 ml of pH 3.5–10 ampholines and 1.05 ml of pH 5–8 ampholines per 40 ml of gel solution.

### Cell transfection

A cDNA encoding full-length PPX catalytic subunit [16] was tagged at its 5′-end using oligonucleotides encoding the HA epitope tag sequence (YPYDVPDYA) as previously described for epitope-tagging PP2A/C [22]. The epitope-tagged PPX/C (TagPPX) cDNA was cloned into the pCW1-neo expression plasmid [22]. COS M6 cells maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum were transiently transfected (10 cm tissue culture dishes) using the DEAE–dextran procedure as described previously [22,23].

### Methylation of COS cell extracts

Transfected COS cells (one 10 cm dish) were dislodged by scraping in 300 $\mu$l of lysis buffer containing 50 mM Tris/HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 $\mu$g of leupeptin/ml, 1 mM benzamidine and 0.5 mM PMSF. Cells were lysed in a glass Wheaton homogenizer (10 strokes) and vortex-mixed briefly, followed by centrifugation at 13000 ***g*** for 5 min. The extracts (250 $\mu$g of protein in 100 $\mu$l) were incubated with 0.1 vol. of 0.55 mCi/ml [$^3$H]SAM (78.6 Ci/mmol) for 30 min at 37 °C, then subjected to SDS/PAGE. Gels were stained with Coomassie Blue and analysed by fluorography. Identical extract samples incubated with 0.1 vol. of vehicle were analysed by immunoblotting.

### Immunoprecipitation of methylated PPX/C

[$^3$H]SAM-labelled cell extracts (approx. 1 mg) were incubated with an equal volume of 2 × IP buffer (20 mM Tris/HCl, pH 7.5, 1.0 % Triton X-100, 1 M NaCl, 2 mM EDTA, 2 mM dithiothreitol, 20 $\mu$g of leupeptin/ml and 2 mM benzamidine) and approx. 5 $\mu$g of HA monoclonal antibody. The mixture was rotated for 1 h at 4 °C. GammaBind Plus Sepharose (5 $\mu$l of a

75% slurry) was added and the mixture was rotated for an additional 30 min. The beads were briefly washed twice with 0.5 ml of 1 × IP buffer, then twice with 1 × IP buffer containing 150 mM NaCl. After the last wash, Laemmli sample buffer was added to the beads and the solubilized proteins were analysed by SDS/PAGE and fluorography.

### Immunocytochemistry

Immunostaining of rat brain sections was performed as previously described [24] except that some of the sections were treated with 0.2 M NaOH before incubation with primary antibodies. Alkali treatment greatly enhanced PP2A/C and PPX/C immunoreactivity (see the Results and discussion section). Control experiments where antibody was incubated with 20 $\mu$g of immunogenic peptide/ml showed no staining.

### Gel electrophoresis and immunoblotting

The indicated amount of protein was solubilized in Laemmli sample buffer, boiled for 10 min, and then subjected to SDS/PAGE (10% gel, 1.5 mm thick). After electrophoresis, proteins were transferred to nitrocellulose membranes [25]. The blots were incubated with the appropriate antisera or affinity-purified anti-peptide antibodies, followed by incubation with goat anti-rabbit IgG–alkaline phosphatase conjugate or biotinylated secondary antibody/streptavidin–biotin–alkaline phosphatase conjugate as indicated. All immunocomplexes were visualized with bromochloroindoylphosphate and Nitro Blue Tetrazolium as substrates for alkaline phosphatase.

### Determination of protein concentration

Protein concentration was determined using the Bio-Rad protein assay with BSA as the standard.

## RESULTS AND DISCUSSION

### Tissue, cellular and subcellular distribution of PP2A/C and PPX/C

Peptides corresponding to the C-termini of PP2A/C and PPX/C, as well as to an internal region close to the N-terminus of PP2A/A, were used to generate specific antisera. Rabbit polyclonal antibodies were affinity-purified from these antisera (see the Materials and methods section) and used for Western-blot analysis of selected rat tissue extracts (Figure 1A). Affinity-purified PP2A/A, PP2A/C and PPX/C antibodies detect single bands of 65, 36 and 34 kDa respectively, the predicted size of these proteins based on their amino acid sequences. These immunoreactive proteins were not observed when incubation of the blot with antibody was carried out in the presence of the corresponding peptide (not shown). Immunoblotting rat tissues with PP2A/C- and PPX/C-specific antibodies revealed a widespread distribution of these enzymes, with highest levels of PP2A/C and PPX/C in brain and testes respectively (Figure 1A). The tissue distribution of PP2A/C protein is in good agreement with a previous report [26]. The tissue distribution of PPX/C protein has not previously been reported, but high levels of PPX/C mRNA have been detected in testes [16]. Thus it appears that the relative levels of PPX/C protein parallel PPX/C mRNA. PP2A/A was detected in all tissues examined, largely paralleling the distribution of PP2A/C, which is consistent with the hypothesis that PP2A/C and PP2A/A form the core PP2A complex [8].

In addition to differential tissue distribution, PP2A/C and PPX/C also exhibit differences in subcellular localization (Figure 1B). Western-blot analysis of RLCE and RLNE revealed that PP2A/C is enriched in the cytosol, whereas PPX/C is more abundant in the nucleus. Similar results were obtained using subcellular fractions from rat brain (not shown). Despite the high specificity of the affinity-purified antibodies, quantification of catalytic subunit level was not attempted because post-translational modification masks antibody recognition in some instances (see below). Nonetheless, the qualitative findings provide strong evidence that PP2A/C and PPX/C are highly expressed in brain and testes respectively, and that PPX/C is predominantly localized to the nucleus, whereas PP2A/C is largely cytoplasmic.

To investigate the cellular and subcellular distribution of PPX/C and PP2A/C in more detail, rat brain sections were examined by light-microscopic immunocytochemistry using the avidin–biotin–horseradish peroxidase staining technique (Figure 2). Immunostaining with either affinity-purified antibody was very weak unless brain sections were first treated with 0.2 M NaOH. In contrast with PPX/C and PP2A/C, PP2A/A immunoreactivity was unaltered after alkali treatment (not shown). These results are consistent with a previous report examining the carboxymethylation of PP2A/C [13] and the biochemical data described below that demonstrate the carboxymethylation of PPX/C.

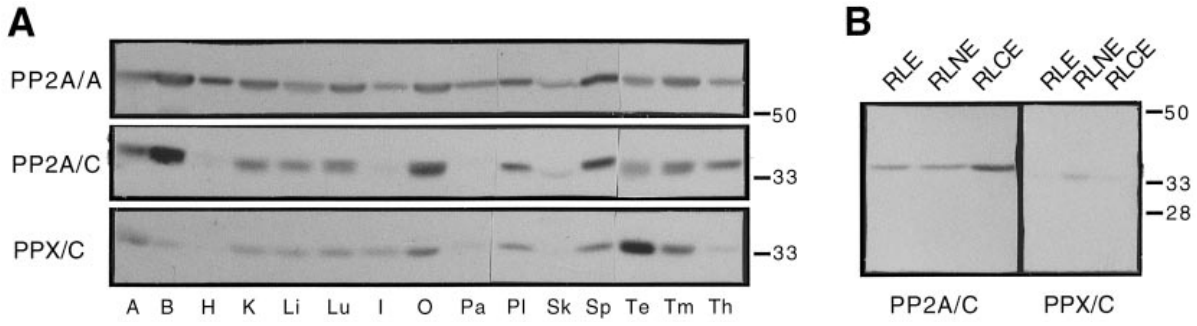


**Figure 1 Immunoblotting rat tissue extracts and subcellular fractions with affinity-purified PP2A and PPX antibodies**

(**A**) Whole-tissue homogenates (75 $\mu$g) from different rat tissues were analysed by SDS/PAGE and immunoblotting with the indicated affinity-purified antibodies specific for PP2A/C, PPX/C and PP2A/A. The tissues analysed were: adrenal gland (A), brain (B), heart (H), kidney (K), liver (Li), lung (Lu), intestine (I), ovaries (O), pancreas (Pa), placenta (Pl), skeletal muscle (Sk), spleen (Sp), testes (Te), thymus (Tm) and thyroid (Th). (**B**) Total rat liver extract (RLE), RLNE and RLCE (75 $\mu$g each) were analysed by SDS/PAGE and immunoblotting with the indicated affinity-purified antibodies specific for PP2A/C and PPX/C. The migration of prestained SDS/PAGE molecular-mass standards (kDa) is shown.

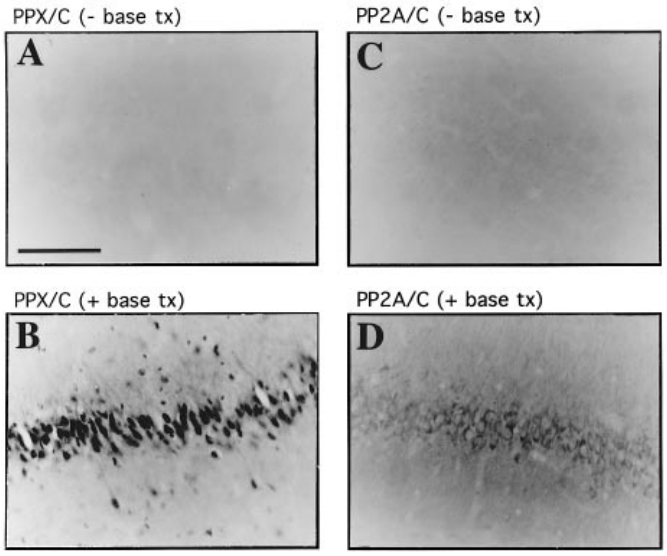


**Figure 2 Immunocytochemical localization of PPX and PP2A in the rat brain**

Coronal sections of adult rat brain were probed with affinity-purified antibodies to either PPX/C (**A**, **B**) or PP2A/C (**C**, **D**), and bound antibody was visualized using the avidin–biotin–horseradish peroxidase staining technique (see details in the Materials and methods section). Sections in (**B**) and (**D**) were treated with 0.2 M NaOH (base tx) before antibody incubation; sections in (**A**) and (**C**) received no pretreatment. Similar areas of the pyramidal cell layer in the CA3 region of the hippocampus are shown. Preadsorbing antibody with the corresponding peptide virtually abolished staining (not shown).

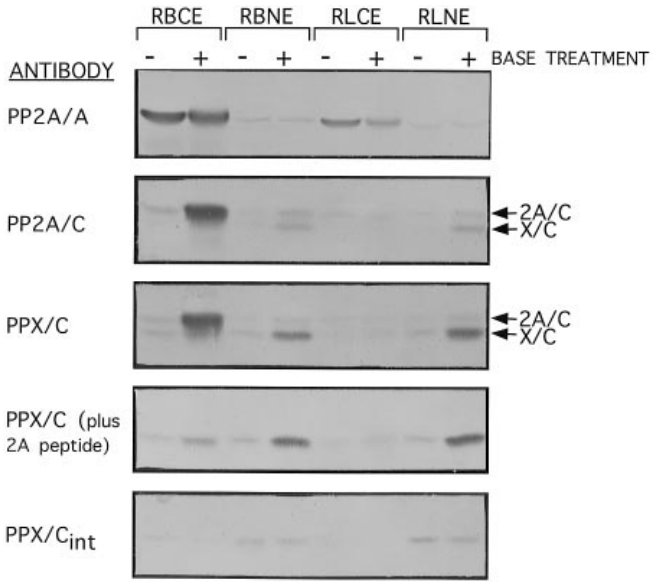


**Figure 3 PP2A and PPX immunoreactivity in alkali-treated extracts**

A 100 $\mu$g sample of RBCE, RLCE and RLNE and 50 $\mu$g of RBNE were incubated with either Tris/HCl (−) or NaOH (+). The samples were analysed by SDS/PAGE and immunoblotted with the indicated affinity-purified antibody (PP2A/A, PPX/$C_{int}$) or the indicated antiserum (PP2A/C, PPX/C) diluted 1:500. The unpurified PP2A/C and PPX/C antisera used in these experiments show some cross-reactivity with the other phosphatase. However, the immunoreactivity of PP2A/C using PPX/C antisera was eliminated by inclusion of the PP2A/C C-terminal peptide (fourth panel).

PPX/C was found to be expressed by most, if not all, neurons and was predominantly localized to the nucleus. At high antibody concentrations, some staining of PPX/C was observed in soma and processes. Glial nuclei were stained, but this staining was less intense than neuronal nuclei. Consistent with a previous report [17], PP2A/C was found to be widely expressed in the brain. Both PPX/C and PP2A/C were expressed in the same neurons, but PP2A/C was enriched in soma and processes. Glial staining was weak or absent with the PP2A/C antibody. No cross-reactivity between the two affinity-purified antibodies was observed, since preincubation of the PPX/C antibody with the PP2A/C peptide or vice versa had no effect on the cell staining pattern or intensity. In other studies, the immunostaining of tissue culture cells (e.g. NIH-3T3, COS M6 and H4IIe cells) also revealed predominant nuclear localization of PPX/C (results not shown). However, at this level of resolution, we were unable to visualize any centrosomal staining of PPX/C as has been previously reported [16].

### Post-translational modification of PPX

Demethylation of the C-terminal leucine residue of PP2A/C by alkali treatment or ethanol precipitation increases antibody binding and thus enhances immunoreactivity [11]. The alkali-induced increase in PPX/C immunostaining (Figure 2) suggests that PPX/C, like PP2A/C, also may be carboxymethylated. Furthermore, comparison of the amino acid sequence of the C-termini of PP2A/C and PPX/C reveals a conserved sequence (i.e. DYFL) implicated in carboxymethylation [11]. On the basis of this homology, it seemed likely that binding of antibodies to the C-terminus of PPX/C would be sensitive to carboxymethylation.

Alkali treatment of rat tissue extracts resulted in increased PP2A/C and PPX/C immunoreactivity; however, the tissue distribution profile of PP2A/C and PPX/C in the alkali-treated extracts (not shown) parallelled their distribution in untreated samples (Figure 1). Similarly, alkali treatment of cytosolic and nuclear extracts from rat brain and liver also resulted in increased immunoreactivity of both PP2A/C and PPX/C, without any change in the molecular mass of these proteins (Figure 3, middle panels). The most dramatic increase in PP2A/C immunoreactivity was observed in RBCE, whereas PPX/C immunoreactivity was most strongly enhanced in RLNE. In contrast with the affinity-purified PPX/C and PP2A/C antibodies which are specific (Figure 1), the PPX/C antiserum exhibits some cross-reactivity with PP2A/C (Figure 3, third panel). This cross-reactivity, which is most apparent in samples containing large amounts of PP2A/C (such as brain), can be eliminated by preincubation of the PPX/C antiserum with the PP2A/C peptide used as antigen for preparation of PP2A-specific antibodies (Figure 3, fourth panel). Affinity-purified antibodies directed against an internal peptide of PPX/C and an N-terminal peptide of PP2A/A did not reveal any change in antigen recognition after alkali treatment (Figure 3, bottom and top panels), indicating that alkali exposure resulted in selectively enhanced immunoreactivity of the C-terminus of PPX/C and PP2A/C.

Although the observed effect of alkali treatment is consistent with demethylation of the C-terminal leucine residue, a phosphate group on serine and threonine residues is also sensitive to hydrolysis by alkali [11]. A serine residue ten amino acids from the C-terminus (Ser-297) of PPX/C is present in the two epitopes recognized by the C-terminus and internal antibodies. The increase in immunoreactivity was only observed with the C-terminal antibody, consistent with the interpretation that this enhanced detectability was not due to the removal of phosphate

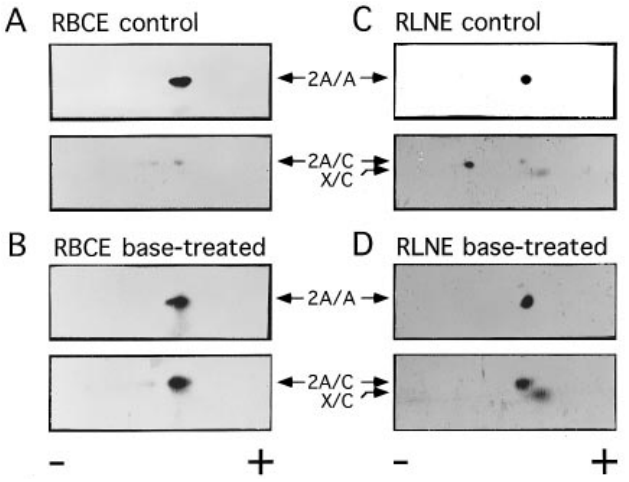


**Figure 4 Two-dimensional gel electrophoresis of alkali-treated nuclear and cytosolic extracts**

RBCE (**A** and **B**) and RLNE (**C** and **D**) (250 $\mu$g of each) were incubated with Tris/HCl (**A**, **C**) or NaOH (**B**, **D**) and analysed by two-dimensional electrophoresis. The proteins were transferred to nitrocellulose and probed with the indicated affinity-purified antibody (2A/A) or antiserum (2A/C, X/C) diluted 1:500. The catalytic subunits were visualized by the amplified alkaline phosphatase immunoblot assay kit. The pH range shown is 5.1–6.7.

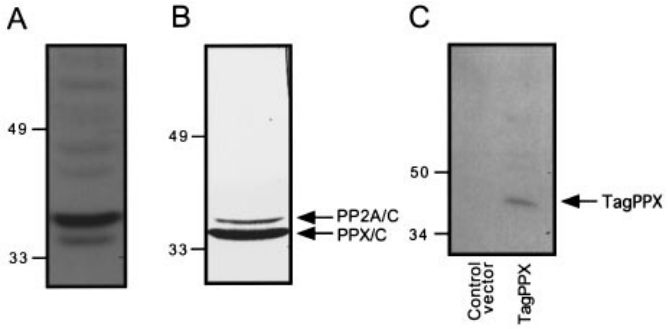


**Figure 5 *In vitro* methylation of PPX/C**

(**A**) COS cell extracts were incubated with [$^3$H]SAM for 30 min at 37 °C and analysed by SDS/PAGE and fluorography. (**B**) Samples of the cell extracts in (**A**) were incubated with water and analysed by immunoblotting with PPX/C antiserum which also detects PP2A/C. (**C**) Cellular extracts prepared from COS cells transfected with the indicated constructs were incubated with [$^3$H]SAM. Epitope-tagged PPX/C was then immunoprecipitated using the HA tag antibody and analysed by SDS/PAGE and fluorography. The migration of prestained SDS/PAGE molecular-mass standards (kDa) is indicated.

from a serine residue. Furthermore, the alkali-induced increase in immunoreactivity of PPX/C using unpurified PP2A/C antiserum (Figure 3, second panel) would also suggest that the serine residue in the peptide sequence recognized by the antibody is not modified since the PP2A antiserum is directed against a peptide that does not contain a serine residue at the corresponding position. Since only the last four amino acids are identical in the PP2A/C and PPX/C peptides, the alkali-induced change in immunoreactivity of PPX detected by both the PPX-directed and PP2A-directed antibody probably occurs in the four shared C-terminal amino acids (DYFL).

Further evidence that the alkali-induced increase in immunoreactivity is due to demethylation and not dephosphorylation was obtained using two-dimensional gel electrophoresis (Figure 4). A shift towards the basic end of the gel should be observed after removal of a negatively charged phosphate group, whereas no change in isoelectric point should be observed after removal of an uncharged methyl group [11]. RBCE and RLNE were selected for these studies as they exhibited the most pronounced increase in immunoreactivity of PP2A/C and PPX/C, respectively, after alkali treatment of these extracts. Two major forms of PP2A/C were observed in untreated RBCE (Figure 4A). This heterogeneity of PP2A/C proteins is consistent with previous reports [11,27]. Alkali treatment of RBCE did not result in any noticeable shift in PP2A/C proteins, as visualized using the PP2A/C C-terminal antiserum, but resulted in a substantial increase in immunodetection of the most acidic form of PP2A/C (Figure 4B). Immunoblotting of untreated RLNE with the PPX/C C-terminal antiserum revealed that PPX/C was slightly more acidic and of lower molecular mass than the two nuclear PP2A/C forms that were detected with the PPX/C antiserum (Figure 4C). The identity of the PP2A/C proteins was established by preadsorbing the PPX/C antiserum with PP2A/C peptide (see Figure 3). Alkali treatment of RLNE did not alter the isoelectric point of PPX/C but did cause a significant increase in immunoreactivity (Figure 4D). Again, the top halves of these blots were probed with the affinity-purified PP2A/A antibodies to control for non-specific effects of alkali treatment and to provide a reference point. The lack of change in molecular mass and isoelectric point of PPX/C after alkali treatment suggests that the predicted modification is a small uncharged alkali-sensitive group.

Interestingly, two-dimensional gel electrophoresis of RLNE revealed an alkali-induced conversion of two PP2A/C charge variants to one protein, concomitant with an increase in immunoreactivity (Figures 4C and 4D). The apparent alkali-induced acidic shift in the one nuclear PP2A/C protein suggests that the observed effect was not due to the removal of a negatively charged phosphate group. It remains to be determined whether these results can be explained by the presence of two nuclear PP2A/C forms, which exhibit different isoelectric points but co-migrate on two-dimensional gels after alkali treatment, or by the presence of novel PP2A/C isoforms.

## *In vitro* methylation of PPX/C

The data above are suggestive of carboxymethylation of PPX/C. To examine the methylation of PPX/C more directly, COS cell extracts were incubated with the methyl donor [$^3$H]SAM and subsequently analysed by SDS/PAGE and fluorography. Two prominent radiolabelled proteins migrating at 34 and 36 kDa were observed (Figure 5A). Western-blot analysis of the same samples showed that PP2A/C and PPX/C immunoreactivity co-migrated with these labelled proteins (Figure 5B). Radiolabelling of the 34 and 36 kDa proteins was not observed after alkali treatment of [$^3$H]SAM-labelled COS cell extracts (results not shown). To evaluate directly whether the catalytic subunit of PPX could be methylated, lysates prepared from COS cells transfected with epitope-tagged PPX/C (TagPPX) cDNA or control vector were incubated with [$^3$H]SAM, and immunoprecipitated using the HA tag antibody. Labelled TagPPX was immunoprecipitated from lysates of cells transfected with TagPPX cDNA, but not from lysates of mock-transfected cells (Figure 5C), demonstrating that PPX/C can be methylated by endogenous methyltransferases. Moreover, the radiolabelled moiety could be removed from immunoprecipitated epitope-tagged PPX after alkali treatment (results not shown). Thus the [$^3$H]SAM labelling of epitope-tagged PPX was sensitive to the

same alkali treatment that resulted in increased PPX immunoreactivity (Figures 3 and 4).

Multiple forms of protein methylation have been reported. Methylation of C-terminal prenylcysteine residues has been demonstrated for several important regulatory molecules including low-molecular-mass GTP-binding proteins such as Ras, the $\gamma$-subunits of heterotrimeric G-proteins and nuclear lamins [28,29]. This post-translational modification of the $\gamma$-subunit of heterotrimeric G-proteins allows the most efficient signal transduction between molecules, but it is not required for transduction to occur [30,31]. Unlike the family of methyltransferases acting on G-proteins and lamins, the carboxymethylation of PP2A/C occurs on a C-terminal leucine residue and is catalysed by a novel methyltransferase, which is thought to be relatively specific for this enzyme [32]. The carboxymethylation of PPX/C may also be catalysed by the same or a related methyltransferase, given the high degree of amino acid sequence identity at the C-termini of PP2A/C and PPX/C.

Although the precise role of carboxymethylation of PP2A/C and PPX/C is not known, previous reports have revealed that the methylation state of PP2A/C changes during the cell cycle [13] and influences its catalytic activity *in vitro* [11]. Carboxymethylation may be necessary for functional expression of these catalytic subunits and/or for the correct assembly of subunits to form holoenzymes. The C-terminus of the catalytic subunit does appear to be important for functional expression of the catalytic subunit since alterations in this region affect expression in clonal cells. PP2A/C epitope-tagged on its N-terminus could be expressed as a functional enzyme after transient expression in COS or stable expression in Rat1a cells, but the C-terminal tagged PP2A/C could not be expressed in these cells [22]. Alternatively, carboxymethylation of the phosphatase catalytic subunit may influence binding of regulatory subunits which are predicted to direct the cellular localization of the catalytic subunit, and thus could indirectly play a role in compartmentalization of these phosphatases. Indeed, our preliminary data indicate that carboxymethylation of PP2A/C may be necessary for association with specific regulatory subunits (J. Bryant and B. Wadzinski, unpublished work).

In summary, the present studies demonstrate that PPX is predominantly localized in the nucleus and that the catalytic subunit of PPX, like the PP2A catalytic subunit, is carboxymethylated. Additional studies, which include purification of the nuclear PPX complex and elucidation of the role carboxymethylation plays in regulating this enzyme, will reveal the subunit composition and regulation of nuclear PPX.

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