Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Bα subunit

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Methylation of the C-terminal leucine residue (Leu[309]) of protein serine/threonine phosphatase 2A catalytic subunit (PP2A$_C$) is known to regulate catalytic activity *in vitro*, but the functional consequence(s) of this post-translational modification in the context of the cell remain unclear. Alkali-induced demethylation of PP2A$_C$ in purified PP2A heterotrimer (ABαC), but not in purified PP2A heterodimer (AC), indicated that a larger fraction of PP2A$_C$ is carboxymethylated in ABαC than in AC. To explore the role of Leu[309] in PP2A holoenzyme assembly, epitope-tagged PP2A catalytic subunit (HA-PP2A) and a mutant of HA-PP2A containing an alanine residue in place of Leu[309] (HA-PP2A-L309A) were transiently expressed in COS cells. Both recombinant proteins exhibited serine/threonine phosphatase activity when immunoisolated from COS cell extracts. HA-PP2A, but not HA-PP2A-L309A, was carboxymethylated *in vitro*. A

chromatographic analysis of cell extracts indicated that most endogenous PP2A$_C$ and HA-PP2A were co-eluted with the A and Bα regulatory subunits of PP2A, whereas most HA-PP2A-L309A seemed to elute with the A subunit as a smaller complex or, alternatively, as free catalytic (C) subunit. The A subunit co-immunoisolated with both tagged proteins; however, substantially less Bα subunit co-immunoisolated with HA-PP2A-L309A than with HA-PP2A. These results demonstrate that the reversibly methylated C-terminal leucine residue of PP2A$_C$ is important for Bα regulatory subunit binding. Furthermore, the results provide evidence for an interrelationship between PP2A$_C$ carboxymethylation and PP2A holoenzyme assembly.

Key words: protein dephosphorylation; protein phosphatase; protein phosphorylation; post-translational modification.

INTRODUCTION

Protein serine/threonine phosphatase 2A (PP2A) performs a variety of regulatory roles in eukaryotic cells (reviewed in [1–4]). Cellular substrates for PP2A include metabolic enzymes, transcription factors, protein kinases, cytoskeletal proteins and cell-surface receptors. Because PP2A has an integral role in so many cellular processes, its activity is most probably tightly controlled *in vivo*. Regulatory mechanisms that have been identified for the control of PP2A catalytic activity include: (1) association of the catalytic subunit with regulatory proteins, (2) targeting of PP2A holoenzymes to specific subcellular structures, and (3) post-translational modifications of the catalytic subunit.

PP2A is a multiprotein complex consisting of a catalytic (C) subunit, a structural (A) subunit, and one of a number of variable (B) regulatory subunits (reviewed in [1–4]). In mammalian cells, the 36 kDa C subunit is tightly complexed with the 65 kDa A subunit to form the core structure of PP2A. This heterodimer associates with one of multiple B subunits (ranging from 54 to 130 kDa), which presumably confer distinct regulatory properties on the PP2A holoenzyme. Currently, three B subunit families have been identified (B, B′ and B″). Little amino acid sequence similarity exists between the different families, but isoforms within the same family are homologous. Although the physiological significance of this heterogeneity is not fully understood, recent results indicate that the B regulatory subunit might modulate substrate selectivity and catalytic activity, and also might direct the localization of the catalytic enzyme to a particular cellular microenvironment.

Targeting of PP2A holoenzymes to specific compartments in the cell is another regulatory mechanism for PP2A. For example, the PP2A holoenzyme consisting of the C subunit and the A and Bα regulatory subunits has been shown to associate with microtubules [5], tau [6], neurofilament proteins [7,8], and Ca²⁺/calmodulin-dependent protein kinase IV [9]. This form of regulation provides a means of tethering the enzyme to a local microenvironment, thereby enhancing access of the catalytic subunit to specific substrates. By analogy, cAMP-dependent protein kinase A, protein kinase C isoforms and protein phosphatase 2B (calcineurin) are targeted to specific cellular loci through their association with A-kinase anchoring proteins (AKAPs) [10]. Similarly, protein serine/threonine phosphatase 1 (PP1) is targeted to distinct cellular structures via its association with specific regulatory subunits [11,12].

The reversible covalent modifications of the PP2A catalytic subunit (PP2A$_C$) that have been described include phosphorylation and carboxymethylation. (The term carboxymethylation is used as a descriptor for O-methylation of the C-terminal α-carboxy group, also referred to as methyl esterification of the C-terminal amino acid.) Phosphorylation of PP2A$_C$ on one or more threonine residues by an autophosphorylation-activated protein kinase inactivates the phosphatase [13]. Phosphorylation of Tyr[307] by a variety of cellular and oncogenic tyrosine kinases also inhibits PP2A activity [14]. Another post-translational modification of PP2A$_C$ is the methylation of its C-terminal leucine residue (Leu[309]) by a specific methyltransferase [15–17]. This modification seems to increase PP2A activity *in vitro* [17,18] and might be important for the cell-cycle-dependent regulation of

PP2A [19] and insulin secretion from pancreatic β-cells [18]. The reversal of this modification is catalysed by a recently identified methylesterase [20]. These post-translational modifications of $PP2A_C$ probably have an important role in modulating phosphatase function, but their biochemical and functional consequences in the cell remain unclear.

Although several regulatory mechanisms have been described for the control of PP2A activity, little is known regarding the co-ordination of these regulatory processes. The C-terminus of $PP2A_C$, which contains sites for phosphorylation and carboxymethylation, does seem to be important for interaction with regulatory B subunits. Ogris et al. [21], have recently reported that the replacement of Thr^{304} or Tyr^{307} with a charged amino acid abolished the binding of the regulatory Bα subunit, whereas the introduction of a conservative amino acid change at either of these positions had little, if any, affect on Bα subunit binding. Because the C-terminal leucine residue of $PP2A_C$ can be modified post-translationally in a reversible manner, it was important to determine whether this residue (or its methylation state) can influence holoenzyme assembly. In the present studies, the C-terminal leucine residue, Leu^{309}, in epitope-tagged $PP2A_C$ was mutated to an alanine residue and the biochemical properties of the mutated catalyst were examined in transfected mammalian cells. The mutant catalytic subunit (HA-PP2A-L309A) was not carboxymethylated *in vitro* and demonstrated diminished interaction with the Bα regulatory subunit. Together, the results provide evidence for a connection between two forms of PP2A regulation: methylation of the C-terminal leucine residue and binding of the regulatory B subunit.

MATERIALS AND METHODS

Materials

The transformer site-directed mutagenesis kit was obtained from Clontech Laboratories (Palo Alto, CA, U.S.A.). Oligonucleotide primers were synthesized on a PerSeptive Biosystems 8900 in the Diabetes Research and Training Center (Vanderbilt University, Nashville, TN, U.S.A.). The HA tag (12CA5) monoclonal antibody was obtained from Berkeley Antibody Co. (Richmond, CA, U.S.A.) and alkaline phosphatase-conjugated goat anti-rabbit antibodies were from Bio-Rad Laboratories (Hercules, CA, U.S.A.). GammaBind Sepharose and chromatography columns were obtained from Pharmacia Biotech (Uppsala, Sweden). *S*-Adenosyl-L-[*methyl*-³H]methionine ([³H]SAM) and ENTENSIFY fluorography reagents were from DuPont–NEN (Wilmington, DE, U.S.A.).

Purification of PP2A complexes from rat brain

The protocol for purification of heterotrimeric (ABαC) and heterodimeric (AC) forms of PP2A from rat brain was based on previously published procedures [22–26]. In brief, five to ten frozen brains from Sprague–Dawley rats were homogenized in a buffer containing 50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 150 mM NaCl, 1 mM PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin and 20 μg/ml soybean trypsin inhibitor. The homogenate was centrifuged at 100 000 g for 1 h. Proteins in the supernatant precipitating at between 25 % and 50 % satn. of $(NH_4)_2SO_4$ were resuspended and PP2A complexes were purified by FPLC by using sequential fractionation on Phenyl-Sepharose [300–0 mM $(NH_4)_2SO_4$ gradient], Mono Q (0–500 mM NaCl gradient), aminohexyl-agarose (200–1000 mM NaCl gradient), Superdex-200, and Mono Q again (150–400 mM NaCl). Fractions from each column were assayed for phosphatase activity towards phosphorylated histone

H1; fractions containing the peak activity were pooled and applied to the next column. Western analysis of the column fractions demonstrated that the peak of phosphatase activity coincided with the peak of $PP2A_C$ immunoreactivity. Purified AC and ABαC were present in fractions that were eluted from aminohexyl-agarose at 1 M NaCl and from the final Mono Q column between 270 and 320 mM NaCl.

Alkali-induced demethylation of $PP2A_C$

Purified PP2A heterotrimer (ABαC) and heterodimer (AC) were adjusted to approximately equal concentrations of A and C protein with 50 mM Tris/HCl, pH 7.4. The samples were incubated with an equal volume of buffer or 0.2 M NaOH for 30 min at 30 °C. After this incubation, the alkali-treated samples were neutralized with the appropriate amount of 1 M HCl; the samples were then subjected to Western analysis with polyclonal antiserum directed against the $PP2A_C$ C-terminus [27].

Mutagenesis

Mutagenesis was performed with the transformer site-directed mutagenesis kit in accordance with the manufacturer's instructions. In brief, a mutagenic primer (5′-CCCCAGACTACTT-CGCGTAATGAAATTTTAAACTT-3′) and a primer that eliminated the *Not*I restriction site in pBluescript SK (5′-GCGGTGGCGGCAGCTCTAGAACTAGT-3′) were annealed to the epitope-tagged PP2A catalytic subunit (HA-PP2A)/pBluescript construct. After a complete cDNA-strand synthesis, the mutated cDNA was propagated in mismatch repair-deficient *Escherichia coli* (BMH 71-18 *mutS*). Mutated plasmids were twice selected by restriction digest; HA-PP2A-L309A cDNA was excised from pBluescript and inserted into the pCW1 mammalian expression plasmid [28]. The orientation and proper construction of HA-PP2A-L309A were verified by restriction enzyme analysis and DNA sequencing.

Cell transfections

COS M6 cells maintained in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum were transiently transfected with each cDNA construct by using the DEAE-dextran procedure as described previously [28].

Carboxymethylation *in vitro*

Transfected COS cells (one 10 cm dish) were dislodged 72 h after transfection by scraping into 300 μl of buffer containing 50 mM Tris/HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM benzamidine and 0.5 mM PMSF. Cells were lysed in a glass Wheaton homogenizer (ten strokes) and centrifuged at 13 000 g for 5 min. Soluble extracts (250 μg of protein in 100 μl) were incubated with 1/10 vol. of 0.55 mCi/ml [³H]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 enhanced fluorography. Similar extract samples were analysed by Western analysis.

Chromatographic fractionation of cell extracts

COS cells (four 10 cm dishes) were dislodged 72 h after transfection by scraping into 100 μl of FPLC lysis buffer [50 mM Tris/HCl (pH 7.4)/0.25 % (v/v) Triton X-100/1 mM EDTA/1 mM dithiothreitol/10 μg/ml leupeptin/1 mM benzamidine/0.5 mM PMSF] per dish. Cells were homogenized as above and centrifuged at 4000 g for 3 min. The supernatant was removed

and the low-speed pelleted material was washed with 100 μl of lysis buffer per dish and centrifuged again. Combined supernatants were centrifuged at 13000 g for 5 min. The resulting supernatant was diluted with 100 ml of TE buffer [10 mM Tris/HCl (pH 7.5)/1 mM EDTA] and solid $(NH_4)_2SO_4$ was added to make a 60%-satd. solution, which was stirred for 30 min. After centrifugation at 27000 g for 30 min, the pelleted proteins were resuspended in 300 μl of TE buffer and clarified by centrifugation at 13000 g for 10 min. The clarified extracts (200 μl) were loaded on a Superdex-200 HR 10/30 FPLC column equilibrated in buffer A [25 mM Tris/HCl (pH 7.4)/1 mM dithiothreitol/1 mM EDTA/10% (v/v) glycerol] containing 150 mM NaCl. The column was developed in the same buffer over a 48 min period at a flow rate of 0.5 ml/min; 0.5 ml fractions were collected. Proteins in the column fractions were precipitated with trichloroacetic acid, washed with acetone and subjected to Western analysis.

Immunoisolations

Transfected COS cells (two 10 cm dishes) were dislodged 72 h after transfection by scraping into 150 μl of lysis buffer [10 mM Tris/HCl (pH 7.5)/0.5% Triton X-100/150–500 mM NaCl/1 mM EDTA/1 mM dithiothreitol/10 μg/ml leupeptin/1 mM benzamidine] per dish. Cells were homogenized and centrifuged at 13000 g for 5 min. HA tag antibody (10 μg) was added to the clarified cell extract and the mixture was rotated for 1 h. GammaBind Sepharose (10 μl) was then added and the mixture was rotated for an additional 1 h. In some experiments, 10 μl of HA tag antibody cross-linked to GammaBind Sepharose [29] was added to the clarified extract; the mixture was then rotated for 4 h. The beads were centrifuged at 4000 g for 1 min, washed three times in lysis buffer and twice in buffer containing 10 mM Tris/HCl, pH 7.5, and 150–500 mM NaCl. After the last wash, approx. 5 μl of packed beads was assayed for phosphatase activity and the remainder was analysed by SDS/PAGE and immunoblotting with subunit-specific antibodies. In some experiments, immunoisolated phosphatases were eluted from the bead–antibody complex with 25 μM HA tag peptide for 1 h at room temperature; 1/10 of the eluate was assayed for phosphatase activity and the remainder was subjected to Western analysis.

Preparation of phosphorylated substrates

Labelled phosphorylase a was prepared by a modification of a method described previously [30]. Phosphorylase b (1 mg) was incubated in a reaction mixture (400 μl) containing 20 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 50 μg of phosphorylase kinase and 50 μM [γ-^{32}P]ATP (4400 c.p.m./pmol) for 2 h at 37 °C. Proteins were precipitated with an equal volume of satd. $(NH_4)_2SO_4$ and pelleted by centrifugation. The protein pellets were washed in buffer B [50 mM Tris/HCl (pH 7.5)/1 mM dithiothreitol/1 mM EDTA] containing 40%-satd. $(NH_4)_2SO_4$. The pellet was resuspended in 1 ml of buffer B and dialysed overnight against 500 ml of buffer B, with one change of dialysis buffer. Protein kinase C-phosphorylated histone H1 was prepared as described previously [31,32].

Phosphatase activity assays

Immunoisolated proteins (5 μl of eluted protein or 10 μl of bead–antibody–protein complex) were assayed for phosphatase activity in a 100 μl reaction volume containing 25 mM Tris/HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA, 4 mM $MgCl_2$, 0.2 mg/ml BSA and 0.2–0.5 μM ^{32}P-labelled phosphorylase a

(4400 c.p.m./pmol). After a 10 min incubation at 37 °C, the reaction was terminated by the addition of trichloroacetic acid [20% (w/v) final concentration] and proteins were pelleted by centrifugation at 13000 g for 10 min. Supernatants were collected and quantified for [^{32}P]P$_i$ released by scintillation counting. Less than 20% of the total phosphorylated substrate was dephosphorylated during the assays, demonstrating that substrate was not limiting. Fractions from each step of the PP2A purification protocol were diluted (typically between 10-fold and 100-fold) and similarly assayed for protein kinase C-phosphorylated histone H1 [31].

Western analysis

Proteins were solubilized in Laemmli sample buffer, boiled for 5 min and separated by SDS/PAGE. After electrophoresis, the proteins were transferred to supported nitrocellulose membranes at 1 A for 1.1 h in 10 mM 3-[cyclohexylamino]propane-1-sulphonic acid, pH 11, containing 10% (v/v) methanol. The membranes were then immunoblotted with subunit-specific polyclonal antibodies or HA tag monoclonal antibody. After incubation with primary antibody, the membranes were washed and incubated with either alkaline-phosphatase-conjugated goat anti-rabbit IgG or alkaline-phosphatase-conjugated goat anti-mouse IgG. The membranes were washed again and immunocomplexes were revealed with 5-bromo-4-chloroindol-3-yl phosphate and Nitro Blue Tetrazolium as substrate for alkaline phosphatase.

Determination of protein concentration

The protein concentration was determined by using the Bio-Rad protein assay, with BSA as the standard.

RESULTS AND DISCUSSION

Previous studies have demonstrated that alkali-induced demethylation of PP2A$_C$ results in the enhanced immunoreactivity of this protein to C-terminus-directed antibodies, a property that can be used to monitor the carboxymethylation status of this enzyme [17,27]. This strategy was utilized to examine the carboxymethylation status of PP2A$_C$ in heterodimeric (AC) and heterotrimeric (ABαC) forms of PP2A purified from rat brain. Partly purified preparations of ABαC and AC were adjusted to approximately equal concentrations of A and C protein (Figure 1A) and analysed for alkali-induced changes in PP2A$_C$ immunoreactivity (Figure 1B). Treatment with alkali significantly enhanced PP2A$_C$ immunoreactivity in the purified ABαC preparation, with little or no change in the immunoreactivity of PP2A$_C$ in the AC preparation. These findings indicate that a larger fraction of PP2A$_C$ is carboxymethylated in the heterotrimeric complex than in the heterodimeric complex, and suggest that there might be a correlation between the carboxymethylation of PP2A$_C$ and the assembly of PP2A holoenzyme.

To explore further the effects of PP2A$_C$ carboxymethylation in cells, a point mutant of the modified amino acid was prepared by using site-directed mutagenesis of HA-PP2A cDNA (see the Materials and methods section). HA-PP2A contains a nine-residue sequence (YPYDVPDYA) fused to the N-terminus of PP2A$_C$, which permits the detection of recombinant PP2A in mammalian cells. Introduction of this epitope tag sequence did not seem to alter the localization, regulation or function of HA-PP2A relative to endogenous PP2A$_C$ [28]. Thus cDNA species encoding HA-PP2A and a C-terminal mutant of HA-PP2A (HA-PP2A-L309A) were cloned into the pCW1 mammalian expression vector and transiently expressed in COS cells. Both HA-PP2A and HA-PP2A-L309A exhibited phosphatase activity when im-
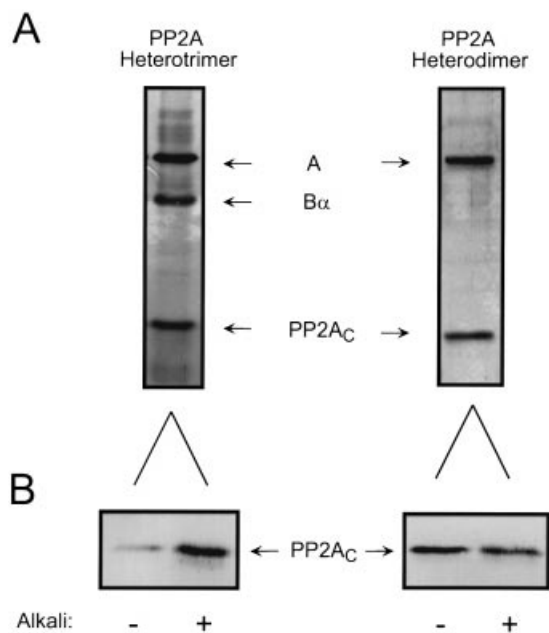
Figure 1   Treatment of partly purified PP2A heterotrimer and heterodimer with alkali

(**A**) Silver-stained SDS-polyacrylamide gel of purified ABαC and AC. (**B**) The ABαC and AC preparations used in (**A**) were incubated with buffer (−) or 0.1 M NaOH (+) followed by SDS/PAGE and immunoblotting with an antibody against the C-terminus of PP2A$_C$. The results are representative of three independent experiments.

Figure 2   Expression and carboxymethylation _in vitro_ of epitope-tagged phosphatases

(**A**) COS cells were transfected with the pCW1 expression plasmid without an insert (pCW1) or with pCW1 containing a cDNA encoding HA-PP2A or HA-PP2A-L309A before immunoisolation with the HA tag antibody. The immunoisolates were assayed for ^{32}P-labelled phosphorylase _a_ phosphatase activity as described in the Materials and methods section. The bars indicate means ± S.D. for three independent experiments. (**B**) Extracts (50 μg) from COS cells transfected with the indicated constructs were incubated with [^{3}H]SAM and analysed by SDS/PAGE and enhanced fluorography. (**C**) Samples of the extracts in (**B**) were incubated with vehicle and analysed by SDS/PAGE and Western blotting with HA tag antibodies. The results in (**B**) and (**C**) are from one experiment and are representative of three independent experiments.

munoisolated from COS cell lysates, demonstrating the expression of a functional phosphatase enzyme (Figure 2A). Interestingly, phosphatase activity directed against ^{32}P-labelled phosphorylase _a_ was approximately twice as high for HA-PP2A-L309A as for wild-type HA-PP2A when equivalent amounts of epitope-tagged protein were assayed (compare Figures 2A and 2C). Similar results were obtained when phosphatase activity was assayed either in the immune complex (Figure 2A) or after peptide elution from the GammaBind Sepharose (results not shown). The total phosphorylase _a_ phosphatase activity in whole lysates of cells transfected with HA-PP2A or HA-PP2A-L309A cDNA was not markedly different from total activity in control lysates, suggesting that the level of expression of recombinant HA-PP2A was very low relative to endogenous PP2A. However, the expression (not over-expression) of functional HA-PP2A is an advantage for exploring the structure–function relationships of PP2A$_C$.

To determine whether HA-PP2A and HA-PP2A-L309A could be carboxymethylated _in vitro_, the corresponding COS cell extracts were incubated with [^{3}H]SAM and analysed by SDS/PAGE and enhanced fluorography (Figure 2B). A major band of tritiated protein was observed migrating at 36 kDa, the predicted size of endogenous PP2A$_C$, in extracts prepared from mock-transfected cells as well as from cells transfected with HA-PP2A and HA-PP2A-L309A cDNA species. This observation is consistent with previous reports demonstrating that PP2A$_C$ is the major methylated (i.e. on its C-terminal leucine residue) protein after the incubation of cytosolic extracts with [^{3}H]SAM [15–17]. However, we were able to take advantage of the differential migration of HA-PP2A and HA-PP2A-L309A (38 kDa; Figure 2C) compared with endogenous PP2A$_C$ (36 kDa) to demonstrate that a tritiated epitope-tagged PP2A protein (38 kDa) was
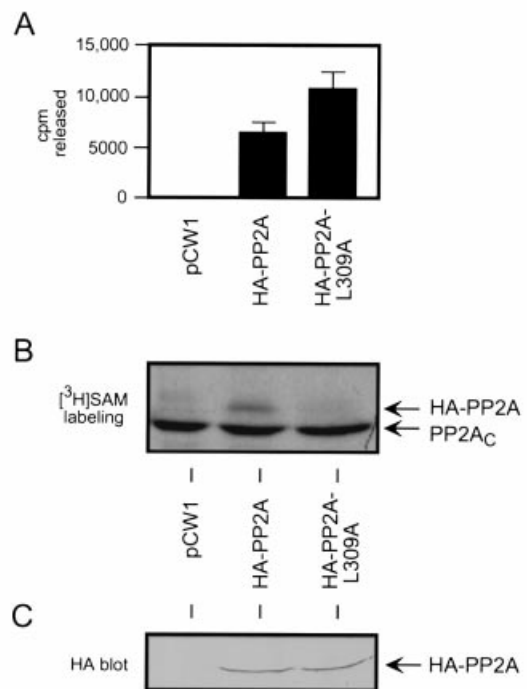
observed after the [^{3}H]SAM labelling of extracts prepared from COS cells expressing HA-PP2A, but not in extracts prepared from cells expressing HA-PP2A-L309A or in control extracts (Figure 2B). The presence of equivalent amounts of both recombinant proteins was verified by Western analysis (Figure 2C). These results demonstrate that both endogenous PP2A$_C$ and HA-PP2A, but not HA-PP2A-L309A, are methylated _in vitro_, presumably at Leu^{309}, by a cellular methyltransferase activity.

The C subunit of PP2A is associated with regulatory subunits _in vivo_; several PP2A oligomeric complexes have been identified and purified from a variety of tissues. The different PP2A complexes are often distinguished on the basis of their chromatographic properties. For example, heterodimeric (AC) and heterotrimeric (ABαC) PP2A can be resolved from each other and from free C by gel filtration or anion-exchange chromatography ([33], and J. C. Bryant and B. E. Wadzinski, unpublished work). To examine the chromatographic properties of HA-PP2A compared with HA-PP2A-L309A, extracts from COS cells expressing these recombinant proteins were analysed by Superdex-200 gel filtration (Figure 3). HA-PP2A (like endogenous PP2A$_C$, not shown) was co-eluted with A and Bα regulatory subunits from the gel-filtration column as a fairly broad peak corresponding to 150–400 kDa. In contrast, HA-PP2A-L309A immunoreactivity was eluted from the gel-filtration column in two peaks, a major
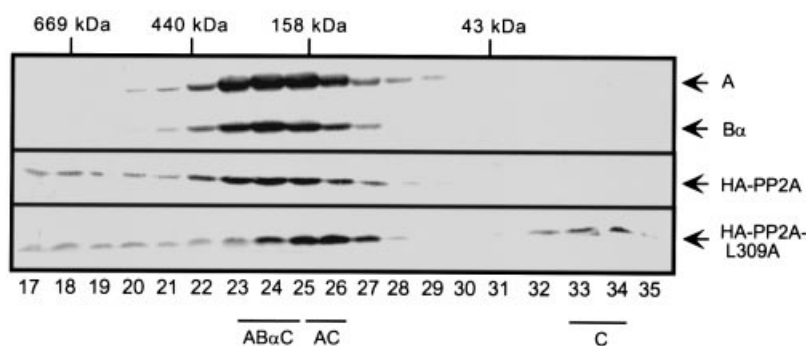
Figure 3   Comparison of the elution profiles of endogenous and epitope-tagged phosphatases

COS cells were transfected with plasmids containing HA-PP2A or HA-PP2A-L309A cDNA species. Lysates prepared from the transfected cells were fractionated by Superdex-200 gel filtration by using FPLC. Fractions were then analysed by SDS/PAGE and immunoblotting with the indicated phosphatase subunit antibodies or the HA tag antibody. The top panel is a representative blot probed with antibodies against the A and Bα regulatory subunits; the elution profiles of these proteins from Superdex-200 were indistinguishable in all experiments. Fraction numbers are shown at the bottom, and the positions of gel-filtration standards at the top. Representative blots from two independent experiments are shown.
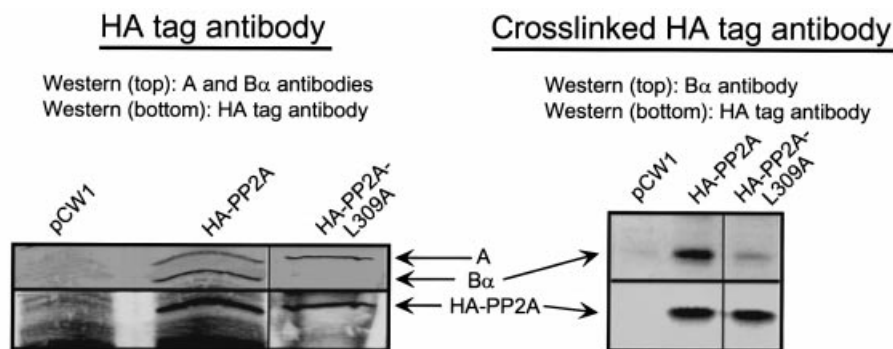

Figure 4   Co-immunoisolation of A and Bα regulatory subunits with epitope-tagged PP2A catalytic subunits

Lysates prepared from COS cells transfected with pCW1 or pCW1 containing HA-PP2A or HA-PP2A-L309A cDNA species were incubated with HA tag antibody and GammaBind Sepharose (left panel), or cross-linked HA tag antibody–GammaBind Sepharose (right panel), followed by Western analysis of the immunoisolates as described in the Materials and methods section. Immunoblotting was performed with the indicated antibodies. Results are representative of two to five independent experiments.

peak corresponding to approx. 100 kDa and a minor peak corresponding to approx. 35 kDa. Purified AC and free C also eluted as approx. 100 kDa and approx. 35 kDa proteins respectively when similarly analysed by gel filtration. Consistent with the gel-filtration results, HA-PP2A-L309A eluted differently from HA-PP2A and endogenous PP2A$_C$ when COS cell extracts were analysed by anion-exchange chromatography (results not shown). Together, these results suggest that the carboxymethylation-deficient HA-PP2A-L309A protein is defective in associating with other cellular proteins, including PP2A regulatory subunits.

To explore further the holoenzyme structure of wild-type HA-PP2A compared with HA-PP2A-L309A phosphatase, these enzymes were immunoisolated from COS cell lysates by using HA tag antibody and GammaBind Sepharose (or cross-linked HA tag antibody–GammaBind Sepharose), resolved by SDS/PAGE and analysed by immunoblotting with phosphatase subunit antibodies (Figure 4). Both A and Bα regulatory subunits were co-immunoisolated with HA-PP2A. In contrast, substantially less Bα protein was co-immunoisolated with the HA-PP2A-L309A mutant enzyme. Similar results were obtained when the immunoisolations were performed in buffers with varying salt concentrations (e.g. 150–500 mM NaCl). Moreover, the ratio of

phosphorylase phosphatase activities of HA-PP2A-L309A to HA-PP2A in the immunoisolates (approx. 2:1; see Figure 2) was similar to the reported ratio of phosphorylase phosphatase activities of AC to ABαC [24]. These findings, together with the chromatographic results, are consistent with the interpretation that HA-PP2A-L309A is defective in its ability to associate with the Bα regulatory subunit.

Post-translational modification of PP2A$_C$ via carboxymethylation could theoretically have a number of biological effects, including direct effects on ther catalytic activity of PP2A or, alternatively, modulation of the phosphorylation, regulatory subunit (or other interacting protein) binding and/or cellular localization of PP2A$_C$. Previous findings have demonstrated, at least *in vitro*, that carboxymethylation has only a moderate effect on the intrinsic catalytic activity of PP2A$_C$ [17]. The present results provide several lines of evidence that the carboxymethylation of PP2A$_C$ and the association of PP2A$_C$ with the Bα regulatory subunit are inter-related. This is both an important and exciting finding in light of the recent reports that the carboxymethylation state of PP2A$_C$ varies during the cell cycle [19] and that the ABαC complex associates with and dephosphorylates multiple proteins including tau [6] and Ca^{2+}/calmodulin-dependent protein kinase IV [9]. The observation that

both purified PP2A$_C$ and PP2A heterodimer (AC) can be carboxymethylated *in vitro* ([17,20,34], and J. C. Bryant and B. E. Wadzinski, unpublished work) indicates that the B subunit is not essential for this modification. However, additional studies are needed to determine whether PP2A$_C$ carboxymethylation regulates holoenzyme assembly. The availability of purified PP2A methyltransferase and methylesterase will probably expedite these studies. Moreover, it will be interesting to determine whether regulated changes in PP2A$_C$ carboxymethylation observed during cell growth and differentiation are correlated with any changes in holoenzyme assembly, the association with other cellular proteins or the cellular localization of PP2A.

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