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A Protein Phosphatase Methylesterase (PME-1) Is One of Several Novel Proteins Stably Associating with Two Inactive Mutants of Protein Phosphatase 2A*

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Abstract

Carboxymethylation of proteins is a highly conserved means of regulation in eukaryotic cells. The protein phosphatase 2A (PP2A) catalytic (C) subunit is reversibly methylated at its carboxyl terminus by specific methyltransferase and methylesterase enzymes which have been purified, but not cloned. Carboxymethylation affects PP2A activity and varies during the cell cycle. Here, we report that substitution of glutamine for either of two putative active site histidines in the PP2A C subunit results in inactivation of PP2A and formation of stable complexes between PP2A and several cellular proteins. One of these cellular proteins, herein named protein phosphatase methylesterase-1 (PME-1), was purified and microsequenced, and its cDNA was cloned. PME-1 is conserved from yeast to human and contains a motif found in lipases having a catalytic triad-activated serine as their active site nucleophile. Bacterially expressed PME-1 demethylated PP2A C subunit *in vitro*, and okadaic acid, a known inhibitor of the PP2A methylesterase, inhibited this reaction. To our knowledge, PME-1 represents the first mammalian protein methylesterase to be cloned. Several lines of evidence indicate that, although there appears to be a role for C subunit carboxyl-terminal amino acids in PME-1 binding, amino acids other than those at the extreme carboxyl terminus of the C subunit also play an important role in PME-1 binding to a catalytically inactive mutant.

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Protein phosphatase 2A (PP2A)[1] is a highly conserved serine/threonine phosphatase involved in the regulation of a wide variety of enzymes, signal transduction pathways, and cellular events (1, 2). Consonant with its diverse roles, subpopulations of PP2A have been found to localize to the nucleus, cytoplasm, cytoskeleton, and membranes (3–6). The smallest functional unit of PP2A thought to exist *in vivo* consists of a heterodimer between a catalytic 36-kDa subunit, termed C, and a constant regulatory 63-kDa subunit, termed A (7). This A/C heterodimer often further complexes with a member of one of three additional cellular regulatory subunit families termed B (or B55), B′ (or B56), and B″ (or PR72/120) (1). In cells stably transformed by the middle tumor antigen (MT) of polyomavirus, MT substitutes for the B subunit in a small portion (~10%) (8)[2] of PP2A complexes (9). MT·PP2A complex formation is known to be important for MT-mediated transformation (10–13), but the precise functional consequences of MT association with PP2A are still being elucidated.

Efforts aimed at understanding PP2A regulation have uncovered a complex set of noncovalent and covalent mechanisms. These include association with different regulatory subunits (1), association with heat stable inhibitors (14), action of a phosphotyrosyl activator protein (15), lipid binding (16), phosphorylation (17), and methylation (18–22). These mechanisms affect the catalytic activity, substrate specificity, and cellular localization of PP2A. However, little is known about the molecular bases of their effects, and even less about how these effects might be coordinated and integrated to orchestrate PP2A functions throughout the cell.

The carboxyl terminus of the PP2A C subunit seems to be a focal point for regulation of PP2A. In addition to containing the amino acids identified as the sites of tyrosine phosphorylation and methylation, this region contains residues essential for stable binding of the B regulatory subunit (23). It is possible that these three events may influence one another. We have recently shown that substitution of tyrosine 307, the site of tyrosine phosphorylation, with a negatively charged amino acid abolishes both B subunit binding (23) and methylation of the C subunit.[3] In contrast, MT does not require these residues to form PP2A heterotrimers (23), raising the possibility that different B-type subunits might be differentially affected by, or differentially affect, covalent modification at the carboxyl terminus.

The first indication that PP2A C subunit was methylated involved two observations. Rundell (18) showed that a 36-kDa SV40 small tumor antigen (ST)-associated cellular protein was a major acceptor of the methyl group from radiolabeled *S*-adenosylmethionine added to cell extracts. Subsequently, this ST-associated cellular protein was reported to be the PP2A C subunit (9). More recently, several groups showed that PP2A C subunit is indeed methylated and reported that this methylation is reversible and occurs on the carboxyl-terminal amino acid leucine 309, forming a methyl ester (19–22). In addition, the PP2A methyltransferase and methylesterase enzymes have been purified and an initial characterization performed (19, 24). However, cloning of cDNAs for these enzymes has not been reported and their primary sequences are unknown.

Although methyltransferase and methylesterase enzymes specific for PP2A have been purified, the regulation and role(s) of PP2A methylation have only begun to be elucidated. Based on differential antibody recognition of methylated and nonmethylated C subunits,

[1]The abbreviations used are: PP2A, protein phosphatase 2A; MT, middle tumor antigen; ST, small tumor antigen; EST, expressed sequence tag; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region; wt, wild type; ORF, open reading frame; PMSF, phenylmethylsulfonyl fluoride.
[2]L. Haehnel and D. C. Pallas, unpublished data.
[3]X. X. Yu, X. Du, E. Ogris, R. E. Green, Q. Feng, L. Clon, and David Pallas, submitted for publication.

PP2A has been reported to undergo cell cycle dependent changes in methylation (6). This suggests that PP2A methylation may participate in the regulation of, or be regulated by, cell cycle progression. cAMP was found to stimulate PP2A methylation in *Xenopus* egg lysates (25), suggesting that this second messenger may be involved in the regulation of PP2A methylation. The activity of PP2A toward phosphorylase *a* and a phosphopeptide substrate was reported to increase approximately 2-fold upon methylation (21). Greater effects might be observed with other substrates, given that the effects of some other mechanisms of PP2A regulation, such as B-type subunit association and heat-stable inhibitor proteins, have been shown to be highly substrate dependent. Another possibility is that PP2A methylation might affect B-type subunit association, or *vice versa*.

The exact determinants on PP2A essential for functional recognition by the PP2A methyltransferase and methylesterase enzymes are unknown, although they must include more than just the highly conserved carboxyl terminus itself. Xie and Clarke (22) showed that a synthetic carboxyl-terminal PP2A C subunit octapeptide functions neither as a PP2A methyltransferase substrate nor as an inhibitor, and Lee and co-workers (24) demonstrated that a methylated synthetic carboxyl-terminal tetrapeptide also functions neither as a PP2A methylesterase substrate nor as an inhibitor. In the latter study, a 600-fold excess of unmethylated PP2A C subunit was found to inhibit the PP2A methylesterase by 50%, while a 10^6-fold excess of a carboxyl-terminal C subunit decapeptide did not inhibit the methylesterase at all. Collectively, these results suggest that both enzymes make essential contacts with C subunit residues that are not in the carboxyl terminus. One hint as to where such contacts might be located comes from the observation that the potent PP2A inhibitors okadaic acid and microcystin-LR also inhibit the PP2A methyltransferase and/or methylesterase enzymes (19, 20, 24). It has been suggested that this inhibition may be due to these inhibitors binding in part to the carboxyl-terminal region of the C subunit, interfering with the binding of the methyltransferase and methylesterase. However, an equally attractive possibility is that these two enzymes interact with residues in or around the active site of PP2A.

In this study, we report that an individual substitution of either of two PP2A C subunit active site histidines with gluta-mine results in a catalytically inactive PP2A mutant that forms a stable complex with several cellular proteins not bound stably by wt C subunit. The formation of this stable complex enabled us to purify, microsequence, and clone the first of these cellular proteins, which we have designated PME-1. PME-1 was identified as a PP2A methylesterase by the ability of the bacterially expressed and purified enzyme to demethylate PP2A C subunit. To our knowledge, this is the first mammalian methyles terase for which protein or cDNA sequences have been reported. Data base searches reveal a single homolog in *Saccharomyces cerevisiae*, as well as complete or partial sequences, respectively, for homologs in *Caenorhabditis elegans* and zebrafish, indicating that this enzyme is conserved across eukaryotes. PME-1 contains a motif found in lipases that utilize a catalytic triad-activated serine as their active site nucleophile, and has other scattered homology with other lipases in which this motif is conserved. Based on a number of results, we propose that the specificity of PME-1 for PP2A may in part be determined by interaction with residues or metals in or near the PP2A active site.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis

Site-directed mutagenesis was performed on a HA-tagged wt C subunit cDNA cloned in the pcDNA I Amp vector (23) using the Muta-Gene Phagemid *In Vitro* Mutagenesis Kit according to the manufacturer's instructions (Bio-Rad). The entire cDNA of both H59Q and H118Q was sequenced to confirm successful mutagenesis and to ensure that no additional

mutation occurred. Mutant C subunit cDNAs including the HA tag coding sequence were cloned into the dexamethasone-inducible vector, pGRE5-2 (26). The construction of a pGRE5-2 vector expressing HA-tagged wt PP2A C subunit has been previously described (23).

To make a PME-1 construct (pPS.PME-1) to be used for sequencing and *in vitro* transcription/translation, the PME-1 cDNA product generated by RT-PCR (see below) was inserted via blunt end ligation into an *Srf*I site in the PCR-Script™ SK(+) vector using the PCR-Script™ SK(+) kit (Stratagene).

Cells and Cell Culture

NIH3T3 lines expressing wt polyomavirus MT and a geneticin resistance gene (27) were transfected by the calcium phosphate precipitation method (28), and individual clones and mixtures of clones expressing wt C subunit (wt C sub), H59Q, H118Q, or empty vector (GREonly) were selected and maintained as described previously (23). H118Q expressed at a level well below that of endogenous wt C subunit, while H59Q expressed at a level equal to or greater than the wt level. Although the inducible vector, pGRE5-2, was used to express these proteins, their levels were substantial in the absence of dexamethasone; for this reason, GREonly cells were used as a negative control in this study rather than uninduced wt or mutant C subunit expressing cells. However, dexamethasone treatment was used throughout to obtain maximal expression of the C subunits.

Radiolabeling of Cells

For metabolic labeling of cells with methio-nine, subconfluent dishes of cells were labeled for 5 h with [^{35}S]methionine (300 μCi/ml) in Dulbecco's modified Eagle's medium minus methionine supplemented with 0.5% dialyzed fetal bovine serum.

Preparation of Cell Lysates and Immunoprecipitation

The details of treating the cells with dexamethasone, preparation of cell lysates, and immunoprecipitation of C subunits have been described previously (23). For experiments quantitating PME-1 binding to different mutants (Fig. 6*B*), immunoprecipitates were washed twice with Nonidet P-40 lysis buffer, twice with phosphate-buffered saline, and once with ddH20. Washed immune complexes were used for phosphatase assays or analyzed by one or two-dimensional gel electrophoresis.

One- and Two-dimensional Gel Electrophoresis and Fluorography

SDS-polyacrylamide gel electrophoresis (10% acrylamide) was performed according to Laemmli (29). Two-dimensional gel analysis was performed as described previously (30). Gels were silver stained by the procedure of Wray *et al.* (31) except that after electrophoresis the gels were sequentially incubated 10 min in distilled water (200 ml), 10 min in 95% ethanol (200 ml), 1 h in 50% methanol (100 ml), and 30 min in distilled water (100 ml) prior to staining.

Immunoblotting

Immunoblotting (32) was performed with mouse monoclonal anti-tag antibody (16B12; 1:5000 dilution of ascites; BAbCO), rabbit anti-B subunit antibody (P16; 1:5000), affinity-purified rabbit (R39; 1:5000) or mouse monoclonal (4G7; 1 μg/ml) anti-A subunit antibodies, mouse monoclonal anti-MT antibody (F4; 0.25 μg/ml) (33), mouse monoclonal anti-C subunit antibody (1D6; 0.25 μg/ml), or rabbit anti-PME-1 antibodies (AR2 or E37; see below). Immunoblots were developed with enhanced chemiluminescence (Amersham or NEN Life Science Products Inc.).

Phosphatase Assay

Phosphatase activity present in anti-HA tag immunoprecipitates from the different cell lines was assayed using phosphorylase *a* and histone H1. γ-^{32}P-Labeled phosphorylase *a* substrate was prepared from phosphorylase *b* according to the manufacturer's (Life Technologies, Inc.) instructions. Histone H1 was phosphorylated by mitotic p34^{cdc2} purified from Nocodazole arrested HeLa cells as described previously (34). Lysates used for immunoprecipitation were equilibrated according to HA-tagged C subunit expression levels. Assays were performed at a linear range and with subsaturating amounts of each substrate.

Purification and Microsequencing of p44A(PME-1)

To obtain p44A(PME-1) protein for microsequencing, H59Q C subunit complexes containing p44A(PME-1) were immunoaffinity purified. In total, 135 confluent 15-cm dishes of MT-transformed NIH3T3 cells expressing HA-tagged H59Q were used. Forty-five separate immunoaffinity purifications were performed on 3 dishes of lysate at a time, reusing the same immunoaffinity matrix at least 15 times. To prepare the immunoaffinity matrix, anti-HA tag antibody (12CA5; obtained from BAbCO) was chemically cross-linked to protein A-Sepharose beads (Pharmacia) by published methods (35). Cell lysates were incubated for 1 h at 4 °C while rocking with 500 μl of the cross-linked antibody-bead complexes. Complexes were washed once with Nonidet P-40 lysis buffer, three times with Tris-buffered saline, and then twice with distilled deionized H_2O. Bound H59Q complexes containing p44A(PME-1) were eluted by three sequential incubations with 300 μl of 20 mM triethylamine. Eluates were quickly frozen on dry ice and stored frozen until all batches of affinity purification had been completed. The antibody-bead complexes were then washed twice with 20 mM triethylamine and twice with Nonidet P-40 lysis buffer prior to reuse. After H59Q complexes had been purified from all 135 dishes of cells, eluates containing p44A(PME-1) were concentrated to dryness by vacuum centrifugation, and the residues were suspended in phosphate-buffered saline and gel buffer and analyzed on three separate SDS-polyacrylamide gels. One-dimensional gels were chosen to avoid losses associated with two-dimensional gel analysis. Because p44A(PME-1) migrates closely to actin, the separation of these two proteins was maximized by the use of an 8% gel run for an extended period of time.

Trypsin Digestion, HPLC Separation, and Microsequencing

After separation of p44A(PME-1) complexes by SDS-PAGE, the proteins were electrotransferred to polyvinylidiene difluoride membrane and stained with Ponceau S. Individual protein bands were excised and subjected to *in situ* digestion with trypsin (36, 37). The resulting peptide mixture was separated by microbore high performance liquid chromatography using a Zorbax C18 2.1 × 150-mm reverse phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Optimum fractions from the chromatogram were chosen based on differential UV absorbance at 205, 277, and 292 nm, peak symmetry and resolution. Peaks were further screened for length and homogeneity by matrix-assisted laser desorption time-of-flight mass spectrometry on a Finnigan Laser-mat 2000 (Hemel United Kingdom), and selected fractions were submitted to automated Edman degradation on an Applied Biosystems 494A, 477A (Foster City, CA) or Hewlett-Packard G1005A (Palo Alto, CA). Details of strategies for the selection of peptide fractions and their microsequencing have been previously described (37).

cDNA Cloning via PCR

To obtain the missing 5′ portion of the PME-1 coding region, nested and semi-nested PCR were performed using human B cell, human hippocampus, and human kidney plasmid libraries. 5′ primers corresponded to vector sequence that flanked cDNA inserts in the

library being used as template, while 3′ primers corresponded to known sequence (EST or newly derived 5′ PME-1 sequence). Southern blotting using an end-labeled 20-base pair oligo-nucleotide corresponding to known PME-1 sequence upstream of the 3′ PCR primer was employed to identify authentic PME-1 products after each reaction. PCR products containing 5′ extensions of the PME-1 sequence were purified using a PCR product purification kit (Roche Molecular Biochemicals), cloned, and sequenced. New primers were designed for PCR and Southern blotting and then the above steps were repeated until the sequence of the remainder of the PME-1 coding region and a portion of the 5′ UTR were obtained.

RT-PCR

Total mRNA was purified from HeLa cells using Trizol Reagent (Life Technologies) according to the manufacturer's instructions. First strand synthesis was performed with avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals) by the manufacturer's protocol using a primer from the PME-1 3′ UTR (TGTTGAGGAGGGGTGGACAG). Using Pfu polymerase (Stratagene), the product was used for PCR with the same 3′ primer and a primer from the PME-1 5′ UTR (TGTATGGGGACCTTCCTCCT) to generate a cDNA containing the entire PME-1 coding region and much of the 5′ UTR.

Computer Analyses

The NCBI BLAST program (38) was used to probe various data bases for p44A(PME-1) ESTs and related proteins. The DNASTAR Lasergene software package was utilized for alignments and identification of the PROSITE data base lipase motif found in p44A(PME-1).

Northern Blot

Adult Balb/c mice were sacrificed and organs removed and flash-frozen in liquid nitrogen. Total RNA from the organs was isolated using the RNeasy kit (QIAGEN), and analyzed on formaldehyde, 1% agarose gels to check for RNA integrity and to estimate the amount of the 18 S and 28 S RNAs. Based on these estimates, similar amounts of RNA were separated on formaldehyde, 1% agarose gels and transferred to GeneScreen nylon membranes. After UV cross-linking, the membranes were stained with a 0.04% methylene blue solution to visualize the RNA. Filters were then hybridized with a ^{32}P-radiolabeled probe generated by random primer labeling of a DNA fragment from the 3′-untranslated region of the mouse PME-1 cDNA. The probe, 395 base pairs in length, is an *Eco*RI-*Not*I fragment of a PME-1 EST clone (accession number W34856). The blots were used for autoradiography with x-ray film and/or analyzed on a STORM PhosphorImager (Molecular Dynamics).

Production of Polyclonal Antibodies Recognizing PME-1

Two different antisera recognizing PME-1 were raised in rabbits. The first, AR2, was raised against a 16-residue PME-1 peptide sequence (RIELAKTEKYWDGWFR) found encoded in the PME-1 cDNA. The peptide was conjugated to keyhole limpet hemocyanin via an added carboxyl-terminal cysteine residue using a Pierce Imject conjugation kit, and the conjugate was used as immunogen. The second antiserum, E37, was raised against a mixture of two nickel agarose-purified, 6xHis-tagged, bacterially expressed human PME-1 fragments that together represent the carboxyl-terminal half of the protein. For each immunogen, a single female New Zealand White rabbit was immunized and boosted multiple times using Freund's adjuvant.

Demethylation Assays

Assays utilizing methylation-sensitive antibodies (6) were performed to evaluate PP2A demethylation. Logarithmically growing wt C subunit-expressing MT-transformed NIH3T3 cells were lysed and C subunit immunoprecipitated as described previously (23). The C subunit immunoprecipitate was divided into equal aliquots for use as substrate in demethylation reactions. C subunit immune complexes from one 10-cm dish of cells could support 8 demethylation reactions. To each aliquot of substrate, 38.75 μl of reaction buffer containing 55 mM Tris, pH 8.0, 55 mM NaCl, 1 mM dithiothreitol, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.55% Nonidet P-40, and 0.2 mg/ml bovine serum albumin was added. Then, 0.5 μl of inhibitor (okadaic acid or PMSF) dissolved in dimethyl sulfoxide or dimethyl sulfoxide without inhibitor was added to the appropriate tubes. After 3 min, 0.75 μl of lysate from bacteria expressing or not expressing PME-1 was added to the appropriate tubes (to obtain bacterial lysate, bacteria were lysed by sonication in 25 mM Tris, pH 8.0, containing 140 mM NaCl, and lysates were cleared by centrifugation at 13,000 × *g* for 5 min). Demethylation reactions were carried out at 32 °C with shaking for 60 min. Then the reactions were combined with SDS-PAGE sample buffer and boiled. Following SDS-PAGE and electrophoretic transfer of proteins to nitro-cellulose, the membrane was immunoblotted as described in the legend to Fig. 5.

RESULTS

Generation and Characterization of Catalytically Inactive PP2A C Subunits

In order to create catalytically inactive PP2A C subunit mutants that retained the maximum amount of structural integrity, single residues likely to be important for catalysis were mutated. To identify such residues, an alignment of PP2A and various related phosphatases was performed to identify highly conserved residues (data not shown). A small number of residues were found that are identical in PP2A, PP1, PPX, PP2B, and PPλ. Of those, two histidines (H) at positions 59 and 118 were chosen as having catalytic potential, and were individually mutated to glutamine (Q), yielding the mutants H59Q and H118Q. Subsequent to the construction of these mutants, the crystal structures of PP1 and PP2B (39, 40) and a mutational analysis of PPλ (41) were reported, the results of which suggested that these two histidines would be involved in PP2A substrate binding and catalysis. As described under “Experimental Procedures,” each C subunit mutant or wt cDNA was constructed with the hemagluttinin (HA) tag at its amino terminus to allow for immunoprecipitation analysis (23). Individual mutants, wild-type C subunit, or vector only were expressed stably in NIH3T3 cell lines with and without coexpression of MT. In the MT expressing cells, most PP2A complexes still contain B subunit because MT is produced at a low level relative to endogenous PP2A (8).[2]

After construction of stable lines, the C subunit mutants were characterized with respect to two properties: 1) competence to form complexes containing the A and B subunits or MT and 2) catalytic activity. To examine complex formation *in vivo*, immunoprecipitates of epitope-tagged wt and mutant C subunits were probed by immunoblotting for the presence of additional subunits and MT (Fig. 1). Both mutants bind substantial A subunit. H118Q also binds a small amount of B subunit, while H59Q binds almost none of this subunit. Although a small amount of MT was found in control immunoprecipitates from cells which do not express any epitope-tagged C subunit, levels of MT well above this were readily detected in the mutant immunoprecipitates, indicating that A·C·MT trimeric complexes had been formed by these proteins. A portion of the MT coimmunoprecipitated with H59Q is shifted relative to the MT associated with wt C subunit; this result is reproducible and will be described in more detail elsewhere.[4] The fact that both mutants bind additional subunits indicates that these mutants have substantial native structure *in vivo*.

To test for catalytic activity, phosphatase assays were performed on anti-tag immunoprecipitates from the various cell lines. Using both phosphorylase *a* and histone H1 as substrates, only wt C subunit immunoprecipitates were found to have increased activity compared with control immunoprecipitates prepared from a cell line containing only empty vector (Table I). Immunoprecipitates of the two mutants showed no activity above the control level toward either substrate. This finding is consistent with the results of others who found that mutation of the corresponding residues in related phosphatases also completely inactivated those enzymes (41–43).

Two 44-kDa Cellular Proteins Associate with the Inactive C-subunit Mutants, H59Q and H118Q

To determine if novel cellular proteins associate with one or both catalytically inactive C subunit mutants, anti-tag immunoprecipitates were prepared from ^{35}S-labeled cells and analyzed on two-dimensional gels. Several proteins were seen, including one prominently labeled protein of 44 kDa with a pI near 7 (p44B), that specifically associate with H59Q and H118Q but not with control immunoprecipitates prepared from cells containing empty vector or cells expressing wt C subunit (data not shown).

To determine if sufficient p44B could be obtained to allow microsequencing, scaled up immunoprecipitates from vector only control cells (GREonly) and from cells expressing H118Q were analyzed on two-dimensional gels and silver-stained (Fig. 2). P44B was not readily visible in these gels (see brackets); however, another 44-kDa protein was seen that also specifically coimmunoprecipitates with H118Q. This protein was present in almost a 1:1 stoichiometry with the A and C subunits and was initially designated p44A because its pI, approximately 6, was more acidic than that of p44B. A similar p44A spot was found in silver-stained immunoprecipitates of H59Q (data not shown). An ^{35}S-labeled spot corresponding to p44A was absent in the two-dimensional gels of ^{35}S-labeled immunoprecipitates described above, suggesting that p44A may be synthesized at a very low rate and may have a much longer half-life than the PP2A C or A subunits or p44B. Alternatively, there may be a delay before newly synthesized p44A forms a complex with the inactive mutants.

Purification, Microsequencing, and cDNA Cloning of p44A Reveals That It Is a Novel Protein (PME-1) Highly Conserved from Yeast to Human

To facilitate the identification or cloning of p44A, sufficient protein for microsequencing was obtained by purifying HA-tagged H59Q complexes on an anti-tag immunoaffinity column as described under “Experimental Procedures.” After analysis on one-dimensional gels, both actin and a clearly separated p44A band migrating just above it were excised for further processing. Microsequencing of two tryptic peptides from the lower band confirmed that it was indeed actin (data not shown). Nine microsequences obtained from the p44A band matched no known protein in GenBank, indicating that it was a novel protein.

Initial searches of the expressed sequence tag (EST) data base also revealed no match for the microsequences from p44A; however, in time, a human EST sequence (accession number H12112) was deposited that encoded three of those microsequences. Because a human EST sequence matched first, we decided to clone the human version of this protein, which we have renamed PME-1 for functional reasons that will become obvious below. Additional DNA sequencing of EST H12112 revealed coding information for two more PME-1 microsequences, and it was determined that this EST encoded most of the carboxyl-terminal half of PME-1 (162 amino acids).

[4]E. Ogris, I. Mudrak, E. Mak, D. Gibson, and D. C. Pallas, submitted for publication.

To obtain the missing 5′ portion of the PME-1 coding region, nested and semi-nested PCR were performed as described under “Experimental Procedures.” The sequence of the remainder of the coding region and a portion of the 5′-UTR were obtained. Because errors may have occurred during the multiple PCR reactions that were necessary to obtain the complete cDNA sequence, RT-PCR was then performed with HeLa cell mRNA as template to provide a reliable PME-1 coding sequence. Sequencing of this cDNA resolved a few questionable nucleotides in the coding region, and confirmed the presence of a stop codon just upstream of the predicted start codon (Fig. 3*A*). The stop codon upstream of the predicted start ATG is in-frame with the PME-1 coding sequence, indicating that the PME-1 cDNA contains the complete PME-1 ORF.

A schematic of a PME-1 cDNA that includes the end of the 3′-UTR deduced from overlapping ESTs is shown in Fig. 3*A*. The entire sequence is approximately 2.5 kilobase pairs in size, with an open reading frame (ORF) of 1164 base pairs including two tandem stop codons. When a probe specific for mouse PME-1 was used to detect transcripts from different mouse organs, a single transcript of 2.6 ± 0.2 kilobase pairs was detected in all tissues (Fig. 3*B*). The level of this transcript was highest in brain and testis. To date, multiple EST sequences which encode PME-1 have been deposited in the DBEST data base. The sequenced portions of those ESTs cover the entire 3′-and 5′-UTRs, but not the entire coding region. Information from the NCBI Cancer Genome Anatomy Project (CGAP) indicates that PME-1 ESTs have been mapped to human chromo-some 11, interval D11S916-D11S911 (80–84 centimorgan). It is not known at this time whether PME-1 is mutated in any of the diseases with defects mapped to this general region of chromosome 11.

The 386-amino acid PME-1 (p44A) protein product encoded by the human PME-1 cDNA ORF is shown in Fig. 3*C*. It has a predicted molecular size of just over 42 kDa, close to that predicted from its migration on SDS-PAGE. In addition, it has a pI of 5.8, consistent with its migration in the isoelectric focusing dimension on two-dimensional gels like the one shown in Fig. 2. All nine mouse PME-1 tryptic peptide sequences (*underlined* in Fig. 3*C*) were accounted for in the human sequence with differences present only at a few positions, indicating that PME-1 is well conserved between these two species. Because no tryptic peptide microsequences potentially corresponding to p44B were found, efforts to identify this protein are still ongoing.

Using the NCBI BLAST program, highly homologous sequences probably corresponding to PME-1 homologs were found for zebrafish, *C. elegans*, and *S. cerevisiae*. A hypothetical 88.4-kDa *C. elegans* protein in chromosome 3, B0464.7, contains some of the *C. elegans* sequence homologous to PME-1, but lacks other highly homologous sequences, suggesting that it may not represent an accurate prediction of exon combinations. A more likely combination of exons that includes all B0464 cosmid exons homologous to PME-1 generates a protein of 364 amino acids and approximately 40 kDa (Fig. 3*D*). *S. cerevisiae* PME-1 (Fig. 3*D*) appears to be a single hypothetical 44.9-kDa protein (PIR accession number S46814) of unknown function encoded by an ORF on chromosome 8R (YHN5; Gen-Bank accession number U10556). Recently, based on a single partially homologous nonapeptide sequence, YHN5 was proposed to be a mitochondrial ribosome small subunit protein and named YmS2 (44). Human PME-1 has approximately 40 and 26%, respectively, identity with the *C. elegans* and yeast sequences (Fig. 3*D*). A highly charged stretch of amino acids is present in human PME-1 but absent in PME-1s from *C. elegans* and *S. cerevisiae*. This stretch of amino acids does not represent a cloning artifact, because ^{35}S-labeled PME-1 *in vitro* transcription/translation product generated using the human PME-1 cDNA comigrates on gels with PME-1 from HeLa cell lysates (data not shown).

In order to facilitate further experiments characterizing PME-1, an anti-PME-1 peptide antibody was raised to a 16-amino acid peptide sequence encoded by the PME-1 cDNA. This peptide antibody detected a 44-kDa protein present in H59Q immunoprecipitates, but absent from immunoprecipitates of wild-type C subunit (Fig. 4). Thus, PME-1, like p44B, associates stably with the catalytically inactive mutant C subunits, but not with wt C subunit. Because B subunit, but not MT, requires the C subunit carboxyl terminus for association with the PP2A A/C heterodimer, we wanted to determine if MT expression might increase the amount of PME-1 bound to H59Q. Similar levels of PME-1 were coimmunoprecipitated from untransformed NIH3T3 cells and polyomavirus MT-transformed NIH3T3 cells (Fig. 4), indicating that MT expression does not greatly affect the level of H59Q·PME-1 complex formation in the cell.

PME-1 Is a PMSF-resistant, Okadaic Acid-sensitive PP2A Methylesterase That Probably Uses Serine as an Active Site Nucleophile

When the human, *C. elegans*, and *S. cerevisiae* PME-1 protein sequences were analyzed for motifs found in the Prosite data base using DNASTAR Lasergene software, a consensus sequence ((L/I/V)*X*(L/I/V/F/Y)(L/I/V/S/T)G(H/Y/W/V)S-*X*G(G/S/T/A/C)) for lipases utilizing an active site serine was found to be conserved. The invariant serine in this motif, corresponding to serine 156 in human PME-1, is the active site serine in these enzymes. In addition, scattered similarities can be seen between other regions of the PME-1 sequence and some of the lipases that have this motif. Therefore, PME-1 is probably a lipase whose active site serine is serine 156.

The various lipases that share this motif are found in both prokaryotes and eukaryotes and include, among others, two *Drosophila melanogaster* carboxylesterases. In addition, CheB, a bacterial glutamate methylesterase, has a similar, but not identical, sequence surrounding its active site serine (45) (Table II). CheB (46) and other lipases utilizing an active site serine (*e.g.* Refs. 47 and 48) have a catalytic triad in their primary sequence in the order Ser-Asp(or Glu)-His. Of the conserved histidines in human PME-1, His-349 is a likely candidate for a putative catalytic triad histidine (Fig. 3*D*). Identification of a putative PME-1 catalytic triad acidic residue by sequence comparison is more problematic because there are multiple acidic residues conserved between species. However, of these, two aspartates in human PME-1, Asp-181 and Asp-324, show conservation in position with putative catalytic triad aspartates in other lipases, and therefore may be more likely possibilities.

A PP2A C subunit carboxyl methylesterase of 46 kDa has recently been purified (24), but no sequence information was reported. To test the possibility that PME-1 might be a PP2A methylesterase, PME-1 was expressed in bacteria and bacterial lysates were tested for methylesterase activity toward PP2A C subunit as described under “Experimental Procedures.” The results shown in Fig. 5 demonstrate that lysates of bacteria expressing PME-1 contain a PP2A methylesterase activity not found in bacterial lysates lacking PME-1. Similar results were obtained with purified recombinant PME-1 (Fig. 5). These results indicate that PME-1 is indeed a PP2A methylesterase. Because its specificity toward other methylated phosphatases (such as PPX) has not been characterized, it was generically named protein phosphatase methylesterase-1 (PME-1).

The 46-kDa PP2A methylesterase reported by Lee and coworkers (24) was inhibited by okadaic acid, a potent PP2A inhibitor, but not by PMSF, a covalent inhibitor of certain serine esterases. To determine if PME-1 displays similar sensitivities to these inhibitors, the above demethylation assay was also conducted in the presence of okadaic acid and PMSF (Fig. 5). The methylesterase activity of bacterially expressed PME-1 was inhibited by 0.1 or 1 μ_M okadaic acid but not by 1 or 5 m_M PMSF, similar to the methylesterase purified by Lee *et al.* (24).

Okadaic Acid and Other PP2A Inhibitors Decrease the Association of PME-1 with H59Q

Because single amino acid changes in the C subunit active site were capable of inducing stable complex formation of C subunit with PME-1, it was of interest to determine if PP2A inhibitors could antagonize the H59Q·PME-1 complex. To assay for this possibility, NIH3T3 cells expressing epitope-tagged H59Q C subunit were lysed in the presence of various phosphatase inhibitors and H59Q was immunoprecipitated via its epitope tag. The amount of endogenous, untagged PME-1 coimmunoprecipitating in each case was assayed by blotting with anti-PME-1 antibody (Fig. 6*A*). Inhibitors to which PP2A is highly sensitive (okadaic acid, sodium fluoride, and sodium pyrophosphate), but not those to which PP2A is less sensitive or insensitive (vanadate and phenylarsine oxide, respectively), decreased the amount of PME-1 bound to H59Q.

The H59Q Carboxyl Terminus Is Important, but Not Essential, for Complex Formation with PME-1

A PP2A methylesterase might be expected to make important contacts with carboxyl-terminal residues. However, Lee and co-workers (24) found that PP2A carboxyl-terminal peptides functioned neither as inhibitors nor as substrates for their 46-kDa PP2A methylesterase, suggesting that, at a minimum, contacts with other parts of the C subunit are essential. To investigate the importance of the H59Q C subunit carboxyl terminus for stable interaction with PME-1, a double mutant, H59Q/301Stop, was created. This mutant combines the H59Q mutation, which induces stable binding of PME-1, with a deletion of the nine C subunit carboxyl-terminal acids, 301–309. Fig. 6*B*, shows the results of an immunoprecipitation assay measuring the relative abilities of H59Q and H59Q/301Stop to bind A subunit and PME-1. Deletion of residues 301–309 from wt C subunit has previously been found to decrease the amount of A subunit bound (23). Fig. 6*B* shows that deletion of these residues from H59Q also reduces the binding of the PP2A A subunit to H59Q. In addition, although similar amounts of H59Q and H59Q/301Stop were immunoprecipitated in this experiment, the double mutant bound less PME-1 than did H59Q, indicating that one or more of the deleted carboxyl-terminal residues is important for H59Q·PME-1 complex formation. PME-1 binding was not completely abolished, however, demonstrating that interactions also exist between PME-1 and other residues in the C subunit.

To address the same question via a different approach, we assayed via immunoprecipitation whether antibodies directed against the C subunit carboxyl terminus would compete with PME-1 for binding to H59Q. If an antibody competes with PME-1 for binding to residues on H59Q that are important for PME-1 association, that antibody would be expected to coimmunoprecipitate reduced amounts of PME-1 with H59Q when compared with an antibody that does not compete with PME-1. The carboxyl-terminal C subunit monoclonal antibodies used for this experiment, 1D6, 4B7, and 4E1, were recently generated against a 15-residue unmethylated carboxyl-terminal peptide.[3] These antibodies are unable to efficiently recognize a C subunit mutant lacking the carboxyl-terminal leucine, indicating that they bind, at least in part, at the very carboxyl terminus.[5] A positive control monoclonal antibody, 12CA5, immuno-precipitates H59Q via its amino-terminal epitope tag and should not interfere with interactions at the C subunit carboxyl terminus (23). Comparison of the relative ratios of the PME-1 and H59Q bands in Fig. 6*C* reveals that, relative to 12CA5, 1D6 and 4B7 immunoprecipitate less PME-1 for the same amount of H59Q C subunit (the band of endogenous, wt C subunit immunoprecipitated by the carboxyl-terminal antibodies can be ignored as wt C subunit does not associate stably with PME-1). Furthermore, although 4E1 immunoprecipitated a substantial amount of H59Q C subunit (within approximately 2-fold of 12CA5), no PME-1 could be detected even on long exposures. These results thus further

[5]D. C. Pallas, unpublished data.

substantiate the conclusions made from Fig. 6*B*. In addition, the fact that 1D6 and 4B7 coimmunoprecipitate similar amounts of PME-1, but dramatically different amounts of A subunit indicates that PME-1 binding does not appear to be dependent on A subunit binding.

DISCUSSION

In this study, we report the identification of the first of a number of cellular proteins that stably associate with catalytically inactive PP2A C subunit mutants, but not with wt C subunit. Two proteins of 44 kDa that differ in their isoelectric points, p44A and p44B, uniquely associated with two different catalytically inactive C subunit mutants substituted individually at two different active site histidine residues. P44A was affinity purified and a cDNA encoding it was cloned. This protein was identified as a PP2A methylesterase (PME-1) by several criteria including: 1) molecular size, 2) the presence of a motif found in lipases that use serine as their nucleophilic catalytic residue, 3) activity assays performed *in vitro* with bacterially expressed protein, and 4) the ability of okadaic acid, a known inhibitor of both PP2A and the PP2A methylesterase, to inhibit its activity and decrease its association with the catalytically inactive C subunit mutant, H59Q.

The cloning of PME-1, to our knowledge, provides the first sequence of a mammalian protein methylesterase. Like the specific bacterial methylesterase CheB (49, 50), which participates in the chemotactic response, PME-1 appears to be a serine esterase which is resistant to PMSF. The molecular basis for resistance to PMSF is presently unknown, but it has been proposed that a unique arrangement of the active site residues in CheB might contribute (46). It will be interesting to see if PME-1 has a similar arrangement of its catalytic residues. It seems likely that PME-1, like CheB (46) and other lipases utilizing an active site serine (*e.g.* Refs. 47 and 48), will be found to have a catalytic triad of serine, histidine, and aspartate (or glutamate) in its active site. We are presently attempting to obtain structural information for PME-1 to determine which residues are in the active site and to gain insight into the molecular basis of PME-1 recognition of PP2A C subunit and into potential regulation of this methylesterase.

Based on its molecular size, sensitivity to okadaic acid, and the lack of effect of PMSF on PME-1 activity, PME-1 is likely to be equivalent to the 46-kDa PP2A methylesterase whose purification and initial characterization was recently reported by Lee and colleagues (24). Its insensitivity to PMSF indicates that it is not the PMSF-sensitive serine esterase/protease activity reported by Xie and Clarke (51), which also could remove PP2A carboxymethyl groups. Lee and co-workers (24) reported that their purified PP2A methylesterase eluted as two different peaks from an anion exchange column, consistent with either differential modification or the existence of two closely related isoforms of the enzyme. The amounts of these two species were within severalfold of each other. Two pieces of evidence from our studies support the idea that those two forms probably represent differentially modified forms of the enzyme. First, probing of the GenBank EST data base with the PME-1 cDNA sequence provides no evidence for a closely related PME-1 isoform, even though numerous ESTs are found which correspond precisely to the PME-1 cDNA sequence. Second, Northern blot analysis yielded a single band in multiple organs. In addition, we have found via immunoblotting that mammalian PME-1 in cell lysates migrates on two-dimensional gels as two spots differing in their isoelectric point in a manner consistent with a single charge difference.[6]

The molecular basis of the cell cycle-dependent regulation of PP2A C subunit methylation is unknown. The poor metabolic labeling of PME-1 in an asynchronous population of cells

[6]X. Du and D. C. Pallas, unpublished data.

relative to a number of other proteins suggests that this protein is quite stable. This result argues against the possibility that cell cycle PP2A methylation is regulated by modulating the amount of the PP2A methylesterase. Whether PME-1 activity is regulated is unknown. In the case of the bacterial chemotactic response, the CheB methylesterase is regulated by phosphorylation (52, 53) while the methyltransferase is thought to be constitutively active. Lee and co-workers (24) found no difference in the activity of their two purified forms of PP2A methylesterase, suggesting that the differential modification likely responsible for generating these two forms might not be involved in regulation of activity of this enzyme. It is possible, however, that effects might be seen under other conditions, or that an additional protein(s) may be necessary for an effect to be manifested. In addition, it is possible that more than one modification occurs. More definitive evidence should be obtained from a genetic analysis of the importance of the site(s) of modification, once identified.

The intriguing possibility exists that PP2A methyltransferase and methylesterase enzymes might achieve their specificity in part by interacting with or near the active site of the PP2A C subunit. This hypothesis is consistent with a number of experimental findings. First, it was reported previously that neither the PP2A methyltransferase nor the PP2A methylesterase can recognize short peptide substrates corresponding to the C subunit carboxyl terminus. Thus, functional recognition by both these enzymes requires additional C subunit structure. Second, as demonstrated in this study, perturbation of the C subunit active site by either of two different mutations can stabilize the interaction with the PME-1 methylesterase. Third, PP2A inhibitors have a destabilizing effect on the PME-1-H59Q interaction. Fourth, the methyltransferase is inhibited by the PP2A inhibitors, okadaic acid and microcystin-LR, and the methylesterase is inhibited by okadaic acid (testing for inhibition of the methylesterase by microcystin has not been reported). Although it has been proposed that this inhibition may be due to the interaction of these inhibitors with carboxyl-terminal C subunit residues, this would not explain the ability of the PP2A inhibitors, sodium fluoride or sodium pyrophosphate, to partially or fully disrupt PME-1·H59Q complexes. The latter effect is more consistent with a role in binding the PME-1 methylesterase for active site residues and/or metals, or nearby residues sensitive to effects on the active site. Yet another experimental finding compatible with the above hypothesis is that four separate catalytically inactive PP2A active site point mutants, including the two described in this study, are methylated at less than 3% of the wild-type level *in vivo* and *in vitro*.[3] Although all these findings are consistent with our hypothesis of an interaction with residues and/or metals in or near the active site, another equally viable possibility is that mutation of active site residues and/or binding of inhibitors may have more distant effects on the C subunit conformation critical for stable complex formation with PME-1.

Contact between the C subunit and PME-1 could theoretically be with PME-1 residues and/or with a phosphorylation site on PME-1. Because H59Q and H118Q are virtually unmethylated,[3] PME-1 apparently can remain bound to these mutants in the absence of a methylated carboxyl terminus. At least with H59Q, PME-1's contacts other than on the C subunit carboxyl terminus are strong enough to result in substantial complex formation in the absence of the nine carboxyl-terminal C subunit residues. This conclusion is further supported by the finding that two C subunit carboxyl-terminal peptide antibodies, known to require Leu-309 for efficient binding, could immunoprecipitate H59Q·PME-1 complexes. However, the amount of PME-1 coimmunoprecipitated by these antibodies was less than that coimmunoprecipitated by an antibody recognizing an amino-terminal epitope tag on the C subunit. The latter result and the fact that a third carboxyl-terminal C subunit antibody could not immunoprecipitate H59Q·PME-1 complexes at all suggest that PME-1 is proximal to the C subunit carboxyl terminus in the H59Q·PME-1 complex. Moreover, the reduced amounts of PME-1 in complex with the H59Q·301Stop double mutant indicate that carboxyl-terminal residues play a role in binding of H59Q to PME-1. The contribution of

these residues to the interaction of wild type C subunit with PME-1 might be even more important in the absence of the complex-stabilizing H59Q mutation.

The significance of the decreased B subunit binding observed with these mutants is unclear, but an attractive hypothesis is that it might be due indirectly to lack of methylation at the carboxyl terminus of these mutants. The fact that H59Q and H118Q bind the structural PP2A A subunit and polyomavirus MT suggests that they are not grossly altered in their structure. Two other catalytically inactive point mutants that bind A subunit and polyomavirus MT, but are highly deficient in methylation,[3] are also deficient in B subunit binding.[4] Given that the B subunit requires the C subunit carboxyl terminus for stable complex formation with the A/C heterodimer, the B subunit might require a methylated carboxyl terminus for efficient binding to C subunit. An alternate, but not mutually exclusive, possibility is that the carboxyl terminus and the active site are proximal in the three-dimensional structure of the C subunit. This model would provide an explanation for how events occurring at the carboxyl terminus (B subunit binding, methylation, phosphorylation, etc.) can affect the active site (activity, specificity), and vice versa. In addition, at least for H59Q and H118Q, PME-1 and B subunit binding might be mutually exclusive, although this remains to be tested.

These catalytically inactive C subunit mutants should be useful for identifying other proteins involved in PP2A signaling. H59Q and H118Q bind multiple proteins not bound stably by wt C subunit. These include, in addition to PME-1, p44B, and several other proteins. Interestingly, initial experiments suggest that p44B binding to H59Q is even more sensitive to phosphatase inhibitors than is PME-1 binding.[7] These proteins could be PP2A substrates or other proteins whose binding is sensitive to the state of the C subunit active site. One of these proteins is the same molecular size as the PP2A methyltransferase reported by Lee and Stock (19). Catalytically inactive mutants of dual specificity and tyrosine phosphatases (54, 55) have been previously used successfully to identify novel substrates, but unlike PP2A, their catalytic mechanisms involve the formation of covalent intermediates with substrates. It will be very interesting to determine whether any of the remaining H59Q/H118Q associated proteins are indeed PP2A substrates.

PME-1 and p44B differ in several characteristics, suggesting that these two proteins are not simply modified forms of one another. They are separated from each other on two-dimensional gels by approximately 1 pH unit, which is unlikely to be accounted for by modification; PME-1 forms sharp spots on these gels while p44B migrates as a smear. In addition, *in vitro* translation of PME-1 yields no product migrating at the position of p44B and we have been unable to detect p44B with antibodies raised against PME-1 sequences.[6]

Finally, because of the high conservation of PP2A with other phosphatases such as PP1, PPX, PPV, etc., it will be of interest to see if similar or different cellular proteins bind stably to these phosphatases when the residues corresponding to PP2A His-59 and His-118 are mutated to glutamine. One question of special interest is whether the corresponding catalytically inactive mutants of PP*X*, which has the same last four carboxyl-terminal amino acids as PP2A and is also methylated at its carboxyl-terminal leucine, will trap its methylesterase. Given the lack of close relatives to PME-1 in the various data bases, it would not be surprising if that methylesterase turns out to be PME-1.

[7]K. C. Nelson and D. C. Pallas, unpublished data.

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REFERENCES

1. Cohen P. Annu. Rev. Biochem. 1989; 58:453–508. [PubMed: 2549856]
2. Mumby MC, Walter G. Physiol. Rev. 1993; 73:673–699. [PubMed: 8415923]
3. Jakes S, Mellgren RL, Schlender KK. Biochim. Biophys. Acta. 1986; 888:135–142. [PubMed: 3017441]
4. Sim AT, Ratcliffe E, Mumby MC, Villa-Moruzzi E, Rostas JA. J. Neurochem. 1994; 62:1552–1559. [PubMed: 8133283]
5. Sontag E, Nunbhakdi-Craig V, Bloom GS, Mumby MC. J. Cell Biol. 1995; 128:1131–1144. [PubMed: 7896877]
6. Turowski P, Fernandez A, Favre B, Lamb NJ, Hemmings BA. J. Cell Biol. 1995; 129:397–410. [PubMed: 7721943]
7. Usui H, Imazu M, Maeta K, Tsukamoto H, Azuma K, Takeda M. J. Biol. Chem. 1988; 263:3752–3761. [PubMed: 2831201]
8. Ulug ET, Cartwright AJ, Courtneidge SA. J. Virol. 1992; 66:1458–1467. [PubMed: 1371166]
9. Pallas DC, Shahrik LK, Martin BL, Jaspers S, Miller TB, Brautigan DL, Roberts TM. Cell. 1990; 60:167–176. [PubMed: 2153055]
10. Grussenmeyer T, Carbone-Wiley A, Scheidtmann KH, Walter G. J. Virol. 1987; 61:3902–3909. [PubMed: 2824823]
11. Pallas DC, Cherington V, Morgan W, DeAnda J, Kaplan D, Schaffhausen B, Roberts TM. J. Virol. 1988; 62:3934–3940. [PubMed: 2845116]
12. Glenn GM, Eckhart W. J. Virol. 1995; 69:3729–3736. [PubMed: 7538175]
13. Campbell KS, Auger KR, Hemmings BA, Roberts TM, Pallas DC. J. Virol. 1995; 69:3721–3728. [PubMed: 7538174]
14. Li M, Guo H, Damuni Z. Biochemistry. 1995; 34:1988–1996. [PubMed: 7531497]
15. Cayla X, Goris J, Hermann J, Hendrix P, Ozon R, Merlevede W. Biochemistry. 1990; 29:658–667. [PubMed: 2159785]
16. Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA. J. Biol. Chem. 1993; 268:15523–15530. [PubMed: 8393446]
17. Chen J, Martin BL, Brautigan DL. Science. 1992; 257:1261–1264. [PubMed: 1325671]
18. Rundell K. J. Virol. 1987; 61:1240–1243. [PubMed: 3029419]
19. Lee J, Stock J. J. Biol. Chem. 1993; 268:19192–19195. [PubMed: 8396127]
20. Li M, Damuni Z. Biochem. Biophys. Res. Commun. 1994; 202:1023–1030. [PubMed: 8048914]
21. Favre B, Zolnierowicz S, Turowski P, Hemmings BA. J. Biol. Chem. 1994; 269:16311–16317. [PubMed: 8206937]
22. Xie H, Clarke S. J. Biol. Chem. 1994; 269:1981–1984. [PubMed: 8294450]
23. Ogris E, Gibson DM, Pallas DC. Oncogene. 1997; 15:911–917. [PubMed: 9285686]
24. Lee J, Chen Y, Tolstykh T, Stock J. Proc. Natl. Acad. Sci. U. S. A. 1996; 93:6043–7. [PubMed: 8650216]
25. Floer M, Stock J. Biochem. Biophys. Res. Commun. 1994; 198:372–379. [PubMed: 8292043]
26. Mader S, White JH. Proc. Natl. Acad. Sci. U. S. A. 1993; 90:5603–5607. [PubMed: 8390672]

27. Cherington V, Morgan B, Spiegelman BM, Roberts TM. Proc. Natl. Acad. Sci. U. S. A. 1986; 83:4307–4311. [PubMed: 3012562]
28. Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning: A Laboratory Manual. 2nd Ed.. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1989.
29. Laemmli UK. Nature. 1970; 227:680–685. [PubMed: 5432063]
30. Pallas D, Solomon F. Cell. 1982; 30:407–414. [PubMed: 7139708]
31. Wray W, Bolikas T, Wray VP, Hancock R. Biochemistry. 1981; 118:197–203.
32. Towbin H, Staehelin T, Gordon J. Proc. Natl. Acad. Sci. U. S. A. 1979; 76:4350–4354. [PubMed: 388439]
33. Pallas DC, Schley C, Mahoney M, Harlow E, Schaffhausen BS, Roberts TM. J. Virol. 1986; 60:1075–1084. [PubMed: 3023660]
34. Mayer-Jaekel RE, Ohkura H, Ferrigno P, Andjelkovic N, Shiomi K, Uemura T, Glover DM, Hemmings BA. J. Cell Sci. 1994; 107:2609–2618. [PubMed: 7844174]
35. Harlow, E.; Lane, D. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1988.
36. Fernandez J, Andrews L, Mische SM. Anal. Biochem. 1994; 218:112–117. [PubMed: 8053543]
37. Lane WS, Galat A, Harding MW, Schreiber SL. J. Protein Chem. 1991; 10:151–160. [PubMed: 1718307]
38. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. J. Mol. Biol. 1990; 215:403–410. [PubMed: 2231712]
39. Kissinger CR, Parge HE, Knighton DR, Lewis CT, Pelletier LA, Tempczyk A, Kalish VJ, Tucker KD, Showalter RE, Moomaw EW, Gastinel LN, Habuka N, Chen X, Maldonado F, Barker JE, Bacquet R, Villafranca JE. Nature. 1995; 378:641–644. [PubMed: 8524402]
40. Goldberg J, Huang HB, Kwon YG, Greengard P, Nairn AC, Kuriyan J. Nature. 1995; 376:745–753. [PubMed: 7651533]
41. Zhuo S, Clemens JC, Stone RL, Dixon JE. J. Biol. Chem. 1994; 269:26234–26238. [PubMed: 7929339]
42. Ansai T, Dupuy LC, Barik S. J. Biol. Chem. 1996; 271:24401–24407. [PubMed: 8798696]
43. Zhang J, Zhang Z, Brew K, Lee EY. Biochemistry. 1996; 35:6276–6282. [PubMed: 8639569]
44. Kitakawa M, Graack HR, Grohmann L, Goldschmidt-Reisin S, Herfurth E, Wittmann-Liebold B, Nishimura T, Isono K. Eur. J. Biochem. 1997; 245:449–456. [PubMed: 9151978]
45. Krueger JK, Stock J, Schutt CE. Biochim. Biophys. Acta. 1992; 1119:322–326. [PubMed: 1547277]
46. West AH, Martinez-Hackert E, Stock AM. J. Mol. Biol. 1995; 250:276–290. [PubMed: 7608974]
47. Winkler FK, D'Arcy A, Hunziker W. Nature. 1990; 343:771–774. [PubMed: 2106079]
48. Brady L, Brzozowski AM, Derewenda ZS, Dodson E, Dodson G, Tolley S, Turkenburg JP, Christiansen L, Huge-Jensen B, Norskov L, Thim L, Menge U. Nature. 1990; 343:767–770. [PubMed: 2304552]
49. Snyder MA, Stock JB, Koshland DE Jr. Methods Enzymol. 1984; 106:321–330. [PubMed: 6387376]
50. Simms SA, Cornman EW, Mottonen J, Stock J. J. Biol. Chem. 1987; 262:29–31. [PubMed: 3539934]
51. Xie H, Clarke S. Biochem. Biophys. Res. Commun. 1994; 203:1710–1705. [PubMed: 7945320]
52. Wylie D, Stock A, Wong CY, Stock J. Biochem. Biophys. Res. Commun. 1988; 151:891–896. [PubMed: 3279958]
53. Hess JF, Oosawa K, Kaplan N, Simon MI. Cell. 1988; 53:79–87. [PubMed: 3280143]
54. Gelderloos JA, Anderson SM. Oncogene. 1996; 13:2367–2378. [PubMed: 8957078]
55. Bliska JB, Clemens JC, Dixon JE, Falkow S. J. Exp. Med. 1992; 176:1625–1630. [PubMed: 1281213]

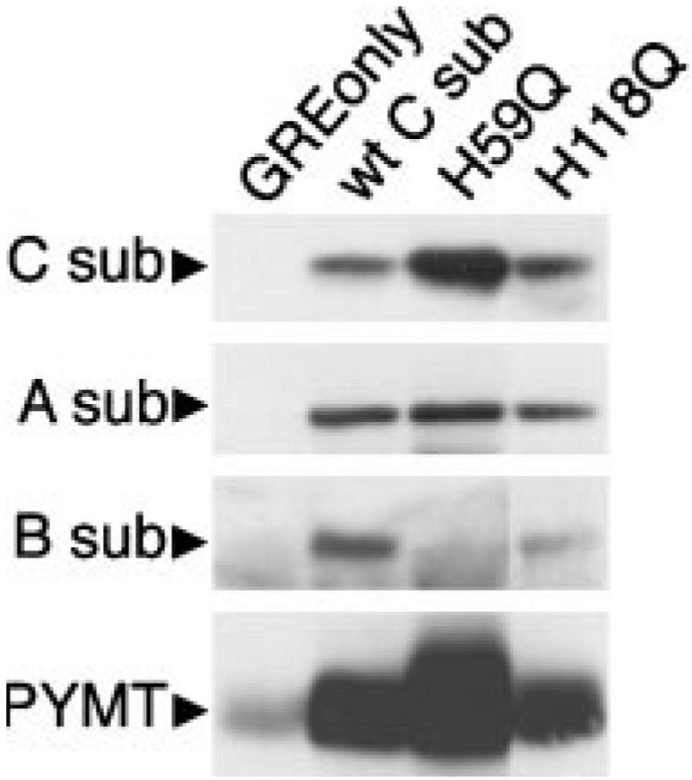

Fig. 1. The catalytically inactive mutant PP2A C subunits can form complexes with A subunit and MT *in vivo*

Lysates from cells containing only control vector (*GREonly*) or HA-tagged wt (*wt C sub*) or mutant C subunits (*H59Q* and *H118Q*) were immunoprecipitated with anti-HA tag antibody (12CA5) and analyzed by SDS-PAGE and immunoblotting. The blot was probed first with anti-MT antibody, and then sequentially with antibodies recognizing the A, C (via the HA tag), and B PP2A subunits. Because a lower level of expression was consistently seen with H118Q, the immunoprecipitate of this mutant was prepared from more cells; to properly control for this, the control immunoprecipitate was prepared from an equivalent amount of cells expressing only the vector. Under these conditions, a small amount of MT can be seen sticking nonspecifically to the immunoprecipitate in the GREonly lane.

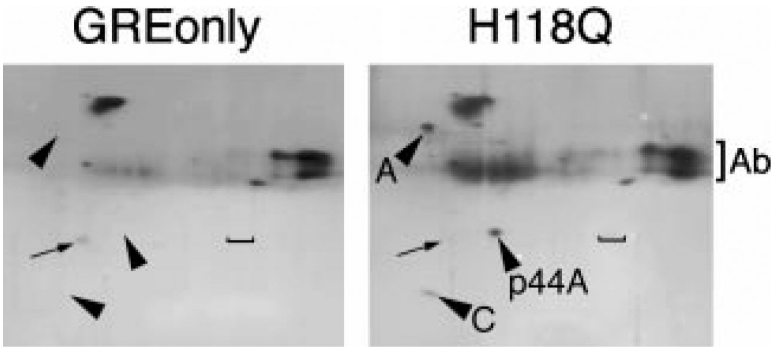

Fig. 2. p44A appears to bind stoichiometrically to H118Q

Silver-stained two-dimensional gels of HA tag immunoprecipitates prepared from unlabeled cells expressing vector only (*GREonly*) or the C subunit mutant, H118Q, are shown. Only the portion of each gel containing the relevant proteins is shown. The A and C subunits, p44A, and anti-HA tag antibody heavy chain (*Ab*) are indicated by labeled *brackets* and *arrowheads*. Unlabeled *arrowheads* indicate the corresponding positions in the GREonly control panel. For reference, actin is indicated in both panels by a *small unlabeled arrow*. The approximate position where p44B would be located on these gels is indicated by the *unlabeled brackets*.

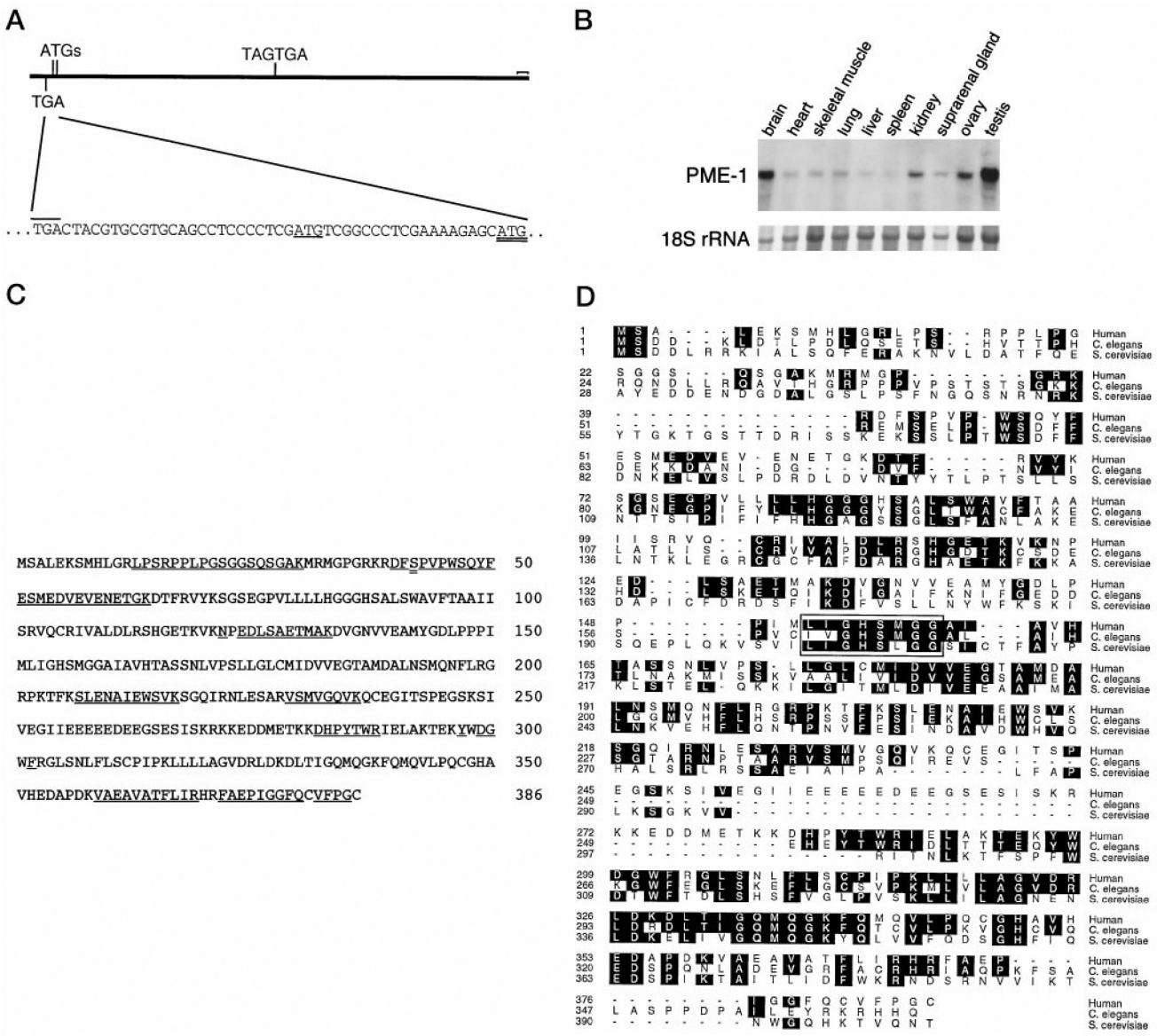

Fig. 3. PME-1 cDNA schematic, mRNA tissue distribution, and predicted protein sequence

A, schematic of a 2.5-kilobase human PME-1 cDNA. On the stick diagram, the positions of the in-frame 5′-UTR stop codon (TGA), of the first two potential start codons (ATGs), of tandem stop codons (TAGTGA) at the end of the PME-1 ORF, and of the poly(A) tail (*bracket*) are shown. The 3′ end of the 3′-UTR, including the position of the poly(A) tail, was deduced by analyzing overlapping PME-1 ESTs; all other regions were directly sequenced. The sequence shown below the stick diagram extends from the in-frame 5′-UTR stop codon (TGA; *overlined*) to the second possible start ATG (*double underlined*). The first possible start ATG (*underlined once* in the sequence shown) was identified as the authentic start site *in vivo* by making constructs whose transcription/translation products *in vitro* would start with one or the other of these two ATGs. ^{35}S-Labeled *in vitro* transcription/translation product starting at the first ATG, but not the product starting at the second ATG, comigrated precisely on two-dimensional gels with PME-1 from HeLa cell lysates (data not shown). *B,* expression of PME-1 mRNA in different tissues. Total RNA from the indicated mouse organs was separated by electrophoresis and hybridized with a mouse PME-1 partial

cDNA probe from the 3′-UTR of mouse PME-1. In a separate experiment, the size of the PME-1 transcript was calculated to be 2.6 ± 0.2 kilobases. The *lower panel* shows the 18 S rRNA from the same blot visualized with methylene blue. *C,* the protein sequence encoded by the human PME-1 cDNA is shown. Amino acid sequence information for murine PME-1(p44A) obtained by tryptic peptide microsequencing is *underlined*. Over 98% of the microsequenced murine residues (107 of 109) were identical to the human sequence. The *double underlined* serine at position 42 corresponds to a threonine in murine PME-1. *D,* alignment of human, *C. elegans* (predicted), and *S. cerevisiae* (YHN5) PME-1 protein sequences. Residues identical with human PME-1 are *shaded*. Residues corresponding to the Prosite motif for lipases employing an active site serine are *boxed*.

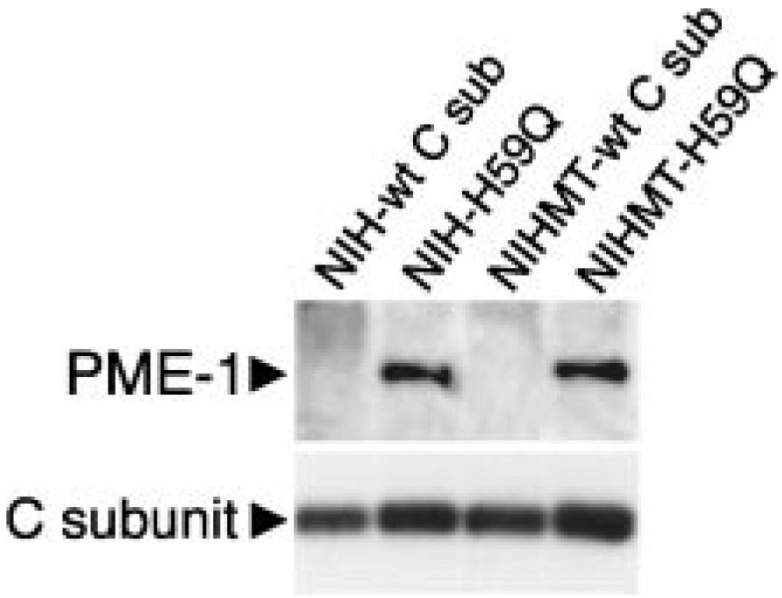

Fig. 4. PME-1 stably associates with H59Q but not wild-type C subunit

HA tag immunoprecipitates prepared from NIH3T3 (*NIH*) or MT-transformed NIH3T3 (*NIHMT*) cell lines individually expressing HA-tagged wt (wt C sub) or mutant (H59Q) C subunits were analyzed by SDS-PAGE and immunoblotting with HA tag antibody and PME-1 anti-peptide antibody. The C subunits migrate as tight doublets in these gels; whether doublets or a single band are seen varies from gel to gel and does not appear to be due to degradation (6, 13, 23). The panels and lanes shown are from the same experiment and gel, but the lanes were not all originally adjacent. Even on long exposure, the 44-kDa protein seen in the mutant lanes is not seen in the wt lanes.

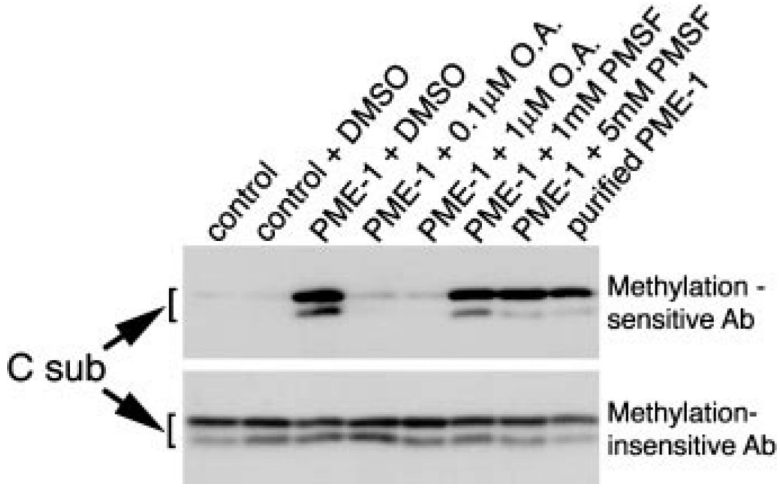

Fig. 5. Human PME-1 is a PP2A methylesterase

Immunoprecipitated PP2A C subunit was incubated with lysates from bacteria either not expressing PME-1 (*control*) or expressing PME-1 (*PME-1*), or with purified bacterially expressed PME-1 (~5 ng). Okadaic acid (*O.A.*) or PMSF was added to the reactions to the indicated final concentrations. Reactions containing 1.25% dimethyl sulfoxide (*DMSO*) as a control to match the level resulting from addition of okadaic acid or PMSF stock solutions are noted. After incubation, the immunoprecipitated PP2A C subunits were analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose and the membrane was probed with 4b7 (methylation-sensitive Ab), an anti-C subunit antibody that only recognizes unmethylated C subunits. Subsequently, the same membrane was probed with Transduction Laboratories anti-PP2A C subunit antibody (methylation insensitive Ab), which is insensitive to the methylation state of PP2A and therefore reveals the total C subunit in each lane. The C subunits migrated as doublets in this gel, but whether double or single bands are seen can vary (see comments in legend to Fig. 4).

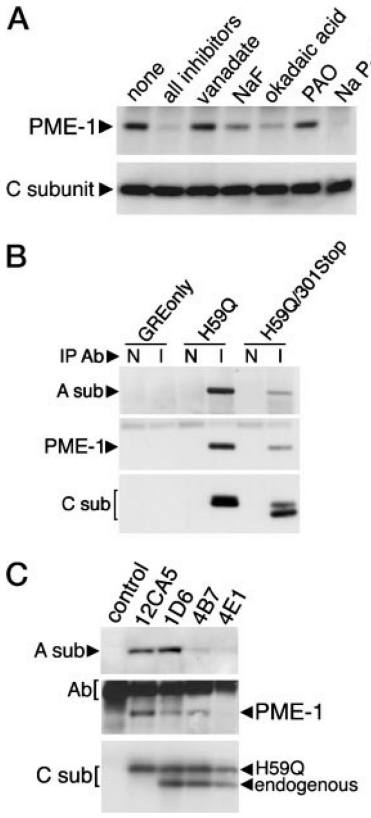

Fig. 6. Analysis of H59Q·PME-1 complex formation

A, the PP2A inhibitors, okadaic acid, sodium fluoride, and sodium pyrophosphate, reduce the amount of PME-1 complexed with the catalytically inactive H59Q C subunit. Seven parallel dishes of NIH3T3 cells expressing HA-tagged H59Q were lysed as described under "Experimental Procedures" in Nonidet P-40 lysis buffer containing the indicated inhibitor(s) at the following concentrations: sodium vanadate (1 mM), sodim fluoride (50 mM), okadaic acid (500 nM), phenylarsine oxide (*PAO*, 10 μM), sodium pyrophosphate (*NaP~P,* 20 mM). Anti-HA tag immunoprecipitates were prepared from these lysates and analyzed by SDS-PAGE and immunoblotting. The blot was probed sequentially with antibodies detecting PME-1 and H59Q C subunit (via its HA tag). In a separate experiment using phosphorylase *a* as substrate (not shown), sodium fluoride, okadaic acid, and sodium pyrophosphate were, respectively, found to inhibit PP2A 91 ± 10, 97 ± 4, and >99%, while phenylarsine oxide and sodium vanadate, respectively, showed no or 25 ± 18% inhibition. *B*, loss of the C subunit carboxyl terminus reduces, but does not abolish, PME-1 binding. Non-immune (*N*) and HA tag (*I*) immuno-precipitates were prepared from MT-transformed NIH3T3 cells expressing vector only (*GREonly*), HA-tagged H59Q, or HA-tagged H59Q/301Stop double mutant which lacks nine carboxyl-terminal amino acids. Immune complexes were analyzed by SDS-PAGE; proteins were transferred to nitrocellulose; and immunoblotting was performed with antibodies directed against A subunit, PME-1, and C subunit (anti-HA tag). The C subunits migrate as doublets in this gel, but whether double or single bands are seen can vary (see comments in legend to Fig. 4). The band seen in all lanes in the PME-1 panel is from the immunoprecipitating antibodies. Chemiluminescent quantitation (using a Bio-Rad Fluor-S Max MultiImager or a Roche Molecular Biochemicals Lumiimager) was used in seven separate experiments with mixtures of clones to quantify the ratio of PME-1 to C subunit signal in each lane. In six of seven experiments with mixes of clones, the double mutant bound less PME-1 than did H59Q, with a mean reduction of 56 ± 30% and a median

value of 39 (range of 8–87%). Thus, PME-1 binding is clearly reduced by loss of the carboxyl terminus. In a seventh experiment, for unknown reasons, the double mutant bound 235% of the H59Q level of PME-1, lowering the overall mean reduction to 28% (median = 40). *C,* C subunit carboxyl-terminal antibodies immunoprecipitate reduced amounts of H59Q·PME-1 complex. Immunoprecipitates were prepared from MT-transformed NIH3T3 cells expressing HA-tagged H59Q using control antibody, HA-tag antibody (12CA5), or carboxyl-terminal C subunit antibodies (1D6, 4B7, 4E1). The immune complexes were analyzed by SDS-PAGE, proteins were transferred to nitrocellulose, and immunoblotting was performed with anti-A subunit antibody (*upper panel*), anti-PME-1 antibody (*middle panel*), and anti-C subunit antibody recognizing both endogenous and HA tagged proteins (*1D6 lower panel*). The positions of A subunit, the immunoprecipitating antibody heavy chains (*Ab*), PME-1, HA-tagged H59Q C subunit, and untagged, endogenous wt C subunit are indicated. The C subunits migrate as single bands in this gel, but whether double or single bands are seen can vary (see comments in legend to Fig. 4). HA-tagged H59Q C subunit migrates more slowly than endogenous wt C subunit because of the HA tag.

Table I

H59Q and H118Q are catalytically inactive

PP2A activity present in anti-C subunit (HA tag) immunoprecipitates was measured using phosphorylase *a* and cdc2-phosphorylated histone H1 as substrates as described under “Experimental Procedures,” and normalized to the wt value. The data represent four independent experiments. Background phosphatase activity is probably due to nonspecific sticking of a small amount of active, non-HA tagged, endogenous C subunit.

C subunit	C subunit-associated phosphatase activity (%wt)	
	Phosphorylase *a*	cdc2-phosphorylated histone H1
	mean ± SD	
None (vector only control)	9 ± 2	2 ± 1
wt	100	100
H59Q	7 ± 1	2 ± 1
H118Q	8 ± 3	2 ± 1

Table II

Comparison of the sequences surrounding the putative or known active site serines of PME-1 proteins and CheB

The PME-1 and CheB residues matching the signature motif ((L/I/V)*X*(L/I/V/F/Y)(L/I/V/S/T)G(H/Y/W/V)S*X*G(G/S/T/A/C)) for lipases utilizing an active site serine are underlined. The serine present in each sequence is the predicted (PME-1) or known (CheB) active site serine.

Species	First residue shown	Sequence
Human PME-1	150	IMLIGHSMGG
C. elegans PME-1	158	VCIVGHSMGG
S. cerevisiae PME-1	199	VILIGHSLGG
S. typhimurium CheB	158	LIAIGASTGG