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

Egon Ogris§§§, Xianxing Du**+, Kasey C. Nelson†††**, Elsa K. Mak‡‡‡, Xing Xian Yu**, William S. Lane§$, and David C. Pallas‡‡‡ **

From the §Division of Cellular and Molecular Biology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115, the $Institute of Molecular Biology, University of Vienna, A-1030 Vienna, Austria, the **Department of Biochemistry and Winship Cancer Center, Emory University School of Medicine, Atlanta, Georgia 30322, and the §§Harvard Microchemistry Facility, Harvard Biological Laboratories, Cambridge, Massachusetts 02138

Carboxymethylation of proteins is a highly conserved means of regulation in eukaryotic cells. The protein phosphatase 2A (PP2A) catalytic (C) subunit is reversibly methylestated 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 demethylestated 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.

Protein phosphatase 2A (PP2A) 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%) 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. 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 methylsterase 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 methylsterase 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, 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 methylsterase 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 methylsterase substrate nor as an inhibitor. In the latter study, a 600-fold excess of unmethylated PP2A C subunit was found to inhibit the PP2A methylsterase by 50%, while a 106-fold excess of a carboxyl-terminal C subunit decapetide did not inhibit the methylsterase 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 methylsterase 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 methylsterase. 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 glutamine 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 methylsterase by the ability of the bacterially expressed and purified enzyme to demethylate PP2A C subunit. To our knowledge, this is the first mammalian methylsterase 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 Mutagen 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, pGREG-2 (28). The construction of a pGREG-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 SphI site in the PCR-Script<sup>®</sup> SK(+) vector using the PCR-Script<sup>®</sup> 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, pGREG-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.

Preparation of Cells—For metabolic labeling of cells with methionine, subconfluent dishes of cells were labeled for 5 h with [35S]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. 6B), immunoprecipitates were washed twice with Nonidet P-40 lysis buffer, twice with phosphate-buffered saline, and once with ddH2O. 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 (ID6; 0.25 μg/ml), or rabbit anti-PME-1 antibodies (AR2 or E37; see below). Immunoblots were developed with enhanced chemiluminescence (Amerham 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 I1. γ-32P-Labeled phosphorylase a substrate was prepared from phosphorylase b according to the manufacturer’s (Life Technologies, Inc.) instructions. Histone H1 was phospho-
rylated by mitotic p34\(^{\text{cd}}\) 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 with the mouse PME-1 cDNA. The probe, 395 base pairs in length, is an EcoRI-NofI 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 (RIELAK-TEKYWDGWFR) 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 Injet conjugation kit, and the conjugate was used as immunogen. The second antisera, E37, was raised against a mixture of two nickel agarose-purified, 6xHis-tagged, bacterially expressed human PME-1 fragments that distinguished between 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 \(\mu\)l of reaction buffer containing 55 mM Tris, pH 8.0, 55 mM NaCl, 1 mM dithiothreitol, 1.0 mM MgCl\(_2\), 1.0 mM CaCl\(_2\), 0.55% Nonidet P-40, and 0.2 mg/ml bovine serum albumin was added. Then, 0.5 \(\mu\)l 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 \(\mu\)l 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 \(\times\) g for 5 min). Demethylation reactions were carried out at 32 \(^\circ\)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 nitrocellulose, 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, PFX, PP2B, and PPA. 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 PP1 (41) were reported, the results of which suggested that these two histidines were involved in PP2A catalytic binding and catalysis. As described in "Experimental Procedures," each C subunit mutant or wt C DNA 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

**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 (QiGEN), and analyzed on formaldehyde, 1% agarose gels for RNA integrity and to estimate the amount of total RNA (18 S and 28 S RNA). 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 32P-radiolabeled probe generated by random primer labeling of a DNA fragment from the alignment region of the mouse PME-1 cDNA. The probe, 395 base pairs in length, is an EcoRI-NofI 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).
immunoprecipitates prepared from cells containing empty vector (GREonly) or HA-tagged wt (ser C sub) or mutant C subunits (H59Q and H118Q) were immunoprecipitated with anti-HA tag antibody (12CA5) and analyzed by SDS-PAGE and immuno blotting. 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 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.

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 coinmunoprecipitated 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. ^3 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 35S-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 coinmunoprecipitates 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 pl, 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 35S-labeled spot corresponding to p44A was absent in the two-dimensional gels of 35S-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 immunofinity column as described under “Experimental Procedures.” After analysis on one-dimensional gels, both actin and histone H1 were absent in the two-dimensional gels of 35S-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.

Initial searches of the expressed sequence tag (EST) data

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**Table I**

H59Q and H118Q are catalytically inactive

| C subunit-associated phosphatase activity (% of wt) | Phosphorylase a | cd2-phosphorylated histone H1 |
|--------------------------------------------------|----------------|-----------------------------|
| None (vector only control) | 9 ± 2 | 2 ± 1 |
| wt | 100 | 100 |
| H59Q | 7 ± 1 | 2 ± 1 |
| H118Q | 8 ± 3 | 2 ± 1 |

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

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.
The PME-1 and CheB residues matching the signature motif ((L/I/V/F/Y)(L/I/V/S/T)G(H/Y/W/V)S) were accounted for in the human sequence homologous to PME-1, 44.9-kDa PME-1 (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. 3D). 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 35S-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 H59Q 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, Okahtacid 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 aligned 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/Y/W/S/X/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 Drosophilia 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. 3D). 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,
bands are seen can vary (see comments in legend to Fig. 4). Subunits migrated as doublets in this gel, but whether double or single
PP2A and therefore reveals the total C subunit in each lane. The C
subunit was assayed by blotting 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).

of these, two aspartates in human PME-1, Asp-181 and Asp-182, 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 co-workers (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 μM okadaic acid but not by 1 or 5 mM 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. 6A). 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 amino acids, 301–309. Fig. 6B, 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. 6B 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. These antibodies are unable to efficiently recognize a C subunit mutant lacking the carboxyl-terminal leucine, indicating that they bind, at least in part, to the very carboxyl terminus. A putative carboxyl-terminal domain has recently been found to decrease the amount of A subunit bound to H59Q (23). Comparison of the relative ratios of the PME-1 and H59Q bands in Fig. 6C reveals that, relative to 12CA5, 1D6 and 4B7 immunoprecipitated 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 substantiate the conclusions made from Fig. 6B. 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 catalyt-

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5 D. C. Pallas, unpublished data.
Protein Phosphatase 2A Methylesterase PME-1

**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), sodium fluoride (50 mM), okadaic acid (500 nM), phenylarsine oxide (PAO, 10 μM), sodium pyrophosphate (NaP, 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 α as a substrate (not shown), sodium fluoride, okadaic acid, and sodium pyrophosphate were, respectively, found to inhibit PP2A 91 ± 10, 97 ± 4, and 99%, while phenylarsine oxid 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 (H) immunoprecipitates were prepared from MT-transformed NIH3T3 cells expressing vector only (GreOn), 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. 6). 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 Multimager or a Roche Molecular Biochemicals Lumimager) 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 reduced when the C subunit is truncated in a manner consistent with either differential modification or the existence of two closely related isoforms of the enzyme. The amounts of these two species were within a twofold 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 database 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.

The molecular basis of the cell cycle-dependent regulation of PP2A C subunit methylation is unknown. The poor metabolic

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*Fig. 6 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.
labeling of PME-1 in an asynchronous population of cells 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 methyltransferase. Whether PP2A-1 activity is regulated is unknown. In the case of the bacterial chemotactic response, the CheB methyltransferase 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 methyltransferase, 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 methyltransferase 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 methyltransferase 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 PP2A-1 methyltransferase. Third, PP2A inhibitors have a destabilizing effect on the PP2A-1-H59Q interaction. Fourth, the methyltransferase is inhibited by the PP2A inhibitors, okadaic acid and microcystin-LR, and the methyltransferase is inhibited by okadaic acid (testing for inhibition of the methyltransferase 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 PP2A-1-H59Q complexes. The latter effect is more consistent with a role in binding the PP2A-1 methyltransferase 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. 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 PP2A-1.

Contact between the C subunit and PP2A-1 could theoretically be with PP2A-1 residues and/or with a phosphorylation site on PP2A-1. Because H59Q and H118Q are virtually unmethylated, PP2A-1 apparently can remain bound to these mutants in the absence of a methylated carboxyl terminus. At least with H59Q, PP2A-1 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-PP2A-1 complexes. However, the amount of PP2A-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-PP2A-1 complexes at all suggest that PP2A-1 is proximal to the C subunit carboxyl terminus in the H59Q-PP2A-1 complex. Moreover, the reduced amounts of PP2A-1 in complex with the H59Q-301Stop double mutant indicate that carboxyl-terminal residues play a role in binding of H59Q to PP2A-1. The contribution of these residues to the interaction of wild type C subunit with PP2A-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, are also deficient in B subunit binding. 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, PP2A-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 PP2A-1, p44B, and several other proteins. Interestingly, initial experiments suggest that p44B binding to H59Q is even more sensitive to phosphatase inhibitors than is PP2A-1 binding. 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.

PP2A-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; PP2A-1 forms sharp spots on these gels while p44B migrates as a smear. In addition, in vitro translation of PP2A-1 yields no product migrating at the position of p44B and we have been unable to detect p44B with antibodies raised against PP2A-1 sequences.

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

7 K. C. Nelson and D. C. Pallas, unpublished data.
Hisp-59 and Hisp-118 are mutated to glutamine. One question of special interest is whether the corresponding catalytically inactive mutants of PPx, which has the same last four carboxyl-terminal amino acids as PP2A and is also methylated at its carboxyl-terminal leucine, will trap its methyltransferase. Given the lack of close relatives to PME-1 in the various data bases, it would not be surprising if that methyltransferase turns out to be PME-1.

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