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Protein Phosphatase Methyltransferase 1 (Ppm1p) Is the Sole Activity Responsible for Modification of the Major Forms of Protein Phosphatase 2A in Yeast[1]

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Protein phosphatase 2A (PP2A) is a major threonine/serine phosphatase that is involved in regulating a variety of cellular processes. It has been shown in both yeast and mammals that the PP2A catalytic subunit (PP2Ac) is methyl-esterified at the conserved C–terminal Leu residue. The recent characterization of a mammalian PP2A carboxyl methyltransferase has led to the identification of two ORFs in *Saccharomyces cerevisiae* as potential orthologues of the mammalian PP2A methyltransferase: protein phosphatase methyltransferase 1 (*PPM1*) and protein phosphatase methyltransferase 2 (*PPM2*). To experimentally identify the PP2A methyltransferase in yeast, we obtained deletion mutants of *PPM1* and *PPM2* and then constructed double mutants. Using *in vivo*-labeling techniques, we demonstrate that only the *PPM1* gene is required for PP2Ac methylation at the C-terminus. Because yeast has at least three homologues of PP2Ac (*PPH21*, *PPH22*, and *PPH3*), we then asked whether all of these catalytic subunits are methylated by the *PPM1* and/or *PPM2* putative methyltransferases. We modified the segment corresponding to the N-terminal coding region of all three PP2Ac genomic genes with a hemagglutinin (HA) tag in the parent, *ppm1*, *ppm2*, and *ppm1ppm2* mutant genetic backgrounds. Using immuoprecipitation with anti-HA antibodies followed by methyl ester analysis, we showed that only in the *ppm1* mutant were both Pph21p and Pph22p not methylated. We did not detect any methylesterification of Pph3p under our conditions. Our results indicate that *PPM1* is the sole methyltransferase responsible for methylating the two major homologues of PP2Ac in yeast. The function of the *PPM2* gene product remains unclear. © 2001 Academic Press

Key Words: **protein phosphatase 2A; methyltransferase; yeast; carboxyl methylation.**

Protein serine/threonine phosphatase 2A (PP2A),[3] a highly conserved protein from yeast to man, has been shown to play various regulatory roles in eukaryotic cells (1, 2). PP2A substrates include phosphorylated transcription factors, metabolic enzymes, cytoskeletal proteins, and a number of protein kinases (1–3). PP2A functions as a holoenzyme; the catalytic subunit (PP2Ac) interacts with a structural PR65/A subunit to form a core dimer, which then associates with one of a number of variable B subunits (4). These B subunits are encoded by at least three gene families in mammals and are thought to exert distinct regulatory properties on the PP2A holoenzyme (2). The budding yeast *Saccharomyces cerevisiae* contains two homologous gene products, Pph21p and Pph22p, which are similar in sequence to mammalian PP2Ac and which share its function (5). The *PPH21* and *PPH22* genes are considered redundant since either one can be deleted without any phenotypic defect. However, a deletion of the two genes causes a severe growth inhibition in the budding yeast (4). A third yeast gene product Pph3p is a more distant homologue of Pph21p and Pph22p. Deletion of

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[3] Abbreviations used: PP2A, protein phosphatase 2A; PP2Ac, catalytic subunit of protein phosphatase 2A; AdoMet, *S*-adenosyl-L-methionine; [^{3}H]AdoMet, *S*-adenosyl-[*methyl*-^{3}H]-L-methionine; YPD, yeast extract, peptone, and dextrose; YPG, yeast extract, peptone, and D-galactose; SDS, sodium dodecyl sulfate; HA, hemagglutinin.

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PPH21 and *PPH22* in the absence of *PPH3* is lethal in *S. cerevisiae* (5).

PP2Ac, in both mammals and yeast, has been shown to be methylesterified at the C-terminus of the α-carboxyl group of a conserved Leu residue (6, 7). The role of carboxyl methylation of PP2A on the catalytic subunit has not yet clearly been determined. *In vitro* studies have suggested that the methylation of the C-terminal leucine can enhance the phosphatase activity of the catalytic subunit in the mammalian enzyme (8). However, when a mutated form of mammalian PP2Ac lacking the conserved Leu residue is expressed in a yeast mutant lacking endogenous PP2Ac, no difference of cell growth was observed from the wild-type form of PP2Ac (9). Moreover, the methylation of the C-terminal Leu residue of PP2Ac has been shown to be important for the assembly of the holoenzymes and the binding of the regulatory B subunits in mammalian systems (10).

We have been studying biological methylation reactions using *S. cerevisiae* as a model organism taking advantage of the availability of its genome sequence and ease of performing biochemical experiments in intact yeast cells. We have recently identified 26 putative methyltransferases in *S. cerevisiae* based on the presence of specific *S*-adenosylmethionine (AdoMet) motifs present in their open-reading frames (11). Using biochemical analyses, we have been able to show that one of these putative methyltransferases modifies the δ-nitrogen atom of arginine residues (11). Additionally, we characterized another of these ORFs as *trans*-aconitate methyltransferase, methylating a small molecule derived from citric acid cycle (12). To identify the enzyme responsible for PP2A methylesterification, we have also examined deletion mutants of a number of the other putative methyltransferases to ask whether PP2A is methylated when compared to their wild-type counterparts. However, none of these mutant strains had any PP2Ac methylation defect.

The recent characterization of the mammalian PP2Ac carboxyl methyltransferase has allowed the identification of candidate yeast genes for the same activity (13). Using sequence homology searches, two yeast ORFs designated as *PPM1* and *PPM2* (protein phosphatase methyltransferase) were found in *S. cerevisiae* that possessed 30 and 26% sequence identity, respectively, to the mammalian PP2A methyltransferase (13). Two recent studies have suggested, on the basis of indirect evidence, that the *PPM1* gene product is the yeast orthologue of mammalian PP2A methyltransferase (14, 21). PP2A carboxyl methylation is a reversible reaction in intact cells, and the half-life of the methyl ester is approximately 20 min (15). A specific carboxyl methylesterase has been found to be responsible for the turnover of the PP2A methyl groups in mammalian cells as well as in yeast cells (16).

TABLE I

Yeast Strains Used in This Work

Strain	Genotype
BY4741	*MAT***a** *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*
BY4742	*MATα his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*
KLY101	BY4741, *ppm1*::KAN
KLY102	BY4742, *ppm2*::KAN
YCY1001	*MATα his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ppm1*::KAN, *ppm2*::KAN
YCY1002	*MAT***a** *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, lys2Δ0, ppm1*::KAN, *ppm2*::KAN
HKY101	BY4742, *pph21*::HIS-*PPH21*-HA
HKY102	KLY101, *pph21*::HIS-*PPH21*-HA
HKY103	KLY102, *pph21*::HIS-*PPH21*-HA
HKY104	YCY1001, *pph21*::HIS-*PPH21*-HA
HKY105	BY4742, *pph22*::HIS-*PPH22*-HA
HKY106	KLY101, *pph22*::HIS-*PPH22*-HA
HKY107	KLY102, *pph22*::HIS-*PPH22*-HA
HKY108	YCY1001, *pph22*::HIS-*PPH22*-HA
HKY109	BY4742, *pph3*::HIS-*PPH3*-HA
HKY110	KLY102, *pph3*::HIS-*PPH3*-HA
HKY111	YCY1001, *pph3*::HIS-*PPH3*-HA

In this paper, we provide direct *in vivo* evidence that only Ppm1p is responsible for methylation of the PP2A catalytic subunit. We have also shown that Pmp1p is able to methylate both the Pph21p and Pph22p yeast PP2Ac subunit homologues, but we find no evidence that the third homologue Pph3p is methylated by Ppm1p.

MATERIALS AND METHODS

Yeast strains. Strains and their genetic backgrounds was listed in Table I. Strains BY4741, BY4742, KLY101 (#4271), and KLY102 (#16650) were obtained from the Saccharomyces Genome Deletion Project via Research Genetics (Huntsville, AL). The double *ppm1ppm2* deletion mutants were made by cross-streaking a *ppm1* MAT**a** *met*-haploid strain (KLY101) and *ppm2* MATα *lys*-haploid strain (KLY102) on a YPD plate. Colonies that grew on synthetic medium lacking lysine and methionine at 30°C were selected as diploids. After 2 days, the colonies were replica-plated on 1% potassium acetate sporulation plate and incubated 2 days at 30°C. Resulting asci were treated in a 0.02% dilution of α-glucosidase (ICN, Irvine, CA) in H_2O at room temperature for 5–10 min to allow digestion of the spore sac. Using a microscope, at least 15 tetrads were dissected as described (17). Individual spores from each tetrad were grown for 2 days at 30°C on YPD plates. Individual colonies were then replica-plated on a G418-containing plate to select for kanamycin-resistant colonies, a Lys-containing synthetic defined medium plate, and Met-containing synthetic defined medium plate. Haploids containing the double knockout were picked among the nonparental ditypes that did not grow on G418 (GIBCO BRL, Grand Island, NY) plates. PCR analysis was performed to confirm the double deletion of *ppm1ppm2* using a primer for the kanamycin cassette and primers specific for *PPM1* and *PPM2* genomic sequences.

Strains were prepared where the wild-type *PPH21, PPH22,* and *PPH3* genes were replaced by homologous recombination with a hemagglutinin (HA)-encoding fragment fused to the 5′ end of each gene under the control of the galactose *GAL1* promoter after the

procedure of Longtine *et al.* (18). Briefly, PCR was performed using a template plasmid containing DNA sequences for *GAL1,* a His-selectable marker, and three tandem HA sequences. DNA for yeast transformation was generated by PCR in 50-μl reaction using *Taq* polymerase (Promega, Madison, WI), 2.0 mM Mg_2Cl, 1 mM of each dNTP, 2 μM of each primer, and 0.1 μg template DNA. Reactions were run for 23 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The 20 cycles were followed by a final 10-min extension at 72°C. The product from six PCR was pooled, precipitated, and resuspended in 50 μl of water. This concentrated amplified DNA was transformed into *S. cerevisiae* cells using a lithium acetate procedure (18). The transformed cells were washed once with 1 ml of water, resuspended in 300 μl of water, and spread onto YPG plates lacking histidine. In addition, PCR analysis was done to confirm the genetic background of each genomically tagged strain.

In vivo labeling and preparation of cell extracts. Yeast strains were grown to early log phase ($OD_{600\ nm}$ between 0.6 and 0.8). An aliquot of 5 $OD_{600\ nm}$ cells was collected by centrifugation at 1000*g* for 5 min and the cells were washed 3 times with 10 ml of medium (1% yeast extract, 2% peptone, and 2% of either D-glucose for YPD medium or D-galactose (Sigma, St. Louis, MO) for YPG medium. The cell pellet was resuspended in 820 μl of YPD or YPG and 180 μl of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet) (80 Ci/mmol, 12.5 μM, in dilute hydrochloric acid/ethanol 9/1 (pH 2.0–2.5), Amersham Life Science) to get a final [^{3}H]AdoMet concentration of 2.2 μM. The cells were incubated on a rotary shaker at 225 rpm for 30 min at 30°C and pelleted as above and washed twice with water.

The cell pellet was then resuspended in 50 μl of lysis solution (1% SDS (w/v) and 0.67 mM phenylmethylsulfonyl flouride). Glass beads (0.2 g, 0.5 mm diameter, Biospec Products, Inc., Bartlesville, OK) were added to the cell suspension and the mixture was vortexed for 1 min followed by an incubation on ice for another 1 min. The vortexing step was repeated 7 times. The extract was collected into a new tube and another 50 μl of lysis solution was added to the beads and vortexed for 30 s to wash the remaining protein from the beads. This washed extract was then pooled with the orginal extract.

SDS-gel electrophoresis and analysis of [^{3}H]methylated polypeptides. Extract (50 μl, about 250 μg protein) was mixed with an equal volume of concentrated gel electrophoresis sample buffer (3.5% (v/v) β-mercaptoethanol, 6% (w/v) SDS, 0.18 M Tris-Cl, pH 6.8, 0.005% (w/v) bromphenol blue) and incubated at 100°C for 3 min, and 35 μl was loaded onto 1.5-mm-thick slab gel containing a stacking gel and 10.5 cm resolving gel. The resolving gel was made from 12.6% (w/v) acrylamide and 0.28% (w/v) *N,N*-methylenebisacrylamide. Molecular mass standards (7 μl of BioRad low molecular weight standard 161-0304; each standard at 2 mg/ml) were diluted to 20 μl using distilled water and mixed with 20 μl of sample buffer; the entire 40 μl was loaded into wells and the electrophoresis buffer as described by Laemmli (19). Electrophoresis was performed at 20 mA until the dye front ran off the end of resolving gel. The gels were then stained for 15 min with 0.1% (w/v) Coomassie brilliant blue in 50% (v/v) methanol and 10% (v/v) acetic acid in water. The gels were then destained overnight at room temperature with 5% (v/v) methanol and 10% (v/v) acetic acid in water. The gels were vacuum-dried at 65°C onto Whatman 3MM chromatography paper. The dried gels were cut into 3-mm slices (8 mm wide). To hydrolyze the [^{3}H]methyl ester from the polypeptides, 150 μl of 1.5 M Na_2CO_3 (pH 12) was added to each dried gel slice in a 1.5-ml polypropylene microcentrifuge tube. The tubes were gently placed into 20-ml scintallation vials containing 5 ml of Safety Solve counting fluid (Research Products International, Mt. Prospect, IL). Vials were incubated at 37°C for 24 h to allow [^{3}H]methanol derived from base hydrolysis of methyl esters to diffuse from the microcentrifuge in the vapor phase to the scintillation fluid. Vials were counted using a Beckman LS6500 scintillation counter. To obtain the total radioactivity of each gel slice, 1 ml of 30% hydrogen peroxide was added gently to each microcentrifuge tube containing the gel slice after they were counted for [^{3}H]methanol. Vials were capped loosely and incubated at 37°C for an additional 24 h. After the vials were shaken to mix the content of the microcentrifuge tube with the scintillation fluid, the samples were recounted.

Immunoprecipitation of HA-tagged PP2Ac homologues. Genomically HA-tagged *PPH21, PPH22,* and *PPH3* were grown in YPG or YPD. Cells (5 $OD_{600\ nm}$) were *in vivo*-labeled as described previously. Cells were subsequently disrupted in 100 μl of buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl flouride, 2 μg/ml pepstatin A, 1 μg/ml aprotinin) and the cytoplasmic fraction was prepared as described above. To the lysate 2 μl of hemagglutinin influenza monoclonal antibodies (anti-HA, Convance Inc., Richmond CA) at 5 mg/ml concentration was added and the mixture was incubated in a rotary shaker for 12 h at 4°C. Twenty microliters of washed protein g plus/protein A agarose bead suspension (Oncogene Research Products) was added to the mixture and incubated for another 2 h at 4°C. The beads were pelleted and washed twice with 1 ml of buffer A for 5 min. To the beads 50 μl of concentrated gel electrophoresis sample buffer was added and vigorously vortexed. The samples were then boiled for 5 min and loaded onto a 12.6% SDS-gel as described above.

RESULTS

To study methylation reactions in intact *S. cerevisiae* yeast cells, one can simply incubate cells with AdoMet radiolabeled on the methyl group. This molecule is rapidly transported into cells and its methyl group can be transferred to methyl acceptors including proteins, lipids, DNA, and small molecules (15, 20). As a result, all AdoMet-dependent methylation products can be studied biochemically in intact cells as they occur *in vivo.* In this work, we were interested in the methylesterification reaction at the C-terminal leucine residue of the catalytic subunit of PP2A which has been shown to occur both in mammalian cells and in yeast (6, 7). Recent work has suggested that the enzyme responsible for this reaction may be the *PPM1* gene product (14, 21). We sought to directly determine the methylation status of PP2Ac in yeast mutant strains disrupted in *PPM1,* its homologue *PPM2,* or in both genes. Yeast cells were incubated with [^{3}H]AdoMet and their polypeptides were fractionated by SDS-gel electrophoresis. Polypeptide-bound [^{3}H]methyl esters were measured by a vapor phase assay after base hydrolysis. This type of analysis has been used previously to identify ester-containing species of both RNA and polypeptides (15, 20).

However, we faced an initial problem because the major PP2Ac forms can comigrate on SDS gels with the more abundant methylesterfied form of eEF1A (15). We thus optimized electrophoresis conditions using 12.6% acrylamide in the resolving gel so that we could separate PP2Ac from eEF1A. As illustrated in Fig. 1, a major 42.5-kDa species corresponding to PP2Ac can be separated from the 49-kDa eEF1A species and is methylesterified in the parental strain and in the *ppm2* mutant. However, no methylesterified species is found in the *ppm1* deletion strain nor in the *ppm1ppm2* double mutant strains. Under our conditions, methyles-

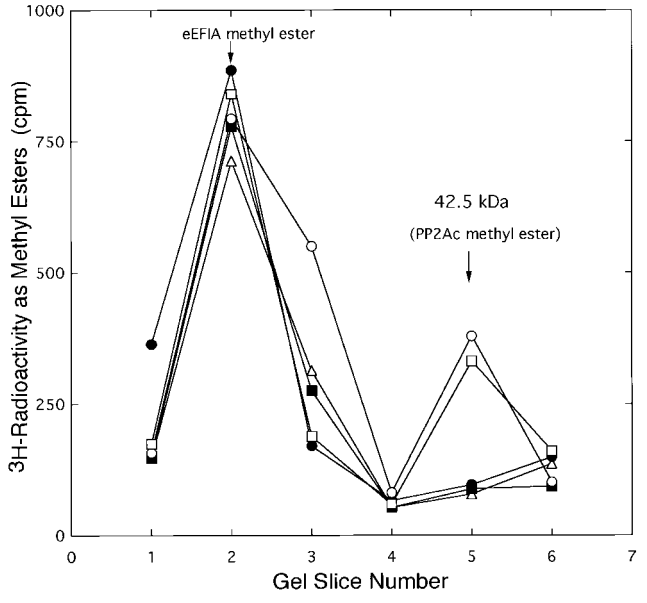

FIG. 1. PP2Ac is not methylated in a *ppm1* mutant in intact cells. *S. cerevisiae* parent and mutant strains lacking the *PPM1, PPM2,* and *PPM1PPM2* genes were grown and labeled *in vivo* with [^{3}H]AdoMet as described under Materials and Methods. Cells were lysed, and polypeptides (about 80 μg protein/lane) were separated by 12.6% polyacrylamide SDS-gel electrophoresis. Gel slices (3 mm) in the portion of the gel corresponding to polypeptide weights of 30–66 kDa were analyzed for [^{3}H]methyl esters as described under Materials and Methods. The position of the ovalbumin 42.5-kDa molecular mass marker electrophoresed in a parallel lane is indicated with an arrow. Parent strain, BY4741 (□); *ppm1* strain, KLY101 (■); *ppm2* strain, KLY102 (○); *ppm1ppm2* strain, YCY1002 (●); and *ppm1ppm2* strain YCY1001 (△).

terification of eEF1A was similar in each strain as was the stable methylation of eEF1A at lysine residues (data not shown).

Because PP2Ac has three homologues in *S. cerevisiae,* we were interested in investigating which of the homologues is methylated by Ppm1p and if Ppm2p might be capable of methylating minor homologues of PP2Ac which would not be detected in the experiment shown in Fig. 1. To determine the methylation status of each PP2Ac homologue in yeast, the genes for *PPH21, PPH22,* and *PPH3* were modified by addition of a segment encoding an hemagglutinin epitope under the *GAL1* promoter in parent, *ppm1, ppm2,* and double deletion *ppm1ppm2* strains as described in Fig. 2 and under Materials and Methods. The N-terminus of each gene product was modified to contain an HA epitope so that the C-terminus can be probed for the presence or absence of ^{3}H-methylation by immunoprecipitation with anti-HA antibodies. In addition, by driving the expression of the PP2Ac homologues under *GAL1* promoter, the tagged genes can be specifically turned on in the presence of galactose or turned off in media containing glucose. In Fig. 3, the expression of PP2Ac homologues Pph21p and Pph22p in the mutant strains as well as the parent strain is shown using specific monoclonal antibodies against the HA epitope. As expected, the expression of tagged genes was dependent upon growth of cells in galactose-containing medium; no expression was found when glucose was used as a carbon source (Fig. 3). The expression of each homologue was similar in each strain background.

To directly examine the extent of methylesterification of each PP2Ac homologue in the *ppm1, ppm2,* and *ppm1ppm2* mutants background as compared to the isogenic parent strain, yeast strains containing genomically tagged HA epitope were *in vivo* radiolabeled using [^{3}H]AdoMet. Each homologue was immunoprecipitated from extracts by anti-HA and the amount of radioactivity as methyl esters was determined for polypeptides separated by SDS gels. As shown in Fig. 4A, there is substantial methyl ester

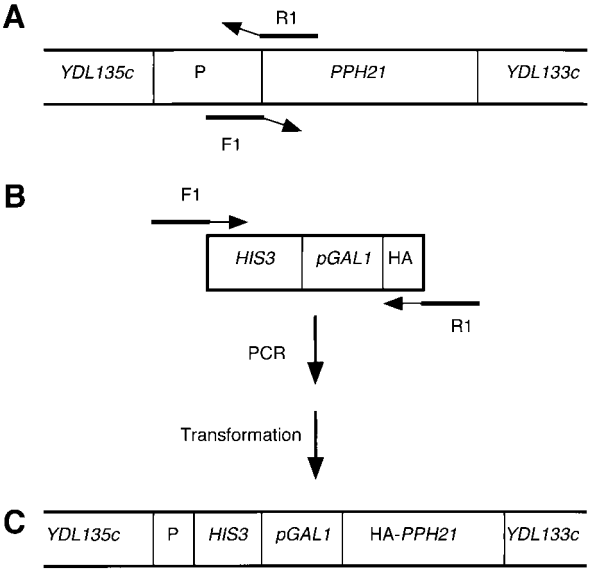

FIG. 2. General scheme of HA epitope tagging of endogenous PP2Ac homologues (18). To prepare strains where the wild-type, *PPH21, PPH22,* and *PPH3* genes were replaced in the genome by genes encoding the catalytic subunits with HA-epitope segment fused to their N-terminus under the control of a galactose *GAL1* promoter, as described under Materials and Methods. For simplicity only the *PPH21* gene is depicted in here (A). Briefly, PCR was performed using a transformation module as a template containing DNA sequences for *GAL1,* the His-selectable marker (His3), and three HA sequences as shown in B (18). Specific PCR primers F1 and R1 contained sequences that can amplify the transformation module: [F1, 5′-GAATTCGAGCTCGTTTAAAC-3′; and R1 sequence, 5′-GCACTGAGCAGCGTAATCTG-3′]. Forty nucleotides that span the region upstream (−90) of the desired ORF were incorporated into 5′ end of primer F1. Forty nucleotides that span the coding region of the gene (+50) were also incorporated into R1 primer. F1 and R1 are used to generate target DNA that can be integrated into genomic DNA via homologous recombination. (A) Genomic organization of *PPH21* and site of homologous recombination. (B) Plasmid template for PCR containing portions F1 and R1 sequences. (C) Recombinant genomic organization of *PPH21* with a HA N-terminal epitope and a selectable His marker.

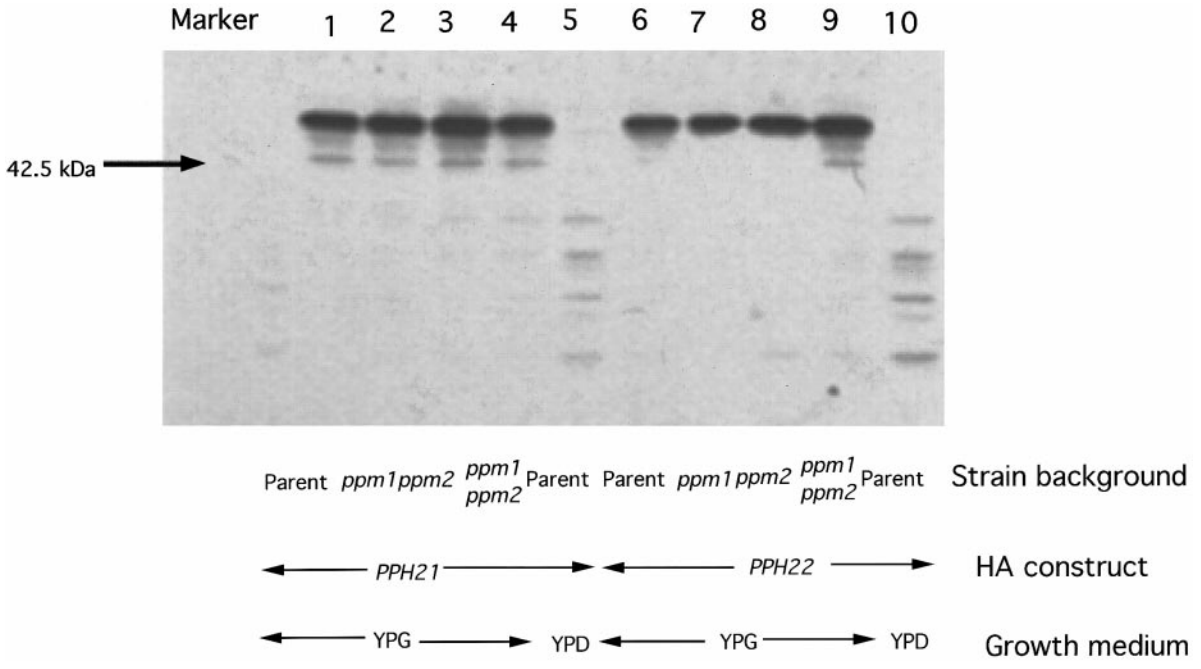

FIG. 3. Expression of HA-tagged PP2Ac homologues. Cells were grown in either YPG or YPD to a log phase. A total of 1 $OD_{600\ nm}$ cells was pelleted and cytoplasmic proteins were extracted as described under Materials and Methods. The cytoplasmic extract was subjected to Western blotting. In brief, after the samples were heated for 3 min at 100°C, approximately 15 μg (0.5 $OD_{600\ nm}$) protein was loaded into each lane of 10% SDS-polyacrylamide gel. Samples were electrophoresed at 125 V for 1.5 h. Proteins then were transferred to Hybond-ECL nitrocellulose membrane (Amersham, Arlington Heights, IL) at 12 V for 1.5 h at room temperature. Membranes were blocked in blocking solution (50 mM Tris, pH 7.5, 0.9% NaCl, 0.05% Tween 20, 10% nonfat dry milk) at room temperature for 2 h. HA monoclonol antibodies (1:3000; Sigma) were diluted into the blocking buffer and incubated with the membrane for 1 h at room temperature. The membrane was then rinsed twice and washed for three times for 15 min with blocking buffer; it was then incubated with the secondary antibody [1:3000 peroxidase anti-mouse antibodies, Amersham Life Sciences]. The membrane was first rinsed twice and then washed three times for 15 min with blocking buffer. The PP2Ac band was detected using the chemiluminescence detection system (NEN Life Sciences Products, Inc., Boston, MA). All strains were grown in YPG except the strains used in lanes 5 and 10 which were grown in YPD. The parent strain is BY4741.

radioactivity in the expected migration position of the Pph21p homologue (45 kDa) in the parent strain ($PPM1^+/PPM2^+$). This radioactivity is absent is in the *ppm1* mutant strain, the parent strain grown on glucose, and *ppm1ppm2* double mutant strain (Fig. 4B). However, the same methyl ester peak is present in the *ppm2* mutant as well as in parent strain grown in galactose. In addition, methyl ester radioactivity was missing in the wild-type strain grown in glucose as the sole source of sugar, indicating that the observed methylation is due to the expression of the tagged gene product Pph21p (Fig. 4A). In Fig. 4C, we demonstrate that Pph22p-tagged protein is also methylated in the wild-type parent strain as well as the *ppm2* mutant but that labile ester radioactivity is not present in the *ppm1* and *ppm1ppm2* mutants. However, as shown in Fig. 4D, no C-terminal methylesterification is detected for Pph3p in any of the strains examined. Therefore, we suggest that *PPM1* gene product is the sole methyltransferase responsible for the carboxyl methylation of Pph21p and Pph22p homologues. Our failure to detect the methylation of Pph3p suggests that this homologue of PP2Ac is not methylated in yeast, at least under our conditions.

DISCUSSION

Among the types of methylation reactions that occur in the cell, those resulting in the esterification of the C-terminus of a protein are of special interest since they are potentially reversible and may be involved in the modulation of the function of the protein. The pivotal protein phosphatase PP2A has been shown to be C-terminally methylated at the conserved leucine residue from yeast to mammals. Several regulatory mechanisms have been proposed for PP2A including the interaction of the catalytic and various regulatory subunits, the localization of the PP2A holoenzyme to specific subcellular structures, and its posttranslational modification by phosphorylation and methylation of its PP2Ac (1–3).

In this work, we provide evidence from *in vivo* studies that Ppm1p is the sole enzyme responsible for the methylation of the Pph21p and Pph22p catalytic subunits of PP2A in the yeast *S. cerevisiae.* First, in ex-

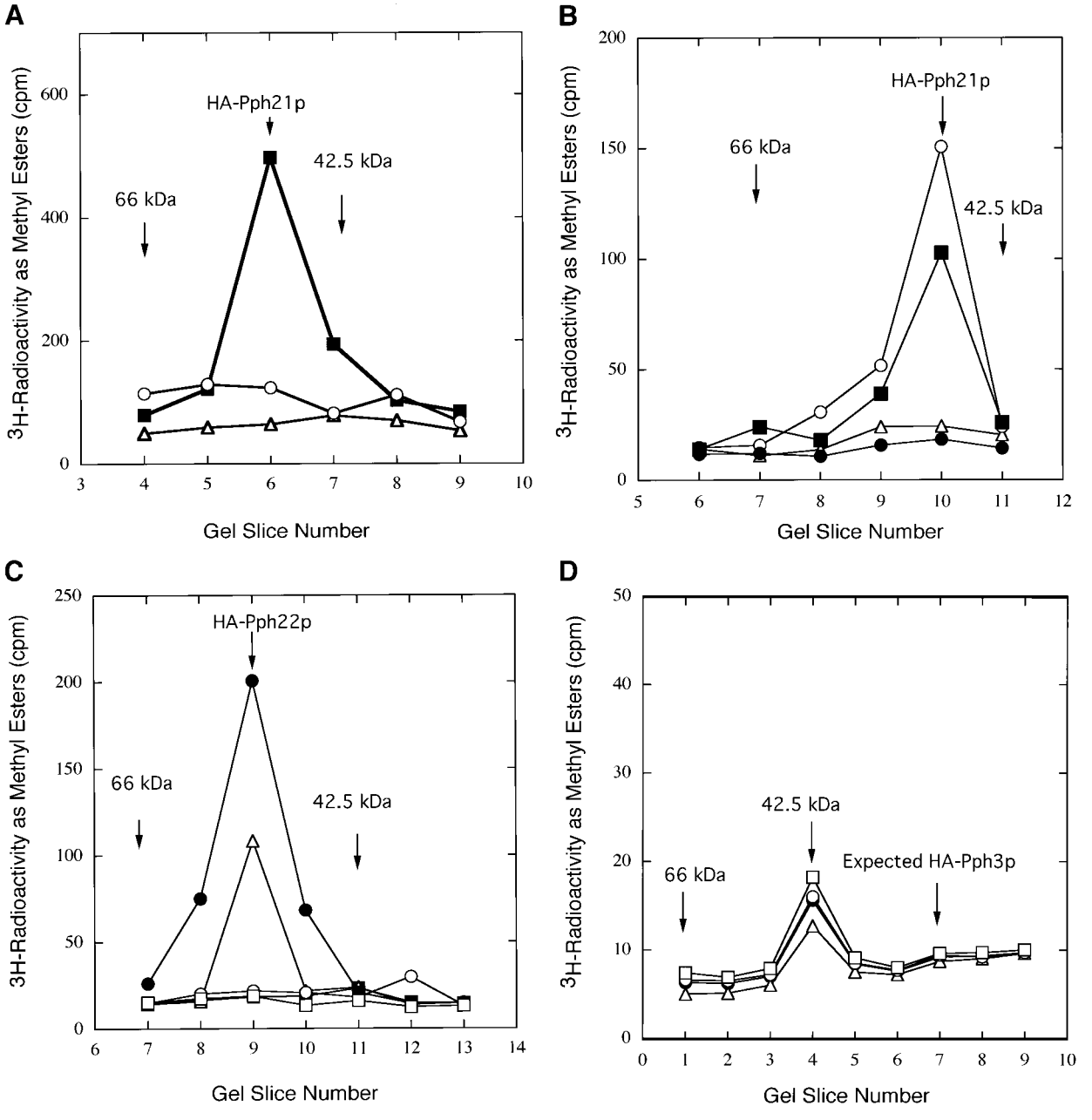

FIG. 4. Methylesterification of HA-tagged PP2Ac homologues in yeast. Yeast strains were grown in YPG unless otherwise indicated and were *in vivo* labeled with [^{3}H]AdoMet and extract prepared as described under Materials and Methods. PP2Ac polypeptides were immunoprecipitated, by incubating with monoclonol HA as described under Materials and Methods. Protein G/A agarose beads were used to pull down the antibody-tagged protein complex. The washed beads were then electrophoresed in 12.6% SDS-polyacrylamide gels as described under Materials and Methods. A portion of the dried gel was sliced into 3-mm fragments and 150 μl of 1.5 M Na_2CO_3 was added to cleave the [^{3}H]methyl ester bond for detection as [^{3}H]methanol. (A) Yeast strain PPH21HA (HKY101, parent) (■); *ppm1,* PPH21HA (HKY102) (△); PPH21HA (HKY101, parent) (grown in YPD) (○). (B) Yeast strain PPH21HA (HKY101, parent) (■); *ppm1,* PPH21HA (HKY102) (●); *ppm2,* PPH21HA (HKY103) (○); *ppm1ppm2,* PPH21-HA (HKY104) (△). (C) Yeast strain PPH22HA (HKY105, parent) (●); *ppm1,* PPH22HA (HKY106) (○); *ppm2,* PPH22HA (HKY107) (△); *ppm1ppm2,* PPH22HA (HKY108) (■); PPH22HA (HKY105, parent) (grown in YPD) (□). (D) PPH3HA (HKY109, parent) (○); *ppm2,* PPH3HA (HKY110) (△); *ppm1ppm2,* PPH3HA (HKY111) (●); PPH3HA (HKY109, parent) (grown in YPD) (□).

tracts from labeled intact cells, we showed the absence of ^{3}H-methylesterified PP2Ac in *ppm1* and *ppm1ppm2* double deletion mutant strains but not in the *ppm2* strain. In order to identify which of the three homologues of PP2Ac (Pph21p, Pph22, and Pph3p) in the budding yeast is methylated by *PPM1* gene product, all

three homologues were N-terminally tagged by HA epitopes, in *ppm1* and *ppm2*, and *ppm1ppm2* mutant genetic backgrounds as well as in wild-type background. It was determined that both Pph21p and Pph22p were methylated by Ppm1p whereas Pph3p did not show any detectable methylation in our assay. Although we did not detect Pph3p C-terminal methylation, it is possible that that Pph3p is only methylated in the absence of Pph21p or Pph22p , or that its methylation requires conditions which may have not met in our assays.

In the course of this work, indirect evidence for the role of Ppm1p in PP2Ac methylation has been presented (14, 21). Moreover, it was shown that methylation of PP2Ac in both yeast and mammalian cells is responsible for the interaction of the B regulatory subunit Cdc55p and B′ regulatory subunit Rts1p to the core dimer of the PP2A holoenzyme (14, 21). However, the fate of methylation of each homologue of PP2Ac was not addressed.

We have shown previously that the C-terminal methylation of PP2Ac in yeast has a half-life of approximately 20 min in intact cells, indicating that this methylation reaction is potentially subject to biological regulation by demethylation (15). Indeed, a protein carboxyl methylesterase has been identified as the enzyme cleaving the C-terminal methyl ester of PP2Ac in yeast and mammals (13, 14, 21). It would be of great interest to identify possible extracellular signaling events that can lead to regulation of C-terminal methylation of PP2Ac, either though changes in the activity of the *PPM1* methyltransferase or the esterase.

Although Ppm2p has a significant sequence similarity to Ppm1p (25% amino acid identity over 342 residues), we found no evidence to suggest that Ppm2p could methylate any of the PP2A catalytic subunits. We have used biochemical analyses to look for any methylesterfication defect in the *ppm2* mutant in other polypeptide or RNA species. However, the methyl ester patterns observed to date are identical in the *ppm2* strain and its isogenic *PPM2* parent. Further studies will be needed to identify the role of this potential methyltransferase.

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REFERENCES

1. Goldberg, Y. (1999) *Biochem. Pharmacol.* **57,** 321–328.
2. Virshup, D. M. (2000) *Curr. Opinion Cell Biol.* **12,** 180–185.
3. Janssens, V., and Goris, J. (2001) *Biochem. J.* **353,** 417–439.
4. Zhao, Y., Boguslawski, G., Zitomer, R. S., and DePaoli-Roach, A. A. (1997) *J. Biol. Chem.* **272,** 8256–8262.
5. Ronnwe, H., Carlberg, M., Hu, G. Z., and Nehlin, J. O. (1991) *Mol. Cell Biol.* **11,** 4876–4884.
6. Xie, H., and Clarke, S. (1993) *J. Biol. Chem.* **268,** 13364–13371.
7. Xie, H., and Clarke, S. (1994) *J. Biol. Chem.* **269,** 1981–1984.
8. Favre, B., Zolnierowicz, S., Turowski, P., and Hemmings, B. A. (1994) *J. Biol. Chem.* **269,** 16311–16317.
9. Evans, D. R. H., Myles, T., Hofsteenge, J., and Hemmings, B. A. (1999) *J. Biol. Chem.* **274,** 24038–24046.
10. Bryant, J. C., Westphal, R. S., and Wadzinski, B. E. (1999) *Biochem. J.* **339,** 241–246.
11. Niewmierzycka, A., and Clarke, S. (1999) *J. Biol. Chem.* **274,** 814–824.
12. Cai, H., Dumlao, D., Katz, J. E., and Clarke, S. (2001) *Biochemistry* **40,** in press.
13. De Baere, I., Derua, R., Janssens, V., Van Hoof, C., Waelkens, W., Merlevede, W., and Goris, J. (1999) *Biochemistry* **38,** 16539–16547.
14. Wu, J., Tolstykh, T., Lee, J., Boyd, K., Stock, J. B., and Broach, J. R. (2000) *EMBO J.* **19,** 5672–5681.
15. Zobel-Thropp, P., Yang, M. C., Machado, L., and Clarke, S. (2000) *J. Biol. Chem.* **275,** 37150–37158.
16. Ogris, E., Due, X., Nelson, K. C., Mak, E. K., Yu, X. X., Lane, W. S., and Pallas, D. C. (1999) *J. Biol. Chem.* **274,** 14382–14391.
17. Spencer, J. F. T., and Spencer, D. M. (1996) *in* Methods in Molecular Biology, Vol. 53, Yeast Protocols (Evans, I. H., Ed.), pp. 51–67, Humana Press, Totowa, NJ.
18. Longtine, M. S., McKenzie III, A., Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle J. R. (1998) *Yeast* **14,** 953–996.
19. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
20. Hrycyna, C. A., Yang, M. C., and Clarke, S. (1994) *Biochemistry* **33,** 9806–9812.
21. Wei, H., Ashby, D. G., Moreno, C. S., Orgis, E., Yeong, F. M., Corbett, A. H., and Pallas, D. C. (2001) *J. Biol. Chem.* **276,** 1570–1577.