Purification of Porcine Brain Protein Phosphatase 2A Leucine Carboxyl Methyltransferase and Cloning of the Human Homologue[†,‡]

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ABSTRACT: The carboxyl methyltransferase, which is claimed to exclusively methylate the carboxyl group of the C-terminal leucine residue of the catalytic subunit of protein phosphatase 2A (Leu^{309}), was purified from porcine brain. On the basis of tryptic peptides, the cDNA encoding the human homologue was cloned. The cDNA of this gene encodes for a protein of 334 amino acids with a calculated M_r of 38 305 and a predicted pI of 5.72. Database screening reveals the presence of this protein in diverse phyla. Sequence analysis shows that the novel methyltransferase is distinct from other known protein methyltransferases, sharing only sequence motifs supposedly involved in the binding of adenosylmethionine. The recombinant protein expressed in bacteria is soluble and the biophysical, catalytic, and immunological properties are indistinguishable from the native enzyme. The methylation of PP2A by the recombinant protein is restricted to Leu^{309} of $PP2A_C$. No direct effects on phosphatase activity changes were observed upon methylation of the dimeric or trimeric forms of PP2A.

PP2A[1] (protein phosphatase 2A) dephosphorylates a wide variety of substrates involved in many cellular processes (*1*−*3*). The modulation of activity and specificity of PP2A toward its various substrates is regulated by a variety of means. The PP2A holoenzyme is a dimeric or trimeric protein built up from a catalytic subunit ($PP2A_C$) (catalytic subunit of PP2A), a second constant regulatory subunit, and a third variable subunit. Three major classes of third subunits are currently described. Each of the three subunits of PP2A exists in at least two isoforms, thereby leading to a great variety of possible holoenzyme conformations, all with potential different substrate affinities and catalytic activities (*4*). $PP2A_C$ is subject to different types of posttranslational modifications. Tyrosine kinases (e.g., src) phosphorylate tyrosine at position 307 (*5*, *6*), while a threonine kinase that is stimulated by autophosphorylation, phosphorylates a yet unknown threonine residue of the catalytic subunit (*7*). Both types of phosphorylation inhibit PP2A phosphatase activity. In addition, the carboxyl group of the C-terminal leucine amino acid (Leu_{309}) is methylated by a specific methyltransferase (*8*−*10*) resulting in a moderate increase in phosphatase activity (*11*). This methylation is alkaline and ethanol labile in vitro (*11*) and is reversible in vivo due to the presence of a specific methylesterase (*12*, *13*).

Methylation of proteins is a rare event, irreversible when occurring at amino groups but reversible when carboxyl groups are involved (*14*). Several types of protein methylation were studied in more detail (*15*). In bacteria, methylation of glutamate is involved in chemotaxis (*16*). Methylation of isoprenylcysteine of G proteins modulates the interaction with other proteins and is, therefore, important in signal transduction (*17*). Methylation of iso-aspartyl is thought to be essential for the repair of damaged proteins (*18*). Arginine methylation is essential for nuclear export of heterogeneous nuclear ribonucleoproteins (*19*). All of these protein methyltransferases show little sequence similarity, although some conserved motifs involved in the binding of AdoMet (S-adenosyl-L-methionine) can be detected, with exception of isoprenylcysteine methyltransferase (*20*). Comparison of the crystal structures of AdoMet binding proteins shows that the structural motifs for AdoMet binding are conserved despite the large difference in primary sequences (*21*).

The methylation of $PP2A_C$ varies over the cell cycle. $PP2A_C$ is methylated during all stages of the cell cycle. A temporary decrease in methylation of $PP2A_C$ occurs at the G_0/G_1 boundary in the cytoplasm and at the G_1/S boundary in the nucleus (*22*). The mechanism of this cell cycle dependent regulation and the physiological consequences of this oscillating methylation are unknown. Zhu et al. (*23*) described a transient methylation of $PP2A_C$ in leukemia cells during S phase. In their experiments, a decrease of phos-

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[1] Abbreviations: AdoHCys, S-adenosyl-L-homocysteine; AdoMet, S-adenosyl-L-methionine; EST, expressed sequence tag; LCMT, leucine carboxyl methyltransferase; MTA, 5′-deoxy-5′-methyladenosine; PIMT, D-aspartyl, L-isoaspartyl protein carboxyl methyltransferase; PP2A, protein phosphatase 2A; $PP2A_C$, catalytic subunit of protein phosphatase 2A ; $PP2A_D$, dimeric form of protein phosphatase 2A ; $PP2A_{T55}$, trimeric form of protein phosphatase 2A with PR55 subunit; $PP2A_{T72}$, trimeric form of protein phosphatase 2A with PR72 subunit; PTPA, phosphotyrosyl phosphatase activator of PP2A.

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phatase activity was measured for the methylated state. Estimations indicate that a large proportion (>50%) of cellular PP2A is methylated (*11*).

Floer and Stock (*24*) described a moderate stimulation of the PP2A methylation in *Xenopus* oocytes extracts by cAMP, whereas Ca^{2+} and calmodulin had no influence. On the other hand, Kowluru et al. (*25*) noticed no influence of cAMP on methylation of $PP2A_C$ in pancreatic rat cells but reported the stimulation of methylation by Ca^{2+}. It was suggested that methylation could interfere with substrate recognition and affinity, catalytic activity, binding specificity to the variable third subunits, and/or intracellular localization of PP2A in the cell (*9*, *11*, *22*, *26*, *27*).

The methylation of PP2A occurs at the C-terminus of an extremely conserved amino acid sequence (T R R/K T P D Y F L), which contains also a target for tyrosine phosphorylation (*5*, *6*). Mutational analysis of this peptide sequence established the importance for binding of the PR55 regulatory subunit (*26*, *27*) but not middle t antigen (*26*). The YFL tripeptide is extremely well-conserved in all of the known $PP2A_C$-like sequences (PP4/PPX, PP6/PPV) in most diverse phyla. Recently, it was shown that PP4, localized in the nucleus, can be methylated as well (*28*).

Here, we present a modified procedure to purify leucine carboxyl methyltransferase from porcine brain and describe the cloning of the cDNA of the human homologue. The in vitro catalytic activity of the bacterially expressed recombinant human protein is indistinguishable from the native porcine enzyme. No activity changes were observed upon methylation of dimeric and trimeric PP2A.

EXPERIMENTAL PROCEDURES

Materials. $PP2A_D$ (dimeric form of protein phosphatase 2A) was purified from rabbit muscle according to ref *29*. Antipeptide antibodies against an internal peptide (AA169−182) and against the carboxyterminus (AA299−309) of $PP2A_C$ (*11*) were obtained from B. A. Hemmings (FMI, Basel). The latter were also newly prepared in our laboratory according to the published method, having very similar properties especially with respect to their methylation sensitivity (see Figure 3A). Recombinant PTPA was made and purified as described in ref *30*, and [^{32}P]-labeled phosphorylase and RCAM lysozyme as in ref *30*. DEAE Sephacel, Phenyl Sepharose, Sephadex G75, and Superdex 200 HR 10/30 were from Pharmacia, DEAE cellulose was from Whatman, and Poros 20HQ was from PerSeptive Biosystems. Sinefungin was a gift of Eli Lily Research Laboratories. AdoHcys (S-adenosyl-L-homocysteine), 5′-S-isobutyl-5′-deoxyadenosine, and AdoMet were from Sigma, scintillation liquid (Lumasafe) was from Lumac-LSC, and S-adenosyl-L-[methyl-^{3}H]methionine (15 Ci/mmol) was from NEN-Dupont. Enzymes for molecular biology were from GIBCO-BRL with the exception of PWO DNA polymerase (Roche). Protein A-TSK was from Affiland, and carboxypeptidase Y was from Roche.

Porcine PP2A LCMT (Leucine Carboxyl Methyltransferase) Purification. One kg fresh porcine brains, collected in a local slaughterhouse, were homogenized in 1 L buffer A (50 mM Tris/HCl, pH 8.0, 0.5 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.5 mM benzamidine, 250 mM sucrose). The homogenate was centrifuged for 20 min at 10000*g* and the supernatant adsorbed twice batchwise on 800 mL DEAE Sephacel in a funnel with sintered glass filter, equilibrated in buffer B (buffer A without sucrose and adjusted to pH 7.4). The DEAE Sephacel was washed five times with 200 mL buffer B and eluted 9 times with 200 mL buffer B containing 0.15 M NaCl. The eluates 3−9 were pooled, and solid ammonium sulfate was added to 70% saturation. After 30 min, the mixture was centrifuged for 30 min at 10000*g*, the pellet resuspended in buffer B and the volume adapted to obtain the same conductivity as 0.8 M ammonium sulfate in buffer B (∼120 mL total volume). This solution was loaded on a Phenyl Sepharose column (2.5 × 15 cm) (equilibrated in buffer B containing 0.8 M ammonium sulfate) and the column washed with 70 mL of the equilibration buffer. LCMT eluted with a descending ammonium sulfate gradient of 0.8−0 M and a concurrent glycerol gradient of 0−10% (v/v) in 600 mL of buffer B. The active fractions were pooled, avoiding contamination with PIMT (D-aspartyl, L-isoaspartyl protein carboxyl methyltransferase) and precipitated with 60% ammonium sulfate. The pellet was resuspended in 10 mL buffer B, dialyzed for 2 h against buffer B, and separated on a Sephadex G-75 column (2.5 × 90 cm), equilibrated in buffer B. LCMT activity eluted as a single peak ($M_r \approx 40\,000$) well-separated from the majority of higher M_r proteins. This fraction was loaded on a DEAE cellulose column (1.5 × 15 cm), equilibrated in buffer B, and developed with a 200 mL gradient from 0 to 0.25 M NaCl in buffer B. The active fractions (peak eluting at 80 mM NaCl) were pooled, concentrated by consecutive dialysis against 50% (w/v) poly(ethylene glycol) and 60% (v/v) glycerol in buffer B, loaded on a Poros ion exchange HPLC column equilibrated in buffer C (buffer B at pH 8.0), washed with 5 mL buffer C and developed with a 40 mL gradient form 0 to 0.5 M NaCl in buffer C.

Porcine PIMT Purification. The unbound protein fraction of the DEAE Sephacel chromatography was precipitated with 70% ammonium sulfate and subjected to Phenyl Sepharose and Sephadex G-75 chromatography as described for LCMT. In the Phenyl Sepharose chromatography, PIMT eluted at the end of the gradient and on the Sephadex G-75, PIMT eluted as one single peak with an estimated M_r of 25 000. The majority of contaminating proteins were separated from PIMT by ion exchange chromatography on a Poros column equilibrated in buffer B where PIMT does not bind. At this stage, more than 60% of the protein represents a doublet of 24−25 kDa, the M_r assigned to PIMT (*31*).

PP2A Methylation Assay. The PP2A specific methyltransferase was assayed by incubating 5 μL of methyltransferase solution with 5 μL PP2A substrate (30−150 ng $PP2A_D$) and 5 μL of 1 μM [^{3}H] AdoMet (5−10 000 cpm/pmol) for 15 min at 30 °C in 20 mM Tris, 0.5 mM DTT, pH 7.4. Subsequently, the reaction was quenched by spotting 12 μL of the reaction mixture on a 1.5 × 1.5 cm Whatman 3 MM filter paper and transferring it to 10% (w/v) TCA at 0 °C. The paper was washed twice with 5% TCA at room temperature, once with acetone, and the radioactivity was counted in 5 mL scintillation liquid. The incorporation of [^{3}H]methyl in $PP2A_C$ was determined by the gel slice methanol vapor phase diffusion assay on 1 mm slices (*9*) or by autoradiography with Hyperfilm-^{3}H (Amersham). The methylation assay by PIMT was performed in a similar way using lactoglobulin (1 mg/mL) as substrate.

Protein Sequencing. Protein bands of interest, collected from 10 identical lanes on a 1D-mini-SDS-PAGE gel, were concentrated by a stacking gel method (modified from ref *32* and described in ref *33*) and subsequently digested in situ with trypsin, essentially as described in ref *34*. The resulting peptide mixture was separated on a μRPC C2/C18 SC 2.1/10 column (SMART System, Pharmacia, Sweden), developed at a flow rate of 80 μL/min using a 83 min linear gradient from 0 to 70% CH_3CN in 0.1% TFA in H_2O. UV-detected (215 nm) peak fractions were analyzed by a 492 Procise (PE Applied Biosystems) amino acid sequencer operating in a pulsed liquid mode.

Cloning of Human LCMT. The peptide sequences determined by Edman degradation were used to screen the EST (expressed sequence tag) database with the BLAST program (*35*). A putative ORF could be constructed with overlapping EST sequences. Three EST clones (AA461616, AA075653, R20826), likely to contain the full length cDNA, were ordered from the HGMP center (Hinxton, England) and used as template for a PCR reaction with primers 2174 and 2175 encompassing the 5′ and 3′ part of the ORF, respectively (2174, ccgggg*aagctt*g*catatg*gccactaggcagagggaatc, *Hin*d III and *Nde* I restriction sites in italics; 2175, ccgggggg*atc-cgggccc*ttaataagttatctccttcag, *Apa* I and *Bam*H I restriction sites in italics). PCR was performed for 30 cycles (1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C) with 1 Unit PWO DNA polymerase.

The 1 kb PCR fragment from generated EST clone AA461616 was digested with *Nde* I and *Bam* HI and cloned in a bacterial expression vector under the control of an IPTG-inducible T7 promoter, pet3C (Novagen), resulting in plasmid pJG145.

Bacterial Expression and Purification of Recombinant LCMT. The bacterial strain BL21(DE3) pLysS transformed with pJG145 was grown at 37 °C to $OD_{600} = 0.7$ and after addition of IPTG to a final concentration of 0.5 mM incubated for an additional 3 h at 30 °C with vigorous shaking. The bacteria were collected by centrifugation and stored at −80 °C. The thawed bacteria were lysed by sonication in 50 mM Tris, pH 8.0, 50 mM NaCl, 10 mM DTT, and 0.1 mM PMSF and centrifuged for 20 min at 50000*g*. Following the addition of ammonium sulfate (0.4 M final concentration), the proteins were loaded on a Phenyl Sepharose column equilibrated with 0.4 M ammonium sulfate in buffer B. Proteins were eluted with a descending ammonium sulfate gradient from 400 to 0 mM and a concurrent glycerol gradient of 0−20% (v/v) glycerol in 600 mL buffer B.

LCMT containing fractions were pooled, concentrated by 60% ammonium sulfate precipitation, and dialyzed against buffer B. This solution was loaded on a DEAE cellulose column (2.5 × 10 cm) equilibrated in buffer B. The column was eluted with a 400 mL gradient from 0 to 0.4 M NaCl in buffer B. The methyltransferase eluted at 0.15 M NaCl and was concentrated by dialysis against 50% (w/v) poly(ethylene glycol) followed by dialysis against 60% (v/v) glycerol. At this stage, the protein was essentially pure. Minor contaminants could be removed by gel filtration (Superdex 200 for analytical or Sephadex G-75 for preparative purposes).

Generation of LCMT Antibodies. Antibodies against LCMT were generated in a standard protocol by several injections of 200 μg purified full length recombinant human protein in rabbits (*36*). In this study, the serum after the fourth boost was used as such.

Table 1: Purification of LCMT from 1 kg of Porcine Brain

	volume (mL)	total units (pmol/min)	total protein (mg)	specific activity (pmol/min/mg)
extract[a]	1200	18.0×10^3	30 000	0.6
0.15 M DEAE[a,b]	160	12.3×10^3	2000	6.2
Phenyl Sepharose[b]	50	7.6×10^3	230	33.0
Sephadex G-75	94	2.7×10^3	33	81.5
DEAE-celllose	7.7	1.0×10^3	3	347
Poros	3	120	0.27	444[c]
LCMT		120	0.026	4600[c]

[a] In the extract and 0.15 M eluate of the DEAE Sephacel, methylation of endogenous proteins was subtracted. [b] This was the activity found in the pooled fractions after precipitation with ammoniumsulfate. [c] This was the specific activity found in the pooled fraction (6−11, see Figure 1B). The specific activity of the pure LCMT is estimated from the quantity of the 38 kDa band found in the Coomassie stained gel and proven to be LCMT (see text).

Phosphatase Assays. Phosphorylase *a* phosphatase activity of PP2A was measured plus and minus 33 μg/mL protamine, 16 mM ammonium sulfate as described (*37*), tyrosyl phosphatase activity of PP2A was measured after 10 min activation with a saturating amount of PTPA in the presence of 5 mM $MgCl_2$ and 1 mM ATP as described (*38*).

Sequence Analysis. Database screening was mainly performed with the BLAST algorithm (*35*) at NCBI (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). Other LCMT-like sequences were retrieved by screening the ongoing sequence projects of puffer fish (*Fugu rubriceps*) (http://fugu.hgmp.mrc.ac.uk) and malaria parasite (*Plasmodium falciparum*) (http://www.ebi.ac.uk/parasites). Multiple alignments were made with the MULTALIN program (*39*) (http://www.toulouse.inra.fr) and manually adjusted.

RESULTS

Assay of LCMT. We developed a simplified assay to specifically measure LCMT. In this assay, the incorporation of [^{3}H] is linear in function of time and increasing LCMT concentration. The purity of $PP2A_D$ is essential in this assay, since crude PP2A preparations contain an excellent substrate for PIMT, present during the initial stages of LCMT purification. We double-checked that indeed $PP2A_C$ is methylated in this assay. The incorporation of [^{3}H] methyl was determined by the gel slice methanol vapor phase diffusion assay (*9*), and radioactivity was found exclusively in the fraction containing the Coomassie stained $PP2A_C$. Alternatively, after SDS-PAGE, the proteins were blotted on a PVDF membrane, $PP2A_C$ immunolocalized by polyclonal anti-$PP2A_C$ antibodies, and the membrane exposed to a Hyperfilm-^{3}H (Amersham) to localize the radioactivity. Both the immunoreactivity and the radioactivity matched.

Purification of PP2A-Leucine Carboxyl Methyltransferase. A novel procedure was developed to purify LCMT as outlined in the methods section and summarized in Table 1. The batchwise adsorption−desorption on DEAE Sephacel (see Methods) prevents binding of the aspecific Protein Methylase I on the resin (refs *31* and *40* and our observations), while LCMT can be eluted with 0.15 M NaCl. After precipitation and concentration by ammonium sulfate, LCMT could be separated completely from Protein Methylase II and

endogenous PP2A by the Phenyl Sepharose purification step (see Figure 1). Three additional purification steps (see Table 1) (gel filtration on Sephadex G-75, anion exchange on DEAE-cellulose, and FPLC anion exchange on Poros) increased the specific activity a further 13-fold, but were accompanied with substantial losses of activity. These losses of activity of the more purified protein prohibited purification to homogeneity. At this stage, it was decided to follow an alternative procedure to identify LCMT.

Analytical gel filtration chromatography on Superdex 200 of the peak activity fractions during purification revealed persistent copurification of transferase activity with protein bands of 40, 38, 36, 28, and 25 kDa. Proteins of 25 and 38 kDa comigrate with the activity on the final ion exchange chromatography in our purification scheme (Figure 1). All these proteins were identified by sequencing of internal tryptic peptides. The 25, 28, and 36 kDa proteins were identified as aldose reductase, ubiquitin conjugating enzyme, and the porcine homologue of a protein related to the non catalytic subunit of glucose-6-phosphate dehydrogenase. Two tryptic peptides of the 40 kDa protein were sequenced but did not show convincing resemblance to any known sequence. From the 38 kDa protein that comigrates with the methyltransferase activity, two tryptic peptides (R/KYFEIDFPMINT and R/KYAIXGADL) were sequenced.

Both sequences were found in the same human EST sequence that, together with several overlapping EST sequences, makes a putative ORF. The location of the tryptic peptides is shown in Figure 2. No biological function was assigned to this protein sequence. The length of the ORF (334 amino acids) and the presence of motifs for AdoMet binding (see discussion) made this protein a likely candidate for human LCMT.

Three EST clones judged to contain the complete cDNA of LCMT (EST clones AA075653, R20826, AA461616) were used for generating the complete open reading frame by PCR with primers encompassing the 5′ and 3′ end of the gene. Two clones contained the DNA fragment of the desired length (R20826, AA461616). Finally, EST clone AA461616 was sequenced for confirmation. The ORF of the human LCMT cDNA clone encodes a protein of 334 amino acids with a calculated M_r of 38 305 and a pI of 5.72. Screening of databases reveals the presence of highly similar proteins in many of the important model organisms currently studied. On the basis of its high sequence similarity in the methyltransferase domain (33% identical), a putative functional homologue of LCMT with a C-terminal extension of 350 amino acids was found in fungi and vertebrates. All sequence data are compiled in Figure 2.

Bacterial Expression, Purification, and Characterization of the Recombinant LCMT. Following the procedure described in the experimental procedures, the recombinant LCMT was essentially pure, with a yield of about 0.4 mg protein/L bacterial culture. On SDS-PAGE, the recombinant LCMT migrates as a 38 kDa protein at the same position as the protein purified from porcine brain. The recombinant protein can incorporate 2.3 nmol [^{3}H]methyl/min/mg in our standard assay conditions (4, 0.7, 2.3 nmol/min/mg for three different and independent preparations), which is in the same range or slightly lower than the native porcine brain methyltransferase (5.8 nmol/min/mg). The specific activities found in three different preparations of native LCMT were

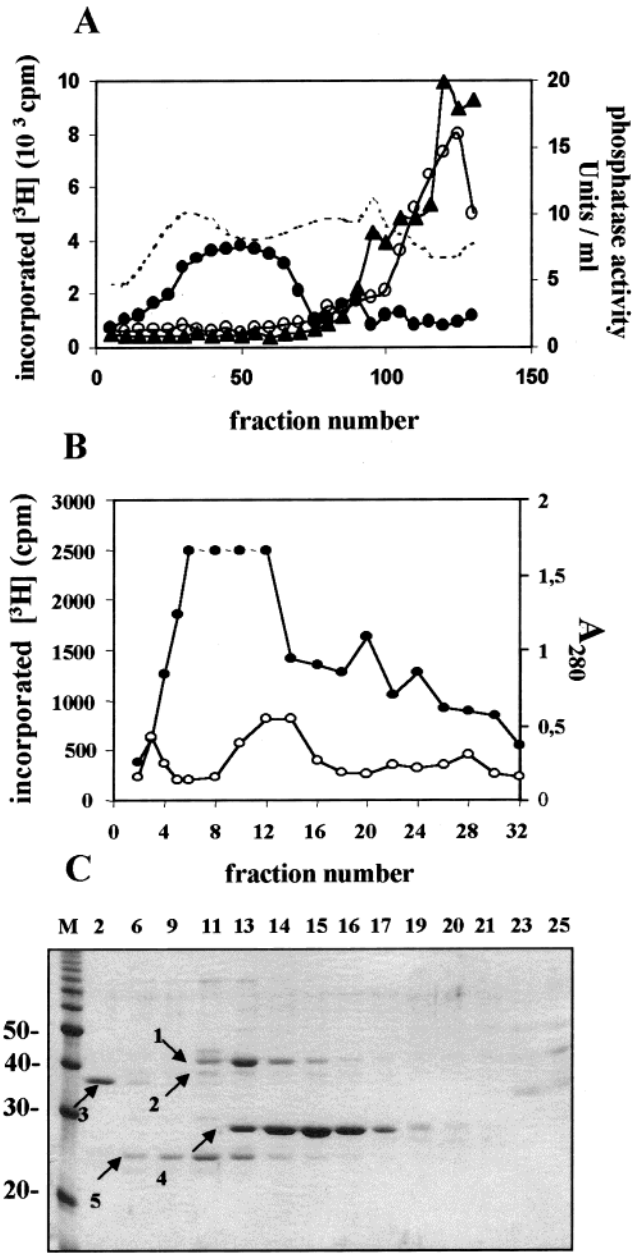

FIGURE 1: Purification of LCMTA. Separation of PIMT and LCMT by hydrophobic interaction chromatography. During the purification of porcine brain LCMT, a phenyl Sepharose column was performed as described in the methods section. Every fifth fraction LCMT (●) and PIMT (○) was measured in the standard assay with, respectively, $PP2A_D$ and lactoglobulin as substrates. Phosphorylase *a* phosphatase activity was measured with (▲) and without protamine (not shown) as described in the methods section. A_{280} is indicated with a dashed line (maximal value: 1.1) and the gradient (0.8−0 M descending ammonium sulfate and a concurrent 0−10% glycerol gradient) is shown with a dotted line. At the end of the gradient (fraction 115), the column was further washed with 20% glycerol. B: Ion exchange chromatography (Poros) of the final porcine methyltransferase purification step. Methyltransferase activity is expressed as cpm [^{3}H] methyl incorporated in purified dimeric PP2A. ○, Absorption at 280 nm; ●, methyltransferase activity. C: SDS-PAGE analysis of Poros column fractions. The numbering of the lanes corresponds to column fractions. Protein lanes indicated by arrows are identified by protein sequencing: 1, unknown M_r 40 000 protein; 2, leucine carboxyl methyltransferase; 3 homologue of glucose-6-phosphate dehydrogenase subunit; 4, ubiquitin conjugating enzyme; 5, aldose reductase.

5.8, 4.6, and 7 nmol/min/mg. Apparent K_m values of 0.1 μM and 1.3 μM for the recombinant protein and 0.16 μM and

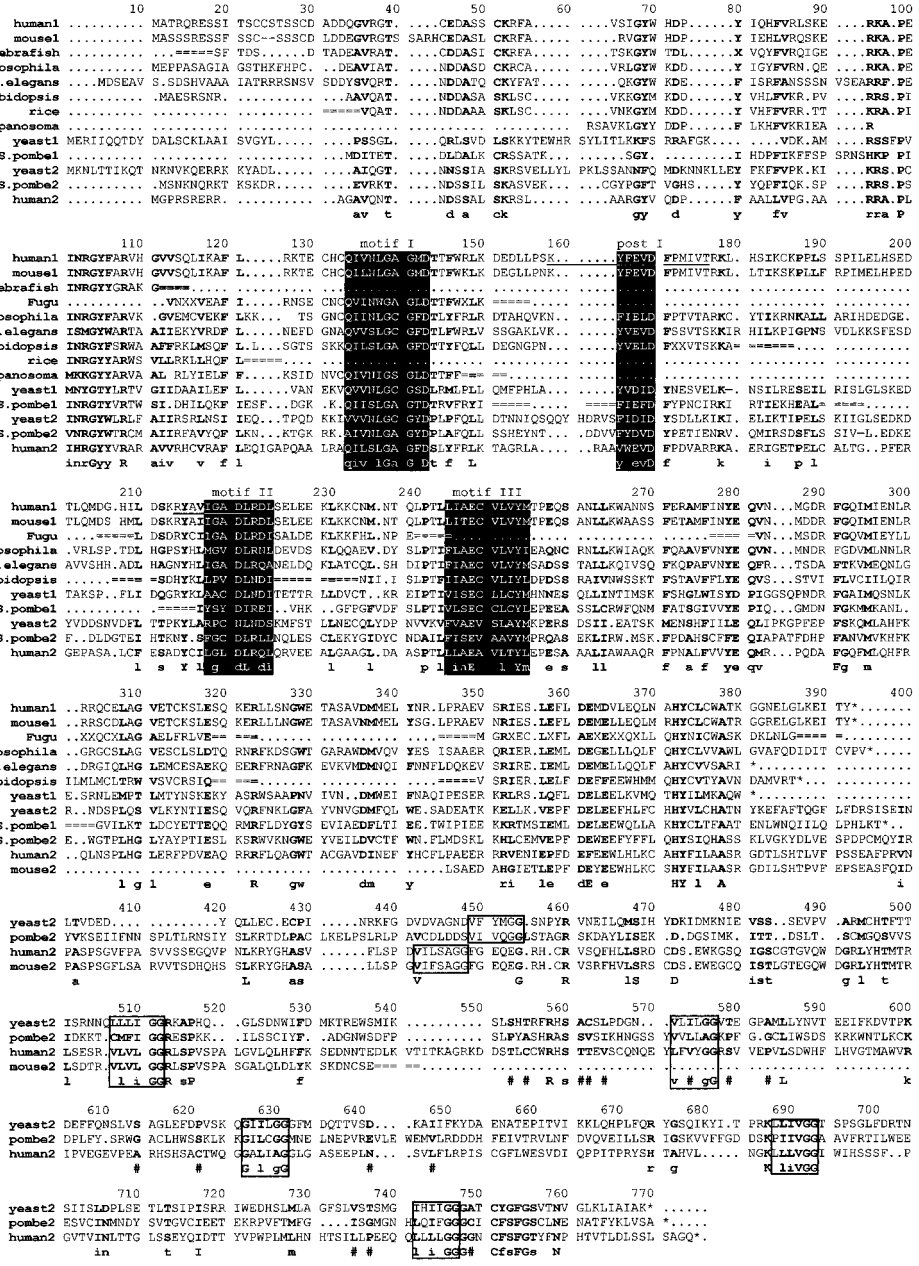

FIGURE 2: Sequence alignment of leucine carboxyl methyltransferases. Human1, (LCMT) translated sequence of leucine carboxyl methyltransferase (gb: AF037601); Human2, translated sequence of cDNA KIAA0547 (gb: AB011119); Mouse1, translated sequence of EST clones (gb: W15851, AA118474, AA154029, AA896126, AA921127); Mouse2, translated sequence of EST clone (gb: AA002765); *Arabidopsis*, translated sequence of nucleotides 40423−41613 of BAC T7I23 (gb: U89959); *Drosophila*, translated sequence of nucleotides 19054−20196 of P1 clone DS09217 (D109) (gb: AC003700); *C. elegans*, hypothetical 37.8 kd protein b0285.4 in chromosome III (sp: P46554); *Fugu*, translated sequence of cDNA clones O12K02aC6 (325−215), O12K02aB10 (108−200), O55M11aB10 (86−166), 012K02aA9; Yeast1, hypothetical protein YDR435c of bakers' yeast (pir: S69715); Yeast2, hypothetical protein YOL141w of bakers' yeast (pir: S61873); *S. pombe*1, hypothetical protein of fission yeast (gb: AL032634); *S. pombe*2, hypothetical protein of fission yeast (gb: AL023859); Zebrafish, translated sequence of EST clone fa07a05.r1. (gb: AA542480); Rice, translated sequence of clone EST clone RICR2964A (gb: D25036); *Trypanosoma*, translated sequence of genomic clone 0448m (embl: T09891, T09892). Putative motifs for AdoMet binding are shaded, conserved sequences in the C-terminal extension are boxed. Partial sequences are preceded and/or followed by (====, stop codons) of full length, proteins are indicated by an asterisk, and conserved amino acids are represented in bold. Absolute conserved residues are listed below the sequence. Amino acids belonging to one of the following groups, occurring more than 80% at a given position are marked by a lower case symbol or by # (1, ACGPST; 2, ILMV; 3, HKR; 4, DENQ; 5, FWY). Corresponding amino acid sequences of experimentally determined porcine tryptic peptides are underlined.

1.25 μM for the native protein were found by varying the $PP2A_D$ and AdoMet concentrations, respectively, very similar as described for bovine brain LCMT (*41*). The specificity of the recombinant protein, exclusively methylating the carboxyterminal Leu^{309}, was shown in several ways.

After methylation of $PP2A_D$ by recombinant LCMT, $PP2A_C$ shows less recognition toward the methylation sensitive $Ab^{299/309}$, which preferentially binds to unmethylated $PP2A_C$ (Figure 3A). Alkali treatment fully demethylates $PP2A_C$ and restores Ab recognition. Notice that the control of unmethylated $PP2A_D$ is hardly affected by alkali treatment, indicating that $PP2A_D$, as isolated, is unmethylated.

Separation on a "SMART" HPLC column of tryptic peptides from maximally [^{3}H]methylated $PP2A_D$ resulted in

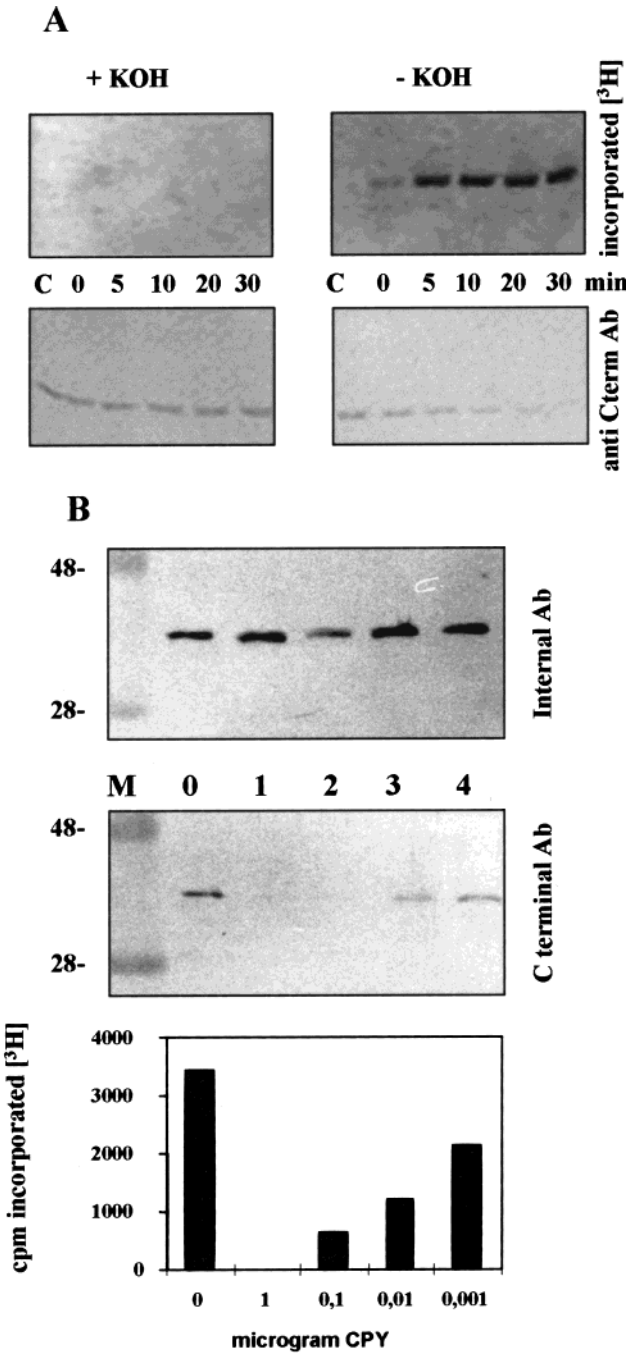

FIGURE 3: Specificity of LCMT. A: Recombinant LCMT methylation of PP2Ac affects binding of the methylation sensitive $Ab^{299/309}$. Right pannels: Incubation (0−30 min) of $PP2A_D$ with [^{3}H]AdoMet and recombinant LCMT increases [^{3}H] incorporation (upper panel) and decreases recognition of $PP2A_C$ with $Ab^{299/309}$ (dilution 1/15 000) (lower panel). Left panels: Alkali treatment after methylation (incubation of the blot at room temperature during 30 min in 0.2M KOH) with recombinant LCMT removes the alkali labile methyl group and restores antibody recognition. B: Methylation by recombinant LCMT occurs at the carboxyterminal Leu^{309}. Dimeric PP2A was methylated by recombinant LCMT with [^{3}H]-AdoMet and was treated with different amounts of carboxypeptidase Y (0, no CPY; 1, 1 μg; 2, 0.1 μg; 3, 0.01 μg; 4, 0.001 μg). The digested protein was (1) run on SDS-PAGE, blotted, and developed with an antibody against an internal peptide of $PP2A_c$ (top panel), (2) run on SDS-PAGE, blotted, and developed with an antibody against a carboxyterminal peptide of $PP2A_c$ (middle panel), and (3) precipitated with TCA and the incorporated [^{3}H] measured by scintillation counting (bottom panel).

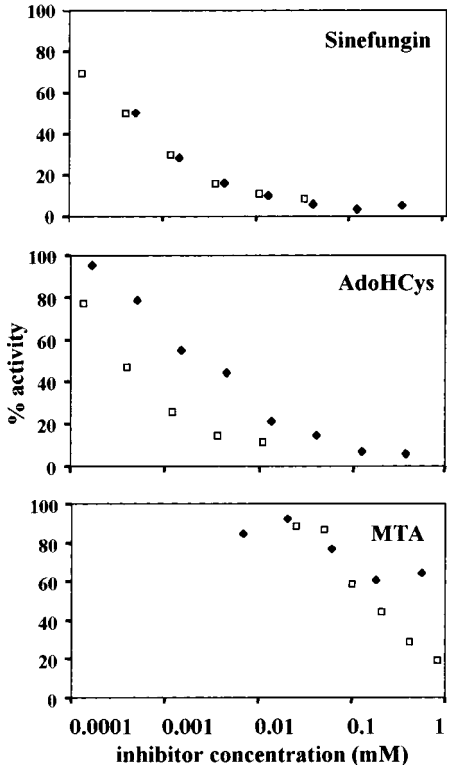

FIGURE 4: Sensitivity of LCMT and PIMT toward methyltransferase inhibitors. Highly purified porcine brain LCMT (◆) and porcine PIMT (□) were incubated in the presence of different inhibitors. The activity is expressed as the percentage of activity in the absence of inhibitor (incorporating, respectively, 1 and 4 pmol/min/mL assay). The data represent typical results obtained in at least three independent experiments.

the recovery of only one radiolabeled peptide. Sequencing identified it as the carboxyterminal peptide of $PP2A_C$ ($_{304}$TPDY...). [^{3}H]Methylated $PP2A_D$ was treated with carboxypeptidase Y; because of its specificity, the hydrolysis of amino acids is impeded when acidic amino acids occur at the penultimate position of a peptide. Thus, in the carboxyterminus of $PP2A_C$ (−RRTPDYFL) only Phe and Leu are released. Figure 3B shows the loss of binding of the Ab against the carboxyterminus, while no gross change in mobility of $PP2A_C$ occurs, confirming the restricted hydrolysis by carboxypeptidase Y. Proteolysis does remove all incorporated [^{3}H], indicating that apart from the carboxyterminus no methylation occurs and that the only site for alkali-labile O-methylation resides at the COOH group of Leu^{309}.

To distinguish LCMT from other methyltransferases, we determined the inhibition characteristics of some methyltransferase inhibitors (Figure 4) and obtained IC_{50} values for highly purified porcine brain LCMT (after Poros FPLC) of 0.5 μM for sinefungin and 3 μM for AdoHCys. MTA (5′-deoxy-5′-methyladenosine) was only slightly inhibitory in the higher concentration range, whereas no inhibition was found by isobutyl deoxyadenosine in concentrations up to 1 mM (data not shown). For PIMT, the IC_{50} values are 0.5 μM for sinefungin, 0.5 μM for AdoHCys, and 0.2 mM for MTA. Isobutyl deoxyadenosine was not inhibitory for PIMT. Moreover, 10 nM okadaic acid completely inhibited the methylation reaction of LCMT as described (*41*), whereas 10 μM okadaic acid was not inhibitory for PIMT. Very similar inhibitory data were obtained with the different

inhibitors for the purified recombinant human LCMT (data not shown).

Immunological Characterization. Rabbit antibodies were produced against the recombinant human LCMT (see method section). In Western blots, these antibodies recognized the 38 kDa protein, identified as LCMT at different stages during the purification of the native porcine as well as the recombinant human protein (data not shown). A 1:2000 dilution of the antiserum could detect less than 2 ng recombinant LCMT on a Western blot. During immune precipitations, LCMT activity measurements in supernatant and pellet revealed that no LCMT activity remained in the supernatant and that the antibodies inhibited LCMT in the pellet since no LCMT activity could be detected in the immunoprecipitate.

Effect of C-Terminal Leucine Methylation on the Protein Phosphatase Activity of PP2A. To test the effect of C-terminal methylation on the phosphatase activity of PP2A, the dimeric form of PP2A was highly methylated (0.8−0.9 mol/mol) by recombinant LCMT and the phosphatase activity was compared with controls treated in an identical way in a parallel experiment with omission of the methyltransferase and/or the AdoMet substrate. Two types of experiments were performed: PP2A was maximally methylated during 60 min at 30 °C and methylation stoichiometry was monitored by sampling as described for the methylation assay in the method section. The phosphatase was subsequently diluted appropriately (20−100-fold) in 10 mM Tris, 0.5 mM DTT, and 1 mg/mL BSA in order to measure the activity in linear conditions in a plus and minus protamine sulfate assay as described (*37*), using phosphorylase *a* as substrate. Alternatively, the phosphatase was re-isolated after methylation by Superdex 200 gel filtration. In none of the conditions, the effect of methylation observed on the phosphatase activity was larger than the stabilizing effect (10−20%) introduced by the protein concentration of LCMT, independent of the presence of AdoMet. This stabilizing effect could be mimicked by BSA. The optimal concentration for protamine stimulation nor the degree of stimulation was affected either. In addition, we also did not find an effect on the activation of the phosphotyrosyl phosphatase activity of $PP2A_D$ by PTPA. By using the methylation sensitive $Ab^{299/309}$, in combination with a plus and minus KOH treatment, we found that only the dimeric form of PP2A as isolated from tissues was always fully demethylated, whereas the $PP2A_{T72}$ was always fully methylated and the degree of methylation of $PP2A_{T55}$ varied from preparation to preparation. As expected, these different levels of methylation correlated with the possibility (or absence of possibility) to further methylate the different preparations with LCMT. So far, no correlation could be found between the degree of methylation and the specific activity (basal or polycation stimulated) or the specific properties of these oligomers (such as a characteristic optimal concentration for polycation stimulation or the possibility to be stimulated by PTPA), further substantiating the idea that methylation has no direct effect on the activity of PP2A.

DISCUSSION

Following the procedure (*8*) for the purification of the methyltransferase that specifically methylates the catalytic subunit of PP2A, a major Coomassie-stained protein with an apparent molecular mass of 40 kDa was detected as predicted. However, it was clear that in several purification steps the activity of the methyltransferase did not copurify with this protein (data not shown).

With our modified purification procedure, we identified in porcine brain a protein of 38 kDa that specifically methylates PP2Ac at its carboxyterminal leucine. On the basis of internal peptide sequences, we found human EST clones containing an open reading frame coding for a protein of 38 305 with a potential AdoMet binding motif.

The recombinant LCMT can be expressed in bacteria as a soluble and active protein. This recombinant protein cannot be distinguished from the tissue-purified protein in terms of chromatographic behavior; they both have very similar K_m values for the substrates AdoMet and PP2A as well as comparable specific activities. Both are equally sensitive to the inhibitors tested and are equally recognized by the antibodies, raised against the recombinant protein. Moreover, antibodies made against the bacterially expressed 38 kDa LCMT could immunoprecipitate a 38 kDa protein from tissue purified protein.

LCMT is clearly distinct from PIMT since both enzymes can be completely separated during purification (see Figure 1) and have a different, nonoverlapping substrate specificity. Although both enzymes are equally well-inhibited by an inhibitor such as sinefungin, other inhibitors such as MTA (Figure 4) and okadaic acid can differentiate among them.

Apart from the AdoMet binding motif, LCMT shows no obvious sequence similarity to any of the other known protein methyltransferases or any other type of AdoMet binding proteins. This is not surprising, considering the great sequence diversity within protein methyltransferases. Sequence alignment of LCMT members shows highly conserved amino acid regions (Figure 2). Kagan and Clarke (*20*) discussed the presence of three conserved motifs responsible for AdoMet binding. Motif I: [VIL]-[LV]-[DE]-[VIG]-[GC]-G-[PT]-G. Motif "post I": h-h-x-h-[DE] (where h stands for hydrophobic). Motif II: [PG]-[QT]-[FYA]-D-A-[IVY]-[FI]-[CVL]. Motif III: L-L-[RK]-P-G-G-[RIL]-[LI]-[LIVF]-[IL]. The spacing between these motifs is restricted. In our alignment (Figure 2), the best agreement is found within motif I and the well positioned "post I" motif, while the other conserved regions coincide fairly well with motifs II and III. Through database screening, sequences were found with high similarity to LCMT (31% identity, 45% similarity in the methyltransferase region) but having an additional C-terminal extension of 350 amino acids (LCMTII in Figure 2). The putative AdoMet binding motifs and other conserved regions of LCMT are also present in the N-terminal part of these sequences and are, therefore, considered to represent a homologue of LCMT. The carboxyterminal extension has an internal repeated sequence, with as hallmark the occurrence of a 3−4 apolar residues followed by two glycines (see Figure 2). These repeats show significant sequence similarity with members of the kelch domain family. The kelch domain has been shown to have actin binding capacities (*42*). Therefore, LCMTII might be directed to this cellular location in order to perform its specific function.

We could not find an activity change induced by methylation of $PP2A_D$. This is at variance with other publications that, depending from cell lines used and experimental

conditions, mention a 25−50% increase of PP2A activity (*11*, *25*), or a decrease in phosphatase activity (*23*). Our maximal methylation of the purified $PP2A_D$ with the recombinant LCMT reached high levels (0.8−0.9 mol/mol); therefore, insufficient methylation cannot be the reason for not observing an effect of methylation in our experiments. As a provisional hypothesis, we can say that methylation probably does not affect phosphatase activity directly but possibly as a consequence of secondary events such as association with other subunits or proteins.

The C-terminal amino acid sequence of $PP2A_C$ (T R/K R T P DY F L) is extremely well-conserved in all species and shown to be target for phosphorylation (*5*, *6*). It is tempting to suggest that the interplay of phosphorylation (*5*, *6*) and leucine carboxyl methylation would modify the activity and subunit composition of the PP2A holoenzyme. Ogris et al. (*26*) mutated Thr^{304} and Tyr^{307} into amino acids, mimicking a constitutively phosphorylated and nonphosphorylated conformation. They noticed indeed an influence on PR55 third subunit binding. However, mutation into other residues also affected the PR55 binding. We suggest that methylation, by changing the size and charge of the carboxyterminal part might also influence subunit binding.

LCMT was claimed to be an enzyme with $PP2A_C$ as the unique substrate (*8*). This is consistent with the observation that in sequenced microbial genomes without $PP2A_C$-like phosphatase, LCMT is not encountered. However, there are also arguments in favor for a less stringent substrate specificity of LCMT. Recently, Kloeker et al. (*28*) showed evidence for the methylation of PP4 (PPX). Methylation of PP6 (PPV), having PYFL at its carboxyterminus, has not been shown yet. There is also evidence for the methylation of different phosphatases of the PP2A type in *Saccharomyces* (*43*).

The mechanism of methylation of PP2A remains unknown in terms of recognition of the substrate. The presence of a third subunit is not essential for methylation, since $PP2A_D$ as well as $PP2A_C$ can be methylated (ref *42* and our observations). The C-terminal 8 or 17 amino acids of $PP2A_C$ as synthetic peptides are not sufficient for methylation (ref *10* and our unpublished results), indicating that substrate recognition probably occurs via other conserved structural parts of $PP2A_C$. For the bacterial methyltransferase CheR, it has been shown that the binding site on the substrate is distinct from the catalytic site (*44*).

Because methylation of PP2A occurs at specific stages of the cell cycle (*22*), LCMT is likely to be regulated in a cell cycle dependent manner. A possible mean of regulation could be phosphorylation. Several tyrosine and threonine residues are conserved within different LCMT sequences, but none of them occurs in an obvious consensus sequence for the major cell cycle regulating protein kinases. Alternatively, the methylation state of PP2A could be influenced by an interplay between carboxyl methyltransferase and carboxyl methylesterase. During the preparation of this manuscript the cloning of PP2A methylesterase as an enzyme that binds to inactive forms of PP2Ac, was published (*45*). By this, all key players of the reversible PP2A methylation are available.

In this paper, we described the cloning of LCMT and showed the specificity of the bacterially expressed enzyme for the C-terminal leucine of $PP2A_C$. It lays a structural basis for further analysis of the function of the specific methylation of PP2A. A more detailed understanding of this specialized modification can generate new insights in the complex regulation of PP2A, an enzyme involved in a myriad of cellular processes.

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