Protein Phosphatase 2A Is Reversibly Modified by Methyl Esterification at its C-terminal Leucine Residue in Bovine Brain*

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We have recently described a novel protein carboxyl methylation system that results in the reversible modification of a 36-kDa polypeptide component of a 178-kDa protein in the cytosol of a variety of eucaryotic cells. This reaction, catalyzed by a cytosolic 40-kDa methyltransferase, results in the methylesterification of the α-carboxyl group of the C-terminal leucine residue. We have now purified the major methylated 36-kDa polypeptide from bovine brain. N-terminal sequence analysis of a tryptic fragment of this polypeptide revealed identity to the catalytic subunit of protein phosphatase 2A. This enzyme exists in the cell predominantly as a trimeric 151-kDa native species containing the 36-kDa catalytic polypeptide that terminates in a leucine residue. We then fractionated bovine brain cytosolic extracts to separate the major phosphatase isoforms 2A1 and 2A2 and found that both could be methylated by a partially purified preparation of the methyltransferase. A synthetic C-terminal octapeptide based on the sequence of the 36-kDa catalytic subunit is neither a substrate nor an inhibitor of this methyltransferase, suggesting that this enzyme recognizes aspects of the tertiary and/or quaternary structure of the native phosphatase. Because this modification reaction is readily reversible in extracts, it may represent a novel strategy of the cell to modulate the function of this protein phosphatase.

The reversible modification of protein function by phosphorylation and dephosphorylation reactions has now been clearly established as a major component of both metabolic regulation and signal transduction pathways. While much effort has been focused on the characterization of the large number of protein kinases, especially with regard to their activation by ligands and second messengers (Hunter, 1987; Blackshear et al., 1988; Ferrigno et al., 1993). Most recently, a cytosolic methylation system has been described in eucaryotic cells that results in the modification of one or more 36-kDa polypeptides in protein(s) of native molecular mass of about 178 kDa (Xie and Clarke, 1993). Here, the methylation reaction results in the formation of a methyl ester on the α-carboxy group of a C-terminal leucine residue (Xie and Clarke, 1993).

We have focused our efforts on understanding the role of this latter methylation reaction. The methylation of cytosolic 36-kDa polypeptides has been observed previously in a variety of human, mouse, and rat tissues (O'Connor and Clarke, 1984; Siegel and Wright, 1985; Chelsky et al., 1985; Ladino and O'Connor, 1990; Volker et al., 1991). In this study, we were interested in identifying the nature of the 178-kDa methyl-accepting species. We have now purified the methylated component from bovine brain and present evidence that the 36-kDa methylated polypeptide is the catalytic subunit of protein phosphatase 2A. This enzyme has a broad substrate specificity for phosphoproteins that are involved in carbohydrate, amino acid, and lipid metabolism as well as in cell cycle control (Cohen, 1989; Ferrigno et al., 1993). The reversible methylation of this enzyme may play a role in coupling its activity to physiological signals in the cell.

EXPERIMENTAL PROCEDURES

Materials—Fresh bovine brain was purchased from the Shamrock Meat Co. (Los Angeles, CA). Rabbit muscle phosphorylase b (twice crystallized; 27 units/mg of protein) and rabbit muscle phosphorylase kinase (170 units/mg of protein) were obtained from Sigma. [γ-32P]ATP (10 Ci/mmol) was purchased from DuPont-New England Nuclear. The peptide RRTD-DYFL was synthesized by Dr. Joe Reeves at the UCLA Peptide Synthesis Facility.

Amino Acid Sequence Analysis—Polypeptides from SDS-PAGE were transferred to a polyvinylidene difluoride membrane by electrobolitig.

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1 The abbreviations used are: [γ-32P]ATP, S-adenosyl-L-[32P]methionine; DTT, dithiothreitol; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; PP-2A, protein phosphatase 2A; PAGE, polyacrylamide gel electrophoresis; bis-Tris(2-[bis[2-hydroxyethyl]amino]-2-[hydroxymethyl]propane)-1,3-diol.
and internal sequence analysis was performed using the procedure of Fernandes et al. (1992). Briefly, the portion of the membrane containing the 36-kDa polypeptide (visualized after Amido Black staining) was incubated in 37°C overnight with trypsin (mass ratio of 36-kDa polypeptide: trypsin = 50:1). The resulting peptides were then separated by HPLC on a C18 reverse-phase microbore column with a nonlinear gradient from 0% to 100% methanol over 20 min. Edman degradation was then performed using an Applied Biosystems Model 470A gas-phase sequencer with on-line HPLC detection. All of these procedures were performed by Dr. Audee Fowler at the UCLA Protein Microsequencing Facility.

**Protein Phosphatase 2A**—The phosphatase substrate [32P]phosphorylase a was prepared from phosphorylase b, [γ-32P]ATP, and phosphorylase kinase as described by Brautigan and Shirran (1988) with the following modifications. Norit A-activated carbon (20 mg) was added directly to the solubilized preparation and then removed by centrifugation. The [32P]phosphorylase a product was precipitated twice by adding an equal volume of saturated ammonium sulfate (pH 6.7) at room temperature, followed by incubation at 0°C for 15 min. The preparation was then dialyzed against 2 liters of 20 mM Tris acetate, pH 6.8, 50 mM β-mercaptoethanol overnight at 4°C. The resultant crystals of [32P]phosphorylase a were dissolved in glycerol:100 mM imidazole HCl, pH 7.2, 10 mM caffeine, 1 mM DTT, (1:1) and stored at −20°C. The specific activity of this preparation was 830 cpm/μg of protein.

Protein phosphatase 2A activity was assayed using [32P]phosphorylase a (final concentration, 0.5 mg/ml) as a substrate in the absence or presence of 10 μg/ml protein kinase in a 50-μl total volume buffered with 50 mM bis-Tris acetate, pH 7.0, 2 mM DTT, and 5 mM caffeine. The reaction was carried out for 30 min at 30°C in the presence of acid-soluble [32P]phosphate was measured after precipitation in 10% trichloroacetic acid as described by Brautigan and Shirran (1988).

**C-terminal Leucine Protein Methyltransferase**—Bovine brain cytosol was prepared as described by Xie and Clarke (1993). This material (50 ml, 20 mg of protein/ml) was dialyzed against buffer C (20 mM bis-Tris acetate, pH 6.8, 1 mM DTT, 0.2 mM EDTA) before fractionation on a DEAE-cellulose column (DE52 resin; 1.5-cm diameter × 15-cm length) equilibrated at 4°C with buffer C at 4°C. After washing the column with 80 ml of buffer C, proteins were eluted at 1 ml/min with a gradient of 0 to 1 M sodium acetate in a volume of 180 ml. The methyltransferase was assayed by mixing 25 μl of column fractions, 5 μl of 37 mM [3H]AdoMet (15 Ci/mmol), and 25 μl of a fraction containing its native polypeptide methyl-acceptor (fraction 46 from a Sepharose CL-4B column as described in Fig. 4 of Xie and Clarke (1993)). The incubation was carried out at 37°C for 30 min followed by separation of the polypeptides by SDS-PAGE. [3H]Radioactivity present as methyl esters in the 36-kDa polypeptide (filled circles) was then separated by HPLC on a C18 reverse phase microbore column with a nonlinear gradient of 0 to 100% methanol over 120 min. The elution profile was monitored by radioactivity eluting at 1 ml/min with a gradient of 0 to 1 M sodium acetate and at 4°C. The resulting crystals were centrifuged and the supernatant brought to 20% (w/w) ammonium sulfate at 0°C. The precipitate was then washed twice with buffer C and dissolved on a Sephacryl S-200 column at 4°C in buffer A. Fractions containing the [3H]methylated 36-kDa polypeptide were identified by SDS-PAGE followed by gel slice vapor-phase diffusion assay (Xie and Clarke, 1995; Xie et al., 1990). The resultant proteins were then loaded on a DEAE-cellulose column (Whatman DE52 resin; 1-cm diameter × 15-cm length) equilibrated at 4°C with buffer A. The column was washed with 50 ml of buffer A before starting a salt gradient (0 to 0.8 M sodium acetate in 300 ml of buffer A) at a flow rate of 15 ml/h. Fractions (3 ml) were collected and 50 μl of each third fraction was analyzed by SDS-PAGE/gel slice vapor-phase diffusion assays to locate the proteins containing the [3H]methylated 36-kDa polypeptide (filled circles). The absorbance at 280 nm is shown (open circles).

**RESULTS AND DISCUSSION**

**Purification of the 36-kDa Methyl-accepting Substrate of the C-terminal Leucine Protein Carboxyl Methyltransferase**—To identify the 36-kDa polypeptide methyl-acceptor, we decided to purify the [3H]methylated protein that was previously characterized in cytosolic extracts of bovine brain (Xie and Clarke, 1993). Initial attempts at purification were limited by the instability of the [3H]methyl ester on the 36-kDa polypeptide. Because we found that the instability persisted under conditions where spontaneous decay had been minimized, we sought conditions where we might inactivate endogenous enzymatic esterase activities. We found that treatment of the cytosoledialysate with PMSF, a common inhibitor of serine proteases and esterases, stabilized the [3H]methyl esters on the 36-kDa polypeptide and allowed us to follow its purification by monitoring the radioactivity in this polypeptide chain (Xie and Clarke, 1993).

As described in Fig. 1, we fractionated [3H]methylated bovine brain cytosol by DEAE-cellulose chromatography after initial ammonium sulfate precipitation and Sephacryl S-200 gel filtration chromatography. A major peak of radioactivity enriched in this material was found to elute at about 0.38 M salt. These fractions (91–101) were combined and further fractionated by hydrophobic chromatography on a phenyl-Sepharose column (Fig. 2A). Here, the [3H]methylated 36-kDa polypeptide eluted in a broad peak from fractions 141 to 150 at a salt concentration of about 0.12 M. All of the radioactivity in these fractions is present as [3H]methyl esters in the 36-kDa polypeptide (Fig. 2A). SDS-PAGE analysis of these fractions revealed a prominent 36-kDa band (Fig. 2B). We find that the specific activity of the 36-kDa polypeptide (determined as the ratio of the radioactivity and the silver-staining intensity in the 36-kDa band) is decreased in later-eluting fractions suggesting the partial resolution of methylated and unmethylated proteins containing the 36-kDa polypeptide. Enzymatic remethylation studies are consistent with this picture. For example, we find a 5.9-fold stimulation of methylation of fraction 157 polypeptides but a 95-fold stimulation of methylation of fraction 171 polypeptides (data not shown).

**Sequence Analysis of the Purified 36-kDa Polypeptide**—To determine the identity of the [3H]methylated polypeptide purified in Figs. 1 and 2, we combined fractions 161 to 169 of the phenyl-Sepharose column and separated the 36-kDa polypeptide from the other polypeptide components by SDS-PAGE. No terminal sequence was found when the intact 36-kDa polypeptide was subjected to automated Edman degradation analysis. The polypeptide was then digested with trypsin, and internal peptides were purified by HPLC as described under "Experimental Procedures." We selected one well-resolved peak eluting at 50 min for N-terminal sequence analysis and obtained the following sequence (picomole yields of each residue are given in subscript): Tyr4Gly3Ala5Asn7Ala5Asn9Val5Trp3Lys. We then searched the translated Genbank database (re-
lese 76) using this sequence. We found 100% identity with amino acid residues 137–144 of the catalytic subunit of protein phosphatase 2A from human, pig, rat, rabbit, Drosophila, and the plant Brassica napus (rape).

Although this database does not contain the bovine sequence of protein phosphatase 2A, it has been reported to differ only at residue 55 from that of rabbit and pig (Cohen, 1989). Residues 137–144 are preceded by a lysine residue and would thus be expected to be contained within a single tryptic fragment.

This result suggests that the 36-kDa polypeptide methyl acceptor for the C-terminal leucine methyltransferase is the catalytic subunit of protein phosphatase 2A. This assignment is also consistent with the known features of both the methylated protein (Xie and Clarke, 1993) and this enzyme (reviewed in Cohen, 1989). The polypeptide chain molecular weight of the methylated species and the phosphatase catalytic subunit are both 36 kDa. The product of methylation is a C-terminal leucine methyl ester; the C-terminal residue of protein phosphatase 2A from all species tested is a leucine residue. The molecular weight of the native methylated protein has been estimated at 178,000 by gel filtration chromatography; the native structure of the major isoform of phosphatase 2A is a 151-kDa trimer. The 36-kDa methylated species is found in the cytosol of a variety of mammalian species and yeast as well as in wheat germ and nematodes (data not shown); highly conserved protein phosphatase 2A species are found in the cytosolic fraction of all eucaryotic tissues examined to date.

**Methylation of Isoforms of Protein Phosphatase 2A in Bovine Brain—** Different forms of protein phosphatase 2A, including PP-2A0, PP-2A1, and PP-2A2, can be resolved by DEAE-cellulose chromatography (Cohen, 1989). While isoforms PP-2A1 and PP-2A2 have been purified from several tissues (although not from bovine brain), PP-2A0 has been found only in rabbit skeletal muscle. Isoforms PP-2A1 and PP-2A2 both contain the 36-kDa catalytic subunit and a 60-kDa subunit, while PP-2A1 contains an additional 55-kDa subunit. To determine which isoforms are substrates for the C-terminal leucine methyltransferase, we fractionated bovine brain cytosol on a DEAE-Sephacel column using the approach described by Erickson and Killilea (1992) (Fig. 3). Using \(^{32}P\) phosphatase a as a substrate, we found two major peaks of phosphatase activity corresponding to the PP-2A1 and PP-2A2 forms. The activity in both peaks was stimulated by protamine sulfate, a characteristic feature of this enzyme (Pelech and Cohen, 1985). As shown in Fig. 3, fractions containing either PP-2A1 or PP-2A2 are excellent methyl-accepting substrates for the C-terminal leucine protein carboxyl methyltransferase. To show that the methyl-acceptor in the PP-2A1 peak in Fig. 3 was in fact the phosphatase, we further fractionated this material using a phenyl-Sepharose column. As shown in Fig. 4, the methyl-accepting activity co-migrated with the protamine sulfate-stimulatable phosphatase activity.

**Specificity of the C-terminal Leucine Methyltransferase—** We tested whether a synthetic peptide RRTPDYFL, containing the
C-terminal sequence of the PP-2A catalytic subunit from all species examined so far, was recognized by the C-terminal leucine methyltransferase. We did not detect methylation of the peptide by the partially purified enzyme at concentrations up to 1 mM, nor did we observe any inhibitory effect on the methylation of PP-2A in cytosolic extracts at concentrations up to 0.5 mM. Coupled with the previous observation that N-acetyl-leucine is not recognized by this enzyme (Xie and Clarke, 1993), these results suggest that the methyltransferase requires additional elements of the phosphatase structure for catalysis.

Physiological Consequences—Our discovery of this novel modification on the C-terminal leucine residue of the bovine brain protein phosphatase 2A suggests the possibility that this enzyme may be regulated via methylation/demethylation reactions. The lability of the methyl ester in extracts, coupled with the stabilization by PMSF treatment, suggests the presence of an esterase activity that could work in concert with the methyltransferase to set the level of C-terminal modification. The methylation of the leucine residue described here is not the only modification of the C terminus of the catalytic subunit of PP-2A. A tyrosine residue at position 307, only two amino acids away from the C-terminal leucine residue, is phosphorylated in a reaction that deactivates the phosphatase (Chen et al., 1992). Protein phosphatase 2A is also modified (and deactivated) by a serine/threonine kinase (Guo et al., 1993). The presence of multiple covalent modifications on a single protein can greatly expand the possibilities for precisely controlling metabolic pathways.

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