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CARBOXYL METHYLATION OF PROTEIN PHOSPHATASE 2A FROM *XENOPUS* EGGS IS STIMULATED BY cAMP AND INHIBITED BY OKADAIC ACID

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Protein phosphatase 2A is the principal carboxyl methylated protein in cytoplasmic extracts derived from *Xenopus* eggs arrested in interphase. Levels of methylation approach stoichiometric values after 2 hr of incubation at 22°C. Rates of PP2A methylation increase by over 50% upon addition of cAMP. Methylation is inhibited by okadaic acid at concentrations that inhibit PP2A phosphatase activity.

Reversible covalent modifications play a central role in regulating protein activities. The most common chemistry involves phosphorylation and dephosphorylation at serine, threonine, and tyrosine residues. Phosphorylation at these side chains has been shown to be essential to the control of a wide range of cellular processes including metabolism, motility, growth, and gene expression. Another type of modification involves methylation and demethylation at carboxyl groups. In bacteria, carboxyl methylation at glutamate side chains regulates chemoreceptor function (1), and in eukaryotic cells the methylation of carboxy-terminal prenylcysteine groups may play a role in signal transduction pathways mediated by guanine nucleotide binding proteins such as the G-proteins and *ras* (2).

We have recently shown that protein phosphatase 2A (PP2A) is also methylated at its carboxy-terminus (3). The modification differs from what occurs in G-proteins (4). Methylation of PP2A is mediated by a distinct 40,000 molecular weight methyl transferase that catalyzes the carboxyl methylation of the carboxy-terminal leucine residue. PP2A is one of the major cytosolic phosphatases in eukaryotic cells. In vitro studies indicate that PP2A has broad specificity, catalyzing the dephosphorylation of phosphorylated serine, threonine, tyrosine, and histidine (5) residues. The enzyme has been purified from a number of sources as a heteromultimer composed of a 36,000 molecular weight catalytic subunit ($PP2A_C$) associated with one or two regulatory subunits. $PP2A_C$ is common to all forms of the enzyme and it is this subunit that is methylated.

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PP2A is thought to function in cell cycle regulation in *Xenopus* eggs (6). To begin to investigate the role of PP2A methylation in this process we have characterized this modification in a system that is thought to mimic *in vivo* conditions in the cytoplasm during interphase (7). Here we show that $PP2A_C$ is carboxyl methylated in this system. The stoichiometry approaches one methylated group per $PP2A_C$ subunit. The rate of methylation is stimulated by cAMP and inhibited by the S-adenosylhomocystein (AdoHcy) homolog, sinefungin, as well as by a specific inhibitor of PP2A, okadaic acid.

MATERIALS AND METHODS

Materials--Mature female *Xenopus laevis* were purchased from Xenopus I, Ann Harbour, Michigan. S-Adenosyl-L-[^{3}H-methyl]methionine (^{3}H-AdoMet), specific acivity, 60-85 Ci/mmol, and adenosine 5'-[γ-^{32}P]triphosphate, specific activity 3000 Ci/mmol, were obtained from New England Nuclear. The pH of the radiolabeled S-adenosylmethionine was adjusted to 7.5 with 1.0 M Tris just prior to use. Hyamine hydroxide and CytoScint were obtained from ICN. Pregnant mare serum gonadotropin, human chorionic gonadotropin, sinefungin, adenosine3':5'-cyclic monophosphate, phorbol12-myristate13-acetate, casein and cAMP dependent protein kinase were obtained from Sigma. Okadaic acid was obtained from Boehringer Mannheim.

Preparation of cytosolic extracts from Xenopus eggs--Cytosolic extracts were prepared as described by Felix et al. (7). Eggs were arrested in interphase in the presence of cycloheximide. The cytosolic extracts obtained contained 40 mg/ml protein with little variation from extract to extract.

Methylation assay--Cytoplasmic extracts were incubated with ^{3}H-AdoMet for the time indicated. The reaction was stopped by addition of Laemmli SDS-sample buffer. Samples were fractionated on SDS-12.5% polyacrylamide gels and analyzed for base-volatilized radioactivity using the methanol diffusion assay as described previously (8), or by incubation of 0.3 cm gel slices with 90% hyamine hydroxide at 37°C for 1 hr. The radioactivity was measured using a Beckmann liquid scintillation system after addition of 10 ml of CytoScint scintillation fluid.

Phosphatase assay--Phosphatase activity was measured using ^{32}P-labelled bovine casein as substrate, specific activity ~10^6 cpm/nmol, prepared by phosphorylation of casein with cAMP dependent protein kinase as described by McGowan and Cohen (9). Phosphatase assays were performed by measuring the TCA soluble radioactivity released after incubation at 30°C for the time indicated.

RESULTS

Methylation of the catalytic subunit of protein phosphatase 2A--In cytosolic extracts prepared from *Xenopus* eggs one protein with an apparent molecular weight of 36,000 corresponding to $PP2A_C$ was carboxyl methylated (Fig.1). This reaction was inhibited by an antibody raised against the carboxy-terminal peptide of $PP2A_C$.

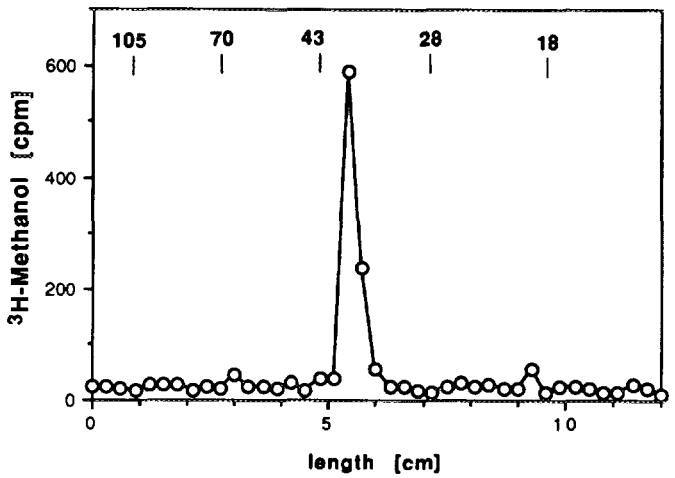

Figure 1. Protein carboxyl methylation in cytoplasmic extracts from *Xenopus* eggs. Extracts were incubated with 10% ^{3}H-AdoMet for 1 hr at 37°C. The reaction was stopped by adding Laemmli SDS-sample buffer. Samples were heated at 100°C for 5 min and fractionated on SDS-12.5% polyacrylamide gels. Gel lanes were cut into 0.3 cm sections and analyzed for base-volatilized radioactivity as described in Materials and Methods. Migration of molecular weight standards in kD is indicated.

Rates of methylation were determined by incubating extracts with ^{3}H-AdoMet (Fig.2). The rate of incorporation of ^{3}H-methyl groups into $PP2A_C$ was approximately constant for up to 1 hr and significant rates of incorporation were observed for up to 3 hr.

To estimate the stoichiometry of $PP2A_C$ methylation it was necessary to determine the final specific activity of ^{3}H-AdoMet in reaction mixtures. The cytosolic extract used in these experiments was not dialyzed nor significantly diluted, and contained an ATP generating system. Endogenous S-adenosyl-methionine (AdoMet) concentrations were estimated by HPLC analysis (Fig.3). The results indicated that extracts contained 40 μM AdoMet. Using this value, theincorporation of ^{3}H-methyl groups into $PP2A_C$ approached 1.0 μM after 3 hr of incubation at 22°C. This is comparable to the total concentration of PP2A in *Xenopus* oocyte cytoplasm (11).

Demethylation of $PP2A_C$--Carboxyl methylation of $PP2A_C$ is a reversible protein modification (12). It is possible, therefore, that changes in methylation could be modulated through alterations in rates of demethylation as well as through changes in methylation. To study the demethylation of $PP2A_C$, sinefungin, an nonhydrolyzable analog of S-adenosyl-L-homocysteine, that inhibits methylation (13), was added to extracts labeled with ^{3}H-AdoMet (Fig.2). The turnover of methyl groups was followed for one hour, but little loss of methyl groups was found. In previous studies of protein methylation in intact oocytes a relatively rapid

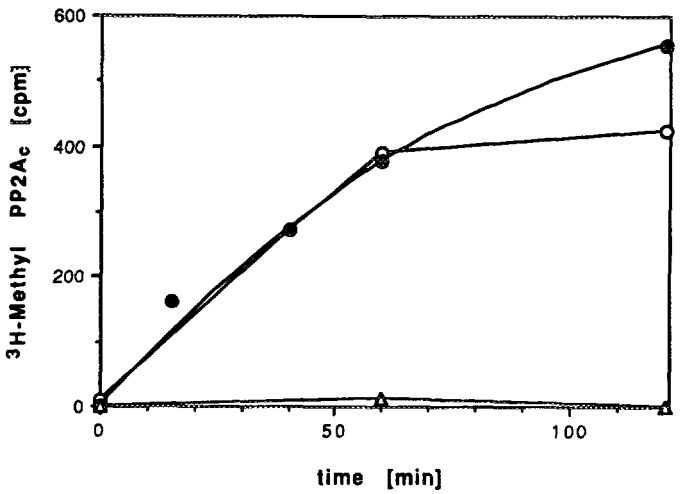

Figure 2. Time course of $PP2A_C$ methylation in the presence and absence of sinefungin. Samples of *Xenopus* egg extract were incubated with 15% ^{3}H-AdoMet for 2 hr at 22°C (●). After the indicated times fractions of the reaction mixture were removed and subjected to SDS-PAGE as described in the legend to figure 1. Samples were preincubated with 15% ^{3}H-AdoMet for 1 hr at 22°C (○). Sinefungin was added to a final concentration of 1.4 mM, and samples were incubated for 1hr. Samples were then subjected to SDS-PAGE and analyzed for base-volatilyzed radioactivity. (Δ) Samples were incubated with 15% ^{3}H-AdoMet and 1.4 mM sinefungin for the time indicated.

demethylation of a 36-kD protein, presumably $PP2A_C$, was observed (13). Apparently this activity is not present under the conditions used for our methylation assay.

Regulation of the methylation of $PP2A_C$ --If carboxyl methylation serves to control PP2A, one would expect methylation to be regulated. To investigate this possibility, we studied the effects of different second messengers on the carboxyl methylation of $PP2A_C$. Samples of frog egg extract were incubated with ^{3}H-AdoMet in the presence of Ca^{2+}, calmodulin, cAMP or phorbol ester for one and two hr (Table 1). cAMP caused a significant increase of methylation of $PP2A_C$. The effects of these second messengers on demethylation were investigated using the procedure outlined in figure 2, but under all conditions tested little demethylation was observed.

Inhibition of the carboxyl methylation of $PP2A_C$ by okadaic acid--Okadaic acid is a specific inhibitor of protein phosphatase 2A. We therefore investigated the effects of okadaic acid on PP2A methylation (Fig.4). Our results show that okadaic acid dramatically inhibits $PP2A_C$ methylation. The levels of okadaic acid required to inhibit methylation are approximately the same as those required to inhibit phosphatase activity using [^{32}P]casein as a substrate, which is specific for PP2A under the conditions used. Okadaic acid has a very high affinity for PP2A, I_{50} = 0.10 nM (6).

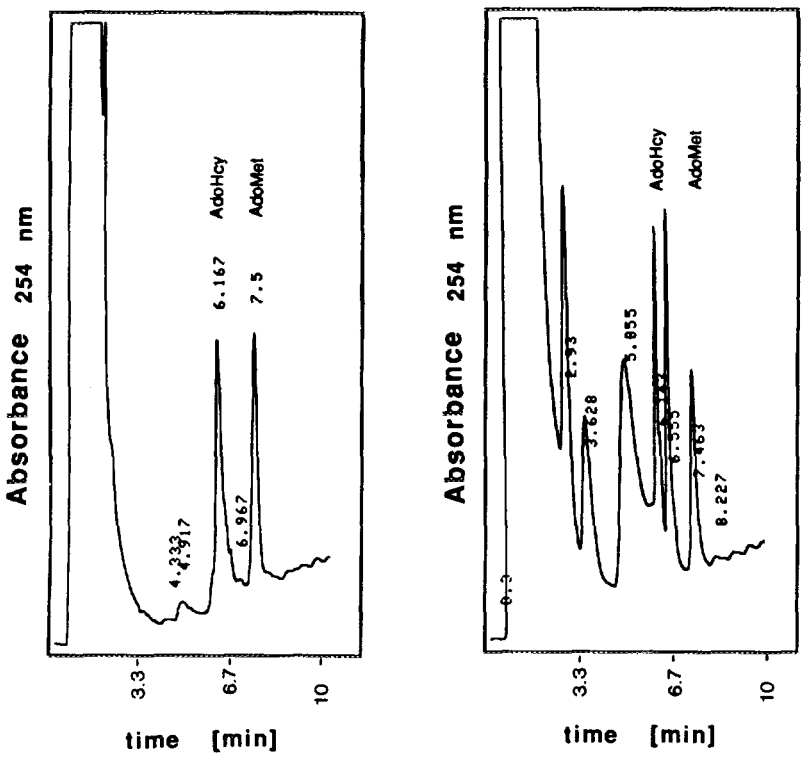

Figure 3. Analysis of AdoMet in *Xenopus* egg extracts. AdoMet was determined by HPLC using a Partisil SCX column (8 x 100 mm) in a radial compression module (Waters Associates) as described by Gordon *et al.* (10). The elution gradient used two buffers, buffer A: 1.0 mM NH_4COOH, pH 4.0; and buffer B: 0.20 M NH_4COOH, 0.80 M $(NH_4)_2SO_4$, pH 4.0. The elution gradient was as follows: 3 min with buffer A followed by a 3-35 min gradient to 80% buffer B. The figure on the left shows the elution profile of standards of AdoMet (0.25 nmoles) and AdoHcy, S-adenosylhomocysteine (0.25 nmoles) in 5% sulfosalicylic acid. The figure on the right shows the profile of a sample of *Xenopus* egg extract after 1 hr incubation with 15% ^{3}H-AdoMet, containing 4.0 µl of extract diluted with 96 µl of 5% sulfosalicylic acid, prior to chromatography. AdoMet levels determined prior to incubation of the sample and after incubation for 2 hr gave approximately the same values corresponding to a concentration of 40 µM.

Given the relatively high concentrations of PP2A in our extracts, one would expect the amount of okadaic acid required to obtain half maximal inhibition to correspond to half the amount of PP2A. This provides an independent estimate of the level of PP2A in our extracts. Using this approach the value obtained for the concentration of PP2A in our extracts is 880 nM, which corresponds to previously reported estimates obtained using other procedures.

DISCUSSION

PP2A is thought to play a role in the early embryonic cell cycle. Previous results showed, that inhibition of PP2A by okadaic acid resulted in premature entry

Table 1. Effects of second messengers on $PP2A_C$ methylation

Second Messenger	Percentage of Control	
	(1 hr)	(2hr)
control	100	100
Ca^{2+}	103 ± 3	78 ± 7
Ca^{2+} + calmodulin	108 ± 6	85 ± 4
cAMP	164 ± 3	154 ± 11
phorbol ester	96 ± 5	83 ± 4

Samples of *Xenopus* egg extract were incubated with 15% ^{3}H-AdoMet at 22°C for 1 and 2 hr in the presence of 2.0 mM Ca^{2+}, 8.0 µg/ml calmodulin + 1.0 mM Ca^{2+}, 2.0 mM cAMP, and 20 ng/ml phorbol ester (phorbol12-myristate13-acetate), as indicated. Samples were fractionated by SDS-PAGE and analyzed for base-volatilized radioactivity as described in Materials and Methods (100% corresponds to the cpm obtained in control samples after the time indicated, relative values obtained in the presence of second messengers are averages of at least 3 independent experiments ± the standard deviation from the mean).

into mitosis in cytosolic extracts derived from *Xenopus* eggs (6). We have investigated carboxyl methylation of PP2A in this system and found a continuous incorporation of methyl groups into its catalytic subunit. Under the conditions used

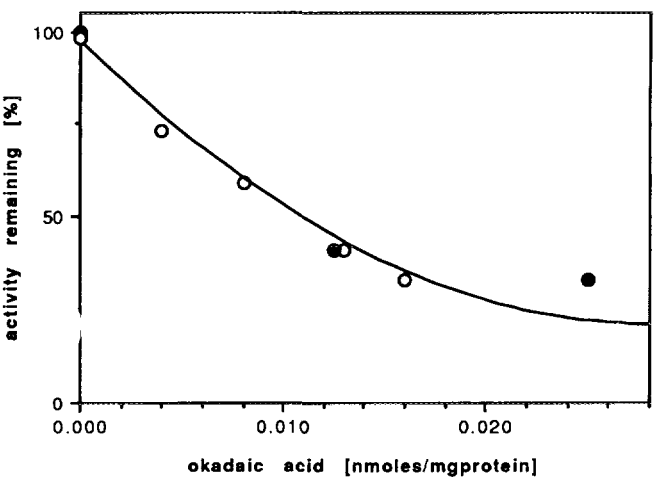

Figure 4. Inhibition of methylation and phosphatase activity by okadaic acid. Samples of *Xenopus* egg extract were incubated for 1hr at 22°C with 15% ^{3}H-AdoMet in the presence of increasing amounts of okadaic acid as indicated (O). Phosphatase activity (●) was assayed in the presence of the indicated amounts of okadaic acid using [^{32}P]casein as substrate (see Materials and Methods). The extract was diluted 1:100 into reaction buffer containing 50 mM Tris-HCl, pH 6.8, 0.10 mM EGTA and 0.10% (v/v) 2-mercaptoethanol and incubated for 5 min at 30°C.

no demethylation occurred, and over a two to three hr incubation period levels of methylation approached stoichiometric values. These results suggest that levels of $PP2A_C$ methylation are low in cytoplasm derived from cells entering interphase, and that substantial levels of PP2A methylation are obtained at later times. In studies of carboxyl methylated proteins in the cytosol of *Xenopus* oocytes arrested in metaphase II of meiotic division a relatively rapid turnover of methyl groups was observed in a 36-kD protein that was presumably $PP2A_C$ (13). The lack of demethylating activity we observe in cytoplasm derived from cells in interphase raises the possibility that the PP2A demethylating activity is a membrane-associated activity

Our results clearly show a stimulation of $PP2A_C$ carboxyl methylation by cAMP. cAMP is known to regulate protein phosphatase 1 (PP1) in glycogen metabolism (14). I-1, a specific inhibitor of PP1 is phosphorylated upon high levels of cAMP. The phosphorylated form of I-1 inhibits PP1. PP2A is thought to be the major I-1 dephosphorylating activity at basal Ca^{2+}-levels, but a signal transduction pathway regulating PP2A through cAMP has not been defined.

The inhibition of methylation by okadaic acid suggests that PP2A conformation is important for methylation, and/or okadaic acid interacts directly with the carboxy-terminal region of $PP2A_C$. Cohen (15) has proposed that okadaic acid interacts with a carboxy terminal domain of $PP2A_C$ since a homologue of $PP2A_C$ missing this portion of the molecule is insensitive to inhibition of phosphatase activity by okadaic acid.

It remains to be determined if carboxyl methylation of the catalytic subunit of PP2A regulates its function. Preliminary results indicate that methylation is not required for phosphatase activity, but rather serves a role in the regulation of substrate specificity. This might be caused by methylation-induced conformational changes at the carboxy terminus of $PP2A_C$ that alter its interactions with other proteins.

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