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Identification of two distinct protein carboxyl methyltransferases in eucaryotic cells

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Two distinct protein carboxyl methyltransferases (PCM) were identified in the electric organ of *Torpedo ocellata*. They were separated from each other in the active form by means of nondenaturing gel electrophoresis and by *p*-(chloromercuri)benzoate-agarose chromatography, and were individually identified by specific polyclonal antibodies. The existence of at least two distinct PCMs in eucaryotic cells raises the possibility that these enzymes are involved in distinct transmethylation reactions.

Protein carboxyl methyltransferase; Protein methylation

1. INTRODUCTION

Protein carboxyl methyltransferases (*S*-adenosyl-L-methionine: protein-*O*-carboxyl methyltransferase, PCM) catalyze the methylation of free carboxyl side chains of methyl acceptor proteins [1–3]. One type of PCM, present in chemotactic bacteria, methylates γ-glutamyl residues of bacterial chemoreceptors [4,5]. A second type, purified from human erythrocytes [6] or bovine brain [7], was shown to catalyze the methylation of protein substrates containing atypical D-aspartyl or L-isoaspartyl residues. This enzyme seems to be involved in the repair of age-damaged proteins [3,6,7], but not in other postulated functions of PCMs in eucaryotic cells [3], such as chemotaxis, regulation of neurotransmitter release and processing of precursor peptides [8,9]. The possible existence of PCM subtypes – which might account for such diverse activities – was suggested in a previous study, where a 26–27 kDa polypeptide was found to copurify with the 29–30 kDa PCM of *Torpedo* electric organ [10]. Here, we show that the two polypeptides are immunologically distinct PCMs, one of 27 kDa and the other being of 30 kDa.

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Abbreviations: PCM, *S*-adenosyl-L-methionine: protein *O*-carboxyl methyltransferase; AdoHcy, *S*-adenosyl-L-homocysteine; NRS, naive rabbit serum

2. EXPERIMENTAL

PCM was assayed essentially as described [10], using 100 μg gelatin, 10–20 μl enzyme preparation, 2.0 μM *S*-[*methyl*-^{3}H]adenosyl-L-methionine (15 Ci/mmol, NEN) and 0.04 M sodium acetate buffer, pH 6.5. Reactions were carried out at 37°C for 10 min. PCM activity is expressed as the amount of [^{3}H]methyl groups transferred to gelatin (in cpm) per 10 min incubation time. Blanks of reactions carried out at 0°C were subtracted. PCM was purified from the electric organ of *Torpedo ocellata* as in [10]. The degree of purification was 600–900. PCM preparations that showed, on 12.5% SDS-polyacrylamide gels [11], a single protein band at 30 kDa [10] were used to raise antibodies against the denatured and nondenatured 30 kDa PCM. In preparations that were found to contain the 30 kDa polypeptide and another of 27 kDa [10], both polypeptide bands were excised from the gel and used for immunization. Rabbits were injected subcutaneously with ~50 μg of protein prepared in complete Freund's adjuvant.

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This was followed after 3 weeks by bimonthly subcutaneous injections of the proteins prepared in incomplete Freund's adjuvant. Three types of antisera were raised: (i) antiserum 01 was raised against the pure 30 kDa polypeptide denatured by the SDS gel; (ii) antiserum 04 was raised against the pure nondenatured 30 kDa polypeptide; (iii) antiserum 02 was raised against the combined 27 kDa/30 kDa polypeptides denatured by the SDS gel. Immunoblotting procedures were as detailed in [12], using the three antisera (at dilutions of 1:500–1:2500) and goat anti-rabbit IgG-peroxidase conjugate diluted 1:2500. The blots were exposed to 0.01% hydrogen peroxide, 0.6 mg/ml 4-chloro-1-naphthol in Tris-buffered saline. Nondenaturing gel electrophoresis was performed according to [13], using 5% polyacrylamide as the running gel. The proteins were prepared in sample buffer (50% sucrose solution, 0.1% bromophenol blue), loaded onto the gel, and electrophoresed at constant power [13]. The gels were then frozen on dry ice and sliced into 2-mm sections each of which was crushed in a small scintillation vial containing 100–200 μl of 25 mM sodium phosphate buffer (pH 7.4), 14 mM β-mercaptoethanol and 0.1% Nonidet P-40. The vials were shaken for 12 h at 4°C and their PCM activity was then measured in 20-μl aliquots. *p*-(Chloromercuri)benzoate-agarose chromatography (Bio-Rad Affi Gel-501) was performed as follows: the column (20 ml matrix) was loaded with 100–120 ml of the cytosolic PCM preparation [10] (500–700 mg protein) so as to maintain a ratio of approx. 1 mg protein/0.2 μmol *p*-(chloromercuri)benzoate. The column was washed with 40 mM sodium phosphate buffer (pH 7.5) containing antiproteases [10] (buffer A) until all non-bound proteins were eluted. Bound proteins were then eluted with 14 mM β-mercaptoethanol in buffer A. All fractions were assayed for PCM activity.

3. RESULTS

Purified PCM preparations as well as the crude cytosol of the *Torpedo* electric organ were used for immunoblot assays, in which each of the anti-PCM antisera was examined (fig.1). As shown, antisera 01 and 04 gave positive responses only with the 30 kDa polypeptide, while antiserum 02 reacted with both the 27 and 30 kDa polypeptides.

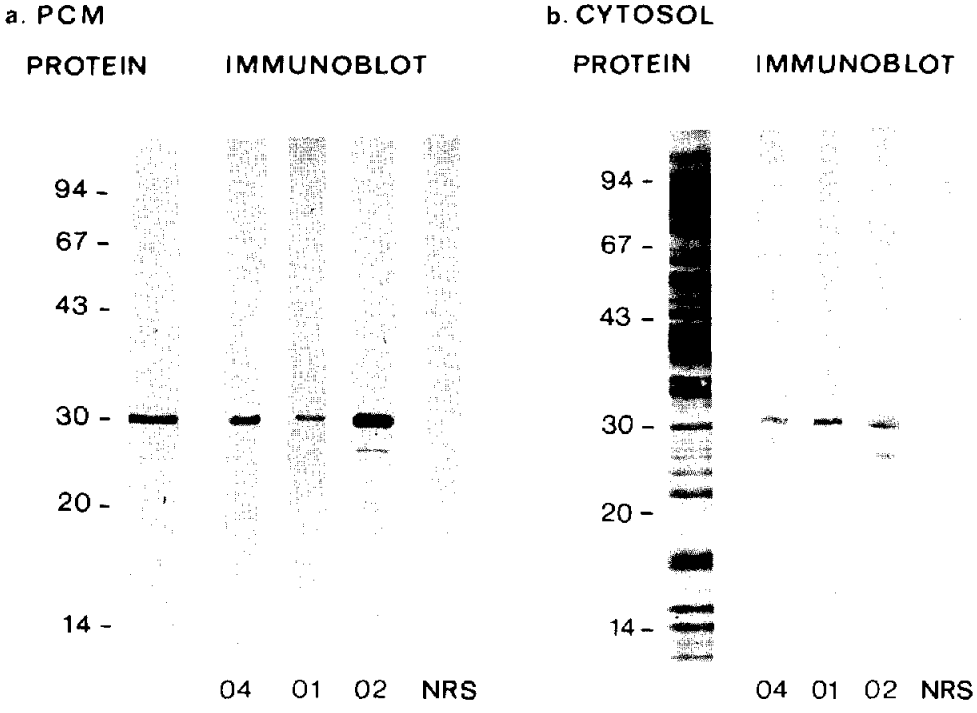

Fig.1. Characterization of anti-PCM antibodies by immunoblot assays. 10 μg purified PCM from *Torpedo* electric organ cytosol (a) and 100 μg electric organ cytosolic proteins (b) were electrophoresed and blotted as detailed in section 2. Protein blots were exposed to one of the three anti-PCM antisera (04, 01, 02) at a dilution of 1:2500, or to naive rabbit serum (NRS) at a dilution of 1:1000. Left lanes in a,b: Coomassie brilliant blue staining pattern of the purified PCM and of the cytosolic proteins, and the migration of molecular mass standards (kDa) on the SDS gels.

The immunoblots shown in fig.1 demonstrate that antibodies raised against the pure nondenatured 30 kDa PCM (antiserum 04) or the denatured enzyme (01) do not cross-react with the copurified 27 kDa polypeptide. Thus, the two polypeptides differ in their immunological reactivity. However, the finding of immunoreactivity associated with the 27 kDa polypeptide (detected with antiserum 02) in the crude cytosolic fraction suggests that this polypeptide does not derive from breakdown of the 30 kDa polypeptide during the purification process.

Fig.2 depicts a typical separation between the 27 and 30 kDa PCMs of *Torpedo* electric organ by nondenaturing gel electrophoresis. Two major activity peaks (designated A and B_1) and a minor activity peak (B_2) were detected. The activity of all three fractions was inhibited by AdoHcy (fig.2). No activity was detected in the absence of exogenous protein substrate (not shown). Samples of the two major PCM activity peaks (A,B_1) were rerun on SDS-polyacrylamide gel, which was then subjected to immunoblot assays using antiserum 04 (specific for the 30 kDa polypeptide) and antiserum 02 (specific for both 27 and 30 kDa polypeptides). As shown in fig.2, samples of peak A exposed to antiserum 02 yielded a clear band at 27 kDa, but no response to either antiserum was detected at 30 kDa. Samples of peak B_1, however, exhibited a strong band at 30 kDa but no 27 kDa band with both antisera (fig.2). The results obtained for peak B_2 were similar to those for peak B_1 (not shown). These immunological experiments demonstrate that the activity residing in peaks B_1 and B_2 corresponds to the previously described 30 kDa PCM [10], whereas that in peak A corresponds to the 27 kDa polypeptide. Fig.3 demonstrates chromatographic separation between the 27 and 30 kDa PCMs of *Torpedo* electric organ by *p*-(chlormercuri)benzoate-agarose. Under the conditions used here, we found that about 60% of the PCM activity flowed through the column (peak A), while 40% (peak B) was bound to the column and eluted by β-mercaptoethanol. Each of the PCM activity peaks was then subjected to an immunoblot assay. The results showed that the nonbound enzyme activity (peak A, fig.3) was associated with the 27 kDa PCM (peak A, fig.2), while that bound (peak B, fig.3) was associated with the 30 kDa PCM (peak B, fig.2).

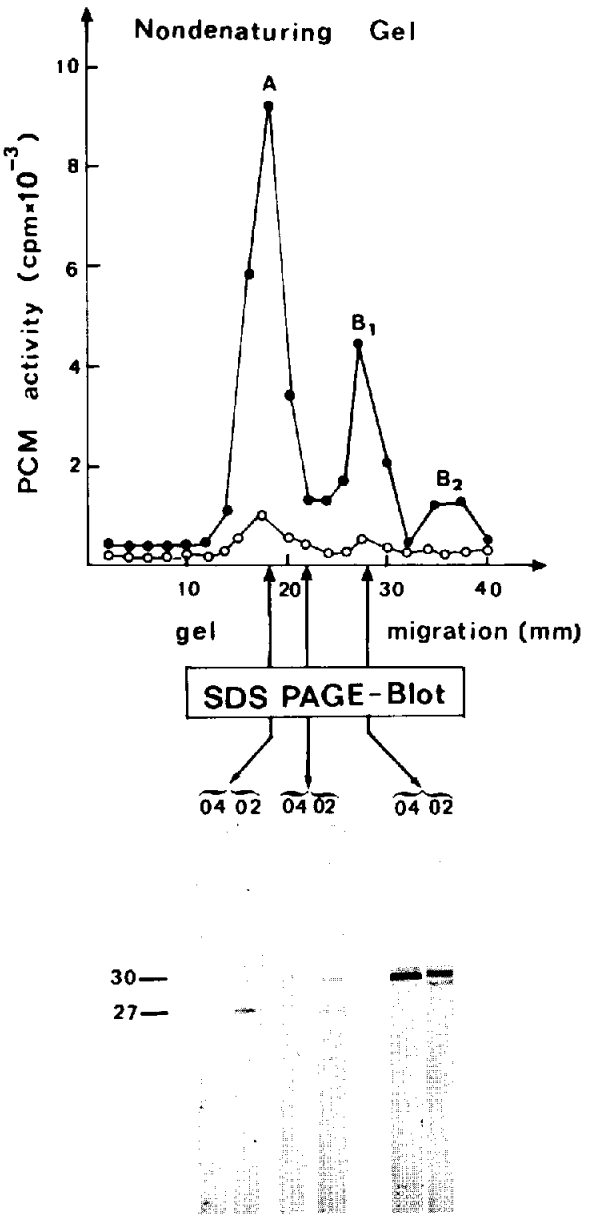

Fig.2. Nondenaturing gel electrophoresis of a crude *Torpedo* electric organ PCM preparation. Ammonium-sulfate-precipitated PCM preparation (~135 μg protein) of the electric organ cytosol [10] was electrophoresed on 5% polyacrylamide gel as detailed in section 2. The gel was then sliced into 2-mm sections and PCM activity determined (see section 2) in the absence (●) and presence (○) of 100 μM AdoHcy (upper). Aliquots (70 μl) of activity peaks A and B, as well as an equivalent sample between the two peaks, were rerun on SDS-polyacrylamide gel and subjected to immunoblot assays with antiserum 04 (diluted 1:1000) or antiserum 02 (diluted 1:1500) (lower).

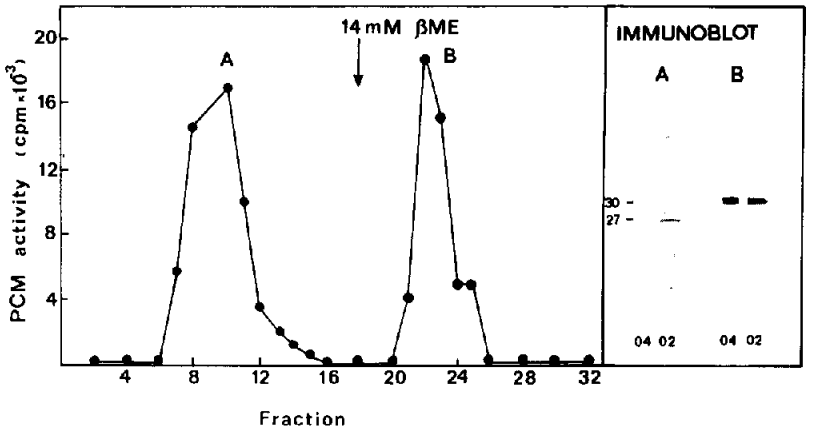

Fig.3. *p*-(Chloromercuri)benzoate-agarose chromatography of *Torpedo* electric organ PCM. Crude electric organ cytosol (500 mg protein) prepared in buffer A was loaded onto the column, which was washed with the same buffer until all of the non-bound protein flowed through (18 × 7.5-ml fractions). The buffer was then replaced by buffer A containing 14 mM *β*-mercaptoethanol (arrow) and additional 7.5-ml fractions were collected. PCM activity was determined in 10-*μ*l aliquots of each fraction (left). Samples (50 *μ*l) of activity peaks A and B were rerun on SDS-polyacrylamide gels and subjected to immunoblot assays with antiserum 04 (diluted 1 : 1000) or antiserum 02 (diluted 1 : 1500) (right).

4. DISCUSSION

Two distinct protein carboxyl methyltransferases, a 30 and a 27 kDa polypeptide, co-exist in the cytosol of *Torpedo* electric organ. Our data strongly suggest that the two polypeptides are distinct entities. This assertion is based primarily on the lack of immunological cross-reactivity between the 27 and 30 kDa PCMs of *Torpedo* electric organ, which was observed with polyclonal antibodies prepared against both denatured and nondenatured 30 kDa *Torpedo* enzyme. Other data show that the interaction of the 30 kDa PCM with *p*-(chloromercuri)benzoate-agarose differs from that of the 27 kDa PCM, and that the 27 kDa enzyme is not a breakdown product formed from the 30 kDa enzyme during its purification. Both the 27 and 30 kDa PCMs are soluble and are present in crude homogenates as well as purified enzyme preparations. Separate immunoreactivities associated with the 27 and 30 kDa polypeptides were also detected in *Torpedo* liver and brain, as well as in the rat brain and human erythrocytes (unpublished). Taken together, these results suggest that both the 27 and 30 kDa PCMs are endogenous constituents of eucaryotic cells.

It now appears that eucaryotic PCMs are much more heterogeneous than was previously thought. This heterogeneity could be associated with biological functions of protein carboxyl methylation in eucaryotic cells, and raises the possibility that PCMs constitute a family of enzymes that modulate a variety of regulatory and non-regulatory proteins. This hypothesis can reconcile previously described data which on the one hand point to the involvement of PCM-catalyzed reactions in neurosecretion, leucocyte chemotaxis, sperm motility and cell differentiation (reviews [8,9]) and, on the other, suggest their involvement in the repair of age-damaged proteins [3].

Future studies on the substrate specificities of the two immunologically distinct eucaryotic PCMs, their tissue distribution, and their manifestations in various pathological conditions will improve our understanding of their biological significance in protein methylation of intact cells.

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