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Protein Carboxylmethylation in Porcine Spleen is Mainly Mediated by Class I Protein Carboxyl *O*-Methyltransferase

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The functional role of protein carboxylmethylation (PCM) has not yet been clearly elucidated in the tissue level. The biochemical feature of PCM in porcine spleen was therefore studied by investigating the methyl accepting capacity (MAC) of natural endogenous substrate proteins for protein carboxyl *O*-methyltransferase (PCMT) in various conditions. Strong acidic and alkaline-conditioned (at pH 11.0) analyses of the MAC indicated that approximately 65% of total protein methylation seemed to be mediated by spleen PCMT. The hydrolytic kinetics of the PCM products, such as carboxylmethylesters (CMEs), under mild alkaline conditions revealed that there may be three different kinds of CMEs [displaying half-times ($T_{1/2}$) of 1.1 min (82.7% of total CMEs), 13.9 min (4.6%), and 478.0 min (12.7%)], assuming that the majority of CME is base-labile and may be catalyzed by class I PCMT. In agreement with these results, several natural endogenous substrate proteins (14, 31 and 86 kDa) were identified strikingly by acidic-conditioned electrophoresis, and their MAC was lost upon alkaline conditions. On the other hand, other proteins (23 and 62 kDa) weakly appeared under alkaline conditions, indicating that PCM mediated by class II or III PCMT may be a minor reaction. The MAC of an isolated endogenous substrate protein (23-kDa) was also detected upon acidic-conditioned electrophoresis. Therefore, our data suggest that most spleen PCM may be catalyzed by class I PCMT, which participates in repairing aged proteins.

Key words: Protein carboxylmethylation, Spleen, Protein carboxyl-*O*-methyltransferase, Natural endogenous protein substrate

INTRODUCTION

Protein carboxylmethylation (PCM) is a biochemically inefficient reaction compared to protein phosphorylation, which requires 1 ATP molecule per reaction, whereas PCM requires 12 ATP molecules (Hrycyna and Clarke, 1993). Nevertheless, PCM is still highly conserved in biological systems, suggesting that it plays an important role in carrying out some special functions or functions that are still unclear. This modification is catalyzed by protein carboxyl *O*-methyltransferase [EC 2.1.1.24] [PCMT, also called as protein methylase II], which transfers a methyl group from *S*-adenosyl-L-methionine (AdoMet) to a free carboxyl group in a methyl accepting polypeptide (Paik and Kim, 1990). Consequently, the alteration of polarity by carboxylmethylation may cause a conformational change that importantly regulates the molecular function of the substrate proteins (Paik and Kim, 1992), as in other post-translational modification reactions. Furthermore, negative physiological regulations by pH change, activation of methylesterase (demethylating enzyme) (Veeraragavant *et al.*, 1989; O'Dea *et al.*, 1981), and the existence of inhibitory molecules such as natural proteinacious inhibitors (Hong *et al.*, 1986; Park *et al.*, 1993; Kwon *et al.*, 1994; Seo *et al.*, 2002) and *S*-adenosyl-L-homocysteine (AdoHcy) (Paik and Kim, 1990) seem to support the importance of this modification.

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To date, PCMT is classified into three distinct forms (Aswad, 1989; Hrycyna and Clarke, 1993) according to target amino acids to be carboxylmethylated. Each class of PCMT is found to play a special role in the post-translational modifications. Therefore, identification of carboxylmethyl esters (CMEs) is regarded as an important work primarily in understanding PCM in target cells or tissues. The first class (class I) is categorized as protein L-isoaspartyl methyltransferase (also named as EC 2.1.1.77), or type II PCMT, which is thought to participate in repairing aged proteins *via* selective carboxylmethylation of atypical L-isoaspartyl or D-aspartyl residues (Ingrosso *et al.*, 2000; Farrar and Clarke, 2002; Clarke, 2003). The class I PCMT-generated CMEs display an acid-resistant and base-labile property. The second class (class II) is known as the type III PCMT, which is exerted in modification of the α-carboxyl group of C-terminal cysteine residues in some signaling proteins including the ras oncogene protein and a number of related guanine nucleotide-binding proteins (Hrycyna and Clarke, 1993; Yamane *et al.*, 1991). With prenylation or farnesylation, class II PCMT-mediated modification has been shown to critically regulate the membrane targeting of these proteins (Hrycyna and Clarke, 1993). Finally, the third class (class III) of PCMT carries out a novel modification reaction by which the α-carboxyl group of C-terminal leucine in cytosolic proteins, such as in protein phosphatase 2A, is methylated (Xie and Clarke, 1994; Lee and Stock, 1993; Vafai and Stock, 2002). These two classes of PCMTs catalyze the generation of base-stable CMEs. At present, class II- or class III-mediated PCM is seen as a new therapeutic intervention for treating cancer, pancreatic β cell impairment, and Alzheimer's Disease (Hrycyna and Clarke, 1993; Zolnierowicz, 2000; Kowluru and Amin, 2002; Vafai and Stock, 2002; Winter-Vann *et al.*, 2003).

In spite of numerous studies, however, there was no clear and direct evidence that explained PCMTs roles in relation to tissue or organ functions. This has been the case even though the increased activity of PCMT was detected as carrying out principal functions of tissues or cells such as the secretion-excitation processes in pancreas, neuron, and brain (Diliberto *et al.*, 1976; Diliberto and Axelrod, 1976; Kowluru *et al.*, 1996; Kowluru and Amin, 2002), and the chemotactic mobility of sperm (Gagnon *et al.*, 1982) and immune cells (Leonard *et al.*, 1978; Pike *et al.*, 1978; Rodriguez *et al.*, 1991; Law *et al.*, 1992). To resolve this key issue, the biochemical features of natural endogenous substrate proteins for PCMT in the target tissues or organs could be investigated. In this study, therefore, we have examined the MAC of splenic natural endogenous substrate proteins upon various conditions, such as different pH levels and propose that the class I PCMT, involved in repairing aged proteins, may mainly mediate the PCM in porcine spleen.

MATERIALS AND METHODS

Materials

Fresh porcine (*Sus scrofa domestica*) tissues (spleen, liver, testis and kidney) were obtained from the Suwon slaughterhouse (Suwon, Korea) and kept at -70°C until used. Cytosolic PCMTs from spleen, liver, testis, and kidney were purified by affinity chromatography as reported previously (Kim *et al.*, 1994; Kim *et al.*, 1978). *S*-adenosyl-L-[methyl-^{14}C]methionine ([methyl-^{14}C] AdoMet, specific activity: 50 mCi/mM) was purchased from ICN Radiochemicals (Irvine, CA, USA). Chymotrypsin, pepsin, phenylmethylsulfonyl fluoride, and trypsin were from Sigma Chemical Co. (St. Louis, MO, USA).

Subcellular fractionation of porcine tissues

Frozen porcine tissues (spleen, liver, testis and kidney) were homogenized in a Waring blender with 4 volumes of a cold 0.3 M sucrose solution and subcellular fractionation was done using the conventional method (Kim *et al.*, 1994). Of the subcellular fractions, each cytosolic fraction was further fractionated to obtain Fraction I after ammonium sulfate precipitation (80%) as well as dialysis (against imidazole buffer, pH 7.0).

Methyl accepting capacity assay (Methanol extraction method)

Reaction mixture composed of a 0.25 M citrate-phosphate buffer (pH 6.5), purified PCMT, 100 mM [methyl-^{14}C] AdoMet (50 mCi), and Fraction I in the final volume of 0.5 mL was incubated at 37°C for indicated times in each experiment. MAC from the reaction mixture was determined by the radioactivity that is converted to a volatile species, i.e. [^{14}C] methanol, by alkaline treatment (Diliberto, *et al.*, 1976). Briefly, the reaction was stopped by the addition of a strong base [0.175 M sodium borate buffer (pH 11.0)], and the radioactive CMEs that were hydrolyzed were extracted with 1 mL of isoamyl alcohol on ice. After centrifugation at 10,000 rpm for 20 min, aliquot (500 μL) of upper layer was applied to the scintillation vial containing 5 mL of scintillation fluid. The radioactivity was measured by the liquid scintillation counter (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

Gel slice methanol vapor phase diffusion assay

After acidic or basic-conditioned gel electrophoresis, the MAC of separated proteins was measured by the Gel slice methanol vapor phase diffusion assay as reported previously (Law *et al.*, 1992; Cho *et al.*, 2001). Briefly, the dried gel lane was cut into a 0.2 cm slice and placed in a 1.5 mL microcentrifuge tube. After adding 100 μL of 1 M

NaOH to the tubes, each tube was then placed in a 5 mL scintillation vial containing 2.5 mL of scintillation fluid. The volatile radioactivity ([^{14}C] methanol) was trapped into the scintillation fluid for 12 h at 37°C and then measured by liquid scintillation counter.

Protein determination

Protein was determined by the method of Bradford (1976), and bovine serum albumin was used as a standard protein.

Treatment of strong base or acid

To quantitatively access total protein methylesters and CMEs from spleen Fraction I, strong acid (6 N HCl) and base [0.175 M sodium borate buffer (pH 11.0)] were added to the reaction mixture. Briefly, the reaction mixture was incubated with 80 μL of 6 N HCl at 37°C for 10 h or 80 μL of 0.175 M borate buffer for 2 h at 37°C. The radioactive materials were then extracted with isoamyl alcohol.

Determination of hydrolysis rate of CMEs

To check hydrolytic kinetics of CMEs upon mild alkaline conditions (pH 8.9), the incubation mixture was treated with 4 volumes of 0.1 M Tris-HCl buffer, which had a pH of 8.9 (O'Connor and Clarke, 1984) and was further incubated at 37°C for as indicated (from 0 to 274 min). To determine the remaining CMEs, the methanol extraction method was used after the precipitation of methyl accepting proteins with trichloroacetic acid. The exponential decay for half-time ($T_{1/2}$) and remaining percent of CMEs were calculated by RSTRIP software (Micromath, Salt Lake City, UT, USA), as reported previously (Cho *et al.*, 2001).

Proteolytic digestion

Chymotryptic, pepsin, and tryptic digestion of Fraction I was performed as described by Rao and Reithmeier (1979). Fraction I was incubated for 2.5 h at 25°C with bovine pancreatic trypsin (0.25 M citrate buffer, pH 6.0) or at 37°C with pepsin (pH 2.0) and α-chymotrypsin (Tris-HCl buffer, pH 8.0). The reactions with chymotrypsin and trypsin were terminated by the addition of phenylmethylsulfonyl fluoride at 4°C for 30 min. Pepsin digestion was stopped by adding phosphate buffer of pH 6.0 (Terwilliger and Clarke, 1981; Janson and Clarke, 1980). The aliquots of 15 μL from the terminated incubation mixtures (50 μL) were used for determining the proteolytic effect of the digestive enzyme judged by the MAC assay. The control was incubated with cytosolic fraction alone.

Identification of natural substrates

Acidic electrophoresis: A modified acidic gel system consisting of 0.05 M sodium phosphate of pH 2.4; 8 M urea; 1% sodium dodesyl sulfate (SDS); and 10% acrylamide was used as described previously (Fairbank and Avruch, 1972). The reaction mixture that was diluted with a freshly prepared sample buffer (0.05 M sodium phosphate, pH 2.4; 3% SDS; 8 M urea; 20% glycerol; 0.05% pyronin Y; and 30 mM 2-mercaptoethanol) was loaded onto slab gels at a constant current (70 mA/slab) until the pyronin Y tracking dye had migrated to the bottom. After gels were stained and destained, the wet gels were dried for 2 h at 50°C. SDS-polyacrylamide gel electrophoresis: base-resistant carboxylmethylated proteins were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Prior to gel electrophoresis, the incubation mixture was diluted with 2x sample buffer, boiled for 5 min, and then loaded onto an 11% polyacrylamide slab gel (acrylamide : bisacrylamide = 30 : 0.8). The measurement of radioactivity derived from CMEs in dried gels was performed by the gel slice methanol vapor phase diffusion assay.

Isolation of natural substrates

The natural endogenous substrate was isolated from the splenic cytosolic fraction. Isolation of the substrate was accomplished using ammonium sulfate precipitation, chromatography on a DEAE Sephacel column and Sephadex G-100, and HPLC on a Protein PAK-125 column. The following buffers were used: Buffer A: 20 mM Tris-HCl (pH 8.0) containing 0.2 mM EDTA, 15 mM 2-mercaptoethanol; and Buffer B: 10 mM phosphate buffer (pH 6.3). All steps in this procedure were carried out at 4°C.

Step 1. Ammonium sulfate precipitation.

The cytosolic fraction from the subcellular fraction was brought to 80% saturation with solid ammonium sulfate and stirred for 30 h. After centrifugation at 39,000×g for 30 min, the precipitate was dissolved in 10 mL of buffer A per 50 g of tissue and was dialyzed against the same buffer overnight to prepare Fraction I.

Step 2. DEAE Sephacel chromatography.

The above dialyzed sample (15 mL of Fraction I) was applied to a column of DEAE Sephacel (3×30 cm) previously equilibrated with buffer A, and the column was washed by linear gradient elution with buffer A containing 0 to 0.5 M NaCl and 1 M NaCl at a flow rate of 62 mL/h. The fractions were pooled by peak pattern and were concentrated by ultrafiltration using an Amicon apparatus (Beverly, MA, USA), YM-10 (molecular cut-off: 10,000) membrane. The MAC of the concentrated peaks was determined by the Methanol extraction method.

Step 3. Sephadex G-100 gel filtration chromatography.

The concentrated fraction (peak II) was applied to a Sephadex G-100 column (1.5×90 cm) that was pre-equilibrated and eluted with buffer B at a flow rate of 7.2 mL/h. Each peak (fraction size: 3.2 mL), concentrated by ultrafiltration, was measured by the MAC assay, and the fraction containing the highest MAC was applied to the

same column for a second and third chromatography. To decrease the total protein concentration, the concentrated peak has been chromatographed two more times under the same conditions, and each subfraction was obtained by pooling the fractions divided into half-A and half-B.

Step 4. High performance liquid chromatography (HPLC) on Protein PAK-125.

To check the MAC profile of the preparation from step 3, the concentrated fraction (Peak II-3-half B-half B) was incubated with purified PCMT and [methyl-^{14}C] AdoMet for 2 h and then applied to the Protein PAK-125 column (7.8×300 mm) that was pre-equilibrated with buffer B. Fractions were collected per min using the fraction collector at a flow rate of 1.0 mL per min. Then, the radioactivity of each fraction (500 μL) was directly measured by liquid scintillation counter. To prepare the unlabeled fraction, all steps were followed using the same conditions and also using un-labeled Peak II-3-half B-half B. The fractions having the same retention time (11 min) were pooled and concentrated by ultrafiltration. The concentrated fraction was then applied to the same column for re-chromatography (second HPLC). The peak, having the same retention time (11 min), was pooled and concentrated by lyophilization.

RESULTS

Subcellular distribution of the MAC in porcine spleen

The distribution of the MAC in porcine spleen subcellular fractions was examined first. As Table I shows, the MAC was widely distributed in spleen subcellular fractions. Cytosolic fraction and 600×g precipitated fraction displayed the highest total MAC and the highest MAC (5.3 pmol/mg protein), respectively. Because not only was the highest spleen PCMT activity found in cytosolic fraction (Kim *et al.*, 1994), but the spleen PCMT used in this study was also purified from the same fraction. We, therefore, continued further experimentation using cytosolic fraction.

Table I. Subcellular distribution of MAC catalyzed by PCMT in porcine spleen*

Fraction	MAC[1] (pmol/mg protein)	Total vol.	Total MAC (pmol/mg protein)	Yield (%)
Whole homogenate	3.6	225.0	814.5	100.0
600×g ppt[2]	5.3	35.4	185.2	22.8
15,000×g ppt	4.4	23.4	100.5	12.3
105,000×g ppt	3.4	7.2	23.9	2.9
105,000×g supn[3]	3.4	133.4	449.5	55.2

*Fractions were incubated for 10 min with purified PCMT in the presence of 10 μM of [methyl-^{14}C] AdoMet, and the MAC was measured by the methanol extraction method as described in Materials and Methods. [1]Methyl accepting capacity, [2]Precipitate and [3]Supernatant.

Quantitative analysis of total protein methylation and carboxylmethylation by a strong acid and base treatment

Because a strong acid treatment has been demonstrated to release volatile materials (such as methylamine and methanol), derived from all methylesters, which are catalyzed by intracellular protein methylase (I, II, and III) (Najbauer *et al.*, 1991). Therefore, we assumed that the comparison of MACs obtained under both a strong acid (6 N HCl) condition and alkaline treatment will allow us to

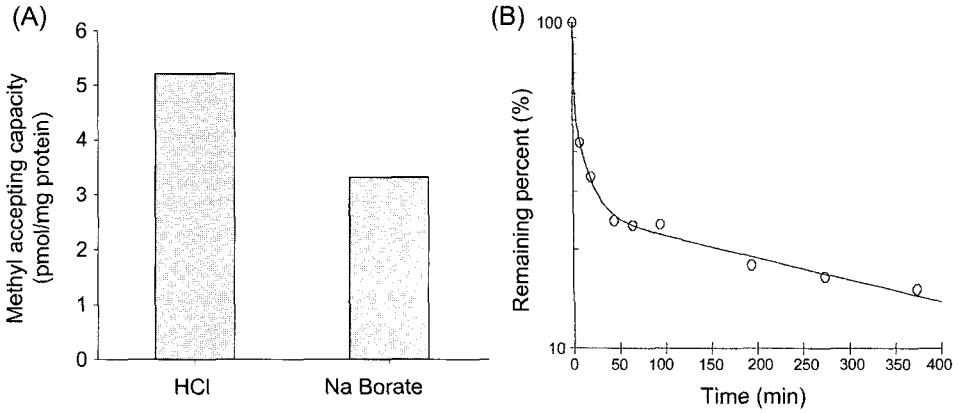

Fig. 1. Quantitative analysis of total protein methylation and carboxylmethylation in spleen cytosolic fraction by strong acid and base treatment, and hydrolytic kinetics of CMEs at pH 8.9. **(A)** Cytosolic fraction (Fraction I) was methylated in the presence of 10 μM [methyl-^{14}C] AdoMet for 2 h at 37°C, and then, the methylated mixture was incubated in a 0.175 M sodium borate buffer (pH 11.0) for 30 min for basic conditions, or hydrolyzed in 6 N HCl for 10 h for acidic conditions. Radioactive materials released were extracted into the isoamyl alcohol layer as described in Materials and Methods. **(B)** Fraction I was methylated in the presence of 20 μM [methyl-^{14}C] AdoMet for 20 min at 37°C, and then, the methylated mixture was further incubated in a 0.1 M Tris buffer (pH 8.9) for various lengths of times. Radioactive materials that remained were determined by the methanol extraction method as described in Materials and Methods.

know the ratio of spleen PCM/PM. As Fig. 1A shows, MACs under acid and base treatments were 5.3 and 3.3 pmol/mg protein, respectively, suggesting that 63.5% of the total MAC seemed to be generated by the PCMT activity in spleen cytosolic fraction.

Hydrolytic kinetics of CME under mild basic condition (pH 8.9)

To quantitatively identify how many CMEs can be generated by spleen PCM, we next analyzed the hydrolytic profile of base-labile CMEs under mild basic conditions (pH 8.9) (O'Connor and Clarke, 1984; Cho *et al.*, 2001). Fig. 1B and Table II indicate that three distinct classes of CMEs were formed in spleen PCM. These CMEs revealed variable half-times ($T_{1/2}$) of 1.1 (82.7% of total CMEs), 13.9 (4.6%), and 478.2 (12.7%) min, respectively, which suggests that most of the CMEs are base-labile.

Identification of natural protein substrates from cytosolic fraction

Next, in order to confirm the base-lability of CMEs and to directly identify the natural endogenous substrates, we investigated the distribution patterns of natural endogenous substrates for PCMT using acidic (pH 2.4) and basic-conditioned gel electrophoreses. It has been known that acidic conditioned gel electrophoresis prevents degradation of CMEs from carboxylmethylated proteins (Terwilliger and Clarke, 1981). Three major endogenous substrates (14, 31, and 86 kDa) and 4 minor proteins were identified from the cytosolic fraction under acidic conditions (Fig. 2). Interestingly, the gel pattern obtained from mild alkaline condition was completely different from the acidic gel. Thus, two proteins (63 and 24 kDa) were newly generated, whereas, three major substrates identified from the acidic conditions were not detected under basic conditions, suggesting that major endogenous substrates from porcine spleen are base-labile. There was no correlation between radiolabeling and protein amount judged by the Coomassie blue staining (data not shown), suggesting that these substrates may be selectively carboxylmethylated by cytosolic PCMT.

Table II. The hydrolytic kinetics of CMEs from spleen cytosolic fraction under mild alkaline condition (pH 8.9)

Half life	Min	Remaining (%)
$T_{1/2}$ 1	1.1	82.7
2	13.9	4.6
3	478.2	12.7

*Fraction I was methylated in the presence of 10 μM of [methyl-^{14}C] AdoMet for 2 h at 37°C, and after precipitation, methylated proteins were incubated in 0.175 M sodium borate buffer (pH 11.0) for 30 min at 37°C. Then hydrolyzed volatile materials were extracted with isoamyl alcohol as described in Materials and Methods.

Effect of proteolytic digestion and pH change on MAC

To examine the PCM pattern in various porcine tissues, we explored the effect of proteolytic digestion and pH changes on the MAC of each cytosolic fraction (Fraction I) from the spleen, liver, testis, and kidney. Fig. 3 shows that PCM of spleen cytosolic fraction was similar to that of the testis, but distinguished from those of the liver and kidney. Thus, chymotrypsin treatment enhanced the MAC of

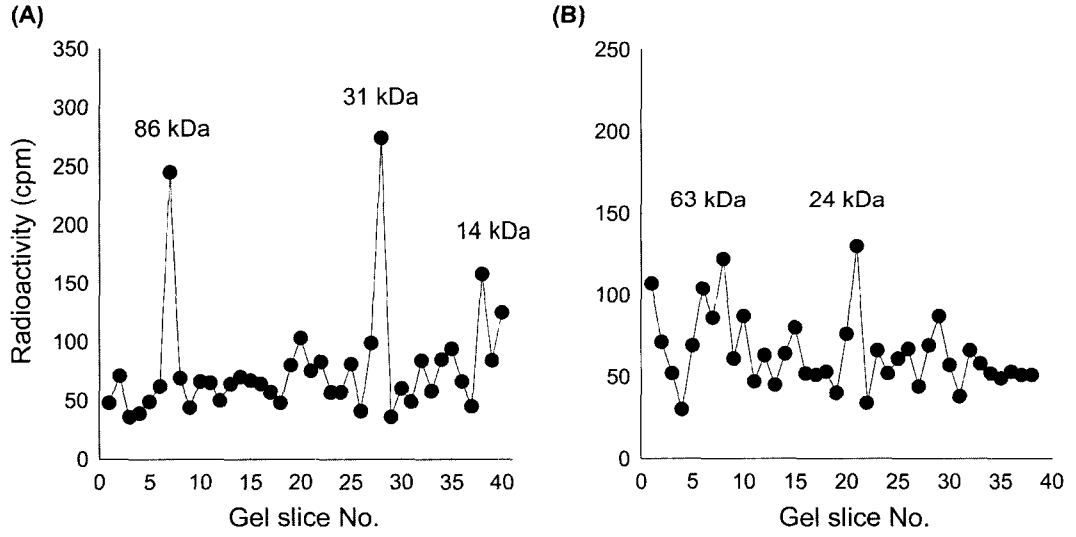

Fig. 2. Effects of proteolytic lysis and pH change on MAC of Fraction I. **(A)** Fraction I was treated with proteolytic enzymes as described in Materials and Methods. After the inactivation of proteases, the treated fractions were methylated with purified PCMT. **(B)** Twenty five mL of Fraction I was incubated in the presence of 10 μM [methyl-^{14}C] AdoMet under different pH conditions (pH 6.0 and 6.5 : 0.25 M citrate buffer; pH 7.0 and 7.5 : 0.25 M phosphate buffer; and pH 8.0 : 0.25 M Tris buffer) for 10 min at 37°C. The MAC was determined as described in Materials and Methods.

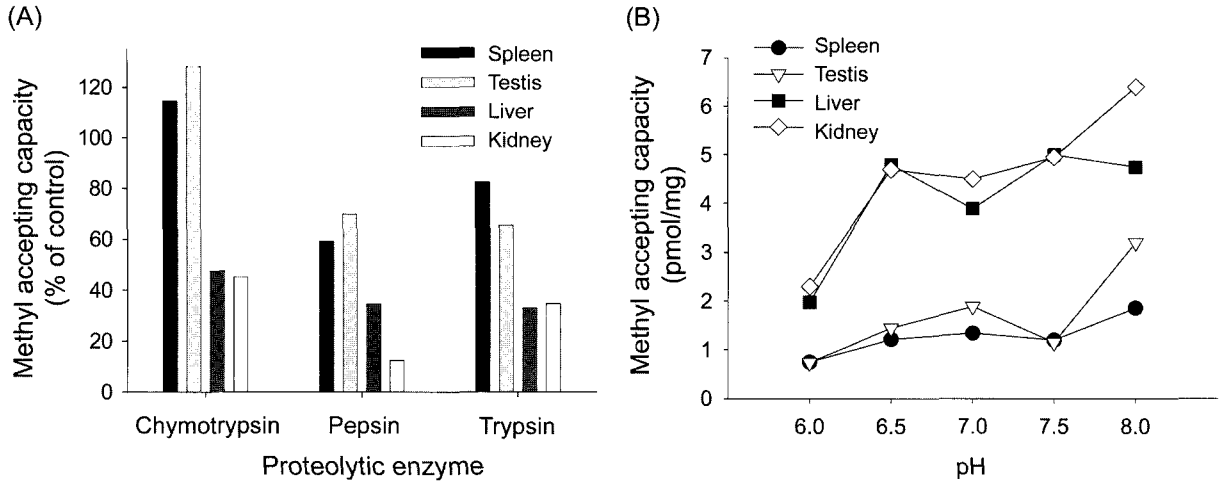

Fig. 3. Distribution of natural endogenous substrates for PCMT in spleen cytosolic fraction. **(A)** Urea/SDS gel electrophoresis of acidic conditions (pH 2.4) was conducted as described in Materials and Methods. **(B)** SDS polyacrylamide gel electrophoresis by the Laemlli method was performed as described in Materials and Methods. Each fraction that contained 120 μg of protein was loaded per lane after incubation with [methyl-^{14}C] AdoMet and PCMT fractions. Dried gel lanes were cut into 0.2-cm slices and mixed with 2 M NaOH in a 1.5 mL micro centrifuge tube. Radioactivity was measured by the Gel slice methanol vapor phase diffusion assay.

cytosolic fractions of the spleen and testis but decreased in the liver and kidney. In the case of the pH effect, sensitivity of the spleen and testis to pH 6.5 and 7.0 was different from that of the liver and testis, suggesting that there might be a difference of the PCM pattern between spleen (and testis) and liver (and kidney).

Methyl accepting capacity in each isolation step

Having established that spleen cytosolic proteins, including PCMT, can be efficiently separated by conventional column chromatography (Kim *et al.*, 1994), we tried a methodological approach of isolating the natural endogenous substrate for the next study. To do this, we set up several purification steps, such as ion-exchange (DEAE Sephacel), size exclusion (G-100), and HPLC (Protein PAK-125), and the selection of each fraction was guided by the MAC assay (Table III). Through ion-exchange chromatography (Fig. 4A), we obtained four fractions from each concentrated peak. As the MACs of three fractions (Peaks II, III and IV) were similar, we, first, chose Peak II, which had enough protein for the next steps. Thus, the concentrated Peak II was further applied to size-exclusion chromatography on G-100, and after repeating two more times, Peak II-2-half-B-half-B was obtained for the next preparation (Fig. 4B and C, and Table III). Through these chromatography processes, the endogenous substrate activity arose up to 18-fold. Using the concentrated Peak II-2-half-B-half-B fraction, two repetitive HPLC steps were further added to prepare a final 11-min fraction.

Verification of 23 kDa substrate

To verify whether our column chromatography steps have been properly performed in isolating the natural endogenous substrate for PCMT, acidic-conditioned gel electrophoresis was used. As Fig. 5 depicts, we eventually isolated a 23-kDa endogenous substrate protein.

Table III. Fractionation of natural substrate proteins for PCMT in porcine spleen*

Fraction	Methyl accepting capacity (pmol/mg protein)
Cytosol (105,000×g supernatant)	3.2
80% ammonium sulfate fraction	10.8
DEAE Sephacel chromatography	
Peak I	16.4
Peak II	26.8
Peak III	26.8
Peak IV	30.0
Sephadex G-100 chromatography (First)	
Peak II-1	38.0
Peak II-2	45.6
Peak II-3	52.4
Sephadex G-100 chromatography (Second)	
Peak II-3-half A	25.6
Peak II-3-half B	59.2
Sephadex G-100 chromatography (Third)	
Peak II-3-half B-half A	23.2
Peak II-3-half B-half B	54.4

*Fractions to test were incubated with purified PCMT and 20 mM of [methyl-^{14}C] AdoMet for 20 min at 37°C. The MAC was measured by the methanol extraction method as described in Materials and Methods.

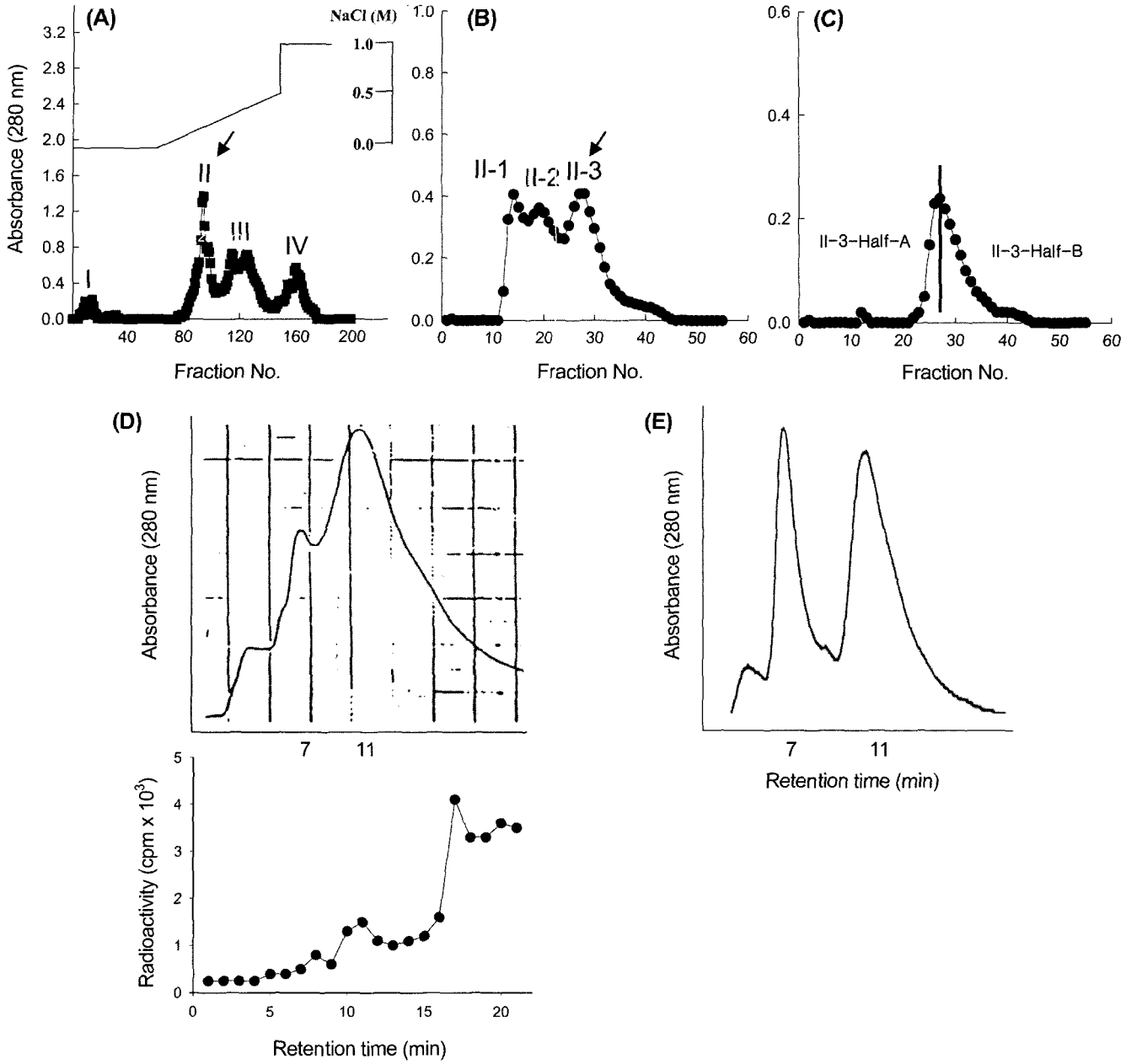

Fig. 4. Column chromatography of natural endogenous substrates for PCMT on DEAE Sepacel, G-100 and protein PAK-125 columns. **(A)** Fraction I (15 mL) was applied to DEAE Sephacel column (3×30 cm), and fractions were obtained as described in Materials and Methods. The pooled fraction was concentrated by ultrafiltration, and the MAC was determined as described in Materials and Method. **(B** and **C)** Peak II-3 (arrow) was applied to Sephadex G-100 column (1.5×90 cm) under the conditions described in Materials and Methods. Re-separation was carried out with the indicated fraction (Peak II-3-half-B) under the same conditions. **(D)** The first HPLC was performed with the concentrated fraction (Peak II-3-half-B-half B) after incubation with purified PCMT and [methyl-^{14}C] AdoMet for 2 h. The incubation mixture was applied to the protein PAK-125 column (7.8×300 mm) and fractionated as described in Materials and Methods (upper panel). Each fraction (500 μL) was directly counted by the liquid scintillation counter, and the radioactivity profile was shown in the lower panel. **(E)** To obtain the fractions having the highest radioactivity, non-labeled fraction (Peak II-3-half B-half B) was applied under the same conditions, and fractions having the same retention time (11 min) were pooled and concentrated by ultrafiltration for applying the second HPLC. The concentrated 11-min fraction was then applied to the same column for re-chromatography, and the second peak (11 min) was further analyzed for testing the MAC and the identification of natural endogenous substrate.

DISCUSSION

Functional role of protein carboxylmethylation (PCM) has not been clearly elucidated yet on the tissue level. In this study, we examined biochemical features of PCM [occupying 65% of total protein methylation (Fig. 1A)] in

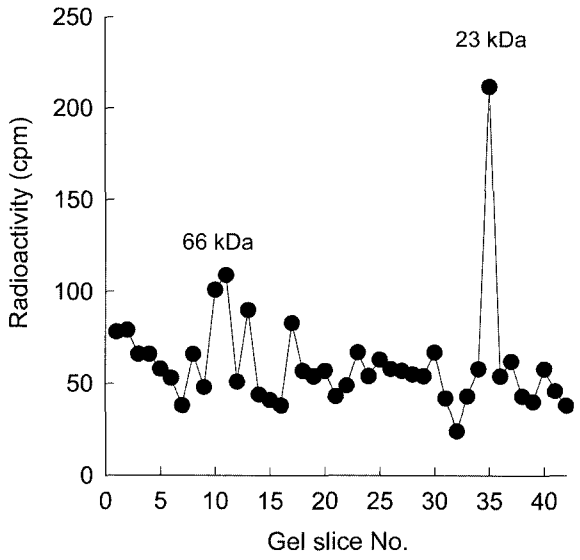

Fig. 5. Identification of the isolated natural substrate prepared from the second HPLC by acidic electrophoresis. The second peak concentrated by lyophilization from second HPLC was incubated with purified PCMT and radioactive [methyl-^{14}C] AdoMet for 2.5 h at 37°C, and then, 160 μL (80 μL of fraction and 80 μL sample buffer) was loaded. Dried gel lanes were cut into 0.2 cm slices and mixed with 75 μL of 2 M NaOH in a 1.5 μL microcentrifuge tube. Radioactivity was assayed by the Gel slice methanol vapor phase diffusion assay.

porcine spleen that was assessed by (i) quantitative analysis of the MAC and PCM products, CMEs under different pH conditions, (ii) identification of natural endogenous substrates for spleen PCMT by electrophoresis, (iii) sensitivity of the MAC to proteolytic digestion and pH changes, and finally, (iv) isolation of one of the natural endogenous substrate proteins.

Many different CMEs that were generated by spleen PCM can be easily speculated by the analysis of hydrolytic kinetics of CMEs upon mild alkaline conditions ranging from pH 7.55 to pH 8.9 (Terwilliger and Clarke, 1981; O'Connor and Clarke, 1984). Because the identification of CMEs could be important for the functional suggestion of PCM, we initially analyzed the hydrolytic kinetics of spleen CMEs by measurement of the radioactive methanol formation from cytosolic fraction, which displayed the highest MAC (Table I). Each $T_{1/2}$ value of the CMEs and the portion of each CME were calculated using a kinetics regression program, RSTRIP software (Cho *et al.*, 2001). Fig. 1B and Table II show that the spleen PCM consists of three different classes of CMEs with half-times of 1.1, 13.9, and 478.2 min, respectively, and with proportions of 82%, 5%, and 13%, respectively. This feature suggests that there are three classes of CMEs, and most of them are base-labile, similar to the results of previous papers (Cho *et al.*, 2001; O'Connor and Clarke, 1984; Terwilliger and Clarke, 1981), although $T_{1/2}$ values are variable. Thus, we previously reported that porcine liver and testis have three distinct CMEs with $T_{1/2}$ ranged from 1 to 800 min (Cho *et al.*, 2001). Under the same conditions, O'Connor and Clarke (1984) reported three methylation sites having half-lives of 6.5 min (60%), 19 min (30%), and 300 min (10%). CMEs displaying 20% ($T_{1/2}$ = 3 min), 70% ($T_{1/2}$ = 30 min), and 10% ($T_{1/2}$ = 360 min) were also found in the erythrocyte membrane, even though hydrolysis kinetics was analyzed at pH 7.55 (Terwilliger and Clarke, 1981). Therefore, our data, as well as previous reports, suggest that mammalian PCM may commonly be composed of three distinct classes of CMEs regardless of cell or organ type.

Compared to the CMEs generated by aspartic acid and glutamic acid, spleen PCM contained two kinds of rapidly hydrolyzed CMEs with $T_{1/2}$ values of 1 and 14 min (Terwilliger and Clarke, 1981). According to previous papers (Aswad and Deight, 1983; Ingrosso *et al.*, 2000; Farrar and Clarke, 2002; Clarke, 2003), these basic-labile carboxylmethylation sites seemed likely to be derived from L-isoaspartyl and D-aspartyl residues which appear in aged proteins and are catalyzed by class I PCMT. In contrast, 10% of the minor CME ($T_{1/2}$ = 478 min) seems to be a stable class which may be generated by class II or class III PCMT. Thus, the third minor site is suggested to be α-carboxylmethyl ester formed in cysteine or leucine residues, which are mainly found in signaling molecules (Hrycyna and Clarke, 1993; Xie and Clarke, 1994; Lee and Stock, 1993; Vafai and Stock, 2002). Similarly, on the substrate level, the strong radioactivity (MAC) of major substrate proteins that were detected upon acidic-conditions (Fig. 2A) disappeared when the basic-conditioned gel electrophoresis was performed. Instead, other endogenous substrates exhibiting weak MAC were newly identified even under alkaline condition (Fig. 2B). Therefore, our data suggest that spleen PCM may be mainly catalyzed by class I PCMT and partly catalyzed by class II or III, as shown in other tissues such as liver and testis (Cho *et al.*, 2001; O'Connor and Clarke, 1984; Terwilliger and Clarke, 1981).

Although properties of CMEs in the spleen were similar to other tissues or cells, distribution patterns of endogenous substrates obtained by acidic- or basic-conditioned gel electrophoresis were distinguishable from other tissues (liver and kidney) or cells. Fig. 2 depicts the cytosolic fraction containing three major methyl accepting proteins that include 14, 31, and 86 kDa polypeptides and 3 to 5 minor proteins under acidic condition. However, the new endogenous substrates (63 and 24 kDa) were only seen in alkaline conditions. The pattern under the same acidic conditions was quite distinct from that of liver (26, 33 and 80 kDa) (Cho *et al.*, 2001) and kidney [34 and 55 kDa (data not shown)]. Also, the difference was clearly found in the cases of other tissues or cells. For example, Gingras

et al. (1991) showed that many of the cytosolic substrate proteins (14, 17, 21, 27, 31, 48, 61, and 168 kDa) were identified from the kidney brush border membrane through acidic electrophoresis. Several proteins (34, 38, 53, and 61 kDa) were found to be carboxylmethylated in PC12 cells (Najbauer *et al.*, 1991). Methyl accepting substrates from erythrocytes (O'Connor and Clarke, 1984; Terwilliger and Clarke, 1981), the pituitary cell (Vincent and Siegel, 1987), and the brain (Aswad and Deight, 1983) were also found to be different. Even though there are some proteins with similar molecular weights, the PCM patterns of the spleen judged by substrate distribution seem to be unique in a certain tissue or cell. Interestingly, however, cytosolic fraction from the testis exhibited a very similar distribution of spleen endogenous substrates (14, 25, 32, and 86 kDa) (Cho *et al.*, 2001), and this similarity was also confirmed by the effect of proteolytic digestion and pH changes on the MAC (Fig. 3). Therefore, our results probably suggest that spleen PCM catalyzed by class I PCMT may be similar to that of the testis and can be distinguished from PCM in other tissues (liver and kidney) and cells in terms of the endogenous substrate level. Furthermore, our results also demonstrate that the PCM pattern can be more clearly distinguished by investigation into the endogenous substrate level

Based on the PCM patterns on the tissue level, we tried a methodological approach in order to isolate one of endogenous substrates using the conventional column chromatography (summarized in Table III) and to examine its MAC in acidic conditions. To do this, we first chased a reasonable higher MAC from cytosolic fractions, which displayed the highest total MAC. Due to low methylation activity and broad substrate specificity, some purification steps were repeated for ensuring a sufficient amount. Using various purification methods (Fig. 4), we eventually confirmed the isolation of a 23-kDa protein by gel electrophoresis followed by staining with Coomassie blue dye (data not shown). Carboxylmethylation of this protein was only detected by acidic [but not basic (data not shown)] electrophoresis after radiolabelling of the final fraction with [methyl-^{14}C] AdoMet and PCMT (Fig. 5), although there was a minor peak that was not detected by staining with Coomassie blue (data not shown). Since a possibility that the minor peak may be formed by a strong molecular association between 41 kDa purified PCMT (Kim *et al.*, 1994) and the substrate protein, similar to the case of cyclic AMP-dependent protein kinase (Krebs and Beavo, 1979), cannot be excluded, we will further continue to improve the purity of these substrate proteins for more biochemical analysis of the endogenous proteins. Taken together, we isolated the 23-kDa protein using column chromatography and confirmed that the protein is a natural endogenous substrate protein that may be catalyzed by class I PCMT, even though it was not detected by the acidic-conditioned electrophoretic analysis of Fraction I (Fig. 2).

In conclusion, we found that spleen PCM (63% of total protein methylation), which is composed of three distinct classes of CMEs was mostly catalyzed by class I PCMT, according to hydrolytic kinetics at pH 8.9 and the distribution of natural endogenous substrates under both acidic and basic conditioned electrophoreses. The MAC of an isolated endogenous substrate protein (23-kDa) was also detected through acidic-conditioned electrophoresis. Therefore, our data suggest that most of the spleen PCM may be catalyzed by class I PCMT. The potential role of PCMT in relevance to the spleen function, such as filtration and removal of spherocytes and other abnormal red cells or even other immune responses (Law, *et al.*, 1992; Leonard, *et al.*, 1978), will be further explored in the next study.

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