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DISTRIBUTIONAL AND DEVELOPMENTAL VARIATIONS OF MULTIPLE FORMS OF CALPASTATIN IN MOUSE BRAIN*

TAKAO HAMAKUBO, MICHIKO UEDA, EMIKO TAKANO,† and TAKASHI MURACHI‡

Department of Clinical Science and Laboratory Medicine, Kyoto University Faculty of Medicine, Kyoto 606, Japan

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DEAE-cellulose chromatography of mouse brain extract demonstrated the occurrence of two calpastatin fractions, CS-0.1 and CS-0.2, with distinctly higher content of the latter. CS-0.1 emerged from the column at 0.1 M NaCl, inhibited calpain II more strongly than calpain I, and identified also immunologically with hitherto known calpastatin. CS-0.2 emerged at 0.2 M NaCl, inhibited calpain I more strongly than calpain II, and did not crossreact with anti-calpastatin antibody used. Fairly consistent amounts of CS-0.2 and calpain II were found in the brain of mice from 10 days to 10 weeks after birth, while CS-0.1 became measurable only after 4-week growth. In adult mice, CS-0.1 was highest in specific activity in brainstem, lower in cerebellum, and not detectable in cerebral hemisphere.Physiological significance of multiple forms of calpastatin and their variations found is not known.

KEY WORDS: Brain development, calpain, calpastatin, mouse brain, multiple forms.

INTRODUCTION

Calpastatin, an endogenous inhibitor protein acting specifically on calpain (EC 3.4.22.17; Ca^{2+}-dependent cysteine endopeptidase) is known to be as widely distributed among various tissues as calpain is, but the enzyme-inhibitor ratios vary greatly from tissue to tissue.[1-3] Apparent molecular diversities of calpastatin preparations from different sources had been noticed,[4-6] but some of them were found to be due to different degrees of association of protomeric calpastatin[7] and also due to proteolytic fragmentation of the parent molecule.[8] Seemingly true or protomeric difference is found between calpastatin from erythrocytes and that from many other tissues including the cardiac muscle, liver and lung.[2,5] However, recent analysis of cloned cDNA for calpastatin has indicated the derivation of erythrocyte-type calpastatin from a longer length cardiac muscle-type species.[9,10] The latter molecule consists of four internaly repetitive domains 1 to 4 and one non-homologous amino terminal domain L, each with approximately 140 amino acid residues.[9-11] Erythrocyte calpastatin was found[12] to lack entire domain L and a part of domain 1. Individual domain

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†Present address: Central Clinical Laboratory, National Kyoto Hospital, Kyoto 612, Japan.
‡To whom correspondence should be addressed.

Abbreviations: EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol bis(β-amino-ethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate.

1 to 4 and also truncated fragments therefrom were found to exhibit inhibitory activity against calpain.[13-15] All these longer or shorter calpastatin species are known[1,13,15] to inhibit calpain II that requires high or mM Ca^{2+} for activation more strongly than they do calpain I that requires low or μM Ca^{2+}.

By contrast, we have recently found[16,17] that the brain tissues contained significant amounts of calpastatin(s) that inhibits calpain I more strongly than calpain II. Since the calpain I-favoring inhibitor fraction emerged from a DEAE-cellulose column at pH 7.5 at a NaCl concentration of approximately 0.2 M, it was termed CS-0.2, compared with CS-0.1 for the hitherto well characterized calpastatin fraction that emerged from the same column at approximately 0.1 M NaCl.[16,17] Partial purification and characterization of CS-0.2 inhibitor(s) from pig brain has revealed that it has a lower molecular weight than CS-0.1, or ordinary calpastatin, and that it does not cross-react with antibodies raised against pig cardiac muscle and human erythrocyte calpastatins (E. Takano *et al.*, to be published elsewhere). The present paper describes the occurrence of CS-0.2 also in mouse brain and its distributional and developmental variations.

MATERIALS AND METHODS

Materials

Casein (Hammarsten grade), and monoiodoacetic acid were obtained from E. Merck, Darmstadt, Germany, dialyzing tubing Spectrapor 3 from Spectrum Medical Industrial Inc., Los Angels, CA, DEAE-cellulose (DE52) from Whatman, Kent, U.K., and electrophoresis calibration kit from Pharmacia Fine Chemicals, Uppsala, Sweden. The nitrocellulose membranes were purchased from Scheicher & Schüll, Dassel, Germany, and peroxidase-conjugated affinity-purified goat anti-rabbit IgG was from Miles, Elkhart, IN. All other reagents were of analytical grade and were products of Wako Pure Chemicals, Osaka, or Nakalai Tesque, Kyoto, Japan.

Animals

Adult A/Jackson mice were obtained from SLC Co., Hamamatsu, Japan. After mating, ofspring ranging in age from 10 days to 10 weeks after birth were produced in specific pathogen-free system at the Institute of Laboratory Animals, Kyoto University. Animals were supplied freely with food and water. Only male mice were used for distribution study.

Tissue preparation

Under diethyl ether anesthesia, mice were perfused from the left ventricle with 10 ml of phosphate-buffered saline and then with 20 ml of buffer A (20 mM Tris-HCl, containing 1 mM EDTA, 1 mM EGTA, and 5 mM 2-mercaptoethanol, pH 7.5) supplemented with 0.25 M sucrose. The brains were rapidly excised after perfusion, and the cerebellum, brainstem, and cerebral hemisphere were dissected.

Preparation of crude extract

Tissues were homogenized with three volumes of ice-cold buffer A, containing 0.25 M

sucrose, and then centrifuged at 105,000 × *g* for 60 min. The supernatant was dialyzed overnight at 4°C against buffer A with 50 mM NaCl using Spectrapor 3 membrane tubing.

Calpain and inhibitor assay

Calpain activity was determined with heat-alkali denatured casein as the substrate.[18] Each reaction mixture, in a final volume of 1.0 ml, contained 0.4% casein, 50 mM imidazole-HCl buffer(pH 7.5), 5 mM cysteine, 5 mM $CaCl_2$, and 0.5 ml of the sample, filled up with buffer A. After an incubation for 30 min at 30°C, the reaction was terminated by adding 1 ml of 5% trichloroacetic acid. Acid-soluble products were determined by the method of Ross and Schatz.[19] The mixture after reaction without $CaCl_2$ was used as the blank. One unit of calpain was defined as the amount of enzyme that caused an increase in absorbance at 750 nm of 1.0 under the above conditions.

Inhibitor samples were heat treated at 100°C for 5 min, and the activity was measured by the method of Murakami *et al.*,[20] using calpain I purified from human erythrocytes[21] or calpain II purified from porcine kidneys.[22] One unit of inhibitory activity was defined as the amount which could inhibit one unit of calpain under the standard assay conditions.

Affinity-purified anti-calpastatin IgG

Affinity-purified anti-calpastatin antibody specific to human erythrocyte 70 kDa calpastatin was obtained as previously described.[5]

Western blot analysis

Marker proteins and inhibitor fractions were first subjected to electrophoresis in the presence of SDS according to the procedure of Laemmli,[23] using 10% separating gel and 3% stacking gel. Proteins on the gel were transferred to nitrocellulose membrane, using a Bio-Rad transblot cell.[24] One half of the nitrocellulose membrane containing marker proteins was stained with Amido Black, and the other half was immunostained using anti-calpastatin antibody. The peroxidase staining was developed using diaminobenzidine as the substrate according to the method of Hawks *et al.*[25]

RESULTS

Separation of CS-0.1 and CS-0.2

The crude extract from the whole brain of 4 A/Jackson mice (6 weeks after birth) was applied to a 5-ml DE-52 column pre-equibrated with buffer A containing 50 mM NaCl, and eluted with a linear gradient of NaCl up to 0.5 M. Each fraction was assayed for calpain and calpastatin activities (Figure 1). Ca^{2+}-dependent caseinolytic activity was observed at 0.25 M NaCl, which corresponds to calpain II activity.[22] Two distinct inhibitory peaks, against human erythrocyte calpain I, were observed, one at 0.1 M NaCl (CS-0.1) and the other at 0.2 M NaCl (CS-0.2). Inhibitory activity *without* the heat-treatment was also assayed, and the result was essentially the same as that *with* the heat-treatment (data not shown), suggesting that calpain I, if present, must be very low in content. Neither CS-0.1 nor CS-0.2 inhibited trypsin and papain (data

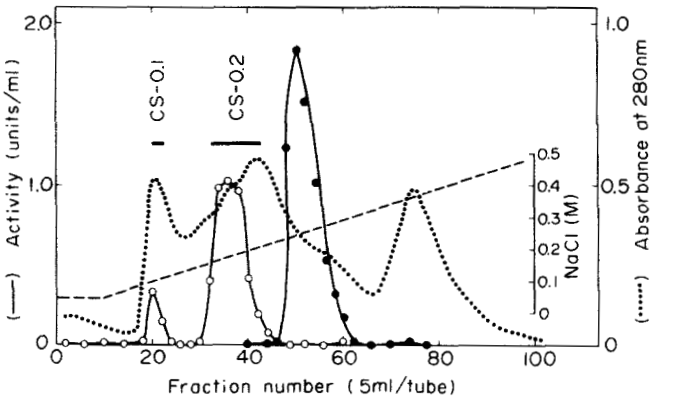

FIGURE 1 DEAE-cellulose (DE52) chromatography of brain crude extract. The crude extract from the whole brain of 30 A/Jackson mice (8 weeks after birth) was applied to a 1.6 × 15 cm DE52 column, which was then eluted with a 0.05–0.5 M linear gradient of NaCl. ●, calpain activity; ○, calpastatin activity as assayed against human erythrocyte calpain I. Horizontal bars indicate the respective fractions collected as CS-0.1 and CS-0.2 materials.

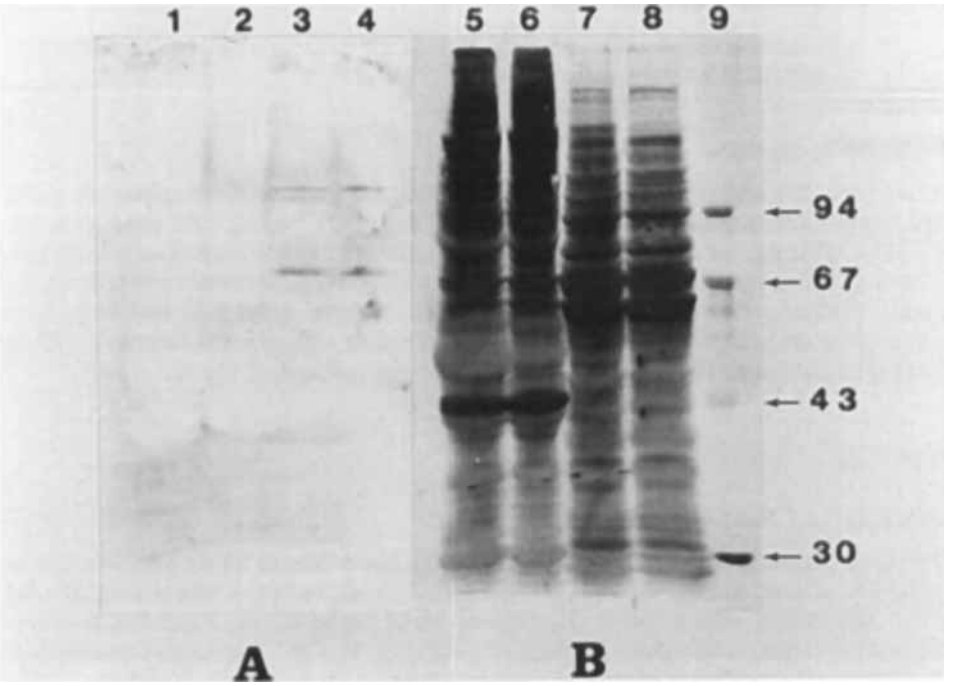

FIGURE 2 Western blot analysis of CS-0.1 and CS-0.2 by anti-calpastatin antibody. A, immunostaining; B, Amido Black staining. Lanes 1 and 5, CS-0.2 (49 μg protein); lanes 2 and 6, CS-0.2 (63 μg protein); lanes 3 and 7, CS-0.1 (38 μg protein); lanes 4 and 8, CS-0.1 (56 μg protein). Lane 9 is for marker proteins: phosphorylase b (94.0 kDa), bovin serum albumin (67.0 kDa), ovalbumin (43.0 kDa) carbonic anhydrase (30 kDa).

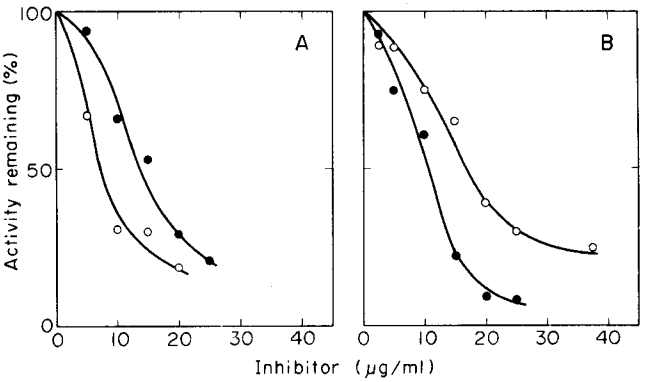

FIGURE 3 Inhibition of calpains I and II by CS-0.1 (A) and CS-0.2 (B). ●, calpain I; ○, calpain II.

not shown). The same inhibitory activities were observed in other strains, Balb/c, C-57/black, and C3H (data not shown).

Characterization of CS-0.1 and CS-0.2

Aliquots of CS-0.1 and CS-0.2 peak fractions were subjected to Western blot analysis. As shown in Figure 2, two major bands, at 110 and 70 kDa, were observed for the CS-0.1 fraction, which are compatible with the reported apparent diversity of calpastatin.[5] The CS-0.2 fraction had no bands that cross-reacted with the calpastatin-specific antibody.

As shown in Figure 3, both CS-0.1 and CS-0.2 inhibited calpains I and II in a dose-dependent manner. There was, however, clear difference between them: CS-0.1 inhibited more strongly calpain II than calpain I, whereas CS-0.2 inhibited more strongly calpain I than calpain II.

Age-related changes

When the young animals (5 weeks after birth) were used, very low CS-0.1 activity was

TABLE I
Age-related changes of CS-0.1, CS-0.2, and calpain II in mouse brain

Age	Specific activity (units/g wet tissue)		
	CS-0.1	CS-0.2	Calpain II
10 days	0.01	4.65	4.49
16 days	0.01	4.46	2.46
3 weeks	0.01	3.71	4.62
4 weeks	0.15	5.87	5.36
10 weeks	0.43	5.01	5.58

The cude extract from 2 g wet weight brain tissues, collected from 5–7 A/Jackson mice, was applied to a 1.3 × 3.8 cm DE-52 column. Fractionation by elution with a NaCl gradient was carried out in the same way as that shown in Figure 1. Inhibitor and enzyme activities were calculated by summing up the activity of each fraction. Inhibitor activity was assayed against human erythrocyte calpain I.

TABLE II
Distribution of CS-0.1, CS-0.2, and calpain II in adult mouse brain.

Tissue	Specific activity (units/g wet tissue)		
	CS-0.1	CS-0.2	CalpainII
Cerebral hemisphere	0.01	4.32	4.62
Cerebellum	0.22	6.24	6.58
Brainstem	1.38	5.78	6.97

Brain tissues were collected from 32 mice of 10 weeks after birth. The other methods were the same as those given under Table I.

observed. Then, we examined the age-related changes of both inhibitors and enzyme, using DE-52 chromatography (Table I). CS-0.1 activity was below the detectable level until 3 weeks after birth, and it gradually increased. CS-0.2 and calpain II activities were relatively stable from 10 days to 10 weeks after birth.

Distribution in mouse brain

Distribution of inhibitors and enzyme was also examined by the same method as above using adult, 10-week-old mice (Table II). CS-0.1 was highest in specific activity in brainstem, and not detectable in cerebral hemisphere. In cerebellum, only a little amount of CS-0.1 was distributed. CS-0.2 and calpain II levels did not show much difference.

DISCUSSION

The chromatographic and also other behaviors of CS-0.1 and CS-0.2 fractions from mouse brain (Figures 1–3) were essentially the same as those for pig brain (E. Takano *et al.*, to be published). CS-0.1 must represent the ordinary calpastatin known in the literature,[1] whereas CS-0.2 seems to be unique in the brain. The brain is known to contain calpain in excess over its content of calpastatin, so that the measurement of calpain acitivity in the brain homogenate could be done without any previous chromatographic separation of calpastatin from calpain.[26] Even so, the occurrence of calpastatin in the elution fractions from a Sephadex G-200 column was demonstrated[27] when each fraction was tested against calpain II. The chromatogram obtained by the present study shows that in adult mouse brain, the total inhibitor activity of the CS-0.2 fraction was higher than that of CS-0.1, while the content of calpain I was very low (Figure 1). This would mean that under such circumstances there will be almost no possibility for calpain I to function in the adult brain, whereas calpain II can be activated, if a sufficiently high concentration of ca^{2+} could be reached *in situ*.

We demonstrated earlier the distributional change of calpain I and calpain II during the development of rat brain[28]. Immunohistochemical studies showed that during the early phase of brain development, neurons contained both calpains I and II, and the disappearance of calpain II from these cells began about 20 days after birth, when the major phase of axon and dendrite growth is ending.[28] The present study demonstrated significant change in the content of calpastatin CS-0.1 during the

development of mouse brain (Table I). Whether our earlier and present findings could in fact be related must await further experimentations.

The distributional uneveness of CS-0.1 and CS-0.2 in different parts in mouse brain (Table II) may not be surprising, since such wide variation of the calpain-calpastatin system in various tissues has been well documented.[1-3] In mouse brain, the relative content of CS-0.1 is highest in brainstem and lower in cerebellum and cerebral hemisphere, but such clear difference was not the case for pig brain (E. Takano *et al.*, to be published).

In the previous studies with nervous tissues, interests were mostly centered on calpain in relation to demyelination,[29] Wallerian degeneration,[30] and Alzheimer disease.[31] Calpastatin in the nervous system had not been well characterized, until our present studies demonstrated the occurrence of multiple forms of calpastatin in the brain. The newly found CS-0.2 seems to be unique in the brain, but it has not been determined whether CS-0.2 is a protein fragment(s) derived from calpastatin CS-0.1 or it is an entirely different genetic product (E. Takano *et al.* to be published). Although CS-0.2 inhibits rather specifically calpain I, it also acts on calpain II (Figure 2). Since CS-0.2 exists in large amount in all areas of the brain (Table II) and at all stages of the development (Table I), it must be the major regulatory factor of calpain in central nervous system.

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