Volume 223, number 1, 174–180 FEB 05219 October 1987

All four internally repetitive domains of pig calpastatin possess inhibitory activities against calpains I and II

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Received 2 September 1987

Complementary DNA portions coding for each domain (domain L and internally repetitive domains, domains 1 4, each composed of approximately 140 amino acid residues) of pig calpastatin were subcloned into *E. coli* plasmids to express the respective portions of the proteinase inhibitor gene in bacteria. Cell extracts of *E. coli* harboring recombinant plasmids were assayed for calpain inhibition. All four internally repetitive domains showed inhibitory activities, essentially similar to one another, against calpains I and II. No inhibition was observed in the case of the N-terminal non-homologous domain (domain L). These results support our previous conclusion that the repetitive region is a functional unit of the proteinase inhibitor.

Calpastatin; cDNA; Expression; Proteinase inhibitor

1. INTRODUCTION

Calpastatin is an endogenous inhibitor protein acting specifically on calpain (EC 3.4.22.17; Ca^{2+}-dependent cysteine proteinase) (review [1]). Both calpain and calpastatin are known to be widely distributed in mammalian and avian cells [2,3]. Although physiological roles of calpain have not yet been clarified, the proteinase-proteinase inhibitor system has been suggested to play important roles in various cellular functions coupled with calcium ion mobilization [4–8].

Recently we determined a primary structure of pig calpastatin by nucleotide sequencing of cloned cDNAs and by the Edman degradation of the purified inhibitor from pig heart muscle [9]. As will be published elsewhere (Takano, E. et al., in preparation), the inhibitor protein is composed of 713 amino acid residues, and contains four internally repetitive sequences (domains 1, 2, 3 and 4) and one non-homologous sequence on the N-terminal side (domain L), each being separated by intervals of approx. 140 amino acid residues. This structural feature is similar to the recently published structure of rabbit calpastatin [10].

Previously we demonstrated that one such repetitive region (domain 3) had calpain-specific inhibitory activity, and concluded that the structural domain also represents a functional unit of calpastatin [11]. In the present study, we further subcloned cDNA portions for all the domains of pig calpastatin and compared their inhibitory activities against calpains I and II.

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2. MATERIALS AND METHODS

2.1. *Materials*

Most restriction endonucleases and other enzymes for molecular biology were obtained from Takara Shuzo Co. (Kyoto, Japan). Plasmids pUC118 and pUC119 [12], and a dideox-

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00145793/87/$3.50

ynucleotide sequencing kit were from the same company. *Sac*II and *Nhe*I were from Toyobo (Osaka, Japan) and Nippon Gene (Toyama, Japan), respectively. Other reagent-grade chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) or Nakarai Chemicals (Kyoto, Japan).

2.2. *Preparation of synthetic oligonucleotides*

Oligodeoxyribonucleotides for in vitro mutagenesis primers were synthesized by the phosphoramidite method [13] with an automatic DNA synthesizer (Applied Biosystems, model 380B). After the synthesis, oligonucleotides were purified by high-performance liquid chromatography under the conditions recommended by Applied Biosystems and phosphorylated by T_4 polynucleotide kinase as described [14].

2.3. *Site-directed mutagenesis*

Oligonucleotide-directed in vitro mutagenesis [15] was performed using a mutagenesis kit supplied by Amersham (Amersham, England) according to the provided protocol except employing single-stranded DNAs of pUC118/119 derivatives as templates and reducing the reaction volume to a half of the original volume. Single-stranded DNAs were produced by infecting transformants of either *E. coli*·MV1304 or MV1184 harboring pUC118/119 derivatives with helper phage M13K07 as described by Vieira and Messing [12]. After polyethyleneglycol precipitation [16], phage particles obtained from a 2-ml culture were treated with 0.1 ml of 50 μg/ml DNase I and 5 μg/ml RNase A in 10 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ at 37°C for 30 min to digest contaminating DNA and RNA derived from host cells. After the addition of 20 μl DNase stop solution (50 mM EDTA in 1.5 M sodium acetate, pH 7), single-stranded DNAs were extracted with phenol, and subsequently with chloroform as described [16].

Filtration of dCTPαS incorporated DNA through nitrocellulose filters was performed with a microfiltration unit S&S009 (Schleicher & Schuell, Dassel, FRG). After exonuclease III digestion, repair synthesis was primed with 5′-phosphorylated synthetic oligonucleotide corresponding to an M13/pUC reverse primer (5′-TGTGGAATTGTGAGCGG-3′). Mutants were screened by restriction analysis of isolated plasmids from transformants of *E. coli* MV1304 or MV1184. Mutation was confirmed by nucleotide sequencing.

2.4. *Subcloning of calpastatin cDNA fragment*

Restriction fragments of calpastatin cDNAs were inserted into multiple cloning sites of pUC118, pUC119 or pUC118N, using T_4 DNA ligase. A *Sac*II/*Eco*RI fragment from pPECS14 and a 2-kb *Pst*I fragment from pOBCS1 was blunt-ended with T_4 DNA polymerase as described [17] before ligation to the *Hin*cII site of pUC119 and the *Sma*I site of pUC118, respectively. A *Kpn*I/*Eco*47III fragment from pPECS146NK was inserted into pUC118N at *Kpn*I/*Bam*HI sites where the *Bam*HI cleaved end was filled with a Klenow fragment of *E. coli* DNA polymerase as described [14]. A 16-mer synthetic double-stranded oligonucleotide of a universal translation terminator (5′-GCTTAATTAATTAAGC-3′, Pharmacia, Uppsala, Sweden) was inserted to the *Hin*cII site of pUC118N derivatives to obtain clones pCSL and pCSD1.

2.5. *Calpain inhibition assay of* E. coli *cell extracts*

A preparation of cell extracts of *E. coli* 545πHR1 transformants was performed as described [11]. Addition of cAMP and isopropyl-β-D-thiogalactoside (IPTG) to a culture medium was omitted in the present study. These inducers had little effect on the level increase of calpastatin activities in *E. coli* 545πHR1 transformants.

Protein concentration was determined by the method of Lowry et al. [18] using bovine serum albumin as a standard protein. Calpastatin activity was measured by the method of Murakami et al. [19] using low- (or μM) Ca^{2+}-requiring calpain I purified from pig erythrocytes or high- (or mM) Ca^{2+}-requiring calpain II purified from pig kidneys [20].

3. RESULTS

Fig.1 illustrates the scheme for the construction of recombinant plasmids which expressed each domain of calpastatin in *E. coli*. Calpastatin cDNA clones pOBCS1 [9] and pPECS14 [Takano et al. (1987), see above] were isolated and used for fur-

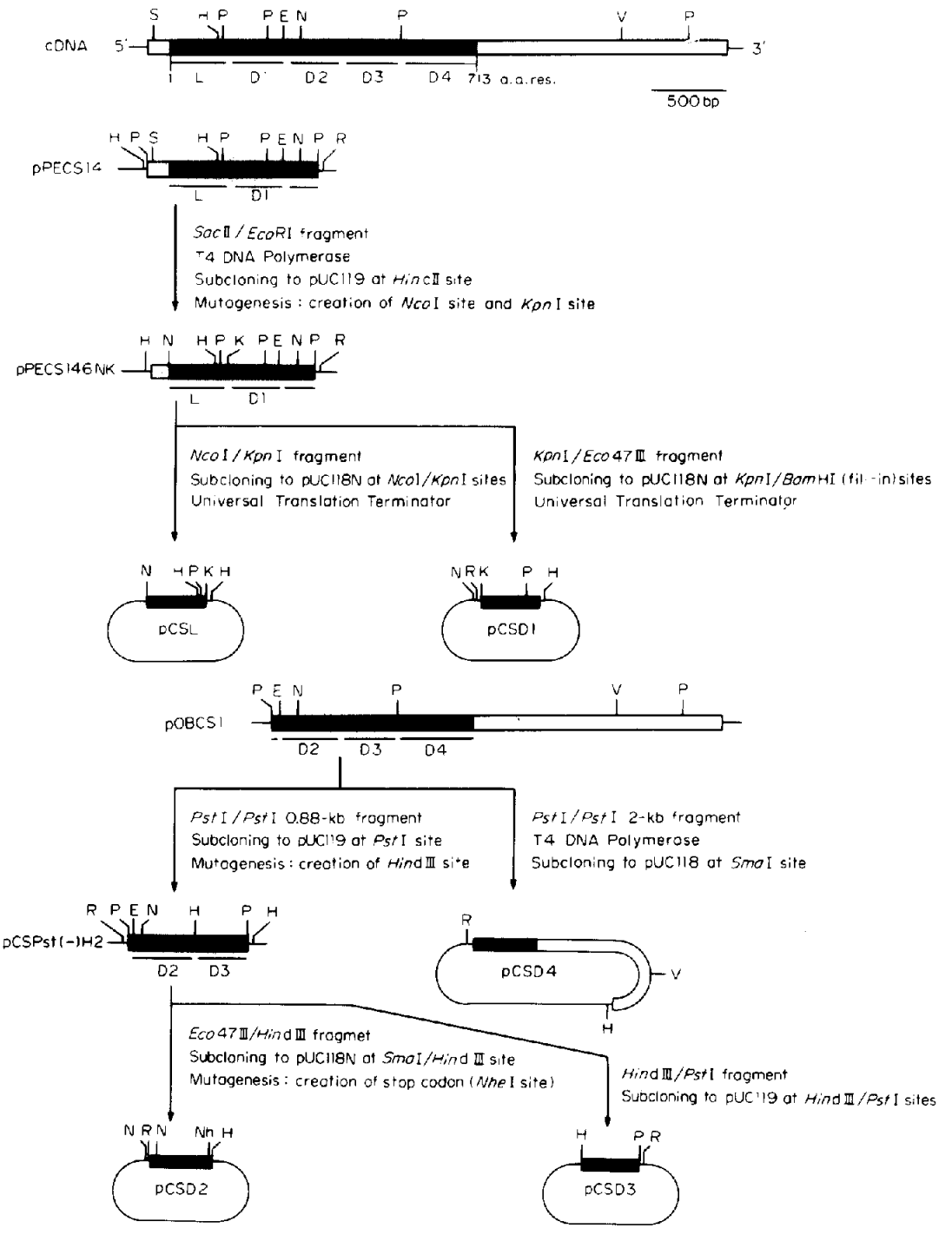

Fig.1. Scheme for the construction of plasmids which express calpastatin domains in *E. coli*. Closed boxes and open boxes indicate translated and untranslated regions of calpastatin cDNAs. Abbreviations are used for restriction endonuclease cleavage sites: E, *Eco*47III; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; Nh, *Nhe*I; P, *Pst*I; R, *Eco*RI; S, *Sac*II; V, *Eco*RV. Restriction sites on vector DNAs are shown only for *Eco*RI, *Hin*dIII and *Nco*I. *Pst*I sites on vector DNAs are not shown except the sites of the dG-dC tail on the cDNAs.

ther subcloning. Restriction endonuclease cleavage sites of *Nco*I, *Kpn*I and *Hin*dIII were created in calpastatin cDNAs by in vitro site-directed mutagenesis as described in section 2. This technique was also employed to obtain plasmids pUC118N and pUC119N by creation of a *Nco*I site surrounding the translation initiation methionine codon of *lacZ'* of pUC118 and pUC119 (fig.2). The distance between the ribosome binding site and the initiation codon was also changed from 7 to 8 nucleotide residues. This structure is similar to that of an efficient expression vector pKK233-2 constructed by Amann and Brosius [21].

Appropriate restriction DNA fragments which contained each domain were subcloned into multiple cloning sites of pUC118, pUC119 or pUC118N (figs 1 and 2). Construction of pCSD3 was described in [11]. Translation termination codons were introduced by a 16-mer double-stranded oligonucleotide of a universal translation terminator (pCSL and pCSD1), by in vitro site-directed mutagenesis (pCSD2) or by frameshift insertion at the *Pst*I site which precedes a stop codon (TAG) in the *Xba*I site (pCSD3). The translation termination codon of pCSD4 is derived from the calpastatin cDNA. Thus the calpastatin segments expressed in *E. coli* contained non-calpastatin amino acid residues at N-termini (pCSD1, pCSD2, pCSD3 and pCSD4) or at C-termini (pCSL, pCSD1 and pCSD3).

Crude cell extracts of *E. coli* transformants were tested for calpain inhibition (fig.3). Both calpains I and II were inhibited in a dose-dependent manner by the extracts from *E. coli* cells harboring pCSD1, pCSD2, pCSD3 and pCSD4. No inhibition was observed in the case of pCSL under the conditions used. Since only crude *E. coli* cell extracts were used, the comparison of the inhibitory activities among the four domains (Panels C–F) may not be quantitative. However, assuming the efficiency of

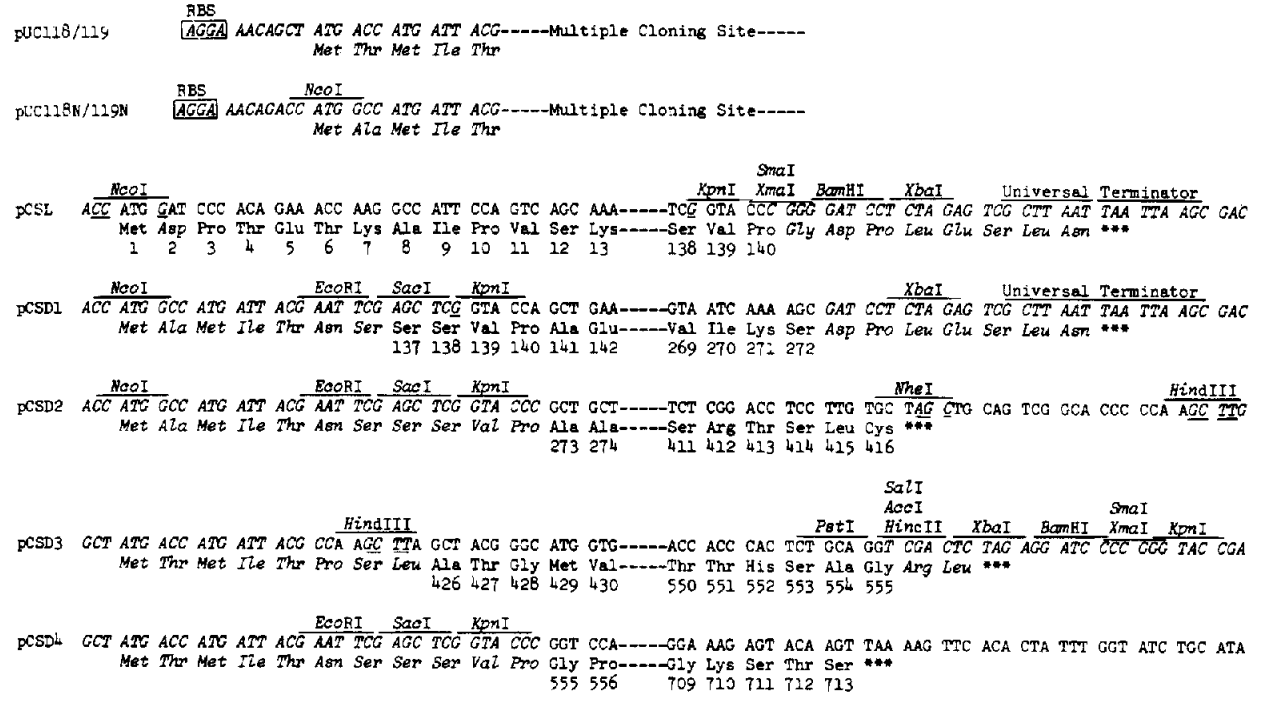

Fig.2. Nucleotide and amino acid sequences around N-termini and C-termini of calpastatin domains which are expressed in *E. coli*. Non-calpastatin residues are italicized. Substituted nucleotides by in vitro mutagenesis are underlined. Numbers below the amino acid residues indicate the residue number of pig calpastatin. Ribosome binding sites (RBS) of *lacZ'* genes of pUC118/119 and of pUC118N/119N are boxed. Asterisks indicate translation termination codons. Recognition sequences of major restriction endonucleases and universal translation terminators (16-mer) inserted at the *Hin*cII sites are indicated by solid lines above the nucleotide sequences.

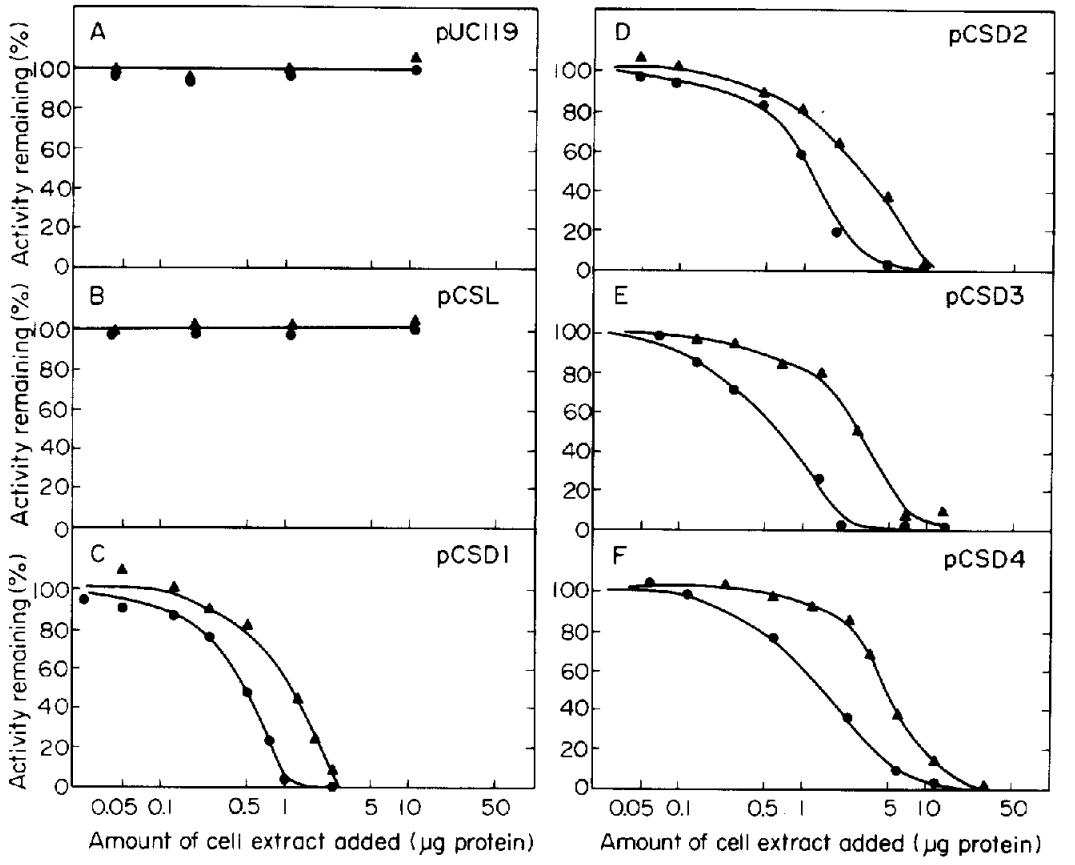

Fig.3. Inhibition profiles of calpains I and II. Proteolytic activities of calpain I (●) and calpain II (▲) were assayed in the presence of various amounts of crude cell extracts of *E. coli* harboring recombinant plasmids which expressed each domain of calpastatin: (A) pUC119, negative control; (B) pCSL, domain L; (C) pCSD1, domain 1; (D) pCSD2, domain 2; (E) pCSD3, domain 3; (F) pCSD4, domain 4.

the synthesis of proteins which includes the expression of these calpastatin segments in *E. coli* harboring different plasmids to be comparable, the four products seem to have similar specific activity. With all the four domain proteins, the inhibition of calpain I is weaker than that of calpain II, which is in agreement with what was previously reported in the case of intact calpastatin molecules [4].

4. DISCUSSION

Previously we claimed that the repetitive region of pig calpastatin is the functional unit of the proteinase inhibitor by demonstrating the production in *E. coli* of an active calpastatin segment which is composed of only one repetitive region (then designated as region Y, corresponding to domain 3 in the present report) [11]. The present study has now clarified that all of the four repetitive regions (domains 1–4), but not the non-homologous region (domain L), possess the capability of inhibiting calpains (fig.3). Domains 1–4 showed inhibitory activities essentially similar to one another, suggesting that these are almost equivalent functional units of a calpastatin molecule. This agrees with previous stoichiometrical observations that calpastatins have multiple binding sites for calpains [22]. Although stoichiometrical analysis of calpain binding to purified calpastatin segments still has to be carried out, each homologous domain is likely to bind to one calpain molecule. Imajoh et al. [23] reported

that the *E. coli*-expressed, truncated rabbit calpastatin, which contained two internally repetitive domains, inhibited 2 mol calpains.

The multiple and internally repetitive substructure of a proteinase inhibitor is not unique to calpastatin. Kininogens contain three cystatin-like sequences in the N-terminal sides of the molecules (heavy chains) [24]. By stoichiometrical analysis, Higashiyama et al. [25] showed two binding sites for papain per one molecule of human high-molecular-mass kininogen as well as of human low-molecular-mass kininogen. Using purified proteolytic fragments of human low-molecular-mass kininogen, Salvesen et al. [24] showed that two of the three repetitive segments have inhibitory activities against papain and cathepsin L. It is postulated that one of the repetitive domains lost its inhibitory function during evolution [26]. In contrast, all four repetitive domains of calpastatin maintain their inhibitory functions as demonstrated in the present study.

Although multiplication of a unit inhibitor domain in calpastatin during evolution must have contributed to the increase of inhibitory capacities per one molecule of protein (4-fold, if disregarding any steric effect), biological significance of this multidomain structure remains to be solved. It is interesting to note that erythrocyte calpastatin may contain only three repetitive domains [Takano et al. (1987), see above]. Yamato et al. [27] reported the change of molecular size of calpastatin (from 280 kDa to 34 kDa, estimated by gel filtration under non-denaturing conditions) in rat liver after the administration of phenylhydrazine. It is not known whether such smaller molecules are formed at the transcriptional or post-transcriptional stage of the biosynthesis of the calpastatin protein. More detailed structure-function relationship studies are required with various molecular forms of calpastatin.

ACKNOWLEDGEMENTS

We thank Tsutomu Osawa for the technical assistance, and Nobuko Ohmori for the preparation of the manuscript. This work was supported in part by Grants-in-Aid for Scientific and Cancer Research from the Ministry of Education, Science and Culture, Japan, and a grant from Muscle Dystrophy Association, USA (to T.M.).

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