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A putative Ca^{2+}-binding protein: Structure of the light subunit of porcine calpain elucidated by molecular cloning and protein sequence analysis

(Ca^{2+}-dependent proteinase/DNA sequence/sequence homology/E–F hand structure/glycine-rich polypeptide)

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ABSTRACT **cDNA clones specific for the light subunit of porcine calpain I have been isolated from a porcine kidney cDNA library. The complete primary structure of the light subunit has been revealed by nucleotide sequence analysis of the cDNA clones isolated and amino acid sequence analysis of peptides isolated from the purified mature protein. We found that the light subunit contains two distinct domains. Domain I, the amino-terminal half, has two unusually long, paired polyglycyl sequences and may serve as a binding site to the heavy subunit. Domain II, the carboxyl-terminal half, is a region highly homologous to the putative Ca^{2+}-binding domain of the heavy subunit of chicken calpain elucidated recently. This region has four potential Ca^{2+}-binding sites, each having the "E–F hand" structure. Our results suggest that the Ca^{2+}-mediated proteolytic activity of calpain is controlled through the cooperative and/or sequential actions of multiple Ca^{2+}-binding sites present in both two-subunit molecules, heavy and light subunits of calpain.**

Ca^{2+}-dependent cysteine proteinases are very widely distributed in mammalian and avian cells (1–3) and collectively called calpain (4) (EC 3.4.22.17). There are two distinct molecular forms: calpain I (μM Ca^{2+}-requiring) and calpain II (mM Ca^{2+}-requiring) (1, 4, 5). Both calpains I and II are heterodimeric, each composed of one heavy (≈80 kDa) and one light (≈30 kDa) subunit (1, 2). The heavy subunit is known to be the catalytic subunit (6, 7), whereas the function of the light subunit is largely unknown. In the course of *in vitro* reconstitution of the heterodimeric calpain molecule from its once denatured-and-separated subunit components, the light subunit exerts a facilitating effect on the heavy subunit to enhance its catalytic activity as well as to lower its Ca^{2+} requirement (6, 7), but it remains uncertain whether this may in fact have any implication for the function of the light subunit. Thus, the role of the light subunit remains to be elucidated. As a first approach to solve the problem, we attempted to elucidate a complete primary structure of the light subunit by means of molecular cloning assisted by protein sequence analysis. We report here the cloning of DNA sequences complementary to the porcine kidney mRNA coding for the light subunit of calpain I. The deduced amino acid sequence has revealed that the light subunit contains a region highly homologous to the putative Ca^{2+}-binding domain of the heavy subunit of chicken calpain (8).

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MATERIALS AND METHODS

Cloning Procedures. Total RNA was extracted from porcine kidney as described (9) and poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography (10). A cDNA library was constructed by the method of Okayama and Berg (11) and screened by hybridization (12) at 36°C with a mixture of 12 oligodeoxyribonucleotides described in *Results*. The oligodeoxyribonucleotides were synthesized by the modified triester methods (13). To isolate cDNA clones coding for the amino-terminal region of protein, a synthetic oligodeoxyribonucleotide primer was elongated by reverse transcription of poly(A)-containing RNA (14, 15), and the resulting cDNA transcripts were cloned into the plasmid pBR322 by using poly(dG)·poly(dC) homopolymeric extensions (15). This library was screened by hybridization with an appropriate restriction fragment derived from the cDNA clone isolated earlier. All of the cloning procedures were conducted in accordance with the guidelines for research involving recombinant DNA molecules issued by the Ministry of Education, Science and Culture of Japan.

Analytical Procedures. Hybridization-selection and cell-free translation were carried out as described (16). DNA sequence analysis was carried out by the procedure of Maxam and Gilbert (17). For clone pPCal 9 (see *Results* and *Discussion*), a *Pst* I restriction fragment was subcloned into plasmid pUC9 (18), and sequence analysis was carried out by the dideoxy method (19). For RNA blot hybridization analysis, porcine kidney poly(A)-containing RNA was denatured with 1 M glyoxal/50% dimethyl sulfoxide, electrophoresed on a 1% agarose gel, and transferred to a GeneScreen membrane (New England Nuclear). Transfer of RNA to the membrane and hybridization were carried out as recommended by the manufacturer.

Amino Acid Sequence Analysis. Calpain I purified from porcine erythrocyte (20) was reduced and *S*-carboxymethylated as described (21). Separation of carboxymethylated subunits was carried out by size-exclusion HPLC, with a Varian 5000 liquid chromatograph, using three TSK gel columns connected in series (G3000SW–2000SW–G2000SW, each 7.5 × 600 mm, purchased from LKB) and equilibrated with 6 M guanidine hydrochloride/10 mM sodium phosphate, pH 6.0. The carboxymethylated light subunit was cleaved with cyanogen bromide in 72% formic acid at room temperature for 15 hr. Digestion of the subunit with endoproteinase

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Lys-C (Boehringer Mannheim) was carried out in 0.1 M ammonium bicarbonate/2 M urea, pH 8.0, at 37°C for 6 hr. Peptides were separated primarily by size-exclusion HPLC, as described for separation of subunits, and purified by reversed-phase HPLC on a column of SynChropak RP-P (SynChrom, Linden, IN) or Ultrapore RPSC (Altex, Berkeley, CA) using a trifluoroacetic acid/acetonitrile system (22) or purified directly by reversed-phase HPLC. Amino acid analysis was performed with a Dionex D500 amino acid analyzer or by reversed-phase HPLC of phenylthiocarbamoyl derivatives (23, 24) using a Waters pico-tag system. Automated Edman degradation was performed with an Applied Biosystems 470A protein sequencer using a program adapted from Hunkapiller *et al.* (25). Phenylthiohydantoin derivatives were identified in a semiquantitative manner by two HPLC systems (26, 27).

A computer alignment of amino acid sequences was performed by the method of Dayhoff *et al.* (28). Secondary structure was predicted according to Chou and Fasman (29) by using conformational parameters of Argos *et al.* (30).

RESULTS

Isolation of cDNA Clones. A porcine kidney cDNA library was screened by hybridization with a mixture of 12 synthetic oligodeoxyribonucleotides, 5′ TTGAT$^{A}_{T}$T$^{A}_{G}$T$^{A}_{G}$TTCCA 3′, which represents cDNA sequences predicted from a partial amino acid sequence of calpain I light subunit, Trp-Asn-Asn-Ile-Lys. Eleven hybridization-positive clones were isolated from about 40,000 transformants. Though the length of the cDNA inserts of these clones differed from one clone to another, restriction mapping of them with the endonucleases *Pst* I, *Pvu* II, and *Eco*RI showed the presence of common restriction sites in 10 of these clones, suggesting that they were derived from the same mRNA. From among these 10 clones, 1 was subjected to partial nucleotide sequence analysis and it was revealed that this clone carried a DNA segment encoding another peptide sequence of the light subunit in addition to the one used for the probe. Then clone pPCal 5, which harbored the largest cDNA insert of this group, was chosen and analyzed by hybridization-selection and cell-free translation. When the translation products of mRNAs hybridized to pPCal 5 were immunoprecipitated with rabbit IgG against human calpain I and electrophoresed, a unique band that comigrated with authentic light subunit of calpain was observed. This band was not observed when mRNAs hybridized to pBR322 were translated as a control (data not shown). This result showed that pPCal 5 contained a cDNA insert corresponding to the mRNA for the light subunit of calpain.

To obtain clones with longer cDNA inserts, an additional 20,000 transformants from the Okayama–Berg cDNA library were screened by hybridization with the restriction fragment derived from the 5′-terminal portion of clone pPCal 5 (Fig. 1). Two hybridization-positive clones were isolated, which seemed to be identical in their size of cDNA inserts and cleavage pattern by several restriction endonucleases. One of them, named pPCal 7, and pPCal 5 were subjected to nucleotide sequence analysis (for sequencing strategy, see Fig. 1). Because clones pPCal 5 and pPCal 7 did not carry the entire protein coding sequence, a cDNA library constructed by elongation of a synthetic oligodeoxyribonucleotide primer, 5′ CTCACTGATGGCGCT 3′ (complementary to residues 193–207), was screened by hybridization with a restriction fragment derived from pPCal 7. Four hybridization-positive clones were isolated. One of them, named pPCal 9 (harboring the largest cDNA insert), was subjected to nucleotide sequence analysis (Fig. 1).

Nucleotide Sequence of mRNA for Calpain I Light Subunit. The primary structures of the mRNAs coding for the light subunit of porcine calpain I were deduced from the nucleo-

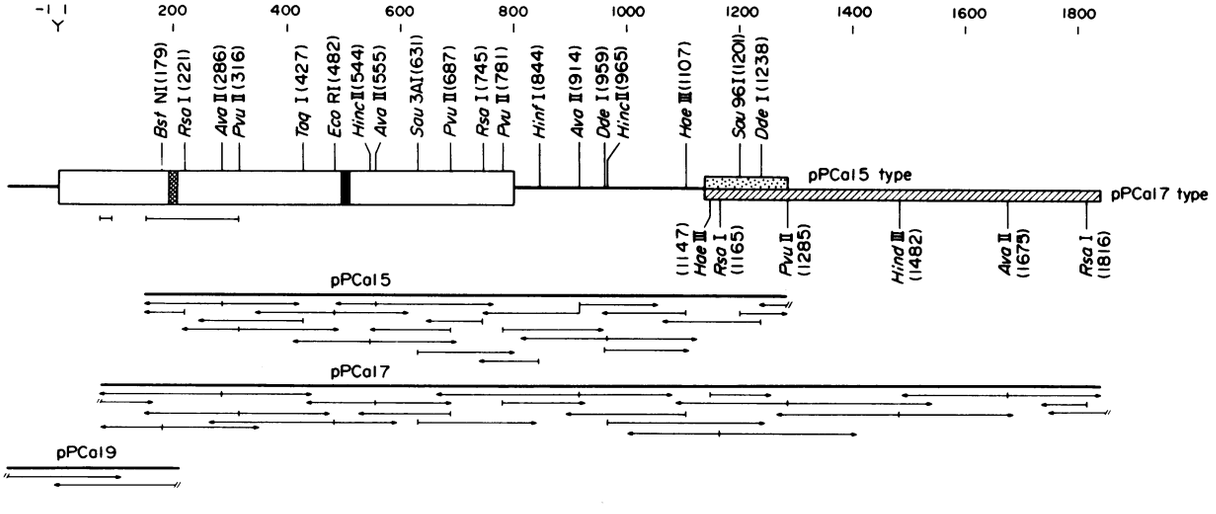

FIG. 1. Strategy of determining the sequences of the cDNA inserts in clones pPCal 5, pPCal 7, and pPCal 9. For isolation of clone pPCal 5, see text. For isolation of clone pPCal 7, the cDNA library was screened by hybridization at 60°C with the nick-translated *Pst* I–*Pvu* II fragment containing nucleotides 153–315 and the poly(dG)·poly(dC) tails from clone pPCal 5. Clone pPCal 9 was isolated from the cDNA library primed by oligodeoxyribonucleotide by hybridization at 50°C with the 5′-^{32}P-labeled *Hae* III fragment containing nucleotides 72–92 from clone pPCal 7. The restriction map displays only relevant restriction endonuclease sites, which are identified by numbers indicating the 5′-terminal nucleotide generated by cleavage; for the nucleotide numbers, see Fig. 2. The sequence corresponding to the coding region is shown by the open box. The closed and crosshatched boxes indicate the sequences of the synthetic oligodeoxyribonucleotides used as the probe and primer, respectively. The dotted and slashed boxes represent regions in the two cDNAs where their DNA sequences differ. The sequences used as hybridization probes for selecting clones are indicated by the lines directly beneath the restriction map. The direction and extent of sequence determinations are shown by horizontal arrows under each clone used; the sites of 5′-end labeling are indicated by short vertical lines at the end of arrows. The slash marks at the end of arrows mean that the site of 5′-end labeling was located on the vector DNA for clones pPCal 5 and pPCal 7 and that the site of subcloning was the *Pst* I site at the end of the poly(dG)·poly(dC) tail for clone pPCal 9.

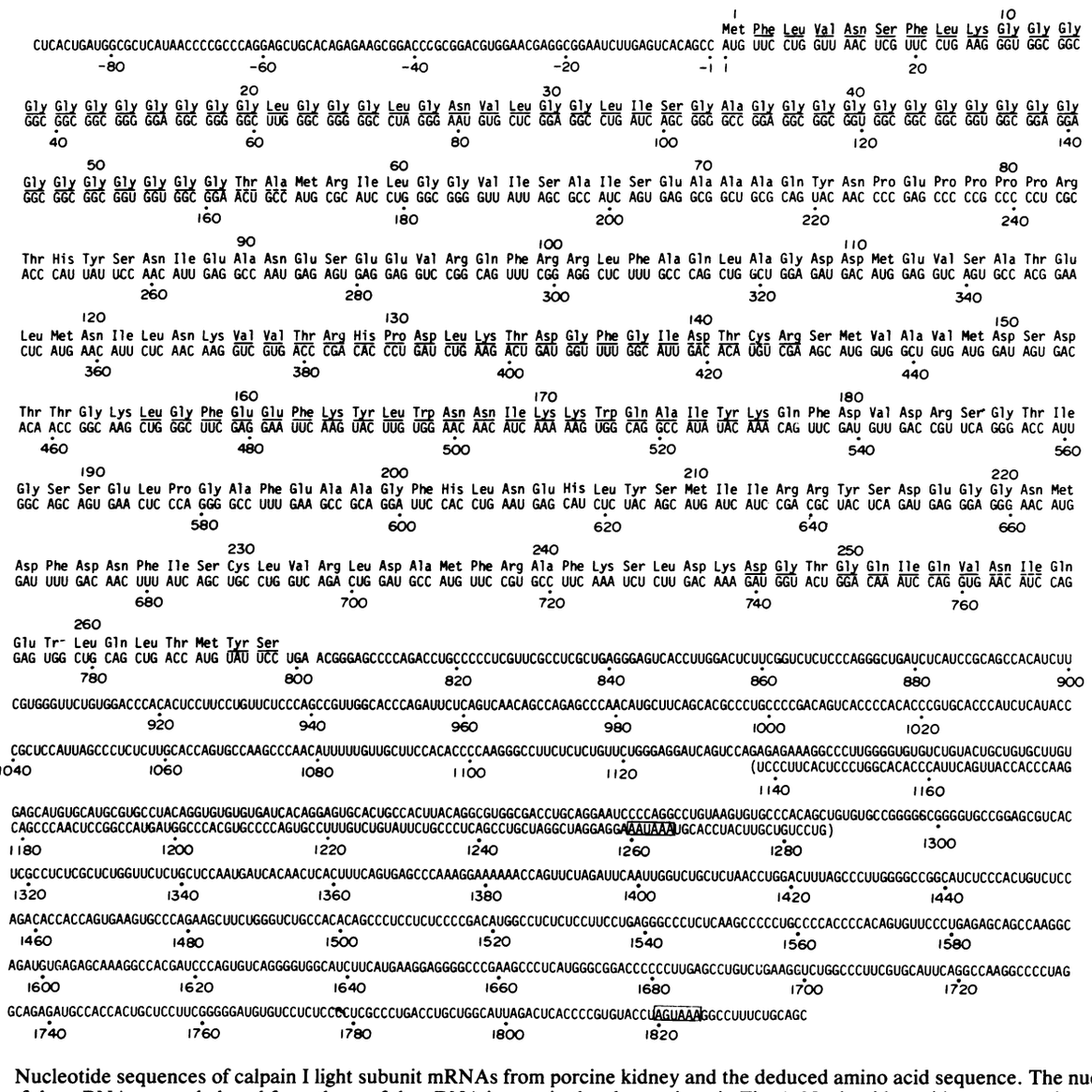

FIG. 2. Nucleotide sequences of calpain I light subunit mRNAs from porcine kidney and the deduced amino acid sequence. The nucleotide sequences of the mRNAs were deduced from those of the cDNA inserts in the clones given in Fig. 1. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of the AUG triplet encoding the initiation methionine, and the nucleotides on the 5′ side of residue 1 are indicated by negative numbers. In the region where the sequences of clones pPCal 5 and pPCal 7 differ, the sequence of the mRNA deduced from clone pPCal 5 is displayed in parentheses below that deduced from clone pPCal 7. The 3′-terminal sequences shown are both followed by a poly(A) tract connected with the vector DNA sequence, thus representing the complete sequences of the 3′-terminal regions. The two putative polyadenylylation signals are boxed. It has not been determined whether the 5′-terminal sequence shown extends to the 5′ end of the mRNA. The predicted amino acid sequence of calpain I light subunit is displayed above the nucleotide sequence, and the amino acid residues are numbered beginning with the initiation methionine. Amino acid sequences of peptides identified by protein sequence analysis are underlined. Broken lines indicate the residues tentatively identified.

tide sequences determined for the cloned cDNAs (clones pPCal 5, pPCal 7, and pPCal 9). These results are summarized in Fig. 2. Clones pPCal 5 and pPCal 7 differ in length and in sequence on their 3′-terminal region. However, to residue 1137, the sequences of the overlapping region between the two clones are completely identical. A single long open reading frame is present beginning with an initiation codon (AUG) at residues 1–3 and terminating with a stop codon (UGA) at positions 799–801. The sequence AAUAAA commonly found near the 3′ end of eukaryotic mRNAs is present at 21 residues upstream from the polyadenylylation site in the mRNA corresponding to clone pPCal 5. In the mRNA corresponding to clone pPCal 7, there exists the sequence AGUAAA at 15 residues upstream from the polyadenylylation site. The same sequence has been found in the mRNA of the human T24 oncogene (31), and it also seems to serve as a signal for polyadenylylation of mRNA.

When porcine kidney poly(A)-containing RNA was analyzed by the blot hybridization technique, only one band was observed corresponding to a length for the mRNA of about 1600–1700 nucleotides (Fig. 3). The number of cDNA clones we isolated (10 clones belonging to the pPCal 5 type, 2 clones belonging to the pPCal 7 type) may reflect less abundance of the mRNA corresponding to clone pPCal 7. That clones pPCal 5 and pPCal 7 harbor the same coding region but differ in the 3′ noncoding region is very unusual. However, this does not seem to result from any errors occurring during cDNA cloning, because the 2 clones independently isolated belong to the pPCal 7 type and because preliminary Southern blot hybridization analysis of total genomic DNA from porcine kidney using two different probes derived from the coding region and 3′ noncoding region of clone pPCal 7 suggests that the coding region and the 3′ end of clone pPCal 7, respectively, can exist on the same genomic DNA seg-

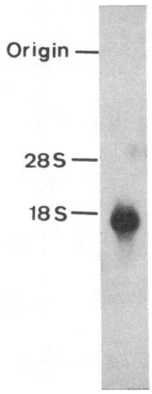

FIG. 3. Autoradiogram of blot hybridization analysis of porcine kidney poly(A)-containing RNA with cDNA probes. A 12.5-μg sample of RNA was analyzed. The hybridization probe used was the 584-base-pair *Hae* II–*Pst* I fragment (residues 198–782) derived from clone pPCal 7. The probe was ^{32}P-labeled by nick-translation. The size markers used were porcine rRNA.

ment. RNA blot analysis in more detail and analysis of genomic DNA coding for the light subunit should be performed to reveal the reason for having obtained these two different types of cDNA clones.

Amino Acid Sequence of Calpain I Light Subunit. The amino acid sequence of the light subunit of porcine calpain I predicted from the cDNA sequence is given in Fig. 2. To confirm this primary structure, the amino acid sequence of about 40% (106 of 266 residues, including 6 tentatively identified residues) of the light subunit of porcine calpain I was obtained by automated Edman degradation of peptides (each about 0.3–1.0 nmol) isolated from cyanogen bromide and endoproteinase Lys-C digests (each 5–10 nmol). Amino acid sequences of these peptides all confirmed the predicted protein sequence (amino acid residues 2–56, 125–143, 157–177, 247–248, 250–256, and 265–266, see Fig. 2).

Duplicate sequence analysis of the intact carboxymethylated light subunit failed to reveal the amino-terminal sequence in six and four cycles of Edman degradation, indicating that the amino terminus of the subunit is blocked. Among the cyanogen bromide peptides, we isolated an amino-terminal blocked peptide by reversed-phase HPLC. This peptide was tentatively identified as *N*-acetylhomoserine, because it showed the same retention time as that of synthetic *N*-acetylhomoserine by reversed-phase HPLC and yielded only homoserine on amino acid analysis. Thus, it appears likely that the mature protein begins with the initiation methionyl residue most possibly *N*-acetylated. The carboxyl terminus of the mature protein was unambiguously identified to be Tyr-Ser as predicted from the cDNA sequence. These results imply that the protein is not proteolytically processed and is composed of 266 amino acid residues. The calculated molecular mass (28,110) well agrees with that reported previously (20). Ohno *et al.* (8) recently reported that the only proteolytic processing event of the heavy subunit of the chicken enzyme is the removal of the initiation methionyl residue and subsequent blocking at the α-amino group of the second methionyl residue.

Homology with the Heavy Subunit. Comparison of the amino acid sequence of the light subunit from porcine calpain I with that of the heavy subunit from chicken calpain (8) revealed that there is a strikingly high degree of homology in their sequences of the carboxyl-terminal region (Fig. 4, amino acid residues 99–266, 52% identity and alignment score of 32). This region corresponds to the putative Ca^{2+}-binding domain in the latter protein and contains four potential Ca^{2+}-binding sites, which have sequences characteristic of the "E–F hand" structure (amino acid residues 150–161, 180–191, 215–226, and 245–258) (32). The secondary structure predicted indicates that these four potential Ca^{2+}-binding loops are located between two α-helical regions (Fig. 4). Comparison of the amino acid sequence of the light subunit with those of known Ca^{2+}-binding proteins by the graphic method of Kubota *et al.* (33) showed that three of these loops (loops I, II, and IV) gave high-density charts with the Ca^{2+}-binding loops of calmodulin, parvalbumin, and troponin C, which suggest significant sequence homology between them.

The amino-terminal region (amino acid residues 1–98) of the light subunit contains unusual polyglycyl sequences (amino acid residues 10–20, 22–24, and 37–54) and does not show any significant homology to any region of the heavy subunit from chicken enzyme. Thus, the alignment of this region in Fig. 4 is rather arbitrary. Among proteins of which amino acid sequences are known, we could not find one having any significant homology to this region of the light subunit.

DISCUSSION

Our present results indicate that the light subunit of porcine calpain contains two distinct domains: domain I, amino acid residues 1–98, and domain II, amino acid residues 99–266. Domain I contains unusual polyglycyl sequences and appears to comprise two extended consecutive β-turn structures (amino acid residues 5–24 and 35–53) (Fig. 4). It is noteworthy that these two portions are almost identical in size and may form a paired polyglycyl structure. This region may serve as a binding site to target molecules that possibly include the heavy subunit of calpain. Domain II contains four potential Ca^{2+}-binding sites, each having the E–F hand structure. Its amino acid sequence shows a high degree of homology to that of the putative Ca^{2+}-binding domain of the

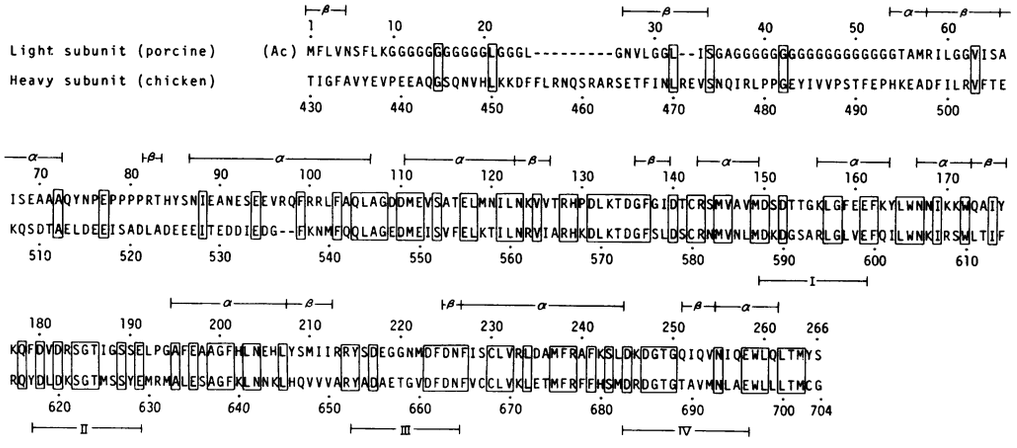

FIG. 4. Comparison of the amino acid sequences of calpain subunits (porcine light and chicken heavy). Identical residues are boxed. Dashes indicate gaps inserted to obtain higher homology. α and β regions indicate those predicted to be α-helix and β-sheet in the porcine light subunit. (Ac) indicates tentative amino-terminal blocking group acetyl. I–IV indicate four potential Ca^{2+}-binding loops.

heavy subunit from chicken origin (8). Thus, the two domains are very different both structurally and functionally.

Ca^{2+}-binding proteins containing the E–F hand structure (for example, calmodulin, troponin C, and parvalbumin) are thought to have arisen from the same evolutionary origin (34). The remarkable homology of the amino acid sequences demonstrated in this study strongly suggests that the putative Ca^{2+}-binding domains of the heavy and light subunits of calpain seem to have arisen from a common origin, which has also served as the origin of the other E–F-handed Ca^{2+}-binding proteins. Ohno *et al.* (8) postulated that the heavy subunit of calpain arose by fusion of genes for cysteine proteinases and Ca^{2+}-binding proteins. From our present results, it is possible to speculate that the heavy subunit has been made up by incorporation of a part of the gene for the light subunit into the gene of a cysteine proteinase. Alternatively, the light subunit could have arisen by duplication of the gene for the Ca^{2+}-binding domain of the heavy subunit. Elucidation of the gene structure of both subunits would provide the answer to these questions.

Ca^{2+} is known to be essential for the proteolytic activity of calpain. It was reported that the heavy subunit of chicken calpain can bind as much as 6 mol of Ca^{2+} per molecule (35). Ohno *et al.* (8) recently established the primary structure of the heavy subunit of chicken enzyme and claimed that the heavy subunit functions for Ca^{2+}-binding as well as for proteolytic catalysis by showing that the molecule contains four potential Ca^{2+}-binding sites. We now can clearly show that the light subunit also contains four potential Ca^{2+}-binding sites and that, in total, calpain has at least eight potential Ca^{2+}-binding sites. Previous results of *in vitro* reconstitution experiments showed that the light subunit lowered significantly the Ca^{2+} requirement of the heavy subunit (7). Thus, it can be postulated that the role of the light subunit is to enhance the proteolytic activity of the heavy subunit in concert with the function of the Ca^{2+}-binding domain of the heavy subunit, when the heterodimer comes into contact with Ca^{2+}.

Two types of regulation of calpain activity by Ca^{2+} may operate; one is the interaction between the catalytic domain and Ca^{2+}-binding domain within the heavy subunit, whereas the other is the interaction between the catalytic heavy subunit and regulatory light subunit that binds Ca^{2+}. Each one of these seems to be analogous in part to the mechanism by which calmodulin activates a target enzyme, but the uniqueness in the case of calpain should be the coordination of these two types of mechanisms, which can be expected to exert more delicate and precise regulation by Ca^{2+} compared with the case for calmodulin alone. It is likely that calpain has acquired such a delicate mechanism of regulation during the course of evolution, which has been directed to create a heterodimeric structure. We wish to propose to call this mechanism the "double-regulation mechanism," signifying the dual control that is achieved by the existence of Ca^{2+}-binding sites in both the catalytic and regulatory subunits. Calpain must be one example of the proposed double-regulation mechanism, and the same mechanism may also hold for some of other Ca^{2+}-regulated but calmodulin-independent enzymes.

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1. Murachi, T. (1983) *Trends Biochem. Sci.* **8,** 167–169.
2. Murachi, T. (1983) in *Calcium and Cell Function*, ed. Cheung,W. Y. (Academic, New York), Vol. 4, pp. 377–410.
3. Kay, J. (1983) in *Proteases: Potential Role in Health and Disease*, eds. Heidland, A. & Hörl, W. H. (Plenum, New York), pp. 519–532.
4. Murachi, T., Tanaka, K., Hatanaka, M. & Murakami, T. (1981) *Adv. Enzyme Regul.* **19,** 407–424.
5. Mellgren, R. L. (1980) *FEBS Lett.* **109,** 129–133.
6. Tsuji, S. & Imahori, K. (1981) *J. Biochem. (Tokyo)* **90,** 233–240.
7. Kikuchi, T., Yumoto, N., Sasaki, T. & Murachi, T. (1984) *Arch. Biochem. Biophys.* **234,** 639–645.
8. Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M. & Suzuki, K. (1984) *Nature (London)* **312,** 566–570.
9. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
10. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.
11. Okayama, H. & Berg, P. (1982) *Mol. Cell. Biol.* **2,** 161–170.
12. Hanahan, D. & Meselson, M. (1980) *Gene* **10,** 63–67.
13. Hirose, T., Crea, R. & Itakura, K. (1978) *Tetrahedron Lett.* **28,** 2449–2452.
14. Agarwal, K. L., Brunstedt, J. & Noyes, B. E. (1981) *J. Biol. Chem.* **256,** 1023–1028.
15. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S. & Numa, S. (1982) *Nature (London)* **295,** 202–206.
16. Parnes, J. R., Velan, B., Felsenfeld, A., Ramanathan, L., Ferrini, U., Appella, E. & Seidman, J. G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2253–2257.
17. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
18. Vieira, J. & Messing, J. (1982) *Gene* **19,** 259–268.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
20. Kitahara, A., Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N., Hatanaka, M. & Murachi, T. (1984) *J. Biochem. (Tokyo)* **95,** 1759–1766.
21. Koide, A., Titani, K., Ericsson, L. H., Kumar, S., Neurath, H. & Walsh, K. A. (1978) *Biochemistry* **17,** 5657–5672.
22. Mahoney, W. C. & Hermodson, M. A. (1980) *J. Biol. Chem.* **255,** 11199–11203.
23. Heinrikson, R. L. & Meredith, S. C. (1984) *Anal. Biochem.* **136,** 65–74.
24. Knoop, D. R., Morgan, E. T., Tarr, G. E. & Coon, M. J. (1982) *J. Biol. Chem.* **257,** 8472–8480.
25. Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J. & Hood, L. E. (1983) *Methods Enzymol.* **91,** 399–413.
26. Hunkapiller, M. W. & Hood, L. E. (1983) *Methods Enzymol.* **91,** 486–493.
27. Ericsson, L. H., Wade, R. D., Gagnon, J., MacDonald, R. M., Granberg, R. R. & Walsh, K. A. (1977) in *Solid Phase Methods in Protein Sequence Analysis*, eds. Previero, A. & Coletti-Previero, M. A. (Elsevier/North-Holland, Amsterdam), pp. 137–142.
28. Dayhoff, M. O., Baker, W. C. & Hunt, L. T. (1983) *Methods Enzymol.* **91,** 524–545.
29. Chou, P. Y. & Fasman, G. D. (1978) *Adv. Enzymol.* **47,** 45–148.
30. Argos, P., Hanei, M. & Gravito, R. M. (1978) *FEBS Lett.* **93,** 19–24.
31. Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. & Goeddel, D. V. (1983) *Nature (London)* **302,** 33–37.
32. Tufty, R. H. & Kretsinger, R. H. (1975) *Science* **187,** 167–169.
33. Kubota, Y., Nishikawa, K., Takahashi, S. & Ooi, T. (1982) *Biochim. Biophys. Acta* **701,** 242–252.
34. Baker, W. C., Ketcham, L. K. & Dayhoff, M. O. (1977) in *Calcium Binding Proteins and Calcium Function*, eds. Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H. & Siegel, F. L. (Elsevier/North-Holland, New York), pp. 73–75.
35. Suzuki, K. & Ishiura, S. (1983) *J. Biochem. (Tokyo)* **93,** 1463–1471.