Isolation and Sequence Analyses of cDNA Clones for the Large Subunits of Two Isozymes of Rabbit Calcium-dependent Protease*

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Two sets of cDNA clones were isolated from cDNA libraries prepared from poly(A⁺) RNA of rabbit lung and spleen by screening with the cDNA probe for the large subunit (80-kDa subunit) of chicken calcium-dependent protease (Ca²⁺-protease; Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M., and Suzuki, K. (1984) Nature 312, 566–570). The two sets of clones were identified as cDNA clones for two Ca²⁺-protease isozymes with high (μ-type) and low (m-type) calcium sensitivities from a comparison of the primary structures deduced from the nucleotide sequences with partial amino acid sequences from the two isozymes. The cDNA clones for the 80-kDa subunits of the μ- and m-type Ca²⁺-proteases contained, in total, about 1.5- and 2.2-kilobase cDNA inserts, respectively, which correspond roughly to the C-terminal halves of the coding regions and the entire 3' non-coding regions.

The two isozymes are encoded by two distinct mRNA species present in all the tissues examined, although the amount of mRNA significantly differs among the various tissues.

Four E-F hand structures, typical calcium-binding structures in various calcium-binding proteins such as calmodulin, were detected in the C-terminal regions of both isozymes, as in the case of chicken Ca²⁺-protease.

Comparison of the amino acid sequences of the two rabbit isozymes and the corresponding region of the chicken enzyme revealed marked homology, which indicates that these three enzymes have the same evolutionary origin. Furthermore, we suggest that the μ-type rabbit Ca²⁺-protease, rather than the m-type, is similar to chicken Ca²⁺-protease, which is regarded as an m-type enzyme in the C-terminal region. The evolution and molecular basis of the differences in calcium sensitivities of the Ca²⁺-proteases are discussed.

Calcium-dependent protease (Ca²⁺-protease [calpain]) is an intracellular protease that shows a wide distribution (1–3) and comprises two subunits. The large subunit (80-kDa subunit) is a catalytic subunit (4, 5) with calcium-binding regions (6), but the function of the small subunit (30-kDa subunit) is not yet clear. In mammals, two isozymes (μ- and m-types) have been isolated (7–12). They are similar in various molecular and enzymatic properties, but differ markedly in their calcium sensitivities. The μ-type enzymes have high calcium sensitivity and are active at micromolar concentrations of calcium. In contrast, the calcium sensitivity of the m-type enzymes is low, and they require millimolar calcium for their activity. On the basis of peptide mapping and amino acid sequencing, we have shown that the small subunits of the rabbit μ- and m-type enzymes are identical. The difference in the calcium sensitivity of the two isozymes is thus ascribed to the large subunits.

In the chicken, however, only a single species of Ca²⁺-protease exists with an intermediate calcium sensitivity (13). As the first step in clarifying the mechanism of action, we determined the structure of the cDNA for the unique 80-kDa subunit of chicken Ca²⁺-protease (6).

As the next step, we determined the cDNA structures for the 80-kDa subunits of the two Ca²⁺-protease isozymes to clarify the basis of the calcium sensitivity. We isolated cDNA clones for the 80-kDa subunit of rabbit Ca²⁺-protease. The amino acid sequences of the C-terminal halves of the μ- and m-type Ca²⁺-proteases were determined from the nucleotide sequences and compared with that of chicken Ca²⁺-protease. These results are presented together with a discussion of the molecular basis of the calcium sensitivity and of the evolution of Ca²⁺-protease.

EXPERIMENTAL PROCEDURES

Materials—Guadinium isothiocyanate and cesium chloride were purchased from Wako Pure Chemical Co. Oligo(dT)-cellulose (Type VII), Escherichia coli DNA ligase, and RNase-H were products of P-L Biochemicals. Reverse transcriptase and SI nuclease were obtained from Takara Shuzo Co. The restriction enzymes used were from Takara Shuzo Co., Toyobo Co., and P-L Biochemicals. The nick translation kit and [γ-³²P]ATP (≈3000 Ci/mmol) were from Amersham Corp. [α-³²P]dCTP (≈3000 Ci/mmol) and PstI-cut and oligo(dG)-tailed pBR322 were purchased from New England Nuclear.

Construction of cDNA Libraries—Total RNA was extracted from rabbit lung or spleen by the guanidinium isothiocyanate/cesium chloride method (14). Poly(A⁺) RNA was purified by oligo(dT)-cellulose column chromatography (14). After single-stranded cDNA synthesis with reverse transcriptase with total poly(A⁺) RNA as a template, double-stranded cDNA synthesis was performed according to Gubler and Hoffman's method (15) using E. coli DNA polymerase, E. coli DNA ligase, and RNase H or the self-primer method (14) using the Klenow fragment and SI nuclease. The double-stranded cDNA was oligo(dC)-tailed at the 3'-ends with terminal transferase, annealed with PstI-cut and dG-tailed pBR322, and then introduced into E. coli

* This work was supported in part by research grants from the Ministry of Education, Science, and Culture, a grant-in-aid for new dictionary origin. Furthermore, we suggest that the μ-type rabbit Ca²⁺-protease, rather than the m-type, is similar to chicken Ca²⁺-protease, which is regarded as an m-type enzyme in the C-terminal region. The evolution and molecular basis of the differences in calcium sensitivities of the Ca²⁺-proteases are discussed.

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MM294 by Hanahan's method (16).

Isolation of Ca\(^{2+}\)-protease cDNA Clones—Tetracycline-resistant transformants (about 10\(^5\) colonies) were screened by in situ hybridization (14) with nick-translated chicken Ca\(^{2+}\)-protease cDNA as a probe in a solution containing 50 mM Tris-HCl (pH 8.0), 1 mM NaCl, 10 mM EDTA, 0.02% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll 400, 0.2% SDS, 100 \(\mu\)g/ml heat-denatured salmon sperm DNA (9). Washing was performed with 2 \(\times\) SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS at 65 \(^\circ\)C. The eight independent clones obtained were subjected to cross-hybridization among themselves, and they could be divided into two types. The longest representative of each type was sequenced, and the deduced amino acid sequences were compared with those determined with peptides obtained from the two isozymes as described below.

Partial Amino Acid Sequence Determination of Peptides of the Two Ca\(^{2+}\)-protease Isozymes—The two isozymes of rabbit Ca\(^{2+}\)-protease (\(\mu\)- and m-types) were purified from rabbit skeletal muscle as described previously (11). The purified enzymes were further fractionated into two subunits as described in the Miniprint Supplement. 3

The purified large subunits were digested with trypsin or lysyl-endopeptidase, and then the digests were fractionated on a C\(_25\) reverse-phase high pressure liquid chromatography column. The amino acid sequences of the purified peptides were determined with a gas-phase protein sequenator (Applied Biosystems, Inc., Model 470A).

RNA Blot Hybridization—Total RNA was extracted from various rabbit tissues as described above. Each RNA sample (2 \(\mu\)g of total RNA) was denatured and electrophoresed in a formaldehyde-containing agarose gel (14). After electrophoresis, the RNA was transferred to a nylon membrane (Bionyne BN1G) and then hybridized with nick-translated cDNA (14) at 42 \(^\circ\)C in a solution containing 50 mM sodium phosphate (pH 7.0), 5 \(\times\) SSC, 50% (v/v) formamide, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400, 0.2% SDS, and 200 \(\mu\)g/ml heat-denatured salmon sperm DNA template. The filter was washed in 0.1 \(\times\) SSC, 0.1% SDS at 50 \(^\circ\)C and then exposed to Fuji RX film at -80 \(^\circ\)C with an intensifying screen.

DNA Sequencing—DNA sequencing was performed by the 5′-end labeling and chemical modification method of Maxam and Gilbert (17). DNA fragments prepared by digestion of recombinant plasmids with restriction enzymes were dephosphorylated with alkaline phosphatase and then 5′-end-labeled with \([\gamma-\text{32P}]\text{ATP}\) with T4 polynucleotide kinase. After the second digestion with an appropriate restriction enzyme, the 3′-end-labeled DNA fragments were recovered from polyacrylamide gels and processed for chemical modification and cleavage reaction.

RESULTS

Identification of Two Isozymes of Calcium-dependent Protease cDNA Clones—From about 10\(^5\) colonies, eight clones were isolated as rabbit Ca\(^{2+}\)-protease cDNA clones using chicken Ca\(^{2+}\)-protease cDNA as a probe. The eight clones were divided into two types on the basis of the results of cross-hybridization experiments. Two clones, representative of the two types (pLU1001 and pLM28, Fig. 1), were first sequenced. The amino acid sequence deduced from the nucleotide sequence of pLU1001 contains the partial amino acid sequences of two peptides obtained from the 80-kDa subunit of the \(\mu\)-type Ca\(^{2+}\)-protease (shown in the Miniprint Supplement (Table I-S) and indicated by horizontal arrows in Fig. 2B). Thus, pLU1001 and pLM28 were assigned as the cDNA clones for the 80-kDa subunits of the \(\mu\)- and m-type Ca\(^{2+}\)-proteases, respectively.

Nucleotide Sequences of \(\mu\)- and m-Type Ca\(^{2+}\)-protease cDNAs—Restriction mapping and nucleotide sequence analyses of the two types of clones gave the following results. The \(\mu\)-type cDNA clones contained the C-terminal sequence of 302 residues, which corresponds to positions 404–705 of chicken Ca\(^{2+}\)-protease 80-kDa subunit (6) (Fig. 4), and the complete 3′-noncoding region (Figs. 1 and 2A). The m-type cDNA clones contained the C-terminal 424-residue sequence, corresponding to residues 282–705 of the chicken enzyme (6) (Fig. 4).

The 3′-noncoding regions of mRNAs for the three enzymes (the chicken enzyme and the \(\mu\)- and m-type rabbit enzymes) are markedly different both in length and sequence. The chicken mRNA has 1227 nucleotides in the 3′-noncoding sequence (6), whereas the rabbit \(\mu\)- and m-type cDNAs have 580 and 887 nucleotides, respectively. No significant sequence homology among them could be detected, although their coding regions are highly homologous, with ~65% homology, as described below.

Identification and Characterization of mRNA for Ca\(^{2+}\)-protease in Various Rabbit Tissues—The presence of mRNAs for the two isozymes was examined by RNA blot hybridization. We used the nick-translated 3′-noncoding regions of the cDNAs for the two rabbit isozymes as probes because their...
cDNA Cloning for Two Isozymes of Rabbit Ca^{2+}-protease

FIG. 2. Nucleotide sequences of Ca^{2+}-protease cDNA inserts and the deduced amino acid sequences. A, α-type; B, μ-type. Each amino acid sequence deduced is shown below the respective nucleotide sequence. The polyadenylation sequence (AATAAA) is underlined. Amino acid sequences determined with a gas-phase protein sequenator are indicated by }\text{sv} horizontal arrows (see Table I-S).
coding regions are homologous and might cross-hybridize with each other. The results in Fig. 3 show that the mRNA for both enzymes is expressed in all the rabbit tissues examined, although the amount differs considerably from tissue to tissue. The Ca\(^{2+}\)-protease mRNA content was the lowest in liver and the highest in lung for both types of isozyme. The length of each mRNA was about 3.5 kilobases, but the m-type mRNA was slightly larger than the \(\mu\)-type, probably due to the different lengths of their 3'-noncoding regions, as described above. In spleen, a smearing around the 3.5-kilobase band was always observed for the \(\mu\)-type enzyme. The same result was seen when its coding region was used as a probe. Furthermore, a faint band of about 2 kilobases was usually detected in brain for the \(\mu\)-type enzyme (as shown in Fig. 3A). The reasons for these phenomena are not clear.

Amino Acid Sequences of the 80-kDa Subunits of the \(\mu\)- and m-Type Rabbit Ca\(^{2+}\)-proteases—We previously determined the total amino acid sequence of the chicken Ca\(^{2+}\)-protease 80-kDa subunit from the cDNA nucleotide sequence (6). The cDNA clones for the 80-kDa subunits of the two rabbit Ca\(^{2+}\)-proteases obtained here contained the C-terminal halves of their coding regions, and their amino acid sequences were compared with that of the chicken Ca\(^{2+}\)-protease 80-kDa subunit. Remarkable sequence homology among the three enzymes was found. Their sequences can be aligned as shown in Fig. 4, with only two deletions in the sequence for rabbit m-type Ca\(^{2+}\)-protease. The per cent homology is summarized in Table I in terms of the amino acid and nucleotide sequences. The highest amino acid sequence homology was found between the rabbit \(\mu\)-type enzyme and the chicken enzyme. The situation was the same when the nucleotide sequences were compared. Namely, the rabbit \(\mu\)-type enzyme is more similar to the chicken enzyme, an enzyme of a different species, than to the m-type enzyme of the same species.

![Fig. 3. RNA blot analysis of total RNA from various rabbit tissues. A, \(\mu\)-type; B, m-type. RNA (2 \(\mu\)g) was transferred to a nylon membrane after agarose gel electrophoresis as described under "Experimental Procedures" and then hybridized with nick-translated cDNA fragments corresponding to the 3'-noncoding regions. The DNA fragments used were from residues 908-1401 (DdeI/RsaI fragment) for the \(\mu\)-type (see Fig. 2A) and from residues 1484-2123 (RsaI fragment) for the m-type (see Fig. 2B). Lane 1, brain; lane 2, heart muscle; lane 3, kidney; lane 4, liver; lane 5, lung; lane 6, skeletal muscle; lane 7, spleen; lane 8, stomach. The positions of ribosomal RNAs (28 S and 18 S) are indicated by arrows.](image-url)

![Fig. 4. Comparison of the amino acid sequences of the two isozymes (\(\mu\)- and m-types) of rabbit and chicken Ca\(^{2+}\)-proteases (6). Identical amino acid residues are shadowed in boxes. The numbers shown are the residue numbers for the chicken enzyme (6). Two gaps (shown as bars) are inserted for maximum homology in the sequence of the m-type enzyme (positions 524 and 705). Four calcium-binding regions (E-F hand structures I-IV) are shown below the sequences.](image-url)

| TABLE I Percentage sequence homology between the C-terminal regions (302 amino acids, 906 nucleotides) of the Ca\(^{2+}\)-protease 80-kDa subunits |
|-----------------|-----------------|-----------------|
| Rabbit \(\mu\)-type | Rabbit m-type | Chicken |
| Amino acid sequence homology (%) | 70.4 | 64.3 |
| Nucleotide sequence homology (%) | 55.3 | 64.9 |
The C-terminal region of about 170 residues of the chicken Ca\(^{2+}\)-protease 80-kDa subunit has been identified as the calcium-binding domain (6). This region, as in calmodulin and parvalbumin, contains four consecutive calcium-binding regions, so-called E-F hand structures (18, 19). Alignment of the two rabbit isozymes with the chicken enzyme (Fig. 4) revealed the presence of four putative calcium-binding regions in the C termini of the two rabbit enzymes, as found for the chicken enzyme. These four regions in both isozymes show high scores for homology when the presence of the E-F hand structures was examined with the test sequence (19, 20). As summarized in Table II, the scores for the \( \mu \)-type enzyme are 12, 12, 11, and 13 and for the m-type enzyme are 12, 11, 11, and 13 in order from the N terminus. The scores for the chicken enzyme are 11 for all four regions (6), which is lower than those for the two rabbit enzymes. Thus, these four regions in both the \( \mu \)- and m-type rabbit enzymes can be regarded as E-F hand structures and putative calcium-binding regions. The spacing of these four E-F hand structures along the peptide chain is similar to that in calmodulin (18), as in the case of the chicken enzyme (6).

The predicted secondary structures based on the sequences (21) indicate that for the putative calcium-binding loops the probabilities of the formation of either \( \alpha \)-helix or \( \beta \)-sheet structures are low and that they are flanked by \( \alpha \)-helix or \( \beta \)-sheet structures (data not shown). These are typical features of the secondary structure for E-F hand structures (18-20).

**DISCUSSION**

We have isolated cDNA clones for the 80-kDa subunits of two isozymes of rabbit Ca\(^{2+}\)-protease and have shown that the two isozymes are evolutionarily related. Amino acid and nucleotide sequence homology reveals that the chicken enzyme (6), which was previously designated as the m-type (13), is similar to the \( \mu \)-type rabbit enzyme rather than to the m-type in the C-terminal region. In chicken tissues, only one type has been isolated, and its calcium sensitivity is intermediate between those of the two isozymes from mammalian sources (13). Although the total structure of the two isozymes and further comparative studies on Ca\(^{2+}\)-protease from various species are required, we can assume that there was once only one type of Ca\(^{2+}\)-protease, as now found in chicken, and it evolved into two species with different calcium sensitivities, as now found in mammals. The chicken enzyme might be a prototype Ca\(^{2+}\)-protease.

In contrast to the marked sequence homology in the coding regions, the 3'-noncoding regions of the cDNAs for the three Ca\(^{2+}\)-proteases show no distinct homology. This suggests that there is a greater pressure to conserve the sequence of the coding regions for functional conservation.

The two species of mRNA for the \( \mu \)- and m-type isozymes showed a wide distribution, being detected in all the rabbit tissues examined. No significant difference in the tissue distributions of the \( \mu \)- and m-type enzymes was found. But, the contents of the mRNAs in various rabbit tissues differed significantly. The content of mRNAs roughly corresponds to the amount of Ca\(^{2+}\)-protease in various chicken and rat tissues determined by measuring the enzyme activities (22, 23). Similar tissue distribution results were reported for the chicken enzyme mRNA (6, 24).

The two isozymes of rabbit Ca\(^{2+}\)-protease differ significantly in their calcium sensitivities. The \( \mu \)-type enzyme requires 40 \( \mu \)M Ca\(^{2+}\) for 50% activity, and the m-type enzyme requires 700 \( \mu \)M Ca\(^{2+}\) (11). However, both enzymes have four consecutive E-F hand structures in their calcium-binding domain, like calmodulin, and the homology scores for the calcium-binding regions of the two rabbit enzymes and the chicken enzyme are similar. Therefore, differences in calcium sensitivity of the Ca\(^{2+}\)-proteases may be ascribed to finer structural differences in the calcium-binding regions. In this respect, it is noteworthy that the two types of rabbit enzyme clearly have different sequences at the C-terminal ends of 5 or 6 residues (Fig. 4). In addition, the sequences of the loop structures in calcium-binding regions IV and III vary (see

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**Table II**

Scores of the E-F hand structures for the putative calcium-binding regions of rabbit Ca\(^{2+}\)-protease

In the test sequence, O denotes oxygen-containing amino acid residues (D, E, N, Q, S, ?), L denotes hydrophobic residues (L, V, I, F, M), E denotes E, and G means G. Residues matching the test sequence are marked by asterisks below the sequences and were counted to obtain the scores. For the positions of the regions shown, see Fig. 4.

|          | Helix | Loop | Helix |
|----------|-------|------|-------|
|          | EL-LL-L | O-O-G-I-O | L-LL-L |
| \( \mu \)-Type |
| Region I | SCRSMVLML | DRDGNGKGLGLVE | FNILWNRI |
| ** | ** ** | ** ** ** ** ** | ** ** |
| Region II | NYLAIFFRKF | DLKSGSMSAYE | MRMAIESA |
| ** | ** | ** ** ** ** ** | ** ** |
| Region III | KLYELITTR | YSEPDLAVFDN | FVCLVLRL |
| ** | ** ** | ** ** ** ** ** | ** ** |
| Region IV | TMFRFFKTIL | DTDLDGVFTFDL | FKWQLTM |
| ** | ** ** | ** ** ** ** ** | ** ** |
| m-Type |
| Region I | TCKIMWOML | DSDGTGKGLGLKE | FVVLWTKI |
| ** | ** | ** ** ** ** ** | ** ** |
| Region II | KYQKIVREI | DVDRSGTMMNSYE | MRKALEKA |
| ** | ** | ** ** ** ** ** | ** ** |
| Region III | QHFEVIVAR | FADDQLTIDFDN | FVRCVLRL |
| ** | ** | ** ** ** ** ** | ** ** |
| Region IV | TLFKIFIQDL | DPDNTGMQLDL | ISMLCFSV |
| ** | ** ** | ** ** ** ** ** | ** ** |

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**Notes:**

1. The peptides were synthesized according to the amino acid sequence of the chicken enzyme.
2. The scores for the positions of the regions shown, see Fig. 4.
3. The calcium sensitivity of the Ca\(^{2+}\)-proteases may be ascribed to finer structural differences in the calcium-binding regions. In this respect, it is noteworthy that the two types of rabbit enzyme clearly have different sequences at the C-terminal ends of 5 or 6 residues (Fig. 4). In addition, the sequences of the loop structures in calcium-binding regions IV and III vary (see...
Table II and Fig. 4). To clarify the basis of the calcium sensitivity, the total amino acid sequences of the two rabbit enzymes should be determined together with those of other species. Studies along these lines are in progress, and the results will be presented elsewhere.

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