J. Biochem. 123, 546-554 (1998)

Caldecrin Is a Novel-Type Serine Protease Expressed in Pancreas, but Its Homologue, Elastase IV, Is an Artifact during Cloning Derived from Caldecrin Gene[1]

Izumi Yoshino-Yasuda,* Keiko Kobayashi,* Masashi Akiyama,† Hirotaka Itoh,† Akito Tomomura,‡ and Takeyori Saheki*,[2]

*Department of Biochemistry, Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8520; †Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., Komakado 1-135, Gotemba, Shizuoka 412-0038; and ‡Department of Dental Biochemistry, Faculty of Dentistry, Meikai University, Keyakidai 1-1, Sakado, Saitama 350-0248

Received for publication, November 28, 1997

As reported previously, caldecrin, a serum calcium-decreasing factor, from pancreas was found to be a serine protease, but the proteolytic activity was not necessary for its serum calcium decreasing activity. The caldecrin cDNA encoded a protease zymogen of the chymotrypsin/elastase superfamily consisting of a signal peptide, an activation peptide and a mature enzyme. On a homology search, we found that the sequence of rat caldecrin is almost identical to that of rat elastase IV (nucleotides: 99.3%, amino acids: 90.3%) with the exception of the central region. However, it is not known whether or not elastase IV is transcribed and translated *in vivo*, and has proteolytic activity. In the present study, we constructed a rat elastase IV cDNA by means of combinatorial PCR, and compared the recombinant elastase IV with the recombinant caldecrin synthesized in a baculovirus expression system. The recombinant caldecrin protein was expressed in the cells and secreted mainly into the medium. In contrast, in the case of elastase IV, no recombinant protein was immunologically or enzymatically detected in the medium, while an immunoreactive protein with much lower protease activity was found in the cells in an amount comparable to that of the caldecrin protein. Using the RT-PCR method to discriminate caldecrin mRNA from elastase IV mRNA, we detected caldecrin mRNA expression in rat pancreas, but no elastase IV mRNA expression in any tissues examined. PCR analysis of rat genomic DNA revealed the presence of caldecrin and the absence of elastase IV sequences. These results indicate that caldecrin is expressed in the pancreas, but that elastase IV is an artifact produced during cloning. Furthermore, we investigated the protein-chemical and enzymological properties of the rat and human caldecrins using their recombinant proteins. Both recombinant proteins were secreted into the medium as proforms and showed protease activity after trypsin treatment. Some differences were found in the activation process and stability between human and rat caldecrins; human caldecrin was more easily activated by trypsin, but was much more labile than rat caldecrin. Although both caldecrins were found to be chymotrypsin-type proteases, on the basis of their substrate and inhibitor specificities, they were not inhibited by TPCK, suggesting that caldecrin is a novel type of serine protease.

Key words: baculovirus expression, caldecrin, chymotrypsin-like protease, elastase, serine protease.

Hypocalcemia is associated with acute pancreatitis, and hypocalcemic factors are postulated to exist in the pancreas (*1*). Yoneda *et al.* reported that a hypocalcemic factor, PX, purified from porcine pancreas resembled human elastase IIIB, and that recombinant human elastase IIIB showed hypocalcemic activity connected with its protease activity (*2-5*). Previously, Tomomura *et al.* purified a serum calcium-decreasing factor, referred to as caldecrin, from porcine and rat pancreas (*6, 7*), and cloned the caldecrin cDNA from rat and human pancreas (*7, 8*). The caldecrin exhibits proteolytic activity, but its serum calcium-decreasing activity does not require its protease activity (*6-8*). The primary structure of caldecrin is different from that

[1] This work was supported in part by the Kodama Memorial Foundation for Research on Medical Science.

[2] To whom correspondence should be addressed. Tel: +81-99-275-5242, Fax: +81-99-264-6274, E-mail: takesah@med2.kufm.kagoshima-u.ac.jp

Abbreviations: MCA, 4-methylcoumaryl-7-amide; pNA, *p*-nitroanilide; TLCK, *N*-tosyl-L-lysine-chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

of elastase IIIB (*9*). On the other hand, Kang *et al.* (*10*) isolated elastase IV cDNA from rat pancreas by means of reverse transcription (RT)-polymerase chain reaction (PCR). Rat caldecrin is almost identical to rat elastase IV in the nucleotide sequence (99.3%), differences being found at 5 positions in the central region of the cDNA; three substitutions, one deletion and one insertion. As a consequence, the deduced amino acid sequences exhibit 90.3% identity. However, no information on mRNA expression *in vivo* or the enzymological properties of rat elastase IV has been reported.

Tomomura *et al.* reported that the purified porcine caldecrin is a chymotrypsin-like serine protease (*6*), and that the purified procaldecrin does not possess serum calcium-decreasing activity but acquires this bioactivity as well as protease activity upon trypsin treatment (*11*). cDNA cloning and sequencing analysis revealed that the predicted caldecrin protein is synthesized as a preproenzyme of 268 amino acids with a signal peptide of 16 amino acids and an activation peptide of 13 amino acids (*7, 8*). The recombinant caldecrin is secreted as a proform into the medium in a baculovirus expression system and requires enzymatic cleavage of its amino terminus for conversion to the active form, like other serine proteases (*7, 8*). On a homology search, caldecrins showed about 60% identity with elastase family members and about 40% with chymotrypsin family members, and on the basis of the predicted protein structure, caldecrin is classified as an elastase-type protease (*7, 8*). Although it is suggested that caldecrin is a protease of the chymotrypsin/elastase superfamily, the enzymatic properties of caldecrin as a protease are not well known.

In order to investigate the enzymological properties and bioactivity of elastase IV in comparison with those of caldecrin, we constructed rat elastase IV cDNA by means of combinatorial PCR (*12*), and compared the recombinant elastase IV protein with that of caldecrin synthesized in a baculovirus expression system. The recombinant elastase IV protein was not found in the medium, although it was synthesized in insect cells, differing from the recombinant caldecrin protein accumulated in the medium. To further examine whether or not caldecrin and elastase IV are really transcribed *in vivo*, we established methods for detecting their mRNAs by means of RT-PCR analysis and for discriminating caldecrin cDNA from elastase IV cDNA, and investigated their mRNA expression in several rat tissues. We report that caldecrin is expressed in rat pancreas, but that no elastase IV mRNA was detected in any tissues examined. Furthermore, we describe that caldecrin is a new-type serine protease, as judged on enzymatic analysis using the recombinant rat and human caldecrin proteins.

MATERIALS AND METHODS

Materials—The 4-methylcoumaryl-7-amide (MCA)-peptides used as substrates were purchased from the Peptide Institute. 4-Amidinophenyl-methanesulfonyl fluoride (APMSF) was from Boehringer Mannheim. L-*Trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64) was provided by Dr. H. Kido, Tokushima University. α-Chymotrypsin from bovine pancreas, phenylmethanesulfonyl fluoride (PMSF), and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were obtained from Nacalai. Bovine pancreas trypsin, *p*-nitroanilide (pNA)-peptides, leupeptin, chymostatin, pepstatin, aprotinin, elastatinal, diisopropyl fluorophosphate (DFP), *N*-tosyl-L-lysine-chloromethyl ketone (TLCK), and soybean trypsin inhibitor were purchased from Sigma.

Construction of Rat Elastase IV cDNA—Since there are a few differences in the nucleotide sequence between rat caldecrin and elastase IV, as shown in Fig. 1A, rat elastase IV cDNA was constructed according to the combinatorial PCR protocol (*12*) using already cloned rat caldecrin cDNA (*7*) as a template. Four primer sets derived from the nucleotide sequence of rat elastase IV cDNA were used in the first PCR; *Eco*RI tagged-F1 (5′-TTGAATTCATGTTGGGAATTACGGTCCTCG-3′, 1 to 22 of mRNA) and B1 (5′-AGAGACCTGCCAAGCCCAGCTGTTGGGGAC-3′, 138 to 109), F2 (5′-GTCCCCAACAGCTGGGCTTGGCAGGTCTCT-3′, 109 to 138) and B2 (5′-GTACACGGAGCCTCCGCATCCTCCACTGTC-3′, 302 to 273), F3 (5′-AGGCTCCGTGTACACTGAGGTGGACACCAT-3′, 290 to 319) and B3 (5′-ATGATAGCGATGTCGGTTCCACAGGAAGAG-3′, 374 to 345), and F4 (5′-CTCTTCCTGTGGAACCGACATCGCTATCAT-3′, 345 to 374) and *Eco*RI tagged-B4 (5′-TTGAATTCTCACAGTTGTATTTTCTCGTTG-3′, 807 to 786). The amplification was performed in 25 mM TAPS buffer (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μg of each primer, and 2.5 units of Ex Taq polymerase (Takara Shuzo), in total volume of 100 μl. The PCR conditions were 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by at 72°C for 7 min. The four kinds of amplified DNA fragments were separated by 2% agarose gel electrophoresis and extracted from the gel with QIAEX (QIAGEN). The extracted first PCR products (15 μl each) were mixed and annealed slowly from 94 to 37°C for 1 h. A part of the annealed sample was used as a template for the second PCR using the primer set, *Eco*RI tagged-F1 and -B4, under the same conditions as for the first PCR. The amplified PCR product of rat elastase IV cDNA was cloned into the pUC19 vector, selected, and confirmed by sequencing using a Thermo sequenase core sequencing kit (Amersham) and Texas-red labeled M13 universal primers (forward and reverse) with a Hitachi model SQ-5500 DNA auto-sequencer (HITACHI Japan).

Selective Detection of Rat Caldecrin and Elastase IV cDNA—Rat caldecrin cDNA was discriminated from elastase IV cDNA by means of the combination of PCR or mismatched-PCR and restriction enzyme digestion, as illustrated in Fig. 1B. The mismatched-PCR was performed using a modified primer (mB: 5′-CTCAGCCAACTTAATGATAGCGATGC-3′; 387 to 362) by the method described by Haliassos *et al.* (*13*). Two methods were used for the selective detection of caldecrin and elastase IV cDNA; one was the combination of PCR, with the F1 and B2 primer set, and digestion by *Hae*III, the other was mismatched-PCR, with the F3 and mB primer set, and *Msp*I digestion. Furthermore, to examine the expression of caldecrin and elastase IV mRNAs *in vivo*, total RNA was isolated from pancreas, liver, kidney, cerebellum, and testis of Wistar strain rats by the acid guanidinium thiocyanate-phenol-chloroform extraction method (*14*). The synthesis of the first-strand cDNA was carried out according to the supplier's recommendations (Pharmacia LKB). The first-strand cDNAs, together with already cloned rat caldecrin cDNA and rat elastase IV cDNA isolated in the

above experiment, were used as templates for the selective detection method, and the amplification of cDNA was performed as described previously (*15*).

Analysis of Genomic DNA—There is no information about a genomic DNA for caldecrin and elastase IV. On the basis of intron-exon boundaries in gene structure of elastase/chymotrypsin families (*9*), rat genomic DNA was amplified with a primer set, Ex4-F (5′-GGCCTGGGGAAGTATAATCT-3′, 253 to 272 nucleotide position of caldecrin cDNA) and Ex4-B (5′-GAAGAGTCGGTTCCACTTCT-3′, 351 to 332). The primer set is presumed to amplify the central region which contains the different nucleotides at 3 positions (2 substitutions and 1 deletion) between caldecrin and elastase IV cDNA. The PCR was performed according to the method described by Saiki *et al.* (*15*). The amplified DNA fragment was cloned into the TA vector and sequenced using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PERKIN ELMER) with M13 universal forward primer on a ABI 310 Genetic Analyzer (PERKIN ELMER Applied Biosystem).

Production of a Recombinant Virus and Expression of the Recombinant Proteins in Insect Cells—The recombinant virus of rat elastase IV was produced according to the manual for the baculovirus expression system (Clontech) and as described previously (*7, 8*). The rat elastase IV cDNA containing the entire coding region (807 bp), *i.e.* the *Eco*RI fragment from the pUC19 clone, was ligated into the *Eco*RI site of the baculovirus transfer vector, pBacPAK9 (Invitrogen). The purified rat elastase IV-BacPAK9 and linearized pBacPAK6 (*Bsu*36 digested) were cotransfected with lipofectin into sf-9 cells. After 5 days, serial diluted viruses were added to the culture medium, and then the plaques were visualized with an overlay of 0.5% SeaPlaque-agarose (FMC Bio Products) containing 0.05 mg/ml neutral red (Sigma). The clear plaques after a further 1 day were picked up and stored as baculovirus stocks until use for the infection experiment with sf-9 cells.

The recombinant rat elastase IV, rat caldecrin and human caldecrin were expressed in the baculovirus expression system (Clontech), as reported previously (*7, 8*).

Purification of the Recombinant Rat and Human Caldecrins—Sf-9 cells infected with a rat or human caldecrin baculovirus stock were cultured for 5 days at 27°C in serum-free medium (Gibco BRL). Since the recombinant caldecrin was secreted into the cultured medium as a proform, the medium was harvested and centrifuged at 3,000 rpm for 5 min. The supernatant was concentrated by Amicon ultrafiltration (YM-10) and then dialyzed against 10 mM sodium phosphate buffer, pH 6.8. The rat and human caldecrins were purified by on a Mono-Q FPLC column (*7*) and a hydroxyapatite CHT-1 column (*8*), respectively.

Enzymatic Analysis and Determination of Kinetic Parameters—Conversion of the purified proforms of rat and human caldecrins (0.375 μg) to their mature forms was performed by incubation in 80 μl of 0.1 M Tris-HCl, pH 7.8, containing 0.01 M $CaCl_2$ with 0.25 μg and 0.05 μg of trypsin for 60 min at 35°C and 20 min at 25°C, respectively. Protease activity was measured at 25°C for 10 min with 800 μl of 0.1 mM succinyl (Suc)-Ala-Ala-Pro-Phe-pNA or various peptide substrates in 0.1 M Tris-HCl and 0.01 M $CaCl_2$, pH 7.8 (final volume, 880 μl), and the reaction was stopped by the addition of 120 μl of 36% acetic acid. The concentration of pNA released was measured photometrically at 280 nm with a double-beam spectrophotometer (Shimadzu), and that of MCA was measured fluorophotometrically, with excitation at 370 nm and emission at 460 nm, with a spectrofluorophotometer (Shimadzu). One unit of activity was defined as 1 μmol of pNA or MCA released per min. Before the measurement of protease activity with Boc-Gln-Gly-Arg-MCA and Boc-Glu-Lys-Lys-MCA, the activated caldecrin was treated with TLCK for 30 min on ice (weight ratio of trypsin to TLCK, 1: 1,000). Kinetic parameters, *i.e.* K_m, k_{cat}, and k_{cat}/K_m values, were calculated from Lineweaver-Burk plots.

Protein concentrations were determined using BCA reagent (Pierce) with bovine serum albumin as a standard.

Electrophoresis—SDS-PAGE (12.5% acrylamide gel) was performed according to the method of Laemmli (*16*), and the gel was stained with the reagents in a Quick-CBB gel staining kit (Wako Pure Chemical Industrial). The samples for SDS-PAGE were pretreated with 1 mM PMSF for 10 min on ice and then heat-treated at 95°C for 5 min after the addition of SDS-buffer containing β-mercaptoethanol. Western blot analysis was performed with anti-porcine caldecrin antibodies, as described previously (*6*).

RESULTS

Construction of Rat Elastase IV cDNA, and Detection of Rat Caldecrin and Elastase IV mRNAs—The nucleotide sequences of rat caldecrin and elastase IV cDNAs differ at only 5 positions; 3 substitutions, 1 deletion, and 1 insertion, as shown in Fig. 1A (*7, 10*). The rat elastase IV cDNA was constructed from the rat caldecrin cDNA, as a template, by means of two-step PCR using the combinatorial PCR method (*12*), as described under "MATERIALS AND METHODS." The sequence was confirmed by sequencing and with the selective detection method, which can discriminate caldecrin cDNA from elastase IV cDNA, as shown in Fig. 1B. In addition, to determine whether or not the elastase IV mRNA is expressed *in vivo* and to confirm that the caldecrin mRNA is transcribed in the pancreas, first strand cDNA was synthesized with total RNA isolated from rat tissues including pancreas, liver, kidney, cerebellum, and testis, and used as a template for the selective detection method.

The rat elastase IV cDNA isolated in this study, the rat caldecrin cDNA already cloned (*7*), and the first strand cDNAs from various rat tissues were used as templates for amplification with 2 primer sets; F1 and B2, or F3 and a modified primer (mB). Figure 2A shows the gel electrophoresis pattern of the PCR products with primers F3 and mB. A 98 bp or 97 bp PCR product was derived from each cloned cDNA of elastase IV or caldecrin, and only detected in the pancreas, *i.e.* not in other tissues. In this experiment, it was difficult to determine whether the PCR product found in the pancreas originated from caldecrin or from elastase IV, because the difference between caldecrin and elastase IV is only one bp. The same results were obtained with the other primer set; F1 and B2 (data not shown).

Therefore, the caldecrin and elastase IV cDNAs were discriminated by two methods; *Hae*III digestion of the PCR products with primers F1 and B2, and *Msp*I digestion of the PCR products with primers F3 and mB, as shown in Fig. 2B. In the PCR product with F1 and B2, the 311 bp from the caldecrin cDNA contains 3 *Hae*III sites, while the 310 bp

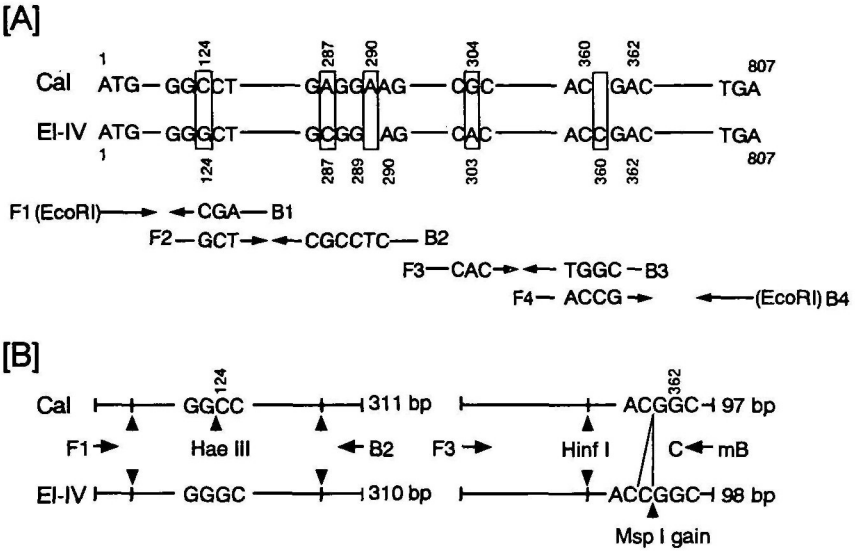

Fig. 1. **Principles for the construction of rat elastase IV cDNA [A], and for the selective detection of rat caldecrin and rat elastase IV mRNA** [B]. [A] The five boxed positions at the top represent the differences in the nucleotide sequences of the coding regions (1-807) of the rat caldecrin (Cal) and elastase IV (El-IV) cDNAs. The nucleotides are numbered with the A residue of the initiation codon taken as +1. The location of each primer used in the combinatorial PCR is indicated schematically by arrows at the bottom. The sequences and detailed positions of the primers, and the construction of rat elastase IV cDNA are described under "MATERIALS AND METHODS." [B] Two methods were used for the selective detection of caldecrin (Cal) and elastase IV (El-IV) cDNA by the combination of PCR and restriction enzyme digestion; one was with primers F1 and B2, and digestion by *Hae*III. The other was with primers F3 and mB, and digestion by *Msp*I. mB, a modified primer, has a mismatched base (from A to G in the sense strand) at nucleotide position 362 to create a *Msp*I site for elastase IV cDNA, as described under "MATERIALS AND METHODS." There are size differences in the PCR products amplified from the cDNAs, because a deletion or insertion is present in elastase IV cDNA compared with in caldecrin cDNA.

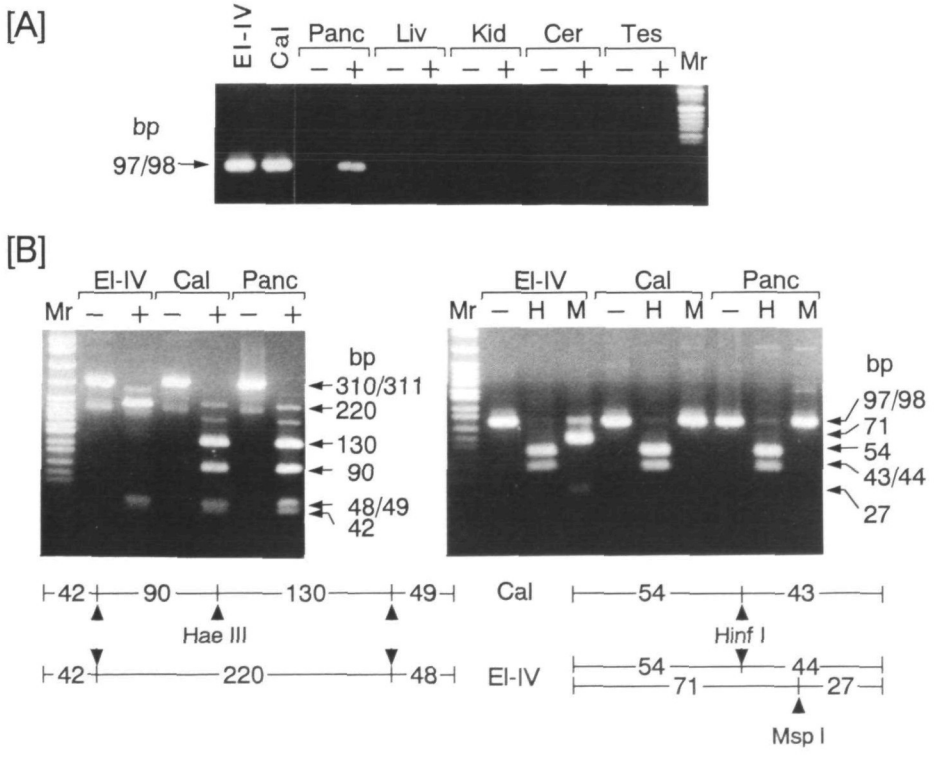

Fig. 2. **Selective detection of caldecrin and elastase IV cDNAs, and confirmation that caldecrin mRNA is expressed selectively in pancreas.** [A] Elastase IV cDNA (El-IV) isolated by the combinatorial PCR method, caldecrin cDNA (Cal) cloned already, and first-strand cDNAs synthesized from total RNA of rat pancreas (Panc), liver (Liv), kidney (Kid), cerebellum (Cer), and testis (Tes) without (−) or with (+) reverse transcriptase were amplified using primers F3 and mB, as indicated in Fig. 1. The PCR products were applied to a 2% agarose gel and stained with ethidium bromide after the run. [B] The PCR products amplified with primers F1 and B2 (left panel) or F3 and mB (right panel) were digested without (−) and with (+) *Hae*III or *Hin*fI (H) and *Msp*I (M), respectively. The digests were separated on a 2.5% agarose gel and stained with ethidium bromide. A schematic representation of the selective detection method is shown in the bottom panel. The diagram indicates the position of the *Hae*III, *Hin*fI, and *Msp*I sites, and the sizes of the restriction fragments obtained on digestion. M_r denotes the size markers of DNA.

from the elastase IV cDNA contains 2 *Hae*III sites, because there is a C to G substitution at nucleotide position 124. The PCR product was digested into four fragments of 130, 90, 49, and 42 bp for caldecrin, and into three fragments of 220, 48, and 42 bp for elastase IV, respectively. The digestion fragments of the PCR product from the pancreas showed the same pattern as caldecrin cDNA (left panel in Fig. 2B). On the other hand, with primers F3 and mB, an *Msp*I site was newly created in the PCR product derived from elastase IV but not in that from the caldecrin. As shown in the right panel of Fig. 2B, the PCR product (98 bp) from elastase IV cDNA was digested by *Msp*I into 71 and 27 bp fragments, while the PCR products from caldecrin cDNA and cDNA from the pancreas were not digested. A *Hin*fI site was found in all three kinds of PCR products. These results indicate that no elastase IV mRNA was detected in any tissues examined and confirmed that the caldecrin mRNA is selectively expressed in the pancreas.

Analysis Using Genomic DNA—Although elastase IV was not expressed in any tissues as above, we can not ruled out the possibility that the gene expression of elastase IV is extremely low. To examine whether either sequence for caldecrin or elastase IV, or both are present on genomic DNA, the central region which involves 3 nucleotide differences between caldecrin and elastase IV cDNA was amplified using rat genomic DNA, cloned and sequenced. As the gene structure of caldecrin and elastase IV is not known yet, we presumed the exon boundary of caldecrin based on the intron-exon junction in serine protease gene such as elastase and chymotrypsin (*9*). A primer set, Ex4-F and Ex4-B, used in the present study was unique sequences for caldecrin and elastase IV compared with other serine proteases. The amplified fragment showed 99 bp size, and the sequences of all 8 clones derived from genomic DNA were completely identical to that from 253 to 351 of caldecrin cDNA (data not shown), indicating that there is no intron in the central region amplified, and suggesting that the intron-exon boundary in caldecrin gene is very similar to that of elastase family and different from chymotrypsin. These results clearly show that the caldecrin sequence is exactly present in genomic DNA, and that elastase IV is an artificial product during RT-PCR and/or cloning.

Expression of the Recombinant Rat Caldecrin and Elastase IV in Sf-9 Cells—To examine the functional properties of rat elastase IV, the recombinant protein was synthesized in sf-9 cells using a baculovirus expression system and compared with the recombinant rat caldecrin. The sf-9 cells infected with each recombinant virus were cultured in serum-free medium at 27°C, and the cultures were harvested every day for 5 days. The cell lysates and cultured medium were used for Western blot analysis and for the determination of protease activity after trypsin treatment.

On Western blot analysis, as shown in Fig. 3A, an immunoreactive band corresponding to 32 kDa in size was detected for the cell lysates derived from both caldecrin and elastase IV from 2 days after infection, the maximum being reached at 4 days. In the cultured medium, immunoreactive materials were found in the caldecrin culture, but not in the elastase IV virus-infected one. The recombinant rat caldecrin was significantly secreted at 2 days after virus infection, the maximal level being reached at 5 days. The recombinant caldecrin in both cultured cells and medium showed protease activity toward a synthetic substrate for chymotrypsin, Suc-Ala-Ala-Pro-Phe-pNA, only after treatment with trypsin, indicating that the major form expressed is a proenzyme or zymogen. Although an immunoreactive protein was detected in the elastase IV virus-infected cells, the cell extract showed much lower protease activity even after trypsin treatment. It is reasonable to use a protease substrate for caldecrin for the detection of elastase IV protease activity because the amino acid residues concerned with the substrate specificity of caldecrin are conserved in elastase IV (see "DISCUSSION"). Very similar results were obtained with a substrate for elastase, Suc-Ala-Ala-Pro-Leu-pNA (data not shown). Thus, the protease activity reflected the results of Western blot analysis fairly well. These results indicate that rat elastase IV was expressed in sf-9 cells, but not secreted, and did not exhibit as much protease activity as caldecrin. As the results, we could not examine the serum calcium-decreasing activity of elastase IV. It was confirmed that rat caldecrin is a secretory protein, which is consistent with the results for BMT-10 cells transfected with an expression vector bearing rat caldecrin cDNA (*7*).

Comparison of the Rat and Human Caldecrins as to Activation by Trypsin—The purified mature form of porcine caldecrin is a chymotrypsin-like serine protease, and its protease activity was measured as the hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA (*6*). The proform of caldecrin was purified from porcine pancreas, and showed chymotrypsin activity after trypsin treatment in time- and dose-dependent manners (*11*). As judged from the sequence homology of the rat and human caldecrin cDNAs with serine proteases and/or elastases, caldecrin is synthesized as a preproenzyme for secretory proteins and is secreted as a proenzyme that requires activation by trypsin for conversion to the active enzyme (*7, 8*). Since the activation of the rat and human caldecrin proforms had not been investigated in detail, dose-response and time-course experiments involving trypsin treatment were performed using the recombinant proteins in the present study.

As shown in Fig. 4A, full activation of human caldecrin (0.375 μg of proform) was achieved in 10 to 30 min at 25°C using 0.05 μg of trypsin, which is the same as the conditions used for activation of the porcine proform, as described previously (*11*), but the level of activated rat caldecrin was very low under the same conditions. On the contrary, for rat caldecrin to become the active form required a higher

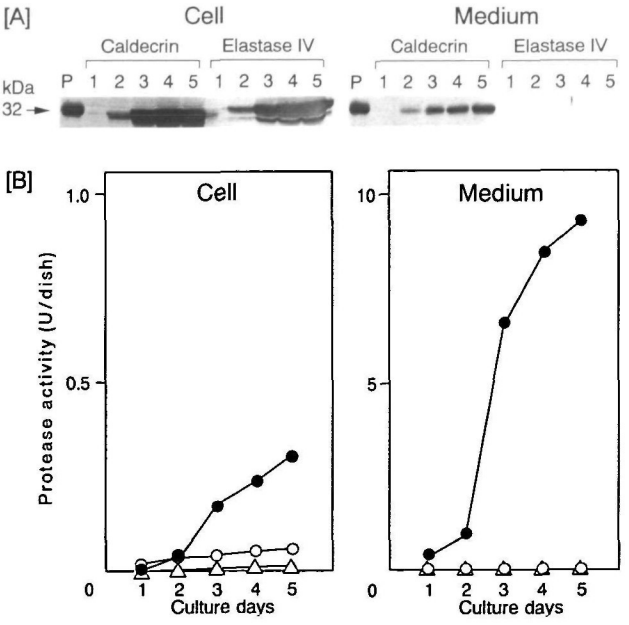

Fig. 3. **Expression of rat caldecrin and rat elastase IV in sf-9 cells, and secretion into the medium.** Sf-9 cells transfected with either rat caldecrin virus (closed circles in [B]) or rat elastase IV virus (open circles in [B]) were cultured for 1 to 5 days. The cell lysates and medium obtained from cultures each day were analyzed by Western blotting [A] using anti-porcine caldecrin antibodies, and their protease activities [B] were measured as the hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA after trypsin treatment under the optimum conditions for rat caldecrin (0.25 μg of trypsin at 35°C for 60 min). The purified recombinant rat caldecrin proform (P) was used for Western blot analysis [A] as a positive control. pBacPAK6 wild-type virus containing only the polyhedrin promoter (open triangles in [B]) was used as a control.

temperature, a higher dose of trypsin, and a longer time than for human caldecrin. These data suggest that the affinity for trypsin differs between rat and human caldecrins. Although the amino acid sequence around the cutting site for activation by trypsin is well conserved in the rat and human caldecrins, the P_2 site being Thr in rat caldecrin, and Ala in the human and porcine enzymes (*8*). With this background, we constructed a mutant cDNA of rat caldecrin in which Ala was substituted for Thr, and synthesized the mutant protein in a baculovirus expression system. As the rate of activation of the rat recombinant mutant, however, was the same as that of the rat wild-type proform (data not shown), unconserved amino acids in other sites might be related to the affinity of trypsin for each caldecrin.

The activated rat caldecrin was much more stable than the human caldecrin; the latter rapidly lost its activity after activation (Fig. 4B). It remains to be determined why the activated human caldecrin is so labile compared with rat caldecrin. The instability or inactivation of the activated human caldecrin was not even prevented by the addition of TLCK at 20 min after trypsin treatment, and no difference in molecular weight on SDS-PAGE was observed between the fully activated (20 min) and completely inactivated (240 min) human caldecrins (data not shown), suggesting that the instability of the activated human caldecrin is not due to further degradation by trypsin or autolysis by caldecrin.

Substrate Specificity of the Rat and Human Caldecrins— The protease activities toward various pNA and MCA substrates were measured in 0.1 M Tris-HCl containing 0.01 M $CaCl_2$, pH 7.8, at 25°C with the activated rat and human caldecrins after trypsin treatment under the optimal conditions for each caldecrin. The substrates for elastase (Suc-Ala-Ala-Ala-pNA, Suc-Ala-Pro-Ala-pNA or -MCA, and Suc-Ala-Ala-Pro-Val-MCA), kallikrein (Z-Phe-Arg-MCA), post-proline cleaving enzyme (Suc-Gly-Pro-MCA), and aminopeptidase (Leu-pNA) were not hydrolyzed by either the rat or human caldecrin. On the other hand, the peptide substrates hydrolyzed by both caldecrins at moderate or high rates are listed in Table I, and the K_m values for the substrates, and the k_{cat} and k_{cat}/K_m values were calculated. For both the rat and human caldecrins, Suc-Ala-Ala-Pro-Leu-pNA, used as a substrate for elastase, was the best substrate. Suc-Ala-Ala-Pro-Phe-pNA, a sensitive substrate for chymotrypsin, was also hydrolyzed well. Suc-Leu-Leu-Val-Tyr-MCA was, however, a poor substrate. When the insulin B chain was used as a substrate for human caldecrin, Tyr as well as Leu and Phe at the P_1 site was cleaved well (data not shown). Arg and Lys at the P_1 site on the insulin B chain were not cleaved by human caldecrin, although those of MCA substrates were hydrolyzed with high K_m values. These results indicate that the rat and human caldecrins are closer to chymotrypsin than to elastase (*17, 18*), although they hydrolyze Boc-Gln-Gly-Arg-MCA and Boc-Gly-Lys-Lys-MCA, and Suc-Leu-Leu-Val-Tyr-MCA poorly.

Effects of Various Inhibitors on the Proteolytic Activity— The activated caldecrins were treated without and with various protease inhibitors for 30 min on ice, and then the protease activity was measured using Suc-Ala-Ala-Pro-Phe-pNA as a substrate. As shown in Table II, the activities of both the rat and human caldecrins were strongly inhibited by serine protease inhibitors, such as DFP and PMSF, and by chymotrypsin-type serine protease inhibitors, such

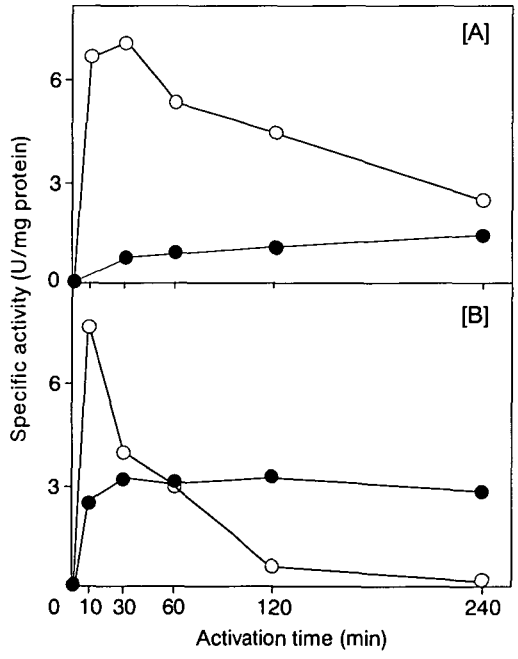

Fig. 4. **Time course of activation of rat and human caldecrins by trypsin.** After the activation of the rat (closed circles) and human (open circles) caldecrin proforms by trypsin, the protease activity was determined as the hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA. The procaldecrins (0.375 μg) from rat and man were incubated with 0.05 μg of trypsin at 25°C [A] or 0.25 μg of trypsin at 35°C [B] for the times indicated.

TABLE I. **Kinetic parameters for rat and human caldecrins.** The purified rat and human caldecrins (0.375 μg) were activated with 0.25 and 0.05 μg of trypsin at 35°C for 60 min and 25°C for 20 min, respectively, and then the protease activity was measured with each substrate at 25°C. The K_m, k_{cat}, and k_{cat}/K_m values were calculated from Lineweaver-Burk plots.

Substrates	Rat			Human		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
pNA-peptide						
Suc-Ala-Ala-Pro-Phe-pNA	0.22	3.81	17.3	0.03	4.74	158
Suc-Ala-Ala-Pro-Leu-pNA	0.14	4.44	31.7	0.01	1.62	232
MCA-peptide						
Suc-Ala-Ala-Pro-Phe-MCA	0.26	0.67	2.57	0.06	0.87	14.5
Boc-Gln-Gly-Arg-MCA	0.31	0.36	1.15	0.63	0.44	0.70
Boc-Gly-Lys-Lys-MCA	1.43	0.02	0.02	1.67	0.20	0.12
Suc-Leu-Leu-Val-Tyr-MCA	0.09	0.01	0.12	0.07	0.03	0.46

TABLE II. **Effects of protease inhibitors on the protease activities of rat and human caldecrins.** The purified rat and human caldecrins were full-activated with trypsin under the same conditions as given in Table I and then incubated with various protease inhibitors on ice for 30 min. The protease activity of caldecrin was measured with Suc-Ala-Ala-Pro-Phe-pNA as the substrate at 25°C.

Inhibitor (concentration)	Relative activity	
	Rat	Human
	(%)	
Without	100	100
DFP (0.1 mM)	0	0
PMSF (1 mM)	0	0
APMSF (50 μM)	98.6	90.5
Leupeptin (100 μM)	93.4	94.4
Chymostatin (100 μM)	0	0
E-64 (2.5 μM)	108.2	65.5
Pepstatin (100 μM)	27.8	9.4
Soy bean trypsin inhibitor (100 μg/ml)	10.5	8.9
Aprotinin (0.2 mg/ml)	65.1	119.2
TPCK (100 μM)	98.4	91.1
TLCK (100 μM)	91.3	92.9
Elastatinal (100 μM)	94.5	96.1

as chymostatin and soybean trypsin inhibitor. The caldecrins were not inhibited by other inhibitors for trypsin, thiol protease, and elastase, such as APMSF, leupeptin, E-64, and elastatinal. Surprisingly, TPCK, which is a well-known chymotrypsin-specific inactivator and which actually dose- and time-dependently inhibits chymotrypsin (data not shown), failed to inactivate either the rat or human caldecrin, and a high concentration of pepstatin, an acid protease inhibitor, had an inhibitory effect on the human and rat caldecrins. These results indicate that rat and human caldecrins can be classified as chymotrypsin-type serine proteases, but show dispositions that are not characteristic for chymotrypsin, as judged from the finding of no inhibition by TPCK.

DISCUSSION

Kang *et al.* (*10*) isolated elastase IV cDNA from rat pancreas poly(A)$^+$ RNA by PCR using primers derived from the consensus amino acid sequences of the active sites of mammalian serine proteases, but did not investigate its function or expression *in vivo*. The nucleotide sequence of elastase IV cDNA showed 99.3% identity with that of the rat caldecrin cDNA cloned by Tomomura *et al.* (*7*) on the screening of a pancreas cDNA library using anti-porcine caldecrin antibodies. As judged from the nucleotide differences at 5 positions of elastase IV cDNA (3 substitutions, 1 deletion, and 1 insertion) as compared with rat caldecrin, the homology of the deduced amino acid sequences was 90.3%. The predicted elastase IV protein is presumed to be synthesized as a preproenzyme with a signal peptide and an activation peptide, and the mature form consists of 239 amino acid residues. The primary structure of elastase IV is almost identical to that of rat caldecrin, there being an amino acid residue substitutions of Pro to Ala at position +13 of the mature form, and of 25 amino acid residues from position +67 to +91 in the amino acid sequence of elastase IV, which differ completely from those of caldecrin. The amino acid residues characteristic of the catalytic triad of the serine proteases (*19*) are conserved at the same positions His-45, Asp-92, and Ser-187, in both caldecrin and elastase IV, and the other key amino acid residues, Gly-209 and Val-221, thought to contribute to the substrate specificity are retained in the deduced amino acid sequences of caldecrin and elastase IV (*7*). We previously reported that the recombinant rat and human caldecrins synthesized in a baculovirus expression system were secreted as proforms into the cultured medium, and that the proform of caldecrin exhibited no proteolytic activity and no serum calcium-decreasing activity (*7, 8*). The mature form and the activated caldecrin derived from its proform on trypsin treatment showed chymotrypsin-like activity, but the proteolytic activity of caldecrin was not necessary for its serum calcium-decreasing activity (*6-8, 11*). These data suggest that elastase IV and caldecrin are derived from a single gene through some unusual splicing or that one of them is an artifact. Anyway, if we could isolate the elastase IV protein and examine its bioactivity, a lot of information about the structure necessary for serum calcium-decreasing activity would be obtained.

In the present study, we constructed elastase IV cDNA by means of combinatorial PCR, and compared caldecrin with elastase IV synthesized in a baculovirus expression system. On Western blot analysis with anti-porcine caldecrin antibodies, an immunoreactive protein corresponding to a size of 32 kDa was detected in sf-9 cells transfected with the rat elastase IV virus. When the recombinant elastase IV from the cells was treated with trypsin, the protease activity was, however, much lower than that of caldecrin. No recombinant rat elastase IV protein was found in the cultured medium, which was quite different from in the case of caldecrin. It is reasonable to presume that the rate of protein synthesis in sf-9 cells is equal between caldecrin and elastase IV, because they have the same promoter and the same preprosequence. Therefore, the much lower total amount of the immunoreactive protein found in the elastase IV virus-infected cells and the medium suggests its instability; the amount of the elastase IV protein was calculated to be about one-thirtieth of that of caldecrin from their protein bands and activities. The rat elastase IV protein is probably degraded easily before or after secretion. As a result, we could not isolate a recombinant elastase IV. On the other hand, we investigated whether or not caldecrin and elastase IV mRNAs are synthesized *in vivo*. No elastase IV mRNA was detected in rat tissues including pancreas, while caldecrin mRNA was selectively expressed only in the pancreas. In addition, we detected caldecrin but not elastase IV sequences in rat genomic DNA. All these results suggest that rat elastase IV cDNA is an artifact arising during cloning due to RT-PCR or a reading mistake in sequencing, and that the caldecrin gene is expressed *in vivo*. This conclusion is supported by our previous data showing that the deduced amino acid sequence of the +67 to +91 region of rat caldecrin cDNA, completely differing from that of elastase IV, was identical to the peptide sequence of a fragment obtained from the purified rat caldecrin, and that it exhibited high homology with the corresponding peptide sequence of the purified porcine caldecrin (*7*).

We have reported that procaldecrin required activation with trypsin for conversion to the active form both as a protease and a bioactive substance (*7, 8, 11*). Surprisingly, the conditions for activation with trypsin differed between the rat and human caldecrins. Rat caldecrin required much

more trypsin, a higher temperature and a longer incubation time than human caldecrin. This may be due to the difference in the affinities of trypsin for them. It may be possible that a difference in glycosylation caused the difference in activation. Ladenheim *et al.* reported that a difference in glycosylation affects the processing of the mouse submaxillary gland prorenin to its active form (*20*). Human caldecrin contains a potential asparagine-linked glycosylation site at Asn-23 of the active form, which is not conserved but substituted by Asp in rat caldecrin (*8*).

The substrate specificities of rat and human caldecrins (Table I) suggests that they belong to the chymotrypsin family, not the elastase family. Synthetic substrates for chymotrypsin are well cleaved by both the rat and human caldecrins. We have already obtained similar results for porcine caldecrin (unpublished data). Among the pNA and MCA substrates examined, the k_{cat} values for the latter are one order of magnitude smaller than those for the former, which have the same peptide sequence (Suc-Ala-Ala-Pro-Phe-), although there is little difference in the K_m values between pNA and MCA substrates. Generally, chymotrypsin catalyzes the hydrolysis of peptide or ester bonds in two distinct steps; acylation and deacylation. The second step (deacylation) is much slower than the first (acylation), so that the deacylation step determines the overall rate of hydrolysis of ester bonds by chymotrypsin. In some cases, however, acylation determines it (*21, 22*). The availability of substrates having the same peptide sequence with different P'_1 sites for caldecrin was different, suggesting that the acylation step, *i.e.* the release of pNA and MCA, may be rate-limiting and that the amino acid residue providing the amino group for the peptide bond (P'_1 site) may be involved in the specificity of caldecrin.

The data given in Table II indicate that rat and human caldecrins as well as the porcine enzyme (*6*) are serine proteases. Although these caldecrins are considered to be chymotrypsin-type rather than elastase-type enzymes from viewpoint of their substrate specificity, it is difficult to determine the type from the inhibitor specificity results. Elastatinal at 100 μM failed to inhibit the caldecrins, while it showed 50% inhibition (ID_{50}) of elastase at 3.5 μM (*23*). In addition, when the molar ratio of TPCK to chymotrypsin was 10, TPCK inactivated chymotrypsin to 50% (ID_{50}) in about 50 min (*24*), but even excess TPCK did not inactivate the caldecrins. Although pepstatin did not inhibit chymotrypsin at 360 μM (*25*), it remarkably inhibited the caldecrins at 100 μM. These results suggest that caldecrin is a novel-type serine protease, which is closely related to chymotrypsin rather than elastase, with characteristic properties including weak but significant substrate specificity for basic amino acid residues at the P_1 site, insensitivity to TPCK and inhibition by pepstatin which concentration was much higher than for acid proteases (*25*). We consider that in addition to serum calcium-decreasing activity, caldecrin probably plays a role also as a digestive enzyme like chymotrypsin, because its proteolytic activity measured with Suc-Ala-Ala-Pro-Phe-pNA was as same as chymotrypsin in human enzyme or one-fifth in rat enzyme.

We are grateful to Dr. Hiroshi Kido, Tokushima University, for supplying the E-64. We also wish to thank Mariko Tanaka for her assistance in preparation of the manuscript and Tomoko Fukushige for her technical support.

REFERENCES

1. Balart, L.A. and Ferrante, W.A. (1982) Pathophysiology of acute and chronic pancreatitis. *Arch. Int. Med.* **142**, 113-117
2. Yoneda, T., Takaoka, Y., Alsina, M.M., Garcia, J., and Mundy, G.R. (1991) Porcine pancreas extract decreases blood-ionized calcium in mice and inhibits osteoclast formation and bone resorption in culture. *FEBS Lett.* **278**, 171-174
3. Yoneda, T., Izbicka, E., Bonewald, L., Jacobs, C., Chavez, J., Takaoka, Y., and Mundy, G.R. (1993) Identification of a novel hypocalcemic factor from the pancreas which inhibits bone resorption. *J. Bone Miner. Res.* **8** (suppl.), 174
4. Yoneda, T., Takaoka, Y., Boyce, B.F., Scott, L., and Mundy, G.R. (1994) Extracts of porcine pancreas prevent progression of hypercalcemia and cachexia and prolong survival in nude mice bearing a human squamous carcinoma. *Cancer Res.* **54**, 2509-2513
5. Izbicka, E., Dunstan, C., Horn, D., Mundy, G.R., and Yoneda, T. (1995) Identification and characterization of a pancreatic elastase IIIB with systemic effects on bone resorption and bone formation *in vivo*. *J. Bone Miner. Res.* **10** (suppl.), 480
6. Tomomura, A., Fukushige, T., Noda, T., Noikura, T., and Saheki, T. (1992) Serum calcium-decreasing factor (caldecrin) from porcine pancreas has proteolytic activity which has no clear connection with the calcium decrease. *FEBS Lett.* **301**, 277-281
7. Tomomura, A., Tomomura, M., Fukushige, T., Akiyama, M., Kubota, N., Kumaki, K., Nishii, Y., Noikura, T., and Saheki, T. (1995) Molecular cloning and expression of serum calcium-decreasing factor (caldecrin). *J. Biol. Chem.* **270**, 30315-30321
8. Tomomura, A., Akiyama, M., Itoh, H., Yoshino-Yasuda, I., Tomomura, M., Nishii, Y., Noikura, T., and Saheki, T. (1996) Molecular cloning and expression of human caldecrin. *FEBS Lett.* **386**, 26-28
9. Tani, T., Ohsumi, J., Mita, K., and Takiguchi, Y. (1988) Identification of a novel class of elastase isozyme, human pancreatic elastase III, by cDNA and genomic gene cloning. *J. Biol. Chem.* **263**, 1231-1239
10. Kang, J., Wiegand, U., and Müller-Hill, B. (1992) Identification of cDNA encoding two novel rat pancreatic serine proteases. *Gene* **110**, 181-187
11. Tomomura, A., Fukushige, T., Tomomura, M., Noikura, T., Nishii, Y., and Saheki, T. (1993) Caldecrin proform requires trypsin activation for the acquisition of serum calcium-decreasing activity. *FEBS Lett.* **335**, 213-216
12. Higuchi, R. (1990) DNA engineering using PCR in *PCR Technology: Principles and Application for DNA Amplification* (Enrlich, H.A. and Katoh, I., eds.) pp. 87-100, TAKARA Syuzou
13. Haliassos, A., Chomel, J.C., Tesson, L., Baudis, M., Kruh, J., Kaplan, J.C., and Kitzis, A. (1989) Modification of enzymatically amplified DNA for the detection of point mutations. *Nucleic Acids Res.* **17**, 3606
14. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
15. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491
16. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
17. Folk, J.E. and Schirmer, E.W. (1965) Chymotrypsin C: I. Isolation of the zymogen and the active enzyme: preliminary structure and specificity studies. *J. Biol. Chem.* **240**, 181-192
18. Folk, J.E. and Cole, P.W. (1965) Chymotrypsin C: II. Enzymatic specificity toward several polypeptides. *J. Biol. Chem.* **240**, 193-197
19. Blow, D.M., Birktoft, J.J., and Hartley, B.S. (1969) Role of a buried acid group in the mechanism of action of chymotrypsin. *Nature* **221**, 337-340
20. Ladenheim, R.G., Seidah, N.G., and Rougeon, F. (1991) *N*-Linked glycosylation affects the processing of mouse submaxil-

lary gland prorenin in transfected AtT 20 cells. *Eur. J. Biochem.* **198**, 535-540
21. Epand, R.M. and Wilson, I.B. (1963) Evidence for the formation of hippuryl chymotrypsin during the hydrolysis of hippuric acid esters. *J. Biol. Chem.* **238**, 1718-1723
22. Williams, A. (1970) Chymotrypsin-catalyzed phenyl ester hydrolysis. Evidence for electrophilic assistance on carbonyl oxygen. *Biochemistry* **9**, 3383-3390
23. Umezawa, H., Aoyagi, T., Okura, A., Morishima, H., Takeuchi, T., and Okami, Y. (1973) Elastatinal, a new elastase inhibitor produced by actinomycetes. *J. Antibiot.* **26**, 787-789
24. Guenther, S. and Elliot, S. (1962) Direct evidence for the presence of histidine in the active center of chymotrypsin. *Biochemistry* **2**, 252-255
25. Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Hamada, M., and Takeuchi, T. (1970) Pepstatin, a new pepsin inhibitor produced by actinomycetes. *J. Antibiot.* **23**, 259-262