Cloning of the gene and cDNA for hamster chymase 2, and expression of chymase 1, chymase 2 and angiotensin-converting enzyme in the terminal stage of cardiomyopathic hearts

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Chymase is responsible for the formation of angiotensin II, which plays crucial roles in the pathogenesis of cardiovascular diseases. In the present study we determined the gene organization of a novel hamster chymase (hamster chymase 2) and analysed the expression of chymase 1, chymase 2 and angiotensin-converting enzyme (ACE) in hamster hearts at the terminal stage of cardiomyopathy. The gene encoding hamster chymase 2 is 3.2 kb in length and has five exons and four intervening sequences. The overall organization of this gene is similar to that of several other serine proteases. The deduced amino acid sequence revealed the existence of a preproenzyme composed of a signal peptide with 19 amino acids, a propeptide with two amino acids and a catalytic domain with 226 amino acids. The predicted full sequence of the catalytic domain was revealed to be very similar to the sequences of mouse mast-cell protease 5 (86%), rat mast-cell protease III (85%) and human chymase (70%) and less similar to hamster chymase 1 (56%). The expression of chymase 1 in heart was higher than that of chymase 2. The cardiac chymase-like activity, as well as the mRNA levels of chymase 1 and 2 of BIO 14.6 cardiomyopathic hamsters at the age of 60 weeks were increased 3.4-, 2.8- and 5.1-fold respectively compared with age-matched BIO F1B control hamsters. The cardiac ACE activity and the ACE mRNA level of cardiomyopathic hamsters were also increased 4.1- and 2.4-fold compared with those of age-matched controls. These results suggest that up-regulation of both ACE and chymases participates in the pathophysiology of the terminal stage of cardiomyopathy.

INTRODUCTION

Chymase is a chymotrypsin-like serine protease mainly localized to the secretory granules of mast cells and most likely involved in the immune and inflammatory responses by hydrolysing a variety of substrates, including extracellular matrix [1,2], vasoactive substances [3–5], cytokines [6,7], metalloproteases [8] and lipoproteins [9]. In addition, chymase might be able to alter the cellular metabolism directly and may have an important role in regulating cellular function [10]. These properties of chymase may be relevant to the mechanisms of tissue remodelling. Although its precise *in vivo* pathophysiological role as well as the actual substrate for chymase have not been determined, recent observations have focused on the function of chymase as an angiotensin II-forming enzyme.

Chymases of human, monkey, dog, and hamster are highly efficient in converting angiotensin (ANG) I into ANG II [3,11–15]. ANG II is a potent bioactive peptide and exhibits diverse actions on cardiovascular tissues. There is substantial evidence that ANG II is formed locally in cardiac tissues, and the augmented formation of tissue ANG II is involved in the paracrine regulation of cardiac functions and in the pathogenesis of heart failure. However, ANG II-forming pathways do not always follow the classical renin–angiotensin system. Many lines of evidence show that the chymase-dependent ANG II-forming pathway is a major pathway for ANG II formation in cardiovascular tissues [11–21]. In a recent study we purified [14] and cloned ANG II-forming chymase (hamster chymase 1) from Syrian hamsters [19] and determined that hamsters possess the same ANG II-forming system as do humans.

The cardiomyopathic Syrian hamster of the inbred strain BIO 14.6 has proven to be a useful animal model for studying the pathophysiology of human idiopathic cardiomyopathy and congestive heart failure. The progression of the disease process has been divided into four phases: premyolytic, myolytic and fibrotic, hypertrophic and the terminal stage of congestive heart failure. In the premyolytic stage, neither histological lesions nor clinical evidence of disease is apparent. The myolytic stage is characterized by spotty myolysis, which begins at 6 weeks of age, and continues for 3–4 months. By about 10–20 weeks of age, many myolytic lesions have healed, reconstructed with the fibrotic connective tissue and followed by a compensatory cardiac hypertrophy. Congestive heart failure becomes apparent after 40 weeks. In our previous study, the cardiac ANG II-forming system was examined in three separate age groups: 4 weeks old, 12 weeks old and 25 weeks old. These age groups corresponded to the stage of premyolysis, myolysis and hypertrophy without any clinical sign of heart failure. The cardiac chymase mRNA level and chymase-like activity in the cardiomyopathic hamsters increased significantly at the age of 12 weeks and 25 weeks, while ACE was not up-regulated in the cardiomyopathic heart up to the age of 25 weeks [19]. These results indicate that chymase-dependent ANG II-forming system is involved in the development of cardiomyopathy without heart failure. However, the precise ANG II-forming system in the terminal stage of cardiomyopathy has not been established. Information at the terminal

Abbreviations used: ANG, angiotensin; ACE, angiotensin-converting enzyme; MCP, mast-cell protease; GMCP, gerbil MCP; MMCP, mouse MCP; RMCP, rat MCP; RT, reverse transcriptase.

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The nucleotide sequence data reported will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AB007622.

stage of cardiomyopathy is extremely important for exploring the pathophysiology of congestive heart failure. Furthermore, the entire hamster chymase family has not yet been clarified.

In the present study we determined the gene organization of a novel hamster chymase (hamster chymase 2) successfully isolated by genomic cloning. Furthermore, we analysed the expression of two chymases and ACE in hamster hearts at the terminal stage of cardiomyopathy and explored the ANG II-forming system in the failing hearts.

EXPERIMENTAL

Genomic and cDNA cloning

Hamster genomic DNA was obtained from the liver and a genomic library was constructed in λ ZAP II vector (Stratagene, La Jolla, CA, U.S.A.). Approx. 5×10^5 phage plaques were screened with the ^{32}P-labelled 147 bp fragment of the hamster chymase-1 gene using standard methods [19]. The fragment was synthesized using PCR. The PCR primers were designed on the basis of the hamster chymase-1 genomic sequence [19] (sense primer: 5′-GGAGACTCCGGGGGACCTC-3′, position 3067-3085; antisense primer: 5′-TCAACTGCTTGCCTTTATGAC-3′, position 3213-3193). This portion of the gene is highly conserved among serine proteases of the chymase family. Clones hybridized with the probe were plaque-purified. The inserts were liberated by digestion with *Eco*RI, subcloned into pBS II KS vector (Stratagene), and subjected to restriction mapping and nucleotide sequencing.

Hamster chymase-2 cDNA was cloned using PCR. Total RNA was isolated from heart by the procedure described previously [22]. A single-stranded cDNA was synthesized from 0.5 μg of the total RNA using a cDNA synthesis kit (Gibco BRL, Gaithersburg, MD, U.S.A.). To amplify hamster chymase cDNA, two oligonucleotide primers were selected from the 5′ and 3′ region of the coding sequence (sense primer: 5′-TCT-GAGAGGATGCTTCTTCCTG-3′, positions 1557–1578; antisense primer: 5′-CCAGAGTTAATTCTCCCTCAAG-3′, positions 4620–4599). A 2 μl portion of the reverse transcriptase (RT) reaction mixture were added to 98 μl of the PCR buffer containing 25 pmol each of the upstream and downstream primers, and 2.5 units of *Taq* polymerase (Boehringer Mannheim G.m.b.H., Mannheim, Germany). The PCR reaction was carried out on a DNA thermal cycler (Perkin–Elmer, Norwalk, CT, U.S.A.). A single 759 bp PCR product was subcloned into pBS II KS vector and subjected to nucleotide sequencing.

Dot-matrix comparisons of chymase genes

Dot matrix comparisons of hamster chymase 2 versus mouse mast-cell protease (MMCP) 5 [23], hamster chymase 1 [19] versus MMCP 1 [24], and hamster chymase 1 versus hamster chymase 2 were performed using Mac Vector software (Oxford Molecular Group, Inc., Campbell, CA, U.S.A.). The dot-matrix analysis was performed with a window size of 20 nucleotides and a minimal match of 75 %.

Southern-blot analysis

A 5 μg portion of liver DNA was digested with *Eco*RI, *Bam*HI and *Sac*I, and the resulting fragments were resolved on a 0.7 %-agarose gel and transferred to a nylon membrane. A 406 bp *Xba*I–*Xba*I fragment of the chymase-2 gene was labelled with ^{32}P and was hybridized to the genomic blot at 60 °C in 5 ml of hybridization solution containing 1 mol/l NaCl, 10 % dextran sulphate and 1 % SDS. The filter was washed twice for 30 min under high-stringency conditions [65 °C in 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/1 % SDS] and then exposed to radiographic film for 3 days.

Experimental-animal preparation

Male cardiomyopathic Syrian hamsters, 60 weeks old (BIO 14.6 strain; BIO Breeders, Inc., Fitchburg, MA, U.S.A.) and age-matched control hamsters (BIO F1B strain: BIO Breeders, Inc.) were used. They were allowed access to regular rat chow (Clea Japan, Osaka, Japan), given free access to tap water, and were housed three animals to a cage under the same conditions throughout the experimental period. The experimental procedures for animals were in accordance with the Guide for the Care and Use of Laboratory Animals (Animal Research Laboratory, Osaka Medical College, Japan). After anesthetization by an intraperitoneal injection of pentobarbital (50 mg/kg), blood samples were obtained through the carotid artery. After the animals had been killed by decapitation, the hearts were collected for determining the mRNA levels of ACE and chymases 1 and 2, as well as ACE and chymase-like activities.

Quantitative RT-PCR analysis

The mRNA levels of hamster chymase 1 and ACE in hearts were quantified using the competitive RT-PCR method described previously [19]. The PCR primers for hamster chymase 2 were selected according to the hamster chymase-2 cDNA sequence (sense primer: 5′-CTGAGAGGATGCTTCTTCCTGC-3′, positions 1558–1579; antisense primer: 5′-AGATCTTATTGAT-CCAGGGCCG-3′, positions 4600–4579). Competitive RT-PCR was performed in 100 μl of PCR buffer containing 2 μl of the RT reaction mixture, 25 pmol of sense and antisense primers for ACE or chymase, 100 μmol/l each deoxynucleotide, 10 μmol/l Tris/HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 2.5 units of *Taq* DNA polymerase and 2-fold serial dilutions of chymase-2 competitor DNA (2.4×10^2–5.0×10^5 molecules). The competitor DNA for hamster chymase 2 was obtained by deleting a fragment of 250 bp using the restriction enzymes *Bam*HI and *Pma*CI. The PCR products for target hamster chymase 2 and the competitor were 738 and 488 bp respectively. The amplification conditions for hamster chymase 2 were 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min over 45 cycles. A 10 μl portion of PCR products was electrophoretically separated on 1.4 % agarose gels, stained with ethidium bromide and photographed. The stained images were scanned on a densitometer (CS-9000; Shimadzu, Kyoto, Japan) and the integrated densities of PCR products of the target and competitor cDNAs were calculated. For correcting the differences in the molecular weight of the target and competitor cDNA products, the integrated areas for chymase-2 competitor were multiplied by 1.51 (738/488 bp). The logarithm of the ratio of competitor to target PCR products was plotted versus the logarithm of the amount of competitor DNA molecules. At the competition equivalent point (log ratio = 0), the original amount of ACE or chymase transcripts was equal to the amount of competitor DNA added to the initial reaction mixture, and calculated by extrapolating from the intersection of the curves to the x-axis.

Measurement of ACE and chymase-like activities

The ACE activity was determined using a synthetic substrate hippuryl-histidyl-leucine (Peptide Institute, Inc., Minoh, Japan), designed specifically for ACE [22]. Chymase-like activity was measured using a modified procedure of Okunishi et al. [12]. A 20 μl aliquot of tissue extract was incubated for 10 min at 37 °C with 770 μmol/l ANG I in 150 mmol/l borax/borate buffer,

pH 8.5, containing 5 mmol/l EDTA, 20 μmol/l aprotinin, 8 mmol/l dipyridyl and 0.77 mmol/l di-isopropyl fluorophosphate as inhibitors for ACE, cathepsin G and angiotensinases. A blank was carried out with further addition of 0.5 mmol/l chymostatin to the inhibitor mixture mentioned above. The ANG II-forming activity inhibitable by chymostatin represented the net chymase-like activity.

Statistical analysis

All numerical data shown in the text are expressed as mean $\pm$S.E.M. Significant differences between the means of different groups were evaluated by the Student's *t*-test for unpaired data and by the modified *t*-test for multiple comparison (Tukey's method) after a one-way analysis of variance.

RESULTS

Genomic organization of hamster chymase 2

Five clones were identified by screening the hamster genomic library. A 5.8 kb insert was isolated from one of these clones. A 4798 bp fragment containing the entire chymase-2 gene was isolated from the insert. The nucleotide sequence of the hamster chymase-2 gene is shown in Figure 1. The gene spans approx. 3.2 kb and has five coding blocks and four introns. The 5′-untranslated region contains consensus elements for the binding of GATA (AGATAA) instead of the TATA box at positions 1525–1530. Two consensus AATAAA polyadenylation motifs are found in the 3′-untranslated region (Figures 1 and 2). The 5′-regulatory region contains consensus elements for the binding of AP-1 (nucleotides 802–808 and 1068–1074), AP-2 (nucleotides 1488–1495), AP-3 (nucleotides 1467–1474), and NF-IL6 (nucleotides 770–778). A comparison of the 5′ region of hamster chymase gene with the corresponding region of the rat mast-cell protease (RMCP) II gene [25] revealed a similar sequence (CAGCTC-TGCCACTCCCCTGCCTG) at nucleotides 1475–1497. This region was similar to a consensus core sequence present in the enhancer region of pancreas-specific genes [26]. A mast-cell-specific enhancer element located further upstream region in the RMCP II gene [27] was not found in the 1.5 kb 5′-regulatory

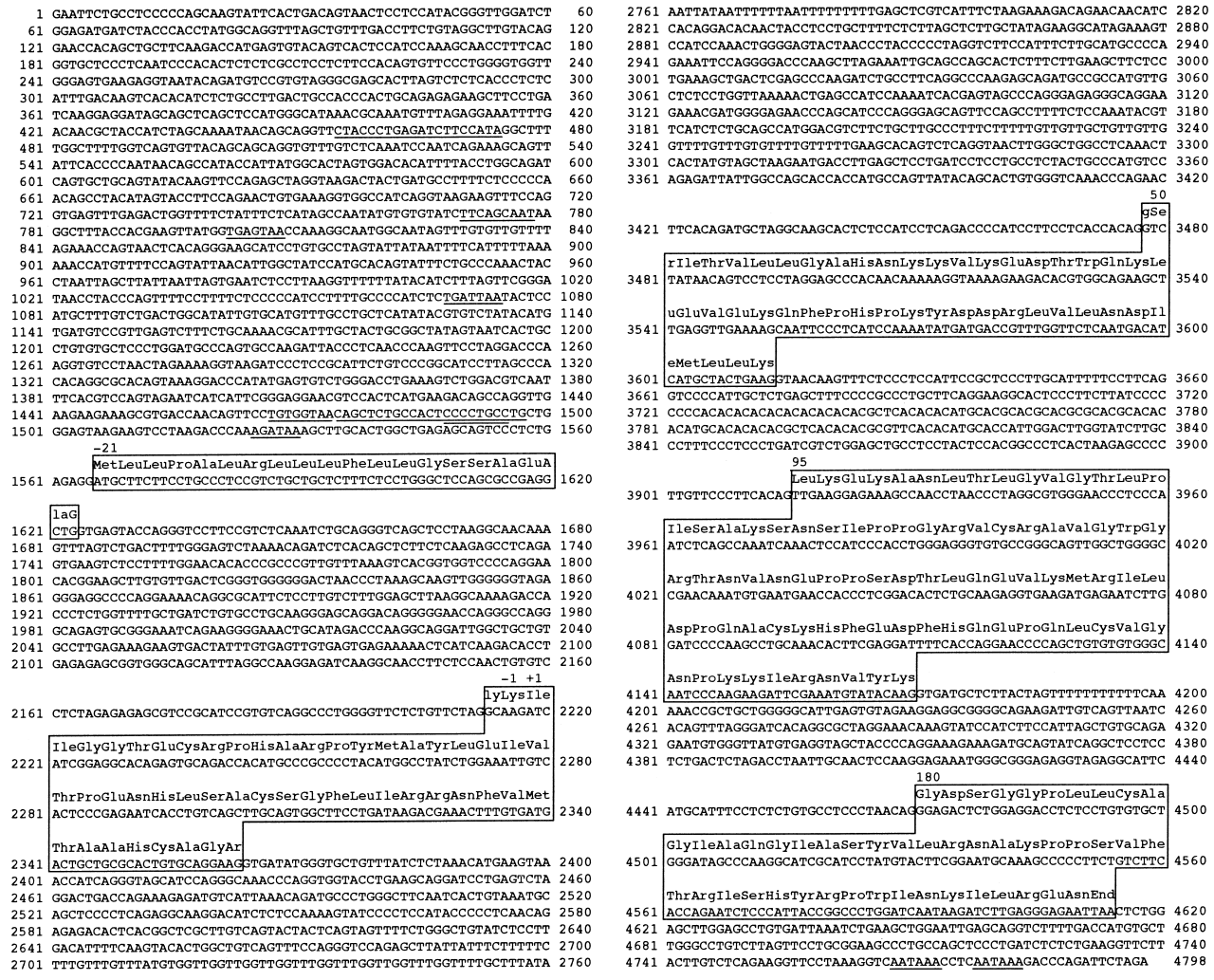

Figure 1 Genomic organization of hamster chymase 2 gene

The nucleotide sequences of five coding blocks are boxed. The deduced amino acids are indicated above the corresponding nucleotides. Right- and left-hand-side numbers refer to the nucleotides, and overhead numbers refer to the deduced amino acids. The GATA-binding motif and the poly(A) addition site are underlined. The putative AP-1, AP-2, AP-3, and NF-IL6 DNA-binding elements, and the region similar to the pancreatic protease enhancer core sequence are underlined.

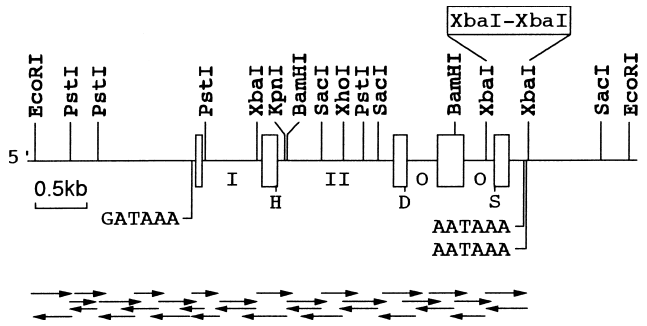

Figure 2. Hamster chymase 2 gene organization and the restriction map

The locations of the coding blocks are represented by rectangles. The GATA-binding motif and the poly(A) addition site are shown in the 5′- and 3′-untranslated regions. The locations of codons corresponding to the catalytic triad residues His (H), Asp (D), and Ser (S) are indicated below the appropriate coding blocks. Intron phase is shown below each intron. The location of the *Xba*I–*Xba*I fragment used in the Southern blot is indicated below the restriction map. Arrows indicate the extent and directions of the sequencing strategy.

region of the hamster chymase-2 gene, while there was an additional region similar to the core sequence of the pancreatic protease gene enhancer at positions 455–474.

Deduced amino-acid sequence

The deduced amino acid sequence suggests that the hamster chymase is synthesized initially as a preproenzyme of 247 amino acids (Figure 3). On the basis of a comparison with other chymases, hamster chymase 2 was predicted to have a hydrophobic signal peptide of 19 amino acids and an activation peptide of two amino acids (Gly-Lys), which were removed to yield a mature enzyme of 226 amino acids. The deduced amino acid sequence predicts an M_r of 25 314 for the mature protease. The amino acid sequence of the mature form of hamster chymase 2 was compared with the sequences of hamster chymase 1 [19], GMCP 1 [28], GMCP 2 [28], MMCP 1 [24], MMCP 2 [29], MMCP 4 [30], MMCP 5 [23], RMCP I [31], RMCP II [25], RMCP III [32], RMCP 3 [33], RMCP 4 [33], RMCP 5 [33] (RMCP 5 is the same protein as RMCP III), dog chymase [34], monkey chymase [15], baboon chymase [35] and human chymase [36,37]. It was found to be highly similar to MMCP 5 (86 %), RMCP III (85 %) and GMCP 2 (82 %), although less similar to hamster chymase 1 (56 %), GMCP 1 (57 %), MMCP 1 (52 %), MMCP 2 (50 %), MMCP 4 (59 %), RMCP I (56 %), RMCP II (52 %), RMCP 3 (54 %) and RMCP 4 (52 %). The similarities to dog, monkey, baboon and human chymases were 71, 70, 70 and 70 % respectively. The catalytic triad found in all serine proteases was present at residues His^{45}, Asp^{89} and Ser^{182}. Hamster chymase 2 contained a single potential N-glycosylation site at Asn^{100}. On the basis of the number of basic (Arg + Lys = 32) and acidic (Asp + Glu = 20) amino acids, the hamster chymase catalytic domain had a predicted net charge of +12.

Dot-matrix comparisons of chymase genes

The inter-genetic relatedness that exists between hamster chymase 2 and MMCP 5, hamster chymase 1 and MMCP 1 and hamster chymase 1 and hamster chymase 2 is depicted in Figure 4. The overall structural similarity between hamster chymase 2 and MMCP 5 known as the counterpart to the α-chymase gene is apparent due to highly conserved exons, and the size and sequence of each intron and 5′-upstream region of hamster chymase 2 are

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                Met Leu Leu Pro Ala Leu Arg Leu Leu Leu Phe Leu  [-10]
TCTGAGAGG ATG CTT CTT CCT GCC CTC CGT CTG CTG CTC TTT CTC     45
                                -1  +1
Leu Gly Ser Ser Ala Glu Ala Gly Lys Ile Ile Gly Gly Thr Glu    [6]
CTG GGC TCC AGC GCC GAG GCT GGC AAG ATC ATC GGA GGC ACA GAG    90

Cys Arg Pro His Ala Arg Pro Tyr Met Ala Tyr Leu Glu Ile Val   [21]
TGC AGA CCA CAT GCC CGC CCC TAC ATG GCC TAT CTG GAA ATT GTC    135

Thr Pro Glu Asn His Leu Ser Ala Cys Ser Gly Phe Leu Ile Arg   [36]
ACT CCC GAG AAT CAC CTG TCA GCT TGC AGT GGC TTC CTG ATA AGA    180

Arg Asn Phe Val Met Thr Ala Ala His Cys Ala Gly Arg Ser Ile   [51]
CGA AAC TTT GTG ATG ACT GCT GCG CAC TGT GCA GGA AGG TCT ATA    225

Thr Val Leu Leu Gly Ala His Asn Lys Lys Val Lys Glu Asp Thr   [66]
ACA GTC CTC CTA GGA GCC CAC AAC AAA AAG GTA AAA GAA GAC ACG    270

Trp Gln Lys Leu Glu Val Glu Lys Gln Phe Pro His Pro Lys Tyr   [81]
TGG CAG AAG CTT GAG GTT GAA AAG CAA TTC CCT CAT CCA AAA TAT    315

Asp Asp Arg Leu Val Leu Asn Asp Ile Met Leu Leu Lys Leu Lys   [96]
GAT GAC CGT TTG GTT CTC AAT GAC ATC ATG CTA CTG AAG TTG AAG    360

Glu Lys Ala Asn Leu Thr Leu Gly Val Gly Thr Leu Pro Ile Ser  [111]
GAG AAA GCC AAC CTA ACC CTA GGC GTG GGA ACC CTC CCA ATC TCA    405

Ala Lys Ser Asn Ser Ile Pro Pro Gly Arg Val Cys Arg Ala Val  [126]
GCC AAA TCA AAC TCC ATC CCA CCT GGG AGG GTG TGC CGG GCA GTT    450

Gly Trp Gly Arg Thr Asn Val Asn Glu Pro Pro Ser Asp Thr Leu  [141]
GGC TGG GGC CGA ACA AAT GTG AAT GAA CCA CCC TCG GAC ACT CTG    495

Gln Glu Val Lys Met Arg Ile Leu Asp Pro Gln Ala Cys Lys His  [156]
CAA GAG GTG AAG ATG AGA ATC TTG GAT CCC CAA GCC TGC AAA CAC    540

Phe Glu Asp Phe His Gln Glu Pro Gln Leu Cys Val Gly Asn Pro  [171]
TTC GAG GAT TTT CAC CAG GAA CCC CAG CTG TGT GTG GGC AAT CCC    585

Lys Lys Ile Arg Asn Val Tyr Lys Gly Asp Ser Gly Gly Pro Leu  [186]
AAG AAG ATT CGA AAT GTA TAC AAG GGA GAC TCT GGA GGA CCT CTC    630

Leu Cys Ala Gly Ile Ala Gln Gly Ile Ala Ser Tyr Val Leu Arg  [201]
CTG TGT GCT GGG ATA GCC CAA GGC ATC GCA TCC TAT GTA CTT CGG    675

Asn Ala Lys Pro Pro Ser Val Phe Thr Arg Ile Ser His Tyr Arg  [216]
AAT GCA AAG CCC CCT TCT GTC TTC ACC AGA ATC TCC CAT TAC CGG    720

Pro Trp Ile Asn Lys Ile Leu Arg Glu Asn End                  [226]
CCC TGG ATC AAT AAG ATC TTG AGG GAG AAT TAA CTCTGG            759
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Figure 3 Nucleotide sequence of hamster chymase 2 cDNA and the deduced amino acid sequence of the preproenzyme

Right-hand-side numbers show the nucleotides. Numbers in square brackets show the amino acids beginning with residue 1 of the mature enzyme. The primers used for cDNA cloning of hamster chymase are underlined.

also similar to those of MMCP 5. On the other hand, the overall structure of hamster chymase 1 is similar to that of β-chymase, MMCP 1. However, the structure of hamster chymase-1 gene is quite different from that of the hamster chymase-2 gene.

Southern-blot analysis

The restriction map of the hamster chymase-2 gene predicts that a 5.8 kb *Eco*RI fragment, a > 1.8 kb *Bam*HI fragment and a 2.2 kb *Sac*I fragment will hybridize to the 406 bp *Xba*I–*Xba*I DNA fragment from the chymase-2 gene. Indeed, the 5.8 kb *Eco*RI fragment, the 4.7 kb *Bam*HI fragment and the 2.2 kb *Sac*I fragment were detected (Figure 5). A 6.5 kb *Eco*RI fragment, a 1.1 kb *Sac*I fragment, and a 3.8 kb *Bam*HI fragment were also detected. These bands were predicted from the restriction map of the hamster chymase-1 gene [19]. No other bands were detected.

Cardiomyopathic abnormalities in experimental animals

The body weight of 60-week-old BIO 14.6 cardiomyopathic hamsters was significantly less than that of age-matched BIO F1B control hamsters (122 ± 3 versus 157 ± 2 g, $n = 6$, $P < 0.001$),

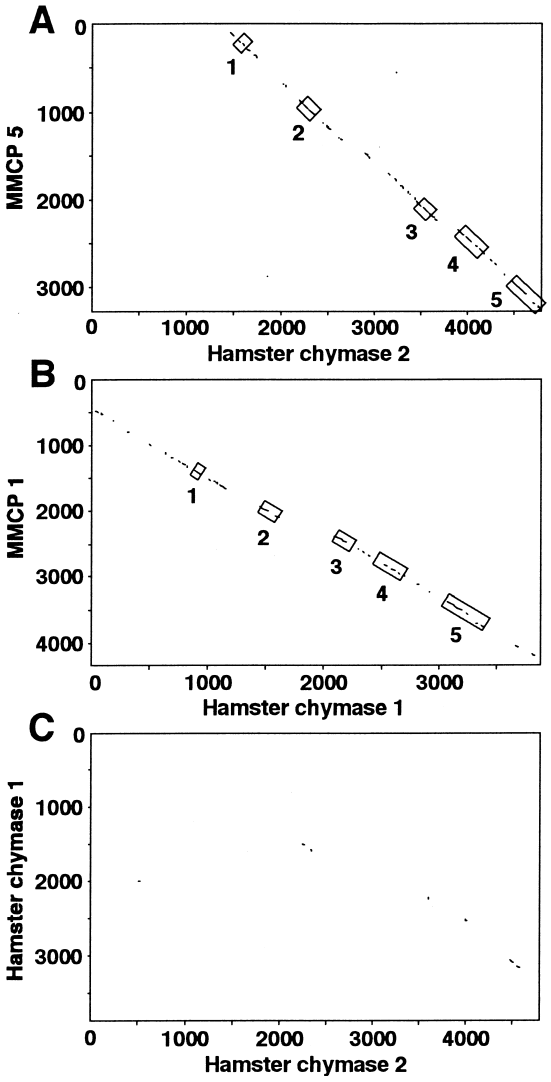

Figure 4 Dot-matrix comparisons of hamster chymase 2 versus MMCP 5 (A), hamster chymase 1 versus MMCP 1 (B) and hamster chymase 1 versus hamster chymase 2 (C)

Each dot represents a 20 nucleotides minimum match with a minimum of 75% identity. The numbers on each axis reflect the original sequence data for each gene. The positions of exons 1–5 are indicated by the rectangles.

whereas the absolute weight of cardiomyopathic hamster hearts was greater than that of the controls (0.98 ± 0.09 versus 0.51 ± 0.01 g, $n = 6$, $P < 0.01$). The index of gross hypertrophy, i.e., the heart weight/body weight ratio of cardiomyopathic hamsters was 2.5 times greater than that of the control hamsters ($P < 0.01$). The hearts of cardiomyopathic hamsters were extremely enlarged, and diffuse fibrosis and calcification were observed.

Quantitative RT-PCR analysis

Figure 6 shows representative competitive RT-PCR analyses that used constant amounts of RT mixture and serially diluted amounts of chymase-2 competitor cDNA. The precise amount of target transcript was determined by generating a standard curve for each sample. The logarithm of the ratio of competitor/target PCR products was plotted on the *y*-axis and the logarithm of the

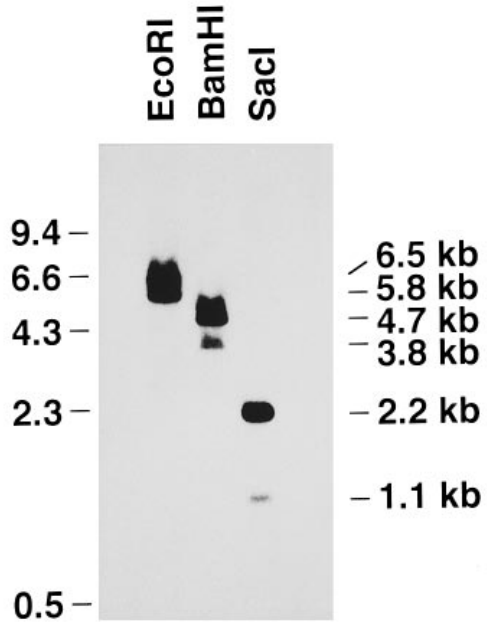

Figure 5 Southern-blot analysis of hamster genomic DNA

Hamster DNA was digested with *Eco*RI, *Bam*HI, and *Sac*I indicated at the top of the Figure. Hybridization was performed under high-stringency conditions with the ^{32}P-labelled 0.4 kb *Xba*I fragment of hamster chymase gene. The sizes and positions of DNA markers are shown to the left.

serial dilutions of the competitor cDNA was plotted on the *x*-axis. The relationship was linear ($r = 0.99$, $P < 0.001$) and permits quantification of the target transcripts.

ACE and chymase-like activities, and ACE, chymase 1 and chymase 2 mRNA levels in the chronic stage of cardiomyopathic hearts

The cardiac ACE activity of 60-week-old BIO 14.6 cardiomyopathic hamsters exhibited a 4.1-fold increase ($P < 0.01$) compared with that of age-matched BIO F1B control hamsters (Figure 7A). The cardiac chymase-like activity of cardiomyopathic hamsters was increased 3.4-fold ($P < 0.01$) compared with that of the controls (Figure 7B). The cardiac ACE, chymase 1 and chymase 2 mRNA levels of cardiomyopathic hamsters were increased 2.4- ($P < 0.01$), 2.8- ($P < 0.001$) and 5.1-fold ($P < 0.001$) respectively compared with those of the control hamsters (Figures 7C, 7D and 7E). The expression of ACE was about 10 times higher than that of chymase 1 in the cardiomyopathic hearts, and the expression of chymase 1 was about 10 times higher than that of chymase 2. Plasma ACE activity of the cardiomyopathic hamsters did not differ significantly from that of the age-matched controls (43.8 ± 1.7 versus 42.7 ± 1.1 munits/ml, $n = 6$).

DISCUSSION

In the present study we cloned the hamster chymase-2 gene and determined the primary structure of hamster chymase 2. On the basis of a comparison with other chymases, hamster chymase 2 was predicted to have a signal peptide of 19 amino acids and an activation peptide of 2 amino acids which were removed to yield a mature enzyme of 226 amino acids. After removal of the signal peptide, the removal of the activation peptide by dipeptidyl

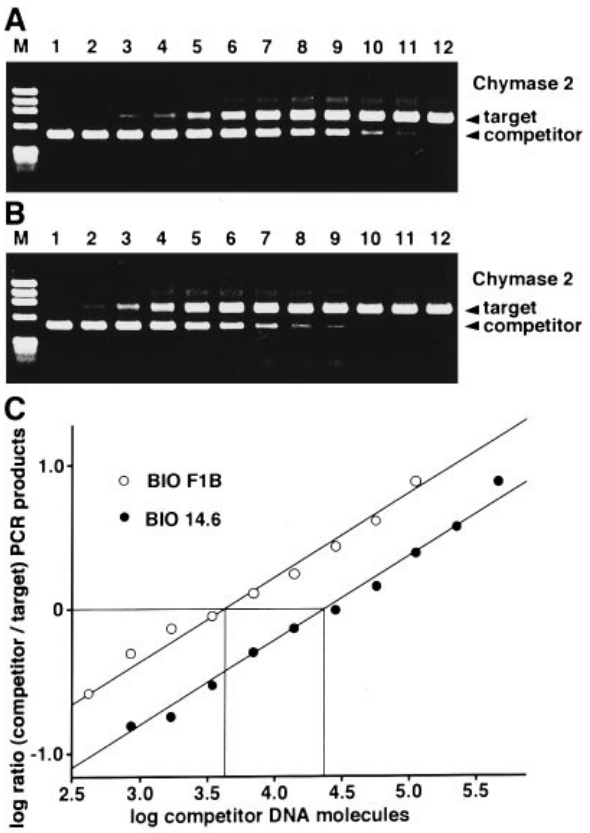

Figure 6 Representative quantitative RT-PCR analyses of heart chymase 2 of the 60-week-old BIO F1B control hamster (A) and BIO 14.6 cardiomyopathic hamster (B)

Lane M contains ϕx174-HaeIII DNA marker. Lanes 1 to 12 contain PCR products amplified with 2 μl of RT reaction mixture and 2-fold serial dilutions of competitor DNA (5.0×10^5, 2.5×10^5, 1.3×10^5, 6.3×10^4, 3.1×10^4, 1.6×10^4, 7.8×10^3, 3.9×10^3, 2.0×10^3, 9.8×10^2, 4.9×10^2, and 2.4×10^2 molecules). The arrowheads indicate the positions of target chymase (738 bp) and chymase competitor (488 bp). The logarithm of the ratio of competitor to target PCR products was plotted versus the logarithm of the amount of competitor DNA molecules (**C**).

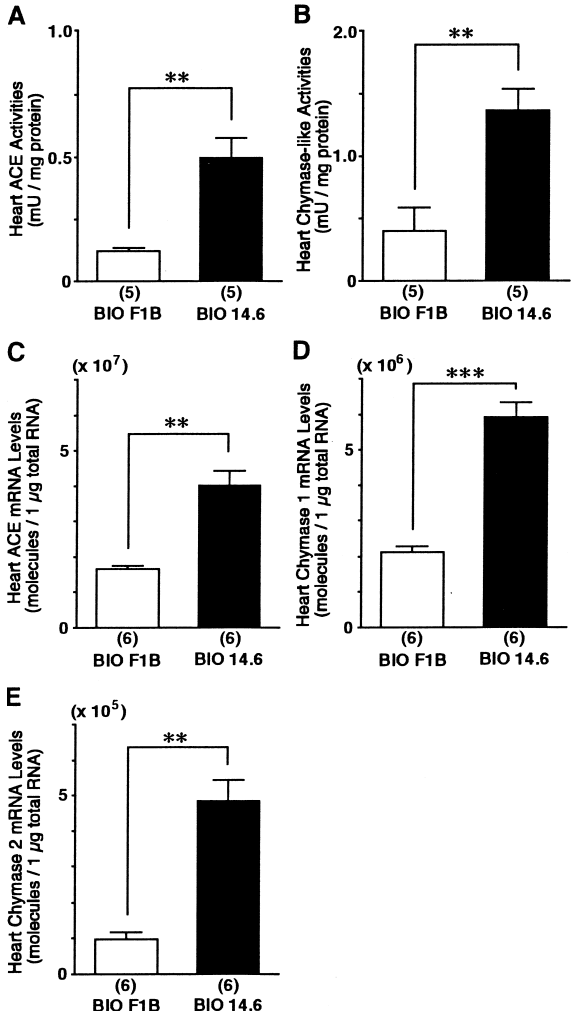

Figure 7 ACE activities (A), chymase-like activities (B), ACE transcripts (C), chymase-1 transcripts (D) and chymase-2 transcripts (E) in the hearts of BIO F1B control and BIO 14.6 cardiomyopathic hamsters at the age of 60 weeks

Vertical bars represent the S.E.M. The numbers of hamsters are shown in parentheses. Statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$ versus respective controls.

peptidase I is necessary for the functional activation of chymases and other granular serine proteases [38]. Hamster chymase 2 differs from the other chymases in that it contains a Lys at position -1 rather than a Glu. The Gly-Lys activation peptide in hamster chymase 2 has not been found in any other mast-cell chymase; however, the Glu-Lys activation peptide is present in human granzyme A [39]. The mature form of hamster chymase 2 is highly similar to MMCP 5, RMCP III, GMCP 2, dog chymase and primate chymases. The evolutionary tree of mammalian chymases shows that chymases are divided into two distinct structural groups, α- and β-chymases [35], and recent studies suggest that α- and β-chymases are different in their substrate specificity [40] and gene structure [41]. Primate and dog chymases belong to the α-chymase group [35,41] and are highly efficient in converting ANG I into ANG II [3,11–13,15]. On the other hand, RMCP I belongs to the β-chymase group, and cleaves the Tyr^4–Ile^5 bond of ANG I and degrades ANG I to inactive fragments [40,42]. Hamster chymase 2 belongs to the α-chymase group. Although we could not purify and determine the enzymological characteristics of hamster chymase 2, it might possess enzymological properties similar to primate chymases. However, our previous study indicated that hamster chymase 1 belonged to the β-chymase group in spite of possessing the ANG II-forming property like humans [19]. Further kinetic studies using purified hamster chymases 1 and 2 would clarify the precise substrate-binding site residues of chymase that influence the selection of the scissile bond of ANG I.

In the 5′-upstream region of the hamster chymase-2 gene, a TATA box was not found, while a conserved GATA-binding motif was located 41 bp upstream of the translation initiation site. The promoter activity of the human mast-cell carboxypeptidase A gene was shown to depend on the GATA-binding motif, which is located 51 bp upstream of the translation initiation site [43]. The human chymase gene contained 11 GATA-binding motifs in the 5 kb 5′-upstream region [44]. GATA *cis–trans* interaction was also shown to be important for transcriptional activation of baboon chymase promoter in mast cells [45]. Although the precise gene regulatory mechanism of chymase is obscure, similar gene-regulatory mechanisms may exist in granular proteases in mast cells. For mast-cell-specific expression

of the RMCP II gene, the presence of both the RMCP II enhancer element located at position –1578 to –1387 and the 5′ region immediately upstream of the TATA box, which contains the core sequence of the enhancer region of the pancreatic protease gene, is thought to be important [27]. The 5′ region immediately upstream of the TATA box is highly conserved among chymase genes, and a similar sequence is also found in hamster chymase-2 gene at a similar position. In the 5′-upstream region of the hamster chymase-2 gene there is an additional region similar to the core sequence of the pancreatic protease gene enhancer at positions 455–474. A recent study indicated that the 571 bp domain of the baboon chymase promoter contains a mast-cell-specific region [45]. The GATA-binding motif and the mast-cell-specific promoter regions might have crucial roles for targeting the expression of chymase to mast cells.

Chymase has been described as highly specific and efficient for ANG II generation and organizes a major pathway for ANG II formation in the human heart [13,21]. These biochemical studies imply the potential role of chymase in the pathogenesis of human cardiac diseases through the production of ANG II. However, recent clinical observations demonstrated no significant differences in cardiac chymase expression and chymase-like activities between failing and non-failing human hearts [46,47], and thus the pathophysiological significance of cardiac chymase has not been established. In these studies, chymase analyses were performed on the end-stage failing hearts of patients undergoing cardiac transplantation. These hearts were accompanied by coronary artery disease and idiopathic cardiomyopathy, and a wide range of variation was observed relative to the levels of chymase gene expression [47], which might obscure changes in levels of chymase expression. Thus, genetically controlled animal models bearing ANG II-forming chymase are necessary for elucidating the precise roles of chymase and ACE in the pathophysiology of myocardial diseases.

The present study demonstrates for the first time that not only cardiac ACE but also cardiac chymases are upregulated remarkably at the terminal stage of cardiomyopathy. In our previous study, the cardiac ANG II-forming system was examined in the hearts of cardiomyopathic hamsters aged 4, 12 and 25 weeks without heart failure. Cardiac chymase mRNA levels and chymase-like activities of 4-week-old cardiomyopathic hamsters were no different from those of age-matched control hamsters; however, chymase mRNA levels and chymase-like activities of cardiomyopathic hamsters were increased 4.5- and 2.3-fold compared with those of controls at 12 weeks, and there were 3.0-fold and 3.9-fold increases in these levels at age 25 weeks respectively. On the other hand, cardiac ACE activities and mRNA levels in the cardiomyopathic hamsters were not altered up to the age of 25 weeks [19]. In the present study, cardiac ACE activities and ACE mRNA levels in 60-week-old cardiomyopathic hamster hearts were 4.1- and 2.4-fold higher than those in 60-week-old control BIO F1B hamsters, and were increased 3.2- and 1.7-fold compared with those in cardiomyopathic hamsters aged 25 weeks. Our results are supported by another report that ACE activities in cardiomyopathic hamsters aged 14 months are 4.1-fold higher than those in age-matched controls [48], and ACE was activated in the human heart with congestive heart failure [47]. Furthermore, many clinical trials confirm that ACE inhibitors reduce mortality and morbidity in patients with chronic heart failure [49–51]. Cardiac ACE exhibits a more prevalent role in the pathogenesis of cardiomyopathy during the terminal stage with heart failure than in the early developmental stage.

Chymase-1 mRNA expression in hearts was 10-fold lower than ACE mRNA. In the human heart as well, chymase was less abundantly expressed than ACE [47]. However, the enzymic activities of chymase and ACE were comparable. This difference could be explained by the following observations. Chymase converts ANG I into ANG II four times more rapidly than ACE [19]. Furthermore, it is suggested that chymase binds to the extracellular matrix such as to heparin or heparin sulphate proteoglycans, and chymase may be more stable and its turnover rate may be slower than ACE [46]. In the terminal stage of cardiomyopathy, heart chymases 1 and 2 were also activated. The mRNA levels of heart chymases 1 and 2 of cardiomyopathic hamsters at the age of 60 weeks were increased 2.8- and 5.1-fold respectively compared with age-matched control hamsters, while the expression of chymase 2 was less than that of chymase 1. Chymase 1 that possesses an ANG II-forming property was predominantly expressed in hamster hearts. Heart chymase 1 was activated concurrently with the development of cardiomyopathy [19], and continuously increased up to the terminal stage of cardiomyopathy. A recent human trial, in which an ANG II receptor antagonist, losartan, and an ACE inhibitor, captopril, were compared relative to their effects on heart failure in the elderly, has shown that losartan provides a more beneficial effect than captopril [52]. Enzymic activities of chymase were not inhibited by ACE inhibitors; therefore, chymase could generate ANG II irrespective of the presence of ACE inhibitors. Inhibition both of the chymase-dependent and ACE-dependent ANG II-forming pathways might be more beneficial in the treatment of heart failure than ACE inhibition alone.

In conclusion, the present study demonstrated for the first time that both chymases 1 and 2, as well as ACE, were remarkably activated in cardiomyopathic hearts at the terminal stage. The hamster is a useful animal model for exploring the ANG II-forming chymase *in vivo* and for analysing the functional significance of α- and β-type chymases. Further studies using specific inhibitors of chymase will clarify the true pathophysiological functions of cardiovascular chymase.

This work was supported in part by Grant-in-Aid for Encouragement of Young Scientists (nos. 07770073 and 08770076) to N.S. from the Ministry of Education, Science, Sports and Culture, Japan.

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Received 5 January 1998/31 Marchn 1998; accepted 28 April 1998