Extended cleavage specificity of the mast cell chymase from the crab-eating macaque (Macaca fascicularis): an interesting animal model for the analysis of the function of the human mast cell chymase

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Abstract

Serine proteases are the major protein constituents within mast cell secretory granules. These proteases are subdivided into chymases and tryptases depending on their primary cleavage specificity. Here, we present the extended cleavage specificity of the macaque mast cell chymase and compare the specificity with human chymase (HC) and dog chymase (DC) that were produced in the same insect cell expression host. The macaque chymase (MC) shows almost identical characteristics as the HC, including both primary and extended cleavage specificities as well as sensitivity to protease inhibitors, whereas the DC differs in several of these characteristics. Although previous studies have shown that mouse mast cell protease-4 (mMCP-4) is similar in its hydrolytic specificity to the HC, mouse mast cells contain several related enzymes. Thus mice may not be the most appropriate model organism for studying HC activity and inhibition. Importantly, macaques express only one chymase and, as primates, are closely related to human general physiology. In addition, the human and macaque enzymes both cleave angiotensin I (Ang I) in the same way, generating primarily angiotensin II (Ang II) and they do not further degrade the peptide like most rodent enzymes do. Both enzymes also cleave two additional potential in vivo substrates, fibronectin and secretory leukocyte protease inhibitor (SLPI) in a similar way. Given the fact that both HC and MC are encoded by a single gene with high sequence homology and that many physiological processes are similar between these species, the macaque may be a very interesting model to study the physiological role of the chymase and to determine the potency and potential side-effects of various chymase inhibitors designed for therapeutic human use.

Keywords: animal model, chymase, cleavage specificity, human chymase, mast cell, macaque

Introduction

Mast cells are resident tissue cells that are distributed along both the external and internal surfaces of the body. They are frequently found in the connective tissue of the skin and around blood vessels and nerves, as well as in the mucosa of the airways and the intestine (1). Following activation, mast cells rapidly exocytose their cytoplasmic granules, which results in the release of a number of potent pre-stored inflammatory mediators (2). The majority of proteins found in these granules are serine proteases, which generally can be subdivided into chymases and tryptases (3–5). Most chymases are chymotrypsin-like proteases and cleave substrates after aromatic amino acids (aa). Phylogenetic analyses of the chymases have led to the identification of two distinct subfamilies of these proteases, which are designated as α-chymases and β-chymases (6–8). The α-chymase is present as a single gene in all species investigated, except for ruminants where two very similar α-chymase genes have been described (Fig. 1) (9, 10). β-Chymases have only been
identified in rodents, except for what appears to be a non-functional relict, a pseudogene in dogs (Fig. 1) (9).

There is a major interest in developing potent chymase inhibitors from the pharmaceutical industry to study their effects on hypertension, vascular degeneration such as atherosclerosis and aortic aneurysm and the use as general anti-inflammatory drugs. However, these attempts have been hampered by the lack of good animal models. It has been very difficult to determine the safety profiles and to obtain relevant efficacy data to evaluate the potential of chymase inhibitors as future drugs without a good animal model. Rodents are commonly used in pre-clinical studies. However, for the mast cell enzymes, and in particular for the chymase, rodents are not good models. This is primarily due to the large differences in the complexity of their chymase loci (Fig. 1). The human locus encodes four enzymes: the mast cell α-chymase, cathepsin G and granzymes B and H (Fig. 1) (9, 10). The mouse and rat loci have instead 15 and 28 functional genes, respectively (Fig. 1) (9, 10). The α-chymases in mice and rats, mouse mast cell protease-5 (mMCP-5) and rat mast cell protease-5 (rMCP-5), respectively, have changed their primary cleavage specificity from aromatic aa (chymotrypsin-like) to aliphatic aa (elastase-like; 11, 12). In addition, rodents express a number of β-chymases to which there are no direct homologues in other placental mammals. These genes are located in a region of the locus between the α-chymase and cathepsin G (Fig. 1). Rodents also have several additional protease genes within this locus. For example, the mouse locus has seven granzyme genes compared with only two in the human locus (Fig. 1) (9, 10).

In a larger attempt to identify an animal model for in vivo studies of the role of the mast cell granule proteases in various physiological processes, we have screened a number of chymases from different animal species for their extended cleavage specificity (12–19). A large panel of mammalian mast cell chymases have also been studied for their sensitivity to various chymase inhibitors (20). These studies have been complemented by studies of the chymase and the tryptase loci from a panel of mammalian species (Fig. 1) (9, 10, 21). Data collected from a number of studies have shown that most animal models are not well suited for analysis of efficacy, potential toxicity and other side-effects from human chymase (HC) inhibitors. The reason that these model systems are not so well suited are that their chymases have either a very different primary and/or extended specificity as well as having several additional chymotryptic enzymes present in their mast cells.

There are also other important differences. One of the most well-studied potential in vivo substrates for the HC is angiotensin I (Ang I). The HC has been shown to almost exclusively generate active Ang II from Ang I, whereas mMCP-4, which has very similar cleavage specificity to HC, also degrades Ang I to inactive shorter peptides (22). The analysis of a large panel of different mammalian mast cell chymases, including the human, macaque, dog, hamster I, hamster II, guinea pig and sheep chymases (II and III) has also shown that the inhibitors developed for the HC are
primarily active against the human and macaque enzymes (20). On the basis of these studies, we can conclude that most animal species tested, including mice, rats, dogs, hamsters, sheep, guinea pigs or opossums do not serve as good models for efficacy and toxicity studies of HC inhibitors. We therefore decided to extend the characterization of the macaque chymase (MC) using a number of additional criteria to evaluate the MC as a potential animal model for studies of the HC.

Here, we present a detailed characterization of the extended cleavage specificity of a recombinant MC and a detailed comparison with the corresponding human and dog enzymes using substrate phage display. The results show that the extended cleavage specificity of the MC is almost identical to the HC. This was confirmed by analysing the cleavage of a selection of recombinant substrates and by the cleavage of a few potential in vivo substrates of the chymase; Ang I, human fibronectin and human secretory leukocyte protease inhibitor (SLPI). The MC locus is also very similar to the human counterpart with four genes in total and with only one α-chymase gene (Fig. 1). The collected information concerning the extended specificity, inhibitor sensitivity and Ang I cleavage as well as the fact that the MC is 97.8% identical in sequence to the HC (Fig. 2) makes macaques an ideal model for studies of potency and potential toxic side-effects of various HC inhibitors.

**Methods**

**Production and purification of recombinant HC, MC and DC**

Chymase sequences were retrieved from the SwissProt/TrEMBL under the following accession numbers: human (P23946) and macaque (P56435). HC, MC and dog chymase (DC) were expressed in baculovirus-infected insect cells, purified on heparin-Sepharose and activated by enteropeptidase as previously described (12, 13). In these T7 phages, the C-terminus of the capsid protein 10 were manipulated to contain a nine aa long random peptide followed by a His6-tag (13). An aliquot of the amplified phages (~10⁹ pfu) was bound to 100 µl nickel-nitriloacetic acid (Ni-NTA) beads by their His6-tags for 1 h at 4°C under gentle agitation. Unbound phages were removed by washing 10 times in 1.5 ml of 0.18 mM in 200 µl PBS. S-2586 hydrolysis was monitored spectrophotometrically at 405nm in a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Determination of cleavage specificity by phage-displayed nonapeptide library**

A library of 5 × 10⁹ unique phage-displayed nonameric peptides was used to determine the cleavage specificity of the HC, MC and DC as previously described (12, 13). In these T7 phages, the C-terminus of the capsid protein 10 were manipulated to contain a nine aa long random peptide followed by a His6-tag (13). An aliquot of the amplified phages (~10⁹ pfu) was bound to 100 µl nickel-nitriloacetic acid (Ni-NTA) beads by their His6-tags for 1 h at 4°C under gentle agitation. Unbound phages were removed by washing 10 times in 1.5 ml of 0.18 mM in 200 µl PBS. S-2586 hydrolysis was monitored spectrophotometrically at 405nm in a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Enzymatic activity was confirmed by cleavage of the chromogenic substrate S-2586 (MeO–Suc–Arg–Ala–Tyr–pNA; Chromogenix, Mölndal, Sweden). Measurements were performed in 96-well microtiter plates with a substrate concentration of 0.18mM in 200 µl PBS. S-2586 hydrolysis was monitored spectrophotometrically at 405nm in a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

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![Protein Alignment](image)

**Fig. 2.** A protein alignment of MC and HC. The figure shows a sequence alignment of MC and HC. When comparing the sequence of HC and MC only five differences in aa sequence is detected in the active enzyme, which result in a sequence identity of 97.8% in the 225 aa long active enzyme. The N-terminus of the active protease is indicated by an arrow.
Generation of a consensus sequence from sequenced phage inserts
Phage insert sequences were aligned by hand assuming a preference for aromatic aa in position P1. Sequences with only one aromatic aa were aligned first and sequences with more than one possible cleavage site were then aligned to fit this pattern. Amino acids with similar characteristics were grouped together as follows: aromatic aa (Phe, Tyr, Trp); negatively charged aa (Asp, Glu); positively charged aa (Lys, Arg); small aliphatic aa (Gly, Ala); large aliphatic aa (Val, Leu, Ile, Pro); and hydrophilic aa (Ser, Thr, His, Asn, Gin, Cys, Met). The nomenclature by Schechter and Berger (24) was adopted to designate the aa in the substrate cleavage region, where P1–P1′ corresponds to the scissile bond.

Generation of recombinant substrates for the analysis of the cleavage specificity
A new type of substrate was developed to verify the results obtained from the phage display analysis. Two copies of the E. coli thioredoxin gene were inserted in tandem into the pET21 vector for bacterial expression (Fig. 6A). In the C-terminal end, a His6-tag was inserted for purification on Ni2+ immobilized metal ion affinity chromatography (IMAC) columns. In the linker region, between the two thioredoxin molecules, the different substrate sequences were inserted by ligating double-stranded oligonucleotides into two unique restriction sites, one BamHI and one SalI site (Fig. 6A). The sequences of the individual clones were verified after cloning by sequencing of both DNA strands. The plasmids were then transformed into the E. coli Rosetta gami strain for bacterial expression (Novagen, Merck, Darmstadt, Germany). A 10 ml overnight culture of the bacteria harbouring the plasmid was diluted 10-fold in LB and Amp and was grown at 37°C for 1–2 h until the OD (600 nm) reached 0.5. IPTG was then added to a final concentration of 1 mM. The culture was then incubated at 37°C for an additional 3 h under vigorous shaking, after which the bacteria were pelleted by centrifugation at 3500 rpm for 12 min. The pellet was washed once with 25 ml PBS and 0.05% Tween 20. The pellet was then dissolved in 2 ml PBS and was sonicated for 6 x 30 s to open the cells. The lysate was centrifuged at 13 000 rpm for 10 min and the supernatant was transferred to a new tube. Five hundred microliter of Ni–NTA slurry (50:50; Qiagen, Hilden, Germany) was added and the sample was slowly rotated for 45 min at room temperature. The sample was then transferred to a 2 ml column and the supernatant was allowed to slowly pass through the filter leaving the Ni–NTA beads with the bound protein in the column. The column was then washed four times with 1 ml of washing buffer (PBS, 0.05% Tween 20, 10 mM Imidazole and 1 M NaCl). Elution of the protein was performed by adding 150 µl elution buffer followed by five 300 µl fractions of elution buffer (PBS, 0.05% Tween 20, 100 mM imidazole). Each fraction was collected individually. Ten microlitres from each of the eluted fractions was then mixed with 1 volume of 2× sample buffer and 1 µl β-mercapto-ethanol and then heated for 3 min at 80°C. The samples were analysed on an SDS bis tris 4–12% PAGE gel and the second and third fractions that contained the most protein were pooled. The protein concentration of the combined fractions was determined by Bio-Rad DC Protein assay (Bio-Rad Laboratories Hercules, CA, USA). Approximately 60 µg of recombinant protein was added to each 120 µl cleavage reaction (in PBS). Twenty microlitres from this tube was removed (0-min time point), the active enzyme was then added (~35 ng) and the reaction was kept at room temperature during the entire experiment. Twenty microlitres samples were removed at the indicated time points (15 min, 45 min and 150 min) and stopped by addition of one volume of 2× sample buffer. One microlitre of β-mercapto-ethanol was then added to each sample followed by heating for 3 min at 80°C. Twenty microlitres from each of these samples were then analysed on 4–12% pre-cast SDS-PAGE gels (Invitrogen). The gels were stained overnight in colloidal Coomassie staining solution and destained for several hours according to the previously described procedures (23).

Analysis of the activity of five HC inhibitors on the human and macaque enzymes
Five patented chymase inhibitors from five different companies were synthesized and purified to almost homogeneity. Compound A is TYS1184 from Tao Eiyo (patent no WO 2002 022595), compound B is from Teijin (patent no WO 2007 068621), compound C from Johnson & Johnson (patent no WO 2005 073214), compound D from Roche (patent no WO 2000 003997) and compound E from Boehringer Ingelheim (patent no WO 2009 023655).

Analysis of the cleavage products of Ang I, human fibronectin and human SLPI after cleavage with HC, MC and DC
Ten micrograms of 95–98% pure Ang I (Sigma-Aldrich, Stockholm, Sweden) were digested with 17 ng of HC, DC or MC for 1 h at 37°C in a PBS buffer. The cleavage products
were then analysed by mass spectroscopy (MS). A second experiment was also performed using the same conditions except with 8-fold more enzyme (137 ng). Purified human fibronectin (a kind gift from Professor Staffan Johansson, Uppsala University, Uppsala, Sweden) and human recombinant SLPI (R&D systems, Abingdon, UK) were used to study the similarity of the cleavage pattern by the HC, MC or DC. Approximately 4 µg of human fibronectin and 4 µg of human recombinant SLPI (per reaction in PBS) were cleaved for 30 min at 37°C with 137 ng of HC, MC or DC. The reaction mixtures were separated on a 4–12% SDS page pre-cast gel (Invitrogen) and then stained with colloidal Coomassie brilliant blue.

Results

Production, purification and activation of recombinant mast cell HC, MC and DC

The coding regions for the HC, the MC and the DC were inserted in the baculovirus vector pAcGP67B. All three chymases were expressed in the baculovirus-infected insect cells and were purified as described previously (20). Following purification, all the mature chymases (25–28 kDa) were >95% pure as determined by SDS-PAGE (Fig. 3). The correct N-termini were also confirmed by N-terminal sequencing. Mass spectrometry analysis suggested that the HC, the MC and the DC are heterogeneously glycosylated (data not shown).

To confirm that the recombinant enzymes were proteolytically active, the three enzymes were tested against the chymotrypsin-sensitive chromogenic substrate S-2586 (MeO–Suc–Arg–Ala–Tyr-pNA; Chromogenix).

Determination of the extended cleavage specificity by phage display technology

The phage library used to determine the extended cleavage specificity of the three chymases contains ~5 x 10^7 phage clones. Each phage clone expresses a unique sequence of nine random aa, followed by a histidine (His_6) tag in the C-terminus of capsid protein 10. Therefore, the phages display a random nonamer on their surface and by interactions of the His_6-tag, the phages can be immobilized on Ni–NTA agarose beads. The HC, MC and the DC were used to screen the phage library for peptides susceptible to cleavage. After the first selection step (biopanning), the phages, released by digestion of their nonapeptides, were amplified in E. coli and subjected to additional biopannings. Selections of nonamers, susceptible to cleavage by the three proteases, were performed with five to seven biopannings, after which they induced the release of 194, 174 and 75 times more phages compared with a PBS control for the HC, MC and DC, respectively (data not shown).

After the last biopanning, 100 individual phage clones were isolated for each of the three proteases and the sequences encoding the randomly synthesized nonapeptides were determined. The nucleotide sequences were then translated into nonapeptides, which were aligned based on similarities to the cleavage specificity of the HC that we have studied in detail previously (17). The DC and HC were also included in this study, but in the previous study these two enzymes were produced in another host cell from the human embryonic kidney (HEK) 293 cell line. We wanted to ensure that the data obtained with the macaque enzyme was fully comparable with the previous studies of the DC and HC (17, 18).

The analysis of 100 sequences from the HC produced in insect cells resulted in sequences that were in agreement with the previous study (17). The difference between the enzyme and the control was greater in this experiment. This indicated that the quality of the data was even better in this study. We observed a slightly higher preference for acidic aa in the P2' position (Fig. 5) than in the previous experiment (17, 18). The results from the phage display analysis...
of the DC produced in insect cells also matched the mammalian cell produced enzyme very well. Similarly to the previous study, we saw a much less pronounced preference for acidic aa in the P2′ position in the DC compared with the HC and MC (Fig. 5) (18). The DC also had a marked preference for arginine in the P2 position that is not seen for the HC or the MC. Biopanning with the MC resulted in sequences that were almost identical to the sequences obtained with the HC (Figs. 4 and 5).

Verifying the consensus sequence with the use of a new type of recombinant protein substrate

In order to verify the results from the phage display analysis, we used a new type of recombinant substrate. The sequence obtained from the phage display analysis was inserted in a linker region between two E. coli thioredoxin proteins by ligating a double-stranded oligonucleotide encoding the actual sequence into a BamHI and a SalI site of the vector construct (Fig. 6A). For purification purposes, a His6-tag was added to the C-terminal of this protein (Fig. 6A). A number of related substrate sequences were also produced with this system, by ligating the corresponding oligonucleotides into the BamHI/SalI sites of the vector. All of these substrates were expressed as soluble proteins in a bacterial host, E. coli, and were purified on IMAC columns to obtain a protein with a purity of 90–95% (based on SDS-PAGE evaluation). These recombinant proteins were then used to study the preference of the MC for the different sequences (Fig. 6B and C). The result shows that the MC efficiently cleaves the HC consensus sequence (VVLFSEVL; 17). By changing the glutamic acid residue in the P2′ position of the HC consensus sequence into a glycine (VVLFSGVL), the efficiency in cleavage by the MC drops by a factor of 4–5 (Fig. 6B and C). The MC cleaved these two substrates with comparable activity to the HC.

The substrate consensus sequence for the DC has recently been determined (18). This site (VVRFLSLL) shows similarities to the preferred site for the HC double mutant but does not contain an acidic P2′ residue. Consequently, the HC was found to cleave the DC consensus sequence 5–7 times less efficiently than the HC consensus sequence. The MC also cleaved this substrate with a comparable activity to the HC (Fig. 6B and C).

The optimal sequence for cleavage by the opossum chymase (OC) has also recently been determined (14). Compared with the HC, this enzyme has a preference for Trp over Phe and Tyr in the P1 position. When analysing the cleavage of this sequence (VGLWLDRV), we observed that both the HC and the MC cleave it ~50-fold less efficiently than the HC consensus sequence (Fig. 6). Based on this analysis and with these substrates, the specificity of MC was almost identical to HC. We observed only minor differences, which all are within the error margin of this type of analysis (Fig. 6B and C).

Analysis of the sensitivity of the HC and MC to five different inhibitors of the HC

In order to obtain a more detailed understanding of similarities and differences between the HC and the MC, we analysed...
their sensitivity to five protease inhibitors. The inhibitors are patented compounds from five companies that are in clinical development. Compound A is TY51184 from Tao Eiyo (patent no WO 2002 022595), compound B is from Teijin (patent no WO 2007 068621), compound C from Johnson & Johnson (patent no WO 2005 073214), compound D from Roche (patent no WO 2000 003997) and compound E from Boehringer Ingelheim (patent no WO 2009 023655). Chemical structures of the five compounds are presented in Fig. 7A.

Four of the inhibitors (B–E) showed very little difference in their activities between the HC and the MC. Inhibitor A, from Tao Eiyo, was more active towards the MC than the HC (Fig. 7B). However, in general, the five inhibitors’ activities towards the MC and HC are very similar. Note that the compound B was tested at 6 µM concentration, whereas the other compound concentrations were <1 µM.

Analysis of the angiotensin conversion activity of the MC in relation to HC and DC

Of all mammalian enzymes studied, the HC is the only mast cell chymase that has a pure Ang activating activity (22). HC cleaves Ang I at the Phe8–His9 bond of and generates almost exclusively Ang II and very little of other cleavage products. Conversely, DC, mMCP-4 and rMCP-1 cleave Ang I also at the Tyr4–Ile5 bond hydrolyzing Ang I to a non-active degradation products (22). This finding has indicated major differences in the biological function of primate mast cell chymases compared with other mammals. Alternatively, Ang I-converting activity may not be one of the main conserved functions of the mast cell chymase. In order to extend the studies of functional similarities between the HC and the MC, we analysed the activity of the MC towards Ang I. Here, we used the HC and the DC as reference enzymes. Ten micrograms of Ang I were cleaved in the presence of 17 ng of the MC, HC or DC for 1 h. The cleavage mixture was then analysed by MS. The data depicted in Fig. 8 show that all three enzymes generate almost exclusively Ang II and very small amounts of other cleavage products except the 2-aa-long peptide that is being generated after cleaving Ang I into Ang II. This is consistent when using an amount of enzyme that is sufficient to fully convert all Ang I to Ang II. Alternatively, Ang I-converting activity may not be one of the main conserved functions of the mast cell chymase. In order to extend the studies of functional similarities between the HC and the MC, we analysed the activity of the MC towards Ang I. Here, we used the HC and the DC as reference enzymes. Ten micrograms of Ang I were cleaved in the presence of 17 ng of the MC, HC or DC for 1 h. The cleavage mixture was then analysed by MS. The data depicted in Fig. 8 show that all three enzymes generate almost exclusively Ang II and very small amounts of other cleavage products except the 2-aa-long peptide that is being generated after cleaving Ang I into Ang II. This is consistent when using an amount of enzyme that is sufficient to fully convert all Ang I to Ang II. However, when adding 8-fold more enzyme, the DC starts to cleave at the second site of Ang II generating two 4-aa-long cleavage products of Ang II. At this higher enzyme concentration, ~30% of the Ang II was converted into smaller cleavage products (Fig. 9). Neither the HC nor the MC shows any tendency to further degrade Ang II to smaller products even after cleavage at 8-fold higher enzyme concentration (Fig. 9).
Analysis of the cleavage pattern of human fibronectin and human SLPI after cleavage with HC, MC and DC

In order to obtain a more detailed picture of the similarities between the HC and the MC in the cleavage of natural substrates, we analysed the cleavage pattern for two additional potential targets for the HC, fibronectin and SLPI. As seen in Fig. 10, the HC, MC and DC generate an almost identical cleavage pattern for fibronectin. When studying SLPI, we can see that only the HC and MC cleave this protease inhibitor. In order to see if the lack of cleavage by the DC was due to a convergent evolution between the enzyme and substrate, we analysed the sequence of the dog SLPI. The potential target region in dog SLPI is identical in both human and dog species (GQCL↓MLNPP) indicating that the DC most likely does not cleave the endogenous SLPI at least not in this region of the dog protein.

Discussion

For many years, studies of the physiological role of the HC have been hampered by the lack of good animal models. Mice and rats are good models for many physiological phenomena due to their small size and the availability of a large number of inbred strains. The large number of transgenic animals or gene knock-outs for many important molecules in mice and rats also adds to their value as animal models. However, for studies of the mast cell chymase, these two rodent species are not optimal due to the large differences in the chymase loci between rodents and primates (Fig. 1) (9, 10). The HC locus contains only one chymase gene, one gene for neutrophil cathepsin G and two T-cell and NK-cell-expressed genes, granzyme B and H, whereas the mouse locus harbours six different chymase genes, one cathepsin G gene and seven different granzyme genes (Fig. 1). In spite...
of the fact that there is a large similarity in the cleavage specificity between the HC and mMCP-4 (15), the complexity of the mouse chymase locus makes a detailed analysis of the role of the chymase very complicated in mice and other rodents (Fig. 1).

On the basis of the phage display analysis, where we observed an almost identical specificity of the HC and the MC, the macaque appears to be an ideal model for studies of physiological effects related to the mast cell chymase. The same kinetics in the cleavage of a number of recombinant substrates, the generation of only Ang II from Ang I and a very similar cleavage pattern of human fibronectin and human SLPI, two additional potential natural substrates of the chymase, further support this conclusion. The very similar activity of five different potent HC inhibitors also supports the macaque as an excellent animal model for studies of the physiological role of the HC. Five additional inhibitors have also previously been studied and all showed almost identical characteristics between the HC and MC, whereas the activity was very low against the mast cell chymases of most other species (20). It appears as if the primary limiting factor for the use of macaques is the cost of maintaining monkeys and the ethical considerations of using primates. However, for complicated questions concerning physiological effects and potential side-effects of compounds that are aimed for human use, the macaque is a very attractive model. The relatively small size (3–9 kg depending on age and sex) and relatively short generation time makes them ideal as a primate model. Primates are sometimes needed for questions of a more...
complex nature. When it comes to safety and efficacy issues, macaques can likely serve an important animal model to identify potent and safe chymase inhibitors that can become important additions in the treatment of hypertension and various mast cell-related inflammatory conditions in humans.

A number of molecules have been identified as potential targets for the chymase including fibronectin \( (25, 26) \), serum albumin \( (27) \), endothelin 1 (and sarafotoxin; \( 28, 29 \)), big-endothelin-1 \( (30) \), thrombin and plasmin \( (26, 31) \), the protease inhibitors \( \alpha 2\)-macroglobulin \( (32) \), C1 inhibitor \( (33) \), tissue inhibitor of metalloproteinases (TIMP; \( 34) \), tissue factor pathway inhibitor (TFPI; \( 34) \) and SLPI \( (35) \), the cytokines IL-1\( \beta \) \( (36) \), IL-18 \( (37) \), stem cell factor (SCF; \( 38) \), latent TGF\( \beta 1 \) \( (39) \) and IL-13 \( (40) \) as well as a number of chemokines including CCL3, CCL5, CCL6, CCL9, CCL15 and CCL23 \( (41) \) and the pro-matrix metallopeptases pro-MMP-2 and pro-MMP-9 \( (42) \). The HC has a relatively broad specificity and this list of potential targets is by no means a complete list, which shows the real complexity of studying this enzyme. However, one major question that remains to be addressed is which of these potential targets actually are important in vivo targets. In addition, there are likely many additional targets not yet identified that are of major importance for the physiological role of the chymase. Here, the macaque may also serve as an excellent model to address such questions for the HC. We have analysed the similarity in cleavage patterns between the HC, MC and DC for three of these potential targets (Ang I, fibronectin and SLPI) and shown that the cleavage pattern is almost identical between the HC and MC for these targets, which supports the conclusion that the macaque may serve as an excellent model also for this type of question.

The MS analysis of the cleavage of Ang I also resulted in an interesting and slightly unexpected finding, namely that the DC primarily generated Ang II. Only after the use of excessive amounts of the enzyme (8-fold more enzyme), we observed cleavage at the second cleavage site (generating Ang II). This finding may indicate that the second site is not cleaved in vivo. After using 17 ng of enzyme an almost complete conversion of Ang I into Ang II was observed also with the DC. In vivo it is likely that the generated Ang II will diffuse away from the site of cleavage and bind receptors before it has a chance to be cleaved at the second site. This finding favours the role of the MC chymase in Ang conversion and opens the question of whether several of the rodent enzymes are also potent Ang converters under physiological conditions. A theoretical modelling of an ancestral chymase also points in this direction, that an early mast cell chymase was a potent Ang converter \( (8) \). In favour of an important role of the HC enzyme in Ang conversion is the fact that other rodent \( \beta \)-chymases have been shown to be better Ang converters than mMCP-4 and rMCP-1. The mucosal mMCP-1 and rat vascular chymase are both efficient Ang converters and these enzymes may compensate for the poor activity of the connective tissue chymases (e.g. mMCP-4 and rMCP-1) in rodents \( (43, 44) \).

This is the first study to highlight the remarkable similarity between the primary and extended cleavage specificity of
the HC and the MC. Additional characteristics, namely the chymase gene loci arrangement and the behaviour of the enzymes with a number of recombinant and natural substrates further strengthens the usefulness of the macaque, over other potentially less relevant animal models. In order to facilitate the development of medical products (such as chymase inhibitors) for the treatment of cardiovascular disease and as more general anti-inflammatory drugs, informative animal models are highly desired. Despite the higher cost and arguable ethical issues surrounding the use of primates in research, the beneficial effect for knowledge gained about the physiological role of the HC in such models is clearly worth contemplating.

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**References**