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ScienceDirect
Molecular Immunology 45 (2008) 2116–2125
Molecular Immunology
www.elsevier.com/locate/molimm

Extended substrate specificity of opossum chymase—Implications for the origin of mast cell chymases

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Received 21 August 2007; received in revised form 8 October 2007; accepted 8 October 2007
Available online 19 November 2007

Abstract

Serine proteases are major granule constituents of mast cells, neutrophils, T cells and NK cells. The genes encoding these proteases are arranged in different loci. The mast cell chymase locus e.g. comprises at least one α-chymase, one cathepsin G, and two granzyme genes in almost all mammalian species investigated. However, in the gray, short-tailed opossum (*Monodelphis domestica*) this locus contains only two genes. Phylogenetic analyses place one of them clearly with the α-chymases, whereas the other gene is equally related to cathepsin G and the granzymes. To study the function of opossum chymase, and to explore the evolutionary origin of mast cell chymases, we have analyzed the cleavage specificity of this enzyme. The protease was expressed in mammalian cells and the extended substrate specificity was determined using a randomized phage-displayed nonapeptide library. A strong preference for the aromatic amino acids Trp over Phe and Tyr in the P1 position was observed. This is in contrast to human chymase and mouse mast cell protease-4, which prefer Phe over Tyr and Trp in this position. However, in most other positions this enzyme shows amino acid preferences very similar to human chymase and mouse mast cell protease-4, i.e. aliphatic amino acids in positions P4, P3, P2 and P1′, and acidic amino acids (Glu and Asp) in the P2′ position. The overall specificity of MC chymase thereby seems to have been conserved over almost 200 million years of mammalian evolution, indicating a strong selective pressure in maintaining this specificity and an important role for these enzymes in mast cell biology.

Keywords: Mast cell; Chymase; Cleavage specificity; Evolution; Marsupial

1. Introduction

Mast cells (MCs) are potent immune effector cells primarily known for their proinflammatory role in atopic allergies. However, their original function is probably in parasite and bacterial defense of the host (Echtenacher et al., 1996; Knight et al., 2000), and as a general inducer of inflammation. More recently, MCs have also been found to have important regulatory functions (reviewed in Bischoff, 2007; Galli et al., 2005).

Upon activation and subsequent degranulation, MCs release a number of preformed and *de novo* synthesized mediators, i.e. histamine, heparin, proteases, cytokines, leukotrienes and prostaglandins. Of these components the major protein constituents of MC granules are various proteases (Schwartz et al., 1987). The majority of the MC proteases belong to the large family of chymotrypsin related serine proteases. Proteases with chymotrypsin-like specificity are termed chymases and proteases with trypsin-like specificity are termed tryptases (Schwartz et al., 1987). Serine proteases secreted by MCs have been shown to be involved in a number of different biological processes, e.g. activation of metalloproteases (Tchougounova et al., 2005), tissue remodeling (Tchougounova et al., 2003), recruitment of inflammatory cells (Meyer et al., 2005) and blood pressure regulation (Li et al., 2004). The large and increasing

Abbreviations: MC, mast cell; Mcpt, mast cell protease; mMCP, mouse mast cell protease; MMC, mucosal mast cell; Ni-NTA, nickel-nitrilotriacetic acid; EK, enterokinase; *p*NA, *para*-nitroanilide acid; aa, amino acid(s); rMCP, rat mast cell protease; Ang, angiotensin; CTMC, connective tissue mast cell; LSMC, lymphatic sinus MC.

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doi:10.1016/j.molimm.2007.10.015

number of functions ascribed to these enzymes, clearly indicates their importance in MC biology.

Chymases and tryptases are encoded from two complex loci, the MC chymase locus and MC tryptase locus, respectively. The MC chymase locus contains at least one granzyme B (*Gzmb*) gene, one cathepsin G (*Ctsg*) gene, and one α-chymase (*Cma1*) gene in all eutherian (placental) mammals investigated (Gallwitz et al., 2006). In human, only one additional gene has been identified, *Gzmh* (Gallwitz and Hellman, 2006; Hanson et al., 1990). In contrast, the chymase loci of rodents have undergone a significant expansion. In mouse (*Mus musculus*), 13 functional genes have been identified and in rat probably as many as 17 (Gallwitz and Hellman, 2006). Moreover, in mouse, much of this expansion is the result of a dramatic increase in the number of granzyme genes, as many as seven functional granzyme genes (*Gzmb-g* and *n*) and one pseudogene (*Gzml*) have been described (reviewed in Grossman et al., 2003). The rat (*Rattus norvegicus*) locus, on the other hand, contains only three functional granzyme genes, *Gzmb*, *Gzmc* and *Nkpt7* (Gallwitz and Hellman, 2006). Two additional gene families have appeared in rodents, the β-chymase- and the mast cell protease-8 (Mcpt8)-families. Four β-chymases (Huang et al., 1991; Hunt et al., 1997; Newlands et al., 1987; Serafin et al., 1990; Trong et al., 1989) and one Mcpt8 gene (Lützelschwab et al., 1998) have been identified in mouse, whereas seven β-chymases (Benfey et al., 1987; Guo et al., 2001; Ide et al., 1995; Kido et al., 1986; Le Trong et al., 1987) and at least six functional genes belonging to the Mcpt8-family have been described in the rat (Gallwitz et al., 2007; Gallwitz and Hellman, 2006; Lützelschwab et al., 1997).

The α- and β-chymases are expressed in MCs, cathepsin G essentially in neutrophils, and granzyme B and H in NK and T cells (Caughey, 2002). One member of the Mcpt8-family, mouse mast cell protease-8 (mMCP-8) has been shown to be expressed in basophils (Lützelschwab et al., 1998; Poorafshar et al., 2000b), whereas five of the rat Mcpt8-family members are expressed by the mucosal MC (MMC) cell line RBL-1 and in MMC related tissues, e.g. large and small intestine and stomach (Gallwitz et al., 2007; Lützelschwab et al., 1997).

To study the evolutionary origin and history of the chymase locus and the proteases encoded therein, we screened the newly annotated genome sequence assembly of the gray short-tailed opossum, *Monodelphis domestica*, for potential MC chymase locus genes. Two genes were identified and were named opossum *Cma1* and opossum *grathepsodenase* (Gallwitz et al., 2006). Phylogenetically, the opossum chymase consistently falls into the α-chymase subgroup. In contrast, the *grathepsodenase* appears to be equally related to the granzymes, cathepsin G, and the ungulate-specific duodenases (also members of the MC chymase locus). A gene resembling the *grathepsodenase* was most likely the ancestor of both cathepsin G and the granzymes during early mammalian evolution. In this recent study we also proposed that opossum *Cma1* and *grathepsodenase* were derived from a duplication of an ancestral chymase locus gene occurring 185–215 million years ago (Kumar and Hedges, 1998; van Rheede et al., 2006; Woodburne et al., 2003). Our conclusion is supported by the presence of the platypus (*Ornithorynchus anatinus*) "granzyme" gene, the single MC chymase locus gene found in this monotreme (Poorafshar et al., 2000a). This gene is equally related to the chymase, cathepsin G, and the granzyme genes and suggests that all MC chymase locus genes present in marsupials and placental mammals arose from a common ancestor similar to that found in modern monotremes (Gallwitz et al., 2006). The fact that the opossum chymase is the first MC chymase locus gene that appears to be the direct homolog of any of the separate families seen in placental mammals of today, makes it a highly interesting enzyme from an evolutionary viewpoint.

The aim of the present study was to determine if this novel marsupial chymase, the opossum chymase, is a true α-chymase. The enzyme was therefore allowed to hydrolyze different chromogenic substrates to determine its primary specificity. We also used a phage display approach to study the extended cleavage specificity. The data presented here supports the phylogenetic analyses that opossum chymase is an α-chymase and indeed has chymotryptic activity. In addition, this enzyme has an extended specificity very similar to the human chymase and the mouse mMCP-4, which indicates that the MC chymase function has been highly conserved through almost 200 million years of mammalian evolution.

2. Materials and methods

2.1. Cloning and expression of recombinant opossum chymase

Total RNA was extracted from opossum skin (pinna) by the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987). cDNA was synthesized and amplified by RT-PCR. The cDNA for the coding region of opossum chymase was further isolated and amplified by PCR using the oligonucleotide primers 1054-OpChy-pCEPF (5′–3′); TAT GAA TTC CAT CAC CAT CAC CAT CAC GAC GAT GAC GAT AAG ATT ATT GGG GGA CAC GAA TCA ATT and 1056-OpChyR (5′–3′); CAG CTC GAG GCC AAG GCC ACA GAG CTA ATT TGA TTT TAG GAC. For purification and activation purposes, the coding region of a polyhistidine tag (His_6) and a site susceptible to enterokinase (EK) cleavage (Asp-Asp-Asp-Asp-Lys) was introduced in the 5′-primer, 1054-OpChy-pCEPF. The PCR product was digested with EcoRI and XhoI and subcloned into the EcoRI/XhoI sites of the pCEP-Pu2 vector (Hallgren et al., 2000; Vernersson et al., 2002). The nucleotide sequence was verified by sequencing on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The opossum chymase/pCEP-Pu2 construct was transfected into human embryonic kidney cells, 293-EBNA. Cells were maintained in puromycin selection medium (DMEM Glutamax containing 5% FBS, 50 μg/ml gentamicin, 5 μg/ml heparin and 1.5 μg/ml puromycin), for seven days. The puromycin was thereafter decreased to 0.5 μg/ml.

2.2. Purification and activation of recombinant opossum chymase

Conditioned medium from cell cultures of 293-EBNA cells carrying the opossum chymase expression vector was