

Mast Cell Carboxypeptidase A, a Secretory Granule Component

**Insights to its Processing, Intracellular Sorting and
Interaction with Serglycin Proteoglycans**

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Abstract

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Mast cells are highly granulated cells of the immune system that upon stimulation release a number of inflammatory mediators including heparin and/or chondroitin sulphate (CS) proteoglycan (PG) and various heparin-binding proteases such as tryptase, chymase and carboxypeptidase A (CPA). Mast cell CPA is a zinc-metalloexopeptidase, cleaving substrates with carboxyl-terminal aliphatic or aromatic amino acids. In this thesis, the storage and activation of CPA was investigated, using bone marrow derived mast cells (BMMCs) from mice lacking heparin, either due to loss of the gene coding for the heparin biosynthesis enzyme, NDST-2, or the gene coding for the PG core protein serglycin (SG).

We found that BMMCs from NDST-2^{-/-} mice that thus lack heparin, but produce CS, lack the chymase, mMCP-5 and mature CPA. Interestingly, the pro-form, but not the active form of CPA could be detected in the heparin-deficient cells, indicating a role for heparin in the processing of CPA. Furthermore, we have shown that the cysteine proteases, cathepsins C and S, are not involved in processing CPA, but rather that lack of cathepsin C or S cause increased levels of CPA as well as mMCP-5. In addition, neither cathepsins B nor L influence CPA processing at all, but instead, an aspartic protease, cathepsin E, plays a role in processing pro-CPA. Further, these studies led to the novel finding that cathepsin E is located inside the mast cell granules, where it is stored in complex with heparin.

The activation of mast cells, which ultimately leads to degranulation, has been studied in detail; however, the process where mast cell granules are formed has not gained as much attention. We addressed this issue by the use of BMMCs from mice lacking SG. Here, we present evidence that secretory granules are formed independently of SG PG but that SG mediates selective condensation of certain granule constituents, while others are independent of SG. Mast cell proteases are correctly sorted into the granules but are subsequently degraded, exocytosed or remain unprocessed when SG is absent. These results indicate a model in which selected granule constituents are sorted into granules by SG-mediated retention.

Keywords: mast cell mediator, protease, proteoglycan, proteolytic activation, granule formation, cathepsin

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To my family

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Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Henningsson F., Ledin J., Lunderius C., Wilén M., Hellman L. and Pejler G. “Altered storage of proteases in mice lacking heparin: a possible role for heparin in carboxypeptidase A processing.” *Biological Chemistry*. (2002) 383: 793-801

II. Henningsson F., Wolters P., Chapman H.A., Caughey C.H. and Pejler G. “Mast cell cathepsins C and S control levels of carboxypeptidase A and the chymase mouse mast cell protease 5.” *Biological Chemistry*. (2003) 384: 1527-1531

III. Henningsson F., Yamamoto K., Saftig P., Reinheckel T., Peters C., Knight S.D. and Pejler G “A role for cathepsin E in the processing of mast cell carboxypeptidase A” *Journal of Cell Science*. (2005) 118: 2035-2042

IV. Henningsson F., Hegert S., Cortelius R., Åbrink M. and Pejler G “A role for serglycin proteoglycan in granular retention and processing of selected granule components in mast cells.” *Submitted*

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Abbreviations

BMMC	bone marrow-derived mast cell
BP	bullous pemphigoid
CPA	carboxypeptidase A
CPB	carboxypeptidase B
CS	chondroitin sulphate
CTMC	connective tissue mast cell
DPPI	dipeptidyl peptidase I
EAE	experimental allergic encephalomyelitis
ER	endoplasmatic reticulum
ET-1	endothelin-1
GAG	glycosaminoglycan
HDC	histidine decarboxylase
Ig	immunoglobulin
IL	interleukin
ISG	immature secretory granule
LDL	low density lipoprotein
LPS	lipopolysaccharide
LT	leukotriene
MIP	macrophage inflammatory protein
MMC	mucosal mast cell
mMCP	mouse mast cell protease
MS	multiple sclerosis
MSG	mature secretory granule
NDST	N-deacetylase/N-sulphotransferase
PG	proteoglycan
PGD ₂	prostaglandin D ₂
PGN	peptidoglycan
RA	rheumatoid arthritis
rMCP	rat mast cell protease
SCF	stem cell factor
SG	serglycin
TGN	<i>trans</i> -Golgi network
TIMP	tissue inhibitor of metalloproteinases
TLR	toll-like receptor
TNF- α	tumour necrosis factor- α

Introduction

The immune system

Everyday we encounter bacteria, viruses and other pathogens. The skin and mucosal tissues act as a first barrier, but many pathogens still enter our body. However, most infections in normal individuals are short-lived and leave little permanent damage. This is due to the immune system, which combats infectious agents. All immune responses involve, firstly, recognition of the pathogen, and secondly, mounting a reaction against it to eliminate it. There are two types of immune responses: innate and adaptive. The adaptive response is highly specific for a particular pathogen and improves with each successive encounter with the same pathogen whereas the innate immune response does not alter on repeated exposure to a given infectious agent.

A number of cells and mediators released by them are involved in immune responses. Leukocytes are the main group of immunological cells consisting of the phagocytic cells (monocytes, macrophages and polynuclear neutrophils) and the lymphocytes (T lymphocytes and B lymphocytes). Phagocytes have a non-specific recognition system and are the first line of defence in the innate responses. Lymphocytes on the other hand, specifically recognize individual pathogens and are thus involved in the adaptive immune response. A number of other cells mediate inflammation, the main purpose of which is to attract leukocytes and the soluble mediators of immunity towards a site of infection. These cells include platelets, basophils and mast cells.

The mast cell

Mast cells are highly granulated cells of the immune system that are especially prominent near surfaces exposed to the environment, including the skin, airways and gastrointestinal tract, where they serve as a first line of defence against pathogens, allergens and other environmental agents. Mast cells were discovered in 1877 (Ehrlich, 1877), and in recent decades it has become more and more evident that the mast cell is a highly important cell that is involved in several different physiological and pathological conditions. The diverse roles of mast cells and their mediators will be reviewed and investigated in this thesis.

Mast cells arise from pluripotent haematopoietic stem cells in the bone marrow (Kitamura *et al.*, 1981, Sonoda, Ohno and Kitamura, 1982, Sonoda *et al.*, 1983, Bender, Van Epps and Stewart, 1988, Kirshenbaum *et al.*, 1991). However, mast cells do not ordinarily circulate in their mature form; instead, they undergo the terminal stages of their differentiation and/or maturation locally, after the migration of their precursors into those vascularized tissues or serosal cavities in which they ultimately will reside. Specific growth factors influencing the differentiation of mast cells include stem cell factor (SCF), interleukin-3 (IL-3)

and nerve growth factor (in humans only) (reviewed in (Metcalf, Baram and Mekori, 1997). Other mediators such as IL-4, IL-9 and IL-10 can also regulate mast cell differentiation.

Mast cell heterogeneity

Both rodent and human mast cells display a high degree of heterogeneity based on their granular location and contents (Galli, 1990). In rodents, two main subtypes have been described (Table 1). The connective tissue mast cells (CTMC) are found in the skin and peritoneal cavity and contain heparin proteoglycan, high amounts of histamine and the proteases tryptase, chymase and carboxypeptidase A (CPA) whereas the mucosal mast cells (MMC) reside beneath mucosal surfaces such as the lamina propria of the respiratory and gastrointestinal tract and contain chondroitin sulphate proteoglycan and chymase, but lacks tryptase and CPA. Human mast cells can be similarly subdivided into two populations based on their granular content (Irani et al., 1986) (Table 1). One phenotype contains only tryptase and is termed MC_T whereas the other (MC_{TC}) contains both tryptase and chymase as well as cathepsin G and CPA. MC_{TC} are found in human dermis and subepithelial sites such as in airway and gastrointestinal submucosa, whereas the MC_T subset is located predominantly in airway and gastrointestinal sites and in alveolar tissue (Irani et al., 1986). It has also been proposed that the two human mast cell phenotypes express different arrays of cytokines (Bradding et al., 1995).

Table 1. Mast cell heterogeneity in mouse and human

	Mouse		Human	
	CTMC	MMC	MC_T	MC_{TC}
Proteoglycan	Heparin	CS	Heparin & CS	Heparin & CS
Tryptase	mMCP-6, mMCP-7	-	+	+
Chymase	mMCP-4, mMCP-5	mMCP-1, mMCP-2	-	+
CPA	+	-	-	+

Mast cell granules

A prominent feature of mast cells is their electron dense secretory granules containing the different preformed mast cell mediators. Mast cells were first described by Ehrlich in 1877 (Ehrlich, 1877), when he detected cells in various organs of several animal species with densely packed (overfed) cytoplasmic granules and therefore named them “mästzellen”, from the German word “mäst”, referring to feeding.

Granule formation

Even though the process and signalling pathways leading to mast cell degranulation have been intensely investigated (reviewed in (Blank and Rivera, 2004), the mechanism by which the mast cell granules are formed is still largely unknown.

Every existing cell contains a secretory pathway to deliver newly synthesised proteins to the cell surface, called the constitutive pathway. Secretory cells, like mast cells, also support another pathway known as the regulated secretory pathway, where proteins destined for the secretory granules are sorted. Most secretory proteins are translated on ribosomes bound to the ER (endoplasmic reticulum) and following the removal of the signal peptide, are transported to the Golgi in vesicles, where they are further processed while they pass the *cis*- and *medial*-cisterns until they finally reach the *trans*-Golgi network (TGN). In a general view, secretory granules (in endocrine, neuroendocrine and exocrine cells, such as mast cells) form in the TGN and are classified initially as immature secretory granules (ISGs) (Tooze, Martens and Huttner, 2001). Several subsequent events convert ISGs to mature secretory granules (MSGs). These changes include a change in size and modification of the composition of the membrane and content of the secretory granule. The TGN has been suggested to be the location where proteins are sorted to their final destination. However, whereas the mannose-6-phosphate receptor has been shown to mediate targeting of lysosomal proteins from the TGN (Kornfeld and Mellman, 1989), no such sorting receptor has been identified for mast cell secretory granule proteins.

In the past 20 years, two different hypotheses have emerged to explain the selection of proteins for storage in secretory granules (Arvan and Castle, 1998). Importantly, these models are not mutually exclusive. In the **sorting-for-entry** model, the TGN is seen as the location where sorting takes place (Figure 1a). Regulatory secreted proteins aggregate in the TGN, bind to a sorting receptor and are thus actively separated from proteins that are constitutively secreted. In the **sorting-by-retention** model on the other hand, proteins are packed into ISGs in an unsorted manner and regulated secretory proteins are retained in the ISG, where they aggregate and bind to a receptor similar to the sorting-for-entry model, while other proteins bud off from the ISG in vesicles in which their granule content is gradually refined (Figure 1B). Which of these apply to mast cell granule formation is not known, but in a recent study it was shown that serglycin proteoglycan is necessary for correct storage of mast cell proteases in mast cell granules (Abrink, Grujic and Pejler, 2004)

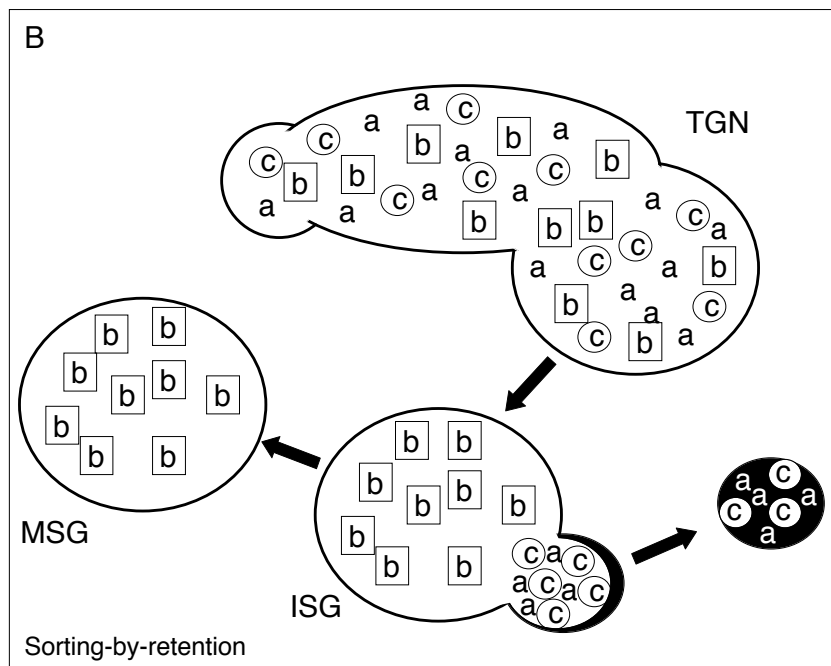
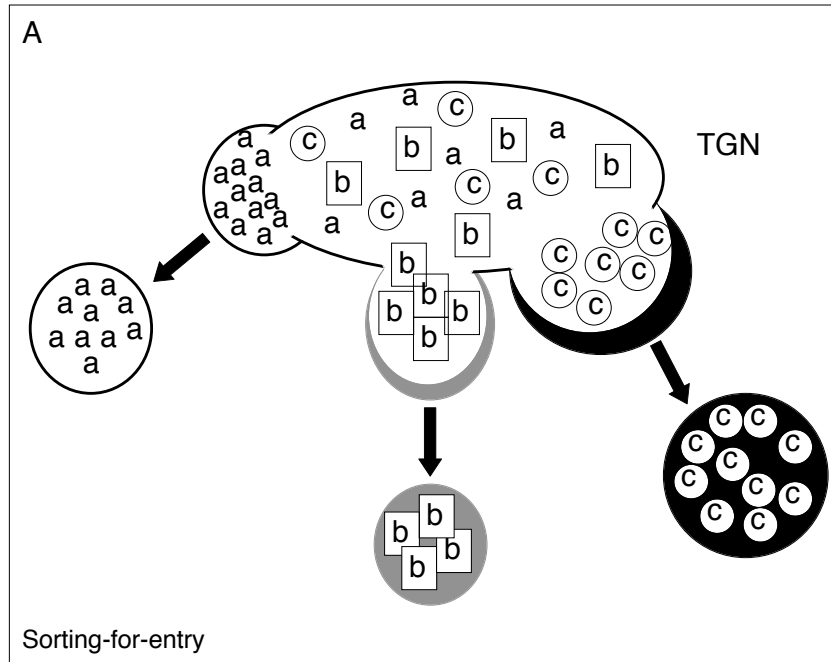


Figure 1. The two proposed models for granular sorting. TGN = *trans*-Golgi network, ISG = immature secretory granule, MSG = mature secretory granule.

Activation/degranulation

When mast cell granules degranulate upon activation, their inflammatory mediators are released into the extracellular milieu

Activation may occur by various mechanisms, including antigen-mediated cross linking of surface-associated IgE or IgG and exposure to complement factors as well as bacterial components, neuropeptides and calcium ionophores (Figure 2).

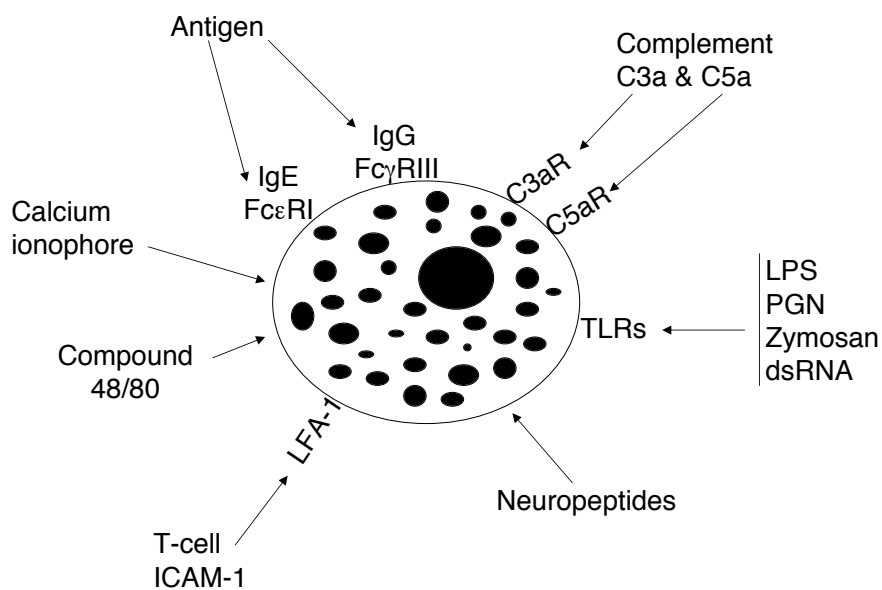


Figure 2. Different ways of activating mast cells. For details, see the text.

IgE-mediated activation

The classical way of activating mast cells, especially in allergic reactions, is through their high affinity IgE receptors, FcεRI. Mast cell FcεRI is a tetrameric protein complex (Blank *et al.*, 1989, Ra, Jouvin and Kinet, 1989), consisting of the IgE-binding α-subunit (Hakimi *et al.*, 1990, Blank, Ra and Kinet, 1991), a single signal-amplifying membrane-tetraspanning β-subunit as well as a homodimeric disulphide-linked γ-subunit (Perez-Montfort, Kinet and Metzger, 1983) that provides the signalling ability of the receptor.

When an antigen enters the body, it may provoke IgE production. The IgE molecules bind tightly to FcεRI on the mast cell surface and the next time the same antigen enters it will cross-link IgE on the mast cell surface. Cross-linking brings the receptors into position to initiate intracellular signalling (Blank and Rivera, 2004), leading to mast cell degranulation and release of mast cell mediators.

IgE-independent mechanisms

IgG

In addition to IgE-dependent activation, mast cells can also be activated through the low-affinity IgG receptor FcγRIII, where aggregation of the receptor is followed by mast cell adhesion, degranulation and an increase in tumour necrosis factor-α (TNF-α) synthesis (Katz et al., 1992, Latour et al., 1992).

Complement

It has long been known that complement components C3a and C5a (also called anaphylatoxins) can activate mast cells (Johnson, Hugli and Muller-Eberhard, 1975). The complement system is involved in a number of processes contributing to host defence and/or inflammation (Muller-Eberhard, 1988). Rodent CTMCs, but not MMCs express receptors binding complement-derived peptides C3a and C5a and thus is the only rodent mast cell subtype that can be activated through the complement system (Mousli et al., 1994).

Human mast cells display differential expression of the C5a receptor (C5aR) but do not express the receptors for C3-derived fragments such as CR1, CR2, CR3 and CR4 (Fureder et al., 1995). However, bone marrow mast cells from patients with systemic mastocytosis have high levels of CR1, CR4 as well as C5a receptor (Nunez-Lopez et al., 2003), indicating a mechanism for up-regulation of these receptors.

Toll-like receptors

Mast cells can also be activated by different bacterial components through their interactions with toll-like receptors (TLRs) expressed on the mast cell surface. TLRs are a highly conserved family of pattern recognition receptors that are type I transmembrane proteins with an extracellular domain consisting of leucine-rich repeats and an intracellular domain with homology to the IL-1 receptor, called the Toll/IL-1R domain (Medzhitov, Preston-Hurlburt and Janeway, 1997).

Eleven different TLRs (TLR1-11) have been identified in mammalian systems (Rock et al., 1998, Takeuchi et al., 1999, Du et al., 2000, Chuang and Ulevitch, 2001, Zhang et al., 2004) and it has been shown that mast cells express TLR2, TLR4, TLR6 and TLR8 (Supajatura et al., 2001). However, a recent study also indicates the presence of TLR3 on mast cells (Kulka et al., 2004). Activators of TLRs include lipopolysaccharide (LPS) (TLR2 and TLR4), peptidoglycan (PGN),

(TLR2 and TLR6) and zymosan (TLR2) as well as other bacterial components and viral dsRNA (TLR3) (Aliprantis *et al.*, 1999, Chow *et al.*, 1999, Takeuchi *et al.*, 2001, Kulka *et al.*, 2004). The natural activator of TLR8 remains to be discovered. Binding of these activators to TLRs ultimately leads to expression and release of cytokines, leukotrienes and prostaglandins, usually without causing degranulation of the mast cell (Poltorak *et al.*, 1998, Chow *et al.*, 1999, Qureshi *et al.*, 1999, Supajatura *et al.*, 2001, Takeuchi *et al.*, 2001, Kulka *et al.*, 2004). Mast cells may thus have the potential to recruit other immune cells to the infected sites by responding to various bacterial and viral components through TLR signalling pathways.

Other mechanisms

As well as responding to bacterial and viral components, mast cells can be activated by **neuropeptides** released from neurons, beside which mast cells often lie in proximity (Bauer and Razin, 2000). The mast cell-activating neuropeptides include substance P, calcitonin gene regulated peptide, vasoactive intestinal peptide and neurotensin (Church *et al.*, 1989). Mast cells can also be activated by different types of **cytokines and chemokines**, such as macrophage inflammatory protein 1 α (MIP-1 α) and monocyte chemoattractant-1 (Alam *et al.*, 1994). In addition, **activated T-cells** can activate mast cells to degranulate and produce cytokines, through the adhesion molecule ICAM-1 and its ligand LFA-1 (Bhattacharyya *et al.*, 1998, Inamura *et al.*, 1998). Other compounds that can directly activate mast cells include calcium ionophores, compound 48/80, morphine, codeine and synthetic adrenocorticotrophic hormone, adenosine and endothelin (Metcalf, Baram and Mekori, 1997).

In this context, it is important to emphasize that individual stimuli of mast cell secretion can elicit distinct, through sometimes overlapping patterns of mediator secretion. For example, although IgE and specific antigen can induce the cells to release large quantities of all classes of mediators, it has been reported that neuropeptides favour the release of preformed mediators (Church, el-Lati and Caulfield, 1991), whereas LPS and other stimuli that are identified by mast cell TLR favour the release of certain cytokines and may induce little or no release of preformed mediators.

Mast cell mediators

Following activation, a number of mast cell mediators are released into the extracellular milieu of the mast cell. The vast majority of them are stored as preformed, active molecules in the mast cell granules, but some, such as cytokines and the lipid mediators are synthesized upon mast cell activation.

De novo-synthesized mediators

Lipid mediators

The lipid mediators, leukotrienes and prostaglandins, are members of the eicosanoid family. Prostaglandins are cyclooxygenase products whereas leukotrienes (LT) are synthesised through the lipoxygenase pathway. Upon activation, mast cells produce the cysteinyl leukotrienes (cysLTs) LTC₄, LTD₄ and LTE₄ which are potent inflammatory mediators that initiate and propagate a diverse array of biological responses (Kanaoka and Boyce, 2004).

The cysLTs exert their biological functions through two G-protein coupled receptors, CysLT₁ and CysLT₂ receptors (Lynch et al., 1999, Heise et al., 2000). The biological effects of the cysLTs include bronchoconstriction (Dahlen *et al.*, 1980, Davidson *et al.*, 1987) and influx of eosinophils and neutrophils into the lungs, as well as macrophage infiltration, haematopoietic progenitor cell recruitment and eosinophil and mast cell cytokine production (reviewed in (Kanaoka and Boyce, 2004)). Several studies have shown that inhibitors of the cysLT-generating pathway (Israel et al., 1996) as well as cysLT receptor antagonists (Altman et al., 1998) decrease symptoms in asthmatic individuals, indicating an important role for cysLTs in acute asthma.

Prostaglandin D₂ (PGD₂), the main cyclooxygenase product in mast cells (Lewis et al., 1982), has also been shown to play an important role in asthmatic responses (Matsuoka et al., 2000). The receptor for PGD₂ was knocked out, and mice lacking this receptor did not develop asthmatic responses in an OVA-induced asthma model, indicating PGD₂ and its receptors as important players in asthma.

Cytokines

Mast cells produce a broad range of cytokines, mainly of Th2 type. It has been shown that activated human lung mast cells express IL-3, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-13, as well as TNF- α and GM-CSF (Okayama *et al.*, 1995, Ishizuka *et al.*, 1999b, Ishizuka *et al.*, 1999a, Kobayashi, Ishizuka and Okayama, 2000), but there is a heterogeneity of mast cells regarding cytokine expression (Bradding et al., 1995). The mast cell cytokines regulate functions of inflammatory cells, giving the mast cell an important role in the course of pathologic inflammation.

Preformed mediators

Histamine

Perhaps the most well-known mast cell mediator is histamine, a biogenic peptide that is formed after decarboxylation of histidine, a reaction catalyzed by the enzyme L-histidine decarboxylase (HDC). Histamine is found in several different cell types throughout the body, but the main sources are mast cells (Riley and West, 1952) and basophils (Ishizaka et al., 1972) where it is stored as a preformed mediator in the secretory granules. Histamine exerts its functions through

histamine receptors, H1-H4 (MacGlashan, 2003). H1 and H2 are widely spread whereas H3 is restricted to the brain. H4 is found in the intestines and in haematopoietic cells. All of the histamine receptors are G-protein coupled receptors and their differential expression on almost all cells in the body makes the cellular response towards histamine highly diverse. However, one of the most studied roles of histamine is in allergic disease, where the interaction of histamine with the histamine H1-receptor mediates the classical allergic effects, including vascular permeability and smooth muscle contraction as well as sneezing and itching (Bachert, 2002).

To study the biological role of histamine the HDC gene was knocked out (Ohtsu et al., 2001). HDC-deficient mice, kept on a histidine-free diet lack histamine in all organs except for the brain. Interestingly, mast cell numbers in these mice are significantly decreased and their granular content is reduced. However, this is not only due to the lack of histamine in the mast cell granules, but the levels of the mast cell proteases mMCP-4, mMCP-5 and CPA as well as heparin PG are also reduced. Possibly, the lack of histamine disturbs the normal electrostatic balance in the mast cell granules, causing the disturbed storage of granule constituents. The HDC-deficient mice have been used in several studies to show the biological effects of histamine, such as angiogenesis (Ghosh et al., 2002), gastric acid secretion (Tanaka et al., 2002) as well as gastric mucosal morphology (Nakamura et al., 2004). In addition, *Helicobacter pylori*-induced gastritis (Klausz et al., 2004) and scratching behaviour in mice with contact dermatitis (Seike et al., 2005) have been shown to be dependent on histamine.

Proteoglycans

One major constituent of the mast cell granules is the highly negatively charged proteoglycans (PGs). A PG is any glycoprotein that consists of a protein core and has glycosaminoglycan (GAG) side chains. GAGs are polysaccharides that have a simple repeating disaccharide unit and are highly anionic in nature because of the presence of many sulphate and carboxyl groups. There are several types of PGs, regarding the protein core as well as the GAG chain. However, it has been suggested that the PG core protein expressed by mast cells is exclusively of the serglycin (SG) type (Stevens *et al.*, 1988a, Kjellen *et al.*, 1989), even though the SG core protein can have different GAG chains attached to it.

Murine CTMCs express only PGs with heparin side chains (Yurt, Leid and Austen, 1977), whereas MMCs express side chains of the chondroitin sulphate (CS) type (Enerback L, 1985). In contrast, human mast cells can contain both GAG types in the same mast cell type (Stevens et al., 1988b). The CS side chains are usually of the CS-E type (Razin *et al.*, 1982, Enerback L, 1985), although CS-B (Seldin, Austen and Stevens, 1985) and CS-D (Davidson et al., 1990) also have been reported in certain subsets of mast cells.

Both heparin and CS consists of repeating disaccharide units with different degrees of modifications. The synthesis of heparin and CS chains is initiated with

a linker region, consisting of xylose, galactose, galactose and glucuronic acid attached to a serine residue in the core protein (Prydz and Dalen, 2000) (Figure 3). The GAG is then elongated by the addition of repeating disaccharide units. In the case of heparin, they consist of N-acetyl-glucosamine and glucuronic acid whereas in CS the disaccharide unit is made up by N-acetyl-galactosamine and glucuronic acid. Subsequent modification steps shape the final structure of the GAG-chains. In heparin, the most highly sulphated GAG, a key modification step is the N-deacetylation/N-sulphation of N-acetyl glucosamine residues. This reaction, catalyzed by the bifunctional enzyme N-deacetylase/N-sulphotransferase (NDST), is the first step in the process that finally renders the fully sulphated heparin chain. Subsequent modifications include C5 epimerization of GlcA to IdoA, and variable O-sulphations at C2 of IdoA and GlcA, at C6 of GlcNAc and GlcNS units, and occasionally at C3 of GlcN residues.

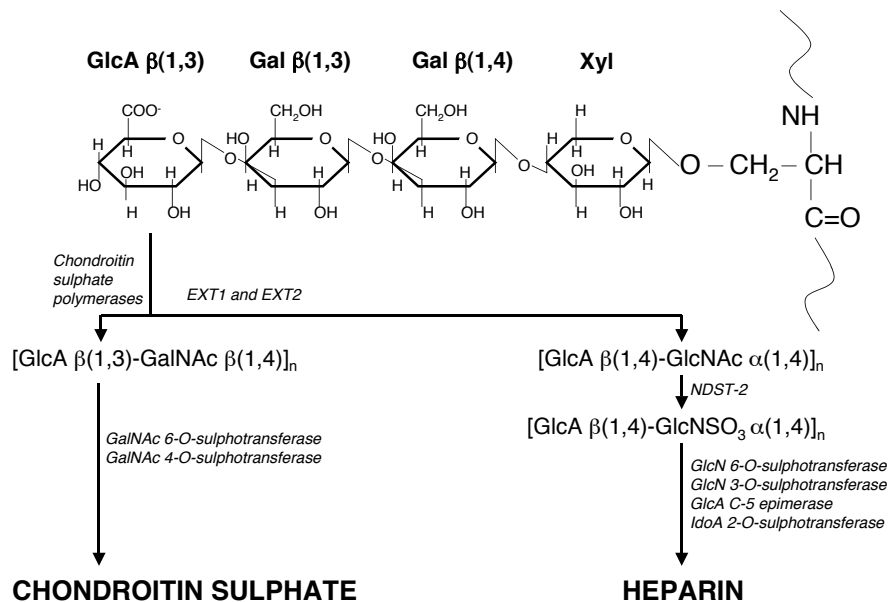


Figure 3. A general scheme for the synthesis of heparin and chondroitin sulphate

Four isoforms of the key enzyme family of NDSTs have been identified (Hashimoto *et al.*, 1992, Eriksson *et al.*, 1994, Orellana *et al.*, 1994, Aikawa *et al.*, 2001). Out of these, CTMCs express predominantly NDST-2. By targeting the gene for this enzyme, mast cells devoid of sulphated heparin were generated (Forsberg *et al.*, 1999, Humphries *et al.*, 1999). Using this approach, several of the functions of the mast cell-specific PG heparin could be investigated.

CTMCs from mice lacking NDST-2 lack chymase, tryptase and CPA in their granules indicating a role for heparin in the storage of mast cell proteases.

Furthermore, heparin was shown to be necessary for the biological function of some of the proteases. For example, the degradation of fibronectin by chymase was shown to be dependent on heparin (Tchougounova et al., 2001) and a chymase-heparin PG complex may modulate extravascular coagulation (Tchougounova and Pejler, 2001). Here, heparin probably works as an attractant for chymase substrates, which in many cases has heparin binding motifs. Furthermore, tryptase is dependent on heparin for formation of enzymatically active tetramers (Hallgren et al., 2000).

However, the targeting of NDST-2 affected only CTMCs since MMCs contain CS PG. Therefore, NDST-2 was not a perfect tool for studying the role of mast cell PGs in general. To address this issue, mice lacking the core protein, SG, were generated (Abrink, Grujic and Pejler, 2004). These mice show a complete lack of mast cell PGs, indicating that SG is indeed the only core protein in mast cell PGs. Moreover, mast cells from the SG knock out had a defect in storage and processing of the mast cell proteases similar to the NDST-2-deficient mast cells. However, the mechanisms behind these granular abnormalities have not been investigated in detail.

Proteases

In terms of mass and molarity, proteases that are enzymatically active at neutral pH are the major protein constituents exocytosed from activated mast cells. Up to 35 % of the total protein content in mast cell granules are proteases (Schwartz et al., 1987). Tryptases, chymases and carboxypeptidase A (CPA) represent the three major families of proteases stored in the secretory granules of mast cells where chymase and tryptase are serine proteases with endopeptidase activity, whereas CPA is a metalloprotease with exopeptidase activity. In addition, the serine protease cathepsin G has also been reported in certain human MC_{TC} (Meier *et al.*, 1985, Schechter *et al.*, 1990).

Chymase

Chymases are serine proteases with chymotrypsin-like substrate specificity, cleaving substrates downstream from aromatic amino acids (Powers *et al.*, 1985). Chymases were first identified and localized in mast cell granules in 1959 (Benditt and Arase, 1959), and they can be divided into two separate groups, the α -chymases and the β -chymases (Chandrasekharan et al., 1996). In humans, α -chymase alone is expressed, whereas even though the β -chymases are ancient, they seem to exist only in rodents where, in mice, they are known as mouse mast cell proteases (mMCP) -1, -2, -4, and -9. In mice, as in humans, α -chymase exists alone, and is called mMCP-5. Not all chymases are expressed in all mast cell subclasses. MMCs in mice express mMCP-1 and -2 whereas CTMCs have been shown to express mMCP-4 and -5. In humans, only the MC_{TC} express chymase.

Chymase is stored as an active enzyme in the mast cell granules in complex with heparin. Activation of pro-chymase occurs by the removal of a two-residue

propeptide by dipeptidyl peptidase I (DPPI, also known as cathepsin C), as shown from experiments using DPPI knockout mice (Wolters et al., 2001). After degranulation, the chymase remains in a tight complex with heparin PG, protecting chymase from protease inhibitors (Pejler and Berg, 1995) as well as increasing the enzymatic activity of chymase (Pejler and Sadler, 1999). It has been suggested that the negatively charged heparin brings positively charged substrates close to the chymase and reduces the level of electrostatic repulsion between chymase and its positively charged substrates (Pejler and Sadler, 1999, Tchougounova and Pejler, 2001). Functions ascribed to mast cell chymase include attraction of neutrophils and eosinophils (He and Walls, 1998, Watanabe, Miura and Fukuda, 2002) and extracellular matrix remodelling by fibronectin degradation (Tchougounova et al., 2001) and matrix metalloproteinase activation (Tchougounova et al., 2005) as well as TIMP (tissue inhibitor of metalloproteinases) inactivation (Frank et al., 2001). Furthermore, chymase may be involved in extravascular coagulation and fibrinolysis, where it can degrade thrombin as well as plasmin (Tchougounova and Pejler, 2001). In addition, chymase seems to play a role in the extravascular processing of angiotensin I to angiotensin II (Chandrasekharan et al., 1996).

Most of these findings are results of *in vitro* investigations. However, knock outs for two of the β -chymases, mMCP-1 (Knight et al., 2000) and mMCP-4 (Tchougounova, Pejler and Abrink, 2003), and the α -chymase mMCP-5 (Abonia et al., 2005) have recently been generated, allowing experiments that have verified some of the *in vitro*-data. Because mMCP-5 is an α -chymase it was thought to be the homologue of human chymase. However, recent studies have indicated that mMCP-5 and its rat counterpart, rMCP-5, have elastase-like, rather than chymotrypsin-like substrate specificities (Kunori et al., 2002, Karlson et al., 2003). Because of these findings, mMCP-4 was chosen as the next candidate for being the human chymase counterpart and indeed, the deletion of mMCP-4 showed that this chymase accounted for the major chymotrypsin-like activity in the peritoneum and ear tissue in mice (Tchougounova, Pejler and Abrink, 2003). Furthermore, mMCP-4 was also proven to play an important role in angiotensin-processing, together with CPA (Lundequist et al., 2004). The knockout of mMCP-1 has also indicated the involvement of this α -chymase in expulsion of the parasite, *Trichinella spiralis* (Knight et al., 2000).

Tryptase

The other mast cell serine protease has tryptic-like substrate specificity and is thus called tryptase. There are two main types of human mast cell tryptases: α -tryptase and β -tryptase. There is approximately 90% sequence identity between the two types. β -tryptases are further classified into β I-, β II- and β III-tryptases (Miller, Moxley and Schwartz, 1990, Vanderslice *et al.*, 1990), and the α -tryptases into α I- and α II-tryptases (Miller, Westin and Schwartz, 1989, Pallaoro *et al.*, 1999). Moreover, other members of the tryptase family include γ -tryptase, which may be anchored to the plasma membrane (Caughey et al., 2000) and δ -tryptase (Wang et al., 2002). β -tryptases appear to be the main type expressed in humans, whereas

the α -tryptases have an amino acid substitution rendering them constitutively secreted. Furthermore, the α -tryptases have no or little enzymatic activity and about 29% of the human population lack α -tryptase, without any obvious defects (Soto et al., 2002). In mice, four mast cell tryptases have been identified to date. These are mMCP-6, mMCP-7, the newly found mouse transmembrane tryptase and mMCP-11. mMCP-6 is the mouse tryptase most closely related to human β -tryptase, with 78% sequence identity. In mice, tryptase is only expressed in CTMC, whereas both types of human mast cells (MC_T and MC_{TC}) contain tryptase.

Tryptases, like chymases, are stored in the mast cell granules as active enzymes. Active tryptase is a homotetramer with the active sites facing a central pore, stabilized by heparin (Schwartz and Bradford, 1986). Activation of tryptase may occur in two steps where the first step is an autocatalytic cleavage into pro-tryptase and the second is removal of an N-terminal dipeptide by DPPI (this enzyme is only partially responsible for mouse tryptase activation (Wolters et al., 2001)) which results in monomeric tryptase (Sakai, Ren and Schwartz, 1996). The tetrameric structure of tryptase, formed and stabilized by interactions with heparin under acidic conditions (Hallgren *et al.*, 2000, Hallgren, Lindahl and Pejler, 2005), gives tryptase its highly selective substrate specificity, cleaving only substrates small enough to enter the central pore. Furthermore, the resistance of tryptase to macromolecular inhibitors can also be attributed to its tetrameric structure. However, heparin antagonists have been shown to be potent inhibitors of tryptase (Hallgren *et al.*, 2001, Lundequist *et al.*, 2003), in accordance with its heparin-dependent activation.

Tryptase has been shown to degrade low molecular weight peptides such as the bronchodilators vasoactive intestinal peptide (Caughey et al., 1988) and peptide histidine-methionine (Tam and Caughey, 1990), which may lead to increased bronchial responsiveness and contribute to the involvement of tryptase in asthma. Tryptase may also increase vascular permeability through activation of prekallikrin and production of bradykinin from kininogens (Imamura et al., 1996). Furthermore, tryptase has been reported to degrade several large matrix proteins, *e.g.* fibronectin, fibrinogen and collagen type VI (Lohi, Harvima and Keski-Oja, 1992, Kielty *et al.*, 1993). However, since these proteins are too large to fit into the central pore of tetrameric tryptase the relevance of these findings have been questioned (Sommerhoff et al., 2000). Interestingly, later studies have indicated the presence of active tryptase monomers, degrading both fibronectin and fibrinogen (Hallgren, Spillmann and Pejler, 2001, Fajardo and Pejler, 2003). The presence of active monomers *in vivo*, however, remains to be demonstrated.

In addition to reacting with the substrates discussed above, tryptase stimulates proliferation of fibroblasts (Ruoss, Hartmann and Caughey, 1991), smooth muscle cells (Brown et al., 1995) and epithelial cells (Cairns and Walls, 1996), which may lead to the thickening of airway walls and increased airway responsiveness, as seen in asthmatic patients. Tryptase also has pro-inflammatory activities, *e.g.* in its function of recruiting inflammatory cells (He, Peng and Walls, 1997, Huang *et al.*, 1998, Huang *et al.*, 2001).

Carboxypeptidase A

Carboxypeptidase-activity in mast cells was first suggested in 1979 when a sediment of high density from rat liver mitochondria was found to contain a carboxypeptidase activity that was shown to originate from mast cells (Haas and Heinrich, 1979, Haas, Heinrich and Sasse, 1979). Further evidence for a mast cell carboxypeptidase came in the following year, when a carboxypeptidase was purified from rat peritoneal cells (Everitt and Neurath, 1980). Further studies on rat, human and mouse mast cell carboxypeptidases showed that this enzyme resides in the mast cell granules in all of these species (Goldstein *et al.*, 1987, Serafin *et al.*, 1987, Cole *et al.*, 1991, Irani *et al.*, 1991). The enzyme was found to be very similar to bovine pancreatic carboxypeptidases. It has a molecular weight of approximately 35 kD and resembles bovine carboxypeptidase B (CPB) in structure and amino acid composition, although its enzymatic properties are more similar to bovine carboxypeptidase A (CPA). The enzyme was thus named mast cell CPA (Goldstein *et al.*, 1989).

Structure and activation

In 1989, murine and human mast cell CPA were cloned, and it was shown that they are highly homologous (Reynolds *et al.*, 1989a, Reynolds *et al.*, 1989b). A few years later rat mast cell CPA was also cloned and was found to be similar to its mouse and human counterparts (Cole *et al.*, 1991). In bovine pancreatic CPA and CPB, the amino acid at position 255 is crucial for positioning substrate amino acids for cleavage. Because mouse, rat and human mast cell CPA have the hydrophobic leucine at this site, they would be expected to more closely resemble bovine CPA (which has an isoleucine) than CPB (which has an aspartic acid) in its substrate specificity, in agreement with the enzymatic properties previously reported for mast cell CPA.

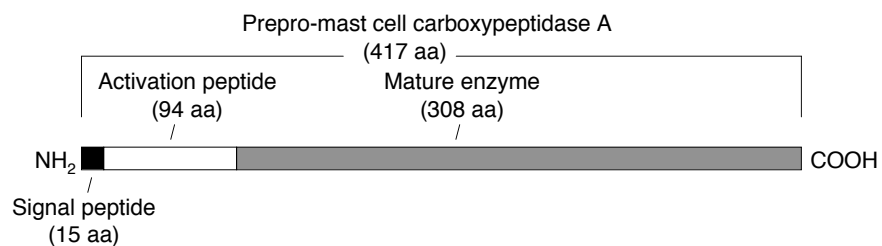


Figure 4. Schematic structure of mast cell carboxypeptidase A.

The prepro-mast cell CPA in both mouse and human is 417 amino acids long and consists of a 15 amino acid N-terminal signal peptide and a 94 amino acid activation peptide (Reynolds *et al.*, 1989a, Reynolds *et al.*, 1989b) (Figure 4). The unusually long activation peptide is common for all CPAs and it assumes a compact globular conformation (Aviles *et al.*, 1982). Molecular modelling of pro-CPA predicts the globular propeptide domain to physically block access to the substrate-binding pocket and the loop connecting the propeptide with the mature enzyme to be highly exposed (Springman, Dikov and Serafin, 1995). Furthermore, the model predicts that the active site of mast cell proCPA is fully formed in the zymogens, but substrate binding is blocked by the propeptide. To activate CPA the activation peptide must be cleaved between Glu⁹⁴ -of the activation peptide and Ile¹ (the first residue of the mature enzyme) and degraded. As discussed above, CPA is stored in the mast cell granules as a mature enzyme, and the location of pro-CPA to mast cell granules indicates that the processing occurs inside the mast cell granules (Dikov *et al.*, 1994, Springman, Dikov and Serafin, 1995, Rath-Wolfson, 2001). Studies using protease inhibitors have implicated cysteine-protease activity in the process (Springman, Dikov and Serafin, 1995), however, the precise mechanism behind the activation has not been investigated.

Localization to the proteoglycan-protein macromolecular complex

Mast cell CPA has a highly positive net charge and has an isoelectric point of more than 9 (Schwartz *et al.*, 1982). This positive net charge makes CPA binding to the highly negative heparin PGs heparin very probable. Indeed, several studies have indicated that CPA is located to the macromolecular PG-protein complex in mast cells (Schwartz *et al.*, 1982, Serafin *et al.*, 1987), to which other mast cell proteases have also been localized (Serafin *et al.*, 1986). Both CPA and chymase remain in this complex even after degranulation and the presence of such complexes may allow both enzymes to remain in close proximity after exocytosis, thereby inclining the likelihood of their cooperation.

Substrates and biological functions

Based on inhibition by *O*-phenanthroline, EDTA and 8-hydroxyquinoline mast cell CPA is a zinc-metallopeptidase, containing a tightly bound zinc atom directly involved in catalysis (Everitt and Neurath, 1980, Goldstein *et al.*, 1987). Further, it is an exopeptidase that catalyzes the hydrolysis of peptide bonds at the C-terminus of peptides (Vendrell, Querol and Aviles, 2000). Both rodent and human mast cell CPA are functionally optimal between pH 7.5 and 9.5 and have a preference for cleavage of substrates that possess carboxy--terminal aliphatic or aromatic amino acids (Everitt and Neurath, 1980, Goldstein *et al.*, 1987). The neutral to basic pH optimum indicates that mast cell CPA cleaves substrates in the extracellular milieu rather than inside the acidic mast cell granules where it is stored.

A number of synthetic dipeptides with carboxyl-terminal aromatic residues, e.g. Z-Ala-Phe, and the His₉-Leu₁₀ bond of angiotensin I have been shown to be good

in vitro substrates for mast cell CPA (Everitt and Neurath, 1980, Goldstein et al., 1987), but little is still known about its biological substrates. It has been suggested that mast cell CPA might catalyze the conversion of the highly potent broncho- and vasoconstrictor LTC₄ into its less potent form, LTF₄, and thereby represent a cellular homeostatic mechanism for eliminating the highly reactive lipid mediators produced by mast cells (Reddanna et al., 2003). Another proposed function of mast cell CPA is the degradation of apolipoprotein B of low density lipoprotein (LDL) (Kokkonen, Vartiainen and Kovanen, 1986). *In vitro* studies using isolated rat peritoneal mast cells together with CPA inhibitors and chymase inhibitors suggested a model where chymase and CPA act in sequence, in which chymase first cleaves peptides from the apolipoprotein B of LDL and CPA subsequently liberates amino acids from the peptides formed. Because chymase is an endoprotease that acts on the carboxyl side of amino acids with aromatic side chains, and CPA is an exoprotease that removes C-terminal amino acids with aromatic side chains, this cooperation seems very likely. The presence of both of these proteases in the PG-protein macromolecular complex further supports this theory. Further support is provided by the finding that pancreatic pro-CPA forms complexes with pancreatic serine proteases (Oppezzo *et al.*, 1994).

Another more recent study also suggests concerted action between mast cell CPA and chymase. Investigations concerning the involvement of mast cells in the degradation of the vasoactive peptide, endothelin-1 (ET-1) indicated that CPA and chymase were involved in this process (Metsarinne et al., 2002). The use of selective inhibitors as well as purified enzymes and synthetic ET-1 in an *in vitro* system showed that chymase and CPA are capable of degrading ET-1. This could represent an important role for CPA *in vivo* in limiting inflammatory reactions by degradation of ET-1. Yet another study indicates the cooperation between mast cell CPA and chymase. Using the knock outs of the heparin-modifying enzyme, NDST-2 and the mouse chymase mMCP-4 together with CPA-inhibitors, it was shown that CPA cooperates with chymase to form as well as degrade angiotensin II (Lundequist et al., 2004). In this study, an *ex vivo* system was used and a model for formation and degradation of angiotensin II was proposed where CPA and chymase closely cooperate (Figure 5).

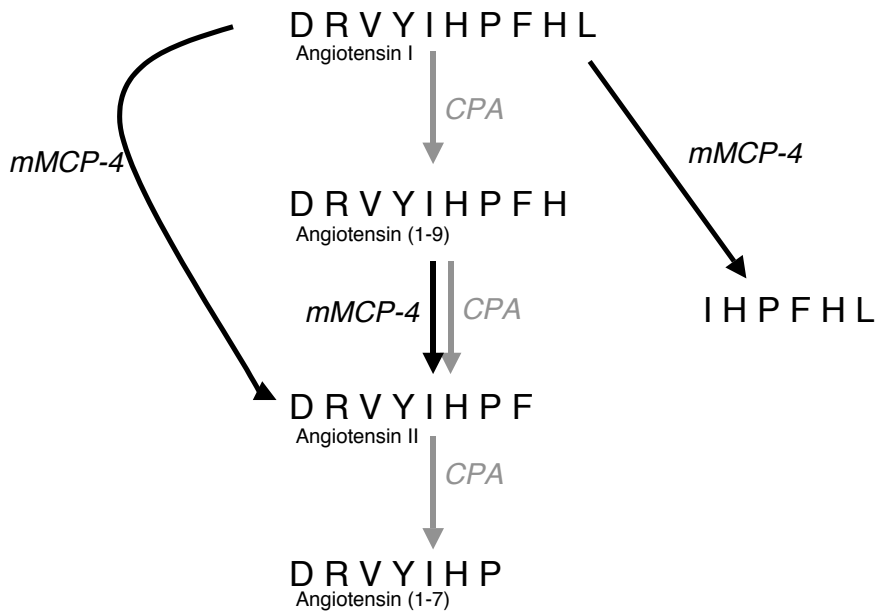


Figure 5. Scheme for the processing of Angiotensin I by CPA and mMCP-4 (adapted from Lundquist *et al.*, 2004)

CPA knockout

These studies all support the theory that mast cell CPA and chymase work in concert to degrade different substrates. However, the true biological substrate for mast cell CPA remains to be discovered. A step in this direction is the newly generated knockout of mast cell CPA (Feyerabend *et al.*, 2005). These mice appear normal and healthy and are born at the expected Mendelian ratio, indicating that CPA is not essential for embryonic development. The peritoneal and skin mast cells appear in normal numbers and they are morphologically normal. Furthermore, passive cutaneous anaphylaxis is not affected by CPA-deficiency, indicating that CPA is not required for immediate mast cell responses.

Histochemical studies showed that the CPA-deficient mast cells seemed immature. Their staining properties resemble those of BMMCs, generally considered as immature mast cells, indicating that CPA is important for maturation of mast cells. However, heparin synthesis is unaffected and there are normal levels of the tryptase mMCP-6 as well as the chymase mMCP-4. Interestingly, mast cells from CPA-deficient mice totally lack the chymase mMCP-5, even though the mRNA levels are unaffected. This further supports the theory that CPA and chymase work in concert, and indicate that they are dependent on each other also for storage inside the mast cell granule. The same pattern has been found in the knock out of the chymase, mMCP-5, where CPA protein is absent from the mast cell granules (Stevens *et al.*, 1996).

This knock out of mast cell CPA is an important tool to help elucidate the biological role of mast cell CPA. However, the lack of mMCP-5 in the CPA knockout is an important consideration when performing experiments on this knockout.

Biological functions: are mast cells “Good Guys” or “Bad Guys”?

Mast cells have mainly been known for their involvement in anaphylactic conditions such as allergy and asthma where they are thought of as “The Bad Guys”. However, mast cells have been described in lower orders of animals (e.g. insects and fishes) (Hakanson et al., 1986) and this persistence through evolution suggests a beneficial and important role for mast cells, in which they can be seen as “The Good Guys”.

Mast cells in host defence – “Good Guys”

Mast cells reside in locations such as the gastrointestinal tract, the skin and nerve endings, where they line up as a first “defence-barrier”. It is not surprising, therefore, that mast cells are important for host defence against certain parasites and bacteria. Infection by helminth parasites is accompanied by eosinophilia, the production of high levels of IgE and a mast cell hyperplasia in the mucosa of the small intestine. Studies using the mast cell-deficient mouse strain WBB6F₁/J-*Kit^W-Kit^{W-v}* (*W/W^v*) which lack fully functional *c-kit* (the receptor for SCF) (Kitamura, Go and Hatanaka, 1978), have shown a critical effector function for mast cells in immunity to the parasitic helminth, *Trichinella spiralis* (Ha, Reed and Crowle, 1983, Alizadeh and Murrell, 1984). Furthermore, using a strain lacking the MMC-specific protease, mMCP-1, it was demonstrated that this β -chymase is involved in the expulsion of the parasite (Knight et al., 2000). Investigating *W/W^v* mice has also revealed a role for mast cells in the expulsion of *Nippostrongylus brasiliensis* (Crowle and Reed, 1981) as well as *Strongyloides venezuelensis* (Khan et al., 1993). In the case of *S. venezuelensis*, the CS PG secreted from MMCs appears to play an important role in parasite expulsion (Maruyama et al., 2000). However, mast cells do not appear to be absolutely necessary for the parasite expulsion in all cases. Rather, the lack of mast cells appears to delay expulsion, indicating that other factors are involved in the defence against parasites. The role of mast cells in protozoan infections is less well described, but the involvement of mast cells in *Giardia lamblia* (Li et al., 2004) as well as *Giardia muris* (Erlich et al., 1983) infections has been described.

The binding of different bacterial components to TLRs on mast cells activates the mast cells and stimulates a response to several different bacteria. Using mast cell-deficient *W/W^v* mice, the importance of mast cells in bacterial infections has been demonstrated (Malaviya et al., 1996). In a model of acute septic peritonitis (cecal ligation and puncture) a critical protective role of mast cells and mast cell-derived TNF- α was demonstrated (Echtenacher, Mannel and Hultner, 1996). TNF-

α enhances neutrophil recruitment during bacterial infection (Malaviya et al., 1996). Moreover, other mast cell mediators such as LTB_4 (Malaviya and Abraham, 2000), tryptase (Huang et al., 2001), MIP-1 α , MIP-1 β , MIP-2 and IL-8 also appear to contribute to the influx of neutrophils to sites of bacterial infection (Krishnaswamy et al., 2001). In addition to initiating site-specific inflammation through the recruitment of neutrophils, mast cells can themselves phagocytose bacteria (Malaviya et al., 1994, Arock et al., 1998), although this is probably not their main role in bacterial clearance.

In addition to host defence, mast cells have been shown to be involved in tissue remodelling activities, for example wound healing. Histamine released from mast cells increases fibroblast migration and proliferation (Kupietzky and Levi-Schaffer, 1996), and histamine, as well as other cytokines and growth factors released from mast cells, can initiate or modulate angiogenesis, another important process in wound healing (Ribatti *et al.*, 2004). However, this angiogenic function of mast cells may also be important in different cancer forms, where a growing tumour is dependent on vascularisation, providing an example of mast cells as “Bad Guys.”

Mast cells in disease – “Bad Guys”

Tumour vascularisation in cancer is one example where mast cells acts as “Bad Guys”, however, asthma and allergy are the classical “mast cell-diseases”. Because of their location in the airway at an interface between the internal and external environment, the mast cells are positioned to respond to exogenous airborne antigens against which they have been sensitized with specific IgE. However, the mast cell can also respond directly to nonimmunologic triggers as described in an earlier section. The preformed granule components that are released upon stimulation contribute to increased vascular permeability and recruitment of inflammatory leukocytes as part of both innate and acquired immunity. Mast cell-derived mediators and cytokines also contribute to the chronic inflammation of the asthmatic response. The specific roles of the different mast cell mediators are reviewed in earlier sections of this thesis.

Lately, mast cells have also been shown to play an important role in several other diseases, most of them autoimmune diseases. Studies on multiple sclerosis (MS) and its animal model, experimental allergic encephalomyelitis (EAE) were among the first in this field. MS is a chronic inflammatory disorder that leads to demyelination, and a similar disorder can be induced in rodents by injecting different myelin components. The involvement of mast cells in these diseases has long been suggested (reviewed in Zappulla *et al.*, 2002), and firm proof came in 2000 when it was shown that W/W^u mice develop EAE later and less severely than control mice (Secor et al., 2000). Complementation of W/W^u mice with wild type immature mast cells derived *in vitro* restores typical EAE susceptibility.

A potential role for mast cells in rheumatoid arthritis (RA) has also been highlighted recently. RA is a chronic joint disease characterized by leukocyte invasion and synoviocyte activation followed by cartilage and bone destruction.

There are several different animal models for this disease and it was shown that mice lacking mast cells are resistant to the onset of arthritis in some of these models (Malfait et al., 1999, Lee et al., 2002). Furthermore, mast cells accumulate in the swollen paws of mice suffering from collagen-induced arthritis, and they degranulate during the disease process (Malfait et al., 1999). Mast cells also accumulate in the synovial tissues and fluids of humans suffering from RA, further indicating an important role for mast cells in this disease (Crisp et al., 1984).

Bullous pemphigoid (BP) is an autoimmune skin disease that is characterized by subepidermal blisters resulting from autoantibodies against two hemidesmosomal antigens, BP230 and BP180. A mouse model of this disease involves the injection of antibodies against BP180 (Liu et al., 1993). Injection of these antibodies into *W/W^v* mice did not induce BP, but mice reconstituted intradermally with mast cells derived *in vitro* showed typical features of disease. Furthermore, it was concluded that the crucial role of mast cells in murine BP is to recruit neutrophils to the developing lesion. A similar process might also occur in the human disease, because degranulated mast cells are a prominent feature of the skin blister of individuals affected with BP (Wintroub et al., 1978).

These are a few examples of physiological and pathological conditions in which mast cells have been shown to participate. However, considering the large amount of potent mediators that are released upon activation, combined with the ability of the mast cell to migrate to different tissues, it is likely that mast cells are involved in several other processes where inflammation and recruitment of other cells are key features. Investigations into the mast cell mediators will hopefully help reveal the biological functions of mast cells.

Present investigations

Aim

The general aim of this thesis was to investigate how heparin PGs affect mast cell proteases and their storage and activation. Particular attention was paid to mast cell CPA, a mast cell protease that has not gained as much attention as chymase and tryptase. Bone marrow derived mast cells (BMMCs) were used as a model system. Granular composition and formation, as well as protease storage was investigated in cells lacking heparin, either due to a mutation in the gene coding for NDST-2 (NDST-2^{-/-}) or in the gene coding for the PG core protein SG (SG^{-/-}). Furthermore, the heparin-dependent activation of mast cell CPA was investigated.

Results and discussion

Paper I: Altered storage of proteases in mice lacking heparin

Heparin-deficient mice, generated by gene targeting of the heparin biosynthesis enzyme, NDST-2, display severe mast cell defects, including an absence of stored mast cell proteases (Forsberg et al., 1999, Humphries et al., 1999). However, the mechanism behind these observations is not clear. In this study, we investigated the importance of heparin in mouse bone marrow-derived mast cells (BMMCs). As it is difficult to obtain large numbers of differentiated mast cells from live animals, a good alternative source is the culture of bone marrow stem cells grown in the presence of appropriate cytokines (IL-3 and stem cell factor), which give rise to a form of immature mast cells

In agreement with previous reports (Humphries *et al.*, 1999), NDST-2^{+/+} BMMCs grown in the presence of IL-3 were shown to contain several mast cell proteases, including mMCP-5, -6 and CPA. In contrast, the IL-3-driven BMMCs from NDST-2^{-/-} animals showed similar levels of mMCP-6 as in wild type cells, but a complete lack of mMCP-5 and mature CPA. Further investigations showed that the wild type BMMCs contain a significant amount of heparin (10% of the total GAG chains), in addition to CS, whereas the NDST-2^{-/-} BMMCs contain only highly sulphated CS. Although this finding conflicts with previous studies (Levi-Schaffer *et al.*, 1986, Dayton *et al.*, 1988), it is consistent with our results, since NDST-2 inactivation only affects heparin biosynthesis and not CS.

Interestingly, the pro-form, but not the active form of CPA could be detected in the heparin-deficient cells. This indicates that CPA is actually translated in the NDST-2^{-/-} cells but is dependent on heparin for subsequent processing events. Thus it seems that heparin, but not CS, is important for the processing of CPA, although the mechanisms behind this are unclear. One possibility is that the processing protease requires heparin for optimal activity against pro-CPA. The fact that both mature CPA and mMCP-5 are missing from the NDST-2^{-/-} BMMCs suggests that these two proteases are interdependent. As previous studies have suggested firstly that both these proteins are necessary for degradation of substrate

and secondly, that they co-localise to the PG-protein macromolecular complex, it is very possible that they are mutually dependent on one another for their storage in mast cell granules.

In conclusion, this study using BMMCs derived from normal and NDST-2^{-/-} mice, shows that the presence of heparin PG is necessary for storage of mMCP-5 and activation of CPA, whereas mMCP-6 is stored and processed also when only CS PG is present.

Paper II: Regulation of carboxypeptidase A and mMCP-5

The enzyme responsible for processing of pro-CPA into the mature enzyme is unknown. However, it has been proposed that cleavage of the 94-residue activation peptide takes place inside mast cell secretory granules (Dikov *et al.*, 1994, Springman, Dikov and Serafin, 1995, Rath-Wolfson, 2001). As it has been suggested that cysteine protease activity is responsible for the process (Springman, Dikov and Serafin, 1995), the influence of two particular cysteine proteases, cathepsins C and S, on the mast cell proteases was investigated here.

Cathepsin S is a cysteine protease with endopeptidase activity which is thought to play an important role in antigen presentation (Riese *et al.*, 1996). In a previous study, it was shown that the cysteine protease cathepsin C (also called dipeptidylpeptidase I, DPPI) is expressed by mast cells (Wolters, Laig-Webster and Caughey, 2000) and it has also been demonstrated that cathepsin C is responsible for the removal of the two-amino acid activation peptide of mast cell chymases (Wolters *et al.*, 2001). However, as the role of specific cathepsins in regulating CPA has not been investigated, this issue was investigated here. As in paper I, BMMCs were used as a model system. BMMCs were obtained from cathepsin C^{-/-}, cathepsin S^{-/-} and corresponding ^{+/+} mice. In mature BMMCs, cellular extracts were assayed for CPA activities. The CPA-like activities as assessed by the substrate, M-2245, were markedly increased in both cathepsin knockouts compared to wild type cells. Western blot analysis revealed that the levels of active, processed CPA were markedly higher in both the cathepsin knockouts, and closely corresponded to the activity measurements. A similar increase was seen in protein levels of the chymase mMCP-5, whereas the tryptase mMCP-6 was unaffected by the knockouts. However, in the cathepsin C^{-/-} BMMCs, mMCP-5 was only seen in its proform, in agreement with previous findings (Wolters *et al.*, 2001). The mRNA levels were not affected by any of the knockouts. The reasons behind the increase in CPA and mMCP-5 levels in the cathepsin C- and S-deficient BMMCs are not clear. However, one may speculate that cathepsin C or S is involved in CPA and mMCP-5 turnover or that cathepsin C or S activate proteases that are involved in CPA and mMCP-5 degradation. These data also lend further support to the hypothesis that CPA and mMCP-5 are physically associated, and where an increase or decrease in either of the proteases may lead to an equivalent change in the corresponding binding partner.

Taken together, the results from this study indicate that neither cathepsin C nor S are involved in the processing of CPA, but rather that the loss of either causes an increase in the levels of CPA and mMCP-5 according to an unknown mechanism.

Paper III: A role for cathepsin E in the processing of mast cell CPA

The enzyme responsible for CPA activation was investigated in this study. We took advantage of existing knockout models in which various cathepsins were targeted. Previous studies using protease inhibitors have suggested that a cysteine protease is involved in mast cell CPA activation (Springman, Dikov and Serafin, 1995). The cysteine protease family includes several lysosomal cathepsins, widely distributed across many species (Turk, Turk and Turk, 2000). In addition to cysteine proteases, the cathepsin family of proteases includes two aspartic proteases, cathepsins D and E. The cathepsins require a slightly acidic environment for optimal activity, a condition that is prevalent in lysosomes but also in other cellular compartments such as the secretory granule of mast cells.

In this study, we assessed the role of two specific cysteine proteases, cathepsins B and L, in the processing of pro-CPA, but neither of these cysteine proteases appeared to have any significant role. Together with the results from the investigation reported in paper II, we were thus unable to provide any further evidence of a major role for cysteine proteases in the processing of pro-CPA. Importantly, however, we cannot exclude an involvement of other cysteine proteases. We instead present evidence that the aspartic protease, cathepsin E, is involved in the processing of pro-CPA. This conclusion is supported both by *in vivo* experiments demonstrating that pro-CPA processing is defective in mice lacking cathepsin E and by *in vitro* experiments in which purified cathepsin E was found to process recombinant pro-CPA. Interestingly, the knockout of another aspartic protease, cathepsin D, did not affect pro-CPA processing, indicating that the ability to process pro-CPA is not a general function of aspartic proteases, but rather a specific property of cathepsin E. However, the lack of cathepsin E did not completely inhibit pro-CPA processing, indicating the involvement of another protease, possibly a cysteine protease, in the process.

Furthermore, this study revealed for the first time the presence of immunoreactive cathepsin E in the secretory granules of mast cells. The granular location of cathepsin E was also strongly supported by the lack of staining for cathepsin E in peritoneal cells lacking heparin due to the lack of the enzyme, NDST-2, which was shown in paper I to lead to defective processing of pro-CPA. Furthermore, using affinity chromatography, we showed that cathepsin E is a high-affinity heparin-binding protein and that the interaction is pH dependent, indicating that histidine residues are involved. Indeed, the model of mouse cathepsin E identified five surface-exposed histidine residues that might be interesting candidates for heparin binding. The association of cathepsin E with heparin in the mast cell granules may bring cathepsin E and CPA (which is also

located to the heparin-complex) into close contact, thus promoting the proteolytic action of cathepsin E on pro-CPA.

To summarize, the present study strongly suggests a role for cathepsin E in processing pro-CPA and that this process occurs in the mast cell granules where cathepsin E is located in complex with heparin.

Paper IV: Role of serglycin in secretory granule assembly

Mast cell granule release is an important component of mast cell-dependent inflammation and other pathological conditions. It is therefore imperative to understand the mechanisms that lead to granular release as well as to define those that lead to the assembly of secretory granules. Indeed, the processes involved in mast cell degranulation, in particular the signal transduction pathways, have been the subject of intense investigation (Blank and Rivera, 2004). In contrast, strikingly little is known about the actual formation of mast cell secretory granules.

This issue was investigated using BMMCs from mice lacking the PG core protein, SG ($SG^{-/-}$) and its wild type counterpart ($SG^{+/+}$). $SG^{-/-}$ BMMCs, which are essentially devoid of all PGs, as well as $SG^{+/+}$ cells, were analyzed at various stages of differentiation for morphology as well as for content, expression, processing and cellular location of the granule components. The morphological characteristics were examined after staining with May-Grünwald/Giemsa. Young cultures of both genotypes displayed cells containing “empty” (May-Grünwald/Giemsa negative) vesicles. On further culture, these vesicles disappeared in both genotypes while May-Grünwald/Giemsa positive cells appeared in cultures of $SG^{+/+}$ cells, but not $SG^{-/-}$ cells. These findings suggest that the formation of secretory vesicles is initiated independently of SG, but that metachromatically stained mature granules are only formed in the presence of SG. Ultrastructural investigations using transmission electron microscopy revealed the existence of secretory granules in similar numbers in both genotypes. However, the morphology of the granules was different; whereas the $SG^{+/+}$ granules contained defined electron-dense core structures the contents of the $SG^{-/-}$ granules were of more amorphous character, with no defined electron dense core. The most plausible interpretation of these morphological findings is that SG PG binds to and concentrates secretory granule compounds. However, the granules from $SG^{-/-}$ cells were still fully capable of undergoing degranulation.

Furthermore, this study indicated that the knockout of SG does not affect cellular differentiation into mast cells in terms of expression of mast cell markers at mRNA levels. However, when the protein levels of mast cell proteases were investigated, it was found that mMCP-5 was not detected at any time point in $SG^{-/-}$ cells and that the levels of mMCP-6 were clearly reduced. Total CPA antigen was present in approximately equal amounts in both $SG^{-/-}$ and $SG^{+/+}$ cells, but only the pro-form was detected in $SG^{-/-}$ cells, as expected in the light of its dependence on heparin for its activation (as showed in paper I and III). The

proteases could still be released upon degranulation, supporting their location in secretory granules even in the absence of SG. We also demonstrated that histamine and β -hexosaminidase are released to the same extent by BMMCs of both genotypes. Together, these data clearly indicate that the intracellular transport of the studied compounds into secretory granules occurs independently of SG PG. Furthermore, our results suggest that mMCP-5 is targeted to degradation if not retained in the granules by SG PG whereas mMCP-6 and possibly CPA seem to be secreted from the cells in the absence of SG PG.

Taken together, our results are consistent with a sorting-by-retention-model of secretory granule maturation in which SG PG is essential for retention of certain granule constituents, including selected proteases and histamine, but is not essential for retention of others, such as β -hexosaminidase.

Concluding remarks and perspectives

Mast cells are important players in different inflammatory conditions as well as in host defence against bacteria and parasites. A prominent feature of mast cells is their densely packed secretory granules, from which a number of potent mediators are released upon stimulation. Among these mediators are the mast cell proteases that constitute up to 35 % of the granular content. However, the biological roles of these proteases are still largely unknown, and it is therefore difficult to understand precisely how they contribute to mast cell function. More knowledge of the proteases, their properties, how they are stored in the mast cell granules as well as identification of their biological substrates should help us improve the treatment of mast cell-related conditions as well as improve our understanding of the beneficial roles of mast cells.

The investigations reported in this thesis have mainly focused on the mast cell protease, CPA, its heparin-dependent activation and storage in mast cell granules as well as co-operation with the chymase, mMCP-5. Furthermore, the influence of a number of cathepsins on mast cell proteases has been investigated as well as the mechanisms for granule formation in mast cells. We have presented evidence for a heparin-dependent activation of pro-CPA and further showed that this activation is performed by the aspartic protease cathepsin E. The finding that cathepsin E is localized in the secretory granules of mast cells in a complex with heparin is new and it may be expected that additional functions will be ascribed to cathepsin E activity in mast cells. The existence of mice devoid of cathepsin E offers great possibilities for further investigations on the involvement of this aspartic protease in mast cell-related conditions.

Even though the studies included in this thesis have clarified a number of issues concerning mast cell CPA, the full range of its biological functions are still unknown. From the studies presented here and elsewhere it is likely that CPA acts in concert with mast cell chymase, and possibly also other mast cell proteases. It is possible that more substrates will be found that are open to cleavage by the cooperative action of this enzyme pair. However, the most successful way to elucidate the role of mast cell CPA in physiological and pathological conditions will probably be to use the newly constructed CPA knockout mouse strain. Using this strain in mouse models for different diseases will be a very powerful tool, although it should be taken into consideration that this knockout strain also lacks mMCP-5. Even though previous research has mainly focused on the roles of mast cell chymase and tryptase, it is very likely that CPA will be proven to play an important role in mast cell-related conditions. More knowledge of the structure, activation mechanisms and biological functions of mast cell CPA may well increase our opportunities to treat the many conditions in which mast cells are involved.

Populärvetenskaplig sammanfattning

Varje dag stöter vår kropp på en stor mängd bakterier, virus och andra sjukdomsalstrande partiklar. Vår hud och våra slemhinnor fungerar som en första barriär, men många sjukdomsalstrande partiklar kommer ändå in i vår kropp. Dock är det en väldigt liten del av dessa som faktiskt gör oss sjuka. Detta har vi vårt immunförsvar att tacka för. Immunförsvaret består av en rad olika celler som på olika sätt oskadliggör de bakterier och virus som kommit in i vår kropp. En av dessa celler är mastcellen. Mastceller är en typ av immunologiska celler med en mängd funktioner i vårt försvar mot bakterier och parasiter. Förutom att hjälpa till att försvara oss mot sjukdomsalstrande partiklar är mastceller även involverade i olika typer av sjukdomstillstånd. Främst tänker man här på astma och olika typer av allergier, men även i andra autoimmuna sjukdomar som reumatism och multipel skleros (ms) har det visat sig att mastceller är involverade.

Det som gör mastcellerna så speciella är dess sekretoriska granula (små korn inuti cellen) som innehåller en stor mängd inflammatoriska mediatorer (typ av molekyler). Vid mastcellsaktivering (tex vid parasitangrepp eller en allergisk reaktion) släpper dessa granula sitt innehåll ut i omgivningen där de inflammatoriska mediatorerna kan utföra sina olika uppgifter. Det finns en stor mängd olika mediatorer i mastcellsgranula, varav den mest kända är histamin. Histamin är involverat i allergiska reaktioner där det ger klassiska allergiska symptom såsom nysningar och kliande. Andra mastcellsmediatorer är cytokiner som fungerar som signalmolekyler för andra celler; de signalerar till dessa celler att det är dags att komma till platsen för tex ett parasitangrepp eller allergisk reaktion för att hjälpa till. I mastcellsgranula finns dessutom proteaser och proteoglykaner, vilka har studerats närmare i denna avhandling. En proteoglykan är en molekyl som består av en proteinkärna med sockerkedjor på sig. I mastceller finns det två olika typer av proteoglykaner; heparin och kondrotinsulfat. Det som skiljer dessa åt är deras sockerkedjor, som är något mer negativt laddade i heparin jämfört med kondrotinsulfat. Dessa proteoglykaner har en mängd olika roller i mastcellen. Bland annat hjälper de till att lagra övriga mastcellsmediatorer, som ofta har en positiv laddning och därmed binder till de negativt laddade sockerkedjorna på proteoglykanerna. I den här avhandlingen har förhållandet mellan proteoglykaner och några andra mastcellsmediatorer, främst proteaser undersökts.

Proteaser är en typ av molekyler som kan klippa sönder andra molekyler i kroppen. Mastcellsgranula innehåller tre huvudtyper av proteaser: tryptaser, kymaser och karboxypeptidas A (CPA). Dessa proteaser har olika klyvningsspecificitet, dvs de kan klippa i olika typer av molekyler. Genom att klippa i olika molekyler kan proteaserna hjälpa till att aktivera dem samt bryta ner vävnader i kroppen där det kan behövas. I den här avhandlingen har vi främst studerat proteaset CPA, som det tidigare inte har funnits så mycket kunskap om.

Genom att använda oss av s.k. knockout-teknik har vi studerat hur mastcell-CPA interagerar med bland annat heparin, hur CPA aktiveras samt hur mastcellsgranula bildas. Knockout-teknik går ut på att man förstör genen för en

viss molekyl, och därmed får möss som saknar den speciella molekylen, s.k. knockout-möss. I den första studien (artikel I) i denna avhandling använde vi oss av knockout-möss som saknar heparin. Från dessa odlade vi fram mastceller och studerade hur mastcellsproteaserna uppför sig i frånvaro av heparin. Vi visar att vissa av proteaserna uppför sig helt normalt även utan heparin (men med kondrotinsulfat) medan mastcells-CPA inte kan aktiveras utan heparin. Denna aktivering sker genom att ett annat proteas klipper bort en bit av det inaktiva CPA, så att det får sin rätta form och kan klippa andra molekyler efter utsläppning ur mastcellsgranula.

Vidare visar vi (artikel III) att denna aktivering av CPA sker av ett annat proteas som heter cathepsin E. Däremot har två andra närbesläktade proteaser, cathepsin C och cathepsin S ingen inverkan på CPA-aktiveringen (artikel II). Snarare gör frånvaron av dessa proteaser (pga knockout) att nivåerna av CPA ökar, något som kan bero på att dessa proteaser vanligtvis bryter ned CPA. Vidare visar vi att cathepsin E finns i mastcellsgranula där det är starkt bundet till heparin, vilket även CPA är. Detta gör troligtvis att cathepsin E kan komma nära CPA och att CPA-aktiveringen mha cathepsin E sker innuti mastcellsgranula.

I avhandlingens sista studie (artikel IV) använde vi oss av en knockout för mastcellsproteoglykanernas kärnprotein, serglycin. När denna molekyl inte finns har mastcellerna i princip inga proteoglykaner överhuvudtaget. Detta leder till att mastcellsgranula är svårt deformerade eftersom proteoglykanerna behövs för lagring samt aktivering av flera av de andra mastcellsmediatorerna. Med hjälp av denna serglycin-knockout undersökte vi hur frånvaron av proteoglykaner påverkar bildningen av mastcellsgranula. Vi visar här att trots att granula i mastceller som saknar proteoglykaner inte kan ses med vanliga färgningsmetoder ändå finns, då de kan ses klart och tydligt mha elektronmikroskopi. Dock ser de inte likadana ut som granula från normala mastceller, och de har mycket låga nivåer av de olika mastcellsproteaserna. När det gäller CPA kunde vi bara se inaktivt CPA, vilket stämmer bra med våra tidigare observationer där vi visat att aktivering av CPA är beroende av heparin. För att ta reda på vad som händer med proteaserna i frånvaro av proteoglykaner undersökte vi några olika möjliga scenarion. Våra studier visar att några av mastcellsproteaserna utsöndras från cellen i frånvaro av proteoglykan, medan andra däremot bryts ned någon av cellens ”avfallskvarnar”, där oönskade molekyler förstörs. Sammantaget visar denna studie att funktionella mastcellsgranula kan bildas utan proteoglykaner, men att dess innehåll inte kan hållas kvar på ett korrekt sätt.

Eftersom mastceller är viktiga både i vårt försvar mot sjukdomsalstrande partiklar samt i olika typer av sjukdomar så är det mycket viktigt att vi vet så mycket som möjligt om dessa celler. Mastcellens främsta kännetecken är dess granula som utsöndrar en rad olika mastcellsmediatorer. I den här avhandlingen har vi studerat hur några av dessa mediatorer, proteoglykaner och proteaser, samarbetar och fungerar, samt undersökt hur mastcellsgranula bildas. Detta är viktig information som kan leda till att vi når en högre förståelse om mastceller och därmed kan förstå hur man tex bättre kan behandla astma och andra

mastcellsberoende sjukdomar. Det finns mycket kvar att göra innan vi helt förstår mastcellen, men med denna avhandling har vi tagit ännu ett steg i rätt riktning.

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