The Role of Heparin in the Activation of Mast Cell Tryptase

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Abstract

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Mast cells play an important role in our immune defense against bacteria and parasites but are also key effector cells in various inflammatory diseases. They act by releasing inflammatory mediators from intracellular granules. Tryptase, one of the most abundant mast cell proteases, is stored in its active form and may therefore act immediately after mast cell degranulation. In this thesis, the activation mechanism of mast cell tryptase has been addressed. Further, the interaction between heparin and tryptase has been thoroughly investigated.

We found that the mouse tryptase, mMCP-6, is critically dependent on heparin and acidic pH for its activation. The critical role of heparin for tryptase activation indicated that displacement of heparin might inactivate tryptase. Indeed, we proved that heparin antagonists, protamine and Polybrene, were potent inhibitors of mMCP-6 and purified human lung tryptase. A closer study of the structural requirements of heparin revealed that its capacity to activate tryptase is dependent on size and high anionic charge density. Further, these studies led to a novel finding in the demonstration of an active tryptase monomer.

The dependence of mMCP-6 activation on acidic pH suggested that histidines were involved in heparin binding. Site-directed mutagenesis of four selected histidines (H35, H106, H108 and H238) demonstrated that H106, positioned closest to the interface, contributed most to heparin binding, indicating that this region may be particularly important. Generally, the single mutants displayed subtle defects compared to when several mutations were combined, which produced large defects in activation, tetramerization and heparin binding. The heparin-induced activation of human β-tryptase was dependent on the size and high anionic charge density of the activator and closely resembled the structural requirements of mMCP-6 for its interaction with heparin. Altogether, we showed that the mechanism for activation of human β-tryptase was very similar to that of mMCP-6. This indicates that the mouse system is a highly relevant model for the analysis of the biological role of tryptase in human mast cell-related diseases.

Keywords: mast cell mediator, serine protease, carbohydrate-protein interactions, oligomerization, inflammation, allergy.

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To Mum and Dad

Success consists of going from failure to failure without the loss of enthusiasm
-Winston Churchill

Contents

Appendix

Papers I-V

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

I. Hallgren J., Karlson U., Poorafshar M., Hellman L., and Pejler G.
”Mechanism for activation of mouse mast cell tryptase: Dependence on heparin and acidic pH for formation of active tetramers of mouse mast cell protease 6.”
Biochemistry. (2000) 39:13068-77.

II. Hallgren J., Estrada S., Karlson U., Alving K., and Pejler G.
”Heparin antagonists are potent inhibitors of mast cell tryptase.”
Biochemistry. (2001) 40:7342-9.

III. Hallgren J., Spillman D., and Pejler G.
”Structural requirements and mechanism for heparin-induced activation of a recombinant mouse mast cell tryptase, mouse mast cell protease-6.”
J. Biol. Chem. (2001) 276:42774-81.

IV. Hallgren J., Bäckström S., Estrada S., Thuvesson M., and Pejler G.
“Histidines are critical for heparin-dependent activation of mast cell tryptase.”
J. Immunol. (2004) 173:1868-75.

V. Hallgren J., Lindahl S., and Pejler G.
“Structural requirements and mechanism for heparin-dependent activation and tetramerization of human βI- and βII-tryptase”
J. Mol. Biol. (2004). In press.

Abbreviations

APCs	Antigen presenting cells
BMMC	Bone marrow derived mast cell
CGRP	Calcitonin gene related peptide
CPA	Carboxy peptidase A
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate proteoglycan
DPPI	Dipeptidyl peptidase
ECM	Extracellular matrix
Heparin PG	Heparin proteoglycan
Ig	Immunoglobulin
IFN-γ	Interferon-γ
LTs	Leukotrienes
LPS	Lipopolysaccharide
mMCP	Mouse mast cell protease
MC	Mast cell
MC_T	Mast cell type containing only tryptase
MC_{TC}	Mast cell type containing tryptase and chymase
MCP-1	Monocyte chemoattractant peptide
MIP-1α	Macrophage inflammatory protein
MMP	Matrix metallo protease
PAR-2	Proteinase activated receptor -2
PCA	Passive cutaneous anaphylaxis
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
VIP	Vasoactive intestinal peptide

INTRODUCTION

General overview

The immune system protects us from a variety of microbes ranging from viruses and bacteria to parasites. The first line of defense is our skin, which protects us from most potentially dangerous organisms. If a microbe succeeds in entering the body, an immune response is necessary. The innate immune response (or non-adaptive), predominant in early immune responses, is a non-specific way of eliminating pathogens. If the innate immune system fails to eliminate the pathogen, adaptive immunity takes over. This response is highly specific towards one particular pathogen and after repeated encounters, the immune response improves further- a memory of how to respond is created.

Mast cells (MCs) are cells of the immune system that are responsible for attracting phagocytes and lymphocytes to a site of infection. Moreover, MCs have a role in the initiation of adaptive immune responses and play an active role in defense against certain pathogens. MCs and basophils are commonly referred to as granulocytes. Basophils are cells of the immune system that share some functions with MCs. Importantly, they share a common distinctive feature: their cytoplasms are filled with granules packed with inflammatory mediators. They differ, however, in that basophils circulate in the blood while MCs reside in mucosal areas and in connective tissues. Although MCs are mostly beneficial by participating in our defense against for example bacteria and parasites, under certain circumstances they can cause considerable damage. Allergies, asthma and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis are MC-mediated diseases that may be initiated if the immune system is activated due to false recognition of either endogenous molecules or harmless exogenous substances as threats. Irrespective of whether the role of the MC is beneficial or harmful to the host, MCs are activated through different pathways, degranulate and release inflammatory mediators, which cause the physiological effect. One of the inflammatory mediators is an enzyme, which due to its trypsin-like activity is named tryptase.

In the present study, we have characterized tryptase in terms of its mechanism of activation and its interaction with heparin proteoglycan (heparin PG), another MC mediator stored in the granules. We have mainly focused on the mouse tryptase, mouse MC protease -6 (mMCP-6), but we have also investigated the corresponding human β-tryptase. An understanding of the fundamental biochemical events leading to activation may be crucial in the fight against MC-related diseases where tryptase is involved.

Mast cells

Knowledge of MCs has greatly increased in recent years. However, they were first described by Ehrlich in the late 19th century. Using aniline dyes, he saw how certain cells were filled with granules. Ehrlich called them “mastzellen”, meaning well fed cells (Ehrlich, 1878).

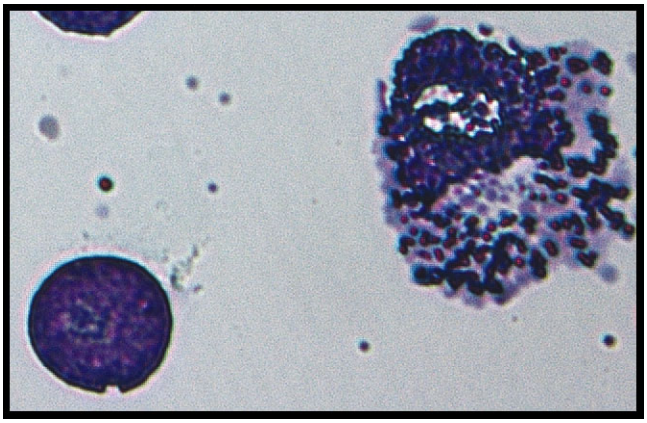

Figure 1. An intact MC and a degranulating MC stained with May-Grünwald Giemsa. The negatively charged proteoglycans bind the dye and make the MC granules densely colored.

Subtypes and heterogeneity

MCs originate in the bone marrow but mature in peripheral tissues (Galli, 1993). Circulating human MC precursors are defined as being $CD34^{+}$, c-$kit^{+}$and $CD13^{+}$ cells (Kirshenbaum *et al.*, 1999). Mouse MC precursors are poorly granulated and are defined as Thy-1^{lo} and c-kit^{hi} cells (Rodewald *et al.*, 1996). Intestinal mucosa in adult mice have been found to constitute a peripheral pool of precursor MCs (Guy-Grand *et al.*, 1984). Mature MCs are distributed throughout the body, often located strategically in tissues that interface the outside world. Different types of MCs arise due to the influence of different microenvironments in various tissues. In mice, two types of mature MCs have been described based on location and granule content. The connective tissue type MC resides in connective tissues in the skin and peritoneum, whereas the mucosal type is typically found in the gastrointestinal mucosa. The connective tissue type MCs contain heparin proteoglycan (heparin PG), high amounts of histamine and, in addition, the proteases tryptase, chymase and carboxypeptidase A (CPA). In contrast, mucosal MCs contain chondroitin sulfate proteoglycans (CSPG) and other types of chymases but lack tryptase and CPA. Human MCs are classified according to their granule contents. MC_Ts contain only tryptase and mostly resemble mucosal MCs in their distribution pattern whereas MC_{TC}s contain tryptase, chymase and CPA and predominate in skin (Metcalfe, Baram & Mekori, 1997; Miller & Pemberton, 2002; Schwartz, 1994a).

Table 1. MC heterogeneity in mouse and human.

	Mouse		Human	
	Connective tissue type	**Mucosal type**	**MC_T**	**MC_{TC}**
Proteoglycan	Heparin	Chondroitin sulfate	Heparin, Chondroitin sulfate	Heparin, Chondroitin sulfate
Tryptase	mMCP-6, mMCP-7		+	+
Chymase	mMCP-4, mMCP-5*	mMCP-1, mMCP-2	-	+
CPA	+	-	-	+

*Recent findings have suggested that mMCP-5 has elastase-like substrate specificity (Karlson *et al.*, 2003; Kunori *et al.*, 2002).

Mechanisms of mast cell activation

IgE-mediated activation

The classical route of MC activation is through the adaptive immune response via antibodies that bind to receptors on the MC surface. This is how MCs act both in our immune defense towards parasites and in mediating hypersensitivity reactions such as allergies and asthma. The response is initiated when an antigen e.g. a pollen or a parasite enters the body. Firstly, parts of the antigen are taken up and degraded by antigen presenting cells (APCs). These cells present antigenic peptides on special cell-surface molecules referred to as MHC (major histocompatibility complex) class II. In the presence of T_{H2} cytokines, the APCs interact with $CD4^+$ T cells and thereby induce them to proliferate. The newly formed T_{H2} cells interact with B cells, which proliferate into plasma cells and secrete specific antibodies of the immunoglobulin E (IgE) isotype. The MCs become sensitized when IgE molecules bind to the high affinity FcεR1 receptors on the MC membrane. Upon a second encounter, the antigen can bind directly to the IgE-FcεR1 receptor complex. The binding of a multivalent antigen induces cross-linking of the FcεRI receptor, which, via a signaling cascade involving tyrosine phosphorylation and Ca^{2+} influx, causes MC degranulation. Besides the release of pre-formed mediators, MC activation also induces production of *de novo* synthesized lipid mediators and various cytokines that are released within hours of activation.

IgE-independent mechanisms

Besides the common IgE-dependent mechanism there are several other pathways that lead to MC activation. IgG antibodies can mediate MC activation through low affinity IgG receptors. Mouse MCs express two isoforms of IgG receptors, FcγRIIb and FcγRIII, while human MCs express the two isoforms, FcγRI and FcγRII (Tkaczyk *et al.*, 2004). Stimulation of FcγRI and FcγRIII induce MC degranulation. However, simultaneous ligand binding of FcγRII and FcεRI result in down-regulation of the degranulation initiated by FcεRI aggregation (Daeron & Vivier, 1999). The biological significance of IgG receptors was demonstrated by

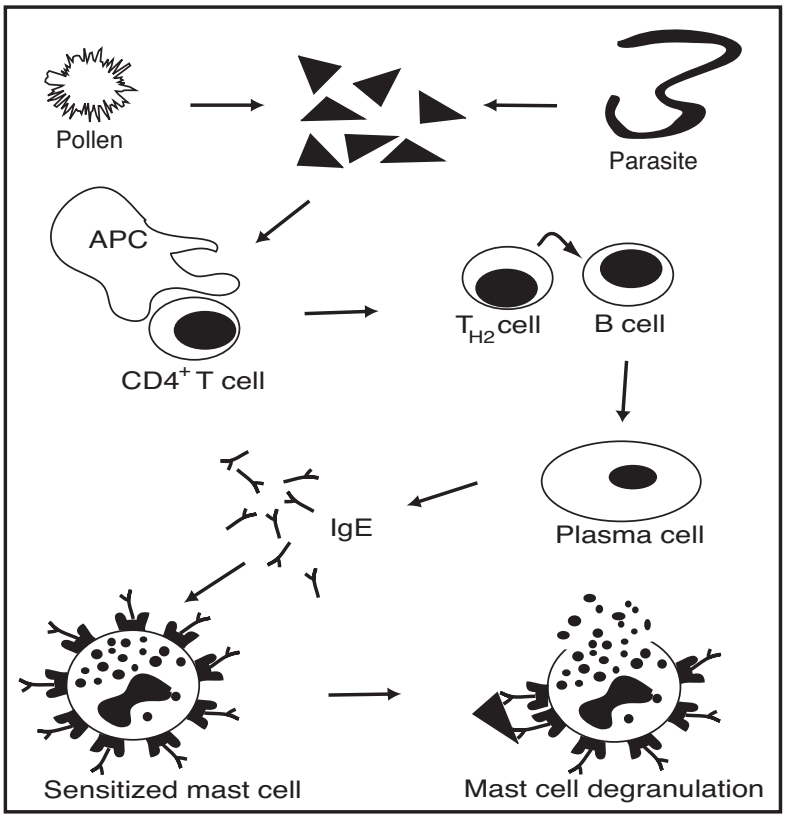

Figure 2. Mechanism for IgE-mediated MC activation.

$IgE^{-/-}$ mice that despite a complete lack of IgE, show an anaphylactic reaction in response to sensitization and allergen challenge (Oettgen *et al.*, 1994).

MCs can be activated in a direct fashion in several other ways. For example, recent studies have demonstrated MC activation by Toll-like receptors (TLRs). The family of TLRs comprises cell-surface molecules that directly recognize different pathogens. TLRs were first found in drosophila and TLR4 was identified as the first mammalian TLR (Medzhitov, Preston-Hurlburt & Janeway, 1997). MCs become activated when TLRs on the MC surface bind to pathogens. MCs express TLR2, 4, 6, and 8 (Takeda, Kaisho & Akira, 2003). The identification of the responsible gene of two mouse strains that failed to respond to lipopolysaccharide (LPS) demonstrated that TLR4 recognizes LPS (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). These results were later verified in a $TLR4^{-/-}$ strain (Hoshino *et al.*, 1999). Bacteria-derived lipopeptides, peptidoglycan and the yeast cell wall component, zymosan, are potent activators of TLR2 (Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999; Means *et al.*, 1999; Schwandner *et al.*, 1999). However, TLR2 seems to require cooperation of other TLR family members e.g. TLR1 and TLR6 for ligand recognition. Accordingly, heterodimers of TLR2/TLR6 are suggested to mediate responses to peptidoglycan and zymosan (Ozinsky *et al.*, 2000). The natural activator(s) of TLR8 remain unknown.

Complement factors such as C3a and C5a have long been known to induce MC activation and were subsequently referred to as anaphylatoxins (Johnson, Hugli & Muller-Eberhard, 1975). However, mucosal MCs do not express receptors for C3a and C5a and fail to respond to complement factors (Mousli *et al.*, 1994). In a model of acute septic peritonitis, complement-mediated MC activation was demonstrated to be crucial for bacterial clearance *in vivo* (Echtenacher, Mannel & Hultner, 1996; Prodeus *et al.*, 1997). Different cytokines and chemokines, e.g. MIP-1α (macrophage inflammatory protein-1α) and MCP-1 (monocyte chemoattractant peptide-1), can also directly cause MC activation (Alam *et al.*, 1994). Moreover, MCs can be activated by cell-cell contact with activated T cells (Baram *et al.*, 2001). This cell-cell contact is mediated at least partly by ICAM-1 (intercellular adhesion molecule-1) and its ligand LFA-1 (leukocyte function-associated antigen-1) (Inamura *et al.*, 1998). Furthermore, the co-localization of MCs with nerve terminals, the ability of neuropeptides to stimulate MC activation and evidence that MC tryptase stimulates release of neuropeptides from neurons has suggested a neurogenic control of MC activation (Bauer & Razin, 2000; Steinhoff *et al.*, 2000). The MC activating neuropeptides include substance P, CGRP (calcitonin gene related peptide), VIP (vasoactive intestinal peptide) and neurotensin (Church *et al.*, 1989). In addition, it has been known for a long time that MC degranulation can be induced by various basic compounds such as compound 48/80, which has been extensively used as a research tool (Metcalfe, Baram & Mekori, 1997).

Function

For many years, MCs were considered effector cells of anaphylactic reactions. Recent evidence, however, suggests that MCs may also have a significant beneficial role.

Role of Mast Cells in the immune response

Host defense against parasites

Parasites such as nematodes, which colonize the gastrointestinal tract, are highly prevalent in the human population, particularly in tropical and sub-tropical areas of the world. MC mastocytosis i.e. MC accumulation and proliferation, can be triggered by nematode infection and is accompanied by eosinophilia and IgE production (Love, Ogilvie & Mclaren, 1976; Maizels & Holland, 1998; Negrao-Correa, 2001). These hallmarks of nematode infection are regulated by cytokines derived from T_{H2} cells. T_{H2} cytokines include IL-4, IL-5, IL-9, IL-10 and IL-13 and promote growth and differentiation of MCs and eosinophils as well as promoting B-cells to produce Ig-E antibodies (Abbas, Murphy & Sher, 1996). MC-dependent immune responses to parasites have been demonstrated by the use of IL-$3^{-/-}$ mice, which lack mature MCs. These mice show delayed expulsion of *Strongyloides venezuelensis* (Lantz *et al.*, 1998). Further, another study identified the mucosal MC-specific chymase, mMCP-1, to be involved in immune responses to parasites. mMCP-$1^{-/-}$ mice show delayed expulsion of *Trichinella spiralis* compared to wild type mice (Knight *et al.*, 2000). Many studies have shown the importance of IgE

in the response towards gastrointestinal nematode infection (Negrao-Correa, 2001). Recently, $IgE^{-/-}$ mice were used to demonstrate that IgE regulates MC responses to *Trichinella Spiralis*. Interestingly, these mice have delayed worm expulsion in combination with markedly diminished MC numbers and reduced serum levels of mMCP-1 (Gurish *et al.*, 2004).

Host defense against bacterial infections

MCs have a critical role in host defenses against certain bacteria. This was demonstrated *in vivo* using MC-deficient mice (Kit^{W}/Kit^{W-v}), which showed impaired clearance and survival to enterobacterial infections compared to wild type or MC-reconstituted (Kit^{W}/Kit^{W-v}) mice (Malaviya *et al.*, 1996), as well as in a model of acute septic peritonitis (Echtenacher, Mannel & Hultner, 1996). The key MC mediator that initiates the host response to bacterial infection is thought to be preformed TNF-α (Tumor necrosis factor-α), which recruits neutrophils to the site of infection. However, MC tryptase can also induce recruitment of neutrophils (Huang *et al.*, 2001; Huang *et al.*, 1998). Furthermore, MCs have an important role in the adaptive immune response to bacteria through MC-derived TNF-α that goes to the lymph nodes and induces recruitment of circulating T cells (Mclachlan *et al.*, 2003). MCs also act directly in the immune defense through their capacity to phagocytose and eliminate bacteria (Malaviya *et al.*, 1994; Sher *et al.*, 1979).

Role of mast cells in diseases

MCs contribute to the pathology of many diseases. It has been known for a long time that MCs play a key role in inflammatory conditions such as asthma and allergies. However, knowledge of MC involvement in other severe diseases has emerged. Lately, MCs have been demonstrated to play a role in autoimmune diseases such as multiple sclerosis and they have also been proposed to participate in some types of cancers.

Allergies and asthma

Inflammatory conditions such as asthma and allergies are typically divided into three effector phases: the early response or acute reaction that occurs within minutes of allergen exposure, the late phase reaction that occurs within a few hours of allergen exposure, and chronic allergic inflammation that is ongoing for days or years. MCs are considered the primary cells responsible for acute allergic reactions such as type I hypersensitivity reactions. For example, MC involvement was shown in a mouse model of passive cutaneous anaphylaxis (PCA). In this study, MC-deficient (Kit^{W}/Kit^{W-v}) mice were unable to express detectable PCA reactions (Wershil *et al.*, 1987). The MC mediators, histamine and leukotrienes are thought to play a part because both antihistamines and antagonists to leukotrienes block the early reaction (Roquet *et al.*, 1997). There are conflicting data on the significance of the role that MCs play in late phase reactions and chronic inflammatory conditions. For example, when MC-deficient mice are sensitized with ovalbumin (OVA) together with adjuvant they become “asthmatic”, however when the same mice are sensitized with OVA without adjuvant they remain

healthy (Williams & Galli, 2000). These results suggest that MCs may have the key role or a non-essential role depending on the asthma model chosen.

Many different MC mediators may contribute to inflammatory conditions. Histamine stimulates smooth muscle contraction and increases vascular permeability but also increases mucus secretion in the lower airway (Hart, 2001). Leukotrienes and prostaglandins mediate bronchoconstriction and vasodilatation. In a mouse model of cutaneous late phase reactions, MCs were responsible for essentially all the leukocyte infiltration after challenge with IgE and specific antigen. TNF-α clearly is important to these reactions because approximately 50% of the leukocyte infiltration was blocked using a neutralizing antibody to recombinant TNF-α (Wershil *et al.*, 1991). Other cytokines that MCs secrete, such as IL-4, IL-5 and IL-13 participate in the inflammatory response (Brightling *et al.*, 2003). In addition, MC tryptase has been demonstrated to contribute to the late phase reaction in atopic asthmatics (Krishna *et al.*, 2001).

Autoimmune diseases

Recent studies have indicated that MCs are important for the onset of several autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA). In a mouse model of MS named experimental allergic encephalomyelitis (EAE), MC-deficient (Kit^{W}/Kit^{W-v}) mice were shown to have significantly reduced symptoms of disease compared to wild type mice (Secor *et al.*, 2000). Subsequently, using MC-deficient mice reconstituted with $Fc\gamma^{-/-}$, $Fc\gamma RIII^{-/-}$ or $Fc\gamma RIIB^{-/-}$ bone marrow derived MCs (BMMCs) it was demonstrated that the activating Fc receptors (FcεRI and FcγRIII) and the inhibitory receptor FcγRIIB regulate EAE (Robbie-Ryan *et al.*, 2003). Other indirect evidence has also suggested MC involvement in MS. For example, MCs accumulate in sites of demyelination in the brain and spinal cord (Ibrahim *et al.*, 1996) and myelin can be degraded by MC proteases (Johnson, Seeldrayers & Weiner, 1988). Increased tryptase levels are found in the cerebrospinal fluid of MS patients (Rozniecki *et al.*, 1995). Further, analysis of MS lesions by microarray techniques showed a high contribution of transcripts derived from MCs including genes for tryptase, the TNF receptor and the high affinity IgE receptor (Lock *et al.*, 2002).

Lately, the importance of MCs in the pathology of RA was demonstrated using two strains of MC-deficient mice ($Kitl^{Sl}$/ $Kitl^{Sl-d}$ and Kit^{W}/Kit^{W-v}). In this study, mice were injected with serum from the K/BxN mice, which caused wild type mice to develop symptoms similar to RA. However, the MC deficient mice were rescued from disease (Lee *et al.*, 2002a). In patients suffering from RA, MCs have been shown to accumulate in synovial tissues and fluid in response to a number of MC chemoattractants e.g. SCF (stem cell factor) and TGF-β (transforming growth factor -β) (Olsson, Ulfgren & Nilsson, 2001) . MCs have also been implicated in other autoimmune diseases such as bullous pemphigoid (Chen *et al.*, 2001) and lupus nephritis (Lin, Gerth & Peng, 2004).

Cancer

There is also some evidence for the involvement of MCs in cancer. MCs accumulate around tumors such as basal-cell carcinoma lesions (Grimbaldeston *et al.*, 2000), invasive melanoma (Reed *et al.*, 1996) and breast cancer (Kankkunen, Harvima & Naukkarinen, 1997). In addition, MC mediators such as histamine, tryptase, heparin and different cytokines/chemokines, particularly VEGF (vascular endothelial growth factor), are implicated as either beneficial to the tumor, or in some cases, detrimental (Theoharides & Conti, 2004).

Inflammatory mediators

MC mediators encompass both preformed mediators stored inside the granules in their active forms and *de novo* synthesized mediators. The preformed mediators include histamine, proteoglycans and proteases whereas leukotrienes and prostaglandins are synthesized upon MC activation. Cytokines may be stored in the granules as well as synthesized upon MC activation.

Leukotrienes and prostaglandins

Leukotrienes (LTs) and prostaglandins (PGs) are lipid mediators derived from arachidonic acid. The LTs include LTA4, LTB4 and the cysteinyl LTs, LTC4, LTD4 and LTE4. However, MCs predominately express cysteinyl LTs. Other cell types such as basophils, eosinophils and macrophages are also important sources of cysteinyl LTs. These act through two G-protein coupled receptors called CysLT1 and CysLT2 (Kanaoka & Boyce, 2004). Originally, the cysteinyl LTs were recognized for their broncho constricting effects (Dahlen *et al.*, 1980) and induction of increased venular permeability (Peck, Piper & Williams, 1981). Recently, a number of additional LT functions have been proposed such as leukocyte recruitment (Medeiros *et al.*, 1999) and migration of dendritic cells (Robbiani *et al.*, 2000). LTs are also suggested to play a role in allergic diseases. This was demonstrated in mice lacking cytosolic PLA2 (phospholipase A2), a key enzyme for the biosynthesis of LTs. $PLA2^{-/-}$ mice showed reduced bronchiolar hyperreactivity after allergen challenge (Uozumi *et al.*, 1997). Further studies have suggested a role for LTs in asthmatic airway remodeling (Henderson *et al.*, 2002) and pulmonary inflammation and fibrosis (Beller *et al.*, 2004; Nagase *et al.*, 2002). Recently, the FLAP (5-lipoxygenase-activating protein) gene, an early enzyme in leukotriene biosynthesis, was identified as the first common gene associated with a greater risk of stroke and heart attack (Helgadottir *et al.*, 2004).

Most cell types express prostaglandins (PGD2, PGE2, PGF2 and PGI2). However, MCs express predominantly PGD2, which can also be produced by macrophages and dendritic cells. PGD2 exerts its effect through two cell surface receptors, DP (prostaglandin receptor D) and CRT_{H2} (chemoattractant receptor-homologous molecule expressed on T_{H2}) (Kabashima & Narumiya, 2003). The importance of PGD2 in inflammatory conditions was shown using $DP^{-/-}$ in a mouse model of asthma (Matsuoka *et al.*, 2000). DP-deficient mice had significantly reduced levels of T_{H2} cytokines such as IL-4, IL-5 and IL-13, and less infiltration of lymphocytes and eosinophils. However, similar serum

concentrations of total and specific IgE were detected, indicating that the primary response was not affected. PGD2 is also associated with other inflammatory diseases such as atopic dermatitis, allergic rhinitis and allergic conjunctivitis.

Cytokines

MCs are a source of many different cytokines. Preformed MC-derived cytokines, e.g. TNF-α, can be stored in the granules. However, most studies indicate up-regulation of cytokine production after MC activation. The MC cytokines include IL-3, IL-4, IL-5, IL-6, IL-10, IL-13 and TNF-α (Hart, 2001). Briefly, IL-3 plays an important role for growth and differentiation of $CD34^+$ progenitor cells into MCs, basophils and dendritic cells (Martinez-Moczygemba & Huston, 2003). Interestingly, IL-3 is needed for protective immunity in mice infected with the nematode, *Stronglyoides venezuelensis*, through the induction of increased numbers of tissue MCs and basophils (Lantz *et al.*, 1998). IL-4 is a well-known mediator of allergic asthma and belongs to the T_{H2} cytokines. The discovery of IL-4 antagonists that prevent the development of allergic reactivity in mice (Grunewald *et al.*, 1998) has stimulated a search for new IL-4 antagonists to be used as a treatment of allergic asthma (Mueller *et al.*, 2002).

IL-5 is a typical T_{H2} cytokine, which stimulates eosinophil production and activation (Martinez-Moczygemba & Huston, 2003). The significant role of IL-5 in asthma was demonstrated in IL-5-deficient mice which were rescued from airway eosinophilia and airway hyper-reactivity after allergen challenge (Foster *et al.*, 1996). IL-6 has a wide range of different biological activities such as its role as a growth factor for T cells and its capacity to induce the differentiation of cytotoxic T cells, macrophages and osteoclasts. Moreover, IL-6 works in synergy with IL-3 to induce proliferation of hematopoetic stem cells (Naka, Nishimoto & Kishimoto, 2002). IL-6 overproduction may be responsible for the clinical symptoms of RA, and antibodies towards IL-6 are currently being evaluated as a new therapeutic strategy (Naka, Nishimoto & Kishimoto, 2002). IL-10 has both pro-inflammatory and anti-inflammatory effects. The pro-inflammatory effects of IL-10 predominate in innate immune reactions whereas the anti-inflammatory effects are features of the adaptive immune response (Mocellin *et al.*, 2003). IL-10 is also a maturation factor for human MC progenitors and posseses anti-tumor properties. IL-13 is a key mediator of allergic asthma (Grunig *et al.*, 1998; Wills-Karp *et al.*, 1998), but also plays a key role in host immunity to gastrointestinal parasites (Mckenzie *et al.*, 1998; Wynn, 2003). TNF-α is a multifunctional cytokine, which mediates key roles in all stages of inflammation, infection and anti-tumor responses (Palladino *et al.*, 2003). For example, mice that overexpress TNF-α develop RA-like symptoms (Douni *et al.*, 1995). Since TNF-α induces other pro-inflammatory cytokines and chemokines it is likely that TNF-α acts both directly and indirectly in the development of RA (Van Den Berg, 2001). In addition, preformed TNF-α is thought to initiate the host response to bacterial infections (Echtenacher, Mannel & Hultner, 1996; Malaviya *et al.*, 1996). Accordingly, anti-TNF-α therapies for a variety of diseases are currently under development (Palladino *et al.*, 2003).

MCs also express a variety of chemotactic cytokines or chemokines such as MCP-1 (monocyte chemoattractant protein -1), RANTES and IL-8 (Ono *et al.*, 2003). MCP-1 and RANTES recruit monocytes/macrophages whereas IL-8 is a neutrophil chemoattractant (Hart, 2001; Mukaida, Harada & Matsushima, 1998). MCP-1 also attracts T cells and RANTES recruits eosinophils.

Histamine

Histamine is one of the most well studied MC mediators. It is stored in the MC granules and is recognized as a central mediator of allergic diseases. Although MCs are the main source, other cell types such as basophils, gastric enterochromafin-like cells, and histaminergic nerves in the brain also produce histamine. Besides these cell types, lymphocytes and monocytes may produce histamine in minute quantities (Macglashan, 2003). The physiological effects of histamine include bronchoconstriction, stimulation of smooth muscle contraction, increased vascular permeability and increased mucus secretion in the lower airway (Bachert, 2002). Histamine mediates its effect through at least four different G-linked receptors (H1-H4). H1 and H2 are widely distributed while H3 expression is restricted to the brain. H4 is found in the intestines and in hematopoetic tissues. Because of the expression of H receptors on almost every cell, the role of histamine at the cellular level is extremely complicated.

Histidine decarboxylase (HDC) catalyzes the synthesis of histamine from the amino acid histidine. A knockout of HDC therefore produces an almost histamine-free mouse although histamine may be taken up from food. MCs from $HDC^{-/-}$ mice have altered morphology and reduced granular content (Ohtsu *et al.*, 2001). Further, IL-3 differentiated BMMC from $HDC^{-/-}$ mice show impaired differentiation compared to those from wild type mice (Wiener *et al.*, 2002). $HDC^{-/-}$ mice have been used in several studies to prove the effect of histamine. For example, in a model of asthma, $HDC^{-/-}$ mice exhibit strongly reduced antigen-induced airway responses as well as reduced eosinophil infiltration and IgE levels (Kozma *et al.*, 2003). However, in this and other studies of different disease models using the HDC knockouts, it is unclear if it is lack of histamine or the presence of a reduced number of MCs, which contain less amounts of other granule constituents, that causes the effect.

Proteoglycans

MCs express two types of proteoglycans, heparin PG and CSPG, in their granules.

Heparin proteoglycan

Heparin PG is exclusively expressed by MCs. However, it closely resembles the broadly expressed heparan sulfate proteoglycan (HSPG). Heparin PG consists of glycosaminoglycans (GAGs) that are linked to the serglycin protein core. The GAG chains consist of repeating disaccharide units of glucuronic acid or iduronic acid and glucosamine. The GAG chain is O-linked to serglycin through a tetrasaccharide linker (Xyl-Gal-Gal-GlcA). A key enzyme in the biosynthetic

pathway leading to the production of the GAG chain is the N-deacetylase/N-sulfotransferase (NDST), which is necessary for subsequent modifications such as C5-epimerization, 2-O-sulfation, 6-O-sulfation and 3-O-sulfation. A heparin disaccharide contains on average 2.7 sulfate groups that give rise to the unusually high negative charge density as well as much of the heterogeneity of the GAG structure (Capila & Linhardt, 2002). There are four isoforms of NDST. NDST-1 is expressed ubiquitously whereas NDST-2 is expressed exclusively in MCs. NDST-3 and -4 are expressed during embryonic development.

Heparin was first discovered in liver extracts on account of its anti-coagulant properties in the beginning of the 20th century (heparin; from hepatic origin). It is primarily known for its use in anti-thrombosis therapy. Later, it was found that the anti-coagulant activity was due to a specific highly sulfated pentasaccharide in the heparin GAGs that binds to antithrombin and thereby induces an allosteric change that increases binding of thrombin and factor Xa (Olson, Bjork & Bock, 2002). As most heparin is located in MC granules and antithrombin is a serum protein, it is likely that HSPGs, which are found at the plasma membrane of endothelial cells lining blood vessels, bind to anti-thrombin *in vivo* (Marcum *et al.*, 1986). A number of other heparin-binding proteins have been described, such as FGFs (fibroblast growth factors), annexins, chemokines and adhesion proteins. For many of these, HSPG is thought to be the endogenous ligand (Capila & Linhardt, 2002).

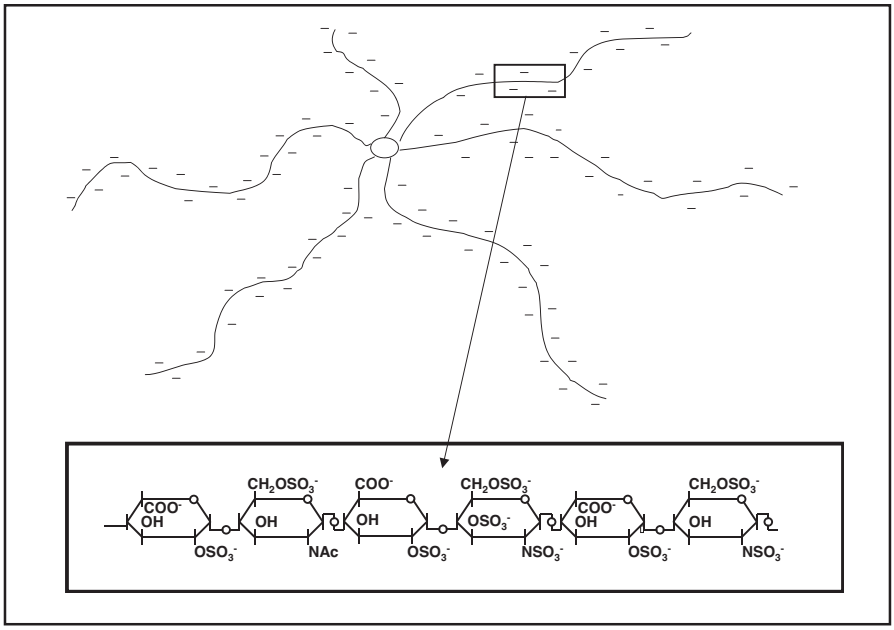

Figure 3. Heparin proteoglycan. The close up shows the structure of the heparin chains.

The main function of heparin PGs in MC granules is to work as a storage scaffold for other MC granule components. This was demonstrated in two separate studies using NDST-2 knockout mice (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). The NDST-$2^{-/-}$ mice showed a drastic reduction in various granule proteases as well as histamine although the mRNA levels were unchanged. Further, morphology of the MCs was distorted, with the cells developing large empty vacuoles. Heparin PG, with its high negative charge, possibly binds the positively charged granule compounds, neutralizes their charge and packs them efficiently in the MC granules. As proteases are stored in MC granules in an active form, packing with heparin PG may also prevent undesired proteolytic cleavage of the granule components. Further, heparin PG may also play a protective role after degranulation. For example, MC chymase remains in complex with heparin PG after degranulation and is protected from plasma protease inhibitors (Pejler & Berg, 1995). Moreover, heparin PG also helps chymase in a more sophisticated way by binding to other heparin-binding proteins, thereby potentiating recruitment of substrate (Pejler & Sadler, 1999). Other granule components, for example histamine, bind to heparin PG in the acidic granule microenvironment (~ pH 5.5) because of the positively charged histidine residues (pKa ~ 6.5). After exocytosis, the higher pH in the extracellular milieu causes deprotonation and dissociation from heparin PG. Furthermore, heparin PG may also be involved in the activation/processing of MC proteases such as CPA (Henningsson *et al.*, 2002) and tryptase (Sakai, Ren & Schwartz, 1996).

Chondroitin sulfate PG

Murine mucosal MCs contain exclusively CSPG whereas human MCs contain both heparin PG and CSPG at a ratio of about 2:1 (Stevens *et al.*, 1988). Chondroitin sulfate (CS) is linked to the same core protein (serglycin) as heparin. Further, CS consists of repeating units of glucuronic acid (GlcUA) and galactosamine (GalNAc) where the GalNAc can be 4-O or 6-O sulfated (Kolset, Prydz & Pejler, 2004).The CS type found in MCs is referred to as CS-E and can be sulfated at both positions. However, CSPG is normally not as negatively charged as heparin PG. It has been demonstrated that CSPG may compensate for the lack of heparin PG under certain circumstances. In fact, BMMCs from NDST-$2^{-/-}$ mice synthesize CSPGs that are as equally negatively charged as heparin PGs (Henningsson *et al.*, 2002).

Proteases

All MC proteases are stored in their active form inside MC granules and have a variably functional relationship to heparin PG. The MC proteases include chymase, CPA and tryptase.

Chymase

Chymases are chymotrypsin-like serine proteases uniquely expressed by MCs. They are further categorized into α- and β-chymases based on structure. In humans, there is only one α-chymase whereas in mice there are five: one α-chymase, mMCP-5 and four β-chymases, mMCP-1, mMCP-2, mMCP-4 and the newly discovered mMCP-9. mMCP-9 is implicated in inflammation of the jejunum during helminth infections and tissue remodeling of the uterus during pregnancy (Friend *et al.*, 2000; Hunt *et al.*, 1997). As mentioned earlier, different MC populations selectively express the different chymases. Thus, mucosal MCs preferentially express mMCP-1 and -2 whereas connective tissue MCs predominantly express mMCP-4 and -5. Recently, it was found that mMCP-5 has elastase-like activity rather than chymotrypsin-like activity (Karlson *et al.*, 2003; Kunori *et al.*, 2002). This implies that the functional homologue of human chymase must be found among the β-chymases. Accordingly, it was demonstrated that the β-chymase, mMCP-4, is responsible for the chymotrypsin-like activity in peritoneum and ear-tissue, whereas human chymase is widely distributed (Tchougounova, Pejler & Abrink, 2003). An important feature of the connective tissue MC chymases is their interactions with heparin PG. In the MC granules, they are stored in complex with each other and even after MC degranulation the chymases remain associated with heparin PG. Outside the MC, heparin PG binds potential substrates for chymase and thereby facilitates the cleavage of these substrates (Pejler & Sadler, 1999). Heparin PG also protects extracellular chymase from protease inhibitors (Pejler & Berg, 1995). However, CSPG-containing mucosal MCs contain chymases that may be constitutively secreted (Brown *et al.*, 2003) and less dependent on negatively charged PGs.

Chymases play an important role in various inflammatory conditions. For example, chymase attracts neutrophils and eosinophils (He & Walls, 1998; Watanabe, Miura & Fukuda, 2002) and may have a role in lung fibrosis (Tomimori *et al.*, 2003). Moreover, chymase has been demonstrated to activate TGF-β, which is a profibrotic cytokine (Lindstedt *et al.*, 2001). Chymases are also involved in atherosclerotic diseases through several different mechanisms. These include inhibition of smooth muscle cell (SMC) mediated collagen synthesis (Leskinen, Kovanen & Lindstedt, 2003), degradation of fibronectin which is necessary for SMC adhesion and survival (Leskinen *et al.*, 2003), activation of MMP-1 (matrix metallo protease –1) and MMP-9, which degrade the collagen matrix (Suzuki *et al.*, 1995), and inactivation of TIMP (tissue inhibitor of metalloprotease) (Frank *et al.*, 2001). Further, chymases (α and β) can convert angiotensin I (AngI) to angiotensin II (AngII), the latter being a peptide with important physiological effects such as vasoconstriction and increased blood pressure, although β chymase may also function in degrading angiotensins (Dell'italia & Husain, 2002). The most important enzyme for AngII formation in

the blood is angiotensin-converting enzyme (ACE). In contrast, chymase may be the predominant AngI converter in tissues (Wei *et al.*, 2002). In a recent study, it was found that mMCP-4 and CPA cooperate in the formation and degradation of AngII (Lundequist *et al.*, 2004). In addition, the mucosal MC type chymase mMCP-1 is involved in defense against gastrointestinal nematode infections (Knight *et al.*, 2000).

Carboxypeptidase A

CPA is a monomeric Zn^{2+}-dependent exoprotease exclusively produced by MCs. CPA is only distantly related to the other MC proteases. However, it is highly similar to pancreatic carboxypeptidases (Reynolds *et al.*, 1989). CPA is stored in the MC granules in complex with heparin PG. Human CPA is found in the class of human MCs denoted MC_{CT} whereas mouse CPA seems to be restricted to the expression by connective tissue type MCs (Irani *et al.*, 1991; Mcneil *et al.*, 1992). CPA is transported into the MC granules with its 94 amino acid long activation peptide attached. In the MC granule, pro-CPA is processed into mature CPA (Rath-Wolfson, 2001). The processing of pro-CPA was demonstrated to be critically dependent on heparin (Henningsson *et al.*, 2002). A recent study has suggested that cathepsin E may process pro-CPA inside the MC granules (F. Henningsson; personal communication). There is also some evidence that mCPA may be physically associated to mMCP-5 in the granules. It was demonstrated that mMCP-$5^{-/-}$ mice cannot store CPA in their granules (Stevens *et al.*, 1996). Accordingly, it was shown that mCPA and mMCP-5 levels were equally increased in cathepsin -C and -S knockout mice (Henningsson *et al.*, 2003). Further, chymase and CPA have been shown to be located in the same macromolecular complex with heparin and are located separately from tryptase in the MC granules (Goldstein *et al.*, 1992). The biological function of CPA has remained largely unknown but recently it was demonstrated that CPA may have a role in extravascular formation of AngII (Lundequist *et al.*, 2004).

Tryptase

In 1960, a trypsin-like activity was found in MCs (Glenner & Cohen, 1960). Since then, much knowledge about MC tryptase has been gathered but little is still known about its true biological function. The predominant form of tryptase is a granular protease stored in its active form and therefore able to act immediately after MC degranulation. Besides the tryptases found in human and mice, several different species such as dog, rat, sheep, cow and gerbil have been shown to produce different types of functional tryptases.

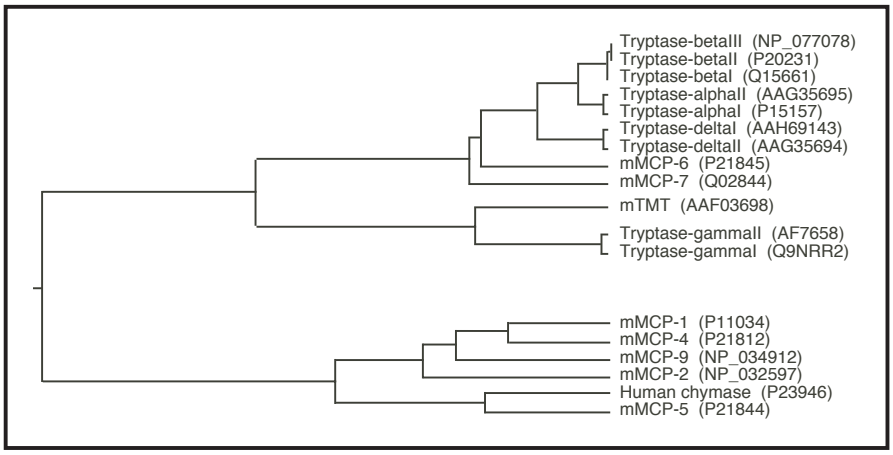

Figure 4. A phylogenetic tree showing the similarity between human and mouse tryptases and chymases. The clustal method with pairwise alignment (DNASTAR) was used to obtain the phylogenetic tree. NCBI protein accession numbers are indicated within brackets.

Human tryptase

Several human MC tryptases are known today. They include α-, β-, δ-, γ-tryptase and TMT (transmembrane tryptase). Tryptase is also expressed by human basophils. However, mean levels of tryptase in basophils are less than 1% of those found in MCs (Jogie-Brahim *et al.*, 2004).

α-tryptase

There are two very similar α-tryptases identified, αI (Miller, Westin & Schwartz, 1989) and αII (Pallaoro *et al.*, 1999). Human α-tryptase was previously considered unable to be processed into its mature form (Sakai, Ren & Schwartz, 1996). In contrast, recombinant α-tryptase was shown to be assembled into an active tetramer, although the activity was extremely low compared to β-tryptase (Huang *et al.*, 1999). Site-directed mutagenesis of Asp216 into Gly, which is the corresponding amino acid in β-tryptase, demonstrated that the difference in activity was partly attributed to this amino acid substitution (Huang *et al.*, 1999). Further, the crystal structure of α-tryptase revealed that the substrate binding region

(Ser214-Gly219) is kinked in the α-tryptase tetramer, which makes substrate binding and processing unproductive (Marquardt *et al.*, 2002). α-tryptase seems to be the predominant form of tryptase in serum under normal conditions (Schwartz *et al.*, 1995). It was suggested that due to the differences in the signal peptide, α-tryptase is continuously secreted rather than directed to the MC granules (Sakai, Ren & Schwartz, 1996). Later, it was found that precursor forms of both α- and β-tryptase are secreted spontaneously (Schwartz *et al.*, 2003). The discovery that the α and βI alleles compete at one locus suggested that there may be individuals with a complete lack of α-tryptase (Caughey, 2002). Surprisingly, α-tryptase deficiency is very common; about 29% of the human population lack α-tryptase (Soto *et al.*, 2002).

β-tryptase

The three β-tryptases identified are almost identical. These are βI, βII and βIII (Miller, Moxley & Schwartz, 1990; Vanderslice *et al.*, 1990). βI and βIII differ from βII in that Asn104 is substituted to a Lys in βII. As a result, βI and βIII are glycosylated whereas βII is unglycosylated at this position. βI and βII differ in only this amino acid. However, βIII is more significantly different from βI and βII in that positions 21-23 consist of RDR in contrast to HGP (Fiorucci & Ascoli, 2004). The β-tryptases preferentially cleave substrates with Lys or Arg in the P1 and P3 positions. For P2 and P4 positions, they have a much broader specificity with some preference for proline (Harris *et al.*, 2001; Huang *et al.*, 2001). Increased β-tryptase can be found in serum during extreme inflammatory conditions such as systemic anaphylaxis (Schwartz *et al.*, 1995).

γ-tryptase

There are two different γ-tryptases, γI and γII (Caughey *et al.*, 2000). In contrast to α- and β- tryptases, γ-tryptases contain an extended hydrophobic C-terminal domain followed by a small cytoplasmic tail, that makes anchoring to plasma membranes possible. Another transmembrane tryptase (TMT) may be identical to γI-tryptase or at least very similar (98-99%) (Wong *et al.*, 2002; Wong *et al.*, 1999). Interestingly, it was demonstrated that TMT migrates to the plasma membrane upon MC degranulation (Wong *et al.*, 2002). When TMT is enzymatically activated it retains its propeptide and forms a disulfide bond linking two TMT chains together. Further, when recombinant TMT is injected into mice trachea, airway hyperresponsiveness (AHR) is induced in combination with increased levels of IL-13 (Wong *et al.*, 2002).

δ-tryptase

Finally, there are two δ-tryptases, δI and δII (Wang *et al.*, 2002). These were previously referred to as mMCP-7-like (I and II), due to homology between their fifth exon and mMCP-7 (Pallaoro *et al.*, 1999). The δI- and δII-tryptases differ in only one amino acid. δ-tryptase contains a premature stop-codon that results in a shorter mature protein that is likely to alter the substrate specificity significantly, although the catalytic triad is intact (Wang *et al.*, 2002). Immunohistochemical

analysis has shown that δ-tryptase is expressed in MCs from tissues such as colon, lung and heart (Wang *et al.*, 2002).

Mouse tryptase

Four murine MC tryptases have been identified to date. These are mMCP-6, mMCP-7, mTMT (mouse transmembrane tryptase) and mMCP-11. All MC tryptases have been localized to mouse chromosome 17A3.3 (Wong *et al.*, 2004).

mMCP-6

mMCP-6 is exclusively expressed in connective tissue type MCs (Reynolds *et al.*, 1990). It is the mouse tryptase that is most closely related to human β-tryptase, with 78% sequence identity. mMCP-6 and -7 are homologous enzymes with 71% sequence identity. Phage-display experiments to define the substrate specificity revealed that mMCP-6 prefers Lys to Arg in the P1 position and has some preference for Pro in the P4 position, closely resembling the substrate specificity of human β-tryptase (Huang *et al.*, 1998). Up to an hour after MC degranulation, mMCP-6 can be found in the adjacent ECM but not in circulation (Ghildyal *et al.*, 1996). This indicates that mMCP-6 exerts its effect locally.

mMCP-7

mMCP-7 was first discovered in early stages of BMMC cultures (Mcneil *et al.*, 1992). Later, expression was found in ear and skin connective tissues of adult mice (Stevens *et al.*, 1994). mMCP-7 was demonstrated to preferentially cleave substrates with Arg in the P1 position and Ser or Thr in the P2 position. Further, mMCP-7 shows an unusually high negative net charge at neutral pH (-10). In contrast to mMCP-6, mMCP-7 can be detected in plasma as early as 20 minutes after MC degranulation, probably due to lack of serglycin proteoglycan-mediated retention (Ghildyal *et al.*, 1996). This may be explained by histidines in mMCP-7 that become neutral extracellularly and no longer mediate heparin PG binding (Matsumoto *et al.*, 1995).

mTMT

Mouse transmembrane tryptase (mTMT), similar to human γI-tryptase/human TMT, was identified by mapping the mouse tryptase locus to chromosome 17 (Wong *et al.*, 1999). mTMT has a C-terminal hydrophobic extension similar to γI-tryptase and probably has similar properties.

mMCP-11

mMCP-11 was recently discovered in BMMCs and in the V3 and C57.1 cell lines (Wong *et al.*, 2004). As the level of mMCP-11 transcripts in BMMCs decrease dramatically after 3 weeks of culture, this protease has long remained unidentified. mMCP-11 has 52% and 54% sequence identity to mMCP-6 and -7, respectively.

Structure and stability

It was early discovered that tryptase is active as a tetramer (Schwartz, Lewis & Austen, 1981). Gel electrophoresis and gelfiltration studies showed that the tryptase tetramer has an apparent molecular mass of approximately 140 kDa, built from four identical subunits of 30-36 kDa. The active tryptase tetramer is stabilized by heparin PG and other polymers with high anionic charge density (Alter *et al.*, 1987; Schwartz & Bradford, 1986). In the absence of heparin, the tryptase tetramer is dissociated into inactive monomers. However, the stability of free tryptase tetramers can be increased at high NaCl concentrations. On the other hand, increasing NaCl concentrations in the presence of heparin-stabilized tryptase has the opposite effect due to dissociation of the tetramer (Alter *et al.*, 1987).

Spontaneous tryptase inactivation was discovered to be associated with structural changes that could be reversed by heparin or dextran sulfate (Schechter *et al.*, 1995). Further, an inactive tetramer intermediate was shown to be re-activable by the addition of heparin (Addington & Johnson, 1996). In a subsequent study, the dissociation of the tetramer was suggested to occur in three steps (Selwood, Mccaslin & Schechter, 1998). The first reversible step involved conformational changes into an inactive destabilized tetramer followed by a second reversible step in which dissociation of the destabilized tetramer occurred. In a third and final slow, irreversible step, the inactive monomers were unable to be reactivated. The same authors later demonstrated that recombinant human βII-tryptase displays stability properties similar to the purified skin tryptase described above (Selwood *et al.*, 2002). In contrast, another study concluded that the dissociation from active tetramer into inactive monomers occurs immediately at the beginning of the inactivation process (Kozik, Potempa & Travis, 1998). In yet another study, it was demonstrated that when dissociation of tryptase into inactive monomers has occurred, addition of heparin at neutral pH failed to reverse the process. However, complete reactivation occurred at acidic pH even without addition of heparin (Ren, Sakai & Schwartz, 1998). It should be noted that almost all of these studies were performed with purified lung or skin tryptase. The occurrence of several different tryptases and the possibility of heterotypic formation of tetramers may explain some of the discrepancies between the investigations (Huang *et al.*, 2000; Pallaoro *et al.*, 1999).

Several attempts have been made to predict the tetramer structure. One model, based on a crystal structure of bovine trypsin (~ 40% identity to β-tryptase) suggested that a group of conserved tryptophans and a proline-rich region could be responsible for tetramer formation and it was speculated that 10-13 histidines on the model surface might be involved in heparin-binding (Johnson & Barton, 1992). In 1998, the crystal structure of human βII-tryptase revealed a fascinating tetramer structure were the monomer units are positioned at the corners of a flat rectangular frame (Pereira *et al.*, 1998). Each monomer has its active site facing a continuous pore in the middle of the tetramer. Access to the wider central cavity is limited due to a loop that projects from each of the monomers. The monomer units have two different interfaces with its neighbors, one consisting of hydrophobic and polar interactions and the other with only hydrophobic interactions. The unique tetramer structure can explain earlier observations such as

the inability of endogenous protease inhibitors to inhibit tryptase and the relatively limited number of protein substrates (Alter *et al.*, 1990).

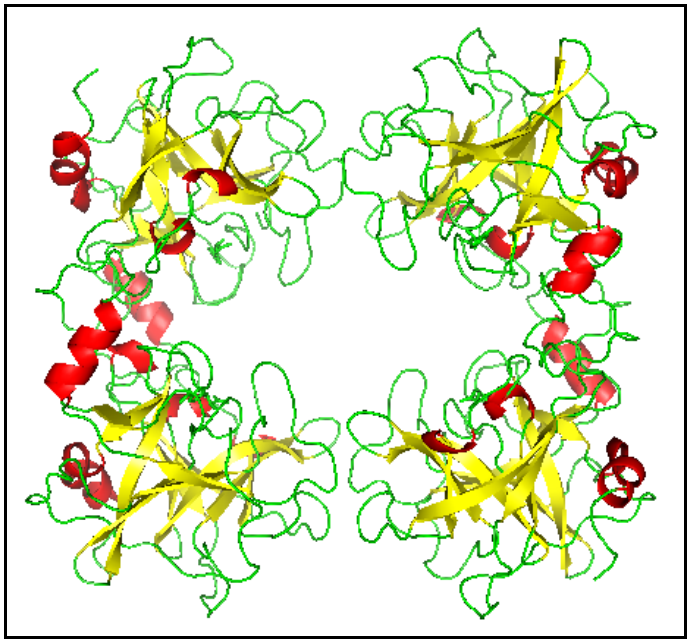

Figure 5. The structure of human β-tryptase. Adapted from Sommerhoff *et al*. (Pereira *et al.*, 1998).

Besides the active tryptase tetramer, the existence of an active tryptase monomer has been suggested (Addington & Johnson, 1996). An active tryptase monomer would explain the observations that tryptase cleaves large substrates that cannot fit into the small central pore of the tetramer. Recently, the formation of an active β-tryptase monomer has been verified (Fajardo & Pejler, 2003a; Fukuoka & Schwartz, 2004). The first study demonstrated that active monomers could be obtained from human β-tryptase tetramers. It was shown that this process occurred at neutral pH and low heparin concentrations at body temperature, suggesting that active monomers are formed *in vivo* after MC degranulation (Fajardo & Pejler, 2003a). In the second study, the formation of active β-tryptase from inactive monomers was demonstrated to occur at acidic pH in the presence of heparin, suggesting an intermediate step in the formation of active tetramers (Fukuoka & Schwartz, 2004). Accordingly, the active β-tryptase monomers represent short-lived states that may occur both inside the MC granule before tetramer formation and extracellularly before inactivation.

Biological function

Tryptase is the most abundant mediator stored in MC granules and plays a pivotal role in several inflammatory conditions.

Proinflammatory properties

Tryptase levels in bronchiolar lavage fluid increase with clinical conditions such as anaphylaxis and bronchial asthma (Jarjour *et al.*, 1991; Schwartz, 1994b). Further, tryptase injected into mouse peritoneum or the tracheas of the lung induces neutrophil infiltration in mouse (He, Peng & Walls, 1997; Huang *et al.*, 1998) and human (Huang *et al.*, 2001). The proinflammatory properties of tryptase may be explained by its role in regulating endothelial cell proliferation by inducing IL-1 and IL-8 mRNA expression and selective IL-8 release (Compton *et al.*, 1998) that can promote neutrophil accumulation (Smart & Casale, 1994). Moreover, inhalation of tryptase causes bronchoconstriction in sheep (Molinari *et al.*, 1996).

Biological substrates

Tryptase has been suggested to be involved in a variety of biological processes through cleavage of different substrates. Fibrinogen was one of the first recognized tryptase substrates, suggesting anticoagulant activity (Schwartz *et al.*, 1985). It has also been demonstrated that tryptase increases vascular permeability through activation of prekallikrin and production of bradykinin from kininogens (Imamura *et al.*, 1996). In addition, tryptase may have a role in artherosclerosis by degrading HDL (high density lipoprotein) and thereby hindering the removal of cholesterol by HDLs (Lee *et al.*, 2002b). Other studies describe the ability of tryptase to degrade neuropeptides such as VIP (vasoactive intestinal peptide) (Caughey *et al.*, 1988), PHM (peptide histidine-methionine) and CGRP (calcitonine gene-related peptide) (Tam & Caughey, 1990). The degradation of these mediators of bronchodilation may lead to increased bronchial responsiveness and contribute to the involvement of tryptase in asthma. Another important feature of asthma is subepithelial fibrosis. Tryptase may contribute to this process through its role as a mitogen for smooth muscle cells and lung fibroblasts. The mechanism behind this effect is debatable; one study shows that the mitogenic effects of tryptase are via non-proteolytic actions, because irreversible inhibition of the activity of tryptase did not abolish the mitogenic effect (Brown *et al.*, 2002). Others have suggested that the ability of tryptase to cleave and activate proteinase activated receptor-2 (PAR-2) is responsible for the mitogenic effect (Akers *et al.*, 2000; Berger *et al.*, 2001).

PAR-2 belongs to a family of four G-protein coupled receptors (PAR-(1-4)) involved in cell signaling. PAR activation occurs when a part of its extracellular N-terminal is cleaved, exposing a new N-terminal that auto-activates the receptor (Dery *et al.*, 1998; Schmidlin & Bunnett, 2001). PAR-2 activation may have a broncho-protective role in the lungs, mediated by epithelial trypsin (Cocks *et al.*, 1999). However, most reports demonstrate a pro-inflammatory response after PAR-2 activation. For example, tryptase was demonstrated to induce inflammation by activation of PAR-2 on sensory nerves, which, upon stimulation,

release proinflammatory neuropeptides such as CGRP and substance P (Steinhoff *et al.*, 2000). The localization of PAR-2 on MCs suggests an amplification of MC degranulation (D'andrea, Rogahn & Andrade-Gordon, 2000). Moreover, neuropeptides can also stimulate MC degranulation (Church *et al.*, 1989). Another indication of the role of PAR-2 in inflammation is that LPS and pro-inflammatory cytokines, including IL-1 and TNF-α, upregulate PAR-2 mRNA (Nystedt, Ramakrishnan & Sundelin, 1996). Further, PAR-$2^{-/-}$ mice demonstrate a delayed onset of inflammation with a defect in P-selectin-mediated leukocyte rolling (Lindner *et al.*, 2000). Another study using $PAR2^{-/-}$ mice demonstrates that stimulation of PAR-2 contributes to allergic inflammation of the airways by mediating hyperreactivity and infiltration of eosinophils (Schmidlin *et al.*, 2002). Furthermore, increased tryptase levels are found in inflammatory bowel diseases such as Crohn's disease (He, 2004). Accordingly, PAR-2 activation was found to induce intestinal inflammation, as indicated by granulocyte infiltration, thickening of the bowel wall and tissue damage (Cenac *et al.*, 2002). Lately, PAR-2 activation by tryptase has been suggested to play a role in a number of different diseases such as contact dermatitis (Seeliger *et al.*, 2003) and arthritis (Ferrell *et al.*, 2003).

Activation of PAR-2 also involves release of MMP-9 from airway epithelial cells, which can have a role in tissue-remodeling in asthma (Vliagoftis *et al.*, 2000). However, tryptase can also activate pro-MMP-3 (Gruber *et al.*, 1989). Once activated, MMP-3 can degrade different ECM components such as proteoglycans, fibronectin and laminin, a feature of inflammatory diseases such as rheumatoid arthritis. In addition, tryptase can directly cleave fibronectin (Lohi, Harvima & Keski-Oja, 1992) and gelatin (Fajardo & Pejler, 2003b). Thus, tryptase has a role in tissue remodeling and promotes angiogenesis (Blair *et al.*, 1997). Angiogenesis is crucial to many pathological conditions including tumor growth. Tryptase may therefore play a role in these conditions. Recently, a correlation between the extent of angiogenesis and tryptase-positive neurons and microvessels was found in a mouse model of duchenne muscular dystrophy, an X-linked genetic disorder characterized by muscle degeneration and brain damage (Nico *et al.*, 2004).

Tryptase inhibitors

The search for selective, high affinity tryptase inhibitors has been intense in recent years. The primary goal has been the discovery of drugs for treatment of tryptase-mediated diseases but also for the ability of selective inhibitors to determine the true physiological role of tryptase.

One of the first tryptase inhibitors was APC-366, a peptide-based inhibitor, which, despite problems of specificity underwent clinical trials as a treatment for asthma. It was shown that APC-366 administered to allergic sheep significantly inhibited late-phase responses (Clark *et al.*, 1995). Immediate cutaneous responses could be partly inhibited with APC-366 (Molinari *et al.*, 1995). Lately, APC-366 was demonstrated to significantly reduce acute airway obstruction in a pig model of asthma (Sylvin *et al.*, 2002). Another early inhibitor was BABIM, a benzamidine derivative that showed effects similar to APC-366 when administered to allergic sheep (Clark *et al.*, 1995). Later it was found that BABIM acts via a Zn^{2+} that is tetrahedrally coordinated between two chelating nitrogens of BABIM and with the catalytic Ser195 and His57 residues in tryptase (Katz *et al.*, 1998).

The crystal structure of human βII-tryptase revealed the unique organization of the tryptase tetramer and the presence of an acidic surface loop in the active site region suggested that fairly simple dibasic inhibitors could be designed (Pereira *et al.*, 1998; Rice *et al.*, 1998). AMG-126737 is a selective dibasic tryptase inhibitor (Ki = 90nM). It blocked the development of airway hyperresponsiveness in allergen-challenged guinea pigs as well as inhibiting both early and late phase bronchoconstriction in a sheep model of asthma (Wright *et al.*, 1999). MOL 6131 is another selective dibasic tryptase inhibitor (Ki = 45 nM), which has anti-inflammatory effects in a mouse model of asthma (Oh *et al.*, 2002). Furthermore, BMS-262084 is a guanidine-based dibasic tryptase inhibitor (IC50 = 4 nM), which was demonstrated to efficiently prevent allergen-induced bronchoconstriction and infiltration of inflammatory cells to the lung in guinea pig models (Sutton *et al.*, 2002). Since then, the same group has developed the guanidine concept further to find compounds that are more selective (Slusarchyk *et al.*, 2002). Recently, they reported very potent (IC_{50} down to 1.8 nM) and highly selective non-guanidine azetidinone based inhibitors of tryptase (Bisacchi *et al.*, 2004). The use of tryptase inhibitors has been adapted to the treatment of inflammatory bowel diseases. APC 2059, another dibasic tryptase inhibitor that is highly specific and selective, was used in a phase II study of ulcerative colitis (Tremaine *et al.*, 2002). Fifty-six adults received APC 2059 daily for 28 days. It was concluded that APC 2059 was safe and half of the patients showed clinical improvement. In addition, some of them showed complete remission. A monobasic inhibitor called gabexate mesylate, which is therapeutically used in pancreatitis, has also been demonstrated to selectively inhibit human MC tryptase with high potency (Ki=3.4 nM) (Erbaa *et al.*, 2001). In a recent study, gabexate mesylate and nafamostat mesilate, which is a structurally related compound, were shown to suppress pulmonary dysfunction in a rat model (Sendo *et al.*, 2003).

LDTI (leech derived tryptase inhibitor), a small protein of 46 amino acids isolated from the leech *Hirudo medicinalis*, was until recently considered the only protein active site inhibitor of tetrameric β-tryptase (Sommerhoff *et al.*, 1994). LDTI interacts with the same acidic loop as dibasic inhibitors but is non-selective and inhibits other related proteases such as trypsin. Interestingly, SERPINB6 is a newly discovered β-tryptase protein inhibitor that belongs to a family of intracellular serpins (serine protease inhibitors). Serpins have a so-called reactive loop region (RSL) that determines the specificity of the serpin. This RSL is cleaved by the protease and thereby the serpin becomes covalently linked to the protease. Meanwhile, the protease becomes inactivated. SERPINB6 was suggested to inhibit trypsin-like proteases such as tryptase because of an Arg in the P1 position in its RSL. Apparently, SERPINB6 is abundantly expressed in human MCs and is thought to regulate intracellular β-tryptase (Strik *et al.*, 2004). SERPINB6 is relatively large (42kDa) and would not fit into the central cavity of a tryptase tetramer. It was therefore suggested that SERPINB6 inhibits monomeric active β-tryptase. This is possible because active tryptase monomers may occur in the granules as an intermediate in the formation of active tryptase tetramers (Fukuoka & Schwartz, 2004). Moreover, several protein inhibitors that are unable to inhibit active tryptase tetramers have been shown to inhibit active tryptase monomers (Fajardo & Pejler, 2003a; Fukuoka & Schwartz, 2004). These include BPTI (bovine pancreatic trypsin inhibitor), STI (soybean trypsin inhibitor), antithrombin, and α2-macroglobulin.

Heparin antagonists

Heparin antagonists use a completely different mode of inhibition. This class of inhibitors exploits the requirement of heparin for stabilization of the active tryptase tetramer. Heparin antagonists may block tryptase irreversibly because when the tetramer has been destabilized to dissociate in the absence of heparin, it will not associate again unless more heparin is added. Antithrombin was the first compound reported to partly inhibit tryptase by binding to heparin (Alter *et al.*, 1990). Later, lactoferrin, a cationic protein released by neutrophils, was shown to potently inhibit tryptase (IC_{50} = 24 nM) (Elrod *et al.*, 1997). In the same study, lactoferrin was also demonstrated to block late-phase responses and airway hyper-responsiveness in a sheep model of asthma. Recently, another study reported that lactoferrin could be taken up into MCs and inhibit degranulation (He *et al.*, 2003). In this study, lactoferrin was a less potent inhibitor of tryptase; the IC_{50} value was demonstrated to increase with increasing heparin concentrations and ranged from 69 nM to 3.1 mM. This indicates that lactoferrin is not acting as a tryptase inhibitor *in vivo* but might instead have a role in regulating MC activation. Myeloperoxidase, another cationic protein secreted from neutrophils, also inhibits tryptase by displacement of heparin (Cregar *et al.*, 1999). Further, protamine, which is widely used in cardiovascular surgery to neutralize the anticoagulant effect of heparin, has also been suggested to inhibit tryptase by the same mechanism (Rice *et al.*, 1998). In a recent study, synthetic polycationic peptides were shown to have excellent inhibiting potency for recombinant βI-tryptase (IC_{50} down to 1 nM) (Lundequist *et al.*, 2003).

Processing and activation

Active β-like tryptases are stored in complex with heparin PG in the MC granule. Like the other MC proteases, tryptase is synthesized as a precursor protein with an N-terminal signal peptide followed by an activation peptide. Human protryptase contains a 12 amino acid long activation peptide whereas the activation peptide of mouse protryptase contains only 10 amino acids (Lutzelschwab *et al.*, 1997; Vanderslice *et al.*, 1990). *In vitro* studies of recombinant β-tryptase produced in a baculovirus/insect cell system have suggested a two-step process (Sakai, Ren & Schwartz, 1996). The first step consists of an intermolecular autocatalytic cleavage at Arg-3/Val-2, which is dependent on acidic pH and heparin. Apparently, monomeric protryptase may have separate substrate specificity and cleaves other protryptases but is unable to cleave small peptide substrates. In the second step, dipeptidyl peptidase I (DPPI; also referred to as cathepsin C) completes the processing to generate the mature monomer form of tryptase. Cleavage by DPPI did not require heparin. However, heparin was found to be required to produce enzymatically active tryptase (Sakai, Ren & Schwartz, 1996). In contrast, recombinant mMCP-6 produced in insect cells seemed not to require heparin for activation (Huang *et al.*, 1998) whereas recombinant human tryptase produced using the same insect cell system was found to require heparin for activation (Huang *et al.*, 2001). *In vivo* studies using mice lacking DPPI demonstrated that tryptase is not solely dependent on DPPI for processing (Wolters *et al.*, 2001). The levels of active tryptase in the $DPPI^{-/-}$ mice were diminished by 75%. This indicates that another enzyme can compensate for DPPI or that an unknown processing enzyme is affected by the lack of DPPI.

SUMMARY OF PRESENT INVESTIGATION

Aim

The aim of this thesis was to define the activation mechanisms of MC tryptase. Two closely related tryptases, mMCP-6 and human β-tryptase, were studied. Further, we wanted establish whether heparin has a role in tryptase activation and how heparin and tryptase interact.

Results and discussion

Paper I. The activation mechanism of mMCP-6.

The fate of tryptase is determined in several ways by heparin, its companion in the MC granule. Tryptase depends on heparin for stabilization of its tetrameric form and its catalytic activity (Schwartz & Bradford, 1986) and heparin is crucial for the storage of tryptase in the MC granule (Forsberg *et al.*, 1999; Humphries *et al.*, 1999). Tryptase activation is divided into two parts. The first part is characterized by proteolytic cleavage of the protryptase into the mature form. *In vitro* studies using recombinant β-tryptase suggest a two-step process consisting of heparin-dependent autocatalytic cleavage followed by cleavage by DPPI (Sakai, Ren & Schwartz, 1996). The assembly of inactive monomers into an active tetramer characterizes the second part of tryptase activation. Although one study suggested that mMCP-6 could be activated in the absence of heparin (Huang *et al.*, 1998), the role of heparin and the actual activation mechanism was not investigated in detail. To address this issue, we decided to study the mouse tryptase, mMCP-6, considered the murine counterpart of human β-tryptase. A recombinant form of mMCP-6 was produced, with an N-terminal histidine tag (for purification) followed by an enterokinase cleavage site replacing the natural activation peptide. The mammalian expression system, human 293 EBNA cells, provided high yields of mMCP-6 protein. Efficient purification was obtained using Ni-NTA agarose and after digestion with enterokinase, we obtained the mature monomeric form of mMCP-6.

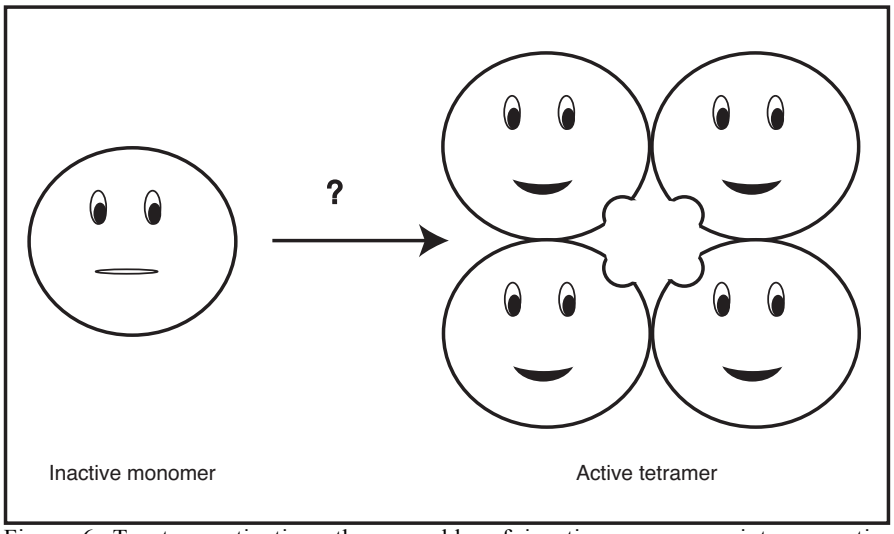

Figure 6. Tryptase activation; the assembly of inactive monomers into an active tryptase tetramer.

We found that heparin was required to interact with tryptase in an acidic environment in order for enzymatic activity to develop. However, maximal activity was obtained if the tryptase-heparin interaction was established at acidic pH followed by transfer of the mixture to neutral pH before the substrate was added. This indicated that when mMCP-6 had been activated, neutral pH enhanced the substrate cleavage. Further, mMCP-6 bound strongly to heparin-Sepharose in acidic pH, whereas binding was undetectable at neural pH. The effects of heparin and pH were correlated to the ability of tryptase to form tetramers. Gel-filtration experiments showed that in the absence of heparin, tryptase eluted as an inactive monomer. Nevertheless, the presence of heparin at acidic pH, but not at neutral pH, induced formation of an active tetramer. Furthermore, the potency of our recombinant mMCP-6 to exert its effect *in vivo* was demonstrated by injection of mMCP-6 into the mouse peritoneal cavity. Only heparin-activated mMCP-6 induced inflammation characterized by increased neutrophil influx.

The behavior of tryptase suggests how it may function *in vivo*. It is likely that it becomes activated into forming tetramers by interacting with heparin inside acidic MC granules or the *trans*-Golgi network. Upon MC degranulation, tryptase is released into the neutral extracellular milieu where it is ready to exert its proteolytical effect. The most likely explanation for the dependence on acidic pH for tryptase activation is that histidines (pKa ~ 6.5), which become positively charged at acidic pH, are involved in heparin binding. Together, the results suggest that heparin plays a critical role in the activation and tetramerization of mMCP-6.

Paper II: Heparin antagonists are potent inhibitors of mast cell tryptase.

The critical role of heparin for tryptase activation indicates that displacement of heparin may inactivate tryptase. In paper II, we investigated whether the polycationic compounds, protamine and Polybrene, could be used as tryptase inhibitors. Polybrene is a synthetic heparin antagonist that is used in the clinic for the reversal of heparin therapy, whereas protamine is an Arg-rich protein involved in packing of DNA in sperm and certain viruses (Brewer, Corzett & Balhorn, 1999). Kinetic studies showed that both heparin antagonists were potently inhibiting mMCP-6 and human lung tryptase (IC_{50} values in the nM range). The most likely cause of this effect is that the heparin antagonists compete with tryptase for binding to heparin. When tryptase loses heparin, the tetramer may destabilize, rapidly monomerize and lose its activity, resembling the events occurring during spontaneous inactivation in the absence of an inhibitor (Schechter *et al.*, 1995). Accordingly, when active tryptase in complex with heparin is co-injected with a heparin antagonist on a gel chromatography column, tryptase elutes as an inactive monomer. This suggests a non-competitive mode of inhibition, because heparin-antagonists would bind heparin rather than occupying the active site. Correspondingly, Polybrene inhibited tryptase with non-competitive kinetics. In contrast, protamine displayed competitive inhibition kinetics. This may be due to the high content of arginine in protamine, and that arginine side-chains may interact with the active sites. However, both Polybrene and protamine were sensitive to increasing heparin concentrations, which reduced their potency. The reason for the sensitivity of protamine, despite competitive kinetics, may be that protamine binds free heparin but fails to challenge tryptase for its associated heparin and instead interacts with the active site of tryptase at low heparin concentration. Further, tryptase inactivated with Polybrene seemed to be mostly resistant to reactivation with heparin. In contrast, when tryptase was inhibited by protamine, all activity could be regained by addition of excess heparin. Spontaneous inactivation is proposed to take place through several reversible steps ending with reactivable monomers and proceeding to an irreversible step to monomers that cannot be reactivated (Selwood, Mccaslin & Schechter, 1998). The reason for the irreversibility of Polybrene may be that its high potency makes the tryptase tetramers monomerize to a stage were they can't be reactivated. Consequently, protamine-inactivated tryptase can be reactivated since protamine is unable to compete with the tryptase-bound heparin and tryptase therefore never dissociates completely from heparin.

In comparison, both lactoferrin, a previously reported heparin antagonist and inhibitor of human tryptase (Elrod *et al.*, 1997), and APC 366, an active site-directed inhibitor (Clark *et al.*, 1995), displayed no or only moderate inhibition of mMCP-6 and human lung tryptase. Interestingly, APC-366 displayed different Ki values over time for human tryptase, but not for mMCP-6. After 40 minutes incubation, the Ki was in the millimolar range whereas after four hours the Ki had decreased 500 times to approximately 0.5 μM. The reason for this behavior might be explained by subtle differences in the active sites of mouse and human tryptase that make human tryptase more susceptible for the reshaped structure of APC-366 appearing after four hours of incubation. However, APC-366 also displayed an

inhibitory effect on MC chymase. This may be exlained by the interaction of an aromatic 1-hydroxy-2-naphthoyl group in the active site of chymase, in contrast to the inhibition of trypsin-like proteases, which involve interaction of an arginine-like structure in APC-366 with the Asp189 of the S1 pocket. Thus, that APC-366 can reduce asthmatic symptoms in sheep and pig models (Clark *et al.*, 1995; Sylvin *et al.*, 2002) may be related to inhibition of chymase as well as tryptase.

Paper III: Structural requirements for heparin-induced activation and identification of active tryptase monomers.

In paper III, we studied what the structures that determine the capacity of heparin to activate mMCP-6. We found that most other structurally related, but less sulfated compounds e.g. heparan sulfate, were unable to substitute for heparin. However, the highly negatively charged synthetic compound, dextran sulfate, efficiently activated tryptase. Further, structurally modified heparins, in which the N-sulfate, 2-O sulfate or 6-O sulfate groups were selectively removed, were all less effective than unmodified heparin. These results suggest that heparin interacts through its high negative charge density rather than through any specific structural motif. Moreover, experiments using heparin oligosaccharides of defined sizes (up to 26 saccharide units) showed that the heparin-tryptase interaction was highly size-dependent. Generally, the longer the heparin chain, the better its ability to induce tetramerization, however, intact heparin remained superior. Although short oligosaccharides consisting of 8-10 units were able to bind to tryptase, they were highly inefficient in inducing tetramerization. Thus, heparin binding does not by itself induce tetramerization. Furthermore, tryptase activation displayed a bell-shaped dose-response curve. Together, these results suggest a model for tetramer formation that involves bridging of tryptase monomers by heparin or other highly sulfated polysaccharides of sufficient chain length.

A completely novel finding was the identification of an active tryptase monomer. Short heparin oligosaccharides that bound to heparin did not produce tetramers but instead induced activation of monomeric tryptase. To ensure that the activities found in the monomer elution position really were due to active monomers and not tetramers that were formed after the chromatography step, the fractions were pooled and re-injected in the same column. The monomer fractions were again recovered in the monomer position. The presence of an active monomer was further proven by BPTI, which inhibited the active monomers but not the active tetramers. Moreover, the ECM component, fibronectin, which was recognized as a substrate for tryptase in earlier studies (Kaminska *et al.*, 1999; Lohi, Harvima & Keski-Oja, 1992), was found to be a substrate for active monomers but not for active tetramers.

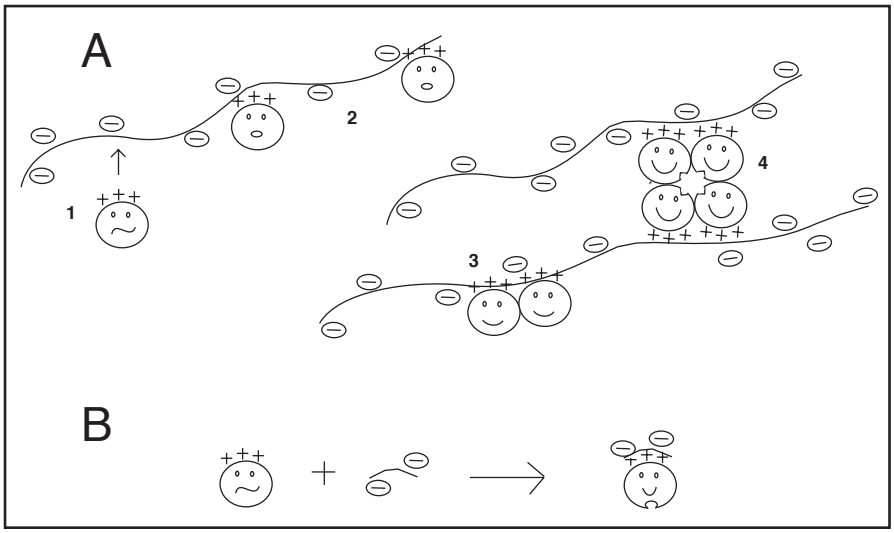

Figure 7. Model of tryptase activation. A) The heparin-dependent mechanism of tetramer formation. B) Short heparin oligosaccharides are unable to bridge two tryptase monomers but instead enable activation of monomeric tryptase.

Paper IV: Heparin binding is mediated by histidines in mMCP-6.

In paper IV, we wanted to test our hypothesis that histidines were involved in heparin binding. Our approach was to use site-directed mutagenesis of histidine residues that were selected on the basis of sequence alignments among pH-dependent tryptases and molecular modeling of the mMCP-6 structure from the crystal structure of human β-tryptase (Pereira *et al.*, 1998). Out of 13 histidines, we selected four (H35, H106, H108 and H238) that were conserved and exposed on the molecular surface.

Nine mutants were prepared: four single, two double, two triples and one quadruple. All except the quadruple mutant were expressed in high yields. Unfortunately, the lysosomal pathway of the 293 EBNA cells degraded the quadruple mutant. The single mutants displayed subtle defects in activation, tetramerization and heparin binding. Of the single mutants, H106A was most affected in its interaction with heparin. H106 is positioned closest to the interface between the subunits, indicating that this region may be particularly important for productive heparin binding i.e. that leading to tetramerization. Importantly, when several mutations were combined, large defects were found in all studied parameters. The triple mutants displayed the most dramatic defects due to the loss of three histidines in each monomer, which causes a reduction of positive charges from sixteen to four in each tryptase tetramer. Moreover, the triple mutants also showed tendencies to misfold into inactive aggregates, although tryptophane fluorescence measurements indicated that the mutations did not cause any changes in overall conformation of the mMCP-6 monomer. It is likely that the quadruple mutant deficient in all its surface-exposed histidines is so defective that it is not secreted and is instead routed to intracellular degradation.

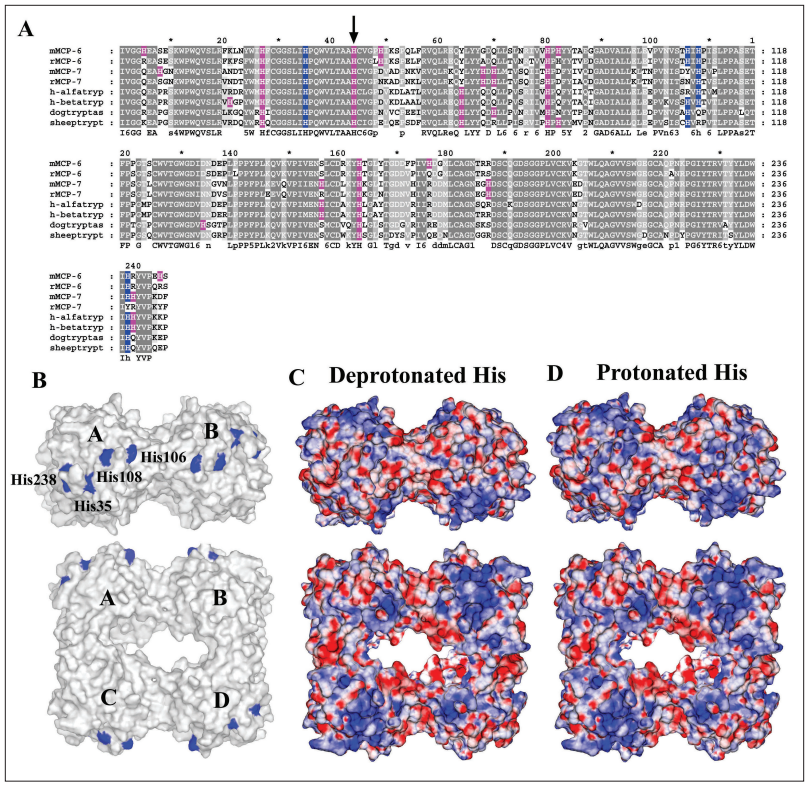

Figure 8. Sequence alignment and model of the mMCP-6 tetramer. A) Sequence alignment of mature tryptases from various species. Mutated His residues are labeled in blue and nonmutated His residues are shown in magenta. B) A surface representation of the mMCP-6 model. C, D) Electrostatic potential surface of the mMCP-6 model at neutral pH (C) and acidic pH (D).

Paper V. Activation mechanisms for human βI- and βII-tryptase.

In order to confidently use mouse models of human MC-related diseases and to assess the potential role of tryptase, it is important to ensure that the basic biochemical behavior of mouse tryptase and its human counterpart, β-tryptase, are similar. We therefore addressed the mechanisms of activation for human βI- and βII- tryptase. These tryptases differ in only one amino acid: Asn102 in βI-tryptase versus Lys102 in βII-tryptase. Asn102 is part of an N-glycosylation site and as a consequence, βI-tryptase is glycosylated at Asn102 whereas βII-tryptase lacks the corresponding glycosylation. We could therefore assess whether glycosylation at Asn102 was involved in the activation mechanism. Although an earlier study did show that recombinant human β-tryptase needed heparin for its activation (Huang *et al.*, 2001), a detailed study of the mechanism of activation and the structural requirement was lacking. We constructed recombinant human βI- and βII-tryptase using the same system for expression and purification as previously used for mMCP-6.

We found that heparin was crucial for the activation of βI- and βII-tryptase. A preference for acidic pH for activation of βI-tryptase, closely resembling the requirements for activation of mMCP-6 was also demonstrated. However, unlike mMCP-6, heparin-dependent tryptase activation was also detectable at neutral pH. This indicates that histidines are important for heparin binding in human βI-tryptase, but not as crucial as for mMCP-6. In contrast, βII-tryptase was much less dependent on acidic pH than βI-tryptase. Nevertheless, both β-tryptases showed similar bell-shaped dose-response curves and approximately equally high affinity for heparin. This means that the reason for the lower degree pH dependence of βII-tryptase is unclear and cannot be explained by lower affinity for heparin. Moreover, gel filtration analysis demonstrated that when βII-tryptase was activated with heparin it showed tendencies to misfold into inactive aggregates, whereas activation of βI-tryptase resulted in only active tetramers. This indicated that glycosylation may play a role in the assembly of tryptase tetramers.

The heparin-induced activation of the human β-tryptases was dependent on the size and high anionic charge density of the activator, and closely resembled the structural requirements in terms of heparin for mMCP-6. We also found evidence that the β−tryptases formed active monomers in the presence of low molecular weight heparin. Together, we found that the mechanism for activation of human β-tryptase was highly similar to that of mMCP-6. Thus, results obtained from studies of mouse tryptase appear to be highly relevant for the situation in humans and vice versa. This indicates that the mouse system is a good system for analyzing the biological role of tryptase and that mouse models of human MC-related diseases might be highly relevant.

In conclusion, we have proven the significance and nature of the heparin-tryptase interaction, shown a new mode of tryptase inhibition, constructed a model for formation of the active tryptase tetramer, demonstrated the presence of an active tryptase monomer and shown that mMCP-6 and human β-tryptase are very similar proteases as regarding their modes of activation.

Future perspectives

Our studies have revealed that the mouse tryptase, mMCP-6, and human β-tryptase show similar properties and that mouse models may be used for searching for the biological role of tryptase. Clearly, if a knockout mouse for mMCP-6 were to be constructed, it would be very interesting to employ various inflammation models to study the biological effects of tryptase *in vivo*. However, in recent years, many new human MC tryptases have been discovered. Some, such as γ/TMT tryptase, have a corresponding mouse variant (mTMT) whereas the mouse variants of other human tryptases such as α- and δ-tryptase may be missing. The near future will be important to judge which of these new tryptases are functional and account for the effects that have been discovered using purified tryptase. To do these studies, it will be important to make recombinant tryptases and characterize their basic biochemical behavior.

In a recent study, the possibility of the existence of active monomers *in vivo* was addressed (Fajardo & Pejler, 2003a). It was demonstrated that active tryptase monomers could be formed from dissociated tetramers under conditions that resemble the *in vivo* conditions to which tryptase is exposed after MC degranulation. This means that the discovery of the active tryptase monomer, recently confirmed by another major group in the field (Fukuoka & Schwartz, 2004), will encourage further characterization of the biological functions that can be attributed specifically to the active monomer, the active tetramer and to both in combination. Interestingly, both active forms of mMCP-6 mediated activation of PAR-2 (J. Hallgren, R. Pike, G. Pejler; unpublished data) and both active forms of human β-tryptase mediated cleavage of fibrinogen (Fajardo & Pejler, 2003a), whereas fibronectin is specifically cleaved by the active monomer (paper III) (Fajardo & Pejler, 2003a). The possibility of active tryptase monomers *in vivo* is of consequence not only to scientists wishing to dissect the true biological role(s) of MC tryptases, but also to those who want to find potent tryptase inhibitors to be used as medical treatment. For some clinical conditions, it may be important to inhibit only one active form, whereas in most cases it may be critical that the inhibitors can potently inhibit both forms. As the heparin antagonist, Polybrene, inhibits both forms of active tryptase (Fajardo & Pejler, 2003a), it may be important to further investigate the potential use of heparin antagonists.

Populärvetenskaplig sammanfattning

Mastcellen är en typ av cell som ingår i kroppens immunförsvar. Mastceller hjälper till att försvara oss mot bakterier och parasiter. Tyvärr är mastceller inte bara till hjälp för oss, utan det har visat sig att de är involverade i flera olika sjukdomar t.ex. allergier och astma. Inuti mastcellen finns en mängd små kapslar, s.k. granula, som innehåller en hel arsenal av olika inflammatoriska ämnen. Det är dessa ämnen som ibland försvarar oss och ibland gör oss sjuka. När mastcellen aktiveras töms innehållet i granula, de inflammatoriska substanserna, ut i kroppen. Ett av dessa inflammatoriska ämnen är det proteolytiska enzymet tryptas. Ett proteolytiskt enzym är ett protein som klyva andra proteiner i mindre bitar utan att själv påverkas. Tryptas är aktiv som en tetramer. Det betyder att för att tryptas ska vara aktivt så måste fyra stycken identiska tryptasmolekyler sitta ihop. Eftersom tryptas medverkar i vissa mastcells-relaterade sjukdomar är det många som forskar för hitta bra tryptasinhibitorer. En inhibitor är en molekyl som blockerar ett enzyms aktivitet.

Den här avhandlingen visar att för att tryptas ska kunna fungera som enzym behöver tryptas aktiveras av en annan molekyl, nämligen heparin. Heparin finns också inne i mastcellens granula och är ett sorts negativt laddat socker. Vi har visat att för att tryptas ska bli aktivt måste det interagera med heparin i en sur miljö. Eftersom vi fann att tryptasets aktivitet var kritiskt beroende av heparin, ville vi testa om man kunde blockera tryptasets aktivitet genom att förhindra att heparin binder till tryptas. Heparinantagonister är positivt laddade molekyler och binder själva starkt till heparin. Vi visade att heparinantagonister är effektiva tryptasinhibitorer. Vad är det då som gör heparin till en så bra tryptasaktivator? Vi kom fram till att det är längden på heparinkedjan och dess mycket negativa laddning som avgör. Vi fann att heparinkedjorna måste vara tillräckligt långa för att kunna binda till två tryptasmolekyler samtidigt och på det sättet binda ihop tetrameren och aktivera tryptaset. Dessutom hittade vi en ny aktiv form av tryptas, en aktiv tryptasmonomer, d v s en molekyl tryptas som blivit aktiv på egen hand. Vi visade att aktiva tryptasmonomerer uppstår när man aktiverar tryptas med korta kedjor av heparin (som inte förmår att brygga över två tryptasmolekyler).

Vilka delar av tryptas binder till heparin? Liksom andra proteiner är tryptas uppbyggd av olika sorters aminosyror. Eftersom vi visste att heparinbindningen måste ske vid lågt pH och att den var av elektrostatisk natur så misstänkte vi att en viss sorts aminosyror som kallas histidiner var inblandade. Det som kännetecknar aminosyran histidin är att det är den enda aminosyran som är positivt laddad vid lågt pH men oladdad vid neutralt pH. Därför kan histidiner bara binda till den negativt laddade heparinkedjan vid lågt pH. För att studera detta konstruerade vi tryptasmutanter, d v s varianter av tryptas där någon eller några aminosyror är förändrade. Våra tryptasmutanter förändrades så att en eller flera histidiner var muterade till den neutrala aminosyran alanin. Vi fann fyra histidiner som var speciellt viktiga för tryptasets förmåga att binda till heparin. I de första fyra studierna använde vi oss av ett rekombinant mustryptas, d v s vi tillverkade mustryptaset utifrån dess DNA-sekvens. För att kunna studera olika mänskliga sjukdomar i möss är det viktigt att undersöka att de grundläggande biokemiska

mekanismerna är lika för motsvarande enzym i mus och människa. I den femte studien visade vi att humant tryptas blir aktivt på samma sätt som mustryptas.

I denna avhandling har vi visat hur och varför tryptas blir aktiverat och hittat ett nytt sätt att inhibera tryptas, samt att tryptas även kan vara aktivt som monomer. Dessutom har vi visat att att heparin via sin negativa laddning binder till positivt laddade histidiner i tryptas och att mus- och humantryptas aktiveras på liknande sätt.

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