Review Letter

STRUCTURE, SPECIFICITY AND LOCALIZATION OF THE SERINE PROTEASES OF CONNECTIVE TISSUE

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1. Introduction

It is the purpose of this review to describe several well characterized proteases present in tissues and cells which appear to be related to the pancreatic serine proteases. Much of the early work concerning the nature of tissue proteases was done during the late 19th century by German physiologists who were studying tissue autolysis [1]. Hedin and Rowland [2] examined homogenates of various mammalian organs for proteolytic activity and observed that, with the exception of muscle, autolysis was greatest at acid pH. The term 'cathepsin' was introduced by Willstätter and Bamann [3] in 1929 to describe proteolytic activity of tissues in the weakly acid pH range. In recent years, however, this term has sometimes been applied to include tissue proteases in general, such as cathepsin G which has an optimum activity at pH 8 and cathepsin E which is most active at pH 2.5. The rapidly growing literature dealing with tissue and cellular proteases has been recently reviewed by Barrett [4].

Tissue and cellular serine proteases, which are most active at the physiological pH (pH 7–8), have been implicated in such processes as cellular chemotaxis [5], endocytosis, exocytosis [6], protein turnover in tissues [4,7] tumorigenesis [8] and fertilization [9], but very few of these enzymes have been purified or characterized in detail. Two notable exceptions are the human neutrophil serine proteases, cathepsin G, and elastase which have been intensely studied because of their implication in emphysema and other respiratory [10] disorders. Imbalances in the levels or regulation of tissue and cellular protease are generally thought to manifest themselves in various disease states, e.g., muscular dystrophy, arthritis, degenerative skin disorders as well as certain respiratory and gastrointestinal diseases [4]. Malignant tissues also show significant changes in proteolytic activity compared to normal, particularly that due to collagenases [11].

As important as abnormal proteolytic activity in tissues may be in disease states, intra- and extracellular proteases are necessary for maintaining normal tissue homeostasis. The steady-state concentration of proteins in cells and tissues is controlled by the rates of their synthesis and degradation [12,13] and although lysosomal proteases play a major role in intracellular protein degradation [14], there is considerable evidence that other proteases are of equal importance [15].

Several hypotheses have been advanced suggesting that specific limited proteolysis initiates the degradation of certain intracellular or extracellular proteins or groups of similar proteins [15,16]. For example, mammalian collagenases, secreted by several types of connective tissue cells exhibit specific and limited activity and so do the cytoplasmic proinsulin degrading proteases [17]. Support for the existence of intracellular degradative pathways of this type was derived from the investigations of Katunuma and coworkers [18] who have isolated several proteases exhibiting relatively restricted specificities and optimal activities at pH 8–9. It was reported that these proteases inactivated by limited proteolysis the apo-forms of pyridoxal phosphate dependent enzymes but not those requiring other cofactors [19]. These intracellular 'group-specific' proteases were shown to possess chymotrypsin-like specificity toward small synthetic substrates, were inactivated by DFP, suggesting that their catalytic sites were structurally related to those of the pancreatic serine proteases [19]. Because of the increasing awareness of the role

of limited proteolysis in many biological control mechanisms [20] and in tissue homeostasis [4,21], we have studied in detail the nature of these group-specific proteases of rat tissues. Some of our findings were unexpected while others emphasize the difficulties in attempting to interpret cellular events on the basis of observations made using tissue homogenates. Wherever appropriate we shall relate our findings about the nature of these enzymes to those which have been described for the other well characterized tissue serine proteases; i.e., neutrophil cathepsin G and elastase.

2. Purification

Katunuma and coworkers [19,22,23] describe the purification and characterization of four similar but distinct 'group-specific' proteases from rat tissue; two from the small intestine (one from mucosa and the other reported to originate in the smooth muscle layer) and one each from skeletal muscle and liver. The liver protease was thought to be a mitochondrial enzyme [19] on the basis of determinations of marker enzyme levels of fractions obtained by differential centrifugation. We decided to study the proteases from the muscle layer of small intestine and from skeletal muscle since these enyzmes could be obtained in sufficient quantity to allow detailed chemical characterization. Affinity chromatographic methods were developed in order to purify the proteases more efficiently and rapidly than previously possible.

The immobilized ligands were potato chymotrypsin inhibitor I immobilized on Sepharose 4B for the purification of the intestinal protease [24] and ovoinhibitor coupled to Sepharose 4B for the rat skeletal muscle protease [25]. The proteases isolated by these methods were chemically, physically, enzymatically and immunologically identical to those prepared by the described procedures [19,22].

3. Localization and identification

During the development of the affinity chromatographic purification method, it became apparent that the intestinal protease was not present in the muscle layer as reported [19]. Immunofluorescent and histochemical studies revealed that, in fact, the protease was contained in the so-called 'atypical' mast cells of the intestinal mucosa [26]. Cells containing the protease were observed in the bronchial and tracheal mucosa as well.

A similar chymotrypsin-like protease, known as chymase, is present in mast cells obtained from rat peritoneum [27]. The amino acid composition (table 1) of this enzyme is clearly different from that of the atypical mast cell protease, but it is remarkably similar to that of the skeletal muscle protease [28]. The amino-terminal sequences of the mast cell protease and the skeletal muscle protease are identical for the first 35 residues [28]. Immunodiffusion tests for cross-reactivity using anti-rat mast cell protease indicated that the mast cell and skeletal muscle proteases are immunologically identical [28]. Finally, immunofluorescent localization of skeletal muscle protease showed that this enzyme was in fact derived from mast cells present in the connective tissue of muscle (N. Katunuma, personal communication). Thus, both of these 'group-specific' proteases are in fact derived from mast cells.

Several other investigators have isolated chymotrypsin-like serine proteases from a variety of rat

Table 1
Comparison of the amino acid compositions of rat mast cell proteases

Amino acid	mol amino acid/mol protein	
	RMCP I[a]	RMCP II[b]
Aspartic acid	18.5	14
Threonine	16.8	13
Serine	12.0	13
Glutamic acid	20.0	17
Proline	16.2	15
Glycine	22.4	18
Alanine	15.6	16
Valine	22.1	22
Methionine	5.7	5
Isoleucine	12.4	18
Leucine	12.5	16
Tyrosine	9.9	9
Phenylalanine	8.0	6
Histidine	8.8	9
Lysine	22.7	13
Arginine	13.3	12
Half-cystine	n.d.	6
Tryptophan	n.d.	2

[a] Determined by analysis of samples after 24 h hydrolysis in 6 N HCl at 110°C [28]
[b] From sequence analysis [37]

n.d., Not determined

tissues such as skin, liver, thymus and skeletal muscle [29–31]. Besides their similar esterase specificities, these enzymes usually require concentrated salt solutions to maintain their solubility. It has now become apparent that many of these proteases are derived from normal mast cells which are widely distributed in rat connective tissue [32,33]. In fact, two similar proteases from liver, separately reported to be mitochondrial enzymes [19,34], have recently been identified as the chymotrypsin-like protease (chymase) of normal mast cells [35]. Subsequently, Katunuma has re-evaluated his observation that homogenates of Morris hepatomas grown in rats have ~100-fold greater activity of the 'mitochondrial' chymotrypsin-like protease compared to that of normal rat liver [36] and has shown that the increase in enzyme activity is due to a similar increase in the number of mast cells present in the hepatoma (N. Katunuma, personal communication).

In recent years, much effort has been made to isolate and study alkaline proteases from tissues in an attempt to elucidate the mechanisms involved in intracellular and extracellular protein turnover. There is now abundant evidence that the rat (or other animals containing large numbers of mast cells) should be avoided for such studies due to the likelihood that homogenates of tissues will contain significant levels of highly active mast cell protease.

4. Chemical structure and evolution

The complete covalent structure of the protease of atypical mast cells, RMCP II (rat mast cell protease II), has been determined [37].

The protease contains 224 amino acid residues in a single polypeptide chain crosslinked by three disulfide bonds (fig.1). A homologous relationship is clearly evident when this structure is compared to those of chymotrypsin, trypsin and elastase (~33% sequence identity to each). In addition, the residues forming the charge-relay system of the active site of chymotrypsin (His-57, Asp-102, Ser-195) are found in corresponding regions of the atypical mast cell protease. These structural features clearly indicate that the mast cell enzyme is structurally and mechanistically closely related to the pancreatic serine proteases. However, other structural features distinguish this protease from the known serine proteases.

(1) An alanyl residue is at position 176 in the mast cell protease corresponding to a residue which participates in the primary substrate binding site in serine proteases; i.e., Asp-177 in trypsin, Ser-189 in chymotrypsin. Thus, this binding site in the mast cell protease is less polar than that of any of the known serine proteases.

(2) Three disulfide bonds are present in the mast cell protease as compared to five in chymotrypsin, six in trypsin and four in elastase. The mast cell enzyme uniquely lacks a disulfide bond which is present in all known serine proteases (fig.2) and links Cys-191 to Cys-220 in chymotrypsin. In view of the close proximity of this bond to both the primary and antiparallel binding sites [38] of other serine proteases, it is likely that its absence in the atypical mast cell protease is related to the substrate specificity of this enzyme.

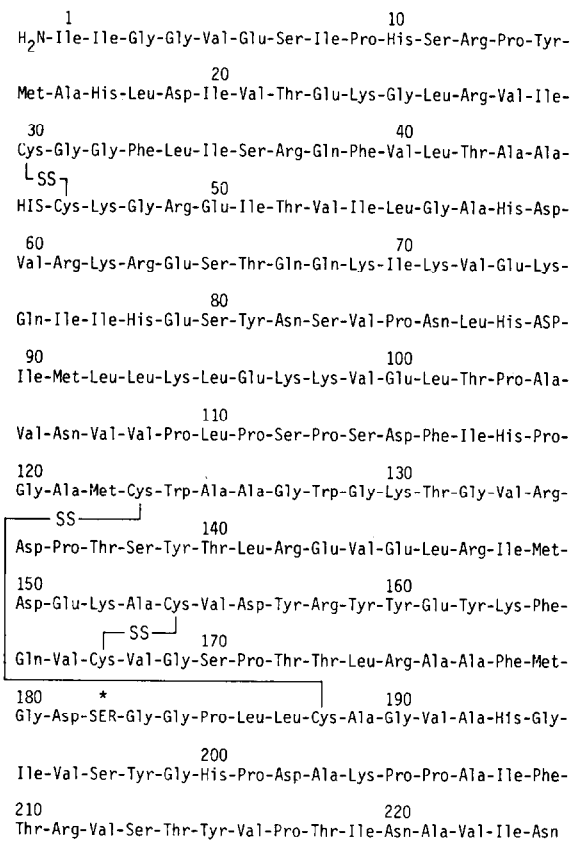

Fig.1. The covalent structure of RMCP II. Residue of the 'charge-relay' system of the active site are in capital letters (HIS-45, ASP-89, and SER-182). (*) indicates the location that radioactivity was observed when the protease was inactivated with [^{14}C]DFP. The disulfide bonds are indicated as –SS–.

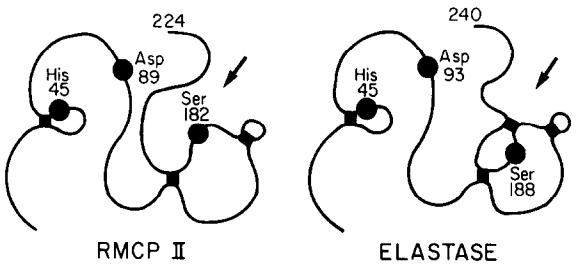

Fig.2. Comparison of the location of the disulfide bonds (■) in RMCP II and pancreatic elastase. Active site residues are indicated (●). Arrows indicate the disulfide bond present in all known serine proteases except RMCP II.

Analysis of the 3-dimensional structure of RMCP II is now being carried out by Matthews and coworkers at the University of Oregon and when completed should provide important information as to how these structural features of the enzyme relate to its activity and specificity.

A comparison of the 52 amino-terminal residues of the normal mast cell proteases with those of the atypical mast cell protease and of bovine chymotrypsin A (fig.3) shows ~75% sequence identity between the mast cell enzymes and ~40% compared to bovine chymotrypsin A [28]. Since the structures of the catalytic and substrate binding regions of the normal mast cell protease (RMCP I) have not been determined, no comparison can be made yet in regard to how structural differences between the two mast cell proteases may relate to known differences in their activities [19].

Since insertions and deletions (gaps) of residues in the sequences of homologous proteins occur less frequently than amino acid substitutions [39], the evolutionary relationship of homologous proteins

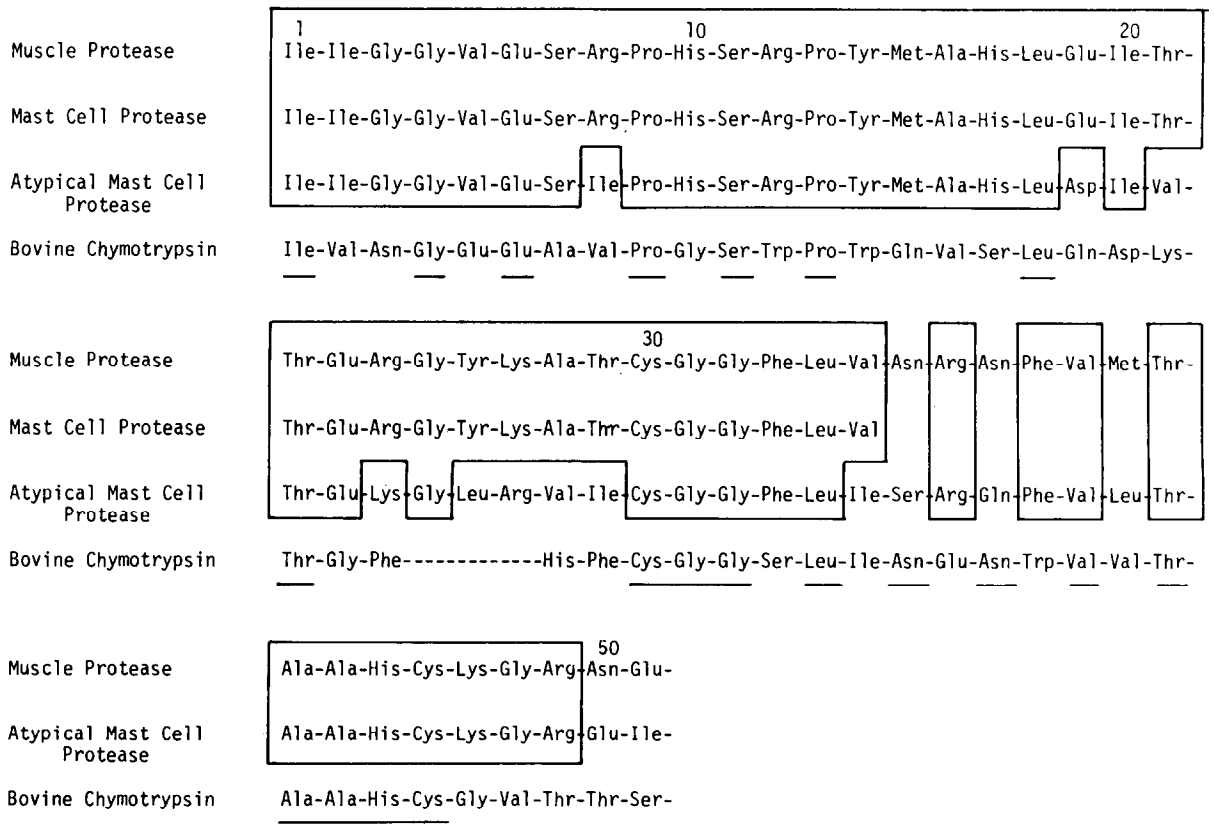

Fig.3. Comparison of the amino-terminal sequences of rat normal mast cell protease (RMCP I), atypical mast cell protease (RMCP II), and bovine chymotrypsin A. Muscle protease is normal mast cell protease. (---) indicates a gap placed in the sequence of chymotrypsin in order to obtain an optimal alignment of this structure with the others. Residues of chymotrypsin that are underlined are identical to those in mast cell protease.

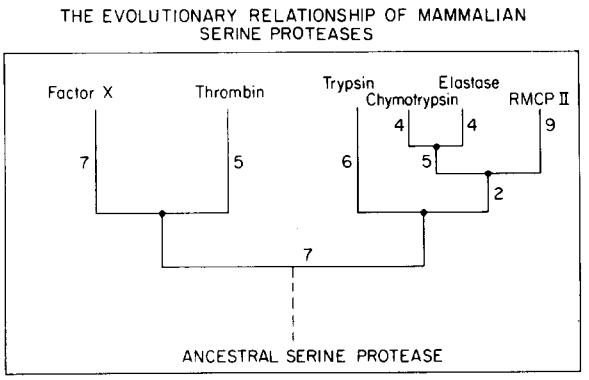

Fig.4. The evolutionary relationship of the serine proteases as determined by the number of insertions and deletions (gaps) arising from optimally aligned sequences of the enzymes. Numbers indicate the sequence gaps occurring when the structures are compared. Points of divergence are indicated (•).

may be more reliably estimated from the number and position of inferred gaps arising when the sequences are optimally aligned [40]. Based on this criterion, the progenitor of atypical mast cell protease may have arisen sometime after that of trypsin but before those of elastase and chymotrypsin (fig.4) [37]. Examination of the amino acid compositions of the two mast cell enzymes shows that the protease from normal mast cell contains twice as much lysine as does the enzyme from the atypical cell. The former enzyme binds strongly to the highly sulfated acid mucopolysaccharide, heparin, present in mast cell granules and requires high salt concentrations (1 M KCl) to disassociate the complex. In contrast, RMCP II is freely extracted from granules without any added salt. The solubility differences of the two enzymes may well be explained by their levels of lysine.

No sequence information has been reported yet for the human neutrophil proteases. Human cathepsin G and normal rat mast cell protease have a number of properties in common, including the requirement of high salt solutions to maintain solubility, chymotrypsin-like esterase activity, similar molecular weights, location in secretory granules, the apparent lack of a zymogen precursor, in situ association with sulfated acid mucopolysaccharides, basic isoelectric points, as well as the fact that each is synthesized by cells believed to play major roles in inflammation. Indeed, Starkey [41] has suggested that cathepsin G and mast cell protease (chymase) represent the same enzyme in different cells. This question will remain unanswered until the human mast cell proteases are characterized. It was concluded from the results of immunofluorescent studies in the rat, however, that neutrophils do not contain a protease which is antigenically similar to mast cell protease [26]. Nevertheless, human cathepsin G and rat normal mast cell protease are similar enough in chemical, physical and enzymatic properties to suggest that perhaps they do carry out a similar function during inflammation, but are triggered by different cellular mechanisms.

5. Specificity

Both RMCP I and RMCP II have chymotrypsin-like esterase activity. The activities of RMCP I (μmol substrate hydrolyzed . min^{-1} . mg $enzyme^{-1}$) toward AcTyrOEt and BzTyrOEt are 3–10-fold higher, respectively, as compared to RMCP II. The esterase activity of RMCP I is comparable to that of bovine chymotrypsin.

Powers and coworkers at the Georgia Institute of Technology are investigating in detail the substrate specificity of the two mast cell proteases toward synthetic polypeptide derivatives of *p*-nitroanilide (in preparation). Their results indicate that the mast cell enzymes possess proteolytic activity which appears unique among the known serine proteases and their findings should be helpful in defining the structure–function relationships of these proteases. The best substrates for both mast cell proteases have the general structure:

P4 P3 P2 P1
| | | |
X–Y–Leu–Phe$_{\downarrow}$–*p*-nitroanilide

where the bond cleaved is indicated by the arrow and the designation for the amino acid residue position, e.g. P1, is that of Schechter and Berger [42]. This observation is consistent with those of Kobayashi et al. [43,44] who observed similar specificity requirements. Chloromethyl ketone derivatives of the same general structure were found to be the most effective inhibitors of mast cell proteases. The best substrates for both proteases were *N*-Suc–Phe–Leu–Phe–*p*-nitroanilide and *N*-Suc–Phe–Pro–Phe–*p*-nitroanilide. The specificity constant k_{cat}/K_m for RMCP I, however, is ~100-times greater than that for RMCP II.

Katunuma et al. [19] observed that RMCP I was ~100-times more efficient than RMCP II in inactivating ornithine amino transferase. The pH optimum for the hydrolysis of both synthetic substrates by the atypical mast cell protease was 6.6. Compared to the values measured at pH 8.0, k_{cat} was ½ as great and K_m only 1/10th as great at pH 6.6 resulting in a 5-fold increase in k_{cat}/K_m. Examination of the amino acid sequence of atypical mast cell protease shows that there is a histidyl residue at position 200 which may interact with the succinyl group at P_4 of the substrate. An interaction of this type would explain the unusual pH profile for these substrates. Perhaps the most interesting observation made during this study was that polypeptides containing a prolyl residue at P_3 were excellent substrates for both mast cell proteases. These types of substrates are not cleaved by other serine proteases in a way which releases *p*-nitroaniline [44,45]. For example, RMCP II catalyzes the release of *p*-nitroanilide from *N*-Suc–Ala–Pro–Leu–Phe–*p*-nitroanilide with a k_{cat}/K_m of 2100 $M^{-1} . s^{-1}$. Cathepsin G or chymotrypsin will not release *p*-nitroaniline from this substrate but the former protease will cleave the Leu–Phe bond with a k_{cat}/K_m of 8.9 $M^{-1} . s^{-1}$. Apparently, most serine proteases cannot accommodate a prolyl residue at position P_3 at the antiparallel binding site of the enzyme and at the same time allow the P_1 residue to fit correctly into the primary substrate binding site [44]. If possible, the enzyme will interact with the substrate in a way which places the prolyl residue at position P_2 with respect to the bond cleaved. The reason that the mast cell enzymes can accept a P_3 prolyl residue in the substrate may well be related to the unique structural feature in the vicinity of the substrate binding site which has already been discussed.

The efficiency of RMCP I as a protease, based on the observed activity toward *N*-Suc–Phe–Leu–Phe–*p*-nitroanilide is quite remarkable. Cathepsin G cleaves this substrate readily to yield free *p*-nitroaniline with a k_{cat}/K_m of 1500 $M^{-1} . s^{-1}$, whereas the specificity constant for RMCP II is ~ 18 000 $M^{-1} . s^{-1}$. Under identical conditions, the k_{cat}/K_m for RMCP I is ~1×10^6 $M^{-1} . s^{-1}$, one of the highest specificity constants recorded for any protease/synthetic substrate system tested!

6. Physiologic role

Mast cells are important mediators of allergic, infective and anaphylactic reactions of tissues in most animals. The secretory granules of these cells contain histamine and/or serotonin or other vasoactive amines in addition to proteases. It has been widely assumed that the proteases contribute to the inflammatory process by degrading ground substances or by causing lesions in the vascular bed [47]. In view of the close proximity of mast cells to blood lymphatic vessels in tissues, it has been suggested that the mast cell enzymes promote and intensify the increase in vascular permeability initiated by the action of histamine [48]. Indeed, a recent study has shown that RMCP I when injected into rats leads to a very rapid increase in vascular permeability [49]. There is no function, however, which has been shown conclusively to be due to the action of mast cell proteases.

Because of the similar specificities of the two mast cell proteases toward synthetic peptides and peptide hormones (J. C. Powers and N. Yoshita, unpublished; [43,44]) one is tempted to suggest that each enzyme carries out similar functions and that the differences between the proteases reflect tissue specificity. There is no evidence whatsoever that this is so. Indeed, accumulated data strongly indicate that the so-called atypical mast cells are, in fact, unrelated to normal mast cells with the exception that both contain basophilic granules rich in chymotrypsin-like protease.

The current view is that the atypical mast cells in mammals (including man) are somehow involved in the local immune or inflammatory response at mucosal surfaces, particularly in parasitic infections [50–52]. Miller and Jarrett [50] observed that in gut these cells increased at least 10-fold at the time when rats spontaneously expelled nematodes from this site. In a recent quantitative study of the levels of protease during nematode infection (R. G. Woodbury and H. R. P. Miller, unpublished), it was observed that a similar increase in enzyme level accompanies the increase in the number of atypical mast cells. Recent evidence suggests that the occurrence of the atypical mast cells, but not normal mast cells, is dependent on T-lymphocytes [53,54]. Kitamura et al. [54] have shown that atypical mast cells of mouse small intestine, but not normal mast cells, arise from precursor cells derived from bone marrow. These observations, together with the morphologic [55] and histologic [56] differences between atypical and normal mast cells strongly suggest that the cells are distinct types.

There is suggestive evidence that atypical mast cells are involved in the local immune response at the

mucosal surface since it was observed that during the development of rats there is a significant proliferation of these cells and an increase in the protease levels precisely at the same time that the levels of I_gA of the gut are synthesized in measurable quantities [27]. Recently, it was observed that RMCP I, but not RMCP II, efficiently produces limited proteolysis of native human placenta type IV collagen which was obtained by mild treatment with pepsin [57]. Type I collagen was resistant to the action of both enzymes. If type IV collagen was prepared without pepsin treatment however, RMCP II cleaved native collagen, resulting in a product which had a molecular weight similar to pepsin-treated type IV collagen. Since type IV collagen is a major component of 'basement membranes' which underlie epithelial and endothelial cells and since both types of mast cells are in close proximity to these structures, it would appear that examinations of the effect of mast cell proteases on native basement membrane collagen would be worthy of further study.

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