The ability of human mast cell chymase and tryptase to process procollagen was examined. Purified human intestinal smooth muscle cell procollagen was incubated with human mast cell tryptase or human mast cell chymase. Purified chymase, but not tryptase, exhibited procollagen proteinase activity in the presence of EDTA. Addition of purified porcine heparin over a range of 0.1–100 μg/ml did not affect either the rate or the products of procollagen chymase cleavage. The cleavage site of chymase on the pro-α1(I) collagen carboxyl terminus was found to be in the propeptide region at Leu-1248-Ser-1249. Cleavage at this site suggested that the collagen products would form fibrils and confirmed the production of a unique carboxyl-terminal propeptide. Turbidometric fibril formation assay demonstrated \textit{de novo} formation of chymase-generated collagen fibrils with characteristic lag, growth, and plateau phases. When observed by dark field microscopy, these fibrils were similar to fibrils formed by the action of procollagen proteinases. Thus, mast cell chymase, but not tryptase, exhibits procollagen peptidase-like activity as evidenced by its ability to process procollagen to fibril-forming collagen with concurrent formation of a unique carboxyl-terminal propeptide. These data demonstrate that mast cell chymase has a potential role in the regulation of collagen biosynthesis and in the pathogenesis of fibrosis.

Mast cells are abundant in connective tissues of skin, lung, and intestine (1, 2). Increased numbers (or increased activity) of mast cells are associated with fibrotic disorders such as scleroderma, pulmonary fibrosis, and Crohn’s disease (3–5). The precise role for mast cells in connective tissue biology and scleroderma, pulmonary fibrosis, and Crohn’s disease (3–5).

Cleavage of Type I Procollagen by Human Mast Cell Chymase Initiates Collagen Fibril Formation and Generates a Unique Carboxyl-terminal Propeptide* (Received for publication, September 12, 1996, and in revised form, November 6, 1996)

Mark W. Kofford§§, Lawrence B. Schwartz§, Norman M. Schechter¶, Dorne R. Yager***, Robert F. Diegelmann**, and Martin F. Graham‡

From the Departments of §Pediatrics, ¶Medicine, and **Surgery, Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia 23298-0529 and the §Department of Dermatology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The abbreviations used are: N-proteinase, amino-terminal procollagen proteinase; C-proteinase, carboxyl-terminal procollagen telopeptide; N-proteinase, amino-terminal procollagen telopeptide; PAGE, polyacrylamide gel electrophoresis; MCTC, tryptase-positive, chymase-positive mast cell type; C-proteinase, carboxyl-terminal procollagen propeptidase; pN-collagen, collagen intermediate following enzymatic removal of the carboxyl telopeptide region; PICP, procollagen type I carboxyl-terminal propeptide; serpins, serine protease inhibitors in the plasma.

1 The abbreviations used are: N-proteinase, amino-terminal procollagen proteinase; C-proteinase, carboxyl-terminal procollagen telopeptide; N-proteinase, amino-terminal procollagen telopeptide; PAGE, polyacrylamide gel electrophoresis; MCTC, tryptase-positive, chymase-positive mast cell type; C-proteinase, carboxyl-terminal procollagen propeptidase; pN-collagen, collagen intermediate following enzymatic removal of the carboxyl telopeptide region; PICP, procollagen type I carboxyl-terminal propeptide; serpins, serine protease inhibitors in the plasma.
**Chymase Processes Procollagen**

**Procollagen Cleavage Assay—**Chymase and mast cell lysate were each assayed for procollagen proteinase activity in 0.01% (v/v) Brij 35 or 2 mM NaCl, 0.05 mM Tris, pH 7.4, 0.02% (w/v) Na$_2$SO$_4$, and 25 mM EDTA at 35°C, a modified form of the assay published by Hójima et al. (18) for the measurement of the specific activity of procollagen carboxy-terminal propeptidase. Trypsin was preincubated on ice with heparin-trypsin (5:1, w/w) before being added to assay solutions containing 0.2 mM NaCl. N-proteinase and C-proteinase were incubated in procollagen cleavage assays containing 0.1 mM CaCl$_2$. Reactions were stopped by the addition of an equal volume of non-reducing sample buffer containing 62.5 mM Tris, pH 6.8, 10% (v/v) glycerol, 10% (v/v) SDS, and 0.001% (v/v) bromophenol blue. In some cases 2% (v/v) 2-mercaptoethanol was added to reduce samples. All samples were heated to 100°C for 3 min and then subjected to polyacrylamide gel electrophoresis using 4% stacking and 6 or 8% separating gels. All gels were fixed for 20 min with a mixture of 10% (v/v) acetic acid and 25% (v/v) 2-propanol, incubated in 1 M sodium salicylate, dried, and exposed to Amersham Hyperfilm-MP (Amersham International, Buckinghamshire, United Kingdom) at −70°C or stained with Coomassie Blue (20, 21). The protein bands on the autoradiographs and Coomassie-stained gels were quantitated using a Molecular Dynamics PDSI 486 laser densitometer. Molecular weights were determined using FragmeNT analysis software (Molecular Dynamics, Inc., Sunnyvale, CA).

**Chymase Assay—**By convention, the specific activity of chymase is 2.7 μmol of product min$^{-1}$nmol of chymase, measured by cleavage of the synthetic substrate succinyl-Ala-Ala-Pro-Phe-nitroanilide (5)(16). Chymase used in these studies had a specific activity of 2.7 μmol of product min$^{-1}$nmol of chymase used and was at a concentration of 0.24 μg/ml, a concentration attainable in tissues with mast cell degranulation (22).

**The Effect of Heparin on Chymase Activity—**Aliquots of human chymase were incubated for 20 min with serial concentrations of purified porcine heparin in siliconized microcentrifuge tubes on ice. Purified human type I procollagen was then added to initiate the reactions, which were carried out in 0.15 M NaCl assay solutions at 35°C for 2 and 4 h. Reactions were quenched by addition of reducing sample buffer. Samples were analyzed by polyacrylamide gel electrophoresis and densitometry as above.

**Amino Acid Sequencing of Reaction Products—**Chymase (50 pmol) was incubated for 15 min with 50 μl of Affi-Gel heparin (Bio-Rad) in 0.12 mM NaCl, 0.05 mM Tris, 0.01% Na$_2$SO$_4$, pH 7.3, at 25°C. Using these conditions, chymase bound tightly to Affi-Gel heparin (23). Bound chymase was then incubated for 24 h at 35°C with 400 μg of purified type I procollagen in 0.12 mM NaCl, 0.05 mM Tris, 0.01% Na$_2$SO$_4$, pH 7.3. After 24 h, chymase bound to Affi-Gel was removed from the reaction mixture by centrifugation at 1,000 g for 1 min. The reaction mixture was then prepared for gel electrophoresis under non-reducing conditions as described above. Cleavage products were purified by slab gel electrophoresis on a 9% polyacrylamide gel and blotted to a polyvinylidene difluoride membrane (Bio-Rad). The polyvinylidene difluoride membrane was stained with a solution of 40% methanol, 60% water, 0.025% Coomassie Blue and protein bands were visualized by destaining with a 50% methanol, 50% water solution. Product bands were labeled, and polyvinylidene difluoride membranes were shipped for commercial sequencing. Amino-terminal sequencing was performed by automated Edman degradation using an ABI 470A gas phase sequencer and an HP GS1000 HPLC (Protein Structure Laboratory, Davis, CA).

**Turbidometric Fibril Formation Assay—**Stock procollagen (150 μg/ml) was twice dialyzed against 500 volumes of physiological fibril formation buffer, pH 7.4, containing 20 mM NaHCO$_3$, 110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 0.18 mM MgSO$_4$, 1,03 mM NaHPO$_4$, and 0.04% (w/v) NaN$_3$. Procollagen solutions were then preheated in a temperature-controlled cuvette, and the reactions were initiated by the addition of chymase, charged with 10% CO$_2$, 90% air, and sealed with a greased rubber stopper. The turbidity of the reactions was monitored at 313 nm using a Shimadzu UV-1604 spectrophotometer as described previously (24).

**Fibril Formation for Dark Field Microscopy—**Collagen fibril formation was determined by de novo fibril formation assay (25). 150 μg/ml procollagen in the fibril formation buffer described above was incubated with either pure chymase or 3 units of each of N- and C-proteinases in siliconized microcentrifuge tubes. Tubes were charged with 10% CO$_2$, 90% air, sealed, and incubated for 24 h at 34°C. 300 μl of each solution was also incubated in the sealed well of a Lab-Tek chamber slide (Nunc, Inc., Naperville, IL) (25). Fibril formation was monitored with a Zeiss dark field microscope, and photographs were taken with an Olympus PM-A10 optical attachment.

**RESULTS**

The Effect of Purified Human Mast Cell Chymase—Incubation of purified human mast cell chymase with type I procollagen resulted in the time-dependent cleavage of procollagen to collagen-size chains. Four discrete intermediates of approximate molecular masses of 162, 143, 130, and 117 kDa were formed during the incubation (Fig. 1, 2 and 4 h). Proteins corresponding in size to α1(I)- and α2(I)-collagen chains were not further degraded under the conditions employed (Fig. 1, 48 h)(9). The intermediates formed by chymase cleavage of procollagen were similar in size to intermediates formed by cleavage of procollagen by the specific amino-terminal and carboxyl-terminal propeptidases (Fig. 1, lanes NP and CP). This result demonstrated that chymase cleaved procollagen in both the N- and C-protepeptid regions, producing collagen products similar to those produced by the combined activity of amino- and carboxyl-terminal propeptidases (Fig. 1, lane N&C). Type I procollagen substrate was not processed in the absence of enzyme (Fig. 1, lane C). Chymase was determined to cleave 0.38 μg of procollagen/pmol of chymase/h at 35°C in a 100-μl assay (assayed for quantitative cleavage using a modified form of a carboxyl-terminal proteinase assay described previously (21)). Although this number gives some indication of the activity of chymase relative to type I procollagen proteinases, it cannot be used as a direct comparison against N- or C-proteinase activity because chymase appears to cleave in both the amino- and the carboxyl-terminal regions. It is also important to note that the type I procollagen concentrations used in the time-course studies presented in Fig. 1 were an order of magnitude greater than that used in the published assay (21). This accounts for the nearly 5-fold increase in the rate of procollagen cleavage demonstrated in Fig. 1 compared to the cleavage by C-proteinase assay.

**Propeptide Products Produced by Chymase Cleavage of Procollagen—**Cleavage of type I procollagen by C-proteinase generated a heterotramer of two α1 carboxy-propeptidases disulfide-linked to one α2 carboxy-propeptide (Fig. 2, lane N&C-PICP). The molecular mass of the PICP molecule was subsequently determined to be 96 kDa by FragmeNT analysis, which corresponds to previous reports (18). In contrast, cleavage of procollagen by mast cell chymase produced two very disparate propeptide chains, one of 67-kDa and another of 25 kDa molecular mass (Fig. 2, lane Chy).

**Carboxyl-terminal Chymase Cleavage Site in Procollagen—**The precise cleavage site of chymase in the carboxyl terminus of the type I procollagen molecule was determined by amino acid sequence analysis of the 67- and 25-kDa peptide products (Fig. 2). The analyses yielded an identical sequence that was
found in the α1(I) C-propeptide region (Fig. 3). The analysis demonstrated the carboxyl-terminal chymase cleavage site on the α1(I) chain to be between Leu and Ser at positions 1248–1249 (Fig. 3). Leu in the P1 position and Ser in the P2 position are compatible with the known preferences of chymase for its substrates (22). The chymase cleavage site, therefore, is 20 amino acids carboxyl to the C-proteinase cleavage site that is between residues 1228 and 1229. The only other potential chymase-compatible cleavage sites more proximal to this one would lie within less than 10 residues of the triple helix region, where steric problems would be expected to hinder chymase approximation. These data strongly suggest that 1248–1249 is where steric problems would be expected to hinder chymase cleavage. It would lie within less than 10 residues of the triple helix region.

The only other potential amino acids carboxyl to the C-proteinase cleavage site that is compatible with the known preferences of chymase for its substrate is Met (Fig. 3). Leu in the P1 position and Ser in the P2 position are the primary candidates for the first 10 amino acids identified for both the 67-kDa and 25-kDa products (Fig. 5). The transient 46-kDa fragment seen in Fig. 2, lane Chy, corresponds to the carboxyl-terminal cleavage site of chymase.

**Effects of Heparin on Chymase Activity**—Chymase has a high affinity for charged proteoglycans and is secreted either bound to heparin or bound extracellularly and immobilized shortly after secretion (23). Such binding may enhance or inhibit proteolysis of a large molecule such as procollagen. Therefore, it was important to test the proteolytic action of chymase-heparin complexes as well as chymase alone. To determine whether the action of purified chymase on procollagen was augmented by heparin (26), chymase was incubated with increasing concentrations of heparin prior to the addition of type I procollagen substrate in the procollagen cleavage assay (0.15 M NaCl). Both the rate of cleavage and the products formed remained unchanged in the presence of heparin over a 1000-fold concentration range (data not shown) suggesting that the binding of chymase to heparin in vivo would not affect the affinity or specificity of chymase for type I procollagen substrate. Thus, procollagen and C-propeptides may be suitable substrates in the extracellular space for free chymase or chymase bound to heparin.

**Turbidometric Fibril Formation Assay**—A turbidity assay was used to determine whether chymase-generated collagen was capable of de novo fibril formation. Following addition of purified chymase to procollagen in fibronectin formation buffer, a lag phase (0–8 h), a growth phase (8–16 h), and a plateau phase (16–24 h) were observed (Fig. 4B). The lag phase represents the time necessary for chymase to generate a critical concentration of collagen molecules before fibril assembly can begin, the growth phase represents the assembly of collagen molecules into collagen fibrils causing increased turbidity of the reaction solution, and the plateau phase represents an equilibrium between collagen fibrils and collagen molecules in solution. The turbidity profile obtained conformed to what has been described previously for collagen fibril formation (14, 24). Aliquots taken from a parallel reaction solution were analyzed by SDS-PAGE in 8% separating gels under non-reducing conditions. The turbidity profile shown in Fig. 4B corresponded to the cleavage of procollagen to collagen-size chains shown in Fig. 4A.

**Fibril Morphology by Dark Field and Electron Microscopy**—Collagen fibrils were generated by the de novo cleavage of type I procollagen by chymase, or N- and C-proteinases. Fibrils were visible to the eye in both enzyme solutions following a 24-h incubation period at 34 °C. When observed by dark field microscopy, fibrils formed by the activity of N- and C-proteinases ranged in diameter from approximately 0.25 to 1 μm (Fig. 5, A and B). Fibrils formed by chymase cleavage of type I procollagen were similar in shape, but tended to be smaller in diameter, ranging from approximately 0.1 to 0.5 μm (Fig. 5, C and D). Type I procollagen incubated without chymase or N- and C-proteinases did not form fibrils. When negatively stained (27) and observed by transmission electron microscopy, fibrils of both types displayed 67 nm D-periods as previously reported for native fibrils (28).

**DISCUSSION**

We have hypothesized that mast cell proteases play a role in connective tissue biology and contribute to the pathogenesis of fibrosis by cleaving secreted type I procollagen to collagen. To...
begin testing this hypothesis, we explored the effect of isolated mast cell enzymes on purified type I procollagen in vitro. Purified human mast cell chymase was found to cleave type I procollagen to collagen-size products that can then spontaneously form fibrils. These results suggest a novel role for chymase and confirm that mast cell chymase does not degrade the helical region of the collagen molecule (9).

Mast cell chymase is selectively present in human mast cells of the MCTC type (tryptase-positive, chymase-positive) and comprises about 4.5 μg of the total protein/mast cell of this type (29). Localization of chymase-containing MCTC cells to intestinal submucosa and dermis of skin is suggestive of a specific role for chymase in the connective tissue of these organs, but the precise function of the chymase is not known. The studies presented here suggest that mast cell chymase may function to augment normal procollagen processing in order to facilitate tissue repair.

The types and concentrations of enzyme inhibitors around the mast cells after degranulation are not known, making it difficult to predict the functional lifetime of chymase. Nevertheless, chymase is inhibited by serine protease inhibitors in the plasma (serpins). The serpins (α1-antichymotrypsin and α1-protease inhibitor) responsible for 80% of the inhibitory effect of plasma on chymase, inhibit chymase at a rate 3000-fold less than rates calculated for their inhibition of either neutrophil proteinase cathepsin G or elastase (30). Because serpins prove to be better substrates than inhibitors for chymase (30), chronic low level mast cell degranulation may deplete local inhibitor concentrations and provide a constant level of active chymase to the affected tissue. In this scenario, microenvironments of chymase would be in contact with secreted procollagen. The results of these studies suggest that such a scenario may lead to accelerated processing of procollagen to fibril-forming collagens and to the formation of as yet unidentified procollagen-derived peptides with potential bioactivity (31, 32).

Telopeptide degradation studies have shown that N- and C-telopeptides function in determining the morphology of the collagen fibril. Specifically, the amino-telopeptide was shown to direct the orientation of collagen monomers and the carboxy-telopeptide was shown to have a role in the determination of fibril diameter (33). Fibrils of smaller diameter form when pN-collagen is allowed to assemble, due to steric hindrance from the N-propeptide folding back on the triple helix region (34). The fibril formation studies reported here demonstrate that de novo cleavage of procollagen by chymase leads to the formation of collagen fibrils smaller in diameter than procollagen proteinase-generated fibrils. This suggests that the 20-

**FIG. 4.** Cleavage of type I procollagen by chymase results in the formation of collagen fibrils. A, type I procollagen (~150 μg/ml) and mast cell chymase (0.24 μg/ml) were incubated in physiologic buffer in a siliconized microcentrifuge tube at 35 °C. 3.5-μg aliquots were removed at 0-, 4-, 8-, 12-, 16-, 20- and 24-h time points and analyzed by SDS-PAGE on an 8% separating gel under non-reducing conditions. The processing of procollagen to collagen-size chains shown in A corresponded to increasing turbidity shown in B. Without chymase, no processing of procollagen was observed (far right lane). B, turbidometric assay of collagen fibril formation de novo. Type I procollagen (~150 μg/ml) and mast cell chymase (0.24 μg/ml) were incubated in physiologic buffer at 35 °C in the cuvette of a temperature-controlled spectrophotometer. Turbidity readings at 313 nm were taken in 5-min intervals and are represented on the y axis of the graph. Lag phase, before the critical concentration of collagen molecules is reached; Growth phase, assembly of collagen molecules into fibrils; Plateau phase, equilibrium of collagen and collagen fibrils (——). There was no increase in turbidity in the sample incubated without chymase (○).

**FIG. 5.** De novo fibril formation. Purified type I procollagen (150 μg/ml) was incubated for 24 h with either 3 units each of N- and C-proteinases (A and B) or 0.24 μg of chymase (C and D) at 34 °C, then visualized by dark field microscopy. Fibril formation was monitored with a Zeiss dark field microscope, and photographs were taken with an Olympus PM-A10 optical attachment (bar, 25 μm).
aminocacid C-telopeptide extension, indicated by sequence data (Fig. 3) may limit fibril diameter by a similar mechanism. Investigation by electron microscopy into the assembly pattern and orientation of chymase-generated collagen monomers has demonstrated parallel assembly and 67-nm D-periodic symmetry of these fibrils as described previously for native collagen fibrils (28). These data demonstrate that chymase-generated collagen monomers may contribute to aberrant fibril architecture. Similarly decreased collagen fibril diameter has been reported in vitro in remodeled anterior cruciate ligament grafts (35, 36).

The data presented in this paper demonstrate clearly that human mast cell chymase directly cleaves type I procollagen and generates novel propeptide products. Classical propeptidases generate amino- and carboxyl-terminal propeptides that are thought to function as feedback inhibitors of procollagen biosynthesis (37–40). Synthetic peptide subfragments derived from the α1 carboxyl-terminal propeptide have been shown to be potent positive and negative effectors of matrix protein biosynthesis (37–40). Synthetic peptide subfragments derived from the α1 carboxyl-terminal propeptide have been shown to be potent positive and negative effectors of matrix protein biosynthesis (37–40).

Sieron for helpful advice and Dr. Rod Watson for the kind gift of chick cell chymase directly cleaves type I procollagen and generates novel propeptide products. Whether chymase-generated propeptides will have functions similar to those generated by classical propeptidases is currently being examined.

The current study demonstrates for the first time that mast cell chymase cleaves type I procollagen to a fibril-forming collagen molecule. This may have important implications with regard to the increased numbers, or activation, of mast cells reported in association with fibrotic diseases such as scleroderma, pulmonary fibrosis, and Crohn’s disease. Initial recruitment of mast cells by chemotactic factors such as transforming growth factor-β (41) and soluble stem cell factor (42) generated at such sites may be followed by the secretion of proteases that contribute to the production and accumulation of collagen. The effect of chymase on procollagen processing, collagen fibril formation, and C-propeptide degradation may prove important for understanding the role of mast cells in connective tissue metabolism and in these fibrotic disorders.

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