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Defining Requirements for Collagenase Cleavage in Collagen Type III Using a Bacterial Collagen System*

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Background: Structural requirements of triple-helical collagen for collagenolysis are not fully understood.
Results: Recombinant bacterial collagens with human collagen III sequence insertions defined the minimum sequence for cleavage by human collagenases.
Conclusion: Susceptibility of bacterial-human collagen chimeras to collagenases mimicked that of human collagen III.
Significance: This recombinant system is useful to investigate biological functions of collagen segments in a triple-helical context.

Degradation of fibrillar collagens is important in many physiological and pathological events. These collagens are resistant to most proteases due to the tightly packed triple-helical structure, but are readily cleaved at a specific site by collagenases, selected members of the matrix metalloproteinases (MMPs). To investigate the structural requirements for collagenolysis, varying numbers of G*XY* triplets from human type III collagen around the collagenase cleavage site were inserted between two triple helix domains of the Scl2 bacterial collagen protein. The original bacterial CL domain was not cleaved by MMP-1 (collagenase 1) or MMP-13 (collagenase 3). The minimum type III sequence necessary for cleavage by the two collagenases was 5 G*XY* triplets, including 4 residues before and 11 residues after the cleavage site (P4-P11′). Cleavage of these chimeric substrates was not achieved by the catalytic domain of MMP-1 or MMP-13, nor by full-length MMP-3. Kinetic analysis of the chimeras indicated that the rate of cleavage by MMP-1 of the chimera containing six triplets (P7-P11′) of collagen III was similar to that of native collagen III. The collagenase-susceptible chimeras were cleaved very slowly by trypsin, a property also seen for native collagen III, supporting a local structural relaxation of the triple helix near the collagenase cleavage site. The recombinant bacterial-human collagen system characterized here is a good model to investigate the specificity and mechanism of action of collagenases.

Enzymatic degradation of collagen is essential in embryonic development and normal tissue remodeling and repair (1), whereas abnormal degradation is implicated in progression of diseases such as cancer, arthritis, atherosclerosis, and abdominal aortic aneurysms (2). The distinctive triple-helical conformation of collagens, with three polyproline II-like chains tightly wound around a common axis (3–6), makes collagens type I, II, and III with this motif resistant to most proteinases (7), but they are readily cleaved by members of a family of enzymes, matrix metalloproteinases (MMPs).[4] Of the 23 MMPs in man, those reported to cleave triple-helical collagen are the collagenases (MMP-1, MMP-8, and MMP-13), gelatinase A (MMP-2), membrane type 1 MMP (MMP-14) (8), and membrane type 2 MMP (MMP-15) with very weak activity (9). However, the nature of this cleavage and substrate requirements of these collagenolytic MMPs are not fully understood.

The structure of most MMPs includes an N-terminal prodomain, a catalytic domain with Zn^{2+} and Ca^{2+} binding sites, a proline-rich linker region, and a C-terminal hemopexin domain (8). MMPs are activated by removing the N-terminal prodomain, which blocks the catalytic domain of MMPs. The C-terminal hemopexin domain of collagenases is considered to play an important role in binding to the native fibrillar collagens because the catalytic domain alone is not capable of cleaving them (10–16). Collagenases cleave native type I, II, and III collagens at a unique Gly-Leu/Ile bond which is located about three quarters from the N terminus (17, 18). Although there are many similar sequences in the collagen triple-helical region, they are not cleaved by collagenases, indicating the importance of the extended local sequence and structure (19). Specifically, the absence of imino acids on the C-terminal side of the collagen cleavage site that could result in a less tight triple helix is considered to be important for collagenase recognition (19, 20). It was shown that MMP-1 locally unwinds the triple-helical collagen before cleavage can take place (21). Alternatively, Stultz and co-workers proposed that the conformation of col-

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[4] The abbreviations used are: MMP, matrix metalloproteinase; Cat, catalytic; MRE, mean residue ellipticity; MMP-1Cat, matrix metalloproteinase 1 Cat domain only; MMP-13Cat, matrix metalloproteinase 13 Cat domain only; SCLIII, streptococcal collagen-like protein with type III sequences.

lagen near the collagenase cleavage site represents an equilibrium between the standard triple helix and a more open state susceptible to collagenases without the enzyme contribution to unwinding (22, 23). The recent study by Salsa-Escat *et al.* (24) showed that the catalytic domain of MMP-8 (collagenase 2) can cleave native collagen I at 25 °C, but this was extremely inefficient. Recent biochemical studies of collagenase-resistant homotrimer collagen I further suggest that, although the collagenase cleavage site is naturally prone to form a less tight triple helix, additional conformational changes occur upon binding of the enzyme that eventually lead to efficient collagenolysis (25).

Characterization of MMP cleavage has been carried out on collagens purified from animal tissues (14, 26–29), collagens expressed in mammalian (30) and yeast cell lines (31), and triple-helical peptide models (32–34). We considered that a recombinant bacterial collagen system developed recently could complement these techniques to investigate MMP specificity by introducing mammalian collagen segments into it. Collagen was thought to be present only in multicellular animals, but the examination of bacterial genomes revealed more than 100 proteins with typical $(GXY)_n$ sequences with high Pro content, and a number of these bacterial collagen-like proteins have been shown to form stable collagen triple helices (35–39). In this study we chose the well characterized Scl2 protein from *Streptococcus pyogenes* (35, 37, 38, 40). An advantage of this recombinant collagen system is the ease of inserting specific sequences (37, 38, 40). The construct utilized here contains a noncollagenous V domain at the N terminus (trimerization domain) followed by a duplicated CL domain with 78 G*X*Y triplets each (38), yielding a V-CL-CL protein with a triple-helical domain about half the length of collagens I, II, and III. These bacterial proteins lack hydroxyproline, which has been shown to be important for the stability of animal collagens (6, 41). Importantly, despite the absence of hydroxyproline, the V-CL-CL proteins have a $T_m = 36–37$ °C, very similar to the thermal stability of mammalian collagens, and this is due in part to a very high content of charged residues (35, 37, 38).

Here, a new chimeric system is introduced for characterizing collagenase specificity and kinetics, by inserting G*X*Y sequences from the human type III collagenase cleavage site between the two CL domains of the bacterial collagen. The total length of the insert was varied from four to six triplets around the collagenase cleavage site. The original bacterial collagen domain was not cleaved, but after introducing a sufficient length of the type III collagen MMP cleavage site, the chimeric collagens became efficiently cleavable by MMP-1 and MMP-13. Varying the number of tripeptide units inserted made it possible to define the minimum cleaved sequence.

EXPERIMENTAL PROCEDURES

Expression and Purification of Matrix Metalloproteinases—ProMMP-1, proMMP-1Cat, proMMP-3, and proMMP-13Cat were cloned in pET3A, overexpressed in *Escherichia coli* BL21 DE3, and refolded from inclusion bodies, purified, and activated essentially as described (42). Full-length MMP-13 was cloned into pCEP4 with a FLAG tag between the signal and propeptide, and expressed in HEK293 EBNA cells. It was purified from the medium using a FLAG column followed by S-200 gel filtration in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ (TNC buffer). To activate proMMP-13 it was incubated with 1 mM 4-aminophenylmercuric acetate at 37 °C for 1 h, then further purified by gel filtration on an S-200 column in TNC. Activation removed the FLAG tag and yielded wild-type protein.

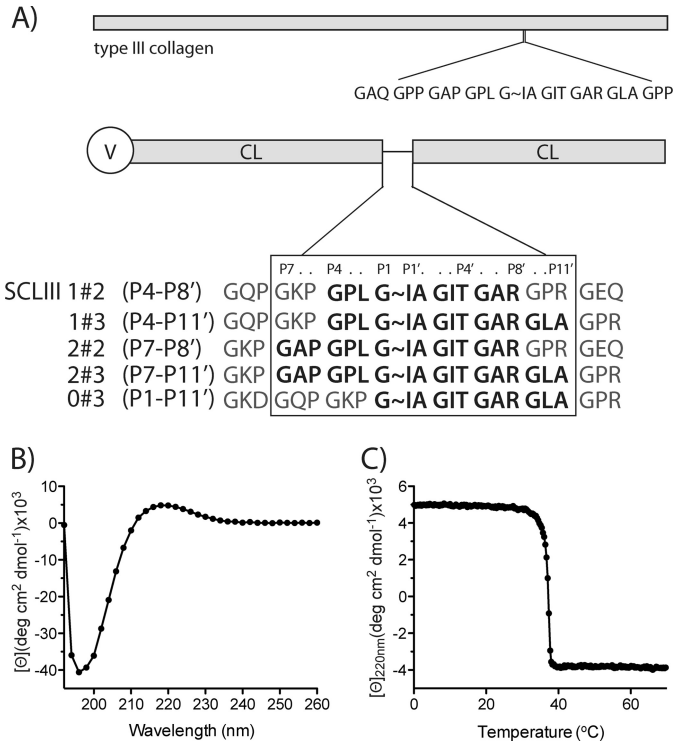

FIGURE 1. **Constructs of bacterial collagen-human collagen III chimeras.** *A*, schematic diagram of type III collagen highlighting its collagenase sensitive sequence (*top*) together with a *diagram* of the bacterial collagen V-CL-CL showing V, the globular domain and CL, the triple-helical domains. Five V-CL-CL constructs were created with insertions of 4–6 Gly-Xaa-Yaa triplets from the collagenase cleavage site of human type III collagen between the two CL units. The 18 residues around the G~I cleavage site in each construct are *boxed*, with the type III sequences (shown in *bold*) flanked by bacterial collagen sequences. The P7-P11′ subsites of the cleavage site are assigned according to the Schechter and Berger protease substrate convention (56). The constructs are named streptococcal collagen-like III (SCLIII), supplemented with numbers of triplets to the N-terminal and C-terminal sides of the triplet containing the collagenase cleavage site (#). For instance, SCLIII1#2 (P4-P8′) indicates a protein containing 1 triplet N-terminal to the G~IA triplet and 2 triplets C-terminal to the G~IA triplet. *B*, CD spectra of the SCLIII2#3 (P7-P11′) construct. The spectra of the other constructs are similar. *C*, CD melting transition of the SCLIII2#3 (P7-P11′) construct. The melting transitions of the other constructs are similar (see Table 1).

Construction and Expression of Recombinant Bacterial-Human Collagen Chimeras—All of the proteins were expressed using the cold shock vector system (43). The five constructs were designed as shown in Fig. 1. The constructs SCLIII1#2 (P4-P8′) with insertion sequence GPL-G~IA-GIT-GAR (# represents the triplet with the cleavage site (~) of collagen III and the figures before and after # indicate the number of triplets N-terminal and C-terminal to the cleavage site triplet, respectively) and SCLIII2#3 with insertion sequence GAP-GPL-G~IA-GIT-GAR-GLA (P7-P11′) were obtained by inserting the annealed oligonucleotides between the DNA sequence of the two CL domains of the V-CL-CL protein, using the *SmaI* and *ApaI* restriction sites. The inserted MMP cleavage sites are flanked by bacterial CL sequences GKD-GKD-GQP-GKP at the

N terminus and GPR-GEQ-GPT-GPT at the C terminus. SCLIII2#2 (P7-P8′) and SCLIII1#3 (P4-P11′) were constructed by adding one tripeptide GAP at the N-terminal or GLA at the C-terminal end of SCLIII1#2, respectively. The construct SCLIII0#3 (P1-P11′) was obtained by deleting one tripeptide GPL from the N terminus of SCL1#3. All of the constructs were confirmed by DNA sequencing and then transformed into *E. coli* BL21.

Purification of Recombinant Bacterial-Human Chimeric Collagens—A single colony of *E. coli* BL21 cells containing the corresponding plasmid was inoculated into 5 ml of M9-casamino acid medium with 50 μg/ml ampicillin. The culture was shaken at 37 °C for 16 h and transferred into 250 ml of M9-casamino acid medium and shaken at 37 °C until A_{600} reached 0.8. 1 mM isopropyl 1-thio-β-D-galactopyranoside was added to induce protein expression, and the cells were shaken at room temperature. After overnight culture, the cells were centrifuged at 4 °C and resuspended in 25 ml of lysis buffer (20 mM sodium phosphate and 500 mM NaCl, pH 7.4), followed by cell disruption using a French press. The cell lysate was centrifuged in a Sorvall RC6+ refrigerated superspeed centrifuge (Thermal Scientific, Amarillo, TX) at 11,500 rpm (using an F21-8x50Y rotor) for 20 min at 4 °C. The supernatant was centrifuged in an Optima™ L-90 Ultracentrifuge (Beckman Coulter) at 45,000 rpm (using a 50 Ti rotor) for 1 h at 4 °C. The supernatant was applied to a nickel-nitrilotriacetic acid-agarose column (25 ml) at room temperature. After washing the column with wash buffer (20 mM $NaPO_4$, 500 mM NaCl, 20 mM imidazole, pH 7.4), buffers with increasing concentrations of imidazole (50, 100, 125, and 400 mM) were used to stepwise elute the proteins. The purity was determined by SDS-PAGE, and the concentration was measured by absorbance at 280 nm with ϵ = 9129 $M^{-1}\cdot cm^{-1}$.

Circular Dichroism (CD) Spectroscopy—CD data were obtained using an Aviv model 62DS spectropolarimeter (Aviv Associates Inc., Lakewood, NJ). Before CD scanning, proteins were kept in 1-mm cuvettes at 4 °C for at least 24 h. CD spectra were collected from 195 to 260 nm at 0.5-nm intervals with an averaging time of 5 s, and each scan was repeated three times to obtain an average. The thermal transition was obtained by monitoring the CD signal at 220 nm with increasing temperatures from 0 to 60 °C in 0.33 °C steps. Proteins were equilibrated at each temperature point for 2 min, and the temperature was increased with an average rate of 0.1 °C/min.

MMP Cleavage—The substrates were dialyzed against 20 mM Tris-HCl, pH 7.5, with 150 mM NaCl. $CaCl_2$ was added to 10 mM, and the substrates (20 μM) were incubated with 2 nM MMPs (MMP-1, MMP-13, MMP-1Cat, MMP-13Cat, or MMP-3) at 25, 30, or 35 °C for different lengths of time. Adding EDTA to a final concentration of 25 mM stopped the reaction, and then the samples were analyzed by SDS-PAGE.

Identification of MMP-1 Cleavage Site by Mass Spectrometry—SCL2#3 was digested with 2 nM MMP-1 and analyzed using a Bruker MicroFlex MALDI-TOF mass spectrometer to define the cleavage site.

Trypsin Digestion—Purified bacterial collagens (20 μM) in Tris buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3) were incubated with 0.01 mg/ml (430 nM) trypsin at 25 °C for 1, 2.5, 4, and 6.5 h and overnight. The reaction was stopped by adding phenylmethylsulfonyl fluoride (PMSF) to 1 mM, and then the samples were run on an SDS-PAGE.

Kinetics Measurement Assays—Each substrate at a concentration of 2, 4, 7, 14 or 20 μM was incubated in TNC buffer, and 0.02% NaN_3 with 2 nM MMP-1 or MMP-13, for 5, 10, 20, 30, and 60 min, respectively. The reaction was stopped by adding EDTA to 20 mM. The samples were then applied on SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Gels were scanned and the collagen degradation assessed by densitometry; the initial velocity of collagen degradation was calculated from the linear section of the plot. Initial velocity of the reaction (the number of collagen molecules degraded/min) was plotted against substrate concentration to obtain typical substrate saturation curve (Michaelis-Menten kinetics). A plot of substrate *versus* rate of cleavage was fitted to the equation rate = V_{max}*[S]/(K_m + rate) using GraphPad Prism (GraphPad Software) to calculate V_{max} and K_m, with the standard error obtained from the curve fit; k_{cat} was calculated from V_{max}.

RESULTS

Insertion of Collagenase Cleavage Site of Human Type III Collagen into Bacterial Collagen Triple Helix—Five recombinant molecules (SCLIIIs) were created by inserting sections of 4–6 G*X*Y triplets (12–18 residues) containing the human type III collagen MMP cleavage site between the two bacterial CL domains (see Fig. 1*A* for a schematic representation). The chimeric proteins were expressed in *E. coli* and purified. The CD spectra of all of the constructs showed a maximum at approximately 220 nm ($MRE_{220\ nm}$ = ~5,000 deg cm^2 $dmol^{-1}$) and a minimum at around 198 nm ($MRE_{198\ nm}$ = ~80,000 deg cm^2 $dmol^{-1}$), indicating the presence of a typical triple-helical structure (44). The CD spectrum of SCLIII2#3 (P7-P11′) is shown as a representative example in Fig. 1*B*. A sharp thermal transition was observed with a melting temperature (T_m) of around 36–37 °C (Fig. 1*C*), which was identical to that of V-CL-CL (38) and just slightly lower than that of type III collagen (45, 46). The CD spectra and thermal transitions of these chimeric proteins together indicated that they all form stable triple-helical structures (see Table 1).

Cleavage of SCLIII Chimeras by MMP-1 and MMP-13—The wild-type bacterial collagen V-CL-CL without any insertion of type III collagen sequences was not cleaved by MMP-1 or MMP-13 (2 nM) at 25 °C (Fig. 2*A*). Incubation at much higher concentrations (up to 600 nM) at 25 °C overnight with MMP-1, MMP-13, MMP-3, MMP-1Cat, MMP-13Cat, and MMP-3Cat also led to no significant cleavage, but denatured V-CL-CL (gelatin) was degraded (data not shown). This indicated that V-CL-CL can serve as a suitable nonreactive scaffold for the introduction of human collagen sequences to investigate the requirements for collagenase cleavage in native mammalian fibrillar collagens. To test this possibility we chose type III collagen because it is a homotrimeric collagen and therefore better suited than type I collagen for a homotrimer recombinant expression system. In addition, among three interstitial colla-

TABLE 1

T_m values of SCLIIIs and kinetic parameters for cleavage by MMP-1 and MMP-13

Enzyme kinetic analyses were carried out at 25 °C.

Collagen	T_m	Collagenase cleavage	MMP-1			MMP-13		
			k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
	°C		s^{-1}	μM	$\mu M^{-1} \cdot s^{-1}$	s^{-1}	μM	$\mu M^{-1} \cdot s^{-1}$
SCLIII1#2	36.9	–						
SCLIII1#3	36.5	+	1.2 ± 0.3	32 ± 12	36,100	0.22 ± 0.05	28 ± 10	7,760
SCLIII2#2	36.5	–						
SCLIII2#3	36.4	+	1.4 ± 0.2	15 ± 4	93,000	0.32 ± 0.07	14 ± 5	22,600
SCLIII0#3	36.3	–						
Human collagen III	37–39	+[a]	0.15[a]	1.4[a]	112,000[a]			
Recombinant collagen III		+[b]	0.08[b]	1.3[b]	59,000[b]			

[a] From Welgus *et al.* (26).
[b] Expressed in *P. pastoris*, from Williams and Olsen (31).

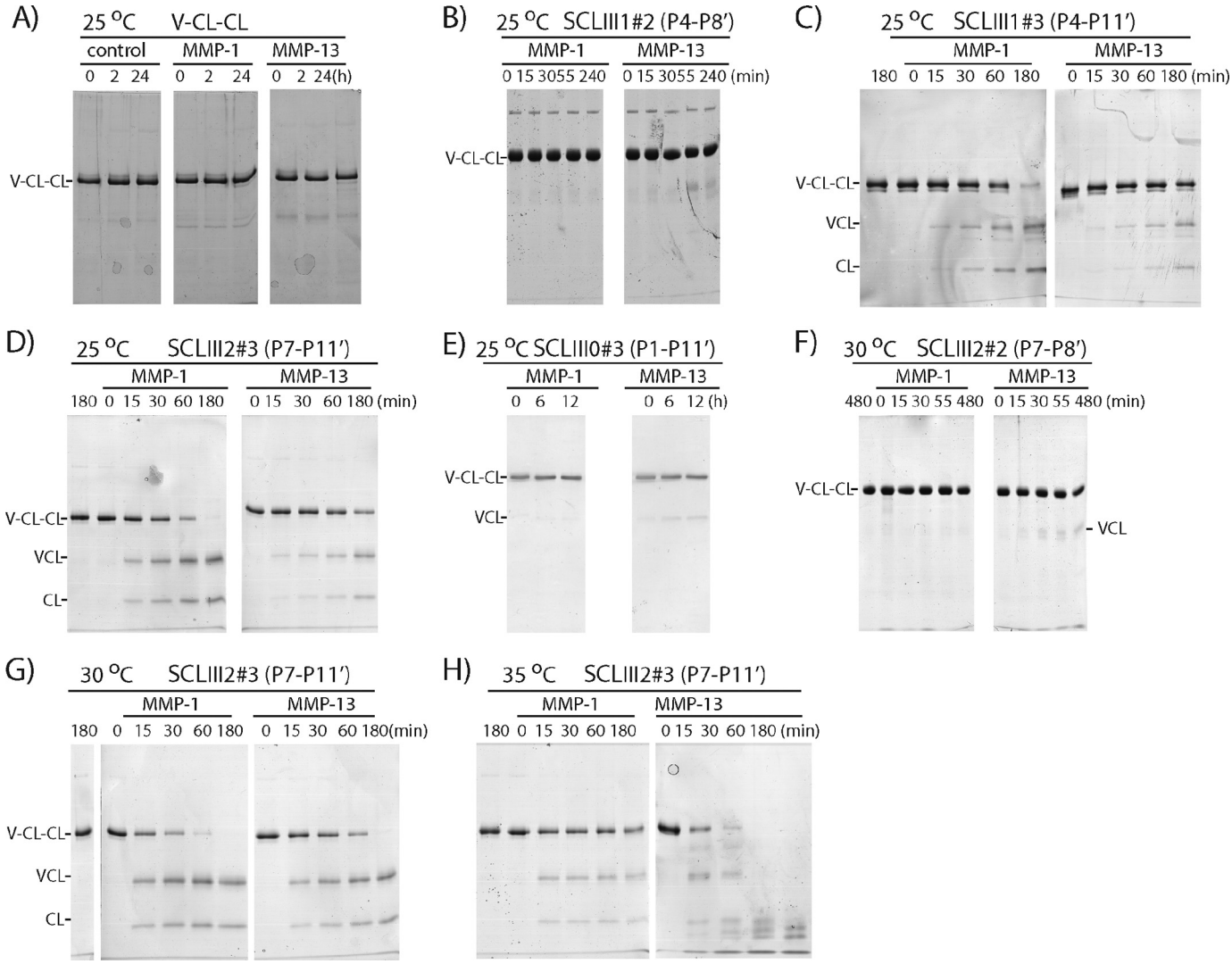

FIGURE 2. **Digestion of the SCLIII substrates with MMP-1 and MMP-13.** *A–E*, time course of the digestion at 25 °C of V-CL-CL (*A*), SCLIII1#2 (P4-P8′) (*B*), SCLIII1#3 (P4-P11′) (*C*), SCLIII2#3 (P7-P11′) (*D*), and SCLIII0#3 (P1-P11′) (*E*) by 2 nM MMP-1 or MMP-13 for the indicated periods of time. *F* and *G*, digestion at 30 °C of SCLIII2#2 (P7-P8′) (*F*) and SCLIII2#3 (P7-P11′) (*G*) with 2 nM MMP-1, MMP-13. *H*, digestion of SCLIII2#3 (P7-P11′) with 2 nM MMP-1, MMP-13 at 35 °C.

gen types it was most readily cleaved by MMP-1 (26), and it was cleaved by MMP-13 less efficiently (29).

The SCLIII1#2 (P4-P8′) consisting of one triplet at the N-terminal side (designated by numbers, without primes) and two triplets at the C-terminal side (designated with primes on numbers) of the triplet with the collagenase cleavage site (designated as #) was not cleaved by MMP-1 or MMP-13 (Fig. 2*B*). One triplet extension at the N-terminal side, SCLIII2#2 (P7-P8′) did not make it susceptible to either collagenase (data not shown). However, the chimeras with three triplets at the C-terminal side, and one or two triplets at the N-terminal side SCLIII1#3 (P4-P11′) and SCLIII2#3 (P7-P11′) were cleaved by MMP-1 and MMP-13 and converted to 33-kDa and 23-kDa fragments corresponding to the V-CL and CL fragments (Fig. 2, *C* and *D*).

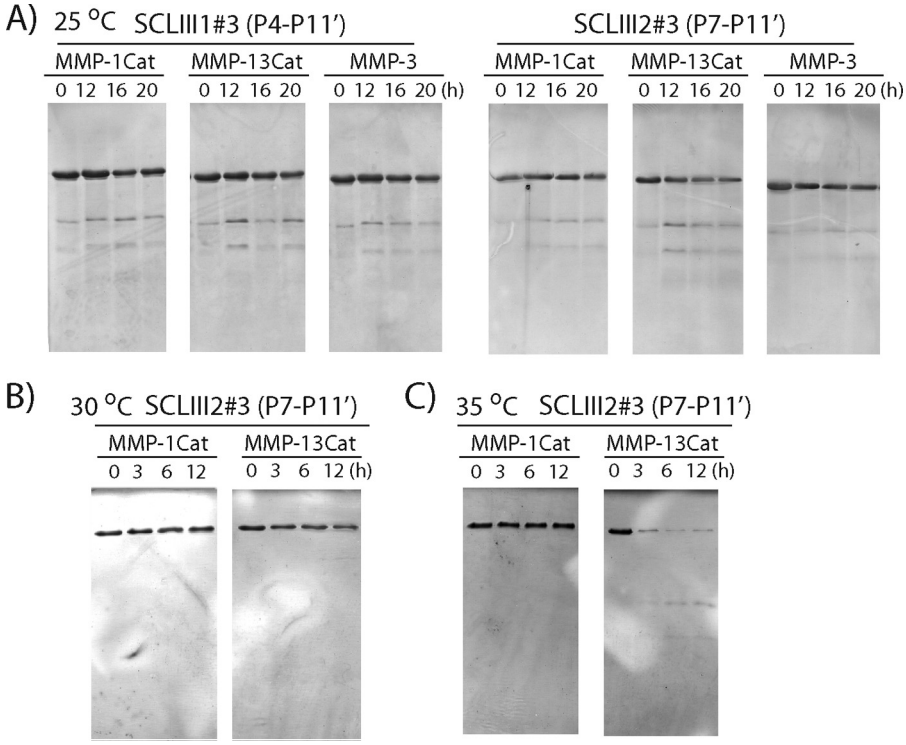

FIGURE 3. **MMP-1Cat, MMP-13Cat, and MMP-3 do not cleave SCLIII2#3.** *A*, SCLIII2#3 (P7-P11′) was digested with 2 nM MMP-13Cat, MMP-3Cat, and MMP-3 at 25 °C for the indicated times. The *weak bands* below V-CL-CL are not cleavage products of MMP-1Cat, MMP-13Cat, or MMP-3 because incubation with these enzymes up to 20 h did not increase the intensity of these bands. These bands likely result from denatured substrate being cleaved during the purification process. *B* and *C*, SCLIII2#3 (P7-P11′) digested with 2 nM MMP-1Cat, MMP-13Cat at 30 °C (*B*) and at 35 °C (*C*).

On the other hand, SCLIII0#3 (P1-P11′), which lacked a triplet N-terminal to the collagenase cleavage triplet, was not cleaved by MMP-1 and very slowly by MMP-13 (Fig. 2*E*). These results indicated that both MMP-1 and MMP-13 require extended sequences around the collagenase cleavage site including the GPL (P4-P2) triplet N-terminal and the GLA (P9′-P11′) three triplets C-terminal to the collagenase cleavage site which were essential for recognition by the two collagenases.

The three chimeras, SCLIII1#2 (P4-P8′), SCLIII2#2 (P7-P8′), and SCLIII0#3 (P1-P11′), that were not cleaved at 25 °C by MMP-1 and MMP-13 still did not show any significant cleavage at 30 °C. For example, at 30 °C, the SCLIII2#2 (P7-P8′) protein was not cleaved by MMP-1 and very slowly cleaved by MMP-13 (Fig. 2*F*). The SCLIII2#3 (P7-P11′) chimera, which is susceptible to cleavage, was degraded more rapidly at 30 °C than at 25 °C by MMP-1 and MMP-13 (Fig. 2*G*). However, at 35 °C MMP-13 degraded this substrate faster and into smaller fragments, whereas MMP-1 still yielded only two fragments and showed slower digestion compared with cleavage at 30 °C (Fig. 2*H*). The trend at 30 and 35 °C for the other cleavable chimera, SCLIII1#3 (P4-P11′), was comparable with SCLIII2#3 (P7-P11′) (data not shown). These results indicated that at 35 °C the triple-helical structure around the cleavage site had considerably loosened or the substrate had at least partly denatured at this temperature close to the T_m. MMP-13, which has a stronger gelatinolytic activity, completely degraded the substrate, whereas MMP-1, which has a weaker activity on gelatin compared with collagen, showed reduced overall activity (Fig. 2*H*) (42, 47). At 35 °C SCLIII1#2 (P4-P8′) and SCLIII2#2 (P7-P8′) were completely degraded by MMP-13 but not MMP-1, again indicating local denaturation of the substrates (data not shown).

Catalytic Domains of MMP-1 and MMP-13 Do Not Cleave SCLIII Chimeras—Full-length MMP-1 and MMP-13 cleaved native collagens efficiently, but the catalytic (Cat) domains of these did not (11, 42). We therefore tested whether the Cat domains of MMP-1 (MMP-1Cat) and MMP-13 (MMP-13Cat) could cleave SCLIII1#3 (P4-P11′) and SCLIII2#3 (P7-P11′) at 25 °C. We also tested full-length MMP-3 (stromelysin 1) which did not cleave collagen types I and II but had a weak activity against type III collagen (48). As shown in Fig. 3*A*, all three enzymes failed to cleave the chimeric SCLIIIs which were susceptible to full-length MMP-1 and MMP-13. At 30 °C, both MMP-1Cat and MMP-13Cat failed to cleave SCLIII2#3. At 35 °C, the temperature close to the T_m of the chimeras, MMP-1Cat did not exhibit any activity, but SCLIII2#3 was completely degraded by MMP-13Cat. Both MMP-13Cat and full-length MMP-13 degraded SCLIII2#3 at 35 °C. These observations were consistent with the substrate being partly denatured at this temperature.

Trypsin Cleavage of SCLIII Chimeras—The native collagen triple helix is highly resistant to trypsin digestion (7), whereas denatured collagen is very susceptible. However, one unique site with a significant trypsin sensitivity was observed in human type III collagen, at the peptide bond following an Arg residue located two triplets C-terminal to the MMP cleavage site (G∼IAGITGAR↓GLA) (18). To investigate whether this unique GAR site maintained trypsin sensitivity in the chimeric constructs, all recombinant proteins were incubated with 10 μg/ml (430 nM) trypsin at 25 °C for different lengths of time

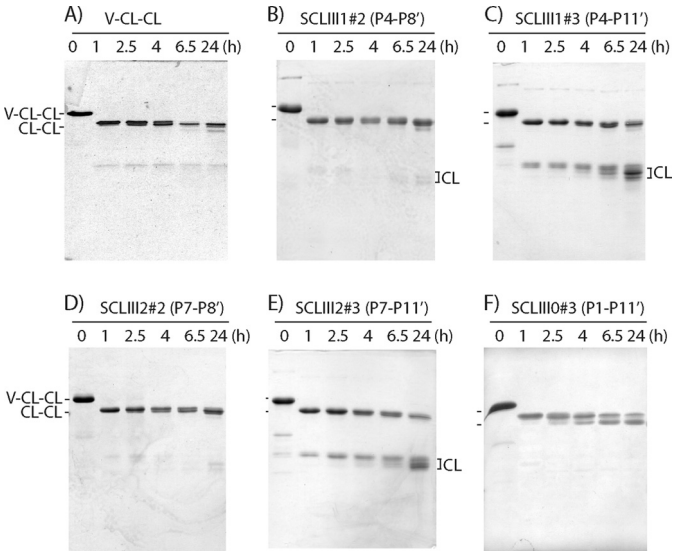

FIGURE 4. **Trypsin digestion of SCLIII chimeras.** V-CL-CL (*A*), SCLIII1#2 (P4-P8′) (*B*), SCLIII1#3 (P4-P11′) (*C*), SCLIII2#2 (P7-P8′) (*D*), SCLIII2#3 (P7-P11′) (*E*), and SCLIII0#3 (P1-P11′) (*F*) were incubated with 430 nM trypsin at 25 °C for up to 24 h, and the products were analyzed by SDS-PAGE. In *A*, the *weak bands* below V-CL-CL remained unchanged with incubation time and were not seen in all preparations, suggesting that CL bands generated by a trace amount of V-CL-CL partially unfolded in the central region. The small decrease in total CL-CL intensity with long incubation times was not seen in all preparations, suggesting that the parent protein V-CL-CL is largely resistant to trypsin. In *C* and *E*, the *weak bands* seen below the control V-CL-CL were likely generated by small amounts of SCLIII1#3 or SCLIII2#3 cleaved between V-CL and CL during protein purification. The multiple *bands* at CL-CL or CL positions seen with increasing digestion times in all lanes are consistent with trypsin susceptibility of fraying ends.

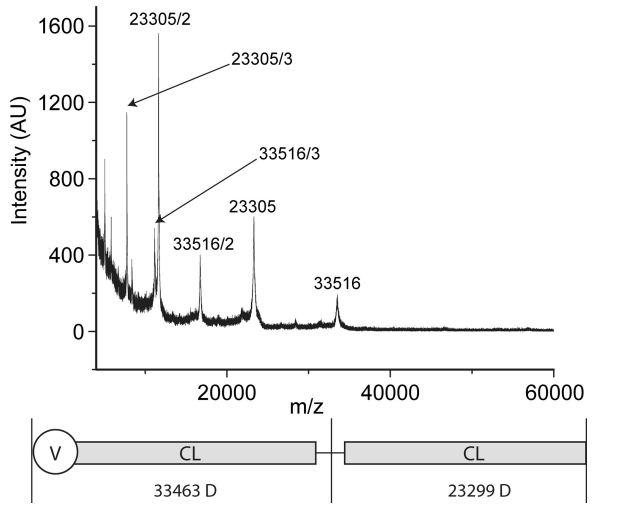

FIGURE 5. **Identification of the collagenase cleavage site of SCLIII2#3 by mass spectrometry.** Mass spectrum of 20 μM SCLIII2#3 (P7-P11′) treated with 2 nM MMP-1 at 25 °C for 3 h indicates that the molecular masses of the V-CL and CL was 33,516 Da and 23,305 Da, respectively. The theoretical molecular masses of the products with MMP-1 cleaving the Gly-Ile bond are 33,463 and 23,299 Da, respectively.

(Fig. 4). At this high trypsin concentration at 25 °C, type III collagen was slowly cleaved, comparable with that reported by Miller *et al.* (18).

Trypsin digestion of the parent V-CL-CL protein and all chimeric constructs resulted in removal of the V domain, as a specific proteolytically susceptible sequence had been inserted between the V and CL domains (38) (Fig. 4). The parent protein and constructs with inserted 1#2 (P4-P8′) or 2#2 (P7-P8′) sequences were largely resistant to trypsin (Fig. 4, *A*, B, and *D*). The chimeras that included the GITGARGLA sequence on the C-terminal primed side in SCLIII1#3 (P4-P11′) and SCLIII2#3 (P7-P11′) were cleaved by trypsin to generate CL fragments which increased with digestion time (Fig. 4, *C* and *E*). However, the sensitivity to trypsin was moderate because after 24 h about one third of the substrate remains intact (Fig. 4, *C* and E). These results showed that the addition of the GLA (P9′-P11′) triplet was essential to convey trypsin sensitivity. Interestingly, the removal of the N-terminal GPL triplet from SCLIII1#3 (P4-P11′) *i.e.* in SCLIII0#3 (P1-P11′), abrogated the trypsin sensitivity (Fig. 4*F*). These results indicated that trypsin cleavage occurs between the CL domains only when a sufficiently long type III sequence around the MMP cleavage site had been introduced, suggesting that the local collagen structure within the triple helix is important for trypsin sensitivity. Interestingly, the trypsin sensitivity directly correlated with the ability of collagenases to cleave the chimeras. The trypsin action, however, was very moderate.

Identification of MMP-1 Cleavage Site in SCLIII2#3—To identify the cleavage site in SCLIII2#3 this substrate was incubated with MMP-1, and the products were analyzed by MALDI-TOF mass spectrometry. The predicted molecular mass of the cleaved V-CL fragment was 33,463 Da and of the C-terminal CL fragment 23,299 Da. Mass spectrometry of 2#3 (P7-P11′) following digestion with MMP-1 showed two fragments of 33,516 Da and 23,305 Da, respectively, very close to the expected size, with no sign of other cleavage products (Fig. 5). The mass spectrometry data confirm that the collagenase cleavage occurred exclusively at the expected G∼I (P1-P1′) site within the type III collagen sequence inserted in V-CL-CL.

Kinetic Analysis of SCLIII1#3 and SCLIII2#3 with MMP-1 and MMP-13—Kinetic analysis was done for the two constructs SCLIII1#3 and SCLIII2#3, which were found to be susceptible to MMP-1 and MMP-13 (Fig. 6), and the parameters are summarized in Table 1. The K_m values of MMP-1 and MMP-13 for SCLIII1#3 were 32 and 28 μM, indicating that their affinity for the two collagenases is similar. The affinity of the two collagenases for SCLIII 2#3 was also similar, but inclusion of the P4-P2 triplet improves the substrate affinity about 2-fold (K_m ∼14–15 μM). The length of the type III collagen sequence inserted (1#3 *versus* 2#3) did not have much effect on the k_{cat} values for a given enzyme (*e.g.* 1.3 s^{-1} for SCLIII1#3 *versus* 1.4 s^{-1} for SCLIII2#3 in the case of MMP-1), but MMP-1 catalyzed both substrates approximately 5-fold faster than MMP-13. This difference in k_{cat} is reflected in the 5-fold higher specificity parameter k_{cat}/K_m for MMP-1 compared with MMP-13.

DISCUSSION

Recombinant chimeric proteins of a bacterial collagen fused with the MMP cleavage site sequences of human type III collagen are proven to be a good system to study collagenase cleavage specificity and kinetics. The triple-helical length of the chimeric collagens is about half that of fibrillar collagens, but they exhibited a thermal stability similar to that of human collagens. Because the original bacterial collagen domain cannot be cleaved by MMP collagenases, the system gives a clean back-

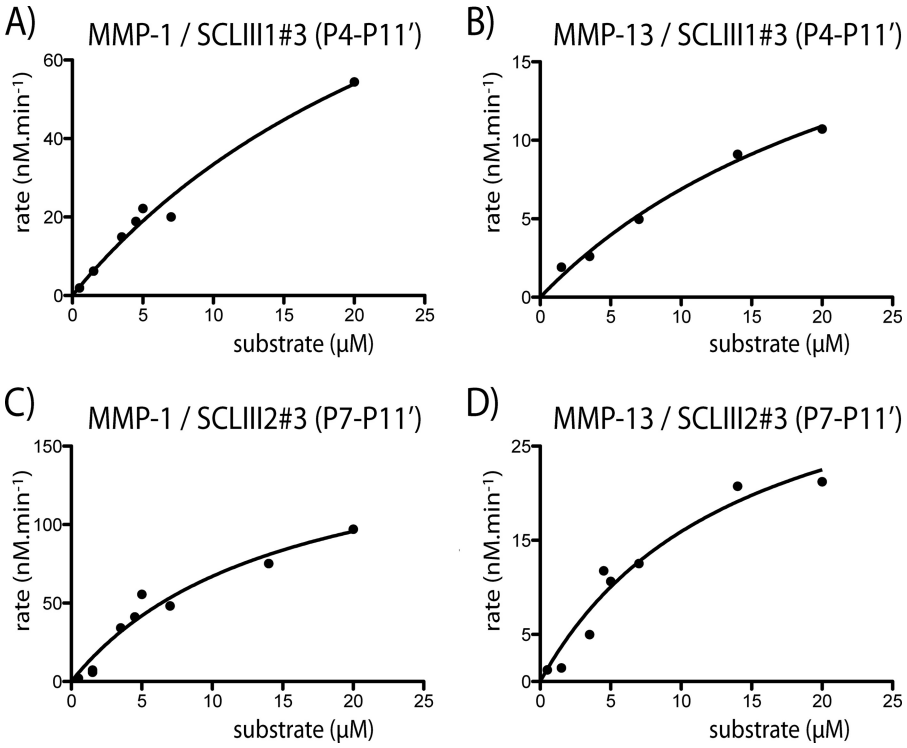

FIGURE 6. **Kinetics analysis of SCLIII1#3 and SCLIII2#3 with MMP-1 and MMP-13.** Various concentrations of each substrate were reacted with MMP-1 or MMP-13 at 25 °C at pH 7.5, and the initial rate of substrate hydrolysis was measured after analysis of the fragments on SDS-PAGE. MMP-1 (*A*) and MMP-13 (*B*), with SCLIII1#3 (P4-P11′); MMP-1 (*C*) and MMP-13 (*D*) with SCLIII2#3 (P7-P11′) are shown. Calculated data are in Table 1.

ground for probing the collagenase susceptibility of introduced sequences. Although the bacterial collagen sequence is more charged than human collagen and lacks hydroxyproline, the incorporation of five triplets from type III collagen made it possible to be specifically recognized and cleaved by human collagenases. This is consistent with the importance of the basic triple-helical structure for enzyme activity and indicates that the amino acid composition outside of the recognition region is not critical for collagenolysis as long as appropriate triple helix stability is present.

The minimal inserted sequence that MMP-1 and MMP-13 could cleave was GPL G∼IA GIT GAR GLA (designated SCLIII1#3; P4-P11′), which includes 4 residues N-terminal to the cleavage site (nonprimed side) and 11 C-terminal (primed side). Sequences shortened by one triplet from either N-terminal or C-terminal end were no longer cleaved by collagenases, and this could not be improved by raising the temperature, indicating that the inability to cleave is related directly to the inserted sequence. Inclusion of an additional GAP (P7-P5) triplet N-terminal to the cleavage site, *i.e.* GAP GPL G∼IA GIT GAR GLA (SCLIII2#3; P7-P11′), further increased the susceptibility to collagenases approximately 2-fold for both enzymes. The increase is solely due to the improvement in affinity of both enzymes for the substrate. This suggests that the two collagenases have similarly extended substrate binding sites in both the primed and nonprimed subsites. SCLIII1#3 has a GKP triplet from bacterial collagen directly N-terminal to the inserted type III collagen sequence, in the same position as GAP (P7-P5) in SCLIII2#3. This suggests that either the Ala is important, or the absence of Lys in this position improves cleavage. Xiao *et al.* (49) recently compared collagenase cleavage sites using triple-helical peptides and proposed that the signature sequences for collagenase sensitivity are, in addition to the cleavable G∼[I/L]A triplet two imino acid-containing triplets (P7-P2), on the N-terminal side and two non-imino acid-containing triplets on the C-terminal side (P3′-P8′) of the cleaved triplet. Proline at position P3 in the GPL (P4-P2) triplet was considered to be particularly important, contributing to the accessibility of P1′ Ile for cleavage (49). The data with bacterial collagen chimeras agree with the importance of the P3 Pro residue for collagenase cleavage. Removal of the triplet with this residue in SCLIII0#3 (P1-P11′) abrogated collagenase cleavage completely. This may be due to an introduction of GKP from bacterial collagen in the P4-P2 subsites. Having Pro in the P2 subsite instead of the P3 subsite may not be compatible with the substrate binding site of collagenolytic MMPs. The neighboring imino acid-containing triplet GAP (P7-P5) seems less important in the bacterial collagen-mammalian collagen chimeric system. It was found that the presence of this triplet improves collagenase sensitivity but it was not essential; SCLIII2#3 (P7-P11′) improved the k_{cat}/K_m only at most 2.9-fold over SCLIII1#3 (P4-P11′). Our study also underlines the importance of C-terminal residues distal from the MMP cleavage site. It clearly demonstrates the requirement for the inclusion of P9′-P11′ (GLA) as the SCLIII1#3 (P4-P11′) chimera was cleaved by MMP-1 and MMP-13, but SCLIII1#2 (P4-P8′) was not. By contrast, the study by Xiao *et al.* (49) with a triple-helical peptide showed MMP-1 cleavage for the P4-P8′ type III collagen sequence which was flanked by four N-terminal GPO triplets and five C-terminal GPO triplets, but the activity was low (reported k_{cat}/K_m value = 1250 M^{-1} s^{-1} at 20 °C). A later study by Robichaud *et al.* (50), using more extended fluorescently

quenched triple-helical peptide substrates, showed a considerable improvement of the MMP-1 activity, but not MMP-13, when the substrate was extended with primed collagen II sequence from P9′-P17′ (reported k_{cat}/K_m value = 37,000 M^{-1} s^{-1} at 27 °C). The activity observed is similar to what we observe here for MMP-1 with SCLIII1#3 (k_{cat}/K_m value = 36,000 M^{-1} s^{-1} at 25 °C). In our system, a further addition of a triplet on the nonprimed side increased the k_{cat}/K_m values 2.6-fold for MMP-1 and 2.9-fold for MMP-13. In the case of the triple-helical peptide substrate, the extension of the nonprime sequence improved catalysis observed with MMP-13, but not MMP-1 (50). These discrepancies may have arisen from the use of different collagen type sequences (type III *versus* type II).

Recent NMR studies of a triple-helical collagen peptide and MMP-1 indicate the interaction of the P10′ Leu with the hemopexin domain of MMP-1 (51). We have also observed the importance of the P10′ residue for MMP-1 binding by collagen peptide binding studies and x-ray crystallography of the MMP-1(E200A) collagen peptide complex.[5] However, P10′ Leu may be less important for MMP-13 as the inclusion of type II P9′-P17′ sequence, which replaces P10′ Pro with P10′ Leu, in a fluorescent triple-helical peptide did not improve cleavage (50).

The selectivity of the cleavage only at the expected G∼I site in the bacterial chimeric system is noteworthy. When recombinant type III collagen was expressed in a strain of *Pichia pastoris* that co-expresses human prolyl-4-hydroxylase, additional cleavage by MMP-1 was observed between P3′ and P4′ (31).

When the GPL G∼IA GIT GAR GLA (P4-P11′) sequence required for collagenase cleavage was inserted into the bacterial collagen, this also led to mild susceptibility of the triple helix to trypsin at the GAR site. An unusual sensitivity to trypsin at this single site in type III collagen was initially reported by Miller *et al.* (18) and indicates that the triple helix is locally more relaxed here than at the other potential cleavage sites. The crystal structure of a triple-helical peptide spanning this region shows that the imino acid-poor region is more relaxed and has a different symmetry than the imino acid-rich flanking regions (52). Recent triple-helical peptide NMR studies suggest that local instability seems to occur in the leading strand, driven by the P3 Pro residue (49). Computational analysis of this region indicates that this region samples distinct sets of local unfolding (23). All of these results are consistent with a local relaxation of the triple-helical structure near the MMP cleavage site, which is likely to play a role in its susceptibility to collagenase.

The importance of the GLA (P9′-P11′) sequence in contributing to this relaxed structure is clear. In the SCLIII1#2 (P4-P8′) and SCLIII2#2 (P7-P8′) constructs, the bacterial collagen triplet C-terminal to the type III sequence insert is GPR. The presence of an imino acid in this triplet must present a tighter helix content leading to the inability of trypsin to cleave these constructs. However, SCLIII0#3 (P1-P11′) which contains G∼IA GIT GAR GLA was not susceptible to trypsin, suggesting that there are requirements on the N terminus as well for this local relaxation that are quite distal from the trypsin cleavage site. It should be reiterated that the trypsin sensitivity is very weak, and prolonged incubation with a high trypsin concentration only resulted in partial cleavage. Under the conditions used, k_{cat} is estimated to be about 2 SCLIII substrate molecules cleaved per trypsin per hour. Interestingly, the type III inserts that show trypsin sensitivity are also sensitive to collagenase cleavage, whereas those that are not sensitive to trypsin are not cleaved by collagenases, a correlation noted previously for type III collagens from different animals (26). The MMP-1 catalytic domain alone does not cleave the substrate at 25 or 30 °C. This indicates that the local loosening of the triple helix itself is not enough to allow proficient cleavage by MMP-1Cat. It indicates that there are additional requirements that are likely mediated by the hemopexin domain, or the Cat and Hpx domain cooperatively, when it binds to collagen, leading to additional changes in the collagen structure that ultimately leads to cleavage of the G∼I peptide bond as we have proposed previously (21).

Human type III collagen expressed in *P. pastoris* has K_m values similar to those of tissue-extracted collagen III, but the k_{cat} values are half that of tissue-extracted collagen III (31). The kinetic data of SCLIII 2#3 (P7-P11′) indicated that the k_{cat}/K_m of this substrate is similar to that of human type III collagen (26), although the K_m value is about 10-fold higher than that of type III collagen and so are the k_{cat} values. The reason for this discrepancy is not clear. One contributing factor could be differences between proline and hydroxyproline (Pro of the GAP triplet (P7-P5) in SCLIII *versus* hydroxyproline in type III collagen). In addition, the coverage of the triplets introduced in bacterial collagen may not be sufficient, and there may be sequences responsible for specificity of different MMPs outside the minimally essential cleavage site. The absence of hydroxyproline and the presence of charged bulky amino acids forming salt-bridges in the flanking triple helical sequence of the bacterial collagen could also be a factor. The highly charged nature of bacterial collagens may also have an effect on MMP processing. The difference between MMP-1 and MMP-13 cleavage of the chimeric bacterial collagens is 5-fold based on the kinetic data. It is known that type III is more susceptible to MMP-1 than MMP-13 (29), but comparative kinetic studies on mature type III collagen degradation by these two enzymes are not available at the moment. It will be interesting to insert longer sequences outside the essential sequence, especially P12-P17′ from type III collagen, to test whether the different kinetic parameters for MMP-1 and MMP-13 cleavage could be observed using the bacterial collagen system.

The bacterial-human collagen chimeras clearly have the triple-helical integrity needed to lead to specificity in collagenase cleavage, as MMP-3 and the MMP-1Cat and MMP-13Cat domains fail to cleave it, whereas synthetic triple-helical peptides are cleaved by the Cat domains of collagenases albeit with reduced activity (32, 53, 54) and longer peptides are less efficiently cleaved by Cat domains compared with shorter peptides (55). This system is a new triple-helical substrate model for collagen cleavage by collagenases. The results presented here clearly confirm the extended nature of the triple helix site for MMP-1 and MMP-13 cleavage. It shows that this sequence inherently has a relaxed triple-helical structure and is likely important for binding of the Hpx domain. The easy manipulation of this system will make it very useful to further delineate

[5] S. W. Manka, F. Carafoli, R. Visse, D. Bihan, N. Raynal, R. W. Farndale, G. Murphy, J. J. Enghild, E. Hohenester, and H. Nagase, submitted.

the relative importance of individual residues in the extended collagenase binding and cleavage site.

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