The Role of the C-terminal Domain of Human Collagenase-3 (MMP-13) in the Activation of Procollagenase-3, Substrate Specificity, and Tissue Inhibitor of Metalloproteinase Interaction

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Recombinant human procollagenase-3 and a C-terminal truncated form (Δ249–451 procollagenase-3) have been stably expressed in myeloma cells and purified. The truncated proenzyme could be processed by aminophenylmercuric acetate via a short-lived intermediate form (N-terminal Leu58) to the final active form (N-terminal Tyr65). The kinetics of activation were not affected by removal of the hemopexin-like C-terminal domain. The specific activities of both collagenase-3 and Δ249–451 collagenase-3 were found to be similar using two quenched fluorescent substrates, but Δ249–451 collagenase-3 failed to cleave native triple helical collagens (types I and II) into characteristic one- and three-quar-...
proenzyme (9), which was earlier demonstrated for the two other human collagenases, MMP-1 and MMP-8 (10–13). Cellular activation of procollagenase-3 could also be demonstrated using cancanavalin A-stimulated fibroblast monolayers, which was shown to be dependent on the activity of MT1-MMP and/or gelatinase A. This mechanism is unique within the collagenase subfamily of MMPs since MMP-1 and MMP-8 were not affected (8).

The catalytic domain of human collagenase-3, which contains the catalytic zinc-binding site, has 55% sequence similarity to the catalytic domains of human MMP-1 and MMP-8. The catalytic domains of these enzymes are all known to consist of a five-stranded β-sheet structure containing three α-helices in a typical sequential order (14-17). The C-terminal domain of MMPs shows homology to hemopexin and vitronectin and is of particular interest in the case of the collagenases MMP-1, MMP-8, and MMP-13 since this domain is essential for their specific triple helicase (collagenolytic) activity (6, 18–23). In the case of MMP-1 (17) and MMP-13 (24), x-ray crystallographic data have shown that this domain displays a four-bladed β-propeller structure that is linked via a short hinge sequence motif to the catalytic domain.

Detailed analyses of fibroblast and neutrophil collagenases in relation to their domain organization are well advanced (18, 21, 22), but there are currently no data available regarding the functions of the collagenase-3 domains. We have expressed full-length procollagenase-3 and a C-terminal deletion mutant (∆249–451 procollagenase-3) and assessed the role of the C-terminal domain of collagenase-3 in relation to activation of the proenzyme, substrate specificity, and TIMP binding.

EXPERIMENTAL PROCEDURES

Materials—The digoxigenin antibody labeling kit was purchased from Boehringer (Mannheim, Germany). All other chemicals were obtained from Sigma and were the purest grade available. Collagens were generously donated by Drs. M. Barnes, K. Kühn, E. Aubert-Foucher, J.-J. Wu, and D. R. Eyre.

Generation of ∆249–451 Procollagenase-3 cDNA—Expression and Purification of the Deletion Mutant and Wild-type Procollagenase-3, and Activation of Proenzymes—The procollagenase-3 cDNA in pSP64 was cleaved with XcmI and EcoRI, which removed the complete C-terminal domain. The purified cleaved plasmid was ligated to the oligonucleotide 5′-CTCTAGGTTAAG-3′ and 5′-AATTTCCTAACCATAGAG-3′, thereby introducing a stop codon following the codon for glycine 248, which was followed by an EcoRI site. The ∆249–451 procollagenase-3 cDNA was sequenced using the dyeoxy method, which confirmed its identity, except for replacement of the C-terminal coding sequences with the BamHI stop codon. The construct was subcloned into the HindIII and EcoRI sites of the mammalian expression vector pEE12. The NaeI-linearized ∆249–451 procollagenase-3 cDNA in pEE12 (50 μg) was transfected into NSo mouse myeloma cells by electroporation. Stable clones were selected through growth in glutamine-free medium as described above for procollagenase-3 and ∆249–451 procollagenase-3. The reaction products were analyzed by silver-stained SDS-PAGE. Three different type IV collagen preparations (10 μg each) were cleaved with 200 ng of wild-type collagenase-3 or 90 ng of ∆249–451 collagenase-3 for 16 h at 25 °C, followed by analysis of the reaction products by silver-stained SDS-PAGE. The concentrations of the active enzymes were estimated by titration against TIMP-1 of known concentration (25). The initial rate of substrate hydrolysis (v) axis) was plotted versus TIMP-1 concentration (x axis), and the active enzyme concentration was determined from the x axis intercept. The kinetics of the inhibition of active full-length collagenase-3 and active ∆249–451 collagenase-3 by wild-type and mutant TIMPs were analyzed by evaluation of the second-order rate constant (kobs) (30).

Determination of TIMP Binding—The concentrations of the active enzymes were estimated by titration against TIMP-1 of known concentration (25). The initial rate of substrate hydrolysis (v) axis) was plotted versus TIMP-1 concentration (x axis), and the active enzyme concentration was determined from the x axis intercept. The kinetics of the inhibition of active full-length collagenase-3 and active ∆249–451 collagenase-3 by wild-type and mutant TIMPs were analyzed by evaluation of the second-order rate constant (kobs) (30).

Determination of TIMP Binding of Procollagenase-3, Active Collagenase-3, and Active ∆249–451 Collagenase-3 to Type I Collagen Films—Full-length procollagenase-3 and ∆249–451 procollagenase-3 were labeled with digoxigenin using a digoxigenin-3-O-succi-nyl-γ-aminocaproic acid-N-hydroxysuccinimide ester according to the manufacturer’s instructions (DIG antibody labeling kit, Boehringer), except that phosphate-buffered saline was replaced with 50 mM HEPES (pH 7.6), 10 mM CaCl2, 150 mM NaCl, and 0.04% Tween 20. It was verified that labeling had no effect on latency and enzymatic activity following APMA activation using the quenched fluorescence substrate assay. The binding of these labeled enzymes to type I collagen was determined using a modification of the enzyme-linked immunosorbent assay method described by Murphy et al. (18). Rat skin type 1 collagen (0.5 mg/ml in phosphate-buffered saline) was plated at 50 μl/well onto a 98-well microtiter plate and allowed to form fibrils before drying at 37 °C. The films were washed three times with Tris-HCl, 10 mM Tris-HCl, 150 mM NaCl, and 0.02% Tween 20 and then blocked in 100 mM Tris (pH 7.4), 150 mM NaCl, 1% bovine serum albumin, 2% nonfat dry milk, and 0.1% Triton X-100 (blocking buffer) for 20 min at room temperature. Latent or active enzymes were applied in blocking buffer (100 μl/well) and incubated at 4 °C for 12 h, and then unbound enzymes were removed by washing. Bound enzymes were detected and quantified using anti-digoxigenin polyclonal antibody Fab fragments conjugated to alkaline phosphatase (Boehringer; 1:2000 in blocking buffer for 1 ha t1 5 ° C ) followed by analysis of the degradation products by silver-stained SDS-PAGE. The final concentration was determined from the absorbance at 405 nm was measured after stopping the reaction with NaOH.
RESULTS

Autoproteolytic Fragmentation of Active Full-length Collagenase-3—Studies of the stability of active collagenase-3 at 37 °C showed that fragmentation occurred, as observed for other members of the MMP family. The enzyme was cleaved into two major fragments, a doublet of M, 29,000, which is due to N-glycosylation, and M, 27,000 (Fig. 1). These represent the catalytic domain as revealed by gelatin zymography (data not shown; doublet of M, 29,000) and the C-terminal domain (M, 27,000) of collagenase-3. N-terminal sequence determination showed that the Ser^{245}–Leu^{248} peptide bond had been autoproteolytically hydrolyzed, thereby releasing the C-terminal domain (Fig. 2). The initial specific collagenolytic activity of 100 μg/min/nmol was lost completely, indicating that the C-terminal domain was essential for the collagenolytic activity of the enzyme.

The catalytic domain of collagenase-3 was also further processed internally, releasing 79 amino acid residues from the initial N-terminal Tyr^{85}. This second fragmentation product displayed the N-terminal sequence LLAHAFPPG, which was the result of the hydrolysis of the Gly^{164}–Leu^{165} peptide bond (Fig. 2). During autoproteolysis, the enzymatic activity versus Mca-PLGL-Dpa-AR-NH₂ decreased to 20% of the initial value (after 16 h) and further declined upon prolonged incubation (>24 h) until no proteolytic activity was measurable, which we deduced was due to cleavage of the Gly^{164}–Leu^{165} peptide bond within the catalytic domain. It was therefore not possible to use the original fragmentation products for analysis of the enzymatic and TIMP binding properties of the catalytic domain of collagenase-3; hence, we produced an intact C-terminal deletion mutant (Δ_{249–451} procollagenase-3) by protein engineering.

Purification of Δ_{249–451} Procollagenase-3, Activation by APMA, and Comparison of the Proteolytic Activity with Wild-type Collagenase-3 against Interstitial Collagens—Δ_{249–451} procollagenase-3 was expressed by NSO cells and purified from the resulting conditioned medium. The recombinant deletion mutant (Δ_{249–451} procollagenase-3) was analyzed by SDS-PAGE and shown to occur as a single band of M, 35,000 (Fig. 3A, lane 1) relative to the M, 60,000 (Fig. 3B, lane 1) for full-length procollagenase-3. The discrepancy between the calculated M, of Δ_{249–451} procollagenase-3 of 25,000 and the observed M, of 35,000 is due to N-glycosylation as earlier demonstrated for the full-length proenzyme (6). No proteolytic activity was detectable prior to Δ_{249–451} procollagenase-3 activation, indicating that the proenzyme was correctly folded. Furthermore, N-terminal sequence analysis confirmed that Δ_{249–451} procollagenase-3 displayed the sequence LPLPSGGD, which was earlier demonstrated for the wild-type proenzyme (6).

Activation by APMA treatment at 37 °C for 30 min resulted in autoproteolytic processing of Δ_{249–451} procollagenase-3 and wild-type procollagenase-3 through one detectable intermediate of M, 28,000 (Fig. 3A, lane 2) and 50,000 (Fig. 3B, lane 2), respectively, to the final active forms of ~24,000 (Fig. 3A, lane 3) and 48,000 (Fig. 3B, lane 3). N-terminal sequence data confirmed that Δ_{249–451} procollagenase-3 was processed via a short-lived intermediate form displaying the sequence LEVTGK that was converted to the final active form through hydrolysis of the Glu^{146}–Tyr^{155} peptide bond. These data confirm that Δ_{249–451} procollagenase-3 was processed in an identical manner as earlier described for wild-type procollagenase-3 (6). Our sequence analysis of active Δ_{249–451} collagenase-3 furthermore confirmed that the enzyme was N-glycosylated at Asn due to a lack of a signal during amino acid sequencing.

Activity measurements using the synthetic peptide substrate Mca-PLGL-Dpa-AR-NH₂ demonstrated the rapid generation of enzymatic activity, which reached a plateau after 30 min and declined to 20% of the initial maximal activity after 16 h at 37 °C (data not shown). This suggests that the secondary cleavage at Gly^{164}–Leu^{165} described for wild-type procollagenase-3 also occurred in the case of active Δ_{249–451} collagenase-3. The k_{cat}/K_{m} values of optimally activated enzymes (30 min at 37 °C) were determined after the enzyme concentrations had been determined by active-site titration using TIMP-1 and gave similar values for both enzymes (Table I). In contrast, Δ_{249–451} collagenase-3 showed no detectable triple helicase activity.

![Image](url1)

**Fig. 1.** Molecular weight determination of the collagenase-3 fragmentation products using SDS-PAGE. The relative molecular weights of the collagenase-3 fragmentation products were determined by SDS-PAGE under reducing conditions (lane 1). The positions of the catalytic and C-terminal domains are indicated on the right. Molecular weight markers are indicated on the left. Only the upper band (doublet at M, 29,000) displayed gelatinolytic activity as assessed by gelatin zymography (data not shown) and therefore corresponds to the catalytic domain.

![Image](url2)

**Fig. 2.** N-terminal amino acid sequence analysis of the fragmentation of active collagenase-3. The fragmentation products of active collagenase-3 were purified by reverse-phase HPLC and analyzed by N-terminal amino acid sequence determination. The cleavage sites are indicated by arrows.

**Autoproteolytic fragmentation of active collagenase-3**

![Diagram](url3)
when incubated with type I or II collagen (Fig. 4A, lanes 2 and 5). It is noteworthy, however, that Δ249–451 collagenase-3 cleaved the β1,2(I) chains of type I collagen, generating α1,2(I) chains that were somewhat smaller than the α1,2(I) chains of the native substrate (Fig. 4B, lane 1). This indicates that the catalytic domain of collagenase-3 is an efficient telopeptidase that is not dependent on the presence of the C-terminal domain and does not require binding of the enzyme to the substrate (see below). This is a unique feature of collagenase-3 since MMP-1 and MMP-8 do not display any significant telopeptidase activity. In the case of type III collagen, partial hydrolysis (10%) by Δ249–451 collagenase-3 was observed, which generated fragments corresponding to one- and three-quarters in size (data not shown). This was due to nonspecific susceptibility of a part of the type III collagen preparation to hydrolysis since identical products were generated by gelatinase B (data not shown). However, if the collagens were heat-denatured prior to cleavage, Δ249–451 collagenase-3 degraded these with the same efficiency as the full-length enzyme (data not shown).

Collagen binding experiments revealed that labeled procollagenase-3 and active collagenase-3 bound to type I collagen films, while Δ249–451 procollagenase-3 and active Δ249–451 collagenase-3 did not bind (Fig. 5). Thus, binding was clearly promoted by the C-terminal domain of the enzyme. The binding experiments were performed over a wide range of concentrations, and saturation was reached at ~200 nM enzyme binding to the substrate. The assay revealed that the active enzyme bound marginally better than procollagenase-3. The binding of the proform of collagenase-3 to type I collagen was somewhat unexpected since the two other human collagenases bind only as active enzymes to type I collagen. To confirm these data, the amount of bound enzymes was also quantitated by activity measurements using the quenched fluorescence substrate Mca-PLGL-Dpa-AR-NH₂ after the proenzyme had been activated by APMA treatment (data not shown), and the results showed that both the active enzyme and the proenzyme bound with comparable efficacy. However, the amount of bound active enzyme was marginally higher compared with the proenzyme, and this was consistent over a wide range of bound enzyme concentrations (50–400 nM) (see Fig. 5).
C-terminal Domain Function of Collagenase-3

Fig. 5. Determination of structural features of collagenase-3 responsible for binding to type I collagen fibrils. Binding experiments of procollagenase-3, active collagenase-3, Δ249–451 procollagenase-3, and active Δ249–451 collagenase-3 to type I collagen fibrils were performed at 4 °C for 12 h using digoxigenin-labeled enzymes. Bound enzymes were detected using anti-digoxigenin antibodies and were quantified by enzyme-linked immunosorbent assay. ○, procollagenase-3; □, active collagenase-3; ▲, Δ249–451 procollagenase-3; ▼, active Δ249–451 collagenase-3.

Fig. 6. Degradation of the large TN-C isoform by collagenase-3 and Δ249–451 collagenase-3. APMA-activated Δ249–451 collagenase-3 and collagenase-3 were incubated with the large TN-C isoform for 8 h at 37 °C, followed by analysis of the cleavage products by silver-stained SDS-PAGE (reducing conditions). Lane 1, large TN-C cleaved by Δ249–451 collagenase-3; lane 2, large TN-C cleaved by collagenase-3; lane 3, large TN-C in the presence of buffer. Molecular weight markers are indicated on the left.

Fig. 7. Degradation of type IV collagen by collagenase-3 and Δ249–451 collagenase-3. The APMA-activated enzymes were incubated with mouse type IV collagen at 25 °C for 16 h. The cleavage products were analyzed by Coomassie Blue-stained SDS-PAGE (reducing conditions). Lane 1, type IV collagen cleaved by collagenase-3; lane 2, type IV collagen cleaved by Δ249–451 collagenase-3; lane 3, type IV collagen in the presence of buffer. The positions of the α1(IV) and α2(IV) chains are indicated on the right. The corresponding cleavage products are indicated by arrows. Molecular weight markers are indicated on the left.

repeats. The C-terminal domain of collagenase-3 had no influence on the rate of TN-C hydrolysis, indicating that specificity is mediated by the catalytic domain alone.

Purified human plasma FN and recombinant FN fragments were hydrolyzed by both enzymes with equal efficiency (data not shown). Intact plasma FN was cleaved into four main fragments with Mr values of 100,000, 43,000, 35,000, and 29,000. In contrast, the recombinant Mr 120,000 FN fragment was hydrolyzed into smaller fragments with Mr values of 48,000, 46,000, 35,000, 32,000, and 28,000, while the Mr 110,000 FN fragment was cleaved into fragments with Mr values of 100,000, 38,000, and 36,000.

Wild-type and Δ249–451 collagenase-3 were able to hydrolyze type IV collagen into fragments, and hydrolysis even proceeded at 25 °C. Both the α2(IV) chain (Mr 247,000) and the α1(IV) chain (Mr 217,500) were cleaved as observed by analysis of the reaction products under reducing conditions. The major cleavage products displayed Mr values of 240,500 and 80,000, respectively (Fig. 7). Under nonreducing conditions, a single cleavage product of Mr 76,000 was visualized (data not shown). In comparison, human gelatinase A was not able to hydrolyze type IV collagen under identical conditions and cleaved only at elevated temperatures (data not shown and Ref. 31). These data indicate that collagenase-3 may play a considerable role in the dissolution of type IV collagen, a major component of the basement membrane.

Furthermore, collagenase-3 and Δ249–451 collagenase-3 cleaved native type XIV collagen with comparable kinetics (data not shown). When the cleavage products were compared with those generated by gelatinase B, a different cleavage pattern was observed. The major cleavage product generated by collagenase-3 or Δ249–451 collagenase-3 showed a Mr of 165,000, being considerably smaller than the NC3 domain of type XIV collagen (Fig. 8). A minor smaller cleavage product of Mr 110,000 was also generated; and furthermore, the type XIV collagen dimer was partially degraded, yielding a product that retained the reduction-resistant disulfide bridge of the NC2 domain.

The minor cartilage collagens (types IX, X, and XI) were also analyzed in cleavage experiments using collagenase-3 or Δ249–451 collagenase-3. Type X collagen was susceptible to collagenase-3 and Δ249–451 collagenase-3, generating a Mr 48,000 fragment (Fig. 9). Type IX collagen was also susceptible to cleavage by collagenase-3 and Δ249–451 collagenase-3, although the differences between cleaved and noncleaved type IX collagens were only very subtle and were dependent on the species source of the substrate under investigation. The intact α3(IX) and α1(IX) chains of bovine type IX collagen were reduced from Mr 175,000 to 170,000, while the low molecular weight Col1 domain, which is a result of pepsin extraction, was resistant. In contrast, rat type IX collagen showed reverse susceptibility, i.e., the high molecular weight α1(IX) chain was resistant, while the low molecular weight Col1 domain was cleaved (Mr 52,000 compared with 53,000 for the noncleaved material). It is furthermore noteworthy that the higher molecular weight material (>175,000) in both type IX collagen preparations was cleaved (Fig. 9). In contrast, the α1(XI), α2(XI), and α3(XI) chains were completely resistant to both enzymes.
Degradation of native bovine type XIV collagen by collagenase-3 and Δ249–451 collagenase-3. APMA-activated Δ249–451 collagenase-3 and collagenase-3 were incubated with bovine type XIV collagen for 14 h at 25 °C. The cleavage products were analyzed by Coomassie Blue-stained SDS-PAGE (reducing conditions). Lane 1, type XIV collagen cleaved by collagenase-3; lane 2, type XIV collagen cleaved by Δ249–451 collagenase-3; lane 3, type XIV collagen in the presence of buffer. The positions of the type XIV collagen dimer and monomer are indicated by arrows. The position of the NC3 domain generated by cleavage with gelatinase B is indicated on the right.

Degradation of native type IX, X, and XI collagens by collagenase-3 and Δ249–451 collagenase-3. Type IX, X, and XI collagens were incubated for 16 h at 25 °C with active Δ249–451 collagenase-3 and collagenase-3. Lane 1, bovine type XI collagen incubated in the presence of collagenase-3; lane 2, bovine type XI collagen incubated in the presence of Δ249–451 collagenase-3; lane 3, bovine type XI collagen in the presence of buffer; lane 4, bovine type IX collagen cleaved by collagenase-3; lane 5, bovine type IX collagen cleaved by Δ249–451 collagenase-3; lane 6, bovine type IX collagen in the presence of buffer; lane 7, rat type IX and X collagens cleaved by collagenase-3; lane 8, rat type IX and X collagens cleaved by Δ249–451 collagenase-3; lane 9, rat type IX and X collagens in the presence of buffer. The positions of the α2(IX) and α1(IX) chains are indicated on the right (high molecular weight material). The α2(IX) chain contains chondroitin and dermatan sulfate hybrid glycosaminoglycan and is therefore normally not visible by SDS-PAGE. The position of the Co1 domain of type IX collagen generated by pepsin digestion is indicated on the right. The positions of the α1(XI), α2(XI), and α3(XI) chains are indicated on the left.

Kinetic Analysis of the Inhibition of Wild-type Collagenase-3 and Δ249–451 Collagenase-3 by Wild-type and Mutant TIMPs—Active site titrations of collagenase-3 and Δ249–451 collagenase-3 with wild-type and mutant TIMPs revealed a stoichiometry of 1:1 (data not shown). It is currently not possible to determine accurate $K_i$ values for collagenase-3 and Δ249–451 collagenase-3 interactions with wild-type and mutant TIMPs due to the tight binding nature of their interaction, with appropriate $K_i$ values well below 200 pM. This is due to the limitations in assay sensitivity, substrate solubility, and fluorescence quenching at high substrate concentrations. Furthermore, the enzymes are unstable at low concentrations, which does not allow analyses during long-term assays. It is possible, however, to determine the apparent first-order rate constant ($k_{on}$) at low reagent concentrations (50 ps enzymes, 0.06–2 nM inhibitors) from the analysis of the curvature in the progress of substrate cleavage. At these low concentrations, inhibition can be treated as “slow binding” as recently discussed (30). The second-order rate constant ($k_{on}$) can be calculated for the interaction of TIMPs with collagenase-3 and Δ249–451 collagenase-3; the data are summarized in Table II (30).

The data presented clearly demonstrate that the C-terminal domain of collagenase-3 contributed significantly to the binding of wild-type TIMP-1 and TIMP-3 and the chimeric inhibitor N.TIMP-2/C.TIMP-1 since the association rates of TIMP-1, TIMP-3, and N.TIMP-2/C.TIMP-1 with Δ249–451 collagenase-3 were significantly reduced (17–33 times slower). In contrast, TIMP-2, N.TIMP-1/C.TIMP-2, and Δ127–194 TIMP-2 showed $k_{on}$ values of 0.3–1.8 × 10^8 M⁻¹ s⁻¹ with either full-length collagenase-3 or Δ249–451 collagenase-3 and thus are not significantly affected by C-terminal domain interactions with the enzyme. The increase in the association rate of collagenase-3/TIMP complexes was also shown to be mediated by the C-terminal domain of the inhibitor since the $k_{on}$ values for the interaction of full-length collagenase-3 with Δ127–194 TIMP-1 and Δ128–194 TIMP-2 were in the range of those obtained with Δ249–451 collagenase-3 and full-length TIMPs. In contrast, the C-terminal domain of TIMP-2 does not contribute significantly to complex formation between enzyme and inhibitor, which is applicable to the charged tail deletion mutant Δ187–194 TIMP-2. The N-terminal domains of both enzyme and inhibitor lead to association rates of 3–8.1 × 10^6 M⁻¹ s⁻¹; and it can therefore be concluded that the N-terminal domain interactions are the major contributors of complex formation between collagenase-3 and TIMPs.

**DISCUSSION**

We have determined the autoproteolytic fragmentation sites in collagenase-3 and assessed the function of the C-terminal domain of the molecule in activation, substrate specificity, and inhibitor interaction. Since collagenase-3 has been implicated in the pathologies of breast cancer and osteoarthritis, we have also examined the ability of collagenase-3 and Δ249–451 collagenase-3 to degrade different components of the extracellular matrix in order to evaluate the possible function of the enzyme in vivo.

Active collagenase-3 is not stable, and autoproteolytic cleavage was observed at the Ser²⁴⁵–Leu²⁴⁶ peptide bond, which is localized at the end of the catalytic domain, thereby releasing the C-terminal domain including the complete hinge sequence motif. Similar autoproteolytic cleavages have been observed earlier for the two other human collagenases (MMP-1 and MMP-8), although these undergo fragmentation farther downstream from the catalytic domain within the hinge sequence motif (19, 23). MMP-1 is hydrolyzed at the Pro²⁵⁰–Ile²⁵¹ locus and MMP-8 at the Glu²⁴²–Leu²⁴³ or Pro²⁴⁷–Ile²⁴⁸ locus, indicating similarities between the three collagenases, but clearly demonstrating differences in their precise autoproteolytic processing. The peptide bonds hydrolyzed by all three enzymes resemble typical collagenase cleavage sites, and autoproteolytic fragmentation could be inhibited by complex for-

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**Table II**

| Inhibitor          | Collagenase-3 | Δ249–451 collagenase-3 |
|--------------------|---------------|------------------------|
| TIMP-1             | 7.84          | 0.46                   |
| TIMP-2             | 1.41          | 0.81                   |
| TIMP-3             | 10.20         | 0.36                   |
| N.TIMP-1/C.TIMP-2   | 0.90          | 0.76                   |
| N.TIMP-2/C.TIMP-1   | 11.87         | 0.35                   |
| Δ127–194 TIMP-2     | 1.80          | 0.69                   |
| Δ127–194 TIMP-1     | 0.32          | 0.50                   |
| Δ128–194 TIMP-2     | 0.29          | 0.32                   |
The catalytic domain of collagenase-3 generated by autoproteolytic fragmentation was itself unstable, and further autoproteolysis was observed by cleavage of the Gly^{164}–Leu^{165} peptide bond, which results in a decrease in the peptidolytic activity to 20 or 0% of the initial value, which was dependent on the incubation time employed. Similar processing has been observed in the case of gelatinase A, although it is not clear whether the activity of the enzyme was affected (32). We have earlier drawn attention to the similarities between collagenase-3 and the gelatinases, and the similarities in autoproteolytic fragmentation of the active enzymes underline our earlier results (6).

To assess the function of the C-terminal domain of collagenase-3, we prepared a recombinant form lacking this domain. Comparison of the activation of wild-type and Δ^{249–451} procollagenase-3 by APMA revealed that the corresponding propeptidases were processed in an indistinguishable way. In addition, activation by the catalytic domain of MT1-MMP was observed for both full-length and Δ^{249–451} procollagenase-3, indicating that these events were not influenced by the C-terminal domain (data not shown). We have previously shown that progelatinase A and a C-terminal deletion mutant were equally well processed by the catalytic domain of MT1-MMP in vitro, indicating that the biochemistry of the reaction is also not influenced by C-terminal domain interactions in solution at high concentrations (33).

We have extended our previous analysis of the substrate specificity of collagenase-3 (6) using a wide range of extracellular matrix proteins and compared the full-length enzyme with the C-terminal deletion mutant in order to determine the role of the C-terminal domain in substrate specificity. Comparison of the enzymatic activities of full-length and Δ^{249–451} collagenase-3 revealed that the collagenolytic activity (triple helicase activity) versus interstitial collagens was mediated by the C-terminal domain, while the gelatinolytic and peptidolytic activities were unchanged. These results confirm earlier observations that the triple helicase activities of the two other human collagenases are dependent on the C-terminal domain (18, 19, 23). We have furthermore established that human collagenase-3 is an efficient telopeptidase and demonstrated that this activity is dependent on its catalytic domain and independent from C-terminal domain interactions. This feature distinguishes collagenase-3 from the two other human collagenases (MMP-1 and MMP-8) since these show no detectable telopeptidase activity. However, an N-telopeptidase activity has been described for the highly homologous rat collagenase (34) using a mutant type I collagen that cannot be cleaved into one- and three-quarter fragments. It was suggested that the N-telopeptidase activity might be sufficient for resorption of type I collagen during embryonic and early adult life, while triple helicase activity is necessary during intense tissue resorption such as observed in the postpartum uterus and in the dermis later in life (34). The telopeptidase activity of collagenase-3 does not require binding of the enzyme to the substrate since Δ^{249–451} collagenase-3 does not bind to triple helical type I collagen (see below), and this activity might be important during bone resorption and cartilage turnover since collagenase-3 is expressed at high levels in these tissues in the human.

We have also demonstrated that the C-terminal domain promotes binding of both procollagenase-3 and active collagenase-3 to type I collagen. Procollagenase-3 and active collagenase-3 bound nearly equally well to triple helical type I collagen, with the amount of active enzyme being marginally higher. Our data are in agreement with earlier data published on the highly homologous mouse collagenase-3 (35). In this case, the proenzyme could be eluted from a type I collagen-Sepharose column at lower salt concentrations than the active form (35), which might indicate that the interaction between the active enzyme and substrate is tighter and in part of ionic nature. The association and dissociation rates for proenzyme and active enzyme binding to triple helical collagen await further detailed analysis. Binding is essential for the triple helicase activity of active collagenase-3, but is not necessary for the telopeptidase activity of the enzyme. In contrast, the two other human collagenases bind only as active enzymes to collagen, while the latent counterparts do not bind to the triple helical substrates (18, 19). From binding data of chimeric N-terminal stromelysin-C-terminal collagenase and N-terminal collagenase-C-terminal stromelysin molecules to fibrillar type I collagen, it is known that binding is promoted by the C-terminal domain of both collagenase and stromelysin (18). However, stromelysin and the chimeric N-terminal collagenase-C-terminal stromelysin mutant also bound as proenzymes, which indicates that the binding motif within the respective C-terminal domain is unmasked in the respective proenzymes, which is also true for procollagenase-3. Furthermore, active stromelysin and the active chimeric proteinases were not able to cleave the triple helical substrates. From further data using a mutant neutrophil collagenase that contained the hinge sequence motif of stromelysin, which is nine amino acid residues longer than the collagenase hinge sequence, it became clear that collagenolysis only proceeds if the connecting “hinge” between the catalytic and C-terminal domains contains the correct number of amino acid residues (22). Thus, the capacity of the collagenases to cleave triple helical substrates obviously depends on the correct interplay between catalytic and C-terminal domains, which appear to be separately folded entities. Our recent x-ray crystallographic analysis of the C-terminal domain of collagenase-3 revealed that the overall structure of this domain shows more similarity to the C-terminal domain of fibroblast collagenase than to gelatinase A, which indicates that those structural features important for efficient triple helicase activity are conserved within the collagenases (24). The triple helicase activity of the collagenases might be coordinated by the hinge sequence motif, but it is not clear how this is mediated specifically. We have noted that none of the residues conserved within the C-terminal domains of the three human collagenases is unique to this subfamily. Recently, Bode (36) suggested that triple helical collagen might be bound between the catalytic and C-terminal domains in such a way that the substrate is bound like a waffle in a waffle iron. The triple helical substrate could be destabilized by this interaction, causing unwinding or relaxing around the cleavable bond. The unwound “single” strands would then be able to fit into the active-site cleft of the molecule, where hydrolysis of each strand would proceed. This hypothesis has been underlined by modeling experiments performed by De Souza et al. (37).

The analysis of the cleavage of the large TN-C isoform, FN, FN fragments, and type IV, IX, X, and XIV collagens by wild-type collagenase-3 and Δ^{249–451} collagenase-3 revealed that these substrates were equally well hydrolyzed, indicating that the C-terminal domain had no affect on specificity. The cleavage products of the large TN-C isoform revealed fragment sizes that were identical to those recently identified for gelatinase A hydrolysis of this extracellular matrix component (38). This indicates that collagenase-3 shows very similar specificity versus large TN-C, with the major cleavage sites being located within the alternatively spliced FN type III repeats, which is confirmed by the resistance of the small TN-C isoform to proteolysis. The increased sensitivity of the large TN-C isoform to degradation could modify its biological activities by unmasking
or abolishing specific functional sites. Thus, a more suitable environment for cellular proliferation and migration may well be established.

Interestingly, collagenase-3 and Δ249–451 collagenase-3 were able to hydrolyze type IV collagen at 25 °C, and these data strongly indicate that collagenase-3 is an efficient type IV collagenolytic enzyme. This observation may have important implications for the pathophysiological role of collagenase-3 during breast cancer pathology, allowing rapid dissolution of the basement membrane, leading to increased ability of tumor cells to extravasate and metastasize. Taking into account that gelatinase A cleaves type IV collagen only at elevated temperatures (≥25 °C) (31), our findings might indeed represent important data for the proteolytic turnover of type IV collagen in vivo.

Type XIV collagen consists of two triple helical domains (Col1 and Col2) interspersed by non-triple helical domains important data for the proteolytic turnover of type IV collagen. Degradation by collagenase-3 and Col1 and Col2, with the cleavage sites being located within the NC2 domain of all three chains (40, 41). Our results suggest that collagenase-3 and Δ249–451 collagenase-3 act as “telopeptidases” on type IX collagen as well as on type I collagen (see above) and type II collagen. Taking into account that collagenase-3 is expressed at high levels in arthritic cartilage and the high specific activity of collagenase-3 on type II collagen, it may indeed be concluded that collagenase-3 contributes considerably to cartilage damage during arthritis. This is furthermore underlined by the fact that aggrecan is cleaved with four times the efficiency of stromelysin-1, which demonstrates that collagenase-3 can hydrolyze the two major cartilage proteins very effectively.

The assessment of the association rates for complex formation of full-length and Δ249–451 collagenase-3 with wild-type and mutant TIMPs revealed that these were affected by N- and C-terminal domain interactions. C-terminal domain interactions were most pronounced in the case of the full-length inhibitors TIMP-3, TIMP-1, and N.TIMP-2/C.TIMP-1, which reacted up to 33 times faster with full-length collagenase-3 than with Δ249–451 collagenase-3. We have recently reported that the association rate constants for complex formation between wild-type gelatinase A and TIMP-1 were increased by a factor of 132 (30). It has been shown that the C-terminal domain and especially the charged tail of TIMP-2 (residues 187–194) are responsible for the formation of a proteolysis A/TIMP-2 complex (30), and this interaction is thought to be vital in the cellular activation of gelatinase A by MT-MMPs. We have recently described the activation of procollagenase-3 in the same system and can only conclude that TIMP-2 is unlikely to be involved in the interaction of procollagenase-3 with the cell surface during activation.

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