Matrix metalloproteinase-2 (MMP-2, gelatinase A) and membrane type (MT)-1-MMP (MMP-14) are cooperative dynamic components of a cell surface proteolytic axis involved in regulating the cellular signaling environment and pericellular collagen homeostasis. Although MT1-MMP exhibits type I collagenolytic but poor gelatinolytic activities, MMP-2 is a potent gelatinase with weak type I collagenolytic behavior. Recombinant linker/hemopexin C domain (LCD) of MT1-MMP binds native type I collagen, blocks MT1-MMP collagenolytic activity in trans, and by circular dichroism spectroscopy, induces localized structural perturbation in the collagen. These changes were reflected by enhanced cleavage of the MT1-LCD-bound collagen by the collagenases MMP-1 and MMP-8 but not by trypsin or MMP-7. Thus, the MT1-LCD alone can initiate triple helicase activity.

In contrast, the native and denatured collagen binding properties of MMP-2 reside in the fibronectin type II modules, accordingly termed the collagen binding domain (CBD). Recombinant CBD (but not the MMP-2 LCD) also changed the circular dichroism spectra leading to increased MMP-1 and -8 cleavage of native collagen. However, recombinant CBD reduced gelatin and collagen cleavage by MMP-2 in trans as did CBD23, which comprises the second and third fibronectin type II modules, but not the CBD25 mutant W316A/W374A, which neither binds gelatin nor collagen. This indicates that MMP-2 and MT1-MMP bind collagen at a different site than MMP-1 and -8. Thus, MMP-2 utilizes the CBD in cis for collagen binding and triple helicase activity, which compensates for the lack of collagen binding by the MMP-2 LCD. Hence, the MMP family has evolved two distinct mechanisms for collagen triple helicase activity using two structurally distinct domains, with triple helicase activity occurring independent of α-chain hydrolysis.
(11), which yields 3/4 and 1/4 fragments that are thermally unstable at body temperature (12, 13). MMP-2 is a potent gelatinase that is believed to be important in the final clearance of degraded and denatured collagen (14, 15) with a controversial role in cleaving native type IV collagen (16–18). However, MMP-2 also has weak native type I collagenase activity in rodents (19, 20), man (5, 21, 22), and on triple helical synthetic peptides (23, 24). The membrane type (MT) MMPs also exhibit collagenase activities (5, 25) with MT1-MMP being considered a critical enzyme of native collagen metabolism (26–28).

The mechanism by which triple helical collagen is cleaved by MMPs has been extensively studied using engineered pro tease, type I collagen, and triple helical peptide analogues. Despite this, it is still far from being understood (29, 30). As the width of the MMP active site cleft (5 Å) (31) cannot accommodate all three α-chains (15 Å diameter) (32), the collagen triple helix must first unwind prior to cleavage in a process termed triple helicase activity. The MMP catalytic domain in isolation cannot cleave triple helical collagen and requires exosites, substrate binding sites outside of the active site cleft, for native collagen cleavage (29). All collagenolytic MMPs contain a hemopexin C-terminal (C) domain, which is linked to the catalytic domain by a flexible linker peptide (10, 29). In the collagenases MMP-1, -8, -13, and -14, the hemopexin C domain binds collagen and is absolutely required for the cleavage of triple helical collagen (5, 33–37). However, soluble collagen has been reported to be cleaved by the isolated MMP-8 catalytic domain at 37 °C (38). As demonstrated for MMP-8 and MMP-14, the linker peptide also plays a role in collagenolysis (39, 40). Interestingly, the MMP-2 hemopexin C domain, does not bind native or denatured type I collagen (41, 42). Instead this functionality is accomplished by the fibronectin type II modules within the MMP-2 catalytic domain which are arranged as a compact collagen binding domain (CBD) (43, 44). Although this domain potentiates gelatin cleavage (18, 22), its role in collagen triple helicase activity is still unclear (29).

In our recent study we reported the critical role of the hemopexin C domain and linker of MT1-MMP in collagen recognition, cleavage and in blocking MT1-MMP and MMP-2 collagenolysis when present as a recombinant domain or natural autolysis product on the cell surface (5). In the present report, using circular dichroism spectroscopy we demonstrate for the first time that the MT1-MMP linker hemopexin C domain and MMP-2 CBD fibronectin type II modules perturb the secondary structure of native type I collagen upon binding. Hence, triple helicase activity is an independent event and separable from collagen α-chain scission. Although this specific interaction cooperatively increased the rate of collagen cleavage by the collagenases MMP-1 and MMP-8 it was not sufficient to allow for collagen cleavage by the noncollagenolytic proteases MMP-7 and trypsin. However, despite the lack of collagen binding by the MMP-2 hemopexin C domain, it was still absolutely required, together with the fibronectin type II modules, for native type I collagen cleavage.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Extraction and purification of rat tail tendon type I collagen; the preparation of biotin-labeled bovine type I skin collagen; and the preparation, purification and characterization of recombinant human MMP hemopexin C domains expressed with the linker (L): MT1-LCD (Gly260-Cys600) and MMP-2 LCD (Gly464-Cys660); or without the linker, MT1-CD (Gly115-Cys508), were previously described (5, 42). Recombinant human MMP-2 collagen binding domain proteins consisting of all three fibronectin type II modules, CBD (Val420,Gln449) (44), or as a N-terminal module 1 deletion, CBD23 (Ala275-Gln290), and the CBD23 mutant, W316A/W374A, are described elsewhere.2 All recombinant domains are numbered from the initiating Met of the full-length enzyme. Human soluble (a) MT1-MMP lacking the stem, transmembrane, and cytoplasmic tail (5), MMP-8,2 and MMP-2 (45) have also been described elsewhere. Human MMP-1 was expressed and purified using the pG-WGH vector kindly provided by British Biotech Pharmaceuticals (Oxford, UK).

**Expression of C-terminally Truncated MMP-2**—The following primers: 5′ (CTCCTATGGGGCTGCCATGCAAGTATGACCCTG) and 3′ (GCCAAGTCTAGTGTCAAGTCGGCCCTACGAG) (target bases are underlined) were used to introduce a STOP codon in pro-MMP-2 (template pGLa/pGWHG) to create a truncated form, N-MMP-2, which lacks the hemopexin C domain (1446–680). The entire DNA sequence was confirmed by sequencing. Stable CHO (Chinese hamster ovary) cell clones were selected using mycophenolic acid as previously described (46) and characterized by Western blotting with rabbit polyclonal antibody α CBD123 (raised against the collagen-binding domain) (44). Roller bottle cultures (850 cm², BD Biosciences) were incubated in 50–100 ml serum-free medium (CHO-S-SFMII, Invitrogen Life Technologies, Inc.) and harvested every 1–2 days for up to 10 days. N-MMP-2 was purified as follows: filtered conditioned medium was passed through 10–15 ml of gelatin-Sepharose 4B equilibrated with column buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM CaCl₂, 0.025% sodium azide, 0.04% Brij 35). After washing with column buffer including 1 mM NaCl, bound proteins were eluted with 10% dimethyl sulfoxide (Me2SO). Peak fractions were pooled, dialyzed into column buffer and passed through a 4-ml column of heparin-Sepharose CL-6B to remove free hematin-hemopexin 2B. The flow-through was loaded onto a small gelatin-Sepharose 4B column (2 ml) and washed and eluted as before. Peak fractions were dialyzed in storage buffer (50 mM Tris-HCl pH 7.8, 10 mM CaCl₂, 0.1 mM NaCl).

**Circular Dichroism**—Collagen and recombinant protein domains were incubated either alone or in combination for 2 h at 28 °C. Samples were transferred to a 1-mm path-length quartz cell and ellipticity (mdeg) was measured from 197 nm to 250 nm using a J810 (Jasco) spectropolarimeter at 28 °C. The CD spectra of native collagen alone were also determined at various temperatures maintained by a computer-controlled Neslab water bath thermal jacket during thermal denaturation. Ellipticity values were averaged between triplicates and plotted against wavelength. CD spectra were measured for proteins alone: native collagen, MT1-LCD, MT1-CD, MMP-2 CBD, MMP-2 LCD, and as mixtures of native collagen with the individual recombinant protein domains. Calculated CD spectra were produced by summing the ellipticity values for collagen and the recombinant domains alone (47) and compared with the measured CD spectra of the corresponding collagen/recombinant domain mixtures to assess the extent of perturbations in collagen secondary structure.

**Enzyme Assays**—Biotin-labeled type I collagen (0.025 pmol) was incubated with MMPs in assay buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM CaCl₂, 3.8 mM NaN₃, 0.05% Brij) for 18 h at 28 °C (5). MMPs were activated with 2 mM APMA for 1 h at 37 °C prior to the addition of substrate. Recombinant protein domains were added for the duration of the assay at various mol ratios of protein:enzyme as indicated. Collagenase assays were terminated with the addition of SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 2.0% urea, 0.05% bromphenol blue) and then analyzed by 7.5% SDS-PAGE and Western blotting using either streptavidin-HRP and ECL detection (Amersham Biosciences) or infrared dye labeled-streptavidin (Molecular Probes) and detection using the Odyssey Infrared Imaging System (LI-COR) at 700 nm. Quenched fluorescent substrates Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH₂ (48) and fluorescein-labeled gelatin (DQ gelatin, Molecular Probes) were incubated with APMA-activated MMP-2 in assay buffer (100 mM Tris-HCL, pH 7.4, 100 mM NaCl, 10 mM CaCl₂, 0.05% Brij) for 1–2 h at 37 °C. Recombinant MMP domains were added for the duration of the experiment as indicated. Fluorescence was measured using the POLARstar Optimma (BMG Labtechnologies) using excitation and emission filters of 320 nm and 405 nm for the Mca-labeled peptide, and 485 nm and 520 nm for DQ gelatin. Substrate cleavage was monitored and reported as arbitrary fluorescence units (units s⁻¹ nm⁻¹).

Metabolically labeled [14C]human type I gelatin (19) was incubated with MMP-2 for 3 h in assay buffer in the presence of MMP-2 LCD, 5% 2 T. R. Moore, E. M. Tam, A. R. Connor, D. Y. Lee, and C. M. Overall, manuscript in preparation.

3 G. Pelman, C. J. Morrison, and C. M. Overall, submitted for publication.

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Comparison of MMP Collagen Triple Helicase Mechanisms

![Figure 1](image)

**Fig. 1. Structural perturbation of native collagen upon binding of recombinant MT1-MMP hemopexin C domain.** A, the measured (---) and calculated (---) CD spectra of native type I collagen (0.02 nmol) in the presence of MT1-LCD (2 nmol, left panel) or MT1-CD (2 nmol, right panel) at 28 °C are shown with ellipticity expressed in millidegrees (mdeg). Calculated spectra of collagen in combination with either MT1-LCD or MT1-CD were generated as described under “Experimental Procedures.” Inset, SDS-PAGE (15%) analysis of recombinant MT1-LCD and MT1-CD (1 μg). B, thermal denaturation of native collagen (5.2 nmol) alone. Spectra were measured at 25, 30, 35, and 40 °C. C, Biotin-labeled collagen (0.025 nmol) was incubated in the absence (C) or presence of collagenases, MMP-1 (0.08 pmol) and MMP-8 (0.03 pmol), for 18 h at 28 °C. MT1-LCD was added at the indicated molar equivalents (1–10) relative to enzyme. D, biotin-labeled collagen (0.025 nmol) was incubated with sMT1-MMP (1 pmol) for 18 h at 28 °C in the presence of buffer, MT1-LCD or MT1-CD at 10 molar equivalents relative to enzyme. Trypsin was incubated with biotin-labeled collagen at the indicated enzyme/substrate ratios for 3 h at 28 °C. Samples were separated by SDS-PAGE (7.5%) followed by Western blotting and enhanced chemiluminescence using streptavidin-HRP. Full-length type I collagen α1(1)-chains (α1, α2), intramolecularly cross-linked α-chains termed β-chains (β), and their 3/4-length cleavage fragments (α1β, α2β, β’β) are indicated.

We have previously shown that the hemopexin C domain of MT1-MMP binds and is required for native collagen cleavage (5). We hypothesized that upon native collagen binding the MT1-MMP hemopexin C domain prepares the collagen for cleavage by localized disruption of the triple helix in the immediate vicinity of the Gly775-Ile/Leu776 cleavage site. To test this, saturating amounts of recombinant MT1-MMP hemopexin C domain with or without the linker (Fig. 1A, inset) were incubated with native type I collagen and changes in protein secondary structure were measured using CD spectroscopy. As shown in Fig. 1A, left panel, a deviation in the spectra was observed in the 199–205 nm region for the collagen/MT1-LCD sample where the measured minimum (203 nm, −45.1 mdeg) was shallower and occurred at a longer wavelength than that of the calculated minimum (202 nm, −48.7 mdeg). Similar spectral changes also occurred upon heat denaturation of native collagen (Fig. 1B). These structural changes were not seen in the collagen/MT1-CD sample (Fig. 1A, right panel) and so are consistent with the inability of MT1-CD compared with MT1-LCD to block MT1-MMP cleavage of collagen (5). Thus these experiments indicate that there are different modes or sites of collagen binding for recombinant MT1-MMP hemopexin C domain with or without the linker.

Given the importance of the hemopexin C domain in MT1-MMP collagenolysis (5), collagen helix perturbation induced by the linker and hemopexin C domain components of full-length MT1-MMP is likely to be an essential component of the triple helicase mechanism. Although the deviation between the measured and calculated spectra predicts a change in protein secondary structure of the collagen/MT1-LCD complex, it does not distinguish in which protein the change occurred. However, the similarity with the change in CD spectra of native collagen upon mild heat denaturation (Fig. 1B) suggests, but does not conclusively show, that the structural alteration originated from or led to perturbations in the structure of collagen. Therefore, as a further indication that the changes occurred in the collagen, we examined its susceptibility to cleavage by two secreted collagenases in the presence of MT1-LCD (Fig. 1C). The absence of collagen degradation by trypsin alone, even at a ratio of 1:10 (enzyme to substrate), confirmed the native structure of the type I collagen in these assays (Fig. 1D). Whereas MT1-MMP activity was blocked by the addition of MT1-LCD (Fig. 1D) as we recently reported (5), the addition of MT1-LCD unexpectedly enhanced collagen cleavage by MMP-1 and MMP-8. This occurred even at low MT1-LCD:enzyme (0.1) mol ratios (Fig. 1C) in a concentration-dependent manner and was particularly apparent when MMP-1 and MMP-8 were incubated at enzyme/substrate ratios that produced minimal cleavage as shown. Hence, the localized disruption of the collagen triple helix by recombinant MT1-LCD rendered it more amenable to cleavage by MMP-1 and -8 in trans in a cooperative manner and shows that triple helicase and cleavage activities are independent, separable events. In the full-length MT1-
MMP, a similar action of the linker and hemopexin C domain in cis is likely the mechanism of triple helicase activity of MT1-MMP. However, when in trans the MT1-LCD blocks MT1-MMP collagen cleavage indicating that the MMP-1 and MMP-8 hemopexin C domains utilize a different binding site which does not compete with MT1-MMP hemopexin C domain binding.

The molecular requirements and domain interactions for MMP-2 cleavage of collagen are not clear. Using recombinant proteins of the MMP-2 fibronectin type II modules (MMP-2 CBD) and the hemopexin C domain (MMP-2 LCD) as competitive inhibitors, we examined the contribution of each in the cleavage of native and denatured type I collagen. As shown in Fig. 2A, addition of MMP-2 CBD reduced the rate of cleavage of internally quenched fluorescein-labeled gelatin by MMP-2 approximately 3-fold compared with the buffer control. These data were confirmed using [14C]-metabolically-labeled human type I gelatin as substrate (Fig. 2B). In contrast, the addition of MMP-2 LCD, MT1-LCD, and MT1-CD had no effect on gelatin cleavage (Fig. 2, A and B). These present results are consistent with the binding properties of these domains we previously reported: MMP-2 CBD binds gelatin and native type I collagen with $K_v$ values in the submicromolar range (44), but neither the MMP-2 LCD (42) nor the MT1-MMP hemopexin LCD or CD (5) domains bind gelatin. Interestingly 5% Me$_2$SO, which is used during biochemical purification to elute MMP-2 bound to gelatin Sepharose by the CBD, only reduced gelatinolysis by $\sim$30% so revealing the intrinsic propensity of the MMP-2 catalytic domain for gelatin cleavage.

To determine the role of the MMP-2 CBD and hemopexin C domain on native type I collagen cleavage, these domains were incubated with collagen and MMP-2 for 18 h. By monitoring the generation of the $\alpha_1^\text{N}$ and $\alpha_2^\text{N}$ fragments we found that recombinant MMP-2 CBD inhibited cleavage of native type I collagen in a concentration-dependent manner whereas the MMP-2 LCD, which does not bind collagen (42), had no effect on cleavage (Fig. 2C). Consistent with this, the cleavage of the collagen $\beta$-components was similarly affected. In comparison to macromolecular substrates, MMP-2 cleavage of the $\alpha_1$ collagen 775-776 cleavage site peptide analogue Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH$_2$ was unaffected by the presence of CBD (Fig. 2D) or MMP-2 LCD (data not shown) in the reactions. This indicates that the exosites on the CBD only potentiate cleavage of macromolecular substrates that can both bind to the CBD and also contact the catalytic center at more N-terminal sites in the bound substrate. Hence, by competitive inhibition experiments, the importance of the collagen binding properties of the MMP-2 exosite domains was demonstrable.

The fibronectin-type II modules both bind and block gelatin and collagen cleavage, whereas the MMP-2 LCD neither binds nor blocks cleavage of gelatin and collagen. Indeed, the CBD (but not the MMP-2 LCD) can quantitatively elute proMMP-2 from collagen and gelatin affinity columns (44). Notably, the collagen binding properties and role of the MMP-2 LCD differs from the MT1-LCD, which binds and blocks native type I collagen cleavage by both MT1-MMP and MMP-2 (5).

Given the similar activities of competitive amounts of exogenous MMP-2 CBD and MT1-LCD proteins in blocking collagen cleavage in trans of the cognate parent proteases, we next investigated whether the fibronectin type II modules of MMP-2 could also perturb the collagen triple helix like MT1-LCD. The CD spectra of native type I collagen/MMP-2 CBD complexes was compared with the calculated spectra generated and analyzed as before (Fig. 3A). A large deviation was observed between the two spectra in the region of 197–227 nm for collagen/MMP-2 CBD (Fig. 3A left panel), where the measured minimum at 198 nm was significantly greater than the corresponding minimum of the calculated spectrum. This general flattening of the absorption curve is similar to that observed for the CD spectra of collagen upon heat denaturation shown here (Fig. 1B) and as previously reported (1). The deviation observed for the collagen/MMP-2 CBD sample is considerably more significant than that seen for MT1-LCD, which may reflect either a different mode of binding or the 2-fold increase in native type I collagen affinity of the MMP-2 CBD (44) compared with MT1-LCD (5). In comparison, there was little or no difference between the measured and calculated CD spectra of collagen/
MMP-2 LCD (Fig. 3A right panel) consistent with the absence of any collagen binding properties of the isolated domain (42).

To probe the biological significance of the secondary structure changes in the collagen/MMP-2 CBD complexes, MMP-1 and MMP-8 were added and incubated for 18 h at 28 °C. As shown in Fig. 3B, MMP-2 CBD binding of collagen enhanced cleavage by both collagenases in trans a concentration-dependent manner. Hence, this suggests that the structural perturbations that occur in the collagen triple helix upon binding the CBD, but not the MMP-2 LCD, is an important component of the triple helicase mechanism of MMP-2. Notably, neither MMP-7 nor trypsin exhibited native collagen cleavage properties in the presence of the MMP-2 CBD or the MT1-LCD (not shown). Since both these enzymes cleave gelatin efficiently, this indicates that upon binding of the exosite domains, the structure of collagen is only perturbed by local denaturation and not completely unwound. In addition, collagen cleavage appears to be only be enhanced in a synergistic manner by proteases that display intrinsic collagen binding and collagenolytic activity. As neither MMP-7 nor trypsin bind collagen, the competitive displacement of the collagen-bound exosite domains by these enzymes is therefore unlikely to occur and so this would restrict access of these proteases to the individual α-chains. Since the CBD had the opposite effect and competitively blocked MMP-2 collagenolysis in trans this also suggests that MMP-1 and MMP-8 bind collagen at a different, but likely nearby, site or sites to that recognized by the CBD.

A hemopexin C domain deleted form of MMP-2 (N-MMP-2) has been previously shown to retain gelatinolytic and type IV collagenolytic activities (49) but not type I collagenase activity (22). Given the effect of the MMP-2 CBD on the structure of collagen and in enhancing collagenolysis by MMP-1 and MMP-8, we investigated whether exogenous MMP-2 CBD could restore the collagenolytic activity of N-MMP-2 in trans. We generated N-MMP-2 and also found that it did not cleave native type I collagen (Fig. 4A) confirming that the MMP-2 hemopexin C domain has a role in triple helical collagen cleavage. However, unlike MMP-1 and MMP-8, the addition of exogenous MMP-2 CBD did not rescue MMP-2 collagenolytic activity, nor did the addition of exogenous MMP-2 LCD. To further explore the mechanics of gelatin degradation we found that the cleavage rate of internally quenched fluorescent-labeled gelatin by N-MMP-2 (Fig. 4B) was comparable with full-length MMP-2 (Fig. 2A), confirming previous reports (41–44) that the hemopexin C domain is not required for gelatinolysis. Also like full-length MMP-2, exogenous MMP-2 CBD effectively reduced gelatin degradation 5-fold (Fig. 4B) confirming the importance of the fibronectin type II modules in gelatin...
binding and cleavage. Hence, these results demonstrate that in
MMP-2 the linker/hemopexin C domain plays a role in collag-
enolysis despite a lack of affinity and ability to induce struc-
tural perturbation in collagen as an isolated domain.

To directly show the importance of the fibronectin type II
modules in MMP-2 gelatinolysis and collagenolysis, we
generated a MMP-2 CBD mutant that does not bind collagen. In
other work we have demonstrated that substitution of Trp316
and Trp374, found at the base of the hydrophobic pit on the
surface of the 2nd and 3rd fibronectin type II modules of
MMP-2, with alanine abrogated gelatin and collagen binding in
each of these modules. Recombinant CBD23 (Ala278-Gln393),
which contains the 2nd and 3rd fibronectin type II modules
only, and the non-collagen binding CBD23 mutant, W316A/
W374A, were compared with recombinant MMP-2 CBD as a
control (which for clarity in describing these experiments is
referred to as CBD123). The recombinant proteins, W316A/
W374A, were incubated with biotin-labeled collagen for 18 h at 28 °C. Samples
were separated by SDS-PAGE (15%) and stained with Coomassie R250
and function of the body, the control of its degradation is a
critical aspect in the homeostasis of most tissues and organs.
However, the role of the collagenolytic MMPs in cleavage of
collagen matrices in vivo is unclear (50) due in part to the
inappropriateness or availability of suitable mouse models.
MMP-1 shows an extremely restricted expression pattern of
two isoforms in mice that until recently were not believed to
exist in rodents (51). The MMP-13 knockout and trans-
genic mice have yet to be reported, whereas the MMP-8 knock-
out was only recently generated and found to have very little
abnormalities in collagen degradation at sites of tissue chal-
lenge (52). Mmp2 knockout mice, though smaller at birth than
wild-type littermates, develop normally without any apparent
deficits in collagen turnover (53). However, in humans the
Mmp2 gene mutations, R101H and Y244X, in three consan-
guineous families leads to a deficiency in MMP-2 expression
expression (54) with afflicted individuals displaying severe growth restric-
tions and many skeletal defects (55, 56). This indicates that in
humans, MMP-2 is critical for the balance of bone matrix
protein synthesis and degradation, in which type I collagen is
the major structural element. The MT1-MMP knockout mouse
also shows the critical importance of this proteinase for colla-
gen metabolism in the skeleton and cartilage where severe
developmental defects are manifested (26, 27). These similar
phenotypes are consistent with the activation of MMP-2 by
MT1-MMP; the spatial and functional association of MMP-2
and MT1-MMP on the cell surface in forming the MMP-2/MT1-
MMP proteolytic axis; and for their synergistic involvement in
the cleavage of collagen in the pericellular matrix (5, 29) as well
as of cell surface receptors and cytokines involved in regulating
connective tissue metabolism (57).

**DISCUSSION**

In view of the pivotal importance of collagen in the structure
and function of the body, the control of its degradation is a
critical aspect in the homeostasis of most tissues and organs.
However, the role of the collagenolytic MMPs in cleavage of
collagen matrices in vivo is unclear (50) due in part to the

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4 R. Dean, G. Butler, and C. M. Overall, manuscript in preparation.
ers that leads to the activation of proMMP-2 (5). MMP-2 then completes the degradation of partially cleaved collagen and gelatin generated by MT1-MMP activity. Although membrane clustering of MT-MMPs increases the efficiency of MMP-2 activation this also results in MT1-MMP autolysis to a cell surface bound 44-kDa form comprised of the linker/hemopexin C domain (65, 66). We recently found that this isolated cell surface form of the MT1-MMP hemopexin C domain retains collagen binding properties and thereby functions in a dominant negative manner to suppress collagenolysis by both MT1-MMP and MMP-2, and to suppress MMP-2 activation (5). Hence, collagen regulates its own levels in the pericellular environment by a unique dynamic feedback mechanism involving its regulation of the expression, activation and activity of the proteases responsible for its degradation.

Despite these spatially and functionally intimately related activities of MT1-MMP and MMP-2 on the cell surface the mechanisms of collagen binding and cleavage occur quite differently in these two proteases. Importantly, this enables complementary, rather than competitive, binding and cleavage interactions with collagen to occur. Whereas MT1-MMP utilizes the hemopexin C domain to bind native collagen and this is an absolute requirement for collagenolysis, MMP-2 binds collagen by the fibronectin type II modules. These modules form an alternate collagen binding domain to the hemopexin C domain, which in previous work (41, 42) was shown not to bind native type I collagen. Hence, we earlier concluded that the mechanisms of triple helicase activity of MMP-2 must be fundamentally different from the collagenases (29, 42). Our present work supports this hypothesis and provides a mechanistic explanation for enzymatic cooperativity (and not competition) in collagenolysis. In MMP-2, a very different interaction with native collagen occurs from MT1-MMP that is driven by the MMP-2 CBD and not the hemopexin C domain. Using recombinant MMP-2 CBD in dominant negative experiments to reduce cleavage of both native and denatured collagen by MMP-2, we demonstrated the pivotal role for the fibronectin type II modules in MMP-2 collagenolysis. Mechanistically, we found by CD spectroscopy that recombinant MMP-2 CBD alone can induce a perturbation in the secondary structure of native collagen. This perturbation was identified to be a destabilization of the helix as the collagen became more susceptible to MMP-1 and MMP-8 cleavage.

The fibronectin type II modules of MMP-2 have been structurally studied by NMR as a separate domain or individual modules (67–69) as well as in the context of the full-length pro-MMP-2 (70). These structures reveal that the three fibronectin type II modules form a compact domain, which we termed the collagen binding domain in view of its native collagen binding properties that distinguish it from gelatin binding domains only (44). It is situated adjacent to the active site cleft extending N-terminal from the S3 subsite, a region identified as being important for collagen cleavage in MMP-1 (71) and MMP-8. The three individual modules are joined by flexible linkers and face outward and can bind more than one collagen α-chain simultaneously (44). Each fibronectin type II module consists of two sets of double-stranded anti-parallel β-sheets perpendicularly arranged, connected by two loops and a short α-helix, stabilized by two disulfide bonds (67–70). The two β-sheets are arranged to form a hydrophobic surface, lined with aromatic residues. Mutational analysis (72) and NMR (67–69) have identified several aromatic residues that may be involved in gelatin binding. By mutagenesis we have also identified the nearby Phe355 and Trp316 in the 2nd fibronectin module and the homologous Phe355 and Trp374 in the 3rd fibronectin module to be critically important in gelatin binding. A substitution to alanine at these sites was found to eliminate the gelatin binding properties of each module. Using a CBD23 construct with the tryptophan mutations W316A/W374A that no longer binds gelatin nor blocks MMP-2 gelatinolytic and collagenolytic activities in competition experiments, we specifically confirmed the importance of the collagen binding properties of the CBD in collagen and gelatin cleavage by MMP-2.

However, our data revealing the critical importance of the fibronectin type II modules in MMP-2 collagenolysis differ from those reported by Patterson et al. (22). Using a deletion approach, these authors reported that a MMP-2 mutant lacking the fibronectin type II modules (ΔCBD) could still cleave native type I collagen and so concluded that the fibronectin type II modules are not involved in this process. We have previously described native 3/4-collagen cleavage as the signature activity of MMP-5 (19) before identifying this protease as MMP-2 (19, 20). The MMP-2 ΔCBD mutant also did not further process the native 3/4-collagen fragments into smaller fragments (22). However, it is unclear how MMP-2 would bind collagen in the absence of the fibronectin type II modules given that the CBD (and not the MMP-2 LCD) can quantitatively elute MMP-2 from collagen and gelatin (44). Moreover, it is unknown what the effects of such a large deletion are on the structural and functional properties of the enzyme toward macromolecular substrates despite MMP-2 ΔCBD showing very similar synthetic peptide cleavage kinetics to that of full-length MMP-2. Patterson et al. (22) therefore proposed that the fibronectin type II modules are only required for gelatinolysis. Although our data is not in agreement with their overall findings, our competition experiments do support the role of the CBD in gelatin degradation as N-MMP-2 degraded gelatin with similar efficiency to MMP-2 and the addition of recombinant CBD inhibited gelatin degradation by both full-length and N-MMP-2.

Despite the importance of the CBD, an enigmatic role for the MMP-2 hemopexin C domain remains since its deletion in the mutant N-MMP-2 eliminates native type I collagen cleavage as previously found (22). The addition of recombinant MMP-2 LCD to N-MMP-2 did not restore collagenolytic activity indicating that a contiguous tertiary structure is required for triple helical collagen cleavage. In this respect, the interaction of the MMP-2 LCD with collagen also differs from that of the MT1-LCD, which enhanced collagen cleavage by MMP-1 and MMP-8, and the hemopexin C domain of MMP-1 (71). In the latter report, these authors found that following the loss of collagenolytic activity upon removal of the MMP-1 hemopexin C domain a minor amount of collagenolytic activity of N-MMP-1 was restored with the addition of the MMP-1 hemopexin C domain in trans.

In isolation, the sMMP-2 hemopexin C domain does not bind native collagens (42). Therefore, this indicates that an interaction with collagen must occur in the context of the full-length enzyme. However, the mode of cooperativity between the MMP-2 catalytic domain and the hemopexin C domain is unclear, but may involve the formation of a collagen-binding groove between the catalytic and hemopexin C domains. In consideration of the three-dimensional structure of MMP-2 (70) and the site of TIMP-2 interaction on the lower surface of the hemopexin C domain at the junction of blades III and IV (63), the most likely site for collagen binding resides on the upper surface of the hemopexin C domain that is immediately juxtaposed with the lower edge of the catalytic domain at the junction of hemopexin blades I and II as previously proposed (29). Near this location the linker peptide could also be potentially involved in collagen interaction by analogy with its role in other collagenolytic MMPs (73) including MT1-MMP (5). How-
ever, the linker peptide itself does not confer the ability to bind collagen alone or in combination with the hemopexin C domain as it is present in the MMP-2 LCD used here and previously (42). Another mechanism of cooperativity in the full-length enzyme may involve the stabilization of the conformation of the lower subdomain of the catalytic domain, which may be required for triple helical collagen cleavage.

We have also revealed the importance of the linker peptide in collagen binding and structural perturbation of native collagen by MT1-MMP. Although the MT1-CD binds collagen, it does not block MT1-MMP collagen cleavage when added in competition experiments reported here and previously (5) nor did it disrupt the secondary structure of collagen as revealed by CD spectroscopy. In contrast, the MT1-LCD protein binds collagen, disrupts its secondary structure and competitively blocks MT1-MMP activity. We also recently found that the linker peptide alone neither binds collagen nor modulates collagenolysis by MT1-MMP (5). Together, this reveals that linker/hemopexin C domain cooperativity only occurs when in contiguous structure. Other work has also revealed the importance of the linker in collagenolysis (39, 40) leading to earlier suggestions that the MMP-1 and MMP-8 collagenase linker intercalates with the individual α-chains of the collagen triple helix, which allows for their sequential cleavage (29, 32, 74). Our data support the hypothesis that the linker, with the hemopexin C domain, perturbs the secondary structure of collagen enabling cleavage of the individual α-chains to occur. Thus, this appears to be the mechanism of collagen triple helicase activity in MT1-MMP. Recombinant MT1-LCD, by binding to the same site that the linker and hemopexin C domain of MT1-MMP does when in context with the full-length MT1-MMP, may mask the cleavage site or compete with MT1-MMP for binding like the CBD does with MMP-2 (Fig. 6, A and C). However, since MMP-1 and MMP-8 showed enhanced cleavage in the presence of MT1-LCD, this also indicates that these proteases may bind collagen at a different site (Fig. 6B) but in the same localized region perturbed by the MT1-LCD domain interaction, thus facilitating collagen cleavage by these enzymes (Fig. 6C). Nonetheless, complete local denaturation of the collagen does not occur since trypsin and MMP-7, which are potent gelatinases, were unable to access individual α-chains in the perturbed collagen structure or displace the exosite domains and initiate cleavage of the native collagen. Thus, these results highlight the importance of the linker in this process since the MT1-CD did not induce structural perturbations in the collagen nor affect collagenolytic behavior of MMP-1, MMP-8, or MT1-MMP.

Notwithstanding our present data and the considerable amount of work from several laboratories, the details of collagenase triple helicase activity remains elusive, including the role of the catalytic domain. Previously, we have proposed several potential mechanisms by which three-point binding of the collagen helix by the catalytic domain, the linker peptide and the hemopexin C domain drives collagen unwinding by the simultaneous binding at all three sites that can only occur upon perturbations being generated in the collagen structure (29) (Fig. 6B). That is, collagenolytic MMPs can bind native collagen at two sites but this alone does not generate helicase activity. In order to simultaneously fulfill the binding propensity of a third site the collagen has to bend or unravel in order to make this contact. In contrast, MMP-2 has evolved a different mech-
anism of collagen triple helicase activity. Because of the spatial orientation of each fibronectin type II module in the MMP-2 fibronectin domain (70), we hypothesize that the three modules bind and interdigitate within the collagen triple helix to splay the α-chains aside in preparation for cleavage (Fig. 6B). This activity would be driven by the binding preference of the fibronectin type II modules for denatured collagen (44) and so provides an alternative mechanism for the molecular tectonics of collagenolysis. However, fully unraveling the mystery of collagen triple helicase activity most likely will only be forthcoming by the development of dynamic imaging techniques or other sophisticated approaches, without which only partial glimpses of the mechanism appear possible.

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