Limited Cleavage of Extracellular Matrix Protein BM-40 by Matrix Metalloproteinases Increases Its Affinity for Collagens*

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The 33-kDa matrix protein BM-40 (SPARC, osteonec- tin) consists of an acidic N-terminal domain I, a central cysteine-rich follistatin-like module, and a C-terminal extracellular calcium-binding (EC) module. Previous studies attributed collagen IV and high affinity calcium binding of BM-40 to its EC module, which was shown by x-ray crystallography to consist of an EF-hand pair surrounded by several α-helical and loop segments. This module was now shown by surface plasmon resonance assay to bind with similar affinities to collagens I, III, and V. Cleavage of recombinant BM-40 and its EC module by collagenase-3, gelatinases A and B, matrixins, and stromelysin-1 showed similar fragment patterns, whereas collagenase-1 was inactive. Some differences were, however, observed in cleavage rates and the preference of certain cleavage sites. Edman degradation of fragments demonstrated only three to four major cleavage sites in the central region of domain I and a single uniform cleavage in helix C of the EC module. Cleavage is accompanied by a 7–20-fold increase in binding activity for collagens I, IV, and V but revealed only small effects on collagen-dependent α-helical changes in the EC module. The data were interpreted to indicate that helix C cleavage is mainly responsible for enhancing collagen affinity by exposing the underlying helix A of the EC module. A similar activation may also occur in situ as indicated previously for tissue-derived BM-40.

The small calcium-binding glycoprotein (33 kDa) referred to as BM-40, SPARC, or osteonectin has been shown to have a widespread occurrence in extracellular matrices of various organs with a particularly high expression found during morphogenesis, tissue remodeling, and repair. The protein exhibits anti-adhesive properties in cell culture and modulates the expression of certain extracellular receptors. Several extracellular ligands have been identified for BM-40 including some collagen types and cytokines (1). This indicated the involvement in collagen V binding of the FS module containing a C-terminal 150 residues (3, 4) demonstrated an acidic and flexible N-terminal domain I (~50 residues) followed by a follistatin-like (FS) module (~75 residues) and a novel extracellular calcium-binding (EC) module (~150 residues). X-ray crystallography of this EC module demonstrated two opposing calcium-binding EF hands, as found in many intracellular proteins (5), which were in close contact to an extended α-helix (4). A combination of adjacent FS and EC modules has been detected in the cDNA sequences of several more extracellular proteins suggesting the existence of a protein family (1, 3, 4). Yet it is not known so far whether they are functionally related to BM-40.

The binding of BM-40 to the fibril-forming collagens I, III, and V and basement membrane collagen IV has been demonstrated and depended on moderate calcium concentrations indicating the involvement of the EC module (6–11). This conclusion was supported by studies with collagen IV and proteolytic fragments and deletion mutants of BM-40 (3, 9, 12). However, further factors may modulate collagen affinities as indicated in studies with BM-40 obtained from human bone and platelets which differed considerably in their binding to collagen V and in their N-linked oligosaccharides being either of the mannos-rich or complex type (11). This indicated involvement in collagen V binding of the FS module containing these sites as also shown by deglycosylation achieved either enzymatically or by mutation (13). This study also provided evidence that the N-terminal domain I and/or the FS module are essential for binding which was subsequently restricted to an N-terminal 17-residue segment in a synthetic and recombinant analysis (14). The controversial nature of these data could still be attributed to the different collagen types used in the binding analyses. A 15-fold affinity difference was noticed in surface plasmon resonance binding assays between recombinant human BM-40 and tissue-derived mouse BM-40 regardless whether human or mouse collagen IV was used as a ligand (3). Since no evidence could be obtained for conformational or glycosylation differences between both preparations of BM-40 (3, 10), the possibility remained that a substantial endogenous proteolytic cleavage in the EC module of mouse BM-40 (15) could be responsible for this effect.

The controversial issue of collagen binding was addressed in the present study by two types of experiments. First, we could demonstrate similar equilibrium dissociation constants for the binding of human BM-40 or its EC module to collagens I, III, IV, and V supporting the interpretation of a single collagen binding site. Furthermore, cleavage of BM-40 by several matrix metalloproteinases mimicked the endogenously observed cleavage of mouse BM-40 and was accompanied by a comparable

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1 The abbreviations used are: FS, follistatin; EC, extracellular calcium-binding; MMP, matrix metalloproteinases.
increase in collagen affinity. This was of particular interest since BM-40 has been shown to increase the production ofstromelysin, collagenase, and gelatinase in cell culture (16) suggesting a positive feedback loop. The structural data of the EC module (4) may now in addition allow us to map the collagen binding site of BM-40 by site-directed mutagenesis.

MATERIALS AND METHODS

Sources of Extracellular Matrix Ligands and Proteases—The production and purification of recombinant human BM-40 (10) and the deletion mutants ΔI and ΔI, II (3) have been described. Collagens I, III, and V were solubilized from human placenta by pepsin and separated from each other by fractional NaCl precipitation (17). Collagen V was further purified by chromatography on a Mono-Q column (18). Collagen IV tetramers that lack the globular NC1 domain were also isolated from a pepsin digest of human placenta (19). Previously described procedures were followed for the recombinant production, purification, and activation of matrix metalloproteinases (MMP) including collagenase-1 (MMP-1) (20), collagenase-3 (MMP-13) (21), gelatinase A (MMP-2) (22), gelatinase B (MMP-9) (23), stromelysin-1 (MMP-3), and matrilysin (MMP-7) (24).

Production of a Deglycosylated BM-40 Mutant—Human BM-40 cDNA encoding mutant ΔI (3) in the pBluescript II SK+ vector (Stratagene) was used to generate two overlapping subfragments by polymerase chain reaction including the vector with Vent polymerase (New England Biolabs) to introduce an N99Q mutation following a previously described strategy (25). The 5'-fragment was generated by polymerase chain reaction using primer 1 GATGCGTACCAAATCCGCGACAC and primer 2 CGAAGGTCTTCTGATCTGTCATTGC and the 3'-fragment was generated using primer 3 GCAATTGAAGAAGACCTGCAG and primer 4 GTGCA-GAATTGCTTGTTCTGACTGTAC and primer 2 CGAAGGTCTTCTGATCTGTCATTGC (in primers 2 and 3, lower-case letters show mutated sequences). About 100 ng of each agarose gel-purified fragment were mixed, denatured, annealed, and initially extended without primers followed by polymerase chain reaction amplification with primers 1 and 4. The product was restricted with NheI and EcoRI and inserted into the vector described above. The insert was then restricted with NheI and Xhol and cloned into the episomal expression vector pCEP-Sh (25) that contained the puromycin instead of the phleomycin resistance gene.2 The correctness of the insert was verified by DNA cycle sequencing. Transfection of human EBNA-293 cells (Invitrogen) and purification of the recombinant mutant followed established protocols (3, 25).

Proteolytic Digestions—Protein samples were dissolved in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM CaCl₂ and digested at 37 °C at different enzyme-substrate ratios (1:10, 1:50, or 1:100) for 5 or 24 h. The reactions were stopped by adding EDTA to a final concentration of 4 mM. The cleavage was then examined by SDS-gel electrophoresis in 10–20% polyacrylamide gradient gels under nonreducing and reducing conditions. Subsequently sufficient CaCl₂ was added to yield a surplus of 2 mM CaCl₂ for the measurements by surface plasmon resonance assay.

Surface Plasmon Resonance Assay—Surface plasmon resonance binding studies (26) were performed with BIAcore instrumentation (BIACore AB). Collagens were coupled covalently via amine coupling to sensor chips CM5 (Pharmacia Biotech). After activation of the carboxymethylated dextran layer by addition of 35 μl of a mixture of 0.05 M N-hydroxysuccinimide and 0.2 μM N-ethyl-N-(3-dimethylaminopropyl)carbodiimide at a flow rate of 5 μl/min, 60 μl of collagen solution in 0.5 M sodium acetate, pH 4.0, at a concentration of 200 μg/ml was added. Residual activated carboxylic groups of the chip were saturated by reaction with 35 μl of 1% ethanolamine, adjusted to pH 8.5. These immobilization reactions resulted in 6,000–12,000 resonance units, equivalent to about 6–12 ng/mm² of immobilized protein on the surface of the chip. Binding assays were performed in neutral buffer containing 2 mM CaCl₂ and 0.05% P20 surfactant, at a flow rate of 20 μl/min. As soluble ligands 10 μM solutions of proteins were applied resulting in signals of 30–650 resonance units. Kinetic rate constants were calculated from the binding and the dissociation curves by BIAevaluation software version 2.1 supplied by the manufacturer.

Circular Dichroism (CD) Spectra—Dissociated and undissociated EC modules from human Tris-HCl, pH 7.4. Protein concentrations were calculated from the absorption at 280 nm as described (12). CD spectra in the far UV region were recorded at 25 °C on a JASCO 715 CD spectropolarimeter in a thermostatted quartz cell of 1-mm optical pathlength. Spectra were measured after adding 2 mM CaCl₂ and after subsequent addition of 6 mM EDTA. Molar ellipticities [θ] (expressed in degrees cm² dmol⁻¹) were calculated on the basis of a mean residue molecular mass of 110 Da.

Analytical Methods—Protein samples were hydrolyzed (16 h at 110 °C) with either 6 or 3% HCl to determine protein or hexosamine concentrations, respectively, on a LC3000 analyzer (Biotronik). Protein digests were separated by electrophoresis and electrophorated onto Immobilon PSQ membranes (Millipore). N-terminal sequences of individual bands were then determined by 3–12 Edman cycles on 475 A or Procise sequencers (Applied Biosystems) following the manufacturer’s instructions.

RESULTS

Binding of BM-40 and Its EC Module to Different Collagen Types—Previous collagen binding studies have all been carried out in solid phase assays using either radioactive labeling or specific antibodies for the detection of binding (6–14). Since such assays are difficult to evaluate in quantitative terms, we have recently used a surface plasmon resonance assay to demonstrate a moderate binding activity of recombinant human BM-40 (Kₐ about 3 μM) and of the BM-40 deletion mutant ΔI, II, which corresponds to its EC module, to collagen IV (3). The same procedure and ligands were now used for a comparative binding analysis including the fibrillar collagen types I, III, and V as immobilized ligands (Table I). This demonstrated similar affinities of BM-40 (Kₐ about 2–5 μM) for the collagens except for a small decrease for collagen III (Kₐ 6.6 μM). Several independent assays with collagens IV and V demonstrated that the error range of single measurements was not larger than ±50% (Table I) indicating a sufficient reproducibility of the assay. All four collagens also bound the BM-40 mutant ΔI, II, either with unchanged affinity, as for collagen III, or with a 5-fold reduced affinity (Table I). The lower affinities could be accounted for by a distinct increase in the dissociation rate constants which was only in part compensated by a smaller increase in the association rate. Further binding tests were performed with BM-40 mutant ΔI which consists of only the FS and EC module. This mutant showed either the same or a slightly reduced affinity to collagens I, IV, and V when compared with BM-40 and a significantly stronger affinity than mutant ΔI, II (cf. Tables I and IV).

Cleavage of BM-40 and Its EC Module by Matrix Metalloproteinases—Stromelysin-1, matrilysin, gelatinase A and B, and collagenase-1 and -3 were used to examine the possibility that

TABLE I

| Immobilized ligand | BM-40 | BM-40 ΔI, II |
|-------------------|-------|-------------|
|                    | kₐ    | kₜ    | Kₐ |
| Collagen I         | 2.4 ± 10⁻³ | 840 ± 10⁻³ | 2.8 |
| Collagen III       | 2.7 ± 10⁻³ | 840 ± 10⁻³ | 6.7 |
| Collagen IV        | 0.6 ± 0.3 ± 10⁻³ | 300 ± 120 | 2.0 ± 1.0 |
| Collagen V         | 1.8 ± 0.8 ± 10⁻³ | 690 ± 310 | 2.6 ± 1.3 |

2 E. Kohfeldt, unpublished observations.
limited tissue proteolysis could enhance collagen affinity of BM-40. Exposure of BM-40 in calcium-saturated form to these proteases at the usual enzyme-substrate ratio of 1:100 for 24 h showed remarkably little cleavage when examined by electrophoresis under nonreducing or reducing conditions (not shown). After a 10-fold increase in the amounts of proteases, distinct cleavage patterns were observed by electrophoresis for five of the matrix metalloproteinases but not for collagenase-1. Analysis under nonreducing conditions revealed a shift of the BM-40 band to various extents to one or two bands of slightly higher mobility (Fig. 1A). Cleavage became more distinct after reduction (Fig. 1B) which showed one to three prominent bands with migration positions corresponding to 28–38 kDa as compared with 40 kDa for intact BM-40. This demonstrated rather complete conversion of BM-40 by stromelysin-1, matrilysin, and collagenase-3, whereas substantial amounts of uncleaved BM-40 were still present in the digests with both gelatinases. Most prominent was, however, the presence of a distinct uniform band of about 10 kDa in these digests indicating its release from a large disulfide-bonded loop.

The nature of the various cleavage products was examined after reduction and electroblotting by Edman degradation (Fig. 2). This demonstrated for all the larger fragments cleavage within the N-terminal domain I (N-terminal residues at positions 21, 31, 33, or 38). A few fragments, however, retained the original N terminus of recombinant BM-40 (APQQEA) indicating that they are generated by an exclusive C-terminal fragmentation. This postulated C-terminal fragment could be identified as the common 10-kDa band which started at position 198 and was a faint band by electrophoresis indicating a very minor cleavage site in BM-40. Both of these cleavage sites are located within a large disulfide-bonded loop of the EC module bordered by Cys-138 and Cys-248 (9, 15), indicating from the size of both small fragments that they extend to the C-terminal end of BM-40 (Ile-286). Together the data demonstrate two routes of BM-40 degradation through a limited number of scissile peptide bonds in the N- and C-terminal region.

Fig. 1. SDS-gel electrophoresis of matrix metalloproteinase digests of BM-40 under nonreducing (A) and reducing conditions (B). Lanes were loaded with undigested BM-40 (lane 1) or digests prepared with collagenase-3 (lane 2), gelatinase A (lane 3), gelatinase B (lane 4), matrilysin (lane 5), collagenase-1 (lane 6), and stromelysin-1 (lane 7). The digests were obtained at an enzyme/substrate ratio of 1:10 at 37 °C for 24 h except for matrilysin (5 h). Migration positions of calibrating proteins are denoted on the left margin (in kDa).

Collagenase-3 executed both cleavages in rather equivalent fashion, whereas matrilysin and stromelysin-1 in increasing order preferred the N-terminal route. The gelatinases showed a preference for C-terminal cleavage that was less efficient with gelatinase B leaving substantial amounts of uncleaved BM-40.

Because of the limited complexity of cleavage patterns we used the C-terminal EC module (mutant ΔI, II) for further studies with stromelysin-1, gelatinase A, and collagenase-3. Electrophoresis patterns under nonreducing conditions demonstrated the appearance of a novel band of slightly reduced mobility that showed the original N-terminal sequence with N denoting the N terminus of recombinant BM-40 (APQQEA). The slowest migrating bands in each digest except in lane 1 correspond to undigested BM-40. Migration positions of calibrating proteins are denoted on the left margin (in kDa). B, partial sequences of BM-40 showing the major cleavage sites in domain I (positions 18–42) and the EC module (positions 321–245). Cleaved peptide bonds are identified by arrowheads and the position number of the novel N terminus. Each sequence was determined by 8–12 Edman degradation cycles.

FIG. 2. Correlation of the reduced electrophoretic band patterns of BM-40 digests (A) with their N-terminal sequences (B) determined after electroblotting. A, lanes were loaded with digests prepared with collagenase-3 (lane 1), gelatinase A (lane 2), gelatinase B (lane 3), matrilysin (lane 4), or stromelysin-1 (lane 5) which were prepared as in Fig. 1. Numbers to the right of each band refer to the novel N-terminal sequence positions with N denoting the N terminus of recombinant BM-40 (APQQEA). The slowest migrating bands in each digest except in lane 1 correspond to undigested BM-40. Migration positions of calibrating proteins are denoted on the left margin (in kDa). B, partial sequences of BM-40 showing the major cleavage sites in domain I (positions 18–42) and the EC module (positions 236–245). Cleaved peptide bonds are identified by arrowheads and the position number of the novel N terminus. Each sequence was determined by 8–12 Edman degradation cycles.

FIG. 3. Domain I, II but with higher efficiency.

Since cleavage in the EC module could interfere with calcium binding to its EF hand pair and thus influence α-helical conformation (3, 12), we compared mutant ΔI, II and the three

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proteolytic digests by CD spectroscopy (Fig. 4). This demonstrated for all samples a typical and similar $\alpha$-helical spectrum when examined in the presence of 2 mM CaCl$_2$. The $\alpha$-helical content, as judged from the ellipticity at 222 nm, of the undigested and stromelysin-treated samples was about the same and was slightly reduced after digestion with gelatinase A or collagenase-3. Furthermore, the addition of an excess of EDTA caused a similar, reversible reduction in $\alpha$-helicity (44–48%) in all samples (Fig. 4). This indicated that the overall structure of the EC module as well as the affinity for calcium were maintained after cleavage.

Modulation of Collagen Binding by Proteolysis—Digests of BM-40 obtained with matrilysin, stromelysin-1, gelatinase A, or collagenase-3, all of them with only little intact protein left (Fig. 2), were examined in surface plasmon resonance assay for their binding to collagen IV (Table II). This demonstrated $K_d$ values in the range of 0.1–0.3 mM and thus a 7–20-fold increase in affinity over BM-40. Similarly low $K_d$ values were previously obtained for collagen IV binding of tissue-derived mouse BM-40 which was also modified in a limited fashion by endogenous proteolysis (3, 15). An increase in the association rate constant was in all these cases mainly responsible for the enhanced binding activity.

Similar binding studies including in addition collagen I and V as ligands were performed with the three matrix metalloproteinase digests of mutant DI, II shown in Fig. 3. This demonstrated a distinct increase in collagen IV affinity to about the same extent as found for BM-40 digests (Table III). A similar activation for collagen I and V binding was, however, only found after treatment with stromelysin-1 or collagenase-3 but not with gelatinase A even though the latter two digests appeared similar by electrophoresis and sequence analysis (Fig. 3). It could indicate some differences at C-terminal regions which we would not detect by these analyses.

Effect of N-Glycosylation on Collagen Binding—Previous studies have shown that BM-40 (osteonectin) binding to collagen V can be modulated by the nature and extent of N-glyco-sylation of the single amide-acceptor site present in the FS module of BM-40 (11, 13). For a comparison to the proteolytic activation described here, we used BM-40 mutant DI that con-
Protease Modulation of Collagen Binding

Table II

| Treatment of BM-40 | kₐ | kᵋ | Kd | μM |
|-------------------|----|----|----|----|
| None              | 0.54 x 10⁻³ | 220 | 2.5 |
| Matrilysin        | 2.8 x 10⁻³  | 9.300 | 0.31 |
| Stromelysin-1     | 1.5 x 10⁻³  | 5.200 | 0.29 |
| Gelatinase A      | 4.7 x 10⁻³  | 1.600 | 0.29 |
| Collagenase-3     | 1.6 x 10⁻³  | 16.000 | 0.29 |

Table III

| Immobilized ligand | Treatment of BM-40 | kₐ | kᵋ | Kd | μM |
|--------------------|--------------------|----|----|----|----|
| Collagen IV        | None               | 1.35 x 10⁻² | 857 | 16 |
|                    | Stromelysin-1      | 2.64 x 10⁻² | 9.300 | 2.8 |
|                    | Gelatinase A       | 1.74 x 10⁻² | 15,000 | 1.1 |
|                    | Collagenase-3      | 1.97 x 10⁻² | 13,000 | 1.5 |
| Collagen I         | None               | 1.6 x 10⁻²  | 1,100 | 14 |
|                    | Stromelysin-1      | 1.4 x 10⁻²  | 13,000 | 1.1 |
|                    | Gelatinase A       | 0.7 x 10⁻²  | 623 | 12 |
|                    | Collagenase-3      | 1.3 x 10⁻²  | 18,000 | 0.7 |
| Collagen V         | None               | 0.91 x 10⁻² | 605 | 15 |
|                    | Stromelysin-1      | 0.28 x 10⁻² | 1,300 | 2.2 |
|                    | Gelatinase A       | 0.90 x 10⁻² | 445 | 20 |
|                    | Collagenase-3      | 0.46 x 10⁻² | 4,400 | 1 |

Discussion

Application of a surface plasmon resonance assay demonstrated a remarkably similar affinity of BM-40 for four different collagen types including several that form larger interstitial fibrils (I, III, and V) or networks in basement membranes (IV). Some larger variability was reported in previous solid phase assays (8, 9), which may be due to differences in coating efficiencies or the detection systems used. Binding was localized to the C-terminal EC module of BM-40 consistent with previous evidence that calcium depletion as well as large deletions in the EC module abolish collagen IV binding (9, 10, 12). The involvement of the same module as well as similarities in the proteolytic enhancement of binding suggests a single collagen binding site for BM-40 that may be represented by a single or several overlapping epitopes. The collagens used in the present study were obtained by solubilization with pepsin which strongly indicates that their BM-40 binding epitopes reside in their triple helical domains. Their number is unknown except for some electron microscopic evidence suggesting two sites along the triple helix of collagen IV (9).

Enhancement of collagen binding by roughly an order of magnitude could be observed following cleavage with several matrix metalloproteinases of either full-length BM-40 or the EC module. This effect can very likely be attributed to cleavage of a single peptide bond, 197–198. Cleavage at this position is not accompanied by large changes in conformation and its calcium-induced change, indicating only minor structural rearrangements in the nicked protein. Similar small changes result from cleavage at position 237–238 in the isolated EC module. This peptide bond is largely protected in full-length BM-40, most likely by interactions with the adjacent FS module, and its cleavage may not have any biological significance.

The recent elucidation of the EC module structure (4) allows us to speculate on the structural consequences of proteolytic cleavage at position 197–198. The central feature of the EC module structure is a pair of EF-hands interacting intimately with a long amphiphilic helix A. The connection between these two substructures is provided by several loops and helices B and C. The crucial cleavage site 197–198 is located at the N terminus of the latter helix, and both residues 197 and 198 are exposed to solvent in the EC module structure. Matrix metalloproteinases are believed to bind their substrates in an extended conformation (27), and some structural rearrangement of helix C and the preceding loop is presumably required for proteolysis. It has been noted that the loop connecting helices B and C makes only weak contacts with the body of the EC module structure (4). Cleavage within the first turn of helix C is therefore likely to result in an increased exposure of the hydrophilic residues on helix A opposite the EF-hand pair. This would in particular expose residues Glu-145, Arg-149, and Asp-152, whose side chains are partly (60–80%) buried by the B-C loop and C helix in the intact EC module. Fig. 5 shows that these three residues, together with Glu-142, form a highly charged and very shallow groove bordered on one side by the B-C loop. We speculate that this is part of the collagen binding epitope.

This proposal, which can now be tested by site-directed mutagenesis, is consistent with the observation that enhanced
binding in the nicked protein is mainly due to an increase in the association rate constants, indicating the removal of steric constraints. Of course, the collagen binding site must already be substantially accessible in BM-40, as collagens do bind to the intact protein. We have previously shown that deletion of helices A to C strongly reduces the affinity of BM-40 for both calcium and collagen IV (12). Although this observation is consistent with the above proposal for the collagen binding site, the drastic consequences of such a large deletion preclude a simple structural interpretation, and more subtle deletions and/or alterations will be required in future mapping studies.

Collagen V binding was also shown for a recombinant human BM-40 fragment lacking the EC module (13, 14). In these studies prevention of N-glycosylation by an N99Q mutation increased, and deletions in the N-terminal domain I abolished collagen V binding. As shown here the N99Q mutation had no effect or slightly decreased collagen binding when mediated through the EC module. Both sets of data are not necessarily in conflict and could indicate two collagen V binding sites in BM-40, one of which is located in domain I. For collagen IV, however, no evidence for a domain I binding site in BM-40 could be obtained in studies with proteolytic and recombinant fragments (3, 9, 12).

Our present study started in part from the observation (3) that tumor tissue-derived mouse BM-40 showed a 15-fold higher affinity for mouse and human collagen IV than recombinant human BM-40 which remained unexplained. Mouse BM-40 was, however, known to be cleaved at position 198/199 to a substantial degree presumably by endogenous proteolysis (15). This site is just one residue apart from the human BM-40 site sensitive to several matrix metalloproteinases described here. This would provide a sufficient explanation for this difference that was supported by recent studies showing identical binding activities of nondegraded recombinant mouse and human BM-40 for several collagen types.3 The small difference in the N-terminal ends could be explained by the further action of aminopeptidases or the involvement of other matrix metalloproteinases than used here since both human and mouse BM-40 show complete sequence identity in the region involved (28). Limited proteolysis of BM-40 accompanied by enhanced collagen affinity was also observed in calvarial cell culture (29, 30). Yet these data need also to be explored in the context of whether similar endogenous cleavages of BM-40 occur in nor-

Fig. 5. Solvent-accessible surface representation of the BM-40 EC module structure. Positive and negative electrostatic potential is shown in blue and red, respectively. The inset shows the same view with a transparent surface and a ribbon representation of the Cα trace. In this view, the N-terminal helix A runs horizontally from left to right; the loop connecting helices B and C and the EF-hand pair are above and below helix A, respectively. The scissile bond for matrix metalloproteinase (MMP) cleavage, 197–198, is indicated, as are the positions of four charged residues proposed to contribute to the collagen binding epitope (see text). Produced with GRASP (39).
mal tissues and thus resemble a physiological process. This would require antibodies specific for the cleavage site or at least for the cleaved 10-kDa fragment released by reduction which are not yet available.

The effect of limited BM-40 cleavage by metalloproteinases on collagen affinity is also of interest in the context of a recent study (16) which showed up-regulation of stromelysin, gelatinase, and collagenase expression in fibroblasts by exogenously added BM-40. This process is apparently dependent on a complex cascade of interactions including a mediator secreted into the culture medium. This suggests an autocrine or paracrine way how BM-40 could regulate its affinity for collagens. The moderate affinity of BM-40 for collagens (\(K_d = 2–6 \mu M\)) and its limited increase after proteolysis (\(K_d = 0.1–0.3 \mu M\)) needs to be considered in relation to the concentrations of the ligands when present in the extracellular space. As discussed previously (3) the concentration of collagen IV in basement membranes is about 5 \(\mu M\), which should be high enough for efficient binding of undegraded BM-40 if present in comparable amounts. Such concentrations are also very likely to exist for fibrillar collagens that are the most abundant proteins in vertebrates. Yet situations of lower BM-40 tissue concentrations could exist and become dependent on proteolytic activation for efficient binding. Of course, this also raises the question of the biological consequences for BM-40 binding to collagens which so far are not known. Possibilities could include a structural function or sequestration of BM-40 and the regulation of its cytokine binding and cell-modulating activities (1).

BM-40 was remarkably stable against several matrix metalloproteinases when compared with other extracellular substrates such as nidogen (31) and fibulin-2 (32), considering the limited number of peptide bonds cleaved and the high protease concentrations required to achieve this. Yet trypsin and leukocyte elastase caused a more extensive degradation particularly of the EC module (9), indicating that BM-40 conformation is not designed to prevent proteolysis. There was also the interesting aspect that most matrix metalloproteinases cleaved identical peptide bonds, particularly that in the EC module equated with collagen binding activation, which is an uncommon observation. Matrix metalloproteinases were for a long time considered to have mainly a catabolic function during tissue remodeling or to activate precursor forms of proteases and cytokines (33, 34). Other functions may include the exposure of cryptic integrin cell binding sites on collagen and fibronectin (35, 36) indicating a more versatile function as also proposed in the present study. More recent data (37, 38) demonstrated the existence of several membrane-type matrix metalloproteinases and other membrane-bound metalloproteinases connected to a disintegrin-like domain (ADAMS family of proteinases). They could be good candidates for examining the modulation of BM-40 in the context of its cellular activities (1).

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