Crystal structure and mapping by site-directed mutagenesis of the collagen-binding epitope of an activated form of BM-40/SPARC/osteonectin

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The extracellular calcium-binding domain (positions 138–286) of the matrix protein BM-40 possesses a binding epitope of moderate affinity for several collagen types. This epitope was predicted to reside in helix α A and to be partially masked by helix α C. Here we show that deletion of helix α C produces a 10-fold increase in collagen affinity similar to that seen after proteolytic cleavage of this helix. The predicted removal of the steric constraint was clearly demonstrated by the crystal structure of the mutant at 2.8 Å resolution. This constitutively activated mutant was used to map the collagen-binding site following alanine mutagenesis at 13 positions. Five residues were crucial for binding, R149 and N156 in helix α A, and L242, M245 and E246 in a loop region connecting the two EF hands of BM-40. These residues are spatially close and form a flat ring of 15 Å diameter which matches the diameter of a triple-helical collagen domain. The mutations showed similar effects on binding to collagens I and IV, indicating nearly identical binding sites on both collagens. Selected mutations in the non-activated mutant Δ I also reduced collagen binding, consistent with the same location of the epitope but in a more cryptic form in intact BM-40.

Keywords: binding assays/collagen triple helix/
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Introduction

Collagens are a large protein family with 19 members (types I–XIX) identified so far. They are localized in the extracellular space or exist as transmembrane forms (Brown and Timpl, 1995; Prockop and Kivirikko, 1995; Olsen and Ninomiya, 1998). They all share a triple-helical domain of variable length which is formed from three chains and requires Gly at every third position and a high imino acid content in order to stabilize a polyproline II-like helix (Brodsky and Shah, 1995). Further stabilization is achieved by a hydrogen-bonded water network, which also requires 4-hydroxyproline, as shown for a synthetic collagen-like peptide (Bella *et al.*, 1994, 1995). As a consequence of this structure, all of the side chains are exposed on the surface of the triple helix and are available for various intermolecular associations.

Interactions mediated through the triple helix include

homo- and heterotypic self-assembly processes, which result in the formation of large fibrils, filaments, networks and some other supramolecular associations, depending on the collagen type (Brodsky and Shah, 1995; Brown and Timpl, 1995; Prockop and Kivirikko, 1995; Olsen and Ninomiya, 1998). To a large extent, these assembly forms determine the mechanical strength of extracellular matrices, but they have many additional functions, frequently mediated through the binding of non-collagenous ligands to the triple helix. These ligands include cell surface receptors such as α 1 β 1 and α 2 β 1 integrins (Vandenberg *et al.*, 1991; Gullberg *et al.*, 1992; Kern *et al.*, 1993; Pfaff *et al.*, 1993) and NG2 proteoglycan (Burg *et al.*, 1996), several growth factors (Taipale and Keski-Oja, 1997), small matrix proteoglycans (Oldberg *et al.*, 1989; Bidanet *et al.*, 1992) and other matrix proteins such as nidogen (Aumailley *et al.*, 1989), BM-40 (Lane and Sage, 1994) and von Willebrand factor (Ruggeri and Ware, 1993). They also include cell surface proteins, adhesins, which are present on some staphylococcal strains and are considered to allow invasion of host tissues (Switalski *et al.*, 1989). The binding of these non-collagenous ligands is either specific for a single collagen type or, more frequently, includes several different collagens (e.g. adhesins, integrins). Most of these interactions require an intact triple-helical conformation and also, as shown for α 1 β 1 integrin (Eble *et al.*, 1993), a specific array of amino acid side chains. Such arrays are thought to interact with specific collagen-binding epitopes, as has been shown in more-detailed molecular analyses of adhesins (Patti *et al.*, 1995; Symersky *et al.*, 1997), α 2 β 1 integrin (Kamata and Takada, 1994; Emsley *et al.*, 1997), von Willebrand factor domain A3 (Bienkowska *et al.*, 1997; Huizinga *et al.*, 1997) and BM-40 (Sasaki *et al.*, 1997).

BM-40, which is also referred to as SPARC or osteonectin, is an abundant 33 kDa extracellular calcium-binding protein and is considered to modulate cellular phenotypes (Lane and Sage, 1994; Sage, 1997). It contains a flexible N-terminal domain I, a follistatin-like (FS) domain and a C-terminal extracellular calcium-binding (EC) domain (Maurer *et al.*, 1995). The structure of the FS and EC domains has been elucidated recently by X-ray crystallography (Hohenester *et al.*, 1996, 1997) and thus set the stage for a molecular analysis of binding epitopes. Calcium-dependent binding of BM-40 to the triple helix of several fibrillar collagen types and basement membrane collagen type IV is of moderate affinity ($K_d \sim 1$ – 2μ M) and has been mapped to the EC domain (Maurer *et al.*, 1995, 1997a; Sasaki *et al.*, 1997). The cleavage of a single peptide bond in helix α C of the EC domain was accompanied by a 10-fold increase in collagen affinity. This suggested that the underlying helix α A should contribute to collagen binding (Sasaki *et al.*, 1997). In the

Fig. 1. Comparison of mutants of BM-40 Δ I by radioimmuno-inhibition assay. The assay system consisted of an antiserum against human BM-40 and 125 I-labelled BM-40 Δ I (1 ng). Inhibitors used were BM-40 Δ I ($\circ$), BM-40 Δ I, α C ($\bullet$), BM-40 Δ I, II ($\triangle$) and BM-40 Δ I, α C mutants M245A ($\blacktriangle$) and D152A ($\square$). The latter two were the only ones which showed a distinct deviation from the parent BM-40 Δ I, α C ($IC_{50} = 0.18$ nM). All other mutants were very similar in slope to Δ I, α C with an IC_{50} in the range 0.13–0.3 nM. Completely reduced and alkylated BM-40 ($\blacksquare$) showed only a weak partial inhibition, demonstrating that the assay detects mainly conformation-dependent epitopes.

present study, a similar increase in affinity could be achieved by recombinant deletion of helix α C. This has allowed us to map the binding epitope in the activated and non-activated forms of BM-40 to two non-contiguous sequence regions of the EC domain and to explain structurally how activation may occur.

Results

Deletion of helix α C of BM-40 increases collagen affinity

Previous data strongly suggested that the increase in collagen affinity of BM-40 after a single proteolytic cleavage in helix α C of its EC domain was due to the removal of steric constraints (Sasaki *et al.*, 1997). In order to verify this prediction, we carried out a recombinant deletion of helix α C (position 196–203) using BM-40 mutant Δ I which lacks the flexible N-terminal domain I (Maurer *et al.*, 1995). This choice was instrumental in allowing crystallographic studies (Hohenester *et al.*, 1997) and facilitating isolation of the new mutant Δ I, α C, as well as further mutants, in a single chromatographic step and in high yields from transfected mammalian cell medium. Purified Δ I, α C showed a single electrophoretic band with slightly higher mobility than Δ I. It also had a defined structure, as shown below. As a second, more versatile assay for structural integrity, we used a radioimmuno-inhibition assay, which detected primarily conformation-dependent epitopes (Figure 1). This demonstrated nearly identical activities for BM-40 mutants Δ I, Δ I, α C and Δ I,II, the latter comprising the EC domain alone (Maurer *et al.*, 1995).

The binding activities of BM-40 Δ I and Δ I, α C were compared by surface plasmon resonance assay with colla-

Table I. Activation of collagen binding of fragment BM-40 Δ I by deletion of helix α C (Δ I, α C)

Immobilized ligand	Soluble ligand (μ M)	$k_{\text{diss}} \times 10^3$ (/s)	$k_{\text{ass}} \times 10^{-3}$ (/M/s)	K_d (nM)
Collagen I	Δ I (2.5–5)	1.5	3.4	441
	Δ I, α C (0.05–2)	2.4	28	86
Collagen II	Δ I (5–10)	2.5	1.7	1470
	Δ I, α C (3–10)	1.1	5.5	200
Collagen III	Δ I (3–5)	0.9	2.1	428
	Δ I, α C (1–3)	0.8	17	47
Collagen IV	Δ I (2.5–5)	1.7	1.5	1130
	Δ I, α C (0.05–2)	1.3	16	81
Collagen V	Δ I (5)	1.8	4.2	428
	Δ I, α C (2–3)	0.5	11.4	43

Measurements were by surface plasmon resonance assay at the concentrations indicated, and average values of 3–4 measurements are given. Uptake of resonance units was in the range 250–650 for Δ I, α C and ~5-fold lower for Δ I.

gens I–V as immobilized ligands (Table I). For BM-40 Δ I, K_d values in the range 0.5–1.5 μ M were observed, slightly better than those (~2 μ M) determined previously for intact BM-40 (Sasaki *et al.*, 1997). A clear 10-fold increase in affinity was observed for BM-40 Δ I, α C ($K_d = 0.04$ –0.2 μ M), mainly due to a 3- to 10-fold increase in the association rate constants (Table I). These observations are consistent with a similar increase in affinity found after proteolytic activation (Sasaki *et al.*, 1997) and also with the interpretation that steric restrictions that impair binding have been removed.

Crystal structure of BM-40 Δ I, α C lacking domain I and helix α C

X-ray crystallography was used to analyse the structural changes accompanying activation of collagen binding. BM-40 Δ I, α C crystallized in a new crystal form and the structure was solved at 2.8 Å resolution by molecular replacement with a truncated BM-40 Δ I model (Hohenester *et al.*, 1997). The BM-40 Δ I, α C structure and a superposition of the structures of BM-40 Δ I, α C and BM-40 Δ I is shown in Figure 2. The overall structures are very similar, but deletion of the major part of helix α C (residues 196–203) in BM-40 Δ I, α C results in a significant structural rearrangement that extends far beyond the immediate site of deletion. If residues 162–205 are excluded from the superposition, the FS–EC domain pairs of BM-40 Δ I, α C and BM-40 Δ I can be superimposed with an r.m.s.d. of 0.6 Å, which drops to 0.4 Å if only the EC domains are compared. This is due to some segmental flexibility in the FS domain, especially in the N-terminal hairpin (Figure 2B; Hohenester *et al.*, 1997). Deletion of α C is accompanied by structural rearrangements in the α B– α D loop, as expected. Surprisingly, however, we observe that these changes are propagated as far as the α A– α B loop. Helix α B is rotated by ~20° relative to the BM-40 Δ I structure and is extended by two additional turns at its C-terminus. Concomitantly, the α A– α B loop in BM-40 Δ I, α C assumes a dramatically different conformation, with an additional helical turn (α A') not present in the BM-40 Δ I structure. Five polar residues at the tip of the newly formed α B– α D loop in BM-40 Δ I, α C are not seen in the electron density map and are presumed to be disordered.

In the BM-40 Δ I, α C crystals, the most prominent

Fig. 2. Structure of BM-40 Δ I, α C. **(A)** Two orthogonal views (Kraulis, 1991) related by a rotation of 90° about the vertical axis. The FS domain is in green and the EC domain is in red. The calcium ions bound to the EF hand pair in the EC domain (Ca1 and Ca2) are in yellow; the calcium ion bound at the tip of the α E– α F loop (Ca3) is in blue. Helices in the EC domain are labelled. Note that five residues around the deletion site (marked by a Δ) have no interpretable electron density, as indicated by the interruption to the red colouring. **(B)** Stereo view (Kraulis, 1991) of a superposition of the C_α traces of BM-40 Δ I (pink; Hohenester *et al.*, 1997) and BM-40 Δ I, α C (black; this work). Residues 162–205, whose conformation differs significantly in the two structures, were excluded from the calculation. The r.m.s.d. for 188 equivalent C_α atoms is $0.6\text{ \AA}$. Residues 196–203, which are deleted in BM-40 Δ I, α C, are shown with thick bonds in BM-40 Δ I and have been labelled. The disordered region in the BM-40 Δ I, α C structure is indicated by broken bonds.

packing interaction involves a bound calcium ion (Ca3; B -factor $11\text{ \AA}^2$) shared by two symmetry-related molecules. Three metal ion ligands are contributed by the first molecule of BM-40 Δ I, α C (the carbonyl oxygens of P241 and I243 and the side chain carboxylate of E246) and two additional ligands are provided by the second molecule (the carbonyl oxygen of T160 and the side chain carboxylate of E163). The average metal–ligand distance is $2.3\text{ \AA}$. The co-ordination geometry is that of an incomplete octahedron; the missing ligand may be a water molecule, and we indeed observe an appropriately positioned peak in the final difference Fourier map (not shown). The calcium-mediated association of two molecules of BM-40 Δ I, α C in the crystals results in 2-fold symmetric dimers, in which

the C-termini of helices α A contact the metal ion bound to the α E– α F loop. We do not know whether such dimers exist in solution. It is noteworthy that in the BM-40 EC domain structure (BM-40 Δ I,II; Hohenester *et al.*, 1996), a metal ion, putatively assigned as potassium, was observed bound to the tip of the α E– α F loop in a location identical to that of Ca3 in the present structure. This suggests the presence of a general cation site on BM-40 that may be involved in ligand binding (see below).

Site-directed mutagenesis of the collagen-binding epitope in the activated form of BM-40

The BM-40 mutant Δ I, α C was chosen for analysis of the collagen-binding epitope for two particular reasons.

Table II. Surface plasmon resonance assay of the binding of BM-40 Δ I, α C point mutants to immobilized human collagens IV and I

Δ I, α C mutants	Kinetic parameters for binding to						Analytical parameters (cumulative)	
	Collagen IV			Collagen I			Concentration range (μ M)	RU
	$k_{\text{diss}} \times 10^3$ (/s)	$k_{\text{ass}} \times 10^{-3}$ (/M/s)	K_d (nM)	$k_{\text{diss}} \times 10^3$ (/s)	$k_{\text{ass}} \times 10^{-3}$ (/M/s)	K_d (nM)		
No mutation ^a	1.3	16	81	2.4	28	86	0.05–2	50–600
L139A	1.6	15	107	2.1	25	84	0.05–3.2	25–380
S141A	1.0	17	59	2.0	22	91	0.1–3.2	70–580
E142A	1.8	18	100	2.3	33	70	0.1–0.8	60–330
T144A	1.2	10	120	2.0	12	167	0.5–8	350–1050
E145A	0.6	22	27	3.2	26	123	0.1–3.2	60–470
R149A	1.4	0.35	4000	1.2	0.23	5200	5–20	14–60
R149K ^b	–	–	–	–	–	–	0.1–6.4	0–15
R149L ^b	–	–	–	–	–	–	0.8–6.4	1–15
R151A	1.8	21	86	2.1	16	131	0.03–0.8	11–310
D152A	1.5	7.4	203	2.1	3.7	568	1–16	290–930
N156A	28	5.7	4900	19	2.8	6900	1–16	170–660
N156Q	13	4.6	2800	16	10	1600	0.2–12.8	30–500
T160A	1.1	15	73	2.0	18	111	0.03–1.6	8–290
L242A ^b	–	–	–	–	–	–	1–8	0–14 ^c
M245A	60	7.8	7700	43	4.3	10 000	2–10	190–390
M245Y	2.8	9.9	283	3.1	14	221	0.1–3.2	30–360
E246A ^b	–	–	–	–	–	–	1–8	0–17
R149/N156A ^b	–	–	–	–	–	–	0.1–6.4	0–14
N156A/M245A ^b	–	–	–	–	–	–	0.1–6.4	0–30 ^c

Kinetic parameters and the resulting K_d values were determined by two consecutive assays using the same batches of collagens by the evaluation described in Materials and methods. Analytical parameters refer to the concentration ranges of soluble BM-40 ligands and the corresponding concentration-dependent uptake of resonance units (RU). The latter showed ~20% deviation between the two collagen ligands.

^aAverage value ($\pm 15\%$ deviation) used in two series of examinations.

^bNB = no binding.

^cThe RU range for collagen I was somewhat larger but the evaluation was questionable.

Because of its higher affinity, it would allow the detection of changes after mutagenesis over a broader range by the available assays. In addition, the mutant may resemble an analogue of biological activation as discussed later. All the mutants designed (Table II, Figure 3) could be obtained in good yields, indicating that there was no folding problem. The purified proteins showed a single band in electrophoresis with a mobility identical to that of BM-40 Δ I, α C (data not shown). In addition, with two exceptions, they did not differ significantly from the parent molecule in the radioimmuno-inhibition assay (Figure 1). Thirteen positions of the EC domain were chosen for alanine mutagenesis, based on several considerations (Figure 3). Three residues in particular are mostly buried in BM-40 Δ I, but are essentially accessible in BM-40 Δ I, α C: R149, D152 and N156 (and, to a lesser extent, L148 and K155). We argued that these residues were good candidates for participating in collagen binding and should be mutated to define this epitope. Additional residues on α A were chosen to map the extent of the epitope (L139, S141, E142, T144, E145, R151, D152 and T160). W153, whose side chain is partly accessible, was not mutated as it contributes significantly to the hydrophobic core of BM-40. We also noted that several residues of the α E– α F loop, as well as Ca3, are directly adjacent to the putative epitope on helix α A. These residues, namely L242, M245 and E246 (a ligand of Ca3), were therefore included in our series of point mutants (Figure 3).

The analysis of a total of 13 alanine mutants revealed very similar changes of binding to collagens I and IV in surface plasmon resonance assays, as evaluated from K_d

values (Table II). Mutants R149A, N156A and M245A showed a 50- to 100-fold reduction in affinity, which was caused either by a strong decrease in the association rate constant (R149) or an increase in dissociation (N156, M245). Two more mutants (L242A, E246A) showed essentially no binding in the concentration range tested. A similar lack of binding was observed for double mutations at positions 149/156 and 156/245, demonstrating an additive effect. Three of these essential residues were examined further by introducing additional mutations. Substitution of R149 by Lys or Leu was even more deleterious than the Ala mutation, indicating a very strict structural requirement. Mutant N156Q still showed a 20- to 30-fold loss of activity, indicating the need for a perfect fit to the length of the side chain. However, mutant M245Y was only of 3- to 4-fold lower affinity compared with the parental Δ I, α C. A similar small reduction of binding was also observed for D152A. Ala mutations of L139, S141, E142, T144, R151 and T160 caused no significant reduction in affinity. Mutant E145A even showed a 3-fold increase in binding to collagen IV, but not to collagen I (Table II).

The binding of these mutants was also analysed by solid-phase assays with immobilized collagens I and IV. A few typical binding curves are shown in Figure 4 for collagen IV, and demonstrate examples of no or only slight changes, moderate loss or activation and a rather drastic inactivation. These assays were evaluated by comparing the concentration of soluble ligands required for half-maximal binding, which showed a 10-fold difference for BM-40 Δ I, α C interactions with collagen I and IV.

Fig. 3. Stereo view (Kraulis, 1991) of a C_{α} trace of the BM-40 $\Delta I, \alpha C$ structure showing the residues that were mutated in this study (see text). Disulfide bridges are shown with thick bonds. Mutated residues are identified by position numbers and their side chains. The three calcium ions are shown as black spheres.

Irrespective of this difference, all mutants showed a similar change in relative binding activity in both assays (Table III). The magnitude and direction of changes were basically the same as observed for the K_d values (cf. Table II). The only exception was mutant M245Y, where the loss of activity was ~10-fold more accentuated in the solid-phase assay. Together, these data demonstrated that five residues (R149, N156, L242, M245 and E246) are crucial for collagen binding. These residues occupy a restricted region constituted by helix αA and a link region on the surface of the activated BM-40 variant $\Delta I, \alpha C$ (Figures 3 and 5A).

Site-directed mutagenesis of a non-activated form of BM-40

Fragment ΔI , which still possesses helix αC , has a low collagen-binding affinity comparable with that of BM-40, as previously shown (Maurer *et al.*, 1995; Sasaki *et al.*, 1997). This fragment was now used to introduce some of the same mutations as in fragment $\Delta I, \alpha C$ as well as a P244A mutation. Analysis of the collagen IV binding of these mutants by surface plasmon resonance assay demonstrated similar but less marked affinity changes (Table IV). These included a distinct decrease for the BM-40 ΔI mutants R149A, N156A and M245A, a more moderate one for S141A and D152A, and no significant change for T144A, E145A and P244A. Similar changes were also observed for collagen I binding and were also confirmed for collagen IV by the solid-phase assay (data not shown). This strongly suggests that most, if not all, critical binding residues are the same for activated and non-activated forms of BM-40.

Discussion

The calcium-binding EC module of BM-40, which is also shared in modified form by five related proteins, was

Fig. 4. Solid-phase binding assays with immobilized human collagen IV and various BM-40 $\Delta I, \alpha C$ mutants. The soluble ligands were BM-40 $\Delta I, \alpha C$ (○) and its point mutations E145A (●), T144A (□), D152A (■), M245Y (▲), M245A (△), N156A (▼) and R149A (▽).

shown previously to harbour the collagen-binding epitope of this abundant extracellular matrix protein (Maurer *et al.*, 1995). It was shown further that cleavage of a single peptide bond of helix αC of the EC domain either by several matrix metalloproteinases (Sasaki *et al.*, 1997) or by unknown endogenous proteases (Maurer *et al.*, 1997a) increased collagen affinity by a factor of 10. Based on the X-ray structure of the EC domain (Hohenester *et al.*, 1996), this was interpreted to indicate the removal of steric constraints imposed by helix αC on the underlying helix αA . In the present study, we have confirmed this hypothesis by deleting helix αC (fragment $\Delta I, \alpha C$) and

showing that this causes a comparable increase in collagen affinity. X-ray crystallography of this deletion mutant demonstrated a rearrangement in the shortened link region between helix α A and the pair of EF hands exposing the central part of helix α A (Figure 2). The effect of the deletion of eight residues of helix α C on the BM-40 structure is not confined to the connection between helices α B and α D. It appears reasonable to assume that a very similar situation exists in BM-40 when proteolytically

Table III. Binding of BM-40 Δ I, α C mutants to immobilized collagens I and IV in solid-phase assays

Δ I, α C mutants	Collagen IV	Collagen I
No mutation	0.8	10
L139A	1	10
S141A	1	10
E142A	1	14
T144A	2	10
E145A	0.2	6
R149A	>350	>350
R149K	>350	>350
R149L	>350	>350
R151A	1.4	14
D152A	3	40
N156A	>350	>350
N156Q	>350	>350
T160A	0.8	12
L242A	>350	>350
M245A	>350	>350
M245Y	18	300
E246A	>350	>350
R149A/N156A	>350	>350
N156A/M245A	>350	>350

The concentrations (nM) required to achieve half maximal binding in comparison with Δ I, α C are recorded.

nicked at the N-terminus of α C (Sasaki *et al.*, 1997). BM-40 Δ I, α C, therefore, seems to be a good model of activated forms of BM-40 as they may exist in tissues (see below).

The collagen-binding epitope of BM-40, as defined by site-directed mutagenesis, is shown in Figure 5A. Two residues on helix α A (R149 and N156) and three residues on the loop connecting helices α E and α F (L242, M245 and E246) were identified as important for binding of both collagen I and IV. These five residues are arranged in a ring of ~ 15 Å diameter, surrounded by residues that were shown not to contribute greatly to collagen binding. Figure 5A suggests that two additional residues may be part of the epitope. The hydrophobic side chains of W153 and P241 are at least partially accessible and are located within the perimeter of the five critical residues. W153

Table IV. Surface plasmon resonance assay of the binding of BM-40 Δ I point mutants to immobilized human collagen IV

Δ I mutant	RU range	$k_{\text{diss}} \times 10^3$ (/s)	$k_{\text{ass}} \times 10^{-3}$ (/M/s)	K_d (μ M)
No mutation	13–40	2.7	1.2	2.2
S141A	16–49	2.0	0.42	4.8
T144A	12–41	3.4	2.0	1.7
E145A	23–99	2.0	1.1	1.8
R149A	3–10	5.8	0.2	29 ^a
D152A	9–33	6.1	1.1	5.5
N156A	34–94	25	2.4	10.4
P244A	69–133	3.2	2.3	1.4
M245A	6–21	no binding ^b		

Soluble BM-40 ligands were used in the concentration range 2 (4)–10 (12) μ M and the corresponding uptake of resonance units (RU) is indicated.

^aEvaluation with a large error range.

^bEvaluation impossible.

Fig. 5. Structure of the collagen-binding epitope and a hypothetical complex with a collagen triple helix. (A) Space-filling representation (Jones, 1991) of the BM-40 Δ I, α C structure in a view similar to Figure 3, showing the extent of the collagen-binding epitope. Residues whose mutation causes a significant reduction in collagen affinity are in red; residues whose mutation causes only a slight or no change in affinity are in brown. W153 and P241 may be part of the binding epitope (see text). (B) Model of a BM-40 Δ I, α C–collagen complex. A collagen triple helix, as represented by a synthetic (Pro-Hyp-Gly)₄-Pro-Hyp-Ala-(Pro-Hyp-Gly)₅ peptide (Bella *et al.*, 1994), was docked manually onto the binding epitope defined by mutagenesis. The single Gly to Ala substitution causes some local untwisting of the collagen triple helix, but this was not considered significant given the strictly qualitative nature of the model. The figure shows one of several possible arrangements related by a rotation of the collagen triple helix about an axis normal to the plane of the paper and passing through the centre of the binding site (see text). BM-40 residues are coloured as in (A) and the collagen triple helix is in yellow, with the N-terminus in the upper left-hand corner.

and P241 were not mutated, as we expected their mutation to Ala to be accompanied by major structural changes.

The surface of BM-40 in the region containing the collagen-binding site is relatively flat and does not feature a pronounced groove, as might have been expected for a binding site for triple-helical collagens. A model showing a complex of BM-40 Δ I, α C with a collagen triple helix is shown in Figure 5B. The extent of the collagen-binding epitope, as defined by site-directed mutagenesis, is seen to match the diameter of the collagen triple helix surprisingly well. The model also suggests that the complementary binding site on collagens should not extend over more than two Gly-X-Y triplet repeats (Brodsky and Shah, 1995). The flatness of the epitope places significant structural constraints on the interaction. The only real variable is the orientation of the axis of the triple helix in the plane defined by the five critical residues, although some arrangements can be ruled out because they would lead to clashes with helix α D and the α A- α D linker region.

The collagen-binding site of BM-40 consists of both polar and non-polar residues, suggesting that collagen binding is due to a combination of van der Waals and electrostatic interactions. Two critical residues, R149 and N156, are not exposed in uncleaved BM-40 (Hohenester *et al.*, 1996, 1997), and this is proposed to account for the relatively low affinity of intact BM-40 for collagens (Sasaki *et al.*, 1997). Binding of collagen by intact BM-40 may first involve the association of collagen with the solvent-accessible part of the epitope (L242, M245 and E246), followed by a conformational change in the region of α C, thereby uncovering R149 and N156 and consolidating the interaction between BM-40 and collagen. Proteolytic cleavage at the N-terminus of α C would remove the second, energetically costly step and thereby increase association rates and, consequently, collagen affinity (Sasaki *et al.*, 1997).

The observation of a calcium ion (Ca³⁺) involved in a crystal contact and co-ordinated by a residue shown to be important for collagen binding (E246) raises the interesting possibility that a metal ion may contribute to collagen binding. The side chain of E246 lies slightly below the surface containing the epitope. E246 could interact with either a lysine or an arginine residue of the collagen ligand. Alternatively, it may provide a crucial ligand for a calcium ion mediating the association of BM-40 and a collagen triple helix. This situation would be rather similar to the metal ion-dependent adhesion site (MIDAS) of integrin I-domains (Lee *et al.*, 1995). The recently determined structure of the collagen-binding I-domain of α 2 β 1 integrin shows a groove around the MIDAS motif, and the authors speculate that collagen binding by the I-domain may involve the co-ordination of the resident magnesium ion by a glutamic acid of the collagen ligand (Emsley *et al.*, 1997). Collagen binding to BM-40 has been shown to be calcium dependent (Maurer *et al.*, 1995), but this may reflect the requirement for calcium bound to the EF hand pair for the structural integrity of the EC domain. Further studies are required to define precisely the role of calcium in collagen binding to BM-40.

Several extracellular matrix proteins share a related EC domain with BM-40 (see Maurer *et al.*, 1995; Hohenester *et al.*, 1996). A sequence comparison of those regions critical for collagen binding (Figure 6) shows that proteins

	Helix α A	Link
	* * *	* **
BM-40, H	149-RMRDWLKN-156	242-LIPME-246
BM-40, CE	RMADWLFQ	VIPME
QR1	RMRDWLKN	LVPME
SC1	RMRDWLKN	LVPME
testican	RLKDWFGA	LDKYE
tsc36	RRIQWLEA	NETAI

Fig. 6. Sequence comparison of BM-40 helix α C and link regions essential for collagen binding with similar regions of related proteins. Critical residues are marked by an asterisk. Sequences are from human (H) and *C.elegans* (CE) BM-40 and from several mammalian proteins sharing an EC domain (Maurer *et al.*, 1995; Hohenester *et al.*, 1996).

QR1 and SC1 (hevin) have a remarkable conservation including all five essential residues. Since these two proteins also have a long connecting region between helices α A and α D, we would predict that they share collagen binding with BM-40 which may also be dependent on proteolytic activation. Testican and tsc36, on the other hand, differ by at least one substitution (N156A) in this region and would not be expected to be active. Recently, the EC domain of testican has been shown to lack significant collagen binding (Kohfeldt *et al.*, 1997). *Caenorhabditis elegans* BM-40 seems to represent a special case as it binds to mammalian collagens through its EC domain with an affinity ($K_d = 0.5\text{--}2.2\ \mu\text{M}$) comparable with mammalian BM-40 (Maurer *et al.*, 1997b). However, our mutant data suggest that an N156Q substitution in the nematode BM-40 (Figure 6) should result in a lower affinity (Table IV). Since the nematode BM-40 lacks a long connecting region (α A- α D), however, it may exist in a permanently activated form and should then have an affinity comparable with BM-40 Δ I, α C mutant N156Q ($K_d = 1.6\text{--}2.8\ \mu\text{M}$), which is in fact the case.

To date, few structural studies of collagen-binding sites have been reported for other proteins. Recently, the structure of the collagen-binding domain of *Staphylococcus aureus* adhesin became available (Symersky *et al.*, 1997). Docking and mutagenesis studies identified, as here, a crucial Arg and suggested a prominent and relatively narrow groove as the binding site for a collagen triple helix (Symersky *et al.*, 1997). Such a pronounced groove may be advantageous for binding generic collagen triple helices, but may not be an element of more specific mammalian collagen-binding proteins. This is suggested by two recent structure determinations of the A3 domain of von Willebrand factor (Bienkowska *et al.*, 1997; Huizinga *et al.*, 1997), where the collagen-binding site seems to reside on a relatively flat face of the domain (Huizinga *et al.*, 1997). By contrast, the related I-domain of α 2 β 1 integrin, which also binds collagen triple helices, has a groove in the corresponding region that also contains the MIDAS motif (Emsley *et al.*, 1997).

The biological consequences of BM-40 binding to collagens in tissues are only poorly understood, but could include a storage function as known for several cytokines (Taipale and Keski-Oja, 1997) or the modulation of various anti-adhesive or anti-proliferative activities reported for

BM-40 (Lane and Sage, 1994; Sage, 1997). A proteolytic activation mechanism in order to increase collagen affinity could therefore represent an important controlling step. This may involve several matrix metalloproteinases, which all cleave at position 197–198 in helix α C (Sasaki *et al.*, 1997), as well as unknown endogenous proteases cleaving at position 198–199 (Mann *et al.*, 1987; Maurer *et al.*, 1997b). BM-40 was shown, furthermore, to increase the production of several matrix metalloproteinases in cell culture (Tremble *et al.*, 1993), suggesting an autocrine or paracrine way of controlling activation. For further biological studies, we have recently generated antisera against the N-terminal part of the two identified cleavage sites using synthetic peptide antigens (T.Sasaki and R.Timpl, unpublished). These antibodies distinguished not only between intact and activated BM-40 but also between the two different cleavage sites. These new reagents and the precise mapping of the collagen-binding site as described here will therefore offer novel molecular approaches to study BM-40 function in a biological context.

Materials and methods

Sources of proteins

Recombinant human BM-40 (Nischt *et al.*, 1991) and its deletion mutants Δ I and Δ I,II (Maurer *et al.*, 1995) have been described previously. Pepsin digests of human placenta were used to purify the fibrillar collagens I, III and V (Miller and Rhodes, 1982) and the basement membrane collagen IV (Vandenberg *et al.*, 1991). Recombinant cartilage collagen II (Fertala *et al.*, 1994) was kindly provided by Dr D.J.Prockop.

Construction of expression vectors

The insert of the construct for human BM-40 deletion mutant Δ I (Maurer *et al.*, 1995) was used to introduce further mutations based on oligonucleotide primers by polymerase chain reaction (PCR) using Vent polymerase (New England Biolabs). The two terminal primers were F1 (GTCAAGCTAGCAAATCCCTGCCAGAAC) for the 5' end and R1 (GTCACTCGAGTTAGATCACAAAGATCCTTGTCG) for the 3' end. Deletion of helix α C was introduced by primers DelC1 (GTTCTTCTCGGGGTGGTCTC) and DelC2 (GAGACCACCCCGAGAAGAAC) which were combined with F1 and R1, respectively, to generate the 5' and 3' fragments. The gel-purified fragments were fused by annealing overlapping ends and amplified with primers F1 and R1 to give the final PCR product (Vallejo *et al.*, 1994). Point mutations were introduced into the inserts encoding BM-40 Δ I or Δ I, α C by using appropriate central overlapping primers (not shown) following the same strategy. A few mutations were close to an internal *Eco*RI site, which was used occasionally for insertion into the parental vector. The PCR products were cloned into blunt-ended vector pUC18 by using the Sure Clone Kit (Pharmacia), and the correctness of the sequence was confirmed by the Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI). The *Nhe*I-*Xho*I-released inserts of correct clones were isolated and inserted into corresponding sites of the expression vector pCEP/Pu, which contains the BM-40 signal peptide sequence (Kohfeldt *et al.*, 1997).

Recombinant production and purification

The expression vectors were used for the episomal transfection of human EBNA-293 cells (Invitrogen). Resistant cells were selected with puromycin (0.5 μ g/ml) and used for the collection of serum-free conditioned medium. The medium (200–300 ml) was passed over a DEAE-cellulose column (2.5 $\times$ 25 cm) which was equilibrated in 0.05 M Tris-HCl, pH 8.6, and eluted with a linear gradient of 0–0.4 M NaCl (500 ml). All recombinant products eluted as a sharp peak around 0.2 M NaCl, and were concentrated by ultrafiltration and dialysed against 0.05 M Tris-HCl, pH 7.4, 0.1 M NaCl. Electrophoresis demonstrated a purity of >95%. The yields were in the range 3–10 mg protein.

Analytical methods

Protein concentrations were determined after hydrolysis with 6 M HCl (16 h, 110°C) on an LC amino acid analyser. SDS-gel electrophoresis

Table V. Data collection and refinement statistics

Data collection (15–2.8 Å)	
Observations	98 015
Unique reflections	17 878
Completeness (%) ^a	99.2 (99.5)
R_{merge} ^b	0.106 (0.445)
$I/\sigma(I)$	6.6 (1.8)
Refinement (6–2.8 Å)	
Reflections ($F > 0$)	16 014
Non-H atoms	2 $\times$ 1772
R -factor ^c	0.255
R_{free} ^d	0.306
Bond lengths (Å)	0.007
Bond angles (°)	1.4
B -factors (Å ²)	7.6

^aThe numbers in parentheses refer to data in the highest resolution shell (2.92–2.80 Å).

^b $R_{\text{merge}} = \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the i -th measurement of reflection h and $\langle I(h) \rangle$ is the weighted mean of all measurements of h .

^c R -factor = $\sum_h |F_{\text{obs}}(h) - F_{\text{calc}}(h)| / \sum_h F_{\text{obs}}(h)$, where F_{obs} and F_{calc} are the observed and calculated factor amplitudes, respectively.

^d R_{free} is the R -factor calculated with 10% of the data excluded from the refinement.

in polyacrylamide gradient gels (10–20%) followed established protocols. A radioimmunoassay with a rabbit antiserum against human BM-40 has been described (Nischt *et al.*, 1991).

Crystallization and X-ray data collection

BM-40 Δ I, α C did not crystallize under the conditions used for BM-40 Δ I (Hohenester *et al.*, 1997). Crystals of BM-40 Δ I, α C were obtained at room temperature by the hanging drop vapour diffusion method: 3 μ l of a protein stock solution (8 mg/ml in 10 mM Tris-HCl pH 7.5, 2 mM CaCl₂) were mixed with 2 μ l of reservoir solution [12–14% (w/v) polyethylene glycol 4000, 10% (v/v) 2-propanol, 100 mM Na-HEPES pH 7.5] and equilibrated against 1 ml of the latter solution. The crystals belong to space group P2₁2₁2₁ with unit cell constants $a = 51.9$ Å, $b = 88.1$ Å, $c = 153.7$ Å. There are two molecules of BM-40 Δ I, α C in the asymmetric unit, resulting in a solvent content of ~65%. The crystals diffracted weakly to 2.8 Å resolution. Diffraction data were collected at 100 K using a MAR image detector mounted on an Elliot GX-21 rotating anode generator operated at 4 kW. Crystals were transferred into artificial mother liquor with 10, 20 and 30% glycerol added (three consecutive soaks of 10 s) and frozen in the cold nitrogen stream. Data were integrated with MOSFLM (Leslie, 1994) and reduced with programs of the CCP4 suite (Collaborative Computing Project No. 4, 1994). Data collection statistics are summarized in Table V.

Solution and refinement of the structure

The self-rotation function did not show any peaks attributable to the non-crystallographic symmetry (NCS) relating the two molecules of BM-40 Δ I, α C in the asymmetric unit. The native Patterson map showed a strong non-origin peak at $\tau(0, 0.5, 0.5)$, suggesting the presence of two similarly oriented molecules. The structure of BM-40 Δ I, α C was solved by molecular replacement with AMoRe (Navaza, 1994). The search model was the BM-40 Δ I structure (Hohenester *et al.*, 1997), with residues 168–208 and the NAG sugar moieties removed. The cross-rotation function (15–3.5 Å) showed one prominent peak at 6.8 σ , compared with 2.9 σ for the highest noise peak. The highest peak in the translation function (6–2.8 Å) resulted in a solution with an R -factor of 0.511. The first molecule was fixed at the respective position and a partial translation function was calculated, which revealed the position of the second molecule, shifted by 0.53 b + 0.47 c relative to the first one, as expected. The R -factor of the two independent molecules after rigid-body refinement (6–2.8 Å) was 0.433 and the correlation coefficient was 0.635.

The electron density map calculated from the molecular replacement solution was improved by 2-fold NCS averaging with RAVE (Kleywegt and Jones, 1994). The resulting map allowed most of the residues missing from the search model to be built, with the exception of a few residues around the deletion site. The structure was refined with X-PLOR (Brünger, 1992) using all observed ($F > 0$) data between 6 and 2.8 Å resolution; strict NCS was imposed throughout. The final model consists

of residues 54–286, three calcium ions and two NAG sugar moieties attached to N99. Residues 192–195 and 204–205 are not defined by the electron density and have been modelled stereochemically. About 10% of the unit cell content is carbohydrate that is not accounted for by the crystallographic model, but is partially ordered and makes a non-negligible contribution to the diffraction at low resolution; therefore, data below 6 Å resolution were excluded from the refinement. A similar problem had been encountered in the structure determination of BM-40 ΔI (Hohenester *et al.*, 1997). According to the definition of PROCHECK (Laskowski *et al.*, 1993), 91.8% of the residues are in core regions of the Ramachandran plot, with the remaining 8.2% in additionally allowed regions. The NCS real-space correlation coefficient of the final averaged map was 0.94 and the *R*-factor of the back-transformed map with the observed amplitudes was 0.194. Refinement statistics are summarized in Table V. Co-ordinates and structure factors have been deposited in the Brookhaven Protein Data Bank (accession code 1NUB) and will be made available 1 year after publication.

Binding assays

Surface plasmon resonance assays were performed with BIAcore instrumentation (BIAcore AB Uppsala) and various immobilized collagens. Collagens were immobilized by covalent coupling to CM-5 sensor chips (research grade) (Maurer *et al.*, 1995), resulting in binding of 6000–8000 RU. Binding assays were performed in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM CaCl₂ and 0.05% P20 detergent (BIAcore) at 25°C and a flow rate of 20 µl/min. Preliminary assays showed that this flow rate was high enough to avoid mass transport problems. The different soluble ligands were applied as far as possible in a concentration range around the *K_d* value, resulting in an uptake of 20–3000 RU. The association curve was followed for 3 min, then the protein solution was replaced by buffer and the dissociation curve was monitored for another 15 min. Since most of the spontaneous dissociations were not quantitative, the chips were completely regenerated by treatment with 10 µl of 10 mM HCl. This treatment did not affect the immobilized layer, as controlled by repeated injections of the same amount of soluble ligands. Non-specific binding to the surface was excluded for all ligands by carrying out binding assays on chips without collagens. The integrity of the immobilized collagens was tested from time to time with known ligands. All measurements were repeated several times with different batches of immobilized and soluble ligands.

Kinetic constants were calculated by non-linear fitting of the association and dissociation curves according to the 1:1 model $A + B = AB$ using BIAevaluation software version 2.1, following the manufacturer's instructions. For evaluation of the dissociation rate constants, the time ranges from ~30–50 s (to avoid bulk refractive index effects at the very beginning of the dissociation) and 300–400 s (analyte rebinding can affect the later part of the dissociation) after the start of dissociation were used. Plots of $\ln(R_0/R)$ against *t* were linear over a wide time span, indicating that a 1:1 dissociation model should be reasonably applicable. For fitting of the association curve, the range where $\ln(dR/dt)$ was linear and the noise level not too high was selected. The fit led to a direct calculation of *k_{ass}*. As a second plot for the association phase, *k_{obs}* [slope of $\ln(dR/dt)$ against *t*] versus *C* and its linear fit were used to calculate *k_{ass}* from the slope and *k_{diss}* from the intercept. This linear fitting allows control of the non-linear fitting and of the applicability of the 1:1 model.

Solid-phase assays with plastic-immobilized collagens followed a previously described protocol (Aumailley *et al.*, 1989). All binding tests were performed in the presence of 2 mM CaCl₂.

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