Dimer model for the microfibrillar protein fibulin-2 and identification of the connecting disulfide bridge

Takako Sasaki, Karlheinz Mann, Hanna Wiedemann, Walter Göhring, Ariel Lustig¹, Jürgen Engel¹, Mon-Li Chu² and Rupert Timpl³

Max-Planck-Institut für Biochemie, D-82152, Martinsried, Germany,

¹Biozentrum, University of Basel, CH-4056, Basel, Switzerland and

²Department of Dermatology and Cutaneous Biology,

Thomas Jefferson University, Philadelphia, PA 19107, USA

³Corresponding author

Fibulin-2 is a novel extracellular matrix protein frequently found in close association with microfibrils containing either fibronectin or fibrillin. The entire protein and its predicted domains were obtained as recombinant products and examined by ultracentrifugation and electron microscopy. This demonstrated a disulfide-linked homodimer of 175 kDa subunits. Partial reduction to monomers identified specifically an odd Cys574 residue responsible for dimer formation in one of three anaphylatoxin-like modules that constitute the central globular domain I (13 kDa) of fibulin-2. Furthermore, a Cys574–Ser mutation abolished disulfide connection but not non-covalent dimerization of fibulin-2. The C-terminal region (85 kDa) was shown to represent a 35-nm-long rod consisting of 11 calcium-binding EGF-like modules (domain II) and a small terminal globe (domain III). The unique N-terminal domain N (55 kDa) was also rod-shaped (~38 nm) and rich in galactosamine indicating extensive O-glycosylation. A dimer model is proposed indicating mainly a rod-like shape of 80 nm length based on an anti-parallel association of two subunits through their domains I. This model also implies alignment of domains II and N between different subunits. This was demonstrated by surface plasmon resonance assay which showed a distinct interaction between domains N and II with a K_d of ~0.7 μ M.

Keywords: electron microscopy/extracellular matrix/recombinant protein/site-directed mutagenesis/ultracentrifugation

Introduction

The extracellular matrix protein fibulin-2 was first identified by cDNA cloning and sequencing (Pan *et al.*, 1993; Zhang *et al.*, 1994) and shown to belong to the family of proteins containing long tandem arrays of epidermal growth factor (EGF)-like modules with a consensus sequence for calcium binding (Campbell and Bork, 1993). This has enabled mouse and human fibulin-2 to be obtained in recombinant form and the generation of specific antibodies (Pan *et al.*, 1993; Sasaki *et al.*, 1995a).

Based on these tools a broad expression and deposition could be demonstrated for fibulin-2 in embryonic and adult tissues. These included in particular blood vessels and endocardial tissues, regions rich in elastin, some basement membranes but also neural and bone-forming tissues (Pan *et al.*, 1993; Zhang *et al.*, 1995, 1996; Miosge *et al.*, 1996; Reinhardt *et al.*, 1996a). Fibulin-2 was also shown to be highly sensitive to various matrix metalloproteinases (Sasaki *et al.*, 1996a) and to be up-regulated during wound repair (Fässler *et al.*, 1996) indicating a strong involvement in tissue remodelling. Binding studies *in vitro* demonstrated distinct affinities of fibulin-2 for the basement membrane components nidogen, perlecan and collagen IV and for fibronectin and fibrillin which in most cases were dependent on calcium (Sasaki *et al.*, 1995a; Reinhardt *et al.*, 1996a). Binding to the latter two ligands was of particular interest since immunogold staining demonstrated a distinct colocalization of fibulin-2 in fibrillin-containing elastic microfibrils (Reinhardt *et al.*, 1996a) and to fibronectin fibrils deposited in fibroblast cultures (Sasaki *et al.*, 1996b). All this information indicated that fibulin-2 could participate in different molecular assemblies of the extracellular matrix and may in addition be a cell-adhesive protein (Pfaff *et al.*, 1995).

These various potential activities made it desirable to establish a precise model of fibulin-2 as a basis for approaching its structure–function relationships. Initial studies of recombinant mouse fibulin-2 demonstrated oligomerization through disulfide bonds and, by electron microscopy, indicated a distinct frequency of three-arm rod-like structures which suggested a homotrimeric assembly (Pan *et al.*, 1993). It was also discussed that a novel N-terminal domain N, which distinguishes fibulin-2 from its closest relative, monomeric fibulin-1, could be the site of oligomerization. Yet these analyses demonstrated also a considerable variability in shape including also two- and four-arm structures. In the present study we have addressed the nature of oligomerization and shape variability by the recombinant production of predicted fibulin-2 domains in various combinations and their analysis by electron microscopy, ultracentrifugation, Edman degradation and site-directed mutagenesis. This led to the proposal of a dimer organization which basically has the shape of a single rod and is stabilized by a single disulfide bond located in a central region of fibulin-2.

Results

Characterization of recombinant fibulin-2 fragments

Previous sequence analysis of mouse and human fibulin-2 (Pan *et al.*, 1993; Zhang *et al.*, 1994) predicted the existence of four distinct domains, N, I, II and III, each one being characterized by a unique set of protein modules

Fig. 1. Domain model of fibulin-2 (top) and schematic outline of the fragments examined. The domains include unique sequences in the N-terminal (N) and C-terminal (III) region and tandem arrays of established modules (I and II). Small letters indicate individual modules or special sections. Fragments ST-1 and ST-3 were obtained by stromelysin proteolysis of fibulin-2 (Sasaki *et al.*, 1996a). All other fragments were recombinant products.

(Figure 1). In order to study the shape, oligomerization and other properties of these domains nine different eukaryotic expression vectors encoding individual domains or various combinations of them were used for the episomal transfection of human embryonic kidney cells (see Materials and methods). Each of the expression vectors contained the same signal peptide region of protein BM-40, giving rise to an artificial N-terminal APLA sequence (Mayer *et al.*, 1993), which allowed purification of the recombinant fibulin-2 fragments from serum-free culture medium in two chromatographic steps as shown by electrophoresis (Figure 2).

Successful production was achieved for three of the four predicted domains, the N-terminal fragment N (sequence positions 1–408 of mouse fibulin-2, Pan *et al.*, 1993), the central fragment I consisting of three anaphylatoxin-like modules (positions 405–525) and fragment II (positions 562–1081) which contains a tandem array of 11 EGF-like modules and two link regions and contributes many calcium-binding sequences (Figure 1). A fragment corresponding to the C-terminal domain III could not be obtained in substantial quantities indicating that the chosen domain borders (positions 1081–1195) do not correspond to an autonomous folding unit within fibulin-2. It was also possible to obtain domain combinations such as fragments N+I, N+I+II and II+III in sufficient purity and quantities. A further fragment I+II+III was also obtained in good yields but showed heterogeneity in size and N-terminal sequence. For this reason we prepared two more fragments by stromelysin cleavage of recombinant mouse fibulin-2

within the central region of domain N (Sasaki *et al.*, 1996a). This included the N-terminal monomeric fragment ST-3 (~210 residues) and the C-terminal disulfide-linked fragment ST-1 (Figure 1).

Hexosamine analyses were used to determine glycoconjugation patterns of recombinant mouse fibulin-2 and several fragments (Table I). This demonstrated 15 residues of glucosamine in fibulin-2 consistent with sequence predictions (Pan *et al.*, 1993) which were, however, obviously insufficient to account for the full substitution of all four N-glycosylation sites (Figure 1). Fragment studies demonstrated full occupation of the site in domain N, presumably of one of the two sites in domain II and no substitution in domain I. However, a high content of galactosamine (20 residues) indicating extensive O-glycosylation was exclusively localized to the C-terminal Nb portion of domain N (Table I). This suggests a mucin-like region within domain N of fibulin-2.

Because of the disulfide-dependent oligomerization of recombinant as well as cell- and tissue-derived fibulin-2 (Pan *et al.*, 1993; Sasaki *et al.*, 1996b) we used SDS gel electrophoresis of non-reduced and reduced fragments for the identification of the domain responsible for oligomerization (Figure 2). This demonstrated that fragments N+I, N+I+II and I (Figure 3) were completely disulfide-bonded. Fragments N, II and II+III, however, apparently lacked such disulfide bonds, strongly suggesting that domain I was exclusively responsible for disulfide linkage. Fragment I+II+III represented a special case since electrophoresis demonstrated only minor amounts of

Fig. 2. SDS gel electrophoresis of fibulin-2 and fibulin-2 fragments under non-reducing (A) and reducing conditions (B). Samples used were fibulin-2 (lanes 1), fibulin-2 mutant C574-S (lanes 2) and the fragments N+I (lanes 3), N+I+II (lanes 4), I+II+III (lanes 5), N (lanes 6), II (lanes 7) and II+III (lanes 8). Runs were calibrated with marker proteins used either in non-reduced (A) or reduced (B) form and their migration positions and molecular masses (in kDa) are indicated at the left margin.

disulfide-bonded oligomers but mainly several bands at the monomer positions which showed the expected N-terminal sequence (APLANSPGD) but also the sequence TIPLP-VPQPN demonstrating proteolytic cleavage between modules IIx and IIa (Figure 1) which would remove domain I. Furthermore, short metabolic labelling of the transfected cells producing fragment I+II+III demonstrated larger amounts of the disulfide-bonded form of fragment I+II+III but also monomers. This suggests that the fragment has more difficulty in forming oligomeric disulfide bonds than others containing domain I. Proteolysis during the long term preparative experiments may have obscured further oligomerization.

Table I. Hexosamine content of mouse fibulin-2 and its fragments

	Glucosamine	Galactosamine
Fibulin-2	15.0 ± 3.9	20.1 ± 3.2
Fragment N	7.3 ± 0.6	17.4 ± 3.2
Fragment ST-3	3.5	<0.2
Fragment I	<0.2	<0.2
Fragment II	5.1	<0.2
Fragment II+III	4.7	<0.2

Values are expressed as residues per monomeric protein and shown as mean ±SD or single determinations.

Analysis by ultracentrifugation and mass spectrometry

Reduced fibulin-2 showed by electrophoresis a molecular mass of 195 kDa (Pan *et al.*, 1993) which exceeded the calculated molecular mass (Table II) by ~35%. Since this anomalous behaviour prevented the unambiguous determination of the molecular mass of the disulfide-bonded forms by electrophoresis, we used ultracentrifugation in neutral buffer either in the presence or absence of EDTA and in 6 M guanidine to examine this particular question (Table II). This demonstrated clearly for fibulin-2 and fragments ST-1 and N+I under dissociating conditions a dimer for the disulfide-bonded forms. Some average higher molecular masses together with evidence for heterogeneous populations were found in neutral buffer, particularly in the absence of EDTA, indicating non-covalent association. Fragments N, ST-3, II and II+III, however, were evidently monomers in neutral buffer and under dissociating conditions. The disulfide-bonded fragment I exceeded the molecular mass of a dimer by 30–43% in both solvents (Table II). MALDI mass spectrometry was therefore used for further confirmation which demonstrated a molecular mass for the non-reduced fragment I of 26 656.8 which was in good agreement with the calculated mass of 26 663.9 for a dimeric structure.

The calculation of axial ratios (a/b) for prolate ellipsoids of revolution from sedimentation coefficients and molecular masses (van Holde, 1971) was also useful to get an estimate of the shape of fibulin-2 and several individual fragments (Table II). This demonstrated ratios between 7 and 30 for the complete protein and most fragments consistent with elongated structures. Fragment I was exceptional with a ratio of 1 indicating a spherical shape. Fragment II+III showed also a low axial ratio of 4 in agreement with the presence of a globular and rod-like structure as demonstrated by electron microscopy (Figure 4).

Localization of a connecting disulfide bridge in domain I

Partial reduction of disulfide bonds in fragment I was used for the identification of cysteines involved in dimer connection. This was achieved by increasing the concentration of dithiothreitol (DTT) from 25 μM to 20 mM which at the lower concentrations caused the increasing conversion of the 22 kDa dimer band to a single 15 kDa band which was followed with some overlap by a slight reduction in the electrophoretic mobility of the monomer band (Figure 3A). This indicated a two-step mechanism where the opening of the dimerizing disulfide bridge is

Fig. 3. Examination of recombinant domain I of fibulin-2 by limited reduction and SDS gel electrophoresis and sequence analysis for the identification of a connecting disulfide bond. (A) Conversion of recombinant fibulin-2 domain I by increasing concentrations of DTT from a 22 kDa to a 15 kDa band followed by a slow decrease in mobility (17 kDa) which was complete at high concentrations of the reducing agent. (B) Comparison with proteolytically obtained domain I of fibulin-1 which migrates non-reduced (Non-R) as a 24 kDa component and has a slightly lower mobility after complete reduction. Both electrophoresis runs (A and B) were calibrated with non-reduced marker proteins (left margin, in kDa). (C) Alignment of the sequence of the three anaphylatoxin-like modules of fibulin-2 domain I (Pan *et al.*, 1993) and sequence determination of a partially reduced fragment by Edman degradation (underlined). The Cys574 identified as responsible for dimerization is marked by an asterisk. All cysteines are shown in bold face. Numbers on top of the six cysteines in module Ia are shown to identify their connectivity (Cys1–Cys4, Cys2–Cys5, Cys3–Cys6) as determined for anaphylatoxin C3a (Huber *et al.*, 1980).

Table II. Sedimentation coefficients ($s_{20,w}$) and molecular masses (M) of fibulin-2 and several of its fragments determined by ultracentrifugation

	Solvent	$s_{20,w}$ (S)	M found (kDa)	M calc (kDa)	Axial ratio ^a a/b
Fibulin-2	TBS	7.2	536 ^b	145	~30
	TBS, E	7.2	353		
	Gu	6.4	280		
Fibulin-2 Mutant C574-S	TBS	7.0	~800 ^b	145	
	TBS,E	7.0	340		
	Gu	4.1	175		
Fragment ST-1	TBS,E	6.6	300	122	~20
	Gu	6.4	243		
	TBS,E	2.0	30	23	
Fragment ST-3	TBS,E	2.6	57	57	7 12
	TBS	2.5	53		
	Gu	2.4	55		
Fragment I	TBS	3.1	32	13	1
	TBS,E	2.9	32		
	Gu	2.4	30		
Fragment N+I	TBS,E	4.6	180	70	~20
	Gu	4.1	123		
	TBS,E	3.3	72	60	
Fragment II	Gu	3.6	70		10
	TBS,E	4.2	85	72	
	Gu	3.9	83		

Samples were analyzed in 0.05 M Tris–HCl pH 7.4, 0.1 M NaCl (TBS), TBS containing 10 mM EDTA (TBS,E) or in 6 M guanidine (Gu). M was also calculated (calc) for monomeric structures based on the amino acid sequence and adding average masses for occupied *O*-glycosylation (0.6 kDa) and *N*-glycosylation (2 kDa) sites. The fragments are explained in Figure 1.

^aCalculated from the frictional ratio f/f_0 assuming the shape of an elongated ellipsoid.

^bSamples contain in addition larger sized material.

Fig. 4. Electron microscopy of fibulin-2 and several of its fragments after rotary shadowing. Representative fields are shown for fibulin-2 (A) and the fibulin-2 mutant C574-S (B) and the fragments N (C), N+I (D), II (E) and II+III (F).

followed by complete reduction of all disulfides at 20 mM DTT. A fragment I homolog obtained from the monomeric fibulin-1 did not show this initial dimer–monomer conversion but only a small shift to a band of slower electrophoretic mobility after full reduction (Figure 3B).

A partially reduced fibulin-2 fragment I sample with ~50% monomer conversion was subsequently alkylated with vinylpyridine followed by complete reduction and alkylation with iodoacetate. Both forms of alkylated cysteines can be clearly distinguished after Edman degradation and this fragment I was used, after appropriate proteolytic cleavage, to determine the whole sequence (Figure 3C). This demonstrated that only a single cysteine (Cys574) in the anaphylatoxin-like module Ib was modified by both alkylating agents in about equal proportions, indicating that it is involved in dimerization. All other cysteines were only carboxymethylated. Interestingly, Cys574 is located in the only anaphylatoxin-like module containing an odd number of cysteines (Figure 3C).

Mutation of Cys574 prevents disulfide bond connection but not dimerization

Based on the sequence data described above the expression vector for fibulin-2 was changed by a single point mutation converting Cys574 to Ser. This mutant C574-S could be as efficiently produced and purified as the natural fibulin-2. Examination of the mutant protein by SDS gel electrophoresis under non-reducing and reducing conditions demonstrated, however, the same band of ~195 kDa (Figure 2, lanes 2) which is the most crucial difference

compared with fibulin-2 (Figure 2, lanes 1). This confirmed the data for fragment I and showed that disulfide bond formation through a single cysteine is sufficient in the stabilization of fibulin-2 dimers. The molecular mass of a monomer was also observed for mutant C574-S by ultracentrifugation in 6 M guanidine. Yet dimers of the mutant are apparently formed in neutral buffer containing EDTA and even higher aggregates in the absence of EDTA (Table II). Non-covalent associations were also indicated by electron microscopy (Figure 4B).

Shape analysis by electron microscopy

Rotary shadowing of fibulin-2 (Figure 4A) and the mutant C574-S (Figure 4B) produced similar complex images of star-like particles consisting of two-, three- and four-arm structures mixed with some larger aggregates. Hallmarks of the structure were rods of ~40 nm length connected in the center by a globular domain and containing at the tips of some rods further, but often smaller, globular structures. Similar pictures were also obtained for fragments ST-1 and N+I+II, the latter often lacking the distal globules (data not shown). It was therefore instrumental to analyze smaller recombinant fragments for the correct interpretation of such complex structures. Fragment N was mainly represented by rods with a length of 38.5 ± 4.8 nm which essentially lacked a globular domain (Figure 4C). Fragment N+I was apparently a dimeric rod connected in the center by a small globular domain (Figure 4D). The entire length of this dimer was 73.8 ± 6.9 nm. Fragment II appeared rather uniformly as rods of 34.8 ± 2.6 nm length

Table III. Analysis of internal interactions of fibulin-2 domains by surface plasmon resonance assay

Soluble ligand	k_{diss} (s^{-1})	k_{ass} ($\text{M}^{-1} \text{s}^{-1}$)	K_{d} (μM)
Fragment II	9.5×10^{-3}	1.3×10^4	0.73
Fragment II+III	6.6×10^{-3}	9.5×10^3	0.70
Fragment ST-1	5.9×10^{-3}	3.3×10^4	0.18
Fibulin-2	7.6×10^{-3}	1.4×10^4	0.54

The immobilized ligand was fragment N and soluble ligands were examined at concentrations of 1–2 μM in neutral buffer containing 2 mM CaCl_2 .

(Figure 4E). Similar rods with the frequent appearance of one terminal globule were observed for fragment II+III (Figure 4F). The average length of the particles with globules was 37.4 ± 3.0 nm. Further electron micrographs of fragment ST-3 demonstrated compact 6–8 nm long rods and for fragment I small globular structures (data not shown).

Binding between different fibulin-2 domains

The dimer model discussed below for fibulin-2 implicated an alignment between domains N and II. Such potential interactions were analyzed by surface plasmon resonance assays using fragment N in immobilized form and several soluble ligands. These soluble ligands showed distinct although weak binding in neutral buffer containing 2 mM CaCl_2 and allowed the determination of rate and equilibrium dissociation constants (Table III). This demonstrated a moderate binding of monomeric fragments II and II+III to domain N ($K_{\text{d}} = 0.7 \mu\text{M}$) and a 4-fold higher affinity for the dimeric fragment ST-1 containing also domain II. Even fibulin-2 bound to domain N but here estimates of $K_{\text{d}} = 0.54 \mu\text{M}$ are tentative since the number of molecules with available fragment II binding sites are unknown. No binding was, however, observed between immobilized and soluble fragment N, underscoring the specificity of the other interactions. All these interactions were also abolished by replacing calcium in the binding buffer by EDTA.

Dimer model of fibulin-2

The entire set of data was sufficiently consistent to let us propose a dimer model for the basic oligomeric unit of fibulin-2 which also provided explanations for the apparent shape variability observed by electron microscopy (Figure 5). The crucial suggestion is the overlapping linear alignment of two subunits in anti-parallel orientation with their disulfide bond connection through domain I being located in the center of the dimer. This indicates in the simplest case basically a rod-like structure of ~80 nm length with a central and small terminal globular domains. It also implies the alignment of domains II and N from opposite subunits through almost the entire length of the two rod-like segments which is supported by interaction data. Spreading of individual fibulin-2 molecules on the mica discs as required for rotary shadowing, may frequently disrupt one or two of these alignments giving rise to three- and four-arm structures. Typical electron microscopical examples for each case can be easily distinguished (Figure 5). Yet the similar length of the rod-like domains N and II and the small size of the terminal

Fig. 5. Dimer models of fibulin-2 and interpretation of their shape variability. Top: schematic models for two-, three- and four-arm structures which are all connected through domain I but vary in the alignment and interaction between domains N and II derived from opposite monomers. Bottom: electron micrographs of selected fibulin-2 particles representative of two-, three- and four-arm structures arranged in the same order as the models on top.

Fig. 6. Comparison of the shapes of monomeric fibulin-1 and fibulin-2. The length of their rod-like domains corresponds to those determined by electron microscopy. The shape of fibulin-2 monomer was predicted from those of individual fragments which are explained in Figure 1.

globular domains often makes it difficult to identify the C- or N-terminal origin of individual arms.

Discussion

The data described in the present study clearly establish that recombinant mouse fibulin-2 is produced and secreted as a disulfide-bonded homodimer and revise our previous

Fig. 7. Outline of the construction of expression vectors for the production of recombinant fibulin-2 fragments and a single-site mutant (C574-S). The strategies included several large cDNA fragments (thick white bars) generated through unique restriction sites indicated on top. Thin hatched bars indicate various segments amplified by PCR with oligonucleotide primers (see text) denoted underneath by arrows.

suggestion that it could be a homotrimer (Pan *et al.*, 1993). The same seems to hold true for tissue-derived fibulin-2 as shown by immunoblotting (Sasaki *et al.*, 1996b). Based on the electron microscopical appearance of recombinant fibulin-2 fragments the monomer structure is represented by a 70-nm-long rod contributed by domains N and II which contains a small central (domain I) and C-terminal (domain III) globular structure (Figure 6). This shape is similar to that of fibulin-1 which contains only homologs of domains I, II and III and appears as a dumb-bell-like structure of ~30 nm length (Figure 6; Sasaki *et al.*, 1995b). Fibulin-1, however, is predominantly a monomer and does not form disulfide-linked dimers although it may non-covalently aggregate into some oligomeric structures (Balbona *et al.*, 1992; Sasaki *et al.*, 1995b).

The short length (80 nm) of fibulin-2 dimers when visualized as two-arm structures by electron microscopy (Figure 5) strongly indicated a considerable topological overlap between the two monomers. This could best be explained by alignment of both rod-like domains over their entire length indicating their non-covalent interaction through distinct epitopes. Partial disruption of these interactions gives rise to three- and four-arm structures (Figure 5) but may in part be induced by the conditions

of rotary shadowing. In support of this model moderate interactions between domains N and II could be determined by surface plasmon resonance assays ($K_d = 0.7 \mu\text{M}$). These interactions became distinctly weaker after calcium depletion consistent with the presence of nine calcium-binding EGF-like domains in domain II of fibulin-2 (Figure 1; Pan *et al.*, 1993). The proposed dimer model favors an anti-parallel association of the monomers which implicates domain II and N interactions between opposite monomers (Figure 5). This would be in agreement with the more frequent appearance of stretched fibulin-2 two-arm dimers compared with those where dimer arms are spread within a short angle. Nevertheless our data can not exclude a parallel association through domain I which would implicate internal domain II and N interactions within each single monomer. A decision between these possibilities will depend on the three-dimensional analysis of dimeric fragment I by X-ray crystallography or NMR, which is feasible through recombinant production.

A tandem array of three anaphylatoxin-like modules in domain I is a common feature of the two fibulins but causes disulfide-bonded dimer formation in fibulin-2 not observed for fibulin-1. A hallmark of this module seems to be the presence of six cysteines which are connected

into the disulfide bridges Cys1–4, Cys2–5 and Cys3–6 as shown for anaphylatoxin C3a (Huber *et al.*, 1980). The central anaphylatoxin-like module Ib lacks either Cys1 in fibulin-2 (Pan *et al.*, 1993) or Cys1 and Cys4 in fibulin-1. This would eliminate one disulfide bridge in fibulin-1 but leave an unpaired Cys4 in fibulin-2. This odd Cys574 has now been shown to be responsible for the covalent dimer stabilization of fibulin-2 by partial reduction of fragment I and by a single point mutation of fibulin-2 (C574-S). The formation of this disulfide bond is, however, not a prerequisite for dimerization since this occurs in fibulin-2 mutant C574-S, as shown by ultracentrifugation and electron microscopy. This also suggests that initial non-covalent associations occur between recombinant fragments I and N+I prior to complete disulfide bonding indicating recognition sites between the anaphylatoxin-like modules of fibulin-2. The lower efficiency of disulfide bonding observed for fragment I+II+III is in part, but not fully, explained by subsequent proteolysis in link regions of domain II which would remove domain I (see Figure 1). This agrees with the analysis of various proteolytic fragments of fibulin-2 which showed that cleavage at the N-terminal site of domain I maintained disulfide bond connectivity but abolished it when occurring at its C-terminal site within the link regions (Sasaki *et al.*, 1996a).

In the N-terminal domain N unique to fibulin-2 a cysteine-rich region Na (22 Cys per 150 residues) and a cysteine-free region Nb (250 residues) can be distinguished which so far have no obvious counterparts in other known proteins. The surprising observation of this study was the rod-like structure of domain N including both regions. This suggests a repeating structure in region Na, which, however, is not supported by any regularities in cysteine patterns (Pan *et al.*, 1993). Region Nb was particularly rich in galactosamine indicating a mucin-like structure which would predict a rod-like structure. There are sufficient Thr and Ser acceptor residues in the Nb sequence to support the analytical data and many of them have a Pro in either the –1 or the +3 position, constituting a consensus sequence for O-glycosylation (Gooley *et al.*, 1991; Wilson *et al.*, 1991). These particular features may also be important for the internal association of domain N.

The elongated structure of fibulin-2 makes it an ideal candidate for associations into fibrillar extracellular structures. Fibulin-2 has been shown to bind with distinct affinities to fibronectin and fibrillin-1 (Sasaki *et al.*, 1995a; Reinhardt *et al.*, 1996a) which are both known to form microfibrillar structures in the extracellular space. Furthermore a close colocalization of fibulin-2 to fibroblast-derived fibronectin fibrils (Sasaki *et al.*, 1996b) and to some but not all fibrillin-containing elastic microfibrils (Reinhardt *et al.*, 1996a) has been shown by immunogold staining, suggesting that such interactions occur also *in vivo*. The dimensions of the fibulin-2 dimer determined here will therefore be instrumental in the analysis of the supramolecular organization of such composite microfibrils. Microfibrillar models, which are still tentative, suggested a staggered alignment for both fibronectin (Hynes, 1990) and fibrillin-1 (Reinhardt *et al.*, 1996b) with a periodicity of ~60 nm. How the somewhat longer fibulin-2 dimers fit into these structures and whether they show a complete alignment or only association through a

restricted binding region remains to be determined in forthcoming studies.

Materials and methods

Construction of expression vectors

All constructs were prepared using the full-length mouse fibulin-2 cDNA inserted into the eukaryotic expression vector pRc/CMV (Pan *et al.*, 1993). The strategy used for producing several deletion and one single-site mutant was based on various combinations of cDNA fragments released through unique restriction sites with other fragments amplified by PCR as outlined in Figure 7. PCR amplification was carried out with Vent polymerase (New England Biolabs) following the manufacturer's instruction and the following oligonucleotide primers with a mutated sequence shown in lower case:

N-1: 5'-GTCAGCTAGCCGTCCCGTGGCAGGATTG
N-2: 5'-GTCACCATGGCAACCAGGAAGCTG
N-3: 5'-GTCAGTACTGACTGAGCCACCA
N-4: 5'-GTCAGTCTGAGTCAGGTCTCGATTAGGTCCTTGG
N-5: 5'-GTCAGTCTGGGGATCTCTGGC
I-1: 5'-GTCAGCTAGCCCTAATCGAGACCTGTTGTG
I-2: 5'-GTCAGTCTGAGTCACACTATGAGGGGCTCTTCAC
II-1: 5'-GTCAGCTAGCCAATAGCCTGCCGGGAGATG
II-2: 5'-GTCAGGGCCCATGTCTTTGCA
II-3: 5'-GTCAGCATGCCAGAGCAAGGGTA
II-4: 5'-GTCAGTCTGAGTCAGCGCTCGCACTTCGTTTGTG
III-1: 5'-GTCAGCTAGCGCGCACCATGCGCAGGATA
III-2: 5'-GTCAGTCTGAGTCATGGGGCAAAAGTGGTG
III-3: 5'-GTCAGATATCACGGAATGTCAAACC
I-mut: 5'-GTCATTGTAGGAGTGAGACGCCGAGGTATCATTGTCCTCTG

Several primers contained in addition to the annealing sequences an *NheI* site at the 5' end (N-1, I-1, II-1, III-1), or a stop codon followed by an *XhoI* site at the 3' end (N-4, I-2, II-4, III-2) in order to allow the in-frame insertion of the constructs into the BM-40 signal peptide (Mayer *et al.*, 1993). The sequences of the PCR fragments as well as of their ligation sites were confirmed by cycle sequencing using Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). All constructs were then inserted through their *NheI* and *XhoI* sites into the modified episomal expression vector pCEP-Sh (Kohfeldt *et al.*, 1996) which contained the puromycin instead of the phleomycin resistance gene (E.Kohfeldt, unpublished).

Recombinant protein production and purification

Human embryonic kidney cells which express the EBNA-1 protein from Epstein-Barr virus (293-EBNA cells; Invitrogen) were used for transfection with the expression vectors (Mayer *et al.*, 1995). Resistant cells were selected in the presence of 0.5 µg/ml puromycin and used for the collection of serum-free culture medium. This medium (~1 l) was passed over a DEAE cellulose column (2.5×20 cm) equilibrated in 0.05 M Tris-HCl buffer of either pH 7.4 (for fragments I, II, II+III, I+II+III) or pH 8.6 (fragments N, I, N+I, N+I+II, mutant C574-S). Bound proteins were then eluted with a linear gradient (800 ml) of 0–0.5 M NaCl. Fragments II, II+III and I+II+III were eluted at 0.1–0.2 M NaCl and fragments N, N+I, I, N+I+II and mutant C574-S were eluted at 0.2–0.3 M NaCl. Pooled fragments were dialyzed against 0.2 M NH₄HCO₃ and finally purified on either Superose 6 or 12 columns (HR 16/50, Pharmacia) equilibrated in 0.2 M ammonium acetate pH 6.8.

Proteolytic fragmentation and chemical modification

Recombinant mouse fibulin-2 (Pan *et al.*, 1993) dissolved in 0.15 M NaCl, 0.05 M Tris-HCl pH 7.4, 2 mM CaCl₂, 1 mM N-ethylmaleimide (1.2 mg/2 ml) was digested with stromelysin at an enzyme-substrate ratio of 1:100 for 2.5 h at 37°C (Sasaki *et al.*, 1996a). Fragments ST-1 and ST-3 were then separated on a Superose 6 column. Recombinant mouse fibulin-1C (Sasaki *et al.*, 1995b) dissolved in the same buffer (1 mg/1.1 ml) was digested with human leukocyte elastase (Calbiochem) at an enzyme-substrate ratio of 1:100 for 3 h at 37°C (Sasaki *et al.*, 1996a). Domain I of fibulin-1 was then purified on a Superose 12 column.

Recombinant fragment I of fibulin-2 dissolved in 0.05 M Tris-HCl pH 8.5, was reduced with 100 µM DTT for 20 min at 25°C followed by alkylation with 4-vinylpyridine for 1.5 h at room temperature. After extensive dialysis against 0.2 M NH₄HCO₃ and lyophilization the sample was dissolved in 6 M guanidine-HCl, 0.05 M Tris-HCl pH 8.5, and reduced with 10 mM DTT (6 h, room temperature) followed by overnight alkylation with 50 mM sodium iodoacetate. After dialysis against 0.2 M

NH_4HCO_3 and lyophilization the material was cleaved with endoproteinase Lys-C (Wako, Osaka) at an enzyme:substrate ratio of 1:80 (overnight, 25°C) and the peptides were purified by reverse phase chromatography (Mayer *et al.*, 1991).

Analytical ultracentrifugation

An analytical ultracentrifuge Optima XL-A (Beckman Instruments) with 12 mm Epon double-sector cells in An-60 Ti rotor was employed. Velocity experiments were performed at rotor speeds of 56 000 r.p.m. at 20°C and short column equilibrium experiments at 10 000–26 000 r.p.m. depending on molecular mass. Molecular masses were determined by two individual sedimentation equilibrium experiments and are given as average values in Table II. All experiments were performed at 20°C and concentrations were monitored by absorption at a wavelength of 232 or 278 nm. Evaluation of molecular masses, frictional ratios and sedimentation coefficients at 20°C and viscosity of water η_{20w} were performed according to standard conditions (Van Holde, 1971). For the partial specific volume of fibulin-2 and its fragments the average value for proteins ($v = 0.73$ ml/mg) was assumed. For the calculation of frictional ratios f/f_0 a degree of hydration of 0.5 was used. Because of these uncertainties the systematic errors of M and f/f_0 values may amount to $\pm 8\%$. Standard deviations were $\pm 5\%$.

Analytical and miscellaneous methods

Samples were hydrolyzed with 6 M or 3 M HCl (16 h, 110°C) to determine protein concentrations and hexosamine compositions, respectively, on a LC 3000 analyser (Biotronik, Maintal, Germany). SDS gel electrophoresis followed standard protocols. Edman degradation of fragments was performed on 473A and Procise sequencers (Applied Biosystems) following the manufacturer's instructions. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry on a Bruker REFLEX II mass spectrometer was carried out as previously described (Mann *et al.*, 1996). Binding analysis was performed with the BIAcore system (Pharmacia Biosensor) following a previous procedure (Sasaki *et al.*, 1995a). Rotary shadowing of proteins and electron microscopy followed established methods (Engel and Furthmayr, 1987). Length measurements of fragments were performed for 20–35 particles and are expressed as mean $\pm$ SD.

Acknowledgements

We thank Dr C.Eckerskorn for mass spectrometry and Dr G.Murphy for a gift of stromelysin and were grateful for the technical assistance of Mrs Vera van Delden. The study was supported by a Max-Planck Research Award and by grants of the Deutsche Forschungsgemeinschaft (SFB 266) and the National Institutes of Health (AR 38923).

References

- Balbona, K., Tran, H., Godyna, S., Ingham, K.C., Strickland, D.K. and Argraves, W.S. (1992) Fibulin binds to itself and to the carboxyl-terminal heparin-binding region of fibronectin. *J. Biol. Chem.*, **267**, 20120–20125.
- Campbell, I.D. and Bork, P. (1993) Epidermal growth factor-like modules. *Curr. Opin. Struct. Biol.*, **3**, 385–392.
- Engel, J. and Furthmayr, H. (1987) Electron microscopy and other physical methods for the characterization of extracellular matrix components: laminin, fibronectin, collagen IV, collagen VI and proteoglycans. *Methods Enzymol.*, **145**, 3–78.
- Fässler, R., Sasaki, T., Timpl, R., Chu, M.-L. and Werner, S. (1996) Differential regulation of fibulin, tenascin and nidogen expression during wound healing of normal and glucocorticoid-treated mice. *Exp. Cell Res.*, **222**, 111–116.
- Gooley, A.A., Classon, B.J., Marschalek, R. and Williams, K.L. (1991) Glycosylation sites identified by detection of glycosylated amino acids released from Edman degradation: the identification of Xaa-Pro-Xaa-Xaa as a motif for Thr-O-glycosylation. *Biochem. Biophys. Res. Commun.*, **178**, 1194–1201.
- Huber, R., Scholze, H., Paques, E.P. and Deisenhofer, J. (1980) Crystal structure analysis and molecular model of human C3a anaphylatoxin. *Hoppe-Seyler's Z. Physiol. Chem.*, **361**, 1389–1399.
- Hynes, R.O. (1990) *Fibronectins*. Springer Verlag, New York.
- Kohfeldt, E., Göhring, W., Mayer, U., Zweckstetter, M., Holak, T.A., Chu, M.-L. and Timpl, R. (1996) Conversion of the Kunitz-type module of collagen VI into a highly active trypsin inhibitor by site-directed mutagenesis. *Eur. J. Biochem.*, **238**, 333–340.
- Mann, K., Mechling, D.E., Bächinger, H.P., Eckerskorn, C., Gaill, F. and Timpl, R. (1996) Glycosylated threonine but not 4-hydroxyproline dominates the triple helix stabilizing positions in the sequence of a hydrothermal vent worm cuticle collagen. *J. Mol. Biol.*, **261**, 255–266.
- Mayer, U., Aumailley, M., Mann, K., Timpl, R. and Engel, J. (1991) Calcium-dependent binding of basement membrane protein BM-40 (osteonectin, SPARC) to basement membrane collagen type IV. *Eur. J. Biochem.*, **198**, 141–150.
- Mayer, U., Nischt, R., Pöschl, E., Mann, K., Fukuda, K., Gerl, M., Yamada, Y. and Timpl, R. (1993) A single EGF-like motif of laminins is responsible for high affinity nidogen binding. *EMBO J.*, **12**, 1879–1885.
- Mayer, U., Pöschl, E., Gerecke, D.R., Wagman, D.W., Burgeson, R.E. and Timpl, R. (1995) Low nidogen affinity of laminin-5 can be attributed to two serine residues in EGF-like motif γ III4. *FEBS Lett.*, **365**, 129–132.
- Miosge, N., Götz, W., Sasaki, T., Chu, M.-L., Timpl, R. and Herken, R. (1996) The extracellular matrix proteins fibulin-1 and fibulin-2 in the early human embryo. *Histochem. J.*, **28**, 109–116.
- Pan, T.-C., Sasaki, T., Zhang, R.-Z., Fässler, R., Timpl, R. and Chu, M.-L. (1993) Structure and expression of fibulin-2, a novel extracellular matrix protein with multiple EGF-like repeats and consensus motifs for calcium-binding. *J. Cell Biol.*, **123**, 1269–1277.
- Pfaff, M., Sasaki, T., Tangemann, K., Chu, M.-L. and Timpl, R. (1995) Integrin-binding and cell-adhesion studies of fibulins reveal a particular affinity for α IIb β 3. *Exp. Cell Res.*, **219**, 87–92.
- Reinhardt, D.P., Sasaki, T., Dzamba, B.J., Keene, D.R., Chu, M.-L., Göhring, W., Timpl, R. and Sakai, L.Y. (1996a) Fibrillin-1 and fibulin-2 interact and are colocalized in some tissues. *J. Biol. Chem.*, **271**, 19489–19496.
- Reinhardt, D.P., Keene, D.R., Corson, G.M., Pöschl, E., Bächinger, H.P., Gambee, J.E. and Sakai, L.Y. (1996b) Fibrillin-1: organization in microfibrils and structural properties. *J. Mol. Biol.*, **258**, 104–116.
- Sasaki, T., Göhring, W., Pan, T.-C., Chu, M.-L. and Timpl, R. (1995a) Binding of mouse and human fibulin-2 to extracellular matrix ligands. *J. Mol. Biol.*, **254**, 892–899.
- Sasaki, T., Kostka, G., Göhring, W., Wiedemann, H., Mann, K., Chu, M.-L. and Timpl, R. (1995b) Structural characterization of two variants of fibulin-1 that differ in nidogen affinity. *J. Mol. Biol.*, **254**, 241–250.
- Sasaki, T., Mann, K., Murphy, G., Chu, M.-L. and Timpl, R. (1996a) Different susceptibilities of fibulin-1 and fibulin-2 to cleavage by matrix metalloproteinases and other tissue proteases. *Eur. J. Biochem.*, **240**, 427–434.
- Sasaki, T., Wiedemann, H., Matzner, M., Chu, M.-L. and Timpl, R. (1996b) Expression of fibulin-2 by fibroblasts and deposition with fibronectin into a fibrillar matrix. *J. Cell Sci.*, **109**, 2895–2904.
- Van Holde, V.E. (1971) *Physical Biochemistry*. 2nd edn. Prentice-Hall Inc., Englewood Cliffs, NJ, pp. 93–136.
- Wilson, I.B.H., Gavel, Y. and von Heijne, G. (1991) Amino acid distributions around O-linked glycosylation sites. *Biochem. J.*, **275**, 529–534.
- Zhang, R.-Z., Pan, T.-C., Zhang, C.-Y., Mattei, M.-G., Timpl, R. and Chu, M.-L. (1994) Fibulin-2 (FBLN2): human cDNA sequence, mRNA expression and mapping of the gene on human and mouse chromosomes. *Genomics*, **22**, 425–430.
- Zhang, H.-Y., Chu, M.-L., Pan, T.-C., Sasaki, T., Timpl, R. and Ekblom, P. (1995) Extracellular matrix protein fibulin-2 is expressed in the embryonic endocardial cushion tissue and is a prominent component of valves in adult heart. *Dev. Biol.*, **167**, 18–26.
- Zhang, H.-Y., Timpl, R., Sasaki, T., Chu, M.-L. and Ekblom, P. (1996) Fibulin-1 and fibulin-2 expression during organogenesis in the developing mouse embryo. *Dev. Dynamics*, **205**, 348–364.

Received on November 20, 1996; revised on January 10, 1997