Properties of the extracellular calcium binding module of the proteoglycan testican

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Abstract The extracellular calcium-binding (EC) module of human testican (115 residues) was obtained in native form by recombinant production in mammalian cell culture and thus shown to represent an independently folding domain. This module showed a large loss in α-helix upon calcium depletion. Apparently only one of the two EF hands binds calcium, with a moderate affinity (K_d = 68 μM) about 100-fold lower than in the homologous BM-40 protein. No clear evidence was obtained for collagen binding, indicating that EC modules found in different proteins may not share similar functions.

Key words: Calcium binding; EF hand; Extracellular matrix; Recombinant protein

1. Introduction

A large number of extracellular proteins are known to bind calcium with moderate to high affinity and calcium-binding consensus sequences exist within at least five different protein modules [1]. One of these consists of a pair of EF hands, as initially shown for the extracellular matrix protein BM-40/osteonectin/SPARC [2,3] and is referred to as the extracellular calcium-binding (EC) module [4,5]. In BM-40 it exists in close association with a follistatin-like (FS) module and the structure of both has recently been elucidated by X-ray crystallography [5,6]. The combination of an FS and EC module seems to be common to several other proteins, as shown by recent sequence analyses. They include the proteoglycan testican [7,8], the quail retina protein QR1 [9], the brain protein SC1 [10], the endothelial protein hevin [11] and the protein TSC-36/FRP [12,13]. The structural and functional relationships of these proteins to BM-40 are still unknown and only a few studies exist on their tissue expression [8,14–16].

Many functions have been identified for BM-40, including regulation of cell shape and gene expression, high affinity binding of calcium and interactions with several extracellular ligands [3,4]. The EC module of human BM-40 was shown to contain two calcium-binding sites and their occupation stabilizes part of the α-helical conformation [4,5]. The same module also promotes calcium-dependent binding to several collagen types [4,17] and calcium-independent binding to platelet-derived growth factor (W. Göhring, T. Sasaki, C.-H. Heldin, R. Timpl, in preparation). Enhanced collagen binding was observed after the cleavage of a single peptide bond in the EC module by matrix metalloproteinases [17], suggesting that similar activation mechanisms may exist in tissues. The EC module of *Caenorhabditis elegans* BM-40 was also shown to bind mammalian collagens as avidly as human BM-40, indicating the conservation of a particular function [18].

In the present study we examined the EC module of human testican [7,8], which shows several sequence deletions when compared to BM-40. By using a modified episomal expression vector we could obtain this EC module in apparently native form. Some but not all of its properties were similar to those of BM-40 and the data can now be interpreted from the sequence differences and the available X-ray structure.

2. Materials and methods

2.1. Construction of an episomal expression vector with puromycin resistance

The eukaryotic episomal expression vector pCEP-Sh of 9.27 kbp [19] was used to produce a modified vector pCEP-Pu (9.51 kbp) by replacing the phleomycin (*Shble*) by the puromycin (*pac*) [20] resistance gene in order to allow a more convenient puromycin selection in mammalian cells. A 8.79 kbp fragment was obtained by digestion of pCEP-Sh with *Rsr*II and partial cleavage with *Bst*EII, which eliminated the EM7 promoter and *Shble*. Vector pSV$_2$pac, containing the *pac* gene [21], was used for the generation of terminal cloning sites and the elimination of internal *Rsr*II and *Bst*EII sites by silent mutagenesis using polymerase chain reaction (PCR) with Vent polymerase (New England Biolabs) according to the supplier's instruction. The following synthetic primers were used (mutated nucleotides in small letters): Pur1a 5′-TGGAGGGTGACCGCCACGACCGGTGCCG; Pur2b 5′-GCCGTCGGACCGGGGTCGTGCGCTCCTT; Pur1b 5′-TCGATGTGGCGGTCgGGGTCGACG; Pur2a 5′-GACCGCCACATCGAaCGcGTCACCGAG. The primers Pur1a/Pur1b and Pur2a/Pur2b matching the restriction sites *Rsr*II and *Bst*EII and mutations were used to produce two overlapping fragments of *pac*, which were purified by agarose gel electrophoresis, annealed and used for five PCR cycles. After addition of Pur1a/Pur2b, 30 more PCR cycles were carried out. The product was digested with *Rsr*II/*Bst*EII, purified, inserted into the restricted pCEP-Sh vector and transformed into *E. coli* XL-1blue (Stratagene). The correct insertion and sequence were verified by cycle sequencing on a 373A DNA sequencer (Applied Biosystems).

2.2. Construction of a testican expression vector and cell transfection

Vector pCEP-Pu (see above) was further modified by inserting some of the 5′UTR and the signal peptide sequence region of human BM-40 to yield pCEP-Pu/BM40s. This region was released as a 105 bp fragment from expression vector pRc/Ac [22] by *Hin*dIII/*Nhe*I restriction and ligated to the same restriction sites downstream of the CMV promoter of pCEP-Pu. A cDNA fragment spanning the EC module of human testican, residues 197–311 [7], was generated by PCR from 2 μl of a human fetal brain 5′ stretch λDR2 library (Clontech) using the 5′ primer 5′-CCTAGCTAGCCTGCACAGACAAGGAGTTG and the 3′ primer 5′-CAGTTAGCGGCCGCTAGAGACCTCCAGGCTTCTGG. The primers introduced new restriction sites (*Nhe*I, *Not*I) and a stop codon [22]. The *Nhe*I/*Not*I restricted PCR product was purified and inserted between the same restriction sites of pCEP-Pu/BM40s in order to obtain the final expression vector pCEP-Pu/EC-test. The correct insertion and sequence was verified by cycle sequencing. Human embryonic kidney cells which constitutively express the EBNA-1 protein from Epstein-Barr virus (293 EBNA, Invitrogen) were used for transfection [23]. The initial selection of transfected cells was ac-

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complished in the presence of 1–5 μg/ml puromycin, which was then reduced to 0.5 μg/ml during the phase of collecting serum-free culture medium.

2.3. Protein purification

Conditioned serum-free medium (1 l) was dialyzed against 0.05 M Tris-HCl, pH 8.6, containing protease inhibitors [24] and passed at 4°C over a DEAE cellulose column (2.5×20 cm) equilibrated in the same buffer. The column was eluted with a linear 0–0.5 M NaCl gradient (500 ml) and the recombinant protein eluted as a major peak at 0.03–0.12 M NaCl. It was further purified on a Superose 12 column (HR16/50, Pharmacia) equilibrated in 0.2 M ammonium acetate pH 6.8. Recombinant EC modules of human [4] and *C. elegans* [18] BM-40 have been previously described.

2.4. Circular dichroism and fluorimetry

Circular dichroism (CD) spectra were recorded with a Jasco 715 CD spectropolarimeter at 25°C in thermostatted quartz cells of optical pathlength 1 mm. The molar ellipticity [θ] (expressed in deg cm^2 $dmol^{-1}$) was calculated on the basis of a mean residue molecular mass of 110 Da. The α-helical content was estimated according to [25]. Fluorescence was measured on a LS50B spectrophotometer (Perkin Elmer, Überlingen, Germany) in 10 mm pathlength rectangular cells. For measurement of intrinsic fluorescence, the excitation wavelength was set at 280 nm and fluorescence was measured at the emission maximum of 346 nm. Protein concentrations were calculated from absorption at 279 nm using $\varepsilon = 23\,000$ M^{-1} cm^{-1}. Calcium titrations and data evaluation were performed as previously described [4].

2.5. Miscellaneous methods

Amino acid and hexosamine compositions were determined on a LC 3000 analyzer (Biotronik) after hydrolysis (16 h, 110°C) with 6 M or 3 M HCl, respectively. SDS gel electrophoresis followed standard protocols. Edman degradation of electroblotted protein bands was performed on a Procise sequencer (Applied Biosystems) following the manufacturer's instructions. Solid phase and surface plasmon resonance binding assays with collagens were those used previously [4,17]. Production of rabbit antisera and radioimmunoassays followed established protocols [26]. Human tissue extracts were from Clontech. An extract of human placenta (50 g) was prepared by homogenization in 200 ml 0.05 M Tris-HCl pH 8.6, 0.5 M NaCl (24 h, 4°C).

3. Results

We prepared a modified, cost-efficient episomal expression vector based on puromycin resistance and used it for the recombinant expression of the EC module of human testican in mammalian cells. The module (residues 197–311) was connected to the BM-40 signal peptide region [22], which directed the product to the culture medium and added an N-terminal APL to the authentic testican sequence. The recombinant EC module could be purified from the culture medium in good yield (1–3 mg/l) by two chromatographic steps. In SDS gel electrophoresis, the purified fragment showed a major 14 kDa band which shifted to a slower mobility after reduction, indicating the opening of internal disulfide bonds (Fig. 1). This agreed with a calculated molecular mass of 13.4 kDa for the recombinant testican EC module, while the same module of BM-40 is about 30 residues larger [5] and has a slower electrophoretic mobility (see Fig. 1). Edman degradation of the 14 kDa testican band demonstrated a single N-terminal sequence APLAXTDKELRN, as expected. Omission of protease inhibitors from the initial purification steps caused partial degradation of the 14 kDa band to a 5 kDa band with a major N-terminal sequence DTSILP, indicating cleavage at position 237/238 of the EC module.

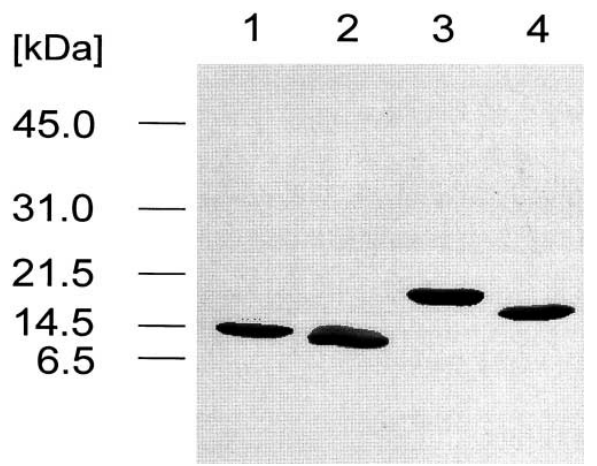

Fig. 1. SDS gel electrophoresis of recombinant EC modules from human testican and BM-40. Lanes were loaded with 2 μg of testican EC (1, 2) and BM-40 EC (3, 4) and examined under reducing (1, 3) and non-reducing (2, 4) conditions. The calibration with reduced protein standards is shown in the left margin.

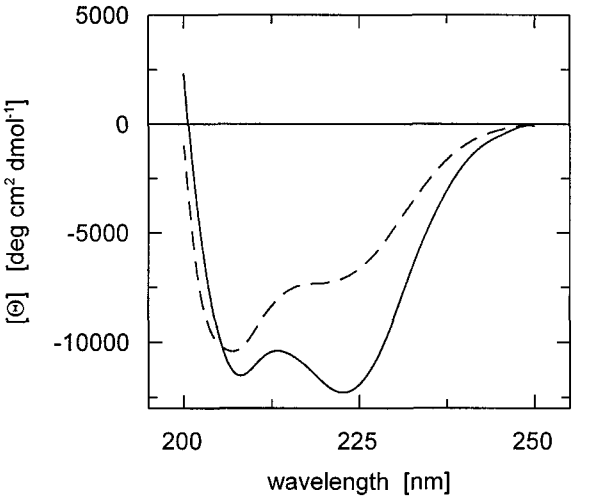

Fig. 2. Circular dichroism spectra of testican EC module. Far UV spectra were recorded at a protein concentration of 0.18 mg/ml in the presence of 2 mM Ca^{2+} (solid line) and 3 mM EDTA (dashed line) in 5 mM Tris-HCl, pH 7.4.

The amino acid composition of the EC module of testican was identical to that predicted from the sequence within the limits of analytical error (data not shown). Hexosamine analysis of the EC module demonstrated substitution by 1.4 ± 0.4 residues glucosamine and 1.9 ± 0.3 residues galactosamine. This indicated the presence of one or two *O*-linked oligosaccharides, in agreement with a molecular mass of 14.4 kDa determined by ESI mass spectrometry, suggesting a 1 kDa mass contribution by post-translational modifications. The recombinant testican EC module showed a distinct conformation, as demonstrated by CD spectroscopy in the far UV region (Fig. 2). The spectra were characteristic of a protein with a high α-helical content, with minima at 208 and 222 nm. In the presence of calcium, the α-helix was estimated to represent 32% of the entire structure.

A rabbit antiserum was raised against the testican EC module and had a moderate ELISA titer (1:200) for the recombinant product but showed no reaction with human BM-40 even at a dilution of 1:10. Immunoblotting of several human tissue extracts (skin, heart, skeletal muscle) demonstrated a distinct band of about 45–50 kDa, which corresponded in

size to that expected for the testican core protein [7] (data not shown). For more quantitative analyses, we also established a sensitive radioimmunoinhibition assay where 50% inhibition could be achieved with 0.2 nM testican EC module (Fig. 3). No inhibition was observed with 1000-fold higher concentrations of BM-40 or its EC module obtained from human or *C. elegans*. Likewise a radioimmunoinhibition assay specific for human BM-40 [23] could not be inhibited by the testican EC module (not shown) demonstrating lack of cross-reactivity. Culture medium from human epithelial HBL-100 cells and a NaCl extract of human placenta could inhibit the assay for the testican EC module (Fig. 3) demonstrating that they contain testican at a low concentration (0.1–0.7 nM). Their inhibition profiles were parallel to that of the testican EC module which indicated that they share identical immunological epitopes [26].

As predicted [4,5], the EC module of testican showed a distinct affinity for calcium. Depletion of calcium by EDTA caused a 41% increase in molar ellipticity at 222 nm (Fig. 2), similar to that observed for the EC module of BM-40 [4]. This increase was used to quantitatively monitor calcium binding. A simple one binding site model could be fitted to the binding curve, with an equilibrium dissociation constant of $K_d = 68$ μM (Fig. 4). A second signal used to monitor calcium binding of the BM-40 EC module was intrinsic fluorescence, which showed an increase of about 100% upon calcium removal [4]. Upon excitation at 280 nm, the testican EC module showed an emission maximum at 346 nm. The intensity of intrinsic fluorescence, however, was only slightly (7%) altered upon addition of excess calcium (data not shown). In order to exclude interference by a putative high affinity calcium binding site 4 mM EDTA was added to the protein but did not change fluorescence intensity.

A further calcium-dependent property of the BM-40 EC module is binding to various immobilized collagens in solid phase and surface plasmon resonance assays [4,17,18]. Similar solid phase tests with the testican EC module showed a weak but calcium-independent binding to collagen IV but no binding to collagen I, laminin and fibronectin. Furthermore, no binding could be detected to either of the two collagens by surface plasmon resonance assay (data not shown).

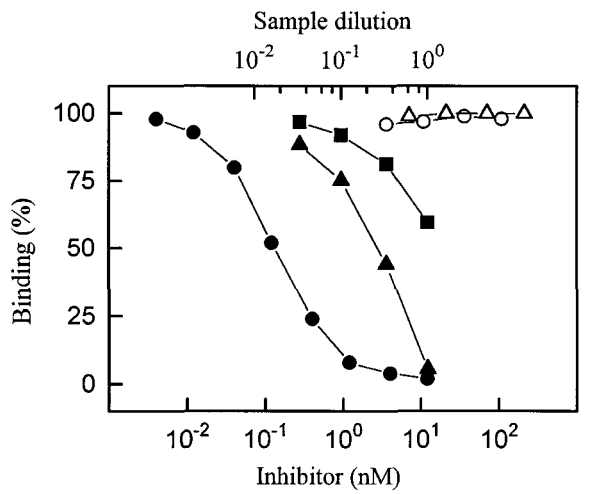

Fig. 3. Radioimmunoinhibition assay with an antiserum against testican EC module. The assay consisted of ^{125}I-labelled testican EC module (1 ng) and a fixed dilution of a rabbit antisera against this module. Inhibitors used were human testican EC module (●), human BM-40 (○), *C. elegans* BM-40 EC module (△), a human placenta extract (▲) and conditioned serum-free medium of human HBL-100 cells (■).

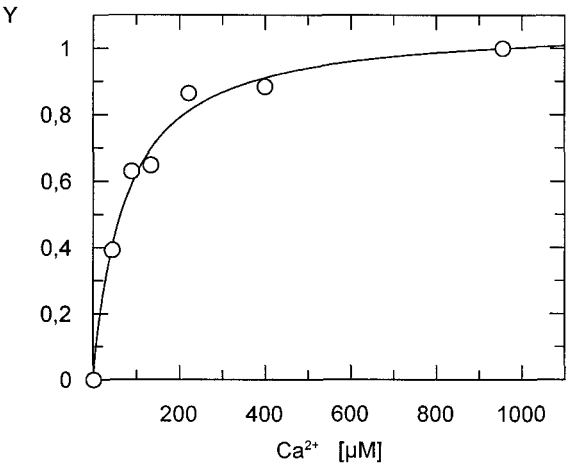

Fig. 4. Calcium binding of testican EC domain as monitored by circular dichroism. Ellipticity at 222 nm was recorded as a function of the total Ca^{2+} concentration and transformed to degree of saturation Y. Best fit curve with a fit parameter $K_d = 68$ μM is shown as a continuous line.

4. Discussion

The calcium-binding EC module represents a novel structure found in extracellular proteins [2,4] and was shown to consist of EF hands 1 and 2 as well as several more α-helical and loop segments which together form a compact globular structure [5]. Extensive characterization of this module for human, mouse and *C. elegans* BM-40 [4,5,17,18,27,28] demonstrated that calcium binds to two sites, including both EF hands, and that a change in α-helical conformation and binding affinity for collagens are both calcium-dependent. Other binding ligands may include oligomeric vitronectin [29] and platelet-derived growth factor (unpublished). The structure of an FS module adjacent to an EC module of BM-40 has also been elucidated and this structure may possess affinity for heparin [6]. A family of at least four more proteins [7–16] exists, all of which share the combination of EC and FS modules but differ in the length of their N-terminal domains. Two members, testican and TSC-36/FRP, differ in addition by C-terminal extensions beyond the EC module and larger deletions within their EC module, the latter being shown for testican in Fig. 5.

The functional relationships of these proteins are still unclear and have been approached here by the recombinant production of the testican EC module. This demonstrated, as for BM-40 [4], that this module represents an autonomously folding unit within the protein. Its folding into a disulfide-bonded, native structure was also ascertained by CD spectroscopy, electrophoresis, calcium binding and immunological assays which demonstrated cross-reaction with testican in tissues and cell cultures. The recombinant module also underwent some post-translational modifications and, like the EC module of BM-40, showed a considerable change in α-helical conformation upon depletion of calcium.

Previous studies have shown that calcium binding to BM-40

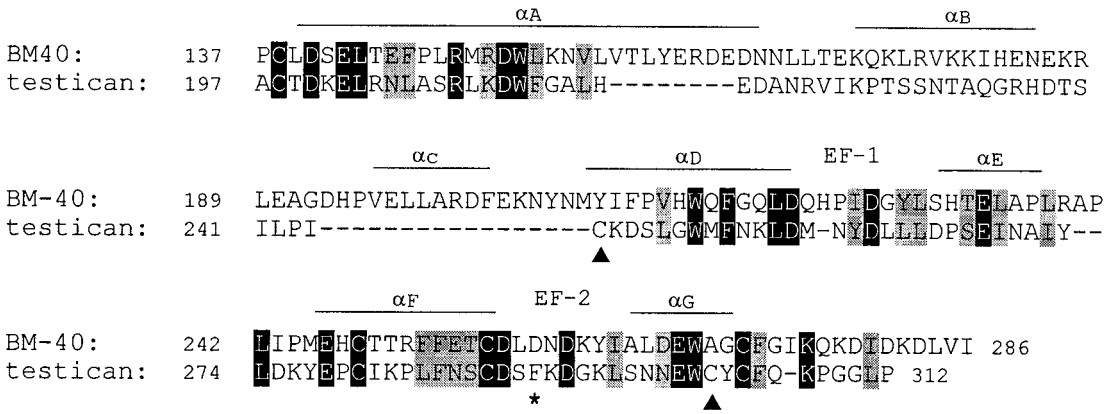

Fig. 5. Sequence comparison of the EC modules of human BM-40 and testican. Identical or conserved amino acids are shown on a black or shaded background, respectively. Triangles indicate two additional Cys residues in testican that may form a disulfide bridge. The star denotes the position of Phe^{291} in testican that corresponds to the Ca^{2+}-coordinating residue Asp^{259} of BM-40. Lines above BM-40 indicate the positions of α-helices (αA-αG) as determined by X-ray crystallography [5].

involves a non-canonical EF hand 1 where the insertion of a single additional residue in the 12 residue consensus sequence [30] is compensated for by a *cis*-peptide bond, providing a novel ligation site [5]. This insertion does not exist in testican (Fig. 5), but nevertheless our data indicate only one calcium binding site for its EC module with a moderate affinity of $K_d = 68$ μM. An affinity about 100-fold higher was previously determined for the BM-40 EC module [4]. We prefer to explain this difference by the substitution of a ligating Asp residue by Phe^{291} in the +Y position of EF hand 2 (Fig. 5), which could prevent calcium binding to this site. Similar Ala substitutions at the same site in troponin C [31] and aequorin [32] were previously shown to perturb calcium binding. This possibility is underscored by recent observations (E. Busch, P. Mauer, unpublished) of a cooperative effect in the calcium binding to EF hands 1 and 2 in BM-40, as revealed by single mutations in individual ligation sites which reduced affinities from $K_d < 1$ μM to 50–200 μM. Proof of our interpretation will still depend on additional equilibrium dialysis experiments and appropriate mutations in the testican EC module, however. Fluctuations in tissue calcium concentrations may exist and could bring the concentration close to the K_d value of testican binding, and this is of particular interest in neuronal tissues [1,33,34] due to the recently documented testican expression in the brain [8].

A further difference with BM-40 is the failure of intrinsic fluorescence to demonstrate calcium binding to the testican EC module, even though the K_d value was not too low and the three Trp residues probably responsible are conserved in BM-40 testican (Fig. 5). We assume that an additional disulfide bridge, Cys^{245}-Cys^{302} (Fig. 5), which could form in testican based on the X-ray structure of BM-40 [5] and which would covalently link EF hands 1 and 2, as well as several deletions may prevent appropriate interactions. The same failure of intrinsic fluorescence to demonstrate calcium binding was observed with *C. elegans* BM-40, but in addition there was no conformational change on calcium depletion [18]. *C. elegans* BM-40 also contains several deletions and an additional disulfide bridge in its EC module, but these are different from that observed for testican [18,35]. Fewer sequence differences exist for the BM-40 related proteins QR1 [9] and SC1/hevin [10,11], which could indicate that their properties more closely resemble those of BM-40.

BM-40 has also been shown to be a versatile binding protein for collagens [3,4,17,18,27], though with moderate affinity ($K_d = 1$–2 μM), through calcium-dependent interactions with the EC module. Binding activity could be increased by proteolysis in helix αC, indicating a controlling mechanism [17,27]. No convincing evidence could so far be obtained for a similar function for the testican EC module which lacks a counterpart of helix αC (Fig. 5) but can apparently be modified at a different position by endogenous proteolysis. This has still to be further examined but suggests that the family of proteins sharing the FS and EC modules have possibly evolved different functions.

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