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Solubilization of protein BM-40 from a basement membrane tumor with chelating agents and evidence for its identity with osteonectin and SPARC

Karlheinz Mann, Rainer Deutzmann, Mats Paulsson* and Rupert Timpl

Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

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Up to 80% of the calcium-binding protein BM-40 could be extracted from a tumor basement membrane with a physiological buffer containing 10 mM EDTA. About half of its amino acid sequence was determined by Edman degradation demonstrating identity with cDNA deduced sequences of bone osteonectin and SPARC.

Extracellular matrix; Ca^{2+}-binding protein; Amino acid sequence; Proteolytic fragment

1. INTRODUCTION

Basement membranes are unique extracellular protein matrices with a broad tissue distribution. They are composed of collagenous protein, several proteoglycans and a diverse group of other glycoproteins which are still incompletely characterized [1]. Among the latter components is also a small protein BM-40 which has recently been purified from 6 M guanidine-HCl extracts of the mouse Engelbreth-Holm-Swarm (EHS) tumor [2]. The protein consists of a single, disulfide-bonded polypeptide chain which is partially cleaved by endogenous proteases during purification. Immunological assays failed to show any relationship to other known basement membrane proteins and demonstrated the widespread occurrence of BM-40 in cell cultures and tissue extracts. It was also present in authentic basement membranes such as lens capsule and Reichert's membrane. During the characterization of BM-40 [2] we noticed a possible relationship to a serum albumin-binding protein obtained from endothelial and other cell cultures [3,4] which was later named SPARC and characterized by a complete amino acid sequence deduced from murine cDNA clones [5]. An almost identical sequence was recently determined for bovine bone osteonectin (M. Bolander, personal communication) which is known as a calcium-binding protein [6,7].

The data suggested that calcium-binding proteins are present in basement membranes and contribute to their special architecture. Evidence for this possibility came initially from the findings that laminin polymerizes in the presence of calcium [8] and that extracts of the EHS tumor obtained with 10 mM EDTA in physiological buffer contain large amounts of the laminin-nidogen complex [9]. In the present study we have applied the same extraction procedure for the purification of BM-40 in a more gentle manner than done before. Partial amino acid sequence analysis provided strong evidence that BM-40 is identical with SPARC and osteonectin.

Correspondence address: R. Timpl, Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

* Present address: Biozentrum, Universität Basel, Basel, Switzerland

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2. MATERIALS AND METHODS

Aliquots (75 g) of EHS tumor were briefly homogenized in 1.5 and 0.8 l of 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, containing 2 mM phenylmethanesulfonyl fluoride (PMSF) and 2 mM *N*-ethylmaleimide (NEM) as protease inhibitors (TBS) and centrifuged. The remaining residue was extracted twice with 375 ml TBS supplemented with 10 mM EDTA by stirring for 1 h at 4°C [9]. Subsequent extracts were obtained with 200 ml of 2 M and 6 M guanidine·HCl containing protease inhibitors (each for 1 day, 4°C). Aliquots (100 ml) of the first TBS-EDTA extract were passed over a Sepharose Cl-6B column (3.2 × 134 cm) equilibrated in TBS with 2 mM EDTA, 0.5 mM PMSF and 0.5 mM NEM. Fractions containing BM-40 were pooled from four runs, concentrated to 200 ml over an Amicon PM10 filter and dialyzed for 1 day at 4°C against 2 × 4 l of 2 M urea, 0.05 M Tris-HCl, pH 8.6, containing protease inhibitors. They were then passed over a DEAE-cellulose column (2 × 20 cm) equilibrated in the same solvent and bound protein was eluted with a linear NaCl gradient (0–0.4 M NaCl, 800–1000 ml). Final purification of BM-40 was achieved on a Sephacryl S-200 column (1.5 × 105 cm) equilibrated in 0.2 M ammonium bicarbonate.

In an alternative procedure 360 ml of the TBS-EDTA extract were mixed with 120 ml of 8 M urea and passed over a DEAE-cellulose column (3 × 25 cm) equilibrated in the same mixture of solvents followed by elution with a linear gradient (total 1000 ml) up to 0.6 M NaCl dissolved in the buffer. Further purification steps included chromatography on Sephacryl S-200 and DEAE-cellulose at pH 8.6 as described above. In addition, BM-40 was purified from 6 M guanidine·HCl extracts as described [2].

Cleavage of BM-40 dissolved in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 2 mM $CaCl_2$ by SV8 protease was performed at an enzyme-substrate ratio of 1:100 for 24 h at 37°C. Other samples were reduced with 2 mM dithioerythritol in 0.1 M Tris-HCl, pH 8.5, 6 M guanidine-HCl, *S*-carboxymethylated and cleaved in 0.2 M ammonium bicarbonate with TPCK-trypsin (24 h, 37°C). Peptides were separated on a Sephadex G75sf column (1.5 × 120 cm) in 0.2 M ammonium bicarbonate and/or by HPLC on a reversed-phase RP 318 column (Biorad, Munich) equilibrated in 0.1% trifluoroacetic acid and eluted with acetonitrile gradients.

Proteins and fragments were characterized by SDS-polyacrylamide gel electrophoresis [20] in 5–15% gradient slab gels and after hydrolysis with 6 M HCl (24 h, 110°C) by amino acid analysis on a Biotronik LC 5001 analyzer. Quantitative determinations of BM-40 were carried out by the radioimmuno-inhibition assay [2]. Edman degradation of samples (0.1–1 nmol) was performed in a gas-phase (model 470A, Applied Biosystems) or a spinning-cup liquid-phase sequencer (model 890 C, Beckman). Released amino acid derivatives were identified and quantitated by HPLC following the manufacturer's recommendation or by using an isocratic HPLC system [11].

3. RESULTS

We have recently developed a fast and gentle extraction protocol for the EHS tumor basement membrane matrix which allowed the purification of large quantities of the laminin-nidogen complex [9]. The same extracts when examined by radioimmunoassay contained also substantial amounts of BM-40 (table 1). Brief homogenization of the tumor in TBS releases about 20% of this protein while about 80% is solubilized by subsequent ex-

Table 1

Solubilization of BM-40 by sequential extraction of the EHS tumor

Extract	Amount of BM-40
1. 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl (TBS)	107 ± 34
2. TBS	30 ± 12
3. TBS, 10 mM EDTA	352 ± 93
4. TBS, 10 mM EDTA	31 ± 27
5. 2 M guanidine·HCl	5 ± 2
6. 6 M guanidine·HCl	9 ± 6
Sum	534 ± 108

Yields (μg/g wet tumor) were determined by radioimmunoassay and are given as average values (± SD) of 4 experiments

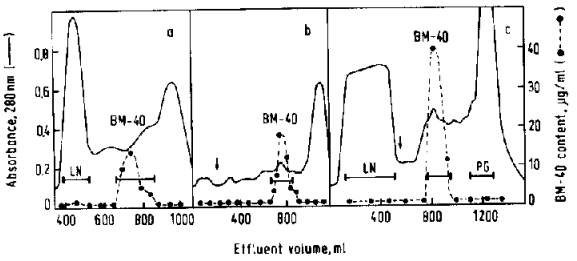

Fig.1. Initial chromatographic steps in the purification of BM-40 from TBS-EDTA extracts of the EHS tumor. (a) Passage of 100 ml extract over Sepharose Cl-6B column (3.2 × 134 cm); (b) chromatography of four BM-40 pools after step a on DEAE-cellulose in 2 M urea, 0.05 M Tris-HCl, pH 8.6, and elution with a gradient from 0–0.4 M NaCl (1 l); (c) chromatography of 360 ml TBS-EDTA extract on DEAE-cellulose in 2 M urea, 0.11 M NaCl, 0.038 M Tris-HCl, pH 7.4, followed by a 1 l gradient up to 0.6 M NaCl. Runs were monitored for absorbance at 280 nm (——) and BM-40 content (●---●) measured in aliquots of individual fractions by radioimmunoassay. Horizontal bars denote pools containing laminin-nidogen complex (LN), BM-40 and heparan sulfate proteoglycan (PG). Arrows indicate start of the gradient.

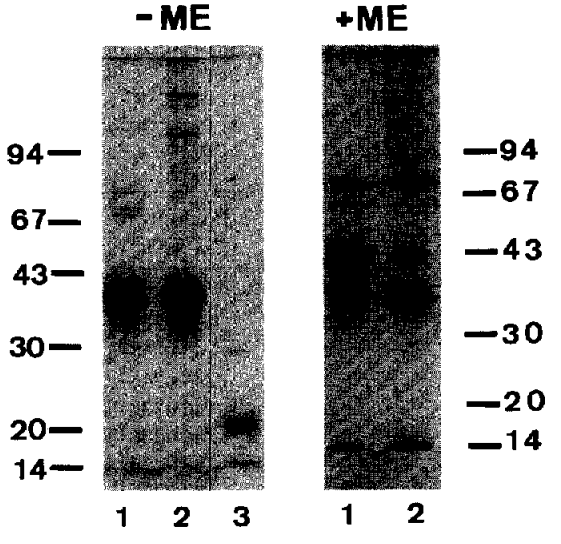

Fig.2. Comparison of BM-40 purified from TBS-EDTA extracts (lane 1) and from 6 M guanidine·HCl extracts (lane 2) and a SV8 protease digest (lane 3) by SDS-polyacrylamide gel electrophoresis. Samples were analyzed non-reduced (−) or after reduction with 2-mercaptoethanol (+). Numbers on each site indicate the migration position of globular marker proteins ($M_r \times 10^{-3}$).

traction with TBS containing 10 mM EDTA. Further extractions with concentrated guanidine·HCl solutions as have been used before in the purification of BM-40 [2] released only insignificant amounts of the protein (table 1). The total yield of BM-40 solubilized by the new extraction scheme was about 2-fold higher than those reported in [2].

The TBS-EDTA extracts were the most convenient source for the purification of BM-40 and contained BM-40 in a 4-fold molar excess compared to laminin-nidogen (table 1 [9]). Both components could be easily separated from each other by molecular sieve chromatography on agarose (fig.1a). Further purification of BM-40 was achieved by chromatography on DEAE-cellulose (fig.1b) followed by a final step on Sephacryl S-200 (not shown). The protein showed a major band in electrophoresis identical in mobility to BM-40 obtained by the previous procedure (fig.2)

Table 2

Comparison of the amino acid composition of BM-40 with that of SPARC deduced from cDNA sequence [5]

	BM-40 (residues/peptide)	SPARC
Aspartic acid	34.0	33[a]
Threonine	17.2	17
Serine	9.6	7
Glutamic acid	47.6	46[b]
Proline	16.4	17
Glycine	16.8	15
Alanine	15.1	14
Half cysteine	11.1[c]	14
Valine	18.9	20
Methionine	2.3	5
Isoleucine	11.0	12
Leucine	23.9	24
Tyrosine	6.7	7
Phenylalanine	12.5	12
Histidine	12.2	12
Lysine	18.3	19
Arginine	8.4	8
Tryptophan	n.d.	3
Sum	282.0	285

[a] Plus asparagine
[b] Plus glutamine
[c] Determined as *S*-carboxymethyl cysteine

n.d., not determined

demonstrating a purity of >95%. An alternative purification procedure was based on initial chromatography of TBS-EDTA extracts on DEAE-cellulose which also separated BM-40 from the laminin-nidogen complex and from proteoglycans (fig.1c) and allowed the processing of larger amounts of material. The final products obtained by both procedures were of equivalent purity and were obtained in yields of 2–3 mg BM-40 from 100 g wet tumor tissue.

The amino acid composition of BM-40 obtained from TBS-EDTA extracts (table 2) was comparable to that reported before for BM-40 obtained by extraction with 6 M guanidine·HCl [2]. A remarkable difference was observed in the extent of polypeptide cleavage due to endogenous proteolysis which results in two disulfide-linked fragments comprising about 75 and 25% of the mass of BM-40, respectively. This cleavage was nearly complete for guanidine-extracted but only in the order of 20–30% for the EDTA-extracted protein (fig.2). In circular dichroism studies, the latter material showed about 30% α-helix which was reduced considerably after treatment with guanidine (Engel, J., personal communication). Together the data demonstrate that extraction of BM-40 with TBS-10 mM EDTA results in a more intact form of the protein than obtained previously.

Purified BM-40 corresponds with a few exceptions (Ser, Cys, Met) in its amino acid composition, within the limits of analytical error (± 10%), to the composition predicted for SPARC (table 2) from cDNA sequences [5]. Determination of 53% of the sequence of BM-40 by Edman degradation (table 3) revealed so far no difference from SPARC confirming the very likely identity of both proteins. The major N-terminal sequence of BM-40 (APQQ) was also identical to that reported for bovine osteonectin [7,12]. A minor N-terminal sequence apparently corresponds to the endogenous cleavage site (fig.2) and located this site at a Leu-Leu bond in position 197/198 of SPARC.

Further sequence data were obtained from proteolytic fragments of BM-40 primarily generated by cleaving the non-reduced protein with SV8 protease. This digestion produced a large, disulfide-bonded fragment SP1 with about half the mass of BM-40 (fig.2) which could be separated from smaller peptides on Sephadex G75sf. This fragment contained two new major N-terminal sequences demonstrating the presence of two disulfide-linked chain fragments and in addition a minor sequence due to a partial cleavage within the

Table 3

Partial amino acid sequences of BM-40

Component[a]	Sequence	Position in SPARC[b]
BM-40	a: A P Q Q T E V A E E I V E E E T V V E E	1– 20
	b: L A X D F E K N Y N M Y I F P V H W Q F	198–217
Fragment SP1	a: V V A D N P X Q N H H	48– 58
	b: L A P L R A P L I P M E	234–245
	m: G T K K G H K (L) H L D Y	116–127
Fragment SP2	a: T C D L D N D K Y I A L E	254–266
	b: W A G C F G I K E	268–276
Fragment SP3	Q H P I D G Y L S H T E	222–233
Fragment SP4	Q D I N K D L V I	277–285
Fragment SP5	K Q K L R V K K I H E	173–183
Fragment T6	L H L D Y I G P C K	123–132
Fragment T7	Y I A P C L D S E L T E F P L R	133–148
Fragment T8	D W L K	151–154

[a] Sequences of components with several chain fragments are classified as major (a,b) or minor (m, 20–30% of a,b) sequences according to the yields of PTH derivatives. Assignment of positions in cases of two major sequences was done according to the known SPARC sequence [5]

[b] Numbering according to [5] without signal peptide (total 285 amino acid residues)

larger chain. Another fragment SP2 also consisted of two chain fragments which are apparently connected by the last two cysteine residues found in the SPARC sequence [5]. All those cleavage sites are preceded in the SPARC sequence by a Glu residue [5] as expected from the specificity of the protease [13]. Several more sequences were determined for some smaller peptides obtained by cleavage with SV8 protease (including the C-terminal peptide SP4) and by cleavage of reduced BM-40 with trypsin (table 3).

4. DISCUSSION

The data so far leave no doubts that mouse BM-40, mouse SPARC [5] and bovine osteonectin (Bolander, M., personal communication) are products of the same gene or at least very similar genes. The osteonectin sequence shows 92% homology to SPARC with substantial differences in the first 20 amino acid residues and an additional Cys residue located between position 191/192 of SPARC. Both sequences are, however, similar in domain structure starting with a region of rather acidic nature (~50 residues), followed by a disulfide-bonded domain (11 Cys; ~90 residues), a Cys-free region (~100 residues) and a second disulfide-bonded region (3 Cys; ~40 residues). Our sequence data on BM-40 fragments are compatible with a covalent connection between both disulfide-bonded domains as predicted from the presence of 14 Cys residues in SPARC [5]. This indicates that fragment SP1 of BM-40 contains two segments encompassing positions 48–140 and 234–253, respectively. The last two cysteine residues form, however, a separate loop as shown by sequence analysis of peptide SP2. How the extra cysteine residue found in bovine osteonectin can be accommodated in this arrangement remains at the moment an open question.

The rather selective extraction of BM-40 with physiological buffer containing EDTA suggests that calcium-binding changes its affinity for other components within the tumor basement membrane. It is in this context noteworthy that the same extraction procedure also solubilizes substantial amounts of laminin and nidogen [9] indicating the possible existence of complexes between BM-40 and these proteins. A distinct loss of α-helical structures has been observed after removal of calcium from BM-40 (Engel, J., personal communication) which could be a factor in changing the interaction potential for other matrix components.

BM-40 has not only been detected as a component of the EHS tumor but is also present in lens capsule, Reichert's membrane and various other tissue extracts and cell cultures [2]. This agrees with similar observations on the widespread distribution of SPARC mRNA [14] and the frequent finding that osteonectin is produced not only by bone but also by several other cells [13,15–17]. The data demonstrate that this unique calcium-binding protein identified as osteonectin, SPARC or BM-40 is in fact a frequent constituent of mineralizing and non-mineralizing connective tissues and of basement membranes suggesting a rather general biological role. It is likely that some of its functions are related to calcium-binding which still remain to be established.

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