Structural variability of BM-40/SPARC/osteonectin glycosylation: implications for collagen affinity

Brigitte Kaufmann, Stefan Müller, Franz-Georg Hanisch, Ursula Hartmann, Mats Paulsson, Patrik Maurer, and Frank Zaucke

2Center for Biochemistry, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, D-50931 Cologne, Germany; and 3Center for Molecular Medicine, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, D-50931 Cologne, Germany

Received on November 26, 2003; revised on February 12, 2004; accepted on February 14, 2004

We performed a detailed investigation of N-glycan structures on BM-40 purified from different sources including human bone, human platelets, mouse Engelbreth-Holm-Swarm (EHS) tumor, and human BM-40 recombinantly expressed in 293 and osteosarcoma cells. These preparations were digested with endoglycosidases and N-glycans were further characterized by sequential exoglycosidase digestion and high-performance liquid chromatography (HPLC) analyses. Bone BM-40 carries high-mannose structures as well as biantennary complex type N-glycans, whereas the protein from platelets and 293 cells has exclusively bi- and triantennary complex type structures. BM-40 derived from the EHS tumor carries biantennary complex type and additional hybrid structures. Using the osteosarcoma-derived MHHE-S1 cell line we successfully expressed a recombinant BM-40 that bears at least in part the bone-specific high-mannose N-glycosylation in addition to complex type and hybrid structures. Using chromatography on Concanavalin-A Sepharose, we further purified a fraction enriched in high-mannose structures. This array of differentially glycosylated BM-40 proteins was assayed by surface plasmon resonance measurements to investigate the binding to collagen I. BM-40 carrying high-mannose structures binds collagen I with higher affinity, suggesting that differentially glycosylated forms may have different functional roles in vivo.

Key words: bone/complex type/high mannose/MHH-ES1 cells/platelets

Introduction

BM-40 (also known as secreted protein, acidic, rich in cysteines [SPARC] or osteonectin) is an extracellular glycoprotein with affinity for calcium ions and collagens (Maurer et al., 1996). Among the numerous functions described for BM-40 are regulation of extracellular matrix organization, binding of growth factors, and inhibition of cell adhesion and spreading (Bradshaw and Sage, 2001). BM-40 has a modular structure made up from an N-terminal domain containing about 50 amino acid residues, out of which 18 are negatively charged, followed by a follistatin-like domain with 10 cysteines in a typical pattern and an extracellular calcium-binding domain with two EF-hand calcium binding motifs, each with a bound calcium in the X-ray structure (Hohenester et al., 1996; Maurer et al., 1995). BM-40 forms the prototype of a protein family characterized by containing both extracellular calcium-binding and follistatin-like domains and often sharing functional features, such as calcium and collagen affinity. Other members of this family are SC1/hevin/QR1 (Johnston et al., 1992), tsc36/Flik/FRP (Shibanuma et al., 1993), testican-1 (Alliel et al., 1993), testican-2 (Vannahme et al., 1999), testican-3 (Hartmann and Maurer, 2001), SMOC-1 (Vannahme et al., 2002), and SMOC-2 (Vannahme et al., 2003).

The glycoprotein nature of BM-40 was recognized directly on discovery of the protein (Termine et al., 1981), and analysis of the bovine and human protein sequences yielded two consensus sequences for potential N-glycosylation at Asn71 and Asn99 (Bolander et al., 1988; Villareal et al., 1989), but there was no evidence for the presence of O-linked oligosaccharides. Later studies indicated that only the N-glycan acceptor site at Asn99 is used (Hohenester et al., 1997; Xie and Long, 1995). This site is widely conserved among species, often with the sequence Asn-Lys-Thr followed by either Phe or Tyr, for example, in chicken (Bassuk et al., 1993), rabbit (Bluteau et al., 2000), mouse (Lankat-Buttgereit et al., 1988; Mason et al., 1986), and Xenopus laevis (Damjanovski et al., 1992). A potential N-glycosylation site in a similar position but with another sequence is found in BM-40 from rainbow trout (Tang and McKeown, 1995) and brine shrimp (Tanaka et al., 2001), and BM-40 from Caenorhabditis elegans contains a site at Asn80 (Schwarbauer and Spencer, 1993). BM-40 from bone and platelets migrate differently in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and enzymatic digestion with N-glycosidase F and endoglycosidase H showed that the difference is due to variable N-glycosylation with the bone-derived protein carrying predominantly high-mannose oligosaccharides, whereas those on platelet BM-40 are mainly of the complex type (Kelm and Mann, 1991). The difference in type of N-glycans appeared to have functional consequences, as the bone form of the protein bound to collagen I, III, and V in solid phase assays, whereas the platelet form did not. The differences were confirmed in independent studies on BM-40 binding to collagen V, and it was also shown that removal of N-linked oligosaccharides by N-glycosidase F bears at least in part the bone-specific high-mannose N-glycosylation in addition to complex type and hybrid structures. Using chromatography on Concanavalin-A Sepharose, we further purified a fraction enriched in high-mannose structures.
F treatment increased the affinity of BM-40 from both bone and platelets to collagen V to reach equal levels. Site-directed mutagenesis was used to show that only the glycosylation of Asn99 affects collagen binding (Xie and Long, 1995).

The collagen affinity of BM-40 is of great functional importance. The collagen I-deficient Mov-13 mouse line does not retain BM-40 in the extracellular matrix (Iruegas et al., 1996) and dermal collagen fibrils in BM-40-null mice have a decreased diameter and a reduced tensile strength (Bradshaw et al., 2003). Not only glycosylation but other forms of posttranslational modification of BM-40 modulate the collagen affinity. Limited proteolytic cleavage of BM-40 occurs in tissues, and treatment with matrix metalloproteinases increases its affinity to collagens 7–20-fold due to a cleavage in helix αC in the extracellular calcium-binding domain (Sasaki et al., 1997). Indeed, deletion of helix αC in recombinant BM-40 gave a similar increase in binding affinity, and by X-ray crystallography this could be shown to be due to the removal of a steric constraint on the binding site, which was mapped to a loop between the two EF-hands in the extracellular calcium-binding domain (Sasaki et al., 1998).

Variable glycosylation provides an opportunity to vastly increase the information of a concise genome, and it has been demonstrated previously that the function of a protein can be modulated by its glycosylation pattern. In experimental tumor models the most common aberrant N-glycosylations are an increase in terminal sialylation and a shift to more highly branched N-linked oligosaccharides; it has been shown that the metastatic potential of tumor cells correlates with these changes (Hakomori, 1996; Varki, 1993; Yamamoto et al., 2000). Glycosylation has also been implicated in the regulation of CD44-mediated cell binding of hyaluronan. Interestingly, N-linked oligosaccharides can both enhance and reduce the CD44 affinity for hyaluronan depending on the specific structure of the glycan (Skelton et al., 1998).

In the present work we analyze the structure of the N-glycans in BM-40 derived from bone, platelets, and a variety of osteosarcoma cell lines. By expressing human BM-40 recombinantly in different cell lines, we were able to prepare BM-40 forms of variable glycosylation and could show that BM-40 carrying high-mannose N-glycans indeed binds collagen I with higher affinity than other forms. Our work shows how tissue-specific variation in glycosylation of a given protein may modulate its biological function.

Results

N-glycosylation of well-characterized forms of BM-40

In a first step we performed a detailed characterization of N-glycosylation of BM-40 purified from human bone, human platelets, the mouse Engelbreth-Holm-Swarm sarcoma (EHS) tumor, and human BM-40 recombinantly expressed in human embryonic kidney 293 cells. These are the most commonly studied preparations of BM-40, and their analysis provides information on the spectrum of N-glycan variants encountered in different mammalian tissues. Initially, highly purified BM-40 preparations were subjected to digestion with N-glycosidase F or endoglycosidase H. BM-40 from human bone (A), human platelets (B), mouse EHS tumor (C), and human BM-40 recombinantly expressed in 293 cells (D) was digested with either N-glycosidase F (+F) or endoglycosidase H (+H) and analyzed by SDS-PAGE on 12% polyacrylamide gels under reducing conditions. In A–C detection was by immunoblot with antibodies to BM-40 and in D by staining with Coomassie brilliant blue. The arrows mark the migration position before and after removal of the N-glycan.

![Image](https://academic.oup.com/glycob/article-abstract/14/7/609/643352/fig1)

**Fig. 1.** Sensitivity of BM-40 from different sources to N-glycosidase F and endoglycosidase H. BM-40 from human bone (A), human platelets (B), mouse EHS tumor (C), and human BM-40 recombinantly expressed in 293 cells (D) was digested with either N-glycosidase F (+F) or endoglycosidase H (+H) and analyzed by SDS-PAGE on 12% polyacrylamide gels under reducing conditions. In A–C detection was by immunoblot with antibodies to BM-40 and in D by staining with Coomassie brilliant blue. The arrows mark the migration position before and after removal of the N-glycan.

In the present work we analyze the structure of the N-glycans in BM-40 derived from bone, platelets, and a variety of osteosarcoma cell lines. By expressing human BM-40 recombinantly in different cell lines, we were able to prepare BM-40 forms of variable glycosylation and could show that BM-40 carrying high-mannose N-glycans indeed binds collagen I with higher affinity than other forms. Our work shows how tissue-specific variation in glycosylation of a given protein may modulate its biological function.
identical to the theoretical mass calculated from the amino acid sequence for BM-40 from the EHS tumor and for the main portion of the recombinant BM-40 sample. BM-40 from bone and platelets and ~40% of the recombinant BM-40 molecules from 293 cells gave masses after deglycosylation that were 0.4 and 0.7 kDa higher than the theoretical one, indicating that additional posttranslational modifications may be present.

N-glycans were cleaved from the various BM-40 samples with N-glycosidase F and labeled at their reducing ends with the fluorescent dye 2-aminobenzamide. The presence and number of terminal sialic acid residues was determined by anion-exchange chromatography on a Q Hyper D10 high-performance liquid chromatography (HPLC) column and comparison of the elution profiles with those of standard glycans (Figure 3, Table I). The extent of sialic acid substitution varied markedly between BM-40 forms, with BM-40 from bone and recombinant BM-40 from 293 cells often carrying neutral N-glycans, whereas more than half of the N-glycans from platelet BM-40 carried up to three sialic acid residues (Table I). The highly variable sialic acid content of various BM-40 preparations is likely to modulate charge-dependent interactions.

In a next step, sialic acid residues were removed by digestion with neuraminidase and the asialo-N-glycans separated according to size and hydrophilic properties by HPLC on a calibrated normal phase column (Figure 4). The N-glycan structures were determined by sequential digestion with different exoglycosidases (β-galactosidase, β-N-acetylhexosaminidase, and α-fucosidase) and the retention times were compared with commercially available carbohydrate standards (Novatec Analytical, Basel, Switzerland).

The analysis suggests that although about half of all N-glycans in both bone and platelet BM-40 are of the complex biantennary type, the other half are of the high-mannose type, and a corresponding portion in platelet BM-40 have a complex triantennary structure (Table II). The N-glycans from EHS tumor BM-40 are to 80% of complex biantennary type, with the rest being of hybrid structure, and the bulk of those on the recombinant BM-40 from 293 cells are of the complex triantennary type with a small contribution of complex biantennary structures. The latter sample showed a marked undergalactosylation, with the N-glycans carrying no or at the most one galactose residue, even in the triantennary structures. Accordingly, the BM-40 N-glycan

Table I. Quantification of sialo-N-glycans derived from BM-40 from different sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Neutral (%)</th>
<th>Monosialo (%)</th>
<th>Disialo (%)</th>
<th>Trisialo (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>71.7</td>
<td>9.8</td>
<td>18.5</td>
<td>ND</td>
</tr>
<tr>
<td>Platelets</td>
<td>43.7</td>
<td>19</td>
<td>26</td>
<td>11.3</td>
</tr>
<tr>
<td>EHS tumor</td>
<td>45</td>
<td>31.8</td>
<td>23.2</td>
<td>ND</td>
</tr>
<tr>
<td>EBNA-293 cells</td>
<td>81.6</td>
<td>18.4</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Relative amounts were calculated from the chromatograms shown in Figure 3. ND, not detected.
Fig. 4. Analysis of asialo-N-glycans from human bone BM-40 by sequential exoglycosidase treatment and normal-phase HPLC. N-glycans were enzymatically released by treatment with N-glycosidase F, labeled with 2-aminobenzamide and digested with neuraminidase (neur) to remove sialic acid residues. The asialo-structures were identified by sequential exoglycosidase treatment and normal-phase HPLC.

The N-glycans on recombinant BM-40 produced in MHH-ES-1 cells were analyzed for sialic acid content and oligosaccharide structure in the same manner as described for other BM-40 forms. Anion-exchange chromatography showed that 47.5% were neutral, and 30.5% carried one sialic acid residue, 15.8% two, and 6.2% three. Sequential exoglycosidase digestion followed by normal-phase HPLC showed the sample to be a mixture of complex bi- or triantennary N-glycans as well as hybrid and high-mannose types (Figure 7A, Table III). Parallel analysis of fraction 2, the BM-40 fraction that bound tightly to Con A-Sepharose, showed this material to be enriched in molecules carrying high-mannose oligosaccharides and in this regard more similar to bone-derived BM-40 than any other fraction (Figure 7B, Table III).

Affinity of different BM-40 glycoforms to collagen I

To test the suggestion that the nature of the N-glycans influences the collagen affinity of BM-40 (Kelm and Mann, 1991), we determined the binding of four different BM-40 preparations to immobilized collagen I in a Biacore apparatus (Figure 8).

First we used increasing concentrations of BM-40 from 293 cells ranging from 2.4 to 4.5 μM to validate the measurements. The resulting curves were aligned and clearly demonstrate a concentration-dependent increase in response units (Figure 8A). Compared to the EBNA-293 derived BM-40 the preparations from bone and fraction 2 from MHH-ES-1 cells showed a markedly slower dissociation (Figure 8B). The binding and dissociation curves obtained allowed the calculation of approximate $K_D$ values (Table IV). Bone-derived BM-40 showed the highest
affinity, with a $K_D$ of 15.5 nM, followed by recombinant BM-40 from MHH-ES-1 cells that had been enriched for high-mannose N-glycan structures by chromatography on Con A-Sepharose (fraction 2), with $K_D$ 130 nM. Fraction 1 from the Con A-Sepharose chromatography, as well as recombinant BM-40 from 293 cells, predominantly carrying complex type bi- and triantennary N-glycans, showed lower affinity for collagen I, with $K_D$ values of 448 and 1.520 nM, respectively. MHH-ES-1 derived BM-40 recovered in the flow-through of the Con A-Sepharose column and devoid of high-mannose N-glycan did not bind to the collagen I coupled chip (data not shown).

**Discussion**

Analysis by MALDI-TOF MS before and after digestion with N-glycosidase F did for all BM-40 variants gave a mass difference between 1.7 and 2.7 kDa, in good agreement with a substitution with a single N-glycan. Our experiments did not address the position of this oligosaccharide, but earlier studies indicate a preferential or unique substitution of Asn99 (Hohenester *et al.*, 1997; Xie and Long, 1995). Comparison of the molecular masses obtained after digestion with N-glycosidase F with the theoretical one, calculated from the amino acid sequence, showed that BM-40 from the MHH-ES-1 cells and from the EHS tumor carry no posttranslational modifications in addition to N-glycans, whereas BM-40 from

Table II. Quantification of asialo-N-glycans derived from BM-40 from different sources

<table>
<thead>
<tr>
<th>Source</th>
<th>glycosylation type</th>
<th>structure</th>
<th>amount [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone</td>
<td>complex, biantennary</td>
<td><img src="image1" alt="structure" /></td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>high mannose</td>
<td><img src="image2" alt="structure" /></td>
<td>47</td>
</tr>
<tr>
<td>Platelets</td>
<td>complex, biantennary</td>
<td><img src="image3" alt="structure" /></td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>complex, triantennary</td>
<td><img src="image4" alt="structure" /></td>
<td>53</td>
</tr>
<tr>
<td>EHS-tumour</td>
<td>complex, biantennary</td>
<td><img src="image5" alt="structure" /></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>hybrid</td>
<td><img src="image6" alt="structure" /></td>
<td>20</td>
</tr>
<tr>
<td>EBNA-293 cells</td>
<td>complex, biantennary</td>
<td><img src="image7" alt="structure" /></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>complex, triantennary</td>
<td><img src="image8" alt="structure" /></td>
<td>93</td>
</tr>
</tbody>
</table>

Relative amounts were calculated from chromatograms as shown for bone-derived BM-40 in Figure 4. Circles, mannose; triangles, fucose; diamonds, galactose; squares, N-acetylglucosamine. The percentages given refer to predominant structures with open symbols being variable.

Fig. 5. Sensitivity of recombinant human BM-40 expressed in MHH-ES-1 to N-glycosidase F and endoglycosidase H. BM-40 recombinantly expressed in MHH-ES-1 cells was digested with either N-glycosidase F (+F) or endoglycosidase H (+H) and analyzed by SDS-PAGE on 12% polyacrylamide gels under reducing conditions. Detection was by immunoblot with antibodies to BM-40. The arrows mark the migration position before and after removal of the N-glycan.
bone and platelets gave masses after deglycosylation that were 0.4 and 0.7 kDa higher than the theoretical ones. The recombinant BM-40 preparation from 293 cells contained both molecules with additional mass after deglycosylation and such without any more modifications.

Even though our study was not primarily aimed at determining the nature of these adducts, we determined the incorporation of 32P-phosphate and 35S-sulfate into BM-40 produced by 293, MHH-ES-1, and SaOS-2 cells, in each case with negative results (data not shown). Even so, phosphorylation has been demonstrated for bone-derived BM-40 (Fisher et al., 1987; Kelm and Mann, 1991; Romberg et al., 1985) and tyrosine sulfation has been suggested (Maillard et al., 1992). There is no evidence from other studies for the presence of O-glycans, even though the software Net-O-Glyc indicates the presence of two potential O-glycosylation sites (data not shown). BM-40 is a substrate for tissue transglutaminase both in vivo (Aeschlimann et al., 1995) and in vitro (Hohenadl et al., 1995), and it is possible that additional mass is contributed by incorporation of low-molecular-mass amines. The presence and potential functions of BM-40 adducts other than N-glycans needs further study.

A starting point for our work were the results from Kelm and Mann (1991) who, through a combination of endoglycosidase digestion and lectin binding studies, proposed that bone BM-40 carries high-mannose N-glycans and platelet BM-40 complex type ones. Our results partially confirm theirs but show a greater variety of N-glycans in BM-40 from each source. Indeed, our experiments showed a rather pronounced sensitivity of bone derived BM-40 to endoglycosidase H when the digests were analyzed by SDS–PAGE (Figure 1), but a detailed analysis of the glycans released by N-glycosidase F showed the presence of about equal amounts of high-mannose and biantennary complex type N-glycans in human bone BM-40 (Figure 4 and Table II).
This also demonstrates that endoglycosidase digestion followed by SDS–PAGE provides basic information but does not allow definitive conclusions on the N-glycan structures present. Human platelet BM-40, on the other hand, carries bi- and triantennary complex type oligosaccharides in equal proportions. In agreement with earlier analysis (Nischt et al., 1991) our results show that the much studied recombinant BM-40 produced by 293 cells is similar to platelet BM-40 in that it carries a mixture of bi- and triantennary complex type N-glysans, but the proportion of biantennary structures is much lower and both forms are markedly undergalactosylated. Therefore 293 cell-derived BM-40 differs from both bone- and platelet-derived BM-40 in its glycosylation and presumably also in such biological features that are affected by glycosylation. BM-40 from the mouse EHS tumor was unique in that it carries hybrid N-glysans in addition to biantennary complex type ones.

The highly variable N-glycosylation of BM-40 derived from different sources indicates that the type of glycosylation is dependent on the glycosyl transferase repertoire of the producing cell type, rather than being directed by the acceptor protein. However, in earlier studies we analyzed the glycosylation of bone sialoprotein expressed in the same 293-cell system as the BM-40 studied in the present work. The N-glysans synthesized onto bone sialoprotein by 293 cells clearly differed from those on BM-40 produced in the same cells and were mainly tetraantennary complex type structures (Wuttke et al., 2001). The glycosylation pattern is obviously determined by both the acceptor protein and by the cell type used for expression.

One purpose of our study was to recombinantly produce a BM-40 variant that as closely as possible mimics the BM-40 found in human bone. On the basis of the arguments above, we expected to be able to do so by adapting our expression system to use in an osteoblast-like cell line. For this purpose we selected a number of well-characterized osteosarcoma cell lines. We were surprised to find that among those only one, MHH-ES-1, produced BM-40 with high-mannose type oligosaccharides. Apparently, the pattern of N-glysans produced is highly sensitive to transformation and/or dedifferentiation. Even in MHH-ES-1 cells a broader variety of N-glysans were produced than found on BM-40 in human bone (Table II and Table III) and chromatography on Con A-Sepharose was needed to prepare a fraction of BM-40 molecules enriched in high-mannose N-glysans that allowed us to test the hypothesis that this kind of glycosylation favors interactions with collagen I. Our Biacore binding studies indeed supported this assumption, showing a higher affinity of the high-mannose N-glycan-containing BM-40 variants from human bone and from the Con A-binding fraction of the MHH-ES-1 derived molecules to collagen I than of other forms, even though the binding by bone-derived BM-40 was for unknown reasons stronger than that of the MHH-ES-1 BM-40. Both preparations showed similar characteristics in circular dichroism spectroscopy, indicating a similar and native fold (data not shown). Possibly the minor additional posttranslational modifications found in bone BM-40 have a favorable influence on collagen affinity. The effect of N-glysans on the binding is likely to be mediated through steric or charge influences on the neighboring collagen-binding site, with the oligosaccharide hindering or promoting the docking of the BM-40 molecule onto the collagen triple helix.

### Materials and methods

#### Purification of tissue-derived forms of BM-40

Human bone powder (300 g) was stirred at 4°C for 48 h in 1 L 4 M guanidine hydrochloride, 50 mM Tris–HCl, pH 7.4,
to remove residues of blood and bone marrow. The residue was washed twice for 24 h with 1.5 L 50 mM Tris–HCl, pH 7.4, and extracted three times for 24 h with 1.5 L 0.5 M ethylenediamine tetra-acetic acid (EDTA), 50 mM Tris–HCl, pH 7.4, containing the protease inhibitors 0.1 M 6-aminohexanoic acid, 5 mM benzamidine hydrochloride, 0.5 mM phenylmethylsulfonyl fluoride, and 5 mM N-ethylmaleimide. The three extracts were pooled, dialyzed against 50 mM Tris–HCl, pH 8.6, and loaded onto a column of DEAE-Sepharose Fast Flow (Amersham Biosciences, Little Chalfont, U.K.). BM-40 eluted at about 0.2 M NaCl in a linear gradient from 0 to 1 M. Pooled BM-40-containing fractions were chromatographed on a ResourceQ column under the same conditions and eluted at 0.25–0.3 M NaCl. BM-40-containing fractions were dialyzed against 50 mM Tris–HCl, pH 7.0, and applied to Hitrap Heparin column. BM-40 bound weakly and was eluted at 0.1–0.15 M NaCl.

Mouse EHS tumor tissue was sequentially extracted with Tris-buffered saline (TBS) and TBS containing 10 mM EDTA and BM-40 chromatographically purified from the EDTA extract as previously described (Mann et al., 1987). Purified human platelet osteonectin (BM-40) was obtained commercially from Calbiochem (San Diego, CA).

Expression and purification of recombinant forms of BM-40

For cloning of the expression vector pCEP-Pu/BM-40, the full-length human BM-40 cDNA including its signal peptide was cut out of the plasmid huBM-40blue (Nischt et al., 1991) using Not I and Xho I restriction sites and inserted between the same restriction sites of the episomal eucaryotic expression vector pCEP-Pu (Kohfeldt et al., 1997). For expression of a His6-Myc-Factor X–tagged fusion protein, the human BM-40 cDNA sequence without signal peptide was amplified using the huBM-40blue plasmid as a template and cloned into a N-terminal His6-Myc-Factor X–tagged expression vector based on the pCEP-Pu (Wuttke et al., 2001). The correct reading frame was confirmed by sequencing both strands using the ABI Prism 377 Automated Sequencer (PE Biosystems, Foster City, CA). Human embryonic kidney cells EBNA-293 (Invitrogen, Carlsbad, CA) were transfected with the expression plasmid encoding the N-terminal His6-Myc-Factor X–tagged BM-40 using the Fugene transfection reagent (Roche, Indianapolis, IN). Transfected cells were selected with puromycin as described (Kohfeldt et al., 1997). Serum-free culture supernatants were collected, dialyzed against 50 mM Tris–HCl, pH 8.6, passed over a DEAE-Sepharose Fast Flow column, and rechromatographed on a ResourceQ column as described for the bone-derived protein.

Table IV. Kinetic evaluation of the interaction between BM-40 glycoforms from different sources with collagen I

<table>
<thead>
<tr>
<th>Source</th>
<th>$k_a$ $(10^{-3})$</th>
<th>$k_d$ $(10^2)$</th>
<th>$K_D$ (nM)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBNA-293</td>
<td>2.42</td>
<td>1.59</td>
<td>1520</td>
<td>0.105</td>
</tr>
<tr>
<td>Bone</td>
<td>0.48</td>
<td>30.7</td>
<td>15.5</td>
<td>0.076</td>
</tr>
<tr>
<td>MHH-ES1 fraction 1</td>
<td>1.78</td>
<td>3.97</td>
<td>448</td>
<td>0.112</td>
</tr>
<tr>
<td>MHH-ES1 fraction 2</td>
<td>0.91</td>
<td>7.02</td>
<td>130</td>
<td>0.066</td>
</tr>
</tbody>
</table>

BM-40 was applied in concentrations between 0.9 and 2.4 μM. Evaluation was done according to a 1:1 binding model.
column of Talon metal affinity resin (Clontech, Palo Alto, CA) with a flow rate of 0.5 ml/min. After washing with five column volumes of the same buffer containing 2.5 mM imidazol, the protein was eluted with a linear gradient between 2.5 and 250 mM imidazol. BM-40-containing fractions were pooled, dialyzed against 50 mM Tris–HCl, pH 8.6, and loaded onto a ResourceQ (Amersham Biosciences) column. BM-40 eluted at about 0.3 M NaCl in a linear gradient from 0 to 1 M.

**SDS–PAGE and western blotting**

SDS–PAGE was performed according to Laemmli (1970) and gels either stained with Coomassie brilliant blue R250 or electrophoretically transferred to nitrocellulose. BM-40 was detected with polyclonal rabbit antisera against either human or mouse BM-40 followed by a peroxidase-conjugated antibody against rabbit IgG (Dako, Highwycombe, U.K.) and the reaction developed with the enhanced chemiluminescence method (Amersham Biosciences).

**Analytical endoglycosidase digestions**

Prior to SDS–PAGE analysis, glycoprotein samples (2–3 μg) were heat-denatured for 15 min at 100°C and incubated overnight at 37°C with 0.2 U N-glycosidase F (Roche) in 0.15 M NaCl, pH 7.4, containing 0.1% SDS and 0.5% Nonidet P-40 or with 2 mU endoglycosidase H (Roche) in 0.1 M sodium acetate, pH 5.8, containing 0.02% SDS and 0.1% β-mercaptoethanol.

N-glycosidase F release of N-glycans, sequential exoglycosidase digestions, and HPLC analyses of 2-aminobenzamide-labeled oligosaccharides

A previously described procedure was followed (Wuttke et al., 2001). Briefly, 200 μg ethanol-precipitated BM-40 was incubated in 2 μl 1% SDS, 0.5% β-mercaptoethanol, 0.1 M EDTA for 30 min at room temperature. After the addition of 40 ml 0.2 M sodium phosphate, pH 8.5, followed by a 5-min denaturation at 100°C, 5 μl 7.5% Nonidet P-40 was added to the mixture. The sample was incubated with 1 U N-glycosidase F (Roche) for 18–20 h at 37°C and then applied to a 150-mg carbon column (Carbograph SPE, Alltech, Unterhaching, Germany) according to the method of Packer et al. (1998). The column was eluted with water and then with 2 ml 25% acetonitrile in 0.05% trifluoroacetic acid to elute the oligosaccharides, which were dried in a SpeedVac evaporator.

The glycans were labeled with the fluorescent dye 2-aminobenzamide according to the method described by Bigge et al. (1995). The dried glycans were resuspended in 2 μl 1 M 2-aminobenzamide in 100% acetic acid and 3 μl 2 M sodium cyanoborohydride in dimethyl sulfoxide. After a 2-h labeling reaction at 60°C, the samples were dotted onto chromatography paper, and excessive labeling reagents were separated by chromatography in n-butanol:ethanol:water (4:1:1). The labeled glycans do not migrate under these conditions. After chromatography the application points were cut out, and the labeled glycans eluted with 200–500 μl water by using centrifugal microfiltration tubes and stored at −20°C.

To determine the monosaccharide sequence of the N-linked oligosaccharides, the 2-aminobenzamide-labeled N-glycans were digested with specific exoglycosidases. Twenty microliters of each sample were incubated sequentially with neuraminidase (New England Biolabs, Beverly, MA), β-N-acetylhexosaminidase (Glyko, Novato, CA), β-galactosidase (Glyko), and α-fucosidase (Glyko) for a total of 18–20 h at 37°C in 50 mM sodium citrate, pH 4.5. The digests were dried in a SpeedVac evaporator, dissolved in 75% acetonitrile in water, and used for HPLC analysis.

For analysis a Beckman System Gold HPLC station was used together with a Shimadzu RF-10A XL fluorescence detector and the Beckman Gold Noveau software. The excitation wavelength was 330 nm and the emission wavelength 420 nm. For anion-exchange HPLC a Q HyperD10 column (10 μm, 4.6 × 100 mm, Beckman, Palo Alto, CA) was used at a flow rate of 1 ml/min. Twenty microliters of the 2-aminobenzamide-labeled oligosaccharides were loaded to the column. Elution buffers were water and 0.5 M ammonium formate, pH 9.0 (buffer B). Sialo-N-glycans were eluted for 1 min with 0% B, for 12 min with 0–5% B, for 13 min with 5–21% B, for 25 min with 25–80% B, and for 4 min with 80–100% B.

Normal-phase HPLC was performed with a polymer-based aminopropyl column (Supelco, 5 μm, 4.6 × 250 mm). The 2-aminobenzamide-labeled oligosaccharides (20 μl) were loaded onto the column dissolved in 75% acetonitrile in water. The linear elution gradient for N-glycans started at 68% acetonitrile and 32% 50 mM ammonium formate, pH 4.4, went over 60 min to 50% acetonitrile and 50% ammonium formate, pH 4.4, and in 3 min to 100% ammonium formate at a flow rate of 0.5 ml/min. Standard glycans (e.g., derived from bovine fetuin) and glucose ladders were used to identify the different structures.

**MALDI-TOF MS**

Samples of BM-40 (3 μg) were analyzed either in intact form or after digestion overnight at 37°C with 0.2 U N-glycosidase F (Roche) in 20 mM Tris–HCl, pH 7.4, or with 2 μU endoglycosidase H (Roche) in 30 mM sodium acetate, pH 5.8. For MALDI-TOF MS analysis, the samples were dissolved in 5 ml 0.1% aqueous trifluoroacetic acid. One milliliter of the sample solution was placed on the target, and 1 ml of a freshly prepared saturated solution of sinapinic acid in acetonitrile/H2O (2:1) with 0.1% trifluoroacetic acid was added. The spot was then recrystallized by addition of another 1 ml acetonitrile/H2O (2:1), which resulted in a fine crystalline matrix. MALDI-MS was carried out in linear mode on a Bruker Reflex IV equipped with a video system, a nitrogen UV laser (λmax=337 nm), and a HiMass detector. For recording of the spectra an acceleration voltage of 20 kV was used, and the detector voltage was adjusted to 1.9 kV. Three hundred to four hundred single laser shots were summed into an accumulated spectrum. Calibration was carried out using the single and doubly protonated ion signal of bovine serum albumin for external calibration.

**Biacore studies of BM-40-collagen I interactions**

Assays were performed using a Biacore 2000 (BIAcore AB, Uppsala, Sweden). Coupling of collagen I (native calf skin, IBFB) to the CM5 chip was performed in 50 mM sodium...
acette, pH 4.0, at a pulse of 5 ml/min. To activate the surface, a 7-min pulse at 0.05 mM N-hydroxy-succinimide/0.2 M N-ethyl-N'-(dimethylaminopropyl) carbodiimide was used. Collagen I (60 µl, 0.2 mg/ml) was injected at the desired amount was coupled (5000–7000 RU), and subsequently excess reactive groups were deactivated by a 7-min pulse of 1 M ethanolamine hydrochloride, pH 8.5. Measurements were carried out in HBS (20 mM HEPES, 150 mM NaCl, 0.005% P20, pH 7.4) containing 2 mM CaCl₂ at a flow of 25 µl/min. The injection of 100 µl BM-40 solution (0.9–4.5 µM) was followed by a 400-s dissociation. The data were analyzed with BIAevaluation software 3.0 according to the Langmuir model for 1:1 binding and association and dissociation rate constants were determined and K_D values were calculated. All binding curves could be fitted with an accuracy of $\chi^2 < 0.2$.

Acknowledgments

This work was supported by the Bundesministerium für Bildung und Forschung (Grant 0311443), the Deutsche Forschungsgemeinschaft (MA 1932/2 and PA 660/6) and the Köln Fortune program of the Medical Faculty of the University of Cologne.

Abbreviations

Con A, Concanavalin A; EDTA, ethylenediamine tetra-acetic acid; EHS, Engelbreth-Holm-Swarm; HPLC, high-performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

References


