Mac-2 binding protein is a cell-adhesive protein of the extracellular matrix which self-assembles into ring-like structures and binds β 1 integrins, collagens and fibronectin

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Human Mac-2 binding protein (M2BP) was prepared in recombinant form from the culture medium of 293 kidney cells and consisted of a 92 kDa subunit. The protein was obtained in a native state as indicated by CD spectroscopy, demonstrating α -helical and β -type structure, and by protease resistance and immunological analysis. It was highly modified by N- and O-glycosylation but not by glycosaminoglycans. Ultra-centrifugation showed non-covalent association into oligomers with molar masses of 1000–1500 kDa. Electron microscopy showed ring-like shapes with diameters of 30–40 nm. M2BP bound in solid-phase assays to collagens IV, V and VI, fibronectin and nidogen, but not to fibrillar collagens I and III or other basement membrane proteins. The protein also mediated adhesion of cell lines at comparable strength with laminin. Adhesion to M2BP was inhibited by antibodies to integrin β 1 subunits but not to α 2 and α 6 subunits, RGD peptide or lactose. This distinguishes cell adhesion of M2BP from that of laminin and excludes involvement of lactose-binding galectin-3. Immunological assays demonstrated variable secretion by cultured human cells of M2BP, which was detected in the extracellular matrix of several mouse tissues.

Keywords: cell adhesion/electron microscopy/lectin-binding/protein–protein binding/protein structure

Introduction

A large oligomeric protein composed of ~90 kDa subunits has been identified as a tumour-associated antigen (Natali *et al.*, 1982; Iacobelli *et al.*, 1986, 1993; Linsley *et al.*, 1986) and in the search for novel ligands of galectin-3 (formerly Mac-2) in several previous studies (Rosenberg *et al.*, 1991; Koths *et al.*, 1993; Inohara and Raz, 1994). Human Mac-2 binding protein (M2BP) has been purified from tumour cell medium, serum and milk and was shown to be a non-covalently linked oligomer with a molecular mass of >1000 kDa (Linsley *et al.*, 1986; Iacobelli *et al.*, 1993; Koths *et al.*, 1993). The sequence of human M2BP (~570 residues) was elucidated after cDNA cloning and shown to contain a signal peptide and a number of cysteines and N-glycosylation sites (Koths *et al.*, 1993; Ullrich *et al.*, 1994). A cyclophilin C-binding mouse protein with 69% sequence identity has also been cloned (Friedman *et al.*, 1993; Chicheportiche and Vassalli, 1994)

and may represent either a mouse analogue, or an isoform, of M2BP. The former possibility is indicated by genetic evidence (Brakebusch *et al.*, 1997).

The functions of M2BP are not yet well defined but may include lipopolysaccharide-dependent binding to CD14 (Yu and Wright, 1995), enhancement of killer cell activity and interleukin production (Ullrich *et al.*, 1994), tumour suppression (Jallal *et al.*, 1995) and the promotion of cell aggregation by cross-linking surface-bound galectin-3 (Inohara *et al.*, 1996). M2BP is present in the μ g/ml range in several biological fluids, where it may exert some of its functions. Elevated levels are often observed in melanomas, some lung, breast and gastrointestinal tumours and in some viral infections, and may be of diagnostic and prognostic value (Longo *et al.*, 1993; Iacobelli *et al.*, 1994). Its expression in normal tissues has been less well established and is in part controversial. Yet, a broad expression in gastrointestinal and other epithelial tissues, muscle and brain was shown by Northern blots (Ullrich *et al.*, 1994). Immunohistology of some of these tissues demonstrated a fine granular pattern which was interpreted to indicate intracellular deposits (Natali *et al.*, 1982; Iacobelli *et al.*, 1986). The possible deposition of M2BP in the extracellular matrix has not yet been considered or adequately examined.

In the present study we have addressed this question by producing recombinant human M2BP, which could be obtained in a native, oligomeric and glycosylated form. The oligomer formed large rings with a diameter of 30–40 nm for the most prominent structures. Binding studies *in vitro* demonstrated β 1 integrin-mediated cell adhesion for M2BP as well as interactions with some collagen types and fibronectin—properties which are consistent with an extracellular matrix localization. This was underscored by immunological analyses of cultured cells and tissues. The amounts of M2BP in these locations are apparently not very high, suggesting that the protein is stored in the matrix rather than being a major structural component.

Results

Purification and properties of recombinant human M2BP

Recombinant human M2BP was purified from serum-free culture medium by ammonium sulphate precipitation and Sepharose CL-6B chromatography and appeared as a single 90 kDa band by SDS gel electrophoresis (Figure 1). It eluted from the molecular sieve column close to the void volume, indicating non-covalent association into large oligomers. In order to obtain M2BP in non-degraded form, the addition of a cocktail of protease inhibitors was required during storage of medium and purification. Purified M2BP was eventually dialysed against 0.2 M

Fig. 1. SDS gel electrophoresis of purified recombinant M2BP and several of its digests. Lanes were loaded with 2 μ g of purified M2BP (lane 1), M2BP digested with N-glycosidase F (lane 2) or plasmin (lane 3). Samples were used either before (–) or after reduction (+) with dithiothreitol. PI-1 and PI-2 denote the plasmin fragments which were subjected to Edman degradation. The positions of calibrating proteins are indicated in the margins in kDa.

NH_4HCO_3 containing protease inhibitors and could thus be kept in a soluble state by storage at -20 or -80°C .

Edman degradation of the non-reduced 90 kDa band of M2BP demonstrated a single N-terminal sequence VNDGDMRLAD, which is identical to those previously determined for serum- and tissue-derived forms (Linsley *et al.*, 1986; Rosenberg *et al.*, 1991; Koths *et al.*, 1993; Inohara and Raz, 1994). Reduction produced a slight decrease in electrophoretic mobility (Figure 1), indicating the opening of internal disulphide bridges. Hexosamine analyses demonstrated 44 ± 9 residues of glucosamine and 16 ± 6 residues of galactosamine per M2BP monomer. A high glycosylation was also demonstrated by a shift of apparent molecular mass from 90 to 60 kDa after treatment with N-glycosidase F (Figure 1). No shift in mobility was observed after digestion with heparitinase or chondroitinase ABC, however (data not shown). A short treatment with plasmin (Figure 1), endoproteinase Lys-C or trypsin completely converted M2BP into 67 and 26 kDa bands. Edman degradation of the plasmin digest revealed the original N-terminus for the 67 kDa band and two sequences starting at positions 424 (YSSDYFQA) and 437 (YYPQS FQ) for the 26 kDa band. The plasmin digest still eluted close to the void volume of a Superose 6 column, indicating that oligomerization was maintained. No substantial cleavage was observed after 24 h treatment with endoproteinase Glu-C.

A sensitive and quantitative radioimmunoassay could be established with M2BP, showing 50% inhibition at ~ 10 ng/ml (data not shown). Reduced and alkylated M2BP was not inhibitory even at 200-fold higher concentrations, indicating that the antibodies bind to conformational epitopes. Analysis of media obtained from EBNA-293 cells transfected with the M2BP expression vector demonstrated 10–15 μ g/ml M2BP. Medium of non-transfected cells contained 0.2 μ g/ml M2BP, demonstrating only a low endogenous production. The same analysis of three normal human sera showed concentrations in the range 4–19 μ g/ml, consistent with previous observations for M2BP (Linsley *et al.*, 1986).

Oligomerization to ring structures

Analytical ultracentrifugation indicated the presence of M2BP monomers only in the presence of strong denatur-

ants like 6 M guanidine HCl. The molar mass was 92 kDa and the sedimentation coefficient ($s_{20,w}$) was 4.1S. However, under non-dissociative conditions and at a neutral pH the average molar mass was 1000–1500 kDa and $s_{20,w}$ was 19.8S, indicating high levels of self-association.

The nature of the aggregates was revealed by electron microscopy (Figure 2). Ring-like structures of 25–45 nm diameter were seen. There is clearly a distribution of size, but rings of 30–40 nm diameter are most abundant. Frequently a segmentation of the circumference of the rings was observed with five to eight segments of ~ 15 nm length for the most prominent particles. The rings exhibited similar dimensions when visualized after rotary shadowing (Figure 2A) or negative staining (Figure 2B) but in addition some linear aggregates and ring-to-ring associations were seen by the latter technique. Negative staining was performed at neutral pH with phosphotungstic acid in order to avoid dissociation of the ring structures. At pH 2.7 in dilute acetic or formic acid, partial to complete dissociation of the aggregates was observed both by electron microscopy (Figure 2C) and by analytical ultracentrifugation (data not shown). The process is completely reversible, as indicated by reformation of rings indistinguishable from those shown in Figure 2A and B after dialysis from acetic acid, pH 2.7, to neutral buffer, pH 7.4.

The conformational state of M2BP in its aggregated form at neutral pH was compared with that at acid pH by circular dichroism (CD) (Figure 3). The CD spectra at pH 2.7 and 7.4 were rather similar and demonstrated the presence of α -helical and β -structures in the protein. The spectra thus indicate that M2BP is in a native state under both conditions. An analysis of different proportions of secondary structure appeared to be of little use because M2BP may consist of at least four rather different domains (see Discussion).

Binding to extracellular matrix ligands

A solid-phase assay was used to examine the binding potential of various plastic-immobilized extracellular matrix proteins to M2BP used as a soluble ligand. Probably because of its large size, at concentrations above 200 nM M2BP already showed some unspecific binding to serum albumin, which was used as a blocking protein (Figure 4). A distinctly higher binding was observed for collagens V and VI, with half-maximal binding achieved at ~ 40 nM (Figure 4A). Weaker binding was observed with collagen IV and none with collagens I and III. These collagens were isolated from pepsin-digested human placenta and their triple-helical domains remained essentially undigested and are therefore potential candidates for M2BP binding. Among several non-collagenous matrix proteins tested, fibronectin was the strongest ligand with half-maximal binding at 10 nM (Figure 4B). The basement membrane protein nidogen was ~ 10 -fold less active. No substantial binding above the albumin background was observed with fibulin-1 and -2 (Figure 4B) and with laminin-1, perlecan and BM-40 (data not shown). This indicated a rather selective interaction of M2BP with components of the extracellular matrix. Galectin-3, however, was the strongest ligand for M2BP (< 1 nM) in the solid-phase assay (Figure 4B), which confirmed previous demonstrations of their high affinity by immunoprecipit-

Fig. 2. Ring-like aggregates of M2BP in PBS, pH 7.4, visualized by electron microscopy after rotary shadowing (A) and negative staining at neutral pH (B). The rotary shadowing technique reveals dissociation of the rings in 0.1 M acetic acid (C). Bar, 100 nm.

ation and affinity chromatography (Rosenberg *et al.*, 1991; Koths *et al.*, 1993; Inohara and Raz, 1994).

Cell adhesion is mediated through $\beta 1$ integrins

When used as a plastic-immobilized substrate, M2BP caused a distinct dose-dependent adhesion and spreading of HBL-100 cells, comparable with that of laminin and perlecan (Figure 5). Similar results, with plateau levels corresponding almost exactly to those of laminin, were obtained for human Saos-2 osteosarcoma cells, human epidermoid A431 cells, rat RN22 Schwannoma cells and rat Rugli glioma cells. Human astrocytoma 251MG cells also showed a distinct adhesion to M2BP but not to laminin. The cell-adhesive activity of M2BP was completely lost after reduction and alkylation (Figure 5). As shown for A431 cells in Figure 6, this adhesion is mediated through integrin receptors, as it could be completely inhibited by a blocking antibody (AIIB2) against $\beta 1$ integrin for both M2BP and laminin substrates. Laminin adhesion was also partially blocked by antibodies against $\alpha 2$ and $\alpha 6$ integrin subunits, consistent with the previous identification of $\alpha 2\beta 1$ and $\alpha 6\beta 1$ integrins as laminin receptors (Aumailley *et al.*, 1996). The latter two antibodies had only marginal effects on M2BP-mediated adhesion, however, indicating that A431 cells adhere to laminin and M2BP through different sets of $\beta 1$ integrins. Involvement of $\beta 1$ integrins in the adhesion to M2BP

Fig. 3. Circular dichroism spectra of M2BP in PBS, pH 7.4 (solid curve), and in dilute acetic acid, pH 3 (broken curve).

could also be demonstrated for HBL-100 and Saos-2 cells (data not shown). Further inhibition studies of RN22 and Rugli cell adhesion to M2BP with lactose (5 mM) and the synthetic peptides GRGDS (0.5 mM) and RGES (0.5 mM) at the maximal concentrations indicated failed to produce significant effects. This is consistent with the lack of a cell-adhesive RGD sequence in human M2BP

Fig. 4. Binding of soluble M2BP to immobilized extracellular matrix ligands in solid-phase assays. (A) Binding to immobilized collagens types I and III (■) which gave superimposable curves, and collagens IV (▼), V (◆) and VI (◇). (B) Binding to galectin-3 (○), fibronectin (●), nidogen (△), fibulin-1C (▲) and fibulin-2 (▽). Bovine serum albumin used for blocking coated wells was used on uncoated wells (×) and is shown in (A) and (B) for the illustration of levels of background binding. In each case, binding was detected with the same dilution (1:2000) of antiserum against M2BP which did not cross-react with the other ligands. The concentrations of M2BP refer to the monomer size.

(Koths *et al.*, 1993; Ullrich *et al.*, 1994) and indicates that galectin-3 is not involved in these adhesions.

Production by cultured cells and deposition in tissues

Conditioned media from human melanoma (Natali *et al.*, 1982; Inohara and Raz, 1994) and other tumour cell lines (Iacobelli *et al.*, 1986, 1993; Linsley *et al.*, 1986; Rosenberg *et al.*, 1991; Koths *et al.*, 1993) had been a convenient source for the identification and the purification of M2BP. We now used similar media as well as an epithelial cell line and fibroblasts for an analysis by radioimmunoassay. This demonstrated highly variable concentrations (0.2–30 µg/ml) for three melanoma cell lines and amounts at the lower end of this range for a fibrosarcoma and an epithelial cell line. The lowest amounts (0.044 µg/ml) were detected in fibroblast medium. Only 3% of the total M2BP was deposited in the fibroblast cell layer, however, as shown by urea extraction, which solubilizes most of the fibrillar fibronectin (Sasaki *et al.*, 1996).

The binding of the affinity-purified antibody against human M2BP to mouse M2BP was examined by immunoblotting of heart and skeletal muscle extracts (data not shown). This revealed staining of a major 100 kDa and a weaker 28 kDa band with a mobility similar to that of the plasmin fragment Pl-2. The somewhat slower mobility of mouse tissue M2BP compared with the recombinant human protein presumably reflects differences in glycosylation.

Double immunofluorescence staining was used for the localization of M2BP in various frozen tissue sections of the mouse using an affinity-purified rabbit antibody against M2BP and a monoclonal antibody against mouse nidogen

Fig. 5. Dose-response profile of the adhesion of human mammary epithelial HBL-100 cells to M2BP, laminin and perlecan. Wells were coated with different concentrations of M2BP (○), reduced and alkylated M2BP (▽), laminin-1/nidogen complex (△) or perlecan (□), and adherent cells measured by a colorimetric assay.

to stain basement membranes (Figure 7). A similar section was used as a control for the background staining by non-immune rabbit IgG. In the testis, nidogen was restricted to the seminiferous tubular basement membranes and some vessels, while M2BP staining was strongest in the interstitial spaces between individual tubules. Weaker M2BP staining, but still above background, was also present around epithelial and/or Sertoli cells within the

Fig. 6. Inhibition of epidermoid A431 cell adhesion to laminin (closed symbols) and M2BP (open symbols) by antibodies specific for integrin subunits. These monoclonal antibodies specifically block $\beta 1$ (○, ●), $\alpha 6$ (△, ▲) or $\alpha 2$ (□, ■) integrin subunits.

tubules (Figure 7A). A similar distinction between basement membrane staining for nidogen and an interstitial staining for M2BP underneath the basement membrane in a punctate and streak-like fashion was observed for the uterus (not shown). Because of the immunostimulatory property of M2BP (Ullrich *et al.*, 1994), we also used spleen and thymus for immunostaining. In the spleen, restricted regions of strong M2BP staining were observed within the white pulpa (Figure 7B) as identified by a parallel haematoxylin–eosin staining. Similar restricted regions of M2BP deposits were also observed in the thymus cortex and medulla, even though background binding was higher than in other organs (Figure 7C). Nidogen staining in skeletal muscle was confined to the endomysium surrounding individual muscle fibres. Distinct staining for M2BP was again restricted to short streaks within or close to the endomysium (Figure 7D). These streaks occasionally showed a co-localization with bungarotoxin-stained synapses (C.Brakebusch, unpublished). Together, this indicates a preferred pericellular and/or extracellular matrix deposition of M2BP, and similar patterns were also observed for intestinal and dermal tissue sections (data not shown).

Fig. 7. Double immunofluorescence staining for M2BP and nidogen on frozen mouse tissue sections. The tissues used were testis (A), spleen (B), thymus (C) and skeletal muscle (D). Staining was with a rat monoclonal antibody against mouse nidogen and the affinity-purified rabbit antibody against M2BP. Control refers to a similar section treated with normal rabbit IgG for the evaluation of background binding. Bar, 100 μ m.

Discussion

Recombinant human M2BP was obtained as a native protein as shown by CD spectroscopy, electron microscopy, protease resistance and immunological epitope analysis. It also formed large non-covalent oligomers as shown previously for M2BP purified from biological fluids and tumour cell culture medium (Linsley *et al.*, 1986; Iacobelli *et al.*, 1993; Koths *et al.*, 1993). Recombinant M2BP was heavily substituted with ~60 hexosamines but apparently did not contain heparan and chondroitin sulfate. This suggested occupation of all seven N-linked and ~16 O-linked oligosaccharide acceptor sites, in agreement with sequence predictions (Koths *et al.*, 1993; Ullrich *et al.*, 1994). There was a loss of ~30 kDa mass after treatment with N-glycosidase F. Recombinant M2BP was shown to promote cell adhesion and to bind selectively to several collagen types and fibronectin as well as to galectin-3. These properties would suggest that, apart from its presence in biological fluids such as serum and milk, M2BP also has a localization in the extracellular matrix, and this has now been confirmed in tissue studies.

A prominent feature of M2BP is its oligomerization to large ring structures. This unusual self-assembly was detected by electron microscopy of M2BP in the present work. Earlier studies by gel chromatography suggested the presence of aggregates in solution without specifying their geometry. Our own ultracentrifugal data indicate a molar mass of 1000–1500 kDa and a sedimentation coefficient of ~20S.

The circular nature of the self-assembly complexes may be explained by a tilted arrangement of complementary binding sites. The rings show a variation of size centred at diameters of 30–40 nm, indicating some flexibility in the arrangement of the interaction sites at the level of M2BP monomers. Some of the rings are segmented at intervals of ~15 nm and five to eight segments can be distinguished at the circumference of the most abundant rings. The exact number of monomers forming rings of five, six, seven or eight segments will need evaluation by mass determination with scanning transmission electron microscopy (STEM). As a rough estimate, 10–16 monomers of molar mass 92 kDa follow from the ultracentrifugal analysis, suggesting that each segment may be composed of two monomers. Further information on the mode of packing of M2BP monomers in the ring-like complexes may also emerge from studies of the reversible pH-induced dissociation and association reactions and by the possible detection of intermediates of the assembly process. In the course of future work, individual domains of M2BP may also be prepared by recombinant methods in order to localize the sites for self-assembly and interaction with other extracellular matrix ligands.

Such domains are indicated from the sequence of human M2BP (Koths *et al.*, 1993; Ullrich *et al.*, 1994) particularly at the N-terminus (positions 19–125), which contains six cysteines and is homologous to the scavenger receptor cysteine-rich domain (Freeman *et al.*, 1990) shared also by the T-lymphocyte receptors CD5 and CD6 (Jones *et al.*, 1986; Aruffo *et al.*, 1991), the complement factor I (Goldberger *et al.*, 1987) and several more proteins (Aruffo *et al.*, 1991). A protein even more closely related to M2BP both in size and sequence identity (69%) is mouse

cyclophilin C-binding protein (Friedman *et al.*, 1993; Chicheportiche and Vassalli, 1994). A sequence comparison of these two proteins indicates to us three more shared modules, two in the central region (126–280 with six Cys; 281–420, Cys-free) and one at the C-terminus (421–585 with four Cys). The latter apparently comprises the 26 kDa fragment PI-2, which is readily released from M2BP but apparently not further degraded by neutral proteinases (Figure 1). Whether these postulated modules also represent autonomous folding units within these proteins remains to be studied. Equally important is the question as to whether cyclophilin C-binding protein also self-assembles into ring-like structures.

Northern blot analyses have demonstrated a moderate to strong expression of M2BP in a large number of normal tissues (Ullrich *et al.*, 1994). Those which contribute to the levels of M2BP observed in the circulation have not yet been identified. The widespread mRNA expression agrees with our immunofluorescence data, which detected M2BP in all normal tissues analysed so far. The data together strongly indicate local production, particularly since extravasation as an origin of interstitial M2BP seems very unlikely considering its size. The staining patterns also strongly suggest a restricted extracellular deposition of M2BP, but in some cell-rich tissues such as testis, spleen and thymus there is also a close pericellular localization. The demonstration of M2BP in the latter two tissues was of particular interest considering the *in vitro* evidence that M2BP is a potent immune stimulator (Ullrich *et al.*, 1994). The restricted nest-like appearance of M2BP in the spleen is reminiscent of germinal centres of clonal immune cell expansion and deserves a more detailed study. Most of the basement membranes, as revealed by double staining with nidogen, seem to contain no, or only little, M2BP. Yet, the precise association of M2BP with specific supramolecular structures of the extracellular matrix remains to be identified by immunoelectron microscopy. Based on our binding studies with extracellular ligands, such structures could include microfibrils formed from collagen VI (Timpl and Chu, 1994) or fibronectin (Hynes, 1990) but would not exclude other structures. Cultured fibroblasts are known to elaborate a dense meshwork of fibronectin fibrils but secrete and deposit M2BP, as shown here, in >10-fold lower amounts compared with fibronectin (Sasaki *et al.*, 1996). This suggests that M2BP is not an essential structural element of such microfibrils but may use them for extracellular storage.

M2BP was hitherto considered to promote cell–cell contacts through cross-linking of surface-bound galectin-3 (Inohara *et al.*, 1996). Our observation of the strong promotion of cell adhesion and spreading by M2BP, which seems to be mediated by β 1 integrins and is independent of galectin-3, adds a new perspective to its potential cellular interactions. This could include the promotion of firm cell anchorage within particular extracellular matrices as well as the action as a cytokine that stimulates the activity of natural killer cells (Ullrich *et al.*, 1994). The latter interpretation would agree with other observations that many identified cytokines are stored in the extracellular matrix which controls their activity, stability and release (Taipale and Keski-Oja, 1997). We therefore consider that our recombinant analysis has revealed not only a unique ring-like self-assembly, but also several new biological

implications for M2BP functions worthy of further examination.

Materials and methods

Recombinant production and purification

An expression plasmid for full-length human M2BP in the pCMV vector (Ullrich *et al.*, 1994) was linearized with *Bam*H1, blunt-ended by filling in the 3'-recessed end with Klenow fragment of DNA polymerase I and then digested with *Xho*I. A 2.3 kb *Bam*H1-*Xho*I fragment containing the whole coding and some 5' and 3' untranslated regions of M2BP was gel purified and inserted into the episomal expression vector pCEP-Pu (Kohfeldt *et al.*, 1997) which was digested with *Pvu*II and *Xho*I. Human embryonic kidney cells which express the EBNA-1 protein of Epstein-Barr virus (EBNA-293, Invitrogen) were used for transfections and transfectants were selected with 0.5 µg/ml puromycin. Serum-free medium collected from cultures was stored at -20°C after adding EDTA and Pefabloc (Boehringer Mannheim) to 1 and 0.4 mM, respectively. M2BP was precipitated from this medium (1 litre) by adding solid ammonium sulphate to 50% saturation (8 h at 4°C). The precipitate was dissolved in 12 ml 0.05M Tris-HCl pH 7.4 and 0.15 M NaCl containing protease inhibitors, and passed over a Sepharose CL-6B column (3×130 cm) equilibrated in the same buffer (Paulsson *et al.*, 1987a). Pooled fractions were then concentrated with Amicon, dialysed against 0.2 M NH₄HCO₃ containing protease inhibitors and stored in small aliquots at -20 or -80°C.

Sources of proteins

Collagens I, III, IV, V and VI were solubilized from human placenta by pepsin and purified following established protocols (Miller and Rhodes, 1982; Odermatt *et al.*, 1983; Vandenberg *et al.*, 1991). Laminin-1 and perlecan were obtained from a mouse tumour (Paulsson *et al.*, 1987a,b). Recombinant mouse nidogen (Fox *et al.*, 1991), fibulin-1 (Sasaki *et al.*, 1995), fibulin-2 (Pan *et al.*, 1993) and human BM-40 (Nischt *et al.*, 1991) were as described previously. Human fibronectin was obtained from Behringwerke, Marburg. Recombinant galectin-3, based on a previously published expression vector (Agrwal *et al.*, 1993), was a kind gift of H.Gabius, Munich.

Sources of antibodies and immunological assays

Monoclonal antibody against human (AII2; Hall *et al.*, 1990) β1 integrin was kindly supplied by C.Damsky. Monoclonal antibodies against α6 integrin (GoH3) were a gift of A.Sonnenberg and those against α2 integrin (Gi9) were purchased (Biomol, Hamburg). Production of rabbit antisera against recombinant M2BP, affinity purification of antibodies and radioimmunoassays followed standard protocols (Timpl, 1982). After elution from a M2BP column, the antibody was further purified on a Superose 12 column. Immunoblotting of tissue extracts and indirect immunofluorescence followed previously described procedures (Pan *et al.*, 1993; Sasaki *et al.*, 1996). In immunofluorescence, the purified antibody and normal rabbit IgG as negative control were used at the same concentrations (5 µg/ml). Double staining was performed with monoclonal antibody BE 1/4 against mouse nidogen (Dziadek *et al.*, 1988).

Enzymatic and chemical modification

Complete reduction and alkylation of disulphide bonds in 6 M guanidine was done as described (Paulsson *et al.*, 1987b). Treatments with heparitinase I and chondroitinase ABC followed a previously used protocol (Costell *et al.*, 1997). Treatment of 20 µg M2BP with 10 units N-glycosidase F (Boehringer Mannheim) was done at 37°C for 18 h in 20 mM phosphate buffer, pH 7.4, containing 0.5% Nonidet P-40 and 0.1% SDS, after denaturation by heating at 97°C (5 min) in the presence of 1% SDS and 1% 2-mercaptoethanol. Proteolytic digestions (enzyme:substrate ratio, 1:100) were for 4 h at 37°C.

Physical and electron microscopic methods

For analytical ultracentrifugation, an Optima XL-A (Beckmann Instruments) with an AnS rotor and 12 nm filled Epon cells was employed. Sedimentation coefficients (*s*_{20,w}) were evaluated from sedimentation velocity experiments at a rotor speed of 52 000 r.p.m. and molecular masses (M) from sedimentation equilibrium experiments at 5200 r.p.m. Measurements were performed in phosphate-buffered saline (PBS; 0.15 M NaCl, pH 7.4) or 0.1 M acetic acid at 18°C, and data evaluation was by standard methods assuming a partial specific volume of

0.697 ml/g, which is the calculated value for a protein with 30% weight glycosylation (Ralston, 1993).

Circular dichroism spectra were recorded on a Cary 61 spectropolarimeter (Varian, Zug, Switzerland) using a thermostatted cell of 0.1 mm path length (Hellma, Müllheim, Germany). Measurements were performed in PBS or 0.1 M acetic acid. The molar ellipticity (in deg-cm²/dmol) was calculated on the basis of a mean residue molecular mass of 110 Da.

Electron microscopy was performed by the rotary shadowing technique as described (Engel, 1994). Protein (25–50 µg/ml) in 0.2 M ammonium bicarbonate, pH 7.4, or 0.1 M acetic acid was mixed with an equal volume of glycerol and sprayed onto freshly cleaved mica discs. These were dried in high vacuum, shadowed with platinum/carbon at an angle of 9° and replicated. Negative staining was performed at neutral pH in order to avoid dissociation of aggregates by the acid pH in the routine procedure (Engel, 1994). Ten µl of a solution in PBS was put on a glow discharged collodium and carbon grid and 5 µl of a 2% sodium phosphotungstate solution of pH 7 was added. After removal of the first stain, incubation was repeated for 2 min.

Protein binding and cell adhesion assays and biological samples

A solid-phase assay with plastic-immobilized ligands and soluble M2BP followed a previous procedure (Aumailley *et al.*, 1989a). The cell adhesion assay as well as the cell lines used have been described previously (Aumailley *et al.*, 1989b; Battaglia *et al.*, 1993). For inhibition assays, cells were mixed with various dilutions of inhibitors before being added to the substrate. Cell culture and collection of serum-free medium followed standard protocols. Two melanoma cell media were kindly supplied by R.Nischt. Tissue extracts of 5- to 7-week-old Balb/c mice were obtained as described previously (Sasaki *et al.*, 1996).

Analytical methods

Samples were hydrolysed with 6 or 3 M HCl (110°C, 16 h) for the determination of protein concentrations or hexosamine compositions, respectively, on a LC 3000 analyser (Biotronik). SDS gel electrophoresis followed standard protocols. Edman degradation of electroblotted proteins was performed on a Procise sequencer (Applied Biosystem) according to the manufacturer's instructions.

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