Mac-2-binding protein (M2BP) is a secreted glycoprotein suggested to have a role in host defense. It forms linear and ring-shaped oligomers, with each ring segment being composed of two monomers. We have produced recombinant human M2BP fragments comprising domains 1 and 2 (M2BP-1,2) and domains 3 and 4 (M2BP-3,4) in 293 human kidney cells to characterize structural and functional properties of M2BP. Both fragments were obtained in a native and glycosylated form, as analyzed by CD spectroscopy, trypsin susceptibility, and enzymatic deglycosylation. These results strongly suggest that both fragments are autonomous folding units. All three potential N-glycosylation sites in M2BP-1,2 and all four in M2BP-3,4 were found to be occupied. M2BP-1,2 expressed in tunicamycin-treated cells contained no glycosyl residues, indicating that O-glycosylation is not occurring. Ultracentrifugation revealed that M2BP-1,2 is homogeneously dimeric in the nanomolar range reflecting the properties of intact M2BP. Domain 2 (BTB/POZ domain) is thus identified as the dimerization domain of M2BP, because it has been formerly shown that recombinant domain 1 is monomeric. M2BP-3,4 showed a concentration-dependent self-association, and aggregates of different size and shape were shown by electron microscopy. In contrast to this irregular aggregation of M2BP-3,4, it has been formerly shown that a fragment comprising domains 2–4 still has the ability to form ring-like structures, although the rings are protein-filled, and thus domain 2 appears to be indispensable for ring formation. Solid phase assays showed that M2BP-3,4 contains binding sites for galectin-3, nidogen, and collagens V and VI, whereas M2BP-1,2 is inactive in binding. Both fragments showed no cell adhesive activity in contrast to native M2BP, suggesting that a concerted binding action and/or multivalent interactions of rings are necessary for cell attachment.

Mac-2-binding protein (M2BP) is a secreted glycoprotein present in the extracellular matrix of several tissues (1) and in extracellular fluids such as serum and milk (2). Elevated levels are observed in some tumors and viral infections (3, 4). In vitro, human M2BP induces production of interleukin-1, interleukin-6, and other cytokines by blood monocytes and stimulates natural killer cell and lymphokine-activated killer cell activity (5, 6). Mouse cyclophilin C-associated protein is 69% identical to M2BP, and it seems likely that the two proteins are functional homologues. Gene-targeted cyclophilin C-associated protein-deficient mice are viable but show an up-regulation of the endotoxin and proinflammatory response (7).

M2BP is extensively glycosylated and interacts with galectin-3 (former name Mac-2), and it also interacts with other extracellular proteins such as collagens IV, V, and VI, fibronectin, and nidogen (1, 2, 8, 9). M2BP binds to galectin-3 on the cell surface and induces homotypic cell aggregation (10). β2-Integrin-mediated cell adhesion to M2BP was also demonstrated (1).

Tissue extracted and recombinant M2BP form linear and ring-shaped oligomers. Investigation by scanning transmission electron microscopy showed that the ring oligomers are comprised of ~14-nm-long segments composed of two 92-kDa M2BP monomers (11). Although the rings vary in size, decamers predominate. The various linear oligomers and dimers are probably ring precursors. It is hypothesized that the multivalency of M2BP provided by its assembly to ring-like structures is of decisive importance for the linkage of different components and for an increase in binding activity.

M2BP consists of 567 residues after cleavage of the signal peptide (2, 5). Four putative domains could be distinguished in its cDNA sequence (11). Domain 1 at the N terminus is a member of the scavenger receptor cysteine-rich domain family (2, 5), and its crystal structure has recently been solved (12). Domains 2 and 3 are putative members of the BTB (broad complex, tramtrack, and bric-a-brac)/POZ (poxvirus and zinc finger) and IVR domain family, respectively. In several proteins, BTB/POZ domains have been shown to mediate homodimerization or multimerization (see Ref. 13 for review). No convincing similarity with other proteins was detected for the C-terminal putative domain 4. Recombinant domain 1 (M2BP-1) is monomeric and is inactive in binding to extracellular matrix ligands and in cell attachment. A fragment consisting of putative domains 2–4 (M2BP-2,3,4) aggregates to heterogeneous, protein-filled ring-like structures and retains the potential for binding to extracellular matrix ligands and for cell adhesion (11).

In the present study we have produced recombinant M2BP fragments consisting of domains 1 and 2 (M2BP-1,2) and domains 3 and 4 (M2BP-3,4) to further characterize the function of the different domains. Both fragments were obtained in native and glycosylated forms, suggesting that they are autonomous folding units. All putative N-glycosylation sites are oc-
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cuped, but there are some modifications in the glycan moieties compared with the parent protein. We show that domain 2 (BTB/POZ domain) is the dimerization domain of M2BP. M2BP-3,4 shows self-association but cannot form ring structures in the absence of domain 2. Binding studies in vitro demonstrated binding sites for extracellular matrix ligands in M2BP-3,4, in contrast to M2BP-1,2. Both fragments are inactive in cell attachment, suggesting that concerted action of several binding sites in both fragments and/or multivalent interactions of rings is necessary for cell adhesion.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant M2BP Fragments—The full-length human M2BP cDNA sequence in the pCEP-Pu vector (1) was used as a PCR template to generate the cDNA constructs encoding human M2BP-1,2 and M2BP-3,4. Oligonucleotide primers corresponding to the domain borders 19 and 254 for M2BP-1,2 and 250 and 585 for M2BP-3,4 were used. Primers were constructed to code for a N-terminal His6 tag and a thrombin cleavage site. Primers used for the M2BP-1,2 protein were GATCGCTAGCTCACCATCATCACCATACGTGGTC-CGGTGGTCTGGAAGGACTGACGCGG and GATCGGATC-CTTGGCGGAGAGGAGATGGG for the M2’ and 3’ ends, respectively. For the M2BP-3,4 protein were GATGCGTACCT-CACACTCATACACTCAGCTGGTGTCGAGGCTC-GAGGACCCTCC and GATCGGATCCTGATGTACACCTGAGGAGT-TGTCG for the M’ and 3’ ends, respectively. Sequences were verified by dye terminator cycle sequencing. They were inserted into the episomal expression vector pCEP-Pu (14) in frame to the BM-40 signal peptide and used for the episomal transfection of human embryonic kidney cells (EBNA-293 cells). Transfectants were selected with 2–10 μg/ml puromycin. Serum-free medium collected from cultures was centrifuged at 2500 × g for 10 min and stored at −20 °C. To the harvested medium 0.05 M HCl, pH 7.9 (final concentration, 20 μM), and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, 1 μg/ml pepstatin, 5 μM EDTA (final concentrations)) were added, and the medium was passed through a 0.45-μm cellulose acetate filter. The solution was dialyzed against 20 mM Tris-HCl, pH 7.9, 1 mM EDTA and passed over a column of DEAE-PhaseL (Amer sham Biosciences). The protein was eluted with 500 mM NaCl and dialyzed against 20 mM Tris-HCl, 50 mM NaCl, 5 mM imidazole, pH 7.9. The proteins were further purified by nickel-Sepharose chromatography (Amer sham Biosciences) and dialyzed against 20 mM Tris-HCl, pH 7.5, or 20 mM sodium phosphate, pH 7.2. Removal of the His tag by treatment with thrombin was not successful. In the case of M2BP-1,2, which is stable to limited trypsin digestion, the His tag was cleaved with trypsin (5 units/μmol of recombinant protein) for 30 min at room temperature. Trypsin and the cleaved His tag fragments were removed by passing the solution over a column of aprotinin-Sepharose (Sigma) and nickel-Sepharose, respectively.

Inhibition of Glycosylation—Transfected cells were incubated in serum-free medium containing 0.1–10 μg of tunicamycin/ml of medium at 37 °C for 36 h before analysis of the expressed recombinant proteins. For protein purification, the cells were incubated in the presence of 0.5 μg/ml tunicamycin and 1 μg/ml of neomycin for 5 min at 95 °C. The reaction products were analyzed by Tricine-SDS-PAGE (15) and stained with silver.

Analytical Methods—The samples were hydrolyzed with 3 μl HCl (110 °C, 16 h) for the determination of hexosamine compositions on a LC 3000 analyzer (Biotronik). Mass spectral data were acquired between 200 and 2000 Da on a Finnigan TSQ7000 mass spectrometer (Finnigan, San Jose, CA) set at single unit resolution; the molecular masses of the proteins were calculated from the raw spectra with the biochemistry applications software provided by the manufacturer. Protein concentrations of M2BP-1,2 and M2BP-3,4 were determined spectrophotometrically by absorbance at 280 nm, using molar extinction coefficients of 30,075 and 80,580 M cm2/dmol. The Sedimentation equilibrium experiments were performed at 20 °C. The molecular masses were calculated from sedimentation equilibrium runs using a floating base-line computer program that adjusts the baseline absorbance to obtain the best linear fit of A versus r2 (A = absorbance from the rotor axis). Partial specific volume of 0.73 cm3/g for nonglycosylated M2BP-1,2 and 0.70 cm3/g for glycosylated M2BP-1,2 and M2BP-3,4 were used for the calculations. The latter value is calculated for proteins with 30% weight glycosylation (16). The Sedimentation coefficients were corrected to standard conditions (water at 20 °C).

RESULTS

M2BP-1,2 and M2BP-3,4: Expression and Investigation of the Oligosaccharide Moieties—Fragments consisting of domains 1 and 2 (M2BP-1,2; residues 19–254) and of domains 3 and 4 (M2BP-3,4; residues 255–585) were produced for the predicted domain boundaries. Full-length M2BP (11) cDNAs were produced by PCR amplification with a code for a N-terminal His tag. Episomal expression vectors containing the BM-40 signal peptide sequence were constructed to express the recombinant proteins in EBNA-293 kidney cells. Both fragments were secreted and detectable by SDS-PAGE (Fig. 1A). A polyclonal antiserum against recombinant full-length M2BP (1) specifically recognized both M2BP fragments in crude culture medium (Fig. 1B). Column chromatography on DEAE-PhaseL and nickel-Sepharose resulted in homogeneous pro-
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Fig. 1. Expression of M2BP-1,2 and M2BP-3,4 in EBNA-293 cells and purification of the recombinant proteins. A and B, aliquots of serum-free culture medium of the cells were chromatographed on 12.5% SDS-polyacrylamide gels. Either the gels were silver-stained, or proteins from the gels were blotted onto nitrocellulose and probed with antibodies against recombinant M2BP (B). Lane 1, secreted proteins from medium of nontransfected cells (negative control); lane 2, medium of cells transfected with cDNA encoding full-length M2BP (positive control); lane 3, medium of cells transfected with cDNA encoding M2BP-1,2; lane 4, medium of cells transfected with cDNA encoding M2BP-3,4. C, recombinant His$_6$-tagged M2BP fragments from serum-free medium were isolated by DEAE-Sepharose chromatography. 1 µg of M2BP-1,2 (lane 1) or M2BP-3,4 (lane 2) were analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels and with silver staining.

Proteins (Fig. 1C) with yields of 10–30 µg/ml of culture medium. The apparent molecular mass of the proteins in SDS-polyacrylamide gels (Fig. 1) was larger than the one calculated from the protein sequence (35–42 kDa as compared with 27.4 kDa for M2BP-1,2 and 50–60 kDa as compared with 40.0 kDa for M2BP-3,4), indicating glycosylation. In addition, the proteins appeared as diffuse bands rather than as sharp bands in the gels, indicating heterogeneity in the oligosaccharide moieties. Hexosamine analyses demonstrated on average 12 residues of glucosamine per M2BP-1,2 monomer and 19 residues of glucosamine and 1 residue of galactosamine per M2BP-3,4 monomer. Fig. 2A shows that treatment of the purified proteins in their denatured form with neuraminidase resulted in small decreases of apparent molar mass (Fig. 2A, lanes 2). By treatment with N-glycosidase F shifts were much larger, and sharp protein bands were obtained, indicating apparent molecular masses close to the calculated ones (Fig. 2A, lanes 3). No further shift in mobility was observed after including O-glycosidase in the digestion mix (data not shown).

Treatment of M2BP-1,2 expressing EBNA-293 cells with tunicamycin, which inhibits N-glycosylation, resulted in a size reduction of the secreted M2BP-1,2 (Fig. 2B) very similar to that of the purified protein after digestion with N-glycosidase F (Fig. 2A, left lane 3). 0.1 µg/ml of tunicamycin resulted in a partial inhibition of N-glycosylation (Fig. 2B, lane 2), whereas 1 µg/ml or higher concentrations of tunicamycin inhibited N-glycosylation completely (Fig. 2B, lanes 3 and 4). Application of this method to M2BP-3,4 was not successful, and addition of tunicamycin stopped expression (data not shown), probably because of misfolding and degradation.

M2BP-1,2 and M2BP-3,4 contain three and four potential N-glycosylation sites, respectively (2, 5). To investigate whether all of these sites are glycosylated, native M2BP fragments were partially deglycosylated with a glycosidase mixture containing neuraminidase, N-glycosidase F, and endoglycosidase F (Fig. 3). This experiment resulted in two and three intermediate protein bands for M2BP-1,2 and M2BP-3,4, respectively (Fig. 3, lanes 3), in contrast to treatment with neuraminidase only (Fig. 3, lanes 2) and with quantitative cleavage of all N-linked glycans by deglycosylation of the proteins in their denatured form (Fig. 3, lanes 4). The stepwise removal of N-glycans suggests that all three potential N-glycosylation sites in M2BP-1,2 and all four sites in M2BP-3,4 are occupied.

Stability and Secondary Structure of M2BP-1,2 and M2BP-3,4—Resistance to protease digestion of correctly folded domains is a common probe for proper folding of recombinant fragments (21, 22). After trypsin incubation (5 units of trypsin/µmol of recombinant protein), M2BP-1,2 undergoes only a very slight reduction of its molecular size in SDS-PAGE (data not shown), which is due to the cleavage of the N-terminal His$_6$ tag at the thrombin cleavage site that was introduced between the His$_6$ tag and the M2BP-1,2 protein sequence (removal of the tag by treatment with thrombin turned out to be unsuccessful).
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Microsequencing showed that the cleaved protein started with the N-terminal sequence GSVND, with the GS being derived from the foreign thrombin cleavage region. M2BP-1,2 from tunicamycin-treated EBNA-293 cells, which contain no N-glycans, was also stable to trypsin digestion, but 10-fold less trypsin was required for cleavage of the His tag. Stability to limited proteolysis of the latter protein indicates that the structural organization in M2BP-1,2 is responsible for this property rather than a protection effect by the glycan moieties. This protein was analyzed by electrospray ionization-mass spectrometry. The molecular mass before cleavage of the His tag was 27,412.0 Da (calculated mass of the nonreduced protein, 27,416.8 Da) and after cleavage of the His tag was 25,769.0 Da (calculated mass of the nonreduced protein, 25,775.9 Da). The agreement between measured and calculated molar masses shows that the protein does not contain any glycosyl residues. The result also proves that the protein is not O-glycosylated in EBNA-293 cells nor does it contain any other posttranslational modification.

Treatment of M2BP-3,4 with trypsin completely converted the protein into two bands of 27 and 35 kDa in SDS-PAGE (data not shown). N-terminal sequencing revealed the two sequences APLAH and GSLLP for the 35-kDa band and the sequences APLAH and GSLLP for the 27-kDa band. The 35-kDa band thus consists of the N-terminal domain 3 with and without His tag, whereas the 27-kDa band consists of the C-terminal domain 4 starting at position 442 of native M2BP. This result is consistent with the finding of several protease cleavage sites within the putative linker region between domains 3 and 4, which have been demonstrated with plasmin and a not identified endogenous protease from human breast carcinoma cells SK-BR-3 (1, 2). Plasmin has been shown to cleave full-length M2BP from EBNA-293 cells into a 67-kDa N-terminal fragment and the 26-kDa C-terminal domain 4 starting at positions 442 and 455 (1). The former N terminus is the same as the one of domain 4 released from M2BP-3,4 by trypsin. Full-length M2BP from SK-BR-3 cells has been shown to be cleaved by an endogenous protease into a 70-kDa N-terminal fragment and the 27-kDa C-terminal domain 4 starting at position 437 (2).

The conformational state of M2BP-1,2 and M2BP-3,4 was analyzed by circular dichroism (Fig. 4). The CD spectrum of M2BP-1,2 shows a dichroic minimum at 209 nm. A nearly identical spectrum was obtained for unglycosylated M2BP-1,2 from tunicamycin-treated cells (data not shown). M2BP-3,4 showed dichroic minima at 209 and 222 nm. The CD spectra demonstrate the presence of α-helical and β-structures in both proteins but with a higher α-helical content in M2BP-3,4. The spectra thus indicate that the proteins are in a native state. An analysis of different proportions of secondary structure appeared to be of little use because both proteins may consist of two rather different domains.

Oligomeric Structures of M2BP-1,2 and M2BP-3,4—Analytical ultracentrifugation was used for the analysis of the oligomeric state of the recombinant proteins (Table I). M2BP-1,2 sedimented with a single sharp profile and a sedimentation coefficient of 4.3 S, and sedimentation equilibrium yielded a molecular mass of 70 kDa. The data indicate a dimeric state of glycosylated M2BP-1,2, which runs on SDS gels with a molecular mass of 35–42 kDa in the monomeric form (Fig. 1C). The frictional ratio $f/f_0 = 1.2$ suggests a globular shape of the dimer, which can be approximated by an ellipsoid of revolution with an axial ratio of 2. Sedimentation equilibrium experiments were performed in a broad range of protein concentrations (3–15 μM), but even at the lowest concentrations near the meniscus of the cells (1 μM) no indication for dissociation of dimers into monomers was detectable. The dissociation equilibrium constant may therefore be estimated to be lower than 100 nM. Strong dimer formation of M2BP-1,2 was confirmed for the unglycosylated form from tunicamycin-treated cells. The sedimentation coefficient was 3.3 S, and equilibrium experiments at 10, 20, and 40 μM yielded a molar mass of 57 kDa. The calculated molar mass of the unglycosylated monomer is 25.8 kDa.

In contrast to M2BP-1,2, M2BP-3,4 forms a heterogeneous distribution of aggregates (Table I). At a concentration of 0.9 mg/ml (23 μM) in 20 mM potassium phosphate, pH 7.2, several sedimentation coefficients between 6 and 11 S were observed. The molecular mass was in the range between 200 and 400 kDa, indicating about 3–8 monomers/aggregate (glycosylated M2BP-3,4 runs on SDS gels with a molecular mass of 50–60 kDa (Fig. 1C)). The aggregation of M2BP-3,4 was found to increase with increasing protein and salt concentration (Fig. 5).

We also investigated the aggregation state of trypsin-digested M2BP-3,4, which consists of the single domains 3 and 4 to possibly separate and characterize the two domains. However, analytical ultracentrifugation experiments revealed that the trypsin digest is still aggregated (data not shown).

The nature of both proteins was also investigated by electron microscopy (Fig. 6). Rotary shadowing of M2BP-1,2 and unglycosylated M2BP-1,2 revealed particles of a homogeneous size (Fig. 6, A and B). Adsorption to the mica surface occurred from an about 1 μM protein solution, at which, according to ultracentrifugal analysis, only dimers are present. The size and shape of the dimers is in agreement with this prediction. Horsehoe-like shapes can sometimes be identified (Fig. 6A). The open ends of this structure may be formed by the noninteracting domains 1 according to the ring model of M2BP (11). M2BP-3,4 shows particles of variable sizes and thus a tendency for aggregation (Fig. 6C), as expected from the ultracentrifugal results.

Binding of M2BP-1,2 and M2BP-3,4 to Extracellular Matrix Ligands—A solid phase assay was used to examine the binding potential of various plastic-immobilized extracellular matrix proteins to soluble M2BP-1,2 and M2BP-3,4 in comparison with native M2BP (Fig. 7). All matrix proteins that were tested showed no or only weak binding to M2BP-1,2. Galectin-3 was the strongest ligand for M2BP-3,4, with half-maximal binding achieved at $−2.5 \mu \text{g} / \text{ml} (−60 \text{ nM})$. The binding to native M2BP was even stronger, with half-maximal binding at $−0.1 \mu \text{g} / \text{ml} (−1.5 \text{ nM})$. No substantial binding of fibronectin to M2BP-3,4 was observed, whereas half-maximal binding to native M2BP...
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TABLE I
Sedimentation coefficients (s_{20, w}) and average molecular masses of M2BP-1,2 and M2BP-3,4 as determined by analytical ultracentrifugation
M2BP-1,2 and M2BP-3,4 were analyzed in 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 20 mM potassium phosphate, pH 7.2, respectively.

| Protein                  | Concentration | s_{20, w} | Molecular mass |
|--------------------------|---------------|-----------|---------------|
| M2BP-1,2 (glycosylated)  | 400 µg/ml     | 4.3       | 25.8          |
| M2BP-1,2 (nonglycosylated)| 500 µg/ml    | 3.3       | 25.8          |
| M2BP-3,4 (glycosylated)  | 900 µg/ml     | 6.11      | 40.0          |
| M2BP-1,2 (nonglycosylated)| 120 µg/ml    |           |               |

a Molecular mass of the monomer based on its amino acid sequence.

b Extrapolated to zero concentration.

FIG. 5. Self-association of M2BP-3,4 monitored by sedimentation equilibrium experiments at different ionic strength. The change of the molecular masses in dependence of the total monomer concentration is shown. M2BP-3,4 was measured in 20 mM potassium phosphate, pH 7.2, with ○ or without ● 120 mM NaCl. The position of the monomer is indicated.

was at \( \sim 1 \) µg/ml (\( \sim 15 \) nM). The basement membrane protein nidogen showed half-maximal binding to M2BP-3,4 at \( \sim 40 \) µg/ml (\( \sim 1 \) µM) and to native M2BP at \( \sim 6 \) µg/ml (\( \sim 95 \) nM). The binding activity of collagens V and VI to M2BP-3,4 was distinctly lower than to native M2BP. Half-maximal binding to the latter was at \( \sim 1.5 \) µg/ml (\( \sim 25 \) nM) in case of both collagens. When used as a plastic-immobilized substrate, both M2BP-1,2 and M2BP-3,4 showed no cell adhesive activity for human HBL-100 and rat Rugli glioma cells (data not shown), in contrast to native M2BP and M2BP-2,3,4 as shown previously (1, 11).

DISCUSSION

For a mapping of functional sites in multidomain proteins, expression of individual domains or domain pairs is a very fruitful approach that was applied to laminin, fibronectin, and many other extracellular matrix proteins. For M2BP, domain 1 and a fragment comprising domains 2–4 were already investigated (1, 11), but a more complete picture was only obtained with domain pair 1 and 2 and domain pair 3 and 4 in the present study. With the exception of domain 2, we were not successful in expressing other single domains (domains 3 and 4) or domain combinations (M2BP-2,3 and M2BP-1,2,3), probably because of misfolding in the ER and subsequent degradation. In all of the unsuccessful cases one of the two domain boundaries selected for cloning was between domains 3 and 4, where a putative linker region has been identified (11). M2BP-2 could only be expressed in low amounts and as a mixture of proteins that differed in their N termini and glycosylation status. Electron microscopy showed that this protein mixture had a high aggregation potential, and it was therefore not characterized further.

Recombinant M2BP-1,2 and M2BP-3,4 were obtained as autonomously folding proteins as shown by CD spectroscopy and protease resistance. This result suggests that a connection between domains 2 and 3 is not necessary for correct folding of M2BP. It also demonstrates that the domain boundary between domains 2 and 3 was predicted correctly (11). Trypsin susceptibility experiments with M2BP-3,4 revealed a trypsin cleavage site within the putative linker region, where cleavage sites for plasmin and an endogenous protease of SK-BR-3 cells have already been localized (1, 2). Trypsin cleaved M2BP-3,4 into domains 3 and 4, which were stable to limited proteolysis. It is not yet clear why expression of M2BP constructs with a domain boundary in this region was not successful. A possible explanation is that both domains are needed for proper folding with each becoming stable against proteolysis.

We showed that folding of M2BP-1,2 is not prevented in the absence of glycosylation. Treatment of M2BP-1,2 expressing EBNA-293 cells with tunicamycin resulted in the production and secretion of unglycosylated M2BP-1,2 in high amounts similar to the glycosylated one. The CD spectra and the resistance to trypsin were very similar for both proteins. In contrast, M2BP-3,4 was not secreted in the presence of tunicamycin, probably because of misfolding.

M2BP-1,2 and M2BP-3,4 were substituted with \( \sim 12 \) and \( \sim 20 \) hexosamines, respectively. This suggested occupation of all three and four N-linked oligosaccharide acceptor sites, respectively. By partial removal of the N-linked glycans we have shown that this is indeed the case. This result proved the previous suggestion that in recombinant full-length M2BP, all seven N-linked oligosaccharide acceptor sites are occupied (1). The almost complete absence of galactosamine residues in both M2BP-1,2 and M2BP-3,4 suggests that O-glycosylation plays a minor role. This result is consistent with our finding that after treatment with N-glycosidase F the molecular mass of both fragments is reduced to values that are very close to the predicted mass of the nonglycosylated fragments. Mass analysis of M2BP-1,2 from tunicamycin-treated cells indeed showed that the protein is completely unglycosylated, i.e. there is no O-glycosylation occurring at all. The demonstration of terminal neuraminic acid residues in the oligosaccharide chains by a small increase of electrophoretic mobility in SDS-PAGE of both M2BP-1,2 and M2BP-3,4 after treatment with neuraminidase points to the presence of N-linked oligosaccharide chains of the complex type.

Analytical ultracentrifugation showed that M2BP-1,2 forms dimers already in the nanomolar range. The dimers were the only species detectable by ultracentrifugation, and the homogeneity was confirmed by electron microscopy. Dimerization is very strong (\( \sim 100 \) nM), and dissociation into monomers could experimentally not be achieved. Dimerization of M2BP-1,2 points to domain 2 (BTB/POZ domain) as the dimer-
The visualization domain of M2BP, because it has been previously shown that recombinantly expressed domain 1 (M2BP-1) is monomeric (11). The observation of dimer formation by M2BP-1,2 is in complete agreement with the strong dimerization tendency of many other proteins containing BTB/POZ domains (reviewed by Aravind and Koonin (13)). The crystal structure of the BTB/POZ domain from the promyelocytic leukemia zinc finger protein revealed a dimer stabilized by an extensive hydrophobic interface (23, 24), a feature typical of obligate dimers. In agreement with this structural evidence, unfolding studies and mutagenesis studies showed that dimerization of the BTB/POZ domain is essential for proper folding (25, 26). Native M2BP exists only in dimeric form because of the putative obligate dimerization of domain 2. This prediction is consistent with mass determinations by scanning transmission electron microscopy, which have shown that the ring segments of recombinant M2BP consist of dimers and that most of the linear oligomers exhibit masses of an even number of monomers (11).
Monomers are apparently only present under strongly denaturing conditions (1).

Unglycosylated M2BP-1,2 from tunicamycin-treated cells was also homogeneously dimeric. Thus the N-linked oligosaccharide structures of M2BP-1,2 are necessary neither for proper folding nor for proper oligomerization.

We have made attempts to crystallize M2BP-1,2 to get a crystal structure of an extracellular BTB/POZ domain. However, N-glycosylated M2BP-1,2 did not form crystals, obviously because of the high oligosaccharide content of this protein. Therefore we now try to crystallize unglycosylated M2BP-1,2 from tunicamycin-treated cells because it has a secondary structure and an oligomeric state indistinguishable from the glycosylated protein.

Analytical ultracentrifugation experiments showed that M2BP-3,4 self-associates in dependence of the protein concentration. No specific oligomeric state was reached within the concentration range used (3–23 μM). Self-association was also dependent on the salt concentration of the buffer. A mixture of aggregates with different sizes and shapes was shown by electron microscopy. It has been previously shown that a M2BP fragment comprising domains 2–4 (M2BP-2,3,4) still has the ability to form rings, although its association is less specific, and the rings were found to be filled with protein (11). Because M2BP-3,4 is not forming ring-like structures, we conclude that domain 2 is indispensable for ring formation. This is in agreement with a previously published model of the rings in which each ring unit must be a dimer (11).

Dimers are formed by the interactions between BTB/POZ domains 2. The scavenger receptor cysteine-rich domain 1 does not directly participate in ring formation, and dimers interact end-to-end via domains 2 and 4. We were not able to show an interaction of M2BP-1,2 with M2BP-3,4 in solid phase and surface plasmon resonance assays (data not shown), which would be expected according to the model. However, this result can be explained by the irregular aggregation of M2BP-3,4.

Previous studies showed that native M2BP and M2BP-2,3,4 bind to several collagen types, fibronectin, and nidogen, as well as to galectin-3. The binding activity of the parent protein and the fragment are similar, and consistently domain 1 was inactive (1, 11). We now have further restricted the binding activities to domains 3 and 4. The binding activity of intact M2BP and M2BP-3,4 was most similar in the case of binding to nidogen. M2BP-3,4, which was used as soluble ligand in the solid phase assays, reached half-maximal binding at a ~7-fold higher protein concentration compared with intact M2BP. In the case of binding to galectin-3, the difference in protein concentration to reach half-maximal binding was ~25-fold, and in the case of binding to collagens V and VI, it was even higher. M2BP-3,4 showed almost no binding activity to fibronectin, whereas M2BP achieved half-maximal binding at ~15 nm. The differences in the binding activity to galectin-3 may be explained by different oligosaccharide modification within domains 2–4 in M2BP-1,2 and M2BP-3,4 on one hand and M2BP and M2BP-2,3,4 on the other hand. The specificity of galectin-3, a β-galactoside-binding lectin, to galactose residues in the sugar moiety of M2BP has already been described (8). Differences in the sugar moieties of M2BP-1,2 and M2BP-3,4 compared with native M2BP are also suggested by their hexosamine content. M2BP-1,2 and M2BP-3,4 contain altogether ~31 glucosamine residues and ~1 galactosamine residue, whereas it has been shown that native M2BP produced recombinantly in the same cells contains ~44 glucosamine and ~16 galactosamine residues (1). The reason for the different oligosaccharide modification in EBNNA-293 cells is not yet clear.

The differences in binding activity of M2BP-2,3,4 and M2BP-3,4 to nidogen, fibronectin, and the collagens point to a supporting role of domain 2. Because domain 2 itself shows no detectable binding to these matrix proteins, its influence appears to be indirect. A putative mechanism for this phenomenon is the prevention of the irregular aggregation of domains 3 and 4 by the presence of domain 2. In conclusion the binding activity of aggregated M2BP-3,4 was lower than that of native M2BP, but the fact that dimeric M2BP-1,2 was inactive in binding strongly suggest that the binding sites are localized within domains 3 and 4.

Both M2BP-1,2 and M2BP-3,4 showed no cell adhesive activity. In former studies it has been shown that M2BP as well as M2BP-2,3,4 strongly promoted adhesion of human HBL-100 and rat Rugli glioma cells, whereas M2BP-1 was inactive (11). It has also been shown that cell adhesion to M2BP is mediated by β1-integrins and is independent of galectin-3 (1). Clearly M2BP-1,2 and M2BP-3,4 do not replace the intact protein in cell adhesion perhaps because of the lack of a concerted action of sites located in different regions or more likely because of the lack of ring-like assembly. For a deeper understanding of the functional advantage of the unusual ring-like structure of M2BP, further studies are required, particularly at the level of the interaction with its ligands.

Acknowledgments—We gratefully acknowledge the help of Dr. Olivier Pertz on the cell culture experiments. We thank Dr. Paul Jenö for N-terminal protein sequencing and the electrospray ionization-mass spectroscopy experiments.

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