Mannan-binding protein (MBP), also known as mannan-binding lectin, is a Ca\(^{2+}\)-dependent (C-type) mammalian lectin exhibiting primary specificity for mannose, fucose, and N-acetylglucosamine (1). Because its discovery as a vital serum component associated with innate immunity, this lectin has been regarded as a multifunctional protein. MBP occurs naturally in two forms, secretory serum MBP (S-MBP) and intracellular MBP (I-MBP). S-MBP activates complement in association with MBP-associated serine proteases via the lectin pathway. Despite our previous study (Mori, K., Kawasaki, T., and Yamashina, I. (1984) Arch. Biochem. Biophys. 232, 223–233), the subcellular localization of I-MBP and its functional implication have not been clarified yet. Here, as an extension of our previous studies, we have demonstrated that the expression of human MBP cDNA reproduces native MBP differentiation of S-MBP and I-MBP in human hepatoma cells. I-MBP shows distinct accumulation in cytoplasmic granules, and is predominantly localized in the endoplasmic reticulum (ER) and involved in COPII vesicle-mediated ER-to-Golgi transport. However, the subcellular localization of either a mutant (C236S/C244S) I-MBP, which lacks carbohydrate-binding activity, or the wild-type I-MBP in tunicamycin-treated cells shows an equally diffuse cytoplasmic distribution, suggesting that the unique accumulation of I-MBP in the ER and COPII vesicles is mediated by an N-glycan-lectin interaction. Furthermore, the binding of I-MBP with glycoprotein intermediates occurs in the ER, which is carbohydrate- and pH-dependent, and is affected by glucose-trimmed high-mannose-type oligosaccharides. These results strongly indicate that I-MBP may function as a cargo transport lectin facilitating ER-to-Golgi trafficking in glycoprotein quality control.

**Subcellular Localization and Physiological Significance of Intracellular Mannan-binding Protein**

Received for publication, February 2, 2007, and in revised form, March 9, 2007 Published, JBC Papers in Press, April 8, 2007, DOI 10.1074/jbc.M700992200

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**Mannan-binding protein (MBP)** is a C-type mammalian lectin specific for mannose and N-acetylglucosamine. MBP is mainly synthesized in the liver and occurs naturally in two forms, serum MBP (S-MBP) and intracellular MBP (I-MBP). S-MBP activates complement in association with MBP-associated serine proteases via the lectin pathway. Despite our previous study (Mori, K., Kawasaki, T., and Yamashina, I. (1984) Arch. Biochem. Biophys. 232, 223–233), the subcellular localization of I-MBP and its functional implication have not been clarified yet. Here, as an extension of our previous studies, we have demonstrated that the expression of human MBP cDNA reproduces native MBP differentiation of S-MBP and I-MBP in human hepatoma cells. I-MBP shows distinct accumulation in cytoplasmic granules, and is predominantly localized in the endoplasmic reticulum (ER) and involved in COPII vesicle-mediated ER-to-Golgi transport. However, the subcellular localization of either a mutant (C236S/C244S) I-MBP, which lacks carbohydrate-binding activity, or the wild-type I-MBP in tunicamycin-treated cells shows an equally diffuse cytoplasmic distribution, suggesting that the unique accumulation of I-MBP in the ER and COPII vesicles is mediated by an N-glycan-lectin interaction. Furthermore, the binding of I-MBP with glycoprotein intermediates occurs in the ER, which is carbohydrate- and pH-dependent, and is affected by glucose-trimmed high-mannose-type oligosaccharides. These results strongly indicate that I-MBP may function as a cargo transport lectin facilitating ER-to-Golgi trafficking in glycoprotein quality control.

**Mannan-binding protein (MBP)**, also known as mannan-binding lectin, is a Ca\(^{2+}\)-dependent (C-type) mammalian lectin exhibiting primary specificity for mannose, fucose, and N-acetylglucosamine (1). Because its discovery as a vital serum component associated with innate immunity, this lectin has been regarded as a multifunctional protein. MBP occurs naturally in two forms, secretory serum MBP (S-MBP) and intracellular MBP, which is termed I-MBP in this study and was called liver MBP (L-MBP) in our previous studies (2–4). Human S-MBP and I-MBP are mainly synthesized in the liver and translated from a single form of mRNA. Both S-MBP and I-MBP are homooligomers composed of 32-kDa subunits. Each subunit has an NH\(_2\)-terminal region containing cysteines involved in interchain disulfide bond formation, a collagen-like domain containing hydroxyproline and hydroxylysine residues, and a carbohydrate-recognition domain (CRD) with an amino acid sequence highly homologous to those of other C-type lectins (5). The CRD is specific for high-mannose oligosaccharide structures on exogenous and endogenous ligands, whereas the collagen-like domain is believed to be responsible for interactions with other effector proteins involved in host defense. S-MBP activates complement through interaction with three novel MBP-associated serine proteases, which is called the lectin pathway (6). Furthermore, S-MBP was shown to exhibit novel cytotoxic activity toward some in vivo colorectal carcinoma cell experiments, which we proposed to term MBP-dependent cell-mediated cytotoxicity (4, 7). However, little is known about the subcellular localization and physiological significance of I-MBP.

Recently, the functions of glycoprotein glycan chains in protein quality control have been attracting more attention, high-mannose-type oligosaccharides especially having been shown to play important roles in this process (8, 9). Glycosylation with an asparagine-linked (N-linked) oligosaccharides is a co-translational process that occurs in the ER. High-mannose-type oligosaccharides, presumably representing nascent core-glycosylated proteins, are subsequently transferred to the Golgi...
apparatus, where multiple processing reactions occur to produce more differentiated oligosaccharides, i.e. complex-type and hybrid-type oligosaccharides, followed by targeting to the respective destinations, i.e. lysosomes, various membranes, and the extracellular milieu (10). Newly synthesized secretory glycoproteins must undergo a complex series of events that results in their correct folding, quality control, and ongoing sorting along the secretory pathway (9). Several intracellular proteins, such as lectins, chaperones, and glycan-processing enzymes, are involved in glycoprotein quality control, including calnexin/calreticulin, UDP-glucose:glycoprotein glucosyltransferase, cargo receptors (ERGIC-53 and VIP36), mannose-like proteins (EDEM and Htm1Pp), and ubiquitin ligase (Fbs) (11). All of them are thought to recognize high-mannose-type glycans similar to I-MBP with subtly different structures, although the precise specificities have yet to be clarified. This prompted us to investigate whether I-MBP is also involved in glycoprotein quality control.

In previous studies, we isolated and characterized MBP ligands from rat liver and primary cultured hepatocytes by affinity chromatography and showed that the carbohydrate portion consisted of high-mannose-type oligosaccharides, Manα6GlcNAc2 and Manα6GlcNAc3 (3). Therefore, we inferred that the possible function of I-MBP may be associated with the intracellular transport of glycoproteins and quality control in the ER. In this study, we have demonstrated that the expression of human MBP cDNA reproduces the native MBP differentiation of S-MBP and I-MBP depending on the different post-translational modifications in human hepatoma cell lines, and have also determined that I-MBP molecules accumulate predominantly as punctate structures in the ER and ER-to-Golgi COPII vesicles. Interestingly, this unique localization profile seems to depend on the carbohydrate binding activity of the lectin and also on the presence of endogenous carbohydrate ligands in the ER network system. Moreover, we have detected the interaction of I-MBP with processing high-mannose-type oligosaccharides on a lysosome-associated membrane protein (LAMP-1) in the ER and on human immunodeficiency virus (HIV) envelope protein gp120 in the cytoplasm. These results suggested that I-MBP, as a putative cargo transport intracellular lectin, is associated with the intracellular transport and sorting of some glycoproteins through recognizing processing high-mannose-type oligosaccharides on them.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal antibodies against calnexin, BiP/Grp78, p230 trans-Golgi, GM130, LAMP-1, and Sec 31A were obtained from BD Biosciences. Rabbit polyclonal antibodies specific for β-COP and green fluorescent protein (GFP) were purchased from ABR (Golden, CO) and Clontech, respectively. Goat polyclonal antibodies specific for gp120 were obtained from CORTEX Biochem (San Leandro, CA). Mouse monoclonal antibodies against ERp57, protein disulfide isomerase (PDI), and GFP were purchased from Stressgen (Victoria, BC, Canada) and Clontech, respectively. Anti-Sec 13 pAb was kindly provided by Dr. Yoshio Misumi (School of Medicine, Fukuoka University, Japan). All Alexa-conjugated and horse-radish peroxidase-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR) and Zymed Laboratories Inc.. Biotin-labeled Glc3Manα6GlcNAc2, Manα6GlcNAc2, and Manα6GlcNAc3, (isomer B) were prepared from corresponding oligosaccharide-glycine conjugates (12). [35S]Met-[35S]Cys was purchased from Amersham Biosciences. N-Glycosylation inhibitors, i.e. castanospermine (CST), deoxy-mannojirimycin (DMJ), and tunicamycin (TM), were obtained from Seikagaku Kogyo (Tokyo, Japan) and Calbiochem. Opti-Prep Density Gradient Media were purchased from Axis-Shield PoC AS (Oslo, Norway). A protease inhibitor mixture and the Ca2+-ATPase inhibitor thapsigargin were obtained from Nacalai Tesque (Kyoto, Japan) and Sigma, respectively. Biacore sensor SA chips were purchased from BIACORE (Upplands, Sweden). Dithiobis(succinimidylpropionate) (DSP) was obtained from Pierce. All chemicals for gel electrophoresis and immunoblotting were supplied by ATTO (Tokyo, Japan) and Bio-Rad.

**Site-directed Mutagenesis and Construction of Expression Vectors**—HIV envelope protein gp120 and wild-type and point mutation human MBPs were modulated with enhanced red fluorescent protein (pDsRed2-N1) and green fluorescent protein (pEFGP), respectively, from Clontech. The gp120 or MBP is located on the NH2-terminal of the fluorescent protein. Site-directed mutagenesis was carried out based on PCR, followed by subsequent sequencing to confirm the desired sequences. The point mutation MBP construct with a cytoplasmic tail CRD was mutated to C236S/C244S in pEFGP, and is termed MBP(C236S/C244S) in this study. It lacks carbohydrate binding capacity, which results in loss of lectin activity.

**Cell Culture and Treatment with N-Glycosylation Inhibitors**—Human hepatoma cell lines, HuH7 and HLF, and human cervix and kidney epithelial cell lines, HeLa and HEK292, were cultured in MEM supplemented with 10% fetal bovine serum under standard tissue culture conditions. HuH7 and HLF cells stably expressing the wild-type and mutant (C236S/C244S) MBP-GFP fusion proteins, HIV gp120 envelope protein-RFP fusion protein, and both MBP-GFP and gp120 fusion proteins were maintained in the presence of 1 mg/ml of G418 sulfate. Fig. 4B shows a schematic representation of the N-linked carbohydrate processing pathway in the ER and demonstrates the specific sites of action of the inhibitors used in this study. N-Linked carbohydrate processing inhibitors were used at optimum concentrations, as described previously (13): CST and DMJ (1 mM) and TM (1 μg/ml). Cells were treated with the inhibitors for 24 h at 37 °C prior to transfection. After transfection, the cells were cultured in the presence of inhibitors again for 24 h under the same conditions prior to harvesting for immunoprecipitation and immunoblotting.

**Confocal Laser Scan Microscopic Study**—All experiments were carried out on two-well chamber glass slides (Nalge Nunc, Naperville, IL). To determine the subcellular localization of the wild-type and point mutation I-MBPs and the interaction of I-MBPs with gp120 in the cells, GFP fusion proteins encoding the wild-type or mutant (C236S/C244S) MBP and RFP fusion protein encoding the gp120 were transfected into HeLa, HuH7, and HLF cells, respectively, with Lipofectamine (Invitrogen). The cells were subsequently incubated overnight at 37°C and monitored by laser confocal microscopy (FV1000; Olympus,
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Tokyo, Japan). For double immunofluorescence, the transfected cells were stained with anti-organellar marker proteins followed by Alexa-conjugated secondary antibodies (Molecular Probes, Eugene, OR). The double stably transfected HLF cells were visualized by laser confocal microscopy for the colocalization.

**Immunoelectron Microscopy**—Cryo-ultramicrotomy and immunogold staining were followed as described previously (14) with a slight modification. Briefly, cells were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 30 min. Fixed cells were processed for ultrathin cryosectioning. Frozen sections were incubated with anti-GFP rabbit polyclonal antibody, followed by incubation with anti-rabbit IgG coupled with 10-nm gold particles. Stained sections were negatively stained, embedded in polyvinyl alcohol (15), and examined under a Hitachi H7600 electron microscope.

**Subcellular Fractionation**—Subcellular fractions of HLF cells stably expressing the wild-type and mutant (C236S/C244S) MBP-GFPs were fractionated by OptiPrep Density Gradient Media centrifugation according to the manufacturer's instructions (Axis-Shield PoC AS, Norway). Briefly, the cells in two confluent 75-cm² flasks were resuspended in 3 ml of homogenization buffer (comprising 10 mM HEPES, pH 7.4, 0.25 M sucrose and protease inhibitors), and then homogenized by 10 strokes in a Dounce homogenizer followed by 5–10 passages through a 27-gauge needle to obtain about 95% broken cells. Nuclei and unbroken cells were pelleted by centrifugation at 1,500 × g for 10 min. The postnuclear supernatant was centrifuged at 65,000 × g for 1 h. The resulting pellet was resuspended in 1 ml of homogenization buffer. The resuspended pellet was loaded onto the top of a linear 5–30% (w/v) Nycodenz gradient. After centrifugation at 126,000 × g for 30 min (model MLS-50 rotor; Beckman Max-E Ultracentrifuge, Fullerton, CA), 13 fractions were collected, and organelle marker proteins were analyzed by SDS-PAGE followed by immunoblotting with specific monoclonal GFP antibodies, respectively. To separate the membrane and cytosol fractions of the ER and Golgi, the ER fractions (numbers 6–10) and Golgi fractions (numbers 1–5) of the wild-type and mutant I-MBP-GFPs were collected and incubated with 0.1 M Na₂CO₃, pH 11.0, to extract soluble proteins from the lumen before collecting membranes, followed by ultracentrifugation at 126,000 × g. The pellet and soluble fractions were analyzed by immunoblotting with GFP antibodies.

**Chemical Cross-linking**—All steps were performed at 4 °C. Cells were washed with cold Tris-buffered saline and cross-linking buffer (10 mM triethanolamine, pH 7.4, 250 mM sucrose, 5 mM CaCl₂), and then incubated for 30 min with 1 mM DSP (Pierce) for cross-linking on a rocking platform. The reaction was quenched by rinsing the cells with 50 mM ethanolamine in the cross-linking buffer, followed by two incubations, 15 min each, in the same ethanolamine buffer.

**Metabolic Labeling and Pulse-Chase Experiment**—Monolayer cultures of HLF-gp120-RFP stable transfected cells followed transfecting wild-type or mutant (C236S/C244S) MBP-GFP, and wild-type and mutant (C236S/C244S) HLF-MBP-GFP stable transfected cells were incubated for 1 h in methionine (Met)- and cysteine (Cys)-free MEM prior to labeling, respectively. Incorporation was initiated with fresh Met- and Cys-free MEM to which [³⁵S]Met and [³⁵S]Cys had been added. For pulse-chase experiments, cells plated into 25-cm² flasks were labeled for 1 h. The pulse was terminated by removal of the [³⁵S]Met- and [³⁵S]Cys-containing medium and washing with MEM, followed by a chase of varying length in the presence of unlabeled 1 mM Met and 1 mM Cys. The medium was collected and concentrated, and the cells were washed with phosphate-buffered saline and solubilized with 1% Nonidet P-40 lysis buffer. S- and I-MBP-GFP and gp120-RFP were immunoprecipitated from the concentrated medium and Nonidet P-40-solubilized cells with mAb against GFP and pAb against gp120, respectively. The immunoprecipitated proteins were resolved on a 5–20% Tris-HCl gradient gel (ATTO) under reducing conditions, followed by transfer to a nitrocellulose membrane, the membrane being analyzed with a BAS 2500 image analyzer (FUJIFILM, Tokyo, Japan) or Typhoon 9400 (GE Healthcare).

**Immunoprecipitation and Immunoblotting**—For the immunoprecipitation of complexes of I-MBP-GFP and glycoproteins (e.g., LAMP-1, gp120, etc.), I-MBP-GFP was immunoprecipitated from 1% Nonidet P-40-solubilized cells with mAb against MBP or pAb against GFP in the presence of 5 mM CaCl₂. The immunoprecipitated proteins were eluted by boiling in SDS-PAGE sample buffer and resolved on a 5–20% Tris-HCl gradient gel (ATTO) under reducing conditions, followed by transfer to a nitrocellulose membrane. The membrane was probed with primary anti-LAMP-1, BiP, gp120, MBP, or GFP antibodies followed by secondary horseradish peroxidase-conjugated anti-mouse IgG (Zymed Laboratories Inc.), and developed by the enhanced chemiluminescent method (SuperSignal West Pico Chemiluminescent Substrate; Pierce). Bands were visualized with a Fuji LAS 3000 (FUJIFILM).

**Carbohydrate-binding Assay of Wild-type and Mutant I-MBPs**—A binding assay for MBP was described previously (4). Briefly, binding buffer containing a wild-type or mutant I-MBP-GFP extract of stable transfected HLF cells was incubated with mannann-Sepharose 4B beads in the presence of 5 mM CaCl₂ for 1 h at room temperature. After incubation, the bound proteins were collected and eluted with elution buffer containing 4 mM EDTA. The eluted proteins were resolved on a 5–20% Tris-HCl gradient gel (ATTO) under reduced or unreduced conditions and then detected by immunoblotting with anti-GFP mAb.

**Solid Phase Binding Assay Based on Surface Plasmon Resonance (SPR)**—The affinity between MBP and Glc₃Man₉GlcNAc₂ or Man₉GlcNAc₂ was measured with a BIACore X instrument (BIACORE). Biotin-labeled Glc₃Man₉GlcNAc₂, Man₉GlcNAc₂, and Man₉GlcNAc₂ (isomer B) were covalently immobilized on a sensor chips SA by the biotin-streptavidin coupling method, respectively, according to the manufacturer’s instructions. All measurements were carried out at 25 °C and with a flow rate of 20 μl/min for both the association and dissociation phases in HBS-P buffer (10 mM HEPES, 150 mM NaCl, and 0.005% polysorbate 20, pH 7.0) supplied by the manufacturer. The interaction was monitored as the change in the SPR response. The association and dissociation...
tion rate constants, \( k_a \) and \( k_d \), were generated from the association and dissociation curves obtained in the BIAcore experiments by nonlinear fitting of the primary sensorgram data using BIAevaluation 3.0 software. The apparent equilibrium dissociation constant \( K_D \) was determined from the ratio of the two rate constants (\( k_d / k_a \)).

**NH₂-terminal Sequencing**—The recombinant wild-type and mutant (C236S/C244S) I-MBP-GFPs expressed in stably transfected HLF cells were purified by affinity chromatography on a Sepharose 4B-mannan column as described previously (4). The NH₂-terminal amino acid sequences of the recombinant I-MBP-GFPs were determined by BIOSUMS Co., Ltd. (Shiga, Japan).

**RESULTS**

**Subcellular Localization of I-MBP in the ER**—To investigate the subcellular localization of I-MBP, we first expressed GFP-tagged human MBP in human hepatoma HLF cells. The GFP tag just showed equally diffuse cytoplasmic and nuclear distributions within cells (data not shown). I-MBP-GFP showed distinct accumulation in cytoplasmic granules, and revealed a pattern of vesicular structures (Fig. 1A, left panels). Although these structures can be found throughout a cell, they are concentrated in the perinuclear region. The two major subcellular compartments in the perinuclear region are the ER and Golgi apparatus. To further identify the subcellular compartment of I-MBP accumulation, multiple markers were used. Double staining of I-MBP and ER- or Golgi-associated protein markers indeed revealed a high degree of co-localization of these two proteins in the ER and a low degree in the Golgi apparatus (Fig. 1B, right panels). However, I-MBP accumulation did not appear to be co-localized directly with mitochondrial and lysosomal markers (data not shown). To verify the immunofluorescent staining of I-MBP, electron microscopy involving immunogold labeling of frozen sections of fixed cells was performed. Immunogold labeling of I-MBP-GFP can be found within the ER and ERGIC, and around the Golgi stacks with low expression (Fig. 1C), and is particularly enriched in the ER lumen with high expression (Fig. 1D) at the ultrastructural level in the same cell. Therefore, consistent with the results obtained at the laser confocal microscopy level, I-MBP is abundant in the ER, distributed less in the ERGIC and Golgi, and not present at all in the mitochondria.

**I-MBP Accumulates at ER Exit Sites and ER-to-Golgi Traffic Vesicles**—Next, we tried to determine the possible functional significance of I-MBP predominantly localized in the ER. Because COPII, ER-to-Golgi traffic vesicles bearing anterograde-targeted cargos, and COPI, Golgi-to-ER traffic vesicles bearing retrograde-targeted cargos, are two major transport vesicles present around the perinuclear region, it was likely that...
I-MBP was localized to these two compartments and associated with ER-to-Golgi transport. This consideration led us to hypothesize that I-MBP could be associated with ER-to-Golgi transport of glycoproteins via anterograde and retrograde traffic vesicles. Therefore, we co-stained I-MBP with various COP-associated marker proteins to more functionally and specifically elucidate the accumulation pattern of I-MBP in these compartments. As shown in Fig. 2, A and B, the subcellular localization of I-MBP revealed a high degree of co-localization in COPII and less in COPI transport vesicles. As mentioned above, the majority of I-MBP-GFP fluorescence accumulated as bright punctate structures throughout the ER network, although more diffuse fluorescence of I-MBP also was observed in the ER network. ER exit sites are defined by the presence of budding COPII vesicles, so COPII components, such as Sec 13 and Sec 24D, are well characterized markers of these structures (16). As shown in Fig. 2, C and D, double staining of I-MBP with Sec 13 indicated that generally the accumulated I-MBP-GFP fluorescence overlapped with similar punctate structures. Taken together, these results demonstrate that I-MBP accumulates at ER exit sites and ER-to-Golgi COPII vesicles, suggesting that it may be associated with ER-to-Golgi transport of glycoproteins via anterograde traffic.

**Newly Synthesized LAMP-1 Interacts with I-MBP**—With respect to the subcellular localization of I-MBP described above, it is possible to speculate that I-MBP may serve as a cargo transport lectin for the trafficking of biosynthetic intermediates of some glycoproteins with high-mannose-type oligosaccharides from the ER-to-Golgi apparatus. Recently, an effort to isolate the endogenous ligands (or the cargo recognition components) of MBP from mouse tissues through affinity chromatography and mass spectrometry resulted in the identification of LAMP-1 (lysosome-associated membrane protein), which is extensively glycosylated with N-linked oligosaccharides. To determine whether LAMP-1, as a cargo recognition component of I-MBP, is conserved in humans, we examined the interaction of the I-MBP with the endogenous LAMP-1 using MBP-GFP-transfected HEK293 cells. As shown in Fig. 3, A and B, immunoblotting following in situ cross-linking, indeed demonstrated the presence of an I-MBP-LAMP-1 complex in the immunoprecipitates, suggesting the interaction of I-MBP with LAMP-1 occurred in the same subcellular compartment. Interestingly, BiP (immunoglobulin-binding protein), which is known as a molecular chaperone that interacts transiently with non-native substrates to enhance and correct their folding in the ER, was found in the I-MBP-LAMP-1 complex (Fig. 3C). The action of BiP involves multiple cycles of association and dissociation, and requires Ca$^{2+}$ and ATP, and it interacts optimally with hydrophobic peptides, which are normally buried in the hydrophobic core of properly folded proteins (17). These results suggest that I-MBP undergoes a specific interaction with newly synthesized LAMP-1 participating in the folding in the ER. There is the possibility that BiP plays a pivotal role in the ER-to-Golgi transport of selected glycoproteins mediated by I-MBP.

5 M. Nonaka, B. Y. Ma, Nana Kawasaki, M. Hirano, Nobuko Kawasaki, and T. Kawasaki, unpublished data.
Effects of Calcium and N-Glycosylation Inhibitor Treatment on the I-MBP/LAMP-1 Interaction—Because it is well known that C-type lectin I-MBP requires Ca\(^{2+}\) for carbohydrate binding, and the ER has a high Ca\(^{2+}\) concentration, we examined whether the binding of I-MBP to LAMP-1 in the ER is Ca\(^{2+}\)-dependent. MBP-GFP-transfected HEK293 cells were cultured in the presence or absence of an ER Ca\(^{2+}\)-ATPase inhibitor, 1 \(\mu\)M thapsigargin, and then incubated for 30 min with 1 mM DSP for cross-linking. The whole cell lysates were immunoprecipitated (IP) with anti-GFP mAb, and immunoblots (IB) were probed with antibodies to LAMP-1 and I-MBP-GFP, respectively, as shown in the figure. The arrows indicate the target proteins.

Effects of Calcium and N-Glycosylation Inhibitor Treatment on the I-MBP/LAMP-1 Interaction—Because it is well known that C-type lectin I-MBP requires Ca\(^{2+}\) for carbohydrate binding, and the ER has a high Ca\(^{2+}\) concentration, we examined whether the binding of I-MBP to LAMP-1 in the ER is Ca\(^{2+}\)-dependent. MBP-GFP-transfected HEK293 cells were cultured in the presence or absence of the ER Ca\(^{2+}\)-ATPase inhibitor thapsigargin. As shown in Fig. 4A, thapsigargin efficiently prevented the I-MBP-LAMP-1 interaction, suggesting that the binding of I-MBP with the biosynthetic glycoprotein intermediates in the ER is Ca\(^{2+}\)-dependent and is probably mediated by the carbohydrate binding activity of I-MBP.

Next, we tried to corroborate the roles of glucose trimming and mannose trimming in the physiological interaction between I-MBP and N-glycosylated cargos by using N-glycosylation inhibitor TM, glucosidase inhibitor CST, or mannosidase inhibitor DMJ (see Fig. 4B). As shown in Fig. 4C, when cells were treated with TM, the LAMP-1 band could not be visualized, indicating that non-glycosylated LAMP-1 could no longer interact with I-MBP. Meanwhile, when the cells were treated with CST, the inhibitor slightly reduced the efficiency of the I-MBP-LAMP-1 interaction, suggesting that glucose trimming in the I-MBP-cargo interaction may not be essential and that the presence of glucose partly prevents optimal binding. However, when the cells were treated with the mannose trimming inhibitor DMJ, it enhanced the efficiency of the I-MBP-LAMP-1 interaction, suggesting that binding to high-mannose-type oligosaccharides is more efficient than that to glucosylated high-mannose-type oligosaccharides, and that the mannose residues are required in cargo binding. Taken together, these data suggest that the binding of I-MBP with precursor LAMP-1, as one of the biosynthetic intermediates of glycosylated cargos, that occurs in the ER is carbohydrate- and Ca\(^{2+}\)-dependent, and is also affected by both untrimmed and trimmed glucose residues.

Carbohydrate-dependent Subcellular Localization of I-MBP—As described above, the LAMP-1 band in the tunicamycin treatment lane almost could not be visualized at all (Fig. 4C), indicating that the binding of I-MBP to a glycoprotein is mediated by carbohydrates on it. In a complementary experiment, we treated MBP-GFP-transfected human hepatoma HLF cells with tunicamycin, which prevents N-linked glycosylation. Confocal microscopy showed that the subcellular localization of I-MBP exhibited an equally diffuse cytoplasmic distribution (Fig. 5B), in contrast to the punctate structures observed in the control cells (Fig. 5A). These results suggest that the unique accumulation of I-MBP in the ER and COPII may be mediated by an N-glycan-lectin interaction.
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Lectin Activity of I-MBP Is Required for Its Functional Localization and Physiological Implications—On the basis of the above findings, an obvious possibility is that the I-MBP accumulated and predominantly localized in ER- and COPII vesicles through recognition of carbohydrates by the CRD may function as a cargo transport lectin for glycoproteins in ER-to-Golgi traffic. To determine whether the lectin activity of I-MBP is directly associated with its unique localization and the transportation of glycosylated cargos, we prepared a mutant MBP construct with a cytoplasmic tail CRD, which had the mutation of C236S/C244S in pEGFP, as shown in Fig. 6A. The two disulfide bonds between the cysteine residues in the center of the CRD are required for the sugar binding activity. The mutant (C236S/C244S) I-MBP lacks the carbohydrate-recognition ability and thus prevents binding of MBP to Sepharose 4B-mannan beads (Fig. 6B). However, there is no big difference in the degree of oligomerization between the intracellular wild-type and mutant (C236S/C244S) I-MBPs (Fig. 6C). Furthermore, the NH₂-terminal amino acid sequences of recombinant I-MBPs were determined with a protein sequencer, and the wild-type and mutant (C236S/C244S) I-MBPs were both cleaved at the same site between the last amino acid, Ser, of the signal peptide and the first amino acid, Glu, of the mature form (Fig. 6D), suggesting that they took the same course of multiple post-translational modification for their maturation. In contrast, as shown in Fig. 7, the subcellular localization of the mutant (C236S/C244S) I-MBP observed on laser confocal microscopy was an equally diffuse cytoplasmic one, unlike the unique accumulation of the wild-type I-MBP but just the same as that in the case of tunicamycin-treated cells (Fig. 5B). Moreover, double staining of the mutant (C236S/C244S) I-MBP with ER-/Golgi-associated or ER-Golgi COP-associated marker proteins indeed revealed a high degree of co-localization of the mutant MBP in the ER, but not in the Golgi apparatus or the COPI and COPII transport vesicles (Fig. 7, A–D), indicating that a functionally active CRD is required for the native subcellular localization of I-MBP.

The Biosynthesis, Maturation, and Differentiation of Human Wild-type and Mutant MBPs in Human Hepatoma Cells—Next, to investigate the maturation and differentiation of the wild-type and mutant (C236S/C244S) MBPs, a pulse-chase experiment was performed using HLF cells that stably expressed the human wild-type and mutant (C236S/C244S) MBP-GFP fusion proteins, respectively. As shown in Fig. 8, both the wild-type and mutant (C236S/C244S) MBP-GFP fusion proteins after a 1-h pulse gave a broad band of a mixture of the precursor and post-translational modified products, and they
were first detected in the medium at 30 min of the chase. The wild-type lectin present in the medium (S-MBP) increased slowly and linearly, and 56% of the total was extracellular between 6 and 48 h of the chase (Fig. 8A). It should be noted that other secretary proteins exited hepatocytes with half-times of 20–60 min (18). These observations indicated that I-MBP, which is predominantly localized in the ER and ER-to-Golgi COPII vesicles, is not a transient precursor of S-MBP, but is a mature resident protein of functional significance in situ. This notion is in good agreement with our previous observation that the biological half-life of I-MBP in rat liver is 2.4 days, which is predominantly in agreement with our previous observation that the biological half-life of I-MBP in rat liver is 2.4 days, but is not a transient precursor of S-MBP and that an active CRD is required for the functional subcellular localization of I-MBP.

Functional Implication of I-MBP, as Analyzed by Subcellular Fractionation—To biochemically confirm the above confocal microscopy finding that the mutant (C236S/C244S) I-MBP does not move from the ER, we used consecutive OptiPrep gradient ultracentrifugation steps to enrich the ER and Golgi apparatus. As shown in Fig. 9, A and B, both the wild-type and mutant (C236S/C244S) I-MBPs were co-fractionated with ER marker proteins PDI and calnexin. The wild-type I-MBP has also co-distributed partially with the Golgi marker protein GM130, in agreement with the immunofluorescent observations described above. However, the mutant (C236S/C244S) I-MBP was only detected within the ER.

Moreover, to determine the topology of I-MBP in the ER, we took ER fraction pools and then performed the ER membrane and lumen-cytosol fractionation. Calnexin and PDI were used as markers for ER membrane-anchored and lumen-resident fractions, respectively. The majority of both the wild-type and mutant (C236S/C244S) I-MBPs was detected in the ER lumen-cytosol (data not shown), indicating that I-MBP is a lumen-resident lectin. Taken together, the results indicate that I-MBP is predominantly localized in the ER lumen and partially in the ERGIC and Golgi lumens, which is consistent with the results of confocal and electron microscopy, suggesting that I-MBP is associated with the transport and sorting of selective glycoproteins through its lectin activity.

pH and Ca$^{2+}$ Dependence of Oligosaccharide Binding of I-MBP in Organelles—To gain insight into the role of I-MBP in complex formation via properly N-linked core oligosac-
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FIGURE 9. Functional implication of I-MBP analyzed by subcellular fractionation. A, wild-type I-MBP is predominantly localized in the ER, and partially in the ERGIC and Golgi. B, mutant (C236S/C244S) I-MBP is localized within the ER. HLF cells stably expressing the wild-type (A) and mutant (C236S/C244S) (B) MBP-GFPs were homogenized and centrifuged to obtain post-nuclear supernatants, which were then subjected to ultracentrifugation on a 5–30% linear OptiPrep gradient, respectively. An aliquot of each fraction was analyzed by SDS-PAGE, and the distribution of I-MBP and organelle marker proteins was determined by direct immunoblotting. Golgi and ER markers are indicated on the left. Fractions 1–5 were determined to contain Golgi components, and fractions 6–13 to contain ER components. C, I-MBP is abundant in the ER lumen. ER fractions (numbers 6–10) of the wild-type (A) and mutant I-MBP (B) GFPs were collected and incubated with 0.1 M Na2CO3, pH 11.0, to extract the soluble proteins from the lumen before collecting membranes, followed by centrifugation. The pellet and soluble fractions were analyzed by immunoblotting (IB) using GFP, PDI, and calnexin antibodies as indicated on the left. Calnexin and PDI were used as markers for the ER membrane-anchored and lumen-resident fractions, respectively.

For the association and dissociation of I-MBP with high-mannose-core oligosaccharides, we explored a possible molecular mechanism for the association and dissociation of I-MBP with high-mannose oligosaccharides of glycoprotein. Fig. 10A shows a schematic representation of the biosynthesis of N-linked core oligosaccharides in the ER, and the different pH values in the ER and cis-Golgi. We quantitatively characterized the MBP-cargo interaction by SPR analysis, Glc1Man9GlcNAc2, Man9GlcNAc2, and Man8GlcNAc2 (isomer B) being immobilized on a BiACore Sensor Chip, respectively. The binding of MBP at different pH values in the flow buffer to such an experimental chip and a control chip was carried out as described under “Experimental Procedures.” There is progressive acidi-

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tein gp120, which plays an important role in the pathogenesis of the acquired immunodeficiency syndrome (AIDS) and contributes to resistance to antibody neutralization (25, 26). In addition to possible roles of these glycans in the synthesis and folding of gp120 during assembly, subcellular traffic and secretion in the infected host cells have recently been hypothesized. To investigate whether I-MBP recognizes gp120 as an exogenous glycoprotein, we co-expressed gp120-RFP and wild-type or mutant (C236S/C244S), which lacks the carbohydrate-recognition ability, MBP-GFP in HLF cells. As shown in Fig. 11A, the wild-type but not the mutant I-MBP-GFP strongly overlapped with gp120-RFP in the cytoplasm observed on laser confocal microscopy, suggesting that gp120 recognition of I-MBP is directly associated with lectin activity. Next, to determine the carbohydrate-dependent subcellular interaction of I-MBP with gp120, immunoprecipitation and immunoblotting experiments were performed using gp120-RFP and wild-type or mutant (C236S/C244S) MBP-GFP-co-transfected HLF cells in the presence of 0.5 mM Ca²⁺, as indicated in the figure. The kₐ (1/Ms) and k₋ (1/s) values were determined by SPR analysis, and Kₛ (M) was calculated from k₋ (k₋/k₊). RU, response units.

FIGURE 10. SPR analysis of MBP binding to high-mannose-core oligosaccharides. A, biosynthesis of N-linked oligosaccharides in the ER. B, SPR analysis of the Glc₃Man₄GlcNAc₂ interaction with MBP. B–J, SPR analysis of the Man₃GlcNAc₂ interaction with MBP. Three sensor SA chips immobilized with biotinylated Glc₃Man₄GlcNAc₂, Man₃GlcNAc₂, and Man₄GlcNAc₂, respectively, were used to analyze the kinetic parameters of the interaction with human MBP, and different MBP concentrations were injected at the flow rate of 30 μl/min at 25 °C. The interaction between oligosaccharides and MBP was observed at pH 7.2 or 6.4 in the presence of 0.5 mM Ca²⁺, as indicated in the figure. The k₊ (1/ Ms) and k₋ (1/s) values were determined by SPR analysis, and Kₛ (M) was calculated from k₋ (k₋/k₊). RU, response units.
performed using HLF cells that co-expressed the gp120-RFP and wild-type or mutant (C236S/C244S) MBP-GFP fusion proteins, respectively. Unexpectedly, there are no significant differences in the levels of gp120 both from cells and medium between the overexpressing wild-type and mutant (C236S/C244S) MBP-GFP HLF cells although the wild-type but not the mutant I-MBP-GFP can interact with gp120 (Fig. 11C). These results imply that other intracellular lectins, chaperones, or glycan-processing enzymes, such as calnexin/calreticulin, cargo receptors (ERGIC-53 and VIP36), UDP-glucose:glycoprotein glucosyltransferase, mannoselike proteins (EDEM and Htm1p), may be also involved in post-translational modification, degradation, or secretion of gp120 because all of them are thought to recognize high-mannose-type glycans (11) similar to I-MBP with subtly different structures. In addition, the subcellular interaction of I-MBP with gp120 may disrupt HIV virion assembly or formation, resulting in restricting HIV replication and transmission during HIV infection in vivo. However, further studies are needed to define the in vivo contributions of I-MBP to transport of specific cargo glycoproteins and destruction of HIV infection.

**DISCUSSION**

MBP occurs naturally in two forms, S-MBP and I-MBP, which are synthesized mainly in the liver and translated from a single form of mRNA. S-MBP activates complement via the lectin pathway. However, subcellular localization and physiological function of I-MBP have been elucidated less. In the present study, we have demonstrated that the expression of human MBP cDNA reproduces the native MBP differentiation and maturation of S-MBP and I-MBP in human hepatoma cell lines (Fig. 8), and I-MBP was first determined to act as a putative cargo intracellular lectin for glycoprotein transport between the ER and Golgi apparatus.

Newly synthesized glycoproteins in the ER must fold and assemble correctly before being transported to their next cellular destination, and they exit the ER in transport vesicles targeted to the Golgi apparatus. The vesicular transport and sorting are often accompanied by post-translational modifications, such as glycosylation of cargo proteins, until they reach the Golgi apparatus (27). Glycoprotein trans-
port from the ER to the Golgi involves the budding of COPII-coated vesicles and their fusion with or maturing into ERGIC-53 (28). Glycoproteins are concentrated in these vesicles, indicating that cargo packaging may be a selective process. Such an active mechanism for the uptake of cargos may involve transport lectins that connect luminal cargo glycoproteins with the COPII vesicle coat and thereby operate as cargo transport lectins. Calnexin and calreticulin, which are ER chaperons with lectin activity, are involved in the quality control of newly synthesized glycoproteins in the ER. They bind to glucosylated high-mannose-core N-linked oligosaccharides and retain misfolded glycoproteins in the ER. A second family of lectins involved in glycoprotein sorting in luminal compartments of animal cells is composed of two members. ERGIC-53 is localized to the ER-Golgi intermediate compartment (29), and VIP-36 is found in the Golgi and post-Golgi portions of the secretory pathway (30).

Although our previous study indicated that I-MBP is localized in the luminal space of the ER and Golgi, and some lysosomal enzymes and secretory glycoproteins were isolated as possible endogenous ligands from rat liver rough microsomes and rat primary cultured hepatocytes (3), the detailed subcellular localization of I-MBP and its functional implication have not been clarified yet. Here, as an extension of that study, we have found the subcellular localization of I-MBP predominantly in the ER and ER-to-Golgi COPII vesicles, and further have detected the interaction of I-MBP with the processing high-mannose-type oligosaccharides on LAMP-1 as an endogenous glycoprotein in the ER and HIV envelope protein gp120 as an exogenous glycoprotein, suggesting its possible function as a novel cargo transport lectin for glycoproteins, it being partially similar to that of cargo transport receptors such as ERGIC-53 and VIP-36. These findings are consistent with our previous observations that the intracellular ligands of I-MBP consist almost entirely of high-mannose-core oligosaccharides, Man₉GlcNAc₂ and Man₉GlcNAc₂, and few or no hybrid-type or complex-type oligosaccharides with or without sialic acid were found, and the carbohydrate portions of these intracellular ligands were mainly composed of biosynthetic intermediates of glycoproteins with rapid turnover rates of an average half-life of 45 min (3).

The protein transport and sorting system is one of the most interesting subjects in the study of vesicular protein traffic processes, but the molecular mechanisms involved remain largely unresolved. N-Linked glycans, as some of the important signals, contribute to the folding, transport, and degradation of glycoproteins via interactions with a variety of intracellular lectins (9, 11, 31). The processing of N-linked oligosaccharides is initiated in the ER by removal of the glucose residues from the Glc₃Man₉GlcNAc₂ oligosaccharide structure. Mannose trimming is subsequently initiated in the ER and continues in the Golgi complex through a series of mannosidases prior to the branching and extension of the oligosaccharides by Golgi glycosyltransferases to produce more differentiated oligosaccharides, i.e. complex-type and hybrid-type oligosaccharides, followed by targeting to the respective destinations, i.e. lysosomes, various membranes, and the extracellular milieu (9).

The data obtained in this study strongly indicate that I-MBP is an abundant component of both ER exit sites and ER-derived COPII vesicles, and that I-MBP possesses a luminal lectin domain that binds specifically to a new synthesized form of LAMP-1 and gp120 with high-mannose-type glycans and acts as a cargo transport lectin. The role of I-MBP at ER budding sites is not clear yet. However, evidence that the mutant (C236S/C244S) I-MBP fails to exit the ER seems to support our conclusion that I-MBP may function as a substrate-specific transport lectin for some selective cargo glycoproteins. I-MBP and cargo binding indeed occurs in the ER, and is prolonged to stay or failed to exit this compartment when I-MBP lost its lectin activity.

Both the ER lumen and ER membrane are often densely packed with glycoproteins. Once folded or assembled properly, secretory glycoproteins must be segregated from ER residents and taken up into transport vesicles. Substrate-specific "travel specialists " could play an essential role in ensuring the quality of this journey. Cargo transport receptors interact with specific secretory glycoproteins in the donor compartment and then "guide " them into appropriate transport vesicles. These receptors interact directly or indirectly with the coat proteins that form transport vesicles and thus serve as adaptors linking the vesicle-forming machinery to cargo recruitment (32). Although not all glycoproteins appear to depend on I-MBP for transport, it is possible that other lectins exist that can also mediate glycoprotein transport. So far, several transport lectins have been reported. The cooperativity of various mannose lectins may be required for all glycoproteins to leave the ER. Alternatively, subsets of glycoproteins may require individual lectin chaperones for more efficient transport, whereas basic transport would be guaranteed by bulk flow (33).

By identifying LAMP-1 as an endogenous model and gp120 as an exogenous model for carbohydrate-dependent cargos for I-MBP in this study together with that in our previous study we identified precursor forms of some secretory glycoproteins and lysosomal enzymes as I-MBP endogenous ligands, we have provided an important piece of evidence supporting the hypothesis that I-MBP operates as a cargo intracellular lectin mediating ER-to-Golgi transport. The results obtained with the glucosidase inhibitor CST and mannosidase inhibitor DMJ (Fig. 4C), and SPR analysis with Glc₃Man₉GlcNAc₂, Man₉GlcNAc₂, and Man₉GlcNAc₂-core oligosaccharides (Fig. 10) suggest that glucose trimming of a bound glycoprotein cargo is required for maximal binding to I-MBP in the ER, and a folded or deglycosylated glycoprotein with a Man₉GlcNAc₂-core oligosaccharide appears to be the best substrate for I-MBP. These results are also consistent with the report of Helenius and Aebi (9) that newly synthesized glycoproteins can undergo mannoside trimming to the Man₉GlcNAc₂ structure by ER mannosidases before leaving the ER. Based on our BIACore SPR analysis data for the association and dissociation of I-MBP with high-mannose-core oligosaccharides, the calcium/neutral dependence of the binding and acid lability of the preformed binding complex substantiate the physiological functioning of I-MBP as a novel intracellular cargo lectin, because they provide a means to control the association-dissociation reaction between the intracellular lectin and some glycosylated cargos within organelles.
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Appenzeller et al. (29) recently reported that ERGIC-53 binds to a glycoprotein cargo in the ER and dissociates before the cis-Golgi, which is consistent with pH dependence of its sugar binding (34). Most recently, Kamiya et al. (35) demonstrated that VIP36 exhibits bell-shaped pH dependence of sugar binding with a maximum near pH 6.5 in the cis-Golgi network and is recycled to the ER, where it releases the cargos due to the higher pH.

In conclusion, the data presented here first demonstrate the subcellular localization of I-MBP and its physiological significance. Our findings strongly suggest that I-MBP may serve as a cargo intracellular lectin facilitating the ER-to-Golgi transport of selective glycoproteins, which may be tightly associated with cargo intracellular lectin facilitating the ER-to-Golgi transport of what cargo glycoproteins do the lectin I-MBP specifically carry, what determines its selectivity for different glycoproteins, how important I-MBP is in general for efficient transport of glycoproteins from the ER, and whether the subcellular interaction of I-MBP with gp120 diminishes HIV replication and infectivity. To address these questions, further investigations are required.

Acknowledgments—We thank Mitsubishi Pharm Corp. (Osaka, Japan) for the generous gift of anti- Sec 13 pAb, and Tomoko Tominaga for secretarial assistance.

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