J. Biochem. 122, 810-818 (1997)

Functional Expression of Human Mannan-Binding Proteins (MBPs) in Human Hepatoma Cell Lines Infected by Recombinant Vaccinia Virus: Post-Translational Modification, Molecular Assembly, and Differentiation of Serum and Liver MBP[1]

Yong Ma,* Hisatoshi Shida,† and Toshisuke Kawasaki*,[2]

*Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, and †Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606

Received for publication, June 2, 1997

Human mannan-binding proteins (MBPs) occur in two forms, serum MBP (S-MBP) and liver MBP (L-MBP), both of which are synthesized in the liver from a single form of human MBP mRNA. To investigate further the mechanisms of post-translational modification, molecular assembly and differentiation of S-MBP and L-MBP *in vitro*, we expressed a full-length human MBP cDNA in three human hepatoma cell lines, using the vaccinia virus expression system. The expression of human MBP cDNA reproduced the native MBP differentiation of S-MBP and L-MBP in human hepatoma cells. The recombinant S-MBP was secreted into the medium, and the recombinant L-MBP retained in the cells. The former had the ability to activate the complement through the classical or lectin pathway but the latter did not. Furthermore, one notable difference between the two MBPs was the degree of oligomerization through interchain disulfide bonds between subunits. In addition, we showed that both S-MBP and L-MBP undergo hydroxylation of lysine and proline residues in collagen-like sequences, and that the hydroxylysine is glycosylated to form glucosylgalactosylhydroxylysine (GluGalHyl) and galactosylhydroxylysine (GalHyl). Hydroxylation was required for S-MBP to be assembled into large complexes, the apparent molecular sizes of which were estimated to be 200-1,300 kDa by SDS-PAGE under non-reducing conditions and gel filtration under non-denaturing conditions. The hydroxylation and subsequent glycosylation and oligomerization were inhibited by α,α'-dipyridyl, an inhibitor of collagen lysyl and prolyl hydroxylases. These results suggested that newly synthesized lectins undergo post-translational modifications unique to the two forms of MBP, S-MBP, and L-MBP, in human hepatocytes and hepatoma cells, and that the collagen-like domains of the MBPs play an important role in promoting molecular assembly.

Key words: complement activation, human hepatoma cell lines, liver MBP, recombinant vaccinia virus, serum MBP.

Mannan-binding proteins (MBPs), which recognize mannose and *N*-acetylglucosamine, are members of the C-type (Ca^{2+}-dependent) animal lectin superfamily, and belong to the collectin (Group III) subgroup along with surfactant protein A (SP-A) and D (SP-D), conglutinin, and CL-43 (*1-3*). Human MBPs are specifically synthesized in the liver in two forms, serum MBP (S-MBP) and liver MBP (L-MBP), both of which are encoded by a single mRNA. Human S/L-MBPs exist as homooligomers of a single subunit of 32 kDa (*4, 5*). Each subunit possesses the following structural features: (1) an NH_2-terminal region containing cysteines involved in interchain disulfide bond formation; (2) a collagen-like domain (CLD) rich in hydroxyproline; (3) a neck region; and (4) a carbohydrate recognition domain (CRD) with an amino acid sequence highly homologous to those of other C-type lectins (*6*). Three subunits form a triple helix or a "structural unit"; S-MBPs consist of 2-6 and L-MBPs 2-3 structural units (*7*). S-MBP is a novel host defense factor involved in innate immunity (*8, 9*), and has been shown to trigger carbohydrate-mediated complement activation through the classical (*10, 11*) and lectin (*12, 13*) pathways, independent of antibodies and C1q, and involving novel Cls-like serine proteases (*14, 15*). S-MBP was also shown to serve as a direct opsonin (*16*), and to function as a β-inhibitor of the influenza virus (*17*). Recently, we (*18, 19*) and others, Kuhlman *et al.* (*20*) and Super *et al.* (*21*), showed that rS-MBPs are assembled into high molecular weight complexes of disulfide-bonded oligomers of the 32 kDa subunit, using the COS-1, Sf9, CHO, and mouse myeloma cell lines, respectively. All these cell lines lack an endogenous MBP message, and the rS-MBPs synthesized and secreted into the media are of a lower

[1] This work was supported by the Japan Health Sciences Foundation.
[2] To whom correspondence should be addressed. Phone: +81-75-753-4572, Fax: +81-75-753-4605
Abbreviations: S-MBP, serum mannan-binding protein; L-MBP, liver mannan-binding protein; CLD, collagen-like domain; CRD, carbohydrate recognition domain; RVV, recombinant vaccinia virus; GalHyl, galactosylhydroxylysine; GluGalHyl, glucosylgalactosylhydroxylysine; ME, sheep erythrocytes with yeast mannan attached.

oligomeric form and exhibit little ability to activate the complement compared to the native human S-MBP. In addition, studies on MBP biosynthesis and assembly have been complicated by overall slower MBP expression by primary rat hepatocytes, and by the low level of MBP production by rat H-4-II-E hepatoma cells (*22*). Given the complexity of the molecular oligomerization of MBP, in this study, we tried to express a full-length human MBP cDNA in three human hepatoma cell lines using the vaccinia virus expression system. We demonstrate for the first time high-level production and molecular assembly of MBPs in a human hepatoma cell expression system, and reproduced the native MBP differentiation of S-MBP and L-MBP from a single mRNA *in vitro*.

EXPERIMENTAL PROCEDURES

Materials—For homologous recombination, a transfection-reagent (DOTAP) was obtained from Boehriger Mannheim (Indianapolis, IN, USA). AP-conjugated anti-mouse IgG and fluorescein (FITC)-conjugated anti-mouse IgG were purchased from Promega (Madison, WI, USA) and Cappel (Durham, NC, USA), respectively. Monoclonal anti-human MBP YM304 was prepared in our laboratory. Guinea pig complement was obtained from Organon Teknika (West Chester, PA, USA). Restriction enzymes and other enzymes for DNA manipulation were obtained from Takara Shuzo (Kyoto), Toyobo (Osaka), and Nippon Gene (Toyama). Collagenase, α,α'-dipyridyl, L-ascorbic acid, hydroxylysine, and hydroxyproline were purchased from Sigma Chemical (St. Louis, MO, USA). Phenyl isothiocyanate, 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt and nitro blue tetrazolium were obtained from Wako Chemical Industries (Osaka).

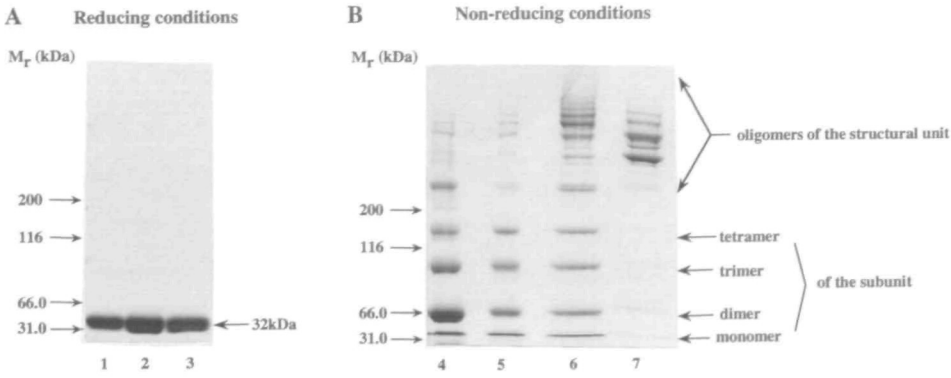
Cells and Viruses—RK13 (ATCC CCL 37) established from rabbit kidney and COS-7 (ATCC CRL 1651) established from African green monkey kidney were obtained from the American Type Culture Collection (Rockville, MD, USA). Three human hepatoma cell lines were used in this study. Chang Liver (JCRB 9066) and HLF (JCRB 0405) were provided by the Japanese Cancer Research Resources Bank (Tokyo), and $HepG_2$ (ATCC HB 8065) was from ATCC. HLF is a non-differentiated hepatoma cell line established from a 68-year-old male (*23*). Chang Liver is an epithelial-like cell line established from human liver (*24*). $HepG_2$ is a hepatoma cell line established from a 15-year-old male (*25*). HLF, $HepG_2$ and COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Nissui) supplemented with 10% fetal bovine serum (FBS) (GIBCO). Chang Liver cells were maintained in minimum essential medium with Earle's salt (MEME) (GIBCO) supplemented with 10% FBS. RK13 cells were maintained in RPMI 1640 (Nissui) supplemented with 10% FBS. Wild-type vaccinia virus strain WR and its IBT (isatin-β-thiosemicarbazone)-dependent derivative (*26*) were gifts from Dr. R. Condit. The viral expression vector, pBSF2-16, was constructed as described (*27*).

Construction of an Expression Vector and Recombinant Vaccinia Virus—The human MBP cDNA (*5*) was subcloned into the expression vector, pBSF2-16, at *Sma*I and *Sac*I sites immediately downstream of the A-type inclusion body of a cowpox virus (ATI) hybrid promoter (the late ATI promoter combined with the tandemly repeated early promoter sequence for the 7.5 kDa protein [the 7.5 kDa promoter], including the upstream consensus sequence and the initiation region). The resultant vector was named pBSF2-16/MBP. pBSF2-16/MBP and the intact genomic DNA extracted from wild-type vaccinia virus strain WR virions were transfected into IBT-dependent vaccinia virus-infected COS-7 cells by use of a transfection-reagent (DOTAP) according to the manufacturer's protocol. The progency viruses generated were screened for the HA (hemagglutinin) phenotype as described, and detected, in RK13 cells by cell ELISA to obtain recombinant vaccinia virus (RVV) (*27*). The viral titer was determined by a plaque-forming assay as described and expressed in plaque-forming units (PFUs).

Immunofluorescence Microscopy—$HepG_2$, Chang Liver, and HLF cells were infected with 10 PFU per cell of recombinant vaccinia virus in six-well tissue culture plates (Becton Dickinson Labware). After 20 h culture, the cells were rinsed once in PBS, and then fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. After being rinsed three times with PBS, the cells were incubated with a 1:500 dilution of anti-human MBP monoclonal antibody YM304 in PBS at room temperature (25°C) for 1 h. The cells were aspirated off and then rinsed three times with PBS before the addition of FITC-conjugated anti-mouse IgG diluted 1:100 with PBS. After incubation at room temperature in the dark for 1 h, the cells were rinsed three times with PBS, and then their immunofluorescence was determined using an OLYMPUS BX50-34-FLADI microscope (OLYMPUS, Tokyo).

Expression and Purification of MBPs in Human Hepatoma Cell Lines—Human hepatoma cell lines HLF, $HepG_2$, and Chang Liver cells were maintained in DMEM and MEME, respectively, supplemented with endogenous MBP-free 10% FBS, which had been prepared by passage through a Sepharose 4B-mannan column. The hepatoma cells were grown at 37°C under 5% CO_2 to a concentration of 5×10^5 cells/ml in 20 ml of medium in 250 ml Falcon flasks. The RVV containing the human MBP cDNA was then added to the cells at a multiplicity of infection (MOI) close to 5. The media and RVV-infected hepatoma cells were harvested 48 h postinfection, respectively. The cell pellets were lysed by gentle sonication or by repeated freezing and thawing in the loading buffer (0.02 M imidazole-HCl, pH 7.8, 1.25 M NaCl, 0.02 M $CaCl_2$) at 0°C, and then centrifuged at $25,000\times g$ for 30 min at 4°C to yield cell lysates. Recombinant S-MBPs (rS-MBPs) secreted into the media and recombinant L-MBPs (rL-MBPs) retained in hepatoma cells were purified, respectively, by affinity chromatography on a Sepharose 4B-mannan column as described (*4*).

Effects of Ascorbic Acid and α,α'-Dipyridyl on Hydroxylation of Lysine and Proline Residues—To enhance the hydroxylation of lysine and proline residues, confluent $HepG_2$ cells infected with RVV expressing MBP were incubated for 48 h with DMEM in the presence of 0.3 mM L-ascorbic acid. To inhibit the hydroxylation of lysine and proline residues, confluent HLF cells infected with RVV expressing MBP were incubated for 48 h with DMEM in the presence of 3 mM α,α'-*dipyridyl*, an iron chelator. The respective cells and media were harvested, and then rMBPs were purified by affinity chromatography as described above.



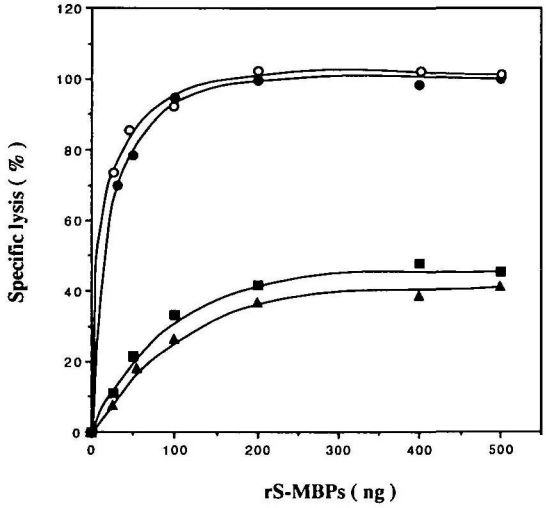


Fig. 1. **Functional expression and biological activities of human S-MBPs expressed in human hepatoma cell lines.** A and B: SDS-PAGE of human S-MBPs expressed in hepatoma cell lines. Samples of rS-MBPs and native S-MBP were electrophoresed on a 3-10% polyacrylamide gel under reducing (A) and non-reducing conditions (B). The positions of the molecular markers (kDa) are indicated on the left, and the arrows on the right of B indicate the positions of oligomers of the subunit. The rS-MBPs in lanes 1 and 4 were expressed in $HepG_2$, those in lanes 2 and 5 in Chang Liver, and those in lanes 3 and 6 in HLF cells. The native S-MBP in lane 7 was isolated from human serum. C: Dose-dependence of the complement activation by human native S-MBP and recombinant S-MBPs. Mannan-coated sheep erythrocytes (ME) were sensitized with 100-500 ng of native S-MBP or rS-MBPs, and then lysed with the complement ($2CH_{50}$), respectively. The symbols are: ○, native S-MBP; ●, rS-MBP expressed in HLF; ■, rS-MBP in $HepG_2$; ▲, rS-MBP in Chang Liver.

Gel Filtration Chromatography—Gel filtration of rS-MBP was performed with a FPLC system (Pharmacia) on a Sephacryl S-300 HR 10/100 column (Pharmacia Biotech, Stockholm, Sweden) at 4°C in 20 mM imidazole-HCl, pH 7.8, 1.25 M NaCl, and 2 mM EDTA. Calibration was carried out using a standard calibration kit (Pharmacia, Uppsala, Sweden).

Complement Activation Assaying by Passive Hemolysis—Sheep erythrocytes were coated with yeast mannan (ME), the ME was sensitized with rL-MBPs or rS-MBPs, and then the sensitized cells were incubated with guinea pig complement as described (*10, 11*). A batch of 1×10^8 cells was coated with 10 μg yeast mannan by the $CrCl_3$ method. The degree of specific lysis was calculated based on the absorption at 541 nm of an equivalent amount of cells totally lysed in water and expressed as a percentage. Correction was made with the blank value obtained in the absence of the complement, which was usually less than 2% of the totally lysed cell value.

Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed according to the method of Laemmli (*28*) in a 3-10% gradient polyacrylamide gel (ATTO, Tokyo) under reducing or non-reducing conditions, and proteins were stained with Coomassie Brilliant Blue R250.

Collagenase Digestion—rS-MBP expressed in HLF cells was incubated with 1.0 unit/ml collagenase in 20 mM imidazole-HCl, pH 7.8, 20 mM $CaCl_2$, 1.25 M NaCl, 10 μM phenylmethane sulfonylfluoride and 0.1% NaN_3 at 37°C for 24 h. The collagenase-digests were applied to a Sepharose 4B-mannan column, and the pass-through and EDTA-eluted fractions were obtained, respectively.

Alkaline Hydrolysis of rMBPs and Preparation of Glycosylated Hydroxylysine Residues—Glycosylated hydroxylysine residues were prepared by a modification of the method used by Spiro (*29*). rS-MBP, rL-MBP, and rS-MBP in the presence of α,α'-dipyridyl, and the pass-through and EDTA-eluted fractions from the affinity column of the collagenase-digests of rS-MBP were hydrolyzed in 2 M NaOH at 110°C for 24 h in glass reactivials (National Scientific), respectively. After adjustment of the hydrolyzates to pH 3.0 with 1 M acetic acid, the mixtures were centrifuged at $12,000\times g$ for 30 min. The supernatants were applied to a column (0.5×6 cm) of Dowex 50-X8 (H^+ form), and the column was eluted successively with 100 ml of 1 mM acetic acid, 100 ml of 8% (v/v) pyridine, and 30 ml of 3 M $NH_3\cdot H_2O$. The 3 M $NH_3\cdot H_2O$ eluates were concentrated with a Servant speed Vac and then lyophilized. Freeze-dried amino acids and amino sugars were derivatized with phenylisothiocyanate (PITC) as previously described (*30*). The PITC-derivatized samples were then dissolved in 50 mM ammonium acetate, pH 6.8, prior to analysis by reverse-phase HPLC on a TSK gel ODS 80_{TM} column (0.6×20 cm) (TOSOH, Tokyo) with a Shimadzu LC-10AD/SPD-M10AV liquid chromatography system (Kyoto).

Ionspray Mass Spectrometry—The identification of glycosylated hydroxylysine residues separated by reverse-phase HPLC was performed with an API III triple-quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) equipped with an ionspray interface (*31*).

NH_2-Terminal Sequencing—The NH_2-terminal amino acid sequences of rS-MBP and rL-MBP were determined with an Applied Biosystems 477A Plus liquid protein sequencer and a 120A PTH analyzer (Foster City, CA, USA).

RESULTS

Expression and Biochemical Characterization of rS-MBPs—Human S-MBPs were expressed in all three human hepatoma cell lines ($HepG_2$, Chang Liver, and HLF) using the vaccinia virus expression system. rS-MBPs were purified from the culture media by Sepharose 4B-mannan affinity chromatography. The yields of rS-MBPs from the culture media of the $HepG_2$, Chang Liver, and HLF cell lines were 7.6, 3.6, and 5.0 mg/liter, respectively, when confluent cells were infected with RVV in a 250 ml flask containing 15 ml of media for 48 h. Figure 1A shows the SDS-PAGE of native S-MBP isolated from human serum and rS-MBPs expressed in the three hepatoma cell lines. Under reducing conditions, rS-MBPs migrated as a 32 kDa protein (Fig. 1A, lanes 1-3) just like the native human S-MBP, as we reported previously (*4*). The SDS-PAGE under non-reducing conditions indicated that rS-MBPs (Fig. 1B, lanes 4-6) and native S-MBP (Fig. 1B, lane 7) are composed of a mixture of several components, corresponding to the monomer, dimer, and trimer of the subunit, and to higher oligomers of the structural unit, respectively. Interestingly, the ratio of higher to lower oligomers differed substantially between the three hepatoma cell lines: the highest ratio with rS-MBP expressed in HLF (HLF rS-MBP), an intermediate one in $HepG_2$ ($HepG_2$ rS-MBP), and the lowest one in Chang Liver (Chang Liver rS-MBP). The complement-activating activity of each rS-MBP was then assayed as the passive hemolysis of mannan-coated erythrocytes with the aid of guinea pig complement, as described under "EXPERIMENTAL PROCEDURES." The results are shown in Fig. 1C. About 200 ng native S-MBP showed 100% and 200 ng HLF rS-MBP about 98% hemolytic activity, while the same amounts of $HepG_2$ rS-MBP and Chang Liver rS-MBP showed 41 and 38% hemolytic activity, respectively. These results suggest that S-MBP is assembled into higher oligomers through disulfide bonds of the structural unit, and that these oligomers play an important role in the activation of complement by S-MBP.

Human S-MBP and L-MBP in HLF Hepatoma Cells—To determine whether or not a single mRNA gives rise into both S-MBP and L-MBP, the RVV encoding the human MBP cDNA was infected into human hepatoma cells, which have an endogenous MBP message. Human hepatoma cells were heavily stained with the YM304 monoclonal antibody followed by FITC-conjugated anti-mouse IgG 20 h after infection with RVV. As shown in Fig. 2, the level of expression of the human MBP was extremely high in the cytoplasm, predominantly in the rough endoplasmic reticu-

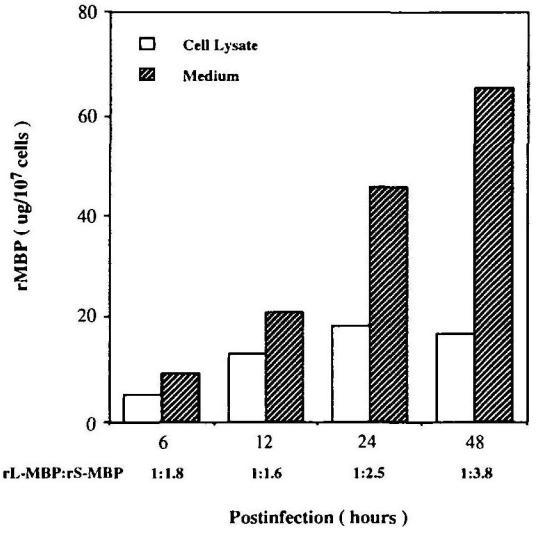


Fig. 3. **Time course of human MBP differentiation in human HLF hepatoma cells.** rS-MBP isolated from the medium and rL-MBP isolated from the cell lysate were subjected to protein quantification using fluorescamine at each time point. The ratio of rL-MBP to rS-MBP at each time point is indicated under the figure.

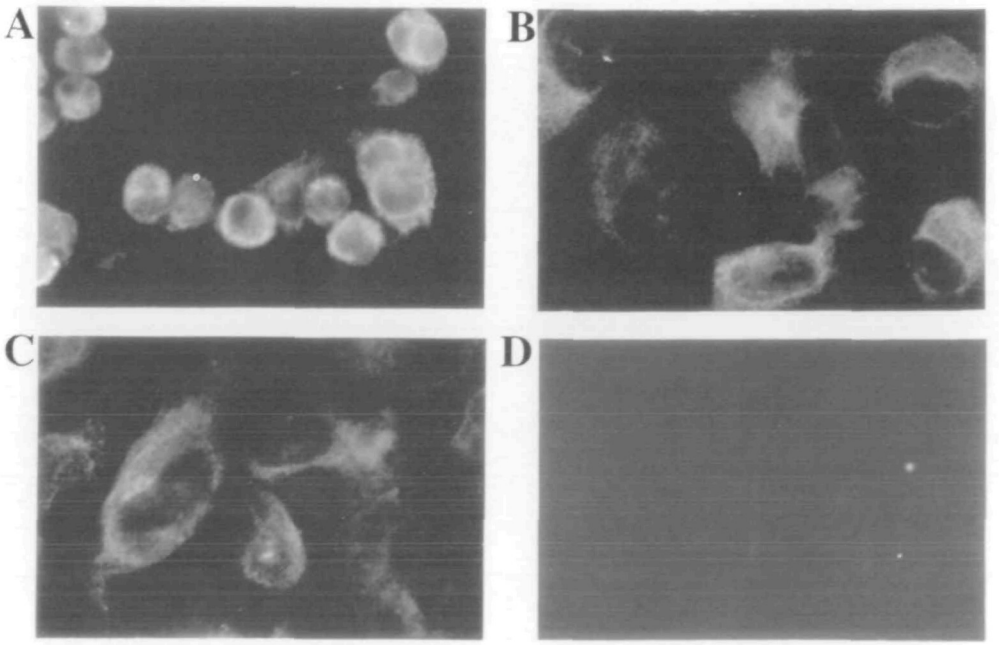
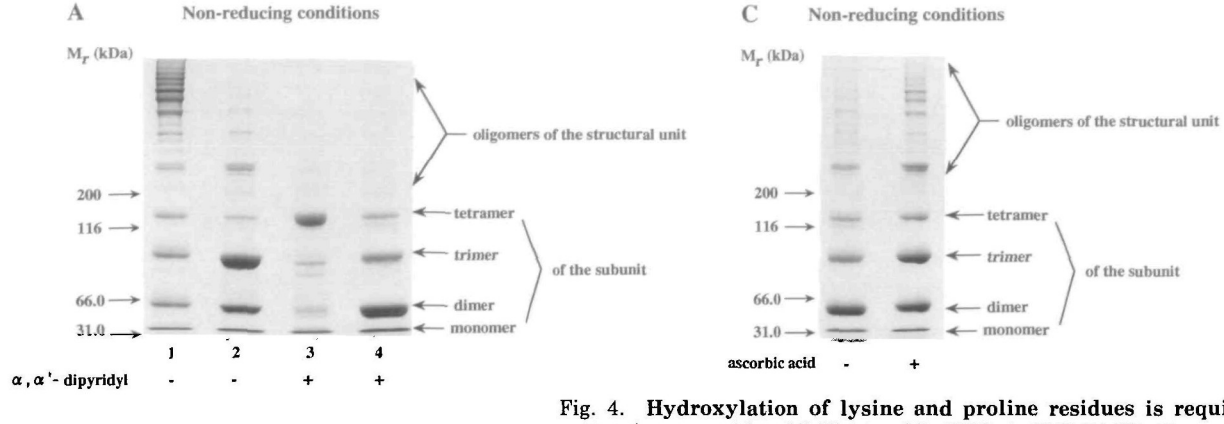


Fig. 2. **Expression of the human MBP on human hepatoma cells infected with RVV.** At 20 h postinfection, expression of the human MBP on RVV-infected hepatoma cells (panel A, $HepG_2$; panel B, Chang Liver; panel C, HLF) and on wild-type vaccinia virus-infected HLF cells (panel D, negative control) was determined by immunofluorescent staining with anti-human MBP monoclonal antibody YM304 followed by FITC-conjugated anti-mouse IgG.

lum (RER) and the Golgi apparatus, in all three hepatoma cell lines, (A) $HepG_2$, (B) Chang Liver, and (C) HLF. In contrast, HLF cells infected with the wild-type vaccinia virus were not stained at all with the YM304 monoclonal antibody (Fig. 2D). Next, we investigated the time course of S/L-MBP production in HLF cells. At each time point, S-MBP and L-MBP were purified from the incubation medium, and the loading buffer extracts of the frozen and thawed cells, respectively, by affinity chromatography on a Sepharose 4B-mannan column. The ratio of S-MBP and L-MBP at each time point is given in Fig. 3. S-MBP accumulated in the medium almost linearly with time up to 48 h, whereas L-MBP appeared to plateau after 24 h incubation. The latter might be related to the cytopathic effect of vaccinia virus, which was observed between 24 and 48 h after infection. The assembly of S/L-MBPs into high molecular weight complexes was analyzed by SDS-PAGE under non-reducing conditions (Fig. 4A, lanes 1 and 2). The proportion of S-MBP migrating as the higher oligomeric form was much higher than that of L-MBP. The NH_2-terminal amino acid sequences of rS-MBP and rL-MBP were determined with a protein sequencer. rS-MBP and rL-MBP were cleaved at the same sites between the last amino acid, Ser, of the signal peptide and the first amino acid, Glu, of the mature MBPs (see Fig. 5). As was previously shown with human native S/L-MBP (5) and



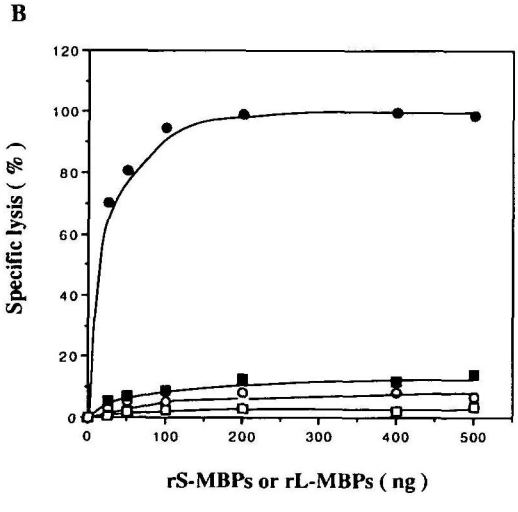


Fig. 4. **Hydroxylation of lysine and proline residues is required for the molecular assembly of S-MBP and L-MBP.** A: SDS-PAGE of human S-MBP and L-MBP in the absence and presence of α,α'-dipyridyl. HLF hepatoma cells infected with RVV were incubated for 48 h with DMEM in the absence (−) or presence (+) of 5 mM α,α'-dipyridyl. The medium and cells were harvested, the rS-MBPs and rL-MBPs were purified by affinity chromatography, respectively, and the purified rS-MBPs and rL-MBPs were analyzed by SDS-PAGE on a 3-10% polyacrylamide gel under non-reducing conditions. The rS-MBPs in lanes 1 and 3 were isolated from medium, and the rL-MBPs in lanes 2 and 4 were isolated from cell lysates. The positions of the molecular markers (kDa) are indicated on the left, and the arrows on the right of B indicate the positions of oligomers of the subunit. B: Dose-dependence of the complement activation by the rS-MBPs and rL-MBPs in the absence (−) and presence (+) of α,α'-dipyridyl. The samples were the same as in A. Mannan-coated sheep erythrocytes (ME) were sensitized with 100-500 ng of rS-MBPs or rL-MBPs, and then lysed with the complement ($2CH_{50}$), respectively. The symbols are: ●, rS-MBP (−): ○, rS-MBP (+); ■, rL-MBP (−); □, rL-MBP (+). C: SDS-PAGE of human S-MBP expressed in $HepG_2$ hepatoma cells in the absence and presence of ascorbic acid. $HepG_2$ hepatoma cells infected with RVV were incubated for 48 h with DMEM in the absence (−) and presence (+) of 0.3 mM ascorbic acid. The medium was harvested, rS-MBPs were purified by affinity chromatography, respectively, and the purified rS-MBPs were analyzed by SDS-PAGE on a 3-10% polyacrylamide gel under non-reducing conditions. The positions of the molecular markers (kDa) are indicated on the left, and the arrows on the right of C indicate the positions of oligomers of the subunit.

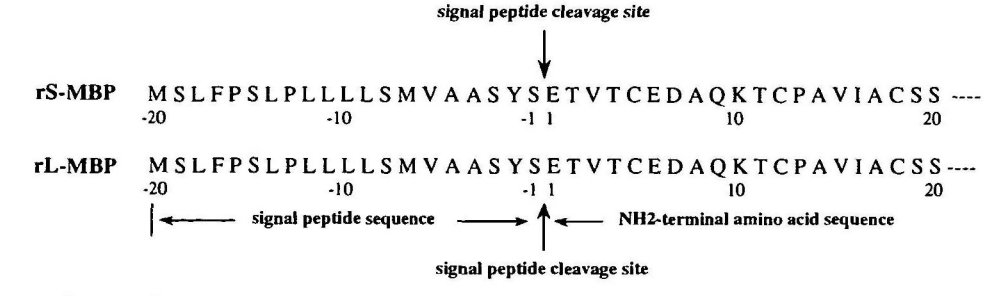


Fig. 5. **The NH_2-terminal amino acid sequences of rS-MBP and rL-MBP determined with a protein sequencer.** The amino acid sequences are shown using the standard one-letter code, with numbering from the NH_2-terminal residue, glutamic acid, of the mature MBPs. The amino acid residues of the signal peptide sequence deduced from the nucleotide sequence of human MBP cDNA are numbered from −20 to −1. The arrows indicate the signal peptide cleavage sites.

confirmed with human recombinant S/L-MBP here (Fig. 4B), human S-MBP activated the complement, as judged in passive hemolysis tests, while L-MBP exhibited little ability to activate the complement. This supports that human S-MBP and L-MBP are encoded by a single mRNA. During the course of the multiple post-translational modification, part of the MBP acquires the ability to activate complement and to be secreted into the medium, while another part acquires the ability to be retained in the cells to become L-MBP.

Oligomer Structures of Human S-MBP Required for Complement Activation—In an attempt to determine the relationship between the oligomeric structure and complement-activating activity, the rS-MBP expressed in HLF cells was analyzed by Sephacryl S-300 gel filtration chromatography. The oligomers of rS-MBP were separated into peaks I-V (see Fig. 6A). Most of the oligomers were eluted in peaks II (1,200 to 540 kDa) and III (540 to 450 kDa), which correspond to the main peaks of native human S-MBP on a Sepharose CL-6B column, as reported previously (*5*). Other small peaks were seen at the void volume (peak I), 270 kDa (peak IV), and 90 kDa (peak V). The protein distribution was 1% in peak I, 52% in peak II, 22% in peak III, 13% in peak IV, and 12% in peak V. The size distribution of the rS-MBP was similar to that of native S-MBP, except for the presence of peaks I, IV, and V. SDS-PAGE analyses of these peaks under non-reducing conditions are shown in Fig. 6B. Each peak shows a ladder pattern of band mobility increasing from peak II to peak IV, consistent with the apparent molecular sizes of the proteins estimated on gel filtration. Peak II is rich in higher oligomers, peak III in pentamer/hexamers, peak IV in trimers, and peak V in monomers of the structural unit. The complement-activating activity of each peak was assayed using the passive hemolysis test (see Fig. 6C). If the hemolytic activity of MBPs is compared as the amount of S-MBP which gives rise to 50% lysis, peak II (about 10 ng) is several-fold more active than peak III (about 30 ng), and greater than 20-fold more active than peak IV (200 ng), peak V having very little activity.

Hydroxylation of Lysine and Proline Residues Is Required for the Molecular Assembly of S-MBP and L-MBP—To determine whether or not the hydroxylation of lysine and proline residues is required for the assembly into high molecular weight complexes (higher homooligomers) of S-MBP, α,α'-dipyridyl, a hydroxylase inhibitor, and

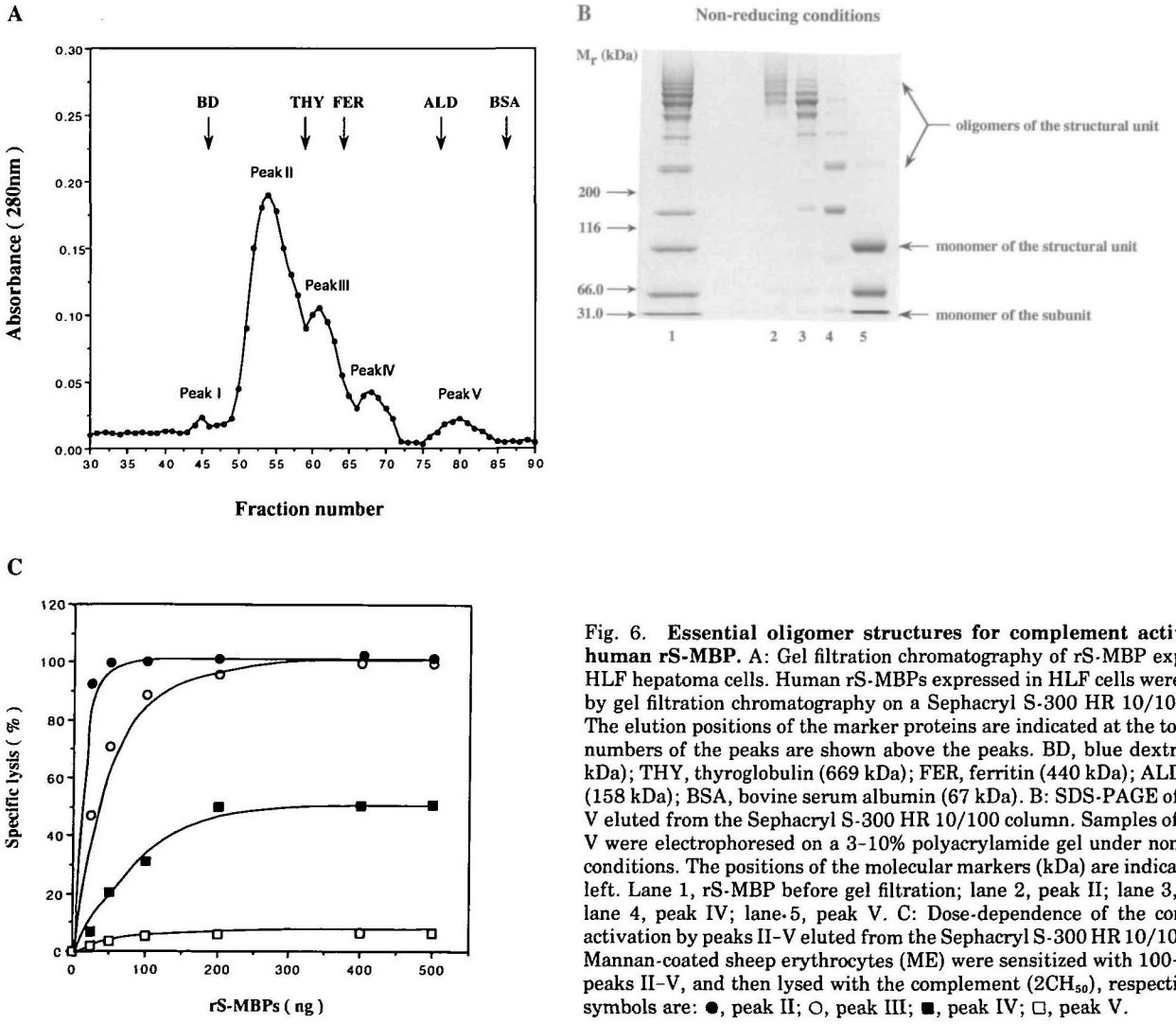


Fig. 6. **Essential oligomer structures for complement activation of human rS-MBP.** A: Gel filtration chromatography of rS-MBP expressed in HLF hepatoma cells. Human rS-MBPs expressed in HLF cells were analyzed by gel filtration chromatography on a Sephacryl S-300 HR 10/100 column. The elution positions of the marker proteins are indicated at the top, and the numbers of the peaks are shown above the peaks. BD, blue dextran (2,000 kDa); THY, thyroglobulin (669 kDa); FER, ferritin (440 kDa); ALD, aldolase (158 kDa); BSA, bovine serum albumin (67 kDa). B: SDS-PAGE of peaks II-V eluted from the Sephacryl S-300 HR 10/100 column. Samples of peaks II-V were electrophoresed on a 3-10% polyacrylamide gel under non-reducing conditions. The positions of the molecular markers (kDa) are indicated on the left. Lane 1, rS-MBP before gel filtration; lane 2, peak II; lane 3, peak III; lane 4, peak IV; lane 5, peak V. C: Dose-dependence of the complement activation by peaks II-V eluted from the Sephacryl S-300 HR 10/100 column. Mannan-coated sheep erythrocytes (ME) were sensitized with 100-500 ng of peaks II-V, and then lysed with the complement ($2CH_{50}$), respectively. The symbols are: ●, peak II; ○, peak III; ■, peak IV; □, peak V.

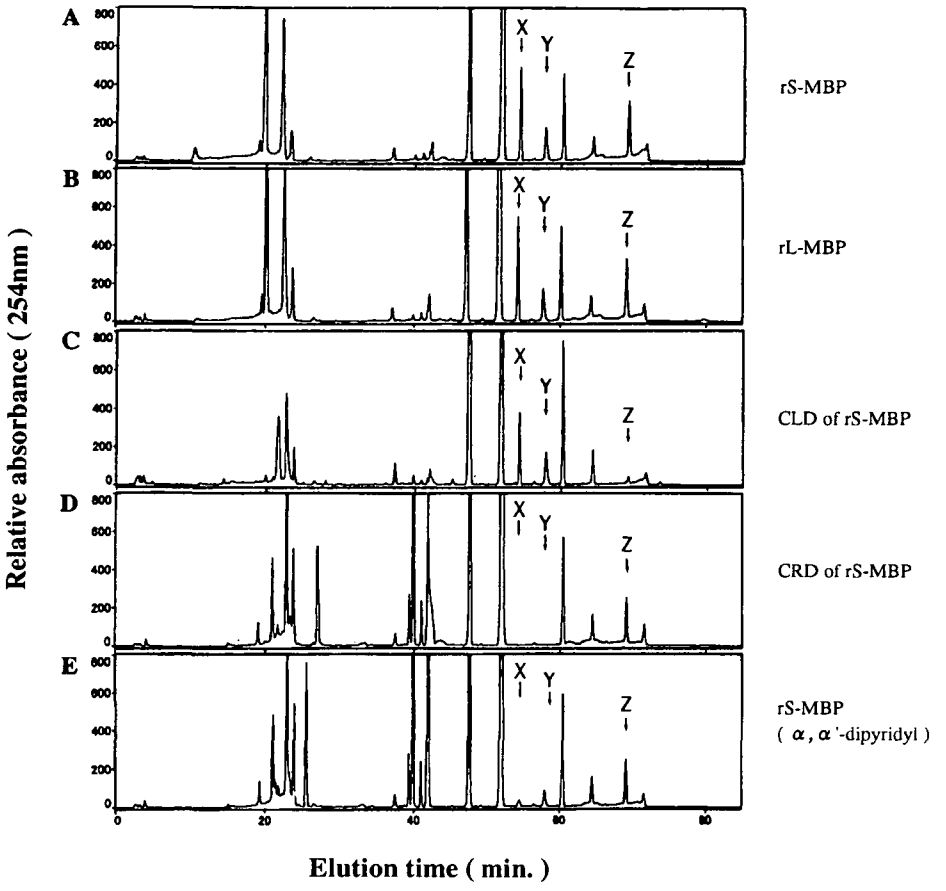


Fig. 7. **The collagen-like domains of human S-MBP and L-MBP contain GluGalHyl and GalHyl.** rS-MBP (A), rL-MBP (B), α,α'-dipyridyl-treated rS- MBP (E), and CLD (C) and CRD (D) of rS-MBP were hydrolyzed in 2 M NaOH at 110°C for 24 h, respectively, and the hydrolyzates were treated with phenylisothiocyanate. Basic amino acids and amino sugars derivatives, which were eluted with 3 M $NH_3 \cdot H_2O$ from a Dowex 50-X_8 ion exchange column, were separated by reverse-phase HPLC, respectively, as described under "EXPERIMENTAL PROCEDURES." Glycosylated hydroxylysine residues separated by reverse-phase HPLC were identified by Ionspray Mass Spectrometry. The arrows at the tops of the HPLC charts indicate the elution positions of X(GluGalHyl), Y(GalHyl), and Z(Lys).

ascorbic acid, a cofactor of hydroxylase, were used to investigate the roles of hydroxylation and subsequent oligomerization. The rMBPs purified from the medium and the cell lysate were examined by SDS-PAGE under non-reducing conditions, respectively. As shown in Fig. 4A, the presence of α,α'-dipyridyl decreased the amount of MBPs migrating as higher oligomers with not only S-MBP secreted into the medium (Fig. 4A, lane 3) but also L-MBP retained in the HLF cells (Fig. 4A, lane 4). It should be noted that the major component of S-MBP was the tetramer of the subunit, and that of the L-MBP the dimer, both of which differ from the normal major constituent of the subunit, the trimer, suggesting that some aberrant disulfide formation took place under these conditions. On the other hand, the incubation of $HepG_2$ cells in the presence of ascorbic acid increased the complement activating activity by several fold (data not shown). α,α'-Dipyridyl at 3.0 mM produced a more than 50% decrease in protein synthesis in HLF cells. In contrast to this, the presence (+) of ascorbic acid in the incubation mixture increased the proportion of higher oligomeric complexes of S-MBP in $HepG_2$ cells (Fig. 4C). The complement activation by the rS-MBPs expressed in HLF cells in the presence or absence of α,α'-dipyridyl was assayed using the passive hemolysis test. As shown in Fig. 4B, HLF rS-MBP produced in the presence of the hydroxylase inhibitor exhibited markedly decreased ability to activate the complement.

The Collagen-Like Domains of Human S-MBP and L-MBP Contain GluGalHyl and GalHyl—GluGalHyl and GalHyl are derived from various collagens through alkaline hydrolysis. To determine whether or not the collagen-like domain of the human S/L-MBPs synthesized in HLF cells contains hydroxylysine residues, and whether or not these residues subsequently undergo glycosylation to form GluGalHyl and GalHyl, purified rS/L-MBP, α,α'-dipyridyl treated rS-MBP, and its collagen-like domain/CLD and carbohydrate recognition domain/CRD were hydrolyzed in 2 M NaOH at 110°C for 24 h as described under "EXPERIMENTAL PROCEDURES." The phenylisothiocyanate derivatives of basic amino acids and amino sugars eluted with 3 M $NH_3 \cdot H_2O$ from the Dowex 50-X8 column were separated by reverse-phase HPLC. The glycosylated hydroxylysine residues were identified by Ionspray Mass Spectrometry as described (*31*). HPLC peaks, with molecular weights corresponding to those of GluGalHyl and GalHyl, were obtained for S-MBP (Fig. 7A), L-MBP (B), and CLD (C). Whereas GluGalHyl and GalHyl were not observed in CRD (D) and S-MBP produced in the presence of α,α'-dipyridyl (E), as might be expected. These results demonstrate that the collagen-like domains (CLDs) of the human S-MBP and L-MBP contained hydroxylysine-glycosides, with an about 3:1 ratio of GluGalHyl to GalHyl.

DISCUSSION

MBPs occur naturally in two forms, S-MBP and L-MBP. There is a marked difference in the control of the biosynthesis of MBP between rat and mouse, and man. As for rat and mouse, S-MBP and L-MBP are known to have distinct amino acid sequences and to be encoded by two similar but

different mRNAs, respectively (*32, 33*). On the other hand, human S-MBP and L-MBP are encoded by a single mRNA (*5*).

In this study, human rS-MBP and rL-MBP were characterized biochemically by expressing a full-length human MBPcDNA in hepatoma cell lines using the vaccinia virus system. A viral vector is an efficient system for the expression of foreign genes in a wide range of host cells. Three human cell lines ($HepG_2$, Chang Liver, and HLF) were chosen, all of which are derived from hepatoma cells. In particular, HLF is a non-differentiated hepatoma cell line judging from its epithelial characteristics and heterotransplantability. The transfection of the MBP cDNA resulted in the high-level production of S-MBP and L-MBP, which are structurally and functionally similar to their native forms, respectively. The mechanisms associated with the differentiation of S-MBP and L-MBP were studied using human HLF hepatoma cells. The processed mature rS-MBP was isolated from the medium following secretion, and rL-MBP, which was essentially retained in the HLF cells, was isolated from the cell lysate. Comparisons of rS-MBP and rL-MBP with regard to structure-function properties indicated that the signal peptide was cleaved at the same site, as determined on NH_2-terminal amino acid analysis. Clear differences between rS-MBP and rL-MBP were in the degree of oligomerization through interchain disulfide bonds, as revealed by SDS-PAGE and gel filtration, and the ability to activate complement; rS-MBP activated the complement, while rL-MBP exhibited very little ability to activate the complement, similar to native S-MBP and L-MBP. Thus, the double localization of human MBPs with different physicochemical properties and biological functions occurs in human hepatocytes or hepatoma cells on post-translational modifications unique to S-MBP and L-MBP, respectively. Next, we examined the hydroxylation of lysine and proline residues as a possible mechanism of differentiation of human S/L-MBPs *in vitro*.

When HLF hepatoma cells were incubated in the presence of α,α'-dipyridyl, an inhibitor of both prolyl and lysyl hydroxylases, the newly synthesized MBP was unable to be assembled into high molecular weight oligomeric complexes. On the other hand, when $HepG_2$ hepatoma cells were incubated with ascorbic acid, which helps complete the hydroxylation of the collagen domain, the oligomerization of the newly synthesized MBP was significantly enhanced. In addition, our previous observation indicated differences in the hydroxylation of proline residues in the collagen-like domains of S-MBP and L-MBP (*5*). These findings suggested that the hydroxylation of proline and lysine residues promotes the triple helical formation of the collagen-like domain, and that this post-translational modification is important for intra- and intermolecular cross-links *via* disulfide bonds.

It is interesting to note that the inhibition of collagen hydroxylation by α,α'-dipyridyl, or under anaerobic conditions, strongly inhibits disulfide bonding between the extension peptides at the carboxyl terminus of interstitual subunits, and prevents the formation of the triple helix (*34*).

As was initially pointed out by Colley *et al.* (*35*), the rat MBP contains hydroxyproline, hydroxylysine, and GluGal-Hyl. Other proteins known to contain collagen-like domains include the complement component, C1q (*36*), SP-D, bovine CL-43 (*37*), and conglutinin; referred to collectively as "collectins." In the present study, we demonstrated that the collagen-like domains of human S-MBP and L-MBP contain the hydroxylysine-glycoside, GalHyl, as well as GlcGalHyl. As the crosslinking of collagen is known to take place between hydroxylysine residues, possible biological functions of the glycosylation could be the inhibition of this modification and the prevention of fiber formation. Further studies are required to establish if this is indeed the case.

Moreover, our results showed that rS-MBP expressed in human HLF hepatoma cells was composed of a mixture of oligomers of about 90 kDa, with a predominance (70% of the preparation) of tetramers (360 kDa) and larger oligomers. The dimer, trimer-rich fraction (peak IV) obtained on gel filtration gave about 1/20% of the full complement-activating activity, as judged with the passive hemolysis test. The pentamer/hexamer-rich fraction of rS-MBP (peaks II and III) appeared to be more efficient in terms of complement activation than the dimer, trimer-rich fraction, indicating the importance of multivalent binding of carbohydrate ligands through the globular heads or the presence of at least five/six triple-helical structures for efficient interaction with the complexes of $C1r_2C1s_2$ or MASP. This observation is consistent with the finding of Lu *et al.* (*38*) that pentamer/hexamer fractions of native S-MBP isolated from normal human serum exhibited effective, zymosan-dependent complement activation.

In general, the complexity of the molecular assembly and post-translational modification of C-type lectins necessitates the utilization of eukaryotic expression systems. In fact, recombinant forms of human MBP have previously been expressed in COS-1 (*5*), CHO (*20*), myeloma (*21*), and Sf9 (*19*) cells, and have been reported to exhibit biologic activities in certain functional assays. However, there is now evidence that these molecules are unable to be assembled into oligomeric complexes, probably because of abnormal post-translational modifications. Also, little is known about the differentiation of human S-MBP and L-MBP, or the structure and function relationship between them, or about the hydroxylation of CLD and subsequent glycosylation of human MBPs.

To our knowledge, this is the first study demonstrating the large scale expression of functionally active human S-MBP and L-MBP in human hepatoma cell lines, using the vaccinia virus expression system. This system should prove a useful model for studying the synthesis and secretion of full-length and fully assembled MBPs, post-translational modifications of and differentiation into L and S-MBPs, and the structure-function relationships of proteins employing molecular mutagenesis.

The authors wish to thank Hiroko Yamaguchi for her excellent secretarial assistance.

REFERENCES

1. Persson, A., Rust, K., Chang, D., Moxley, M., Longmore, W., and Crouch, E. (1988) CP4: a pneumocyte-derived collagenous surfactant-associated protein. Evidence for heterogeneity of collagenous surfactant proteins. *Biochemistry* **27**, 8576-8584
2. Shimizu, H., Fisher, J.H., Papst, P., Benson, B., Lau, K., Mason, R.J., and Voelker, D.R. (1992) Primary structure of rat pulmonary surfactant protein D. cDNA and deduced amino acid

sequence. *J. Biol. Chem.* **267**, 1853-1857
3. Holmskov, U., Teisner, B., Willis, A.C., Reid, K.B.M., and Jensenius, J.C. (1993) Purification and characterization of a bovine serum lectin (CL-43) with structural homology to conglutinin and SP-D and carbohydrate specificity similar to mannan-binding protein. *J. Biol. Chem.* **268**, 10120-10125
4. Kawasaki, N., Kawasaki, T., and Yamashina, I. (1983) Isolation and characterization of a mannan-binding protein from human serum. *J. Biochem.* **94**, 937-947
5. Kurata, H., Sannoh, T., Kozutsumi, Y., Yokota, Y., and Kawasaki, T. (1994) Structure and function of mannan-binding proteins isolated from human liver and serum. *J. Biochem.* **115**, 1148-1154
6. Hoppe, H.J. and Reid, K.B.M. (1994) Trimeric C-type lectin domains in host defence. *Structure* **2**, 1129-1133
7. Yokota, Y., Arai, T., and Kawasaki, T. (1995) Oligomeric structures required for complement activation of serum mannan-binding protein. *J. Biochem.* **117**, 414-419
8. Thompson, C. (1995) MBP and innate immunity. *Science* **269**, 301-302
9. Jensenius, J.C. (1995) MBP and innate immunity (letter; comment). *Science* **270**, 1104
10. Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T., and Yamashina, I. (1987) Serum lectin with known structure activates complement through the classical pathway. *J. Biol. Chem.* **262**, 7451-7454
11. Ohta, M., Okada, M., Yamashina, I., and Kawasaki, T. (1990) The mechanism of carbohydrate-mediated complement activation by the serum mannan-binding protein. *J. Biol. Chem.* **265**, 1980-1984
12. Malhotra, R., Lu, J., Holmskov, U., and Sim, R.B. (1994) Collectins, collectin receptors and the lectin pathway of complement activation. *Clin. Exp. Immunol.* **97** (Suppl. 2), 4-9
13. Thiel, S., Vorup-Jensen, T., Stover, C.M., Schwaeble, W., Laursen, S.B., Poulsen, K., Willis, A.C., Eggleton, P., Hansen, S., Holmskov, U., Reid, K.B.M., and Jensenius, J.C. (1997) A second serine protease associated with mannan-binding lectin that activates complement. *Nature* **386**, 506-510
14. Matsushita, M. and Fujita, T. (1992) Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. *J. Exp. Med.* **176**, 1497-1502
15. Sato, T., Endo, Y., Matsushita, M., and Tujita, T. (1994) Molecular characterization of a novel serine protease involved in activation of the complement system by mannose-binding protein. *Int. Immunol.* **6**, 665-669
16. Ezekowitz, R.A.B., Kuhlman, M., Groopman, J., and Byrn, R. (1989) A human serum mannose-binding protein inhibits in vitro infection by the human immunodeficiency virus. *J. Exp. Med.* **169**, 185-196
17. Hartley, C.A., Jackson, D.C., and Anders, E.M. (1992) Two distinct serum mannan-binding lectins as β inhibitors of influenza virus: Identification of bovine serum β inhibitors as conglutinin. *J. Virol.* **66**, 4358-4363
18. Kurata, H., Chen, H.M., Kozutsumi, Y., Yokota, Y., and Kawasaki, T. (1993) Role of the collagen-like domain of the human serum mannan-binding protein in the activation of complement and the secretion of this lectin. *Biochem. Biophys. Res. Commun.* **191**, 1204-1210
19. Ma, Y., Yokota, Y., Kozutsumi, Y., and Kawasaki, T. (1996) Structural and functional roles of the amino-terminal region and collagen-like domain of human serum mannan-binding protein. *Biochem. Mol. Biol. Int.* **40**, 965-974
20. Kuhlman, M., Joiner, K.A., and Ezekowitz, R.A.B. (1989) The human mannose-binding protein functions as an opsonin. *J. Exp. Med.* **169**, 1733-1745
21. Super, M., Gillies, S.D., Foley, S., Sastry, K., Schweinle, J.E., Silverman, V.J., and Ezekowitz, R.A.B. (1992) Distinct and overlapping functions of allelic forms of human mannose-binding protein. *Nature Genet.* **2**, 50-55
22. Colley, K.J. and Baenziger, J.U. (1987) Biosynthesis and secretion of the rat core-specific lectin. Relationship of post-translational modification and assembly to attainment of carbohydrate binding activity. *J. Biol. Chem.* **262**, 3415-3421
23. Doi, I. (1976) Establishment of a cell line and its clonal sublines from a patient with hepatoblastoma. *Jpn. J. Cancer Res. (Gann)* **67**, 1-10
24. Chang, S. (1954) Continuous subcultivation of epithelial-like cells from normal human tissues. *Proc. Soc. Exp. Biol. Med.* **87**, 440-443
25. Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B. (1979) Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell lines. *Nature* **282**, 615-616
26. Fathi, Z., Shidhar, P., Pacha, R.F., and Condit, R.C. (1986) Efficient targeted insertion of an unselected marker into the vaccinia virus genome. *Virology* **155**, 97-105
27. Jin, N.Y., Funahashi, S., and Shida, H. (1994) Constructions of vaccinia virus A-type inclusion body protein, tandemly repeated mutant 7.5 kDa protein, and hemagglutinin gene promoters support high levels of expression. *Arch. Virol.* **138**, 315-330
28. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
29. Spiro, R.G. (1972) Study of the carbohydrates of glycoproteins. *Methods Enzymol.* **28**, 3-43
30. Heinrikson, R.L. and Meredith, S.C. (1984) Amino acid analysis by reverse-phase high performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Anal. Biochem.* **136**, 65-74
31. Liu, J., Volk, K.J., Kerns, E.H., Klohr, S.E., Lee, M.S., and Rosenberg, I.E. (1993) Structural characterization of glycoprotein digests by microcolumn liquid chromatography-ionspray tandem mass spectrometry. *J. Chromatogr.* **632**, 45-56
32. Drickamer, K., Dordal, M.S., and Reynolds, L. (1986) Mannose-binding proteins isolated from rat liver contain carbohydrate-recognition domains linked to collagenous tails. *J. Biol. Chem.* **261**, 6878-6887
33. Sastry, K., Zahedi, K., Lelias, J.M., Whitehead, A.S., and Ezekowitz, R.A.B. (1991) Molecular characterization of the mouse mannose-binding proteins. The mannose-binding protein A but not C is an acute phase reactant. *J. Immunol.* **147**, 692-697
34. Kivirikko, K.I. and Myllyla, R. (1985) Post-translational processing of procollagens. *Ann. N.Y. Acid. Sci.* **460**, 187-201
35. Colley, K.J. and Baenziger, J.U. (1987) Identification of the post-translational modifications of the core-specific lectin. The core-specific lectin contains hydroxyproline, hydroxylysine and glucosylgalactosylhydroxylysine residues. *J. Biol. Chem.* **262**, 10290-10295
36. Shinkai, H. and Yonemasu, K. (1979) Hydroxylysine-linked glycosides of human complement subcomponent C1q and various collagens. *Biochem. J.* **177**, 847-852
37. Rothmann, A.B., Mortensen, H.D., Holmskov, U., and Hojrup, P. (1997) Structural characterization of bovine collectin-43. *Eur. J. Biochem.* **243**, 630-635
38. Lu, J., Thiel, S., Widemann, H., Timpl, R., and Reid, K.B.M. (1990) Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme $C1r_2C1s_2$ complex, of the classical pathway of complement, without involvement of C1q. *J. Immunol.* **144**, 2287-2294