Molecular Cloning and Characterization of a Novel Mouse Macrophage C-Type Lectin, mMGL2, Which Has a Distinct Carbohydrate Specificity from mMGL1

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RUNNING TITLE: Characterization of mMGL2

SUMMARY

A novel mouse macrophage galactose-type C-type lectin 2 (mMGL2), was identified by BLAST analysis of expressed sequence tags. The sequence of mMGL2 is highly homologous to the mMGL, which should now be called mMGL1. The open reading frame of mMGL2 contains a sequence corresponding to a type II transmembrane protein with 332 amino acids, having a single extracellular C-type lectin domain. The 3' untranslated region included long terminal repeats of mouse early transposon ETn. The *mMGL2* gene was cloned from a 129/SvJ mouse genomic library and sequenced. The gene spans 7,136 base pairs and consists of 10 exons, which is similar to the genomic organization of mMGL1. The RT-PCR analysis indicates that mMGL2 is expressed in cell lines and normal mouse tissues in a macrophage-restricted manner, also very similar to that of mMGL1. The mMGL2 mRNA was also detected in mMGL1-positive cells, which were sorted from thioglycollate-induced peritoneal cells with a mMGL1 specific monoclonal antibody, LOM-8.7. The soluble recombinant proteins of mMGL2 exhibited carbohydrate specificity for α- and β-GalNAc-conjugated soluble polyacrylamides, whereas mMGL1 preferentially bound Lewis X-conjugated soluble polyacrylamides in solid phase assays. These two lectins may function cooperatively as recognition and endocytic molecules on macrophages and related cells.

INTRODUCTION

Macrophages (MØs) and related cells are widely distributed throughout the body, displaying a morphological and functional diversity. They are found in the lymphoid organs, liver, lungs, gastrointestinal tract, central nervous system, serous cavities, bones, synovia, and skin. Resident MØs mediate clearance of senescent or apoptotic cells, produce and secrete cytokines, are involved in hemopoiesis and bone resorption, transport and present antigens, and regulate neuroendocrine processes. Activated MØs are recruited to sites of infection, tissue injury, inflammation, and neoplasia and play crucial roles in tissue repair and pathogenesis (1).

The distribution and functional heterogeneity of MØs derive in part from their specialized plasma membrane receptors (2). Cell surface markers such as F4/80, sialoadhesin, MØ mannose receptor (MMR) and scavenger receptor (SR-AI/SR-AII) have significantly contributed to the current understanding of MØ ontogeny and function (3). Yet, in comparison to other immune cells such as B and T lymphocytes, relatively few MØ-restricted cell surface molecules have been identified. The physiological and pathological roles of these putative markers remain unknown.

Protein-carbohydrate interactions serve a variety of functions in the immune system. A number of lectins (carbohydrate-binding proteins) mediate both pathogen recognition and cell-to-cell interactions using structurally-related carbohydrate recognition domains (CRDs). One of the most diversified families of these CRDs is Ca^{2+}-dependent and termed C-type CRDs (4, 5). MØs and related cells such as dendritic cells are known to express several subfamilies of C-type lectins: type 1 multilectin such as MMR, and lectins having type II transmembrane configurations such as MØ galacotose-type (previously known as galactose/*N*-acetylgalactosamine-specific) C-type lectin (MGL). These lectins seem to mediate carbohydrate-specific endocytosis (6-11). Little is known regarding the expression of selectins, another subfamily of C-type lectins, in MØs and related cells.

MGL was previously cloned from rats, mice, and humans, and characterized. This lectin from mice was shown to have specific affinity for highly-branched N-linked carbohydrate chains with terminal β-galactosyl groups, and glycopeptides carrying three constitutive α-GalNAc-Ser/Thr (Tn-antigen) (12). A human MGL was

also shown to have affinity for glycopeptides carrying three constitutive Tn-antigens (13). Surface plasmon resonance revealed that affinity of recombinant hMGL for immobilized glycopeptides increased in parallel with the number of GalNAc residues(14). Tn-antigen was known as a marker of malignant cells and MGL was shown to play a role as a recognition molecule on MØs for tumor cells (15-17). Immunohistochemical localization of mouse MGL (mMGL) with specific monoclonal antibodies revealed that this lectin has a strong association with MØs residing in connective tissue and those infiltrated into tumor tissues (18-20). Recent studies demonstrated that this lectin is also expressed on the surface of immature dendritic cells and is involved in the uptake of glycosylated antigens in mice and humans (21, 22).

A C-type lectin highly homologous to MGL and expressed mainly on hepatocytes, the hepatic asialoglycoprotein receptor, has two isomers in mice, rats, and humans, apparently due to recent gene duplication. MGL was previously believed to have a single gene. Southern blotting analysis supported this notion. However, in this paper we describe the properties of another novel MØ galactose-type C-type lectin, mMGL2. As an obvious consequence, the previous mMGL must now be called mMGL1. These lectins are highly homologous to each other except in their cytoplasmic domains and CRDs. We found that mMGL2 has a distinct carbohydrate specificity from mMGL1. These lectins seem to be expressed on the same cells and therefore seem to function cooperatively.

EXPERIMENTAL PROCEDURES

Cells- The following cell lines were provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer Tohoku University: L929, JLS-V9, EL4, RL♂1, YAC-1, BCL1-B20, P815, P388, and M1 cells. RAW 264.7 cells were purchased from ATCC. All cells were cultured in RPMI1640 media with 10% fetal calf serum (FCS) at 37°C with 5% CO_2.

Thioglycollate-induced peritoneal cells (TG-PEC) were obtained from 6-8 weeks old female C57BL/6J mice from Clea Japan, Inc (Kawasaki, Japan). These were maintained under pathogen-free conditions. These

mice received 1 ml of 4% thioglycollate broth (Difco, Detroit, MI) by intraperitoneal injection. Four days later, all mice were sacrificed by neck dislocation, and peritoneal exudate cells were harvested by lavage with 5 ml of RPMI1640 media on ice.

TG-PEC were suspended in chilled 0.1% BSA/Dulbecco's modified phosphate-buffer saline (DPBS: 137 mM NaCl, 13.4 mM KCl, 40.5 mM $Na_2HPO_4 \cdot 12H_2O$, 7.35 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.905 mM $CaCl_2$) containing phycoerythrin-labeled mAb LOM-14 (reactive with mMGL1 and mMGL2 as described below), biotin-labeled mAb LOM-8.7 (reactive with mMGL1), and then incubated with streptavidin-fluorescein isothiocyanate (Zymed, South San Francisco, CA) on ice. The cells were sorted for the expression of mMGL1 (mAb LOM-8.7 staining) by Epics Elite (Beckman Coulter, Fullerton, CA).

Mice- *Mgl1-/-* mice were prepared in Hedrick's laboratory at the University of California, San Diego. The detail will be published in a separate paper (23).

RNA preparations and RT-PCR analysis- Total RNAs were extracted by using Ultraspec RNA zol (Biotecx, Houston, TX), according to the manufacturer's instructions. First-strand cDNA synthesis was carried out using oligo $(dT)_{12\text{-}18}$ and Superscript II (Gibco BRL, Rockville, MD). The cDNA was used as the template in PCR reactions using Ampli Taq Gold polymerase (Applied Biosystems). PCR was performed with specific primers for mMgl1 (5'-TCTCTGAAAGTGGATGTGGAGG-3', 5'-CACTACCCAGCTCAAACACAATCC-3'), mMgl2 (5'-TCTCTGAAAGTGGATGTGGAGG-3', 5'-GCTATAAGTTGTGGGGAGTGGGC-3'), and G3pdh (5'-TGAAGGTCGGTGTGAACGGATTTGGC-3', 5'-CATGTAGGCCATGAGGTCCACCAC-3'). The specific primers were designed with Genetyx-Mac (Software Development Co. Ltd., Tokyo). The conditions were 94°C for 30 sec, 65°C for 30 sec, and 72°C for 2 min for 40 cycles. Amplifications of G3pdh were used as a control for cDNA quantity. The condition was 94°C for 30 sec, 61°C for 30 sec, and 72°C for 1 min for 25 cycles. The PCR products were then separated on 1% agarose gels, stained with ethidium bromide and visualized with the Fluor-S image analyzer (Bio-Rad, Hercules, CA).

Rapid Amplification of cDNA Ends (RACE)- The poly(A)$^+$ RNA from RAW 264.7 cells were isolated using a μMACS mRNA isolation kit (Miltenyi Biotec, Germany). Adapter-ligated cDNA were synthesized using the Marathon cDNA amplification kit (Clontech Laboratories UK Ltd., Basingtoke, UK). To obtain a full-length cDNA of mMGL2, an antisense primer, AS3 (5'-TCCTCCACATCCACTTTCAGAG-3') and a sense primer, 3S1 (5'-TTGGAGCGGGAAGAGAAAAACCAG-3') were designed from the EST clones for 5'-RACE and 3'-RACE reactions, respectively. Reactions were incubated at 95°C for 10 min, followed by 43 cycles at 94°C for 45 s, 60°C for 45 s, and 72 °C for 2 min. The resulting 1.5 kbp and 700 bp products were subcloned into the pGEM-T easy vector (Promega UK, Southampton, UK) and sequenced with the dye-primer method.

Screening of the genomic clone coded the mMgl2 gene- A 129/SvJ mouse genomic library (Stratagene; vector λFIXII) was screened with the complete mMGL1 cDNA coding sequence labeled by random priming with ^{32}P by plaque hybridization. The fragments of positive clones were subcloned into pBluescript SK(+) (Stratagene). These clones were sequenced with 24 specific primers with the dye-terminator method. These sequences were assembled and aligned using Genetyx-Mac.

Production and Isolation of Soluble mMGL2- cDNA encoding mMGL1 and mMGL2 was cloned into pGEM-T easy vector (Promega). The vector was digested with *Bam* H I and *Sac* I (for mMGL1) or *Bam* H I and *Not* I (for mMGL2). The fragments encoding these neck and CRD domains were separated with agarose gel electrophoresis and inserted into each site of expression plasmid vector pET-21a (Novagen). The BL21(DE3) cells containing the plasmid were grown to mid log-phase at 37°C in 2 X TY medium (1.2 L) and then treated with isopropyl β-D-thiogalactoside (IPTG) at a concentration of 1 mM. After IPTG induction, the cultured cells were washed with 50 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl (TBS) and suspended in TBS containing phenylmethane-sulfonyl fluoride. The cell lysates were prepared by freezing and thawing, and after the addition of DNase I (final 50 U/ml) and lysozyme (final 0.2 mg/ml), incubated 1 h at 37°C, centrifuged at 15,000 g for 10 min at 4°C. Expressed proteins formed inclusion bodies. The pellets were washed with TBS containing 0.5% Triton X-100 and 10 mM EDTA, and then with H_2O. The washed pellets were solubilized with 2 M NH_4OH (20

ml) and then added to 25 mM MOPS buffer, pH 7.0, containing 2 mM glutathione, reduced form, 0.2 mM glutathione, oxidized form, 20 mM $CaCl_2$, 0.5 M NaCl, and 0.02% NaN_3 (20 ml). These solutions were then dialyzed against 25 mM MOPS buffer, pH 7.0, containing 20 mM $CaCl_2$, 0.5 M NaCl, and 0.02% NaN_3. Soluble recombinant mMGL1 and mMGL2 were purified by affinity-chromatography on a column of galactose-Sepharose 4B, as described previously (24).

Solid Phase Binding Assays- Absorption of the purified soluble recombinant mMGL1 or mMGL2 onto ELISA plates (655061, Griner, Germany) was carried out by adding 100 μl of solution (3 μg/ml in DPBS) to each wells and incubating the plates for 18 h at 4°C. After blocking of the wells using 3% bovine serum albumin (BSA) in DPBS for 2 h at room temperature, 150 μl solution of biotinylated soluble polyacrylamide with attached mono- or oligosaccharides (GlycoTech, Rockville, MD) or hybridoma culture supernatant, diluted into varying concentrations with DPBS containing 3% BSA, were added to each well. After incubation for 2 h at 4°C (at room temperature for mAb reactions), the wells were washed three times with DPBS to remove unbound materials, and then 100 μl of HRP-conjugated streptavidin solution (1.25 μg/ml in DPBS), or HRP-conjugated goat anti-rat IgG (H+L) solution (0.375 μg/ml in DPBS) for rat mAbs, was added to detect bound materials. After incubation for 1 h at room temperature, the wells were washed three times with DPBS. Subsequently, 100 μl of 1 mM 2, 2'-amino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ammonium (ABTS) solution containing 0.34% H_2O_2 in 0.1 M sodium citrate buffer (pH 4.3) was added and the absorbance was measured at 405 nm on a microplate reader (25).

To determine pH dependence of the binding of mMGL1 and mMGL2, the incubation conditions were modified as follows. After the blocking buffer was removed, the wells were washed three times with DPBS. Aliquots (50 μl) of biotinylated soluble polyacrylamide in 2 X incubation buffer (274 mM NaCl, 26.8 mM KCl, 0.98 mM $MgCl_2$, 1.810 mM $CaCl_2$, 2% BSA) were mixed with equal volumes of 2 X pH buffer (50 mM sodium acetate buffer at pH 4.5-6.0, 50 mM MES buffer at pH 6.0-7.0, 50 mM Tris-HCl buffer at pH 7.0-8.0) and transferred to each well. Incubation was performed at 4°C for 1.5 h. The wells were emptied and washed three times with DPBST (DPBS contained 0.1% Tween-20), and 100 μl of HRP conjugated streptavidin solution (Zymed, 1000 X

in DPBS contained 1% BSA) was added and incubated for 1 h at 4°C. The wells were emptied and washed three times with DPBST, and then 100 μl of ABTS solution containing 0.034% H_2O_2 (1 mM ABTS dissolved in 0.1 M sodium citrate buffer, pH 4.2) was added and absorbance at 405 nm was determined.

Immunohistochemical staining-- MGL-positive cells were immunohistochemically detected in the skin as described previously (19). In brief, skin samples freshly prepared from Mgl1-/- mice and their littermates were embedded in O.C.T. Compound (Miles, Elkhart, IN) and frozen in a liquid nitrogen bath. Cryostat sections (10 μm thick) were picked up on poly-L-lysine-coated slides, and fixed in ice-cold acetone for 10 min. Nonspecific bindings were blocked using a blocking solution (2% normal mouse serum and 3% BSA in DPBS) for 10 min. The sections were treated with the first antibodies for 1 h, then with biotinylated mAb mouse anti-rat κ and λ (1/50 dilution) for 30 min, and finally with alkaline phosphatase-streptavidin (1/100 dilution) for 30 min. The staining was visualized using Histomark Red, and the cell nucleus was counterstained in Mayer's hematoxylin after post-fixation using 2% glutaraldehyde in DPBS. The sections were observed under a light microscope (TMD-300, Nikon, Tokyo, Japan).

RESULTS

Cloning of a Novel Macrophage C-type Lectin- We found four clones (AA511511, AA537107, AA671707, and AA498512) similar to mMGL (mMGL1) (S36676) and hMGL (HML-2) (D50532) in the database of mouse EST. To obtain the full sequences of a novel C-type lectin, 5' and 3'-RACE reactions were performed using mRNA from RAW264.7 cells as a template and with specific primers designed to match these EST clones. The full-length cDNA was prepared by RT-PCR of poly(A)+ RNA from RAW264.7 cells. The obtained full-length cDNA (GneBank® accession number AY103461) encodes an open reading frame of 996 base pairs, predicting a protein of 332 amino acid residues (38,067 Da), which we subsequently termed mMGL2 (Fig. 1). The 3'-untranscription region has a sequence of LTR (long terminal repeat) of mouse early transposon (ETn) (26, 27).

The nucleotide sequence of mMGL2 has 79.0% and 54.9% identity with that of mMGL1 and hMGL (HML-2), respectively. The amino acid sequence of mMGL2 has 91.5% and 51.8% identity with that of mMGL1 and hMGL (HML-2), respectively. The neck domain is highly homologous to mMGL1 at 95.3%. Only five residues (Ile 87, Asn 108, Leu 144, Glu 166, and Thr 167) were different in the neck domain from Arg 78 to Gly 183. Amino acids within the CRD (corresponding to exons 8-10) particullarly those corresponding to the last exon (exon 10) showed differences between mMGL1 and mMGL2. In the sequence of cytoplasmic domain (corresponding to exon 2), mMGL2 has a putative internalization signal (YxxΦ) (28) which is also present in mMGL1 and an insertion of extra 14 amino acid residues. A consensus sequence for polyadenylation signal is present in nucleotides 1467-1472 followed by a poly(A) tail. There are two potential N-glycosylation sites (Fig. 1) both in mMGL1 and 2.

Genomic Structure and Chromosome Location of mMGL2- Screening of a 129/SvJ mouse genomic library lead to the isolation of three clones. Two clones (termed 92b and 41b) were included in the *Mgl* (*Mgl1*) gene (AF132744) (29), but the other clone (termed 41a) was included in the *Mgl2* gene, confirmed by PCR using primers of mMGL2. The latter gene spans 7136 bp and consists of 10 exons, and is similar to *Mgl* (*Mgl1*) in genomic organization (Fig. 2A). GneBank® accession number of MGL2 gene is AY103462. The intron/exon boundaries were defined using DNA sequencing. All splice sites conform to the AG/GT rule (Table 1). This clone contains 5'-upstream sequences. In order to identify the promoter sequences of the *Mgl2* gene, transcription factor binding site consensus sequences were searched using the transcription factor database (http://www.cbrc.jp/research/db/TFSEARCH.html). The promoter lacks a classical TATA box but contains several binding sites for other transcription factors, C/EBPβ, CF1/, AML-1, c-Ets, PU.1,c-Rel, Oct-1, Lyf-1, AP-1, GATAs, and STATs. These features are almost identical to that of the 5' flanking motifs of the *Mgl1* gene. These are the sites found upstream of genes expressed preferentially by cells of monocyte/MØ lineages (Fig. 2C).

We identified the BAC clone #RPCI-23-172M21, which coded *Mgl2*, *Asgr1*, and *Asgr2* genes. The other clone #RPCI-23-198E14 encodes *Mgl2* and *Mgl1* genes. These BAC clones have been constructed from genomic DNA of female C57BL/6J mice. Therefore, these four C-type lectin genes should be located within

about 150 kb on mouse chromosome 11 (Fig. 2B).

Reactivity with Monoclonal Antibodies- We have previously obtained mAbs specific for MGL1 using purified mMGL (likely to be a mixture of mMGL1 and mMGL2) and recombinant MGL1 as immunogens. Screening to obtain specific monoclonal antibodies was previously performed with recombinant mMGL1 (25). The antibodies are mAb LOM-4.7, mAb LOM-8.2, mAb LOM-8.7, all shown to have blocking activity, mAb LOM-11 shown to recognize the ligand induced binding site, and a non-blocking mAb LOM–14 (25, 30). As shown in Figure 3, mAb LOM-4.7, mAb LOM-8.2, mAb LOM-8.7, and mAb LOM-11 were shown to be specific for MGL1. LOM-14 bound to both MGL1 and MGL2 (Fig. 3). These results suggested that the most diverse region of these lectins was the ligand binding site.

Distribution of mMgl2 mRNA in Tissue and Cells- As a means of testing the expression profiles of this putative gene and *mMgl1*, RT-PCR analysis was performed on 10 mouse cell lines including L929 (fibroblast line), JLS-V9 (fibroblast-like bone marrow derived cell line), EL4 (thymoma cell line), RL♂1 (lymphoma cell line), YAC-1 (lymphoma cell line), BCL1-B20 (malignant B cells), P815 (mastocytoma cells), P388 (MØ-like lymphoid cell line), M1 (myeloblastic leukemia cells), and RAW 264.7 (MØ-like cell line). The 644-bp bands indicating mMgl2 were found only in the cell lines P388 and RAW 264.7 (Fig. 4A). These cell lines were also high expressers of mMgl1. To assess the expression patterns of mMgl1 and mMgl2 in vivo, RT-PCR analysis was conducted on RNA isolated from 13 different normal mouse tissues and embryos (Fig. 4B). These genes were expressed almost throughout the body and the apparent relative intensities among different organs were similar between mMgl1 and mMgl2. To assess whether the expression levels of these genes correspond at single cell levels, TG-PECs were sorted for the binding of mAb LOM-8.7, an mAb specific for mMgl1, then reacted with mAb LOM-14 reactive with both mMgl1 and mMgl2. As shown in Figure 4C, cells strongly reactive with mAb LOM-8.7 were also reactive with mAb LOM-14. The ratios of expressed mRNAs corresponding to *mMgl1* and *mMgl2* shown by RT-PCR analysis were almost identical when unsorted mAb LOM-8.7-positive and mAb LOM-8.7-negative cells were compared. These results indicate that the *mMgl1*-positive cells also express the *mMgl2*

mRNA.

mMGL2-Positive Cells in Tissue Sections from Mgl1-/- Mice- Frozen sections of skins from *Mgl1-/-* mice were stained with mAb LOM-8.7 and mAb LOM-14 (Fig. 5). The results revealed that *Mgl1-/-* mice expressed epitopes reactive with mAb LOM-14 at lower levels than *Mgl1+/-* mice but not with mAb LOM-8.7. These epitopes are likely to represent mMGL2. The subcellular localization of the mAb LOM-14 staining in *Mgl1-/-* mice did not appear to be distinct from that of mAn LOM-8.7 staining in *Mgl1+/-* mince.

Carbohydrate Specificity of mMGL2- A variety of biotin-labeled soluble polyacrylamides with mono- or oligosaccharides were applied to determine the carbohydrate specificity of mMGL1 and mMGL2 (Fig. 6). The carbohydrates tested are listed in the figure. The recombinant forms of mMGL1 had the highest affinity with Le^X residues among all carbohydrate-modified polyacrylamides tested. mMGL2 showed a very low affinity with Le^X, but showed the highest affinity with β-linked GalNAc residues (Fig. 6). The bindings were inhibited with 5 mM EDTA (data not shown).

pH-dependent Binding of Ligands to mMGL- ELISAs were performed under various pH conditions to determine the binding of soluble polyacrylamides with Le^X residues or β-GalNAc residues to immobilized recombinant mMGL1 or mMGL2 respectively. Under a Ca^{2+} concentration similar to that found in extracellular fluids (0.905 mM), the binding decreased when pH was lowered from extracellular (pH 7.3) to endosomal (pH 5.4). The profiles of the pH-dependent binding were similar to that observed with intact hepatic asialoglycoprotein receptors (31). The pH value of a half-maximal ligand binding, designated as pH_B, was 5.5 for MGL1 and 5.7 for MGL2 (Fig. 7).

DISCUSSION

In the present study, we found a novel MØ C-type lectin, mMGL2, and characterized its genomic structure, expression patterns, and carbohydrate specificity. The *Mgl2* gene spans 7136 bp and consists of 10 exons, which is a similar genomic organization to that of the *Mgl* gene now renamed the *Mgl1* gene. We reported that the *Mgl1* gene linked to *Trp53* and is located 1.8 ± 1.2 cM distal to *D11Mit5* and 1.8 ± 1.2 cM proximal to *Htt* on mouse chromosome 11. Using a panel of DNA samples from two parental mice, C3H/HeJ-*gld* and (C3H/HeJ-*gld* X *Mus spretus*) F1 were digested with various restriction endonucleases and hybridized with a mMGL1 cDNA probe to determine the restriction fragment length polymorphism and to allow haplotype analyses (29). The hepatic asialoglycoprotein receptor genes (*Asgr1* and *Asgr2*) were also known to be linked to *Trp53* (32). We found BAC clones which included these two C-type lectins. These results indicate that the highly homologous *Mgl2* gene is linked within about 50 kb. Their homology and close genomic localization indicate that the original gene was duplicated recently. The human *MGL* (*HML-2*) gene was mapped in chromosome 17p and linked to *ASGR1* and *ASGR2.* These results show that there is a cluster of type 2 C-type lectin genes on mouse chromosome 11 and on human 17p12-13 Likewise, the natural killer gene complex resided on mouse chromosome 6 and on human 12p (33, 34).

Although there was a high degree of sequence homology between mMGL1 and mMGL2, the sequences corresponding to cytoplasmic and CRD showed differences, suggesting that cytoplasmic tail associations and the carbohydrate recognitions are unique between mMGL1 and mMGL2. We showed that recombinant mMGL1 bound soluble polyacrylamide containing Le^X oligosaccharides. mMGL2 had affinity with polyacrylamide containing α- and β-GalNAc. It has been believed that C-type lectins contain a peptide segment that corresponds to the carbohydrate-binding specificity within the CRD, and that the sequence in Gal and GalNAc-binding lectins, such as ASGRs, is Gln-Pro-Asp (QPD). In contrast, the sequence in mannose, fucose, and GalNAc-specific lectins, such as serum mannose-binding proteins, was Glu-Pro-Asn (EPN). The sequence in both mMGL1 and 2 was QPD. However, it was obvious from our results that their fine specificities depended on other amino acids in the CRD (Figs. 6 and 8). Some of the amino acid residues important in the carbohydrate recognitions were conserved in mMGL1, 2, hMGL, RHL-1 and 2, mASGR-1, and hASGR-1. Crystallographic determination of the structure of human ASGR-1 revealed amino acid residues important in sugar binding (35).

Furthermore, rat hepatic asialoglycoprotein receptor was subjected to site directed mutation. As a result, the pH dependence of ligand binding was shown to be mediated by His 256, Asp 266, and Arg 270 (36). Because these residues are also conserved in mMGL1 and 2, they should also be responsible for their pH-dependency (Fig. 8). Amino acid residues responsible for the differential carbohydrate specificity between mMGL1, 2, and other members of C-type lectin family and phylogeny of these lectins, (Fig. 8) are yet to be elucidated.

There is a possibility that mMGL1 and 2 form heterooligomers. These lectins are known to form trimeric structures through the interactions of the stalk domain in a similar manner to that of ASGRs. ASGRs are abundantly expressed on the sinusoidal surface of hepatic parenchymal cells (37, 38). Its primary role is the removal and degradation of desialylated glycoproteins from circulation. High-affinity binding requires the receptor to be assembled as a heterooligmer consisting of two highly homologous subunits, termed hepatic lectin 1 and 2 (39). Experiments with recombinant rat hepatic lectins suggested that the binding properties of the major subunit (RHL-1) and minor subunit (RHL-2/3) were optimized for different ligands (40). In the case of mMGL1 and 2, the recombinant form corresponding to each one showed affinity with different carbohydrates, indicating that hetero-oligomer formation was not required for their carbohydrate recognition. However, these lectins were likely to be expressed on the same cells at the single cell level and cooperatively functioned to recognize and uptake extracellular molecules.

mMGL1-positive cells are abundant in connective tissues throughout the body (19). mMGL1 was shown to be expressed on the surfaces of bone marrow-derived immature dendritic cells (21). hMGL is also shown to be expressed on monocyte-derived immature dendritic cells and monocyte-derived immature MØs in humans (41) We have shown that mMGL1-positive cells migrate from dermis to regional lymph nodes during the sensitization phase of contact hypersensitivity (42). The migration was initiated by cytokine-mediated release of mMGL1-positive cells from dermis (42-44). The cells homed to the boundary of the T-cell area in the regional lymph nodes and the prevention of migration seemed to interfere with sensitization. Involvement of coordinated functions of mMGL1 and 2 in such pathogenic processes should be the most important subject of future investigations.

FOOTNOTES

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The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank®/EBI Deta Bank with the accession numbers AY103461 for mMGL2 mRNA and AY103462 for *Mgl2* gene.

Abbreviations used in this paper: ABTS, 2,2'-amino-bis(3-ethylbenzthiazoline-6-sulfonic acid); ASGR, hepatic asialoglycoprotein receptor; BAC, bacterial artificial chromosome; BLAST, basic local alignment search tool; BSA, bovine serum albumin; C-type lectin, calcium-type lectin; CRD, carbohydrate-recognition domain; DC, dendritic cell; DPBS, Dulbecco's phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tag; FCS, fetal calf serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; Gal, galactose; GalNAc, *N*-acetyl-D-galactosamine; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactopyranoside; MGL, macrophage galactose-type C-type lectin; MØ, macrophage; MMR, macrophage mannose receptor; RHL, rat hepatic lectin; RT-PCR, reverse transcriptase-polymerase chain reaction; TG-PEC, thioglycollate-induced peritoneal cell.

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TABLE 1: Splice junction sequences, exon sizes, and estimated intron sizes of the mouse *Mgl2* gene.

Exon No.	Position[a]	Size (bp)	Exon/Intron Junction Donor Sequence	Acceptor Sequence	Intron Size (bp)	Intron Phase
1	1~23	23	-	CACAGgtaac	3684	-
2	24~168	145	ttcagTTCTG	TCTAAgtatg	787	1
3	169~279	111	agcagTTCCT	CCAAAgtggg	428	1
4	280~375	96	atcagATTCC	CAGGGgtgag	83	1
5	376~447	72	tgcagCTGAC	GGCAGgtgag	79	1
6	448~519	72	ttcagGCCGA	AACAGgtgag	258	1
7	520~606	87	cccagATCTG	CAATGgtgag	103	1
8	607~758	152	cgtagGCTCG	AGCAGgtgat	77	0
9	759~865	107	tttagAATTT	TTCAAgtacg	144	2
10	866~1530	629	ttcagGAATT	-		

Note: Exon sequences are in capital letters; intron sequences are in lowercase letters. [a]Exon positions in coding sequence.

FIGURE LEGENDS

Fig. 1: The nucleotide sequence of the mMGL2 cDNA. (A) The nucleotide sequence of the mMGL2 cDNA and its deduced amino acid sequence. The putative transmembrane domain is underlined. The poly-A addition signal is underlined with a broken line. The potential N-glycosylation site is underlined with a wavy line. The putative internalization signal is boxed. **(B)** Amino acid sequences of mMGL1 and mMGL2. Asterisks mark the amino acid residues conserved in both lectins. Arrows indicate boundaries of exons.

Fig. 2: Genomic structure of *Mgl2* gene. (A) The gene spans 7136 bp and consists of 10 exons, and is a similar to *Mgl1* in genomic organization. The UTR regions are shown as closed boxes and the coding regions are shown as hatched boxes. The initiation codon is within the second exon. **(B)** The linkage of BAC clones. We identified the BAC clone RPCI-23-172M21, which encodes the *Mgl2*, *Asgr1* and *Asgr2* genes. RPCI-23-198E14 encodes *Mgl2* and *Mgl1*. **(C)** The promoter sequences of *Mgl2 gene*. Transcription factor binding site consensus sequences were searched using the transcription factor database (http://www.cbrc.jp/research/db/TFSEARCH.html).

Fig. 3: Binding of mAbs to the recombinant mMGL1 and mMGL2. The binding of mAbs LOM-4.7, LOM-8.2, LOM-8.7, LOM-11, and LOM-14 to the immobilized recombinant mMGL1 **(A)** and mMGL2 **(B)** was measured according to mAb concentration. The binding of mAbs was detected using HRP-conjugated goat mAbs specific for rat IgG(H+L). Absorbance at 405 nm was measured on a microplate reader. The values represent means of triplicate determinations and the error bars indicate SD.

Fig. 4: RT-PCR analysis of *mMGL1* and *mMGL2* mRNA expression in cells and tissue. (A) The cDNA were prepared from mouse cell lines (L929, JLS-V9, EL4, RL♂1, YAC-1, BCL1-B20, P815, P388, RAW 264.7, and M1). **(B)** Tissue cDNA (CLONTECH) was used after normalizing the relative amounts according to the levels of G3PDH cDNA. **(C)** Cells in peritoneal exudates (PEC) after elicitation with thioglycolate (TG-PEC)

were analyzed for the binding of mAbs LOM-14 and LOM-8.7. The cells highly or poorly reactive with mAb LOM-8.7 were separated and tested for mAb LOM-14 binding by flow cytometry. cDNA was prepared from the sorted cells, and RT-PCR analysis was performed. The PCR products were then separated on 1% agarose gels, stained with ethidium bromide and visualized with the image analyzer. The ratios of band intensity of PCR products were measured. The panels represent a typical result of two separate experiments including cell fractionations. The results of these two separate experiments were almost identical.

Fig. 5: Distribution of cells expressing mMGL1 and mMGL2 in skins. Frozen sections of skins from *Mgl1*$^{-/-}$ and *Mgl1*$^{+/-}$ mice were stained with mAb LOM-8.7 (specific for mMGL1) and mAb LOM-14 (cross-reactive between mMGL1 and mMGL2).

Fig. 6: Carbohydrate specificity of recombinant mMGL1 and mMGL2. Binding of biotin-labeled soluble polyacrylamides containing mono- or oligosaccharides to immobilized recombinant mMGL1 **(A, C)** and mMGL2 **(B, D)**. Amounts of bound polymers incubated at various concentrations were quantified using HRP-conjugated streptavidin. ABTS was used as a substrate and absorbance at 405 nm was measured on a microplate reader. The panels represent a typical result of three separate determinations, which showed almost identical results. Each assay was performed in duplicate and the mean value is shown.

Fig. 7: Effects of pH on binding of recombinant mMGL1 and mMGL2. The binding of biotin-labeled soluble polyacrylamides (0.22 μg/ml Le^x-polyacrylamide or 0.67 μg/μl α-GalNAc-polyacrylamide) to the immobilized recombinant mMGL1 **(A)** or mMGL2 **(B)** was determined under varied pH conditions. The assays were performed in the presence of 0.905 mM $CaCl_2$. The values obtained in different buffers (25 mM sodium acetate buffer for pH 4.5-6.0, 25 mM MES buffer for pH 6.0-7.0, 25 mM Tris-HCl buffer for pH 7.0-8.0). The panels represent a typical result of two separate determinations, which showed almost identical results. The values represent means of duplicate determinations.

Fig. 8: Comparison of the amino acid sequences of the CRD of C-type lectins. (A) Multi-alignment of the CRD sequences of C-type lectins. (**B)** Evolutionary tree of sequence of CRD of C-type lectins. Abbreviations used in this figure are: mMGL, mouse macrophage galactose-type C-type lectin; rMGL, rat macrophage galactose-type C-type lectin; hMGL, human macrophage galactose-type C-type lectin previously termed as HML; hASGR, human hepatic asialoglycoprotein receptor; RHL, rat hepatic lectin; MHL, mouse hepatic lectin; rKCR, rat Kupffer cell receptor; mKCR, mouse Kupffer cell receptor.