L-MBP Is Expressed in Epithelial Cells of Mouse Small Intestine

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The mannan-binding proteins (L-MBP and S-MBP, also denoted MBL-C and MBL-A), mainly produced in liver and existing in liver and serum, play important roles in the innate immunity against a variety of pathogens. Total RNA from mouse tissues were screened for MBP mRNA by RT-PCR. In addition to liver, S-MBP mRNA was detected in lung, kidney, and testis, and L-MBP mRNA was detected in kidney, thymus, and small intestine. Quantitative RT-PCR revealed that the small intestine is a predominant site of extrahepatic expression of L-MBP. Western blotting with polyclonal Abs against rat L-MBP demonstrated this protein in Triton X-100 extracts of the small intestine obtained from mice that had undergone systemic perfusion. Immunohistochemical staining with an mAb against mouse L-MBP and in situ hybridization revealed that L-MBP is selectively expressed in some villous epithelial cells of the small intestine. These findings suggest that L-MBP plays a role in mucosal innate immunity.
The resulting cdNA, corresponding to 100 ng of total RNA, was subjected to PCR amplification in a total volume of 25 μl AmpliTaq buffer (PE Applied Biosystems, Foster City, CA) including 0.2 μM of each primer, 0.32 mM of each dNTP, and 2.5 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems). The primers used for amplification of the S-MBP cDNA were as follows: 5′-GGGAAATTGGGGCCTCCAGG-3′ (S-MBP sense) and 5′-CAGTCTTCCTCCAGGACCTATG-3′ (S-MBP antisense), which amplified a 452-bp fragment corresponding to nt 307–758 (3). The primers used for amplification of the L-MBP sequence were 5′-GCAGTTCTCAGGGCTGAAATCA-3′ (L-MBP sense) and 5′-AGGCCGAGGGAATGAGGAGCAG-3′ (L-MBP antisense), which amplified a 262-bp fragment corresponding to nt 303–564 (3). β-Actin was used as a control. The primers used for amplification of the β-actin sequence were 5′-AAGAGGTATGAGCTGCTTGTA-3′ (β-actin sense) and 5′-CAGGAGGAGCAATGATCCTG-3′ (β-actin antisense), which amplified a 270-bp fragment corresponding to nt 811-1080 (19). Oligonucleotide primers were purchased from Genset (Kyoto, Japan). The PCR conditions used for amplification of the S-MBP cDNA were as follows: 98°C for 4 min, followed by 35 cycles of 98°C for 30 s (denaturation), 64°C for 1 min (annealing), and 72°C for 1 min (extension), with 3-min incubation at 72°C after the last extension cycle. The conditions used for L-MBP and β-actin were the same as those used for S-MBP, except that the annealing temperatures were 59 and 60°C, respectively. The PCR products were run on 2% agarose gels and visualized by staining with ethidium bromide. For samples positive for S-MBP or L-MBP cDNA, the PCR products were run on 2% agarose gels and visualized by staining with ethidium bromide. For samples positive for S-MBP or L-MBP cDNA, the PCR products were run on 2% agarose gels and visualized by staining with ethidium bromide.
Frozen sections (10 or 16 μm) of mouse tissues were mounted on glass slides with positively charged surfaces (MAS-coated slides; Masumani Glass, Kishiwada, Japan) and stored at −80°C until use. The sections were fixed in PBS containing 4% formaldehyde for 30 min at room temperature, rinsed in PBS, and then treated with 5 μg/ml of proteinase K in 20 mM Tris-HCl, pH 7.4, for 5 min at 37°C. After rinsing in PBS, the sections were immersed in 0.25% acetic anhydride in 0.1 M triethanolamine/0.09% NaCl for 10 min and then dehydrated through an ethanol series (70, 85, 95, and 100%). The sections were prehybridized for 1 h at 55°C in 5× SSC containing 50% formamide, 5× Denhardt’s solution, 10 mM EDTA, 20 mM DTT, and 0.25 mg/ml of yeast tRNA, followed by hybridization with 35S-labeled RNA probes for 18 h at 55°C in 5× SSC containing 50% formamide, 2.7× Denhardt’s solution, 10 mM EDTA, 20 mM DTT, 0.25 g/ml yeast tRNA, 10% dextran sulfate, and 0.02% heat-denatured salmon sperm DNA. Thereafter, the sections were rinsed three times in 2× SSC containing 10 mM DTT, and then treated with 50 μg/ml of RNase A in 10 mM Tris-HCl, 0.5 M NaCl, and 1 mM EDTA, pH 8.0, for 30 min at 37°C. Washed sections were dehydrated in an ethanol series and then air-dried. The sections were dipped in a liquid emulsion (NTB3; Eastman Kodak, Rochester, NY) diluted 2-fold with water, exposed for 4 wk, and developed. Counterstaining was performed with hematoxylin only or H&E.

Results

Expression of S-MBP and L-MBP in mouse tissues, as analyzed by RT-PCR

The previous study by Sastry et al. (3) showed that the mRNAs of mouse S-MBP and L-MBP are expressed only in the liver, as determined with Northern blotting using total RNA from a limited number of tissues. To explore the extrahepatic expression of mouse MBP, we investigated the tissue distribution of mouse MBP mRNAs by RT-PCR with specific primers using total RNA obtained from 19 different mouse tissues. A fragment of 452 bp corresponding to the carboxyl-terminal portion of the collagen-like domain, the neck domain, and the major portion of the carbohydrate recognition domain of S-MBP was amplified for the detection of S-MBP mRNA, and a fragment of 262 bp corresponding to the carboxyl-terminal portion of the collagen-like domain and the neck domain of L-MBP was amplified for the detection of L-MBP mRNA. As shown in Fig. 1, S-MBP mRNA was found in lung, kidney, and testis as well as in liver, whereas L-MBP mRNA was found in kidney, thymus, and small intestine as well as in liver. All PCR products were checked by digestion with restriction enzymes, because it was found that the lengths of the digested fragments were identical with those deduced from the reported sequence for S-MBP or L-MBP (3) (data not shown).

Quantitative RT-PCR analysis

To quantify the levels of extrahepatic expression of MBPs mRNA revealed above, we employed a quantitative real time RT-PCR method (TaqMan RT-PCR), as described in Materials and Methods. As shown in Table I, the relative levels of expression of S-MBP mRNA in the lung, kidney, and testis were −1.0, 0.52, and 0.23%, respectively, compared with that in the liver. On the other hand, marked extrahepatic expression of L-MBP mRNA was observed in the small intestine, in which the relative level of expression of L-MBP mRNA was −8.7% that in the liver. The relative levels of expression of L-MBP mRNA in kidney and thymus were −0.06 and 0.02% compared with that in the liver, respectively. These results indicate that the extrahepatic expression of mouse MBP is most pronounced in small intestine.

Expression of L-MBP protein in small intestine

We next examined whether L-MBP protein is synthesized in small intestine, which was revealed to be a major site of extrahepatic expression of MBP at the transcriptional level. To do this, an extract of small intestine was enriched with MBP using a mannan-Sepharose 4B column and was then analyzed by Western blotting with anti-rat L-MBP polyclonal Abs. To prevent the detection of L-MBP in serum (23, 24), the mice had undergone systemic peritonitis. As shown in Fig. 1, a 29-kDa band corresponding to L-MBP was detected for the extract of the small intestine as well as for that of the liver. On the other hand, no specific bands were detected in brain extracts. This agrees with the results of the RT-PCR and indicates the efficacy of the systemic perfusion with buffer for washing out the plasma proteins, and thus also the liver-derived L-MBP, before removing the organs. These results indicated that the L-MBP mRNA in the intestine detected in the RT-PCR analysis was, in fact, translated into a protein with mannan-binding activity and with the same molecular size as that of L-MBP in liver.

Immunohistochemical localization of mouse L-MBP

To reveal the location of the L-MBP-producing site, we conducted immunohistochemical staining using mAb 16A8 specific for mouse L-MBP (22) on PFA-fixed cryosections of various gastrointestinal tissues obtained from mice that had undergone systemic perfusion. As shown in Fig. 3, strong staining was observed in some villous epithelial cells throughout the small intestine, whereas the other parts of the small intestine, including crypts, Peyer’s patches, and lamina propria, were not stained (Fig. 3, b–d). Goblet cells were also negative. Villous epithelial cells strongly stained with mAb 16A8 were found to occur much more frequently in the jejunum (47%) than in duodenum (20%) or ileum (14%). Obvious staining was not observed in either stomach or colon (Fig. 3, a and e); this was consistent with the results of RT-PCR shown in Fig. 1. No staining was observed in controls using normal rat IgG (data not shown). These results indicate that mouse intestinal L-MBP is exclusively expressed in some villous epithelial cells.

In situ hybridization of MBP mRNAs

The localization of MBP mRNAs in small intestine as well as liver was confirmed by in situ hybridization using 35S-labeled RNA probes specific for L-MBP and S-MBP. The expression of L-MBP

FIGURE 1. Expression of L-MBP and S-MBP mRNAs in mouse tissues. Total RNA obtained from various tissues of C57BL6 mice was subjected to RT-PCR using primers specific for L-MBP, S-MBP, or β-actin. All PCR reactions were performed for 35 cycles. The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide. Although only gel regions containing specific products are shown, nonspecific bands were absent in all gel regions.

In situ hybridization of MBP mRNAs

The localization of MBP mRNAs in small intestine as well as liver was confirmed by in situ hybridization using 35S-labeled RNA probes specific for L-MBP and S-MBP. The expression of L-MBP
mRNA was found in some intestinal epithelial cells as well as in nearly all hepatocytes (Fig. 4, a and c). On the other hand, the expression of S-MBP mRNA was not detected in small intestine, although it was found in nearly all hepatocytes (Fig. 4, e and f).

Control hybridization with sense strand RNA probes for L-MBP and S-MBP showed only background levels of silver grains, confirming the specificity of the signals (data not shown). These results are consistent with the results of RT-PCR and immunohistochemistry, as described above, suggesting that intestinal L-MBP expression is mainly regulated at the transcriptional level. The densities of silver grains in some L-MBP-positive cells were comparable to those in the hepatocytes (Fig. 4, b and d), suggesting that the expression of L-MBP mRNA in some intestinal villous epithelial cells is as high as that in hepatocytes at the cellular level.

Discussion

It has been well established that liver is the major site of synthesis of both S-MBP and L-MBP (3, 25, 26). Early reports demonstrated mannan or mannose-BSA binding proteins with molecular masses of ~30 kDa in rat mesenteric lymph node or mouse spleen, respectively (27, 28), and a more recent study involving RT-PCR and in situ hybridization demonstrated the expression of rat S-MBP mRNA in renal capsules and distal convolutions (18). In the present study we examined the tissue expression of mouse MBPs in 19 tissues, including liver, by RT-PCR. The extrahepatic expression of S-MBP mRNA was demonstrated in lung, kidney, and small intestine. These results are consistent with the results of RT-PCR and immunohistochemistry, as described above, suggesting that intestinal L-MBP expression is mainly regulated at the transcriptional level.

Table I. Relative quantification of MBP mRNA in mouse tissues

<table>
<thead>
<tr>
<th>MBP (ng standard RNA)</th>
<th>MBP Normalized to GAPDH</th>
<th>MBP (% of liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-MBP mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>82.7</td>
<td>144</td>
</tr>
<tr>
<td>Lung</td>
<td>0.546</td>
<td>37.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.08</td>
<td>146</td>
</tr>
<tr>
<td>Testis</td>
<td>0.116</td>
<td>35.9</td>
</tr>
<tr>
<td>L-MBP mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>83.0</td>
<td>57.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.128</td>
<td>146</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.038</td>
<td>112</td>
</tr>
<tr>
<td>Small intestine</td>
<td>6.85</td>
<td>54.8</td>
</tr>
</tbody>
</table>

*The relative amounts of MBP mRNA in mouse tissues were measured in triplicate by real time PCR in two independent experiments as described in Materials and Methods.

a Total liver RNA obtained from C57BL6 mice was used as a standard. The amounts of MBP and GAPDH mRNA in mouse tissues were expressed as equivalents to those in the standard RNA. Values are the means of two independent experiments performed in triplicate.

The normalized amounts of MBP mRNA were calculated by dividing the amount of MBP mRNA by those of GAPDH mRNA and multiplying by 100.

The relative amounts of normalized MBP mRNA are expressed as percentages of that in the liver.
L-MBP in kidney and thymus was mRNA at a level of 8.5% to that in liver, while the expression of L-MBP was distinguishable from the other tissues by the expression of L-MBP. By quantitative real-time RT-PCR, the small intestine was obviously distinguishable from kidney, thymus, and small intestine (Fig. 1). On measurement by quantitative real-time RT-PCR, the small intestine was obviously distinguishable from the other tissues by the expression of L-MBP mRNA at a level of 8.5% to that in liver, while the expression of L-MBP was distinguishable from the other tissues by the expression of L-MBP mRNA at a level of 8.5% to that in liver. The extrahepatic expression of S-MBP was approximately or less than 1% of that in liver. (Table I). This suggests that L-MBP may have some biological significance in the small intestine.

The biological function of L-MBP is a bit controversial compared with the well-documented function of S-MBP in innate immunity. The rat L-MBP was found in the lumen of the endoplasmic reticulum and the Golgi apparatus in hepatocytes, and it was shown to bind to biosynthetic intermediates of various glycoproteins with high mannose-type glycans (29, 30). Based on these findings, it was suggested that L-MBP might be involved in the intracellular trafficking of biosynthetic intermediates of glycoproteins. On the other hand, mouse L-MBP has been detected in sera at higher concentrations than S-MBP and was also demonstrated to activate complement, although the activity is 5- to 10-fold weaker than that of S-MBP (23). These findings suggest that L-MBP may, like S-MBP, be involved in innate immunity. It should be noted here, however, that these two possible functions are not virtually exclusive. L-MBP may have different functions depending upon the location of the lectin.

Western blotting of tissue extracts using anti-L-MBP polyclonal Abs revealed that the small intestine expressed L-MBP protein of the same size as that in liver under reducing conditions. These results and the fact that L-MBP in the tissue extracts had been enriched by affinity chromatography on a mannan-Sepharose 4B column suggest that the L-MBP protein synthesized in small intestine is structurally and functionally equivalent to that in liver.

Immunohistochemical staining and in situ hybridization of the small intestine revealed that L-MBP is selectively expressed in some intestinal villous epithelial cells. Within those cells, strong immunoreactivity to mAb16A8 was preferentially found in the apical part of the cells (Fig. 3). The villous localization of mouse L-MBP in gastrointestinal tissues is similar to that of rat surfactant protein A (31) and human surfactant protein D (32), both of which are members of the collectin (collagen-like lectin) family involved in regional innate immunity. Many other humoral innate immune factors, for instance, antimicrobial defensins, lysozyme, and phospholipase A2, are preferentially expressed in the secreting granules of intestinal Paneth cells at the bottom of crypts (33), where little or no L-MBP was detected in the present study. It is interesting to note this distinct compartmentalization between MBP and the other bactericidal proteins in intestinal epithelium. Although the primary roles of villous epithelial cells are the absorption of nutrients and presenting a physical barrier against invasion by microorganisms, villous epithelial cells are known to play an active role in immunity; for example, transportation of secretory IgA into the lumen from the lamina propria (34), production of cytokines and chemokines in response to bacterial invasion (35), and Ag presentation via various MHC molecules, including class II and nonclassical class I molecules (36). Thus, it may be reasonable to speculate that L-MBP secreted from villous epithelial cells acts as a humoral immune factor in the intestine, just as does secretory IgA.

In addition to the localization of intestinal L-MBP along the crypt-villus axis, a unique localization along the proximal-distal axis was also found in this study. The villous epithelial cells that express L-MBP were most abundantly distributed in the jejenum, less abundantly in duodenum and ileum, and not at all in colon and stomach. It is well known that a large number of bacteria reside in colonic contents (10^{11} organisms/ml), and that the number of bacteria resident in small intestine decreases markedly (10^{5}-10^{6} organisms/ml in distal ileum and 10^{5} organisms/ml in jejunum and duodenum) (37). It is likely that mucosal factors are responsible for maintaining the relative sterility of jejenum despite the abundance of nutrients and neutral pH. The maximal expression of intestinal L-MBP in jejunum may contribute to the relative sterility of the jejenum. The mechanisms of the host defense mediated by L-MBP in the small intestine remain to be studied. However, L-MBP may act as a defense factor by simply masking the surface of microorganisms, enhancing phagocytosis (14), or inhibiting the attachment of microorganisms on intestinal cell surfaces.

Human MBP deficiency has been associated with recurrent infections, including persistent diarrhea (16). The etiology of diarrhea in patients with MBP deficiency is not fully understood. It has been reported that MBP was found in duodenal fluid (38). In that report it was suggested that serum MBP might be trans-exuded into the intestinal lumen due to abnormal permeability of infected mucosa. Our current findings may provide an explanation for the causal relationship between MBP deficiency and diarrhea.

In summary, we have examined the expression of mouse MBPs and found a relatively abundant expression of L-MBP in small intestine. The expression of L-MBP was found to be restricted to villous epithelial cells.

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INTESTINAL EXPRESSION OF MANNAN-BINDING PROTEIN

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