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Baculovirus expression of mouse lactoferrin receptor and tissue distribution in the mouse

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Abstract

Lactoferrin (Lf) has been shown to have a role in the immune system and in early development of the mouse embryo. A specific receptor for Lf has been suggested to mediate the functions of Lf. We have recently identified a Lf receptor (LfR) in human fetal small intestine. We therefore hypothesized that the mouse homologue of this protein functions as a LfR. We expressed mouse Lf (MLf) and the mouse homologue (MLfR) in a baculovirus-insect cell system. The recombinant MLfR (rMLfR) was purified by immobilized recombinant MLf (rMLf) affinity chromatography, demonstrating an interaction between rMLf and the rMLfR. RT-PCR revealed that MLfR was expressed in various tissues and during embryonic development. Immunohistochemical analysis revealed that the MLfR was localized in various tissues including small intestinal epithelium, stomach, kidney, ovary, and various regions of brain. In summary, the MLfR functions as a receptor for MLf, is expressed and localized in various tissues, and may be involved in the indispensable function of MLf during early embryonic development.

Introduction

Lactoferrin (Lf) is a single-chain glycoprotein, binds iron tightly, and is abundant in milk of some species such as humans, rhesus monkeys, and mice and has been suggested to facilitate iron absorption in infants (Lönnerdal & Iyer 1995). Lf is also found in high concentration in most exocrine secretions and in the secondary granules of neutrophils, from which it is released following activation of these cells (Lash *et al.* 1983; Martins *et al.* 1995). Lf has been suggested to be involved in a variety of biologically important mechanisms such as defense against a variety of pathogens and stimulating cell proliferation (Lönnerdal & Iyer 1995).

The mouse has been used as a model to study iron absorption (Fransson *et al.* 1983), anti-microbial (Teraguchi *et al.* 1995a, b), and immuno-modulating effects of Lf (Lu *et al.* 1987, Zagulski *et al.* 1989, Machnicki *et al.* 1993). The anti-microbial effect may be partially explained by the ability of Lf to interfere with the CD14-dependent pathways for lipopolysaccharide (LPS), which induce mortality in mice (Elass-Rochard *et al.* 1998). Some of these studies on the anti-microbial effect of Lf indicate the existence of a specific receptor for Lf (LfR) in various tissues. Intravenous administration of Lf has been shown to protect mice against lethal doses of *E. coli* (Zagulski *et al.* 1989). This effect was observed even when Lf was administered before a challenge with *E. coli*, suggesting that the receptor-mediated mechanism activates immune function. Oral administration of Lf enhances the secretion of IgA and IgG, indicating that Lf could act as a stimulating factor on the mucosal immune system and that activation of the mucosal immune system is modulated by the LfR in the intestinal mucosa (Debbabi *et al.* 1998). Furthermore, histological examination of the intestine revealed that severe villous atrophy, edema and epithelial vacuolation, which are normally observed in LPS-treated animals, were not observed when mice were pretreated

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with Lf before LPS treatment (Kruzel *et al.* 2000). The detailed mechanism by which Lf protects mice against the lethal effect of LPS is not known, but a receptor-mediated activation of immune function is a feasible hypothesis. Indeed, a mouse LfR (MLfR) has been found in the small intestine and its biochemical characteristics were investigated to some extent (Hu *et al.* 1990). A receptor-mediated mechanism may therefore be involved in the biological functions of Lf in the mouse. We thus find it important to disclose molecular information on the MLfR and to provide insights into the mechanisms involving the MLfR, which may help elucidating the primary biological functions of Lf.

We have recently identified a human lactoferrin receptor (HLfR) in fetal small intestine (Suzuki *et al.* 2001). A mouse homologue of this protein with unknown function was previously found in mouse small intestine and named intelectin because its sequence was somewhat similar to a lectin and it is expressed at high levels specifically in the small intestine (Komiya *et al.* 1998). We therefore hypothesized that the mouse homologue functions as a LfR and may mediate some of the important functions of Lf.

Most studies on Lf *in vivo* were conducted by using bovine Lf, but homologous Lf should be used to elucidate the true biological function of Lf, mediated through the interaction with the LfR. In this study, we expressed recombinant MLf (rMLf) and recombinant MLfR (rMLfR), isolated both proteins to obtain evidence for interactions between rMLf and rMLfR, and investigated tissue expression of MLfR and compared it to those of HLfR and MLf.

Experimental procedures

Cloning of MLf and MLfR cDNA

Total RNA was extracted from mouse small intestine and spleen by the SV total RNA isolation system (Promega, Madison, WI). First-strand cDNA was synthesized from 1 μg of total RNA by using the cDNA Cycle Kit (Invitrogen, Carlsbad, CA) according to the manufacturers instructions and suspended in 20 μl of TE buffer. Primers for MLf and MLfR were designed as 5-GTCTCCCAAGACCACAGACATGAGG-3 (sense for MLf), 5-TGGCTCCGGATGAGACTTCCTG-3 (antisense for MLf), 5-AGGGAAGACCACCATGAC CCAACTG-3 (sense for MLfR), 5-CCTTGGAGCCC ACAATGGAGAAGTC-3 (antisense for MLfR). PCR amplification was performed on 1 μl of cDNA in the presence of 0.2 M sense and antisense primers, 0.2 mM dNTP and 0.4 μl of Advantage cDNA Polymerase Mix (Clontech, Palo Alto, CA) in a total volume of 20 μl. The conditions for PCR were as follows: initial denaturation at 94 °C for 1 min, 20 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 3 min, and final extension at 72 °C for 10 min. The PCR products were cloned into the plasmid vector pCR-XL-TOPO (Invitrogen) and sequenced for both strands of the entire region by using automated DNA sequencing on an ABI Prism 377 DNA sequencer (PE Biosystems, Foster City, CA) at Davis Sequencing Inc. (Davis, CA)

Generation of recombinant baculovirus

The Bac-to-Bac Expression System (Life Technologies Inc., Rockville, MD) was used for expression of rMLf and the rMLfR. The MLf cDNA and MLfR cDNA were excised from the pCR-TOPO-XL vector at the *Spe*I and *Xho*I restriction sites and ligated into the donor plasmid pFastBac via the same restriction sites. The recombinant baculoviruses containing MLf and MLfR cDNA were generated according to the manufacturer's instructions.

Expression of recombinant proteins

Tricoplusia ni (T. ni) cells were routinely maintained at 28 °C in 250-ml Erlenmeyer flasks containing 100 ml of a serum free medium (ESF921; Expression System, Woodland, CA) and sub-cultured every three or four days. For expression of recombinant proteins, *T. ni* cells were seeded at 0.5×10^{10} cells/ml and infected by the recombinant baculovirus at a multiplicity of infection of 0.1. At three days post-infection, the infected cell culture was harvested and centrifuged at $1000 \times g$ for 10 min at 4 °C to separate supernatant and cells.

Isolation of rMLf

The supernatant of infected *T. ni* cells was further centrifuged at $10,000 \times g$ for 15 min and filtered though a Nylon filter cartridge with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, MI). The filtered supernatant of infected *T. ni* cells was loaded onto a Hi-Trap SP column equilibrated with 50 mM Tris buffer, pH 8.0. The bound proteins were eluted by a gradient of 1 M NaCl. The eluted fraction was applied to a Con-A column equilibrated with 20 mM phosphate buffer, pH 7.0, containing 0.05%

cetyltrimethylammonium chloride (CETAC; Aldrich Chemical Company, Inc., Milwaukee, WI) and 0.3 M NaCl. Recombinant MLf was eluted by a gradient of 0.2 M methyl α-D-mannopyranoside (Sigma) in the equilibration buffer. Purified rMLf (5 mg) was then immobilized to Affi-gel 10 (Bio-Rad) as previously described (Kawakami & Lönnerdal 1991).

Isolation of the rMLfR

T. ni cell culture medium infected with the rMLfR baculovirus was applied to the immobilized rMLf affinity column equilibrated with 25 mM Tris, pH 7.4, containing 5 mM $CaCl_2$, 0.1% CHAPS, and 0.5 M NaCl. The bound protein was eluted with 0.2 M sodium acetate buffer, pH 3.7, containing 5 mM EGTA and 0.1% CHAPS. Antibody (P2AB) was purified by using an immobilized antigen peptide (CTVGDRWSSQQGSKAD: peptide 2) affinity chromatography from the antiserum P247AS that had previously been raised in rabbits against synthetic peptides corresponding to parts of amino acid sequences of the HLfR (Suzuki *et al.* 2001). Briefly, peptide 2 ($\sim$0.5 mg) was immobilized to 1 mL of Affigel 15 (Bio-Rad). P247AS (5 mL) was applied to the column and the column was washed with PBS. P2AB was then eluted by 0.2 M glycine buffer, pH 2.7. The eluate was immediately adjusted to pH 7.4 with 1 M NaOH.

SDS-PAGE, Western blot, and amino acid sequencing

Purified proteins were subjected to SDS-PAGE. Gels were stained either with Coomassie Brilliant Blue R solution or with Silver Stain Plus (Bio-Rad). For Western blotting, electrophoresed proteins were transferred to PVDF (Bio-Rad) membrane. Non-specific binding sites on the membrane were blocked by incubation in a blocking buffer (PBS containing 0.05% Tween-20 and 5% skim milk). The membrane was washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tween) for 7 min each, then incubated with 0.4 g/mL of P2AB in PBS for 1 h at room temperature, and washed 4 times with the blocking buffer. The membrane was incubated with a 1:20,000 dilution (in the blocking buffer) of horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (Biodesign, Kennebunk, ME) for 1 h at room temperature and washed 4 times with PBS-Tween for 7 min each. Bound antibody was visualized by SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL) and analyzed with the Chemi-Doc system (Bio-Rad).

RT-PCR

PCR was performed on cDNA from various adult mouse organs and various developmental stages of embryos (Clontech). PCR conditions were same as described above. The products were applied to agarose gel. The intensity of each band was analyzed with the Chemi-Doc system (Bio-Rad)

Immunohistochemical localization of MLfR

Mouse tissue array slide (VastarrayTM; ResGen, Memorial Parkway, SW) was deparaffinized and rehydrated through a graded series of ethanol solutions. The sections were incubated for 1 h in blocking solution (10% normal goat serum), followed by incubation for 3 h with 1.0 g/mL of P2AB. Sections were next incubated with a horse radish peroxidase-conjugated goat anti-rabbit secondary antibody. Immunoreactivity was detected using DAB (3,3-diaminobenzidine) as substrate chromogen (Sigma). Sections were counterstained using hematoxylin. Images were acquired using an Olympus Provis microscope (Olympus, Melville, NY) coupled with a CCD camera.

Results

Cloning of MLf and MLfR

Sequences of the PCR products for MLf and for MLfR were matched to the sequences previously reported as MLf (accession #: D88510) and as intelectin (accession #:NM010584), respectively. These clones were subsequently used for the baculovirus expression experiment.

Isolation of recombinant MLf

Recombinant MLf was expressed in the baculovirus system and isolated by two step chromatography. After the cation exchange chromatography, a major band was revealed at $\sim$70 kD by SDS-PAGE (Figure 1, lane 1) with a significant level of other minor bands. Thus, this fraction was applied to Con A chromatography. A single band with the size $\sim$70 kD was obtained after Con A chromatography (Figure 1, lane 2). The N-terminal amino acid sequence determined from this protein, ^{20}KATTVRXXAVSN^{31}, matched the sequence of the mature form of MLf, indicating successful expression and purification of rMLf.

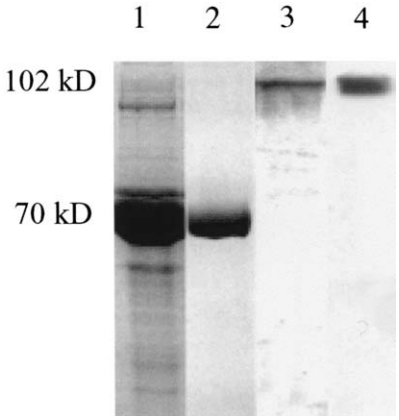

Fig. 1. SDS-PAGE and Western blot analysis of rMLf and rMLfR. Lane 1: SDS-PAGE stained with Coomassie Blue separated by Hi-Trap SP chromatography. 10 μg of protein were loaded. Lane 2: SDS-PAGE stained with Coomassie Blue after Con A affinity chromatography. 5 μg of rMLf were loaded. Lane 3: Silver staining of SDS-PAGE of the MLfR after immobilized rMLf affinity chromatography. Lane 4: Western blot analysis of the MLfR after immobilized rMLf affinity chromatography.

Isolation of recombinant MLfR

The rMLfR was purified by the immobilized rMLf affinity column. SDS-PAGE of the eluted fraction revealed a single band of 102 kD (Figure 1, lane 3). Western blot exhibited a single band at the same size (Figure 1, lane 4), indicating successful expression and purification of the rMLfR. The antibody used in this experiment was affinity-purified with a peptide that is present in the predicted amino acid sequence of MLfR. These results demonstrated that the rMLfR interacted with rMLf, providing evidence that the mouse homologue of the HLfR gene is coding a functional MLfR.

RT-PCR

Expression of MLf mRNA was compared with that of MLfR mRNA (Figure 2). Relative intensity of the RT-PCR products from each organ was adjusted using the intensity of liver equal to one. Testis, skeletal muscle, lung, spleen, and heart expressed MLf at levels relatively higher than MLfR, while small intestine and kidney showed the opposite trend. The MLfR was expressed at high levels in 7-day embryos, but became much lower in 11- and 15-day embryos, while it was expressed again at intermediate levels in 17-day embryos (Figure 3). A similar trend was observed for MLf expression.

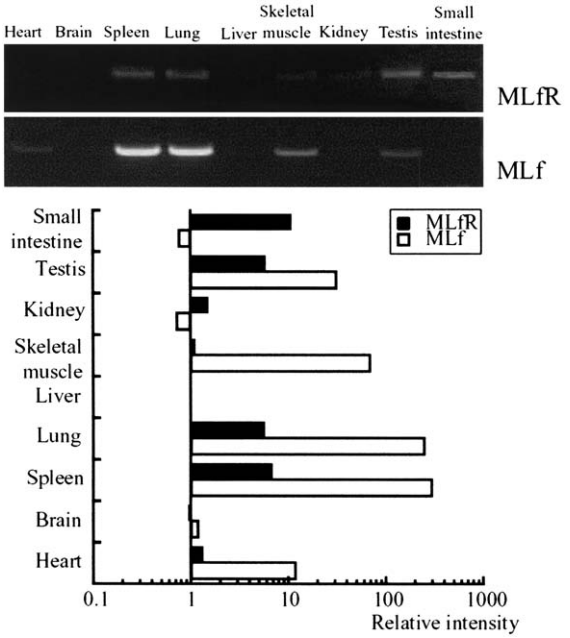

Fig. 2. Tissue expression of MLf and MLfR. Relative intensity of the RT-PCR products from each organ was adjusted using the intensity of liver equal to one.

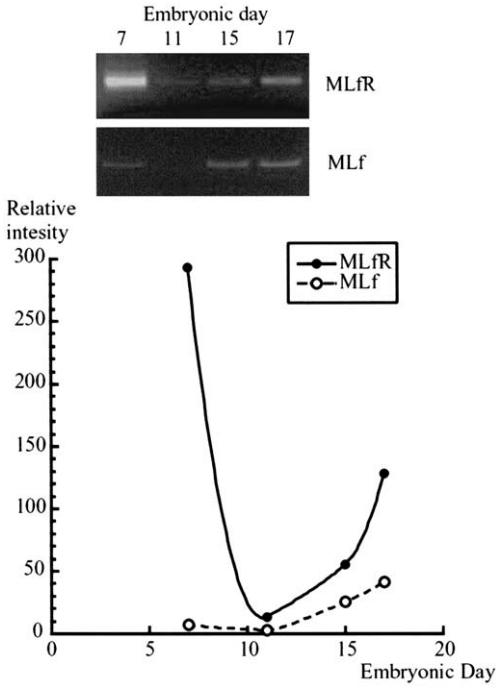

Fig. 3. Developmental expression of MLf and the MLfR in mouse embryos. Relative intensities of the PCR products were normalized against the intensity of G3PDH.

Immunohistochemistry

Negative controls in which no primary antibody was added did not show any nonspecific staining (data not shown). Results for the digestive and urinary systems are shown in Figure 4. Most prominent staining was observed in the brush border of small intestinal epithelium (Figure 4D), which is in agreement with our previous identification of the HLfR originally purified from the brush border membrane of small intestinal epithelium, and suggests that MLfR functions similar to HLfR in the small intestine. There was also strong staining observed in the brush border membrane of the proximal convoluted tubules at both renal cortex (Figure 4H) and renal medulla (Figure 4I). In stomach, the MLfR appears to be associated with chief cells (Figure 4C). Other tissues also showed positive staining but to less extent. In salivary gland (Figure 4A) and pancreas (Figure 4F), the MLfR is mostly localized to acinar cells. Pancreatic islets of Langerhans also showed the MLfR localized in a limited number of cells. In the esophagus (Figure 4B), the MLfR was observed in the stratified squamous epithelium, columnar cells, and muscle layer. Transitional epithelium is faintly stained in the urinary bladder (Figure 4G). Positive staining in the large intestine was much lower than in other digestive tissues (Figure 4E).

Various regions of brain cells also exhibited positive staining (Figure 5). Pyramidal cells in basal ganglia were strongly stained (Figure 5A). In cerebellum, Purkinje cells and outer stellate cells were selectively stained but not any other cells in the granular layer (Figure 5B). Oligodendrocytes and small neurons in prefrontal cortex were also stained (Figure 5C). Corpus striatum, hippocampus, hypothalamus, medulla, and pineal gland exhibited positive staining (Figure 5E–5I). Pons, pituitary neuron and spinal cord were stained to less extent than other brain regions, but many of the neuron cells such as motor neurons in the spinal cord were only faintly stained (Figure 5J–5L).

The MLfR was also localized to the reproductive system (Figure 6). In ovaries, theca interna was strongly and selectively stained, regardless of its pregnancy status (Figure 6A & 6B). Syncytiotrophoblasts that cover the fetal placental villi were stained in placenta (Figure 6C), suggesting that the protein may mediate transport of maternal MLf to the fetus. Embryonal tissues at day 14 p.c. were also stained (Figure 6D). Prostate was also positively stained in its epithelium (data not shown). Testis, mammary gland, and uterus were not stained distinctively (data not shown).

Results from other organs are shown in Figure 7. Liver was lightly stained on the cell membrane but not intracellularly (Figure 7A). The MLfR was detected selectively in some cells in the lung (Figure 7B). Muscle fibers of the heart (Figure 7C) and skeletal muscle (Figure 7D) were faintly stained. Several regions of the wall of the eyeball were selectively stained (Figure 7E). Chromatophores in the choroid and approximately half nuclei of rods and cones in the retina, were most clearly stained. Cones were lightly stained, but rods were not. 10–20% of small lymphocytes in the lymph node (Figure 7F) and those in spleen (Figure 7H) were stained. In thymus, however, lymphocytes were not stained, and stroma was selectively stained (Figure 7G). No staining was detected in the skin and adrenal glands (data not shown).

Discussion

We here for the first time identified a gene encoding mLfR and demonstrated an interaction between rMLf and the rMLfR. We found that the mouse homologue of the HLfR interacts with MLf, and therefore we prefer calling this protein mouse lactoferrin receptor (MLfR) instead of intelectin, which it was previously called without defining its functional properties (Komiya *et al.* 1998). We have recently identified a cDNA for HLfR, expressed the encoded protein in the baculovirus system, and showed a direct interaction between HLf and the recombinant HLfR (rHLfR) (Suzuki *et al.* 2001). The size of the rHLfR was 34 kD under reducing conditions and 102 and 136 kD under non-reducing conditions. The native HLfR (nHLfR) was also found to form a homo-trimer (Kawakami & Lönnerdal 1991). We found that the rMLfR forms oligomers, which is similar to both nHLfR and rHLfR. Recombinant HLf was previously expressed in the baculovirus system and found to retain iron-binding characteristics similar to nHLf (Salmon *et al.* 1997). Thus, rMLf expressed in the baculovirus system could retain structure and functions similar to nMLf. We observed that rMLf was able to bind iron, suggesting that protein folding was appropriate (data not shown). Successful isolation of the rMLfR by the immobilized rMLf affinity column indicated a specific interaction between rMLf and the rMLfR.

We have previously performed iron uptake and transport studies on HLfR-transfected Caco-2 cells,

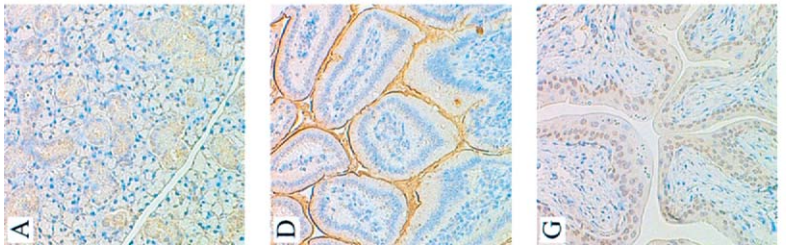

Fig. 4. Immunohistochemical analysis of MLfR localization in the digestive tract and the urinary system. Salivary gland (A), esophagus (B), stomach (C), small intestine (D), large intestine (E), pancreas (F), urinary bladder (G), renal cortex (H), and renal medulla (I).

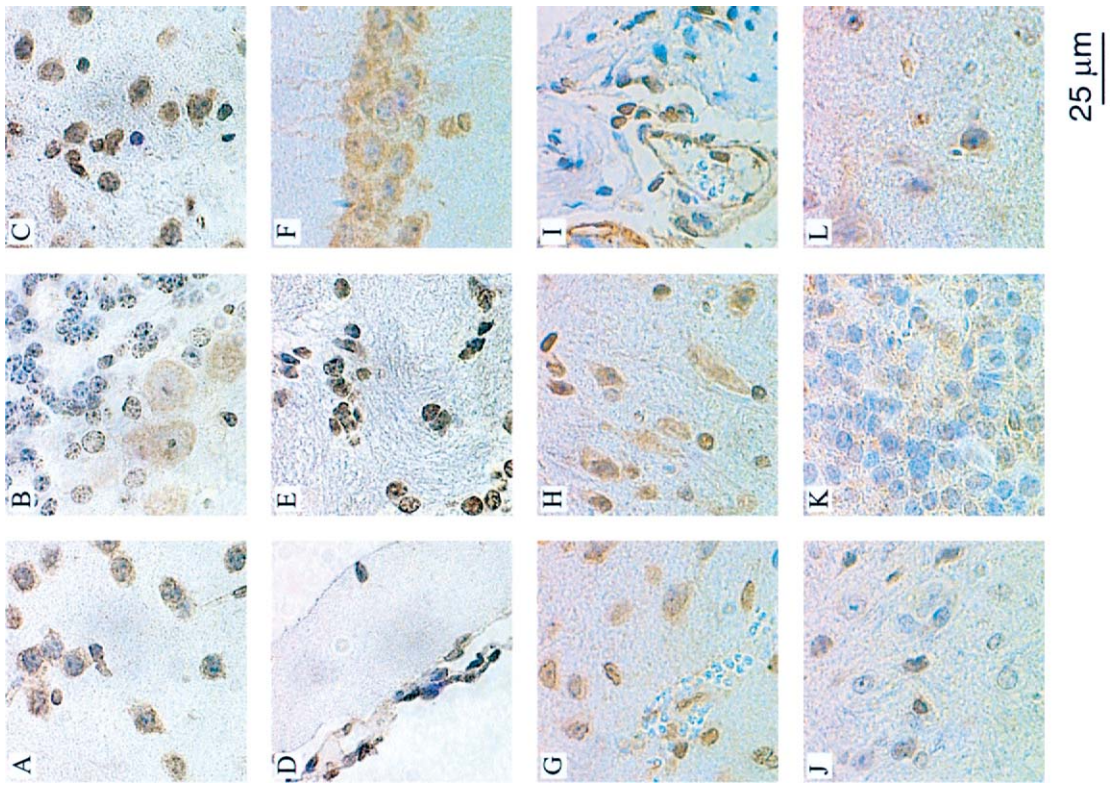

Fig. 5. Immunohistochemical analysis of MLfR localization in the nervous system. Basal ganglia (A), cerebellum (B), prefrontal cerebral cortex (C),temporal cerebral cortex (D), corpus striatum (E), hippocampus (F), hypothalamus (G), medulla (H), pineal gland (I), pons (J), pituitary gland (K), and spinal cord (L).

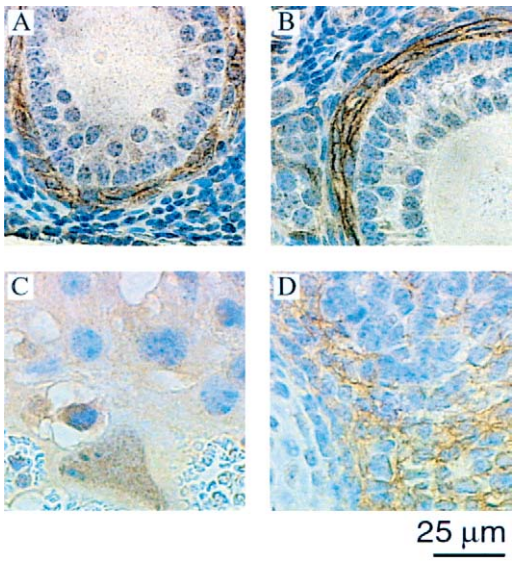

Fig. 6 Immunohistochemical analysis of MLfR localization in the reproductive organs. Non-pregnant ovary (A), pregnant ovary (B), placenta (C), embryo (D).

which showed significantly higher uptake of HLf-bound iron by HLfR-transfected Caco-2 cells than by mock-transfected cells (Suzuki *et al.* 2001). This result suggested that HLf facilitates iron absorption in human infant intestine through the HLfR-mediated HLf uptake pathway. In fact, the bioavailability of human milk iron is unusually high (Saarinen *et al.* 1977), and the incidence of iron deficiency in breast-fed infants is low, which correlates well with the much higher Lf content in human milk as compared with cow's milk. Uptake of Lf-bound iron by human duodenal biopsies has been demonstrated by an *in vitro* assay (Cox *et al.* 1979). We have previously documented that a significant proportion of HLf survives digestion in the gastrointestinal tract of human newborns (Davidson & Lönnerdal 1987), providing a strong support that Lf could play a role in the infant digestive system, possibly by receptor-mediated iron absorption. There is, however, no direct evidence supporting this idea *in vivo*. A specific receptor for Lf has been demonstrated on the small intestinal brush border of several animal species such as rabbits (Mazurier *et al.* 1985), pigs (Gislason *et al.* 1995), rhesus monkeys (Davidson & Lönnerdal 1988, 1989) and mice (Hu *et al.* 1988, 1990), indicating that these animals may be useful for *in vivo* studies on the functions of the LfR in the small intestine.

We studied tissue distribution of the LfR in the mouse. Expression of MLf in various tissues was previously well documented at the protein level (Teng *et al.* 1989). Lung, vagina, mammary gland, oviduct, spleen, lymph node, and uterus express MLf, but duodenum had no detectable immunoreactive material (Teng *et al.* 1989). Since MLf protein was not found in the small intestine by immunochemical analysis (Teng *et al.* 1989), which agrees with our observation (Figure 2), the MLfR on the apical membrane in the small intestine (Figure 4D) is likely to interact with MLf from oral ingestion, pancreatic or bile secretions. Thus, the MLfR, together with MLf, may facilitate

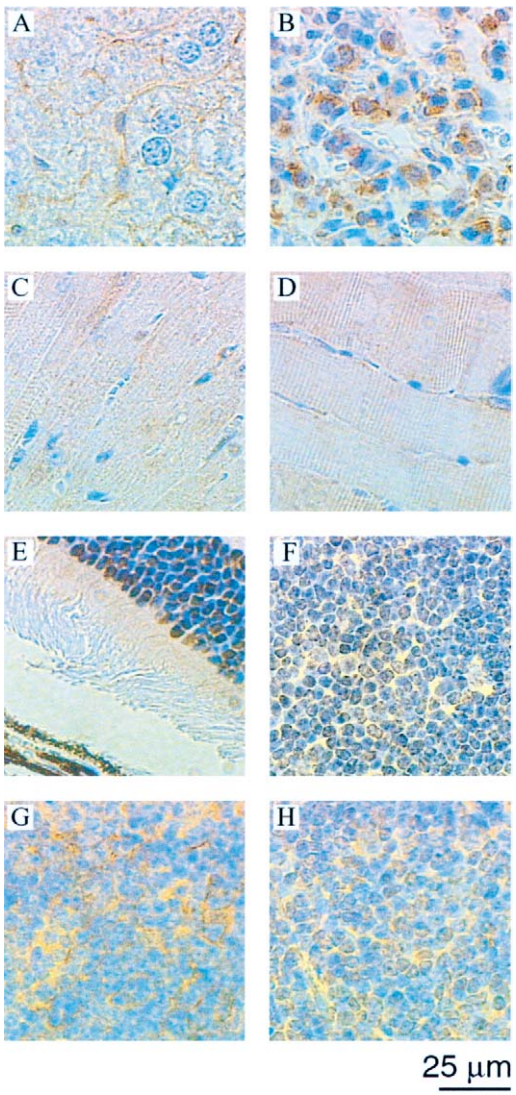

Fig. 7 Immunohistochemical analysis of MLfR localization in the other tissues. Liver (A), lung (B), heart (C), skeletal muscle (D), optic (E), lymph node (F), thymus (G), spleen (H).

iron absorption in mouse small intestine or facilitate other suggested biological activities.

We previously documented the expression patterns for human LfR (HLfR) mRNA in various tissues (Suzuki *et al.* 2001). When expression of MLfR mRNA was compared with that of HLfR (after adjusting the relative expression levels using human and mouse liver equal to one, respectively), the MLfR was expressed at higher levels than HLfR in spleen but lower in heart and skeletal muscle, while it was similar in testis. These differences in the expression pattern of the LfR suggest that humans and the mouse may have different primary purposes to possess Lf and its receptor.

The Lf-LfR interaction may be required in multiple pathways corresponding to multiple functions of Lf since most of the suggested functions of Lf could be well explained if receptor-mediated mechanisms are present. Those include facilitating iron absorption, anti-infective properties such as stimulating cytokine production, and enhancing cell proliferation. Recently, oral administration of bovine Lf was shown to increase production of interleukin-18 (IL-18), interferon-gamma, and caspase-1 in the epithelial cells of the small intestine (Wang *et al.* 2001), which likely requires a receptor-mediated mechanism to occur, and to stimulate intestinal mucosal immunity. Mouse intelectin, which here turns out to be MLfR, has been speculated to be involved in antibacterial systems because it appears to be specifically localized in Paneth cells in terms of mRNA expression (Komiya *et al.* 1998). Since Lf has been known to be involved in the host-defense mechanism, it is quite possible that MLfRs mediate antimicrobial effects of Lf in the small intestine. The primary pathway after the Lf-LfR interaction, however, still needs to be elucidated.

Expression of HLf in stomach was studied by immunocytochemistry, showing that chief cells, but not adjacent parietal cells, were strongly stained (Luqmani *et al.* 1991). Interestingly, we observed that chief cells apparently expressed the MLfR, suggesting that MLf may have a specific role in the stomach. BLf and HLf were reported to inhibit Helicobacter pylori or Helicobacter felis infection in mice (Dial *et al.* 2000, Wang *et al.* 2001), and thus it is possible that MLfR mediates the antimicrobial function of Lf in the stomach. In human kidney, Lf was localized only to the distal part of tubuli. Although a large amount of Lf protein was found in the tubuli, it was present at a very low level in urine. The MLfR therefore could mediate reabsorption of iron bound to Lf by endocytosis.

MLf immunostaining was found on microvessels in the cerebral cortex and in the hippocampus, which was apparently associated with pyramidal neurons and fibers even though the level of Lf transcripts in mouse brain tissue determined by RT-PCR was very low (Fillebeen *et al.* 1999). This research group also reported that Lf expression in the brain was markedly increased when the brain was stressed by the neurotoxin MPTP, which induces Parkinson's disease in mice. In humans, HLf was suggested to participate actively in the mechanism of neuronal degeneration in Parkinson's disease (Leveugle *et al.* 1996). The MLfR in the brain seems to be expressed at a very low level (Figure 2), but the protein was found in most areas of brain (Figure 5), suggesting that there may be some regulation for MLfR expression in the brain as well as for the MLf expression.

Expression of MLfR changed dramatically during different embryonic developmental stages with a trend similar to the change in MLf expression. Spatiotemporal expression of MLf during embryogenesis has been examined and it was found that MLf expression is strictly regulated (Ward *et al.* 1999). These investigators found that in the post-implantation stage, MLf mRNA was not detected until the 11.5-day embryo, when myelopoiesis occurred in the liver, and was primarily localized to neutrophils at day 15.5, coinciding with the highest hematopoietic activity of the liver. We found that the MLfR mRNA expression increased from day 11 to day 15, which matched the MLf mRNA expression pattern well (Figure 3), and MLfR protein was also detected in embryos at day 14 (Figure 6D). Thus, the MLfR may be important for the function of MLf in the late post-implantation stage, which possibly is correlated to hematopoiesis in the liver. In the pre-implantation stage, MLf mRNA was synthesized only by the inner cell mass and the protein was transported, in a specific manner, to the outer trophoectodermal cells, where no MLf mRNA was detected. These results suggest that MLf may have a novel paracrine role in the development of the trophoectodermal layer during murine pre-implantation embryonic development (Ward *et al.* 1999). MLfR mRNA expression in the pre-implantation embryo was not examined in this study, but it is quite possible that the outer trophoectodermal cells express the MLfR for transport of MLf. However, the exact function of Lf in embryonic development and its mechanism still needs to be elucidated.

In conclusion, the cloning and functional expression of the MLfR performed in this study provide a

valuable foundation for future studies to elucidate the mechanisms behind LfR-mediated functions of Lf *in vivo*.

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