Mouse LYVE-1 Is an Endocytic Receptor for Hyaluronan in Lymphatic Endothelium*

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The glycosaminoglycan hyaluronan is a key substrate for cell migration in tissues during inflammation, wound healing, and neoplasia. Unlike other matrix components, hyaluronan (HA) is turned over rapidly, yet most degradation occurs not locally but within distant lymph nodes, through mechanisms that are not yet understood. While it is not clear which receptors are involved in binding and uptake of hyaluronan within the lymphatics, one likely candidate is the lymphatic endothelial hyaluronan receptor LYVE-1 recently described in our laboratory (Banerji, S., Ni, J., Wang, S., Clasper, S., Su, J., Tammi, R., Jones, M., and Jackson, D.G. (1999) J. Cell Biol. 144, 789–801). Here we present evidence that LYVE-1 is involved in the uptake of hyaluronan by lymphatic endothelial cells using a new murine LYVE-1 orthologue identified from the EST data base. We show that mouse LYVE-1 both binds and internalizes hyaluronan in transfected 293T fibroblasts in vitro and demonstrate using immunoelectron microscopy that it is distributed equally among the luminal and abluminal surfaces of lymphatic vessels in vivo. In addition, we show by means of specific antisera that expression of mouse LYVE-1 remains restricted to the lymphatics in homozygous knockout mice lacking a functional gene for CD44, the closest homologue of LYVE-1 and the only other Link superfamily HA receptor known to date. Together these results suggest a role for LYVE-1 in the transport of HA from tissue to lymph and imply that further novel hyaluronan receptors must exist that can compensate for the loss of CD44 function.

The extracellular matrix glycosaminoglycan hyaluronan (HA) is a large polymer of N-acetyl-D-glucosamine and D-glucuronic acid (molecular mass 10⁵–10⁷ Da) which plays an important role in maintaining tissue integrity as well as facilitating the migration of cells during inflammation, wound repair, and embryonic development (1, 2). By comparison with other macromolecules of the extracellular matrix, HA undergoes rapid turnover with a half-life of ~24 h (1). Intriguingly, most degradation occurs not locally, but within distant lymph nodes. During this process, tissue HA enters the afferent lymphatic vessels and is transported with the lymph fluid to the draining lymph nodes where ~90% of the glycosaminoglycan is degraded by unknown mechanisms (3, 4). The remaining 10–15% of the HA exits via the efferent lymphatics to the blood vasculature where it is rapidly endocytosed by the liver sinusoid endothelial HA receptor (5), a 300-kDa heterotrimERIC complex of α, β, and γ subunits that clears not only HA but also chondroitin and heparan sulfate from the circulation (6). While it is clear that HA can rapidly permeate the lymphatics in skin and other tissues (7), the mechanisms responsible for its transport across lymphatic endothelium, and the receptors involved in its uptake and transport within lymphatic vessels are all unknown.

The majority of HA-binding proteins (8, 9) identified to date belong to the Link protein superfamily, defined by the presence of a conserved HA-binding domain known as the Link module (10, 11). This is a unit of ~100 amino acids that contains four conserved cysteine residues interspersed with tracts of both hydrophobic and charged residues. The three-dimensional structure of the Link module closely resembles that of the C-type lectin fold, comprising two β sheets flanked by two short α helices and stabilized by two disulfide linkages enclosing a central hydrophobic core (12, 13). Members of the Link superfamily include versican (14), the cartilage structural proteins aggrecan and link protein (15), the brain proteoglycans neurocan (16) and brevican (17), the inflammation-associated TSG-6 protein (18), and the integral membrane glycoprotein CD44; until recently the only known cell surface (Link superfamily) HA receptor (19). Expressed on a variety of cell types, the CD44 molecule engages in multivalent HA binding that is tightly regulated by glycosylation, so-called “inside-out” signaling and receptor clustering/oligomerization (20–24). Although inactive on normal circulating leukocytes, CD44 can be induced to bind HA in response to inflammatory cytokines (25–27). Binding to HA is thought to direct the extravasation of leukocytes in inflamed tissues, where CD44 engages HA induced on or below the surface of vascular capillary endothelial cells (28–31) and to mediate dendritic cell migration in inflamed skin (32). Yet the unique involvement of CD44 in the aforementioned processes has been called into question by the demonstration that homozygous CD44−/− mice contain no obvious defects in either the vascular or lymphatic systems (33, 34). These factors have
highlighted the likelihood that novel HA receptors must occur within the immune system and elsewhere and have prompted a search for such candidates within the human genome.

Recently we identified a novel Link superfamily HA receptor termed LYVE-1 (LYmphatic Vessel Endothelial HA receptor-1) from a homology search of the human EST data base and showed its expression was exclusive to lymphatic endothelial cells within normal adult tissues (35). These initial studies revealed that the human LYVE-1 molecule binds HA with a high degree of specificity and suggested a role for the receptor in sequestering HA on the luminal surface of lymphatic vessels. To explore such possibilities in an animal model (see also Refs. 36–40, 69–70) we have isolated a murine LYVE-1 orthologue and here we describe its detailed characterization together with sequence comparisons that predict important similarities with the related CD44 molecule. Intriguingly, we have found that mouse LYVE-1 mediates internalization of HA and is located on both the luminal and abluminal faces of lymphatic endothelial cells. The implications of these findings for the function of LYVE-1 in vivo are discussed.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, Plasmids, and Reagents—**The transformed human primary embryonic kidney cell line 293T was obtained from the Imperial Cancer Research Fund Cell Bank, Clare Hall, United Kingdom. The eukaryotic expression vector pRCMV was from Invitrogen, Groningen, The Netherlands. The pCDMT7g vector for soluble Ig fusion protein expression was kindly provided by Dr. Alejandro Arruffo, Bristol-Myers Squibb (50). The pPPI-CD44-1 of 957 base pairs to mouse CD44 (RAM34) was obtained from Pharmingen. Texas RedTM-conjugated goat anti-rabbit was purchased from Southern Biotechnologies. FITC-conjugated goat anti-rat was from Bio-Rad, and Saponin was obtained from Sigma. Alexa 488-conjugated goat anti-rat was from Molecular Probes (Eugene, OR). Horseradish peroxidase-conjugated goat anti-rabbit and horseradish peroxidase-conjugated goat anti-human from Pierce were used.

High molecular weight hyaluronan from rooster comb (catalog number H-5388), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), and Saponin were obtained from Sigma. Biotin-LC-hydrazide was obtained from Pierce. Chondroitin 4-sulfate, chondroitin 6-sulfate, and heparan sulfate were from Sigma. An intracellular adhesion molecule-2 fusion protein containing the extracellular domain of intracellular adhesion molecule-2 fused to the Fc domain of human IgG, kindly donated by Dr. S. Adams (Molecular Parasitology Group, University of Oxford).

**Tissue Sections from CD44–/– Knockout Mice—**Homozygous CD44–/– knockout mice (33) bred on the C57Bl/6 background and wild-type C57Bl/6 controls were obtained from the MRC Center for Inflammation Research, University of Edinburgh, United Kingdom. These were prepared for microscopy as described below.

**Cloning of Full-length Mouse LYVE-1—**The amino acid sequence of human LYVE-1 was used to search for murine homologues within the mouse EST data base using the NCBI BlastSearch tool via the TBlastN program. The search yielded four overlapping ESTs, AI006667, AI391129, AI226003, and AA8820234, which together encoded a conserved domain (MET36–40, 69–70) we have isolated a murine LYVE-1 orthologue and here we describe its detailed characterization together with sequence comparisons that predict important similarities with the related CD44 molecule. Intriguingly, we have found that mouse LYVE-1 mediates internalization of HA and is located on both the luminal and abluminal faces of lymphatic endothelial cells. The implications of these findings for the function of LYVE-1 in vivo are discussed.

**Northern Blot Analysis—**A Northern blot containing a variety of normal mouse tissue RNAs (2 μg of poly(A)– mRNA/lane) was pur- chased from CLONTECH and hybridized in ExpressHyb solution (CLONTECH) with a probe spanning exons 3 and 4 of mouse LYVE-1 (694 base pairs) labelled with [α-32P]dCTP (Amersham Pharmacia Biotech) by random hexamer-primer labeling (Merck). Blots were washed according to the manufacturer's instructions in 2 × SSC, 0.05% SDS at room temperature for 30 min and in 0.1 × SSC, 0.1% SDS at 50 °C for 40 min followed by autoradiography. After stripping in 0.5% SDS (30 °C), blots were re-probed with human β-actin cDNA (supplied by the manufacturer).

**Cloning of a Soluble Mouse LYVE-1 Fusion Construct and Purification of the Fc Fusion Protein—**The extracellular domain of mouse LYVE-1 including the cleavable N-terminal leader (residues 1–228, MLHHTS . . . FKNEAAAG in Fig. 1B) was amplified from mouse stomach cDNA (41) by nested PCR. Reactions (1 μl of cDNA template) were performed as described above for full-length LYVE-1 using the primers 190F (GTCTCCTTTACTGCGGGTGTTG) and 994R (CAGCCGACGCA- CAGCGGCAAG), 1 min 94 °C, 1 min 59 °C, 3 min 72 °C, 33 cycles) followed by the primers 229F Hind (CGCGAAGCTTGGGATCTGCACAATGCTCCAG) and 923R Bam (GGTCGGGATCCCCAGCTGCTGTCGTTCTTGAATG; 1 min 94 °C, 1 min 57 °C, 3 min 72 °C, 33 cycles) using 2.5 units of Pfu polymerase (nucleotides numbered as in Fig. 1A). After digestion with HindIII and BamHI the PCR product was ligated into HindIII/BamHI cut pCDMT7g vector yielding a construct encoding the first 228 amino acids of mouse LYVE-1 fused to the Fc region of human IgG. The cloned construct was sequenced on both strands to confirm integrity.

For expression and purification of the Fc fusion protein, the construct was transfected into 293T cells using calcium phosphate and transfections grown in UltraCHO medium (Bio-Whittaker) for 3 days prior to harvesting the culture supernatant. After adjustment of the pH by the addition of 0.05 volumes of 2 × Tris-HCl buffer, pH 8.0, the fusion protein was purified by affinity chromatography on a column (1–ml bed volume) of protein A-Sepharose (Sigma) eluted with 0.1 M glycine-HCl (pH 2.0). Fractions containing Fc fusion protein were neutralized by the addition of 0.05 volume of 2 × Tris-HCl buffer, pH 8.0, and the purity confirmed by SDS-polyacrylamide gel electrophoresis.

**Biotinylated HA and FITC-conjugated HA—**High molecular weight HA was biotinylated by a modification of the method of Yu and Toole (42) exactly as described previously (35). Conjugation of HA with FITC was carried out using the method of De Belder and Wik (43).

**ELISA Titration Assays—**Binding of mouse LYVE-1 fusion protein to immobilized HA was tested in 96-well ELISA plates (Nunc Maxisorp) as described previously (35). Plates were coated by overnight incubation with 1 mg/ml HA in coating buffer (15 mM sodium carbonate and 34 mM sodium bicarbonate, pH 9.3). Wells were blocked for 2 h in PBS, 1% (v/v) bovine serum albumin, 0.05% (v/v) Tween 20, and subsequently incubated with purified mouse LYVE-1 Fc fusion protein (62.5–1000 ng/ml) in PBS, 0.05% Tween 20 for 1 h at room temperature. Human LYVE-1 Fc (35) and intracellular adhesion molecule-2 Fc fusion proteins were used as positive and negative controls, respectively. After washing (3 times with PBS, once with PBS, 0.05% Tween 20), bound fusion protein was detected with horseradish peroxidase-conjugated goat anti-human IgG (1:4000; Pierce) followed by o-phenylenediamine (Sigma). Subsequently, absorbance at 490 nm was measured in a Bio-Rad microplate reader.

**Competition experiments with free glycosaminoglycans including HA, chondroitin 4-sulfate, chondroitin 6-sulfate, and heparan sulfate were performed by preincubating the mouse LYVE-1 Fc fusion protein (10 μg/ml) with the appropriate glycosaminoglycan (3.13–100 μg/ml) for 30 min in PBS, 0.05% Tween 20. The mixtures were subsequently added to 96-well HA-coated plates and the fusion protein was detected as described above.

**Binding of LYVE-1 to soluble HA, 96-well plates were first coated overnight with either mouse or human LYVE-1 fusion protein or a control syndecan-2 Fc fusion protein (62.5–1000 ng/ml in coating buffer) followed by blocking and washing as described above. The wells were then incubated with biotinylated HA (5 μg/ml) in PBS, 0.05% Tween 20 (with or without a 20-fold molar excess of unlabeled HA as a control for specificity) and bound biotinylated-HA detected using horseradish peroxidase-conjugated avidin (1:500; DAKO) with o-phenylenediamine as substrate. Binding was measured as the absorbance at 490 nm.

**For binding of HA to LYVE-1-transfected cells (see below), these were incubated (20 min, 25 °C) in PBS, pH 7.5, containing FITC-conjugated HA (25 μg/ml, 0.1% azide, and 5% fetal calf serum followed by

Mouse LYVE-1 and Lymphatic HA Metabolism 19421
FIG. 1. Nucleotide and deduced amino acid sequence of murine LYVE-1 and comparison with the human orthologue. Panel A shows nucleotide (nucleotides 1–1440) and deduced amino acid sequence from the 1516-base pair mouse LYVE-1 cDNA identified by Blast Searching the mouse EST database and subsequently cloned from mouse lung cDNA (see “Experimental Procedures”). The predicted N-terminal leader and C-terminal transmembrane anchor are underlined and two motifs for potential N-glycosylation are boxed. Panel B shows an alignment of the amino acid sequences for mouse LYVE-1, human LYVE-1, and human CD44 generated with the GCG programs Pileup and PrettyPlot with similar residues highlighted in yellow. The solid blue line indicates the consensus Link module. Key cysteine residues are highlighted and indicated by colored circles. These are as follows; the four highly conserved structural cysteines of the Link module (red); the two conserved flanking cysteines essential for folding and function of CD44 Link (green); a seventh free cysteine unique to LYVE-1 (blue). Conserved residues within the LYVE-1 Link module equivalent to those implicated in CD44 HA-binding are highlighted with orange arrowheads. In addition, a conserved tract of basic residues downstream of the Link module in mouse and human LYVE-1 and a similar tract in CD44 that forms an extension to the HA-binding domain are highlighted with pink arrowheads.
washing (×3) in PBS alone. Cells were then fixed in 2% formaldehyde, mounted with fluorescent mounting medium, and viewed under a Zeiss Axioskop microscope equipped with epifluorescence illumination.

**Transient Transfection of 293T Cells and Assays for LYVE-1-mediated HA Internalization—** Human 293T cells in 12-well plates were transfected with full-length mouse or in some cases human LYVE-1 (35) in pRcCMV (2 μg/well) using calcium phosphate precipitation. For flow cytometric analysis of ligand internalization, triplicate wells were incubated with FITC-HA (1–10 μg/ml) alone or in the presence of a 500-fold molar excess of unconjugated HA (control) for 0–5 h at 37 °C. After appropriate time intervals, cells were washed briefly with ice-cold PBS and detached by suspension in ice-cold PBS, 5 mM EDTA with gentle pipetting. One-half of each detached cell sample was then fixed directly in 4% (w/v) paraformaldehyde in PBS (to determine total HA accumulation) and the other digested with papain (0.5 mg/ml) for 45 min at 37 °C to remove cell surface LYVE-1-HA complexes followed by fixation (to determine internalized HA). The efficiency of LYVE-1 cleavage under the latter conditions was assessed by staining cells before and after papain treatment with polyclonal anti-mouse LYVE-1 (1/500) followed by phycoerythrin-conjugated goat anti-rabbit Ig. In each case fluorescence was quantitated by flow cytometry using a Becton-Dickinson FacScan.

For analysis of HA internalization by immunofluorescence microscopy, cells were prepared as described for flow cytometry except that cell surface LYVE-1 was stained using Texas Red-conjugated goat anti-rabbit Ig rather than a phycoerythrin conjugate. Slides were then viewed using a Zeiss Axioskop microscope equipped with epifluorescence illumination. For analysis of LYVE-1/HAI internalization by confocal microscopy, transfecants were incubated (5 h, 37 °C) with FITC-HA (10 μg/ml) and antibodies to either mouse or human LYVE-1 (1/500). Cells were then fixed (10 min, room temperature) by paraformaldehyde and permeabilized with 0.25% (w/v) saponin, in PBS, pH 7.5, containing 1% bovine serum albumin and 1% fetal calf serum (30 min, room temperature) prior to the addition of Texas Red™ anti-Ig conjugate. Slides were viewed on a Bio-Rad Radiance 2000 laser scanning confocal microscope equipped with argon and green helium/neon lasers.

**Generation of Mouse LYVE-1 Antisera in Rabbits—** Rabbits were immunized by subcutaneous injection with purified mouse LYVE-1 fusion protein (100 μg) in complete Freund’s adjuvant followed by two further injections in Freund’s incomplete adjuvant at 14-day intervals. Antiserum was tested in ELISAs by assessing reactivity with immobilized mouse LYVE-1 fusion protein or CD44Fc fusion protein as a negative control. Sera were then subjected to affinity chromatography on human IgG-agarose to deplete antibodies directed against the Fc portion of the immunogen.

**Generation of Mouse Lymphangiomas—** Female Balb/c mice were injected (×2) intraperitoneally with 0.1 ml of a 1:1 emulsion of Freund’s incomplete adjuvant in PBS, pH 7.5, at 2-week intervals as described in Ref. 44. Two weeks after the second injection, the mice were sacrificed by cervical dislocation and lymphangiomas which developed on the abdominal surfaces of the diaphragm and in the liver resected for fixation and staining as described below.

**Preparation of Tissues and Cells for Immunoperoxidase/Immunofluorescent Antibody Staining—** Tissues were removed from female Balb/c mice or C57B16 homozygous CD44^{−/−} knockout mice, fixed in PBS, 4% paraformaldehyde, and embedded in paraffin wax. Prior to staining, sections were dewaxed and rehydrated by successive incubation in Citroclear™ (2 × 5 min) 100% industrial methylated spirit (2 × 5 min), 50% industrial methylated spirit (5 min), and water (5 min). Antigen retrieval was performed by microwave treatment (95–100 °C, 10 min) in 0.1 M Tris, 2 mM EDTA, pH 9.0. Sections were then blocked by incuba-
tion in PBS, 5% fetal calf serum for 5 min and treated with a peroxidase quenching agent (Dako) for 5 min prior to incubation with rabbit polyclonal anti-mouse LYVE-1 (1:400) for 45 min. After washing with PBS, slides were incubated with anti-rabbit Ig peroxidase conjugate (Envision kit, Dako) for a further 45 min and developed with diaminobenzidine (Dako) before counterstaining with hematoxylin. All incubations were performed at room temperature.

For double-immunofluorescent staining, tissues (Balb/c mice) were snap frozen in liquid N₂, cut into thin sections using a cryotome and fixed in acetone (room temperature, 10 min) prior to incubation with polyclonal anti-mouse LYVE-1 and rat anti-mouse CD34 (1/100). Sections were then treated with a mixture of Texas Red TM-conjugated goat anti-rabbit Ig (1:50) and Alexa 488-conjugated goat anti-rat Ig (1:200). Slides were fixed in 2% formaldehyde, mounted with fluorescent mounting medium (Vectashield), and viewed under a Zeiss fluorescence microscope. For fluorescent staining of LYVE-1-transfected 293T cells, these were incubated with rabbit anti-mouse LYVE-1 (1/100) in PBS, 5% fetal calf serum, 0.1% azide for 30 min prior to washing in PBS and re-incubation with FITC-conjugated goat anti-rabbit Ig (1/100).

Immunoelectron Microscopy—For immunoelectron microscopy, sections of formaldehyde-fixed mouse small intestine (see above) were washed (3 times) in 0.1M phosphate buffer and cut into 2-mm cubes. These were then incubated (room temperature, overnight) with LYVE-1 polyclonal serum (1/100 dilution), washed, and stained with either immunogold or horseradish peroxidase-conjugated anti-rabbit Ig. Samples were post-fixed in osmium tetroxide in 0.1 m phosphate buffer, dehydrated, and embedded in Spurr’s epoxy resin. Thin sections were cut and examined in a JEOL 1200EX electron microscope.

RESULTS

Cloning of Murine LYVE-1 and Comparison with Human LYVE-1 and CD44—We originally identified the human LYVE-1 cDNA by searching the EST data base for homologues with the amino acid sequence of the Link HA-binding domain of the human CD44 molecule. Here we used the human LYVE-1 amino acid sequence to identify the mouse orthologue by Blast-Searching the mouse EST data base with the program TBlastN. The search yielded four overlapping ESTs, AI006667, -391129, and -226003, and AA8820234 that formed a contiguous sequence of 1516 base pairs. The cDNA which was subsequently cloned from mouse lung using nested PCR (Fig. 1A) contains a large open reading frame of 318 amino acids (four residues shorter than the human receptor) starting with a hydrophobic leader sequence. 2) A 211-residue hydrophilic segment corresponding to the extracellular domain containing seven cysteines, four of which (Cys⁵⁰, Cys⁸⁴, Cys¹⁰⁵, and Cys¹²⁷) are predicted to form the conserved disulfide bridges that stabilize the link module (see below), two N-glycosylation sites (Asn⁶² and Asn¹¹⁷), and a serine/threonine-rich tract (residues 144–188) likely to be heavily O-glycanated. 3) A 21-residue hydrophobic transmembrane anchor. 4) A 63-residue cytoplasmic tail.

Alignment of the murine and human LYVE-1 sequences (Fig. 1B) revealed an overall similarity between the two orthologues of 74% (69% identity). Within the extracellular domain, the regions corresponding to the HA-binding Link homology unit (residues 44–89) are 79% similar, while the downstream, serine/threonine-rich membrane-proximal domains are only 48% similar. Nevertheless eight of the 13 potential O-glycanation sites in this region are completely conserved, as are both the N-glycosylation motifs bracketing the Link module, suggesting that glycosylation, which is known to regulate HA-binding in CD44, may also be important for LYVE-1 function.

The three-way alignment of mouse LYVE-1, human LYVE-1, and human CD44 (Fig. 1B) highlights a number of potentially important similarities between the HA-binding domains of the two receptors, both within the Link module and in the down-stream membrane-proximal/transmembrane domains. For example, the three residues Lys⁴⁶, Tyr⁷⁷, and Asn¹⁰⁹ in human LYVE-1 Link previously shown to correspond to the known CD44 HA-binding residues Lys⁸⁸, Tyr⁷⁹, and Asn¹⁰⁰ (45, 46) are fully conserved in mouse LYVE-1 Link. This interspecies conservation further strengthens the prediction that these amino acids bind HA in the LYVE-1 link domain, a prediction supported by preliminary results from site-directed mutagenesis experiments (data not shown).

Downstream of the Link module, both mouse and human LYVE-1 contain tracts of basic residues (RRKK and RRKK, respectively) conserved in identical locations. Indeed a virtually identical tract (RRKK) is also conserved in a bovine LYVE-1 orthologue identified in a further search of the EST data base (not shown). This feature may have functional significance in light of the fact that a tract of basic residues in the membrane-proximal domain of CD44 has been shown to contribute to HA binding (45). We also note that an adjacent cysteine residue which is absent in CD44 is conserved in both mouse and human LYVE-1 (Cys¹⁹⁷ and Cys²⁰¹, respectively). This may in turn be functionally important as this cysteine is predicted to be unpaired and thus to form a free thiol that could form an intermolecular disulfide bond leading to LYVE-1 dimerization. In addition, the transmembrane anchors of mouse and human LYVE-1 both have a conserved cysteine residue (Cys²⁵³ and Cys²⁵⁷) in the same position as Cys²⁸⁹ in the CD44 molecule that is implicated in covalent dimerization and HA binding (47, 48). Together these sequence comparisons raise the interesting possibility that LYVE-1, like CD44, may possess a regulated HA-binding domain that extends beyond the immediate link module (46).

Binding to Hyaluronan and Other Glycosaminoglycans—To assess the functionality of the murine receptor we first transfected 293T human fibroblasts with full-length cDNA in pRcCMV and measured binding of FITC-HA to the cell surface. As shown by the data in Fig. 2D, the majority of LYVE-1 transfectants but none of the control mock-transfected cells bound FITC-HA. Surface expression of the receptor in these experiments was confirmed clearly by immunofluorescent antibody staining with mouse LYVE-1-specific polyclonal sera (see below). To investigate HA binding in more detail we expressed the extracellular domain of murine LYVE-1 as a solubleFc fusion protein and measured binding to ligand immobilized in 96-well microtiter plates. The results (Fig. 2, A and B) confirm the mouse molecule binds both immobilized high molecular weight HA and soluble biotinylated HA in a concentration-dependent fashion. Furthermore, binding to immobilized HA was inhibited only by free hyaluronan and not by the glycosaminoglycans chondroitin 4-sulfate, chondroitin 6-sulfate, or heparan sulfate (Fig. 2C). These latter results indicate that the murine LYVE-1 receptor has a specificity for hyaluronan that is similar to that of the human orthologue but is distinct from the closely related CD44 molecule which binds both HA and chondroitin sulfates.

LYVE-1 Mediates HA Internalization—The capacity of LYVE-1 to function as a receptor for HA internalization was assessed in experiments where LYVE-1-transfected 293T cells were incubated with FITC-HA and the accumulated ligand assayed by flow cytometry. Internalized ligand was distinguished from surface bound ligand in these assays by measuring FITC-HA fluorescence both before and after treatment of cells with papain to cleave exposed LYVE-1-HA complexes. As shown by the progress curve in Fig. 3A, LYVE-1-transfected 293T cells bound and internalized FITC-HA rapidly. The rate of total accumulation (the sum of both cell surface and internalized components) was logarithmic, reaching a plateau.

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within 1–2 h, while the rate of internalization was linear, reaching a plateau after 4 h. Indeed some 50% of the accumulated HA was internalized by LYVE-1-transfected cells within this time interval. As binding of FITC-HA was blocked (>80%) by a 500-fold excess of unlabelled HA and no uptake was observed with untransfected 293T cells (Fig. 3A), the internalization of FITC-HA most likely represents LYVE-1 receptor-mediated endocytosis rather than simple pinocytosis. Further quantitation of the FITC-HA internalized by cells in these experiments showed that protease treatment sufficient to cleave 90% of cell surface LYVE-1 released only 56% of the accumulated FITC-HA (Fig. 3B). The intracellular localization of the residual papain-resistant HA was confirmed by immunofluorescent microscopy which revealed FITC-HA within numerous small vesicles beneath the plasma membrane (Fig. 3C). Furthermore, these vesicles were also shown to contain internalized LYVE-1 when FITC-HA-loaded cells were permeabilized with saponin for fluorescent antibody staining and confocal microscopy (Fig. 3D, top panels). Similar uptake of FITC-HA was also observed in experiments with human LYVE-1-transfected 293T cells (Fig. 3D, bottom panels, and data not shown). These results provide clear evidence that LYVE-1 promotes both binding and internalization of HA through receptor-mediated endocytosis.

**Tissue-specific Expression of Murine LYVE-1 mRNA**—As an initial test for the tissue specificity of mouse LYVE-1 expression we compared transcript levels in different tissues by Northern blotting. As shown by the blot in Fig. 4, hybridization to a single 2.6-kilobase transcript was seen only in lung, liver, and heart, while no mRNA was detected in either spleen, muscle, kidney, testis, or brain, even after extended exposure (data not shown). This contrasts markedly with the human orthologue where the mRNA is readily detected in each of these tissues and is particularly abundant in spleen (35). These results suggest there are significant differences either in the pattern of LYVE-1 gene expression or in LYVE-1 mRNA stability in humans and mice.

**Immunohistochemical Detection of Murine LYVE-1 in Normal and Neoplastic Lymphatics**—In order to immunolocalize the LYVE-1 polypeptide in murine tissues we generated a polyclonal serum by immunizing rabbits with soluble murine LYVE-1 Ig Fc fusion protein. The resulting antiserum was highly specific for the murine receptor even at high dilution (>1/5000) as assessed by an ELISA and failed to bind either the human LYVE-1 or human CD44 fusion proteins (Fig. 5). Correspondingly, antibodies to the human LYVE-1 molecule failed to recognize the mouse orthologue (data not shown). The specificity of the LYVE-1 antiserum was additionally confirmed by immunofluorescent staining of 293T cells transfected with full-length LYVE-1 which detected the receptor in an intense ring-staining pattern at the cell surface (Fig. 2D). Furthermore, the antiserum blocked binding of LYVE-1 Fc fusion protein to immobilized HA, indicating the presence of antibodies directed against the ligand-binding domain (data not shown).

Immunoperoxidase staining of a range of paraffin-embedded normal C57Bl6 mouse tissues including large intestine, lung, and cardiac smooth muscle (Fig. 6) and stomach, skin, and skeletal muscle (not shown) using LYVE-1 polyclonal serum revealed strong staining of vessels. These were identified as lymphatics on the basis of their characteristic flattened, irregular morphology, empty lumena (lacking erythrocytes), and the absence of a basement membrane. The LYVE-1-stained vessels were particularly abundant in tissues such as the lamina propria of large intestinal mucosa that have an extensive lym-
phatic network (Fig. 6, panel A) and in experimental lymphangiomas induced by intraperitoneal injection of Freund’s adjuvant (Fig. 6, panel D). Moreover the LYVE-1-stained lymphatics could be clearly distinguished from blood capillaries by double immunofluorescence staining with LYVE-1 and the vascular endothelial marker CD34 (Fig. 7, panels A–C) which revealed two discrete populations of single positive vessels (green CD34 and red LYVE-1). Together these results confirm mouse LYVE-1, like its human orthologue is largely restricted to lymphatic vessel endothelium.

**LYVE-1 Expression in Homozygous CD44<sup>−/−</sup> Knockout Mice**—Recent reports demonstrating apparently normal HA metabolism in CD44<sup>−/−</sup> knockout mice suggest the possibility that additional compensatory HA receptors may be up-regulated in these animals. To test the hypothesis that LYVE-1 might fulfill such a compensatory role we compared its expression pattern in adult tissues prepared from wild-type and CD44<sup>−/−</sup> animals by means of immunohistochemical staining. The results revealed equally intense staining of lymphatic vessels in both C57/Bl6 wild-type and C57/Bl6 CD44<sup>−/−</sup> mouse tissues (Fig. 6 compare panels A and B with panels E and F). Moreover no gross differences in the level or distribution of LYVE-1 staining or in the numbers of lymphatic vessels were apparent when a comprehensive panel of different tissues was compared between the two mouse populations (not shown).

Importantly, no CD44 expression was detected in the lymphatics of wild-type mice or in any tissues in the CD44<sup>−/−</sup> mice as assessed by immunostaining with the monoclonal antibody IM-7 (not shown). Although our analyses do not exclude the possibility of more subtle alterations in LYVE-1 expression in CD44<sup>−/−</sup> tissues or of alterations occurring during embryogenesis, the results generally indicate that LYVE-1 is unlikely to compensate for the loss of CD44 expression and that additional as yet unidentified HA-receptors are likely to be involved.

**Murine LYVE-1 Is Located on the Luminal and Abluminal Faces of Lymphatic Endothelial Cells**—To distinguish between the possibilities that LYVE-1 functions as a receptor for the uptake of HA from the lymph or as a receptor for HA in the tissues immediately underlying the lymphatics, we used immunoelectron microscopy to determine whether LYVE-1 is exposed to the luminal or the basolateral face of lymphatic endothelial cells.

Intriguingly, the analyses (Fig. 8) revealed expression of LYVE-1 (detected both with immunogold and horseradish peroxidase conjugates) on both faces of lymphatic endothelium. Individual endothelial cells were visible as thin elongated cells containing many intracellular vesicles, that were devoid of basement membrane and formed characteristic overlapping cell:cell junctions. The bipolar distribution of LYVE-1 was consistently observed in multiple different sections, further supporting the possibility that the receptor is engaged in transport of HA into the vessel lumen.
In this article we have described the cloning, expression, and functional characterization of the mouse lymphatic endothelial HA receptor, LYVE-1, and shown that it mediates both binding and internalization of HA from the surrounding medium. In addition, we have shown that mouse LYVE-1, similar to its human orthologue, is expressed almost exclusively on endothelial cells in lymphatic vessels and capillaries in normal tissues and in a primary lymphatic endothelial tumor and have demonstrated, using immunoelectron microscopy, that the receptor is present on both the luminal and abluminal endothelial surfaces.

We have detailed the degree of sequence homology between the HA receptors CD44 and LYVE-1 from different species and have predicted on the basis of key conserved structural features that LYVE-1, in common with CD44, contains an extended HA-binding domain that is potentially subject to regulatory control. Finally, we have presented evidence that the pattern of LYVE-1 expression is not significantly altered in homozygous CD44<sup>−/−</sup> knockout mice, implying that additional as yet unidentified HA receptors are present within the genome.

In a previous article (35) we showed that human LYVE-1 functions as a specific high-affinity receptor for HA in lymphatic vessel endothelium. In this present article we have...
shown using the mouse orthologue that LYVE-1 also mediates the endocytosis of HA. Using flow cytometry to quantitate the accumulation of FITC-HA we showed LYVE-1-transfected 293T fibroblasts bind and internalize ligand relatively rapidly, with ~50% of total HA partitioning into a protease-resistant intracellular compartment within 4 h (see also below). Moreover, analysis of FITC-HA-loaded cells using both standard fluorescence and laser scanning confocal microscopy clearly demonstrated that HA and LYVE-1 co-localize within numerous small vesicles beneath the plasma membrane. The co-localization of ligand and receptor, coupled with the observations that HA binding/internalization is inhibited by LYVE-1 polyclonal sera2 and that untransfected 293T cells fail to internalize FITC-HA, all point to uptake by means of receptor-mediated endocytosis rather than fluid phase pinocytosis.

Classical receptor-mediated endocytosis, during which receptor-ligand complexes become concentrated within membrane pits coated with the geodesic lattice protein clathrin, is exemplified by transferrin, low density lipoprotein, and immunoglobulin Fc receptors and receptor tyrosine kinases such as epidermal growth factor receptor and fibroblast growth factor receptor (49–51). In common with other receptors that utilize the coated pit pathway these all have cytoplasmic tails that contain either tyrosine-based motifs (Y\(\times\)F, or N\(\times\)Y, where \(\times\) represents any amino acid and \(\Phi\) represents a bulky hydrophobic residue) or dihydrophobic repeats (e.g. LL, LV) which promote the formation of coated vesicles through binding the AP2 clathrin-adapter/clathrin protein complex (51, 52). However, no such sequences are present within the cytoplasmic tail of either mouse LYVE-1 or human LYVE-1. Furthermore, in preliminary studies, incubation of LYVE-1-transfected 293T cells in hyperosmolar conditions (0.4M buffered sucrose) similar to those previously shown to inhibit clathrin-mediated endocytosis in fibroblasts (53) did not significantly affect LYVE-1 mediated HA uptake.3 These properties are similar to CD44, which mediates uptake of HA by fibroblasts, macrophages, and chondrocytes by a mechanism that is not understood but which involves neither coated pit formation nor pinocytosis (54–56).4 Hence the possibility exists that both LYVE-1 and CD44 mediate endocytosis via novel pathways.

An alternative possibility is that LYVE-1 mediates the up-

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2 R. Prevo and D. G. Jackson, unpublished observation.
3 R. Prevo and D. G. Jackson, unpublished data.
4 R. Tammi, personal communication.
take of HA via caveolae. These are small invaginations of the plasma membrane lined with the 22-kDa membrane-anchored protein caveolin (57) that mediate endocytosis and transcellular transport by a mechanism that is distinct from the coated pit pathway (58). It is interesting to note that caveolin-mediated transport is particularly apparent in endothelial cells and that the LYVE-1 cytoplasmic tail contains the hydrophobic motif YXXXFP, that approximates the consensus found within proteins that bind to caveolin (59). Further experiments to investigate the possible association between LYVE-1 and caveolae are currently in progress.

The capacity to mediate HA-internalization suggests a physiological role for LYVE-1 in lymphatic HA turnover. The majority of HA turnover in tissues such as the skin and digestive tract is known to occur in draining lymph nodes. This process is highly efficient and studies have shown that some 80% or more of the HA entering afferent lymphatics is degraded during passage through the nodes, the remaining 10–20% being rapidly cleared by the liver and hydrolyzed to the level of small saccharides (1, 3, 4). However, recent evidence points to the oligomeric HA receptor “HARE” (HA receptor for endocytosis, see Ref. 60) rather than LYVE-1 as the major receptor for HA-uptake in lymph node.

Originally thought to be a liver-specific endothelial HA receptor (LEC HAR, Refs. 5, 6, and 61), HARE is also expressed abundantly in spleen and lymph node medullary sinuses (60). In common with HARE-mediated uptake of HA in rat liver cells (62), the uptake and degradation of HA in lymph node (63) can be blocked by heparin and chondroitin sulfate, yet as shown in this present article neither glycosaminoglycan blocks binding/uptake of HA by LYVE-1. Moreover HARE, which recycles rapidly via clathrin-coated pits, can transport HA at a rate (250 molecules/s/cell) similar to that reported for professional scavengers such as the asialoglycoprotein receptor and the macrophage mannose receptor (62). Although it is difficult to make a quantitative comparison of endocytic rates between the HARE receptor and LYVE-1, we estimate using previously published data (62) that the rate of LYVE-1-mediated HA uptake is considerably lower (50% of total HA internalized/4 h, 37 °C) than that of HARE (90% of total HA internalized/1.5 h, 37 °C). Indeed LYVE-1 appears to mediate HA internalization at a rate that is closer to that reported for its closest homologue CD44 in primary chondrocytes (14% of total HA/8 h; 20% of total HA/8 h) and chondrosarcomas (55, 56). Based on these considerations we consider it unlikely that LYVE-1 plays a significant role in the rapid uptake and degradation of HA within lymph nodes and suggest HARE rather than LYVE-1 is the primary receptor for lymphatic HA degradation.

What then is the likely role of LYVE-1 in the lymphatic system? Rather than facilitating the degradation of HA in lymph node, we suggest LYVE-1 may be involved in its transport across lymphatic endothelium, specifically the movement of tissue HA from interstitium to lymph. This hypothesis is supported by our finding (using immunoelectron microscopy) that LYVE-1 is present on both the luminal and abluminal faces of lymphatic capillaries, a disposition that suggests shuttling across the endothelium or “transcytosis.” Prominent examples of receptors involved in transcytosis are the α2-acidic glycoprotein receptor on vascular endothelium (64) and the polymeric Ig receptor on mucosal epithelium (65). Recent observations that HA applied to the skin can rapidly enter the dermal lymphatics (7) are also consistent with the occurrence of a pathway for rapid transendothelial transport of this glycosaminoglycan in vivo. Efficient macromolecular transport is of course one of the key roles of the lymphatic endothelium (66) and the process has been studied in detail by means of tracer labeling experiments with isolated perfused renal lymphatics (67). Moreover, ultrastructural analyses of lymphatic endothelium have consistently revealed an abundance of intracytoplasmic vesicles, and clusters of these have been suggested to form stable channels that facilitate trans-endothelial transport (68). Regardless of whether HA permeates the lymphatic endothelium by means of channels or by conventional vesicular transfer, it is quite likely that LYVE-1 is directly involved in the process. Clearly, experiments with primary LYVE-1−/− lymphatic endothelial cells will help clarify these issues.

Lastly, it is possible that LYVE-1 regulates the entry of leukocytes or tumor cells into the lumen of afferent lymphatic capillaries. For example, in the skin, resident CD44+/+ epidermal Langerhans cells are known to migrate to draining lymph nodes in response to proinflammatory cytokines and HA breakdown products produced during tissue inflammation (32). The initial entry of these cells to the lumen of lymphatic capillaries could conceivably be facilitated by interaction with LYVE-1-HA complexes on the abluminal face of the endothelium or in overlapping cell junctions. A similar mechanism could regulate the entry of metastasizing tumor cells, many examples of which disseminate to regional lymph nodes in human cancers. Such hypotheses are amenable to testing by experiments in animal models using LYVE-1 antibodies (see e.g. Refs. 36–40) or soluble LYVE-1 Fc fusion protein as adhesion blocking reagents. These studies and the construction of a LYVE-1 knockout mouse will ultimately determine the true physiological function of LYVE-1 in vivo.
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