The major lymphatic endothelial hyaluronan receptor LYVE-1, a Link superfamily glycoprotein similar to the hyaluronan-binding/inflammatory leukocyte homing receptor CD44, was initially implicated in hyaluronan (HA)-mediated cell adhesion and lymph-borne hyaluronan metabolism. However, the apparently normal phenotype of Lyve-1 knock-out mice and the recent demonstration that the receptor undergoes cytokine-induced endocytosis independent of HA uptake have cast doubt on such functions. Here we present new data that reconcile these anomalies by showing that LYVE-1 is functionally “silenced” in a cell-specific fashion by autoinhibitory glycosylation. We demonstrate that LYVE-1 transfects in HEK 293T fibroblasts and Jurkat T cells is competent to bind HA, whereas the endogenous receptor in cultured lymphatic endothelial cells or the receptor transfected in Chinese hamster ovary and HeLa cells is not. Moreover, through a combination of mutagenesis and functional analysis in HEK 293T fibroblasts and glycosylation-defective Chinese hamster ovary cell lines, we reveal that the inhibitory mechanism is reversible and is exerted by terminal sialylation, most likely through α2–3 or α2–6 linkage to O-glycans. Finally, we provide evidence that the mechanism operates in vivo by showing that native LYVE-1 in primary lymphatic endothelial cells is extensively sialylated and that HA binding can be reactivated by neuraminidase treatment of the soluble ectodomain. These results reveal unexpected complexity in the regulation of LYVE-1 function and raise the possibility that this receptor, like CD44, may become active after appropriate unmasking in vivo.

LYVE-1, a recently discovered member of the hyaluronan-binding Link protein superfamily is expressed abundantly on the surface of lymphatic and liver sinus endothelial cells and some populations of tissue macrophages. In light of these characteristics, LYVE-1 has been employed extensively as a molecular marker to distinguish lymphatic vessels from their blood vascular counterparts in normal and tumor tissue (2–6). Composed of a 212-residue glycosylated extracellular domain, a 21-residue transmembrane anchor, and a 63-residue cytoplasmic tail, the LYVE-1 molecule resembles the major form of the leukocyte hyaluronan receptor CD44 (7, 8), with which it shares some 46% amino acid sequence similarity, within the N-terminal ectodomain (9, 10). In common with CD44, the extracellular domain of LYVE-1 contains a single hyaluronan binding unit based around a core consensus Link module. This is supplemented by an additional disulfide linkage and elements of the β-stranded Link extension that form a critical adjunct in the CD44 molecule implicated in allosteric regulation of HA binding affinity by inflammatory cytokines and inflammation-associated HA-protein complexes (11–13). Consistent with these structural characteristics, recombinant LYVE-1 expressed either on the surface of HEK 293T fibroblasts or as a soluble ectodomain fusion protein displays specific saturable binding to both free and immobilized high molecular weight HA that is inhabitable with monoclonal antibodies directed to the putative ligand interaction surface (13, 14).

Based on its ligand binding specificity and the known involvement of the lymphatic system in the transport and turnover of extracellular matrix glycosaminoglycans (15, 16), LYVE-1 was initially predicted to function as a professional receptor for HA uptake and degradation within adherent lymphatic vessels and lymph node sinuses (17). Furthermore, the observed capacity of recombinant LYVE-1 to form stable complexes with HA and soluble CD44 in vitro (9) suggested that LYVE-1 might play a role in trafficking of leukocytes to the lymph nodes in vivo, by supporting HA-mediated adhesion of CD44-expressing cells to the basolateral and luminal surfaces of lymphatic endothelium. Recently, however, doubt has been cast on such hypotheses by gene knock-out studies, which have revealed apparently normal levels of HA in serum and peripheral tissues of Lyve-1-deficient mice and no obvious impairments of lymphatic endothelium function.4

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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4 D. G. Jackson (2004) Glycoforum, www.glycoforum.gr.jp/science/hyaluronan/HA28/HA28E.html.

5 The abbreviations used are: HA, hyaluronan; bHABP, biotinylated hyaluronan-binding protein; CHO, Chinese hamster ovary; Fl-HA, fluorescein-conjugated hyaluronan; HDLEC, human dermal lymphatic endothelial cell(s); Ab, antibody; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; MOPS, 4-morpholinepropanesulfonic acid; SNA, S. nigra agglutinin; MAA, M. amurensis agglutinin.
Sialylation Regulates LYVE-1 Function

ment of leukocyte entry or trafficking through afferent lymphatics (18, 19). Indeed, wild-type mice subjected to conditions of inflammation that might be expected to enhance leukocyte trafficking and HA turnover promoted endocytosis and targeted degradation of LYVE-1 in lysosomes without any attendant uptake of HA (14). It is difficult to reconcile these whole animal studies with the functional data obtained for LYVE-1 in vitro. Hence the true physiological role of LYVE-1 remains unclear, prompting speculation that the receptor is either inactive as an HA receptor or that it binds additional ligands besides HA in vivo.

Here we have investigated the interaction of LYVE-1 with HA and its regulation in some detail. Whereas recombinant LYVE-1 is functionally active in transfected HEK 293T fibroblasts, we show that the endogenous receptor is constitutively inactive both in lymphatic vessels in vivo and in lymphatic vessel endothelial cells in vitro. Using a combination of site-directed mutagenesis, enzymatic deglycosylation, and expression in leukocyte and mutant CHO cells, we show that inactivation of HA binding is exerted by autoinhibitory sialic acids, most likely those at the termini of O-linked glycans, and that binding can be unmasked by neuraminidase cleavage. Such regulation of HA binding by charged sugars either blocking the ligand-binding site or reducing avidity through effects on self-association is reminiscent of CD44 and indicative that LYVE-1 is similarly cryptic. We predict that LYVE-1, like CD44, may indeed be active in vivo but only after appropriate unmasking.

EXPERIMENTAL PROCEDURES

Cells—HEK 293T fibroblasts were obtained from the CRUK cell bank (Clare Hall, London, UK), and HeLa, CHOK1, and Jurkat cells were from the Medical Research Council Human Immunology Unit Cell Bank (Oxford, UK). Primary human dermal lymphatic endothelial cells (HDLEC) were isolated from surgically resected skin tissue by LYVE-1 mAb MACS® bead immunoselection and cultured in EGM-2MV on gelatin (0.1%, w/v)-coated plastic, as described previously (20). CHOK1 cells and the mutant lines Lec1, Lec2, and Lec3.2.8.1 (21–23) were generously provided by Dr. Pauline Rudd (University of Oxford, UK) and Prof. Simon Davis (Human Immunology Unit, Oxford, UK) with full permission from the originators. LDL-D CHO cells were kindly donated by Dr. Monty Krieger (MIT Institute of Biology).

Antibodies, Lectins, and Other Reagents—Mouse anti-human CD44 (BRIC235) was from the International Blood Group Reference Laboratory (Bristol, UK). Rat anti-mouse CD44 (KM81) was a kind gift from Jayne Lesley (The Salk Institute, San Diego, CA). Goat anti-human IgG (Fc-specific) antibody was from Sigma. Affinity-purified rabbit anti-human/rabbit anti-mouse LYVE-1 polyclonal antibodies and the monoclonal anti-human LYVE-1 antibodies 8C and 3A have been described previously (9, 14). The rat monoclonal anti-mouse LYVE-1 antibody C1/8 was generated against a soluble murine LYVE-1 ectodomain-human IgG Fc fusion protein and purified from hybridoma supernatants using Protein A-Sepharose. Biotinylated HA-binding protein was from Seikagaku. Alexa 488- or Alexa 568-conjugated goat anti-rabbit Ig and goat anti-mouse Ig and Alexa 488- or Alexa 594-conjugated streptavidin were from Molecular Probes, and goat anti-rabbit Cy5 was from Chemicon. R-phycocerythrin-conjugated rabbit anti-mouse Ig and peroxidase-conjugated streptavidin were from DAKO. Fluorescein isothiocyanate-conjugated sheep anti-digoxigenin antibody and alkaline phosphatase-conjugated sheep anti-digoxigenin antibody were from Roche Applied Science. Peroxidase-conjugated anti-rabbit IgG was from Pierce. Peroxidase-conjugated Sambucus nigra agglutinin and Maackia amurensis agglutinin were from EY Laboratories. Digoxigenin-conjugated S. nigra agglutinin and M. amurensis agglutinin were from Roche Applied Science. Purified neuraminidases from Macrobodella decora and Streptococcus pneumoniae (α2–3-linked sialic acid-specific) and from Arthrobacter ureafaciens (α2–3- and α2–6-linked sialic acid-degrading) were obtained from Calbiochem. High molecular weight HA (Hylumed Medical grade; Genzyme) was biotinylated by a modification of the method of Yu and Toole (24) exactly as described previously (9). High molecular weight HA was also conjugated with fluorescein, using the method of de Belder and Wick (25).

Immunofluorescence Microscopy and LYVE-1 HA Co-localization Studies—Tissues (BALB/c mice) were snap-frozen in liquid N₂, cut into 10-μm sections using a cryotome (Leica), and fixed in acetone (room temperature; 10 min) prior to blocking with 5% (v/v) goat serum and mounting on glass microscope slides. For co-localization with endogenous HA, slides were first incubated (overnight, 4 °C) with biotinylated HA-binding protein (bHABP; 3 μg/ml) in PBS, 5% (v/v) goat serum and then washed and stained with affinity-purified polyclonal rabbit anti-mouse LYVE-1 Ig (10 μg/ml), followed by a mixture of Alexa 488-conjugated goat anti-rabbit Ig (1:200) and Alexa 594-conjugated streptavidin (1:200). In an alternative procedure, small intestinal tissue from C57/B16 mice was fixed in a 4% (w/v) paraformaldehyde solution containing 1% (w/v) cetylpyridinium chloride prior to paraffin embedding to prevent leaching of endogenous HA. Sections were dewaxed and heated at 95 °C in antigen retrieval buffer (100 mM Tris-HCl, pH 8.5, 2 mM EDTA) for 3 min. Staining was carried out as described above except that bound bHABP was detected by Alexa 488-conjugated streptavidin (1:200), and anti-LYVE-1 was detected by goat anti-rabbit Cy5 (1:50). In separate controls for bHABP staining specificity, tissue sections were subjected to an identical procedure with the exception that an excess of unlabeled high molecular weight HA was included during the bHABP incubation step, or they were pretreated (1 h, 37 °C) with ovine testicular hyaluronidase (10,000 units/ml) prior to staining with bHABP (see Fig. S1). Slides were counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole solution in ethanol, mounted with fluorescent mounting medium (Vectorshield), and viewed under a Zeiss fluorescent microscope (digital camera). As a control for the specificity of the HA-binding probe (bHABP), sections were digested for 1 h at 37 °C with 10,000 units/ml ovine testicular hyaluronidase (Calbiochem) before staining as described above.

For co-localization with exogenously applied Fl-HA, frozen sections of ear pinnae were first digested with ovine testicular hyaluronidase (10,000 units/ml, 37 °C, 1 h) prior to incu-
bation with FL-HA (5 μg/ml) and polyclonal rabbit anti-mouse LYVE-1 Ig (10 μg/ml), followed by Alexa 568-conjugated goat anti-rabbit Ig (1:200 diluted) and counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole solution in ethanol. As a separate control for binding specificity, tissues were subjected to an identical staining procedure with the exception that a 100-fold molar excess of soluble high molecular weight HA was included during the FL-HA incubation step (see Fig. S2). Sections were mounted with fluorescent mounting medium (Vectashield) and viewed under a Zeiss fluorescent microscope.

Preparation of LYVE-1 Ectodomain Fc Fusion Proteins—Human LYVE-1 ectodomain Fc fusion protein (residues 1–232 cloned in the expression vector pCDM7Ig) was prepared by transient transfection of HEK 293T or CHOK1 cells and purification on Protein A-Sepharose, as described previously (9). For expression in primary HDLEC, cells were transfected with the same construct using the Amaxa primary endothelial cell basic nucleofector kit according to the manufacturer’s protocol. Typically, 7 × 10⁶ cells were transfected with 42 μg of LYVE-1 Fc vector. Levels were estimated in a standard antigen capture ELISA using rabbit anti-human LYVE-1 Ig with detection by LYVE-1 mAb 8C and horseradish peroxidase-conjugated goat anti-mouse IgG/o-phenylenediamine substrate.

Preparation of LYVE-1 N-Glycosylation Mutants—LYVE-1 mutants N53A and N130A disrupting the two sites for N-linked glycosylation were generated by oligonucleotide-directed mutagenesis using the method of Fisher and Pei (45). The forward and reverse primer pairs (N53AF (GCAAAAAGGCGACGTGGCTTTTACAGCTCAG), N53AR (CCTTAGCTTCTGTGAAAGCGACGTGGCTTTTACAGCTCAG)); N130F (GCAGCCTATTGTTACGTGGCTTTTACAGCTCAG), N130R (GCAGCCTATTGTTACGTGGCTTTTACAGCTCAG)) were used to amplify full-length Lyve-1 cDNA in the plasmid pRcCMV using Pyrococcus furiosus Pfu DNA polymerase (94 °C, 1 min, 55 °C, 1.5 min, 68 °C, 10 min, 20 cycles). The methylated, nonmutated, parental DNA template was digested with DpnI (20 units) for 4 h at 37 °C.

Preparation of LYVE-1 O-Glycosylation Mutants—LYVE-1 mutants with graded deletion of the O-glycosylated membrane-proximal domain were generated by truncation mutagenesis, exploiting a unique downstream Accl site (nucleotide 655) in the juxtamembrane region, using a common forward primer containing a unique HindIII site (Lyve-1 140F (CCGGAAGCTTATACAGTGACCAGCATTCTGAG)) and a series of alternative reverse primers containing Accl (boldface type) and XbaI sites (italic type) as follows: 0194R (GCCTCTAGATAGCTAGTGAAGCTGG), 0189R (GCCTCTAGATAGCTAGTGAAGCTGG), 0181R (GCCTCTAGATAGCTAGTGAAGCTGG), 0175R (GCCTCTAGATAGCTAGTGAAGCTGG), and 0167R (GCCTCTAGATAGCTAAGTAGAAGCTGG). Appropriate products were amplified (94 °C for 1.5 min, 58 °C for 2 min and 68 °C for 7 min; 30 cycles) from a full-length Lyve-1 cDNA template in pBluescript using Pfu DNA polymerase and ligated into HindIII/XbaI-cut pBluescript. After subsequent ligation with an Accl/XbaI fragment encoding the transmembrane domain and cytoplasmic tail, the individual constructs were excised with HindIII/XbaI and cloned into the expression vector pRcCMV.

Preparation of LYVE-1 Stable Transfectants—Wild-type or mutant Lyve-1 constructs were individually transfected into HEK 293T, CHO-K1, Lec1, Lec2, Lec3, Lec8, or ldl-D CHO cells as appropriate using Lipofectin (Invitrogen) according to the manufacturer’s instructions, followed by selection in G418 (1.25 mg/ml). Lines were immunoselected for high expression by magnetic cell sorting (MACS®; Miltenyi Biotec) with the LYVE-1 mAb 8C and anti-mouse IgG-coated MACS beads according to the manufacturer’s instructions. Transfectants were cultured in RPMI 1640, 10% fetal calf serum or, in the case of ldl-D CHO, in Ham’s F-12 medium, 3% fetal calf serum, appropriately supplemented with galactose (20 μM), GalNAc (400 μM), or both sugars prior to use.

Western Blotting—For analysis of LYVE-1 in transfectants and primary HDLEC, cells were lysed in SDS-PAGE sample buffer and electrophoresed on 4–12% polyacrylamide SDS-polyacrylamide gels (NuPAGE; Invitrogen) with MOPS buffer and then transferred to nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences) and incubated with rabbit anti-LYVE-1 polyclonal antibody (2 μg/ml in PBS, 5% (w/v) dried milk powder, 0.2% (v/v) Tween 20). Blots were developed with peroxidase-conjugated anti-rabbit Ig and visualized by chemiluminescence detection (SuperSignal; Pierce).

HA Binding Assays—Binding to HA was assessed for both LYVE-1 ectodomain Fc soluble fusion proteins and Lyve-1 transfected or native HDLEC using either plate assays or flow cytometry as described below.

Im mobilized HA Plate Binding Assay—Binding of LYVE-1 fusion protein to immobilized HA was measured in 96-well ELISA plates (Nunc Maxisorp) as described previously (9). Briefly, plates were coated overnight with HA (1 mg/ml in 50 mM sodium bicarbonate buffer pH 9.3) prior to blocking (1 h) with bovine serum albumin (10 mg/ml) in PBS, 0.05% (v/v) Tween 20 and incubation (1 h) with purified LYVE-1 Fc fusion protein (1.25–40 μg/ml) in the same buffer. Bound LYVE-1 was detected by incubation (1 h) with polyclonal rabbit anti-human LYVE-1 Ig (2 μg/ml) and developed with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; Pierce) and o-phenylenediamine substrate (Sigma). Binding was measured as absorbance at 490 nm. In some experiments, samples of LYVE-1 Fc fusion protein (40 μg/ml) were mixed with excess unlabeled HA (0.5 mg/ml) and preincubated for 30 min prior to performing the plate assay in order to establish binding specificity.

Soluble HA Plate Binding Assay—For measurement of binding to soluble HA, 96-well plates were precoated overnight (25 °C) with anti-human IgG (10 μg/ml), washed, and then coated (1 h) with LYVE-1 Fc fusion protein 39–10,000 ng/ml. After further washing, wells were incubated with biotinylated HA (5 μg/ml) in PBS, 0.05% Tween 20 (with or without a 100-fold molar excess of unlabeled HA as a specificity control), and bound bHA was detected using horseradish peroxidase-conjugated avidin (1:500; DAKO) and o-phenylenediamine substrate. Binding was measured as the absorbance at 490 nm.

Flow Cytometric Assay—For determination of HA binding to LYVE-1 transfecteds and primary HDLEC, cells were detached where appropriate in ice-cold PBS, 5 mM EDTA or incubated (37 °C) with Accutase™, followed by gentle pipetting prior to
Sialylation Regulates LYVE-1 Function

centrifugation and resuspension in ice-cold PBS, 5% fetal calf serum, and 0.1% sodium azide. Cells were then incubated with a mixture of Fl-HA (5 μg/ml) and the nonblocking LYVE-1 mAb 8C (10 μg/ml), followed by phycoerythrin-conjugated anti-mouse Ig. In the case of CHO cells, which express significant levels of endogenous CD44, this component of HA binding was blocked with inhibitory antibodies (either KM81 or BRIC235; 50 μg/ml). After fixation in PBS, 4% (w/v) paraformaldehyde, cells were subjected to flow cytometry using a BD Bioscience FACSCalibur, followed by division of the fluorescence channel into 15 equal sized gates to allow analysis of Fl-HA binding as a function of LYVE-1 expression level (phycoerythrin fluorescence intensity).

Enzymatic Desialylation—For treatment of LYVE-1 Fc fusion proteins, samples were digested overnight (37 °C) with purified neuraminidases from Vibrio cholerae, A. ureafaciens, or M. decora (125 milliunits/ml) in 50 mM sodium acetate buffer, pH 5.5, 150 mM NaCl, 4 mM CaCl₂. For primary HDLEC or LYVE-1 transfectants, intact cells were treated for 1 h (37 °C) with neuraminidase (125 milliunits/ml) in culture medium, pH 7.5, or alternatively were lysed in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, containing 1% (w/v) Nonidet P-40 and protease inhibitor mixture (Roche Applied Science) prior to immunoprecipitation with LYVE-1 mAb 8C (10 μg/ml) and protein G-Sepharose. Immunoprecipitates were boiled in 0.5% (w/v) SDS, diluted 1:1 in 2× lysis buffer, supplemented with 4 mM CaCl₂, and then treated for 1 h (37 °C) with neuraminidase (125 milliunits/ml).

Lectin Binding ELISA—For analysis of LYVE-1 glycan modification by lectin binding, LYVE-1 Fc fusion proteins were immobilized in 96-well plates exactly as described for soluble HA binding assays. Wells were then incubated with horseradish peroxidase-conjugated S. nigra agglutinin or M. amurensis agglutinin (10 μg/ml), followed by o-phenylenediamine substrate (Sigma), and absorbance was measured at 490 nm. The unglycosylated fusion protein DR5Fc (provided by Dr. Fiona Kimberley (University of Cardiff) was used as a negative control.

RESULTS

Evidence That LYVE-1 Is Not Constitutively Activated within Lymphatic Vessels in Vivo—We showed previously by means of cDNA cloning and expression in HEK 293T fibroblasts that LYVE-1 is a bona fide HA receptor in vitro by demonstrating specific saturable binding to high molecular weight HA with an affinity comparable with that of the closely related homing receptor CD44 (9, 10). To investigate the HA binding properties of the native receptor in lymphatic endothelium, we prepared frozen tissue sections from mouse skin and small intestine and looked for co-localization of receptor and ligand in lymphatic vessels by fluorescent immunostaining with affinity-purified LYVE-1 Ab and bHABP. As shown by the representative images in Fig. 1, bHABP decorated connective tissue within the dermal layers of the ear and stained the supporting stromal tissue in the lamina propria and in some cases the villi of small intestine. However, the extent to which the bHABP staining coincided with that of LYVE-1 either in dermal lymphatic vessels or in intestinal lamina propria or lacteal lymphatics was negligible or at best marginal (Fig. 1, A–F). The staining pattern was not significantly altered by inclusion of cetylpyridinium chloride fixation, a procedure used to prevent leaching of HA during tissue preparation (Fig. 1, E and F; data not shown). Moreover, a lack of significant co-localization was

FIGURE 1. Co-localization studies of LYVE-1 and hyaluronan in mouse tissues. Endogenous, native LYVE-1 in murine tissue lymphatics was assessed either for coincident localization with endogenous HA (A–F) or for its capacity to bind exogenous Fl-HA (G), as described under “Experimental Procedures.” A–D show both single and merged images of mouse ear frozen sections immunostained with a mixture of affinity-purified rabbit anti-mouse LYVE-1 Ig (red), biotinylated HA-binding protein complex (bHABP; green), and the nuclear dye 4’,6-diamidino-2-phenylindole (blue) viewed at either ×40 (A and B) or ×100 magnification (C and D). The arrows indicate positions of individual lymphatic endothelial cells forming prominent LYVE-1-positive lymphatic vessels that lack significant bHABP staining. The positions of the keratinized epidermal layers (Epi), which have been removed during mounting, are indicated. E and F show images of mouse small intestinal sections subjected to similar staining conditions, viewed at either ×40 (E) or ×10 (F), with the exception that E represents tissue fixed in paraformaldehyde/cetylpyridinium chloride, and F represents a frozen section (note that LYVE-1 is stained green and HA is stained red in F). Structures corresponding to villi (V) that contain lacteal lymphatic vessels and to the underlying lamina propria (LP) are indicated. G shows an image of mouse ear pinna stained with Fl-HA and affinity-purified rabbit anti-mouse LYVE-1 Abs (red) following predigestion with hyaluronidase (see “Experimental Methods”) to remove endogenous HA (magnification ×20). Staining is confined to the central cartilage tissue (arrows) and both faces of the keratinized epidermis (Epi).
found also in intestinal tissue from mice with inflammatory bowel disease and in peripheral lymph nodes.\(^7\)

Next, we assessed the capacity of LYVE-1 to bind exogenously added Fl-HA in frozen sections of mouse ear that had received prior treatment with hyaluronidase to unmask any latent binding sites occupied by endogenous HA or HA-proteoglycan complexes. As shown in Fig. 1, Fl-HA decorated connective tissue, most notably the central cartilage within the ear pinnae, an area rich in chondrocytes that express high affinity HA-binding proteoglycans, such as aggrecan and versican. However, in common with bHABP, there was little if any decoration of LYVE-1-positive lymphatic vessels (Fig. 1G and Fig. S2). Although fluorescent histological methods for detecting HA binding may have limitations in terms of sensitivity, these initial findings nevertheless indicated that native LYVE-1 may not be constitutively active in lymphatic endothelium.

The HA Binding Status of LYVE-1 Is Dependent on Cell Background—One obvious explanation for the lack of HA binding to LYVE-1 in tissues is denaturation or proteolytic degradation during sample preparation and manipulation. To address this concern, we isolated primary lymphatic endothelial cells from both human and mouse tissue (HDLEC and mouse dermal lymphatic endothelial cells, respectively) using LYVE-1 immunomagnetic bead selection and assessed HA binding with fluorescent GAG by flow cytometry. Whereas both LEC populations expressed LYVE-1 abundantly, neither displayed significant binding to Fl-HA (Fig. 2). This result suggests that LYVE-1 in lymphatic endothelium is inactive by default. Moreover, transfection of Lyve-1 cDNA into a range of LYVE-1-negative cell lines, including human HEK 293T fibroblasts, human Jurkat T cells, HeLa cells, and CHO K1 cells revealed a range of binding states (Fig. 2B) that were ranked as completely nonpermissive (HeLa) through weakly permissive (CHO K1 and Jurkat) to highly permissive (HEK 293T). Such an influence of cell background on HA binding is reminiscent of CD44, in which binding is known to be regulated by cell type-specific glycosylation (26, 27). Consistent with this notion, LYVE-1 in the different cell backgrounds showed considerable differences in electrophoretic mobility during SDS-PAGE, diagnostic of differences in post-translational modification (Fig. 2C). However, there was no obvious correlation between either a slow or fast migrating protein pattern and HA binding capacity, indicating the likelihood that cell-specific LYVE-1 modification is complex.

Role of N- and O-Linked Glycosylation in the Control of HA Binding—To investigate the possible role of glycosylation in HA binding, we first employed a combination of site-directed and truncation mutagenesis to selectively disrupt either the two N-glycosylation motifs (Asn\(^{53}\) and Asn\(^{130}\)) that are located within the LYVE-1 Link domain or the multiple O-glycosylation motifs within the LYVE-1 membrane-proximal stalk (see the schematic diagram in Fig. 3A; see Ref. 9).

In the case of N-glycosylation, we constructed two full-length mutants, N53A and N130A, which were independently transfected into HEK 293T cells. As shown in Fig. 3B, both mutants displayed similar levels of expression (assessed by reactivity to LYVE-1 antibodies) as the wild-type receptor, and both exhibited a small increase in electrophoretic mobility relative to wild-type receptor, consistent with the loss of covalently linked N-glycan chains. When subjected to functional assays, however, the N53A mutant showed a partial reduction in HA binding, whereas the N130A mutant showed a complete loss of HA binding compared with wild-type LYVE-1 (Fig. 3C). Similar findings were observed when Lyve-1 transfected cells were treated with tunicamycin, an inhibitor of N-glycan biosynthesis.

\(^7\) T. Nightingale and D. G. Jackson, unpublished results.
These results indicate that N-glycosylation of LYVE-1 plays a positive role in HA binding and is obligatory for function, a feature shared with other prominent N-glycosylated receptors, including ICAM-1, ICAM-2, and β5 integrins (28, 29). However, we cannot completely exclude the possibility that additional modifications to the intact N-linked glycans (e.g. sialylation) might themselves be inhibitory. Interestingly, these properties reveal a contrast with CD44, in which N-linked glycan chains have overtly negative effects on HA binding; specifically, site-directed mutagenesis of Asn25 and Asn120 in murine CD44 and biosynthetic inhibition of glycosylation with tunicamycin have both been reported to activate HA binding (30, 31).

We next considered the role of O-linked glycans, focusing particularly on the serine/threonine-rich tracts (residues 165–214; 36% Ser/Thr) in the membrane-proximal region, downstream of the HA-binding Link domain, where the majority of O-glycosylation is predicted to occur (see schematic diagram in Fig. 4A). Initial experiments using the biosynthetic inhibitor benzoyl α-galactoside (BAG; 2 mM) yielded small but significant increases in HA binding (~30% relative to controls), limited perhaps by low membrane permeability of the reagent (data not shown). We therefore considered ablation of O-glycosylation by conventional mutagenesis. Because the large number of individual serine and threonine residues (27 in total) in the LYVE-1 ectodomain rendered disruption by individual mutation impractical, we deleted the serine/threonine-rich region instead in discrete segments, yielding the five graded truncation constructs Lyve-1Δ164–214, Lyve-1Δ172–214, Lyve-1Δ178–214, Lyve-1Δ186–214, and Lyve-1Δ191–214, relative to wild type receptor (WT). B shows the effects of truncation on electrophoretic mobility of LYVE-1 in lysates from HEK 293T cells transfected with either wild-type LYVE-1 (WT), LYVE-1 N53A, or LYVE-1 N130A or untransfected controls (untran), as assessed by SDS-PAGE and Western blotting with polyclonal LYVE-1 Abs. Positions of molecular weight calibration markers are shown at the left. C shows the levels of Fl-HA binding for the same HEK 293T transfectants as a function of LYVE-1 expression by flow cytometry, as described under “Experimental Procedures.” Mean fluorescence intensity (MFI) values represent the mean ± S.E for three independent replicate determinations.
Sialylation Regulates LYVE-1 Function

JΔ178–214, Lyve-1Δ186–214, and Lyve-1Δ191–214 that lacked 21–48 residues of this domain (Fig. 4; see also “Experimental Procedures”). Consistent with heavy O-glycosylation of the deleted segments, the truncated LYVE-1 mutants exhibited a disproportionately large reduction in apparent molecular weight compared with a similarly sized nonglycosylated deletion control (Lyve-1Δcyto261–322, which lacks 61 residues from the cytoplasmic tail), as assessed by SDS-PAGE/Western blotting. For example, removal of a 21-amino acid segment in LYVE-1Δ191–214 caused a shift of ~6 kDa, and removal of a 48-amino acid segment in LYVE-1Δ164–214 caused a shift of ~15 kDa (Fig. 4B). Significantly, when assayed for receptor function, the mutants displayed a graded increase in HA binding that ranged from 2-fold for LYVE-1Δ191–214 to more than 3-fold for LYVE-1Δ178–214 and LYVE-1Δ164–214 compared with the intact receptor (Fig. 4C). Furthermore, in preliminary transfection studies with O-glycosylation-defective Ldl-D CHO cells in which O-linked sugar chain synthesis was metabolically restricted to core Ser/Thr-GalNAc stubs (32), we observed a 4-fold increase in LYVE-1 HA binding relative to intact control (data not shown). Together, these results suggest that O-glycan chains rather than N-glycan chains of LYVE-1 are responsible for functional masking of the receptor.

Identification of Sialic Acid as an Inhibitory Moiety for HA Binding—To further elucidate the role of glycosylation in modifying HA binding, we transfected full-length Lyve-1 cDNA into a panel of Lc CHO cell mutants lacking key enzymes involved in core and terminal glycan chain biosynthesis (23). These included Lec1, which lacks N-acetylgalactosaminyltransferase 1, required for the synthesis of complex, and hybrid N-glycans Lec2, which lacks CMP sialyltransferase required for terminal glycan sialylation (21), and Lec 3.2.8.1 (LecR) a compound mutant that lacks both these enzymes in addition to the UDP- N-acetylgalactosamine-2-epimerase required for sialic acid biosynthesis and the UDP-galactose transporter (33, 34). Importantly, each of the mutant CHO transfectants expressed comparable levels of LYVE-1 at the surface as assessed by LYVE-1 immunostaining and flow cytometry, and each showed a significant change in mobility relative to wild type during SDS-PAGE (Fig. 5, A and B). When assayed for receptor function, the Lec1 transfecteds showed an ~2-fold reduction in HA binding relative to wild-type CHO, in line with the effects of site-directed mutagenesis at Asn53 and Asn130 (Fig. 5C). Dramatically, however, Lec2 transfecteds showed a 4-fold increase in HA binding, suggesting for the first time that terminal sialylation can mask LYVE-1 function. The neutral effect of the LecR background most likely reflects a counterbalance between the positive and negative influences of N-glycosylation and terminal sialylation, respectively.

To confirm inhibition of LYVE-1 by sialylation using a different method and to gain more detailed information on the nature of the inhibitory sialic acid moiety, we probed LYVE-1Fc from HEK 293T cells by ELISA with the lectins M. amurensis (MAA) and S. nigra (SNA) specific for Sia2–3Gal- and Sia2–6Gal-capped glycans, respectively. In addition, we monitored HA binding both before and after digestion of the LYVE-1 Fc fusion protein with corresponding linkage-specific neuraminidases (Fig. 6, A–C). The results of the lectin-based ELISA (Fig. 6B) indicated covalent modification with both α2–3- and α2–6-linked sialic acids. This was further confirmed by treatment with α2–3–specific neuraminidase either from M. decora.
or *S. pneumoniae*, which exclusively removed MAA-reactive sialic acid, and with the *A. ureafaciens* enzyme, which cleaves both α2–3 and the rarer α2–6 linkages and which removed both MAA- and SNA-reactive sialic acids (Fig. 6B). As expected, both treatments also provoked an increase in electrophoretic mobility of LYVE-1 when assessed by SDS-PAGE (Fig. 6A). Significantly, however, only the *Arthrobacter* enzyme unmasked HA binding (Fig. 6C). Given the cleavage specificities of the enzymes, these results imply that in 293T cells, the inhibitory moiety probably corresponds to Siaα2–6Gal rather than Siaα2–3Gal or polysialylated structures, such as Siaα2–8Sia. Furthermore, since inhibition by sialic acid was demonstrable with purified receptor protein, it follows that inhibition in intact cells most likely derives from glycans on LYVE-1 (autoinhibition) rather than glycans on discrete neighboring surface proteins or glycolipids. Curiously, neuraminidase treatment of LYVE-1 in intact HEK 293T (unlike soluble LYVE-1) failed to unmask HA binding (data not shown), indicating that the sialylated moieties might be inaccessible when the receptor is anchored to the cell surface. This possibility is explored further below.

**Sialylation Inhibits Endogenous LYVE-1 in Lymphatic Endothelial Cells**—Having established autoinhibitory sialylation as a mechanism for masking LYVE-1 HA binding in transfected fibroblasts, it was important to determine whether the same mechanism silences the endogenous receptor in lymphatic endothelial cells. To address the question, we first tested the ability of neuraminidase treatment to unmask HA binding in HDLEC. As observed with *Lyve-1* transfected HEK 293T cells, treatment of HDLEC with the *V. cholerae* enzyme had little or no effect, and HA binding remained negligible. Moreover, such treatment of HDLEC induced no change in electrophoretic mobility of LYVE-1, as assessed by SDS-PAGE, despite dramatically reducing the overall level of sialic acid attached to other components at the cell surface as detected by fluorescence-activated cell sorting analysis with SNA and MAA lectins (Fig. 7, A and B). Significantly, however, when LYVE-1 was first immunoprecipitated from HDLEC lysates and then subjected to neuraminidase treatment, there was a marked increase in electrophoretic mobility (Fig. 7A). Thus, we conclude that endogenous LYVE-1 in HDLEC is sialylated, but the sialic acid is physically inaccessible to enzymatic cleavage in intact cells.

To overcome this apparent accessibility barrier, we transfected HDLEC with *Lyve-1* Fc to generate soluble LYVE-1 ectodomains bearing the native HDLEC glycosylation pattern (*i.e.*, modification with both α2–3- and α2–6-linked sialic acids; see Fig. 7B). As expected, the HDLEC-derived LYVE-1 fusion protein bound little if any biotinylated HA; however, binding was significantly unmasked after treatment with *V. cholerae* neuraminidase (Fig. 8). Thus, we conclude that LYVE-1 is indeed masked by sialic acid in lymphatic endothelium and that the receptor can be functionally silenced *in vivo* by a mechanism of autoinhibitory sialylation.

**DISCUSSION**

Despite the specific and abundant expression of *Lyve-1* in lymphatic vessel endothelium, the true physiological function of this receptor has remained elusive. Confounding earlier predictions that LYVE-1 might play a role in lymphatic trafficking analogous to that played by...
Sialylation Regulates LYVE-1 Function

CD44 in blood vascular trafficking, the early indications from mice with targeted disruption of the Lyve-1 gene are that the animals have no obvious defects in such functions and no significant alteration in HA homeostasis (18, 19, 35). Among possible explanations for this anomaly, we therefore considered the possibility that LYVE-1 might have a cryptic function or one that is manifest only under selective physiological conditions.

In addressing these issues, we set out to reappraise the hyaluronan-binding properties of LYVE-1 and uncovered intriguing new evidence that the native receptor is functionally masked or “silenced” in vivo by a mechanism of sialylation. Using transfection with full-length cDNA, we showed initially that hyaluronan binding competence is strictly dependent upon cell background, consistent with regulation of LYVE-1 function by post-translational modification. This was confirmed by further studies using glycosylation-defective CHO cell lines, which defined an inhibitory role for LYVE-1 sialylation. Finally, by selectively disrupting either N-glycosylation or O-glycosylation through mutagenesis and enzymatic desialylation with linkage-specific neuraminidases, we determined that the inhibitory sialic acid moieties (in 293T transfectants and control untransfected HDLEC) are most likely in an α2–6 linkage, a configuration found on both N- and O-linked glycans but particularly abundant in O-glycan-rich proteins (NeuNAc α2–6 GalNAc Ser/Thr), such as the submaxillary gland mucins.

Furthermore, in the case of CD44, the surface-exposed receptor can be reactivated in situ by treatment of cells with bacterial neuraminidases (31). In contrast, LYVE-1 was found to be susceptible to cleavage only when the receptor was isolated from cells and presented in soluble form (i.e. as an ectodomain Fc fusion protein). The limited accessibility of the inhibitory LYVE-1 sialic acids in intact cells (e.g. primary LEC) is consistent with their attachment to the serine/threonine-rich mucin-like stalk region of the receptor, which would be more deeply buried than the asparagine-linked sugar chains in the N-terminal domain.

The precise mechanism by which sialylation inhibits HA binding to LYVE-1 is not clear. The simplest explanation would be that sialylated glycans physically block HA binding through coordination with basic ligand-binding residues or through charge repulsion. This is supported in principle by comparisons between a three-dimensional model for the LYVE-1 Link module and the crystal structure of the CD44 Link module (12, 13). However, other explanations, such as effects on receptor self-association, similar to those advanced for CD44 are equally likely. Indeed, preliminary investigations using bioluminescent resonance energy transfer indicate that LYVE-1 may indeed form dimers at the cell surface (data not shown). Further work in this area is ongoing in our laboratory.

Besides LYVE-1 and CD44, there are many other examples where receptor sialylation has been shown to act as an on/off switch for biological function, particularly during immune and inflammatory responses. For example, developmentally regu-
Sialylation Regulates LYVE-1 Function

luted α2–3 sialylation of the Ig superfamily co-receptor CD8 in thymocytes has been shown to dampen peptide-MHC I signaling and thus to alter the repertoire of surviving cytotoxic T cells during induction of central tolerance (41, 42). In addition, sialylation has been shown to positively regulate protein-tyrosine phosphatase activity in the leukocyte receptor CD45, by maintaining the protein in the active monomeric state (43). Differential sialylation of IgG molecules within the Fc region can alter their binding affinity for Fcγ receptors and thus affect their contribution to the inflammatory response (44). Last and most recently, α2–6 sialylation of lymphocyte cell surface proteins in response to antigen-specific induction of Th2 T helper cell proliferation has been shown to mask N-acetyl-lactosamine binding sites for Galectins and protect Th2 cells from Galectin-induced apoptosis (1).

In many of these examples, the trigger for protein desialylation is the induction of appropriate sialyltransferase activity in response to a developmental program. The same mechanism may well operate within the lymphatic system. However, by analogy with CD44, it is also possible that LYVE-1 undergoes more localized activation in response to local environmental cues. Our investigations so far have failed to identify cytokines or chemokines that activate HA binding. However, our preliminary studies indicate that phorbol ester treatment can induce LYVE-1 desialylation and simultaneous activation of HA binding in transfected fibroblasts, implying that a stimulus-dependent mechanism for LYVE-1 activation does exist in vitro. Moreover, we have observed that phorbol ester also induces metalloprotease-mediated shedding of functionally active LYVE-1 ectodomains, and we have detected such ectodomains by Western blotting in human sera and biological fluids. This raises the intriguing possibility that shed rather than membrane-bound LYVE-1 may be the physiologically active HA-binding form of the receptor and that desialylation and shedding may be concomitant events. Our current efforts are directed toward elucidating the molecular mechanisms by which sialylation inhibits HA binding to LYVE-1 and the factors that regulate sialylation/desialylation in vivo.

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REFERENCES

1. Toscano, M. A., Bianco, G. A., Ilarregui, J. M., Croci, D. O., Correale, J., Hernandez, D. Z., Zwirner, N. W., Poirier, F., Riley, E. M., Baum, L. G., and Rabinovich, G. A. (2007) Nat. Immunol. 8, 825–834
2. Jackson, D. G. (2007) Cancer Treat. Res. 135, 39–53
3. Mouta Carreira, C., Nasser, S. M., di Tomaso, E., Padera, T. P., Boucher, Y., Tomarev, S. I., and Jain, R. K. (2001) Cancer Cell 1, 8079–8084
4. Maruyama, K., Ii, M., Cursiefen, C., Jackson, D. G., Keino, H., Tomita, M., Van Rooijen, N., Takenaka, H., D’Amore, P. A., Stein-Streilein, J., Losordo, D. W., and Streilein, J. W. (2005) J. Clin. Invest. 115, 2363–2372
5. Schledzewski, K., Falkowski, M., Moldenhauer, G., Metharom, P., Kzhyskhowska, J., Gans, R., Demory, A., Falkowska-Hansen, B., Kurzen, H., Ugurel, S., Geginat, G., Arnold, B., and Goerdert, S. (2006) J. Pathol. 209, 67–77
6. Witte, M. H., Jones, K., Wilting, J., Dictor, M., Selg, M., McHale, N., Gershwin, J. E., and Jackson, D. G. (2006) Cancer Metastasis Rev. 25, 159–184
7. Aruffo, A., Stamenkovic, I., Molnick, M., Underhill, C. B., and Seed, B. (1990) Cell 61, 1303–1313
8. Ponta, H., Sherman, S., and Herrlich, P. A. (2003) Nat. Rev. Mol. Cell Biol. 4, 33–45
9. Banerji, S., Ni, J., Wang, S. X., C1asper, S., Su, J., Tammi, R., Jones, M., and Jackson, D. G. (1999) J. Cell Biol. 144, 789–801
10. Prevo, R., Banerji, S., Ferguson, D. J., C1asper, S., and Jackson, D. G. (2001) J. Biol. Chem. 276, 19420–19430
11. Banerji, S., Day, A. J., Kahlmann, J. D., and Jackson, D. G. (1998) Protein Expression Purif. 14, 371–381
12. TeriPete, P., Banerji, S., Noble, M., Blundell, C. D., Wright, A. J., Pickford, A. R., Lowe, E., Mahoney, D. J., Tammi, M. I., Kahlmann, J. D., Campbell, I. D., Day, A. J., and Jackson, D. G. (2004) Mol. Cell 13, 483–496
13. Banerji, S., Wright, A. J., Noble, M., Mahoney, D. J., Campbell, I. D., Day, A. J., and Jackson, D. G. (2007) Nat. Struct. Mol. Biol. 14, 234–239
14. Johnson, L. A., Prevo, R., C1asper, S., and Jackson, D. G. (2007) J. Biol. Chem. 282, 33671–33680
15. Fraser, J. R., Kimpton, W. G., Laurent, T. C., C1ahill, R. N., and Yakakis, N. (1988) Biochem. J. 256, 153–158
16. Tengblad, A., Laurent, U. B., Lillja, K., Cahill, R. N., Engstrom-Laurent, A., Fraser, J. R., Hansson, H. E., and Laurent, T. C. (1986) Biochem. J. 236, 521–525
17. Jackson, D. G., Prevo, R., C1asper, S., and Banerji, S. (2001) Trends Immunol. 22, 317–321
18. Valenzuela, D. M., Murphy, A. J., Frenedewey, D., Gale, N. W., SEconomides, A. N., Auerbach, W., Poueymiroud, W. T., Adams, N. C., Rojas, J., Yasar, J., Chernomorsky, R., Boucher, M., Elsasser, A. L., Esau, L., Zheng, J., Griffiths, J. A., Wang, X., Su, H., Xue, Y., Domiguez, M. G., Nogueria, I., Torres, R., MacDonald, L. E., Stewart, A. F., DeChiate, T. M., and Yancopoulos, G. D. (2003) Nat. Biotechnol. 21, 652–659
19. Gale, N. W., Prevo, R., Espinosa-Femmat, J., Ferguson, D. J., Domiguez, M. G., Yancopoulos, G. D., Thurston, G., and Jackson, D. G. (2007) Mol. Cell Biol. 27, 595–604
20. Johnson, L. A., C1asper, S., Holt, A., Loral, P., Baban, D., and Jackson, D. G. (2006) J. Exp. Med. 203, 2763–2777
21. Eckhardt, M., Gotza, B., and GerdaShahn, R. (1998) J. Biol. Chem. 273, 20189–20195
22. Chen, W. F., and Stanley, P. (2003) Glycobiology 13, 43–50
23. Patnaik, S. K., and Stanley, P. (2006) Methods Enzymol. 416, 159–182
24. Yu, Q., and Tooile, B. P. (1995) BioTechniques 19, 122–128
25. de Belder, A. N., and Wik, K. O. (1975) Carbohydrate Res. 44, 251–257
26. Katoh, S., Miyagi, T., Taniguchi, H., Matusbara, Y., Kadota, J., Tomina, A., Kincade, P. W., Matsukura, S., and Kohno, S. (1999) J. Immunol. 162, 5058–5061
27. Kincade, P. W., Zheng, Z., Katoh, S., and Hanson, L. (1997) Curr. Opin. Cell Biol. 9, 635–642
28. Jimenez, D., Roda-Navarro, P., Springer, T. A., and Casasnovas, J. M. (2005) J. Biol. Chem. 280, 5854–5861
29. Isaji, T., Sato, Y., Zhao, Y., Miyoshi, E., Wada, Y., Taniguchi, M., and Gu, J. (2006) J. Biol. Chem. 281, 33258–33267
30. English, N. M., Lesley, J. F., and Hyman, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 85, 4335–4339
31. Oelmann, S., Stanley, P., and GerdaShahn, R. (2001) J. Biol. Chem. 276, 26291–26300
32. Hong, Y., and Stanley, P. (2003) J. Biol. Chem. 278, 53045–53054
33. Huang, S. S., Liu, I. H., Smith, T., Shah, M. R., and Huang, J. S. (2006) FEBS Lett. 580, 6259–6268
34. Pure, E., and Cuff, C. A. (2001) Trends Mol. Med. 7, 213–221
35. De Grendele, P., Picker, L. J., and Siegelman, M. H. (1996) J. Exp. Med. 183, 1119–1130
36. De Grendele, H. C., Estess, P., and Siegelman, M. H. (1997) Science 278,
672–675
39. Mohamadzadeh, M., DeGrendele, H., Arizpe, H., Estess, P., and
Siegelman, M. (1998) J. Clin. Invest. 101, 97–108
40. Gee, K., Kozlowski, M., and Kumar, A. (2003) J. Biol. Chem. 278,
37275–37287
41. Moody, A. M., Chui, D., Reche, P. A., Priatel, J. J., Marth, J. D., and Rein-
herz, E. L. (2001) Cell 107, 501–512
42. Moody, A. M., North, S. I., Reinhold, B., Van Dyken, S. J., Rogers, M. E.,
Panico, M., Dell, A., Morris, H. R., Marth, J. D., and Reinherz, E. L. (2003)
J. Biol. Chem. 278, 7240–7246
43. Xu, Z., and Weiss, A. (2002) Nat. Immunol. 3, 764–771
44. Kaneko, Y., Nimmerjahn, F., and Ravetch, J. V. (2006) Science 313,
670–673
45. Fisher, C. L., and Pei, G. K. (1997) Biotechniques 574, 570–571