Rapid Plasma Membrane-Endosomal Trafficking of the Lymph Node Sinus and High Endothelial Venule Scavenger Receptor/Homing Receptor Stabilin-1 (Feel-1/Clever-1)*

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The sinusoidal endothelia of liver, spleen, and lymph node are major sites for uptake and recycling of waste macromolecules through promiscuous binding to a disparate family of scavenger receptors. Among the most complex is stabilin-1, a large multidomain protein containing tandem fasciclin domains, epidermal growth factor-like repeats, and a C-type lectin-like hyaluronan-binding Link module, which functions as an endocytic receptor for acetylated low density lipoprotein and advanced glycation end products. Intriguingly, stabilin-1 has also been reported to mediate both homing of leukocytes across lymph node high endothelial venules and adhesion of metastatic tumor cells to peritumoral lymphatic vessels. Currently, however, it is not clear how stabilin-1 mediates these distinct functions. To address the issue, we have investigated the tissue and subcellular localization of stabilin-1 in detail and assessed the functional status of its Link module. We show that stabilin-1 is almost entirely intracellular in lymph node high endothelial venules, lymphatic sinus endothelium, and cultured endothelial cells but that a finite population, detectable only by fluorescent antibody or fluorescein-labeled (Fl)-acetylated low density lipoprotein uptake, cycles rapidly between the plasma membrane and EEA-1** (early endosome antigen 1) early endosomes. In addition, we show using full-length stabilin-1 cDNA and a stabilin-1/CD44 chimera in HeLa cells that intracellular targeting is influenced by the transmembrane domain/cytosplasmic tail, which contains a putative dileucine (DXXLL) Golgi to endosomal sorting signal. Finally, we provide evidence that the stabilin-1 Link domain binds neither hyaluronan nor other glycosaminoglycans. These properties support a role for stabilin-1 as a rapidly recycling scavenger receptor and argue against a role in cell adhesion or lymphocyte homing.

Vertebrates have evolved a bewildering array of different scavenger receptors for the cellular uptake and degradation of “waste” molecules, the products of cell, extracellular matrix, and protein turnover that would otherwise accumulate and interfere with normal homeostasis. The list of “waste” molecules is extensive and includes collagen α-chains and N-terminal propeptides, oxidized low density lipoprotein (LDL), high density lipoprotein, oligodeoxynucleotides, tissue plasminogen activator, immune complexes, atherogenic lipids, advanced glycation end products, and glycosaminoglycans (1, 2). These disparate macromolecules are recognized and bound by scavenger receptors present primarily on the surface of endothelial cells of liver sinusoids but present also on spleen and lymph node sinuses and on phagocytic leukocytes (3, 4). The number of different scavenger receptors is itself very large and includes a wide variety of different protein types. The scavenger receptors SR-A and SR-B, for example, have extracellular domains containing collagen-like domains, α-helical coiled-coil domains, and scavenger receptor cysteine-rich domains, whereas SR-C and LOX-1 (Lectin-like oxidized LDL receptor-1) contain complement-control protein, somatomedin, and C-type lectin domains (2). An additional group of scavenger receptors on endothelial cells has extracellular domains containing multiple epidermal growth factor (EGF) repeats (5).

One unusually complex group of scavenger-like receptors recently identified on sinusoidal endothelia comprises the Link superfamily proteins stabilin-1 and stabilin-2 (also known as FEEL-1 and FEEL-2), which have the capacity to internalize conventional scavenger ligands such as modified LDL, whole bacteria, and advanced glycation end products (6–9). Both FEEL-1 and FEEL-2 are large multidomain molecules comprising multiple EGF-like repeats, tandem fasciclin-like domains, and a single membrane-proximal Link module, a conserved C-type lectin-like domain that can bind the matrix glycosaminoglycans, hyaluronan (HA) and chondroitin sulfate (7, 8, 10). However, the presence of the Link domain is particularly interesting in view of the fact that other receptors containing this polypeptide unit (e.g. CD44 in leukocytes and LYVE-1 in lymphatic endothelium) mediate key functions including cell migration, tissue differentiation, and tumor metastasis (11–13). For example, stabilin-2 (also known as HARE, HA receptor for endocytosis) functions as the major liver and lymph node scavenging receptor for hyaluronan and the related glycosaminoglycans, chondroitin and heparan sulfate (6, 14). This uptake

* The abbreviations used are: LDL, low density lipoprotein; BSA, bovine serum albumin; TM, transmembrane; CLEVER-1, common lymphatic endothelial and vascular endothelial receptor-1; EAA-1, early endosome antigen 1; EGF, epidermal growth factor; FEEL-1, Fasciclin, EGF-like, laminin-type EGF-like, Link domain-containing scavenger receptor-2: HA, hyaluronan; FITC, fluorescein isothiocyanate; HARE, HA receptor for endocytosis; HEV, high endothelial venule; HUVEC, human umbilical vein endothelial cell; LEC, lymphatic endothelial cells; LSEC, liver sinusoidal endothelial cells; LYVE-1, lymphatic vessel endothelial HA receptor-1; PBS, phosphate-buffered saline; TSG-6, tumor necrosis factor-α-stimulated gene-6; F, forward; R, reverse; CT, C-terminal; mAb, monoclonal antibody; Fasc, Fasciclin; MES, 4-morpholineethanesulfonic acid; IL, interleukin.
represents a key step in the metabolism of HA, which begins with its initial release from the tissue matrix through the action of metalloproteinases and active oxygen intermediates, continues with its transport in lymph and partial degradation in lymph nodes, and ends with its terminal hydrolysis in the liver (15, 16). Current evidence indicates that stabilin-2 is located at the cell surface in liver and lymph node sinus endothelium where it internalizes glycosaminoglycans via the conventional clathrin-dependent endocytic pathway (7, 14, 17).

Stabilin-1 is remarkably similar to stabilin-2 in terms of size, domain structure, and tissue expression (7, 18). Nevertheless, its function appears to be significantly different. For example, there is no clear evidence for the involvement of stabilin-1 in HA binding or uptake (7), although the receptor does appear to mediate uptake of acetylated LDL, whole bacteria, and advanced glycation end products (8). In addition, some earlier reports have indicated that stabilin-1 (unlike stabilin-2) may be concentrated in intracellular vesicles rather than present at the cell surface (7, 18). Therefore, the ligand-binding specificity of stabilin-1 and its subcellular localization are unclear.

Recently, an intriguing paper by Irjala et al. (19) has reported the expression of stabilin-1 and its subcellular localization are unclear.

The extra-cellular domain of human CD44H (Met 1-Ile266) was fused to the predicted transmembrane (TM) domain and cytoplasmic tail of stabilin-1 (Ala2467-Lys2470) through a SaIl site. The stable-1 TM and cytoplasmatic tail were amplified by PCR from human stabilin-1 HDPLM44 DNA in pcMV-Sport (supplied by Human Genome Sciences) using Pfu polymerase and 7453F Sal (5'-GGCGGTGCAGCGATGCTGCGG-3') as the forward primer and T7F (5'-AGCCTGAATTCGTGCGCTGAACG-3') as the reverse primer. The restriction sites in each case are underlined. The PCR product was purified and digested with SaIl and NotI. The PCR product was purified and digested with HindIII, and cloned into a HindIII SalI linker between the start codon and the end of KIAA0246 through a three-way ligation into the pCMVSport plasmid. The full-length cDNA of stabilin-1 (position 5'1030 bp from the 5' end) was subcloned into pEGFP-N1 for expression in HEK293 cells.

Cloning and Expression of a CD44-Stabilin-1 Chimera—The extra-cellular domain of human CD44H (Met1-Ile266) was fused to the predicted transmembrane (TM) domain and cytoplasmic tail of stabilin-1 (Ala2467-Lys2470) through a SaIl site. The stable-1 TM and cytoplasmatic tail were amplified by PCR from human stabilin-1 HDPLM44 DNA in pcMV-Sport (supplied by Human Genome Sciences) using Pfu polymerase and 7453F Sal (5'-GGCGGTGCAGCGATGCTGCGG-3') as the forward primer and T7F (5'-AGCCTGAATTCGTGCGCTGAACG-3') as the reverse primer. The restriction sites in each case are underlined. The PCR product was purified and digested with SaIl and NotI. The PCR product was purified and digested with HindIII, which cut 155 bp upstream of the ATG start codon, and SaIl. The CD44 and stabilin-1 inserts were cloned through a three-way ligation into a HindIII NotI digested pcCMV vector.

Cloning and Expression of Stabilin-1 Link Module and Seven Fasciculin Domain (Stabilin-1 Link FC)—A protein comprising amino acids 1–251 of the Link domain (spanning the Link domain and Fascin-1 domain) fused to the C terminus of human IgG1 Fc was generated by PCR amplification of a 849-bp fragment from stabilin-1 (position Cys2391 through Ala2470) using the primers 6624F Aps (5’-CATGCTGGTATCCCTCGCATCAGTGGCATTGTC-3’) and 7472R Bam (5’-GGTGCGGATCCCTCGCATCAGTGCCAGCTG-3’) as the forward primer and T7F (5’-AGCCTGAATTCGTGCGCTGAACG-3’) as the reverse primer. The restriction sites in each case are underlined. The PCR product was purified and digested with SaIl and NotI. The CD44 extra-cellular domain was amplified by PCR from CD44H in pRcCMV (28) using the T7F and CD44 798R Sal primers (5’-CCGCCTGCAGACAATTTGGGCTGTCCTTATAGGAC-3’ and 6909R Sal (5’-GGCGGTGCAGCGATGCTGCGG-3’) as the reverse primer. The restriction sites in each case are underlined. The PCR product was purified and digested with HindIII, which cut 155 bp upstream of the ATG start codon, and SaIl. The CD44 and stabilin-1 inserts were cloned through a three-way ligation into a HindIII NotI digested pcCMV vector.

Cloning and Expression of Stabilin-1 Link Module (Anti-stabilin-1 LINK)—An immunogen comprising the stabilin-1 Link module alone (residues Gly2391Val2501) was generated by PCR amplification of a 293-bp fragment from stabilin-1 (position Cys2391 through Ala2470) using the primers 6624F Aps (5’-CATGCTGGTATCCCTCGCATCAGTGGCATTGTC-3’) and 7472R Bam (5’-GGTGCGGATCCCTCGCATCAGTGCCAGCTG-3’) as the forward primer and T7F (5’-AGCCTGAATTCGTGCGCTGAACG-3’) as the reverse primer. The restriction sites in each case are underlined. The PCR product was purified and digested with SaIl and NotI. The Link domain was amplified by PCR using the primers 6676F Aps (5’-GGTGCGGATCCCTCGCATCAGTGCCAGCTG-3’) and 6698R Bam (5’-GGTGCGGATCCCTCGCATCAGTGCCAGCTG-3’) as the forward primer and T7F (5’-AGCCTGAATTCGTGCGCTGAACG-3’) as the reverse primer. The restriction sites in each case are underlined. The PCR product was purified and digested with HindIII, which cut 155 bp upstream of the ATG start codon, and SaIl. The CD44 and stabilin-1 inserts were cloned through a three-way ligation into a HindIII Notl digested pcCMV vector.
comprising the third and fourth Fasciclin domains of stabilin-1 (Glu\(^{994}\)–Gly\(^{1256}\)) fused to human IgG Fc was generated by PCR amplification using the primers 3033F Asp (5′-CGACGGGATCCGGCAACTGGGGCCAGGCGTCAGGACCCCCGGCTGAGGATCCGGGACCCCGG-3′) and cloned into the Asp718/BamHI site of pCDM7Ig vector. The Fc fusion protein was produced in 293T cells as described previously (27). Injection into rabbits and determination of antibody specificity was the same as described in A.

(C) Stabilin-1 Cytoplasmic Tail (anti-stabilin-1 CT)—The synthetic peptide-(CEEDDFPTQRLTVK) encompassing the last 14 residues from the cytoplasmic tail of stabilin-1 preceded by a cysteine residue was conjugated to keyhole limpet hemocyanin using the Inject maleimide-activated immunogen conjugation kit from Pierce according to the manufacturer's instructions. New Zealand White rabbits were immunized as described in A. The same peptide conjugated to ovalbumin was immobilized on enzyme-linked immunosorbent assay plates to assess antibody reactivity. An irrelevant peptide conjugated to ovalbumin was used as a negative control.

Cell Surface Biotinylation and Western Blotting—Human 293T cells stably expressing LYVE-1 (transfected with Lipofectin from Invitrogen) were transiently transfected with stabilin-1 using calcium phosphate precipitation and subjected to cell surface biotinylation with or without prior permeabilization in 2 mg/ml saponin in PBS (10 min at room temperature). Cells were biotinylated using EZ-Link sulfo-N-hydroxysuccinimide-biotin (Pierce) according to the manufacturer's instructions. Cells were washed three times in PBS and resuspended in PBS at a concentration of 2.5 × 10\(^6\) cells/ml. EZ-Link sulfo-N-hydroxysuccinimide-biotin was added to a final concentration of 0.5 mg/ml, and cells were incubated at room temperature for 30 min. Cells were subsequently washed three times with PBS to remove any remaining biotinylating reagent. Cells (8.8 × 10\(^6\) cells in 1 ml) were then lysed in radioimmune precipitation assay buffer (1% (v/v) Nonidet P-40, 0.5% deoxycholate, 0.1% (w/v) SDS in PBS). Stabilin-1 was immunoprecipitated with rabbit polyclonal anti-stabilin-1 C-terminus and protein A, and LYVE-1 was immunoprecipitated with mouse monoclonal anti-human LYVE-1 and protein G as described previously (26). After Western blotting, membranes were stained with peroxidase-conjugated av- idin and then developed using SuperSignal chemiluminescent substrate (Pierce).

For analysis of stabilin-1 in primary LSECs, cells were lifted in PBS, 5 mM EDTA and lysed in SDS-PAGE sample buffer. Lysates equivalent to 1.5 × 10\(^7\) cells/lane were subjected to SDS-PAGE (7.5% gel). For analysis in whole liver (kindly provided by Dr. David Adams), tissue (4 mm\(^3\)) was first solubilized in 200 μl of sample buffer and 10 μl of sample was subjected to SDS-PAGE. After transfer to nitrocellulose, the membrane was stained with rabbit polyclonal anti-stabilin-1 LINK (5 μg/ml).

Hypotonic Lysis and Crude Cell Fractionation—Transient calcium phosphate stabilin-1-transfected 293T fibroblasts or primary LSECs were lifted in PBS, 5 mM EDTA and pelleted by brief centrifugation. Cells were resuspended in hypotonic buffer (20 mM HEPES, 100 mM NaCl, 1 mM protease inhibitors, Roche Applied Science), and membranes were ruptured by three cycles of freeze-thawing. The membrane fraction was isolated by centrifugation for 15 min at 20,000 × g, and the membrane pellet was re-suspended in 100 μl of 1× SDS-PAGE loading buffer. The supernatant (cytoplasmic fraction) was concentrated 10× to 50 μl using an Amicon Microcon concentrator (10-kDa cutoff, Millipore) before the isolation of membrane fraction. Wells were incubated with the appropriate primary antibodies (stabilin-1 CT (1/400), stabilin-1 LINK (5 μg/ml), anti-Fasc3-4 (1/100), monoclonal anti-human LYVE-1 (10 μg/ml), anti-human CD31 (1/500), or anti-human CD31 (1/200)) followed by Alexa 488-, Alexa 594-, or Alexa-568-conjugated goat anti-mouse or rabbit IgG (1/500) as appropriate. Confluent microscopy was performed on a Bio-Rad Radiance 2000 laser-scanning confocal microscope equipped with argon and green helium/neon lasers and analyzed using LaserSharp2000 software. All of the images were taken in sequential scanning mode.

LSECs, LECs, and HUVECs were grown on collagen-coated 8-well chamber slides, fixed and permeabilized as above, and stained with mouse anti-human CD31 (1/20) and anti-stabilin-1 CT (1/200). Slides were mounted in Vectashield containing 4',6-diamidino-2-phenylindole (Vector Laboratories) and visualized on a Zeiss Axiovert microscope equipped with epifluorescence illumination.

To assess the subcellular localization of the CD44-stabilin-1 chimera, HeLa cells were transfected with the CD44-stabilin-1 chimera using FuGENE 6. The following day, cells were lifted, transferred to 8-well chamber slides, and stained the next day. Cells were fixed and permeabilized in ice-cold (−20 °C) methanol for 5 min on ice and subsequently blocked with PBS, pH 7.5, containing 1% (w/v) bovine serum albumin and 5% (v/v) goat serum and viewed as described above.

For the flow cytometry of LECs, cells were fixed in 4% paraformaldehyde and stained with mouse anti-human CD31 or anti-stabilin-1 (CLEVER-1 mAb 3-372) diluted in PBS, 5% (v/v) fetal calf serum, and 0.1% (w/v) azide followed by F-phycoerythrin-conjugated rabbit anti-mouse Ig.

Internalization of Acetylated LDL, Antibody, and Fluorescein-labeled HA—HeLa or 293T cells transiently transfected with full-length stabilin-1 were grown in 8-well chamber slides. Cells were washed with prewarmed culture medium and incubated with Alexa 488-conjugated acetylated LDL (10 μg/ml, Molecular Probes), anti-stabilin-1 (1 μg/ml, CLEVER-1 mAb 3-372), or fluorescein-labeled HA (FI-HA, 6 μg/ml) (prepared as described above) at 37 °C for different time periods, washed in PBS, fixed and permeabilized in ice-cold methanol, and stained with Alexa 594-conjugated goat anti-mouse Ig or stained with anti-stabilin-1 CT (1/500) followed by Alexa 594-conjugated goat anti-rabbit IgG. Alternatively, cells were fixed in 4% (w/v) paraformaldehyde and permeabilized in 2 mg/ml saponin as described above prior to staining with mouse anti-human EEA-1.

HA-binding Assays—The binding of stabilin-1 fusion protein to immobilized HA was tested in 96-well enzyme-linked immunosorbent assay plates (Nunc Maxisorp) as described previously (25). Plates were coated by overnight incubation with 1 mg/ml HA or goat anti-human Fc (10 μg/ml) as control in coating buffer (15 mM sodium carbonate and 34 mM sodium bicarbonate, pH 9.3) prior to blocking (2 h in PBS containing 1% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20) and incubation with culture supernatant from stabilin-1-LINK Fc-transfected cells. Bound protein was detected with peroxi-dinylconjugated goat anti-human IgG (1:4000; Pierce) followed by O-phenylenediamine substrate (Sigma), and absorbance was measured at 490 nm in a Bio-Rad microplate reader. Analysis of pH dependence of stabilin-1 LINK Fc binding was carried out in a 25 mM Tris-HCl, 25 mM MES mixed buffer system, pH 5–8, containing 100 mM NaCl and 0.05% (v/v) Tween 20.

Immunohistology—Paraffin-embedded sections of human lymph node were obtained from the Department of Histopathology, Oxford Radcliffe Hospital. Frozen sections of human lymph node and paraffin-embedded sections of human colon were obtained from the Nuffield Department of Clinical Laboratory Sciences, Oxford, Radcliffe Hospital.

Results from previous studies suggested that stabilin-1 may be associated with intracellular vesicles in monocyte/macrophages and myeloid cell lines (7, 18, 30). To establish whether such localization is a feature of the receptor in endothelial cells, we used confocal microscopy to examine lymph node sinus and lymph node HEV in situ using a mixture of paraformaldehyde-fixed and frozen tissue sections and a panel of novel receptor-specific antibodies (Fig. 1). These included an anti-peptide antibody to the stabilin-1 cytoplasmic tail (anti-stabilin-1 CT), an antibody to the stabilin-1 Link domain (anti-stabilin LINK), and the monoclonal antibody 3-372 to the stabilin-1/CLEVER-1 ectodomain.

RESULTS

Stabilin-1 Is Predominantly Intracellular in Tissue Vessels and Primary Endothelial Cells—Results from previous studies have suggested that stabilin-1 may be associated with intracellular vesicles in monocye/macrophages and myeloid cell lines (7, 18, 30). To establish whether such localization is a feature of the receptor in endothelial cells, we used confocal microscopy to examine lymph node sinus and lymph node HEV in situ using a mixture of paraformaldehyde-fixed and frozen tissue sections and a panel of novel receptor-specific antibodies (Fig. 1). These included an anti-peptide antibody to the stabilin-1 cytoplasmic tail (anti-stabilin-1 CT), an antibody to the stabilin-1 Link domain (anti-stabilin LINK), and the monoclonal antibody 3-372 to the stabilin-1/CLEVER-1 ectodomain.
FIG. 1. Confocal microscopy of intracellular stabilin-1 in lymph node HEV and lymphatic sinuses. Panel A shows a diagramatic representation of the 2570 residue stabilin-1 polypeptide coded by the cloned stabilin-1 cDNA. Within the extracellular domain, tandem repeats of a fasciclin-like domain (F1–F7) are shown in blue, EGF-like repeats are shaded gray, and the membrane-proximal Link module (LINK) is shaded red. The transmembrane domain and cytoplasmic tail are colored black and white, respectively. The regions of the polypeptide used in the generation of the stabilin-1 antibodies, stabilin-1 Fasc3–4, stabilin-1 LINK, and stabilin-1 CT (see “Experimental Procedures”) are indicated above the figure. Panels B–F show frozen (B and D) and paraffin sections (C, E, and F) of human lymph node stained with monoclonal or polyclonal antibodies directed to different regions of stabilin-1 and either the HEV marker peripheral lymph node addressin (MECA79 mAb) or the lymphatic endothelial marker LYVE-1. These were as follows: panels B–C, HEV stained with MECA79 mAb (FITC, green) and stabilin-1/CLEVER-1 mAb 3-372 (Alexa 594, red) with each channel shown separately or merged; panels D–E, individual HEV stained with MECA79 mAb (FITC, green) and either stabilin-1 CT (D) or stabilin-1 LINK (E) (Alexa 594, red); panel F, lymphatic sinuses stained with stabilin-1/CLEVER-1 mAb 3-372 (Alexa 488, green) and LYVE-1 (Alexa 594, red) with channels shown separately and merged. Sections were viewed by confocal microscopy (bar = 10 μm).

(19). All three revealed a diffuse perinuclear staining pattern for the receptor within the distinct “high” cuboidal endothelium of lymph node HEV, strongly indicative of an intracellular rather than a plasma membrane localization for stabilin-1 in this location (Fig. 1). This localization contrasted markedly with the lymph node homing receptor/addressin (the counter-receptor for L-selectin on naïve lymphocytes) detected with the mAb MECA79 (31), which stained the plasma membrane of the post-capillary venules. An analysis of a total of 21 HEVs present in five separate sections, including those from “reactive” antigen-activated lymph node, yielded similar results (data not shown). In addition, stabilin-1 was concentrated in intracellular vesicles within lymph node subcapsular and cortical lymphatic sinuses, the endothelial structures that surround the HEV-containing parenchyma and regulate traffic entering or exiting the T cell zones. This localization contrasted markedly with the lymphatic endothelial HA receptor LYVE-1, which was restricted to the plasma membrane (Fig. 1). Curiously, however, stabilin-1 was either absent or only weakly detected in the lymphatic vessels of non-lymphoid tissues, such as colon, stomach, or skin, despite characteristically abundant expression of LYVE-1 (Fig. 2). Indeed, stabilin-1 was only detected in these vessels with the CLEVER-1 mAb 3-372 using frozen tissue sections (Fig. 2E), indicating the likelihood that the receptor, when present, is at very low abundance. However, stabilin-1 was expressed strongly on individual macrophage-like cells in these tissues as reported previously by Goerd et al. (18, 30). Hence, the expression of stabilin-1 in tissues appears to be intracellular and largely restricted to lymphatic sinuses and HEV in secondary lymphoid tissue, rather than being present throughout the lymphatic vasculature in both lymphoid and non-lymphoid tissue, as is the case for LYVE-1.

We next examined stabilin-1 localization in isolated primary endothelial cell populations. As shown by the results in Fig. 3, the receptor was detected in primary liver sinusoidal endothelial cultures as well as lymphatic endothelial cells derived from primary dermal microvascular endothelium and HUVEC. However, in each case, detection required prior permeabilization of the cells and no stabilin-1 was detected at the cell surface. The discrepancy between abundant stabilin-1 expression in isolated lymphatic endothelial cells and its relative absence from tissue lymphatics is reminiscent of proteins such as CD44, which is inappropriately expressed in lymphatic endothelial cells after prolonged culture, a phenomenon most probably due to deregulation of gene expression.3 In comparison with the homotypic adhesion receptor PECAM-1 (CD31), which was concentrated at cell-cell junctions in all three endothelial cell types, stabilin-1 appeared to be entirely perinuclear, signifying location within an intracellular membrane or vesicle population (Fig. 3). The virtual absence of cell surface stabilin-1 was confirmed by flow cytometry with the mAb 3-372.

3 S. Clasper and D. G. Jackson, unpublished observation.
which showed only a minor increase in fluorescent intensity compared with the isotype control (Fig. 3D). Finally, we investigated whether the appearance of stabilin-1 at the cell surface could be influenced by treatment with cytokines (IL-1, IL-4, and tumor necrosis factor-α/H9251) and bacterial lipopolysaccharide or by “activation” with phorbol ester. However no increase in cell surface stabilin-1 expression was seen (as detected by staining with the stabilin-1/CLEVER-1 mAb 3-372), even after overnight or longer treatment with any of these agents (data not shown). Therefore, the intracellular “retention” of stabilin-1 is a consistent feature of the receptor in liver/lymph node sinus and HEV that is not altered by tissue disruption, cell culture, or cell activation.

Investigation of Stabilin-1 Intracellular Retention in Transfected 293T Cells—To investigate the subcellular localization of stabilin-1 and its intracellular targeting in more detail, we studied the properties of the receptor in transfected 293T cells. Because of difficulties in isolating the full-length stabilin-1 cDNA, which exceeds 7900 bp in length, we resorted to splicing two separate cDNAs, one that contained the mature N terminus but carried an insertion that contained a premature stop codon and the other that contained an uninterrupted reading frame but lacked the first 1030 bp from the 5’ end (for complete details see “Experimental Procedures”). The sequence of this cDNA was virtually identical to that published by Politz et al. (7) apart from four single amino acid substitutions (including G913R, D2200L, V2282I, and T2506M). The potential significance of these apparent polymorphisms is not yet clear. Transfection of full-length stabilin-1 cDNA into 293T and HeLa cells followed by Western blotting with the anti-stabilin LINK antibody yielded a pattern of protein bands comprising a major doublet of 250 kDa and further bands of 140 kDa or lower, similar to that displayed by endogenous stabilin-1 both in cultured liver sinusoidal endothelium and whole liver (Fig. 4A). Hence, the proteolytic processing of the recombinant and native receptors appears to be similar.

In common with endogenous stabilin-1 in endothelial cells described above, the recombinant stabilin-1 in 293T cells re-
Stabilin-1 Localization in Endothelial Cells

Queried membrane permeabilization for detection and displayed the same characteristic perinuclear localization. This was demonstrated by fluorescent staining with the two antibodies, anti-stabilin LINK and anti-stabilin CT, as well as a third antibody, anti-stabilin Fas3-4, generated against the third and fourth fasciin-like repeats. The intracellular location of stabilin-1 was further underlined by staining cells co-transfected with LYVE-1, which by contrast specifically decorated the plasma membrane (Fig. 4B). The specificity of all three antibodies was underlined by staining control 293T cells transfected with LYVE-1 alone, which detected no signal in each case (Fig. 4B). The absence of recombinant stabilin-1 from the plasma membrane of 293T cells was also apparent using cell surface biotinylation, which showed that the receptor was labeled only after prior permeabilization of the cells. LYVE-1, in contrast, was efficiently biotinylated with or without prior permeabilization (Fig. 5A).

Finally, to determine whether a proportion of the intracellular stabilin-1 was present in the cytoplasm or destined for secretion, we followed the fate of the receptor in transfected 293T cells and liver sinusoidal endothelial cells following crude cell fractionation induced by repeated freeze-thawing in hypotonic buffer (10 mM Tris, pH 8.0). As shown by the Western blot in Fig. 5, B–C, both the major 250-kDa doublet band and the smaller 140-kDa bands were detected only in the particulate fraction and not in the soluble cytoplasmic fraction. Hence, the majority of intracellular stabilin-1 is probably membrane-anchored and little if any is secreted, at least under the cell culture conditions used in our experiments. Interestingly, both the 250-kDa doublet and 140-kDa bands were recognized by the anti-stabilin CT and anti-stabilin LINK antibodies, whereas the anti-stabilin Fas3-4 antibody recognized only the 250-kDa doublet. These results indicate the 140-kDa stabilin-1 species retains the membrane anchor and the Link domain but lacks the distal portion of the extracellular domain.

Stabilin-1 Internalizes Ligand via Early Endosomes—Previously, it has been shown that stabilin-1 can mediate internalization of the scavenger receptor ligand, acetylated LDL, in transfected Chinese hamster ovary-K1 cells (8). To confirm internalization in our own studies using transfected HeLa cells and characterize the endocytic pathway involved, we studied the uptake of acetylated LDL and the stabilin-1/CLEVER-1 mAb 3-372 by confocal microscopy. As a preliminary experiment, we took transfectedants incubated without added ligand and stained for stabilin-1 and markers for endoplasmic reticulum (protein-disulfide isomerase), trans-Golgi network (TGN46), lysosomes (CD63), vesicles of the clathrin-mediated endocytic pathway (transferrin receptor), and the early endosome marker EEA-1 (32). The results showed co-localization of a small population of stabilin-1 with EEA-1-positive vesicles but no co-localization with the other markers (Fig. 6A and data not shown). This finding suggests that a small proportion of stabilin-1 associates constitutively with early endosomes. We next incubated stabilin-1 transfectants at 37 °C with a mixture of acetylated LDL and mAb 3-372 added together. The results showed that both ligands were co-internalized, accumulating in a large number of intracellular vesicles (Fig. 6B). Furthermore, a moderate proportion of the internalized acetylated LDL appeared to associate with the EEA-1-positive vesicles (Fig. 6C). We could also observe rapid internalization of stabilin-1 antibodies alone, which accumulated in similar perinuclear vesicles after 5 min at 37 °C (Fig. 6D and data not shown). Importantly, neither acetylated LDL nor stabilin-1 antibodies were internalized by untransfected cells and no internalization of isotype control antibodies was detected in stabilin-1 transfectants (data not shown). The finding that acetylated LDL was also internalized in the absence of antibodies suggests that the internalization of stabilin-1 is not dependent on receptor cross-linking (i.e. through the secondary uptake of receptor-Ab complexes). Although further analyses will clearly be required to substantiate such conclusions, these results suggest that stabilin-1 cycles constitutively between the plasma membrane and early endosomes and that trafficking to endosomes is significantly increased in response to ligand binding. Given that the steady-state levels of stabilin-1 at the cell surface are below the limits of detection by fluorescence microscopy, we conclude that the rate of trafficking must be extremely fast.

Intracellular Targeting of Stabilin-1 Is Directed by the Transmembrane Domain and Cytoplasmic Tail—The cytoplasmic tail of stabilin-1 contains a potential di-leucine-based intracellular sorting signal within the motif DXXLL (see Fig. 7A) (37). To investigate whether the cytoplasmic tail indeed plays a role in vesicular targeting, a chimera, CD44<sub>ecto</sub>-stabilin-1<sub>TM-tail</sub>, was constructed comprising the transmembrane domain and cytoplasmic tail of stabilin-1 (Ala<sup>2467</sup>-Lys<sup>2570</sup>) fused to the extracellular domain (Met<sup>1</sup>-Ile<sup>296</sup>) of the cell surface HA receptor CD44 (see Fig. 7B). The localization of the chimera was then compared with that of full-length CD44 (whose cytoplasmic tail lacks conventional intracellular-targeting motifs) after co-transfection in HeLa cells and staining with the anti-CD44...
ectodomain mAb IM7 by confocal microscopy. The result (Fig. 7C) showed that the CD44-ecto-stabilin-1(TM-tail) chimera, similar to full-length stabilin-1, was targeted almost exclusively to intracellular vesicles, whereas full-length CD44, as expected, was targeted exclusively to the cell surface. These data provide the first evidence of an important role for the stabilin-1 transmembrane domain/cytoplasmic tail in directing intracellular targeting.

Stabilin-1 Does Not Bind Hyaluronan—Given that the related receptors CD44, LYVE-1, and stabilin-2 that carry the

Fig. 4. SDS-PAGE analysis and confocal microscopy of recombinant stabilin-1 in transfected cells. Panel A, lysates of control (untransfected) and stabilin-1-transfected 293T or HeLa cells, human dermal LEC, LSEC, and whole liver were electrophoresed on 7.5% (w/v) polyacrylamide SDS-PAGE gels and immunoblotted with anti-stabilin-1 LINK antibody. Positions of molecular mass markers (in kDa) are as indicated. Panel B shows comparative localization of stabilin-1 and the cell surface HA receptor LYVE-1 in LYVE-1/stabilin-1 doubly transfected 293T cells stained with a LYVE-1 mAb (Alexa 568, red) in combination with stabilin-1 CT, stabilin-1 LINK, or stabilin-1 anti-Fasc3–4 (Alexa 488, green). The column of panels on the right show 293T transfected with LYVE-1 alone stained with the three different stabilin-1 antibodies indicated. Transfectants were viewed by confocal microscope.
lectin-like Link module can bind hyaluronan, we reinvestigated the functional status of the stabilin-1 Link module. One earlier report alluded to the finding that detergent-solubilized stabilin-1 failed to bind hyaluronan-Sepharose (7). Therefore, we assessed the capacity of stabilin-1-transfected cells to bind and internalize hyaluronan in vitro. However, incubation of stabilin-1-transfected 293T cells with Fl-HA either alone or together with the stabilin-1/CLEVER-1 mAb 3-372 resulted in no detectable binding or internalization of Fl-HA even after several hours at 37 °C (Fig. 8 and data not shown). In contrast, LYVE-1 transfected 293T, bound, and internalized Fl-HA as expected (Fig. 8). Importantly, the 293T transfecants efficiently internalized both stabilin-1 antibody and acetylated LDL (Fig. 8), similar to that observed with HeLa (Fig. 6). To avoid the possibility that chemical modification of HA with fluorescent somehow prevented interaction with the stabilin-1 Link module, we repeated the assay using unlabeled HA followed by detection with biotinylated HA-binding protein. Once again, however, no internalization of HA was detected (data not shown). These results indicate that stabilin-1 Link module is either inactivated in intact cells, or inherently non-functional.

To explore these possibilities using a cell-free system, we expressed the isolated stabilin-1 Link module as a soluble Fc fusion protein and characterized its HA-binding properties in a conventional plate-binding assay. The Fc fusion protein encompassed the stabilin-1 Link module and the adjacent Fasciclin domain (position of Cys2191–Ala2473) fused at the C terminus with the Fc domain of human IgG1 (Fig. 9A). The results of these analyses (Fig. 9B) revealed no binding of stabilin-1 Link fusion protein to HA, in contrast to the positive control LYVE-1 Fc fusion protein, which exhibited specific binding to HA under these conditions. Similar results were obtained with other Link-module constructs including Δ2191–2316, Δ2206–2316, and Δ2206–2473 that included adjacent fasciclin-like domains and EGF-like repeats, none of which was found to bind HA (data not shown). In addition, the stabilin-1 Fc fusion protein failed to bind to other glycosaminoglycans including chondroitin 4-sulfate, chondroitin 6-sulfate, heparan sulfate, and heparin (data not shown).

We next examined the effect of varying the H⁺ ion concentration on HA binding. However, we observed no significant binding over the range of pH 5.0–8.0, indicating that the stabilin-1 Link module is “inactive” at physiological pH values and most probably also when present within weakly acidic endosomal vesicles (Fig. 9C).

Finally, we investigated the possibility that the stabilin-1 Link module might bind acetylated LDL. To address this issue, we tested the ability of stabilin-1 Fc to block acetylated LDL uptake by full-length stabilin-1-transfected 293T. As shown in Fig. 8C, stabilin-1 transfectants alone internalized acetylated LDLs as evidenced by co-localization of ligand and receptor within the intracellular compartment after 5 min at 37 °C. However, the addition of stabilin-1 Link Fc protein had no effect on acetylated LDL uptake (Fig. 8D).

**DISCUSSION**

First identified by the mAb MS-1 as an abundant high molecular weight glycoprotein in sinusoidal endothelium of spleen, liver, and lymph node (18), the molecule now known variously as stabilin-1/FEEL-1/CLEVER-1 has been cloned and sequenced by a number of groups including our own (7, 8, 20, 33). In each case, the cloning and sequencing have revealed the same complex multidomain structure comprising a total of seven fasciclin-like domains, twenty-two EGF-like repeats, and a single membrane-proximal Link domain, the C-type lectin-like module found in proteins that bind the matrix mucopolysaccharide HA. The same studies that identified stabilin-1 also led to the parallel identification of a second closely related protein termed stabilin-2/FEEL-2 with a remarkably similar domain structure and expression profile. This is now considered to be identical to the major endocytic HA receptor in liver, spleen, and lymph node, also known as HARE (7, 8, 34). Yet, despite the similarities between the two stabilin molecules, those studies that have been carried out to date suggest dis-
FIG. 6. Stabilin-1 internalizes ligand via early endosomes. The figure shows confocal microscopic images of stabilin-1-transfected HeLa cells incubated either alone (panel A) or with antibody or acetylated LDL (1 h, 37 °C) prior to permeabilization and staining with the indicated markers (panels B, C, and D) to identify the location of internalized ligand. In panel A, cells were permeabilized and double-stained with anti-stabilin CT (Alexa 594, red) and the early endosome marker EEA-1 (Alexa 488, green) to visualize (total) stabilin-1 localization in the absence of ligand. In panel B, cells were allowed to bind and internalize the non-blocking stabilin-1/CLEVER-1 mAb 3-372 together with Alexa 488-labeled acetylated LDL (green) prior to permeabilization and staining with Alexa 594-conjugated goat anti-mouse Ig to allow visualization of
Stabilin-1 Localization in Endothelial Cells

Alexa 488-labeled acetylated LDL (5 min, 37 °C) and non-blocking mAb conjugated goat anti-mouse Ig; 3-372 followed by detection of internalized stabilin-1 with Alexa 594-transfected 293T cells co-incubated with Fl-HA and non-blocking mAb surface); 293T incubated with Fl-HA (note the binding of Fl-HA to the cell surface); panels C and D, stabilin-1-transfected 293T co-incubated with Alexa 488-labeled acetylated LDL (5 min, 37 °C) and non-blocking mAb 3-372 either alone (panel C) or with stabilin-1 LINK Fc (40 μg/ml) added as potential blocker (panel D) prior to fixation, permeabilization, and detection of internalized ligand using Alexa 488-conjugated goat anti-mouse Ig. Bar = 10 μm. Insets show boxed regions at higher magnification.

Fig. 7. The transmembrane domain and cytoplasmic tail of stabilin-1 directs intracellular targeting of a CD44/stabilin-1 chimera. The role of the transmembrane domain and cytoplasmic tail in stabilin-1 intracellular targeting was studied by confocal microscopy using a CD44c(stabilin-1)4Mcherry chimera in transfected HeLa cells. Panel A shows the amino acid sequence of the stabilin-1 tail with the DXXLL-type di-leucine repeat underlined. Panel B shows a diagram of the CD44c(stabilin-1)4Mcherry chimera with the details of sequences at the fusion junction indicated (residues originating from the SalI site (S) are shown in boldface). Panel C shows confocal microscopic images of CD44c(stabilin-1)4Mcherry and full-length control CD44-transfected HeLa permeabilized and stained with the CD44 mAb IM7 (Alexa 488, green).

Fig. 8. Full-length stabilin-1 neither binds HA nor mediates its internalization in transfected 293T cells. 293T cells transfected with stabilin-1 were incubated with Fl-HA (6 μg/ml Fl-HA for 1 h at 37 °C) and the non-blocking anti-stabilin-1 (anti-CLEVER-1) mAb 3-372, or with Alexa 488-labeled acetylated LDL (5 min, 37 °C) before fixation, permeabilization, and detection of internalized ligand using fluorescence microscopy. Images are as follows: panel A, stabilin-1-transfected 293T cells co-incubated with Fl-HA and non-blocking mAb 3-372 followed by detection of internalized stabilin-1 with Alexa 594-conjugated goat anti-mouse Ig; panel B, control LYVE-1-transfected 293T incubated with Fl-HA (note the binding of Fl-HA to the cell surface); panels C and D, stabilin-1-transfected 293T co-incubated with Alexa 488-labeled acetylated LDL (5 min, 37 °C) and non-blocking mAb 3-372 either alone (panel C) or with stabilin-1 LINK Fc (40 μg/ml) added as potential blocker (panel D) prior to fixation, permeabilization, and detection of internalized ligand using Alexa 594-conjugated goat anti-mouse Ig.

Different properties for stabilin-1 and a different function, even though its precise nature remains uncertain. For example, stabilin-1 was reported to be “deposited” at zones of contact between sinusoidal endothelial cells in spleen and to be induced in cytokine-treated macrophages in which it was present within cytoplasmic granules rather than at the cell surface (18, 30). Second, stabilin-1, unlike stabilin-2/HARE, was reported to be expressed on lymph node post-capillary HEV, structures that were specialized for the recruitment of naïve lymphocytes from the blood circulation as well as the endothelium of lymphatic vessels (19). Based on these latter properties, Ijala et al. (19) renamed stabilin-1/FEEL-1 as CLEVER-1 (19). Third, although both stabilin-1 and stabilin-2 contain a membrane-proximal Link module, only stabilin-2/HARE has been demonstrated to bind HA (7, 14). However, both stabilin-1 and stabilin-2 have been shown to internalize ligands such as acetylated LDL, Gram-negative and Gram-positive bacteria (E. coli and Staphylococcus aureus), and advanced glycation end products (8). These latter properties have led to the proposal that stabilin-1 functions as a general scavenger receptor, similar to but distinct from endothelial cell surface receptors such as SREC (scavenger receptor expressed by endothelial cells) and the smaller C-type lectin scavenger receptor LOX-1 (35). Very recently, a further completely novel function has been proposed for stabilin-1, that of a lymph node-homing receptor. This intriguing role has been inferred from the observation that antibodies to the receptor block adhesion of T cells to HEV in lymph node-frozen sections in vitro and block lymph node functions associated with trafficking, such as the increase in lymph node size resulting from injected foreign antigen (discussed below) (19).

In light of these rather different proposals for stabilin-1 function and reports of its possible intracellular location, we set out in this paper to rigorously characterize the subcellular localization of stabilin-1 both in tissues and primary endothelial cells and to properly assess the glycosaminoglycan-binding properties of its membrane-proximal Link module. Our results showed that stabilin-1 is indeed intracellular in each of the endothelial cells types with which it has been associated, both in whole tissue and after isolation as primary cells in culture. Specifically, we showed by confocal microscopy using three different antibodies (anti-stabilin-LINK, anti-stabilin-CT, and anti-CLEVER-1 mAb 3-372) that stabilin-1 is almost entirely retained within intracellular vesicles in lymphatic sinuses and HEV in tissue sections from normal and inflamed lymph node tissue and found little evidence for expression at the luminal surface of either vessel structure. The same localization was apparent in primary endothelial cells of each type including those isolated from liver sinus, dermal lymphatics, and umbilical vein. In addition, we showed using the lymphatic endothelial marker LYVE-1 that stabilin-1 is present only at very low levels in lymph vessels of peripheral non-lymphoid tissues such as skin, colon, and stomach, indicating a more restricted pattern of tissue expression than previously documented (18, 19).

Although we could not detect appreciable cell surface expression of full-length stabilin-1 either by microscopy or surface biotinylation, we could confirm that stabilin-1 transfected HeLa cells and that 293T cells mediated internalization of acetylated LDL and stabilin-1 antibody, both of which were rapidly targeted to early endosomes as assessed by co-localization with the EEA-1 marker. Whereas the steady-state cell surface levels of stabilin-1 appear to be very low, the receptor clearly shuttles rapidly between the plasma membrane and endosomal vesicles and internalizes scavenger ligands via early endosomes.

By constructing a chimera of the extracellular domain of the adhesion receptor CD44 and the transmembrane and cytoplasmic domain and cytoplasmic tail of stabilin-1 directs intracellular targeting of a CD44/stabilin-1 chimera. The role of the transmembrane domain and cytoplasmic tail in stabilin-1 intracellular targeting was studied by confocal microscopy using a CD44c(stabilin-1)4Mcherry chimera in transfected HeLa cells. Panel A shows the amino acid sequence of the stabilin-1 tail with the DXXLL-type di-leucine repeat underlined. Panel B shows a diagram of the CD44c(stabilin-1)4Mcherry chimera with the details of sequences at the fusion junction indicated (residues originating from the SalI site (S) are shown in boldface). Panel C shows confocal microscopic images of CD44c(stabilin-1)4Mcherry and full-length control CD44-transfected HeLa permeabilized and stained with the CD44 mAb IM7 (Alexa 488, green).
mic domain of stabilin-1, we showed that the latter mediates vesicular targeting. The cytoplasmic tail of stabilin-1, unlike that of the HA scavenger receptor stabilin-2/HARE, contains neither of the classical YXXØ (where Ø is a bulky hydrophobic residue) or NPXY consensus binding motifs for AP adapter binding and clathrin-mediated endocytosis (7, 8, 36). However, the stabilin-1 tail does contain a di-leucine motif within the sequence EPFDDSLLEED. Interestingly, this sequence is of the (DXXLL) pattern, which has been shown to bind the VHS domain of GGAs (Golgi-localized/H9253-ear containing) rather than AP adapter proteins and to direct TGN to endosomal sorting rather than TGN to plasma membrane sorting (37). Although more detailed mutagenesis will be required to confirm this assignment unambiguously, such a role for the di-leucine motif is certainly consistent with the largely intracellular localization of stabilin-1, as distinct from the cell surface location of the HA receptor stabilin-2, which contains the plasma membrane to endosomal sorting motifs NPLY and YDPF in the cytoplasmic tail (19).

How in the light of these findings can stabilin-1 mediate the homing of lymphocytes to lymph node HEV as reported by Irjala et al. (19)? In their paper, these workers presented evidence that mAb 3-372 to the stabilin-1 (CLEVER-1) extracellular domain blocked the adhesion of lymphocytes to post-capillary venules in human lymph node-frozen sections. They also showed that intravenous injection of these antibodies in rabbits led to their concentration in lymph node HEV and to a reduction in antigen-induced lymphoproliferation in lymph node parenchyma (19). These data were interpreted to signify that stabilin-1/CLEVER-1 is expressed on the luminal surface of lymph node HEV where it was proposed to mediate adhesion/transmigration of circulating T cells. Although they do show that stabilin-1 plays some role in the generation of the immune response, we suggest a different interpretation based on our own findings in this paper. Firstly, the adhesion of lymphocytes to endothelia in frozen tissue sections might be explained by interactions with intracellular rather than cell surface stabilin-1, the former being unmasked in the process of frozen-sectioning. This important point has also been stressed by others and is a potential shortcoming of the so-called Stamper-

![Fig. 9. Stabilin-1 Fc fusion proteins do not bind HA. Panel A shows a diagram of the stabilin-1 Link Fc fusion protein, which comprises the stabilin-1 Link module and the adjacent Fasciclin domain (Cys2191-Ala2477) fused to the Fc region of human IgG1. The amino acid sequences at the fusion junction are shown below the diagram with the residues originating from the Asp718 (A) and BamHI site (B) shown in boldface. Panel B shows the binding of the stabilin-1 Link Fc fusion protein and the LYVE-1 Fc fusion protein (positive control) to microtiter plates coated with HA, goat anti-human IgG (loading control), and bovine serum albumin (BSA; negative control) as detected with peroxidase-conjugated goat-anti-human IgG. Panel C shows the binding of stabilin-1 Link Fc to immobilized HA, goat anti-human IgG, or BSA at different pH values. Values for bound protein are the mean absorbance at 490 nm ± S.E. for three replicate determinations.](image-url)
Woodruff assay (38). Secondly, the apparent labeling of HEV by intravenously injected stabilin-1 mAb could be explained by rapid internalization of the stabilin-1/mAb complex. Indeed, we have shown here that such internalization is very rapid in 293T/HeLa transfectants and occurs within 5 min of incubation with mAb 3-372 at 37 °C, consistent with a more orthodox role for the protein as a scavenger. Lastly, the effect of injected stabilin-1 mAb on antigen-induced lymph node cellular proliferation may also be secondary to internalization and could have many possible explanations ranging from cellular cytotoxicity to indirect effects on antigen-processing and presentation, rather than to direct effects on lymphocyte-endothelial cell interactions. The application of stabilin-1 antibodies to HUVEC cultured in Matrigel, for example, has been reported to alter endothelial tube formation and morphology in vitro (8).

In terms of more general considerations, one might expect that an adhesion receptor on lymph node HEVs would be relatively selective, allowing the recruitment of only naïve lymphocytes and not monocytes or neutrophils to extravasate. However, the observation by Irjala et al. (19) that their stabilin-1 mAb blocks the adhesion of lymphocytes as well as granulocytes and monocytes to HEVs in inflamed synovium argues against selectivity for stabilin-1. Moreover, the finding that stabilin-1 has ligands as diverse as acetylated LDL, advanced glycation end products, and bacterial particles (8) further suggests that cell adhesion to stabilin-1, if it does occur, is not likely to be specific. Lastly, it is difficult to conceive how a scavenger molecule such as stabilin-1, which is concentrated in endosomes and whose steady-state cell surface expression level is very low, could efficiently mediate cell adhesion. Although examples of scavenger receptors (e.g. LOX-1 and SR-A) that can support leukocyte adhesion under flow do exist, these are conventional cell surface expressed molecules (39, 40). An alternative, perhaps a more conventional hypothesis, is that stabilin-1 acts as a scavenging receptor in HEV, as it is envisaged to do in liver and spleen sinusoids. There is growing evidence that HEVs do have a scavenging function. For example HEVs express receptors such as the Duffy antigen receptor for chemokines that is thought to take up these potent chemoattractant molecules from the lymph node microcirculation (41). Moreover, HEVs have the capacity to endocytose apoptotic leukocytes from among the recirculating populations in post-capillary venules and may even induce apoptosis in appropriate cells through their expression of Fas ligand and its engagement.
with the death domain-containing counterreceptor, Fas (42, 43). It is even conceivable that stabilin-1 may participate in the uptake of such cellular debris.

Finally, we have shown that full-length stabilin-1 protein as well as a soluble Fc fusion protein of the Link module fail to bind fluorescein-labeled or immobilized HA or other glycosaminoglycans. This contrasts with the closely related stabilin-2 (HARE) molecule, which is reported to bind various glycosaminoglycans (7, 14, 34, 44), and with the Link modules present in the cell surface receptors CD44 and LYVE-1, both of which can be demonstrated to bind Fl-HA prepared by the method used here (13, 25, 45). However, we cannot rule out the possibility that stabilin-1 binds HA with a much lower affinity than these latter receptors and that more highly conjugated HA preparations might be required for its detection. If this turns out to be the case, the possibility remains that additional sites remote from the Link module might also be involved.

Interestingly, a comparison of amino acid sequences within the Link superfAMILY shows that the Link modules in stabilin-1 and stabilin-2 are most similar to those in the small inflammation-induced TSG-6 (tumor necrosis factor and IL-1-inducible gene six) protein (Fig. 10A) (46). Indeed, all three Link modules share an equal degree of similarity (64–67%) and sequence identity (46–48%). The TSG-6 Link module has been characterized extensively, and its three-dimensional structure has been determined by NMR spectroscopy in both its free and HA-bound conformations (10, 47). This has revealed the HA-binding site as a groove lined with tyrosines and hydrophobic and basic residues (see Fig. 10B). The mapping of these residues onto the space-filling model of the TSG-6 Link and a comparison with a model of the stabilin-1 Link module based on the TSG-6 coordinates (47) indicate that several are well conserved in stabilin-1 (i.e. Tyr12, Arg55, and Trp59) and indeed within a patch that partly resembles the TSG-6 HA-binding surface. Interestingly, however, Tyr59 and Tyr78 are substituted by histidine and asparagine, respectively, in stabilin-1, whereas both residues are conserved in the Link domains of stabilin-2 and TSG-6. Together with Tyr12, Tyr55, and Tyr78, they are exchanged for histidine and asparagine, respectively, in stabilin-1, whereas both residues are conserved in the Link domains of stabilin-2 and TSG-6. Together with Tyr12, Tyr55, and Tyr78, they are exchanged for histidine and asparagine, respectively, in stabilin-1, whereas both residues are conserved in the Link domains of stabilin-2 and TSG-6. Together with Tyr12, Tyr55, and Tyr78, they are exchanged for histidine and asparagine, respectively, in stabilin-1, whereas both residues are conserved in the Link domains of stabilin-2 and TSG-6. Together with Tyr12, Tyr55, and Tyr78, they are exchanged for histidine and asparagine, respectively, in stabilin-1, whereas both residues are conserved in the Link domains of stabilin-2 and TSG-6. Together with Tyr12, Tyr55, and Tyr78, they are exchanged for histidine and asparagine, respectively, in stabilin-1, whereas both residues are conserved in the Link domains of stabilin-2 and TSG-6. Together with Tyr12, Tyr55, and Tyr78, they are exchanged for histidine and asparagine, respectively, in stabilin-1, whereas both residues are conserved in the Link domains of stabilin-2 and TSG-6.