Identification of a Carbohydrate-based Endothelial Ligand for a Lymphocyte Homing Receptor

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Abstract. Lymphocyte attachment to high endothelial venules within lymph nodes is mediated by the peripheral lymph node homing receptor (pnHR), originally defined on mouse lymphocytes by the MEL-14 mAb. The pnHR is a calcium-dependent lectin-like receptor, a member of the LEC-CAM family of adhesion proteins. Here, using a soluble recombinant form of the homing receptor, we have identified an endothelial ligand for the pnHR as an ~50-kD sulfated, fucosylated, and sialylated glycoprotein, which we designate Sgp^{50} (sulfated glycoprotein of 50 kD). Recombinant receptor binding to this lymph node–specific glycoprotein requires calcium and is inhibitable by specific carbohydrates and by MEL-14 mAb. Sialylation of the component is required for binding. Additionally, the glycoprotein is precipitated by MECA-79, an adhesion-blocking mAb reactive with lymph node HEV. A related glycoprotein of ~90 kD (designated as Sgp^{90}) is also identified.

THE trafficking of lymphocytes from the blood into secondary lymphoid organs, such as lymph nodes (LN)[1] and gut-associated Peyer's patches (PP), is initiated by an adhesive interaction with the specialized endothelial cells of high endothelial venules (HEV) (Berg et al., 1989; Duijvestijn and Hamann, 1989; Woodruff et al., 1987; Yednock and Rosen, 1989; Stoolman, 1989). Considerable evidence indicates that the lymphoid organ-selective migration or "homing" of lymphocytes is dictated in large part by organ-specific binding of lymphocytes to HEV (Butcher, 1986). Operationally, the lymphocyte-associated molecules underlying the organ-selective interaction with HEV are termed "homing receptors" while the cognate endothelial molecules are known as "HEV ligands" (Gallatin et al., 1986; Rosen, 1989). These endothelial ligands are postulated to be distinctive for the different lymphoid organs and as such are proposed to be responsible for regulating the lymphocyte populations that enter each class of lymphoid organ (Butcher, 1990). Strong support for organ-specific HEV determinants has come with the discovery of the mouse "vascular addressin" antigens, defined by the panel of MECA mAbs (Streeter et al., 1988*a*, *b*). MECA-79 differentially stains and selectively blocks the adhesive function of HEV in peripheral lymph nodes (PN) while MECA-367 shows the converse pattern of reactivity with HEV of Peyer's patches (PP).

Of the homing receptors, the most thoroughly studied is the peripheral lymph node homing receptor (pnHR), initially defined in the mouse by the mAb MEL-14 (Gallatin et al., 1983). This antibody selectively recognizes a cell surface protein of ~90 kD ($gp90^{MEL}$), blocks lymphocyte attachment to PN HEV in the Stamper-Woodruff in vitro adherence assay and prevents in vivo migration to lymph nodes. A homing function for $gp90^{MEL}$ was definitively shown by the finding that the receptor, as a detergent-solubilized molecule or as a soluble recombinant protein, can selectively block adhesive sites for lymphocytes on PN HEV but not those on PP HEV (Geoffroy and Rosen, 1989; Watson et al., 1990).

A lectin activity for $gp90^{MEL}$ and its homologs in other species was initially inferred based upon the ability of specific phosphorylated monosaccharides, such as mannose-6-phosphate (M6P), and specific polysaccharides to prevent lymphocyte attachment to HEV (Stoolman and Rosen, 1983; Stoolman et al., 1984; Yednock et al., 1987*a*; Stoolman et al., 1987; Stoolman and Ebling, 1989). Notable among the active polysaccharides are PPME (a M6P-rich mannan core) and fucoidin (a sulfated, fucose-rich polymer). Subsequently, it was shown that $gp90^{MEL}$ either on the lymphocyte surface (Yednock et al., 1987*a*, *b*), as a purified molecule (Imai et al., 1990), or as a recombinant molecule (Watson et al., 1990) can directly bind to PPME. In all cases, MEL-14 completely inhibits the interaction. Importantly, this carbohydrate-binding activity depends on the presence of calcium, thus mimicking the calcium requirement for the attachment of lymphocytes to HEV. The lectin nature of $gp90^{MEL}$ was confirmed by molecular cloning of the receptor (Lasky et al., 1989; Siegelman et al., 1989) which revealed a transmembrane protein with a calcium-type (C-type), lectin-like domain at its extracellular amino terminus, homologous to domains found in a series of

1. *Abbreviations used in this paper*: C-type, calcium type; HEV, high endothelial venule; LN, lymph nodes; M6P, mannose-6-phosphate; MLN, mesenteric lymph node; PN, peripheral lymph node; pnHR, peripheral lymph node homing receptor; PP, Peyer's patches.

calcium-dependent animal lectins (Drickamer, 1988). Following the lectin motif are an EGF motif, two complement regulatory motifs related to those found in proteins with complement-binding activity, a transmembrane domain, and a short cytosolic tail. That the PPME interaction with gp90^{MEL} is attributable to the lectin domain is confirmed by the mapping of the MEL-14 epitope to this domain (Bowen et al., 1990). The same basic organization of motifs found in gp90^{MEL} has been discerned for two other adhesion molecules, ELAM-1 and GMP-140/PADGEM (Bevilacqua et al., 1989; Johnston et al., 1989), as well as the human homolog of gp90^{MEL} (Bowen et al., 1989; Tedder et al., 1989; Camerini et al., 1989; Siegelman and Weissman, 1989; Kishimoto et al., 1990). On the basis of sequence homology, domain organization, and function, the pnHR, GMP-140, and ELAM-1 constitute a new gene family of cell–cell adhesion proteins, designated as LEC–CAMs (Stoolman, 1989) or selectins (Geng et al., 1990; Springer, 1990).

From the lectin nature of the pnHR, the cognate HEV-ligand is presumed to bear a carbohydrate-based recognition determinant. Early studies demonstrated that the adhesive sites on PN HEV are periodate sensitive (Rosen et al., 1985), indicating a requirement for carbohydrate. Subsequently, it was demonstrated that sialidase treatment of HEV, in vitro or in vivo, selectively eliminates lymphocyte attachment to PN HEV but has no effect on the binding to PP HEV (Rosen et al., 1985, 1989). In addition, exposure of PN tissue sections to Limax flavus agglutinin, a sialic acid–specific lectin, prevents lymphocyte attachment to HEV (True et al., 1990). These early observations have recently been confirmed and extended with the use of a recombinant chimeric receptor, consisting of the extracellular domains of gp90^{MEL} joined to the Fc region of human IgG_1 and designated as LEC–IgG to indicate the presence of the (L)ectin, (E)GF, and (C)omplement-regulatory motifs (Watson et al., 1990). This reagent, when used as the basis of a histochemical stain, reacts selectively with PN HEV. Treatment of PN tissue sections with either sialidase or Limax agglutinin eliminates staining of PN HEV by LEC-IgG (True et al., 1990).

While these previous studies provide direct evidence for the existence of a specific HEV-ligand for the pnHR and indicate a functional requirement for sialic acid, the biochemical nature of this ligand has heretofore not been defined. Based upon adhesion-blocking activity and selective staining of PN HEV by MECA-79 mAb, a candidate for the ligand exists among the complex of proteins recognized by this antibody (Butcher, 1990). In the present study, we use LEC-IgG to identify a ligand for the pnHR as a sulfated, fucosylated and sialylated glycoprotein of 50 kD. A glycoprotein of 90 kD with similar characteristics is also identified.

Materials and Methods

Reagents

Fucoidin, keratan sulfate (bovine cornea), chondroitin sulfate A (bovine trachea), chondroitin sulfate B (porcine skin), α_1-acid glycoprotein (human serum), trypsin (TPCK-treated), soybean trypsin inhibitor, PMSF, and aprotinin were purchased from Sigma Chemical Co. (St. Louis, MO); Triton X-100, CHAPS, and pepstatin were from Boehringer Mannheim Biochemicals (Indianapolis, IN). PPME was purified from Hansenula holstii core mannan, kindly provided by Dr. M. E. Slodki (Department of Agriculture, Northern Regional Research Center, Peroria, IL) and mnn 2 mannan was a gift from Dr. C. E. Ballou (Department of Biochemistry, University of California, Berkeley, CA). MEL-14 mAb (rat IgG_{2a}) was purified on a column of recombinant Protein G-Sepharose 4B (Zymed Laboratories Inc., South San Francisco, CA) and purified anti-CD45 mAb (M1/9.3.4. HL. 2, rat IgG_{2a}) was a gift of Dr. J. Curtis (VA Medical Center, San Francisco, CA). MECA-79 mAb (rat IgM), OZ-42 mAb (rat IgM), and anti-rat Ig-agarose were gifts of Drs. E. Berg and E. Butcher (Department of Pathology, Stanford University, Stanford, CA). The mouse peripheral lymph node homing receptor–human IgG_1 chimera (LEL-IgG) and CD4-human IgG_1 chimera (CD4-IgG) were prepared as previously described (Watson et al., 1990; Capon et al., 1989).

Metabolic Labeling of Organs with ^{35}S-sulfate or ^{3}H-sugars

Mesenteric or peripheral (cervical, brachial, and axillary) lymph nodes were collected from 8–16-wk-old female ICR mice. The LN were cut into 1-mm-thick slices with a razor blade and the slices (typically 0.2 g of wet weight) were suspended in 1 ml of RPMI-1640 containing 25 mM Hepes, 100 U/ml Penicillin G, 100 μg/ml streptomycin, and 200 μCi carrier-free [^{35}S] sodium sulfate (ICN Radiochemicals, Irvine, CA) according to the procedure of Ager (1987). For metabolic labeling with sugars, mesenteric and peripheral LN slices (0.07 g of wet weight) were labeled in 1 ml of medium containing 100 μCi of either [5,6-^{3}H]-L-fucose,[2-^{3}H]-D-mannose, or [6-^{3}H]-glucosamine hydrochloride (New England Nuclear, Boston, MA). The above medium was also used for fucose labeling. For glucosamine and mannose labeling, glucose-free RPMI-1640 with 1 mM sodium pyruvate was used. After incubation at 37°C for 4 h, the slices were washed extensively in Dulbecco's PBS, and then homogenized in 1 ml of lysis buffer (2% Triton X-100 in PBS containing 1 mM PMSF, 1% [vol/vol] aprotinin, 10 μg/ml pepstatin, 0.02% NaN_3) with a Potter-Elvehjem homogenizer on ice. The other tissues (Peyer's patches, spleen, thymus, liver, kidney, and brain) were labeled and homogenized by the same procedure with the volumes of labeling medium and lysis buffer based on the wet weight of the tissue. Lysis was continued for 1 h on a rocker at 4°C. The lysate was centrifuged at 10,000 *g* for 1 h at 4°C. EDTA was added to the supernatant at a final concentration of 2 mM and the supernatant was precleared by rocking with Affi-Gel Protein A (250 μl of packed beads, Bio-Rad Laboratories, Richmond, CA) overnight at 4°C.

Identification of the Components Adsorbed to LEC-IgG Beads

Affi-Gel Protein A (10 μl packed beads) was incubated with 30 μg of either LEC-IgG, CD4-IgG, or human IgG_1 (Calbiochem-Behring Corp., San Diego, CA) in 1 ml of PBS rocking overnight at 4°C. The beads (referred to as LEC-IgG beads, CD4-IgG beads, and huIgG-beads) were washed 3× in PBS and once with lysis buffer. The precleared lysate was centrifuged at 10,000 *g* for 10 s, $CaCl_2$ was added to the supernatant at a final concentration of 5 mM, and the supernatant was mixed immediately with either LEC-IgG beads, CD4-IgG beads, or huIgG-beads (typically 200 μl of precleared lysate per 10 μl packed beads), and incubated for 4 h at 4°C on a rocker. The beads were washed 6× with lysis buffer, transferred to a new tube, and then washed once more with lysis buffer. The materials bound to the beads were solubilized by boiling in SDS in the presence of 2-mercaptoethanol, electrophoresed on SDS–polyacrylamide gels (9 or 10%) and subjected to fluorography with ENTENSIFY or EN^{3}HANCE (New England Nuclear). By fluorography, the 50-kD component tended to be more diffuse with ENTENSIFY than EN^{3}HANCE. In the reprecipitation experiment, the SDS-solubilized sample was electrophoresed on a 7.5% SDS-gel with prestained standards (BioRad; high range) as markers. The region ~50 kD on the gel was excised by using prestained ovalbumin (49.5 kD) as a position marker, and the protein electroeluted (model 422; BioRad) into Laemmli running buffer at 60 mA overnight. The eluate was concentrated and the buffer was exchanged into 10 mM CHAPS in PBS on a Centricon 30 unit (Amicon Corp., Danvers, MA), followed by incubation with LEC-IgG beads or CD4-IgG beads as described above. For the analysis of crude lysate, 200 μl of the precleared lysate was precipitated with cold acetone (80% vol/vol) and then subjected to electrophoresis as above.

Effects of MEL-14 mAb and Polysaccharides

For testing the effect of antibodies, LEC-IgG beads (10 μl packed beads), prepared as described above, were incubated with rat serum (0.5 ml, 1:5 dilution in PBS) for 40 min at 4°C. The beads were washed with lysis buffer

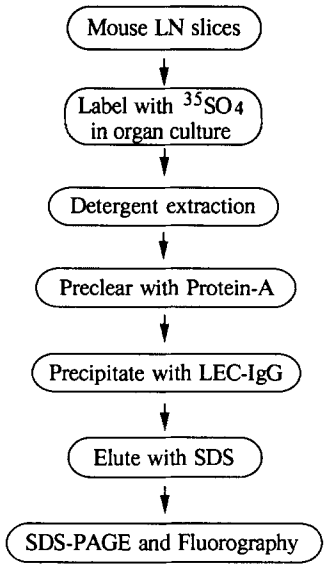

Figure 1. Protocol for identification of 50-kD component.

and resuspended in 0.5 ml of the same buffer. 100 μg of rat mAb (MEL-14 mAb or anti-CD45 mAb) was added to the beads and incubated for 30 min at 4°C. The calcium-restored precleared lysate was then added to the antibody-treated LEC-IgG beads, and the beads were processed as described above. For testing the effect of polysaccharides, LEC-IgG beads (10 μl packed) were preincubated with polysaccharides in lysis buffer for 30 min at 4°C before adding the calcium-restored precleared lysate.

Effect of Sialidase

Material bound to the LEC-IgG beads (50 μl packed) was eluted by treatment with 300 μl of elution buffer (3 mM EDTA, 0.2% Triton X-100 in PBS containing 1 mM PMSF, 1% [vol/vol] aprotinin, 10 μg/ml pepstatin) overnight at 4°C. The beads were removed by centrifugation and the pH of the eluate was adjusted to pH 5 with acetic acid. To 100 μl aliquots of the eluate, 0.1 U of Arthrobacter ureafaciens sialidase (Calbiochem-Behring Corp.) in 10 μl of 0.5 M acetate buffer, pH 5.0, or 10 μl of the acetate buffer alone was added. After incubation for 2 h at 37°C, 45 μl aliquots were removed for SDS-PAGE analysis. To the remainder (65 μl), $CaCl_2$ was added to a final concentration of 6 mM. The calcium-restored eluate was diluted in the lysis buffer (1 ml), incubated with LEC-IgG beads (10 μl packed), and processed as described above.

Effect of Trypsin

The eluate from LEC-IgG beads was prepared as above except that protease inhibitors were not included in the elution buffer. The pH of the eluate was adjusted to pH 8.0 with Tris (Bio-Rad Laboratories, Richmond, CA). The eluate was treated with trypsin (100 μg/ml) in the presence or absence of soybean trypsin inhibitor (200 μg/ml) for 4 h at 37°C and then analyzed by SDS-PAGE.

Immunoprecipitation by MECA-79 Antibody

Anti-rat Ig-agarose (20 μl packed beads) was armed with either MECA-79 mAb or class-matched control OZ-42 mAb (13 μg protein) by overnight incubation at 4°C. The beads were washed three times with PBS and then once with 0.2% Triton X-100 in PBS. 60 μl of the EDTA eluate from LEC-IgG beads (containing protease inhibitors), after calcium restoration (5 mM), was incubated either with MECA-79 beads or with OZ-42 beads for 2 h at 4°C. 45 μl of the supernatant and the material bound to the beads were subjected to SDS-PAGE analysis.

N-glycanase Treatment

The eluate from LEC-IgG beads was prepared as above except that 10 mM CHAPS was used in place of Triton X-100, followed by concentration and exchange of buffer into 10 mM CHAPS, 0.15 M NaCl (containing 1 mM PMSF, 1% [vol/vol] aprotinin) on a Centricon 30 unit. For N-glycanase treatment, 20 μl aliquots of the concentrated sample or α_1-acid glycoprotein (24 μg) were boiled for 5 min in 0.5% SDS with 0.1 M 2-mercaptoethanol. The denatured samples were treated with or without 16 Genzyme U/ml of recombinant N-glycanase (Genzyme Corp., Boston, MA) in the presence of 0.2 M sodium phosphate, pH 8.4, 10 mM 1,10-phenanthroline (Sigma Chemical Co.), 1.25% Triton X-100, 0.17% SDS, 0.03 M 2-mercaptoethanol in a volume of 60 μl for 16 h at 37°C.

Results

Mouse PN Homing Receptor-IgG Chimera (LEC-IgG) Precipitates a 50-kD Component from [^{35}S]Sulfate-labeled Lymph Nodes

Strong sulfate incorporation into macromolecules is a distinctive characteristic of lymph node high endothelial cells in situ and in cell culture (Ager, 1987; Andrews et al., 1982; Ise et al., 1988). Furthermore, among the most active substances in competing the lectin activity of gp90^{MEL} or LEC-IgG are fucoidin, sea urchin egg jelly fucan, and sulfatide, all of which are sulfated macromolecules (Imai et al., 1990; Watson et al., 1990). In light of this information, we examined whether LEC-IgG immobilized on beads could bind $^{35}SO_4$-labeled macromolecules from lymph nodes, which had been metabolically labeled in organ culture in the presence of [^{35}S]sodium sulfate (Fig. 1). Fig 2 *a* shows that LEC-IgG beads precipitated a diffuse 50-kD component (range is 40–58 kD) from [^{35}S]sulfate-labeled mesenteric lymph nodes (MLN) or PN. A band of ~90 kD (83–102 kD), relatively minor in terms of sulfate incorporation, was also observed in most analyses. In control precipitations, human IgG_1-conjugated beads did not recognize the 50-kD major component or the 90-kD component in PN or MLN lysates. When crude lysates of MLN and PN were directly analyzed (Fig. 2 *a*), the 50-kD component represented the major constituent among several other bands. The tissue distribution of the 50-kD component was further examined by applying the identical protocol for [^{35}S]sulfate labeling and precipitation with LEC-IgG to a number of organs. Among lymphoid tissues, only PN and MLN showed the 50- and 90-kD bands, while PP, spleen, and thymus were negative for both (Fig. 2 *b*). Nonlymphoid organs such as kidney, liver, cerebrum, and cerebellum were also completely negative (Fig. 2, *b* and *c*).

Characteristics of the Interaction between LEC-IgG and the 50-kD Component

The interaction between pnHR and its HEV ligand is calcium dependent, since the specific staining of PN HEV by LEC-IgG is eliminated by EGTA (Watson et al., 1990). Moreover, a calcium requirement has been shown in an ELISA assay that measures the interaction between pnHR and PPME, a carbohydrate-based ligand analog (Imai et al., 1990). In accordance with these results, LEC-IgG beads precipitated the 50-kD component when calcium was present, but not in its absence (Fig. 3 *a*). The specificity of the interaction was further examined with the use of MEL-14 mAb, which is directed to the lectin domain of pnHR (Bowen et al., 1990). As shown in Fig. 3 *b*, preincubation of LEC-IgG beads with this antibody completely blocked the binding of the 50-kD band to the beads, whereas a class-matched control antibody (anti-CD45) had no effect. Previously, the histochemical staining of PN HEV by LEC-IgG was shown to be inhibited

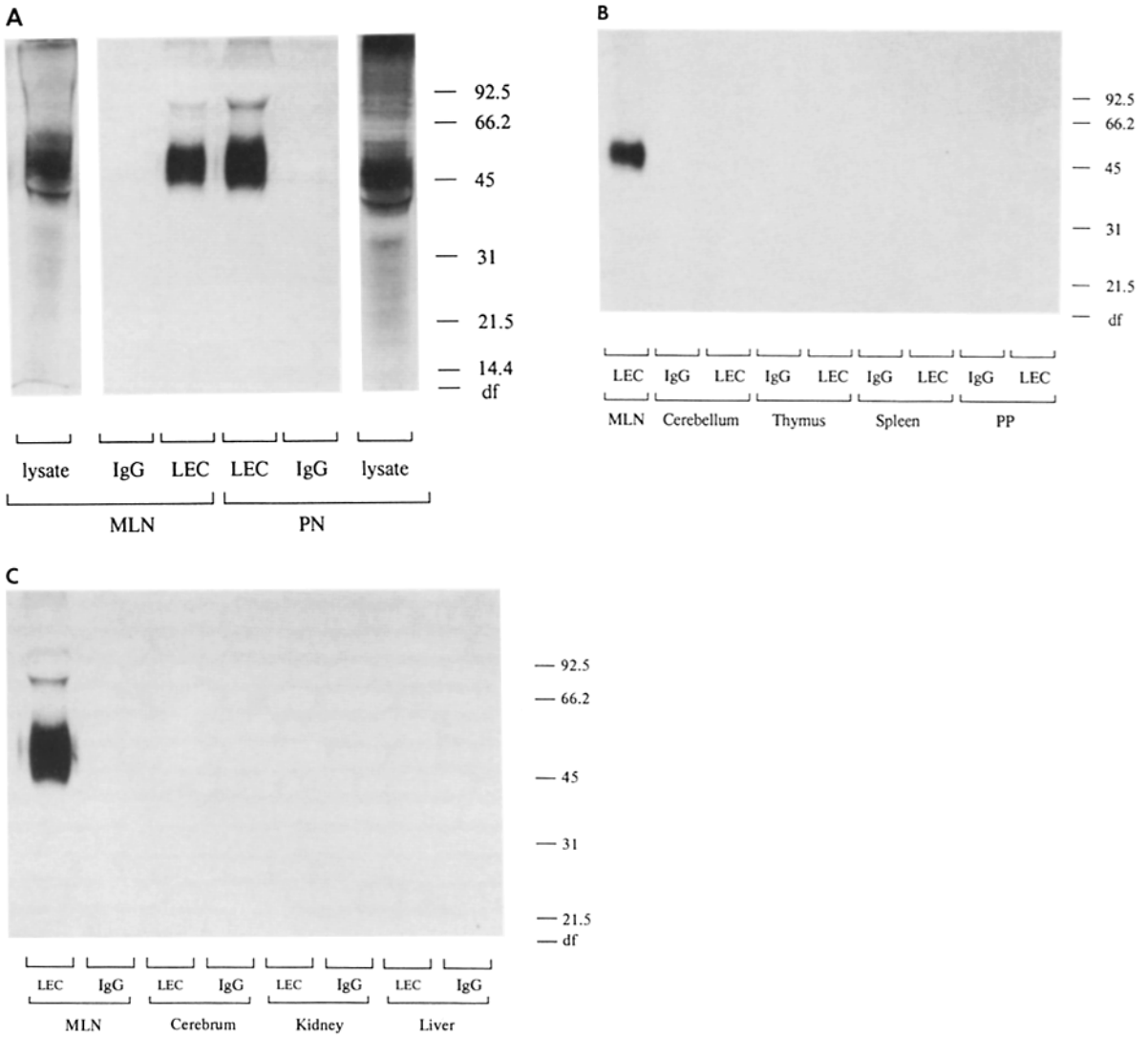

Figure 2. Tissue distribution of sulfated components recognized by LEC-IgG. Tissue slices from various organs were incubated with [^{35}S]sulfate and detergent lysates were prepared and precleared with Affi-Gel Protein A in the presence of EDTA. After calcium was restored, the lysates were precipitated with Affi-Gel Protein A coated with human IgG_1 (*IgG*), or LEC-IgG. (*a*) Peripheral lymph nodes (*PN*) and Mesenteric lymph nodes (*MLN*). The labeling and extraction conditions were as follows: 0.12 g of tissue slices was labeled with 200 μCi of [^{35}S]sulfate and extracted with 1 ml lysis buffer; 200 μl lysate was used for precipitation; for analysis of crude lysates, 200 μl of each lysate was precipitated with 80% cold acetone before electrophoresis. (*b*) MLN, cerebellum, thymus, spleen, PP. 0.2 g of tissue slices was labeled with 200 μCi of [^{35}S]sulfate, extracted with 1 ml lysis buffer and processed as above except 400 μl was used for precipitation. (*c*) MLN, cerebrum, kidney, and liver. 0.15 g of tissue was labeled and extracted as above with 450 μl used for precipitation. The precipitates were solubilized with SDS under reducing conditions and analyzed by SDS-PAGE on a 10% gel (*a*) or on 9% gels (*b* and *c*) and subjected to fluorography using EN^{3}HANCE (*a* and *c*) or ENTENSIFY (*b*). The molecular weight markers (Bio-Rad Laboratories) are shown on the right: phosphorylase B (92.5 kD), BSA (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), lysozyme (14.4 kD). (*df*) The position of the dye front. In *a* the higher intensity of the 50-kD component in PN as compared to that in MLN may reflect the greater abundance of the molecule in PN. This result would be predicted for the biological ligand for the pnHR, since PN HEV exhibits a single-adhesive specificity, whereas MLN HEV appear to represent a hybrid specificity of both PN and PP (Butcher et al., 1980; Rosen et al., 1985; Streeter et al., 1988*a*).

by fucoidin (Watson et al., 1990), consistent with the ability of this polysaccharide to block lymphocyte attachment to PN HEV (Stoolman and Rosen, 1983; Yednock et al., 1987*a*) as well as to compete for PPME binding to the pnHR in the ELISA assay. As shown in Fig. 4, fucoidin completely blocked the precipitation of the 50-kD component by LEC-IgG beads, while control polysaccharides (chondroitin sulfate B, chondroitin sulfate A, keratan sulfate) were completely inactive. Furthermore, the presence of PPME significantly reduced the intensity of the 50-kD band (Fig. 4), although a relatively high concentration was required. A control yeast mannan (mnn 2) had no effect at the same concentration. The precipitation of the minor 90-kD band by LEC-IgG beads was also calcium dependent (Fig. 3 *a*), inhibitable by MEL-14 mAb (Fig. 3 *b*), and blocked by fucoidin and PPME (Fig. 4).

Effect of Sialidase Treatment

Our previous experiments have shown that sialic acid on the

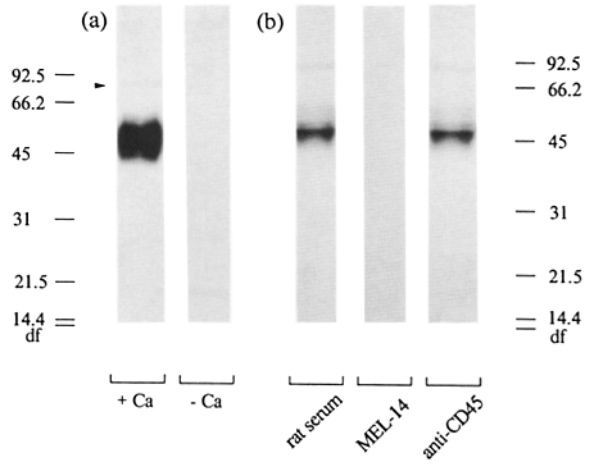

Figure 3. Requirement for calcium and the MEL-14 epitope in the interaction between LEC-IgG and the 50-kD component. (*a*) [^{35}S]sulfate-labeled lysate from MLN was precleared and then precipitated with LEC-IgG in the presence (*+Ca*) or absence (*−Ca*) of calcium. (*b*) Precleared lysate was precipitated with LEC-IgG that had been treated with rat serum alone (*rat serum*), rat serum and MEL-14 mAb (*MEL-14*), or rat serum and anti-CD 45 mAb (*anti-CD45*). The precipitates were analyzed by SDS-PAGE (10% gel) and fluorography. The 50-kD band in *b* is compacted as a result of unlabeled immunoglobulin heavy chain, released from the beads, which migrates just above the sulfate-labeled component. In *a* (*+Ca*), the faint 90-kD band is indicated by an arrowhead.

HEV-ligand itself or on closely associated molecules is critical for recognition by the pnHR. We, therefore, sought to determine if the 50-kD component was sialylated and if so, whether sialic acid was necessary for its binding to LEC-IgG. After EDTA elution of the 50-kD band from LEC-IgG, it was treated with Arthrobacter ureafaciens sialidase or control buffer. Sialidase treatment caused a marked increase in electrophoretic mobility, while the buffer-treated material was unaffected (Fig. 5). Upon reprecipitation with fresh LEC-IgG beads, the control-treated material was adsorbed, whereas the sialidase-treated material was no longer recognized by LEC-IgG (Fig. 5). A similar dependence on sialic acid was observed for the 90-kD component (Fig. 5).

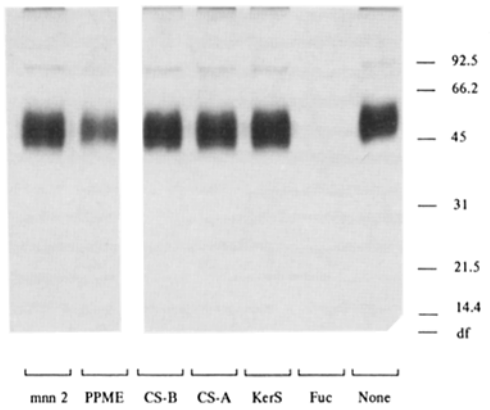

Figure 4. Effect of polysaccharides on the interaction between LEC-IgG chimera and the 50-kD component. Precleared lysate prepared from a mixture of [^{35}S]sulfate-labeled MLN and PN was precipitated with LEC-IgG in the presence of polysaccharides. The LEC-IgG beads were preincubated with mnn2 mannan (510 μg), PPME (510 μg), chondroitin sulfate B (*CS-B*; 51 μg), chondroitin sulfate A (*CS-A*; 51 μg), keratan sulfate (*KerS*; 51 μg), fucoidin (*Fuc*; 51 μg), or without polysaccharides (*None*) in 670 μl of the lysis buffer. After 30 min at 4°C, 170 μl of the precleared lysate was added to each of the bead mixtures and allowed to react for 4 h. The bound material was analyzed by SDS-PAGE (10% gel) and fluorography. In this experiment, several distinct bands are evident within the 50-kD region of the gel. This banding pattern may reflect heterogeneity in glycosylation of the molecule.

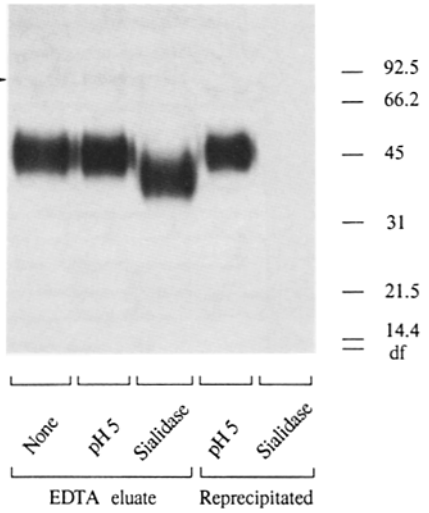

Figure 5. Effect of sialidase on the 50-kD component. [^{35}S]sulfate-labeled lysate was prepared from MLN and was adsorbed to LEC-IgG beads. Bound material was eluted with EDTA. Eluted material (*EDTA eluate*) was analyzed by SDS-PAGE (10% gel) and fluorography after treatment with Arthrobacter ureafaciens sialidase (Sialidase), treatment with buffer alone (*pH 5*), or without any treatment (*None*). Separate aliquots of buffer- and sialidase-treated eluate were reexposed to fresh LEC-IgG (*Reprecipitated*) and the bound material was subjected to analysis by SDS-PAGE and fluorography. The 90-kD band is diffuse in EDTA eluates because there is no compaction in the gel by the LEC-IgG component which runs at a similar position. The diffuse band for the control material (*None*) is indicated by an arrowhead. Sialidase treatment changed the mobility of the 90-kD band and inhibited its reprecipitation by LEC-IgG.

The Protein Nature of the 50-kD Component

To determine whether the 50-kD component was a protein, its sensitivity to trypsin was examined. The component was released from LEC-IgG beads with EDTA and treated with trypsin in the absence or presence of soybean trypsin inhibitor. The molecular weight of the sulfate-labeled band was reduced to ~24 kD after trypsin treatment (Fig. 6). The inclusion of trypsin inhibitor completely blocked this degradation. The 90-kD band was also substantially degraded by the trypsin treatment (Fig. 6).

Precipitation by MECA-79

The MECA-79 mAb recognizes a complex of heterogeneous glycoproteins, for which only a preliminary characterization has been published to date (Butcher, 1990). To determine if the 50-kD component could be precipitated by MECA-79, an EDTA eluate from LEC-IgG, obtained as described above, was exposed to MECA-79–conjugated beads. As shown in

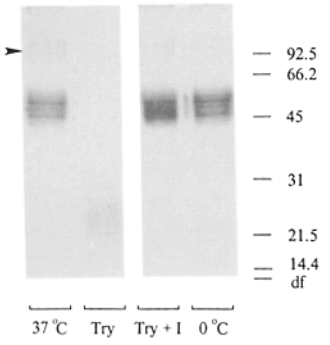

Figure 6. Effect of trypsin on the 50-kD component. Lysate was prepared from a mixture of [^{35}S]sulfate–labeled MLN and PN and was adsorbed to LEC-IgG beads. Bound material was eluted with EDTA in the absence of protease inhibitors. The eluate was analyzed by SDS-PAGE (10% gel) and fluorography after treatment with trypsin (*Try*), treatment with trypsin plus soybean trypsin inhibitor (*Try + I*), exposure to buffer at 37°C (*37°C*), or incubation with buffer on ice (*0°C*). The diffuse 90-kD band is indicated by an arrowhead in the control condition (37°C). Trypsin substantially reduced the intensity of the 90-kD component.

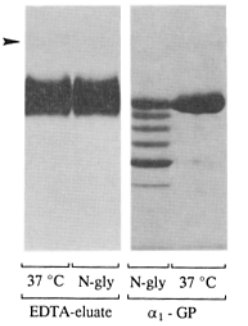

Figure 8. Effects of N-glycanase treatment on the 50-kD component. [^{35}S]sulfate–labeled lysate from a mixture of MLN and PN was adsorbed to LEC-IgG beads with elution of bound material by EDTA. The EDTA eluate was analyzed by SDS-PAGE (10% gel) and fluorography after treatment with N-glycanase (*N-gly*), or exposure to the appropriate control buffer (*37°C*). As a control, α_1-acid glycoprotein (α_1-*GP*) was analyzed after the treatment, and protein was detected by Coomassie brilliant blue G (Sigma Chemical Co.). The faint 90-kD band is indicated by an arrowhead in the control condition (*37°C*).

Fig. 7, the band was adsorbed to the beads and was quantitatively depleted from the supernatant fraction. In contrast, when beads conjugated to a class-matched antibody (mAb OZ-42) were used, the component remained in the supernatant. The 90-kD band was also specifically precipitated by MECA-79 mAb (data not shown).

Nature of Sugar Chains on the 50-kD Component

To characterize further the glycoprotein nature of the 50-kD component, we tested the sensitivity of an EDTA eluate from LEC-IgG beads to N-glycanase treatment. N-linked sugar chains are known to be cleaved by the treatment. As shown in Fig. 8, N-glycanase treatment did not affect either the intensity of the 50-kD band or its electrophoretic mobility, indicating that N-linked sugar chains were not a significant carbohydrate constituent. The minor 90-kD band was also unaffected by the N-glycanase treatment (Fig. 8). In contrast, a control glycoprotein (α_1-acid glycoprotein) yielded a series of deglycosylated products upon N-glycanase treatment performed under the same conditions (Fig. 8).

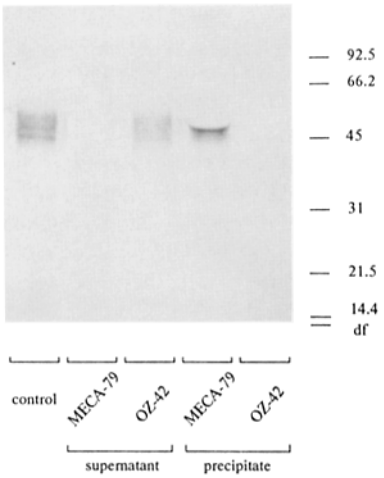

Figure 7. Precipitation of the 50-kD component with MECA-79 mAb. Lysate prepared from a mixture of [^{35}S]sulfate–labeled MLN and PN was adsorbed to LEC-IgG beads with elution of the bound material by EDTA. The eluate was incubated with immobilized MECA-79 mAb (*MECA-79*) or control class-matched mAb (*OZ-42*). The material remaining in the supernatant (*supernatant*) and that bound to the immobilized mAbs (*precipitate*) were analyzed by SDS-PAGE (10% gel) and fluorography. Untreated eluate was also analyzed in parallel (*control*). The band shown in the MECA-79 "precipitate" is compacted as a result of unlabeled immunoglobulin heavy chain, which migrates just about the sulfate-labeled component. MECA-79 mAb also precipitated the minor 90-kD band, which was visible with longer exposure (data not shown).

Metabolic Labeling of Lymph Nodes with Sugars

To characterize its sugar chains further, we determined whether [^{3}H]precursor monosaccharides could be metabolically incorporated into the 50-kD component. Lymph nodes were labeled with [^{3}H]fucose, [^{3}H]glucosamine, [^{3}H]mannose, or [^{35}S]sulfate. The extracts were subjected to two cycles of adsorption to either LEC-IgG beads or CD4-IgG beads with elution by EDTA. As shown in Fig. 9 *a*, LEC-IgG beads bound the 50-kD component from both the fucose- and sulfate-labeled lysates but no bands were precipitated from the glucosamine-labeled or mannose-labeled lysates. CD4-IgG beads failed to react with components in any of the lysates. In another experiment, in which one cycle of adsorption to LEC-IgG beads was used, a fucose-labeled 90-kD component was observed in addition to the 50-kD component (data not shown). As expected, numerous glycoproteins of various molecular weights incorporated fucose and glucosamine when crude lysates (in amounts comparable to those used for precipitation) were directly analyzed (Fig. 9 *b*). Thus, the 50-kD fucose–labeled component that was precipitated by LEC-IgG represented a minor component among many fucosylated glycoproteins.

Reprecipitation of the 50-kD Component after Electroelution from SDS-gel

To establish if the 50-kD band could be recognized directly by LEC-IgG, we carried out the following reprecipitation experiment. LEC-IgG beads that had been incubated with [^{35}S]sulfate–labeled lymph node lysate were boiled with SDS in the presence of 2-mercaptoethanol at 100°C to elute the bound material. The eluted materials were electrophoresed on an SDS-gel, and the region corresponding to 50 kD was excised. The materials were electroeluted from the gel

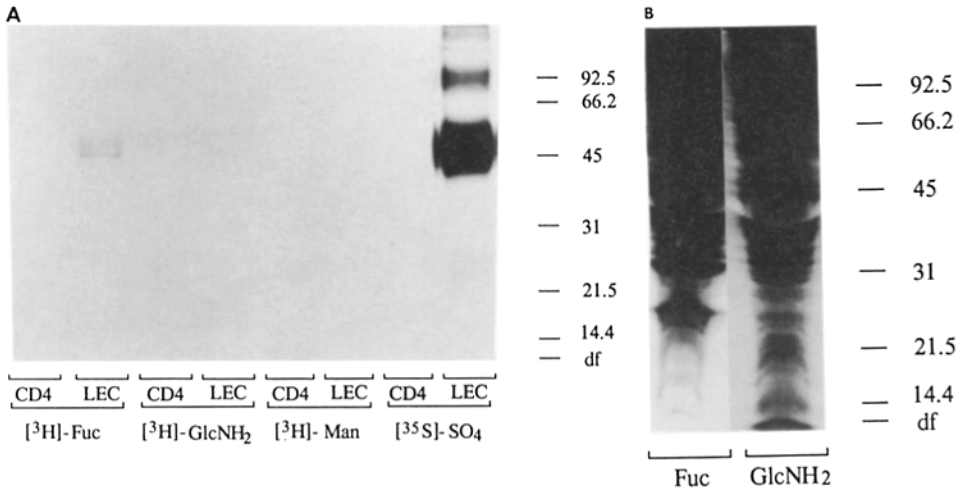

Figure 9. Precursor sugar incorporation into the 50-kD component. MLN and PN were labeled in organ culture with [^{3}H]fucose (*Fuc*), [^{3}H]glucosamine hydrochloride ($GlcNH_2$), [^{3}H]mannose (*Man*), or [^{35}S]sulfate (SO_4). (*a*) Precipitation of labeled lysates with LEC-IgG or CD4-IgG. The labeled lysates were adsorbed to either LEC-IgG beads or CD4-IgG beads (an amount of lysate corresponding to 0.035-g wet tissue weight was used for each lane), and the bound material was eluted with EDTA, followed by reprecipitation with either LEC-IgG beads or CD4-IgG beads after calcium restoration. The bound material was again eluted with EDTA and then analyzed by SDS-PAGE (10% gel) and fluorography. (*b*) Analysis of crude lysates. An amount of each lysate, equivalent to that used in *a*, was precipitated with cold acetone and analyzed by SDS-PAGE and fluorography.

slice and subjected to reprecipitation by LEC-IgG beads or by CD4-IgG beads as a control. As shown in Fig. 10, the 50-kD band was reprecipitated by LEC-IgG beads, whereas CD4-IgG beads showed no reaction.

Discussion

The LEC-IgG chimeric receptor represents a seemingly ideal reagent for the identification of the pnHR HEV-ligand, since it was engineered to contain all of the extracellular domains of gp90^{MEL}, the bonafide pnHR in mouse. Extensive characterization has established that the recombinant molecule does, indeed, reflect all of the known recognition properties of the native receptor: (*a*) it binds to PN HEV in an organ-specific manner; (*b*) its interaction with HEV is blocked by the same carbohydrates that inhibit lymphocyte attachment to PN HEV; (*c*) its binding to HEV is both calcium dependent and MEL-14 mAb inhibitable as observed for the binding of lymphocytes to HEV; and (*d*) it retains the calcium-dependent, carbohydrate-binding activity of gp90^{MEL}, as measured in a quantitative ELISA assay. Using this highly validated reagent as the basis of an affinity matrix, we have precipitated a 50-kD component from [^{35}S]sulfate-labeled lymph nodes. We designate the component as Sgp^{50} (sulfated glycoprotein of 50 kD). The precipitation of Sgp^{50} depends on an interaction with the C-type lectin domain of LEC-IgG as deduced from MEL-14 inhibition, the requirement for calcium, and competitive inhibition by the specific polysaccharides fucoidin and PPME. It is inferred that Sgp^{50} is associated with HEV based on the known propensity of SO_4 to incorporate into HEV-associated macromolecules (Andrews et al., 1982) and the selective staining of HEV by LEC-IgG (Watson et al., 1990). Furthermore, as predicted for an organ-specific HEV ligand, the component was not detected in other organs, including several lymphoid organs. In further support of its ligand function, the binding of Sgp^{50} to LEC-IgG was critically dependent upon its sialylation. Thus, the functional requirement for sialic acid, originally deduced from cell-binding assays (Rosen et al., 1985) and supported by histochemical staining experiments with LEC-IgG (True et al., 1990) is now confirmed at the level of an identified HEV-associated molecule. Finally, Sgp^{50} draws important support as an adhesive ligand by virtue of its precipitation with MECA-79, an adhesion-blocking mAb reactive with PN HEV (Streeter et al., 1988*b*). Heretofore, the functional target of this antibody has not been clear, since it precipitates a heterologous set of proteins (Butcher, 1990).

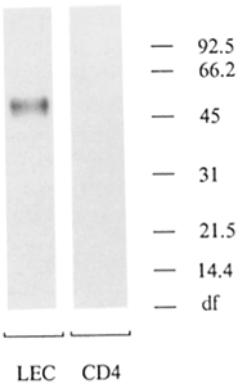

Figure 10. Reprecipitation of the 50-kD component after its electroelution from an SDS gel. [^{35}S]sulfate-labeled lysate from a mixture of MLN and PN was adsorbed to LEC-IgG beads. Bound material was boiled in Laemmli sample buffer (containing 2% SDS and 0.7 M 2-mercaptoethanol) and electrophoresed on a 7.5% SDS-gel. The 50-kD region was excised and electroeluted material was reexposed to fresh LEC-IgG (*LEC*) or CD4-IgG (*CD4*). The bound material was boiled in Laemmli sample buffer and analyzed by SDS-PAGE (10% gel) and fluorography.

That the precipitation of Sgp^{50} with LEC-IgG was because of the contribution of LEC to the chimera is clearly indicated by the inhibition results obtained with MEL-14, specific carbohydrates, and calcium depletion. As further evidence for the specificity of this interaction, we showed that Sgp^{50} was not precipitated by either human IgG or the CD4-IgG chimera. The latter control established directly that the precipitation of Sgp^{50} was not attributable to an unusual characteristic intrinsic to cell-adhesion molecule–immunoglobulin chimeras (Capon et al., 1989). With respect to the selectivity of the interaction between LEC-IgG and Sgp^{50}, we found that the chimera precipitated Sgp^{50} not only from [^{35}S]sulfate-labeled lymph node lysates but also from a [^{3}H]fucose-labeled lysate, but did not precipitate any detectable bands from either [^{3}H]glucosamine- or [^{3}H]-mannose-labeled lysates. In the case of sulfate labeling, the 50-kD band was a major component among a limited number of bands, and thus one might argue that precipitation of

the 50-kD band simply reflected the prevalence of the component in the lysate. However, as shown for fucose and glucosamine and as presumed for mannose, there are numerous labeled components. Yet, only the 50- and 90-kD bands, which represented minor components of the [^{3}H]fucose–labeled lysate were precipitated, while there were no detectable bands precipitated from either the [^{3}H]glucosamine– or [^{3}H]mannose–labeled lysate. Despite the fact that glucosamine is a precursor of sialic acid, the 50-kD band was not significantly labeled with glucosamine. This result may be explained by a dilution effect, since glucosamine is incorporated into several monosaccharides (Cummings et al., 1989).

It might be argued that proteins that are not sulfated or fucosylated could tightly associate with Sgp^{50}, and its precipitation could result from an interaction between LEC-IgG and one of these associated proteins. However, when the denatured Sgp^{50} was electroeluted from an SDS-gel and the eluted material reexposed to LEC-IgG, the reprecipitated material again showed the 50-kD band by SDS-PAGE analysis. The fact that CD4-IgG did not reprecipitate the 50-kD band after electroelution again assured the specificity of interaction. Thus, the possibility of co-precipitation of Sgp^{50} is essentially ruled out. This result also shows that Sgp^{50} precipitates independently of the 90-kD component. A direct interaction between LEC-IgG and Sgp^{50} is clearly indicated.

Taken together, the evidence argues strongly that Sgp^{50} is an HEV ligand for the pnHR. At this point, although limited information is available about its structure, we can conclude that the ligand is a sulfated, fucosylated, and sialylated glycoprotein with trypsin-sensitive core protein. The nature of sugar chains of Sgp^{50} requires further study. The present study suggests that N-linked sugar chains are not a major constituent, since N-glycanase treatment did not affect either the mobility or the intensity of the [^{35}S]sulfate–labeled Sgp^{50}. It remains a possibility that the presence of sulfate on an N-linked sugar chain could potentially interfere with the action of N-glycanase. However, examples are known of sulfated N-linked sugar chains that are susceptible to N-glycanase (Roux et al., 1988; Green and Baenziger, 1988). When the sulfate- or fucose-labeled Sgp^{50} was subjected to alkaline borohydride, a treatment designed to release O-linked chains, labeled oligosaccharide chains were detected by gel filtration analysis (unpublished observations). These results suggest the presence of sulfated, fucosylated, O-linked sugar chains on Sgp^{50}.

The LEC-IgG chimera also precipitated a 90-kD sulfated, fucosylated glycoprotein from MLN and PN lysates. We designate this molecule as Sgp^{90}. As is the case for the interaction with Sgp^{50}, the precipitation of Sgp^{90} was calcium dependent, inhibitable by MEL-14 mAb, inhibitable by specific polysaccharides, and dependent on the presence of sialic acid. In addition, Sgp^{90} was also precipitated by MECA-79. This component may be identical to the prominent 90-kD component recognized by MECA-79 among several other proteins in a Western blot analysis of lymph node (Butcher, 1990). To understand the relationship between Sgp^{90} and Sgp^{50}, it will be necessary to compare their protein and carbohydrate structures, kinetics of synthesis, steady state concentrations, and subcellular localizations.

The exact role of sialic acid in the adhesive function of Sgp^{50} (and Sgp^{90}) is open to speculation. It is likely that a sialyloligosaccharide of Sgp^{50} comprises a recognition determinant for the lectin domain of the pnHR. Specificity could derive from the uniqueness of the sialyloligosaccharide, which is plausible in view of the enormous potential for diversity in sialylated structures (Schauer, 1985). One speculation, for example, is that the novel structure is a heretofore undescribed sulfated and fucosylated sialyloligosaccharide, for which M6P and fucoidin (a sulfated, fucose-rich polysaccharide) are structural mimics. A second contribution to specificity might be based on a valency effect in which a sialyloligosaccharide ligand is repeated several times with precise spacing on a polypeptide backbone, thus allowing for multipoint attachment between pnHR molecules and each Sgp^{50} molecule. The exact role of sialic acid in the function of Sgp^{50} awaits a detailed analysis of its carbohydrate chains and a definition of the minimal oligosaccharide unit for binding to the pnHR. Interestingly, all of the LEC-CAMs (pnHR, ELAM-1, and GMP-140) exhibit an unusually high concentration of lysine residues within their lectin domains (Bevilacqua et al., 1989; Bowen et al., 1989; Johnston et al., 1989; Lasky et al., 1989) which had suggested (Rosen, 1989) a common theme of anionic sugar (e.g., sialic acid) recognition for this family. In fact, it is now known that the ligands for GMP-140 (Corral et al., 1990) and ELAM-1 (Phillips et al., 1990; Tiemeyer et al., 1990), also require sialic acid. Detailed structures have even been proposed for the ELAM-1 ligand (Lowe et al., 1990; Tiemeyer et al., 1990; Phillips et al., 1990; Walz et al., 1990).

Considerable attention will also be directed at the protein component of Sgp^{50}. One possibility is that it serves as a backbone or scaffolding for the presentation of carbohydrate recognition units. As mentioned above, such a function need not be trivial, as the spacing and number of carbohydrate units could be critical for optimal multivalent presentation. Another possibility is that the protein component of Sgp^{50} participates directly in recognition by the pnHR. The ability of Sgp^{50} to be recognized by LEC-IgG after extensive denaturation (boiling and reduction in SDS) supports the notion that carbohydrate binding is sufficient for a productive interaction, which also seems to be the case for ELAM-1 (Lowe et al., 1990; Tiemeyer et al., 1990). A direct recognition role for the protein component of Sgp^{50} is, however, not ruled out. In fact, recent evidence suggests that the EGF motif of the pnHR may have a direct role in HEV binding, independent of the lectin activity (Siegelman et al., 1990). An appropriate analogy for the LEC-CAMs may be the pituitary N-acetylgalactosamine transferase, which appears to have one binding site for an oligosaccharide chain and a separate site for a peptide determinant (Smith and Baenziger, 1988). It will be of great interest to determine whether the primary structure of the Sgp^{50} protein reveals a potential recognition domain for EGF, such as an EGF-receptor motif or another EGF motif. Also of interest will be the structural comparison of Sgp^{50} with the other LEC-CAM ligands. The intriguing possibility is that these ligands, like their cognate receptors, may also comprise a new gene family.

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