the amplitude of the population spike, which attained average increases of 259% and 237% in freely moving and in anesthetized rats, respectively. The population spike height in stimulated controls was unchanged.

14. After in situ hybridization, quantification of the amount of GR33 mRNA in the dentate gyrus of rat brain sections was performed with the BIOCOM computer program (System Photometrique d'Analyse des Autoradiogrammes RAG 200, France). The densities of silver grains in the test areas were calculated separately for the dentate gyrus (d_{dg}) and the CA1 region (d_{CA1}) such that

$$d = \log[ng_c/ng_t] \times A_t$$

where ng_c is the number of grains in the control area, ng_t is the number of grains in the area of the test, and A_t is the test area. The relative amount of GR33 mRNA (d_{dg}/d_{CA1}) on the potentiated side of the hippocampus was compared with that on the contralateral side or that from comparable sections from animals that received unilateral control stimulation.

15. Two additional adult male Sprague-Dawley rats were anesthetized with urethane (1.5 g/kg, given intraperitoneally), and electrodes were positioned bilaterally to stimulate the perforant path. Glass microelectrodes were advanced bilaterally into the dorsal granule cell layer of the dentate gyrus. Test shocks of 50-μs monopolar pulses were given at 30-s intervals throughout the experiment. Three sets of trains (250 Hz for 200 ms) at 1-min intervals were used to induce LTP. The stimulus intensity was doubled during the tetanus on the conditioned side (a total of 150 strong shocks). On the control side, the same number of strong shocks was given by interpolating a single strong shock between weak test shocks for a period of 75 min. Thus, the control side received a substantial number of strong shocks, which produced population spikes that were as large as the potentiated responses on the tetanized side. The APV (Sigma) was injected intraventricularly (20 μl of 200 μM solution) 30 min before the tetanus. After either 2 or 5 hours, the animals were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed for 2 hours at 4°C, and saturated with 30% sucrose phosphate buffer before being stored at −70°C.

16. K. Moriyoshi *et al.*, *Nature* **354**, 31 (1991).

17. Immunohistochemistry was performed with GR33-specific antibodies (1:400) on 15-μm fixed brain slices after pretreatment with 0.2% Triton X-100 in phosphate-buffered saline solution. Antibody binding was revealed with DAB (3,3′-diaminobenzidine) in the presence of nickel with the Vectastain kit (Vector) following the manufacturer's instructions.

18. T. V. P. Bliss *et al.*, *J. Physiol. (London)* **377**, 391 (1986); J. H. Williams, M. L. Errington, M. A. Lynch, T. V. P. Bliss, *Nature* **341**, 739 (1989); D. M. Lovinger, P. A. Colley, R. F. Akers, R. B. Nelson, A. Routtenberg, *Brain Res.* **399**, 205 (1986).

19. Supported by Foundation S. & C. Del Duca, Centre National de la Recherche Scientifique, Institut de la Santé et de la Recherche Médicale, Ministère de la Recherche et de la Technologie, Association de la Recherche contre le Cancer, the Bioavenir Program (Rhône-Poulenc Rorer, Ministère de la Recherche et de l'Espace, Ministère de l'Industrie et du Commerce Extérieur), and the Human Frontier Science Program.

15 March 1993; accepted 12 August 1993

Binding of L-Selectin to the Vascular Sialomucin CD34

Susanne Baumhueter, Mark S. Singer, William Henzel, Stefan Hemmerich, Mark Renz, Steven D. Rosen, Laurence A. Lasky*

The adhesive interactions between leukocyte L-selectin and the endothelium are involved in the migration of lymphocytes through peripheral lymph nodes and of neutrophils to sites of inflammation. A recombinant L-selectin stains high endothelial venules (HEVs) in lymph nodes and recognizes sulfated carbohydrates found on two endothelial glycoproteins, Sgp50 and Sgp90. Amino acid sequencing of purified Sgp90 revealed a protein core identical to that of CD34, a sialomucin expressed on hematopoietic stem cells and endothelium. A polyclonal antiserum to recombinant murine CD34 stains peripheral lymph node endothelium and recognizes Sgp90 that is functionally bound by L-selectin. Thus, an HEV glycoform of CD34 can function as a ligand for L-selectin.

The ability of leukocytes to adhere efficiently to the endothelium under conditions of vascular flow is a key event in the inflammatory response (*1–4*). Leukocyte adhesion and extravasation appear to constitute a multistep phenomenon in which the initial, relatively low-affinity binding event (leukocyte rolling) is mediated by the selectin family of adhesion molecules (*3, 4*). Chemotactically activated leukocytes then induce a higher avidity binding that is mediated by the leukocyte integrins and their cognate endothelial ligands, the immunoglobulin (Ig) superfamily glycoproteins including the intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecule–1 (VCAM-1) (*1–4*). Although the adhesion that is mediated by the leukocyte integrins is due to protein-dependent binding (*2*), the selectins bind through the calcium-dependent lectin recognition of carbohydrate ligands on specific endothelial or leukocyte glycoproteins (*4*). Two such endothelial ligands that are specifically recognized by leukocyte L-selectin are sulfated glycoproteins of 50 and 90 kD that have been termed Sgp50 and Sgp90 (*5–9*). The interactions between recombinant L-selectin and Sgp50 and Sgp90 appear to exactly mimic the L-selectin–mediated binding of lymphocytes to the HEVs of peripheral lymph nodes (PLNs) (*5–9*). The nature of Sgp50 was determined by the cloning of a complementary DNA (cDNA) encoding the protein backbone of this glycoprotein (*10*). The results revealed a potentially soluble mucin-like molecule that could function as a scaffold for the high-density presentation of the appropriate, sulfated carbohydrates to cell surface L-selectin on lymphocytes. The clustering of carbohydrate ligands in a mucin organization was an obvious mechanism to enhance the avidity of this ligand for L-selectin. Previous analyses did not define the molecular nature of Sgp90.

The earlier biochemical characterization of the Sgp50 and Sgp90 L-selectin ligands was done with a chimeric molecule containing the extracellular domain of L-selectin and human IgG1 (L-selectin–IgG) (*5–9*). Isolated Sgp50 interacts with L-selectin–IgG in a calcium-dependent, carbohydrate-mediated manner, as is the case for the binding of lymphocytes to HEVs (*5–7*). To prove that Sgp90 also has independent ligand activity that is carbohydrate-dependent, and is not merely coprecipitated with Sgp50, we electroeluted Sgp90 from an SDS gel and examined its ability to interact directly with L-selectin–IgG. As shown in Fig. 1, purified Sgp90 was quantitatively reprecipitated by L-selectin–IgG only in the presence of calcium, demonstrating the involvement of the calcium-dependent lectin domain of L-selectin (*4*). A CD4-IgG chimera did not react with the 90-kD component. We used affinity chromatography on wheat germ agglutinin and L-selectin–IgG columns to purify Sgp90 from detergent lysates of mouse mesenteric lymph nodes (*7, 10*). The final EDTA-released fraction from the L-selectin affinity column was electrophoresed on an SDS gel, and the region of the gel containing Sgp90 was isolated. Electroblotted material was subjected to amino acid sequence analysis.

A weak (~5 pmol) 12-residue NH_2-terminal sequence was determined that contained a number of gaps ("X"). Comparison of the NH_2-terminal sequence of the purified ligand with the deduced NH_2-terminus of the murine sialomucin CD34 (mCD34) (*11*) revealed an exact match at 7 out of 12 positions (Sgp90 sequence: XXETSXQGIXPT, CD34 sequence: TTE-

S. Baumhueter, M. Renz, L. A. Lasky, Department of Immunology, Genentech, Inc., South San Francisco, CA 94080.
M. S. Singer, S. Hemmerich, S. D. Rosen, Department of Anatomy and Program in Immunology, University of California, San Francisco, CA 94143.
W. Henzel, Department of Protein Chemistry, Genentech, Inc., South San Francisco, CA 94080.
*To whom correspondence should be addressed.

TSTQGISPS) (*12*). Four of the positions that did not match were gaps in the Sgp90 sequence and threonines or serine in the mCD34 sequence. Many of the threonine and serine residues on CD34 are O-glycosylated (*11*, *13–16*), and the lack of an interpretable signal at these positions would be consistent with O-glycosylation of these sites. The final sequence difference was a threonine in the sequence of Sgp90 and a serine in the mCD34 sequence, a conservative, potentially polymorphic substitution. In addition, a tryptic peptide of the isolated ligand exactly matched an internal sequence of mCD34 (residues 253 to 259: KLQLMEK) (*11*). This sequence analysis suggested that Sgp90 was mCD34.

A rabbit polyclonal antiserum to recombinant mCD34 (anti-mCD34) was produced. Flow cytometric analysis revealed that the antiserum specifically stained normal rat kidney (NRK) cells that were stably transfected with a full-length mCD34 cDNA, and a protein of ~90 kD could be immunoprecipitated from the transfected cells (Fig. 2A). The antiserum specifically reacted with NIH 3T3 cells, which are known to express CD34 (*11*), as well as with a small fraction of fetal liver and bone marrow progenitor (or stem) cells that were capable of reconstituting lethally irradiated mice. The latter finding is consistent with the occurrence of CD34 on hematopoietic progenitor cells (*17*).

The antiserum stained capillaries and HEVs within mouse PLNs (Fig. 2B). This is consistent with the expression of human CD34 on the endothelium of capillaries and some larger vessels (*18*), and our result extends these data by showing that the antigen is also expressed in the postcapillary HEVs of PLNs. These data are also consistent with the HEV localization of ligands for L-selectin–mediated lymphocyte adhesion (*1*, *5*). To directly test whether the antiserum to mCD34 reacted with Sgp90, we did an immunoprecipitation analysis (Fig. 3) (*10*). Functional Sgp50 and Sgp90 were precipitated from a [^{35}S]sulfate-labeled lymph node lysate with L-selectin–IgG and eluted with EDTA (*7–10*). An antibody to a peptide of Sgp50 selectively precipitated Sgp50 but not Sgp90 (*10*). Conversely, the

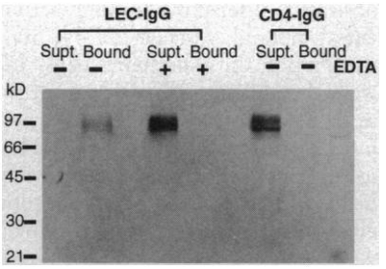

Fig. 1. Reprecipitation of purified Sgp90 with L-selectin–IgG. L-selectin–IgG (LEC-IgG) or CD4-IgG was reacted with SDS–polyacrylamide gel electrophoresis (PAGE)–purified Sgp90 in the absence (−) or presence (+) of EDTA. Supt., unbound material in the supernatant, and Bound, precipitated material. Molecular size standards are shown on the left. We prepared [^{35}S]sulfate-labeled Sgp90 as described (*7–9*) and eluted it from an L-selectin–IgG affinity column with 5 mM EDTA and 0.5% Triton X-100 in tris-buffered saline. The eluate was subjected to reducing SDS-PAGE, and the position of Sgp90 was determined by autoradiography. The Sgp90 band was excised, eluted, and subjected to reprecipitation with L-selectin–IgG in the absence or presence of 5 mM EDTA or with CD4-IgG. Both supernatant and bound material were analyzed by 10% SDS-PAGE under reducing conditions, followed by autoradiography. To purify Sgp90 for amino acid sequencing, we pooled and homogenized the mesenteric lymph nodes from 1965 mice in 2% Triton X-100 in phosphate-buffered saline (PBS), and the Sgp90 ligand was purified by boiling and sequential affinity chromatography on columns of wheat germ agglutinin and L-selectin–IgG (*10*). After preparative SDS-PAGE, proteins were electrophoretically transferred onto a ProBlott membrane, and Sgp90 was localized by autoradiography (*10*). The NH_2-terminal amino acid sequence was determined by a model 470A Applied Biosystems sequencer equipped with an on-line phenylthiohydantoin analyzer. The internal peptide sequence was obtained after tryptic digestion of the ProBlott membrane (*28*). Several of the peptides were sequenced, but all gave very weak signals except for peptide 9 that gave a clearly interpretable sequence.

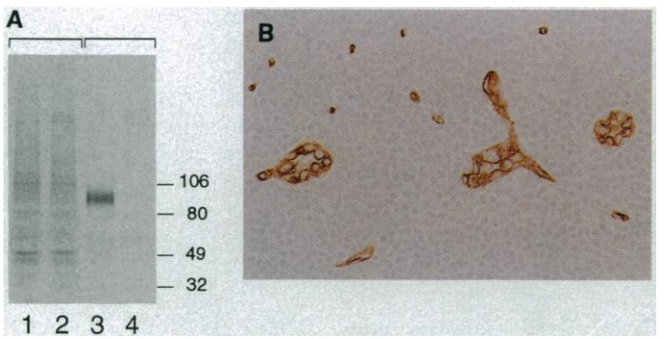

Fig. 2. Immunoprecipitation analysis and immunohistochemical staining of murine PLN with anti-mCD34. (**A**) Immunoprecipitation of mCD34 from [^{35}S]methionine- and [^{35}S]cysteine-labeled lysates of stably transfected NRK cells. Lane 1, mCD34 transfected, preimmune serum precipitated; lane 2, mock-transfected, preimmune serum precipitated; lane 3, mCD34 transfected, anti-mCD34 precipitated; and lane 4, mock transfected, anti-mCD34 precipitated. Molecular sizes are indicated to the right in kilodaltons. (**B**) Specific staining of PLN HEVs (large vessels) and capillaries (small vessels) with anti-mCD34. Neither preimmune serum nor anti-mCD34 incubated with recombinant mCD34 showed any vascular staining (*29*). Recombinant mCD34 was produced as an IgG chimera (*5*). A cDNA of mCD34 (*12*) that terminated at Lys^{286} was ligated to the hinge, the CH2 and CH3 domains of human IgG (*5*). This construct was transfected into human embryonic kidney (293) cells, and a permanent cell line producing mCD34-IgG was isolated. Serum-free supernatants conditioned by this cell line were concentrated and passed over protein G–Sepharose to purify the mCD34-IgG chimera. The purified chimera was cleaved with immobilized papain (Pierce), and the extracellular domain of mCD34 was separated from the bulk (~80%) of contaminating human IgG1 Fc by protein A–Sepharose chromatography. Antiserum specific for mCD34 was produced by immunization of rabbits at multiple sites with ~100 μg of recombinant mCD34. Anti-mCD34 titers were determined in an enzyme-linked immunosorbent assay (*5*). The resultant polyclonal antiserum was depleted of residual antibodies to human IgG by passage over a human IgG affinity column. Immunoprecipitation analysis was done (*10*) with NRK cells stably transfected with a mCD34 expression construct. For immunohistochemistry, 5- to 6-μm sections were cut from PLP-fixed, paraffin embedded murine PLN, and stained (*10*).

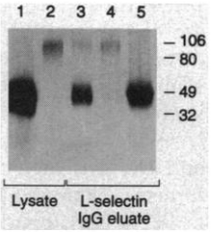

Fig. 3. Immunoprecipitation analysis of [^{35}S]sulfate-labeled glycoproteins from murine PLN. Lane 1, immunoprecipitation of sulfate-labeled PLN lysates with antiserum to GlyCAM-1 (anti–GlyCAM-1) (*10*); lane 2, immunoprecipitation of sulfate-labeled PLN lysates with anti-mCD34; and lane 3, immunoprecipitation of sulfate-labeled PLN lysates with L-selectin–IgG chimera (*7*). The L-selectin–purified ligands were released by EDTA and reprecipitated with L-selectin–IgG in the presence of Ca^{2+}. Lane 4, immunoprecipitation of L-selectin ligands with anti-mCD34 and lane 5, immunoprecipitation of L-selectin ligands with anti–GlyCAM-1. Molecular size standards are shown on the right in kilodaltons. PLNs were labeled with [^{35}S]sulfate in organ culture as described (*7–10*), and the Triton X-100 extract was divided into three equal aliquots. Immunoprecipitation was done for 5 hours at 4°C with rabbit anti-mCD34 or rabbit anti–GlyCAM-1 (*10*) in the presence of protein G–Sepharose beads, or Sepharose beads conjugated to L-selectin–IgG. Material bound to the L-selectin–IgG–coated Sepharose beads was eluted by incubation in PBS, 0.02% NP-40, 0.05% Triton X-100, and 5 mM EDTA overnight at 4°C. We added 10 mM $CaCl_2$ to the eluted material and reprecipitated separate aliquots with either anti–GlyCAM-1, anti-mCD34, or L-selectin–IgG Sepharose beads, as described above.

anti-mCD34 quantitatively precipitated Sgp90 but not Sgp50. Each antiserum precipitated only its cognate antigen from a detergent lysate of lymph nodes (Fig. 3). These data, together with the protein sequence and immunohistochemical analyses, demonstrate that CD34 is an endothelial-associated glycoprotein in PLNs and that Sgp90 is a sulfated, HEV-restricted glycoform of this molecule (*7–9*). Finally, additional evidence for the functional importance of Sgp90 (mCD34) comes from the demonstration that this glycoprotein reacts with the MECA-79 monoclonal antibody (*7*), a reagent that stains the HEVs of PLNs and blocks lymphocyte adhesion (*19*). However, the relation of CD34 to the ~90-kD component recognized by MECA-79 among a complex set of human PLN glycoproteins (*19*) remains to be examined.

We demonstrate here that a second, mucin-like glycoprotein can function as an endothelial-associated ligand for L-selectin. The inferred structures for GlyCAM-1 (Sgp50) (*10*) and CD34 (Sgp90) (*11, 13*) both show mucin-like domains containing a high degree of O-linked glycosylation. We previously hypothesized that such mucin-like domains, which are predicted to be highly rigid and extended structures (*20*), could function in the polyvalent presentation of specific sulfated and sialylated carbohydrate chains to the lectin domain of L-selectin (*4, 10*). The data reported here are consistent with the hypothesis that a diverse family of mucin-like adhesion molecules may present specific carbohydrate ligands to the selectins (*4*).

A major difference between GlyCAM-1 and CD34 is their tissue distribution. The former is expressed predominantly in HEVs of PLNs (*10*), consistent with its proposed function as an endothelial-associated ligand for L-selectin involved in lymphocyte homing to lymph nodes (*1*). In contrast, human CD34 has a much broader tissue distribution with apparently constitutive expression on endothelial cells in a diversity of blood vessels as well as on other tissue elements (*18*). Northern (RNA) blot (*11*), polymerase chain reaction, and immunohistochemical analyses (*21*) suggest a similarly broad constitutive pattern of expression for CD34 in the mouse vasculature. Neutrophil L-selectin participates in the initial rolling response that is a prerequisite for integrin-mediated firm adhesion and extravasation events during inflammation (*3, 4, 22–24*). Although we have not demonstrated L-selectin binding activity for extralymphoid vascular CD34, its broad distribution on endothelial cells, together with the ability of the PLN form to serve as a ligand, are suggestive of a potential function for extralymphoid CD34 in the rolling of neutrophils that is mediated by L-selectin (*23, 24*). Thus, appropriately glycosylated CD34 at other, nonlymphoid vascular sites could potentially function as an L-selectin ligand used by leukocytes during acute and perhaps chronic inflammatory responses (*3, 4*).

Various pathways for the regulation of CD34 ligand activity must be considered, including differential, vessel-specific glycosylation, translocation to the endothelial cell surface, and oligomerization at the cell surface. For example, vessel-specific glycosylation may explain why leukocytes only adhere to postcapillary venules, even though CD34 appears to be expressed on capillaries as well as on venules. It also remains to be seen whether CD34 is subject to biosynthetic regulation under cytokine control, as has been reported for L-selectin ligands on cultured endothelial cells (*25–27*). Finally, our results suggest that different glycoforms of CD34 may be involved in adhesive functions in other contexts. For example, another form of CD34 on hematopoietic stem cells may serve as a ligand for a bone marrow stromal lectin.

REFERENCES AND NOTES

1. L. Stoolman, *Cell* **56**, 907 (1989).
2. T. A. Springer, *Nature* **346**, 425 (1990).
3. E. C. Butcher, *Cell* **67**, 1033 (1991).
4. L. A. Lasky, *Science* **258**, 964 (1992).
5. S. Watson *et al.*, *J. Cell Biol.* **110**, 2221 (1990).
6. D. True, M. S. Singer, L. A. Lasky, S. D. Rosen, *ibid.* **111**, 2757 (1990).
7. Y. Imai, M. S. Singer, C. Fennie, L. A. Lasky, S. D. Rosen, *ibid.* **113**, 1213 (1991).
8. Y. Imai, L. A. Lasky, S. D. Rosen, *Glycobiology* **2**, 373 (1992).
9. _____, *Nature* **361**, 555 (1993).
10. L. A. Lasky *et al.*, *Cell* **69**, 927 (1992).
11. J. Brown, M. Greaves, H. Molgaard, *Int. Immunol.* **3**, 175 (1991).
12. Single letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
13. D. L. Simmons, A. Satterthwaite, D. Tenen, B. Seed, *J. Immunol.* **148**, 267 (1992).
14. C. Civin *et al.*, *J. Immunol.* **133**, 157 (1984).
15. F. E. Katz *et al.*, *Leukemia Res.* **9**, 191 (1986).
16. D. R. Sutherland *et al.*, *Leukemia* **2**, 193 (1988).
17. R. J. Berenson *et al.*, *J. Clin. Inv.* **81**, 951 (1988).
18. L. Fina *et al.*, *Blood* **75**, 2417 (1990).
19. E. Berg, M. Robinson, R. Warnock, E. Butcher, *J. Cell Biol.* **114**, 343 (1991).
20. N. Jentoft, *Trends Biochem. Sci.* **15**, 291 (1990).
21. S. Baumhueter, N. Dybdal, L. A. Lasky, unpublished data.
22. S. Watson, C. Fennie, L. A. Lasky, *Nature* **349**, 164 (1991).
23. K. Ley, P. Gaehtgens, C. Fennie, L. A. Lasky, S. D. Rosen, *Blood* **77**, 2553 (1991).
24. U. von Andrian *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7538 (1991).
25. O. Spertini *et al.*, *J. Immunol.* **147**, 2565 (1991).
26. H. Brady *et al.*, *ibid.* **149**, 2437 (1992).
27. D. Delia *et al.*, *Blood* **81**, 1001 (1993).
28. W. J. Henzel, H. H. Bourell, J. T. Stults, *Anal. Biochem.* **187**, 228 (1990).
29. S. Baumhueter *et al.*, unpublished data.

30. We thank M. Greaves for providing the mCD34 cDNA; A. Joves, M. Joaquin, S. Mallett, M. Janatpour, and W. Lagrimes for assistance with mouse dissection; C. Fennie for help with the production of CD34-IgG; C. Kyle and N. Dybdal for immunohistochemistry; K. Andow for help with the preparation of figures; and S. Watson for helpful suggestions and discussion. Supported by grants from NIH (GM 23547) and Genentech, Inc. (to S.D.R.) and a postdoctoral fellowship from Deutsche Forschungsgemeinschaft (to S.H.).

28 June 1993; accepted 18 August 1993