PHYSIOLOGICAL REVIEWS
Vol. 79, No. 1, January 1999
Printed in U.S.A.

Mechanisms That Regulate the Function of the Selectins and Their Ligands

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Vestweber, Dietmar, and James E. Blanks. Mechanisms That Regulate the Function of the Selectins and Their Ligands. *Physiol. Rev.* 79: 181–213, 1999.—Selectins are a family of three cell adhesion molecules (L-, E-, and P-selectin) specialized in capturing leukocytes from the bloodstream to the blood vessel wall. This initial cell contact is followed by the selectin-mediated rolling of leukocytes on the endothelial cell surface. This represents the first step in a cascade of molecular interactions that lead to leukocyte extravasation, enabling the processes of lymphocyte recirculation and leukocyte migration into inflamed tissue. The central importance of the selectins in these processes has been well documented in vivo by the use of adhesion-blocking antibodies as well as by studies on selectin gene-deficient mice. This review focuses on the molecular mechanisms that regulate expression and function(s) of the selectins and their ligands. Cell-surface expression of the selectins is regulated by a variety of different mechanisms. The selectins bind to carbohydrate structures on glycoproteins, glycolipids, and proteoglycans. Glycoproteins are the most likely candidates for physiologically relevant ligands. Only a few glycoproteins are appropriately glycosylated to allow strong binding to the selectins. Recently, more knowledge about the structure and the regulated expression of some of the carbohydrates on these ligands necessary for selectin binding has been accumulated. For at least one of these ligands, the physiological function is now well established. A novel and exciting aspect is the signaling function of the selectins and their ligands. Especially in the last two years, convincing data have been published supporting the idea that selectins and glycoprotein ligands of the selectins participate in the activation of leukocyte integrins.

I. INTRODUCTION

The migration of leukocytes from the blood vessel into inflamed tissue is the central step in the process of inflammation. Binding of leukocytes to the blood vessel wall is strictly controlled by a complex cascade of molecular interactions between the leukocyte and the endothelial cell layer, mediated by cell adhesion molecules and leukocyte-activating factors (56, 306) (Fig. 1). These molecules allow leukocytes to recognize sites of extravasation, where they attach to and migrate across the endothelial barrier. Emigration of leukocytes from the blood is initiated by the capture of leukocytes from the bloodstream followed by their rolling along the endothelial cell surface. This process is mediated by the selectins, a special family of three cell adhesion molecules.

A variety of inflammatory mediators such as chemokines (18) or platelet-activating factor (PAF) (383), presented on the endothelial cell surface, are recognized by the leukocytes after initial contact. This leads to the activation of leukocyte integrins, which support stable cell attachment and enable leukocyte migration on the endo-

0031-9333/99 $15.00

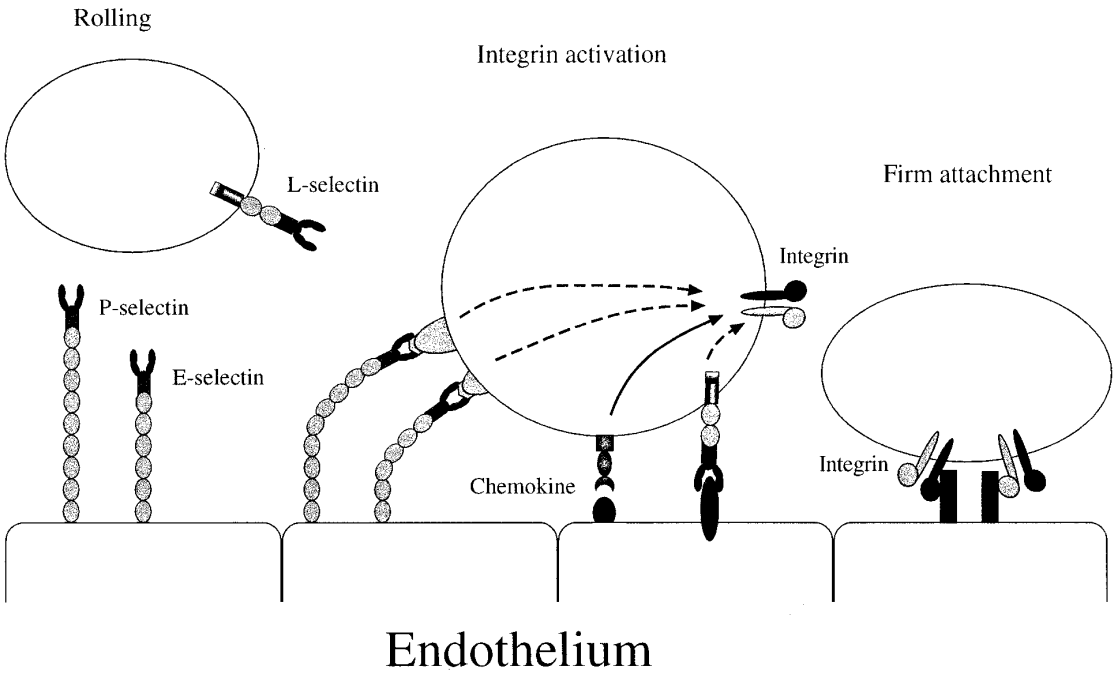


FIG. 1. Entry of leukocytes into tissue is controlled by a cascade of multiple molecular interactions. Initial tethering of leukocytes to the endothelial cell surface is mediated by selectins. This enables leukocytes to roll along the blood vessel wall and to sense activating factors such as chemokines that are deposited on the endothelial cell surface. This leads to activation of leukocyte integrins that bind to members of the immunoglobulin superfamily and mediate firm adhesion, a prerequisite for directed migration of leukocytes on the endothelial cell surface. An increasing number of recent reports suggest that in addition to chemokines, selectins are also directly involved in integrin activation.

thelial cell surface. Finally, the leukocyte transmigrates through the endothelial cell layer and the underlying basal membrane and enters into the tissue.

In contrast to most other cell adhesion phenomena, especially those during embryonal development, the recruitment of leukocytes from the flowing bloodstream is a very rapid process that requires special mechanisms for the establishment of cell contacts. The selectins represent a class of cell adhesion molecules that is specialized for this purpose. Their distribution is restricted to the leukocyte-vascular system. In contrast to the vast majority of most other cell adhesion molecules, the selectins function as lectins, binding carbohydrate ligands. The individual members of the selectins are designated by prefixes, which were chosen according to the cell type where the molecules were first identified: L-selectin is expressed on most types of leukocytes, E-selectin is expressed on activated endothelium, and P-selectin was first found in storage granules of platelets and is also expressed by endothelial cells.

L- and E-selectin were found as antigens for cell adhesion blocking antibodies. L-selectin was first defined by the monoclonal antibody (MAb) MEL14, which blocked the binding of lymphocytes to high endothelial venules (HEV) in mouse lymph nodes, a process called lymphocyte homing (104). Parallel to these studies, it was found that carbohydrate determinants were important for lymphocyte-endothelial interactions during lymphocyte recirculation (317, 318). Later, a mannose-6-phosphate-rich polysaccharide was shown to block the binding of lymphocytes to lymph node HEV (378). Furthermore, this polysaccharide was shown to bind to the MEL14-defined antigen, later named L-selectin. Cloning revealed that L-selectin does indeed carry an NH_2-terminal lectin domain with homology to Ca^{2+}-dependent mammalian lectins (188, 294).

E-selectin was identified by MAb that had been raised against cytokine-activated human endothelial cells and blocked the binding of neutrophils (31, 267). Cloning of this selectin revealed the close relatedness to L-selectin (32).

P-selectin was originally found as a membrane protein of storage granules in human platelets (141, 221). Cloning of P-selectin revealed the selectin nature of this molecule (150) and stimulated experiments that demonstrated the ability of this molecule to mediate neutrophil binding to platelets (127, 184) and to endothelial cells (107).

The extracellular part of all selectins is composed of three different types of protein domains also found in proteins of very diverse function (Fig. 2). The NH_2 terminus of each selectin is formed by a 120-amino acid domain that shares some features with the lectin domain of the C-type animal lectins (88). This domain is followed by a sequence of ~35–40 amino acids similar to a repeat structure, which was first found in epidermal growth fac-

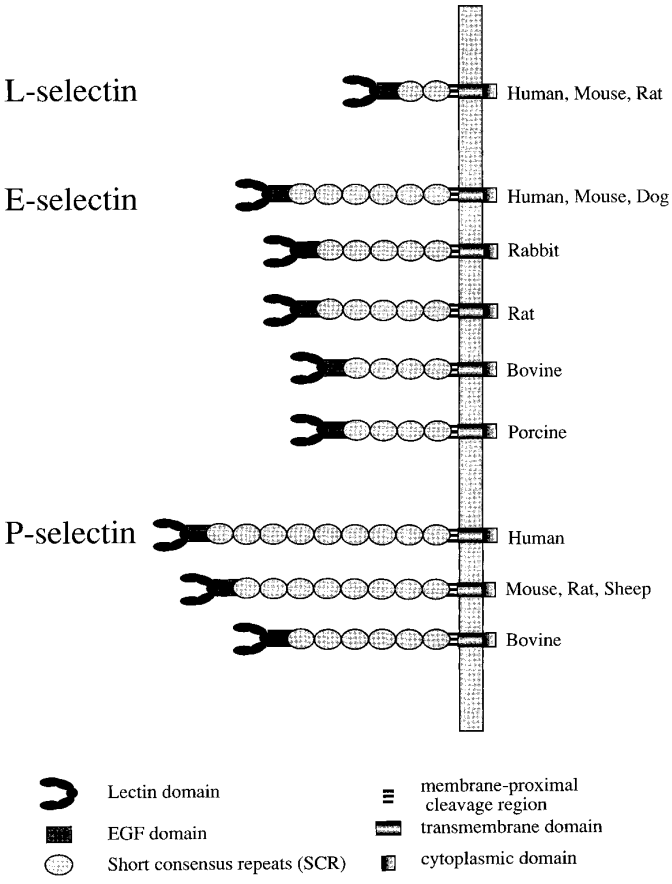


FIG. 2. Structural organization of selectins. Selectins are composed of an NH_2-terminal lectin domain, a single epidermal growth factor (EGF)-type repeat, and various numbers of consensus repeats or so-called complement binding domains, which share sequence homology with a domain structure often found in proteins with complement binding activity. Proteins have a single transmembrane region and a short cytoplasmic tail. E- and P-selectins have different numbers of complement binding domains in different species. [Modified from Huang et al. (142).]

tor (EGF). The six cysteines in this element are located at equivalent positions in so-called "EGF repeats" of several proteins. A truncated, recombinant form of human E-selectin containing only the lectin domain and the EGF repeat has been crystallized, and the three-dimensional structure was determined (119).

The single EGF element that is found in each selectin is followed by a varying number of repetitive elements, each ~60 amino acids long, which resemble protein motives found in complement regulatory proteins. The specific function of these so-called "complement binding" (CB) elements is yet undefined. It was shown that truncating increasing numbers of these domains impaired the efficiency with which P-selectin could support rolling of leukocytes (260), suggesting that the CB domains are important to extend P-selectin a sufficient length from the plasma membrane. Four of the six cysteine residues in these repetitive elements are conserved in the complement-related proteins. The size variation of the three selectins is due to the different numbers of CB domains (Fig. 2). Although L-selectin has two such domains in human, mouse, and rat, the number of CB domains in E- and P-selectin varies between different species. Human, mouse, and dog E-selectin has six such domains, rabbit and rat E-selectin has five, and bovine and pig E-selectin has four. Human P-selectin has nine CB domains; mouse, rat, and sheep P-selectin has eight; and bovine P-selectin has six. All three selectins are anchored in the membrane by a single transmembrane region that is followed by a short cytoplasmic tail consisting of only 17 amino acids for L-selectin and 32 and 35 for human E- and P-selectin, respectively. The functional analysis of the structural organization of the selectins has been reviewed (142).

On the basis of in vivo studies in various species with adhesion blocking antiselectin antibodies and of studies of mice deficient in the selectin genes, the important role of the selectins for the rolling of leukocytes on the blood vessel wall and for lymphocyte recirculation as well as leukocyte entry into inflamed tissue has been well established in numerous reports. The studies on selectin gene-deficient mice have been recently reviewed (51, 102) as well as the function of the selectins as rolling receptors (200), in lymphocyte homing (126), in lung inflammation (360), and in ischemia-reperfusion injury (333).

This review gives an overview of recent studies on the interactions of selectins with their ligands and on the regulation and the function(s) of these molecules. Earlier reviews summarize previous work (161, 186, 222, 280, 331, 348).

II. SELECTINS AS ROLLING RECEPTORS AND INITIATORS OF LEUKOCYTE ENTRY INTO TISSUE

L-selectin was the first of the selectins that was shown to be important for the entry of leukocytes into tissue. On the basis of the in vivo inhibitory effect of the MAb MEL14 on lymphocyte homing into peripheral lymph nodes of the mouse, L-selectin was defined as a lymphocyte-homing receptor (104). L-selectin was also the first selectin that was shown to be involved in the migration of neutrophils into inflamed tissue, again based on the inhibitory effect of the MAb MEL14 on neutrophil migration into inflamed skin (198) and into inflamed peritoneum of the mouse (156). Another class of leukocyte adhesion molecules that is important for leukocyte extravasation is the leukocyte integrins, a group of three integrins sharing the same β_2-chain (316). It was soon shown that activation of neutrophils was accompanied with the downregulation of L-selectin and the upregulation of one of the leukocyte integrins, $\alpha_M\beta_2$ or Mac-1 (171). This suggested that L-selectin may act before the β_2-integrin in the process of adhesion. Indeed, in two different animal models,

it was soon shown that L-selectin mediates leukocyte rolling, the first interaction between leukocytes and the blood vessel wall. A recombinant fusion protein carrying the extracellular part of mouse L-selectin and the Fc part of human IgG_1 (L-selectin-Ig) blocked leukocyte rolling in rat mesenteric venules (202). Similarly, the MAb DREG 200 against human L-selectin (172), when injected into rabbits, inhibited the rolling of leukocytes in vivo while an anti-β_2-integrin antibody did not interfere with the rolling process but blocked the subsequent firm attachment of leukocytes to the venular endothelium (351). This work established a two-step model for leukocyte adhesion to endothelial cells under flow conditions in vivo, with the selectin mediating the rolling process and the β_2-integrin acting subsequently. In very elegant and well-defined in vitro experiments, it was demonstrated that P-selectin, but not intercellular adhesion molecule-1 (ICAM-1; the major endothelial ligand for β_2-integrins), could support rolling of neutrophils under flow conditions on a lipid bilayer containing the purified proteins (193). In contrast, a static incubation of the cells with the lipid bilayer resulted in a cell binding, which was 100 times more shear resistant if ICAM-1 was incorporated into the bilayer, than when P-selectin was incorporated.

Since these initial studies, numerous reports have clearly established and confirmed that all three selectins are involved in leukocyte rolling in vivo and the initiation of physical leukocyte endothelial interactions. E-selectin was soon shown to support rolling of neutrophils in in vitro adhesion assays under flow conditions (1, 165, 194). Both endothelial selectins, E- and P-selectin, were demonstrated to function as rolling receptors in vivo (83, 254). Rolling of leukocytes on the endothelial selectins was not restricted to neutrophils but was also demonstrated for bovine γ/δ T cells (154) and for human α/β and γ/δ T cells (81).

Apart from lymphocytes and neutrophils, other leukocytes utilize the selectins as rolling receptors. Monocytes were found to roll via L-selectin and P-selectin on tumor necrosis factor (TNF)-activated endothelial cells in vitro (214). Eosinophils were shown to roll via L-selectin (309), but not on E-selectin (308). However, all three selectins were reported to be involved in eosinophil recruitment in vivo (135). Most leukocytes that can bind to P-selectin can also bind to E-selectin. However, mouse bone marrow-derived mast cells were found to roll on P-selectin (307) but not on E-selectin (312). In addition to the selectins, α_4-integrins on lymphocytes can also support rolling under physiological flow (29), and it was suggested that the integrin $\alpha_4\beta_7$ would function in L-selectin-mediated rolling as well as in $\alpha_L\beta_2$-mediated firm adhesion, forming a "bridge" between both steps (19).

As can be expected from the in vivo rolling data, all three selectins are involved in the entry of leukocytes into tissue. The homing of lymphocytes into lymph nodes is the only process that is exclusively mediated by L-selectin. E- and P-selectins have not been reported to be involved in this process. Entry of lymphocytes as well as neutrophils into inflamed tissue is mediated by all three selectins. This was shown by antibody-blocking studies for L-selectin in the mouse, as mentioned above (156, 198), and for E-selectin in a peritonitis model in rat with an anti-human E-selectin antibody reported to be cross-reactive with rat E-selectin (238). Antibodies against each of the three mouse selectins were shown to block neutrophil infiltration into chemically inflamed mouse peritoneum, although blocking of L- and P-selectin was more efficient than blocking of E-selectin (38). The involvement of E-selectin in neutrophil-mediated damage of lung endothelium during acute airway inflammation could be demonstrated with anti-E-selectin antibodies in rat (238) and in monkeys. A protective effect of an anti-human P-selectin MAb against cobra venom factor induced pulmonary injury in rats could also be demonstrated (238).

Three mouse mutants have been generated that is each deficient in one of the selectin genes (Table 1). Lymphocyte homing was significantly reduced in L-selectin-deficient mice (12, 311). Similarly leukocyte rolling and peritoneal emigration of neutrophils in response to thioglycolate were reduced (12, 201). L-selectin deficiency also affected the successful execution of an immune response (58, 332, 374). P-selectin-deficient mice showed reduced neutrophil emigration in chemically inflamed peritoneum especially at early time points, 1 and 2 h after stimulation (53, 218). In contrast to P- and L-selectin mutants, E-selectin null mutants unexpectedly have no obvious abnormalities of the inflammatory response (52, 183). A more detailed analysis of E-selectin null mutants revealed a subtle defect in these mice: the slow-rolling granulocytes ($\sim$5 μm/s) were missing in these mice (182). Severe defects were observed when P-selectin was blocked in these animals by antibodies. This led to a strong reduction of neutrophil emigration into inflamed peritoneum and of edema formation in a delayed type hypersensitivity (DTH) model at late time points when anti-P-selectin antibodies had no effect in wild-type animals (183). These findings suggest that E-selectin and P-selectin share overlapping functions.

Interesting results were obtained with double deficient mice. Because all three selectin genes are closely linked in a gene cluster covering $\sim$300 kb on chromosome 1 (362), mice deficient in several selectins cannot simply be generated by breeding single mutant strains. Despite this difficulty, double deficient mice, lacking E- and P-selectin, were generated (52, 100). In contrast to the single-mutant mice, double-mutant mice displayed an increased susceptibility to bacterial infections, with the majority of the animals developing chronic inflammatory lesions of the oral mucosa and skin. Interestingly, neutrophil accumulation in *Streptococcus pneumoniae*-stimu-

TABLE 1. *Defects of selectin-deficient mice*

Mutation	Health Status	Leukocyte Counts	Neutrophil Emigration in Peritonitis		Delayed Type Hypersensitivity	Leukocyte Rolling
			0–4 h	24 h		
P-selectin	Normal	↑	Reduced	Normal*	Normal	↓↓
E-selectin	Normal	Normal	Normal	Normal	Normal	Normal
L-selectin	Normal	Normal	Reduced	Reduced	Impaired	↓↓
P-selectin/ICAM-1	Normal	↑↑	Absent	Normal*	Not determined	↓↓↓
P-/E-selectin	Spontaneous infections	↑↑↑↑	Absent	Normal*	Impaired	↓↓↓↓

ICAM-1, intercellular adhesion molecule-1. * May depend on chemicals versus bacterial stimulus and does not imply "normal" physiology. [From Bullard and Beaudet (51), with permission from Harwood Academic Publisher.]

lated peritoneum was completely blocked 4 h after instillation, whereas the number of emigrated neutrophils was normal compared with wild type at 24 h after stimulation, arguing for other adhesive mechanisms (e.g., L-selectin) mediating neutrophil emigration at later time points.

Mice double deficient in P-selectin and ICAM-1, in contrast to single P-selectin mutants and single ICAM-1 mutants, showed complete absence of surgically induced rolling in cremaster venules for at least 2 h (181). This effect was not seen if P-selectin-deficient mice were treated with an anti-ICAM-1 antibody. It is not known how the lack of ICAM-1 could affect leukocyte rolling. Early emigration of neutrophils into *S. pneumoniae*-stimulated peritoneum was completely blocked at 2–4 h after stimulation in contrast to only partial effects in single mutant mice (53). Surprisingly, neutrophil accumulation in the alveolar space after intratracheal instillation of *S. pneumoniae* was not significantly inhibited in P-selectin/ICAM-1 double-mutant mice (53). Not in all organs are the selectins important for leukocyte-endothelium interactions. For the liver, it was shown that leukocytes activated by the chemoattractant peptide formyl-methionyl-leucyl-phenylalanine (FMLP) adhered to the wall of sinusoids in E/P-selectin double-deficient mice as well as in wild-type mice, even when L-selectin was blocked by antibodies (373). Rolling and adhesion were completely blocked in these animals in cremaster venules.

Numerous reports have demonstrated that the selectins are involved in ischemia/reperfusion injury, as reviewed in Reference 333. Antibodies against P-selectin significantly protected attenuated myocardial necrosis in a feline model of myocardial ischemia-reperfusion (365). A similar protective effect was seen when the selectin binding oligosaccharide sialyl Lewis X (see sect. v*A*) was administered (50). Similar results were obtained in a rat myocardial ischemia-reperfusion model (337). In a very careful study, the rolling and adhesion of leukocytes was analyzed in postischemic mesenteric venules of cats (176). The results demonstrated that antibodies against L-selectin and P-selectin as well as the polysaccharide fucoidin blocked reperfusion-induced leukocyte rolling. However, rolling needed to be blocked by >90% to achieve reasonable (~50%) attenuation in leukocyte adhesion in postischemic venules.

A human genetic disease was described, causing, in addition to other defects, a markedly reduced ability of neutrophils to adhere to endothelium, recurrent episodes of bacterial infection, and localized cellulitis without pus formation (92). This disease is believed to be based on defect(s) in fucose metabolism (92). No sialyl Lewis^x is found in these patients, which is a fucose-containing tetrasaccharide known to bind to all three selectins (see sect. v*A*). Although this tetrasaccharide is not necessarily a physiological ligand for the selectins, it is now well established that the physiological selectin ligands contain α-(1,3)-fucose as an essential structural element (217) (see sect. v*A*). Indeed, neutrophils of the patients do not bind to E- or P-selectin-expressing endothelial cells in vitro (92, 264) and do not roll in venules under shear force (349).

Another human genetic disease, called leukocyte adhesion deficiency (LAD), is due to the lack of functional integrin β_2-chains (CD18), essential for neutrophil extravasation into sites of inflammation. Such patients suffer from life-threatening infections (11). In analogy to this disease, the deficiency described by Etzioni et al. (93) has been named LAD II. This defect demonstrates the importance and the essential role of carbohydrate recognition, probably via the selectins, for host defense mechanisms and inflammation.

III. CELLULAR MECHANISMS OF SELECTIN REGULATION

A. Various Mechanisms for the Regulation of Selectin Expression

Because contact formation between most leukocytes and endothelium is initiated by the selectins, the regulation of their presence on the cell surface is important for the control of leukocyte extravasation. The two endothe-

lial selectins, E- and P-selectin, are absent from the cell surface of nonactivated endothelial cells and become induced upon exposure of the endothelium to various proinflammatory stimuli. This ensures that these selectins are only present on endothelium in inflamed tissues. L-selectin, in contrast, is constitutively expressed on leukocytes, which is in agreement with its special function in the continuous process of lymphocyte homing. The function of L-selectin in the initiation of leukocyte-endothelial contacts in inflamed areas is controlled by the regulated appearance of its ligand(s).

E-selectin is induced by cytokines such as TNF-α or interleukin (IL)-1β and by lipopolysaccharide (LPS) as was first found for human umbilical vein endothelial cells (HUVEC) (31, 267). Induction occurred on the transcriptional level, and within 3–4 h after stimulation, maximal levels of E-selectin protein are expressed at the cell surface (32). Basal levels are reached again after 16–24 h, in contrast to other cytokine-inducible adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). A similar mechanism and similar kinetics of the regulation of mouse E-selectin were found on mouse endothelioma cells (124, 363).

The 5′-flanking regions of human E-selectin were cloned and sequenced (66), and the regulatory elements of the gene were studied intensively. The results were comprehensively summarized in a recent excellent review (219). Some of the most important reports are mentioned here. Four regulatory elements were found in the human E-selectin promoter of which three are NFκB binding sites (199, 232, 289, 367, 368) and one is an ATF-binding element (167, 368). Although the NFκB elements are not sufficient for the cytokine-stimulated induction of E-selectin transcription (367), they are necessary, since proteasome inhibitors, which block the degradation of IκB and thereby block the activation of NFκB also block transcriptional activation of E-selectin (276). In addition to the NFκB elements, the activating transcription factor (ATF) element is involved in cytokine-stimulated expression of E-selectin as well (167, 368). Stimulation with TNF-α activates two signaling pathways, NFκB and the kinases c-Jun NH_2-terminal kinase (JNK1) and p38, which are both required for maximal expression of E-selectin (277).

In addition to TNF-α and IL-1β, several other stimuli were found to activate expression of E-selectin. Interleukin-10 was shown to induce transcription of E-selectin in cultured human endothelial cells (353). On human dermal microvascular endothelial cells, induction was as efficient as with IL-1β, whereas induction on HUVEC was less efficient. Similar strong expression was seen at 4 and 24 h after stimulation, while baseline levels were reached again at 48 h. Interleukin-3 induced E-selectin with the same kinetics as IL-1β, but the amount of E-selectin was roughly one-half that induced by IL-1β (45). Oncostatin M, a cytokine belonging to the IL-6 family, was found to stimulate E-selectin expression with similar kinetics as TNF-α (231). In addition to LPS from gram-negative bacteria, lipoteichoic acid from gram-positive bacteria was also found to induce E-selectin expression (168). Immune complexes were shown to stimulate E-selectin expression via the heat-labile complement component C1q (213), although it is not known whether this is a direct or indirect effect.

The stimulation of E-selectin expression can be suppressed by various mediators. Inhibition of E-selectin expression can be achieved with IL-4 (334). Interleukin-4 induced suppression of TNF-α-stimulated E-selectin expression is mediated by STAT6, which antagonizes the binding of NFκB (23). Furthermore, glucocorticoids (70), transforming growth factor-β (TGF-β) (105), and elevation of cAMP (110, 268) can counteract cytokine-induced expression of E-selectin. The effect of glucocorticoids is mediated by affecting NFκB and not by interfering with ATF or c-Jun (46). The dual cyclooxygenase/lipoxygenase inhibitor tepoxaline was recently shown to suppress LPS-induced E-selectin expression and to block neutrophil migration into inflamed murine skin in vivo (382).

In addition to soluble factors, leukocytes contacting the endothelial cell surface can modulate the expression of E-selectin. Coincubation of HUVEC with human blood monocytes induced E-selectin expression and prolonged E-selectin expression for more than 24 h (274). Cell contact was needed for this effect, and antibodies against TNF-α were partially inhibitory (247). T cells from *Leishmania*-infected mice or from mice sensitized to the contact allergen trinitrochlorbenzene stimulated E-selectin when cocultured with mouse endothelioma cells (326). Again, cell contact was necessary, but anti-TNF antibodies could not block the effect. It is possible that the CD40 ligand was partially responsible, since binding of a soluble recombinant form of CD40 ligand to CD40 on endothelial cells leads to the induction of E-selectin (140, 166, 379).

P-selectin is inducible by two different mechanisms. It is stored in granules inside of platelets (α-granules) and endothelial cells (Weibel-Palade bodies) and can rapidly be mobilized to the cell surface of endothelial cells within minutes (within seconds in platelets) upon stimulation with histamine or thrombin or with pharmacological compounds such as Ca^{2+} ionophores or phorbol esters (107, 130). Expression is maximal at ~5–10 min after stimulation, and the protein is rapidly cleared from the cell surface within the next 30–60 min by endocytosis. Both endothelial selectins are rapidly internalized, but only P-selectin molecules can be recycled from endosomes into the *trans*-Golgi network, where they are targeted to Weibel-Palade bodies (325). Aside from this pathway, a considerable amount of the internalized P-selectin molecules (121) and all of the endocytosed E-selectin molecules (325) are delivered from endosomes into lysosomes. Endocytosis occurs via clathrin-coated pits (292). The cyto-

plasmic tail of P-selectin is responsible for endocytosis and intracellular targeting (82, 121, 292). The transmembrane domain of P-selectin enhances targeting into storage granula as was shown with E-selectin/P-selectin fusion proteins (98). P-selectin as well as E-selectin contain Tyr residues in their cytoplasmic tails, which were thought to be putative internalization signals. However, a clearly defined internalization signal has yet to be defined. The tyrosine residue in the cytoplasmic tail of E-selectin is not necessary for endocytosis (63), and it was shown that residues throughout the cytoplasmic domain of P-selectin affect the internalization efficiency (292).

A second regulation mechanism for P-selectin is similar to the one observed for E-selectin. Tumor necrosis factor-α was initially found to stimulate the transcript level and protein level of P-selectin in mouse and bovine endothelial cells with similar kinetics as that of E-selectin (124, 286, 363). This stimulation of P-selectin synthesis could be confirmed in vivo for the mouse (118) and the rat (16). Studies of a cytokine-induced meningitis model in wild-type mice and mice deficient in P-selectin or for both endothelial selectins revealed that cytokine-induced E- and P-selectin cooperatively contributed to meningitis and leukocyte accumulation in the cerebrospinal fluid (329). However, in experimental autoimmune encephalomyelitis, E- and P-selectin were not induced on blood-brain barrier forming endothelium and, consequently, were not involved in the infiltration of inflammatory cells (90).

In HUVEC, P-selectin expression could neither be stimulated by LPS nor by TNF-α or IL-1β (377). Instead, IL-4 and oncostatin M were found to induce P-selectin transcription and protein expression, which lasted 72 h (377). Interleukin-4 or oncostatin M stimulated P-selectin expression more slowly than TNF-α in the mouse system. Interestingly, oncostatin M was also reported to stimulate P-selectin transport from storage granules to the cell surface (231). Together with the effect on E-selectin transcription (231) (peak at 4 h), oncostatin M induces a tripartite increase of leukocyte adhesion, based first on P-selectin, then E-selectin, and then P-selectin again (223). In addition to oncostatin M, IL-3 may increase similar mechanisms, since it also leads to an immediate upregulation of P-selectin (169), an E-selectin-dependent delayed adhesion (45), and a very small increase in P-selectin on human endothelial cells over a period of days (169).

Both endothelial selectins also seem to be constitutively expressed in certain tissues. Using immunohistochemistry on sections of human hematopoetic tissue showed constitutive expression of E-selectin on endothelium of such organs (290). Similarly, noninflamed skin venules support significant rolling interactions that are mediated in part by P-selectin (248, 375), indicating that some blood vessels do not require inflammatory stimuli for P-selectin expression.

A very interesting novel endothelial adhesion mechanism for neutrophils was recently found to be inducible on HUVEC by IL-1 (151). This mechanism is probably a lectin, since it depends on sialic acid on the neutrophil cell surface. Most importantly, the expression kinetics of this mechanism are different from those of the endothelial selectins, since the novel adhesion activity is maximally expressed only at 24 h after stimulation. A mechanism that may be similar was identified on bovine endothelial cells. This adhesion activity was maximally induced 24 h after stimulation, supported lymphocyte and neutrophil rolling, and could be blocked with a MAb against a 110- to 120-kDa glycoprotein (157). A soluble form of this endothelial protein binds to lymphocytes, and this binding could be blocked by EDTA and *O*-sialoglycoprotease, but not by neuraminidase treatment of the target cells. Thus this novel adhesion molecule could be a novel, lectinlike, and cytokine-inducible adhesion molecule on endothelium.

L-selectin is constitutively expressed on myeloid cells and a large subset of lymphocytes (198). It can be downregulated at the transcriptional level during lymphocyte differentiation from a naive to memory cell phenotype. Mitogen stimulation of T lymphocytes leads to a transient increase of L-selectin on the cell surface paralleled by an increase in L-selectin mRNA and followed by a decrease in L-selectin transcription and L-selectin protein exposed on the cell surface over the next 7 days (160). Stimulation of L-selectin activity by qualitative changes in receptor activity was reported (304) but has not yet been verified in other reports.

L-selectin is involved in the process of lymphocyte recirculation as well as in the migration of neutrophils and lymphocytes into inflamed tissues. Induction of L-selectin-mediated adhesion in inflammatory processes is probably achieved by the upregulation of L-selectin ligands. On the basis of indirect evidence, yet unidentified ligands were upregulated by cytokine activation on human endothelial cells (39, 305). Furthermore, L-selectin is an important adhesion molecule in the so-called "secondary tethering" process that describes the rolling of leukocytes on blood vessel wall-associated leukocytes (20) (see sect. IV*B*). This process depends on L-selectin ligands on the endothelium-associated leukocytes, most likely P-selectin glycoprotein ligand-1 (PSGL-1) (357; see sect. III*B1*).

On lymphocytes as well as on neutrophils, cell activation causes rapid downregulation of L-selectin within minutes (171), by proteolytic activity cleaving L-selectin at an extracellular site proximal to the cell membrane (228). Proteolytic shedding occurs on neutrophils within 1–5 min and can be induced by a variety of chemoattractants and activating factors such as C5a, FMLP, leukotriene B_4, IL-8, TNF, granulocyte-macrophage colony stimulating factor (CSF), and calcium ionophores (122, 156, 171), but not by granulocyte CSF, macrophage CSF, IL-1, or interferon-γ (122). Furthermore, L-selectin shedding is also

stimulated by cross-linking L-selectin with immobilized MAb (257) and by incubating neutrophils with IL-1-activated HUVEC monolayers for 30 min (299).

Proteolytic shedding of L-selectin from the cell surface leaves an intact 6-kDa transmembrane cleavage fragment on the cell surface that can be detected with a serum against the cytoplasmic tail of L-selectin (158). The cleavage site was determined to be located between Lys-321 and Ser-322. Although the membrane proximal region of L-selectin was found to be essential for cleavage, extensive mutations of this region revealed an extremely relaxed sequence specificity surrounding the cleavage site (60, 227). L-selectin shedding is resistant to a large variety of protease inhibitors such as inhibitors of serine proteases, metalloproteases, aspartic proteases, and cysteine proteases. Finally, hydroxamic acid-based metalloprotease inhibitors were found to be able to block proteolytic shedding of L-selectin (13, 24, 95, 270), revealing that the proteolytic activity which led to L-selectin shedding was based on a metalloprotease.

As soon as L-selectin was found to be rapidly lost from the surface of leukocytes after activation, it was speculated that L-selectin shedding facilitates detachment of leukocytes from the endothelial cells as they start migrating through the endothelial cell layer. The identification of protease inhibitors that could block the shedding allowed the testing of the functional significance of this process in leukocyte rolling. In an elegant study, Walcheck et al. (356) showed that neutrophils, rolling on immobilized L-selectin ligands (purified peripheral lymph node addressins, PNAd, see sect. IV*B2*), rolled at considerably lower velocities when treated with a hydroxamic acid-based protease inhibitor. The neutrophil accumulation rate increased. These studies suggest that L-selectin shedding occurs within seconds after stimulation and that L-selectin shedding is an important determinant for rolling velocities. However, in more complex systems, a shedding-blocking inhibitor could not influence the rate of initial attachment, rolling velocity, or transendothelial migration of neutrophils incubated with TNF-activated HUVEC monolayers under flow conditions (5). Thus the means by which regulated shedding of L-selectin contributes to the physiological process of leukocyte extravasation has not yet been established in all detail. Interestingly, it was recently demonstrated that the intracellular association of calmodulin with L-selectin regulates L-selectin shedding (159).

Rapid shedding was also observed for other important cell surface molecules, such as TGF-β, IL-6-receptor, angiotensin converting enzyme, the β-amyloid precursor protein, TNF-α, and several other proteins. Interestingly, a Chinese hamster ovary (CHO) cell mutant defective in the ability to process pro-TGF-β was also not able to shed L-selectin and the IL-6 receptor (13). Recently, the protease that cleaves the membrane-bound TNF-α precursor and releases mature TNF-α from the cell surface was identified and cloned as a disintegrin metalloproteinase (33, 236). This protease, called the TNF-α converting enzyme (TACE), can also be blocked by hydroxamic acid-based inhibitors, but unlike the L-selectin shedding enzyme, TACE can also be blocked by EDTA. Tumor necrosis factor-α converting enzyme is a protein of 85 kDa, contains a disintegrin domain, and is a member of the family of mammalian adamalysins or ADAM (371); TACE is certainly a good candidate for a protease that is involved in L-selectin shedding. It was found that thymocytes from mice deficient in the TACE gene failed to shed L-selectin; however, in a cell-free assay, TACE could not directly cleave L-selectin, possibly arguing for an indirect involvement of TACE in L-selectin shedding (298).

B. Physicochemical and Biophysical Parameters of Selectin-Ligand Interactions

The recruitment of leukocytes from the rapidly flowing bloodstream is a special form of contact formation between cells that requires special molecular mechanisms. The selectins seem to be ideally suited for this purpose. To support leukocyte rolling, selectins have been proposed to have rapid bond association (k_{on}) and dissociation (k_{off}) rate constants and special mechanical properties linking tensile forces and bond dissociation (78, 128, 193). It was often argued that the affinity of the selectins for their ligands does not need to be high. Indeed, selectins have been shown to bind synthetic oligosaccharides such as the tetrasaccharides sialyl Lewis^x (sLe^x) [NeuAcα-2,3Galβ1,4 (Fucα1,3)-GlcNAc] or its stereoisomer sialyl Lewis^a (sLe^a) with very low affinities [dissociation constant (K_d) 0.1–5 mM] (40, 67, 99, 147, 244, 287). Other, more complex carbohydrate compounds such as the tetraantenary *N*-linked carbohydrate with an unusual sialylated di-Le^x on one arm (261) were estimated to bind with a 1,000 times higher affinity to E-selectin. Soluble recombinant forms of P-selectin (344) and of E-selectin (136) were reported to bind leukocytes with affinities of a $K_d \geq 1$ μM. However, it was not completely ruled out that these measurements were possibly influenced by the presence of oligomeric forms of the E- and P-selectin molecules.

Very recently, affinity constant and binding kinetics were determined for the interaction of L-selectin with its soluble glycoprotein ligand glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) (245). With the use of surface plasmon resonance, it was shown that a soluble monomeric form of L-selectin binds to purified immobilized GlyCAM-1 with a K_d of 108 μM. L-selectin dissociates from GlyCAM-1 with a very fast dissociation rate constant of ≥ 10 s^{-1}. The calculated association rate constant is $\geq 10^5$ $M^{-1} \cdot s^{-1}$. Similar studies with a soluble monomeric form of human P-selectin and isolated purified PSGL-1

TABLE 2. *Binding parameters for the interactions of selectins with their ligands*

Selectin	Ligand	K_d	k_{on}, $M^{-1} \cdot s^{-1}$	k_{off}, s^{-1}	Reference No.
L-selectin	GlyCAM-1 purified from mouse serum	108 μM	$\geq 10^5$	≥ 10	245
P-selectin	PSGL-1 from human neutrophils	200 nM	$\geq 7 \times 10^6$	≥ 1.5	226
P-selectin	19-Amino acid glycopeptide of PSGL-1 NH_2 terminus	~800 nM	ND	ND	57

Binding parameters were determined based on surface plasmon resonance measurements. K_d, dissociation constant; k_{on}, association rate constant; k_{off}, dissociation rate constant; GlyCAM-1, glycosylation-dependent cell adhesion molecule; PSGL-1, P-selectin glycoprotein ligand-1.

from human neutrophils revealed a K_d of 200 nM, a k_{on} of $\geq 7 \times 10^6$ $M^{-1} \cdot s^{-1}$, and a k_{off} of ≥ 1.5 s^{-1} (226). With the use of a 19-amino acid, sulfated PSGL-1 glycopeptide and a recombinant form of P-selectin consisting of the lectin and the EGF domain, a K_d of ~800 nM was determined (57; see Table 2).

In addition to fast dissociation rates, a high tensile strength of the selectin-ligand bonds was suggested to support the rolling function of the selectins (9). Measurements were performed in laminar flow chambers by visualizing tethering and release of neutrophils on lipid bilayers containing incorporated P-selectin at a density at which the cells did not roll but transiently adhered (tethered) to the support. The kinetics of these transient binding events (tethers) were analyzed. Because flow subjects the neutrophils to a shear force that increases the k_{off}, the k_{off} in the absence of an applied force ("intrinsic" k_{off}) was estimated by extrapolation to zero flow rate. This intrinsic k_{off} was determined for P-selectin as 1 s^{-1}. The bond interaction distance was determined as 0.5 Å; this is the distance at which separation of selectin and ligand weakens their interaction (9). Similar measurements for L-selectin, using L-selectin expressing neutrophils flowing over substrates coated with L-selectin ligand (PNAd), revealed an intrinsic k_{off} of ~7 s^{-1} (6), which is in very good agreement with the solution k_{off} determinant for the L-selectin/GlyCAM-1 interaction, obtained by surface plasmon resonance measurements (245). L-selectin-mediated leukocyte rolling is clearly faster than rolling mediated by E- or P-selectin (153, 182, 272). These rolling kinetics are in excellent agreement with the ~10 times higher k_{off} of L-selectin versus E- or P-selectin. The specialization of the selectins, cell rolling and tethering, was elegantly demonstrated when leukocyte rolling on immobilized antibodies against Le^x and sLe^x was examined (61). In contrast to selectins, antibodies supported rolling only within a restricted range of site densities and wall shear stresses, outside of which firm adhesion or detachment occurred (61). On the basis of in vitro adhesion assays under flow, other adhesion molecules were shown to support leukocyte rolling, such as tenascin (65), very late antigen (VLA)-4/VCAM-1, and $\alpha_4\beta_7$/mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (29) an CD44/hyaluronan (64, 76). The physiological role of tenascin and CD44/hyaluronan in leukocyte emigration from blood vessels is yet unclear.

The molecular dynamics of the transition from L-selectin- to β_2-integrin-dependent neutrophil adhesion was analyzed under defined hydrodynamic shear (330). Neutrophils were allowed to aggregate (a process which depends on L-selectin and β_2-integrin) in a cone-plate viscosimeter. From this study, the binding kinetics of selectin and integrin appear to be optimized to function at discrete shear rate and stress, providing an intrinsic mechanism for the transition from neutrophil tethering to stable adhesion (330). A mathematical model for cellular aggregation under these conditions was formulated (243).

Although the flowing bloodstream drags on leukocytes that try to bind to the blood vessel wall, surprisingly, it was found that shear above a critical threshold was necessary to promote and maintain rolling interactions through L-selectin (97). Although this was first thought to be a special requirement for L-selectin and not for E- or P-selectin, it was recently reported that all three selectins share this requirement for a threshold level of fluid shear (192). This could even be demonstrated in vivo in L-selectin-deficient mice, under conditions where rolling was exclusively mediated by P-selectin (192). It was suggested that at low shear forces "the fluid shear may generate a moment which induces additional bond formation as the cell experiences a torque into the wall of the flow chamber during the lifetime of existing bonds. . . . Fluid shear may stabilize leukocyte rolling by deforming the cell slightly after the first bond cluster forms, thereby increasing the time and cell/substratum contact area to favor further bond formation" (192).

C. How the Cell Surface Distribution of a Selectin Affects Its Function

Soon after the selectins were found to be responsible for the initiation of leukocyte endothelial contact formation, leading to the rolling of leukocytes (193, 202, 351), L-selectin was found to be located on tips of microvilli,

as was first examined by immunogold electron microscopy on frozen thin sections of neutrophils (266) and then by immunogold scanning electron microscopy (91, 129). On the basis of the analysis of sectioned cells, 78% of neutrophil, 72% of monocyte, and 71% of lymphocyte L-selectin was observed on microvilli (48).

The presentation of adhesion receptors on microvilli has been shown to facilitate the establishment of primary interactions between leukocytes and the vascular lining under physiological shear forces (352). This report examined the distribution of L-selectin and CD44 on transfected lymphoid cells. Although L-selectin was concentrated on microvilli, CD44 was restricted to the planar cell surface. With the use of chimeric molecules, it was demonstrated that the transmembrane and intracellular domains of CD44 targeted the extracellular part of L-selectin to the planar body. Analogously, the extracellular part of CD44 was directed to microvilli when fused to the transmembrane and intracellular domain of L-selectin. These experiments establish a mechanism for the specific targeting or anchoring of surface proteins to the two cell-surface domains on leukocytes. In addition, this study suggests that the expression of L-selectin on microvilli strongly improves its ability to initiate contacts of the transfected cells to ligand bearing substrates under flow. L-selectin-CD44 chimeric molecules that were excluded from microvilli initiated leukocyte rolling under flow only very inefficiently. In agreement with these findings, other adhesion molecules that have been demonstrated to mediate cell contact formation under flow are also found to be enriched on microvillous processes. This was shown for the P-selectin ligand PSGL-1 (234) and for the integrin $\alpha_4\beta_7$ (29). In contrast, β_2-integrins, which are essential for leukocyte adhesion to endothelium but which are not able to initiate contacts under flow conditions, are excluded from microvillous processes.

Deletion of the COOH-terminal 11 amino acids of the 17-amino acid cytoplasmic tail of L-selectin eliminated binding of transfected cells to HEV in frozen sections of lymph nodes and also abolished rolling of these cells in vivo in exteriorized rat mesenteric venules (162). Interestingly, carbohydrate recognition was not affected, arguing for a function of the COOH-terminal amino acids in the correct anchoring to the cytoskeleton and for the importance of cytoskeleton interactions for the rolling process. In line with these results, treatment of the cells with cytochalasin B, which disrupts actin microfilaments, had the same effects as observed for the mutant (162). Pavalko et al. (262) showed that L-selectin binds directly to α-actinin. Lack of the COOH-terminal 11 amino acids of L-selectin disrupted the binding of L-selectin to α-actinin. However, this mutant L-selectin still localized normally to the microvillar projections on the cell surface (262). Thus L-selectin does not only have to be positioned on the tips of microvilli to be able to support leukocyte rolling; it also has to be anchored to the cytoskeleton. It is still unknown which molecular interactions lead to the presentation of L-selectin on the microvilli tips.

Direct binding of E- or P-selectin cytoplasmic tails to α-actinin was not observed, although similar conditions were tried as had been successfully used for L-selectin (163). Furthermore, deletion of the cytoplasmic tails of E- and P-selectin neither affected the cell surface expression of these selectins nor their adhesion function as was tested in nonstatic/rotation assays with transfected COS cells (163). However, a function for the interaction of E-selectin with the cytoskeleton could well be important for events downstream of the leukocyte docking process. Yoshida et al. (380) showed that leukocyte binding to activated HUVEC could increase the fraction of E-selectin that was detergent insoluble, i.e., could not be extracted and presumably was associated with the cytoskeleton. This was not seen with a cytoplasmic deletion mutant of E-selectin. In addition, cross-linking of E-selectin with antibodies allowed to coprecipitate cytoskeleton proteins such as α-catinin, vinculin, paxillin, filamin, and even focal adhesion kinase (FAK) in E-selectin immunoprecipitations. These proteins did not copurify if the cross-linking step was omitted (380). Cytoskeletal linkage of E-selectin might be important for cell-cell signaling or for mechanical stabilization of leukocyte-endothelial interactions immediately after the first interaction of leukocytes with E-selectin.

Examining the effect of cell shape on neutrophil tethering and rolling on endothelial selectins revealed that microvilli are essential to allow neutrophil's initial binding to a support under flow conditions, but are not important for the subsequent rolling movement (96). Disruption of microfilaments by cytochalasin B caused an ~50% reduction of the numbers of microvilli, whereas hypotonic swelling reduced the number of these protrusions by 80%. Both treatments almost completely wiped out tethering, but when tethering was allowed at subphysiological levels of shear stress of ≤ 0.35 dyn/cm^2, subsequent increase of shear stress removed control cells at much lower shear from the support than cytochalasin B-treated or hypotonically swollen cells.

IV. SELECTIN LIGANDS: CARBOHYDRATE MOIETIES THAT ARE PRESENTED ON A SELECTED NUMBER OF CARRIER MOLECULES

Unlike most other cell adhesion molecules that bind to their ligands on the basis of protein-protein interactions, the ligands of the selectins are composed of a scaffold protein, or perhaps a lipid carrier molecule, which is modified by certain carbohydrates. Thus the carrier molecule is not sufficient to define a selectin ligand; it needs

to be expressed in the right cellular background that provides the necessary repertoire of glyosylation enzymes which confer selectin-binding activity to the carrier molecule. Lectin recognition systems have been described as functional triads: receptors, ligands, and carriers. The receptors are the lectins, the ligands are the oligosaccharides, and the carriers are molecules on which these oligosaccharides are optimally assembled and presented for binding to the lectin (68). Because the physiological binding partners of the selectins are most likely glycoproteins and most publications in the field refer to such binding partners as ligands, the term *ligand* will be used for the glycoproteins that bind to the selectins throughout this review.

Some of the uncertainty about which glycoproteins are the physiological selectin ligands is based on the fact that oligosaccharides that can bind with some affinity to a selectin can indeed transfer selectin-binding activity to many different carrier proteins (347). Even BSA, chemically modified with the selectin binding oligosaccharide sLe^x, can bind to a selectin (25) (27). Furthermore, depending on the technique with which binding is detected, ligands with physiologically irrelevant, low affinities could be mistaken for physiological ligands (347).

Physiological ligands are most likely distinct glycoproteins that actively take part in the formation of the ligand molecule. Evidence is evolving that oligosaccharides are not the only modifications that are necessary for selectin binding on a certain carrier, as was shown for the tyrosine sulfation of the P-selectin ligand PSGL-1 (269, 284, 370). Furthermore, some carrier molecules seem to be preferential targets for the generation of certain carbohydrate modifications that enable this molecule to bind to a selectin, as was shown for the E-selectin ligand-1 (ESL-1) and for PSGL-1 (36, 369, 385). Thus a limited number of discrete glycoproteins, modified with certain oligosaccharides and in some cases with other posttranslational modifications, define physiological ligands of the selectins.

This section mainly focuses on those glycoprotein ligands, which have been identified as so-called high-affinity ligands, based on their specific and selective isolation from cellular detergent extracts with selectin affinity probes (Fig. 3). Several excellent reviews were published recently about these ligands (220, 280, 347, 361). Here we focus on the most recent results.

A. Glycolipids as Binding Partners for the Selectins

Numerous reports have documented that glycolipids bind specifically to the selectins. Fucosylated monosialogangliosides mediating binding to E-selectin were isolated from human myeloid cells (239, 323). Sialyl $Lewis^x$-carrying glycolipids and sialyl $Lewis^a$-carrying neoglycolipids were shown to support rolling of E-selectin-transfected cells and of L-selectin-expressing leukocytes (7). An unusual class of sulfated glycosphingolipids, sulfoglucuronyl-containing neolactosyl-ceramides (SGNL lipids), that are recognized by the mouse MAb HNK-1 bind to L- and P-selectin, but not to E-selectin (242). Sulfatides bind to P-selectin (14) as well as to the other two selectins (242), although they only support weak tethering of L-selectin-expressing cells and do not support rolling (7). It is still uncertain to what extent glycolipids are relevant for selectin-mediated adhesive events. Because several selectin ligands are mucins that are rigid and highly extended molecules, likely to project from the leukocyte surface, and L-selectin is exposed on the tips of microvilli, it has been emphasized that projection above the cell surface may facilitate cellular interactions under flow (193, 266). Furthermore, several studies have demonstrated that protease treatment blocks the binding of myeloid cells to P-selectin (185, 315). Treatment of myeloid cells with tunicamycin blocked cell binding to E- and P-selectin (185). This argues against glycolipids as ligands involved in selectin-mediated leukocyte capturing. However, it is possible that glycolipids might be involved in the rolling process after initial tethering has occurred, strengthening selectin-mediated cell contacts during rolling.

B. High-Affinity Glycoprotein Ligands of the Selectins

1. P-selectin ligands

In contrast to the other selectin ligands, PSGL-1 was identified by expression cloning, using a P-selectin-Ig fusion protein as panning reagent and an expression library from the human monocytic cell line HL-60 transfected into COS cells (283). The same protein had been identified as a 250-kDa disulfide-linked dimeric protein 1 year earlier by affinity isolation using purified P-selectin as affinity probe (235). This protein was demonstrated to be identical to PSGL-1 (233). A 230/130-kDa pair of glycoproteins, which had been identified on mouse neutrophils by affinity isolation with P-selectin-Ig and E-selectin-Ig chimeras (196), turned out to be the mouse homolog of PSGL-1 (35) that had been cloned with the help of a human PSGL-1 cDNA probe (376). The PSGL-1 polypeptide chain is broadly expressed on cells of myeloid, lymphoid, and dendritic lineage and on some nonhematopoetic cells, such as the epithelium of the fallopian tube and sporadically on microvascular endothelium in some pathologic tissues (189). P-selectin glycolipid-1 was also reported to be expressed on the zona pellucida of porcine oocytes, and P-selectin was found on the acrosomal membrane of porcine sperm cells (108).

P-selectin glycolipid-1 requires carbohydrate modifica-

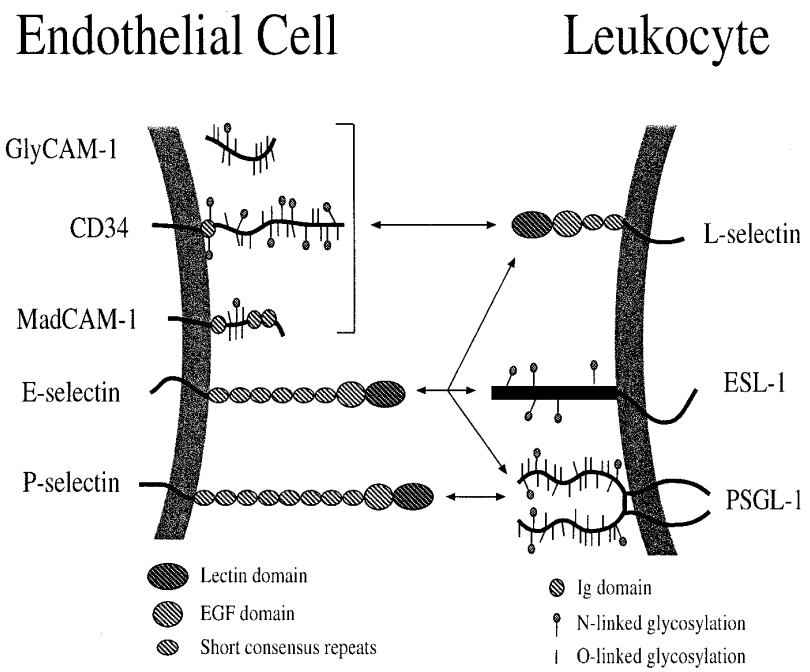


FIG. 3 Selectin ligands that have been identified by affinity isolation with respective selectin as affinity probe. Except E-selectin ligand-1 (ESL-1), depicted ligands are sialomucins or contain at least a sialomucin domain. L-selectin ligand mucosal addressin cell adhesion molecule-1 (MAdCAM-1) was originally found as a ligand for integrin $\alpha_4\beta_7$. Sequencing revealed a sialomucin domain. A subpopulation of MAdCAM-1 molecules in high endothelial venule of mesenteric venules can indeed be expressed as an L-selectin binding glycoform, carrying posttranslational modifications that define peripheral node addressins. L-selectin is a major carbohydrate-presenting ligand for E-selectin on human neutrophils; however, L-selectin of human lymphocytes or mouse neutrophils is unable to bind E-selectin. P-selectin glycoprotein ligand-1 (PSGL-1) is the only selectin ligand so far that has been demonstrated to mediate leukocyte rolling on endothelium (251) and leukocyte recruitment into inflamed tissue in vivo (35, 37). Ig, immunoglobulin; GlyCAM-1, glycosylation-dependent cell adhesion molecule.

tions such as sialic acid and fucose (283) as well as branched carbohydrate side chains generated by the core-2 β-1,6-*N*-acetyl-glucosaminyltransferase (core-2 enzyme) for its binding activity (180, 204, 233, 369). Detailed analysis of the *O*-linked carbohydrate side chains revealed an interesting trifucosylated *O*-glycan, β-1,6-linked to core-2 structures on PSGL-1 of HL-60 cells, which was not found on a control sialomucin of this cell line (369) (Fig. 4). In addition to carrying the correct oligosaccharides, PSGL-1 needs to be sulfated at one of the three tyrosine residues at its NH_2 terminus for binding to P-selectin (269, 284, 370) and probably also for binding to L-selectin (303). P-selectin glycolipid-1, as almost all other glycoprotein ligands of the selectins, is a sialomucin. The clusters of *O*-linked carbohydrate side chains make it a rigid and extended molecule (203). The protein is processed in the endoplasmic reticulum by a paired basic amino acid converting enzyme (PACE) that cleaves off the pro-peptide (345). The following 19 amino acids are important for the binding of PSGL-1 to P-selectin, since they carry the tyrosine residues that can be sulfated, harboring the epitopes of several adhesion blocking antibodies against human and mouse PSGL-1 (35, 37, 234, 300, 376). Furthermore, cleaving the 10 NH_2-terminal amino acids of the mature form of human PSGL-1 with a cobra venom metalloproteinase, mocarhagin, abolishes binding to P-selectin (77).

P-selectin glycolipid-1 is the best-characterized selectin-ligand to date, which fulfills all criteria for a physiologically relevant ligand. It is the major ligand for P-selectin on human neutrophils, as the MAb PL-1 to PSGL-1 completely blocks rolling of these cells on P-selectin (234), and it is the major ligand for P-selectin on stimulated T cells (345). A soluble, recombinant form of human PSGL-1, produced in FucTIII- and core-2 enzyme-transfected CHO cells, can block ischemia/reperfusion injury in the rat (328). The MAb PL-1 can also block rolling of human neutrophils in venules of exteriorized rat mesentery (251). Polyclonal antibodies against mouse PSGL-1 were found to block the entry of Th1 cells into inflamed skin of the mouse (37), and a MAb against mouse PSGL-1 was able to inhibit the migration of neutrophils into chemically inflamed peritoneum (35). Thus, in vitro as well as in vivo, PSGL-1 has been demonstrated to be necessary for neutrophil and lymphocyte binding to P-selectin, despite the fact that PSGL-1 only presents $<1\%$ of the sLe^x on the cell surface (204, 249, 369). The demonstrated functional importance of PSGL-1 establishes that a single glycoprotein can be responsible for the generation of high-affinity and biologically relevant cellular interactions with a selectin, despite the lectin character of the selectins and their ability to bind to many sLe^x-bearing glycoproteins when presented at high density in certain in vitro assays. Because PSGL-1 could be affinity isolated as a major ligand by P-selectin-based affinity probes (196, 235), this ligand is a good example for the validity of this approach to identify physiologically relevant ligands.

E-selectin can also bind to PSGL-1, as has been demonstrated in numerous reports (15, 114, 196, 204, 233, 259). This binding requires the same carbohydrate modifications as the binding to P-selectin; however, sulfation of tyrosine residues is not necessary for the binding to E-selectin (114, 204). With the use of recombinant fragments of human PSGL-1, coated on microspheres, it was shown that P- and E-selectin both bind to the first 19 amino acids

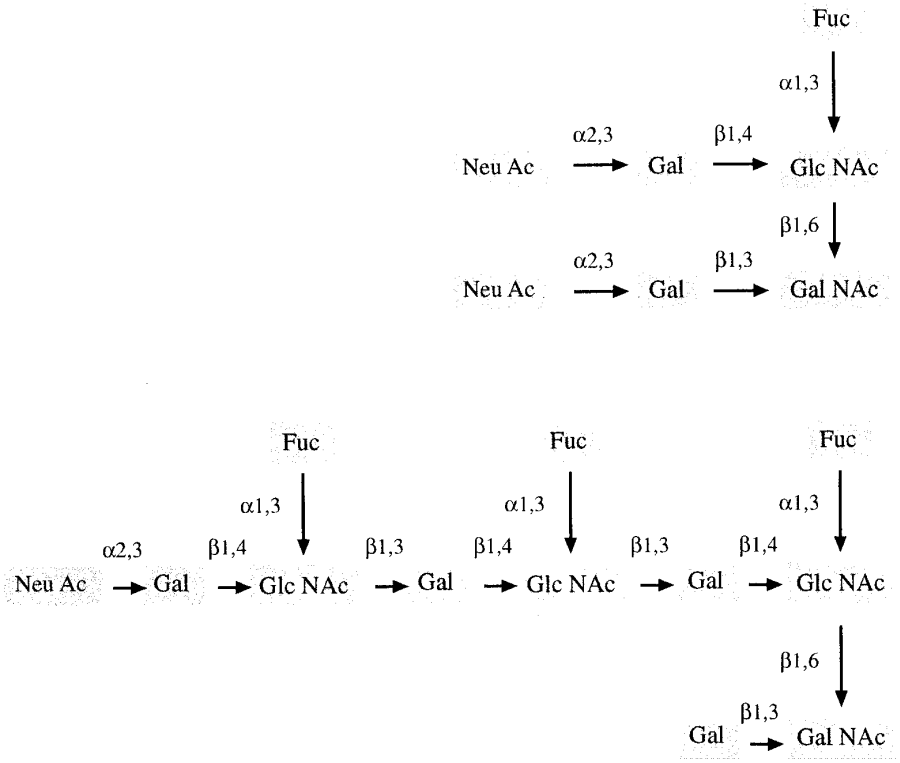


FIG. 4 Analysis of *O*-glycans of human PSGL-1, isolated from HL-60 cells, revealed a minority of α-1,3-fucosylated structures that occurred as 2 core-2-based species, depicted here (369).

of PSGL-1 and that E-selectin can additionally bind to a site located between amino acids 19 and 148 (114). Whether PSGL-1 is indeed relevant for the binding of cells to human E-selectin has been questioned. First, the binding affinity of human PSGL-1 to human E-selectin was reported to be 50-fold lower than to P-selectin (233). Second, the binding of human T-cell clones to E-selectin was reported to be independent of sialomucins (10). Third, the inhibitory effect of the anti-PSGL-1 MAb PL-1 on the rolling of human neutrophils on E-selectin (259) was not due to the blocking of direct interactions of PSGL-1 with E-selectins, but rather due to the blocking of leukocyte-leukocyte interactions (258). This interaction between leukocytes, the so-called secondary tethering, is mediated in part by PSGL-1 (357). Thus evidence for a direct involvement of PSGL-1 in cellular interactions with E-selectin is still lacking.

Despite this lack of evidence for the physiological relevance of PSGL-1 as a ligand for E-selectin, it was shown recently that PSGL-1 on activated T cells is the major if not even the only glycoprotein carrier for a carbohydrate epitope, called cutaneous lymphocyte antigen (CLA) (36, 103), which is thought to be relevant for lymphocyte binding to E-selectin but not to P-selectin. This carbohydrate epitope, defined by the MAb HECA452, is further described in section IV*A*. Borges et al. (36) used a CD8+ mouse T-cell clone that expressed CLA transiently after antigen-specific activation. Cells could only bind to E-selectin when they expressed CLA, whereas cell binding to P-selectin was independent of CLA expression. P-selectin glycolipid-1 was found to be the only ligand that could be affinity isolated from these cells with E-selectin-Fc, and this was only observed at an activation stage when PSGL-1 was modified with the CLA carbohydrate epitope (36) (see also sect. IV*B*). Binding of PSGL-1 to P-selectin occurred in the presence as well as in the absence of the CLA epitope, arguing for the independent regulation of the binding activity of PSGL-1 for E- and P-selectin on activated T cells.

The P-selectin-binding glycoform of PSGL-1 on CD4+ T cells seems to be induced by activation stimuli which also lead to the differentiation of these cells to Th1 cells. In a mouse DTH model, it was recently shown that Th1 cells, generated by in vitro differentiation of primary isolated mouse CD4+ T cells, can migrate into inflamed skin, whereas Th2 cells cannot. Th1 cell migration into the skin was almost completely blocked by antibodies against E- and P-selectin (17). In agreement with this, only Th1 cells, but not Th2 cells, bound readily to the P-selectin-Ig chimera. Biochemical analysis revealed that Th1 and Th2 cells carried similar amounts of PSGL-1 molecules on their cell surface, but only the PSGL-1 on Th1 cells was modified in a way that allowed binding to P-selectin (37).

In addition to P-selectin and perhaps E-selectin, L-selectin seems to be an important receptor for PSGL-1. In adhesion assays under flow, neutrophils (20) and also lymphocytes (155) were found to roll on leukocytes that had already established contact to the selectin coated sup-

port. This secondary tethering (8) was found to be at least in part dependent on L-selectin on the adherent leukocytes (8, 20) and on PSGL-1 as an L-selectin ligand on the flowing leukocytes (303, 342, 357), although one of the reports did not confirm PSGL-1 as an L-selectin ligand in this process (8).

Platelets were reported to be involved in the interactions of lymphocytes with HEV of mouse peripheral lymph nodes, thereby mediating lymphocyte homing. P-selectin on platelets was suggested to mediate this process. It was shown that activated platelets expressing P-selectin on their surface could "rescue" lymphocyte homing in mice treated with the anti-L-selectin MAb MEL14 (80). Circulating activated platelets could reconstitute lymphocyte homing and immunity in L-selectin-deficient mice (79). Platelets bound via P-selectin to PSGL-1 on mononuclear lymphocytes and to peripheral node addressin on HEV, suggesting that the peripheral node vascular addressins can function as P-selectin ligands.

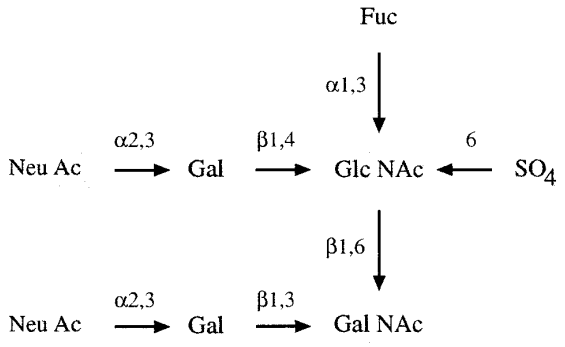
Another ligand that was described for P-selectin is CD24, also called heat-stable antigen (HSA). Although it has not yet been possible to directly affinity isolate this molecule from myeloid cells with a P-selectin probe, its binding to P-selectin has been analyzed in several reports. Heat-stable antigen is a cell surface glycoprotein expressed by neutrophils, B cells, immature thymocytes, and red blood cells. It consists of a very small polypeptide chain of only 27 amino acids, which is phospholipid anchored and highly glycosylated resulting in molecular masses of up to 70 kDa in lymphoid cells. Glycosylation varies strongly among different types of leukocytes. Different isoforms of HSA were purified from different types of leukocytes, coated on microtiter plates, and binding of E- and P-selectin-Ig chimeras was analyzed. No binding was observed with E-selectin-Ig, whereas P-selectin-Ig chimera bound well to HSA from neutrophils, B cells, and a monocytic cell line, and binding was not seen to HSA from red blood cells (285). Antibodies against mouse HSA could partially block the binding of mouse neutrophils and monocytic cells to LPS-activated mouse endothelioma cells (bEND.3) and blocked the binding of HSA-coated latex beads to endothelioma cells or platelets (3). All these interactions were also sensitive to a P-selectin blocking antibody. The relevance of HSA/CD24 for the binding of neutrophils to P-selectin is not yet clarified, especially since PSGL-1 appears to be the dominant ligand. However, a breast and a small cell lung carcinoma cell line (both of human origin) that are negative for PSGL-1 were shown to bind to P-selectin. CD24 purified from these cells and coated onto latex beads bound to P-selectin-Ig chimeras and P-selectin-transfected cells in a Ca^{2+}-dependent way (4). CD24 transfected human adenocarcinoma cells showed increased binding to P-selectin-expressing platelets. Adding soluble, purified CD24 to the assay and removing CD24 with phospholipase C from the cell surface was recently shown to block rolling of the PSGL-1 negative breast carcinoma cell line on P-selectin; the ability to roll was positively correlated with the expression level of CD24 (2). It is possible that CD24 is also involved in the rolling of the PSGL-1-negative human colon carcinoma cell line KM12-L4 on P-selectin (113).

2. L-selectin ligands

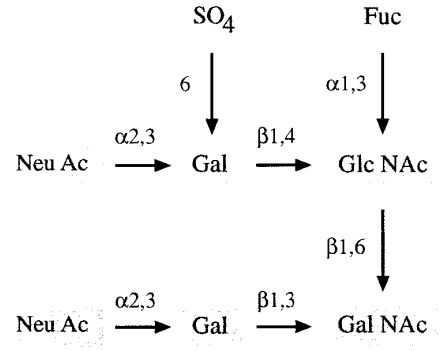
Four glycoprotein ligands have been identified for L-selectin: GlyCAM-1, CD34, MAdCAM-1, and Sgp200 (the latter is not yet cloned). All of them are expressed as L-selectin-binding glycoforms by HEV of lymph nodes. Both GlyCAM-1 and CD34 were identified by affinity isolation with an L-selectin-Ig chimera from $^{35}SO_4$-labeled mouse lymph node tissue (146). The two proteins were first named Sgp50 and Sgp90, according to their apparent molecular weights. Sufficient quantities of these ligands were purified, and microsequencing led to the identification of Sgp50 as a new soluble sialomucin (GlyCAM-1) (187) and Sgp90, a known antigen (CD34) (22).

Glycosylation-dependent cell adhesion molecule-1 is a 50-kDa secreted sialomucin that is specifically synthesized by HEV endothelial cells where its expression is affected by afferent lymphatic flow (224). It is also expressed as a nonbinding glycoform by mammary epithelial cells, where its expression is induced by lactation (85, 246). The gene for murine GlyCAM-1 was cloned and characterized (84, 87), and the GlyCAM-1 homologs were cloned in the rat (86) and in bovine (148, 149). Glycosylation-dependent cell adhesion molecule-1 can bind to all three selectins (225, 297).

The posttranslational modifications of GlyCAM-1 have been intensively studied. Glycosylation-dependent cell adhesion molecule-1 needs to be sulfated on oligosaccharide side chains to bind to L-selectin (145). The major sulfated mono- and disaccharides on GlyCAM-1 were identified as Gal-6-SO_4, GlcNAc-6-SO_4, (SO_4-6)Galβ1→4GlcNAc, and Galβ1→4(SO_4-6)GlcNAc (131). With the use of lectins and exoglycosidases, a major capping structure of GlyCAM-1 was identified that contained all three structural elements known to be critical for L-selectin binding: sialaic acid, fucose, and sulfate. This capping structure was determined as 6′-sulfated sialyl Lewis^x: Siaα2→3(SO_4-6)Galβ1→4(Fucα1→3)GlcNAc (134). By examining the complete structure of β-eliminated oligosaccharide side chains of GlyCAM-1, two sulfated *O*-glycans were identified which represent 6′-sulfo-sLe^x (with SO_4 linked to position 6 of Gal) and 6-sulfo-sLe^x (with SO_4 linked to position 6 of GlcNAc) (133) (Fig. 5). Both these oligosaccharides were found in core-2 structures, i.e., β1→6 linked to GalNAc. Comparison of various sulfated Le^x analogs revealed that 6-sulfo-sLe^x can block the binding of L-selectin-Ig to GlyCAM-1 better than sLe^x or 6′-sulfo-sLe^x (287). In another report, in contrast, CHO cells modified on their cell



Core-2 based 6 sulfo sialyl Lewis x



Core-2 based 6´ sulfo sialyl Lewis x

FIG. 5. Major capping structures of mouse GlyCAM-1 were found to be core-2-based *O*-linked 6-sulfo sialyl Lewis x and 6′-sulfo sialyl Lewis x (133). 6-Sulfo sialyl Lewis x was found to block binding of L-selectin Ig to GlyCAM-1 (287).

surface with 6′-sulfo-sLe^x were reported to bind to plastic-coated L-selectin-Ig chimera, whereas 6-sulfo-sLe^x-modified cells did not (341). Modification of the surface of the CHO cells with these oligosaccharides had been achieved by in vitro transfer of either of the two oligosaccharides, linked to C-6 of the fucose residue of GDP-fucose using a milk-derived fucosyltransferase. Analyzing the binding of recombinant soluble forms of the selectins to immobilized carbohydrate molecules, Koenig et al. (174) reported that 6′-sulfo-sLe^x was not a better inhibitor of L-selectin than sLe^a or 3′-sulfo-Le^x. However, inhibitory activities as measured in these assays may not exclusively reflect binding affinities.

In an attempt to identify endothelial antigens on HEV in peripheral lymph nodes (PLN) or in the mucosa associated Peyer's patches (PP), MAb were raised against these tissues. Two MAb were found, MECA79 (322) and MECA367 (241, 321), which blocked lymphocyte binding to HEV of PLN or PP, respectively. The antigens were described as "vascular addressins." The MAb MECA79 defined an HEV-specific carbohydrate epitope that was seen on a panel of glycoproteins, including GlyCAM-1 and CD34 (146) as well as Sgp200 (132). Binding of the MAb MECA79 requires the presence of SO_4 on the antigen, whereas neither sialic acid (132) nor fucose (217) is necessary for binding.

Glycosylation-dependent cell adhesion molecule-1 is a secreted glycoprotein (49) that is not found on the cell surface but in cytoplasmic granula (170) and in mouse serum at 1.3–1.6 μg/ml (297). These localization studies argue against a direct function of GlyCAM-1 as cell adhesion molecule. However, elegant studies have shown recently that GlyCAM-1 binding to lymphocytes can stimulate the activation of β_2-integrins (143) as well as of β_1-integrins (111). Thus GlyCAM-1 could be a secreted regulator, participating in the recruitment of lymphocytes into peripheral lymph nodes. Mice deficient in the gene of GlyCAM-1 (made by L. Lasky and M. Moore at Genentech) have enlarged lymph nodes, but lymphocyte trafficking to PLN still occurs, as was stated in a review by Watson (361).

Glycosylation-dependent cell adhesion molecule-1, the MECA79 antigens (PNAd), and the MECA367 antigen (MAdCAM-1) were recently found to be induced on endothelium in a model of autoimmune response and tumorigenesis (255). In the mouse model, the oncogene Tag was expressed in islet β-cells of the pancreas, which led to hyperplasia with subsequent progression to tumor and lymphocyte infiltration. Glycosylation-dependent cell adhesion molecule-1, PNAd, and MAdCAM-1 were induced on endothelial cells of infiltrated islands but not in tumors, which were devoid of lymphocyte infiltration. In contrast, endothelial adhesion molecules such as platelet endothelial cell adhesion molecule-1, ICAM-1, and VCAM-1 were expressed on endothelium of both noninfiltrated tumors and infiltrated islets. This argues for a possible function of the L-selectin ligands in autoimmune-related lymphocyte infiltration in inflamed tissue.

Glycosylation-dependent cell adhesion molecule-1 is the best characterized L-selectin ligand so far. Less is known about the other ligands. CD34 is expressed throughout the endothelial cells of the vasculature, on hematopoietic precursor cells, on a number of embryonic fibroblast cell lines, and in the brain (21); however, it only seems to be correctly glycosylated for L-selectin recognition in HEV of lymph nodes (22). Human (295) as well as mouse CD34 (47) have been cloned. CD34 is a type I transmembrane sialomucin and belongs to the group of proteins that is recognized by MAb MECA79. MECA79 affinity-purified glycoproteins from human tonsil, immobilized in laminar flow chambers were found to support rolling of lymphocytes. CD34 was reported to be an important component of these antigens (271). However, genetic disruption of CD34 expression in mice does not give an obvious loss of lymphocyte trafficking to lymph nodes (62).

Mucosal addressin cell adhesion molecule-1 is the mucosal vascular addressin that was defined by MAb MECA367 (321, 241) and cloned in mouse (44), rat (144), macaque, and human (293). It is a ligand for the lymphocyte integrin $\alpha_4\beta_7$ (30). A subpopulation of MAdCAM-1 molecules in HEV of mesenteric lymph nodes carries carbohydrate modifications that are recognized by MAb MECA79 and by L-selectin (26). Mucosal addressin cell adhesion molecule-1 can support rolling of L-selectin-expressing cells (26) as well as of $\alpha_4\beta_7$-expressing cells (19, 29).

Sgp200, the fourth sulfated glycoprotein in mouse HEV which can be affinity isolated with L-selectin Fc chimera (132), is secreted and found in normal mouse serum as well as cell associated (139). Levels of GlyCAM-1 and secreted Sgp200 are downregulated 3–4 days after the induction of an immune response, whereas the levels of the cell-associated ligands, CD34 and Sgp200, remain largely unaltered throughout the immune response (139).

L-selectin-dependent binding of lymphocytes to the CD34+ hematopoetic progenitor cell line KG1a is not mediated by CD34 (256). KG1a cells were found to express a P-selectin-binding glycoform of PSGL-1 (339), and PSGL-1 has been described as ligand for L-selectin (303, 342, 357). Surprisingly, metabolic inhibition of sulfation in KG1a cells did not inhibit the binding of these cells to L-selectin-expressing lymphocytes, suggesting that an unknown, sulfate-independent ligand on KG1a cells may be recognized by L-selectin (281, 282). Evidence for a mucinlike L-selectin ligand activity that is independent of PSGL-1 was recently demonstrated for other human myeloid cells (275).

Several reports have demonstrated L-selectin-mediated binding of leukocytes to cytokine-activated endothelial cells (39, 112, 305). L-selectin-mediated binding of monocytes to unstimulated endothelial cells was partially inhibited by treatment with heparin lyase, indirectly suggesting that heparan sulfate proteoglycans on the endothelial surface can serve as ligands (112). A heparin- or heparan sulfate-containing ligand had been affinity isolated before with an L-selectin affinity probe from cultured calf pulmonary artery endothelial cells (250). However, direct evidence for a physiological role of heparan sulfates as L-selectin ligands is still missing.

3. E-selectin ligands

The structural requirements that are necessary for the recognition of a ligand by E-selectin are different from those necessary for P- and L-selectin. Sulfation, which was found to be essential for P- and L-selectin ligands, is dispensable for E-selectin ligands. Furthermore, several cells can already bind to and even roll on E-selectin just upon transfection with a fucosyltransferase, whereas they do not yet bind to the other two selectins (212, 301). This could either argue for "more simple" recognition structures on E-selectin ligands that are easily expressed on many different scaffold glycoproteins, or for discrete, but broadly expressed scaffold proteins in many different cells. A third, less likely explanation could be that fucosyltransferases might modify glycolipids that might support rolling on E-selectin.

Despite this seemingly lower level of selectivity of E-selectin for various ligands, discrete glycoproteins have been identified by affinity isolation with E-selectin-Ig fusion proteins from total detergent extracts of myeloid cells. Two such glycoprotein ligands were identified on mouse myeloid cells, the ESL-1 (197) and the P-selectin ligand PSGL-1 (196). Interestingly, PSGL-1 expressed on the mouse T cell clone 4G3 was able to bind to P- and E-selectin at early stages of T-cell activation, but at later activation stages, it lost its ability to bind to E-selectin, although it still bound to P-selectin. This shows that the structural requirements for the binding of a ligand to E-selectin can even be more complex than the modifications that are necessary for the binding to P-selectin. Thus E-selectin binds to discrete high-affinity glycoprotein ligands with at least similar high specificity and selectivity as the other two selectins.

E-selectin ligand-1 was found as a 150-kDa glycoprotein on mouse neutrophils and a mouse myeloid cell line (32Dcl3) by affinity isolation with an E-selectin-Ig chimeric fusion protein (197). In contrast to sialomucin-type selectin ligands described so far, ESL-1 requires *N*-linked carbohydrates for binding to E-selectin and only binds to E- but not to P-selectin (196). Whether *N*-linked carbohydrates are necessary for leukocyte binding to E-selectin is controversial, since some inhibitors of glycoprotein processing (e.g., deoxymannojirimycin) did not impair binding of HL-60 cells to E-selectin (195), whereas tunicamycin, an inhibitor of *N*-glycosylation, did inhibit the binding of HL-60 cells to E-selectin (185). Cloning of ESL-1 revealed that it is not a sialomucin and that it contains five putative *N*-glycosylation sites and 16 cysteine-rich repeats (314). Polyclonal antibodies against ESL-1, affinity purified against an ESL-IgG fusion protein, partially inhibited the binding of mouse myeloid cells to E-selectin in nonstatic rotation adhesion assays (314). Most of the ESL-1 protein located on the cell surface (80%) was found on microvillous processes of a lymphoma cell by immunogold labeling and scanning electron microscopy (313). However, in contrast to PSGL-1 and L-selectin, ESL-1 was not found to be concentrated on the tips of microvilli, but rather along the surface of these processes. This may suggest that ESL-1 differs in its function from PSGL-1 and L-selectin. Although the latter two are involved in the capturing of leukocytes to endothelial cells under flow, ESL-1 may function in a step after the capturing. This may strengthen the rolling interaction or, alternatively, this could convey a receptor function monitoring cell binding to E-selectin. To study this, it will be necessary to generate

MAb against ESL-1 that can disrupt the interaction with E-selectin. Attempts have shown that the generation of MAb against ESL-1 is extremely difficult, possibly because the protein is remarkably conserved among species.

In addition to its cell surface expression, ESL-1 is strongly expressed in the Golgi. Furthermore, the protein is expressed in many different cell types including fibroblasts, epithelial cells, and endothelial cells, although only in myeloid cells is ESL-1 expressed as the E-selectin-binding glycoform. Whether it has a function in the Golgi, unrelated to its binding activity for E-selectin, is unknown. A rat homolog, called MG160, was recently identified with an overall amino acid sequence similarity of 98% (116). This protein had originally been identified as a Golgi protein in neuronal tissue and in several other cell types (72, 115), and the human homolog has been recently cloned (237). The function of MG160 is not yet known.

Two other closely related homologs of ESL-1 were cloned that interact with growth factors. A chicken homolog of ESL-1, called CFR, was identified as a receptor for fibroblast growth factor (FGF)-1 and FGF-2 (54) and subsequently cloned (55). It has been suggested that CFR could regulate the intracellular level of exogenously added FGF-1 and FGF-2 (386). Transforming growth factor-β was found in a complex with the hamster homolog of ESL-1, possibly representing a latent TGF-β complex in the supernatant of CHO cells (253). Whether these interactions with FGF or TGF-β are related to the E-selectin binding function of ESL-1 is unknown. Mouse ESL-1, in contrast to the chicken CFR, contains a 70-amino acid domain near its NH_2 terminus for which no equivalent is found in CFR, the rest of the sequence is 94% identical (over 1,078 amino acids). Thus ESL-1 looks like a splicing variant of the mouse homolog of chicken CFR. Interestingly, the 70-amino acid domain contains a peculiar stretch of glutamines interrupted by a few prolines, a putative site for protein oligomerization (109, 319). The hamster and human homologs of mouse ESL-1 contain equivalents to this domain, although the sequence conservation in this domain is much lower than for the rest of the protein.

E-selectin ligand-1 seems to be a protein with more than one function. The other identified selectin ligands, although not as broadly distributed as ESL-1, are also expressed in more cell types than just leukocytes or endothelial cells. It seems to be the rule rather than the exception that proteins that serve a selectin-unrelated function become selectin ligands if expressed in the right environment, i.e., if they are coexpressed with the right combination of glycosyltransferases and/or other protein-modifying enzymes that generate selectin-binding epitopes on these scaffolds. E-selectin ligand-1 is only expressed in myeloid cells as a glycoform that binds to E-selectin. E-selectin ligand-1 seems to be a strongly preferred target molecule for the generation of E-selectin-binding carbohydrate (385). The fucosyltransferases VII and IV, when expressed in CHO cells, generated the carbohydrate epitope HECA452/CLA only on ESL-1 and not on other glycoproteins (see sect. IV*A* for this epitope). Likewise, exclusively ESL-1 could be affinity isolated from such transfected cells with an E-selelctin-Fc fusion protein. In contrast, the fucosyltransferase III generated the HECA452/CLA epitope on a large panel of proteins and enabled these proteins to bind with high affinity to E-selectin (385). This argues for the existence of a glycosylation machinery that selectively generates selectin-binding carbohydrate epitopes on a few or even on just a single protein scaffold. The selectivity and specificity of this machinery is yet unexplained.

In addition to ESL-1, two further ligands can directly be affinity isolated from neutrophils with E-selectin-Ig chimeras and have been identified on sequence level. One of the two is PSGL-1, as has been described in section III*B1* (15, 114, 196, 204, 233, 259). However, direct evidence for the relevance of this binding for cellular interactions with E-selectin is still lacking. The third high-affinity ligand for E-selectin is L-selectin itself. On the basis of adhesion blocking studies with antibodies, human neutrophils had been found to interact with E-selectin via L-selectin (173, 191, 266). Direct evidence for the interaction of L-selectin with E-selectin was provided in affinity isolation experiments with E-selectin-Ig (152, 384). Interestingly, only L-selectin from human neutrophils, but not from mouse neutrophils, was recognized by E-selectin. Binding was dependent on sialic acid on L-selectin. Although human L-selectin obviously binds with high affinity to E-selectin and is presented to E-selectin on the tips of microvilli, it has been questioned whether L-selectin is directly involved in the capturing of human neutrophils when flowing over an E-selectin-coated support. In two reports, the inhibitory effect of anti-L-selectin antibodies in such assays was explained as an effect on the secondary tethering, mediated between L-selectin on the flowing leukocytes and L-selectin ligands on already attached leukocytes (8, 258). Zöllner et al. (384) reported that the inhibitory effect of anti-L-selectin antibodies in such assays affects the primary interaction between L-selectin and E-selectin.

Through analysis of various types of leukocytes from various species, further glycoproteins that can be isolated with E-selectin affinity probes have been identified, but not yet cloned. A 250-kDa protein was isolated from bovine γ/δ T cells of 1- to 4-wk old cattle, using isolated E-selectin immobilized to beads by a nonblocking anti-E-selectin antibody (358). With the help of an E-selectin-Ig chimera, different glycoproteins were identified on bovine γ/δ T cells, human lymphocytes, and neutrophils (152).

Other suggested E-selectin-ligands include members of the nonspecific cross-reactive antigen (NCA) family present on human neutrophils (179), a subpopulation of

the β_2-integrins which carry sLe^x (175), and the heavily sLe^x-modified lysosomal membrane protein lamp-1 (288). However, it is not yet known for any of these ligands whether they can bind to E-selectin with sufficient affinity to allow affinity isolation with E-selectin, as described above for the "high-affinity" ligands.

V. STRUCTURAL DETERMINANTS AND REGULATION OF SELECTIN LIGAND GLYCOSYLATION

A. Oligosaccharides That Bind to the Selectins

The carbohydrate components of selectin ligands have recently been described in an excellent review by Lowe (210). This section briefly summarizes older data and mainly focuses on the most recent developments.

Numerous reports demonstrated that the tetrasaccharide sLe^x Siaα2→3Galβ1→4(Fucα1→3)GlcNAc binds to selectins. It was first reported as a ligand for E-selectin (263, 359) and later shown to bind to all three selectins (99). The stereoisomer sLe^a, where the positions of the linkages of fucose and galactose are exchanged, was also found to bind to E-selectin (27, 343). However, this structure is not found on leukocytes, but rather on colon carcinoma cells, where it might be involved in E-selectin binding (327). Other structurally related oligosaccharides were also found to bind to E-selectin. The VIM-2 epitope, which carries α(1,3)-fucose in the penultimate position, was reported to bind to E-selectin (335), in agreement to the E-selectin-binding activity of other internally fucosylated, α(2,3)-sialylated glycolipids (324). Structures related to sLe^x or sLe^a in which the sialic acid moiety is replaced by a sulfate group also bind to E-selectin (120, 381). In a human genetic defect called LAD II, the lack of leukocyte recruitment into inflamed tissue was found to correlate with the lack of fucosylated carbohydrate epitopes such as Le^x and sLe^x (92, 93), arguing for the central importance of fucose on the physiological selectin ligands.

A subpopulation of human T lymphocytes that avidly binds to E-selectin and that is enriched in sites of chronic inflammation in the skin was reported to express a carbohydrate antigen defined by the MAb HECA452 (265). On the basis of the enrichment of this antigen on skin-located T cells, it was termed CLA. The MAb HECA452 was shown to block lymphocyte binding to E-selectin (28, 75), as well as rolling of human neutrophils in cytokine-stimulated mesenteric venules (350), suggesting a role of this epitope as a ligand for E-selectin. The tetrasaccharide moieties sLe^x and sLe^a, its stereoisomer, are recognized by HECA452. However, the exact nature of the carbohydrate epitope that is recognized by this antibody on T cells localized in the skin is still unknown, since these cells are largely negative for sLe^x and sLe^a (28, 210).

Although the CLA/HECA452 epitope is likely to be of functional relevance for the migration of T cells into inflamed skin, surprisingly, immune responsiveness and lymphocyte recruitment was recently found to be normal in a LAD II patient who did not express this carbohydrate epitope. However, the clinical symptoms of skin inflammation (redness and swelling) were clearly reduced in the patient (177). The basis for this apparent discrepancy is still unclear.

Although the precise structure of the HECA452 epitope is not yet known, it was found recently that the HECA452 antibody and two other antibodies called 2H5 and 2F3 can recognize 6-sulfo-sLe^x and 6,6′-bis-sulfo-sLe^x (230). The anti-sLe^x antibodies CSLEX-1 and FH6 did not recognize sulfated sLe^x. The CD8+ mouse T-cell clone 4G3, which transiently expresses the HECA452 epitope after antigen-specific activation, expresses the 2H5 and 2F3 epitopes parallel to the HECA452 epitope (36). Another antibody that is thought to recognize sLe^x-like structures is KM93; it binds to rat polymorphonuclear neutrophils, whereas the anti-sLe^x MAb CSLEX-1 does not (229).

As was pointed out above, the binding affinity of sLe^x and its simple derivatives is quite low. Other carbohydrate compounds were found that bind with much better affinity. An sLe^a derivative in which the 2-*N*-acetyl substituent of the GlcNAc was replaced by an amino group resulted in a 36-fold more active inhibitor than sLe^x (244). Other inhibitors are even more potent. Based on the SELEX (systematic evolution of ligands by exponential enrichment) process, which uses combinatorial chemistry and in vitro selection to yield high-affinity oligonucleotide ligands, Ca^{2+}-dependent antagonists were found that bound to L-selectin with 10^5 higher affinity than sLe^x (252). Interestingly, these compounds only show little binding to E- and P-selectin. Because these antagonists showed high affinity at 4 and 22°C, but much lower affinities at 37°C, the same technique was used again to generate DNA aptamers that bind better to L-selectin at 37°C. Antagonists were found that bound with nanomolar affinity and functioned in vivo to prevent the homing of human lymphocytes to lymph nodes in severe combined immunodeficiency mice (137). On the basis of polylactosamine backbones carrying multiple sLe^x moieties, other nanomolar inhibitors of L-selectin-dependent lymphocyte-endothelial interactions were generated, as was shown for an enzymatically synthesized tetravalent sLe^x glycan of a branched polylactosamine backbone (291). Because this compound is too complicated to allow synthesis of large samples, an alternative tetravalent sLe^x glycan derived from a linear polylactosamine was produced purely by enzymatic methods, but could also be produced by combined chemical and enzymatic synthesis (278). Branched and linear forms were similarly potent inhibitors of lymphocyte binding to HEV in lymph node frozen sections (Stamper-Woodruff assay) with IC_{50} values in the nanomolar range (338).

The physiological ligand structures that are recognized by the selectins are still not known in detail. The best-known candidate structures are based on the carbohydrate analysis of PSGL-1 and GlyCAM-1 (Figs. 4 and 5). The major capping structures of GlyCAM-1 were found to be 6-sulfo-sLe^x (with SO_4 linked to position 6 of GlcNAc) and 6′-sulfo-sLe^x (with SO_4 linked to position 6 of Gal), and in a complete structural analysis of β-eliminated oligosaccharides, two sulfated *O*-glycans were found representing these structures in core-2-branched side chains (133). 6-Sulfo-sLe^x was found to block the binding of L-selectin-Ig to GlyCAM-1 (287). Analysis of the *O*-glycans of human PSGL-1 revealed that only a minority of the *O*-glycans are α-1,3-fucosylated, and they occur as two major species both based on core-2-branched chains. One is a disialylated core-2 heptasaccharide containing a single α-1,3-fucose, and the other is a monosialylated, trifucosylated glycan presented on a polylactosamine backbone (369). The multiple sLe^x structures are likely to bind to P-selectin with higher affinity.

In a very interesting approach, complex tetra-antenary carbohydrate structures could be affinity isolated with a human E-selectin affinity matrix from carbohydrate extracts that had been released from membranes of myeloid cells (261). In this work, tetra-antenary oligosaccharide side chains were isolated with a linear di-sLe^x structure on the arm linked to the C_4 residue on the mannose, demonstrating that unique, complex oligosaccharide side chains can bind to E-selectin with an affinity sufficient for affinity isolation. A protein backbone was not required to present these carbohydrates in a certain spatial arrangement. According to another hypothesis, the *O*-linked carbohydrate side chains on sialomucins are presented on the protein backbone in unique spacing and/or clustered combinations that determine the binding specificity and high affinity (71, 346). This hypothesis is partly based on the fact that *O*-linked oligosaccharides released from L-selectin binding mucins did not show any detectable binding to L-selectin. It was also reported that the major capping group 6-sulfo-sLe^x does not inhibit binding better than sLe^a or 3′-sulfo-Le^x, further supporting the idea that unusual oligosaccharide sequences are not sufficient to generate strong selectin binding (174). Although these data indeed suggest that single, unusual oligosaccharide side chains on mucins are not alone sufficient for high-affinity binding and that clustering may be important, they do not argue against the necessity of a combination of both features: the clustering of *O*-linked side chains with the necessary participation of unique carbohydrate structures.

For E-selectin, the situation may indeed be different. Although E-selectin also binds to sialomucins such as PSGL-1, it is the only selectin that can bind to a nonsialomucin ligand such as ESL-1. This ligand only requires *N*-linked oligosaccharide side chains for binding and contains only five putative *N*-glycosylation sites. The fact that single tetra-antenary oligosaccharides can be affinity isolated by E-selectin (261) argues for the possibility that ESL-1 might carry unique oligosaccharide side chains that enable this protein to bind to E-selectin. Indeed, evidence for unique glycosylation of ESL-1 has been provided. The fucosyltransferases VII and IV, when transfected into CHO cells, selectively generate the HECA452 epitope (also called CLA) on ESL-1 and not on other glycoproteins (385). In addition, only ESL-1 was modified by these fucosyltransferases in a way that it could bind to E-selectin. In contrast, expression of fucosyltransferase III generated the HECA452 epitope on many different proteins, and these were all able to bind to E-selectin. Thus many proteins are able to bind to E-selectin with high affinity, and all they need is to be modified by α1,3/4-fucosylation. The specificity and selectivity with which E-selectin binds to ESL-1 (in CHO cells and in mouse myeloid cells) seems to rely on the selectivity with which the myeloid fucosyltransferases modify ESL-1. The mechanism for this remarkable selectivity is still unknown.

A similar selectivity seems to exist for PSGL-1. The HECA452 carbohydrate epitope was selectively expressed on PSGL-1 as the major if not only glycoprotein on human T cells (103) and on a mouse CD8+ T-cell clone (36). The human peripheral blood T cells expressed the epitope after stimulation by an unknown mechanism induced by culturing the cells in a special serum-free medium (XVIVO15) and the mouse T cells expressed the epitope after antigen-specific activation. Interestingly, the CLA epitope was only transiently expressed on PSGL-1 upon antigen-specific activation and E-selectin only bound to PSGL-1 when the CLA epitope was expressed, contrary to P-selectin which bound to PSGL-1 even in the absence of this epitope. Thus, apart from the tyrosine sulfation, which is a specific requirement for the binding to P-selectin, PSGL-1 requires specific modifications that are only necessary for the binding to E-selectin, and the expression of these modifications correlates with the expression of the CLA antigen (36).

B. Regulation of Selectin Ligand Expression by Glycosyltransferases

The central regulatory mechanisms that enable a cell to express functional ligands for a selectin act on the level of posttranslational modification of certain scaffold molecules. Most of these modifications affect the glycosylation of these scaffold molecules. Exceptions are the sulfation of tyrosine residues of PSGL-1, which is necessary for the binding to P-selectin (269, 284, 370). Because protein sulfation occurs in most cells, it is not yet clear whether sulfation of PSGL-1 is regulated. Another modification necessary for binding is the cleavage of the propep-

tide of PSGL-1 (345). It was reported that on B-lymphocytic cells, surface-expressed PSGL-1 exists that still contains the propeptide but is nonfunctional. Cleaving off the propeptide with a soluble recombinant form of PACE generated a functional form of PSGL-1. All other modifications known to be necessary for the expression of functional selectin ligands occur on the carbohydrate side chains. The core-2 branching structure is necessary on PSGL-1 for P-selectin binding, and the major capping groups on GlyCAM-1 are also found on core-2 branched *O*-glycans. α-2,3-Linked sialic acid is necessary for selectin binding on each defined ligand so far. Sulfation of carbohydrates has been shown to be essential for the function of GlyCAM-1, although the sulfotransferase responsible for this step has not yet been identified. This enzyme also seems to regulate the expression of the MECA79 epitope in HEV (132), an epitope which is relevant for L-selectin binding, but does not depend on fucosylation (217).

Probably the most important modifying enzymes are the fucosyltransferases (FucT). Five different human FucT have been cloned so far (211). Two of them, FucTIV and FucTVII, are expressed in myeloid cells. Mice deficient in the gene of FucTVII show severe defects in lymphocyte homing and in neutrophil migration into inflamed peritoneum (217). Lack of FucTVII leads to the absence of E- and P-selectin ligands on leukocytes as well as the absence of L-selectin ligands on HEV. This establishes FucTVII as a central and essential glycosyltransferase for the generation of selectin ligands. However, other ligands may exist that can bind to the selectins even in mice double-deficient in FucTVII and FucTIV. As was shown more recently, platelets of mice deficient in both FucT are still able to bind to P-selectin and roll on P-selectin-expressing endothelium in vivo (101).

VI. SELECTINS AND THEIR LIGANDS AS SIGNALING RECEPTORS

In addition to a direct role in leukocyte capturing, selectins and selectin ligands also function as signal-transducing receptors. This function is best characterized for L-selectin and PSGL-1.

First evidence for L-selectin as a signal transducing receptor was provided by showing that sulfatides binding to the surface of human neutrophils stimulate an increase of cytosolic free Ca^{2+} and expression of TNF-α and IL-8 mRNA (190). In the same report, an increase of cytosolic free Ca^{2+} was also seen by triggering L-selectin with cross-linked MAb. Antibody cross-linking of L-selectin also stimulated H_2O_2 production in human neutrophils, although only in the presence of suboptimal concentrations of FMLP (354). Ligation of L-selectin on human neutrophils with three different antibodies led to increased tyrosine phosphorylation of several proteins including mitogen-activated protein kinase (MAPK) (355). Cross-linking of L-selectin with a secondary antibody was not necessary for this effect. Signaling via L-selectin was also studied in T-lymphocyte cell lines, peripheral blood lymphoblasts, and L-selectin transfectants. These studies established a signaling cascade from L-selectin via the tyrosine kinase $p56^{lck}$, Grb2/SOS, Ras, and Rac2 to the activation of MAPK and the synthesis of O_2^- (43). Interestingly, L-selectin-mediated rolling of Jurkat T cells was abolished if these cells were deficient in $p56^{lck}$ (42), giving a possible explanation for the necessity of the cytoplasmic tail of L-selectin for its function in rolling (162). In addition, it was shown that triggering of L-selectin led to a 10-fold increase of actin filament polymerization via activation of the small G protein Rac-2 (41).

Crockett-Torabi et al. (69) were the first to show that L-selectin ligation with antibodies upregulates the cell surface expression of the β_2-integrin Mac-1. With the use of albumin-coated fluorescent latex beads that bind to Mac-1, it was soon shown that L-selectin ligation with antibodies increased the stimulatory effect of low concentrations of formyl peptide on the binding of these beads to the cell surface (296). The same antibody treatment also increased Mac-1 avidity and adhesion and transmigration of human neutrophils across HUVEC. A major problem in all studies where complete antibodies were used to stimulate L-selectin is the possibility of Fc-receptor stimulation. Therefore, it was very important to show that signaling events could also be triggered by antibody-independent stimulation of L-selectin. Hwang et al. (143) showed that binding of GlyCAM-1 to L-selectin on human lymphocytes activated Mac-1-mediated binding of these cells to an ICAM-1-transfected melanoma cell line. In the same way, GlyCAM-1 can also activate the β_1-integrin-mediated binding of peripheral blood lymphocytes and Jurkat T cells to fibronectin (111). Binding of soluble fibronectin to these cells also increased, clearly demonstrating that GlyCAM-1/L-selectin-mediated effects on β_1-integrin activity were independent of later effects on cell shape and spreading. Antibody ligation of L-selectin on human, rat, and mouse leukocytes with some but not all MAb of a panel of different antibodies triggered intercellular aggregation (310). Lymphocyte aggregation independent of lymphocyte function-associated antigen-1 (LFA-1) can also be enhanced by ligation of L-selectin (320).

If the L-selectin-transmitted effect on integrin activation is physiologically relevant, it should be possible to detect this effect under flow conditions. Therefore, neutrophil tethering, rolling, and arrest on a monolayer of L cells transfected with E-selectin and ICAM-1 was analyzed (117). Under shear stress conditions of 2.0 dyn/cm^2, neutrophils rolled but did not arrest. However, when the neutrophils were treated with anti-L-selectin MAb and secondary antibodies, cell arrest was induced. Arrest depended on LFA-1 and Mac-1. Suboptimal levels of IL-8 and

anti-L-selectin antibodies, which alone were insufficient to activate the cells, potentiated the effect when applied together. L-selectin signaling and chemotactic activation acted synergistically on the activation of neutrophil binding to albumin-coated latex beads and on transendothelial migration (340).

The combination of all these reports clearly establishes that L-selectin functions as a signaling receptor on leukocytes. In two recent reports, L-selectin was even identified as a receptor for LPS on neutrophils (215, 216). Lipopolysaccharide binding to neutrophils resulted in superoxide production that could be blocked with antibodies against L-selectin (215) and was independent of Ca^{2+}.

Signaling functions of E- and P-selectin are less well analyzed. Triggering of E- and P-selectin by antibodies was reported to affect endothelial cell shape (164). Cross-linking of E-selectin with antibodies allowed coprecipitation of cytoskeletal proteins such as α-catenin, vinculin, paxillin, filamin, and even FAK with E-selectin in immunoprecipitations. These proteins did not copurify if the cross-linking step was omitted (380). Activation of platelets by agonists such as thrombin or collagen leads to shape changes that are accompanied by the rapid phosphorylation and dephosphorylation of P-selectin (73). The amino acids that were found to be phosphorylated on the cytoplasmic tail of P-selectin were identified as threonine, serine, tyrosine, and histidine (74).

Signaling effects that the endothelial selectins stimulate in leukocytes by triggering their ligands on the leukocyte cell surface have been intensively analyzed. Two early reports described the activation of β_2-integrins on human neutrophils by E-selectin. In one of the reports, integrin activity was measured by analyzing the binding of C3bi-coated erythrocytes to human neutrophils (206). Neutrophils were stimulated in this study by incubating them either with activated endothelial cell monolayers or with immobilized recombinant E-selectin. In the other study, the evidence was only indirect, based on the analysis of neutrophil binding to IL-1-activated endothelial cells (178). Of course, analyzing such complex systems, in which E-selectin on activated endothelium is used as stimulator for E-selectin ligands, cannot exclude other stimuli such as endothelial chemokines as possible stimulators. Using E-selectin-transfected CHO cells and cytokine-activated endothelial cells, other reports could not provide evidence for human neutrophil activation by E-selectin (279).

Binding of monocytes to cytokine-activated endothelial cells stimulated the expression of tissue factor by the monocytes and was partially inhibited by anti-E-selectin antibodies (205). Binding of the colon carcinoma cell line HT-29 to a substrate coated with an E-selectin-Ig fusion protein stimulated tyrosine phosphorylation of several proteins (302).

More is known about signaling effects triggered by the binding of P-selectin to the leukocyte cell surface. Some earlier reports suggested that binding of purified P-selectin to human neutrophils can inhibit β_2-integrin-mediated binding of TNF-stimulated neutrophils to endothelium (106) and superoxide generation by neutrophils (372). However, such inhibitory effects were never confirmed in other reports on P-selectin signaling.

Various mechanisms for a role of P-selectin in influencing the activity of β_2-integrins have been suggested. Several reports presented evidence that P-selectin cannot directly stimulate integrin activation on human leukocytes (89, 208, 209). Instead, these reports describe P-selectin as an anchoring molecule that allows leukocytes to bind to activated endothelium, facilitating the binding of surface-bound PAF to its receptor on the leukocyte cell surface. Triggering by the immobilized chemoattractant PAF stimulates integrin activation. This juxtacrine signaling mechanism would explain how antibodies against P-selectin could inhibit integrin activation induced by contacts between activated endothelial cells and neutrophils.

In addition to juxtacrine signaling mechanisms, where P-selectin would only have an anchoring function, a costimulatory function of P-selectin has been demonstrated. Tethering of human monocytes to purified, immobilized P-selectin was shown to sensitize the cells for PAF-triggered secretion of monocyte chemotactic protein-1 (MCP-1) and TNF-α (366). In a similar study, immobilized P-selectin sensitized human monocytes for regulated on activation, normal T cell expressed and secreted (RANTES)-stimulated secretion of MCP-1 and IL-8 (364). P-selectin could induce the expression of tissue factor in human monocytes, although strong expression was only seen in the presence of costimuli (59). Activated platelets can activate β_2-integrin-mediated binding of neutrophils to platelets. Activation of β_2-integrins in this process could be blocked by anti-P-selectin antibodies, arguing for a direct or indirect participation of P-selectin in the integrin activation process (94). Likewise, participation of P-selectin in platelet membrane-induced stimulation of superoxide production by human monocytes and neutrophils could be demonstrated (240).

Binding of P-selectin-IgG to mouse neutrophils followed by a secondary antibody was found to be sufficient to stimulate LFA-1- and Mac-1-mediated binding to ICAM-1-transfected CHO cells (34). The process was rapid (2 min) and did not require additional costimuli. P-selectin glycolipid-1 was necessary and sufficient for this process, since P-selectin-IgG-stimulated integrin activation was blocked by MAb against mouse PSGL-1 and integrin activation could be induced by such antibodies when cross-linked by a secondary antibody. In agreement with studies by the group of Zimmerman, stimulation of human PSGL-1 by antibody or by P-selectin-IgG on human neutrophils was not sufficient to generate such effects (34). In summary, P-selectin can trigger signals in human and in mouse

neutrophils, but only mouse neutrophils seem to have a signal transduction cascade directly connecting the triggering of PSGL-1 with the activation of β_2-integrins. Additional costimulatory signals seem to be necessary for the same effect in human neutrophils.

Some steps in the signal transduction cascade triggered by PSGL-1 have been identified. Binding of human peripheral blood T cells to substrate-coated P-selectin-Ig stimulated tyrosine phosphorylation of a 68- and a 125-kDa protein of which the latter was identified as FAK (125). Binding of a MAb against human PSGL-1 on human neutrophils as well as binding of the cells to substrate-coated P-selectin stimulated tyrosine phosphorylation of proteins of 105–120, 70–84, and 42–44 kDa (138). Focal adhesion kinase was not among these proteins, whereas the 42- to 44-kDa bands were identified as MAPK. As for antibody triggering of L-selectin, cytochalasin B did not block the antibody effects, suggesting that an intact cytoskeleton was not necessary for this response.

Contact of human neutrophils with P-selectin seems to stimulate the velocity with which the cell can migrate on a solid support. In a report by Rainger et al. (273), neutrophils were allowed to roll on P-selectin (presented either on the surface of immobilized platelets or in purified form coated on glass capillaries), and migration was started by exposing the cells to FMLP or PAF. Migration velocity decreased by antibody blockade of P-selectin and increased if the number of P-selectin molecules was increased, suggesting that the binding of ligands to P-selectin modifies the rate of integrin-supported neutrophil migration.

With the examination of signaling effects that modify the function of P-selectin ligands, it was found that treatment of human neutrophils with the phorbol ester phorbol 12-myristate 13-acetate or the complement component C5a for more than 20 min drastically increased the ability of these cells to bind a P-selectin-Ig fusion protein (336). The mechanism of selectin ligand upregulation did not involve protein biosynthesis. In another report, PAF activation of neutrophils that adhered to P-selectin-expressing CHO cells led to the loosening of these interactions (207). In the same study, it was shown that P-selectin coated microspheres, which bind evenly distributed on the surface of unactivated neutrophils, become redistributed to the surface of uropods upon activation of the cells, a process which required an intact actin cytoskeleton. This redistribution upon phorbol ester treatment was not found for PSGL-1 on CD34+ human hematopoetic progenitor cells, which were demonstrated to express PSGL-1 as a P-selectin-binding glycoform (339).

VII. CONCLUSIONS AND FUTURE DIRECTIONS

The selectins are now well established as important vascular adhesion molecules that initiate leukocyte extravasation in many different physiological and pathophysiological processes. Within the last 2 years, one of the selectin ligands, PSGL-1, has clearly been defined as an important physiological ligand involved in leukocyte rolling and extravasation in vivo. P-selectin glycolipid-1 could even be a central ligand for all three selectins, since it mediates the P-selectin-dependent primary tethering of leukocytes on endothelium and the L-selectin-dependent secondary tethering of leukocytes on rolling leukocytes as well as binds to E-selectin with high affinity and selectivity. The physiological relevance of the PSGL-1 interaction with E-selectin, however, still has to be demonstrated.

Despite considerable efforts to characterize the carbohydrate moieties that are recognized by the selectins, the precise structures of the physiological ligands are not yet known. Similarly, it is still unknown why distinct glycoproteins are recognized in a very selective manner. Do they carry unusual carbohydrate structures or rather common oligosaccharides that are presented in a unique spatial arrangement? Studies on ESL-1 indirectly argue for the existence of unique oligosaccharide side chains on specific polypeptide backbones, and it is an interesting question what machinery might determine such a selective glycosylation. Furthermore, unusual oligosaccharide side chains were found on PSGL-1 and GlyCAM-1. Knowledge of the precise recognition motif of the selectins will be helpful for the generation of selectin inhibitors.

Other exciting questions are as follows: What mechanism keeps L-selectin and PSGL-1 on the tips of microvilli? What is the proteolytic machinery that mediates the rapid shedding of L-selectin, and what is its relevance in vivo? In this context, identification of the protease TACE that generates TNF from its membrane-bound immature form is an important breakthrough. This protease might also be involved in L-selectin shedding, although the mechanism may be indirect.

Another very interesting new development is the possible participation of selectins in the activation of leukocyte integrins. Binding of GlyCAM-1 to L-selectin as well as binding of P-selectin to PSGL-1 can activate leukocyte integrins. Of course, this does not render chemokines obsolete. Very recently, an interesting new chemokine was found that is probably important for the entry of lymphocytes into lymph nodes (123). Numerous reports demonstrate the importance of other chemokines for leukocyte infiltration into inflammatory sites. It is likely that the signals triggered by selectins and selectin ligands act in concert with the chemokine-stimulated signals to activate leukocyte migration and extravasation.

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