A Role For the Epidermal Growth Factor-like Domain of P-Selectin in Ligand Recognition and Cell Adhesion

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Abstract. The selectin family of adhesion molecules mediates the initial interactions of leukocytes with endothelium. The extracellular region of each selectin contains an amino-terminal C-type lectin domain, followed by an EGF-like domain and multiple short consensus repeat units (SCR). Previous studies have indirectly suggested a role for each of the extracellular domains of the selectins in cell adhesion. In this study, a panel of chimeric selectins created by exchange of domains between L- and P-selectin was used to directly examine the role of the extracellular domains in cell adhesion. Exchange of only the lectin domains between L- and P-selectin conferred the adhesive and ligand recognition functions of the lectin domain of the parent molecule. However, chimeric selectins which contained both the lectin domain of L-selectin and the EGF-like domain of P-selectin exhibited dual ligand-binding specificity. These chimeric proteins supported adhesion both to myeloid cells and to high endothelial venules (HEV) of lymph nodes and mesenteric venules in vivo. Exchange of the SCR domains had no detectable effect on receptor function or specificity. Thus, the EGF-like domain of P-selectin may play a direct role in ligand recognition and leukocyte adhesion mediated by P-selectin, with the lectin plus EGF-like domains collectively forming a functional ligand recognition unit.

THE selective recruitment of specific leukocyte subsets in a variety of normal and pathological situations is regulated principally at the level of leukocyte-endothelial recognition. The selectin family of adhesion molecules mediates the initial interactions of leukocytes with endothelium which precedes leukocyte extravasation into tissues (8). The selectins have a unique and characteristic domain structure that includes an extracellular C-type lectin domain, a single EGF-like domain, and between two and nine short consensus repeat (SCR)[1] units homologous to domains found in complement-binding proteins (10, 23, 33, 54, 60). The three selectins (L-selectin, E-selectin, and P-selectin) are closely related to each other in amino acid sequence, ranging from ~40% identity in the SCR domains up to ~65% in the lectin and EGF-like domains. Although each of these motifs can be found in a variety of proteins, the selectins are the only example in which these three domains are found in immediate juxtaposition, suggesting that this spatial relationship serves an important role in the function of this receptor family.

Although closely related in structure, the selectins have distinct patterns of expression and perform distinct adhesive functions. L-selectin is constitutively expressed on all classes of leukocytes and mediates the binding of lymphocytes to the specialized high endothelial cells present in postcapillary venules (HEV) of lymph nodes, and leukocyte rolling on endothelium at sites of tissue injury or inflammation (19, 35, 56, 61, 63, 65). In contrast, P-selectin is rapidly mobilized to the surface of endothelium or activated platelets in response to agonists such as histamine or thrombin, and binds myeloid cells and a subset of T cells (7, 20, 29, 40). E-selectin is expressed primarily on endothelium after activation with proinflammatory cytokines, and also mediates adhesion of myeloid cells and a subset of memory T cells (9, 37, 46, 48, 52). The distinct pattern of expression of the selectins allows for control of both tissue specificity and the kinetics of immune and inflammatory responses.

Previous studies on the molecular basis of adhesion mediated by selectins have focused principally on carbohydrate recognition by the lectin domains. Cell adhesion mediated by each of the selectins can be blocked by certain simple or complex carbohydrates (22, 30, 58, 67), or by monoclonal antibodies (mAb) which define epitopes within the lectin do-

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1. *Abbreviations used in this paper*: HEV, high endothelial venules; OD, optical density; SCR, short consensus repeat.

main (12, 20, 25, 55, 64). Lactosaminoglycans and related carbohydrates such as sLe^x have been identified as candidate ligands for both P- and E-selectin (47, 50, 64). A heavily glycosylated mucin-like protein, Glycam-1, that expresses an L-selectin carbohydrate ligand has been identified (32). These observations emphasize an important role for carbohydrate recognition in cell adhesion mediated by the lectin domain of selectins.

The EGF-like and SCR domains of selectins may also play an important role in cell adhesion. Deletion of either the EGF-like or SCR domains of L-selectin prevents recognition of ligands on HEV by a soluble form of the molecule (12, 66). Deletion of either the EGF-like or SCR domains of E-selectin abolishes or sharply reduces the ability of these receptors expressed in COS cells to mediate HL-60 cell binding (49). Removal of these structural units may eliminate a ligand-binding component of the receptor or alternatively may alter the conformation of the remaining lectin domain, thereby accounting for reduced or absent receptor function. In addition, adhesion-blocking mAb have been identified which define epitopes in the EGF-like domain of L-selectin (25, 53, 55) and in the SCR domains of L-selectin and E-selectin (24). Moreover, both EGF-like (4, 28, 69) and SCR (1, 2, 14, 27) domains present in a diverse array of proteins directly mediated protein–protein interactions. In spite of these observations, the role of the EGF-like and SCR domains in ligand recognition and cell adhesion by selectins remains essentially unknown.

In this study, a domain swapping approach was used to identify the adhesive domains of L-selectin and P-selectin. In addition to confirming an important role for the lectin domains of these selectins, the results provide evidence that the EGF-like domain of P-selectin also plays an important role in ligand recognition and cell adhesion.

Materials and Methods

Construction of Chimeric Selectin cDNA

Chimeric selectin cDNA were constructed as described (25). Restriction endonuclease cleavage sites were introduced by PCR-based site-directed mutagenesis into the L-selectin cDNA close to the borders between the lectin and EGF-like domains (*Pst I*) and between the EGF-like and SCR domains (*Sac I*) of L-selectin; these sites are naturally present in the P-selectin cDNA (23). Exchange of only the lectin domains at the Pst I site created the LPP and PLL cDNA, and exchange of the lectin plus EGF-like domains at the Sac I site created LLP and PPL (see Fig. 1). Finally, replacement of the Pst I–Sac I segment of L-selectin, corresponding to the EGF-like domain of L-selectin, with the Pst I–Sac I segment of P-selectin, created LPL which contains only the EGF-like domain of P-selectin on a background of L-selection (see Fig. 1). A P-selectin cDNA lacking the lectin domain was generated by two-step recombinant PCR using oligonucleotides which bridged the leader sequence and the EGF-like domain. The leader region up to the Tyr residue (residue number 4 as described in reference 23) was fused directly with amino acid residue 121 (Ser) at the beginning of the EGF-like domain. The cDNA was subcloned into pSP65 and sequenced to determine that the junctional border between the leader and EGF-like domains was intact and to verify that no spurious nucleotide substitutions occurred. These cDNA were all subcloned into the pMT-2 vector (26) and used to transiently transfect COS cells by the DEAE-dextran method as described (25).

Quantitative Analysis of Selectin Expression in Transfected COS cells

Twenty-four hours before analysis, cDNA-transfected COS cells (2.5×10^4/well) were replated into 96 well plates. Expression of specific epitopes by chimeric selectins was assessed using mAb reactive with specific domains and a surface immunofluorescence assay as described (38). Values given are means from triplicate wells of absolute optical density (OD) units from which mean OD unit values obtained with a control non-binding mAb were subtracted. Standard deviations for triplicate determinations were <15%. Experimental values less than the control values are represented as zero.

Selectin expression in transfected COS cells was also assessed by indirect immunofluorescence with flow cytometry analysis. Two days after transfection, COS cells were removed from culture dishes by incubation in 2 mM EDTA at 37°C for 20 min. Cells were incubated with mAb reactive with specific selectin domains (as ascites fluid diluted 1:200 or purified mAb at 5 μg/ml), followed by FITC-conjugated rabbit antibodies reactive with mouse immunoglobulins. Analysis was performed on a Profile flow cytometer (Coulter Electronics, Hialeah, FL).

COS Cell Adhesion Assays

COS cells at ~50% confluency were transfected with 3 μg of cDNA. One day after transfection, the COS cells were replated at ~50% confluency onto 35-mm Primeria culture dishes (assay plates) (Becton-Dickinson, Lincoln Park, NJ). The following day, HL-60 cells were washed twice in cold RPMI 1640 media (GIBCO BRL, Gaithersburg, MD), resuspended in RPMI 1640 at a final concentration of 3.3×10^6 cells/ml, and 0.6 ml (2×10^6 cells) was added to assay plates which had been washed twice with cold RPMI 1640 media. The plates were gently rocked for 20 min at 4°C, washed five times with cold RPMI medium and overlayered with RPMI containing 2% (vol/vol) formalin. The number of HL-60 cells bound to individual COS cells were counted on a minimum of 100 COS cells. Where indicated, HL-60 cells were incubated in RPMI 1640 medium at 37°C for 3 h with either 0.25 U/ml *Clostridium perfringens* neuraminidase (Type VI, Sigma Chem. Co., St. Louis, MO), 50 μg/ml chymotrypsin or papain (Sigma Chem. Co.), washed twice, and the assay was performed as described above. In some experiments, COS cells were preincubated with either 5 μg/ml G1 mAb (Provided by Rodger McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK), which is directed against P-selectin and which blocks P-selectin-mediated cell adhesion (20), or ascites fluid containing the LAM1-3 mAb, which is a blocking mAb directed against the lectin domain of L-selectin (55), diluted 1:200.

Construction of Cell Lines Stably Expressing Parental and Chimeric Selectins

The cDNA encoding native L-selectin, LPP, and LPL were subcloned into the Bam HI site of the pZIPneoSV(X) vector (15), and were used to transfect the mouse pre-B cell line 300.19 (3). Stable transfectants were selected in medium containing 0.5–1.0 mg/ml G418 (geneticin; Sigma Chem. Co.), and cells expressing the proteins were selected by panning with the LAM1-3 mAb (55). To generate P-selectin transfectants, the P-selectin cDNA in the pMT-2 vector was cotransfected with the pSV2neo vector containing the neomycin resistance marker.

Immunoprecipitation Analysis

Cells were surface labeled by the glucose/glucose oxidase/lactoperoxidase method. Immunoprecipitations were performed using anti-LAM1-3 mAb conjugated to Affigel (BioRad, Melville, NY) and analyzed by SDS-PAGE.

HEV Assays

Rat lymph nodes were obtained from freshly euthanized Lewis rats, snap frozen in isopentane/liquid nitrogen, and stored at −70°C in isopentane until use. The HEV assay was as described (13). Each cell type (5×10^6 cells) was incubated on three 12 μm sections/slide at 64 rpm for 25 min at ~4°C, the excess cells were gently removed, and the slides were placed vertically in ice-cold fixative (PBS/2.4% glutaraldehyde) overnight. The slides were then counterstained with Gill's hematoxylin, overlaid with glycerol gelatin, and coverslips were applied. Each slide was scored for the number of test cells bound/HEV and 100–200 HEV were counted for each experiment.

In Vivo Rolling Assays

Rolling of 300.19 cells labeled with carboxyfluorescein diacetate (Sigma Chem. Co.), at 30 μg/ml in M199 medium, (GIBCO BRL) was investigated

in venules of the exposed mesentery of anesthetized Sprague-Dawley rats as described (36). Through an abdominal midline incision, a small polyethylene catheter was inserted retrogradely into the ileocecal artery. The exposed mesentery was superfused with a physiological salt solution at low $_pO_2$ and observed through a Leitz intravital microscope (objective SW 25/0.60) modified for telescopic imaging. Both rolling and freely flowing cells were observed with stroboscopic (50 s^{-1}, Strobex 236, Chadwick Helmuth, Mountain View, CA) epifluorescence illumination (filter block Leitz I2) and recorded on video tape. The number of rolling fluorescent cells is expressed as leukocyte flux, defined as the mean fraction of injected leukocytes observed to be rolling in a given length of venule.

Statistical Analysis

Statistical analysis was performed by the two tailed Student's *t* test.

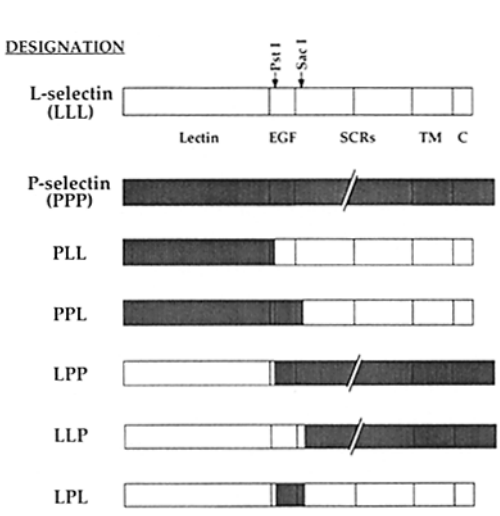
Results

Construction of Chimeric Selectins and Expression in COS Cells

A panel of chimeric selectins was constructed by exchange of the lectin, EGF-like, or both the lectin and EGF-like domains of L- and P-selectin (Fig. 1). These chimeric selectin cDNAs were used to transiently transfect COS cells. Expression of these chimeric proteins at the surface of transfected COS cells was confirmed and quantitated using a panel of domain-specific mAbs (Fig. 1). The level of expression of the chimeric selectins varied as a function of the composition of the hybrid molecules. In general, expression in transfected COS cells correlated with the 3′ region of the chimeric selectin cDNA. Thus, LPP, which contains the lectin domain from L-selectin and the EGF-like, SCR, transmembrane and cytoplasmic domains from P-selectin, and LLP, which contains both the lectin and EGF-like domains from L-selectin and the SCR and other domains from P-selectin, were each expressed at approximately the same level as parental P-selectin (Fig. 1). Similarly, PLL, which contained the lectin domain of P-selectin on a background of L-selectin, was expressed at similar levels as PPL, which contained both the lectin and EGF-like domains from P-selectin with the remainder of the molecule from L-selectin. Expression of LPL, which contained only the EGF-like domain from P-selectin on a background of L-selectin, was equal to or lower than that of native L-selectin (Fig. 1). The level of expression of these molecules was independent of which EGF-like domain they contained. Evaluation of the expression of the cDNA by indirect immunofluorescence staining with flow cytometry analysis correlated closely with the results summarized in Fig. 1, with a similar frequency of positively stained COS cells in each instance. The appropriate expression of domain-specific epitopes within each domain of L- and P-selectin by these chimeric selectins demonstrated that the overall structure of the hybrid proteins was intact, and suggests that domain-specific structural features were retained in the chimeric proteins.

Identification of Functional Adhesive Domains of P-selectin

HL-60 cells express the P-selectin ligand(s) and were therefore used in assays to identify adhesive domains of P-selectin (20, 29). Analysis of the binding of HL-60 cells to COS cells transfected with the cDNA encoding the chimeric selectins depicted in Fig. 1 revealed a specific, domain-dependent pattern of cell adhesion (Fig. 2). HL-60 cells bound to COS cells expressing P-selectin but not to COS cells expressing L-selectin (Fig. 2 *A*). These results demonstrate that although the lectin domain of recombinant soluble L-selectin appears to have a low but measurable affinity for sLe^x (18), which is expressed on HL-60 cells and may form part of a ligand for P-selectin (16, 31, 42, 50), this interaction by itself



	Monoclonal antibody reactivity				
	G1 (P lectin)	LAM1-3 (L lectin)	LAM1-1 (L lectin/EGF)	LAM1-14 (L SCR)	S12 (P SCR)
L-selectin (LLL)	0	525	374	576	0
P-selectin (PPP)	1712	0	0	0	1059
PLL	839	0	68	419	0
PPL	714	0	0	350	0
LPP	19	1484	0	0	1093
LLP	0	1779	1503	0	1018
LPL	0	363	0	266	0

Figure 1. Structure and expression of L-selectin/P-selectin chimeric proteins. Novel *Pst I* and *Sac I* restriction endonuclease sites were introduced into the L-selectin cDNA at the indicated locations, allowing for exchange of the lectin, EGF-like (*EGF*), or both the lectin plus EGF-like domains with those of P-selectin. The first letter of the three letter designations of the cDNA constructs corresponds to the cDNA encoding the lectin domain of L-selectin (*L*) or P-selectin (*P*), the second letter is for the EGF-like domain and the third letter for the SCR domains. P-selectin domains are shaded, and junctional boundaries at the introduced restriction sites, or at domain borders (as determined by the exon/intron boundary of the gene) are drawn as vertical lines. *TM*, transmembrane domain; *C*, cytoplasmic domain. The double slanted lines indicate that all of the nine P-selectin SCR domains are present, but are not drawn. Surface expression of these selectins was analyzed by fluorescence ELISA as described in Materials and Methods. Experimental values less than the control values are represented as zero. These data represent one of four experiments and similar results were obtained when the transfected COS cells were analyzed by flow cytometry. Note that the LAM1-1 mAb fails to recognize either the LPP or PLL proteins, and therefore defines an epitope composed of residues in both the lectin and EGF domains.

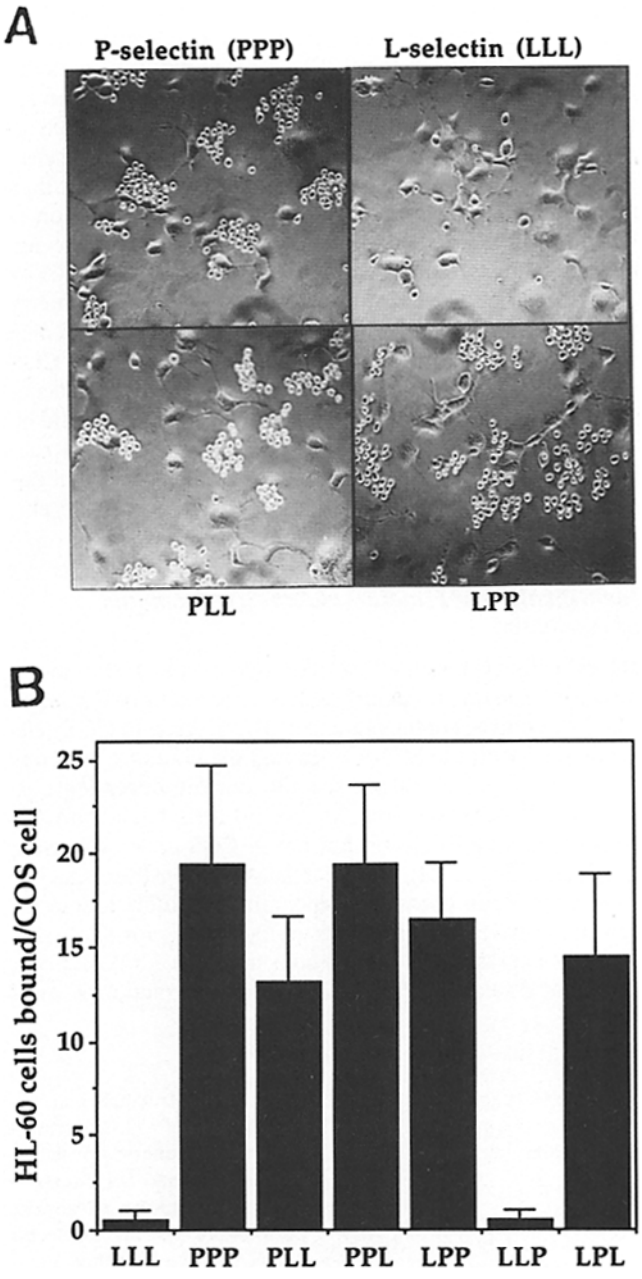


Figure 2. Domains of P-selectin which mediate leukocyte adhesion. Binding of HL-60 cells to transfected COS cells was performed at 4°C, as described in Materials and Methods. (*A*) Binding of HL-60 cells to COS cells transfected with parental and chimeric selectins. Original magnification, 200 ×. The relative intensities of expression of the recombinant proteins was similar to that represented in Fig. 1. (*B*) Mapping of P-selectin domain(s) responsible for adhesion of HL-60 cells. Bars represent the means ± SD, and are representative of at least six experiments.

was insufficient for any detectable cell adhesion in this system. HL-60 cells also bound to COS cells expressing PLL, which contained only the lectin domain from P-selectin (Fig. 2 *A*). The lectin domain of P-selectin alone, when attached to the EGF-like and other domains of L-selectin, is therefore capable of mediating adhesion of HL-60 cells to transfected COS cells.

Surprisingly, HL-60 cells also bound at high levels to COS cells expressing LPP, which contained the lectin domain of L-selectin attached to the EGF-like and SCR domains of P-selectin (Fig. 2 *A*). This result suggests that in addition to the lectin domain, the EGF-like and/or the SCR domains of P-selectin could also mediate HL-60 cell adhesion. Therefore, the domain(s) within LPP responsible for HL-60 adhesion were mapped using additional chimeric selectins. HL-60 cells bound at high levels to COS cells expressing LPL (Fig. 2 *B*), which contained only the EGF-like domain from P-selectin, even though LPL was expressed at the lowest levels of any of the chimeric selectins in this study (Fig. 1). In contrast, HL-60 cells did not bind to COS cells expressing LLP (Fig. 2 *B*), which contained only the SCR domains from P-selectin, and which was expressed at high levels (Fig. 1). The adhesive activity within LPP is therefore within the EGF-like, not the SCR domains. Consistent with this, binding of HL-60 cells to PPL, which contained both the lectin and EGF-like domains from P-selectin, was approximately equivalent to that of parental P-selectin and significantly ($P < 0.01$) higher than binding of HL-60 cells to PLL (Fig. 2 *B*), even though PLL and PPL were expressed at nearly equal levels (Fig. 1). The EGF-like domain of P-selectin was therefore necessary for maximal adhesion of HL-60 cells to transfected COS cells. These results indicate that, in addition to the lectin domain, the EGF-like domain of P-selectin, but not the SCR domains, can also play a role in HL-60 cell adhesion.

Role of the Lectin Domain in Adhesion Mediated by the P-selectin EGF-like Domain

Lectin domain-mediated recognition of sialic acid-containing carbohydrates is an important component of P-selectin mediated cell adhesion (16, 42, 50, 68). The contribution of the lectin domain to the adhesion mediated by P-selectin and these chimeric selectins was therefore examined. Neuraminidase treatment completely removed the sLe^x tetrasaccharide from the surface of HL-60 cells as determined using the CSLEX1 mAb in indirect immunofluorescence staining with flow cytometry analysis (data not shown). However, neuroaminidase treatment inhibited only 60–70% of the binding of HL-60 cells to COS cells expressing native P-selectin or PPL which contained both the lectin and EGF-like domains from P-selectin (Fig. 3). In contrast, pretreatment of HL-60 cells with neuraminidase inhibited >95% of the adhesion mediated by PLL, which contained only the lectin domain from P-selectin (Fig. 3). Pretreatment of cDNA-transfected COS cells with the G1 mAb directed against the lectin domain of P-selectin (Fig. 1) inhibited HL-60 cell adhesion to COS cells in a similar manner. HL-60 adhesion to COS cells expressing P-selectin or PPL was inhibited by only ~70% (Fig. 3), while HL-60 adhesion to COS cells expressing PLL was inhibited by >95% (Fig. 3).

The role of the L-selectin lectin domain in HL-60 cell adhesion mediated by LPL was similarly examined. Interestingly, neuraminidase treatment of the HL-60 cells inhibited 50–60% of the adhesion mediated by LPL (Fig. 3 *A*), as did pretreatment of LPL-expressing COS cells with LAM1-3 mAb, which recognizes the L-selectin lectin domain (25). These results closely parallel the results described above and indicate that the lectin domain of L-selectin present in LPL

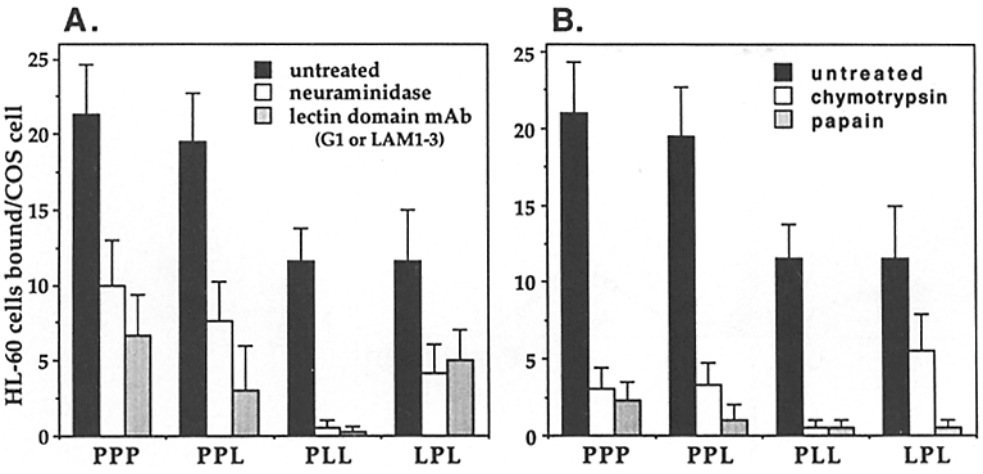


Figure 3. Role of the lectin domain and specificity of adhesion mediated by chimeric selectins. Assessment of HL-60 cell attachment to cDNA-transfected COS cells after various treatments. (*A*) HL-60 cells were treated with medium or neuraminidase, or the COS cells were preincubated with the G1 mAb directed against the lectin domain of P-selectin or the LAM1-3 mAb directed against the lectin domain of L-selectin. Values obtained using isotype-matched control mAb were similar to those obtained using cells treated with media alone. (*B*) HL-60 cells were treated with medium, chymotrypsin, or papain. Adhesion assays were performed as described in Materials and Methods. Bars represent the means ± SD, and results represent one of at least four independent experiments.

mediates a portion of the adhesion mediated by LPL. HL-60 cell adhesion mediated by chimeric selectins which included the P-selectin EGF-like domain was therefore partially resistant to elimination of lectin domain activity. In addition, although recognition of carbohydrates on HL-60 cells by the lectin domain of L-selectin was not sufficient for cell adhesion (Fig. 2 *A*), this interaction appeared to contribute to HL-60 cell adhesion when the EGF-like domain of P-selectin was also present (Fig. 3 *A*). Similarly, removal of Ca^{++} from the assay medium with 2.5 mM EGTA abolished HL-60 attachment in all cases (data not shown). Thus, in addition to the Ca^{++}-dependent lectin domain-carbohydrate interaction, the EGF-mediated interactions may also be Ca^{++} dependent.

Adhesion mediated by P-selectin is sensitive to protease treatment of ligand-bearing cells (31, 41, 42), while adhesion mediated by E-selectin is resistant to proteases (31). Therefore, HL-60 cells were treated with protease to determine whether the production of chimeric selectins altered the ligand-binding activity of P-selectin. Treatment of HL-60 cells with chymotrypsin sharply reduced adhesion mediated by constructs containing both the lectin and/or EGF-like domains of P-selectin, and treatment with papain inhibited adhesion by >90% (Fig. 3 *B*). Binding mediated by the lectin domain and/or the EGF-like domains of P-selectin is therefore protein dependent, consistent with the existence of a protein ligand.

P-selectin lacking the lectin domain was expressed or the surface of COS cells and assessed for its ability to mediate HL-60 cell adhesion. The portion of the P-selectin cDNA encoding the lectin domain was deleted, leaving the amino-terminal leader sequence fused directly with the EGF-like domain. This cDNA produced a cell surface protein in COS cells that was readily identified in immunofluorescence assays by mAb which bind to the SCR domains of P-selectin, but not by mAb which bind to the lectin domain. Although this molecule was expressed on the cell surface at levels similar to LPP and LLP, it was not capable of mediating HL-60 cell attachment (data not shown).

Analysis of Functional Domains of L-selectin

It is possible that introduction of the P-selectin EGF-like domain into L-selectin changed the conformation or other properties of the L-selectin lectin domain. This could conceivably cause such chimeric selectins to bind HL-60 cells through this putatively altered lectin domain, rather than directly through the EGF-like domain. To address this issue, it was essential to determine if the L-selectin lectin domain present in LPP and LPL retained the ability to recognize its natural ligands. Towards this end, cell lines stably expressing LPP, LPL, L-selectin, or P-selectin were produced using a murine cell line which does not express L-selectin. The parental and chimeric selectins were expressed at similar levels on the surface of the transfected cell lines, with only a ~3-fold difference between the mean fluorescence levels of L-selectin and P-selectin transfectants (Fig. 4 *A*). In addition, the transfected gene products exhibited the appropriate mobility with SDS-PAGE analysis (Fig. 4 *B*). L-selectin isolated from human lymphocytes has a M_r of 74,000 while L-selectin from mouse lymphocytes has a M_r of ~90,000 due to differences in posttranslational processing. P-selectin isolated from human platelets has a M_r of 140,000. Therefore, the chimeric human proteins expressed in mouse cells are appropriately sized.

Transfected cells expressing L-selectin, P-selectin, LPP, or LPL were examined for their ability to bind to lymph node HEV in the Stamper-Woodruff frozen section assay (57). Untransfected cells or cells expressing P-selectin did not bind at significant levels (Table I). In contrast, cells expressing L-selectin, LPP or LPL, all of which contain the L-selectin lectin domain, bound to HEV equivalently and at high levels. Cell binding to HEV was completely blocked by LAM1-3 mAb (Table I), which is directed against the lectin domain of L-selectin (25). Therefore, the lectin domain from L-selectin alone, on a background of P-selectin, was capable of mediating the HEV-binding properties of L-selectin.

Since L-selectin also mediates leukocyte rolling along postcapillary venules at sites of tissue injury or inflammation

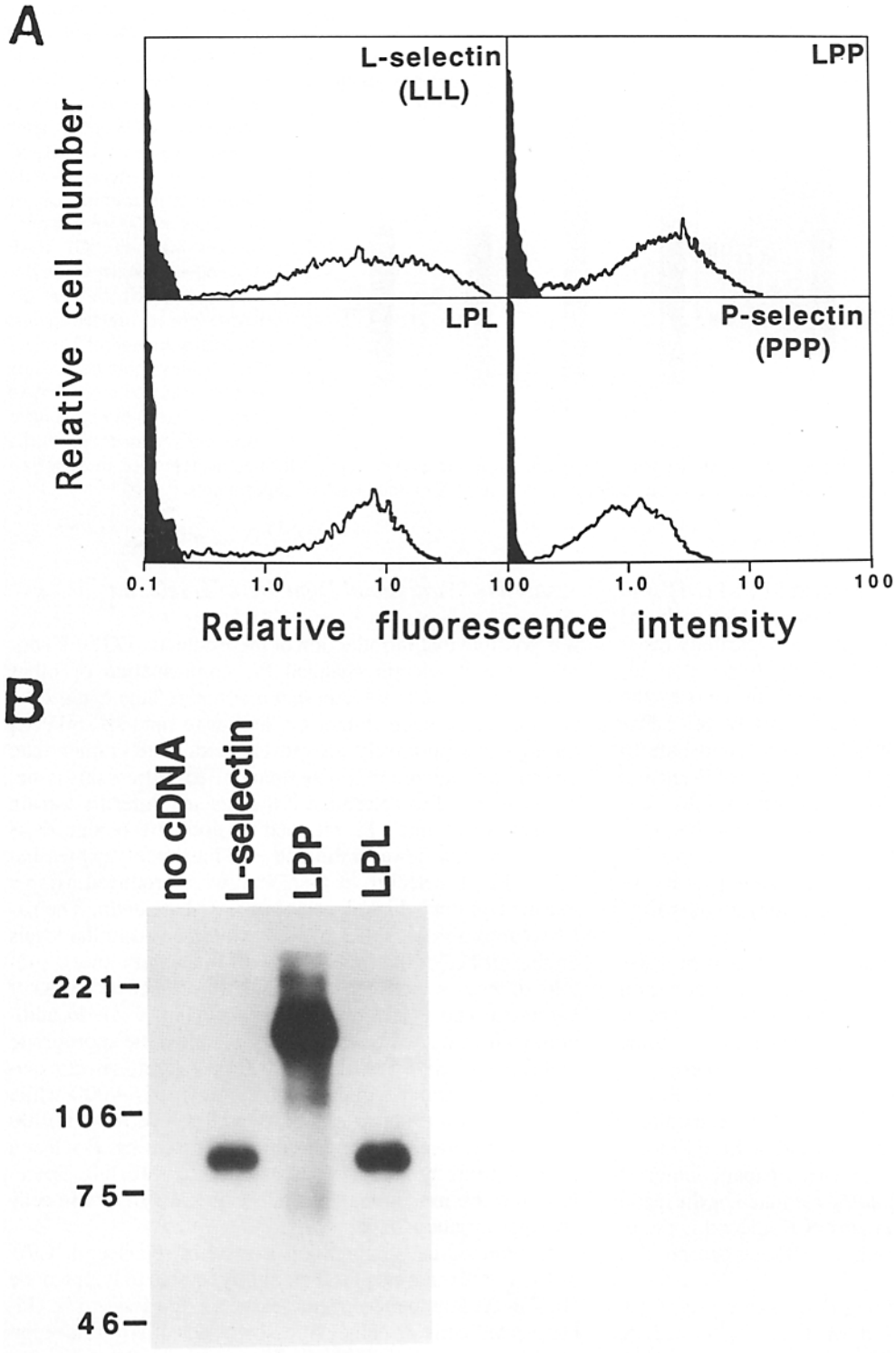


Figure 4. Expression of selectins in transfected 300.19 cells. (*A*) Flow cytometry analysis of cell surface expression of cDNA-transfected 300.19 cells stably expressing L-selectin, P-selectin, LPP, or LPL. Results are depicted on a three decade logarithmic scale. The filled in histograms represent background staining obtained with a nonbinding negative control mAb of the same isotype. (*B*) Immunoprecipitation and SDS-PAGE analysis of natural and chimeric selectins from stably transfected 300.19 cells that were surface labeled with ^{125}I. Molecular weight standards (in kD) are indicated and the gel was run under reducing conditions. P selectin contains nine SCR domains and L-selectin contains two, accounting for the size differences in the chimeric proteins.

(35, 36, 62), the ability of cells transfected with chimeric selectins to roll in vivo was examined. Untransfected or P-selectin-transfected cells did not roll at detectable levels. Similar to the results in the HEV-binding assay, cells expressing L-selectin, LPP, or LPL exhibited significant and equivalent rolling in vivo in postcapillary venules of the exteriorized rat mesentery (Fig. 5). As with HEV binding, mAb directed against the lectin domain of L-selectin completely blocked rolling, confirming the activity of this domain in adhesion. These data demonstrate that the specificity and function of the L-selectin lectin domain present in LPP and LPL were preserved. The LPP and LPL chimeric selectins therefore exhibited a dual ligand recognition specificity, binding to both HL-60 cells bearing P-selectin ligands and to L-selectin ligands present on lymph node HEV and mesenteric endothelium.

Table I. Adhesion of cDNA-transfected Cells to Lymph Node HEV Is Mediated by the Lectin Domain of L-selectin

cDNA	LAM1-3 mAb	expt 1	expt 2	expt 3
None	−	0.047 ± 0.02	0.12 ± 0.025	<0.01
	+	<0.01	ND	ND
L-selectin	−	3.43 ± 0.42	6.85 ± 0.45	2.44 ± 0.51
	+	0.05 ± 0.2	ND	<0.01
LPP	−	3.71 ± 0.26	6.64 ± 0.43	4.40 ± 0.3
	+	0.074 ± 0.03	ND	<0.01
LPL	−	5.71 ± 0.33	6.85 ± 0.18	3.94 ± 0.27
	+	0.08 ± 0.03	ND	<0.01
P-selectin	−	<0.01	<0.01	ND

Transfected mouse 300.19 cells were prepared and HEV assays were performed as described in Materials and Methods. LAM1-3 mAb was used as ascites fluid diluted 1:200. Data are presented as the mean ± SD of cells bound/HEV from >100 HEV examined for three independent experiments. Similar results were obtained in three additional experiments. Values obtained using an isotype-matched control mAb were similar to those obtained using cells treated with media alone. The variation seen in the levels of binding is typical for these assays. ND, not determined.

Discussion

The functional analysis of chimeric proteins produced by exchange of homologous segments between members of the same gene family has proven to be a powerful approach towards identifying the functions of specific domains and amino acid residues in a diverse array of biological systems. The advantage of such an approach is that the overall secondary and tertiary structure of the hybrid proteins is maintained, while the potential problems associated with deletion mutants such as gross alterations of structure, expression and function are avoided. This approach was applied to the identification of adhesive domains of L- and P-selectin. The most striking and important conclusion from these studies is that the EGF-like domain of P-selectin is involved in ligand recognition and cell adhesion. Chimeric selectins which contained both the lectin domain from L-selectin and the EGF-like domain from P-selectin exhibited the adhesive properties of both L-selectin and P-selectin: LPP and LPL mediated binding of HL-60 cells, a property of P-selectin but not L-selectin, and also mediated cell binding to lymph node HEV and mesenteric venules, a characteristic of L-selectin but not P-selectin. The LPL chimeric selectin which contained only the EGF-like domain of P-selectin on a background of L-selectin bound HL-60 cells (Fig. 2 *B*) even though it was expressed at the lowest level of any of the cDNA constructs used in this study (Fig. 1). Most importantly, each of the chimeric selectins which contained the EGF-like domain from P-selectin supported significant levels of HL-60 cell adhesion that was partially resistant to blocking by lectin domain-specific mAb or neuraminidase treatment of HL-60 cells (Fig. 3). Each of these treatments completely eliminated adhesion mediated by the lectin domain of P-selectin (Fig. 3; PLL) or L-selectin (Table I). The dual specificity of these chimeric L- and P-selectins constitutes direct and powerful evidence that the EGF-like domain of P-selectin participates in ligand recognition and cell adhesion.

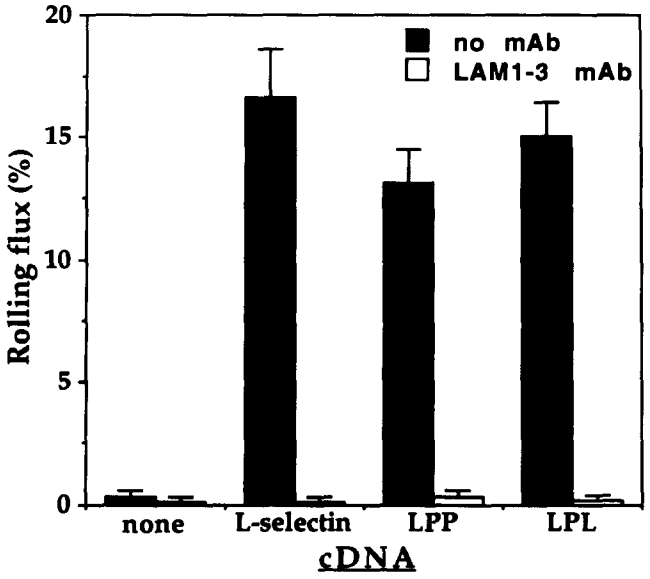


Figure 5. The lectin domain of L-selectin mediates leukocyte rolling in mesenteric venules. 300.19 cells stably transfected with the indicated cDNA were tested for their ability to roll in vivo in exteriorized venules of the rat mesentery. Results obtained using isotype-matched control mAb were similar to those obtained using cells treated with media alone as described (36).

Two mechanisms by which the EGF-like domain of P-selectin participates in ligand recognition are possible, both are novel and of major significance. First, placement of the P-selectin EGF-like domain next to the L-selectin lectin domain may induce a change in the conformation of the adjoining L-selectin lectin domain resulting in a change in affinity or in the acquisition of a novel carbohydrate ligand recognition capability. Second, the P-selectin EGF-like domain may participate directly in ligand recognition via protein–protein interactions. The results of this study support the second mechanism. Juxtaposition of the lectin domain of either L- or P-selectin with the EGF-like domain of the other selectin did not appear to affect the specificity or adhesive activity of the lectin domains for their known physiologic ligands (Figs. 2 and 6, Table I). Both L-selectin and LPP bind PPME, a complex polysaccharide which specifically blocks L-selectin-dependent binding of lymphocytes to HEV, while P-selectin exhibits no binding activity for PPME (25). The overall structure of the L-selectin lectin domain was not significantly altered by juxtaposition with the EGF-like domain of P-selectin in that seven mAb-defined epitopes lo-

cated in the L-selectin lectin domain are conserved in these chimeric proteins (55). Also, LPP, which contained only the lectin domain from L-selectin, mediated adhesion to HEV and rolling as well as native L-selectin (Fig. 5), even though it was expressed at twofold lower levels (Fig. 1). Similarly, PLL expressed by transfected COS cells mediated HL-60 cell adhesion at significant levels (Fig. 2). Thus, the activities and specificities of the lectin domains were not dramatically altered in these chimeric selectins.

Direct protein–protein interactions mediated by the EGF-like domain of P-selectin are suggested by the ability of native P-selectin and chimeric (PPL and LPL) selectins containing the P-selectin EGF-like domain to support significant levels of HL-60 binding in the absence of lectin domain activity (Fig. 3 *A*). Additionally, HL-60 cell adhesion mediated by PLL expressed by transfected COS cells was only ~70% of that mediated by PPL (Figs. 2 and 3), even though these two chimeric selectins were expressed at nearly identical levels (Fig. 1). Furthermore, only a portion of HL-60 binding by LPL was blocked by abrogating L-selectin lectin domain function (Fig. 3 *A*). This suggests that the L-selectin lectin domain in this construct is mediating a portion of the adhesion, even though no adhesion was observed with native L-selectin (Fig. 2) which was expressed at the same or higher levels (Fig. 1). A likely explanation for these observations is that the L-selectin lectin domain may naturally bind sLe^x or other carbohydrates on the HL-60 cell surface with low affinity. This binding alone does not support cell adhesion (Fig. 2), but is sufficient for cell adhesion when this interaction is supported by the adhesive activity of an adjoining P-selectin EGF-like domain. While the finding that deletion of the lectin domain from P-selectin resulted in the concomitant loss of ligand-binding activity argues against a direct ligand-binding activity for the EGF-like domain, it is possible that deletion of the proximal lectin domain affects the tertiary structure of the EGF-like domain, thereby inhibiting ligand-binding activity. Nonetheless, the results of this study suggest an important role for the EGF-like domain for maximal P-selectin-mediated cell adhesion.

The EGF-like domain of L-selectin does not appear to be required for maximal adhesion since LPL and LPP have functional activities similar to L-selectin (Fig. 5, Table I). Therefore, the adhesive function of the EGF-like domain of P-selectin may be unique to this selectin. This is also consistent with the strikingly higher degree of amino acid sequence conservation of the EGF-like domain in P-selectin between human, mouse, and cow (89% identical) compared with either E-selectin (58%) or L-selectin (69%) (6, 10, 11, 23, 33, 51, 59, 60). Dual recognition by P-selectin of both a carbohydrate and a protein ligand would define a cellular interaction of higher affinity and specificity than recognition mediated by the lectin domain alone. The P-selectin EGF-like domain may mediate protein–protein interactions in a manner similar to thrombomodulin EGF-like domains which bind thrombin (28, 69), and plasminogen activator where the EGF-like domain contains the ligand-binding site (4). The cooperative action of two adhesive domains may explain the ability of P-selectin to mediate intercellular adhesion even though there are only low levels of P-selectin expressed on the surface of activated platelets and endothelium (~50 molecules/μm^2) (21, 39, 40). This model would also explain why the carbohydrate recognition capabilities of E- and P-selectin appear to be either closely overlapping or identical (31, 50, 68), while the observed molecular and cellular ligand specificities are distinct (5, 17, 31, 34, 42).

This study did not reveal a direct role for the SCR domains of L- or P-selectin in cell adhesion. The reciprocal exchange of nine P-selectin SCR domains for two L-selectin SCR domains had no detectable effect on ligand binding. Furthermore, these results suggest that no stringent requirement exists for any particular number of SCR. This is consistent with the variable number of SCR found in both P-selectin and E-selectin of different animal species. In addition, the levels of homology found between SCR domains of different selectins and among different species are lower than for the other extracellular domains. However, certain structural features of selectin SCR domains may be required for function since deletion of the SCR domains from E-selectin sharply impairs cell adhesion, and deletion of the SCR domains from soluble forms of L-selectin impairs carbohydrate recognition by the lectin domain (49, 66). Thus, the SCR domains may contribute indirectly to lectin domain function, perhaps by stabilizing receptor structure or oligomerization.

In summary, the results presented here indicate that both the lectin and the EGF-like domains of P-selectin are directly involved in cell adhesion. Furthermore, the dual ligand-binding specificity of the chimeric selectins described in this report may also be clinically useful in blocking selectin function and consequent inflammation and tissue damage in settings where multiple selectins are involved. In this regard, acute lung injury in rats induced by infusion of cobra venom factor depends in part on P-selectin and in part on L-selectin (43, 45); similarly, IgG-immune complex mediated disease is dependent on both E-selectin and L-selectin (44, 45). In each case, inhibitory reagents directed against only a single selectin give only partial protection (43–45). Soluble proteins derived from chimeric selectins with dual specificity may therefore be potent inhibitors of leukocyte-mediated tissue damage.

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