THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 27, Issue of July 5, pp. 16160–16170, 1996

Printed in U.S.A.

Single Amino Acid Residues in the E- and P-selectin Epidermal Growth Factor Domains Can Determine Carbohydrate Binding Specificity*

(Received for publication, January 31, 1996, and in revised form, April 26, 1996)

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E-selectin and P-selectin are two closely related vascular cell adhesion proteins. Each selectin has an amino-terminal C-type lectin domain that is thought to possess the carbohydrate binding site that binds the sialylated Lewis^x antigen (sLe^x or CD15s) (Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc). In addition to the sLe^x carbohydrate, P-selectin binds sulfated proteoglycan, 3-sulfated galactosyl ceramide (sulfatide), and heparin. Both E- and P-selectin have an EGF-like (EGF) domain that is immediately adjacent to and COOH-terminal to the lectin domain. We report that mutagenic substitution of single amino acid residues in either the P- or E-selectin EGF domain can dramatically alter selectin binding to sLe^x, heparin, or sulfatide. Substitution of E- and P-selectin EGF domain residue Ser^{128} with an arginine results in E- and P-selectin proteins that have lost the requirement for α1–3-linked fucose and are thus able to bind to sialyllactosamine. A similar phenotype is reported for an E-selectin mutation within the lectin domain. Additionally, we have determined that conservative substitution of EGF domain residues 124 and 128 can alter E-selectin binding such that it is able to adhere to heparin or sulfatide and can reduce P-selectin adherence to these ligands. The distance between the substituted EGF domain amino acid residues and the primary carbohydrate binding site within the lectin domain and their relative positioning as determined by the three-dimensional crystal structure of the E-selectin lectin and EGF domains (Graves, B. J., Crowther, R. L., Chandran, C., Rumberger, J. B., Li, S., Huang, D.-S., Presky, D. H., Familletti, P. C., Wolitzky, B. A., and Burns, D. K. (1994) *Nature* 367, 532–538) suggest that there is little direct contact between the two domains. However, we report mutant binding characteristics which indicate that selectin oligosaccharide binding may be modulated by both domains and that wild-type E- and P-selectin/sLe^x binding interactions may be significantly different from those previously hypothesized.

The selectins (E-, P-, and L-selectin) make up a family of three vascular cell adhesion proteins that appear to modulate the migration of leukocytes from blood into extravascular tissue. Each of these proteins is composed of an amino-terminal lectin domain followed by an EGF[1]-like domain, variable numbers of short consensus repeats, a single membrane spanning region, and a cytoplasmic tail (for review, see Refs. 1 and 2). The lectin domain of each selectin is thought to possess the carbohydrate binding site and E-, P-, and L-selectin share approximately 70% sequence identity over each of their three individual lectin domains. The best known E- and P-selectin ligand is the sialylated Lewis^x antigen (sLe^x or CD15s) (3–7). This carbohydrate is found on the surface of neutrophils, eosinophils, monocytes, and some lymphocytes. E-selectin binding to sLe^x and P-selectin binding to sLe^x or several other carbohydrate structures (sulfated proteoglycan, 3-sulfated galactosyl ceramide (sulfatide), or heparin) (see Fig. 1*A*, for reference) found on neutrophils and monocytes is thought to be a preliminary step in the recruitment of leukocytes to areas of tissue damage or inflammation. Evidence generated using blocking antibodies or soluble sLe^x carbohydrate to stop selectin-dependent recruitment of leukocytes indicates that inhibiting selectin binding to neutrophils and monocytes can ameliorate or reduce leukocyte-mediated tissue damage and inflammation (for review, see Ref. 8).

The *N*- and *O*-linked glycoproteins synthesized by many cells contain a terminally sialylated lactosamine unit (Neu5Acα2–3Galβ1–4GlcNAc) (9) which is the substrate for the α1–3-fucosyl transferase that catalyzes the addition of fucose to form the sLe^x moiety (10, 4). The final addition of this fucose residue occurs only in certain tissues (10–13) and is a specific determinant required for E-selectin binding. A rare human genetic syndrome, LAD II (leukocyte adhesion deficiency II), has been described in which affected individuals do not possess α1–3-fucosylated carbohydrates and appear to be immunocompromised (14). Individuals with this syndrome display an increased susceptibility to severe chronic and recurrent infection, a characteristic that has also been attributed to a lack of selectin binding and recruitment of leukocytes to infected areas (14, 15).

A second human mutation is known to directly effect the E-selectin gene. This is a polymorphism in which the nucleic acid encoding amino acid, Ser^{128}, is altered such that it encodes an arginine residue (16, 17). Individuals possessing this alteration appear to have a higher incidence of early onset, severity, and frequency of atherosclerotic disease (16, 17). This disease phenotype is itself interesting because one might predict that the increased severity and frequency of atherosclerosis might reflect an amplified rather than a reduced inflammatory response. Likewise, the positioning of this mutation within the E-selectin EGF domain is also intriguing since while the P-selectin EGF domain has previously been implicated in determining binding specificity (18, 19) no E-selectin mutations within the EGF domain have previously been reported to effect

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[1] The abbreviations used are: EGF, epidermal growth factor; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

E-selectin adherence.

Therefore, to better understand the structural and functional relationships that may be responsible for the atherosclerotic phenotype of the Ser^{128}-Arg-affected individuals, we have used site-directed mutagenesis to recreate the S128R mutation in both E- and P-selectin IgG chimeric fusion proteins. Our results indicate that although the S128R mutation effects the EGF domain in a region of E-selectin not previously believed to contact the E-selectin lectin domain (20), the S128R substitution has a profound affect on selectin ligand recognition and binding. Additionally, it appears that a second E-selectin mutation, R84A, which was originally reported by Graves *et al.* (20) to increase E-selectin adhesion, has a similar phenotype. The data reported indicate that both mutant proteins are able to recognize and bind the non-fucosylated sLe^x carbohydrate precursor, sialyllactosamine (Neu5Acα2–3Galβ1–4GlcNAc).

In an attempt to elucidate the molecular mechanism(s) by which the S128R mutation alters selectin binding, we have completed a more extensive mutagenesis of several amino acid residues within the E- and P-selectin EGF domains and report that P-selectin sulfatide binding is dependent upon amino acids that are located within the EGF domain of that protein. Results obtained using site-directed mutagenesis to modify P-selectin residue Asp^{124} indicate that conservative modification of this residue can reduce P-selectin adherence to sulfatide. Additionally, mutagenic modification of the analogous E-selectin residue, Asn^{124}, or of the Ser^{128} residue can change E-selectin binding such that it is able to recognize and bind to heparin or sulfatide although wild-type, unmodified E-selectin binds to neither of these ligands. These results indicate that amino acid substitution within the EGF domain can modulate selectin sLe^x, heparin, and sulfatide adherence.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media, dialyzed fetal calf serum, phosphate-buffered saline (PBS), and antibiotics were obtained from Life Technologies, Inc., and fetal calf serum was from Hyclone. The anti-P-selectin antibody (AC1.2), anti-L-selectin antibody (SK11), anti-sLe^x (CSLEX1), and anti-Le^x (MMA) were purchased from Becton-Dickinson. Anti-E-selectin antibodies (BBA1, BBA2, and BBA8) were purchased from R & D Systems. Magnetic beads conjugated to goat anti-mouse IgG and magnetic separators were obtained from Dynal Inc. (Great Neck, NY). Unless specifically stated, other immunochemicals were purchased from Calbiochem. Sulfatides, fetuin (catalog number F-2379), and heparin albumin were purchased from Sigma. L-Fucose was purchased from Aldrich. Flexible 96-well assay plates and Probind 96-well ELISA plates were purchased from Falcon. The sLe^x tetrasaccharide, Neu5Acα2–3Galβ1–4GlcNAc, and Neu5Acα2–6Galβ1–4GlcNAc were purchased from Oxford GlycoSystems. Neu5Acα2–3Galβ1–4Glc and Neu5Acα2–6Galβ1–4Glc were purchased from Calbiochem and Sigma. Synthetic oligonucleotides were purchased from Oligo Therapeutics Inc. Restriction enzymes and T4 DNA ligase were from Life Technologies, Inc. and New England Biolabs, *Taq* DNA polymerase was obtained from Perkin Elmer.

Cell Culture and Transfection—HL-60 and K562 cells were grown at 37 °C with 5% CO_2 in RPMI supplemented with 10% fetal calf serum. COS-1 cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Prior to electroporation, COS-1 cells were trypsinized, harvested, washed twice with PBS, and 10^6 cells (0.8 ml) were mixed with 10 μg (1 μg/μl) of plasmid DNA in a 0.4-cm electrode gap cuvette. Transfections were performed using a Bio-Rad electroporator following the manufacturer's recommendations and using 0.22 mV, 960 microfarads. After transfection, the cells were grown in 100-mm dishes for 72 h, at which time the culture media was collected, buffered to a final concentration of 10 mM Tris-HCl (pH 7.3), and centrifuged for 5 min at 600 $\times$ *g* to remove cell debris. HL-60 and K562 cell lines were obtained from the ATCC and COS-1 cells were from the lab of J. F. Sambrook and M.-J. Gething.

Construction and Expression of Selectin mutants—Recombinant DNA techniques and mutagenesis were performed as described (21) and references therein. Plasmid DNA for transfection or sequencing was purified as per manufacturer's recommendation using Qiagen reagents. All DNA amplified by polymerase chain reaction or subjected to site-directed mutagenesis procedures were sequenced by the Sequenase method using reagents supplied by U. S. Biochemical Corp. The chimeric selectin mouse IgG_{2A} heavy chain fusion proteins were modeled after those described by Walz and co-workers (6). The P-selectin IgG construct and mutant proteins were constructed and expressed as described (22, 23), using the reported P-selectin nucleotide sequence (24). The E-selectin IgG construct and mutant proteins were made and expressed as described previously (22) using the reported E-selectin nucleotide sequence (25) with the exception of the lectin domain IgG fusion protein which was constructed using the following oligonucleotides 5′-GCTCCTGAATTC**ATG**ATTGCTTCAC-3′ and 5′-TATT**GGTACC**GGCAGCTGT-3′. The *Eco*RI site (GAATTC) just precedes the initiation codon which is denoted by bold print. The *Kpn*I site at the 3′ end of the lectin domain was used to ligate the lectin domain directly to the mouse IgG hinge synthetic *KPN*I site that was constructed as described previously (22). This results in a protein that contains E-selectin amino acids Trp^1 to Ala^{121}, inserts Gly and Thr fusion residues encoded by the *Kpn*I site, and continues with the mouse IgG_{2A} heavy chain hinge amino acid residues RGPTIKPCPPCK . . . and extends to the COOH terminus of the mouse IgG_{2A} heavy chain protein. The protein synthesized from this particular E-selectin lectin domain construct is recognized by both BBA1 and BBA2 (R & D Systems) anti-E-selectin antibodies as determined by binding inhibition (BBA2) and ELISA (BBA1/BBA8).

Protein Purification and Cell Binding Assays—Recombinant protein levels in the cell supernatants were monitored by ELISA using alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Calbiochem). Typically, transfected COS-1 supernatants were either directly coated onto ELISA plates or the recombinant IgG fusion proteins in these supernatants were captured by incubation in ELISA wells that were precoated with rabbit anti-mouse IgG_{A2} antibody (Cappel catalog number 50228). For either method, cell supernatants were incubated in wells overnight. ELISA plates were subsequently washed repeatedly with PBS and then incubated with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Calbiochem). To affinity purify the recombinant proteins, 10 μl (4×10^8 beads/ml) of goat anti-mouse-coated Dynabeads (Dynal Inc.) were added to 10 ml of culture media harvested from transfected cells and incubated overnight with rocking at 4 °C. The beads were concentrated by centrifugation at 600 $\times$ *g* for 5 min and the culture media was removed after the incubation tubes were placed on a magnetic separator. The beads were resuspended in 1 ml of PBS (4×10^6 beads/ml final concentration) and stored at either 0 °C or 4 °C.

The amount of recombinant protein recovered on the beads was monitored by ELISA using anti-P-selectin antibody, AC1.2 (Becton Dickinson), or anti-E-selectin antibody BBA8 (R & D Systems). ELISAs were performed as follows, 8×10^4 beads were added to duplicate wells of 96-well flexible assay plates that had been previously blocked with PBS supplemented with 3% BSA, equivalent amounts of wild-type E-selectin or P-selectin coated beads together with beads prepared from mock transfected cell lysates were tested at the same time on the same plate as positive and negative controls. Using a magnetic separator (Dynal Inc.) to retain the beads inside the wells, the wells were washed once with 50 μl of PBS, then incubated in a solution of 6% formaldehyde in PBS for 20 min at room temperature. This was followed by two sequential PBS washes and a second 20-min incubation in 100 mM ammonium chloride supplemented PBS. The beads and wells were then blocked overnight in 3% BSA and 1% rabbit serum supplemented PBS.

When the blocking buffer was removed, three sequential PBS washes were performed and the primary anti-selectin antibodies were added at final concentrations of 0.2 μg/ml in PBS, 3% BSA and incubated for a minimum of 2 h at 4 °C. The sample wells were then washed three times with PBS and in the case of BBA8, incubated with a 1:2000 dilution of alkaline phosphatase-conjugated streptavidin (Calbiochem, catalog number 189732). For AC1.2, a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse κ light chain (Caltag, catalog number M33008) or for some experiments, a 1:5000 dilution of alkaline phosphatase-conjugated rabbit anti-mouse IgG_1 (Cappel, catalog number 59569) was added and incubated in a solution of 3% BSA, PBS for a minimum of 2 h at 4 °C. The sample wells were then washed two times with PBS and once with alkaline phosphatase ELISA buffer solution (pH 9.4) of 10 mM diethanolamine, 0.5 mM $MgCl_2$, 10 mM NaCl. The alkaline phosphatase colorimetric assays were typically developed overnight at 4 °C using standard buffers and substrates (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium) (26). The absorbance was monitored with a 405 nm filter in a Bio-Rad model 450 microplate reader.

To ensure that the same amount of recombinant selectin protein was coated onto the beads, the goat anti-mouse IgG on the beads were saturated with selectin protein. This was typically done by a single incubation of 10 μl (4×10^8 beads/ml) of goat anti-mouse-coated Dynabeads (Dynal Inc.) in 10 ml of culture media harvested from transfected cells. However, on occasion when the beads were not saturated with selectin protein, a second incubation with harvested transfected culture media was performed. The amount of recombinant selectin fusion protein in the transfected cell medium was very consistent and only appeared to vary when transfected plasmid DNA purity varied. The precipitated, bead-bound selectin IgG proteins could be stored at 4 °C for several months without alteration in concentration.[2] However, to maximize consistency, all experiments reported in this work were performed with selectin-coated beads that were typically stored for less than 1 week. Additionally, to minimize preparation errors, all experiments were repeated with selectin-coated beads that were prepared on a second occasion from an independent COS-1 transfection. The number of times that beads can be used to immunopurify selectin IgG fusion protein from the same 10-ml culture supernatant is dependent upon the concentration of selectin IgG fusion protein in that supernatant. This concentration will vary with the success of transfection.

P- and E-selectin HL-60 cell binding assays were performed in Falcon 96-well flexible assay plates as described (22). The wells were first blocked by incubating briefly with PBS supplemented with 3% BSA. After aspirating the blocking buffer, 10 μl of HL-60 cells (10^7 cells/ml RPMI 1640, 10% fetal calf serum) that had been fluorescently labeled with Calcein AMC-3099 (Molecular Probes) were added to each well followed by 10 μl of beads (4×10^6 beads/ml). Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone. The cells and beads were incubated together at room temperature for 10 min. If sLe^x tetrasaccharide inhibition was being tested, the tetrasaccharide was added immediately prior to this incubation. Following incubation, the assay plate was placed on a magnetic separator and incubated for 2 more minutes. While the plate remained on the separator, excess unbound HL-60 cells were removed and the wells were washed twice with PBS to remove any remaining unbound cells. The HL-60 cells remaining bound to the beads were inspected by microscopy and then lysed by adding 50 μl of a 1% solution of Nonidet P-40 in PBS. Binding was quantitated fluorimetrically using a Millipore Cytofluor 2350 fluorimeter.

Fetuin Binding Assay—Stock solutions containing 2.5 mg/ml (lyophilized weight/volume) of: yeast invertase (Sigma) and bovine fetuin (Sigma catalog number F-2379) in 25 mM Tris (pH 7.5), 1 mM 2-mercaptoethanol, 0.5% SDS, and 5% glycerol were boiled for 10 min, cleared by brief centrifugation, chilled to 0 °C, diluted to final concentrations of 25 μg/ml in PBS, and coated onto 96-well flexible assay plates. Plates were subsequently blocked with 3% BSA in PBS. Beads conjugated with various E- and P-selectin IgG proteins (2×10^4 beads) were added to each well in a 55-μl volume of PBS supplemented with 0.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ and incubated for 1 h. Wells were then washed sequentially with PBS until unbound beads were removed. The number of beads retained in the wells was determined by ELISA using an alkaline phosphatase-conjugated rabbit anti-goat IgG heavy and light chain antibody (Calbiochem catalog number 401512) antibody (to detect the goat antibody that is present on the beads when they are purchased from Dynal). The amount of goat antibody associated with the beads is a reliable indicator of the number of beads that are present. The ELISA absorbance 405-nm readings were curve fit to serially diluted mock, E-selectin IgG, and P-selectin IgG-coated bead standards, all of which had essentially identical amounts of goat IgG. The binding of the mutant proteins to fetuin in the presence of increasing concentrations of free oligosaccharide was performed as described above except that serial dilutions of the carbohydrates were included during the 1-h incubation.

Heparin Binding Assay—A stock solution of heparin-albumin (5 mg/ml) (lyophilized weight/volume) was made in 25 mM Tris (pH 7.5), 1 mM 2-mercaptoethanol, 0.5% SDS, and 5% glycerol. This solution was boiled for 10 min, cleared by brief centrifugation, chilled to 0 °C, diluted to a final concentration of 25 μg/ml in PBS, and coated onto 96-well flexible assay plates. Plates were subsequently blocked with 3% BSA in PBS. Beads conjugated with various P- and E-selectin IgG proteins (2×10^4 beads) were added to each well in a 55-μl volume of PBS supplemented with 0.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ and incubated for 1 h. Wells were then washed sequentially with PBS until unbound beads were removed. The number of beads retained in the wells was determined by ELISA using an alkaline phosphatase-conjugated rabbit anti-goat IgG heavy and light chain antibody (Calbiochem catalog number 401512) to detect the goat IgG that is present on the beads when they are purchased from Dynal. The ELISA absorbance 405 nm readings were curve fit to serially diluted mock, E-, and P-selectin IgG-coated bead standards, each of which had essentially identical amounts of goat IgG.

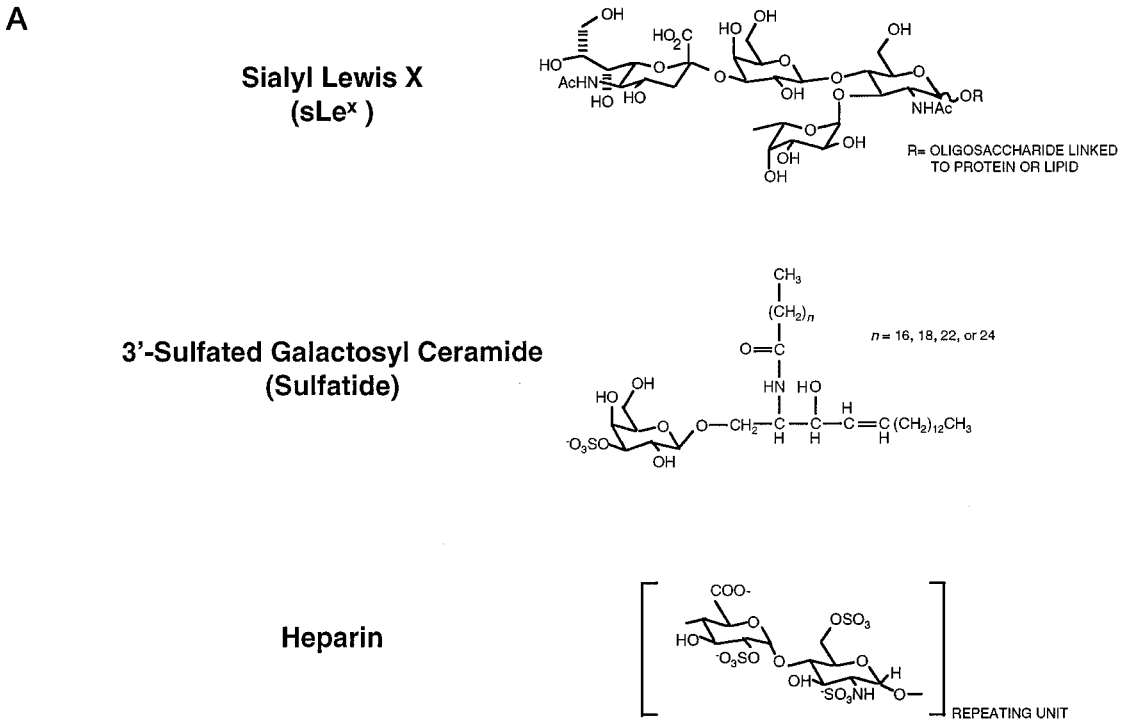
Glycolipid and Sulfatide Binding Assays—sLe^x containing glycolipids were prepared from HL-60 cells as described (7). Glycolipid or sulfatide (0.1 ml of a 1 μg/ml stock solution) were coated onto 96-well plates as described (27, 28). Beads conjugated with various selectin IgG proteins (2×10^4 beads) were added to each well in a 55-μl volume of PBS and incubated for 1 h. The plate was then inverted and unbound beads were blotted away. The plate was then washed once with PBS and unbound beads were removed by vacuum aspiration. The number of beads retained in the wells was determined by ELISA using an alkaline phosphatase-conjugated rabbit anti-goat IgG heavy and light chain antibody (Calbiochem catalog number 401512) to detect the goat IgG that is present on the beads when they are purchased from Dynal. The ELISA absorbance 405-nm readings were curve fit to serially diluted mock, E-, and P-selectin IgG-coated bead standards, all of which had essentially identical amounts of goat IgG.

RESULTS

To determine how the E-selectin S128R mutation might cause an increase in the incidence, early onset, and severity of atherosclerotic disease in individuals that possess this allele, we used site-directed mutagenesis to make the same adenine to cytosine mutation (16, 17) in both E- and P-selectin cDNAs and assessed binding of the altered proteins to several common selectin ligands (see Fig. 1*A*, for reference). These mutations changed amino acid codon 128 of each protein from serine to arginine (Fig. 1*B*). Selectin/mouse IgG fusion constructs were used to express the mutant and equivalent wild-type fusion proteins in COS-1 cells. Each expression cassette contained the coding sequence for the amino-terminal lectin, EGF, and the first two short consensus or complement repeats (SCR) of either E- or P-selectin (22) and were modeled after the selectin/IgG fusion proteins described by Walz *et al.* (6). The results of initial HL-60 cell binding experiments using the affinity purified recombinant proteins are shown in Fig. 2*A*. Interestingly, the S128R substitutions increase both E- and P- selectin binding 2–3-fold over that measured for each wild-type counterpart. This initial assessment was performed using HL-60 cells because binding of wild-type E- and P-selectin can be detected readily, consistently, and in an sLe^x-dependent manner (22, 23).

Although it is a relatively simple matter to rationalize increased E-selectin *in vitro* binding with the known *in vivo* atherosclerotic phenotype, the increased HL-60 cell adherence that was reproducibly measured by this assay was unexpected because as noted, the Ser^{128} residue is located in the EGF-like domain of the selectins. Although a small portion of this domain (residues 135–139) does contact the lectin domain (Gln^{30}), the Ser^{128} residue is not predicted by the E-selectin three-dimensional x-ray crystallographic structure to touch it (20). Since the lectin domain is believed to contain the carbohydrate binding site (20, 22, 29, 30) and wild-type selectin adherence to the HL-60 cell line can be blocked by soluble sLe^x tetrasaccharide or anti-sLe^x antibody which indicate that the adherence may be completely carbohydrate-dependent (Fig. 2*B*), it is difficult to understand how this mutation is able to enhance adhesion. Consequently, to better understand the mechanism by which this mutation increased adherence, we investigated the binding of the S128R mutation and several other E-selectin mutants with increased binding phenotypes. Since each of these mutations, like the S128R mutation, increases selectin binding to HL-60 cells, it was hoped that they might work through a common mechanism and that further detailed binding characterization would help to elucidate that mechanism. The enhanced binding phenotype of the R84A substitution was

[2] B. M. Revelle, and P. J. Beck, unpublished observation.



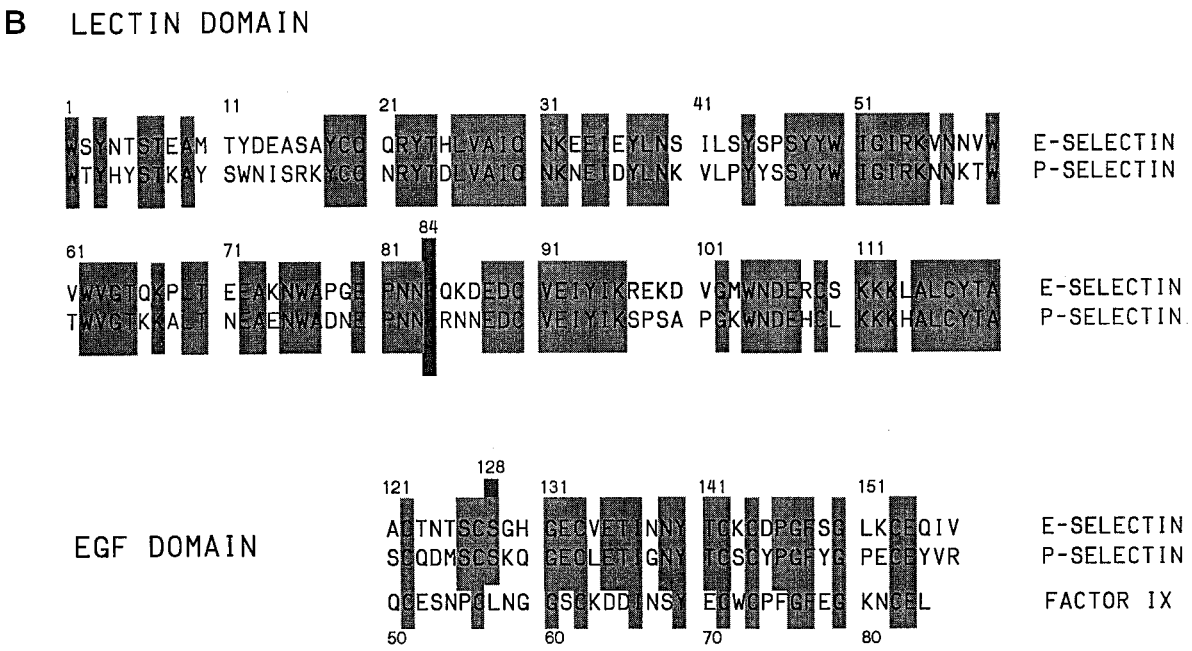


FIG. 1. *A*, selectin carbohydrate ligands. The three major selectin carbohydrate ligands are illustrated. *B*, amino acid sequence alignment of the E- and P-selectin lectin and EGF domains. Amino acid numbering begins with the tryptophan residue at the site of signal cleavage. Residues that are identical in both proteins are *shadowed*. Specific residues discussed in the text have been highlighted and labeled with the appropriate amino acid sequence numbers. The factor IX EGF domain is aligned as described previously by others (20).

originally published by Graves *et al.* (20) while the E107K and D89N mutations were noted by Kogan *et al.* (22).

To determine if the selectin mutant adherence to HL-60 cells was indeed carbohydrate-mediated, binding was evaluated with the sLe^x tetrasaccharide, anti-sLe^x antibody, or anti-Le^x antibody present in the assay. The results shown in Fig. 2*B* indicate that while both E- and P-selectin wild-type binding can be completely blocked by the anti-sLe^x antibody or significantly reduced by a 3 mM concentration of sLe^x tetrasaccharide, neither E- nor P-selectin S128R or E-selectin R84A substitution mutant binding is sensitive to these reagents. Alternatively, several other mutants with enhanced HL-60 cell binding phenotypes were sensitive to both the blocking antibody and to the tetrasaccharide at this concentration (Fig. 2*B*) which illustrates that the change in sLe^x binding affinity is dependent upon the precise mutation and not contingent upon an enhanced binding phenotype. None of the proteins tested could be inhibited by anti-Le^x antibody. However, the binding of each recombinant selectin protein tested appeared to be Ca^{2+} dependent since it could be completely inhibited by 5 mM EDTA (data not shown). Analyzed together, these results indicated that both the R84A and S128R mutants might have altered ligand binding specificities and that this changed specificity might result in their enhanced HL-60 cell binding

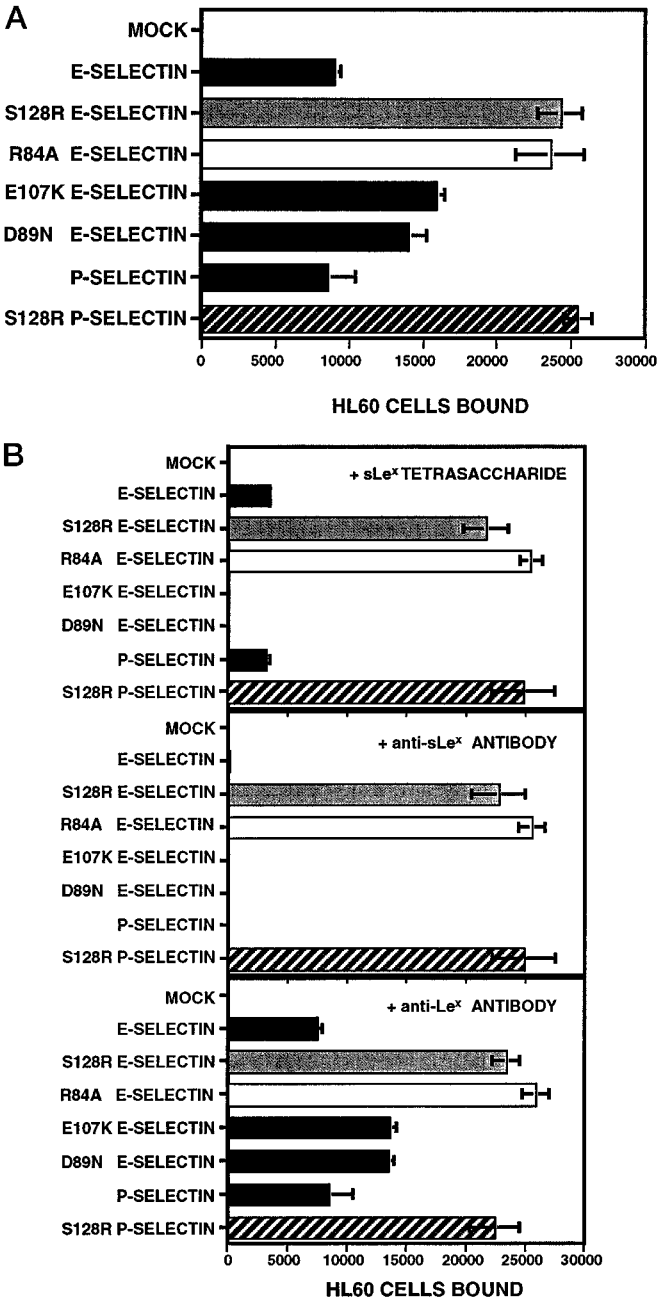


FIG. 2. *A*, assessment of selectin mutant binding to HL-60 cells. Selectin IgG recombinant proteins were immunoprecipitated using goat anti-mouse-conjugated magnetic beads. After ELISA quantitation, 4×10^4 selectin-IgG adsorbed beads were mixed with 10^5 fluorescently labeled HL-60 cells and incubated together at room temperature for 10 min. A magnetic separator was used to separate the bead-bound HL-60 cells. Unbound cells were removed by sequential PBS washes. The number of cells bound by protein was determined by curve fitting to a standard curve that was generated by serially diluting the labeled HL-60 cells. All values were background subtracted for the fluorescence generated by the mock control wells. All assays were performed in duplicate and the results shown are the average of two independent experiments. Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone. *B*, sensitivity of selectin HL-60 cell binding to sLe^x tetrasaccharide and anti-sLe^x antibody. Selectin protein binding to HL-60 cells was assayed as described in *A* with the exception that 3 mM sLe^x tetrasaccharide, 25 μg of anti-sLe^x antibody, or 25 μg of anti-Le^x antibody were included during the 10-min incubation at room temperature. All values were background subtracted for the fluorescence generated by the mock control wells. Each assay was performed in duplicate and the results shown are the average of two independent experiments. Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone.

phenotypes.

To establish if the R84A and S128R ligand specificity had indeed been altered from that of wild-type selectins, the mutants were assayed for the ability to bind the K562 cell line. This cell line does not express the sLe^x antigen and does not typically bind either E- or P-selectin. The results of the K562 assays are shown in Fig. 3*A*. Although neither wild-type selectin is able to bind, both R84A and S128R mutants adhere to and retain K562 cells. The K562 cell line is a human proerythroblastic leukemic cell line (31) with well characterized *N*-linked glycosylation that does not express the sialyl Lewis X antigen (32–34). As with the HL-60 cell line, mutant protein adherence to K562 cells is not inhibited by the anti-sLe^x antibody, anti-Le^x antibody, or 3 mM concentrations of soluble sLe^x tetrasaccharide (Fig. 3, *B* and *C*). However, it is sensitive to 2 mM concentrations and can be completely inhibited at higher concentrations of the trisaccharide, Neu5Acα2–3Galβ1–4GlcNAc (sialyllactosamine) (Fig. 3*C*).

To further investigate R84A and S128R ligand binding, bovine fetuin, a protein with thoroughly analyzed carbohydrate modification (35–37), was coated onto polystyrene dishes and the recombinant selectin proteins were tested for adherence. As a negative control, binding to a second protein, yeast invertase which is highly mannosylated but lacks typical mammalian complex carbohydrate modification, was also tested for selectin binding. As shown in Fig. 4*A*, both the R84A and S128R mutants bind to the boiled and denatured bovine fetuin-coated wells while neither of the wild-type selectin proteins do. The mutant adherence appeared to be carbohydrate-dependent since it was sensitive to concentrations of the sialyllactosamine trisaccharide that were higher than 0.25 mM (Fig. 4*B*), to 4-fold greater concentrations (>1 mM) of the (α1–3)-fucosylated sialyllactosamine tetrasaccharide (sLex) (Fig. 4*C*), and was not inhibited by L-fucose when present at an 80 mM concentration (Fig. 4*D*). None of the selectin proteins tested adhered to the invertase-coated wells.

Since bovine fetuin carbohydrate structure is well documented (35–37) and contains many differentially modified or branched sialyllactosamine units that are not α1–3-fucosylated, this result further supports the hypothesis that both the S128R and R84A mutants have altered ligand binding specificity. It also indicates that the mutants no longer require fucose for adherence. To further substantiate this result, glycolipids were isolated from HL-60 cells using the procedure of Tiemeyer and co-workers (7), coated onto polystyrene dishes, and assayed for selectin binding. The results of these glycolipid experiments (Fig. 5) also indicate that the S128R and R84A mutant adhesion is not dependent upon sialyl Lewis^x since it is not inhibited by coincubation with anti-sLe^x antibody. In contrast, the binding of the wild-type proteins is completely inhibited by this antibody.

Previous mutagenesis of amino acid residues located within the P-selectin lectin domain indicated that several residues could dramatically influence binding of the protein to sLe^x but that none of those same amino acids appeared to specifically change P-selectin binding to sulfatide (23). Those experiments also indicated that P-selectin binds sLe^x in a shallow grove that is defined by amino acid residues 75–80, 95–99, and 104–107. Although we have performed extensive mutagenesis of both of the E- and P-selectin lectin domains, none of the substituted residues appeared to specifically alter the binding capability of either selectin to sulfatide (22, 23). Alternatively, although substitution of E- or P-selectin EGF domain residue Ser^{128} with an arginine residue did not alter sulfatide binding (data not shown), this substitution did reduce both E- and P-selectin adherence to sLe^x and changed selectin ligand preference from

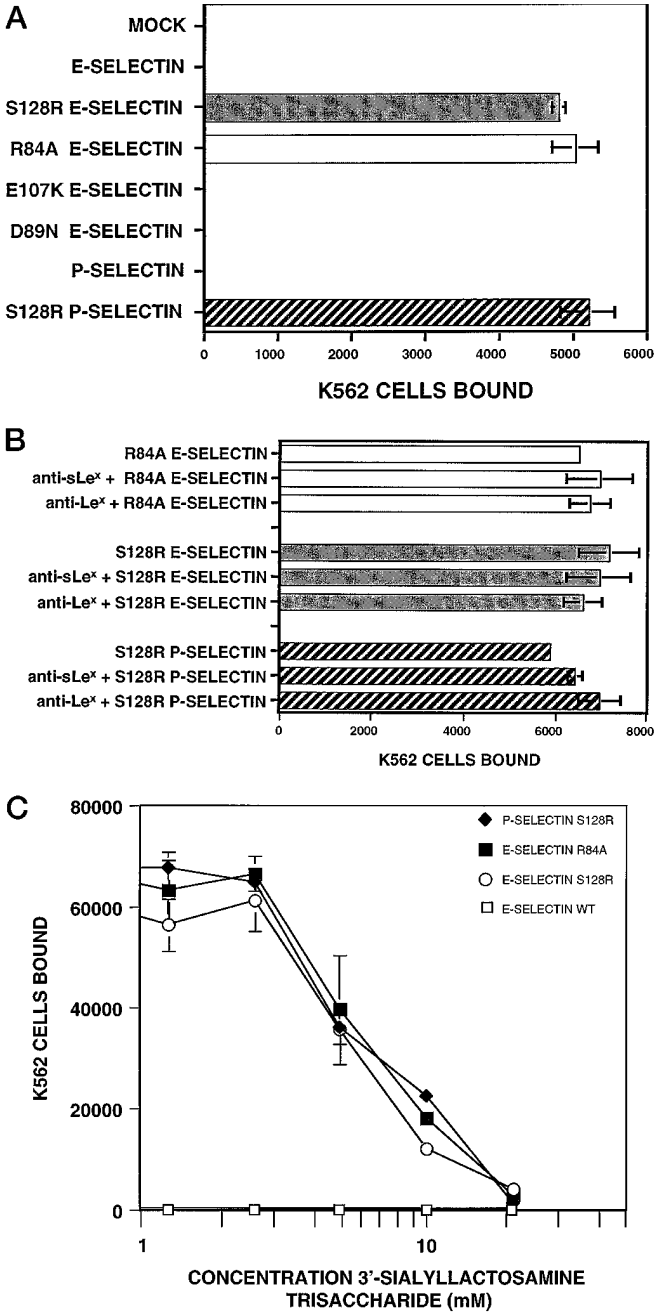


FIG. 3. *A*, R84A and S128R mutants bind K562 cells. Selectin protein binding to K562 cells was assessed precisely as described for HL-60 cells in the legend to Fig. 2. The number of K562 cells bound by protein was determined by curve fitting to a standard curve that was generated by serially diluting the labeled K562 cells. All values were background subtracted for the fluorescence generated by the mock control wells. All assays were performed in duplicate and the results shown are the average of two independent experiments. Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone. *B*, R84A and S128R mutant binding to K562 cell is not sLe^x-dependent. Selectin protein binding to K562 cells was assayed as described for HL-60 cells in the legend to Fig. 2 with the exception that 25 μg of anti-sLe^x antibody or 25 μg of anti-Le^x antibody were included during the 10-min incubation at room temperature. All values were background subtracted for the fluorescence generated by the mock control wells. Each assay was performed in duplicate and the results shown are the average of two independent experiments. Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone. *C*, sialyllactosamine inhibition is concentration-dependent. Selectin protein binding to K562 cells was assayed as described in the legend to Fig. 4*B* with the exception that increasing concentrations of trisaccharide (sialyllactosamine (Neu5Acα2–3Galβ1–4GlcNAc) were included during the 10-min incubation at room temperature. Data are defined as follows: E-selectin WT (*open squares*), E-selectin R84A (*filled squares*), E-selectin S128R (*open circles*), and P-selectin S128R (*closed diamonds*). All values were background subtracted for the fluorescence generated by the mock control wells. Each assay was performed in duplicate and the results shown are the average of two independent experiments. Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone.

sLe^x to sialyllactosamine. Since sulfatide binding has also been reported to inhibit P-selectin sLe^x binding (38), it seemed possible that residues involved in P-selectin sulfatide binding might be located in this same region of the selectin EGF domain and that the Ser^{128} mutation might be affecting a common regulatory mechanism that results in reduced sLe^x binding. Consequently, we undertook a more extensive analysis of the amino acids that comprise this area of both E- and P-selectin and monitored the binding of the recombinant mutant proteins to HL-60 cells, sialylated HL-60 glycolipids, sulfatide, and heparin. For clarification and reference, the chemical structures of each of these selectin carbohydrate ligands are described in Fig. 1*A*.

Since we had previously characterized the binding of the S128R mutant and knew that alteration of this residue could change both E- and P-selectin carbohydrate binding specificity, the HL-60 cell adherence of these additional residue 124 and 128 mutated recombinant proteins were tested for sensitivity to anti-sLe^x blocking antibody (CSLEX-1). As shown in Fig. 6*A*, two P-selectin and two E-selectin residue 124 substitutions specified proteins that bound to HL-60 cells in an sLe^x-dependent manner. However, like the S128R substitution, the HL-60 cell adherence of an E-selectin S128T mutant could not be blocked with anti-sLe^x antibody. Similar binding sensitivities were observed when the adherence of each of the proteins to sialylated HL-60 cell glycolipid extracts was tested (Fig. 6*B*). Additionally, although unable to bind to HL-60 cells using standard assay conditions, an E-selectin lectin domain IgG fusion protein that has no EGF domain (see "Experimental Procedures" for details) was also able to bind to sialylated HL-60 glycolipids. The binding of this protein could also be abolished with anti-sLe^x antibody but like the other selectin proteins tested, adherence was not sensitive to the effects of an anti-Le^x isotype control antibody. To further characterize the effects of each of these mutations upon selectin binding, the mutant proteins were tested for sulfatide binding. As illustrated in Fig. 7*A*, the two P-selectin Asp^{124} mutants displayed reduced adherence to sulfatide (80% and 30%) while each of two E-selectin Asn^{124} substitution mutants and one Ser^{128} mutant were able to bind to sulfatide. These data indicate that residues 124 and 128 are critical for selectin sulfatide binding.

Although the P-selectin heparin binding capability has been well documented (39), to our knowledge, the location of the actual heparin-binding site has not been identified. Since, like sulfatide, P-selectin binds heparin and E-selectin does not (39), we attempted to assess the effect of these same mutations upon selectin-heparin binding. As shown in Fig. 7*B*, under these assay conditions, the heparin binding capacity of each of the P-selectin mutants was not reduced from that measured for the wild-type IgG fusion protein. However, each of the three E-selectin mutants that bind sulfatide had also gained the ability to bind heparin. Additionally, the E-selectin S128R mutant which is unable to bind sulfatide, is able to bind heparin (Fig. 7*B*). It is notable that the R84A mutant is unable to bind either sulfatide or heparin (data not shown). Since the same amino acids that influence sulfatide binding also contribute to heparin binding, it appears that these two ligands are bound at the same or overlapping sites and that binding is dependent upon both EGF domain amino acid side chains 124 and 128.

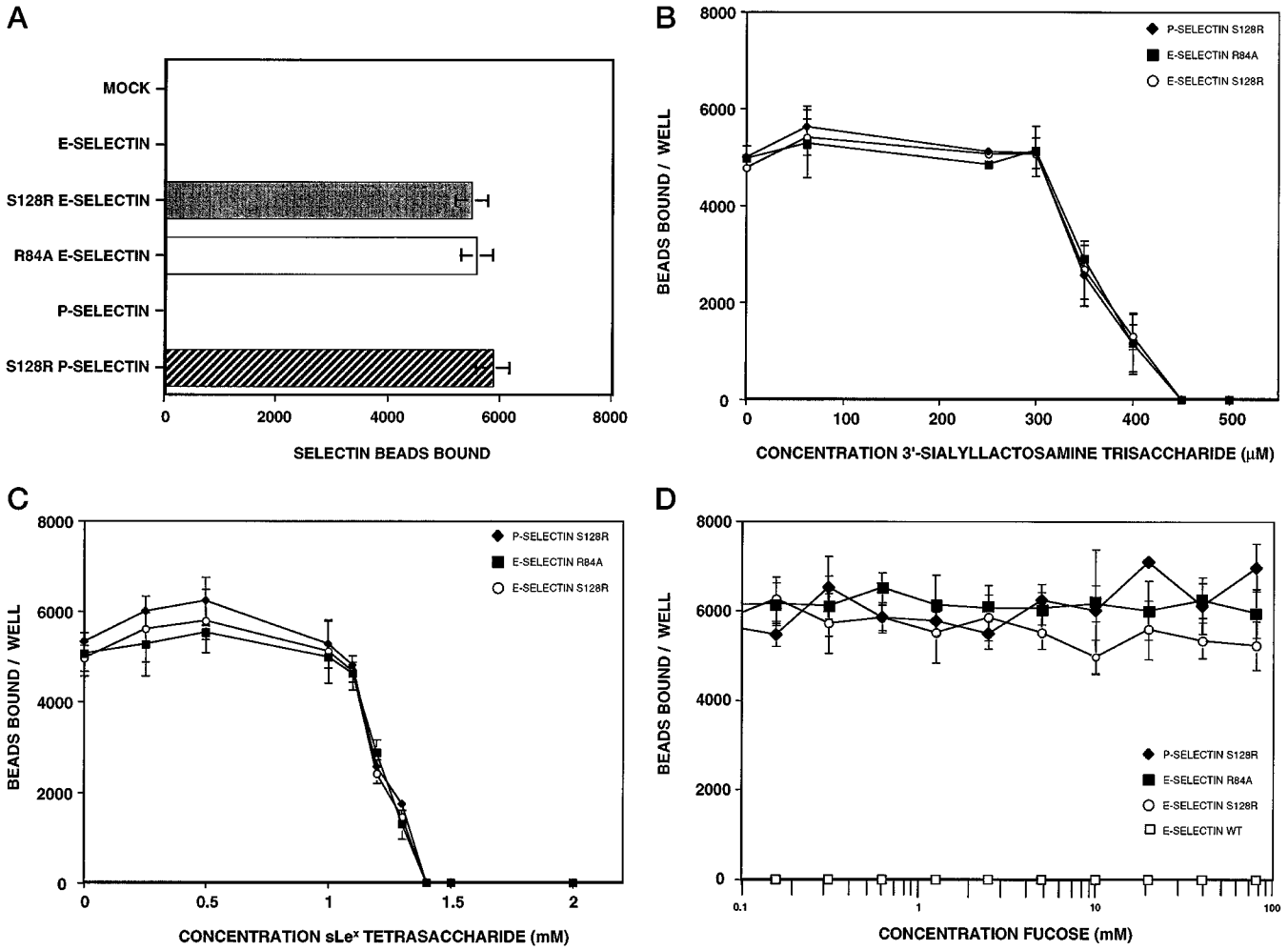


FIG. 4. *A*, assessment of selectin adherence to bovine fetuin. Following boiling, a solution containing 2.5 mg/ml (lyophilized weight/volume) of bovine fetuin in 25 mM Tris (pH 7.5), 1 mM 2-mercaptoethanol, 0.5% SDS, and 5% glycerol was diluted and coated onto 96-well flexible assay plates. Beads conjugated with various E- and P-selectin IgG proteins (2×10^4 beads) were added to each well and incubated for 1 h at room temperature. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate. *B*, selectin mutant binding to fetuin can be inhibited by sialyllactosamine. Selectin binding to fetuin in the presence of increasing concentrations of trisaccharide (sialyllactosamine (Neu5Acα2–3Gal(β1–4GlcNAc) was determined as described in the legend to Fig. 4*A*. Data are defined as follows: E-selectin R84A (*filled squares*), E-selectin S128R (*open circles*), and P-selectin S128R (*closed diamonds*). The results shown are the average of two independent experiments run in duplicate. *C*, selectin mutant binding to fetuin is sensitive to increasing concentrations of sLe^x tetrasaccharide. Selectin binding to fetuin in the presence of increasing concentrations of tetrasaccharide (sLe^x (Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc), Oxford GlycoSystems) was determined as described in the legend to Fig. 4*A*. Data are defined as follows: E-selectin R84A (*filled squares*), E-selectin S128R (*open circles*), and P-selectin S128R (*closed diamonds*). The results shown are the average of two independent experiments run in duplicate. *D*, selectin mutant binding to fetuin is not sensitive to increasing concentrations of fucose. Selectin binding to fetuin in the presence of increasing concentrations of L-fucose was determined as described in the legend to Fig. 4*A*. Data are defined as follows: E-selectin R84A (*filled squares*), E-selectin S128R (*open circles*), P-selectin S128R (*closed diamonds*), and E-selectin WT (*open squares*). The results shown are the average of two independent experiments run in duplicate.

To determine if heparin could competitively inhibit the adherence of the recombinant proteins to HL-60 cells (Figs. 8, *A* and *B*), to HL-60 cell-extracted sialylated glycolipids (Fig. 9*A*), or to sulfatide (Fig. 9*B*), the binding of the recombinant proteins to each of these substrates was tested in the presence or absence of heparin. In each of the assays described (Figs. 8*A* and 9, *A* and *B*), P-selectin binding could be completely inhibited with a 500 μg/ml concentration of heparin. Although still sensitive to heparin binding inhibition, the binding of each of the P-selectin mutants to HL-60 cells and sulfatide appeared to be less sensitive than the wild-type protein (Fig. 8*A*). Alternatively, wild-type E-selectin binding to HL-60 cells and sialylated glycolipids was not effected by this concentration of heparin (Figs. 8*B* and 9, *A* and *B*). However, the binding of each of the E-selectin sulfatide binding mutants was at least partially inhibited by a 500 μg/ml concentration of heparin. The adherence of the E-selectin N124D mutant appeared to be phenotypically identical to wild-type P-selectin.

DISCUSSION

The results presented indicate that changing E-selectin amino acid 84 from arginine to alanine or E- and P- selectin residue 128 from serine to arginine increases binding of the proteins to HL-60 cells. The data further indicate that binding of the mutant selectins to that cell line is not dependent upon the established, wild-type HL-60 cell selectin ligand, the sialyl Lewis X antigen. This alteration in ligand identity was detected while attempting to characterize several selectin mutants that appeared to have altered binding phenotypes (increased binding) and was first noted by the incapacity of anti-sLe^x antibody to block mutant adherence to HL-60 cells. This initial observation was further supported by the inability of 10 mM concentrations of the sLe^x tetrasaccharide to interfere with mutant HL-60 cell binding although that concentration of the tetrasaccharide completely inhibits binding of the wild-type selectins to those cells.

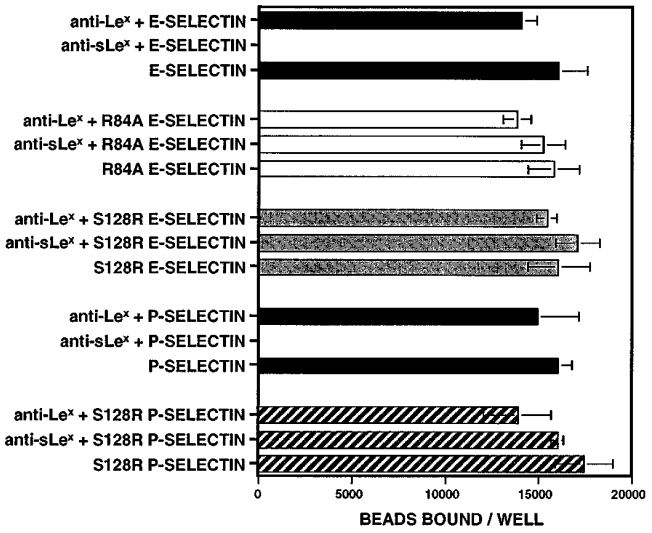


FIG. 5. **Selectin mutant glycolipid binding.** Selectin IgG fusion proteins were assayed for adherence to sialylated HL-60 cell glycolipid extract. Stock solutions containing glycolipids were prepared and coated onto 96-well flexible assay plates as described (24). Beads conjugated with various selectin IgG proteins (2×10^4 beads) were added to each well and incubated in the presence or absence of 10 μg/ml anti-sLe^x or anti-Lex antibody for 1 h. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate.

Bovine fetuin and K562 cell carbohydrate modifications have been extensively analyzed by other researchers (32–37) and neither are known to possess either α1–3- or α1–4-fucosylation. To further substantiate and characterize the observed alteration in selectin mutant ligand specificity, the selectin mutant proteins were tested and found to bind to both K562 cells and bovine fetuin. Adherence to K562, fetuin, and sialylated HL-60 glycolipid extracts was not sensitive to inhibition by either anti-sialyl Lewis X or anti-Lewis X blocking antibody. Furthermore, binding could be competitively inhibited by sialyllactose or sialyllactosamine trisaccharides at 3–4-fold lower concentrations than the sLe^x tetrasaccharide. Taken together, these data indicate that both the R84A and S128R substitutions confer an alteration in selectin ligand binding specificity such that mutant binding is no longer dependent upon the presence of an α1–3-linked fucose moiety for high affinity, calcium-dependent binding.

The method by which substitution of E-selectin Arg^{84} with alanine exerts such a dramatic effect on E-selectin carbohydrate binding is not particularly obvious. Since the R84A and S128R mutants have both lost the standard selectin requirement for fucose and still maintain high affinity binding, an obvious explanation is that selectin sLe^x binding typically occurs through ligation of the terminal sialic acid residue to the primary lectin domain calcium and that the Arg^{84} side chain that appears from the crystal structure to extend away from the bound calcium molecule (20) either directly interacts with the sLe^x fucose residue or alters the local protein secondary structure in such a way that other amino acids must directly contact the α1–3-linked fucose to stabilize and maintain selectin sLe^x selectivity and binding. It seems plausible that the alanine substitution at residue 84 allows the *N*-acetylglucosamine and/or galactose moieties to form more stable, higher affinity contacts with other amino acid side chains or backbone atoms and thus negates the necessity for fucose binding and subsequent sugar stabilization. In any case, it is important to realize that each of these proposals is highly speculative and that further work will be necessary to prove or disprove any of

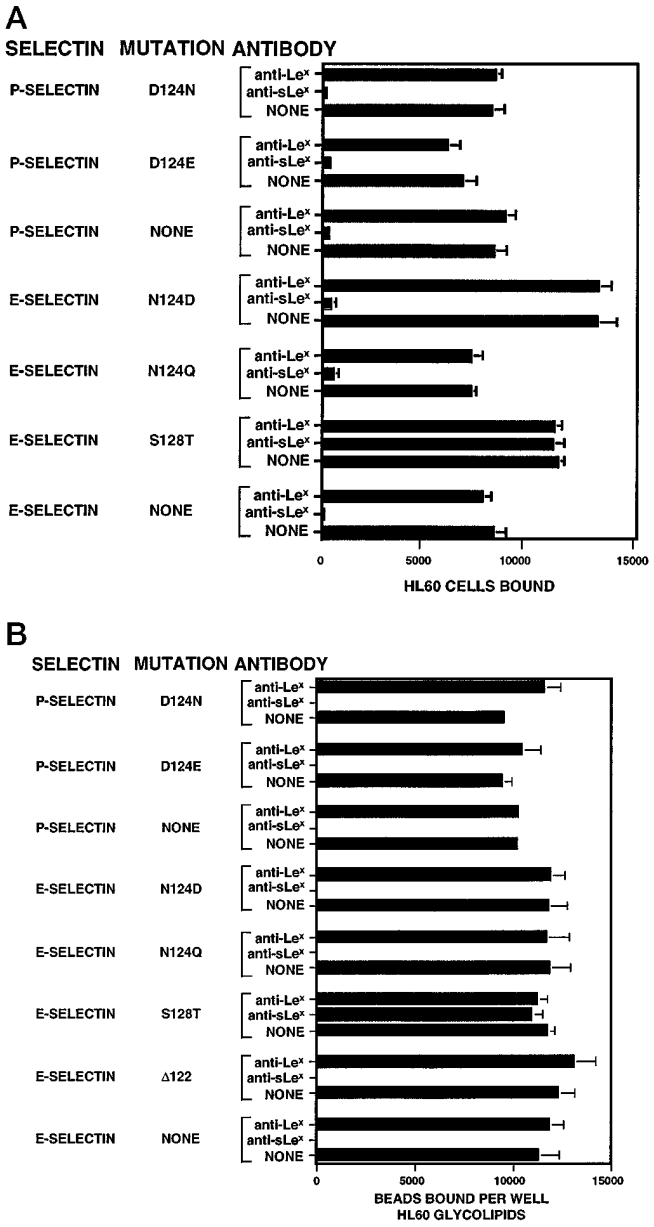


FIG. 6. *A*, assay of selectin HL-60 cell binding. Recombinant selectin proteins were assayed for binding to HL-60 cells. Selectin proteins were immunoprecipitated from transfected COS-1 cell supernatants using goat anti-mouse-conjugated magnetic beads. After ELISA quantitation to ensure that the same amount of recombinant selectin protein was attached to the beads, 4×10^4 selectin IgG adsorbed beads were mixed with 10^5 fluorescently labeled HL-60 cells and incubated together at room temperature for 10 min in the absence or presence of 50 μg/ml anti-sLe^x or anti-Le^x IgM antibodies. A magnetic separator was used to separate the bead-bound HL-60 cells. Unbound cells were removed by sequential PBS washes. The number of cells bound by the wild-type E- or P-selectin IgG fusion protein in the absence of antibody is defined as 100% and that for the mock control wells is designated 0%. All assays were performed in duplicate and the results shown are the average of two independent experiments. Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone. Binding was quantitated fluorimetrically. *B*, selectin mutant glycolipid binding ability. Selectin IgG fusion proteins were assayed for adherence to sLe^x glycolipid. Stock solutions containing sLe^x glycolipid were prepared and coated onto 96-well flexible assay plates as described (24). Beads conjugated with various P-selectin IgG proteins (2×10^4 beads) were added to each well in the absence or presence of 50 μg/ml anti-sLe^x or anti-Le^x IgM antibodies. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate. The Δ122 mutant lacks the E-selectin EGF domain. For a more detailed description of this protein, see "Experimental Procedures."

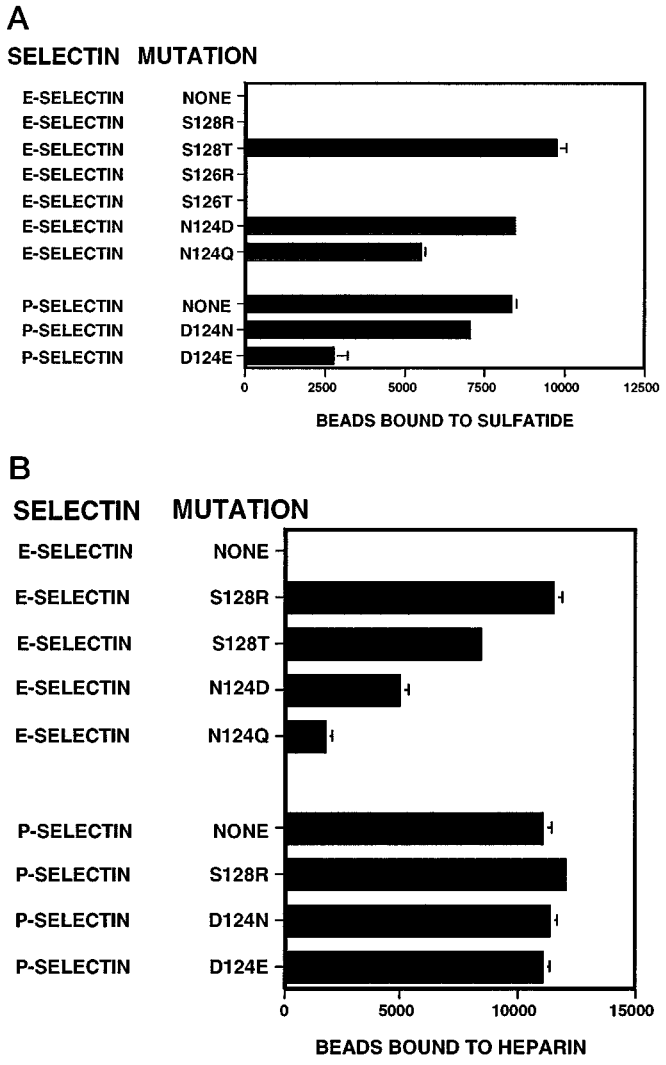


FIG. 7. *A*, selectin mutant sulfatide binding ability. Selectin IgG fusion proteins were assessed for the ability to adhere to sulfatide-coated 96-well flexible assay plates. Beads conjugated with various proteins (2×10^4 beads) were added to each well. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate. *B*, selectin mutant heparin binding ability. Selectin IgG fusion proteins were assessed for the ability to adhere to heparin albumin-coated 96-well flexible assay plates. Beads conjugated with various proteins (2×10^4 beads) were added to each well. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate.

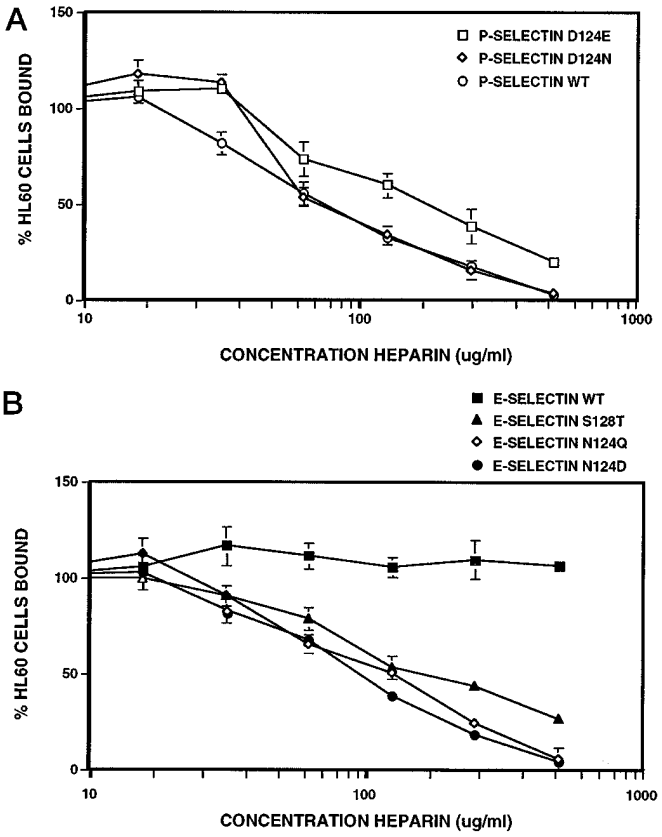


FIG. 8. *A*, heparin can inhibit selectin adherence to HL-60 cells. The binding of the P-selectin (*open circles*), D124E (*open squares*), and D124N (*open diamonds*) to HL-60 cells in the presence of increasing concentrations of free heparin was determined. Selectin proteins were immunoprecipitated from transfected COS-1 cell supernatants using goat anti-mouse-conjugated magnetic beads. After ELISA quantitation to ensure that the same amount of recombinant selectin protein was attached to the beads, 4×10^4 selectin IgG adsorbed beads were mixed with 10^5 fluorescently labeled HL-60 cells and incubated together at room temperature for 10 min in the presence of serially diluted heparin. A magnetic separator was used to separate the bead-bound HL-60 cells. Unbound cells were removed by sequential PBS washes. The number of cells bound by the wild-type P-selectin IgG fusion protein in the absence of heparin is defined as 100% and that for the mock control wells is designated 0%. All assays were performed in duplicate and the results shown are the average of two independent experiments. Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone. Binding was quantitated fluorimetrically. *B*, heparin can inhibit selectin adherence to HL-60 cells. The binding of the E-selectin (*filled squares*), N124Q (*open diamonds*), N124D (*filled circles*), and S128T (*filled triangles*) to HL-60 cells in the presence of increasing concentrations of free heparin was determined. Assays were performed as described in the legend to Fig. 3*C*. All assays were performed in duplicate and the results shown are the average of two independent experiments.

the suggested possibilities.

The results reported here imply that the R84A and S128R altered ligand specificity may in fact cause the enhanced adherence that was measured for each protein when binding to HL-60 cells was tested. While there is little available evidence that directly implicates E-selectin in the pathology of atherosclerotic disease, it is certainly possible that cytokines released from leukocytes recruited to the atherosclerotic region by other means (perhaps binding to P-selectin or VCAM-1) might induce transient E-selectin expression. In fact, it is difficult to believe that this does not occur. Since the E-selectin S128R mutant phenotype has been reported by Wenzel *et al.* (16, 17) to be associated with an increase in frequency, early onset, and severity of atherosclerotic disease in affected individuals, it seems possible that both the changed specificity and enhanced binding capacity might be responsible for the disease phenotype. One might easily hypothesize that the S128R substitution increases monocyte and neutrophil adherence to the activated endothelium in vascular areas that are already susceptible to atherosclerotic plaque formation and that the binding of these activated leukocytes and the cytokine mediators which they release may exacerbate and accelerate atherosclerotic disease progression. If this is indeed the case, one might further speculate that pharmaceuticals that are structurally identical or very similar to the sLe^x tetrasaccharide may not be the most effective prophylactic treatment for individuals possessing the S128R allele. However, further research is needed to better define and characterize this novel mutant ligand alteration before the most suitable pharmaceutical inhibitors can be determined.

In addition to the S128R and R84A binding characterization, we targeted the 124, 126, and 128 amino acid residues in the E-

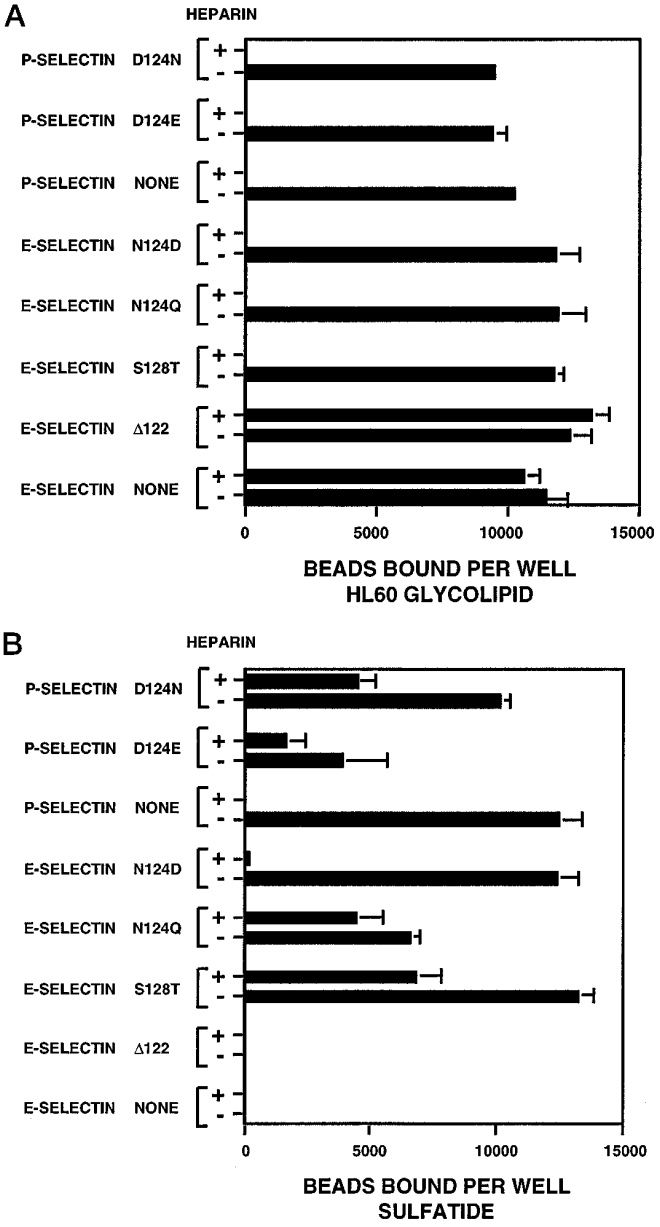


FIG. 9. *A*, heparin can inhibit selectin adherence to glycolipid. Selectin IgG fusion proteins were assessed for the ability to adhere to HL-60-derived glycolipid-coated 96-well flexible assay plates in the presence or absence of 500 μg/ml heparin. Beads conjugated with various selectin proteins (2×10^4 beads) were added to each well. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate. *B*, heparin can inhibit selectin adherence to sulfatide. Selectin IgG fusion proteins were assessed for the ability to adhere to sulfatide-coated 96-well flexible assay plates in the presence or absence of 500 μg/ml heparin. Beads conjugated with various selectin proteins (2×10^4 beads) were added to each well. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate.

and P-selectin EGF domains for further side chain substitution to determine if amino acids in this region of the protein are involved in P-selectin sulfatide binding. The data presented indicate that P-selectin sulfatide binding, while not completely abolished, can be severely compromised by the conservative substitution of a glutamic acid side chain for aspartic acid residue 124. Additionally, E-selectin, a protein that does not bind sulfatide or heparin can be induced to bind both of these sulfated carbohydrates by substituting either an aspartic acid or a glutamic acid side chain for Asn^{124}. A second conservative amino acid substitution, S128T, is also able to change E-selectin so that it binds both sulfatide and heparin proficiently and at the same time alters sLe^x-dependent binding while the S128R substitution displays altered sLe^x-dependent binding, binds heparin proficiently, and is unable to bind sulfatide. We do not know why these mutants display these particular binding specificities. Furthermore, it is difficult to understand how a mutation in the selectin EGF domain can effect carbohydrate binding that is mediated by the lectin domain. However, these results are consistent with the finding that heparin can competitively inhibit both sulfatide and sLe^x-dependent P-selectin adherence (39). Furthermore, there is substantial evidence that supports a functional role for the selectin EGF and complement-regulatory domains in cellular adherence. These data include domain switching mutagenesis that indicates that the EGF domains effect P-selectin binding specificity (18, 19), peptides derived from sequences within the EGF domain and antibodies with epitopes located within the EGF or complement-regulatory domains that are able to inhibit P-, E- or L-selectin cellular adherence (40, 41). It is further supported by the work of Li and co-workers (42) who have reported that the E-selectin complement repeat domains enhance the binding of E-selectin to its ligands. These studies together with the phenotypes of the mutants presented in this article lend support to the hypothesis that typical selectin-carbohydrate binding interactions are more complex than those predicted by a model depicting a single sLe^x oligosaccharide monomer docking into the lone lectin domain calcium site.

One explanation for the possible role of the Asn^{124} and Ser^{128} side chains during ligand recognition and binding might be the existence of a second metal binding site similar to that recently identified in the EGF-like domain of factor IX (43). This newly discovered factor IX calcium site is believed to ligate calcium in such a way that a second protein subunit or domain may ligate with and share the same calcium molecule. This shared calcium binding is thought to stabilize protein-protein interactions in a manner similar to that identified for the CD11b integrin I (A) domain (44). While there is no direct evidence to support this explanation for the residue 124 and 128 alteration of selectin ligand specificity, Graves *et al.* (20) have reported that the factor IX EGF domain is able to substitute for the E-selectin EGF domain and that such a chimeric protein supports selectin binding. Additionally, as determined by amino acid sequence homology, many of the factor IX amino acids involved in ligating the shared calcium are found in close proximity to selectin amino acid Ser^{128} (Fig. 1*B*). In this regard, it is also of interest that the CD11b integrin magnesium binding amino acid consensus sequence, D*X*S*X*S, which ligates and shares a Mg^{2+} ion with a second protein in a similar manner to that described for the factor IX EGF domain (44) is also present at the amino terminus of the P-selectin EGF-domain (amino acid residues 124–128) and that E-selectin has a similar sequence (Fig. 1*B*).

However, as noted, such a site is not predicted by the E-selectin crystal structure, nor would it be consistent with the work of Anostario *et al.* (45), who have very thoroughly analyzed E-selectin metal ion binding *in vitro*. So while it is attractive to invoke the presence of a metal-binding site at this position in the selectin EGF domain to rationalize the observed mutant phenotypes, there is currently no experimental evidence to support this hypothesis. Nevertheless, it should be noted that one cannot rule out the possibility that different physical proximities and presentations of individual selectin monomers might occur *in vivo* as a result of membrane tethering, membrane fluidity, or *in vitro* as a result of IgG fusion

protein dimerization and that these different conformations may result in physical associations that might not necessarily be observed under different physiological concentrations of monomeric selectin protein, metal ions, or buffers *in vitro*.

Although we are currently attempting to determine if one of several hypothetical selectin inter-domain binding interactions occur, it is notable that the E-selectin protein sequence that forms the contiguous sequence $N_{124}XSXS$ is also an *N*-linked glycosylation consensus sequence. Nevertheless, it seems unlikely that the positioning of this *N*-linked consensus site is relevant to selectin ligand binding since its presence or absence does not seem to correlate with the ability of the protein to bind sulfatide or heparin. This is best illustrated by the E-selectin S128T mutant that retains the *N*-linked consensus sequence and yet is able to bind sulfatide while a second mutant, S126R, that does not possess the *N*-linked consensus is phenotypically identical to the wild-type E-selectin IgG fusion protein and does not bind sulfatide.

In summary, the results reported here characterize the cellular and carbohydrate adherence of several selectin mutants. The affected amino acid residues have not previously been implicated in the modulation of selectin binding. We report the identification of several amino acid side chains within the E- and P-selectin EGF domains that modulate the adherence of the proteins to sLe^x, sulfatide, and heparin. Conservative substitution of P-selectin residue 124 reduces the binding of that protein to sulfatide. Alternatively, conservative substitution of E-selectin residues 124 and 128 alters the protein so that it gains the ability to bind sulfatide and heparin. It is hoped that the mutagenic structural and functional information presented here will contribute to the elucidation of selectin binding and the molecular regulation of that binding. How these mutations exert the observed modifications upon binding is difficult to rationalize. It seems probable that only a ligand bound three-dimensional structure of the wild-type and perhaps the mutant proteins will be able to resolve the mechanism(s) of action. However, the presented data do offer new insight into selectin/ligand interactions and perhaps identify the necessity for further structural and functional analyses of those interactions and their modulation. It is hoped that this information will aid in the design and identification of high affinity selectin inhibitors that may be used for the treatment of selectin-mediated inflammatory disease.

Acknowledgments—We thank Richard Dixon, Tim Kogan, Bill Weis, and Ed Yeh for helpful discussions and/or critical reading of this manuscript.

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