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 LewisX antigen (sLex or CD15s) (Neu5Ac2-3Gal1-4(Fucα1-3)GlcNAc). In addition to the sLex 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 sLex, heparin, or sulfatide. Substitution of E- and P-selectin EGF domain residue Ser128 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., Famielli, 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/sLex binding interactions may be significantly different from those previously hypothesized.

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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 Ser128Arg-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 sLex 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 Asp124 indicate that conservative modification of this residue can reduce P-selectin adherence to sulfatide. Additionally, mutagenic modification of the analogous E-selectin residue, Asn124, or of the Ser128 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 sLex, heparin, and sulfatide adherence.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media, dialyzed fetal calf serum, phospho-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-sLex (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 immunochromatographic procedures were purchased from Calbiochem. Sulfatides, fetuin (catenulin number F-3739), and heparin albumin were purchased from Sigma. L-Fucose was purchased from Aldrich. Flexible 96-well assay plates and Porin 96-well ELISA plates were purchased from Falcon. The sLex tetrasaccharide, Neu5Acα2→3Galβ1→4GlcNAc, and Neu5Acα2→6Galβ1→4GlcNAc were purchased from Oxford GlycoSystems. Synthetic oligonucleotides were purchased from Calbiochem and Sigma. Synthetic oligodeoxynucleotides 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% CO2 in RPMI supplemented with 10% fetal calf serum. COS-1 cells were grown at 37 °C with 5% CO2 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 106 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 using 0.22 μl 960 microfarcads. After electroporation, the cells were grown in 100-mm dishes for 72 h, at which time the culture media was collected, buffer to a final concentration of 10 mm Tris-HCl (pH 7.3), and centrifuged for 5 min at 600 × 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 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 IgG4, 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 previously (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'-GCTCTGGAATTCATGATGGTCAC-3' and 5'-TATTGGATATTCTGT-3'. The Bgl II site (GAATT) used 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 KPNI site that was constructed as described previously (22). This results in a protein that contains E-selectin amino acids Trp2 → Ala13, inserts Gly and Thr fusion residues encoded by the KpnI site, and continues with the mouse IgG, heavy chain hinge amino acid residues RGPTIKPCPPCK... and extends to the COOH terminus of the mouse IgG4, 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).
Selectin Mutants

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 × 10^4 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, incubations were performed, and the final 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. However, to maximize consistency, all experiments reported herein 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 could 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 blocking, the assay buffer, 100 µl of HL-60 cells (10^5 cells ml^-1 RPMI 1640, 10% fetal calf serum) that had been fluorescently labeled with Calcein AM-3099 (Molecular Probes) were added to each well followed by 10 µl of beads (4 × 10^4 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 the tetrasaccharide inhibitor, sLex, was 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 concentrated by centrifugation and then blotted dry by adding 50 µl of methanol to each well. The plate was then inverted 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, each of which had essentially identical amounts of goat IgG.

Glycolipid and Sulfatide Binding Assays—sLex 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 removed. 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.

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 MgCl2, 2.5 mM CaCl2, 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 as described (23). The plate was then washed once with PBS and incubated for 1 h. The plate was then inverted 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. 1A, for reference). These mutations changed amino acid codon 128 of each protein from serine to arginine (Fig. 1B). 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 cytosine mutation (16, 17) in both E- and P-selectin DNAs and assessed recombinant proteins are shown in Fig. 2A. 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 sLex-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 elastic 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 (Gln10), the Ser128 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 sLex tetrasaccharide or anti-sLex antibody which indicate that the adherence may be completely carbohydrate-dependent (Fig. 2B), 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.
TodetermineiftheselE- and P-selectin wild-type binding can be completely blocked by the anti-sLex antibody or significantly reduced by a 3 mM concentration of sLex 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. 2B) which illustrates that the change in sLex 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^a 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. 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).
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 SL\textsuperscript{e}\textsuperscript{ \(a\) } antigen and does not typically bind either E- or P-selectin. The results of the K562 assays are shown in Fig. 3A. 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-SL\textsuperscript{e}\textsuperscript{ \(a\) } antibody, anti-Le\textsuperscript{a} antibody, or 3 mM concentrations of soluble SL\textsuperscript{e}\textsuperscript{ \(a\) } 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, Neu5Ac\textsubscript{2}-3Gal\beta1-4GlcNAc (sialyllactosamine) (Fig. 3C).

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. 4A, 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. 4B), to 4-fold greater concentrations (>1 mM) of the (\(\alpha1–3\))-fucosylated sialyllactosamine tetrasaccharide (sLex) (Fig. 4C), and was not inhibited by \(\beta\)-fucose when present at an 80 mM concentration (Fig. 4D). 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 \(\alpha1–3\)-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\textsuperscript{X} since it is not inhibited by coincubation with anti-SL\textsuperscript{e}\textsuperscript{ \(a\) } 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 SL\textsuperscript{e}\textsuperscript{ \(a\) } 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 SL\textsuperscript{e}\textsuperscript{ \(a\) } in a shallow groove that is defined by amino acid residues 75–80, 95–99, and 104–107. Although we have performed extensive mutagenesis of both 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\textsuperscript{129} with an arginine residue did not alter sulfatide binding (data not shown), this substitution did reduce both E- and P-selectin adherence to SL\textsuperscript{e}\textsuperscript{ \(a\) } and changed selectin ligand preference from...
transfected with vector alone.

average of two independent experiments. Beads used in the mock assays were performed in duplicate and the results shown are the 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.

Selectin Mutants

A

MOCK
E-SELECTIN
S128R E-SELECTIN
R84A E-SELECTIN
E107K E-SELECTIN
D89N E-SELECTIN
P-SELECTIN
S128R P-SELECTIN

K562 CELLS Bound

0 1000 2000 3000 4000 5000 6000

B

R84A E-SELECTIN
anti-Le⁺ +
R84A E-SELECTIN
anti-Le⁻ +
R84A E-SELECTIN
S128R E-SELECTIN
anti-Le⁺ + S128R E-SELECTIN
anti-Le⁻ + S128R E-SELECTIN
S128R P-SELECTIN
anti-Le⁺ + S128R P-SELECTIN
anti-Le⁻ + S128R P-SELECTIN

K562 CELLS Bound

0 2000 4000 6000 8000

C

P-SELECTIN S128R
E-SELECTIN R84A
E-SELECTIN S128R
P-SELECTIN S128R
P-SELECTIN WT

K562 CELLS Bound

0 1 2 3 4 5 6 7 8 9 10

CONCENTRATION 3-SIALyllACTOSAMINE TRISACCHARIDE (mM)

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 cells is not sLeα-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-sLeα antibody or 25 μg of anti-Leα 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. 4B 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.

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-sLeα blocking antibody (CSLEX-1). As shown in Fig. 6A, two P-selectin and two E-selectin residue 124 substitutions specified proteins that bound to HL-60 cells in an sLeα-dependent manner. However, like the S128R substitution, the HL-60 cell adherence of an E-selectin S128T mutant could not be blocked with anti-sLeα antibody. Similar binding sensitivities were observed when the adherence of each of the proteins to sialylated HL-60 cell glycolipid extracts was tested (Fig. 6B). 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-sLeα antibody but like the other selectin proteins tested, adherence was not sensitive to the effects of an anti-Leα 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 Fig. 7A, the two P-selectin Asp124 mutants displayed reduced adherence to sulfatide (80% and 30%) while each of two E-selectin Asn124 substitution mutants and one Ser128 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. 7B, 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. 7B). 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.

sLeα to sialyllactosamine. Since sulfatide binding has also been reported to inhibit P-selectin sLeα 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 Ser128 mutation might be affecting a common regulatory mechanism that results in reduced sLeα 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. 1A.

(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.
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. 9A), or to sulfatide (Fig. 9B), 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. 8A and 9A and B), P-selectin binding to fetuin can be inhibited by sialylactosamine. Selectin binding to fetuin in the presence of increasing concentrations of trisaccharide (sialylactosamine (Neu5Acα2–3Galβ1–4GlcNAc) was determined as described in the legend to Fig. 4A. 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 SLε tetrasaccharide. Selectin binding to fetuin in the presence of increasing concentrations of tetrasaccharide (SLε (Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc), Oxford GlycoSystems) was determined as described in the legend to Fig. 4A. 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 fucose was determined as described in the legend to Fig. 4A. 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.

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 sialylactosamine. Selectin binding to fetuin in the presence of increasing concentrations of trisaccharide (sialylactosamine (Neu5Acα2–3Galβ1–4GlcNAc) was determined as described in the legend to Fig. 4A. 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 SLε tetrasaccharide. Selectin binding to fetuin in the presence of increasing concentrations of tetrasaccharide (SLε (Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc), Oxford GlycoSystems) was determined as described in the legend to Fig. 4A. 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 fucose was determined as described in the legend to Fig. 4A. 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.

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 inability of anti-SLeα antibody to block mutant adherence to HL-60 cells. This initial observation was further supported by the inability of 10 mM concentrations of the SLeα 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.
Bovine fetuin and K562 cell carbohydrate modifications have been extensively analyzed by other researchers (32–37) and neither are known to possess either \( \alpha_1-3 \)- or \( \alpha_1-4 \)-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 sLex 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 \( \alpha_1-3 \)-linked fucose moiety for high affinity, calcium-dependent binding.

The method by which substitution of E-selectin Arg84 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 sLex binding typically occurs through ligation of the terminal sialic acid residue to the primary lectin domain calcium and that the Arg84 side chain that appears from the crystal structure to extend away from the bound calcium molecule (20) either directly interacts with the sLex fucose residue or alters the local protein secondary structure in such a way that other amino acids must directly contact the \( \alpha_1-3 \)-linked fucose to stabilize and maintain selectin sLex 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 these ideas.

**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 \( \mu \)g/ml anti-sLex 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.

**Fig. 6.** A, assay of selectin HL-60 cell binding. Recombinant selectin proteins were assayed for binding to HL-60 cells. Selectin IgG fusion 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 × 10^5 selectin IgG adsorbed beads were mixed with 10^6 fluorescently labeled HL-60 cells and incubated together at room temperature for 10 min in the absence or presence of 50 \( \mu \)g/ml anti-sLex or anti-Lex antibody for 1 h. After washing, 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 carbohydrate binding. Selectin IgG fusion proteins were assayed for adherence to sLex glycolipid. Stock solutions containing sLex 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 and incubated in the presence or absence of 10 \( \mu \)g/ml anti-sLex 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. The S122R mutant lacks the E-selectin EGF domain. For a more detailed description of this protein, see “Experimental Procedures.”
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\(^4\) 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-
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 Asn124. 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 SLex-dependent binding while the S128R substitution displays altered SLex-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 SLex-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- or 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 SLex oligosaccharide monomer docking into the lone lectin domain calcium site.

One explanation for the possible role of the Asn124 and Ser128 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 Ser128 (Fig. 1B). In this regard, it is also of interest that the CD11b integrin magnesium binding amino acid consensus sequence, DXSXS, which ligates and shares a Mg²⁺ 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. 1B).

However, as noted, such a site is not predicted by the E-selectin crystal structure, nor would it be consistent with the work of Anastario 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 occurs, it is notable that the E-selectin protein sequence that forms the contiguous sequence N₁₂₄-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 sLex, 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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