Peptides Which Bind to E-selectin and Block Neutrophil Adhesion*

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E-selectin is an inducible cell adhesion molecule which mediates rolling of neutrophils on the endothelium, an early event in the development of an inflammatory response. Inhibition of selectin-mediated rolling is a possible means for controlling inflammation-induced diseases, and several classes of compounds have been tested for this use. We describe here the use of recombinant peptide library screening for identification and optimization of novel ligands which bind to E-selectin. Several of these peptides bind with \( K_d \) values in the low nanomolar range and block E-selectin-mediated adhesion of neutrophils in static and flow-cell assays. Administration of the peptide to mice undergoing an acute inflammatory response reduced the extent of neutrophil transmigration to the site of inflammation, demonstrating the utility of this compound as a potential therapeutic. The identification of a peptide ligand for E-selectin suggests that the complete natural ligand for this adhesion molecule may include protein as well as carbohydrate moieties.

E-selectin is a cell adhesion molecule which is induced on the surface of endothelial cells in response to inflammatory cytokines (1, 2). Binding of E-selectin to its ligand expressed on the surface of circulating neutrophils initiates rolling, an early step in the recruitment of these cells to a site of injury or inflammation (3, 4). The sequence of E-selectin (2, 5, 6) shows that it is a member of the mammalian C-type lectin family (7), and its three-dimensional structure shows strong similarity to mannos-binding protein (8). The carbohydrate structure sialyl Lewis x (sLex; 1 Neu5Ac

1–2Galβ1–3Galβ1–4[Fucα1–3]GlcNAc has been identified as a ligand which binds to the lectin domain at the N terminus of E-selectin (9–12). However, natural ligands may also contain protein (13, 14) or other carbohydrate structures. Inhibition of neutrophil adhesion to endothelium is an attractive approach to controlling inflammation-mediated diseases such as rheumatoid arthritis or psoriasis (15). Several potential therapeutics have been tested for their ability to inhibit the E-selectin-neutrophil adhesion event, including carbohydrate-based molecules (16, 17), antibodies (18), soluble E-selectin (19), and selectin-L chimeras (20). While these molecules have been useful to show the utility of selectin blockers for treating inflammation, each has significant drawbacks as a therapeutic, including short in vivo half-life, potential immunogenicity, high cost, and other possible side effects. A further limitation of these approaches is the lack of an efficient means to improve the pharmaceutical properties of these molecules.

In the past few years, several methods for creating and screening vast libraries of recombinant peptides have been developed (21–24). These libraries have been used to discover novel peptide ligands for several proteins, including antibodies (21, 24, 25), receptors (26, 27), and lectins (28, 29), as well as novel enzyme substrates (30–33). We report here the use of recombinant peptide display technology (21) to identify and optimize peptide ligands for E-selectin. Members of this peptide family block E-selectin-mediated neutrophil adhesion in vitro and reduce influx of neutrophils to a site of inflammation in vivo.

EXPERIMENTAL PROCEDURES

Receptor Preparations—The extracellular domain of E-selectin was expressed in a phosphatidylinositol-glycan-linked form in Chinese hamster ovary cells. Human E-selectin cDNA purchased from R&D Systems served as template for polymerase chain reaction amplification of the extracellular domain using primers which provided restriction sites for ligation in frame with a PI-glycan linkage signal sequence from human placental alkaline phosphatase in a mammalian expression vector (34). This construct was electroporated into CHO cells, and transfected cells were selected by growth in G418. Cells expressing the PIG-linked selectin were stained with a fluorescein isothiocyanate conjugate of a monoclonal antibody (mAb179) which binds tightly to the C-terminal PIG-linkage signal sequence, and the most brightly stained cells were cloned by FACS. Soluble extracellular domain of E-selectin was cleaved from the cell surface using phosphonatitol-dependent phospholipase C (34) and was used as the crude harvest without further purification. PI-PLC-harvested E-selectin immobilized on mAb179-coated microtiter wells mediated adhesion of HL-60 cells, and this adhesion could be blocked by BBA2 (R&D Systems), a blocking antibody specific for human E-selectin. Extracellular domains of human L-selectin (35), P-selectin (36), ICAM-1 (37), VCAM-1 (38), and TNF receptor p55 (39) were expressed in CHO cells as the PI-glycan linked form using the same vector. All these receptors retained at their C termini the epitope for mAb179, which is part of the PI-glycan linkage signal sequence. These receptors were immobilized on mAb179 for cell adhesion, ELISA, and binding assays.

Phage libraries were constructed in the phage vector fAff1 as described (21). Mutagenesis libraries used the phagemid vector pAFF2, which was constructed by cloning the phl gene from fAFF1 into the phagemid expression vector pBAd18 (a gift from L. Guzman and J. Beckwith). Expression of the phl gene is under the control of the arabinose promoter and begins with an ATG start codon. The phage libraries were constructed and screened as described (24, 32). The 70:10:10 mutagenesis strategy indicates use of a mixture of 70% correct nucleotide with 10% each of the other three nucleotides during synthesis of the oligonucleotide used for construction of the library. For panning, phage were incubated in microtiter wells coated with mAb179 at 5 μg/ml followed by a 1:50 dilution of the PI-PLC harvest of

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‡ The abbreviations used are: sLex, sialyl Lewis x; FACS, fluorescence-activated cell sorter; PIG, phosphatidylinositol-glycan; PI-PLC, phospholipase C; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; TNF, tumor necrosis factor; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; BSA, bovine serum albumin; IL, interleukin; rpm, counts/min; HUVEC, human umbilical vein endothelial cell; LPS, Escherichia coli lipopolysaccharide.
E-selectin extracellular domain. Phage were incubated in wells at 4°C for 2 h, washed with 30 min with cold PBS, and eluted with 0.1 M HCl adjusted to pH 2.2 with glycine and containing 0.1% BSA. Eluted phage were amplified, and screening was repeated over three, four, or five rounds. Individual phage clones were picked from agar plates and amplified, and receptor-specific clones were identified using a phage ELISA (40) in microtiter wells coated with mAb179 (5 µg/well) followed by a 1:50 dilution of E-selectin harvest (Fig. 1). Negative control wells contained mAb179 alone or an unrelated PIG-linked receptor (TNF receptor p55) immobilized on the mAb. DNA was prepared from clones found to be positive on E-selectin-coated wells but negative on control wells, and DNA sequencing was carried out as described previously (20).

Competition Binding Assay—Synthetic peptides prepared by standard solid-phase methods were purchased from SynPep Corp. A synthetic peptide of sequence LLRASLG (41, 42), was labeled with cyclic AMP-dependent protein kinase from bovine heart (Sigma) and [32P]ATP (>1000 Ci/mmol; Amerham Corp.) (41, 42). Labeled peptide was separated from unlabeled peptide and unincorporated ATP by passage through a C18 high performance liquid chromatography column (Vydac, 300 Å, 0.5 × 25 cm) and elution with a gradient of 10–90% acetonitrile in 0.1% trifluoroacetic acid. For direct binding assays, microtiter wells were coated sequentially with streptavidin at 5 µg/well, biotin-conjugated mAb179 at 1 µg/well, and E-selectin receptor harvest at a 1:50 dilution. Approximately 50,000 cpm of [32P]ATP-labeled peptide was added in a buffer of PBS, BSA, 0.02% Tween 20, and 0.1% BSA, 10 µM EDTA, 0.02% Tween 20, and were added and allowed to bind at 4°C for 2 h. Wells were washed and counted in a Packard TopCount scintillation counter. Non-specific background was determined by preincubating immobilized receptor with 10 µM unlabeled AF10166 (H2N-DITWDQLWDLMK-COOH) for competition assays. Test peptides at concentrations ranging from 10 µM to 100 µM and 50,000 cpm of labeled peptide were added to wells containing receptor immobilized on streptavidin and biotinylated mAb179 as described. Assay buffer was PBS, 1% BSA, 10 µM EDTA, 0.02% Tween-20. Conditions were used which allowed a maximum of 10% of the radioligand to bind to wells, to avoid depletion of the radioligand and nonspecific binding. These conditions were maintained for 2 h, washed with cold PBS and counted to determine the relative test peptide concentration needed to inhibit 50% of radioligand binding. The IC50 value was calculated by non-linear least-squares regression to a four-parameter logistic equation (44). For sLex competition experiments, receptor was preincubated with the carbohydrate (Oxford GlycoSystems) at concentrations ranging from 10 µM to 50 µM, and F(ab’2)2 fragments of CL2/6, an anti-human E-selectin (fCG1) monoclonal antibody, were used at 20 µg/ml. HUVEC were harvested by collagenase treatment as described previously (46) and plated onto gelatin (0.1%, Sigma)-coated flasks. Cells were grown in M199 medium containing 10% fetal calf serum and 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 1% penicillin-streptomycin, 1% fungizone (all from Life Technologies, Inc.), 1% heparin (Sigma), and endothelial cell growth factor (Sigma). HUVEC were passaged at confluence by trypsin-EDTA and plated onto fibronectin (5 µg/ml)-coated 35-mm tissue culture dishes. These cells were maintained in the medium without endothelial cell growth factor, with 0.001% hydrocortisone and 12.5 µg/ml polymyxin (Sigma). HUVEC monolayers were stimulated with LPS (Sigma) at 30 ng/ml for 4 h.

Cell Adhesion Assays—E-selectin-binding Peptides

The flow assay was conducted in a parallel plate flow chamber as described previously (46). A HUVEC monolayer was mounted on the flow chamber, and PBS with Ca2+ and glucose was perfused over the monolayer for 2 min. Peptide AF11677 was added at various concentrations to this perfusion buffer. AF11793, an unrelated peptide which does not bind to E-selectin (H-N-NTCDGKWCTGGGGS-CONH2), was used as a negative control at 100 µM, and F(ab’2)2 fragments of the monoclonal anti-E-selectin antibody CL2/6 were used at 20 µg/ml. The neutrophil suspension was diluted to 107 cells/ml in PBS with Ca2+ and glucose. The peptide was added and the suspension was passed over the HUVEC monolayer at a wall shear stress of 1.85 dyne/cm2. The interaction was observed for 11 min under a phase-contrast microscope (Diaphot-TMD, Nikon Inc., Garden City, NY) and videotaped. OPTIMAS, an imaging program (Biocsan Inc., Edmonds, WA), was used to determine the average number of neutrophils that rolled on the HUVEC monolayer in five different fields of view. Data were tested for statistical significance using an unpaired one-tailed Student’s t test.

Leukocyte Transmigration in Vivo—Female BALB/c mice (retired breeders, Simonsen, approximately 30 g) were given an intraperitoneal injection of 5 ml of 0.1% oyster glycogen (Sigma) at t = 0. Peptides were administered as a 2-mg intravenous injection 3 h later. Neutrophils were collected from peritoneal lavage 4 h after injection of glycogen, stained with Diff-Quik (Sigma) to determine the percentage of neutrophils, and counted. Data were tested for statistical significance using a nonparametric unpaired two-tailed t test.

RESULTS

Screening of Recombinant Peptide Libraries against E-selectin—The soluble extracellular domain of E-selectin was expressed in a phosphtidylinositol (PI)-glycan-linked form on CHO cells, and was released from the cell surface with 2 E. Whitehorn, L. M. Kochersperger, E. Tate, S. Yanofsky, A. Davis, R. B. Mortensen, S. Yongovich, K. Bell, and R. W. Barrett, manuscript in preparation.

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PI-PLC. A monoclonal antibody, mAb179, recognizes a portion of the PI-glycan linkage signal sequence at the C terminus of the released receptor and can be used to immobilize active receptor in microtiter wells. Using E-selectin immobilized on mAb179, three plII phage display libraries were screened for peptides which bound to E-selectin: a random 8-mer, a random 12-mer, and a library with a variable number of random amino acids (ranging from two to six) between two cysteine residues. Clones which bound specifically to E-selectin and not to negative control wells were identified in the random 12-mer library after four rounds of affinity selection, using a phage ELISA assay (Fig. 1). DNA sequence analysis of these clones revealed a single nucleic acid sequence, from which the peptide sequence HITWDQLWNVMN was deduced. This initial sequence formed the basis for the design of nine phage mutagenesis peptide libraries (listed in Table I) comprising a total of approximately $10^9$ analogs of the original sequence. These libraries were designed with different levels and distributions of sequence diversity to evaluate a very large number of analogs of the original sequence and to assess the roles played by different positions conserved in every clone isolated. No E-selectin-specific clones were isolated from the mutagenesis libraries, it appears that the structural motif which is responsible for binding to E-selectin spans at least eight amino acids and requires tryptophan at position 11; hydrophobic amino acids are preferred at position 3 and also contained a mutagenesis of the core sequence yielded phage which bound to E-selectin, thus demonstrating that an N-terminal extension did not abolish binding. However, no consensus sequence was recognized outside the core 12-amino-acid sequence. A library (M10) that contained these four conserved positions and randomized the other eight again showed strong preferences for related amino acids at several positions. Based on analysis of all E-selectin-binding peptide sequences selected from the mutagenesis libraries, it appears that the structural motif which is responsible for binding to E-selectin spans at least eight amino acids and requires tryptophan at positions 4 and 8, leucine at position 7, and methionine at position 11; hydrophobic amino acids are preferred at position 10, and serine or threonine is always found at position 3 in these phage clones.

A mutagenesis library (M11, Table I) was constructed in a peptides-on-plasmids vector, in which peptides are displayed at the C terminus of the lac repressor protein (24). E-selectin-binding clones were identified in this library, thus demonstrating that a free C terminus is tolerated. The characteristics of peptides isolated from this library were somewhat different from those expressed at the N terminus of the phage plII protein. Most striking was the finding that peptides-on-plasmids clones were much more likely to encode positively charged amino acids at the N terminus (Table I), while phage clones more frequently had a negatively charged N-terminal amino acid. This may reflect biological biases against positive charges at the plII N-terminus.
of carbohydrate ligands to E-selectin requires calcium (49), because binding to E-selectin has been determined to be about 750 nM (40). We tested whether sLex would compete with peptide binding to E-selectin. At concentrations of up to 10 μM, sLex did not inhibit the binding of the radiolabeled peptide to immobilized E-selectin (data not shown). AF10166 may therefore bind to a site on E-selectin which recognizes a protein or other structure on the neutrophil surface structure rather than the sLex carbohydrate determinant.

A series of peptides was synthesized to test the effects of truncating one or two amino acids from the N and C termini of the highest affinity peptide, AF10166 (Table III). Removal of the N-terminal aspartic acid reduced binding affinity by about 3-fold; removal of a second N-terminal residue diminished affinity by an additional 220-fold. Truncation of the C-terminal lysine residue decreased binding affinity by 120-fold, and further truncation abolished receptor binding. Amidation at the C terminus of the full-length peptide did not alter binding affinity, while acetylation at the N terminus caused a small decrease. The truncation series shows that 11 or 12 amino acids provide the optimal structural features for high-affinity binding to E-selectin.

Receptor Specificity—E-selectin is a member of a related family of cell adhesion molecules which also includes L-selectin and P-selectin (3, 50). These three adhesion molecules show a high degree of homology in their N-terminal lectin domains, and all three appear to bind specifically to sLex (10, 11, 51–54). We tested whether the peptides selected on E-selectin would bind to L- or P-selectin, or to ICAM-1 or VCAM-1, adhesion molecules which are not structurally related to the selectins. These adhesion molecules were expressed in PI-glycan-linked molecules which were PI-glycan-linked, made soluble by PI-PLC cleavage, and immobilized on mAb179, in the same manner as described for E-selectin. In an ELISA, phage bearing the E-selectin-binding peptide AF10172 bound specifically to both immobilized L-selectin and P-selectin, but not to low-affinity nerve growth factor receptor (Fig. 3A) or to ICAM-1, VCAM-1, or TNF receptor p55 (data not shown). Because each phage particle carries four to five copies of the peptide, the ELISA can detect phage bearing peptides with affinities as low as 100 nM (40). We then tested whether radiolabeled AF10166 could bind to L-

### Table II

| Peptide no. | Display format | Peptide sequence | IC50 (μM) |
|-------------|----------------|------------------|-----------|
| AF 10166    | pIII           | H2N–DITWDQLWDLMK–COOH | 4.0       |
| AF 10168    | pIII           | H2N–DYTWQLEMDMK–COOH | 4.4       |
| AF 10172    | pIII           | H2N–QITWDQLWDMK–COOH | 11        |
| AF 10176    | pIII           | H2N–DMTWQLDLMS–COOH | 23        |
| AF 10177    | pIII           | H2N–DYSWQLEDMS–COOH | 57        |
| AF 10177    | pIII           | H2N–EITWDQLWDMK–COOH | 67        |
| AF 10175    | pIII           | H2N–HVSQWLEDMS–COOH | 76        |
| AF 10173    | pIII           | H2N–HITWDQLWDMK–COOH | 83        |
| Affy 4      | pIII           | H2N–HIVTWQLEMDMK–COOH | 420       |
| AF 10180    | pIII           | H2N–DIWQLEDMS–COOH  | 620       |
| AF 10181    | pIII           | H2N–QITWDQLWDMK–COOH | 910       |
| AF 10454    | Lad            | H2N–RNKWMLEWDMK–COOH | 5.4       |
| AF 10455    | Lad            | H2N–AENKWDQLWYNEPAEQ–COOH | 23       |
| AF 10452    | Lad            | H2N–HRWNLWADLQSP–COOH | 47       |
| AF 10453    | Lad            | H2N–KRWNLWADLQSM–COOH | 71       |
| AF 10456    | Lad            | H2N–KKKRWLEWDMK–COOH | 1200      |
| AF 11678    | Ac–           | WKLWLTLMWDQ–COOH  | >30,000   |
| Affy 9      |                | H2N–HITWDQLWDMKLLRASLG–COOH | >11,000   |

Several sequences inferred from DNA sequencing of clones isolated from phage displayed on E-selectin were selected for synthesis and further characterization. "Display format" indicates the type of peptide library in which the sequence was originally identified. Bold letters identify the four amino acids which were constant in all E-selectin-binding clones identified. A scrambled sequence was synthesized as a negative control in some experiments; other experiments used Affy9, which lacks the correct amino acid at position 12 and has a C-terminal extension, and was found to have very low affinity for E-selectin.

### Competition binding assays

A, radiolabeled peptide binding assay was performed on immobilized E-selectin as described under "Experimental Procedures." Nonspecific background was determined by preincubating immobilized receptor with 10 μM unlabeled AF10166. Representative curves are shown from assays used to determine the IC50 of synthetic peptides binding to E-selectin. ε, AF10166; Ω, AF10172; λ, AF10181. B, calcium is not required for peptide binding to E-selectin. Competition assays were carried out as described under "Experimental Procedures" with radiolabeled AF10166 as the tracer. Assays were performed in buffer containing either 10 mM EDTA (69) or 424 μM calcium and 406 μM magnesium (83). AF10185 (H2N–DITWDQLWDLMK–COOH2) was added to wells at the concentrations shown (8 x 10–10 to 2.1 x 10–10 M).

We then tested whether sLex would compete with peptide binding to E-selectin. At concentrations of up to 10 μM, sLex did not inhibit the binding of the radiolabeled peptide to immobilized E-selectin (data not shown). AF10166 may therefore bind to a site on E-selectin which recognizes a protein or other structure on the neutrophil surface structure rather than the sLex carbohydrate determinant.

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P-selectins. No convincing specific signal was seen either on P- or L-selectin (Fig. 3B) or on the unrelated receptors. These results suggest that AF10166 binds specifically to L-selectin and to P-selectin, but with a much lower binding affinity than to E-selectin.

Blocking of Cell Adhesion by Synthetic Peptides—Synthetic peptides were tested for their ability to inhibit neutrophil adhesion to E-selectin. Three static adhesion assay formats were used: HL-60 cells, a human promyelomonocytic cell line, adhering to soluble E-selectin immobilized on mAb179; normal human granulocytes adhering to immobilized E-selectin; and HL-60 cells adhering to cytokine-induced human umbilical vein endothelial cells. In all three formats, peptide AF10166 was shown to inhibit specifically the adhesion of HL-60 cells or granulocytes to E-selectin (Fig. 4, A-C). The IC50 for the inhibition of this multivalent interaction was approximately 5–10 μM in all three formats. As a further control for specificity, carboxyamidated AF10166 (AF10185) at concentrations up to 300 μM was found to have no effect on adhesion of HL-60 cells to immobilized P-selectin (data not shown). Thus this peptide appears to interact with E-selectin at a site which blocks binding of the natural ligand expressed on the surface of neutrophils.

To confirm that this peptide family is effective at blocking the interaction of neutrophils with E-selectin, one compound was tested for its ability to block rolling of normal neutrophils on LPS-stimulated HUVECs in a flow assay. Neutrophil rolling in this assay format is known to be mediated by E-selectin and L-selectin (46). Addition of the high-affinity peptide at concentrations of 2.5 μM and higher inhibited leukocyte rolling (Fig. 5). Lower peptide concentrations inhibited rolling to a lesser extent in a dose-dependent manner. The apparent IC50 for this assay was approximately 5 μM, similar to that determined in the static adhesion assays. The peptide decreased rolling to a greater extent (>90% at high concentrations) than did the blocking anti-E-selectin antibody CL2/6 (40%). Since multivalent peptide expressed on phage was found to interact with L-selectin (Fig. 3A), it is possible that at high concentrations the peptide binds to L-selectin on the neutrophils and thus blocks rolling more completely than does anti-E-selectin alone. Addition of a different peptide of similar size which does not bind to E-selectin (AF11793) did not reduce rolling. Thus this family of E-selectin-binding peptides is effective at preventing E-selectin-mediated neutrophil rolling on cytokine-activated endothelial cells in both static and flow assays.

The murine analog of E-selectin has recently been identified, and sequence analysis showed that this receptor has a high degree (~73%) of amino acid homology to human E-selectin in the lectin and EGF domains (6). To determine whether the peptides isolated by affinity for human E-selectin would cross-react with the mouse homologue, we tested whether AF10166 would inhibit binding of HL-60 cells to a cytokine-stimulated mouse brain endothelial cell line. Titration of AF10166 into wells containing TNF-α-stimulated MBEC inhibited adhesion of HL-60 cells in a dose-dependent fashion, with an IC50 of approximately 10 μM (Fig. 4D), a dose-response similar to that found for human endothelial cells (Fig. 4C). Radiolabeled AF10166 bound specifically to a mouse E-selectin-Fc fusion protein (6) immobilized on protein A, but did not bind to a human type I IL-1 receptor-Fc fusion (data not shown). Thus
AF10166 appears to bind to mouse E-selectin with high affinity. E-selectin is up-regulated during the early stages of an inflammation reaction, and blocking E-selectin with antibody (18, 55, 56), soluble E-selectin-Ig chimeras (16, 17, 57) prevents some acute inflammation reactions. Because AF10166 was able to bind to mouse E-selectin, we tested whether administration of an E-selectin-binding peptide to glycogen-injected mice would diminish the influx of neutrophils into the chemically irritated peritoneum (58, 59). Mice which had been given intraperitoneal glycogen received an intravenous injection containing 2 mg of AF10185 3 h after the glycogen injection, and peritoneal neutrophils were harvested and counted 1 h later. Peptide treatment significantly reduced the number of neutrophils in peritoneal lavage fluids (Fig. 6A). Control experiments administering the same dose of a closely related peptide sequence, Affy 9, which did not bind to E-selectin (Table I), showed no decrease in neutrophil influx relative to buffer-injected mice (Fig. 6B). Thus the E-selectin-binding peptide can block an acute inflammation reaction in vivo when administered intravenously. The requirement for this relatively high dose of peptide may reflect a short in vivo serum half-life of this peptide. A complete study of the pharmacokinetic properties of derivatives of this peptide is in progress.

**DISCUSSION**

We have used peptide display libraries to identify novel ligands for E-selectin, which was previously known to bind only to carbohydrates. Peptides in this family bind with high affinity to the selectin and block cell adhesion in both static and rolling assays. Intravenous administration of peptide blocks neutrophil influx into the glycogen-stimulated peritoneal cavity of mice. Thus peptides in this family can serve as lead structures for the development of E-selectin-blocking therapeutics.

The peptide family which was identified in these experiments has very clear structural relationships and suggests that the requirements for binding to E-selectin are stringent. Of more than $10^9$ analogs of the original sequence which were tested in mutagenesis libraries, only a tiny fraction bound to E-selectin. All active peptides contained four conserved residues, which appear to be required for binding with high affinity, but many phage which retained these four residues failed to bind to E-selectin (e.g. mutagenesis libraries M10 and M11). Truncations demonstrated that the highest affinity interaction occurs with 11 or 12 amino acids, though smaller fragments bind with lower affinity. These structural requirements suggest that the peptides in this family may take on a defined structure in solution, despite their small size. Circular dichroism and NMR studies are in progress to address this possibility.

Binding of AF10166 to human E-selectin blocks neutrophil adhesion in both static and flow-cell assays, demonstrating that the peptide blocks the binding of the natural ligand present on the surface of neutrophils. The carbohydrate sLex has been identified as a critical part of the ligand structure which binds to E-selectin (10–12), and thus it is possible that AF10166 may mimic a carbohydrate structure. Peptides have been identified by phage display technology which bind to the lectin concanavalin A and which block binding of its carbohydrate ligand, methyl α-D-mannopyranoside (28, 29). However, our data suggest that the peptides described here are not acting as glycomimetics. AF10166 and sLex do not compete for binding to E-selectin, and AF10166 binding does not require calcium as does sLex. Thus AF10166 may bind to a different site on the receptor which is involved in recognition of the natural ligand, perhaps a protein moiety. Alternatively, binding of the peptide might cause a conformational change in the receptor which then prevents it from recognizing the ligand on the neutrophil surface. It is clear that sLex is not the entire ligand for E-selectin. sLex is found on many cells which do not adhere to E-selectin (60, 61), and many sLex-containing glycoproteins do not bind to P-selectin or E-selectin (14, 62). Further, sLex alone is a poor inhibitor of cell adhesion in vitro, even at concentrations up to 1 mM (17). A glycoprotein has been characterized as the E-selectin ligand on mouse myeloid cells by affinity purification of cell extracts on murine E-selectin (14, 63), and a glycoprotein P-selectin ligand, PSGL-1, binds to E-selectin as well (64). This and other evidence suggest that additional elements, possibly complex carbohydrate structures or protein determinants, make up the complete E-selectin ligand (14).
However, there is no direct evidence to date that any selectin actually interacts with a natural protein determinant.

In addition to the possible E-selectin ligands described above, several groups have shown evidence that L-selectin presents carbohydrate determinants to E-selectin and thus acts as an E-selectin ligand (46, 65, 66). The peptide family described here binds to L-selectin as well as to E-selectin, albeit with low affinity (Fig. 3). The dramatic inhibition of rolling observed in the flow cell assays suggested that some binding to L-selectin may have occurred at the high concentrations used for these experiments and led to more complete inhibition of the interaction between neutrophils and endothelial cells. While it is formally possible that this peptide interacts with some other surface structure on the activated endothelial cells to inhibit adhesion under conditions of shear flow, this seems unlikely since the peptide was isolated on purified E-selectin and was shown to have no detectable binding to ICAM-1, VCAM-1, or several other cytokine receptors. Addition of the peptide to a P-selectin-mediated static adhesion assay had no effect, and neutrophils were not directly activated by the peptide. Thus the most likely explanation for the strong inhibition of neutrophil rolling is that the peptide is bound by both E-selectin and L-selectin, resulting in a level of inhibition similar to that seen with antibodies against both adhesion molecules. While L-selectin-dependent adhesion mechanisms are clearly important for rolling on endothelial cells under conditions of shear flow, experiments with E-selectin-transfected L cells suggested E-selectin interaction with other yet undefined ligands might be important in the initial steps of rolling (46). Whether this is true for endothelial cells as well as L cells has not been established. In contrast to the rolling assays, peptide inhibition of static adhesion assays on activated HUVECs was about the same as anti-E-selectin (Fig. 4C), suggesting that the mechanism responsible for rolling differs from that for static adhesion.

While the complete natural ligand(s) for E-selectin has not been fully characterized, several mucin-like glycoproteins have been identified as ligands for L- and P-selectins (63, 64, 67–70). Notably, the first four residues of the mature PSGL-1 sequence, which binds to both P-selectin and E-selectin, are identical to those of the first four residues of the mature PSGL-1 sequence, which binds to both P-selectin and E-selectin, are identical to those of the first four residues of the mature PSGL-1 sequence, which binds to both P-selectin and E-selectin, are identical to those of the first four residues of the mature PSGL-1 sequence, which binds to both P-selectin and E-selectin, are identical to those of the first four residues of the mature PSGL-1 sequence, which binds to both P-selectin and E-selectin, are identical to those of the first four residues of the mature PSGL-1 sequence, which binds to both P-selectin and E-selectin, are identical to those of the first four residues of the mature PSGL-1 sequence, which binds to both P-selectin and E-selectin, are identical to those of the first four residues of the mature PSGL-1 sequence, which binds to both P-selectin and 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