The Soluble Form of E-selectin Is an Asymmetric Monomer

EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF THE RECOMBINANT PROTEIN

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Preston Hensley‡§, Patrick J. McDevitt†, Ian Brooks‡, John J. Trill, John A. Feild**, Dean E. McNulty†, Janice R. Connor**, Don E. Griswold‡§, N. Vasant Kumar‡§, Kenneth D. Kopple‡§§, Steven A. Carr‡§§, Barbara J. Dalton**, and Kyung Johanson†

From the Departments of ‡Macromolecular Sciences, †Protein Biochemistry, §§Gene Expression Sciences, **Cellular Biochemistry, ¶Inflammation and Respiratory Pharmacology, and §§Physical and Structural Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406-0939

The gene coding for a soluble form of human E-selectin (sE-selectin) has been expressed in Chinese hamster ovary (CHO) cells. Cells seeded into a hollow fiber reactor secreted protein at a level of 160 mg/liter. The protein was purified to >85% pure and low endotoxin (<2 ng/mg), using physiological pH and buffers. The amino acid composition and N-terminal sequence were as predicted from the cDNA sequence. HL-60 cells bound to sE-selectin-coated plates in a dose-dependent manner, and this binding could be blocked up to 100% by pretreatment of HL-60 cells with sE-selectin. The concentration of sE-selectin required for 50% inhibition was 1 μg. This value puts an upper limit for the affinity of E-selectin for its natural receptor. sE-selectin also inhibited inflammatory migration of neutrophils in a selective fashion. Purified sE-selectin exhibited a broad band of Mₐ ~ 75,000 on nonreducing SDS-PAGE. sE-selectin eluted with Mₐ ~ 310,000 from size exclusion chromatography at physiological pH and buffers, suggesting an oligomeric state. Matrix-assisted laser-desorption MS gave a molecular weight of 80,000, while the minimum monomer molecular weight from the gene sequence should be 58,571, demonstrating that the monomeric molecule thus expressed had 27% carbohydrate. Equilibrium analytical ultracentrifugation gave an average solution molecular weight of 81,600 (± 4,500). Velocity ultracentrifugation gave a sedimentation coefficient of 4.3 S and, from this, an apparent axial ratio of 10.5:1, assuming a prolate ellipsoid of revolution. An analysis of the NMR NOESY spectra of sE-selectin, sialyl-Lewis X, and sE-selectin with sialyl-Lewis X demonstrates that the recombinant protein binds sialyl-Lewis X productively. Hence, in solution, sE-selectin is a functional elongated monomer.

The specific recruitment of leukocytes from the blood through endothelial cells in postcapillary venules is the first step in the pathophysiology of a wide range of acute and chronic inflammatory conditions, as well as wound healing and tissue repair. At sites of inflammation, leukocytes, which have been shown to roll along the surface of the venules, become attached to the endothelium, flatten, and pass between adjacent cells into the subendothelium in a process called diapedesis (Atherton and Born, 1972). This weak attachment is stimulated by cytokines, such as tumor necrosis factor-α and interleukin-1, as well as by lipopolysaccharide, which induce the expression of cell adhesion molecules (Wellitome et al., 1990).

Selectins are a class of cell adhesion molecules which mediate this rolling behavior via low-avidity transient interactions between leukocytes and endothelial cells (Lawrence and Springer, 1991; von Andrian et al., 1991). To date, three classes of selectins, E-selectins, P-selectins, and L-selectins have been identified. They are distinguished by the cell type with which they are primarily associated. E-selectin is found on endothelial cells, P-selectin is found on platelets, and L-selectin is found on leukocytes (Benvilacaqua et al., 1991). All mature selectins share similar structural motifs. These consist of an ~120-amino acid calcium-dependent mammalian lectin domain at the N termini, followed by an epidermal motif, and are followed by a variable number of short consensus repeats found in complement regulatory proteins (six for E-selectin), a transmembrane region, and a C-terminal cytoplasmic domain. The selectins are predicted to be glycosylated, and the sites of N-linked glycosylation vary among the three classes (Benvilacaqua et al., 1989; Johnston et al., 1989; Siegelman et al., 1989; Hession et al., 1990).

E-selectin is expressed on the endothelial cells within 4–6 h following induction by cytokines and helps to mediate the initial rolling and adhering of resting neutrophils (Benvilacaqua et al., 1987, 1989), eosinophils (Kyan-Aung et al., 1991), basophils (Bochner et al., 1991), a subpopulation of T-lymphocytes (Graber et al., 1990; Picker et al., 1991; Shimizu et al., 1991), and monocytes (Carlos et al., 1991). The ligand for E-selectin on leukocytes has been determined to be a blood group antigen, sialyl-Lewis X (sLê) (Goelz et al., 1990; Lowe et al., 1990; Phillips et al., 1990; Walz et al., 1990) and is found on many leukocyte cell surface molecules. Because of the central role that E-selectin plays in the initial stages of inflammation, a significant effort has been undertaken to identify analogs of sLê that will antagonize this transient interaction. Such agents, if identified, may play an important role in reducing inflammation after myocardial infarction, in asthma, arthritis,

1 The abbreviations used are: sLê, sialyl-Lewis X; CHO, Chinese hamster ovary cells; G418, Geneticin; sE-selectin, soluble form of E-selectin; ELAM-1, E-selectin; MTX, methotrexate; PBS, phosphate-buffered saline; BCECF/AM (2',7'-bis-(2-carboxyethyl)5-(and-6)-carboxyfluorescein, acetoxymethyl ester; HBSS, Hank's buffered saline solution; BSA, bovine serum albumin; HBSS*, Hank's buffered saline solution containing 1% BSA; PBST, phosphate-buffered saline with 0.05% Triton X-100, pH 7.5; ABTS, 2,2'-azino-di-3-ethylbenzthiazoline sulfonate; MALD-MS, matrix-assisted laser desorption mass spectrometry; NOESY, nuclear Overhauser enhancement spectroscopy; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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† To whom correspondence and reprint requests should be addressed: Dept. of Macromolecular Sciences (UE-0447B), SmithKline Beecham Pharmaceuticals, 709 Swedeland Rd., King of Prussia, PA 19406-0939. Tel.: 215-270-7897; E-mail: HENSELEYCP@PHVAX.DNET@SB.COM.
stroke, acute respiratory distress syndrome, and potentially other inflammatory conditions.

As a first step in this drug discovery effort, a soluble form of human E-selectin has been produced. Such a molecule will be a valuable tool in establishing screens to identify SLE antagonists, and, once they are identified, it will play a role in high-resolution structure-function studies. However, an important question in the study of selectin-cell surface receptor or selectin-ligand interactions, even with purified macromolecules, is the question of valency. It has been suggested that P-selectin (GMP 140) is a tetramer on the basis of its behavior in gel permeation chromatography experiments (Skinner et al., 1989). SLE-selectin has been also proposed to the multivalent (Lobb et al., 1991) based on gel permeation chromatography experiments as chemical cross-linking studies. This potentially complicates the interpretation of ligand binding data. However, it has been recently reported that soluble P-selectin is an elongated rod-like monomer (Ushiyama et al., 1993) as determined by hydrodynamic and thermodynamic methods as well as electron microscopy. To establish the valency of SLE-selectin and to facilitates purification, a recombinant secreted form of E-selectin lacking both transmembrane and cytoplasmic regions has been expressed in Chinese hamster ovary (CHO) cells. The present study describes the expression, large scale purification, biological, biochemical, and physical characterization of a recombinant soluble form of human E-selectin.

MATERIALS AND METHODS
cDNA Cloning and Mutagenesis—The cDNA for full-length E-selectin, was obtained from British Biotechnology Limited (Oxford, UK) in the pCDM8 expression vector (here denoted pCDM8ELAM). The gene was excised on a XhoI fragment and cloned into pSelectTM (Promega). A stop codon and unique EcoRV site were introduced by site-directed mutagenesis (Alterne Site System, Promega). The gene was then ligated into the boundary between the sixth consensus repeat and the transmembrane domain, using the following oligonucleotide, which starts at nucleotide 1776: 5′-CCGCGGCCTCTCTTCTGAAGCTC-3′ (Fig. 1, A and B). E-selectin was inserted into the mammalian expression vector, RK3, as a KpnI/EcoRI fragment to yield the vector pKLMAMdn (Fig. 1, A and B). In addition to 80 base pairs of 5′-untranslated E-selectin DNA, this fragment also contains 55 base pairs of DNA from the p-Selectin vector.

COS Expression—COS-1 cells were transfected with 10 μg of DNA from either rELAMdn or pCDM8ELAM as described previously (Calkhoven et al., 1989) with the following modifications. After the cells were shocked with a 10% dimethyl sulfoxide in PBS solution (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and washed, the cells were refed with MR1 MOD3 medium (an in-house proprietary serum-free medium, here called maintenance medium) containing nucleosides and then incubated overnight on ice, the cells were plated into 96-well plates at 6 × 10^5 cells/ml and cultured for 72 h at 37 °C in 5% CO2, 95% humidified air.

CHO Expression—The CHO cell line, DG-44 (Urlaub et al., 1983), was adapted for suspension growth in maintenance medium. The cells were grown at 37 °C in a 5% CO2, 95% air-humidified incubator and were passaged at 4 × 10^5 cells/ml per 100 ml spinner flask twice weekly. The rELAMdn expression vector was linearized by digestion with Not I and electroporated, using a Bio-Rad Gene Pulser (Bio-Rad Laboratories), into DG-44 cells as described (Trill et al., 1990) with several modifications, i.e. 1 × 10^6 cells were suspended into 0.8 ml of ice cold PBS (500 mM sucrose, 7 mM sodium phosphate, pH 7.4, 1 mM MgCl2). The Gene Pulser was set at 380 V and 25 microfarads. After a 10-min incubation on ice, the cells were plated into 96-well plates at 6 × 10^5 cells/well in maintenance medium for 48 h prior to selection. Cells were selected for resistance to 400 μg/ml G418 (Geneticin™, Life Technologies, Inc.) in maintenance medium. 24 h prior to assay, the cells were grown in the selective medium. Conditioned medium from individual colonies was assayed by standard ELISA assay (see below).

Individual colonies stably expressing SLE-selectin were selected for resistance to methotrexate (MTX, see Fig. 1B) by plating 5 × 10^5 cells/well in a 96-well plate containing nonselective medium without nucleosides but with 10 μg MTX. Colonies with high SLE-selectin expression levels were then subjected to an additional selection in 100 μg MTX. An individual colony was scaled into a 100-ml spinner at 4 × 10^5 cells/ml in medium containing 100 μg MTX and passaged twice weekly to determine the growth and productivity parameters of this cell line. To generate conditioned medium for purification, CHO cells were seeded in a density of 3 × 10^6 cells into the extracapillary side of the reactor. Cells were cultured in BR1970 (Mw = 70,000 cutoff, 19 ft²) Hollow Fiber Bio reactor in a Cell Pharm Cell Culture System II (Unysyn Fibercet, San Diego, CA). Four days post incubation, 50–200 ml of medium was collected daily from the extracapillary side of the reactor. Cells were removed by centrifugation, and the medium was pooled and frozen at −70 °C.

Protein Concentration—Protein concentrations in crude extracts were determined by the bicinchoninic acid method (Pierce Chemical Co.) using bovine serum albumin as a reference. Protein samples (10 μl) were mixed with the 250 μl of BCA reagent in microtiter wells and incubated for 20 min at 45 °C. Absorbance at 560 nm was measured in a Molecular Devices V Max microplate reader. Concentrations of purified SLE-selectin were determined by absorbance at 280 nm (εmax = 102000 M^-1 cm^-1). Relative amounts of SLE-selectin present in CHO media and at each stage of purification were determined by densitometric scanning of silver-stained SDS-PAGE gels of various amounts of samples compared to various amounts of purified protein, in an LKB Model 2222 Scanning Densitometer. The values were compared with the results of ELISA.

Quantitation of SLE-selectin by ELISA—An ELISA was established to identify COS and CHO cells expressing SLE-selectin and to quantitate the amount of SLE-selectin during purification. A recombinant fusion protein, soluble E-selectin (sE-selectin), was produced by the following oligonucleotide, which starts at nucleotide 1776: 5′-CCGCGGCCTCTCTTCTGAAGCTC-3′ (Fig. 1, A and B). Relative amounts of sE-selectin present in CHO media and at each stage of purification were determined by densitometric scanning of silver-stained SDS-PAGE gels of various amounts of samples compared to various amounts of purified protein, in an LKB Model 2222 Scanning Densitometer. The values were compared with the results of ELISA.

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with a Beckman 12/16 system for on-line phenylthiohydantoin analysis. Data were acquired using System Gold chromatography software. Samples were spotted directly onto Polybrene-coated GF filters (Applied Biosystems, Inc.), and standard ABI sequencing cycles were used.

**SDS-PAGE and Western Immunoblotting—**Purification was monitored using SDS-PAGE, a method employed in the three cycles of freezing and thawing described previously (Gimbrone et al., 1989). Blots were probed with rabbit polyclonal antisera directed to Escherichia coli-expressed sE-selectin, which was purified from SDS-PAGE. They were stained with protein A horseradish peroxidase using 4-chloro-1-naphthol substrate.

**Endotoxin Assay—**Levels of endotoxin contamination in sE-selectin samples were measured using a Limulus amoebocyte lysate gelation assay (Associates of Cape Cod Inc., Woods Hole, MA). The concentration of endotoxin in a sample was calculated from the absorbance values of solutions containing known amounts of endotoxin standards. When the level was >2 ng of endotoxin/mg of protein, the sE-selectin sample was rechromatographed on Superose 6. Endotoxin eluted in void fractions separated from sE-selectin.

**Coated Plate Cell Binding Assay—**To assess biological activity of recombinant sE-selectin, a coated plate assay modified from a previously described method (Gimbrone et al., 1989) was employed. Microtiter plates (96-well Immunolon-2, Dynatech, Chantilly, VA) were coated with purified sE-selectin for 16 h at 4°C. Typically, sE-selectin was diluted to 0.5 µg/ml in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4), and 50 µl was added per well. To reduce nonspecific binding, plates were blocked with 2% heat-inactivated bovine serum albumin (BSA). Cells in HBSS* were centrifuged at 100 g for 6 min at 4°C and resuspended at 2 x 10^5 cells/ml in complete Hank's buffer saline solution (0.3 mM sodium phosphate, dibasic, 0.4 mM potassium chloride, 1.25 mM calcium chloride, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 4.2 mM sodium bicarbonate, 138 mM sodium chloride, 10 mM HEPES, pH 7.1) containing 1% BSA (HBSS*). Prior to assay, coated plates were washed once with 0.1 ml of HBSS*, then 0.1 ml of dye-labeled cell suspension was added to coated wells. Cells were allowed to settle and adhere to plates for 30 min at room temperature. Following adhesion, plates were washed twice with HBSS* and coated plates were shaken through a 19 gauge needle and gently adding HBSS* with an 8-channel multipipette. To quantitate adhesion, adherent cells were lysed for 10 min in 0.1 ml of 0.1 M NaOH containing 0.1% SDS, and plates were read in a Fluoroskan II plate reader (Labsystems, Helsinki, Finland) at excitation wavelength 540 nm and emission wavelength 590 nm. In some experiments, sE-selectin-coated wells were pretreated with 0.1% of mono- clonal antibody diluted in HBSS* for 30 min at room temperature. Labeled cells were added directly to wells containing antibody. Hybridomas producing anti-E-selectin were obtained from D. Haskard (Wellesley, MA, 1990), and Fib/1, fragments were prepared and purified by Maine Biotechnology Service (Scarborough, ME). Data were analyzed directly using nonlinear least squares methods and the equation, % input cells bound = B(x) X'(K_n + x) + NS (Johnson and Frasier, 1985; Johnson and Faunt, 1992). Where B(x) is the maximum bound, X is the concentration of sE-selectin in mg/ml, K_n is the concentration of sE-selectin added to the well producing 50% cell adhesion, and NS is the proportion of cells that are nonspecifically bound. To demonstrate inhibition of adhesion with sE-selectin, dye-labeled HEL60 cells were pretreated with increasing concentrations of purified protein for 30 min at room temperature prior to plating onto sE-selectin-coated plates.

**Arachidonic Acid-induced Inflammation—**The method used was as described previously (Griswold et al., 1993) with minor modifications. Arachidonic acid (Sigma) in acetone (1 mg/20 µl) was applied to the inner and outer surfaces of the left ear. The thickness cm) between treated and untreated ears. The thickness of both ears was measured with a thickness gauge (Mitutoyo, Japan) 1 h after application of arachidonic acid, and the data were expressed as the change in thickness (10^-2 cm) between treated and untreated ears. The application of acetone does not cause an appreciable response, and, therefore, the difference in ear thickness represented the response to arachidonic acid. The sE-selectin was administered intravenously in a volume of 0.15 ml/mouse just prior to the application of arachidonic acid. The total amount of sE-selectin in this volume is given in Fig. 7.

**Assay of Myeloperoxidase Activity in Inflamed Tissue—**The method of Bradley (Bradley et al., 1982) was used with modification. Inflamed ear tissue was minced and homogenized (10% w/v) with a Tissumizer (Tekmar Co.) in 50 mM phosphate buffer (pH 6) containing 0.5% hexadechloro-p-toludine and 10 mM hydrogen peroxide. The appearance of a colored product from the myeloperoxidase-dependent reaction of o-dianisidine (0.167 mg/ml; Sigma) and hydrogen peroxide (0.0005%; Sigma) was measured spectrophotometrically at 460 nm. Supernatant myeloperoxidase activity was quantified kinetically (change in absorbance measured over 3 min, sampled at 15-s intervals) using a Beckman DU-7 spectrophotometer and a kinetics analysis package (Beckman Instruments). One unit of myeloperoxidase activity is defined as that degrading 1 µmol of peroxide per min at 25°C.
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\[ f_t = f_f \times f_{fr} \quad (Eq. 3) \]

Here, \( f_t \) is the frictional factor for a sphere of the equivalent molecular weight and partial specific volume, \( i.e., \)
\[ f_f = 6\pi \eta (3M_w/4\pi N)^{1/3} \quad (Eq. 4) \]

\( f_f \) is the component of the frictional ratio contributed by hydration, which is defined as,
\[ f_f = (1 + w/\rho \nu)^{1/2} \quad (Eq. 5) \]

where \( w \) is the grams of H₂O bound per g of protein. This leaves \( f_{fr} \), which is the component of the frictional ratio contributed by shape. The value of \( f_{fr} \) can now be determined from Equation 2. The component of the frictional ratio due to shape can then be related to the axial ratio, \( p = b/a \) (assuming a prolate ellipsoid), where \( b \) is the short semi-axis and \( a \) is the long semi-axis, from the following equation (Cantor and Schimmel, 1980)
\[ f_{fr} = (1 - p^3)^{1/2}(p/p_f)^n \ln((1 + (1 - p^3)/p)) \quad (Eq. 6) \]

The reason why prolate and not oblate ellipsoids were considered is discussed below (see “Results and Discussion”).

NMR—A derivative of sialyl-Lewis X (Neu5Ac α-(2-3)-Galβ-(1-4)-[Fucα-(1-3)]GlcNAcβ-1-NAc (sLeα)) was purchased from Oxford Glyco-systems (Abington, UK). NMR samples consisted of 1–2 mg of sLeα, and of 5 mg of sE-selectin in 0.5 ml of deuterated Tris buffer, pH 7.8, and 1 mM CaCl₂. All NMR experiments were carried out on a Bruker AMX (Karlsruhe, Germany) spectrometer operating at a frequency of 500.14 MHz. Phase-sensitive NOESY experiments were performed using standard pulse sequence (Kumar et al., 1980) using time proportional phase incrementation (TPPI) for quadrature detection along \( \omega_z \) direction. Residual HDO signal was suppressed by presaturation during relaxation incrementation (TPPI) for quadrature detection along \( \omega_z \) direction. Residual HDO signal was suppressed by presaturation during relaxation incrementation (TPPI) for quadrature detection along \( \omega_z \) direction.

RESULTS AND DISCUSSION

Construction of Expression Vector—The construction of sE-selectin is described in Fig. 1A and the expression vector in Fig. 1B. The insertion of a stop codon at amino acid 537 (leucine) creates a soluble molecule which lacks the transmembrane and cytoplasmic domains of the protein. These results were confirmed by transfection of the rsELAMdn (producing soluble E-selectin) and pCDMSELAM (producing membrane-bound E-selectin) plasmids into COS-1 cells and the subsequent analysis of both the conditioned medium and the cells. FACS analysis, using a fluorescent monoclonal antibody to sE-selectin, indicates that the rsELAMdn transfected cells have a mean channel fluorescence equal to that of the untransfected cells. Finally, the ELISA assay results indicate that the conditioned medium from the rsELAMdn transfected cells contains six times more sE-selectin than either the untransfected or pCDMSELAM transfected cells (data not shown).

CHO Expression—After the initial G418 selection and two rounds of amplification, a 100 nm MTX cell line (here called Acc-2351), which secreted in excess of 100 µg/ml in a 96-well plate, was scaled up for growth in spinner flasks and eventually grown in a hollow fiber reactor. Over a 5-day period in spinner flasks, this cell line secreted in excess of 100 mg/liter sE-selectin. However, to produce sufficient quantities of the protein for purification purposes, the hollow fiber was used to generate the conditioned medium. Over a 17-day period, the reactor was fed initially at a rate of 50 ml of medium per day for days 4–8 to allow the cells to establish and populate the reactor. This rate was expanded to 100 ml per day for days 8–19 and finally at 200 ml/day for the last 4 days of the run. In total, 2 liters of medium were collected at a concentration range of 90–220 µg/ml/day with a peak concentration of 273 µg/ml/day. Over the 23-day reactor run, the concentration of sE-selectin produced averaged 200 µg/ml/day, based on ELISA assays.

Purification—One of the major problems in the purification of sE-selectin involved the removal of the large quantity of albumin present in the conditioned medium. For this reason, a Zn²⁺-chelate column was used as the first step of purification. As shown in Fig. 2, the majority of contaminating proteins including albumin bound to the column (lane 3 of Fig. 3A) while sE-selectin flowed through (lane 4 of Fig. 3A). In order to
reducing conditions (Laemmli, 1970). The gels were fixed with formaldehyde and stained with silver nitrate. The gels were analyzed by SDS-PAGE using 10% acrylamide gels under reducing conditions. The proteins (soybean trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; bovine serum albumin, 67 kDa; phosphorylase b, 94 kDa) were used as molecular weight standards. The relative intensities of the protein bands were measured with a scanning densitometer (LKB Model 2222) and converted to mass (mg) by cutting and weighing the scanner output peaks. Using the linear portion of the standard curve, the concentration of sE-selectin in the media was estimated as 157 mg/filter.

The recovery was markedly reduced compared to that from Zn2+ IDA bound; lanes 6 and 7 in Fig. 3B). Both bands were present in the original media and were positive in Western blot analysis. Therefore, broad banding pattern and an extra band in the gel may be due to heterogeneity in carbohydrate composition. The analytical reverse-phase HPLC profile suggests that one major protein component was present in the purified fraction (Fig. 5). Determination of Covalent Structure—One N-terminal sequence (15 cycles) was detected, and it confirmed the sequence predicted for the first 15 residues of the mature protein. The absence of an Asn residue in the fourth cycle may indicate N-linked glycosylation. This was seen previously (Lobb et al., 1991). The sequence is given,

1 5 10 15
Predicted: WSYXT STEAM TYDEA
Observed: WSYNT STEAM TYDEA

The amino acid composition is given in Table II and agrees well with the published sequence.

In Vitro Activity of sE-selectin—HL60 cells bind to sE-selectin-coated plates in a dose-dependent manner (Fig. 6A). Up to 91% of input cells will adhere under assay conditions used here. Background levels in uncoated wells were ~14%. Half-saturation was achieved at ~2.8 µg/ml or 35 nM sE-selectin. The binding of HL60 cells shows a plateau at 100 µg/well, with as little as 10 ng/well supporting adhesion of cells at levels above background. Pretreatment of coated wells with F(ab)2 fragments of monoclonal antibody to E-selectin will block binding to 52% of control levels (Fig. 6B). An anti-ICAM F(ab)2 fragment had no effect. The reason for the lack of complete inhibition of binding in the presence of anti-E-selectin monoclonal antibody (1.2B6) is not clear. Concentrations of up to 400 µg/ml F(ab)2 did not significantly increase inhibition. In contrast, the binding of HL60 cells was completely inhibited by the pretreatment of cells with purified sE-selectin. The midpoint for inhibition
was 1 µm and reached completion. It is worth noting that Ushiyama et al. (1993) have reported that the apparent affinity of sP-selectin for HL60 cells is 70 nM. These experiments were done by direct titration of cells with radiolabeled sP-selectin. This number is only 14-fold lower than the number reported here, which is determined by indirect measurements. This value (1 µm) is probably an underestimate of the intrinsic affinity of sE-selectin for its true receptor as the competitive affinity of HL60 cells for sE-selectin coating the well surface is not considered in the analysis. The intrinsic affinity of sE-selectin for its natural ligand may well be closer to the value for sP-selectin for its natural ligand. Another group has reported only partial inhibition of binding when HL60 cells are pretreated with sE-selectin and then allowed to adhere to sE-selectin-coated plates. They observed maximum inhibition of up to 60% of control (Lobb et al., 1991).

Effect of sE-selectin on the Inflammatory Response, in Vivo—In order to evaluate the in vivo functional activity of recombinant sE-selectin, the protein’s ability to interfere with an inflammatory response was evaluated. As seen in Fig. 7, the administration of sE-selectin markedly blunted the influx of neutrophils into tissue inflamed by the application of arachi-

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**TABLE I**

| Purification steps | Total protein a | Total sE-selectin b | Yield |
|--------------------|-----------------|---------------------|-------|
| Media              | 874             | 67                  | 100   |
| Diafiltered/centered| 352             | 78                  | 87    |
| Zinc-chelate flow-through | 89             | 34                  | 38    |
| Superose 12 pooled fractions | 29             | 30 b | 45    |

a Protein concentrations in crude samples were determined by using Pierce BCA reagent. The concentration of pure sE-selectin was determined by measuring the absorbance at 280 nm (ε280 = 102,000 m-1 cm-1).

b sE-selectin contents were estimated by densitometric scanning of silver-stained SDS-PAGE gels and ELISA.

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**TABLE II**

| Amino acid | Expected | Composition | Obtained |
|------------|----------|-------------|----------|
|            | mol amino acids/mol protein |             |          |
| Alanine    | 33       | 33.1        |          |
| Arginine   | 14       | 13.4        |          |
| Asx        | 41       | 43.1        |          |
| Glx        | 72       | 80.6        |          |
| Glycine    | 42       | 43.7        |          |
| Histidine  | 8        | 7.0         |          |
| Isoleucine | 15       | 13.6*       |          |
| Leucine    | 25       | 24.9        |          |
| Lysine     | 24       | 24.6        |          |
| Methionine | 9        | 7.2         |          |
| Phenylalanine | 20     | ND*         |          |
| Proline    | 32       | 31.5*       |          |
| Serine     | 55       | 54.6        |          |
| Threonine  | 39       | 38.1        |          |
| Tyrosine   | 17       | 19.8        |          |
| Valine     | 29       | 26.9*       |          |

a Values reported are for a 72-h hydrolysis.

b Value not determined due to co-elution of hexosamine sugars.
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FIG. 7. Effect of intravenous sE-selectin on arachidonic acid-induced inflammation. Balb/c mice (n = 5/group; saline control n = 8) were administered sE-selectin at the doses indicated intravenously via tail vein just prior to the application of arachidonic acid (1 mg/ear). One hour later, the edematous response was measured using a thickness gauge (edema values represent the difference between the untreated ear and the inflamed ear). The animals were then sacrificed and myeloperoxidase (MPO) was extracted from the inflamed tissue, the activity measured was spectrophotometrically, and the values were used as a quantification of neutrophil infiltration. The statistical significance was judged using Student’s t test. The saline control values were 0.1578 ± 0.009 units/ear for myeloperoxidase activity and 18 ± 0.5 x 10^-3 cm for ear edema.

FIG. 8. Molecular weight determination by gel permeation chromatography. Molecular weight standards were run on the same Superose 12 column as described in Fig. 3. Data were analyzed by standard methods (Whitaker, 1983; Andrews, 1964).

donic acid, as measured by the inhibition of myeloperoxidase activity. This effect was observed at low concentrations of sE-selectin which had little effect upon the edematous response. In this model, the edematous response should not be affected by sE-selectin as it is mediated largely by vasoactive amines and peptidoleukotriene production (Crummey et al., 1987). In contrast, the higher concentration (10 μg of sE-selectin/mouse) did not inhibit neutrophil influx, but did blunt the edematous response. This reciprocal relationship may suggest a nonspecific effect on the edematous response at higher sE-selectin concentrations. In multiple experiments using a wide range of concentrations, the predominant effect of sE-selectin has been the inhibition of neutrophil infiltration with minimal effect upon edema. The evanescent inflammatory response induced by arachidonic acid is well-characterized, and the neutrophil influx appears to be mediated by leukotriene B4 production (Grishwold et al., 1991). From other studies, it is not clear, at present, what adhesion molecules mediate leukotriene B4-induced inflammatory cell infiltration, although CD18 and CD54 have been implicated in vitro (Plamblad and Lerner, 1992). These new data suggest an additional role for sE-selectin, in vivo.
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Molecular Mass and Hydrodynamic Properties—SDS-PAGE of purified sE-selectin under nonreducing conditions showed a band of $M_r \sim 75,000$ (Fig. 3C, lane 8), which is bigger than the value predicted from the cDNA sequence (58,600). Analysis of the protein after pretreated with dithiothreitol gave a single band of $M_r \sim 100,000$ (Fig. 3C, lane 9). During purification, the protein was eluted from a Superose 12 column as $M_r \sim 331,000$ (Fig. 8).

Mass Spectrometry—The measured $M_r$ of sE-selectin based on the ($M + H^+$) peak is 80,000 (Fig. 9). The molecular mass of sE-selectin based on its peptide sequence alone is 58,826 Da. The mass difference of 21,474 Da suggests that sE-selectin is 27% carbohydrate. The width of this peak suggests significant carbohydrate heterogeneity.

Analytical Ultracentrifugation—The results of equilibrium analytical ultracentrifugation of sE-selectin is shown in Fig. 10. Simultaneous analysis of data from two rotor speeds in terms of Equation 1 fit best to a single sedimenting species yielding an average apparent molecular weight of 81,600 $\pm$ 4,500. Weis et al. (1991) solved the structure of the lectin domain of a man-
nose binding protein which has similarity to the lectin domain of sE-selectin. Inspection of the structure yielded no apparent sugar binding site. A possible explanation is that the sugar binds between two lectin domains, one from each to two molecules of sE-selectin. To test this hypothesis, the molecular weight of sE-selectin was also determined in the presence of sLe\(^\text{a}\). The best fit of the data obtained from four repeats of sE-selectin in the presence of 6 mM sLe\(^\text{a}\) is to a single species with a molecular weight of 79,500. Hence, sLe\(^\text{a}\) does not promote self-association, and the hypothesis is not supported.

Velocity sedimentation experiments gave a sedimentation coefficient for sE-selectin of \(s_{20,w} = 4.3\) and a diffusion coefficient of \(4.1 \times 10^{-10}\) cm\(^2\) s\(^{-1}\) (data not shown, Muramatsu and Minton (1988)). Using a value of 0.35 g of H\(_2\)O/g of protein for the hydration (Kuntz and Kauzman, 1974) and assuming the shape to be a prolate ellipsoid, sE-selectin was shown to have an axial ratio of 10.5:1 and dimensions of approximately 25 Å by 270 Å (see Tinoco et al. (1978) and Cantor and Schimmel (1980)). From these measurements alone, prolate and oblate ellipsoids cannot be distinguished. However, Ushiyama et al. (1993) have shown by electron microscopy that sE-selectin, a highly homologous protein, is an extended rod-like molecule, so this is not an unreasonable assumption. Additionally, these calculations assume the ellipsoid is smooth and that the relative decrease in sedimentation rate, compared to a sphere, can be interpreted solely in terms of extended dimensions. However, sedimentation properties of the proteins are also a sensitive function of the rugosity (roughness) of the protein surface (Teller et al., 1979). Hence, interpretation of the sedimentation properties of sE-selectin in terms of a smooth ellipsoid will give an upper limit to the dimensions.

**NMR—Binding of sialyl-Lewis X (sLe\(^\text{a}\)) to sE-selectin was studied by the two-dimensional version of transferred nuclear Overhauser effect (Clare and Gronenborn, 1982; Campbell and Sykes, 1993). The NOE information in the bound state is transferred to the free ligand by chemical exchange if binding is weak, and the transferred NOE spectrum thereby provides structural information of bound ligand (Campbell and Sykes, 1991, 1993). Fig. 11A shows the NOE spectrum of sLe\(^\text{a}\) at 5 °C. NOE cross-peaks in the spectrum under these conditions are very weak, suggesting that \(\text{o}_{\text{125}}\) is close to 1.1. Fig. 11B shows the NOE spectrum of 10^{-4} M sE-selectin under similar conditions. Normally, protein resonances in a large molecule of the size of sE-selectin are expected to be too broad to be observed. However, several well resolved cross-peaks corresponding to the sialic acid moiety are observed in the NOE spectrum of sE-selectin. The cross-peak 3a-3e (Fig. 11B) corresponds to the NOE between the geminal protons on carbon-3 of sialic acid, and both these protons show NOE contacts with the H4 and H5 protons of the sialic acid. These NOE cross-peaks are relatively intense and reflect high abundance and/or mobility of the sialyl group associated with glycosylation of sE-selectin. These results are consistent with mass spectral data, which show that the carbohydrate content in the sE-selectin is 27%. The two-dimensional version of transferred NOE spectrum of sLe\(^\text{a}\) in the presence of 0.12 mM sE-selectin is shown in Fig. 11C. It shows several cross-peaks which correspond to "negative" NOE and are relatively more intense than the sE-selectin or sLe\(^\text{a}\) spectra. Since free sLe\(^\text{a}\) does not contribute significantly to the NOE spectrum (Fig. 11A), these cross-peaks essentially result from the transferred NOE and arise from the conformation of sLe\(^\text{a}\) when bound to sE-selectin. In addition to several intraresidue contacts, NOE cross-peaks between protons belonging to different monosaccharide residues were observed. Anomeric protons (H1s) of fucose and galactose residues show "sequential" NOEs to H4 and H3 protons, respectively, of the GlcNAc residue (see Fig. 11D). Furthermore, H5 and CH\(^3\) protons of the fucose residue show NOEs to H2 proton of the galactose residue. These "contacts" suggest a compact conformation of the Le\(^\text{a}\) trisaccharide in which the fucose moiety is packed in close proximity to the galactose residue (Fig. 11D). Wormald et al. (1991) and Miller et al. (1992) showed that the Le\(^\text{a}\) trisaccharide in LNFP-3 has a rigid structure which shows these interproton contacts. In addition to these NOE contacts, a weak NOE between the axial proton H3a of sialic acid and the galactose-H3 proton was observed in our studies. This is the only contact observed between the sialic acid moiety and the Le\(^\text{a}\) trisaccharide. A number of independent investigations (Ball et al., 1992; Lin et al., 1992; Miller et al., 1992; Mukhopadhyay et al., 1994) reported similar results in their studies on the structure of sialyl-Lewis X in solution. Our results implicate that the bound conformation of sLe\(^\text{a}\) is similar to its structure in solution.

**Summary**—In summary, sE-selectin has been expressed at high levels in CHO cells and an efficient large-scale purification protocol, using only two chromatography steps under mild aqueous conditions, has been developed. The protein thus produced is active in vitro, in that HL-60 cells will bind to surfaces coated with it and monoclonal antibodies to sE-selectin or pre-treatment of the cells with sE-selectin will compete for this adhesion. This competitive effect puts an upper limit on the affinity of sE-selectin for its natural receptor of ~1 μM. sE-selectin will inhibit neutrophil influx in a mouse inflammation model, demonstrating that it is also active in vivo. The protein has been shown to have a monomer molecular weight of 80,000 by mass spectroscopy and the same molecular weight in solution as determined by analytical ultracentrifugation. Hydrodynamic experiments show that the molecule is an extended monomer as was found for sP-selectin, with an axial ratio of 10.5:1. Finally, NMR data show that this protein productively binds its normal carbohydrate ligand, sLe\(^\text{a}\). This molecule is well behaved and is suitable for configuration in screens for the identification of sLe\(^\text{a}\) antagonists.

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