Affinity, kinetics and thermodynamics of E-selectin binding to E-selectin ligand-1*

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Summary

E-selectin is an endothelial adhesion molecule which mediates the tethering and rolling of leukocytes on vascular endothelium. It recognizes the glycoprotein E-selectin ligand-1 (ESL-1) as a major binding partner on mouse myeloid cells. Using surface plasmon resonance (SPR) we measured the kinetics and affinity of binding of monomeric E-selectin to ESL-1 isolated from mouse bone marrow cells. E-selectin bound to ESL-1 with a fast dissociation rate constant of $4.6 \text{ s}^{-1}$ and a calculated association rate constant of $7.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. We determined a dissociation constant ($K_d$) of 62 µM which resembles the affinity of L-selectin binding to glycosylation-dependent cell-adhesion molecule-1 (GlyCAM-1). The affinity of the E-selectin - ESL-1 interaction did not change significantly when the temperature was varied from 5 to 37ºC, indicating that the enthalpic contribution to the binding is small at physiological temperatures, and that, in contrast to typical protein - carbohydrate interactions, binding is driven primarily by favourable entropic changes. Interestingly, SPR experiments with recombinant ESL-1 from α1,3-fucosyltransferase IV-expressing Chinese hamster ovary cells showed a very similar $K_d$ of 66 µM, suggesting that this fucosyltransferase is sufficient to produce fully functional recombinant ESL-1. Following the recent description of the affinity and kinetics of the selectin – ligand pairs L-selectin – GlyCAM-1 and P-selectin – P-selectin glycoprotein ligand-1 this is the first determination of the parameters of E-selectin binding to one of its naturally occurring ligands.
Introduction

Leukocyte extravasation into tissues is a multistep process involving different classes of adhesion molecules (1,2). The selectins are a family of lectins which mediate the initial step of tethering and rolling of leukocytes on vascular endothelium (3,4). Whereas L-selectin is expressed on most leukocytes, E- and P-selectin are found on activated endothelial cells (5). Each of these type-I transmembrane molecules displays an N-terminal C-type (i.e. Ca$^{2+}$ binding) lectin domain followed by an epidermal growth factor domain as well as several consensus repeat domains (4). Although the selectins have been shown to bind to a variety of carbohydrates related to the tetrasaccharide sialyl Lewis$^{x}$ (sLe$^{x}$) (6-8) they preferentially bind to a limited number of glycoproteins most of which are sialomucins (4,8,9).

It has been proposed that the ability of selectins to mediate the rapid and transient interactions typical of leukocyte tethering and rolling under shear is a consequence of the fact that they bind to their physiological ligands with fast kinetics and/or a high tensile strength (10,11). Recently the affinities and kinetics of two selectin/ligand interactions that can mediate rolling have been measured using SPR technology, namely L-selectin binding to glycosylation-dependent cell-adhesion molecule-1 (GlyCAM-1) (12) and P-selectin binding to P-selectin glycoprotein ligand-1 (PSGL-1) (13). GlyCAM-1 is a mucin-like glycoprotein that is secreted by endothelial cells and is found in serum (14-16). Although the function of GlyCAM-1 as an adhesion molecule in vivo is not yet firmly established, it has been demonstrated that immobilized GlyCAM-1 can support L-selectin dependent rolling of leukocytes in vitro (17). L-selectin binds to mouse GlyCAM-1 with an affinity or dissociation constant ($K_d$) of 108 µM (12). The kinetics were too fast to measure precisely but it was possible to show that the dissociation rate constant ($k_{off}$) was $\geq 10$ s$^{-1}$, and the calculated association rate constant ($k_{on}$) $\geq 10^7$ M$^{-1}$s$^{-1}$ (12). PSGL-1 is a homodimeric sialomucin and the major P-selectin ligand on leukocytes (18-23). The human P-selectin - PSGL-1 interaction displays a much higher affinity ($K_d \sim 0.3$ µM), a $k_{off}$ of 1.4 s$^{-1}$ and a very high $k_{on}$ of $4.4 \times 10^6$ M$^{-1}$s$^{-1}$ (13). The exceptionally high association rate constant of the P-selectin - PSGL-1 interaction is in good agreement with the well documented function of PSGL-1 as a selectin ligand that mediates leukocyte capturing under flow in vitro as well as in vivo (20,24,25).

Thus far, affinities and kinetics of E-selectin – ligand interactions have only been measured on a cellular level. The inhibition of leukocyte binding to immobilized E-selectin by
soluble E-selectin molecules indicated a $K_d$ of $< 1\mu M$ (26). However, the soluble E-selectin that was used in that study was oligomeric suggesting that the true affinity of monomeric E-selectin – ligand interactions may be considerably lower. When transient tethering of leukocytes to immobilized E-selectin was analysed a cellular $k_{off}$ of 0.7 s$^{-1}$ was estimated (27). Here again, it was difficult to formally exclude that the cellular interactions represented more than one E-selectin – ligand bond. Hence, the affinities and kinetics of monomeric E-selectin – ligand interactions have not yet been directly determined.

Two major glycoprotein ligands for E-selectin have been identified on mouse neutrophils by affinity isolation experiments, the E-selectin ligand-1 (ESL-1) and PSGL-1 (28-30). ESL-1 is a 150 kDa glycoprotein which binds specifically to E-selectin but not to P-selectin (28,29). ESL-1 is a type-1 transmembrane protein with a glutamine-rich N-terminal segment of 70 amino acids followed by 16 cysteine-rich repeats, a transmembrane and a short cytoplasmic domain. The ectodomain contains 5 potential N-glycosylation sites (30). In contrast to almost all other selectin ligands that are sialomucins and require O-linked carbohydrates for selectin binding, ESL-1-binding to E-selectin depends on N-linked carbohydrates (28,29). However, as for the other known ligands, binding requires sialic acid as well as $\alpha$1,3-fucosylation (28,31). ESL-1 is expressed by a large variety of cell types, although the glycoform which binds to E-selectin has only been found on myeloid cells (4).

Data from in vitro cell adhesion assays in which polyclonal antibodies to ESL-1 partially inhibited binding of mouse myeloid cells to E-selectin suggested that ESL-1 is involved in the binding of myeloid cells to E-selectin (30). In addition to its cell surface expression on leukocytes, ESL-1, like its rat and chicken homologues, MG-160 (32) and cysteine-rich fibroblast growth factor receptor (CFR) (33,34), is also found in the Golgi (35). The function of ESL-1 in this compartment is unknown.

In the present study we used SPR technology to analyse the interaction of monomeric recombinant forms of E-selectin with native ESL-1 from mouse bone marrow cells. We find that E-selectin binds to ESL-1 with an affinity similar to that of the L-selectin - GlyCAM-1 interaction. However, the affinity is much lower than the affinity of the P-selectin - PSGL-1 interaction due mainly to a 90-fold lower $k_{on}$ indicating that unusually fast association rate constants are not a general characteristic of selectin – ligand interactions. Furthermore, we show that at physiological temperature the E-selectin - ESL-1 interaction is primarily entropically-driven, again resembling the interaction between L-selectin and GlyCAM-1. This
characteristic distinguishes the two selectin - ligand interactions from most protein - carbohydrate interactions previously studied.
Experimental procedures

Antibodies
The following antibodies were used: 10E9.6, rat-anti-mouse E-selectin, IgG2a (36,37); UZ4, rat-anti-mouse E-selectin, IgM (38-40); R4-22, rat IgM isotype control mAb (BD Pharmingen, Heidelberg, Germany); Mel-14, rat-anti-mouse L-selectin, IgG2a (BD Pharmingen); 4RA10, rat-anti-mouse PSGL-1, IgG1 (41); 9E10, mouse-anti-c-myc, IgG1 (BD Pharmingen); OX12, mouse-anti-rat κ light chain, IgG2a (42). The polyclonal rabbit-anti-ESL-1 serum 99060 was raised against an ESL-1-IgG chimeric protein and affinity-purified as described (30). The polyclonal rabbit-anti-mouse PSGL-1 serum 703 has been described elsewhere (43).

Expression of recombinant E-selectin and ESL-1 constructs
Two E-selectin constructs were produced: a long construct (E-Sel.L) consisting of the entire ectodomain of mouse E-selectin and a short construct (E-Sel.S) containing the C-type lectin domain, the epidermal growth factor domain and the first two N-terminal consensus repeat domains. Both constructs carried a C-terminal hexahistidine-tag. For production of E-Sel.L the cDNA of mouse E-selectin (accession No. M87862) (44) was cloned into pBluescript (Stratagene, Amsterdam, NL) and used as template in a PCR using the 5′ primer AGTCTTGACGTCCCGGGAAAGATG (complementary to positions 1622-1645 including an internal AatII restriction site) and the 3′ primer ctactaggtaccagatctaatgatggtggtgatgatgACGGGTGGGGCTGACTGG which is complementary to positions 1787-1804 (upper case) and, additionally, codes for a hexa-histidine-tag followed by a stop codon, as well as BglII and KpnI restriction sites (lower case). The mouse E-selectin cDNA in pBluescript was digested with AatII and KpnI (KpnI restriction site in MCS 3′ of cDNA) and ligated with the AatII/KpnI-digested PCR product. This ligation replaced the sequence coding for the last amino acid (Pro) N-terminal of the transmembrane domain, the transmembrane as well as the cytoplasmic domain by the hexa-histidine sequence. The construct was excised from pBluescript and cloned into the glutamine synthetase expression vector pEE14 (45,46).

For production of E-Sel.S a PCR was performed using mouse E-selectin in pBluescript as a template as well as the 5′ primer GAATGTGAAGCTTTGACCCACCCTGCC (complementary to positions 860-885 including an internal HindIII restriction site) and the 3′ primer ctactaggatcctataatggtggtggtggtctAGCTTTGCATGATGGCGTCTCG which is
complementary to positions 1021-1041 (upper case) and, additionally, codes for an arginine and a hexa-histidine-tag followed by a stop codon and a BamHI restriction site (lower case).

A sequence coding for an E-selectin-Ig fusion protein (47) was cloned into vector pCMV5 (obtained by deleting a HpaI/EcoRI fragment from pCMV1 (48), digested with HindIII and BamHI (BamHI restriction site in MCS 3' of insert) and ligated with the HindIII/BamHI-digested PCR product. The resulting sequence coded for a construct containing the first four domains of mouse E-selectin ending with ‘PSCK’ in the fourth domain, adding an arginine and the hexa-histidine tag. The construct was excised from pCMV5 and cloned into pEE14.

The sequences of the E-Sel.L and E-Sel.S constructs were confirmed by dideoxy sequencing. CHO-K1 cells were transfected with the constructs by standard electroporation and selected in GMEM.S medium (First Link, Brierley Hill, UK) containing 10 % dialysed FCS (First Link), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Karlsruhe, Germany) and 20 µM L-methyl sulfoximine (Sigma-Aldrich, Deisenhofen, Germany). CHO clones expressing the E-selectin constructs were detected by reaction of anti-E-selectin mAb 10E9.6 with CHO cell supernatants in slot blot assays. For protein production clones were grown to confluency before switching to serum-free medium in which FCS was replaced by 2 mM Na-butyrate (Sigma-Aldrich).

The constructs were then purified from the spent tissue culture supernatants by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The constructs were further purified by size exclusion chromatography on a Superdex S75 HR10/30 column (Amersham Pharmacia, Freiburg, Germany) using Heps-buffered saline (150 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM Heps, pH 7.4) as running buffer. The extinction coefficients (e.c.) of E-Sel.L and E-Sel.S were determined by amino acid analysis as described (12). Calculation of e.c. was based on an E-Sel.L protein $M_r$ of 59197 and an E-Sel.S protein $M_r$ of 32434 (both excluding carbohydrate). E-Sel.L and E-Sel.S had an e.c. of 1.48 cm$^2$ mg$^{-1}$ and 1.72 cm$^2$ mg$^{-1}$, respectively.

The proportions of purified E-selectin constructs that were recognized by mAbs UZ4 and 10E9.6 were estimated in depletion experiments essentially as described (12). Briefly, Protein A-Sepharose beads (packed volume 100 µl / sample) were incubated for 1 h at 4°C with 1 mg of rabbit-anti-rat IgM or rabbit-anti-rat IgG, respectively (both from Dianova, Hamburg, Germany) in 200 µl Tris saline (150 mM NaCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 10 mM Tris, pH 7.5). The beads were washed four times with Tris saline before 1 mg of mAb UZ4
was added to anti-rat IgM-coated beads and 1 mg of mAb 10E9.6 was added to anti-rat IgG-coated beads in 200 µl Tris saline. The beads were rotated for 1 h at 4°C. Control antibodies were mAbs R4-22 and Mel-14, respectively. Following four washing steps with 1.5 ml Tris saline 40 µl mAb-coated beads were added to 20 µl of E-selectin construct (1 mg/ml) and incubated for 4 h at 4°C. The beads were pelleted and the supernatants (8 µl) were analysed by SDS-PAGE.

A recombinant ESL-1myc construct was made by ligating the sequence coding for a spacer of 3 glycines and a c-myc tag (EQKLISEEDL) to the 3′ end of the sequence coding for the first 850 amino acids of ESL-1 (accession No. X84037) containing all five potential N-glycosylation sites that are implicated in E-selectin binding (30). The construct was cloned into an expression vector and used for transfections of CHO-Pro5 cells (49) as described (31). Additionally, the CHO-Pro5 cells were co-transfected with the cDNA for mouse FucTIV in pcDNA3 (43) to achieve fucosylation of ESL-1myc. CHO clones were selected in the presence of 0.8 mg/ml G418 (Sigma-Aldrich) and assayed for the production of functional ESL-1myc by means of precipitation using anti-c-myc mAb 9E10 and E-selectin-IgG chimeric protein. ESL-1myc-expressing CHO-Pro5 cells were also produced without co-transfection with FucTIV. Finally, ESL-1myc from positive clones was purified by anti-c-myc affinity chromatography using mAb 9E10 coupled to CNBr-Sepharose.

**Purification of native mouse ESL-1**

Prior to purification of ESL-1 from mouse bone marrow cells three batches of Protein A-Sepharose beads were prepared: 1.25 ml drained Protein A-Sepharose CL-4B (Amersham Pharmacia, Freiburg, Germany) was incubated with 2.5 mg mouse P-selectin-Ig chimeric construct (47) at 4°C for 16 h in 15 ml wash buffer I (50 mM Tris-HCl, pH 8.4, 400 mM NaCl, 1 mM CaCl₂, 0.1 % Triton X-100, 6.2 mM sodium azide). Subsequently, the beads were washed four times in wash buffer I and once in wash buffer II (50 mM Tris-HCl, pH 8.4, 150 mM NaCl, 0.05 % Triton X-100) before they were washed three times with 5 ml of EDTA-elution buffer (50 mM ammonium acetate, pH 7.0, 3 mM EDTA, pH 8.0, 0.05 % Triton X-100). Finally the beads were washed four times in wash buffer I. Protein A-Sepharose beads coated with E-selectin-Ig (47) were prepared in an analogous way. A third batch of Protein A-Sepharose (250 µl drained beads) was incubated with 0.6 mg rabbit-anti-rat IgG (Dianova, Hamburg, Germany) in 3 ml wash buffer I for 2 h at 4°C. The beads were washed three times
in the same buffer before 0.5 mg anti-PSGL-1 mAb 4RA10 was added in 3 ml wash buffer I and incubated with the beads at 4°C for 16 h. Subsequently, the beads were washed four times in wash buffer I and three times in cell lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, 1% Triton X-100, 6.2 mM sodium azide, 2 mM phenyl-methyl-sulfonyl-fluoride, 1 mM benzamidine (Sigma-Aldrich) and 0.1 U/ml α₂-macroglobulin (Boehringer Mannheim, Mannheim, Germany) before the beads were incubated for 16 h in lysis buffer and washed another two times in the same buffer.

Femur and tibia bones were prepared from fifty 6 to 8 weeks old NMRI mice (Charles River, Sulzfeld, Germany). The bone marrow was purged into ice-cold PBS using a syringe with a 21 G needle. Bone marrow cells were resuspended and passed through a 60 µm mesh before they were washed twice in PBS and counted. Usually, 3.5 x 10⁹ cells were obtained from 50 mice. The cells were lysed for 30 min at 4°C in cell lysis buffer at 4 x 10⁷ cells / ml. The lysate was vortexed briefly before nuclei were sedimented at 10,000 x g for 20 min at 4°C. For depletion of PSGL-1, the supernatant was then added to 1.25 ml drained Protein A-Sepharose coated with P-selectin-Ig (47) and incubated at 4°C for 16 h. The beads were sedimented at 2000 g for 5 min at 4°C. For a second round of depletion, the supernatant was added to 250 µl drained Protein A-Sepharose coated with mAb 4RA10 and incubated for 16 h at 4°C. Subsequently, the beads were sedimented and any remaining beads were removed by passing the supernatant over an empty PolyPrep column (Bio Rad, München, Germany). At this stage the lysate was free from PSGL-1 and could be used to specifically precipitate ESL-1 with E-selectin-Ig. To this end the lysate was added to 1.25 ml drained Protein A-Sepharose coated with E-selectin-Ig and incubated at 4°C for 16 h. Subsequently, the beads were divided into 24 micro-test tubes, washed four times with wash buffer I and once with wash buffer II. 60 µl EDTA-elution buffer was added to each of eight micro-test tubes containing drained beads. After 3 min of incubation the eluate was transfered into another eight tubes, then into the remaining eight tubes before the eluate was pooled. This elution protocol was repeated twice. A total of 1.44 ml of eluate was collected. Remaining traces of Protein A-Sepharose beads were removed by passing the eluate over an empty PolyPrep column. The ESL-1 containing eluate was either directly used for immobilization in a biosensor or frozen in liquid nitrogen and concentrated five-fold by lyophyllisation before immobilization. Directly used or concentrated ESL-1 samples gave identical results in measurements of affinities and kinetics. Using this purification protocol ~4 µg of ESL-1 were obtained from fifty mice. For analytical
purposes some preparations were performed with depletion of ESL-1 prior to incubation of cell lysates with E-selectin-Ig. To this end 0.6 ml Protein A-Sepharose coated with 1.2 mg polyclonal rabbit-anti-ESL-1 antibodies (affinity-purified from serum 99060) or irrelevant rabbit control serum were added in the P-selectin-Ig- as well as in the 4RA10-based PSGL-1 depletion step. Removal of PSGL-1 from ESL-1 preparations was analysed by running ESL-1 samples obtained from 1 x 10^8 bone marrow cells on reducing SDS-PAGE before blotting onto nitrocellulose membranes and detection with anti-PSGL-1 mAb 4RA10 (20 µg/ml), secondary conjugate and ECL substrate (Amersham Pharmacia, Freiburg, Germany).

**Surface Plasmon Resonance experiments**

Binding experiments were performed on a BIAcore™ 2000 biosensor (BIAcore AB, Uppsala, Sweden) with Hepes-buffered saline (see above) as running buffer. All injections were done with 0.2 µm-filtered and degassed samples. Proteins were directly coupled to research grade CM5 sensor chips (BIAcore) via primary amine groups using the Amine Coupling Kit (BIAcore) according to the manufacturer's instructions with variations of the pH of the coupling buffer (10 mM ammonium acetate) and the flow rate (FR) for injections: native ESL-1, pH 3.5, FR 1µl/min; ESL-1myc, pH 3.5, FR 5 µl/min; CD2.His, pH 5.0, FR 10 µl/min; mAb OX12, pH 5.0, FR 10 µl/min. Measurements of affinities and kinetics were performed with at least two different levels of immobilization (ranging from 750 to 5000 response units) to make sure that the data were independent of immobilization density. Injections of analytes were performed for 6 s at a flow rate of 100 µl/min unless otherwise indicated. Bound E-selectin was removed by injection of 10 mM EDTA. Sialidase treatment was performed by injecting 200 mU/ml of *Arthrobacter ureafaciens* sialidase (Roche Diagnostics, Mannheim, Germany), diluted in 50 mM sodium acetate, pH 5.0, for 30 min at a flow rate of 1 µl/min. This treatment removed ~80% of the sialic acid from native or recombinant ESL-1. Prolonged treatment (up to 16 hrs) was not more effective. To monitor desialylation the sialic acid-specific *Sambucus nigra* agglutinin (SNA) was injected at 2 mg/ml in 10 mM HEPES, 150 mM NaCl, 0.1 mM CaCl_2_, pH 7.5, for 7 min at a flow rate of 1 µl/min.

Data were collected from four flow cells of the chip simultaneously at a collection rate of two points/s, except for analyses of dissociation phases where data were collected from one flow cell at a time at a collection rate of ten points/s. Analyses were performed at 25°C unless indicated otherwise.
Dissociation rate constants were obtained by a nonlinear fit of a single exponential decay curve to the E-selectin dissociation data using the BIAevaluation program (Version 3.1, BIAcore), which deploys the Marquardt-Levenberg algorithm. Other curve fitting was performed in Origin version 3 (MicroCal). Affinity constants were derived by Scatchard analysis or by non-linear curve fitting of the standard Langmuir binding isotherm (A + B ⇌ AB). Thermodynamic data were obtained by fitting to the affinity data the non-linear form of the van't Hoff equation (50)

\[ \Delta G^o = \Delta H_{To} - T \Delta S_{To}^o + \Delta C_p (T - To) - T \Delta C_p \ln \left( \frac{T}{To} \right) \]

where \( T \) is the temperature in Kelvin (K); \( To \) is an arbitrary reference temperature (e.g. 298.15 K); \( \Delta G^o \) is the standard free energy of binding at \( T \) (kJmol\(^{-1}\)), and is calculated from the \( K_d \) using the equation \( \Delta G^o = R \cdot T \cdot \ln K_d \) where \( R \) is the gas constant; \( \Delta H_{To} \) is the enthalpy change upon binding at \( To \) (kJmol\(^{-1}\)); \( \Delta S_{To}^o \) is the standard state entropy change upon binding at \( To \) (kJmol\(^{-1}\)); and \( \Delta C_p \) is the specific heat capacity (kJmol\(^{-1}\)K\(^{-1}\)), and is assumed to be temperature-independent.
Results

Expression and characterization of monomeric E-selectin constructs
Two recombinant, monomeric E-selectin constructs were produced in CHO cells: a long construct (E-Sel.L) containing all of eight extracellular domains of mouse E-selectin, and a short construct (E-Sel.S) containing only the lectin domain, EGF domain and the first two consensus repeats (Fig. 1A). Using a hexa-histidine tag expressed at the C-terminus of either construct, E-Sel.L and E-Sel.S were purified by Ni-NTA affinity chromatography. As analysed by SDS-PAGE the constructs were highly purified and exhibited apparent molecular weights of 105 and 45 kDa, respectively (Fig. 1B), which were predicted from the molecular weight of full length native mouse E-selectin (115-120 kDa) (44). Moreover, with a polypeptide mass of 59 kDa for E-Sel.L and 32 kDa for E-Sel.S the apparent molecular weights in SDS-PAGE indicated extensive glycosylation of the constructs.

In order to assess the proportions of the E-selectin constructs which were correctly folded we used two E-selectin antibodies, including one (UZ4) that blocks ligand binding, to deplete E-Sel.L and E-Sel.S, respectively. Fig. 1C shows that mAb UZ4 depleted ∼90% of either construct. E-selectin mAb 10E9.6 which binds to the first and/or second consensus repeat domain (36,37) depleted equally well (not shown). These data indicated that at least 90% of each recombinant protein expressed the respective antibody epitopes suggesting correct folding.

Measuring affinity and kinetics of an interaction requires monovalency of the interaction making it neccessary to obtain monomeric E-selectin for incubations with immobilized ESL-1 in SPR experiments. Therefore, the E-selectin constructs were subjected to size-exclusion chromatography in order to separate monomeric fractions from aggregated material. For E-Sel.S two peaks were detected, a small peak near the elution position of a 150 kDa calibration protein indicating a small proportion of di- or trimeric material and a major peak between the elution positions of calibration markers of 29 kDa and 66 kDa consistent with the mobility of monomeric E-Sel.S (not shown). Fractions from the 29-66 kDa peak were pooled and re-analysed by size-exclusion chromatography which resulted in the monomeric peak only, indicating that no re-aggregation occured (Fig. 1D). Only the monomeric material was used for SPR studies. E-Sel.L eluted in a single peak between the elution positions of 66
and 150 kDa calibration markers consistent with a monomeric protein. No aggregated material was detected in E-Sel.L preparations (not shown).

**Purification and analysis of E-selectin ligand-1**

E-selectin binding to ESL-1 is dependent on the correct glycosylation of the E-selectin ligand (30). Hence, native ESL-1 had to be used for affinity measurements. ESL-1 was purified from mouse bone marrow cells which consist to 75-80% of polymorphnuclear granulocytes (28,51), the main source of the E-selectin binding glycoform of ESL-1 (30). In order to avoid co-purification of glycoforms of ESL-1 which do not bind to E-selectin an antibody-like form of E-selectin (E-Sel-Ig) was used to precipitate functional ESL-1. Contamination of ESL-1 preparations with the second glycoprotein ligand on neutrophils, PSGL-1, was precluded by PSGL-1-depletion using a P-selectin-Ig construct (P-Sel-Ig) in a first and an anti-PSGL-1 mAb in a second depletion step.

The resulting preparations of the 150 kDa protein ESL-1 exhibited a highly enriched protein migrating at the correct position in SDS-PAGE stained with silver (Fig. 2A) and Coomassie (not shown). Additional weak bands were also seen in control lanes with sample buffer only (Fig. 2A).

In order to formally prove that the purified 150 kDa protein was ESL-1 Western blots of ESL-1 preparations were performed in which anti-ESL-1 antibodies were able to stain the 150 kDa band (not shown). More importantly, depletion of cell lysates with anti-ESL-1 antibodies prior to precipitation with E-Sel-Ig strongly reduced the 150 kDa band in comparison to a negative-control depletion with irrelevant control antibodies, showing that the purified 150 kDa protein was ESL-1 (Fig. 2B).

Many mucin-like molecules (like PSGL-1) are difficult to detect in silver- or Coomassie-stained SDS-PAGE. Therefore, depletion of PSGL-1 was controlled by sensitive Western blots using a high-affinity PSGL-1 mAb for detection. Typically, the murine P-selectin ligand PSGL-1 migrates with two bands at 130 and 230 kDa, the second band being due to incomplete reduction of the dimeric protein (Fig. 2C lane 1) (29). When high amounts of P-Sel-Ig were used to deplete PSGL-1 from lysates of bone marrow cells before ESL-1 was precipitated with E-Sel-Ig some residual PSGL-1 was detected in ESL-1 preparations (Fig. 2C lane 2). However, when depletion was done in two steps, using P-Sel-Ig first followed by anti-PSGL-1 mAb, depletion of PSGL-1 from ESL-1 preparations was complete (Fig. 2C lane 3). Hence, this two-step depletion protocol was used for ESL-1 purification. Moreover, in SPR
analyses PSGL-1 mAb 4RA10 (50 µg/ml) did not bind to ESL-1 preparations immobilized to sensor surfaces, further excluding contaminations of ESL-1 preparations with PSGL-1 (not shown).

**Specificity of E-selectin – ESL-1 interactions in SPR analyses**

ESL-1 was immobilized in a flow cell (FC) of a sensor chip using an amine coupling procedure. An equal amount of a control protein was immobilized in another FC of the same chip. Two control proteins were used: a recombinant construct containing the extracellular part of mouse CD2 and a hexa-histidine tag at the C-terminus (52) as well as the monoclonal mouse antibody OX12 which is directed against an unrelated antigen (rat κ immunoglobulin light chain) (42). Injection of the two E-selectin constructs over the sensor chip resulted in a response in the ESL-1 flow cell as well as in a lower background response in the control flow cell. As shown in Fig. 3A, the response reaches equilibrium within a second of the start of the injection and returns to baseline within seconds of the end of the injection. Subtracting the control FC response from the ESL-1 FC response gave the specific binding of E-selectin to ESL-1. CD2 and OX12 control proteins gave identical background signals.

The interaction between E-selectin and ESL-1 had been shown to be Ca$^{2+}$-dependent (28,30). In accordance with this, co-injection of EDTA with E-selectin abrogated the specific binding showing the dependence of the interaction on divalent cations (Fig. 3B).

We next examined ESL-1 specificity of the E-selectin interaction with immobilized ESL-1 preparations. It remained the theoretical possibility that although PSGL-1 had been removed efficiently an additional E-selectin ligand had been co-purified with ESL-1 and was not detected in silver- or Coomassie-stained SDS-PAGE. However, when ESL-1-preparations, either depleted of ESL-1 or mock depleted (shown in Fig. 2B), were immobilized on sensor chips (using equal volumes and injection times), binding of E-selectin was reduced by >80 % in the flow cell with ESL-1-depleted material showing that the bulk of the signal obtained in SPR-experiments was ESL-1-specific (Fig. 3C). The residual binding can at least in part be explained by some residual ESL-1 which was not depleted since an anti-ESL-1 serum showed 10% residual specific binding to the immobilized ESL-1-depleted material as compared to mock-depleted material (not shown). Further support for this notion comes from the fact that residual ESL-1 could clearly be seen in SDS-PAGE (Fig. 2B) and...
that E-selectin-binding to the control- and ESL-1-depleted samples showed similar affinities (expressed as $K_d$) (Fig. 3C).

Different murine adhesion molecule constructs were tested for binding to ESL-1. Only injection of E-selectin gave considerable binding whereas injection of L- and P-selectin as well as a CD22-Fc construct (53,54) produced responses just above background (Table I).

**Affinity of the E-selectin – ESL-1 interaction**

Binding affinities were measured by injecting E-selectin constructs in serial dilutions over ESL-1- and control protein-surfaces. The signals produced by a representative series of injections performed with E-Sel.L are shown in Fig. 4A and B. Specific equilibrium responses were plotted against concentrations of E-Sel.L and E-Sel.S, respectively (Fig. 4C and D). Direct fitting of a Langmuir binding isotherm to the data indicated saturable binding for both, E-Sel.L and E-Sel.S, to ESL-1 and allowed calculation of the dissociation rate constants $K_d$. The mean $K_d$ value for binding of E-Sel.L to ESL-1 at 25°C was 62 µM (Table II). A very similar mean dissociation rate constant ($K_d = 56$ µM) was obtained for binding of E-Sel.S to ESL-1 at the same temperature suggesting that domains 5-8 of E-selectin are dispensable for the interaction with ESL-1 (Table II). Scatchard plots of the same data were linear and gave very similar $K_d$ values to those obtained from the binding isotherms (insets in Fig. 4C and D).

We also measured the affinity of E-selectin binding to a recombinant ESL-1myc construct that had been expressed in α1,3-fucosyltransferase IV (FucTIV)-bearing CHO Pro$^5$ cells. Interestingly, the affinities of E-Sel.S for native and recombinant ESL-1 were very similar ($K_d = 56$ µM versus 66 µM at 25°C) (Fig. 4E and Table II). Binding of E-selectin to recombinant ESL-1 was strongly decreased (by 88%) when ESL-1myc from FucTIV-negative CHO Pro$^5$ cells was used (Fig. 5A). These data show that co-expression of FucTIV in CHO Pro$^5$ cells is sufficient to produce fully functional ESL-1. We have shown before that E-selectin binding to native ESL-1 is dependent on the sialylation of the ligand. In those precipitation experiments (28) treatment of the ligand with sialidase reduced E-selectin binding by ~80%. As expected, this result could be reproduced by sialidase-treating native bone marrow cell-derived ESL-1 immobilized to sensor surfaces leading to 75% reduction of E-selectin binding (not shown). More importantly, desialylation of the recombinant form of ESL-1 had a similar effect giving 81% reduction of E-selectin binding at an equivalent decrease of sialylation as judged by the binding of the sialic acid-specific lectin SNA (Fig.
Taken together, these results show that the glycosylation requirements for native and CHO cell-derived recombinant ESL-1 for binding to E-selectin are very similar if not identical.

**Kinetics of the E-selectin – ESL-1 interaction**

The kinetics of the E-selectin – ESL-1 interaction were determined by analysis of the dissociation phase with report points taken at short (0.1 s) intervals. Fig. 6 shows the ESL-1 specific responses of E-Sel.S binding during the dissociation phase at temperatures ranging from 5 to 37°C. At physiological temperature the dissociation rate constant (k\textsubscript{off}) was 4.6 s\textsuperscript{-1}, falling to 1.1 s\textsuperscript{-1} at 5°C (Fig. 6 and Table III). Dissociation of E-Sel.L and E-Sel.S from ESL-1 was compared at 25°C and gave very similar dissociation rate constants of 3.0 and 2.7 s\textsuperscript{-1}, respectively (Table III). As with other interactions between cell surface molecules (12,13,55) the association phase was too brief to allow direct measurement of the association rate constant (k\textsubscript{on}). Instead the k\textsubscript{on} was calculated using the relationship k\textsubscript{on} = k\textsubscript{off} / K\textsubscript{d}. At 25 and 37°C the k\textsubscript{on} for binding of E-Sel.S to ESL-1 was calculated as 4.8 x 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1} and 7.4 x 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1}, respectively (Table III). Similar k\textsubscript{on} and k\textsubscript{off} values were obtained with high (4000 – 5000 RU) and low (750 and 200 RU) levels of immobilized ESL-1, indicating that these values are not limited by mass transport of the soluble E-selectin to the sensor surface.

The k\textsubscript{on} of the E-selectin - ESL-1 interaction was 90-fold slower than the one reported for P-selectin binding to PSGL-1 (Table IV). One possible explanation for this difference would be that the E-selectin - ESL-1 interaction requires greater conformational adjustments than the P-selectin - PSGL-1 interaction, for which a large association activation energy should be an indication. Activation energies associated with association (E\textsubscript{a}\textsuperscript{ass}) and dissociation (E\textsubscript{a}\textsuperscript{diss}) represent the thermal energy that is required to overcome a hypothetical energy barrier that impedes association and dissociation of a molecular complex. E\textsubscript{a}\textsuperscript{diss} was determined from the slope of a plot of ln k\textsubscript{off} against 1/T (Arrhenius plot, Fig. 7A), which equals – E\textsubscript{a}\textsuperscript{diss} / R, where R is the gas constant. Linear regression of such a plot for the E-selectin - ESL-1 interaction over the temperature range 5 to 37°C (Table III) yielded an unremarkable E\textsubscript{a}\textsuperscript{diss} of 31.4 kJmol\textsuperscript{-1}. Similarly, the k\textsubscript{on} changed a modest 3.9-fold when the temperature was decreased from 37 to 5°C (Table III). An Arrhenius plot of the k\textsubscript{on} data gave an E\textsubscript{a}\textsuperscript{ass} of 29.9 kJmol\textsuperscript{-1} (Fig. 7A). Since this value includes the effect of temperature on water viscosity (16-20 kJmol\textsuperscript{-1}) it indicates that there is only a small energy barrier impeding association of the E-selectin - ESL-1 complex. This argues against large conformational
adjustments in the E-selectin – ESL-1 interaction and the difference in $k_{on}$ between this interaction and P-selectin binding to PSGL-1 should rather be due to specific features of the latter interaction.

**Thermodynamics of the E-selectin – ESL-1 interaction**

The binding energy or free energy change ($\Delta G$) associated with an interaction is made up of enthalpic ($\Delta H$) and entropic ($-T\Delta S$) contributions ($\Delta G = \Delta H - T\Delta S$). The binding energy under standard conditions ($\Delta G^o$), where all reactants and products are at a concentration of 1 molL$^{-1}$, can be calculated from the $K_d$ using the equation $\Delta G^o = R \cdot T \cdot \ln K_d$ (where the $K_d$ is in units molL$^{-1}$). This yielded a $\Delta G^o$ at 25ºC of -24 kJmol$^{-1}$ for the E-selectin - ESL-1 interaction. The enthalpic contribution to an interaction can be estimated by van’t Hoff analysis, which involves measuring the dependence of binding affinity on temperature change. In such an analysis a strong increase in affinity with falling temperature indicates a large enthalpic contribution. However, when binding of E-Sel.S to ESL-1 was studied over a temperature range of 5 to 37ºC no significant change in affinity was seen (Table II). The $K_d$-data were transformed into $\Delta G$-values as described above and used for an analysis employing the non-linear form of the van’t Hoff equation. The van't Hoff analysis gave a $\Delta H$ of -3.8 ± 2.8 kJ mol$^{-1}$ (mean ± error) (Fig. 7B). These data show that enthalpic changes contribute only ~10-25% of the binding energy $\Delta G^o$ and that the E-selectin - ESL-1 interaction is primarily entropically driven.
Discussion

Accuracy and specificity of measurements

Accurate affinity measurements require that both the concentration and the activity (i.e. the proportion able to bind to ligand) of the soluble binding partner has to be known. Nearly complete immunodepletion of E-Sel.L and E-Sel.S with monoclonal antibodies directed either against the lectin or the consensus repeat domains of E-selectin suggested that almost all of the material was correctly folded. Furthermore, no degraded material was detected in SDS-PAGE or in size exclusion chromatography. On the basis of these results affinities were calculated assuming 100% activity.

Oligo-histidine tags had been reported to cause aggregation in some cases (56). However, no aggregation of E-Sel.L was observed by size exclusion chromatography and only a small amount of aggregated material was detected in E-Sel.S preparations. These aggregates could be removed by chromatography. The resulting monomeric material did not appear to re-aggregate and the SPR signals that were obtained with this material showed no signs of multivalent binding.

More critical was the purification of functional ESL-1 from mouse bone marrow cells. Since an E-selectin-Ig construct had to be used to specifically purify the E-selectin-binding form of ESL-1, care had to be taken to remove the second neutrophil ligand for E-selectin, PSGL-1. An efficient two-step protocol was developed to deplete lysates of PSGL-1 prior to affinity isolation with E-selectin-Ig. PSGL-1 mAbs detected no PSGL-1 in ESL-1 preparations analysed by Western blot and SPR. Moreover, recombinant P-selectin and L-selectin, for which PSGL-1 is a ligand (18,29,57), did not bind to immobilized ESL-1 preparations (Table I) further indicating that PSGL-1 had been removed completely.

ESL-1 preparations only contained very minor contaminations as shown by SDS-PAGE. At least 80% of the E-selectin-binding activity of these preparations was indeed due to ESL-1 since depletion of ESL-1 from cell lysates with specific antibodies prior to the isolation with the E-selectin-Ig affinity matrix resulted in preparations which showed less than 20% E-selectin binding in SPR assays as compared to non-depleted preparations. We cannot completely rule out that this residual binding might have been partially due to the presence of ligands other than ESL-1 and PSGL-1. However, the similar affinity of E-Sel.S binding to ESL-1-depleted and mock-depleted preparations (Fig. 3C) was consistent with an incomplete
depletion of ESL-1 which was confirmed by SDS-PAGE of the depleted preparation (Fig. 2B) and by residual reactivity of an anti-ESL-1 serum with the depleted material. Importantly, even if the residual material contained some contaminants, their similar affinity for E-selectin and the low signals they produced could only cause minor effects on measurements of kinetics and affinity of E-selectin binding to ESL-1. This is in particular illustrated by the monophasic dissociation of E-selectin from its ligand (Fig. 6).

These results underscore the selectivity with which E-selectin binds to PSGL-1 and ESL-1 in mouse bone marrow cells and indicate that additional glycoprotein ligands constitute at most only a very small proportion of binding. Thus, sensitive SPR assays confirm previous studies which were based on the detection of labelled E-selectin ligands in SDS-PAGE (28,29,58).

While 75-80% of bone marrow cells are polymorphonuclear granulocytes (51), different myeloid cells may also express the E-selectin binding form of ESL-1. However, our SPR experiments did not indicate different functional components in ESL-1 preparations. Thus, ESL-1 from different bone marrow cell subpopulations either binds E-selectin with uniform affinity or the proportion of ESL-1 from cells other than granulocytes is very small so that it does not detectably contribute to E-selectin binding.

ESL-1 is not only expressed on the cell surface but also in the Golgi compartment of granulocytes (35). At present it is not clear if the Golgi contains the E-selectin binding form of ESL-1 and this study cannot answer this question. However, as stated before, ESL-1 preparations exhibited uniform binding characteristics. Thus, if E-selectin binding material from the Golgi was present in ESL-1 preparations its binding affinity / kinetics were indistinguishable from that of cell surface ESL-1.

**Affinity**

Analysis of the affinity of monomeric E-selectin binding to ESL-1 gave a dissociation constant ($K_d$) of 62 µM which is only slightly higher than the affinity measured for L-selectin binding to GlyCAM-1 ($K_d = 108 \mu M$) (12) and Table IV). The $K_d$-value is also reminiscent of a variety of interactions of cell surface molecules other than selectins (reviewed in 59,60). However, the P-selectin - PSGL-1 interaction has a much higher affinity, largely due to a faster $k_{on}$ (Table IV). The rather ordinary affinity of the monomeric interaction between E-selectin and ESL-1 which we determined here is in sharp contrast to the high efficacy with
which ESL-1 can be affinity-isolated using an E-selectin based affinity probe (this study and 28,29,58). A possible explanation for this would be the presence of multi-molecular clusters of ESL-1 on the cell surface which may allow multimeric interactions of high stability with immobilized E-selectin in affinity-isolation experiments. Such multimeric interactions may also occur in vivo since it has been shown that E-selectin clusters on the surface of endothelial cells during contact with leukocytes (61,62). Therefore, the stability of E-selectin – ESL-1 interactions in a cellular context may be much higher than could be deduced from our measurements of monomeric interactions. In this respect it would also be interesting to know the stoichiometry of the E-selectin – ESL-1 interaction. This aspect could not be addressed in this study because this requires the quantification of functional ESL-1 on the sensor surface using anti-ESL-1 monoclonal antibodies which are not available.

It is still an open question which molecular entity is recognized by E-selectin on ESL-1. A variety of studies have analysed binding of E-selectin to sLe\textsuperscript{x} and indicate dissociation constants between 107 and 1800 µM (7,63-66). Some of these studies were inhibition studies in which monomeric ligands were used to inhibit multivalent interactions, and these tend to underestimate the binding affinity. Three of the studies avoided this problem by studying direct binding of sLe\textsuperscript{x} to E-selectin (63,64,66). Still, these studies reported rather different K\textsubscript{d} values of 107, 720, and 1100-1800 µM, respectively. This makes it difficult to decide whether the K\textsubscript{d} of 62 µM for the E-Selectin – ESL-1 interaction is indicative of sLe\textsuperscript{x} being the major E-selectin binding structure on ESL-1 or whether other carbohydrate structures of higher affinity for E-selectin or the protein backbone itself contribute to binding. A study by Patel et al. (67) showed that from a pool of membrane-associated glycostructures of myeloid cells preferentially N-linked tetra-antennary carbohydrates containing sialyl-diLe\textsuperscript{x} structures could be affinity isolated by E-selectin. However, the K\textsubscript{d} for binding of these structures to E-selectin was roughly estimated to be < 1 µM. Based on this rough estimate, ESL-1 with a K\textsubscript{d} of 62 µM is probably not decorated with such structures. It will be interesting to determine which carrier molecules might be decorated with these tetra-antennary carbohydrates.

Whatever carbohydrate structures are recognized by E-selectin on ESL-1, they can be generated in CHO cells and seem to require the co-expression of only one exogenous glycosyltransferase, the α1,3-fucosyltransferase IV (FucTIV). This ability of Fuc-TIV to generate a recombinant glycoform of ESL-1 with full binding activity similar to authentic ESL-1 confirms our previous results demonstrating that Fuc-TVII deficient mouse neutrophils
(still expressing Fuc-TIV) can generate a glycoform of ESL-1 that can be isolated with E-selectin with a similar efficiency as ESL-1 from wild-type neutrophils. In contrast, the lack of Fuc-TIV strongly impaired E-selectin - ESL-1 binding (by 80%), despite the presence of Fuc-TVII in these cells (58). Interestingly, only neutrophils that express Fuc-TVII can generate a selectin-binding form of PSGL-1, while Fuc-TIV alone is insufficient (58). Whether this dependence on different fucosyltransferases is an indication for different sLe\(^x\)-related structures on the two ligands still needs to be elucidated.

Earlier studies showed that a mouse E-selectin-Ig construct lacking the four membrane-proximal consensus repeat (CR) domains was able to bind to E-selectin ligands (28,29). However, it remained the possibility that without these CR domains E-selectin binds more weakly to glycoprotein ligands. The present study answers this question in that it shows that E-selectin constructs containing or lacking the four membrane-proximal CR domains bind ESL-1 with virtually the same affinities and kinetics. Thus, these four CR domains are entirely dispensable for ligand binding in soluble systems where the sizes of interacting molecules play a minor role. Like in P-selectin (68) these CR domains may rather help the E-selectin molecule to protrude the cellular glycocalyx and/or provide more flexibility to the selectin, thereby promoting adhesion.

**Kinetics**

The \(k_{on}\) of the E-selectin - ESL-1 interaction is marginally slower than is typical for protein - protein interactions \((10^5 - 10^6 \text{ M}^{-1}\text{s}^{-1})\) (60) but within the range of reported values for protein - carbohydrate interactions (discussed in 69). While the \(k_{on}\) was less than two-fold slower than the value measured for L-selectin binding to GlyCAM-1 (12), it is important to stress that, because of technical limitations, the \(k_{on}\) for the latter interaction was only a lower limit, and the true \(k_{on}\) may have been considerably faster. The more precise \(k_{on}\) determination in this study for the E-selectin - ESL-1 interaction allows a comparison with the P-selectin - PSGL-1 interaction. It is striking that the P-selectin - PSGL-1 \(k_{on}\) is nearly two orders of magnitude faster than the \(k_{on}\) measured for the E-selectin - ESL-1 interaction (Table IV). This shows that the former interaction has a uniquely fast \(k_{on}\), and raises the question as to the structural basis and physiological consequences of these striking differences in \(k_{on}\).

One possible explanation for these differences was that the E-selectin - ESL-1 interaction requires greater conformational adjustments than the P-selectin - PSGL-1
interaction. Our finding that the E-selectin - ESL-1 \( k_{on} \) is not unusually temperature-dependent (resulting in a moderate \( E_a \)) argues against this, although similar data are not available for P-selectin binding to PSGL-1. Also arguing against this explanation is the finding by Somers et al. (70) that P-selectin undergoes substantial conformational changes upon binding to PSGL-1. It is more likely that the faster P-selectin - PSGL-1 \( k_{on} \) is the result of favourable electrostatic interactions between the tyrosine-sulfated N-terminus of PSGL-1 (which is negatively charged) and its positively charged binding site on P-selectin (70). Because of their ability to act over long ranges as well as to 'steer' approaching molecules, electrostatic interactions can dramatically accelerate binding (71,72).

It was proposed that high \( k_{on} \) values are a prerequisite for the capturing of leukocytes to endothelium (10,11). Whereas it is unclear if the \( k_{on} \) of L-selectin binding to GlyCAM-1 is above what is typical for protein-protein interactions (12), the extraordinarily high \( k_{on} \) of P-selectin binding to PSGL-1 (13) together with its well documented role in capturing (20,24,25) is in support of the notion that capturing is \( k_{on} \)-dependent. According to this view the much lower \( k_{on} \) of E-selectin binding to ESL-1 would suggest that this interaction may be considerably less efficient than the P-selectin – PSGL-1 interaction in leukocyte capturing. In line with this, ESL-1 was not found on the tips of leukocyte microvilli (35), an exposed site on the cell surface ideally suited for the initiation of very first cell contacts, where PSGL-1 (20) and also L-selectin (73,74) have been localized. Instead, ESL-1 was localized along the lateral surfaces of microvilli (35). Such a cell surface localization would be consistent with a function of ESL-1 in strengthening E-selectin-dependent rolling of leukocytes and/or mediating signaling, rather than initiating the tethering of leukocytes. In fact, E-selectin itself is relatively poor in capturing leukocytes when compared to P-selectin (3). E-selectin deficient mice show normal rolling flux (proportion of rolling leukocytes), while the only detectable defect is the lack of slow rolling leukocytes at velocities below 10 \( \mu \text{m/s} \) (75). In conclusion, the kinetics of E-selectin binding to ESL-1 are compatible with a possible role of this interaction in a step after the initial capturing of leukocytes.

The \( k_{off} \) measured here for the mouse E-selectin - ESL-1 interaction (2.7 – 3.0 s\(^{-1}\) at 25\(^\circ\)C) is slightly faster than that reported for the human P-selectin - PSGL-1 interaction (1.4 s\(^{-1}\) but slower than the \( k_{off} \) measured for the murine L-selectin - GlyCAM-1 interaction (\( \geq 10 \) s\(^{-1}\) at 25\(^\circ\)C) (Table IV). It is noticeable that the E-selectin - ESL-1 \( k_{off} \) is approximately four times higher than the \( k_{off} \) of transient human leukocyte tethers to immobilized E-selectin (0.7 s\(^{-1}\) at
25°C) as deduced from flow adhesion assays (27). One explanation for this difference could be based on principle limitations in the biochemical determination of $k_{\text{off}}$ values on the biosensor surface and the measurements of cellular $k_{\text{off}}$ values determined in flow adhesion assays, as has been discussed in detail by Mehta et al. (13). However, since the $k_{\text{off}}$ values did not change when the level of immobilized ESL-1 was varied up to 25-fold (Table III), mass transport or rebinding artifacts are unlikely to play a substantial role in these SPR measurements. A second explanation may be that the binding properties differ between species. The third explanation would be the existence of additional E-selectin ligands. One such additional ligand is of course PSGL-1. Although PSGL-1 does not seem to contribute significantly to the binding of neutrophils to E-selectin (23) it seems to be important for the binding of Th1 cells to E-selectin (76). If further ligands exist, they must either be far less abundant than ESL-1 and PSGL-1 or their binding parameters do not allow direct affinity isolation with E-selectin.

**Thermodynamics**

The key observation in the thermodynamic analysis of the E-selectin – ESL-1 interaction was that there was no significant change in affinity with temperature implying that binding was for the most part entropically-driven with only a small contribution from enthalpic changes. These data contrast with the more usual finding that enthalpic contributions are considerable in protein – protein interactions (77), including interactions of cell-surface molecules like CD48 and CD80 with their respective cell-surface ligands (52,56,78). It is noteworthy, however, that the affinity of another selectin – ligand interaction, L-selectin - GlyCAM-1, is the same at 25 and 37°C, suggesting that this interaction is also entropically-driven (12). The thermodynamic properties of the E-selectin - ESL-1 and L-selectin – GlyCAM-1 interactions also differ from typical protein - carbohydrate interactions, which are generally enthalpically-driven and characterised by unfavourable entropic changes (79). These unfavourable entropic changes can be explained by the high conformational entropy of many oligosaccharides that might be lost upon binding to proteins. The favourable entropic changes observed here for the E-selectin - ESL-1 interaction suggest either that the entropic penalty associated with formation of the E-selectin - ESL-1 complex is less than anticipated from carbohydrate data and/or that solvent entropic effects (a consequence of the ejection of bound water from the interface) are unusually favourable. Conversely, the small contribution of enthalpic changes
suggests either that there are relatively few contacts between E-selectin and ESL-1 and/or that binding is accompanied by the breaking of a significant number of favourable interactions involving solvent.

It has been shown in a study of avidin binding to biotin analogues that there is a better correlation between the mechanical strength of an interaction and its enthalpic change than with its affinity constant (80). Since enthalpic changes arise from the formation of bonds this would seem plausible. However, our results suggest that this correlation is unlikely to extend to selectin - ligand interactions, which have favourable mechanical properties (81). The likely reason for this is that enthalpic changes are the sum of unfavourable contributions from bonds lost between solvent and the binding interface and and favourable contributions from bonds formed between the two interfaces. While it is the latter that would presumably contribute to mechanical strength these two components cannot be independently measured. Given that all selectin - ligand interactions are likely to possess a high degree of mechanical strength, our findings imply that many strongly bound water molecules are ejected from the E-selectin – ESL-1 interface.

In conclusion, we have determined the first affinity and binding kinetic parameters of an authentic glycoprotein ligand of E-selectin, ESL-1. We find an affinity that is similar to that of the L-selectin - GlyCAM-1 interaction but is considerably lower than the affinity of P-selectin binding to PSGL-1 due mainly to a much lower $k_{on}$. This finding shows that exceptionally fast association rate constants are not a general feature of selectin – ligand interactions. It also underscores the unique mechanism with which P-selectin interacts with PSGL-1 involving sulfated tyrosine residues of the ligand. It will be interesting to determine the structural units of ESL-1 that are recognized by E-selectin.
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Footnotes

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†The abbreviations used are:
CHO, Chinese hamster ovary; ESL-1, E-selectin ligand-1; FC, flow cell; FucT, \( \alpha 1,3 \)-fucosyltransferase; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1; RU, response unit; \( sLe^x \), sialyl Lewis\( ^x \); SNA, Sambucus nigra agglutinin; SPR, surface plasmon resonance.
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Figure legends

FIG. 1. Production and analysis of E-selectin constructs. A, schematic diagram of the E-selectin constructs E-Sel.L and E-Sel.S. CL, EGF, and C refer to C-type lectin domain, epidermal growth factor domain, and consensus repeat domain, respectively. B, E-selectin constructs (5 µg each) were analysed by SDS-PAGE (10 %) under reducing conditions. C, E-selectin constructs were incubated with Protein-A Sepharose coated with E-selectin mAb UZ4 or a control mAb (R4-22). The beads were sedimented and the supernatants were analysed for the presence of residual amounts of the respective construct by reducing SDS-PAGE. D, size exclusion chromatography of E-Sel.S. 15 mg of the protein were run on a Superdex S75 HR10/30 column at 0.5 ml/min. The calibration markers shown were alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The indicated fractions (*) were pooled, stored at 4°C and used in SPR assays within 48 h. The E-Sel.L construct eluted in a single peak between 66 and 150 kDa proteins (not shown) and was used equivalently.

FIG. 2. Purification and analysis of E-selectin ligand-1. A, analysis of ESL-1 by SDS-PAGE. An aliquot of purified ESL-1 (obtained by precipitation with E-selectin-Ig from 1 x 10^8 mouse bone marrow cells after PSGL-1 depletion) was separated on an 8 % polyacrylamide gel under reducing conditions and stained with silver. The second lane contains sample buffer only. B, identification of the purified 150 kDa band by ESL-1 depletion. Conditions were as in A, except that cell lysates were incubated with Protein A-Sepharose-bound control serum or anti-ESL-1 serum before precipitation with E-selectin-Ig. C, analysis of the efficiency of PSGL-1 depletion from bone marrow cell lysates by Western blotting. Lysates were either not depleted (lane 1), depleted by one incubation with P-selectin-Ig (lane 2) or by one incubation with P-selectin-Ig followed by a second round with the mAb 4RA10 against PSGL-1 (α-PSGL-1). The lysates were then subjected to precipitation with P-selectin-Ig (lane 1) or E-selectin-Ig (lanes 2 and 3). Precipitated material from 1 x 10^8 cells was separated by 6 % reducing SDS-PAGE, transferred to nitrocellulose membranes and analysed for the presence of PSGL-1 by incubating the filter with anti-PSGL-1 mAb 4RA10, secondary conjugate and ECL substrate for detection.
FIG. 3. **Specificity of E-selectin – ESL-1 interactions in binding experiments.** A, binding of E-Sel.S to immobilized ESL-1. Binding is measured in arbitrary response units. E-Sel.S (0.35 mg/ml) was injected (indicated by the bar above the graph) into a flow cell (FC) with 5000 RU of immobilized ESL-1 (ESL-1 FC) and a FC with an equivalent amount of immobilized OX12 control protein (control FC). Injection of E-Sel.L gave identical binding profiles (not shown). B, dependence of E-selectin binding to ESL-1 on divalent cations. Different concentrations of E-Sel.S (0.5 - 3.0 mg/ml) were injected in the absence or presence of 10 mM EDTA over sensor surfaces with 1000 RU of immobilized ESL-1 and an equivalent amount of immobilized control protein, respectively. Specific responses (ESL-1 FC minus control FC) in the absence of EDTA were normalized to 100 %. Binding in the presence of EDTA is expressed as mean percentage of binding ± S.D. of four experiments. C, ESL-1 specificity of E-selectin binding. Control- and ESL-1-depleted preparations were obtained as described for Fig. 2B and immobilized to sensor surfaces using equal volumes and injection times. The specific binding of E-Sel.S (eight injections with concentrations ranging from 0.5 to 3.0 mg/ml) to the control depleted material was normalized to 100 %. At each concentration binding of E-Sel.S to the ESL-1-depleted material was measured. The mean percentage of this residual binding ± S.D. is given. Dissociation rate constants (K_d) were determined as described in Fig. 4.

FIG. 4. **Measuring the affinity of the E-selectin – ESL-1 interaction.** A and B, eight two-fold dilutions of E-Sel.S (74.9 µM – 0.59 µM) were injected over an ESL-1 FC and a control protein FC (injections are indicated by bars). C, D, and E, specific equilibrium responses (ESL-1 FC minus control FC) from representative experiments as explained in A and B were plotted against the concentrations of injected E-selectin construct. The curves represent nonlinear fits of the Langmuir binding isotherms to the data. C, binding of E-Sel.L to bone marrow cell derived ESL-1. D, binding of E-Sel.S to bone marrow cell derived ESL-1. E, binding of E-Sel.S to recombinant ESL-1myc. Immobilization levels were 750 RU in A-D and 2000 RU in E. Insets, Scatchard plots of the data in C, D, and E. K_d values obtained by nonlinear curve fitting and Scatchard plot analysis, respectively, are given.
FIG. 5. **Dependence of E-selectin binding to ESL-1myc on fucosylation and sialylation of the ligand.** A, dependence of E-selectin binding on fucosylation of ESL-1myc. Different concentrations of E-Sel.S (0.1 – 0.3 mg/ml) were injected over sensor surfaces with 2000 RU of immobilized ESL-1myc obtained from CHO cells expressing (+) or not expressing (-) FucTIV. Specific responses in the FC with fucosylated ESL-1myc were normalized to 100%. Binding to non-fucosylated ESL-1myc is expressed as mean percentage of binding ± S.D. of eight injections. B, dependence of E-selectin binding on sialylation of ESL-1myc. ESL-1myc obtained from FucTIV-expressing CHO cells was immobilized to sensor surfaces (1600 RU on each FC). E-Sel.S (0.1 – 0.3 mg/ml) and the sialic acid-binding lectin SNA (2 mg/ml) were then injected over unmodified (-) or sialidase-treated (+) ESL-1myc. Specific E-Sel.S binding (ESL-1myc FC minus control FC) was measured at equilibrium whereas the binding response of SNA was measured 30 s after the injection to eliminate the bulk phase effect. Specific responses to unmodified ESL-1myc were normalized to 100%. Binding to sialidase-treated ESL-1myc is expressed as mean percentage of binding (± S.D. of five injections of E-Sel.S or ± range of two injections of SNA).

FIG. 6. **Kinetics of E-selectin dissociating from ESL-1.** E-Sel.S was injected at the indicated temperatures at a concentration of 7 µM over 4000 RU of immobilized ESL-1 and control protein, respectively. Shown are ESL-1 specific responses (ESL-1 FC - control FC) which were normalized so that maximum binding (at the start of the dissociation phase) equals 100%. Nonlinear fits of exponential decay curves to the dissociation data gave the dissociation rate constants depicted in Table III.

FIG. 7. **Temperature-dependence of kinetics and affinity.** A, Arrhenius plot of $k_{off}$ and $k_{on}$ data for binding of E-Sel.S to ESL-1. The $k_{off}$ (circles) and $k_{on}$ data (squares) are taken from Table III. The slope was determined by linear regression (correlation coefficient ≥ 0.99), and used to calculate the respective activation energy $E_a$ (slope = - $E_a$ / R, where R is the gas constant). B, estimation of enthalpy by non-linear van't Hoff analysis. $K_d$ values for E-Sel.S binding to ESL-1 (Table II) were converted into free energy of binding ($\Delta G$). The enthalpic change ($\Delta H$) was derived by fitting the non-linear form of the van't Hoff equation to these data.
Table I

Only E-selectin but not other adhesion molecules bind to immobilized ESL-1

| Sample injected | Specific response units |
|-----------------|-------------------------|
| E-Sel.S         | 1449                    |
| L-Sel.His       | 40                      |
| P-Sel.His       | 40                      |
| CD22 d1-3 Fc    | 13                      |

a All samples were injected at a concentration of 1 mg/ml for 30 s at a flow rate of 20 µl/min. L-Sel.His and P-Sel.His constructs contained the entire extracellular domain of the respective murine adhesion molecule and a C-terminal hexahistidine-tag. They were produced in a way analogous to E-Sel.L. Function blocking mAbs against L- and P-selectin were able to deplete > 90% of the respective construct indicating correct folding (not shown). CD22 d1-3 Fc contained the first three domains of mouse CD22 and the Fc part of human IgG1.

b Specific response = response on ESL-1 surface minus response on control surface. 5000 RU of ESL-1 and control protein OX12, respectively, were immobilized.
### Table II

**Summary of affinity measurements of E-selectin binding to immobilized ESL-1**

| Sample immobilized<sup>a</sup> | Sample injected | Temperature (°C) | \(K_d\)<sup>b</sup> (µM) |
|---------------------------------|-----------------|-----------------|-------------------|
| ESL-1                           | E-Sel.L         | 25              | 62 ± 10 (n = 8)   |
|                                 | E-Sel.S         | 37              | 62 ± 9 (n = 5)    |
|                                 | E-Sel.S         | 25              | 56 ± 9 (n = 12)   |
|                                 | E-Sel.S         | 15              | 56 ± 1 (n = 2)    |
|                                 | E-Sel.S         | 5               | 57 ± 9 (n = 2)    |
| recombinant ESL-1myc            | E-Sel.S         | 25              | 66 ± 1 (n = 2)    |

<sup>a</sup> At each temperature E-selectin binding to a low and a high density of immobilized ESL-1 (750 RU and 4000 – 5000 RU, respectively) was measured. Binding of E-Sel.S to a very low immobilization level of ESL-1 (200 RU) at 25 °C was also measured and gave a very similar \(K_d\) of 45 µM. 2900 RU of ESL-1myc were immobilized.

<sup>b</sup> Mean ± S.D. for \(n > 2\); mean ± range for \(n = 2\). \(n\) = No. of determinations. Affinities were measured by equilibrium binding as described in Fig. 4.
**Table III**

Summary of kinetic measurements of E-selectin binding to immobilized ESL-1

| Sample injected | Temperature (°C) | \( k_{\text{off}}^a \) (s\(^{-1}\)) | \( k_{\text{on}}^b \) (M\(^{-1}\)s\(^{-1}\)) |
|-----------------|-----------------|-----------------------------------|-----------------------------------|
| E-Sel.L         | 25              | 3.0 ± 0.1 (n = 4)                 | 4.8 x 10\(^4\)                   |
| E-Sel.S         | 37              | 4.6 ± 0.7 (n = 3)                 | 7.4 x 10\(^4\)                   |
|                 | 25              | 2.7 ± 0.5 (n = 6)                 | 4.8 x 10\(^4\)                   |
|                 | 15              | 1.9 ± 0.1 (n = 2)                 | 3.4 x 10\(^4\)                   |
|                 | 5               | 1.1 ± 0.1 (n = 3)                 | 1.9 x 10\(^4\)                   |

\(^a\) Mean ± S.D. for \( n > 2 \); mean ± range for \( n = 2 \). \( n \) = No. of determinations. At each temperature dissociation of E-selectin from a low and a high density of immobilized ESL-1 (750 RU and 4000 – 5000 RU, respectively) was measured. No significant change in \( k_{\text{off}} \) for the dissociation of E-Sel.S from ESL-1 was noted when the density of immobilized ESL-1 was further reduced to 200 RU and E-Sel.S was used near saturation (180 µM). The \( k_{\text{off}} \) values were obtained from nonlinear fits of exponential decay curves to the dissociation data.

\(^b\) \( k_{\text{on}} \) was calculated using the equation \( k_{\text{on}} = k_{\text{off}} / K_d \).
Table IV

Comparison of affinities and kinetics of selectin – ligand interactions measured by SPR

| Interaction     | Species   | Temp. (°C) | K_d (µM) | k_on (M⁻¹ s⁻¹) | k_off (s⁻¹) | Refs.     |
|-----------------|-----------|------------|----------|----------------|-------------|-----------|
| E-selectin - ESL-1 | Mouse    | 37         | 62       | 7.4 x 10⁴      | 4.6         | This study|
|                 |           | 25         | 56       | 4.8 x 10⁴      | 2.7         | This study|
| L-selectin - GlyCAM-1 | Mouse    | 25         | 108      | ≥ 1 x 10⁵      | ≥ 10        | (12)      |
| P-selectin - PSGL-1  | Human    | 25         | 0.32     | 4.4 x 10⁶      | 1.4         | (13)      |
Fig. 1

A

CL EGF
his-tag COOH
E-Sel.L

CL EGF
his-tag COOH
E-Sel.S

B

E-Sel.L E-Sel.S

C

Depletion with control UZA control UZA

E-Sel.L E-Sel.S

D

O.d. (280 nm)

Elution Volume (ml)

5 6 7 8 9 10 11 12 13

5 4 3 2 1 0

150 66 29
Fig. 4

A

ESL-1 FC

Response Units

Time (s)

E-Sel.S

B

Control FC

Response Units

Time (s)

E-Sel.S

C

K_{d} = 60 \mu M

Response Units

Concentration of injected sample (\mu M)

0 10 20 30 40

130 230 330 430

D

K_{d} = 53 \mu M

Response Units

Concentration of injected sample (\mu M)

0 50 100 150 200

100 200 300 400

E

K_{d} = 65 \mu M

Response Units

Concentration of injected sample (\mu M)

0 60 120 180 240

500 1000 1500

Injected:

E-Sel.L

E-Sel.S

E-Sel.S

Immobilized:

ESL-1

ESL-1

recombinant ESL-1myc
Fig. 5

A

Specific binding of E-Sel.S (%)

FucTIV: + -

100 80 60 40 20 0

B

Specific binding (%)

Sialidase: - + - +

E-Sel.S SNA
Fig. 6
Fig. 7

A

\[ \ln k_{\text{off}} \text{ vs. } \frac{1}{T} (\times 10^4 \text{ K}^{-1}) \]

- \( E_a^{\text{diss}} = 31.4 \text{ kJ mol}^{-1} \)
- \( E_a^{\text{ass}} = 29.9 \text{ kJ mol}^{-1} \)

B

\[ \Delta G^o (\text{kJ mol}^{-1}) \text{ vs. Temperature (K)} \]

\( \Delta H = -3.8 \text{ kJ mol}^{-1} \)
Affinity, kinetics and thermodynamics of E-selectin binding to E-selectin ligand-1
Martin K. Wild, Min-Chuan Huang, Ursula Schulze-Horsel, P. Anton van der Merwe and Dietmar Vestweber

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