The Carbohydrate-Recognition Domain of E-Selectin Is Sufficient for Ligand Binding under Both Static and Flow Conditions

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ABSTRACT: Selectins are a family of adhesion molecules with a well-defined domain structure comprised of a lectin or carbohydrate-recognition domain (CRD), an epidermal growth factor (EGF)-like motif, and a variable number of consensus repeats (CRs). While it is clear from various lines of evidence that the CRD plays a pivotal role in selectin−ligand interactions, little is known about the role of the non-lectin selectin domains. We expressed a series of soluble chimeric proteins with various domains switched between E- and L-selectin and measured binding of the resulting chimeras to sialyl Lewis^a and sulfatide, two carbohydrate structures which are specific for the E- and L-CRDs, respectively. Both CRDs bind to their respective ligands with the same affinity regardless of the origin of the other domains they are attached to. The domain-switched chimeras were assayed for their ability to support static binding and rolling of various cell lines which bind specifically to E-selectin. In these assays, the E-CRD was indispensable for both static binding and rolling under physiological flow conditions. The E-CRD alone, when substituted into L-selectin, supported rolling without the requirement for additional ligand-recognition elements. We conclude that the EGF domain or the CRs of E- and L-selectin have no influence on the CRD's specificity to carbohydrates. Furthermore, at least in the case of E-selectin, they do not contribute to the specificity of binding to cell surface ligands.

Selectins are a group of adhesion molecules which play a crucial role in leukocyte recruitment by mediating leukocyte rolling, the first obligatory step in a cascade of events leading to leukocyte extravasation. The family consists of E- and P-selectins, on the endothelial side, and L-selectin on leukocytes [recently reviewed by Rosen and Bertozzi (1994) and McEver (1994)]. Between them, these receptors mediate the reversible rolling of leukocytes on endothelium in a variety of normal and disease states acting either alone or in concert (Tedder et al., 1995; Lefer et al., 1994). An essential factor in selectin binding to their ligands is carbohydrate recognition. Extensive work by several laboratories has demonstrated that selectins bind with various affinities to the tetrasaccharides sLe^x [1] and sLe^a and various derivative structures [reviewed by Varki (1994)]. Distinct binding preferences have been demonstrated for E-selectin, which seems to bind preferentially to extended sLe^x structures (Patel et al., 1994), and L-selectin, which binds to sulfated sLe^x derivatives (Hemmerich et al., 1995), but not yet for P-selectin, which seems to bind both sulfated and nonsulfated structures.

A series of glycoproteins have been identified as carbohydrate bearing counterligands for the selectins with various levels of certainty and biological significance. P-Selectin has the highest ligand specificity and seems to have a single physiological ligand, a glycoprotein termed PSGL-1 (Sako et al., 1993). L-Selectin binds to several mucins, including Glycam-1 (Lasky et al., 1992), CD34 (Baumhueter et al., 1993), MAdCAM-1 (Berg et al., 1993), and Sgp200 (Hoke et al., 1995). E-Selectin binds to a wider number of counterligands, with a number of reports implicating glycolipids (Pinola et al., 1994) and a variety of glycoproteins, including CD66 (Kuijpers et al., 1992), CD11/CD18 (Kotovuori et al., 1993), Glycam-1 (Mebius & Watson, 1993), and PSGL1 (Asa et al., 1995) as E-selectin ligands. Recently, a new N-linked glycoprotein, called ESL-1, has been cloned after affinity purification with E-selectin (Steegmaier et al., 1995).

The fact that selectins bind to only a small number of glycoproteins raises the question of how specificity is achieved. Two different hypotheses may be invoked to explain selectin ligand specificity. One is that selectin ligands possess glycosylation distinct from other surface proteins, or possibly present their carbohydrates in such a manner as to create novel epitopes not found on other glycoproteins. The other hypothesis is that additional, noncarbohydrate-based, interactions between selectins and their ligands take place.

All three selectins have a domain structure, composed of a single CRD, an EGF-like domain, and a variable number of CRs. The CRD plays an indisputably central role in selectin function as shown by the absolute requirement of appropriate glycosylation for binding of all ligands. Additionally, most selectin-blocking antibodies have been mapped to CRD epitopes, and lethal mutations of E-selectin are located in the CRD (Erbe et al., 1992).

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[1] Abbreviations: sLe^x, Neu5Acα2,3Galβ1,4(Fucα1,3)GlcNAc; sLe^x-Lemieux, Neu5Acα2,3Galβ1,4(Fucα1,3)GlcNAc-O-$(CH_2)_8$COOMe; sLe^a, Neu5Acα2,3Galβ1,3(Fucα1,4)GlcNAc; CRD, carbohydrate-recognition domain; EGF, epidermal growth factor; CR, consensus repeat; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; PAGE, polyacrylamide gel electrophoresis; MTT, thiazolyl blue tetrazolium bromide; SOE, splicing by overlap extension; PCR, polymerase chain reaction; OPD, *o*-phenylenediamine dihydrochloride; DAB, diaminobenzidine tetrahydrochloride; PMN, polymorphonuclear leukocyte.

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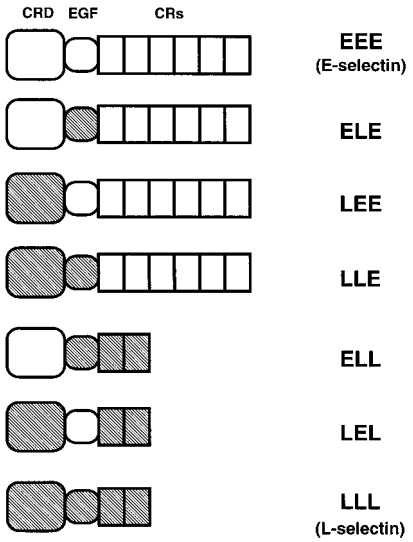

FIGURE 1: Domain structure of wild type and domain-switched E- to L-selectin chimeras. The first letter in the name of each chimera represents the CRD, the second the EGF-like domain, and the third the CRs. According to this scheme, wild type E- and L-selectins are called EEE and LLL, respectively. Chimeras were constructed by PCR, as indicated in the Experimental Procedures. All DNA constructs carried the leader sequence corresponding to the lectin domain they contained and on the 3′ end were spliced to human IgG at exactly the position where the transmembrane domain starts in the native gene.

Diverse, but not so clear, evidence exists for the involvement of the other selectin domains in ligand recognition. Two monoclonal antibodies, one which binds to the EGF domain of murine L-selectin (Siegelman et al., 1990) and one which binds to the CRs of both E- and L-selectin (Bargatze et al., 1994a), are known to block binding. In both E-selectin (Li et al., 1994) and L-selectin (Watson et al., 1991), the CRs seem to enhance selectin affinities. And most convincingly, the EGF domain of P-selectin not only seems to enhance binding (Gibson et al., 1995), but in static binding assays, it is also able to impart P-selectin-like binding specificity when swapped into L-selectin (Kansas et al., 1994).

In order to determine the function of the various domains of E-selectin in ligand recognition and measure their contribution to binding, we used a domain-switch approach in which we exchanged domains between E- and L-selectins. These two lectins, despite their high homology which suggests a similar tertiary structure, have no common high-affinity ligands, and they bind distinctly different oligosaccharide structures, thus making it possible to establish assays which clearly characterize and distinguish between the two selectins. Our reasoning was that, by switching domains between E- and L-selectins, any basic elements required for the correct three-dimensional structure of the proteins would be conserved and interactions which are responsible for specificity will be revealed.

We constructed a series of E- to L-selectin domain-switched chimeras, as shown in Figure 1, and expressed them as soluble dimeric human IgG fusion proteins. We quantitated the binding of chimeras to defined carbohydrates in a molecule/molecule type of assay and measured their ability to recognize and bind to more extended ligands present on cell surfaces in cell/molecule static assays. Since the hallmark of selectin physiological function is rolling (Lawrence & Springer, 1993; Abbassi et al., 1993), we also evaluated the capacity of the domain-switched chimeras to support cell rolling under flow conditions at physiological levels. Our results show that substitution of either the EGF domain alone, the CRs alone, or both simultaneously has no influence on the specificity of either the E- or L-CRD and that the determinants of E-selectin specificity to cell surface ligands, under both static and flow conditions, are contained entirely within the CRD.

EXPERIMENTAL PROCEDURES

Materials. All assays were performed in Immulon B plates (Dynatech), unless otherwise indicated. All cell lines were purchased from ATCC, and all media, transfection reagents, and antibiotics were purchased from GIBCO. CHO-K1 cells were maintained in MEMα and 4% FBS and U937, HL-60, and Colo 205 cells in RPMI 1640 with 10% FBS. The gene for human E-selectin (BBG 57) was purchased from British Biotechnology, and a vector containing the complete coding sequence of human L-selectin was a gift from Dr. B. Bowen. Plasmid 94/4.7HHb containing the genomic sequence for human IgG1 was a gift from Dr. N. Hardman. sLe^x-Lemieux was a gift from Dr. R. Oehrlein. Antibodies BBA2 and BBA8 were purchased from R&D Systems, and SK11 was purchased from Becton Dickinson.

Construction of Selectin Chimera. The various E to L domain-switched chimeras were prepared by a PCR-based strategy. The transitions between the various domains in both E- and L-selectins are unambiguous; amino acids 1−120 constitute the CRD and 121−155 the EGF domain in both E- and L-selectins (Bevilacqua et al., 1989; Bowen et al., 1989). Position 535 of E-selectin and position 332 of L-selectin mark the end of the CRs. These positions were taken as boundaries for the construction of chimeras. Using 10−15 PCR cycles to keep the number of polymerase errors at a minimum, the appropriate 5′ and 3′ cDNA pieces were amplified using primers with complementary overlaps. In the case of ELE and LEL, the overlaps reached across the complete EGF-like domain. The resulting PCR products were then spliced together by SOE-PCR (Horton et al., 1989) to produce the complete soluble chimera cDNA. Human IgG fusion proteins of each domain-switched chimera were constructed by PCR splicing of the selectin chimera cDNAs to a PCR-amplified product consisting of a few bases from the 3′ end of the CH1 domain and the complete hinge and CH2 and CH3 domains of genomic human IgG1. The resulting chimeric selectin/IgG cDNAs were cut with *Not*I and subcloned into the single *Not*I site of expression vector pcDNAI/neo (Invitrogen).

Chimera-Producing Cell Lines. For the production of permanent lines, CHO cells were plated overnight in a 3 cm tissue culture dish at a density of 20×10^3 cells/mL. Dishes were transfected with 3 μg of linearized expression vector DNA using Lipofectamine according to the manufacturer's instructions, with the difference that only half the suggested Lipofectamine reagent was used. After 24 h, cells were trypsinized and plated in two 96-well plates, and 1 day later, 0.4 mg/mL G418 was applied for the selection of transformants. After about 2 weeks, wells with surviving cells were evaluated for the production of human IgG and further subclonned by limiting dilution.

For chimera production, clones were grown in T175 flasks using normal media. After the cells reached confluency, the medium was changed to OptiMEM containing 2% FBS and

supernatant was harvested every 48 h for a period of 3−4 weeks.

Chimera Purification. Frozen pooled supernatants from CHO clones were concentrated using an Amicon YM-100 membrane and purified on a 2 mL protein A Sepharose column (Pharmacia) using the MAPS II buffer system (Bio Rad). Fresh protein A was used for every chimera purified in order to avoid any possibility of cross-contamination. After elution, the buffer was exchanged to PBS and the purified chimeras were stored aliquoted at −20 °C at a concentration of 0.1−1 mg/mL. The purified chimeras were subjected to SDS−PAGE under reducing conditions and either stained with coomassie or, alternatively, blotted on a PVDF membrane and bands visualized with metal-enhanced DAB (Pierce) after incubation with the peroxidase-conjugated anti-human IgG antibody used in the human IgG ELISA.

ELISA Assays. Human IgG was quantitated using a sandwich ELISA. A polyclonal rabbit anti-human IgG $F(ab')_2$ fragment (Jackson) at a concentration of 2 μg/mL was employed for capture, and a polyclonal goat anti-human IgG $F(ab')_2$ fragment peroxidase-conjugated (Jackson) for detection (1:5000 dilution). A standard curve with purified human IgG (Sigma) was performed for every analysis. Developed with OPD, the assay has a linear range from 3 to 100 ng/mL human IgG. Serial dilutions of each sample were analyzed, and the concentration was determined from several dilutions which fell within the linear range.

Glycopolymer Binding Assay. Cell culture plates (96-well) were blocked for 30 min at room temperature with 300 μL of 0.5% BSA and 0.05% Tween-20 in PBS. Then, 50 μL of HBSS containing defined concentrations (0−400 ng/mL) of sLe^a-biotinylated polymer [30−40 kDa molecular mass poly[*N*-(2-hydroxyethyl)acrylamide] containing 20 mol % SLe^a (Bovin et al., 1993) was purchased from Syntesome, Munich] was added to each row. After addition of 50 μL of each chimera (2.2 μg/mL in HBSS containing 1:2500 streptavidin/peroxidase, Boehringer), the plate was incubated for 2 h at room temperature with shaking. The equilibrated mixture (80 μL) was aliquoted on maxisorb plates (Nunc) coated with 0.5 μg of goat anti-human IgG (Fc-specific; Sigma) and incubated for 1 h. After three washes with HBSS, the wells were developed with OPD and absorption (490−650 nm) was measured with a plate reader. All experiments were run in duplicate. Apparent binding constants were determined by using the implemented curve-fitting software.

Sulfatide Assay. Polysorb 96-well plates (Nunc) were filled with 100 μL of a solution consisting of 200 μg/mL sulfatides (Sigma) in ethanol. The plates were left uncovered at room temperature overnight, washed three times with PBS, and blocked with 0.1% BSA. Various concentrations of selectin chimeras were added in a total volume of 100 μL of HBSS and 0.1% BSA and the plates incubated on a rotatory shaker at room temperature for 1 h. The wells were then washed with HBSS and incubated with 100 μL of HBSS containing a 1:5000 dilution of the polyclonal goat anti-human IgG $F(ab')_2$ fragment peroxidase conjugated (Jackson). After being washed, the wells were developed with TMB. All wells were run in triplicate and averaged.

Static Cell Binding Assays. Microtiter plates were coated for 1 h at room temperature with 100 μL of 5 μg/mL polyclonal rabbit anti-human IgG $F(ab')_2$ fragment (Jackson) and then blocked with 250 μL of 0.1% BSA. Each well was then coated for 1 h with 200 ng of selectin/IgG chimera in a total volume of 100 μL of PBS and 0.1% BSA. For controls, wells were coated with purified human IgG at the same concentration. After being washed two times with PBS and one time with HBSS, the wells were ready for the binding assay.

Cells, in logarithmic growth, either were from suspension cultures (HL-60, U937) or were released from the plate with PBS/EDTA at 4 °C (Colo 205). In order to facilitate quantification of binding, cells were first stained with MTT using a procedure similar to that described by Dressen et al. (1992). Briefly, cells were washed in RPMI 1640 media and brought to a concentration of 1×10^6 cells/mL. MTT stock solution in PBS was added to a final concentration of 1 mg/mL and the solution incubated at 37 °C for 30 min. The stained cells were washed two times and suspended in HBSS (containing 1 mg/mL human IgG in order to passivate any Fc receptors present on the cell surface) at the original concentration.

Chimera-coated plates were filled with 100 μL of stained cell suspension and shaken at room temperature on a rotatory shaker at 150 rpm for 30 min. Wells were washed with HBSS two times and inspected visually with a microscope. To each well was added 100 μL of 1-propanol, and the solution was mixed well in order to completely lyse the cells and dissolve all MTT/formazan. Absorbance at 600 minus 650 nm was measured in a microtiter plate reader. All samples, including controls, were run in triplicate. Negative controls were plates coated with only human IgG and also wells with 5 mM EDTA added in the cell suspension.

Rolling Assays. A parallel plate flow chamber was used to study the rolling behavior of cells in contact with selectins in the presence of hydrodynamic flow. The chamber, outfitted with a silicon rubber gasket, was of a circular design to accommodate 35 mm tissue culture dishes (Corning) on which the domain-swapped chimeras were coated. The dimensions of the gasket used in the flow chamber and the volumetric flow rate through the chamber define the wall shear stress for the cells rolling on the chimeras by the following relationship. The wall shear stress (t_w, dynes per squared centimeter) is given by $t_w = 6\mu Q/a^2 b$, where μ is the apparent viscosity of the media (for H_2O at 37 °C = 0.76 cP), a is the channel height (i.e. gasket thickness of 254 μm), b is the channel width (i.e. gasket width of 1 cm), and Q is the volumetric flow rate (milliliters per second).

Domain-switched chimeras were coated onto a 19.6 mm^2 circular area in the center of the 35 mm dishes. To improve the presentation of the chimeras, an anti-human IgG antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was first coated on the dish in order to capture the chimera (20 μL of 50 ng/μL for 1 h at 37 °C). The coating area was then blocked with 2% BSA and coated with a total of 200 ng of chimera per dish (1 h at 37 °C).

The flow assay was performed by placing a chimera-coated 35 mm dish on the flow deck held in place by a vacuum. The cell suspension of HL-60 cells (2×10^5 cells/mL containing 0.2 mg/mL human IgG to block the Fc receptors on the HL-60 cells) was perfused through the chamber at shear rates corresponding to a wall shear stress of 1.2 dynes/cm^2. The cell suspension was allowed to flow through the chamber for 3 min before digital images were collected to quantify each experiment.

Digital Image Acquisition and Analysis. The digital image system consisted of a Silicon Graphics Indigo2 workstation interfaced to an Inovision IC300 digital image system. The cells interacting with the coated chimera were visualized using a Zeiss inverted stage microscope operated in the phase contrast mode using a 10 × objective. A CCD camera (Dage-MTI CCD72) was mounted on the microscope to provide the signal to the digital image system, and the experiments were recorded on a video recorder.

After 3 min of perfusing cells through the flow chamber, digital images were acquired at seven different locations on each dish. The image acquisition program collected ten images at each location to provide sufficient image data for subsequent image processing. Each of the ten images was a result of a real time minimization function (Patton et al., 1993) of three frames to remove all moving cells in the bulk flow which are not in contact with chimeric surface in the flow chamber. Once the ten images were collected, image analysis was performed to generate composite images containing only rolling cells, only arrested cells, or images with the total number of interacting cells (i.e. both the rolling and arrested cells). The images with the rolling cells are created to show the rolling cells as vertical streaks corresponding to the distance traveled by the cells during image acquisition. The number of interacting cells is equal to the number of rolling cells when there are no arrested cells (i.e. no stationary cells), as was the case for these experiments.

The number of rolling cells was determined by a segmentation program based on pixel intensity and size. Quantification of the rolling behavior was performed by analysis of images containing the rolling cells as vertical streaks. The first measure of rolling was the rolling index defined as the total area (i.e. total pixel count) of all the vertical streaks in each image. The second measure of rolling was the rolling velocities defined as the average length of the streaks in each image in the 10 s time period to collect the composite image.

RESULTS

Construction and Purification of Chimeras. Starting with wild type E- and L-selectins, designated EEE and LLL in Figure 1, we constructed a series of domain-switched chimeras in which the CRD, EGF-like, and CRs of the two selectins were interchanged as indicated. All chimeras retained the export sequence associated with the CRD they carried and were fused to the hinge region of human IgG1 immediately after the last CR. The resulting proteins are expected to be exported, soluble IgG dimers.

Each one of the constructed cDNAs was transfected in CHO-K1 cells, and clones were selected by resistance to G418. Several clones were evaluated for each domain-switched chimera in order to find stable, high-producing lines. Production levels varied, ranging from a high level of about 2 mg/L for the wild type proteins (EEE and LLL) to 0.1 mg/L for LEL. All clones were stable and produced the same amount for several weeks after reaching confluency.

Several liters of combined supernatants for each clone were concentrated and purified as a single batch over a protein A Sepharose column. Recoveries, as measured by human IgG ELISA, were in the 80−90% range. SDS−PAGE analysis of the purified chimeras revealed that all samples contained considerable amounts (50−90%) of an extraneous protein (probably bovine IgG), which migrated as a single band close to 200 kDa in nonreducing gels and as a double band at 75 and 25 kDa in reducing gels (data not shown). When, however, Western blots were performed using an anti-human IgG peroxidase-conjugated antibody, all chimeras were shown to be functionally pure and contain a single human IgG species, which in all cases migrated at the expected molecular mass (Figure 2).

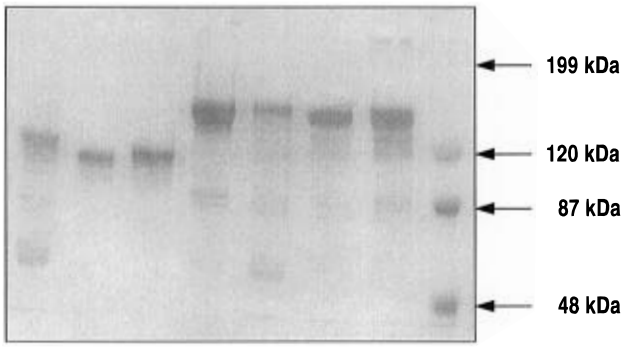

FIGURE 2: Purified domain-switched chimeras were run on 8% SDS−PAGE under reducing conditions and blotted on a PVDF membrane. After being blocked with 1% BSA, the membrane was incubated with a 1:1000 dilution of anti-hIgG/peroxidase conjugate and subsequently visualized with DAB. Coomassie prestained standards were from BioRad. Monomers from chimeras containing the E-selectin six CR domains migrate at about 140 kDa, and monomers from chimeras containing the L-selectin two CR domains migrate at about 110 kDa.

Table 1: Recognition of Domain-Switched Chimeras by Specific Antibodies[a]

	BBA2	BBA8	SK11
EEE	++++	++++	−
ELL	++++	−	+
ELE	++++	++++	+
LLL	−	−	++++
LEE	+	++++	+++
LEL	+	+	++++
LLE	−	++++	++++

[a] Microtiter wells precoated with anti-hIgG antibody and blocked with 1% BSA were incubated with various purified domain-switched chimeras and then developed according to the following sequence: 50 ng of purified selectin chimera per well, 100 ng of the indicated antibody per well, 1:5000 dilution of an anti-mouse IgG/peroxidase conjugate, and OPD substrate. The indicated response represents the relative values obtained with the particular antibody. BBA2 binds to the lectin domain of E-selectin, BBA8 binds to one of the CR domains of E-selectin (Pigott et al., 1991), and SK11 blocks L-selectin binding.

Purified chimeras were further analyzed with several antibodies of known specificities. The results, shown in Table 1, indicate that they are recognized only by antibodies specific against domains which they are meant to contain. Both BBA2 and SK11 are blocking antibodies and bind to the native forms of the E- and L-CRD. The fact that these antibodies bind to their respective epitopes in all domain-switched chimeras suggests that, in comparison to the wild type molecules, no major changes have occurred in the three-dimensional folding of the lectin domains.

Binding to Carbohydrates. Wild type E- and L-selectins have overlapping but quantitatively distinct carbohydrate specificities. We developed assays which measured binding of defined carbohydrates to chimeras in order to determine the influence of domain switching on only the CRD. For this purpose, it was important to use two clearly distinguish-

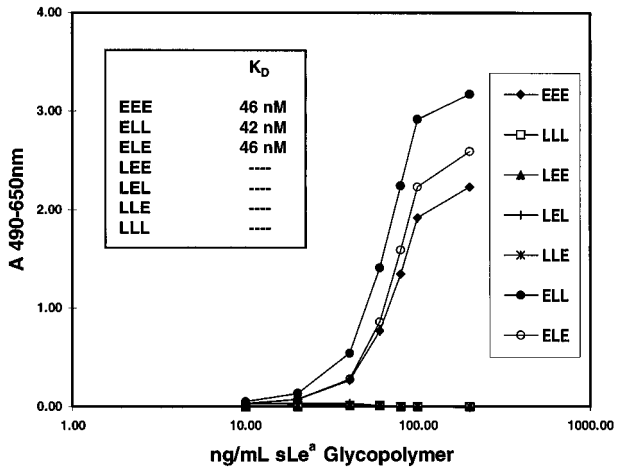

FIGURE 3: Binding of domain-switched chimeras to the sLe[a] glycopolymer in solution. Assays of the purified chimeras were performed as indicated in the Experimental Procedures. The K_D values indicated are per sLe[a] monomer and were calculated using an average molecular mass of 35 kDa for the polymer and a 20 mol % content of sLe[a].

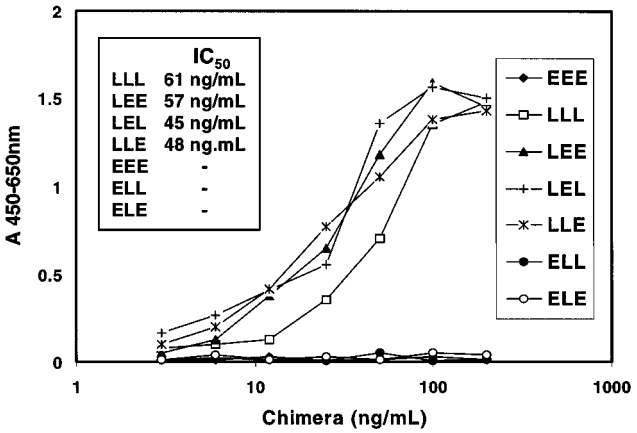

FIGURE 4: Binding of domain-switched chimeras to immobilized sulfatide. The indicated amounts of chimera were incubated in sulfatide-coated plates, and binding was quantitated with an anti-human IgG/peroxidase conjugate.

able carbohydrate ligands, one which binds only to E-CRD and one which binds only to L-CRD. We chose sLe[a] as a specific E-CRD-binding carbohydrate. sLe[a] binds to E-CRD in a manner competitive with that of sLe[x] (Berg et al., 1991) but with higher affinity, thus providing a better signal in our assay. The L-CRD also binds to sLe[a] (Dr. J. Magnani, personal communication), but the affinity is more than 20-fold lower and our assay clearly distinguishes between the two selectin CRDs, as shown in Figure 3. As an L-CRD specific probe, we used the glycolipid sulfatide. Although sulfatide is not a physiologically relevant ligand for L-selectin, it has been previously shown to bind to the L-CRD (Rosen et al., 1991) and to be inhibited both by calcium chelators and by a L-selectin-blocking antibody. In this manner, it represents a good measure of L-CRD function.

For quantification of E-CRD binding, we used a sLe[a] polymer which is additionally labeled with biotin. This high-molecular mass, soluble, polyacrylamide polymer with covalently bound sLe[a] (an average of 20 sLe[a] units per molecule) binds to the E-CRD with much higher avidity than monomeric sLe[a], thus allowing an ELISA format assay. Constant amounts of domain-switched selectin chimeras, mixed with avidin/peroxidase, were incubated together with various amounts of polymer. The incubation mixture was then put in microtiter wells precoated with a standard amount of anti-human IgG antibody and, after washing, developed with a peroxidase substrate. In this manner, the signal obtained from the peroxidase reaction is directly proportional to the amount of polymer bound to the selectin chimera. As shown in Figure 3, this assay gives typical saturation type curves describing the binding of the selectin chimera to sLe[a] polymer in free solution. The calculated K_D values are independent of the absolute amount of selectin chimera used. Indeed, assays performed with double or half the amount of chimera indicated in the Experimental Procedures gave the same inflection points, but with different absolute absorbance values.

No binding of sLe[a] polymer was observed in the presence of 5 mM EDTA or when the E-selectin-blocking antibody BBA2 was included in the assay mix. Furthermore, sLe[x]-Lemieux is a competitive inhibitor in this assay, exhibiting a K_D of 0.7 mM, thus confirming that sLe[a] and sLe[x] bind at overlapping sites on the E-CRD.

Chimeras possessing an E-CRD bound sLe[a] glycopolymer with almost identical affinities. Analysis of the data in Figure 3 indicated K_D values on a per sLe[a] basis of 46 nM for EEE, 42 nM for ELE, and 46 nM for ELL, suggesting that the CRD is totally unaffected by the presence or absence of other domains from the same selectin. On the other hand, chimeras possessing the L-CRD domain do not bind at all to sLe[a] in the range of concentrations tested irrespective of which other E-selectin domains they contain.

Analogous results were obtained from the sulfatide binding assay (Figure 4), which gives a quantitative measure of the affinity of the domain-switched chimeras to immobilized sulfatide. In this case, only chimeras possessing an L-CRD are able to bind to sulfatide. Again, all chimeras display a very close range of affinities, with an IC_{50} of 53 ± 15 ng of chimera/mL.

To further understand the function of the various domains in determining ligand specificity, we tested the ability of the chimeras to support static cell binding. In this case, the ligand presented on the cell surface is more complex than the defined carbohydrates used so far and would presumably uncover any protein−protein interactions important for binding of the selectin chimera. Initial experiments were attempted using human PMN in the static cell assay. While PMNs bind well to E-selectin-coated plates, they also bind to L-selectin-coated plates, giving a signal marginally lower than that produced by E-selectin. This result was unexpected for us since no L-selectin ligands capable of supporting static binding have been so far described on PMN, although L-selectin plays a role in PMN aggregation and rolling (Rochon et al., 1994; Bargatze et al., 1994b). From various attempts to reduce static binding of PMN to L-selectin, including addition of higher amounts of human IgG in the incubation mixture and also addition of azide during PMN purification and assay, the most successful was formaldehyde fixing which decreased binding to L-selectin to about 30% of the signal obtained with E-selectin. Fixing, however, increased the variability of the assay dramatically and after several assays of the various chimeras was abandoned. Data obtained with formaldehyde-fixed PMN (not shown) were analogous to those in Figure 5.

Chimeras were tested for supporting the binding of U937 and Colo 205 cell lines, both of which are well-known for

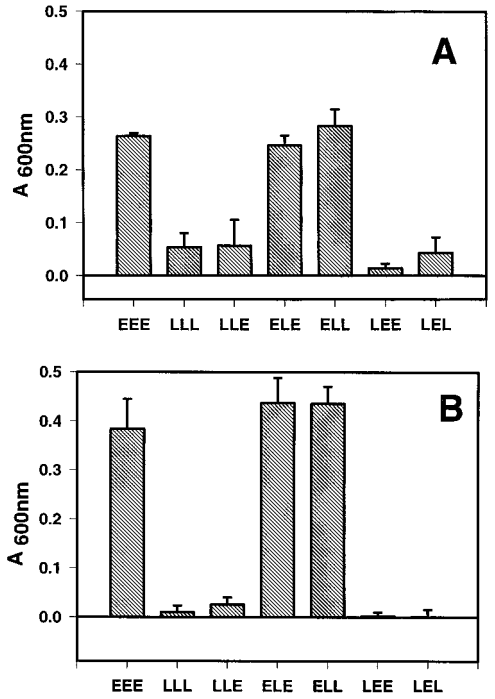

FIGURE 5: Static binding of (A) U937 cells and (B) Colo 205 cells to domain-switched chimeras. Assays were performed as indicated in the Experimental Procedures. All assays were performed in triplicate.

binding to E-selectin. U937 binding is sLe^x-mediated, and Colo 205 binding is sLe^a-mediated (Takada et al., 1993). Both cell lines (Figure 5) bind well to E-selectin but not to L-selectin. U937 cells show a small amount of binding to L-selectin, reminiscent of PMN, but it is clearly much lower than binding to E-selectin. It is interesting to note that U937 binding to L-selectin is not unspecific, as it is blocked by addition of SK11, an L-selectin-blocking antibody. The results in Figure 5 are similar to those obtained with the sLe^a glycopolymer binding assay; the E-CRD is necessary and sufficient for recognition and binding to both cell lines tested.

When examined under physiological flow conditions, the chimeras gave a picture analogous to that obtained in the other assays; only chimeras containing an E-CRD are able to support rolling of HL-60 cells. Remarkably, the number of rolling cells (Figure 6A) is the same with all E-CRD-containing chimeras (EEE, ELE, or ELL), irrespective of the identity of the non-lectin domains. A significant difference was, however, observed in the rolling index and rolling velocities of cells. These two parameters are related, since the rolling index representing the area covered by rolling cells during the observation period is directly proportional to the speed of the cells. According to these two measures (Figure 6B,C), cells roll faster on chimeras with replaced EGF domains or CRs. It is additionally evident from Figure 6 that chimeras possessing an L-CRD are completely unable to support rolling irrespective of which other E-selectin domains they may carry.

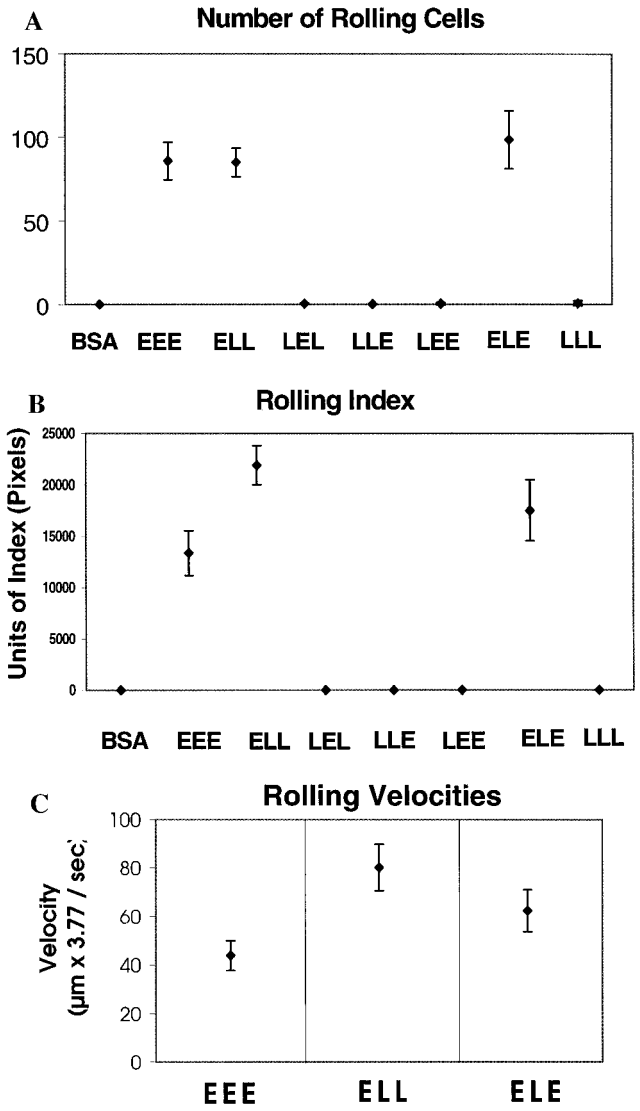

FIGURE 6: Rolling of HL-60 cells on coated domain-switched chimeras was measured as described in the Experimental Procedures.

DISCUSSION

E- and L-selectins are the most divergent of the three selectins, in terms of carbohydrate specificity and counter-ligand recognition. We examined how the various selectin domains contribute to ligand specificity by constructing chimeras with domains switched between the two molecules. The domain-switched chimeras were expressed as soluble human IgG fusion proteins so we would be able to measure their binding both in solution and immobilized and also avoid any artifacts introduced by either low or high levels of expression on cell surfaces. The availability of soluble domain-switched chimeras allowed us to directly measure the binding to defined carbohydrates.

The results we obtained in our carbohydrate binding assays for all domain-switched chimeras show that selectins are true modular proteins and their avidity to specific carbohydrates remains unchanged after exchange of the EGF-like domain and/or CRs. It has been previously reported that deletion of the E-selectin EGF-like domain leads to inactivity (Piggot et al., 1991). In view of our data, it seems that the EGF-like domain plays only a structural role without any direct binding to carbohydrate. This agrees well with the three-dimensional structure of E-selectin, which shows that the EGF-like domain and proposed carbohydrate binding site within the CRD are on opposite sites (Graves et al., 1994). Of course, the lack of influence on carbohydrate binding does not exclude the possibility that the non-lectin domains may directly bind to an extended "natural" ligand and by that

influence specificity.

Therefore, we also tested the binding of the domain-switched chimeras to cell lines known to bind E-selectin. These molecule/cell assays indicate that, in the case of E-selectin, all ligand specificity resides in the CRD of this molecule. All chimeras with an L-CRD were unable to bind E-selectin binding cells, even when the L-CRD was attached to the complete EGF-like domain and CRs of E-selectin (LEE). Thus, the E-selectin EGF-like domain and CRs are incapable of CRD-independent cell binding. In addition to underscoring the importance of lectin−carbohydrate recognition for the initiation and sustenance of binding, these findings also show that the L-CRD is not able to substitute for the E-CRD in recognition of cellular ligands.

Using a similar domain-switch approach between L- and P-selectins, Kansas et al. (1994) showed that a chimera with the P-CRD substituted into L-selectin (PLL) was able to support P-selectin-like binding in static cell assays and that chimeras with the P-selectin EGF-like domain and CRs grafted into L-selectin (LPP and LPL) also supported binding. The same authors also find that a chimera containing only the L-CRD (LPP) supports L-selectin ligand-dependent cell binding to high endothelial venules. Taken together with our findings, these results imply that in the case of P-selectin both lectin−carbohydrate and protein−protein interactions operate in ligand recognition, but in the case of E- and L-selectin, the lectin−carbohydrate interaction is sufficient for recognition and binding. This may explain why for P-selectin there is a single glycoprotein implicated as the natural ligand (PSGL-1) while for E- and L-selectin several glycoproteins and even glycolipids have been shown to be ligands in various experimental systems.

Since rolling and not static binding is the hallmark of selectin function, the results obtained with the flow assay are most physiologically significant. Under physiological shear stress, only chimeras with E-CRDs support rolling, suggesting that lectin−carbohydrate interactions are not only required for initial recognition between selectin and rolling cell but also sufficient to maintain rolling behavior. Interestingly, even though the EGF-like domain and CRs of E-selectin are not required for rolling, they influence the rolling index and rolling velocities of cells. The rolling velocity is greatest when both domains of E-selectin are absent (ELL). When only the E-selectin EGF-like domain is absent (ELE), cells roll with a velocity intermediate between the velocities of EEE and ELL. It is not possible from our data to determine whether the ability of the E-selectin EGF-like domain and CRs to decrease the cell-rolling velocity depends on interaction with cell surface ligands or simply reflects differences of lectin domain accessibility due to the lower number of CRs present in the ELL chimera (see Figure 1).

In view of the exclusive dependence of E-selectin on its CRD for ligand recognition, an interesting question which arises is what is the size and structure of the carbohydrate epitope recognized by the E-CRD. While sLe^x and sLe^a are the minimum structures recognized by E-selectin, more extended structures on the cell surface, such as sialyl dimeric $Lewis^x$ (Pinola et al., 1994; Bochner et al., 1994) and also tetra-antennary structures (Patel et al., 1994), are thought to be interacting more tightly. From the crystal structure of the E-CRD (Graves et al., 1994), it is not clear how a more extended carbohydrate epitope could interact with the protein surface. The "clustered saccharide patch" model proposed by Varki (1994) in which oligosaccharide chains are presented by a protein in such a way as to create a new three-dimensional epitope is the model most consistent with the available binding data. Such a model would also explain the observations that selectins do not interact with an unlimited number of glycoproteins and do so in a specific manner.

The glycoproteins so far described as E- and L-selectin ligands in many cases also have selectin-unrelated functions and act as selectin ligands only if properly glycosylated. This underscores the importance of glycosyltransferases as key regulating factors for selectin-dependent binding. Two such transferases seem to be key elements for selectin-dependent adhesion. In the case of L-selectin, one of the putative counterligands, CD34, is expressed globally on vascular endothelium (Baumhueter et al., 1994), but L-selectin-bearing lymphocytes bind only to peripheral lymph nodes. The crucial factor which allows lymphocyte capture on peripheral lymph nodes seems to be their capacity for specific carbohydrate sulfation (Hemmerich et al., 1995). Furthermore, while there exists a five-member family of α-1,3-fucosyltransferases, only one of them, fucosyltranferase VII, is responsible for selectin ligand fucosylation as shown recently by gene knock-out experiments in mice (Lowe et al., 1995).

In the work described here, we have used several types of assays in order to evaluate the contributions of the various selectin domains to binding. Although many of the parameters of these assays are not physiologically relevant, they nevertheless provide a measure of factors crucial for physiological selectin function. Thus, while it may be argued that the cell lines used may not present the same ligands as PMN, it remains a fact that the E-CRD can support E-selectin specific rolling. This finding, together with the plethora of suggested E-selectin physiological ligands, leads us to propose that E-selectin, unlike P-selectin, does not require distinct counter-receptors to support rolling. Rather, it probably interacts with many cell membrane structures as long as they present the appropriate carbohydrates. Specific counter-receptors for E-selectin may still exist for functions of E-selectin other than supporting rolling, such as leukocyte activation or, as has been recently indicated, angiogenesis (Nguyen et al., 1993; Koch et al., 1995).

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