Monospecific and Common Glycoprotein Ligands for E- and P-Selectin on Myeloid Cells

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Abstract. E- and P-selectin are inducible cell adhesion molecules on endothelial cells, which function as Ca^{2+}-dependent lectins and mediate the binding of neutrophils and monocytes. We have recently identified a 150-kD glycoprotein ligand for E-selectin on mouse myeloid cells, using a recombinant antibody-like form of mouse E-selectin. Here, we report that this ligand does not bind to an analogous P-selectin fusion protein. Instead, the chimeric P-selectin-IgG protein recognizes a 160-kD glycoprotein on the mouse neutrophil progenitor 32D cl 3, on mature mouse neutrophils and on human HL60 cells. The binding is Ca^{2+}-dependent and requires the presence of sialic acid on the ligand. This P-selectin-ligand is not recognized by E-selectin. Removal of N-linked carbohydrate side chains from the 150-kD and the 160-kD monospecific selectin ligands abolishes the binding of both ligands

to the respective selectin. Treatment of HL60 cells with Peptide:N-glycosidase F inhibited cell binding to P- and E-selectin.

In addition, glycoproteins of 230 and 130 kD were found on mature mouse neutrophils, which bound both to E- and P-selectin in a Ca^{2+}-dependent fashion. The signals detected for these ligands were 15–20-fold weaker than those for the monospecific ligands. Both proteins were heavily sialylated and selectin-binding was blocked by removal of sialic acid, but not by removal of N-linked carbohydrates. Our data reveal that E- and P-selectin recognize two categories of glycoprotein ligands: one type requires N-linked carbohydrates for binding and is monospecific for each of the two selectins and the other type binds independent of N-linked carbohydrates and is common for both endothelial selectins.

T HE selectins form a family of three structurally related cell adhesion molecules which mediate early events in the binding of leukocytes to the endothelial lining of blood vessels (Lasky, 1992; Vestweber, 1992). Two of them, E- and P-selectin, are expressed on endothelial cells where they support the binding of neutrophils, monocytes and subsets of lymphocytes (Bevilacqua et al., 1987, 1989; Geng et al., 1990; Picker et al., 1991a; Shimizu et al., 1991; Moore and Thompson, 1992). The third, L-selectin, is found on almost all types of leukocytes (Siegelman et al., 1989; Lasky et al., 1989; Tedder et al., 1990) and is also involved in the migration of neutrophils into sites of inflammation (Lewinsohn et al., 1987; Watson et al., 1991). In addition, L-selectin mediates lymphocyte homing into lymph nodes (Gallatin et al., 1983; Hamann et al., 1991) during lymphocyte recirculation.

The selectins are the only family of cell adhesion molecules whose members function as Ca^{2+}-dependent lectins (Hynes and Lander, 1992). All three selectins were shown

to bind to the tetrasaccharide sialyl Lewis[x] (sLe)[1] (Phillips et al., 1990; Polley et al., 1991; Foxall et al., 1992). Other carbohydrate compounds like the stereoisomer sialyl Lewis[a] (Berg et al., 1991a; Handa et al., 1991; Tyrrell et al., 1991; Berg et al., 1992) or derivatives of both compounds which carry a SO_3^--group instead of sialic acid (Yuen et al., 1992; Green et al., 1992) bind as well or even better. A derivative of sialyl Lewis[a] conjugated to neoglycoproteins was recently demonstrated to be 36-fold more active in E- and P-selectin binding than underivatized, protein-conjugated sLe[x] (Nelson et al., 1993). However, the detailed carbohydrate structure of the physiological ligands is still unknown. Most likely, the physiological ligands of the selectins are glycoproteins.

The first glycoprotein ligands for which binding to a selectin was directly demonstrated were identified using an antibody-like L-selectin fusion protein as an affinity probe. With this approach, a 50-kD mucin-like, soluble glycoprotein (GlyCAM-1) and a 90-kD membrane glycoprotein (Sgp90 or

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1. *Abbreviations used in this paper:* CB, complement-binding domains; CP, clostridium perfringens; endo F, endoglycosidase F; PNGase F, Peptide:N glycosidase F; (sLe[x]), tetrasaccharide sialyl Lewis[x].

CD34) were isolated from lymph node endothelium (Imai et al., 1991; Lasky et al., 1992; Baumhueter et al., 1993). Both ligands were also recognized by the monoclonal antibody MECA79 which defines the vascular addressin for peripheral lymph nodes (Berg et al., 1991b; Streeter et al., 1988). Based on cell adhesion assays, E- and P-selectin have also been described as counterreceptors for the neutrophil type L-selectin (Kishimoto et al., 1991; Picker et al., 1991b). However, the affinity of this interaction is too low to directly demonstrate the binding of L-selectin to purified P-selectin or an antibody-like E-selectin fusion protein (Moore et al., 1992; Levinovitz et al., 1993).

Instead, using purified P-selectin from human platelets, a glycoprotein ligand of 120 kD molecular weight under reducing conditions and of 250 kD under non-reducing conditions was identified on human neutrophils and the monocytic cell line HL60 (Moore et al., 1992). With the help of an antibody-like E-selectin fusion protein, we have recently isolated a 150-kD glycoprotein ligand for E-selectin from mouse neutrophils and the mouse neutrophilic progenitor 32D cl 3 (Levinovitz et al., 1993). In addition, a minor component of 250 kD molecular weight was detected on mature mouse neutrophils.

From the published data, it is difficult to compare the P- and E-selectin ligands since different cells were analyzed and the affinity probes, the isolation procedures and the labeling techniques differed significantly. Here, we have directly approached the question whether E- and P-selectin recognize different or similar ligands. Using analogous antibody-like fusion proteins for mouse E- and P-selectin, we have performed affinity isolation experiments on detergent extracts of mouse neutrophils, the mouse neutrophilic progenitor 32D cl 3 and the human monocytic cell line HL60. We have found two glycoproteins of which each is a specific ligand for only one of the two endothelial selectins. Two additional, more weakly detectable glycoproteins were found which were common ligands for both selectins.

Materials and Methods

Cells

The neutrophilic progenitor 32D cl 3 (Valtieri et al., 1987; Migliaccio et al., 1989) provided by Dr. Rovera (Wistar Institute, Philadelphia) was grown in DMEM with 20% FCS, supplemented with 10% conditioned medium of WEHI-3B cells (as source for IL-3). The human monocytic cell line HL60 was obtained from Amer. Type Culture Collection (Rockville, MD) and cultured in DMEM containing 10% FCS. J558L cells secreting either the P-selectin-IgG, the E-selectin-IgG, or CD4-IgG fusion protein were grown in DMEM supplemented with 10% BMS (Seromed, Berlin, Germany). Mouse neutrophils (polymorphonuclear granulocytes) were freshly isolated from the femurs of 10 wk old NMRI mice as described (Levinovitz et al., 1993).

For the isolation of human neutrophils, 10 ml of heparinized blood was mixed with 20 ml of sterile HBSS, underlayed with 10 ml of Histopaque 1077 (Sigma Chem. Co., St. Louis, MO) and centrifuged for 30 min at 2,300 rpm. Pelleted cells were washed once in RPMI containing 2% FCS and resuspended in HBSS (original blood volume). 20% (vol/vol) of a 6% Dextran 500 (Pharmacia LKB Biotechnology, Piscataway, NJ) solution in PBS was added and the cell suspension was loaded into a vertical syringe, needle upright. Erythrocytes were allowed to settle for 20 min. Leukocytes were expelled and washed twice in RPMI.

Antibodies

The anti-mouse P-selectin rabbit antiserum was raised against the purified

P-selectin-immunoglobulin chimeric protein as described (Hahne et al., 1993). Specific antibodies were affinity purified on the fusion protein conjugated to CNBr-Sepharose (Pharmacia Uppsala, Sweden). Antibodies against the Fc part of human IgG were removed by a second CNBr-Sepharose column with conjugated human IgG (Sigma Immunochemical, St. Louis, MO).

The rat IgM mAb 21KC10 against mouse E-selectin was recently described (Hahne et al., 1993) as was the rat IgM mAb 28AG6 which had been raised against the E-selectin-IgG chimeric protein and recognizes the Fc-region of human IgG1 (Levinovitz et al., 1993).

Cell Adhesion Assay with Plastic-coated P- and E-selectin-IgG

96 well microtiter plates (Falcon, Heidelberg, Germany) were coated with 20 μg/ml P-selectin IgG, E-selectin-IgG, or CD4-IgG in HBSS (Biochrom, Berlin, Germany) and subsequently blocked with 10% FCS in HBSS. 0.5-1 × 10⁶ cells in 200 μl were added to each well and allowed to bind for 20 min at 7°C under mild rotation (50 rpm). Unbound cells were removed by flicking out the plates and washing five times with HBSS. Bound cells were fixed with 2% glutaraldehyde in HBSS for 1 h at 7°C, followed by one additional wash with HBSS. Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas for each well. Each determination was done for four wells. Antibody inhibition was tested by preincubating the coated and blocked wells with antibodies at the indicated concentrations for 45 min at 37°C. Unbound antibodies were washed away before cells were added.

Enzyme treatments of intact HL60 cells were performed in 1 ml DMEM without FCS at a cell density of 5 × 10⁶/ml for 1 h at 37°C with 1 U endoglycosidase F (endo F) or 2 U Peptide:N glycosidase F (PNGase F) (both from Boehringer, Mannheim, Germany) or 20 μl O-sialoglycoprotease from *Pasteurella hemolytica* (Cedarlane, Hornby, Ontario, Canada, purchased from Camon, Wiesbaden, Germany). The lyophilated enzyme, containing Hepes buffer salts and bovine serum proteins, was reconstituted with water to a final protein concentration of 2.5 mg/ml with a specific activity of 5 mg human glycophorin A cleaved per mg protein per hour at 37°C. For controls, cells were incubated in the same way in the absence of the enzymes. To control whether the amount of PNGase F and endo F were sufficient, enzyme treatment of cells was done in the presence of 20 μg of an L-selectin-IgG fusion protein. This protein was affected by the glycosidases in the presence of the cells as efficiently as in the absence of the cells.

Selectin-Immunoglobulin Chimeric Proteins

The construction as well as the production of the P- and E-selectin-IgG chimeric proteins has been described (Hahne et al., 1993). In both fusion proteins, the selectin part includes the signal sequence, the lectin domain, the EGF-like repeat and the first two "complement-binding (CB)" domains, except for the last two amino acids of the second CB-domains.

Affinity Isolation of the Metabolically Labeled P- and E-Selectin Ligands

HL 60 and 32D cl 3 cells were labeled for 4 h with 400 μCi [³⁵S]methionine and 200 μCi [³⁵S]cysteine in 1 ml medium (5 × 10⁶ cells/ml) in MEM without methionine and cysteine (Gibco/BRL, Karlsruhe, Germany), supplemented with 10% FCS which had been dialyzed against PBS. 4 × 10⁷ freshly isolated PMNs from mouse bone marrow were labeled in 500 μl of the same medium with 600 μCi [³⁵S]methionine and 600 μCi [³⁵S]cysteine for 4 h.

Labeled cells were lysed at a density of 0.5-3 × 10⁷ cells/ml in lysis buffer (3% CHAPS; 50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 1 mM CaCl₂, 1 mg/ml Ovalbumin, 1 mM PMSF) for 10 min, insoluble material was pelleted at 10,000 g for 10 min; these and all subsequent steps were performed at 4°C. The lysate was preincubated for 30 min with 50 μl of packed protein A-Sepharose. After removal of the Sepharose beads, carrying unspecifically bound proteins, the cell extract was aliquoted and fractions routinely corresponding to 10⁶-10⁷ cells were incubated with 10-20 μl of protein A-Sepharose preloaded overnight with 10-20 μg of P-selectin-IgG, E-selectin-IgG, or CD4-IgG. After an incubation period of 4 h, the resin was washed five times with washing buffer (0.05% Triton X-100; 50 mM Tris-HCl pH 8.5; 400 mM NaCl; 1 mM CaCl₂) and two more times with a similar washing buffer, lacking CaCl₂ and containing 150 mM instead of 400 mM NaCl. Elution was done twice with 60 μl 3 mM EDTA in 50 mM sodium acetate pH 5.2; 0.05% Triton X-100. Eluted proteins were sepa-

rated by SDS-PAGE and detected either by fluorography using Kodak X-AR X-ray films (Kodak, Stuttgart, Germany) or by analysis in a Fujix BAS 1000 Bio-Imaging Analyser (Fuji, Japan).

In cross-precipitation experiments the cell extract of metabolically labeled mouse neutrophils (see above) was incubated with the P-selectin-IgG affinity matrix, the EDTA eluate was added to 500 μl 50 mM Tris pH 7.4, 150 mM NaCl, 4 mM CaCl₂, 1% Triton X-100 and equal aliquots were again incubated with P-selectin-IgG or E-selectin-IgG affinity matrices. Bound proteins were again eluted with EDTA and analyzed as described above.

Sialidase, Endoglycosidase F, and PNGase F Treatment of Selectin Ligands

All enzymes were purchased from Boehringer (Mannheim, Germany). For sialidase treatment, the P-selectin ligands were eluted from the affinity matrix in 3 mM EDTA; 50 mM sodium acetate pH 5.2; 0.05% Triton X-100. 50 μl EDTA-eluate corresponding to 1.5 × 10⁷ [³⁵S]methionine [³⁵S]cysteine labeled neutrophils or to 2 × 10⁶ HL60 cells were digested either with 500 mU of neuraminidase from Arthrobacter ureafaciens, or 20 mU neuraminidase from New Castle disease virus or 100 mU neuraminidase from Clostridium perfringens overnight at 37°C. Mock treatment was done under identical conditions in the absence of the enzyme. 40% of the digest was set aside for PAGE while 60% were subjected to reprecipitation by overnight incubation with P-selectin-IgG protein A-Sepharose after adding 500 μl of 1% Triton X-100; 50 mM Tris-HCl pH 7.4; 150 mM NaCl; 4 mM CaCl₂. Subsequent washing and elution was done as described above. For digestion with endo F and with PNGase F, the selectin-ligands were affinity isolated from labeled HL60 cells, 32D cl 3 cells or neutrophils as described above using E- or P-selectin-IgG and eluted with EDTA. 50 μl EDTA-eluate corresponding to 2 × 10⁶ HL60 or 32D cl 3 cells or 1.5 × 10⁷ neutrophils was supplemented with 0.05% Triton X-100 and digested overnight at 37°C without (control) or with 0.5 U (10 μl) of endo F or with 2 U (10 μl) of PNGase F. Reprecipitation was done as described above.

O-sialoglycoprotease Treatment of Selectin Ligands

Ligands were affinity-isolated from [³⁵S]methionine [³⁵S]cysteine labeled HL60 cells, 32D cl 3 cells or mature mouse neutrophils with E-selectin-IgG or P-selectin-IgG and eluted with 60 μl 3 mM EDTA, 50 mM ammonium acetate pH 7.0. The eluate was substituted with 60 μl 100 mM Hepes pH 7.4 split into halves and one half was incubated with 1 μl O-sialoglycoprotease (1 μl cleaved 12.5 μg glycophorin A, per h at 37°C) and the other half without the enzyme for 1 h at 37°C. Proteins were analyzed by PAGE and fluorography or phosphorimaging.

Results

E-Selectin-IgG and P-Selectin IgG Specifically Support the Binding of Myeloid Cells

We have recently identified a 150-kD glycoprotein ligand for E-selectin on mouse myeloid cells using a mouse E-selectin-IgG fusion protein (Levinovitz et al., 1993) as affinity probe. We have now used an analogous mouse P-selectin-IgG fusion protein to identify P-selectin ligands and to directly compare the ligands of both selectins. To test whether the P-selectin-IgG fusion protein functions in a selectin-specific way, we analyzed whether it supports the binding of myeloid cells when coated to plastic. The myeloid cells used in this assay were the mouse neutrophilic progenitor 32D cl 3, the human monocytic cell line HL60 and mature mouse neutrophils. All three cell types bound to P-selectin-IgG as well as to E-selectin-IgG while no binding was observed to CD4-IgG (Fig. 1). Binding was dependent on the presence of Ca²⁺ since it was completely blocked by EDTA (Fig. 1). Affinity purified, polyclonal antibodies to P-selectin blocked cell binding to P-selectin-IgG but not to E-selectin-IgG. Similarly, the mAb 21KC10 against mouse E-selectin only blocked cell binding to E-selectin-IgG but not to P-selectin-IgG. A control IgM

mAb (28AG6) against the Fc-part of both fusion proteins did not block cell binding. We conclude that similar to the E-selectin-IgG chimeric protein, the P-selectin-IgG fusion protein also binds via its selectin part in a Ca²⁺-dependent way to myeloid cells. Binding occurs across the species barrier between man and mouse.

E-Selectin-IgG and P-Selectin-IgG Bind to Two Different Glycoprotein Ligands on Myeloid Cells
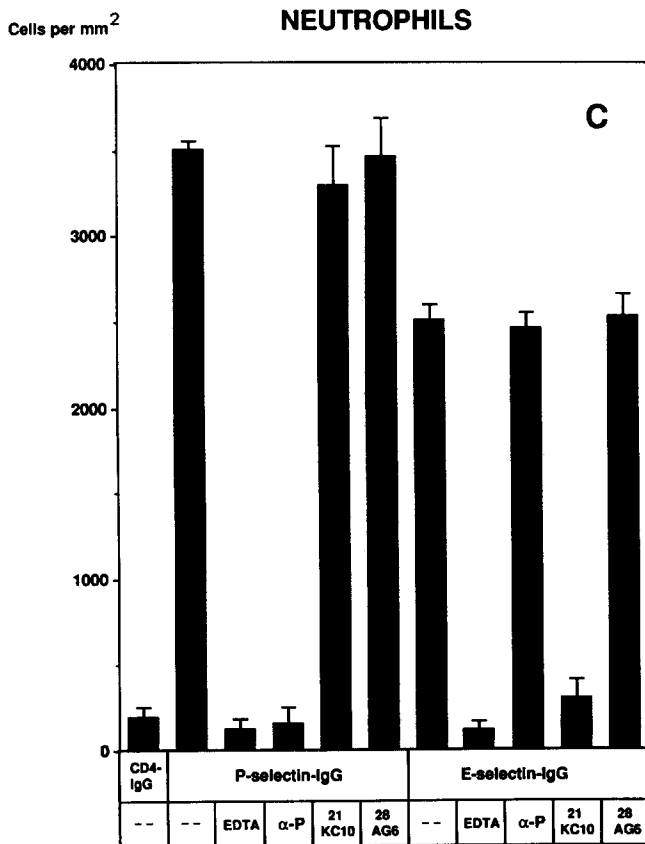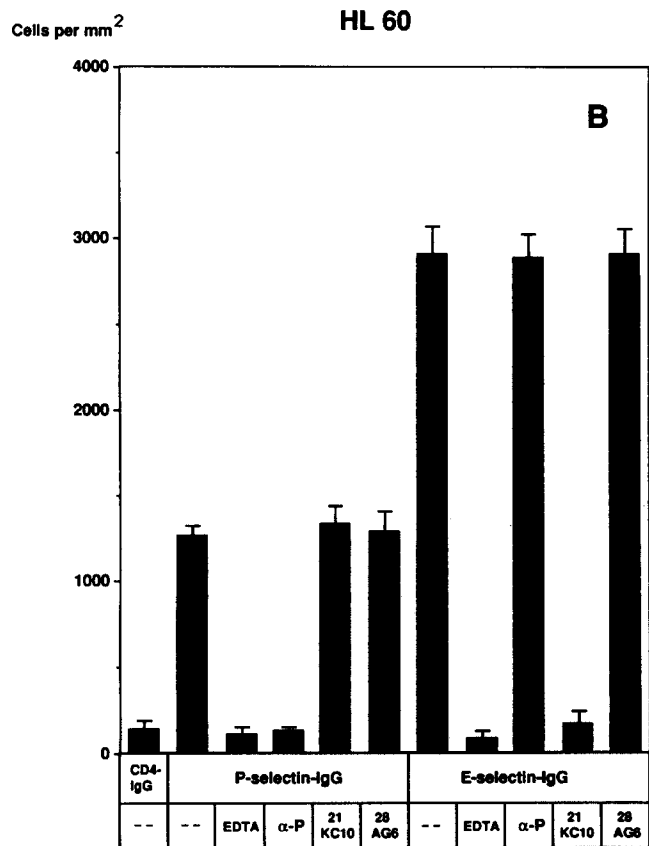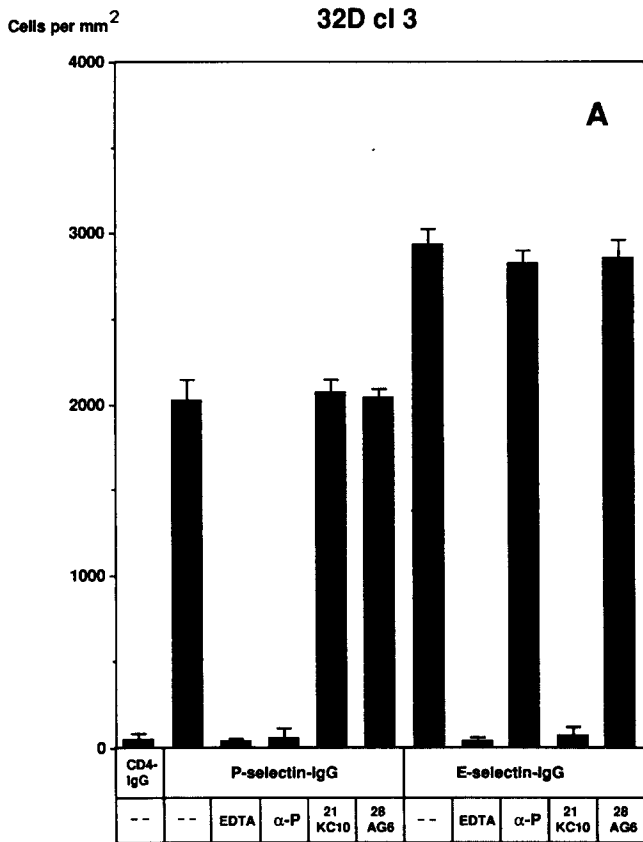
The selectin fusion proteins were bound to protein-A Sepharose and used as affinity matrix to identify and isolate ligands from detergent extracts of the mouse neutrophilic progenitor 32D cl 3. Cells were metabolically labeled with [³⁵S]methionine [³⁵S]cysteine and aliquots of a detergent extract of these cells were incubated with the affinity-matrices. Specifically bound proteins were eluted with EDTA. As expected, the 150-kD E-selectin ligand was eluted from the E-selectin-IgG matrix (Fig. 2 A). This protein, however, did not bind to P-selectin-IgG. Instead, a single protein, migrating at an apparent molecular weight of 160 kD under non-reducing conditions was eluted from the P-selectin-IgG matrix (Fig. 2 A). Neither the E-selectin ligand nor the 160-kD P-selectin ligand bound to CD4-IgG.

Affinity isolation experiments on cell lysates of [³⁵S]methionine/[³⁵S]cysteine labeled human HL60 cells gave similar results. The two isolated glycoproteins had the same molecular weight as the mouse ligands and each of them was only bound by one of the two selectin fusion proteins (Fig. 2 B). Thus, the two endothelial selectins each recognize a different glycoprotein ligand on myeloid cells. These ligands were recognized on mouse as well as on human cells.
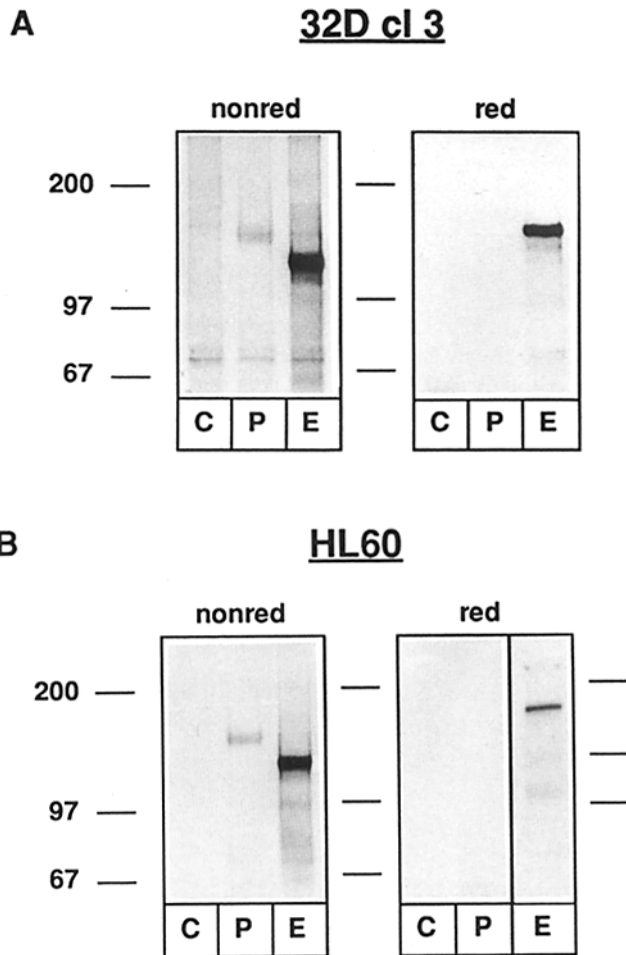
In contrast to the E-selectin ligand which migrates at 130 kD apparent molecular weight under non-reducing conditions and at 150 kD under reducing conditions the P-selectin ligand could only be detected in its non-reduced form. When the EDTA-eluted 160-kD protein isolated from either mouse or human cell lines was boiled in PAGE-loading buffer in the presence of DTT, the protein was no longer detectable on a 10% polyacrylamide gel. Instead, a signal at the top of the stacking gel was found, indicating that reduction of the protein caused the formation of insoluble aggregates (not shown). However, when the non-reduced 160-kD proteins, isolated from both cell lines, were excised from a polyacrylamide gel after the first electrophoresis and reelectrophoresed on a second gel under reducing conditions, both proteins migrated at an apparent molecular weight of 80 kD (Fig. 3). This indicates, that the 160-kD P-selectin ligand may be a disulfide-linked dimer.

The 160-kD P-Selectin Ligand Requires Both Sialic Acid and N-linked Carbohydrates for the Binding to P-Selectin

Since selectins bind to carbohydrate structures, we tested whether the 160-kD ligand is glycosylated and whether carbohydrates would be involved in the binding to P-selectin. The 160-kD protein was isolated from [³⁵S]methionine/[³⁵S]cysteine labeled HL60 cells as described above and treated with three different sialidases at 37°C overnight. Two of them, the neuraminidases from Arthrobacter ureafaciens and from New Castle disease virus, did not affect the elec-

Cells per mm^2

32D cl 3

A

4000

3000

2000

1000

0

CD4-IgG	P-selectin-IgG					E-selectin-IgG				
--	--	EDTA	α-P	21 KC10	28 AG6	--	EDTA	α-P	21 KC10	28 AG6

Cells per mm^2

HL 60

B

4000

3000

2000

1000

0

CD4-IgG	P-selectin-IgG					E-selectin-IgG				
--	--	EDTA	α-P	21 KC10	28 AG6	--	EDTA	α-P	21 KC10	28 AG6

Cells per mm^2

NEUTROPHILS

C

4000

3000

2000

1000

0

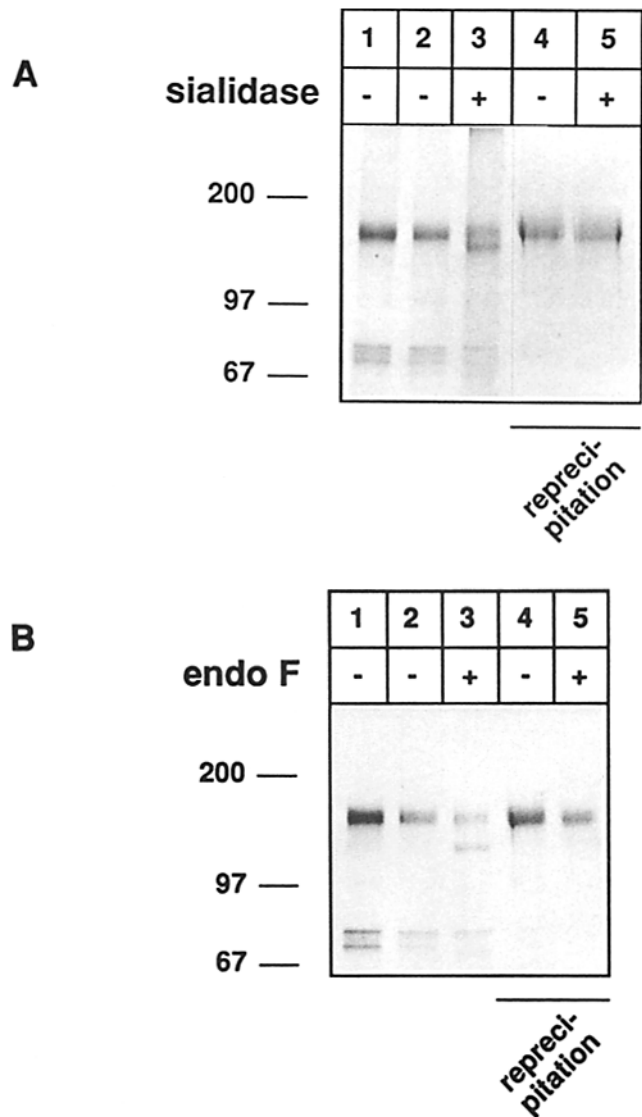| CD4-IgG	P-selectin-IgG					E-selectin-IgG				
--	--	EDTA	α-P	21 KC10	28 AG6	--	EDTA	α-P	21 KC10	28 AG6

Figure 1. Specific binding of myeloid cells to plastic-coated P- and E-selectin-IgG. Cell adhesion assays were performed with 32D cl 3 cells (*A*), HL60 cells (*B*), and mature mouse neutrophils (*C*) in 96 well microtiter plates coated with CD4-IgG, P-selectin-IgG, or E-selectin-IgG (as indicated). Before the addition of cells, the selectin-IgG coated wells were incubated with HBSS (--), HBSS containing 1 mM EDTA (EDTA) or with 2 μg/ml affinity purified rabbit antibodies against P-selectin (α-P), 100 μg/ml mAb 21KC10 against mouse E-selectin (21KC10) or 100 μg/ml control mAb 28AG6 against Fc-part of human IgG-1 (28AG6). Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas of defined size (per well) in four different wells for each determination. The depicted experiment represents one of three similar experiments.

A **32D cl 3**

B **HL60**

trophoretic mobility of the ligand (not shown). However, the neuraminidase from Clostridium perfringens (CP) clearly reduced the apparent molecular weight of the ligand (Fig. 4 A, lane 3), although also in this case ~50% of the ligand molecules were unaffected. If aliquots of the mock and the

A sialidase

B endo F

Figure 2. Affinity isolation of two different glycoprotein ligands of which each is specific for either P- or E-selectin. 32D cl 3 cells (*A*) and HL60 cells (*B*) were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine and detergent extracts were incubated either with immobilized CD4-IgG (*C*), P-selectin-IgG (*P*), or with E-selectin-IgG (*E*). Specifically bound proteins were eluted with EDTA and electrophoresed on a 6% polyacrylamide gel under nonreducing (*non-red*) or reducing (*red*) conditions, except for panel *B*, last lane, where the proteins were separated on an 8% gel. Molecular mass markers (in kD) are indicated.
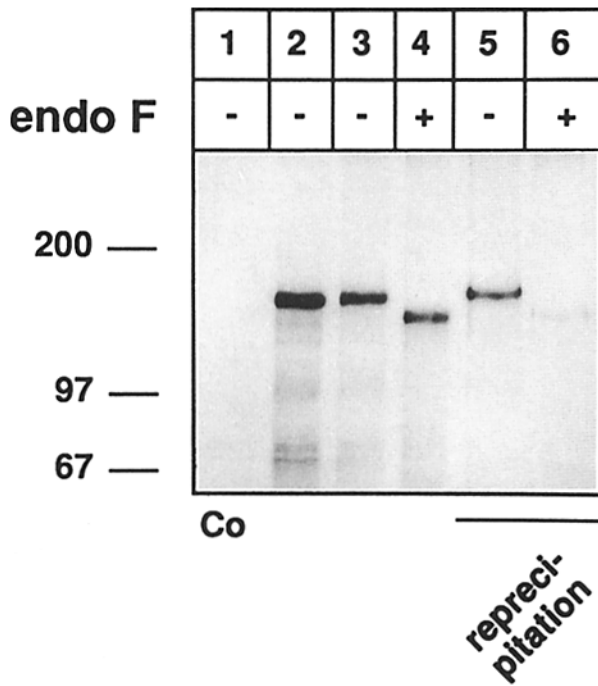
Figure 3. The 160-kD P-selectin ligand is cleaved to an 80-kD monomer by reduction. The 160-kD P-selectin ligand was affinity-isolated from [^{35}S]methionine/[^{35}S]cysteine labeled 32D cl 3 cells or HL60 cells, separated on a polyacrylamide gel under nonreducing conditions, excised from the gel and reelectrophoresed under reducing conditions on a second 8% polyacrylamide gel giving rise to proteins migrating at 80 kD apparent molecular weight. Labeled proteins were visualized by phosphorimaging. Molecular mass markers (in kD) are indicated on the left.

Figure 4. The 160-kD P-selectin ligand requires both sialic acid as well as N-linked carbohydrates for binding to P-selectin. The 160-kD P-selectin ligand was affinity-isolated from [^{35}S]methionine/[^{35}S]cysteine labeled HL60 cells with immobilized P-selectin-IgG. (*A*) The isolated ligand was either directly electrophoresed (lane *1*) or treated with (lanes *3* and *5*) or without (lanes *2* and *4*) 100 mU of sialidase from Clostridium perfringens overnight at 37°C. 40% of the treated samples were directly electrophoresed (lanes *2* and *3*) and 60% reprecipitated with P-selectin-IgG (lanes *4* and *5*). (*B*) Similar as panel *A*, except that endo F instead of sialidase was used. The ligand molecules which were affected by the two enzymes in their electrophoretic mobility (lane *3* in both panels) were in each case not reprecipitated by P-selectin-IgG (lane *5* in both panels). Proteins were electrophoresed on 6% polyacrylamide gels under non-reducing conditions and labeled proteins were visualized by fluorography. Molecular mass markers (in kD) are indicated on the left.

CP-neuraminidase-treated ligand were reprecipitated with P-selectin-IgG, only the unaffected molecules bound the fusion protein while binding of those molecules which were shifted in molecular weight was completely abolished. Thus, sialic acid on the 160-kD ligand is required for binding to P-selectin.
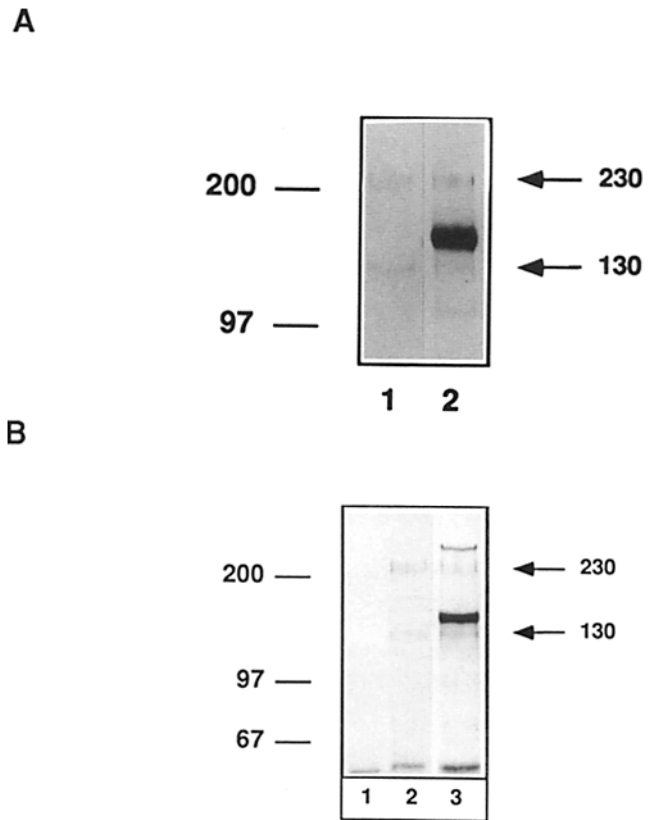
We tested whether N-linked carbohydrates on the ligand would be relevant for the binding process. Treatment of the isolated, [³⁵S]methionine/[³⁵S]cysteine labeled ligand with endo F (Fig. 4 *B*) and also with PNGase F (not shown) caused a similar reduction of the apparent molecular weight of the ligand (Fig. 4 *B*, lane *3*) although again not all molecules were susceptible to the enzymes. Reprecipitation of ligand molecules from which N-linked carbohydrates had been removed, either with endo F (Fig. 4 *B*) or with PNGase F (not shown), was completely abolished in both cases, while the mock-treated material and also undigested ligand molecules, which had been left intact by endo F, were still reprecipitated normally (Fig. 4 *B*, lane *5*). Thus, oligosaccharides on N-linked carbohydrate side chains of the 160-kD glycoprotein are essential for the binding to P-selectin.

The 150-kD E-Selectin Ligand Also Requires N-linked Carbohydrates for the Binding to E-selectin

We have recently shown that removal of sialic acid from the 150-kD E-selectin-ligand strongly reduces (by 80%) its binding to the E-selectin-IgG fusion protein (Levinovitz et al., 1993). The results with the 160-kD P-selectin-ligand prompted us to test whether the E-selectin ligand would also require N-linked carbohydrates for binding. The 150-kD E-selectin ligand was affinity isolated with E-selectin-IgG from [³⁵S]-methionine/[³⁵S]cysteine labeled 32D cl 3 cells and treated with endo F. This treatment reduced the apparent molecular weight of the isolated ligand (Fig. 5, lane *4*). The shifted form of the ligand could only be reprecipitated with E-selectin-IgG with an efficiency of 20% as compared to the mock-treated sample (Fig. 5, lane *6*). Thus, N-linked carbohy-

Figure 5. The 150-kD E-selectin ligand requires N-linked carbohydrates for binding to E-selectin. 32D cl 3 cells were metabolically labeled with [³⁵S]methionine/[³⁵S]cysteine and detergent extracts were incubated either with immobilized CD4-IgG (*Co*) or E-selectin-IgG (all other lanes). The isolated 150-kD E-selectin-ligand was directly electrophoresed (lane *2*) or treated with (lanes *4* and *6*) or without (lanes *3* and *5*) 1 U endo F overnight at 37°C. Half of the treated samples were directly electrophoresed (lanes *3* and *4*) or reprecipitated with E-selectin-IgG (lanes *5* and *6*). Electrophoresis was performed under reducing conditions on a 6% polyacrylamide gel and labeled proteins were visualized by fluorography. Scanning of the depicted x-ray film revealed that the endo F treatment decreased the signal of the reprecipitated 150-kD ligand (lane *6*) to 20% when compared with the signal of the mock-treated, reprecipitated ligand (lane *5*). Molecular mass markers (in kD) are indicated on the left.

Figure 6. Affinity isolation of monospecific and of common glycoprotein ligands for P- and E-selectin from mouse neutrophils. Mouse neutrophils were metabolically labeled with [³⁵S]methionine/[³⁵S]cysteine and detergent extracts were incubated either with P-selectin-IgG (*A*, lanes *1* and *2*; *B*, lane *2*), E-selectin-IgG (*B*, lane *3*), or CD4-IgG (*B*, lane *1*). Specifically bound proteins were eluted with EDTA and electrophoresed on a 6% polyacrylamide gel under nonreducing (*A*, lane *2*) or reducing (*A*, lane *1*; *B*, lanes *1–3*) conditions. In addition to the monospecific 160-kD P-selectin ligand (non-reduced) and 150-kD E-selectin ligand (reduced), two common ligands of 230 kD and 130 kD apparent molecular weight were identified (marked by arrows on the right) which bind to both selectins. Scanning of the depicted x-ray film revealed that the signals of the two common ligands were 15–20-fold weaker than that of the 150-kD E-selectin ligand and 10–15-fold weaker than the 160-kD P-selectin ligand. Molecular mass markers (in kD) are indicated on the left.

drates on the 150-kD ligand are involved in the binding to E-selectin.

## Two Additional Weakly Detectable Ligands on Mature Mouse Neutrophils Bind to Both Endothelial Selectins

Like on the two cell lines described above, we detected the 160-kD P-selectin ligand in affinity isolation experiments with P-selectin-IgG on detergent extracts of [³⁵S]methionine/[³⁵S]cysteine labeled mature mouse neutrophils (Fig. 6 A, lane 2). The neutrophil-derived ligand, like the 160-kD ligands from the two cell lines, was undetectable if the eluted protein was reduced in solution before electrophoresis (Fig. 6 A, lane 1). Again, this protein migrated with an apparent molecular weight of 80 kD if the 160-kD non-reduced form of the protein was excised from a gel, and then reduced before a second electrophoresis (data not shown).

In addition to this protein we found two more ligands which migrated at apparent molecular weights of 230 and 130 kD under reducing as well as under non-reducing conditions (Fig. 6 A, lanes 1 and 2). The signals for these proteins were 10–15-fold weaker than the signal for the 160-kD ligand. Proteins of identical molecular weight were detected in affinity isolation experiments with E-selectin-IgG (Fig. 6 B, lane 3) in addition to the 150-kD E-selectin ligand and the 250-kD ligand, of which the latter was exclusively found on mature mouse neutrophils (Levinovitz et al., 1993). The signals for the 230- and 130-kD proteins were 15–20-fold weaker than the signal for the 150-kD ligand. These two additional proteins had not been detected before with E-selectin-IgG and were discovered now due to improving the label-

ing conditions of neutrophils and due to exchanging the detergent Triton X-100 by CHAPS (see Materials and Methods section). On human neutrophils, isolated from peripheral blood, E- and P-selectin-ligands of similar molecular weight were identified as on mouse neutrophils (data not shown).

To test directly whether the 230/130-kD ligand pairs recognized by each selectin are indeed identical, we affinity-isolated the proteins with P-selectin-IgG, and reincubated equal aliquots (after compensating the EDTA with Ca²⁺) with P-selectin-IgG or E-selectin-IgG, respectively. As shown in Fig. 7, the affinity-purified 230/130-kD ligand pair was equally well recognized by both selectin fusion proteins (lanes 3 and 4), demonstrating that this pair of proteins represents common ligands for both endothelial selectins.

In different affinity isolation experiments, using P-selectin-IgG as affinity probe, the ratio of the 230- and 130-kD common selectin ligands varied slightly. In most cases, the 130-kD protein was more easily detectable under reducing than under non-reducing conditions, although it was always detectable under both conditions. Because of such variations, we tested whether reduction of the 230-kD protein could give rise to the 130-kD band. The 230-kD protein was electrophoresed under non-reducing conditions, cut from the gel and reelectrophoresed under reducing conditions. Under such conditions, reduction did not influence the migration of the 230-kD protein and did not give rise to a protein of 130 kD apparent molecular weight (data not shown). However, it is possible that this reduction procedure at room temperature was not sufficient to fully reduce the protein.
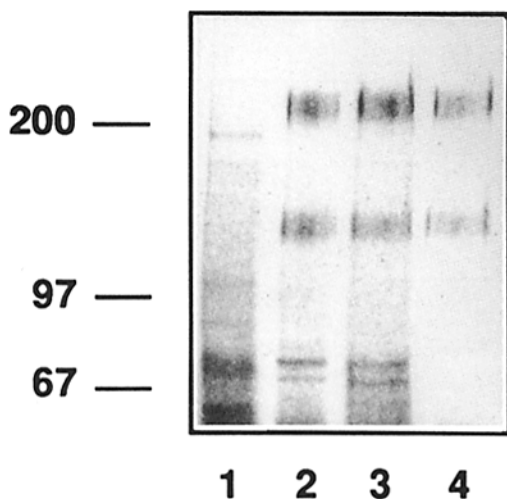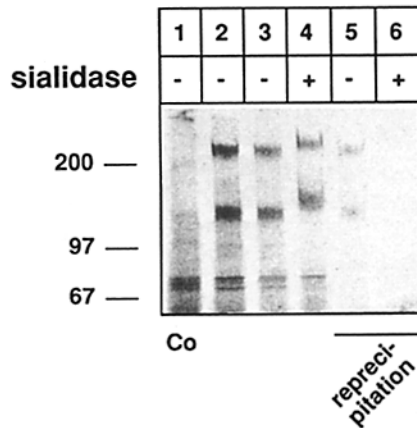

Figure 7. The 230/130-kD ligands recognized by P-selectin are identical with those recognized by E-selectin. Mouse neutrophils were metabolically labeled with [³⁵S]methionine/[³⁵S]cysteine and 20% of the detergent extract was incubated with CD4-IgG (lane 1) and 80% with P-selectin-IgG. Proteins bound to P-selectin-IgG were eluted by EDTA and aliquots were either directly electrophoresed (lane 2) or substituted with Ca²⁺ and reprecipitated with P-selectin-IgG (lane 3) or E-selectin-IgG (lane 4). The aliquots loaded per lane corresponded to 20% (lanes 1 and 2) or 30% (lanes 3 and 4) of the total cell extract. Molecular mass markers (in kD) are indicated on the left.

The 230-kD and the 130-kD Ligands Are Heavily Sialylated and Require Sialic Acid, But No N-linked Carbohydrates for Selectin-Binding
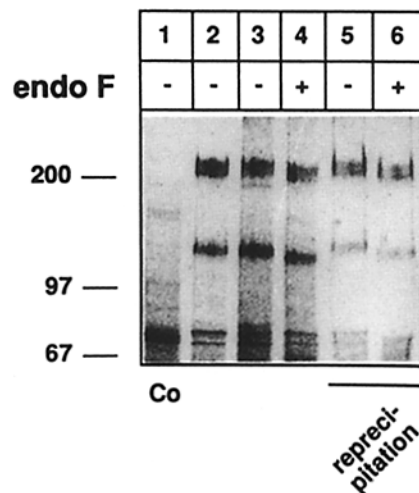
To test whether the two ligands, common to both selectins, contained sialic acid, the metabolically labeled proteins were purified with P-selectin-IgG as described and the EDTA-eluted material was treated overnight at 37°C with sialidase from Arthrobacter ureafaciens. As shown in Fig. 8, this treatment caused a similar increase of the apparent molecular weight of the 230- and the 130-kD ligands while the mock-treatment had no effect. When aliquots of the sialidase and the mock-treated samples were subjected to reprecipitations with P-selectin-IgG affinity beads, binding of the sialidase-treated 230-kD and the 130-kD ligands was completely abolished (Fig. 8 A, lane 6). Thus, sialic acid on the 230-kD and the 130-kD ligands is essential for the binding to P-selectin.

In the same way we tested the effect of endo F-treatment on the binding of the ligands to P-selectin-IgG. The affinity isolated proteins were clearly reduced in apparent molecular weight by treatment with endo F (Fig. 8 B, lane 4), however the endo F-digested molecules were as efficiently reprecipitated with the selectin fusion protein as the mock-treated ligand molecules (Fig. 8 B, lane 6). Similarly, the endo F-treatment did not interfere with the binding of the 230/130-kD ligands to E-selectin-IgG (data not shown). This indicates that in contrast to the two monospecific selectin ligands, N-linked carbohydrates are not essential for selectin-binding of the 230/130-kD glycoproteins.

A

	1	2	3	4	5	6
sialidase	-	-	-	+	-	+

200 —

97 —

67 —

Co

repreci-pitation

B

	1	2	3	4	5	6
endo F	-	-	-	+	-	+

200 —

97 —

67 —

Co

repreci-pitation

Figure 8. The 230- and 130-kD common selectin-ligands require sialic acid but no N-linked carbohydrates for the binding to P-selectin. Mouse neutrophils were metabolically labeled with [³⁵S]methionine/[³⁵S]cysteine and detergent extracts were incubated either with immobilized CD4-IgG (*Co*) or P-selectin-IgG (all other lanes). (*A*) The isolated 230- and 130-kD ligands were either directly electrophoresed (lane *2*) or treated with (lanes *4* and *6*) or without (lanes *3* and *5*) 1 U of sialidase from Arthrobacter ureafaciens overnight at 37°C. Half of the treated samples were directly electrophoresed (lanes *3* and *4*) or reprecipitated with P-selectin-IgG (lanes *5* and *6*). (*B*) Similar as panel *A*, except that endo F instead of sialidase was used. Electrophoresis was performed under reducing conditions on a 6% polyacrylamide gel and labeled proteins were visualized by phosphorimaging. Molecular mass markers (in kD) are indicated on the left.

The 230/130-kD Ligand-Pair and the 160-kD P-Selectin Ligand, But Not the 150-kD E-Selectin Ligand Are Sensitive to O-sialoglycoprotease

The O-sialoglycoprotease from *Pasteurella hemolytica* was described to specifically cleave O-glycosylated sialoglycoproteins (Sutherland et al., 1992). We have analyzed which

of the described E- and P-selectin ligands would be sensitive to this protease. To this end the ligands were isolated from [³⁵S]methionine/[³⁵S]cysteine labeled cells: the 230- and 130-kD ligands were isolated with P-selectin-Ig from mouse neutrophils, the 160-kD P-selectin ligand was isolated in the same way from HL60 cells and the 150-kD ligand was isolated with E-selectin-IgG from 32D cl 3 cells. In each case the EDTA eluates were split into halves and one half was incubated for 1 h at 37°C without and the other half with 2.5 µg of the protein-stabilized enzyme (see Materials and Methods). As shown in Fig. 9, the protease degraded all of the 230-kD/130-kD ligands and 70% of the 160-kD P-selectin ligand molecules while the 150-kD E-selectin ligand was unaffected. This indicates that the 150-kD E-selectin ligand is not heavily O-glycosylated.
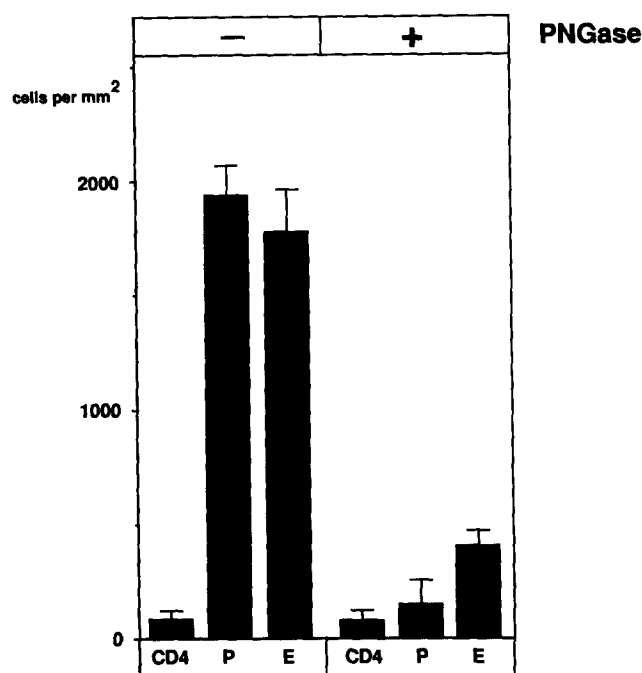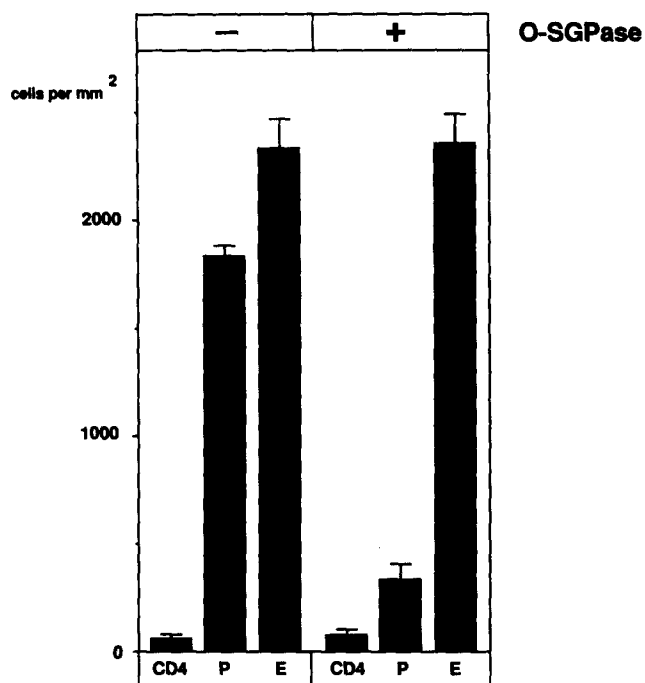
PNGase F-Treatment of HL60 Cells Blocks Binding to E- and P-Selectin, While O-sialoglycoprotease-treatment Only Blocks Cell Binding to P-selectin

If the monospecific selectin-ligands, which require N-linked carbohydrates for selectin-binding, indeed represent cell adhesion ligands for the selectins, removal of N-linked carbohydrates from the surface of intact cells should inhibit cell-binding. Therefore, the adhesion of HL60 cells to plastic-coated P- or E-selectin-IgG was tested after treating the cells with PNGase F for 1 h at 37°C. As shown in Fig. 10 *A* binding of HL60 cells to P- as well as to E-selectin was inhibited by this enzyme treatment when compared to mock-treated cells. The same result was observed for endo F-treated cells (not shown). In contrast, treatment of intact HL60 cells

O-SGPase

-	+	-	+	-	+

200 —

97 —

67 —

| 1 | 2 | 3 | 4 | 5 | 6 |
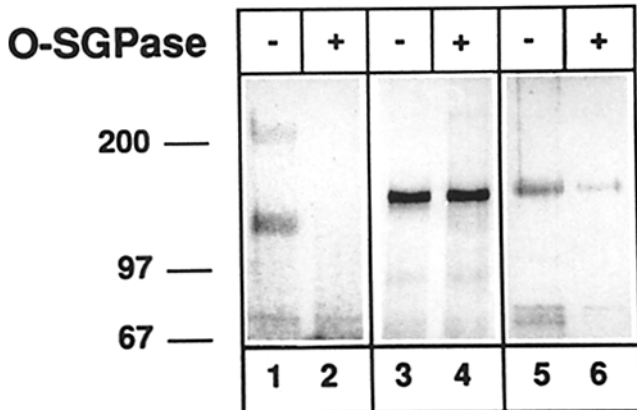
Figure 9. The common but not the monospecific E-selectin ligand is sensitive to O-sialoglycoprotease. Mouse neutrophils (lanes *1* and *2*), 32D cl 3 cells (lanes *3* and *4*) and HL60 cells (lanes *4* and *6*) were metabolically labeled with [³⁵S]methionine/[³⁵S]cysteine and detergent extracts were incubated either with immobilized P-selectin-IgG (lanes *1*, *2*, *5*, and *6*) or with E-selectin-IgG (lanes *3* and *4*). Specifically bound proteins were eluted with EDTA and half of the eluate was mock treated and the other half was treated with O-sialoglycoprotease (O-SGPase) from *Pasteurella hemolytica*. Proteins were electrophoresed on a 6% polyacrylamide gel under reducing (lanes *1–4*) and non-reducing conditions (lanes *5* and *6*) and visualized by phosphorimaging (lanes *1*, *2*, *5*, and *6*) and fluorography (lanes *3* and *4*). Molecular mass markers (in kD) are indicated on the left.

A

B

Figure 10. PNGase F-treatment of HL60 cells blocks binding to E- and P-selectin, while O-sialoglycoprotease-treatment only blocks cell binding to P-selectin. Cell adhesion assays were performed with HL60 cells in 96 well microtiter plates coated with CD4-IgG, P-selectin-IgG, or E-selectin-IgG (as indicated by *CD4, P, E*). Before the assay, cells were incubated with PNGase F (panel *A*, +), with O-sialoglycoprotease (panel *B*, +), or in the respective incubation buffers without the enzymes (panels *A* and *B*, −). Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas of defined size (per well) in four different wells for each determination. The depicted experiment represents one of three similar experiments. Bound cells were quantitated by counting the cells under the microscope in 10 randomly chosen areas of defined size (per well) in four different wells for each determination. The depicted experiment represents one of three similar experiments.

with O-sialoglycoprotease only blocked the binding of cells to P-selectin-IgG but not to E-selectin-IgG (Fig. 10 *B*). This is in agreement with the sensitivity of the P-selectin ligand and the resistance of the E-selectin ligand to this enzyme. We conclude that both monospecific selectin ligands are good candidates for cell adhesion ligands of P- and E-selectin.

Discussion

In this study we describe a 160-kD glycoprotein ligand for P-selectin on two myeloid cell lines and mouse neutrophils. This protein is clearly distinct from the recently identified 150-kD E-selectin ligand which is also present on these cells. Each of the two ligands is specific for only one of the two endothelial selectins. In addition to these ligands, two more weakly detectable glycoproteins of 230 and 130 kD were found, which bind to both endothelial selectins.

The 160-kD P-selectin ligand and the 150-kD E-selectin ligand fulfill the requirements for specific selectin ligands since they bind in a Ca^{2+}-dependent fashion, binding requires the presence of sialic acid on the ligands and no binding to the CD4-IgG control protein was observed. Since both ligands are specific for only one of the two endothelial selectins, the structural entities on each ligand which are recognized by the respective selectin must be different. This

implies that ligand-binding sites on E- and P-selectin can be selective for different structural elements. Furthermore, both monospecific ligands require N-linked carbohydrate side chains for binding to the respective selectin. Although this demonstrates the lectin-type character of the binding mechanisms, the necessity of N-linked carbohydrates for binding clearly distinguishes this type of selectin-ligand-interaction from the one which was described for sialomucin-type selectin-ligands like the L-selectin ligands GlyCAM-1 and CD34. They are heavily O-glycosylated mucins which require sialic acid for the binding to L-selectin. Also, the 120-kD ligand for human P-selectin which was identified on human neutrophils and HL60 cells (Moore et al., 1992) is heavily sialylated on O-linked oligosaccharides and binds in a sialic acid dependent fashion (Moore et al., 1992; Norgard et al., 1993). The two monospecific 150-kD and 160-kD selectin ligands represent the first examples of glycoprotein ligands that require N-linked oligosaccharides for selectin-binding.

In addition to the two monospecific ligands for E- and P-selectin, we have found two glycoproteins of 230 and 130 kD which bind to both selectins in a Ca^{2+}-dependent fashion and do not bind to CD4-IgG. Both proteins were heavily sialylated, as judged from the decrease in electrophoretic mobility caused by sialidase treatment, and binding to the

selectins was dependent on the presence of sialic acid. In contrast to the two monospecific ligands, the 230 and 130-kD ligands were only weakly detectable (15–20-fold weaker signals) and could only be isolated from mouse neutrophils and not from the cell lines 32D cl 3 and HL60 (obtained from Amer. Type Culture Collection, see below). The weak detectability does not necessarily imply a low abundance of these proteins on neutrophils. Other reasons for the weakness of the signals could be a low turnover rate of the ligands (labeling was only done for 4 h) or a lower affinity for the binding to the selectins.

It is likely that the 130-kD glycoprotein which we isolated from mouse neutrophils with both mouse selectin fusion proteins is the homolog of the human 120-kD P-selectin ligand (Moore et al., 1992). Both proteins have a similar apparent molecular weight, show a similar strong decrease in electrophoretic mobility after sialidase treatment, and require sialic acid for the binding to P-selectin. In addition, the 130-kD mouse ligand was susceptible to digestion with O-sialoglycoprotease as was reported for the human 120-kD P-selectin ligand (Norgard et al., 1993), which is characteristic for sialomucins. A 110-kD sialomucin ligand for human P-selectin (PSGL-1) was recently identified by expression cloning (Sako et al., 1993). Similar to the 120-kD ligand described by Moore et al. (1992), the 110-kD protein forms a disulfide-linked dimer.

Although we could not detect the 230- and 130-kD ligands from HL60 cells of ATCC-origin, we detected proteins of similar size on the HL60 cells which we obtained from Genetics Institute (not shown). These were the same cells from which PSGL-1 was cloned, indicating the possibility that the 230/130-kD ligand-pair may be identical with PSGL-1. Indeed, HL60 cells from ATCC do not express PSGL-1 (Trudi Veldman, Genetics Institute, personal communication).

The following arguments support the idea that the 150- and 160-kD monospecific ligands are good candidates for cell adhesion ligands of the two selectins: First, both ligands are the only ones which we could detect on 32 D cl 3 cells and HL60 cells (of ATCC origin). Second, the inhibitory effect of PNGase F and endo F on the binding of these cells to the selectins correlates with the necessity of N-linked carbohydrates on both ligands for selectin-binding. This is in line with results obtained by Larsen et al. (1992), who found that tunicamycin treatment of HL60 cells partially blocked the binding to P-selectin and E-selectin. Third, O-sialoglycoprotease inhibits the binding of HL60 cells to P-selectin and degrades the 160-kD P-selectin ligand, but does not affect the binding of HL60 cells to E-selectin and does not cleave the 150-kD E-selectin ligand. Similarly, Steininger et al. (1992) found that this protease blocks HL60 binding to P- but not to E-selectin.

The sensitivity of the 160-kD P-selectin ligand to O-sialoglycoprotease suggests that this protein is O-glycosylated. This does not necessarily imply that its O-linked carbohydrates are involved in the binding to P-selectin. Also other glycoproteins, such as CD44 or CD45, are sensitive to O-sialoglycoprotease (Sutherland et al., 1992), although they do not carry carbohydrates which support selectin-binding. However, at present we cannot exclude that O-linked carbohydrates on the 160-kD ligand may also be involved in P-selectin binding.

The selectin ligands which we have described in this report

seem to belong to two different categories. The first category is formed by the two monospecific ligands which each bind to only one type of selectin. Both ligands require sialic acid and N-linked carbohydrate side chains for binding. This looks like a different type of interaction than the binding of selectins to sialomucin-type ligands like GlyCAM-1, CD34, or the human 120-kD ligand for P-selectin. The mouse 230- and 130-kD glycoproteins which we have found as ligands for both endothelial selectins belong to the second category of ligands. The sensitivity to O-sialoglycoprotease, the irrelevance of N-linked carbohydrates for selectin-binding and the similarities between the 130-kD mouse protein and the 120-kD human P-selectin-ligand (Moore et al., 1992) and PSGL-1 (Sako et al., 1993) suggest, that the 230- and 130-kD ligands are of the sialomucin-type. This type of ligand seems not to be monospecific for one single selectin. Also GlyCAM-1 can bind to L-selectin and E-selectin (Mebius and Watson, 1993; M. Steegmaier and D. Vestweber, unpublished observation). For the sialomucin-type selectin ligands, it has been suggested that the selectins may recognize common O-linked oligosaccharides which are presented on such ligands as specific epitopes by forming unique "clustered saccharide patches" (Norgard et al., 1993). Such clusters are probably not present on the 150-kD E-selectin ligand, since it is resistant to O-sialoglycoprotease. It will be important to identify the structural motif on the two monospecific ligands which is recognized by each of the two selectins. The involvement of N-linked carbohydrates as well as the monospecificity for only one selectin argues for binding-sites on these ligands which are different from those of the typical sialomucin-type of ligands.

Whether the different types of ligands, which are coexpressed on neutrophils, reflect differences in their physiological role is still unknown. The common ligand(s) on neutrophils could allow these cells to use one and the same mechanism to bind to the two endothelial selectins. The monospecific ligands could provide the basis for different functions which the two selectins may exert on neutrophils when they bind to the blood vessel wall. Indeed, binding of E-selectin was reported to activate neutrophils (Lo et al., 1991; Kuijpers et al., 1991) while for soluble P-selectin, inhibitory effects on the CD11/CD18 dependent activation of neutrophils was reported (Gamble et al., 1990; Wong et al., 1991). Elucidation of the molecular function(s) of the identified E- and P-selectin ligands will be of central importance for the understanding of the physiological role of the endothelial selectins.

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