Identification of a Specific Glycoprotein Ligand for P-selectin (CD62) on Myeloid Cells

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Abstract. P-selectin (CD62, GMP-140, PADGEM), a Ca^{2+}-dependent lectin on activated platelets and endothelium, functions as a receptor for myeloid cells by interacting with sialylated, fucosylated lactosaminoglycans. P-selectin binds to a limited number of protease-sensitive sites on myeloid cells, but the protein(s) that carry the glycans recognized by P-selectin are unknown. Blotting of neutrophil or HL-60 cell membrane extracts with [^{125}I]P-selectin and affinity chromatography of [^{3}H]glucosamine-labeled HL-60 cell extracts were used to identify P-selectin ligands. A major ligand was identified with an ≈250,000 M_r under nonreducing conditions and ≈120,000 under reducing conditions. Binding of P-selectin to the ligand was Ca^{2+} dependent and was blocked by mAbs to P-selectin. Brief sialidase digestion of the ligand increased its apparent molecular weight; however, prolonged digestion abolished binding of P-selectin. Peptide:N-glycosidase F treatment reduced the apparent molecular weight of the ligand by ≈3,000 but did not affect P-selectin binding. Western blot and immunodepletion experiments indicated that the ligand was not lamp-1, lamp-2, or L-selectin, which carry sialyl Le^x, nor was it leukosialin, a heavily sialylated glycoprotein of similar molecular weight. The preferential interaction of the ligand with P-selectin suggests that it may play a role in adhesion of myeloid cells to activated platelets and endothelial cells.

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THE selectins are three structurally related membrane glycoproteins that participate in leukocyte adhesion to vascular endothelium and platelets (McEver, 1991). P-selectin (CD62), previously known as GMP-140 or PADGEM protein, is a receptor for neutrophils and monocytes that is rapidly translocated from secretory granule membranes to the plasma membrane of activated platelets (Hamburger and McEver, 1990; Larsen et al., 1989) and endothelial cells (Geng et al., 1990; Lorant et al., 1991). E-selectin (ELAM-1) is a cytokine-inducible endothelial cell receptor for neutrophils (Bevilacqua et al., 1987), monocytes (Hession et al., 1990), and memory T cells (Picker et al., 1991*a*; Shimizu et al., 1991). L-selectin (LAM-1, LECAM-1), a protein expressed on myeloid cells and most lymphocytes, participates in neutrophil extravasation into inflammatory sites and homing of lymphocytes to peripheral lymph nodes (Lasky et al., 1989; Siegelman et al., 1989; Kishimoto et al., 1989; Watson et al., 1991).

Each selectin functions as a Ca^{2+}-dependent lectin by recognition of sialylated glycans. Both E- and P-selectin interact with sialylated, fucosylated lactosaminoglycans on opposing cells, including the sialyl Le^x tetrasaccharide (Phillips et al., 1990; Walz et al., 1990; Lowe et al., 1990; Tiemeyer et al., 1991; Goelz et al., 1990; Polley et al., 1991; Zhou et al., 1991). However, the precise carbohydrate structures on myeloid cells recognized by these two selectins under physiologic conditions are not known. Such ligands might have unique structural features that enhance the binding specificity and/or affinity for their respective receptors.

P-selectin isolated from human platelets binds with apparent high affinity to a limited number of sites on neutrophils (Moore et al., 1991; Skinner et al., 1991) and HL-60 cells (Zhou et al., 1991). Binding is abolished by treatment of the cells with proteases (Moore et al., 1991), suggesting that the glycans on myeloid cells recognized preferentially by P-selectin are on glycoprotein(s) rather than on glycolipids. The number of binding sites for platelet P-selectin on neutrophils has been estimated at 10,000–20,000 per cell (Moore et al., 1991; Skinner et al., 1991), suggesting that these sites constitute a small component of the total cell surface protein. The protein portion of this ligand(s) may be crucial for binding by presenting the glycan in an optimal configuration, clustering glycans to enhance avidity, favoring the formation of specific oligosaccharide structures by cellular glycosyltransferases or modifying enzymes, and/or stabilizing the lectin–carbohydrate interaction through protein–protein interactions with P-selectin.

The potential importance of protein components in en-

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hancing ligand affinity is supported by studies of CHO cells transfected with a specific fucosyltransferase (Zhou et al., 1991). These cells express higher amounts of the sialyl Le^x antigen than do HL-60 cells and have protease-sensitive binding sites for P-selectin. However, the interaction of P-selectin with these sites is of much lower apparent affinity than with those on myeloid cells, and adhesion of transfected CHO cells to immobilized P-selectin is weaker than that of neutrophils and HL-60 cells (Zhou et al., 1991). These observations suggest that myeloid cells express one or more membrane glycoproteins not found on CHO cells that enhance the lectin-mediated interaction with P-selectin. Alternatively, myeloid cells may express a glycosyltransferase or modifying enzyme not present in CHO cells.

In this study we demonstrate that P-selectin binds primarily to a single major glycoprotein ligand on neutrophils and HL-60 cells, when assessed by blotting assays and by affinity chromatography of [^{3}H]glucosamine-labeled HL-60 cell extracts on immobilized P-selectin. We present preliminary characterization of this molecule and demonstrate that it can be distinguished from other well-characterized neutrophil membrane proteins with similar apparent molecular mass.

Materials and Methods

Materials

Wheat germ agglutinin (WGA)[1]-agarose, pepstatin, aprotinin, N-acetylglucosamine, leupeptin, antipain, benzamidine, MOPS, Pipes, BSA, EDTA, EGTA, and Ponceau S were purchased from Sigma Chemical Co. (St. Louis, MO). Diisopropylfluorophosphate, dichloroisocoumarin, Triton X-100 (protein grade), and sialidase (neuraminidase) from *Arthrobacter ureafaciens* (75 U/mg, EC 3.2.1.18) were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Micro BCA protein assay kits and Lubrol PX (Surfact Amp PX) were purchased from Pierce Chemical Company (Rockford, IL). Enzymobeads™, Tween-20, Affigel-15, and high molecular weight protein standards were from Bio-Rad Laboratories (Richmond, CA). Endo-β-galactosidase (150 U/mg, EC 3.2.1.103) from *Bacteroides fragilis*, 4-methyl-umbelliferyl-α-*N*-acetylneuraminic acid, and 2,3-dehydro-2,3-dideoxy-*N*-acetylneuraminic acid (Neu2en5Ac) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Peptide:*N*-glycosidase F (PNGaseF) from *Flavobacterium meningosepticum* (EC 3.2.2.18, N-glycanase) and endo-α-*N*-acetylgalactosaminidase from *Diplococcus pneumoniae* (EC 3.2.1.97, O-glycanase™) were purchased from Genzyme (Cambridge, MA). HBSS was obtained from Gibco Laboratories (Grand Island, NY). VectaStain ABC kits were purchased from Vector Laboratories Inc. (Burlingame, CA). Phycoerythrin-streptavidin was obtained from Becton Dickinson & Co. (San Jose, CA) and phycoerythrin-conjugated anti-mouse IgG_1 was from Caltag (South San Francisco, CA). Rabbit anti-mouse IgG was purchased from Organon Teknika (Durham, NC) and protein A-Sepharose CL4B was from Pharmacia Fine Chemicals (Piscataway, NJ). [6-^{3}H]glucosamine was obtained from Dupont/New England Nuclear (Boston, MA). All other chemicals were of the highest grade available.

Antibodies and Proteins

The anti-P-selectin murine mAbs S12 and G1, and goat anti-human P-selectin IgG were prepared and characterized as previously described (McEver and Martin, 1984; Geng et al., 1990; Lorant et al., 1991). Rabbit polyclonal antisera and murine mAbs to human lamp-1 (CD3) (Carlsson et al., 1988) and lamp-2 (BB6) (Carlsson and Fukuda, 1989), and rabbit polyclonal anti-human leukosialin antiserum (Carlsson and Fukuda, 1986) were provided by Dr. Sven Carlsson (University of Umeå, Umeå, Sweden). Anti-human leukosialin (CD43) mAb (Leu-22) was purchased from Becton Dickinson & Co. (San Jose, CA). The anti-L-selectin murine mAb antibodies DREG-56, DREG-55, and DREG-200 (Kishimoto et al., 1990) were provided by Dr. Takashi Kei Kishimoto (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT). All mAbs are of the IgG_1 subtype and were used in purified form. Leukosialin purified from HL-60 cells (Carlsson and Fukuda, 1986) was provided by Dr. Sven Carlsson (University of Umeå). P-selectin was purified from human platelets as previously described (Moore et al., 1991).

Preparation of Membranes

Erythrocyte membranes were isolated from leukocyte-depleted human erythrocytes as previously described (Rollins and Sims, 1990) and extracted with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1% Lubrol PX. Detergent-insoluble material was removed by centrifugation at 16,000 *g* for 10 min.

Human neutrophils isolated by discontinuous leukopheresis from volunteer donors were purchased from the Oklahoma Blood Institute (Oklahoma City, OK). Each product contained 1.5–3.3 × 10^{10} leukocytes (≈85% neutrophils). The neutrophil product was centrifuged at 200 *g* for 20 min and the platelet-rich plasma removed. Erythrocytes were lysed by resuspending the pellets with 5 mM EDTA, pH 7.5, in H_2O for 20 s. An equal volume of 1.8% NaCl, 5 mM EDTA, pH 7.5, was then added to restore isotonicity. The cells were centrifuged at 500 *g* for 5 min and resuspended in ice-cold HBSS containing 5 mM EDTA and 10 mM MOPS, pH 7.5. Diisopropylfluorophosphate was then added to a final concentration of 2 mM and the cell suspension incubated for 10 min on ice. The cells were centrifuged at 500 *g* for 5 min at 4°C and resuspended in ice-cold 100 mM KCl, 3 mM NaCl, 1 mM Na_2ATP, 3.5 mM $MgCl_2$, 10 mM Pipes, pH 7.3 (relaxation buffer). To this suspension the following protease inhibitors were added at the indicated final concentrations: 2 mM diisopropylfluorophosphate, 20 μM leupeptin, 30 μM antipain, and 1 mM benzamidine. The cell suspension was pressurized with N_2 at 350 psi in a cell disruption bomb (model 4635; Parr Instrument Company, Moline, IL) for 40 min at 4°C with constant stirring as described previously (Borregaard et al., 1983). The cavitate was collected into EGTA (2 mM final concentration) and nuclei and undisrupted cells were pelleted at 500 *g* for 10 min at 4°C. The cavitate was fractionated as previously described (Eklund and Gabig, 1990). Briefly, it was layered over 40% sucrose in relaxation buffer containing 2 mM EGTA, 20 μM leupeptin, 30 μM antipain, and 1 mM benzamidine, and centrifuged at 104,000 *g* (at r_{av}) for 45 min at 4°C in a rotor (model SW28; Beckman Instruments, Inc., Palo Alto, CA). The top layer (FX_1), the 40% sucrose layer (FX_2), and the granule pellet (FX_3) were collected and assayed for lactate dehydrogenase as a cytoplasmic marker, alkaline phosphatase as a plasma membrane marker, and myeloperoxidase as a marker for azurophilic granules as previously described (Borregaard et al., 1983; Geng et al., 1990). Table I shows the distribution of marker enzymes in the various fractions. FX_2, enriched for alkaline phosphatase, was diluted with four volumes of 0.1 M NaCl, 10 mM MOPS, pH 7.5, and centrifuged at 111,000 *g* (at r_{av}) for 60 min at 4°C in a rotor (model 50.2 Ti; Beckman Instruments, Inc.). The supernatant was collected and the membrane pellet was extracted with 1% Lubrol PX, 0.1 M NaCl, 10 mM MOPS, pH 7.5, 0.02% sodium azide, 20 μM leupeptin, 30 μM antipain, 1 mM benzamidine, and stored at 4°C.

HL-60 cells, maintained in suspension culture in RPMI-1640 supplemented with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin were washed in HBSS, 10 mM MOPS, pH 7.5, and membranes were isolated exactly as described for neutrophils.

Partial Purification of P-selectin Ligand

Neutrophil or HL-60 cell membrane extracts were applied to a WGA affinity column (0.9 × 20 cm, 7.6 mg lectin/ml resin) equilibrated at room temperature with 0.5 M NaCl, 10 mM MOPS, pH 7.5, 0.02% sodium azide, 0.1% Lubrol PX. The column was washed with five column volumes of equilibration buffer, followed by two column volumes of 0.1 M NaCl, 10 mM MOPS, pH 7.5, 5 mM EDTA, 0.02% sodium azide, 0.01% Lubrol PX. The column was then eluted with the above buffer containing 100 mM N-acetylglucosamine. Protein-containing fractions were pooled and extensively dialyzed against 0.1 M NaCl, 10 mM MOPS, pH 7.5, 0.02% sodium azide, 0.01% Lubrol PX at 4°C. The dialyzed WGA column eluate was made 1 mM in $CaCl_2$ and $MgCl_2$ and applied to a human serum albumin Affigel-15 precolumn (0.9 × 11 cm, 25 mg protein/ml resin) hooked in series to a P-selectin-Affigel 15 column (0.6 × 13 cm, 2 mg protein/ml resin). The columns were equilibrated with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% sodium azide, 0.01% Lubrol PX. After the samples were applied the columns were washed with 100 column volumes of equilibration buffer, and eluted with equilibration buffer containing 5

1. *Abbreviations used in this paper*: Lamp, lysosomal-associated membrane protein; Neu2en5Ac, 2,3-dehydro-2,3-dideoxy-*N*-acetylneuraminic acid; PNGaseF, Peptide:N-glycosidase F; WGA, wheat germ agglutinin.

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Table I. Distribution of Marker Enzymes from Subcellular Fractions of Nitrogen-cavitated Human Neutrophils

	Lactate dehydrogenase	Myeloperoxidase	Alkaline phosphatase
FX_1 (cytosol)	95.6 ± 0.5	0	29.0 ± 2.7
FX_2 (membrane)	4.1 ± 0.5	2.6 ± 1.0	56.8 ± 8.7
FX_3 (granule)	0	97.4 ± 1.0	14.1 ± 5.5

Results are expressed as the percentage of the total enzyme activity in the cavitate (mean ± SD, n = 3).

mM EDTA. Yields were estimated by protein assays with the Micro BCA protein assay kit using BSA as a standard.

P-selectin Blotting Assay

Samples were electrophoresed on 7.5% SDS-polyacrylamide gels and proteins electrophoretically transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) for 4-5 h at 0.5 A. The positions of the molecular weight standards were marked with a pen after staining the membranes with Ponceau S. The membranes were blocked overnight at 4°C in 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% sodium azide, 10% (wt/vol) Carnation™ nonfat dry milk, and then washed with the same buffer containing 0.1% Tween-20 without milk. The membranes were incubated with [^{125}I]P-selectin (0.5–1.0 nM), iodinated as previously described (Moore et al., 1991), in 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% Lubrol PX, 1% human serum albumin for 1 h at room temperature. After extensive washing the membrane was dried and exposed to Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY) for 6–18 h at −70°C. All the [^{125}I]P-selectin blots shown are autoradiograms of the entire blot, corresponding to the area from the stacking gel interface to beyond the dye front on the original gel.

Metabolic Radiolabeling of HL-60 Cells and Isolation of [^{3}H]glucosamine-labeled P-selectin Ligand

HL-60 cells (1–2 × 10^6 cells/ml) in 100-mm tissue culture dishes were labeled for 48 h with 50 μCi/ml [6-^{3}H]glucosamine at 37°C in RPMI-1640 containing 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. At the end of the labeling period the cells were washed three times by centrifugation and resuspension in ice-cold PBS. The cell pellet was solubilized with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 1% Triton X-100, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 8 μg/ml pepstatin, 2 mM PMSF, 10 mM benzamidine, and 0.5 mM dichloroisocoumarin. The solubilized cells were allowed to sit on ice for 1-2 h and then sonicated for 20 min at 4°C in a water bath sonicator. The cell extract was centrifuged for 5 min at 16,000 g and the supernatant was applied to a P-selectin-Affigel-15 column (0.25 × 13 cm, 2 mg protein/ml resin) equilibrated with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% Triton X-100. The column was washed with 10–20 column volumes of equilibration buffer and bound material was eluted with equilibration buffer containing 10 mM EDTA. Fractions (1 ml) were collected and monitored for radioactivity by liquid scintillation counting.

Analysis of [^{3}H]glucosamine-labeled P-selectin Ligand

Metabolically labeled proteins eluted from the P-selectin column were precipitated in the presence of 0.1 mg/ml BSA by addition of cold TCA (10% final concentration). The resulting pellets were washed with 1 ml acidified acetone (0.2%), solubilized in 0.1 M NaOH and electrophoresed under reducing and nonreducing conditions on 10% SDS-polyacrylamide gels. The gels were stained with Coomassie blue and then processed for fluorography with EN^{3}HANCE (Dupont/New England Nuclear, Boston, MA) according to the manufacturer's instructions. The dried gels were then exposed to Kodak X-OMAT AR film at −80°C.

Enzyme Digestion

In certain experiments, samples analyzed by P-selectin blotting were pretreated with exo- or endo-glycosidases before SDS-PAGE. For sialidase and endo-β-galactosidase digestions of P-selectin ligand, samples were dialyzed against 0.15 M NaCl, 50 mM acetate, pH 6.0, 9 mM $CaCl_2$, 0.02% azide, 0.01% Lubrol PX, and incubated for various times at 37°C in the presence or absence of 200 mU/ml of enzyme. For PNGaseF and endo-α-*N*-acetylgalactosaminidase digestions, samples were first reduced and denatured by boiling in 0.5% SDS, 0.5% β-mercaptoethanol for 5 min, and then a 7.5-fold molar excess of NP-40 was added. The samples were incubated for 16 h at 37°C with either PNGaseF (20 U/ml at pH 8.6) or endo-α-*N*-acetylgalactosaminidase (70 mU/ml at pH 6.5) in the presence of 5 mM PMSF and 5 mM 1,10-phenanthroline.

Affinity-purified [^{3}H]glucosamine-labeled P-selectin ligand was incubated for 24 h in 25 mM sodium acetate, pH 5.5 at 37°C under a toluene atmosphere in the presence or absence of 1 U/ml of *A. ureafaciens* sialidase for 18 h. For PNGaseF digestion of metabolically labeled ligand, samples were denatured by boiling in 0.25% SDS, 25 mM β-mercaptoethanol for 5 min, and NP-40 was added in eight-fold excess (wt/wt) over SDS. The samples were incubated for 24 h with PNGaseF (3.3 U/ml) in a toluene atmosphere. The samples were then precipitated with TCA and subjected to SDS-PAGE and fluorography as described above.

Flow Cytometry

Human neutrophils, isolated as previously described (Hamburger and McEver, 1990), were suspended (10^6/ml) in HBSS containing 1% FCS and 0.1% sodium azide (HBSS/FCS/Az). 1 ml of neutrophil suspension was underlaid with 100 μl FCS and centrifuged at 500 g for 5 min. The neutrophil pellet was resuspended in 50 μl of purified P-selectin (10 μg/ml, in HBSS/FCS/Az), and then incubated sequentially with 50 μl of biotin-conjugated S12 (10 μg/ml, in HBSS/FCS/Az) and 20 μl of phycoerythrin-streptavidin (neat). In certain experiments, the neutrophils were preincubated for 10–15 min with antisera or antibodies before the addition of P-selectin. Between each step the cells were diluted with one ml of HBSS/FCS/Az, underlaid with 100 μl FCS, and centrifuged at 500 g for 5 min. All steps were performed at 4°C. After the last wash, the cells were fixed with 1 ml of 1% paraformaldehyde in HBSS and analyzed in a FACScan flow cytometer (FACScan is a registered trademark of Becton Dickinson & Co., Mountain View, CA) formatted for two-color analysis as previously described (Moore et al., 1991). Binding of P-selectin to intact neutrophils as assessed by this assay was Ca^{2+}-dependent, was blocked by GI, and was abolished by pretreatment of the cells with trypsin or sialidase (data not shown).

Immunoprecipitations

WGA eluate was incubated with 10 μg of anti-leukosialin (Leu22) or an isotype matched control monoclonal antibody for 1 h at 37°C. The mixture was then incubated with protein A-Sepharose CL4B beads saturated with rabbit anti-mouse IgG for 1 h at 37°C. The beads were pelleted, washed four times with 1 ml of 0.1 M NaCl, 20 mM Tris, pH 7.5, 1% Triton X-100, and bound material eluted by boiling 5 min in 2% SDS, 60 mM Tris, pH 6.8, and 5% β-mercaptoethanol. Immunoprecipitates and immunosupernatants were then analyzed by P-selectin blotting and by Western blotting using Leu22 as a probe.

Assay of Sialidase Activity in Commercial Enzyme Preparations

The sialidase activity in O-glycanase (endo-α-N-acetylgalactosaminidase) or *A. ureafaciens* sialidase was assayed by incubation of dilutions of the enzymes with 50 nmol 4-methyl-umbelliferyl-α-*N*-acetylneuraminic acid in 50 μl of sodium cacodylate, pH 6.5, 10 mM calcium acetate, for various time periods. Incubations were quenched by addition of 0.95 ml 0.1 M sodium bicarbonate, pH 9.3, and assayed for released 4-methylumbelliferone by fluorescence (excitation = 365 nM, emission = 450 nM).

Results

Identification of a P-selectin Ligand

To identify proteins from myeloid cells which bind P-selectin, neutrophil and HL-60 cell membrane extracts were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to Immobilon membranes, and probed with [^{125}I]P-selectin. When samples were analyzed without reduction, P-selectin bound preferentially to a glycoprotein species with an

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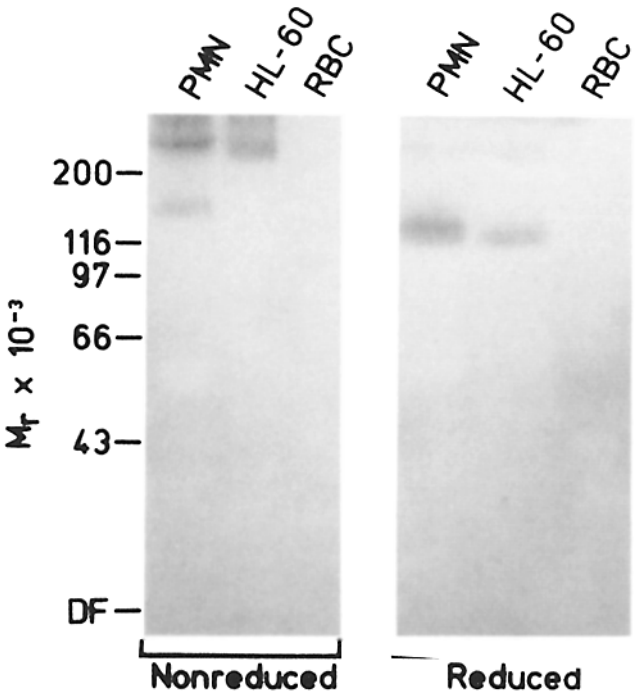

Figure 1. P-selectin blot analysis of neutrophil (*PMN*), HL-60 cell, and erythrocyte (*RBC*) membrane extracts. Cell membrane extracts (80 μg protein/lane) were electrophoresed on 7.5% SDS–polyacrylamide gels under nonreducing or reducing conditions, transferred to Immobilon membranes, and probed with [^{125}I]P-selectin as described in Materials and Methods. *DF*, dye front.

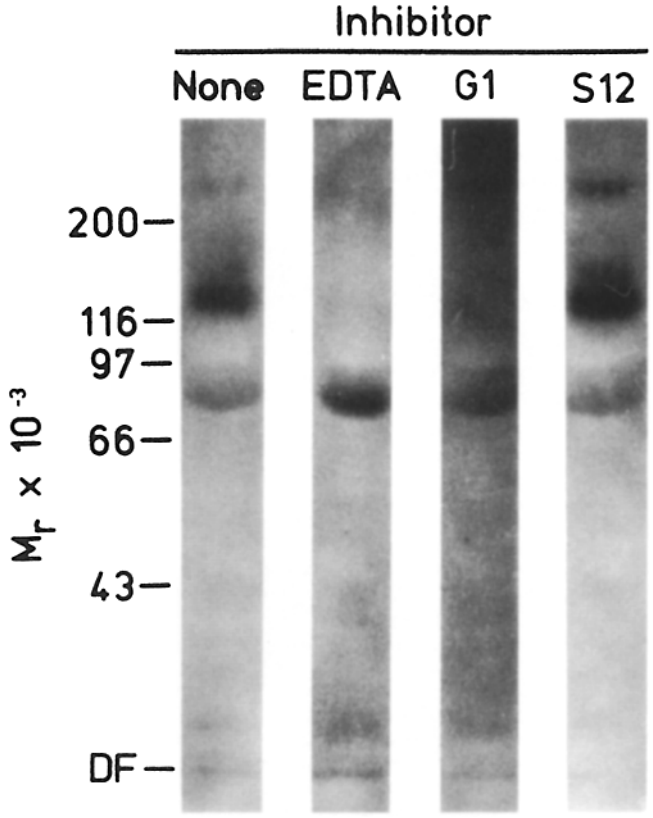

Figure 2. Effect of EDTA or anti-P-selectin antibodies on [^{125}I]P-selectin binding to the neutrophil ligand. Neutrophil membrane extracts (200 μg protein/lane) were electrophoresed on 7.5% SDS–polyacrylamide gels under reducing conditions, transferred to Immobilon membranes, and probed with [^{125}I]P-selectin alone, in the presence of 10 mM EDTA, or in the presence of 20 μg/ml of the anti-P-selectin mAbs G1 or S12.

≈250,000 M_r from both neutrophil and HL-60 cell membranes (Fig. 1). Under nonreducing conditions P-selectin also bound to proteins at the stacking gel interface and to a minor species with an ≈160,000 M_r. When samples were analyzed after reduction, P-selectin preferentially bound to a glycoprotein with an ≈120,000 M_r (Fig. 1). Minor bands were observed at ≈250,000 and ≈90,000. Under both reducing and nonreducing conditions P-selectin also bound to the blots at the dye front (Figs. 2 and 3 *a*). P-selectin binding proteins were not detected when an equivalent amount of erythrocyte membrane protein was analyzed in parallel (Fig. 1). We also directly solubilized the total proteins in the neutrophil cavitate with SDS and analyzed their ability to interact with P-selectin with the blotting assay. P-selectin bound only to proteins with apparent molecular weights of 120,000 and 90,000 under reducing conditions (data not shown). Although the sensitivity of this analysis was limited by the amount of protein that could be run on the gel, the results indicate that we did not exclude major ligands that were either not enriched in the membrane fraction (FX_2) or not effectively solubilized by nonionic detergent.

To further assess the specificity of the blotting assay, neutrophil membrane extracts electrophoresed under reducing conditions were probed with [^{125}I]P-selectin in the presence or absence of EDTA or anti-P-selectin mAbs. Fig. 2 shows that [^{125}I]P-selectin binding to the major 120-kD and the minor 250-kD species was Ca^{2+}-dependent, a characteristic of all selectin-dependent cellular interactions (McEver, 1991). Binding to both species was also blocked by G1, a mAb to P-selectin that inhibits adhesion of myeloid cells to P-selectin, but not by S12, a mAb to P-selectin that does not block adhesion. Binding of [^{125}I]P-selectin was also inhibited by a 100-fold excess of unlabeled P-selectin (data not shown). The binding of [^{125}I]P-selectin to the dye front and to the 90-kD protein was not blocked by EDTA or G1, suggesting that these interactions were nonspecific or used a specific Ca^{2+}-independent recognition mechanism.

Partial Purification of P-selectin Ligand from Neutrophils

Neutrophils were disrupted and the membrane fraction (FX_2) isolated by fractionation of the cavitate as described in Materials and Methods. The membrane fraction constituted ≈5–7% (n >10) of the protein in the cavitate. This fractionation depleted both cytosolic proteins and azurophilic granules (Table I). Proteins binding P-selectin were not detected in the cytosolic fraction (FX_1) with the blotting assay. The final membrane pellet was solubilized with nonionic detergent and applied to a WGA column which bound 4–5% of the protein in the membrane extract. P-selectin blotting assays of reduced proteins demonstrated that both the major 120-kD and the minor 250-kD ligands bound quantitatively to WGA. However, the 90-kD band and the band at the dye front observed in the membrane extract were not bound by WGA (Fig. 3 *a*). After extensive dialysis, the WGA eluate was applied to an Affigel-15 precolumn in series with a P-selec-

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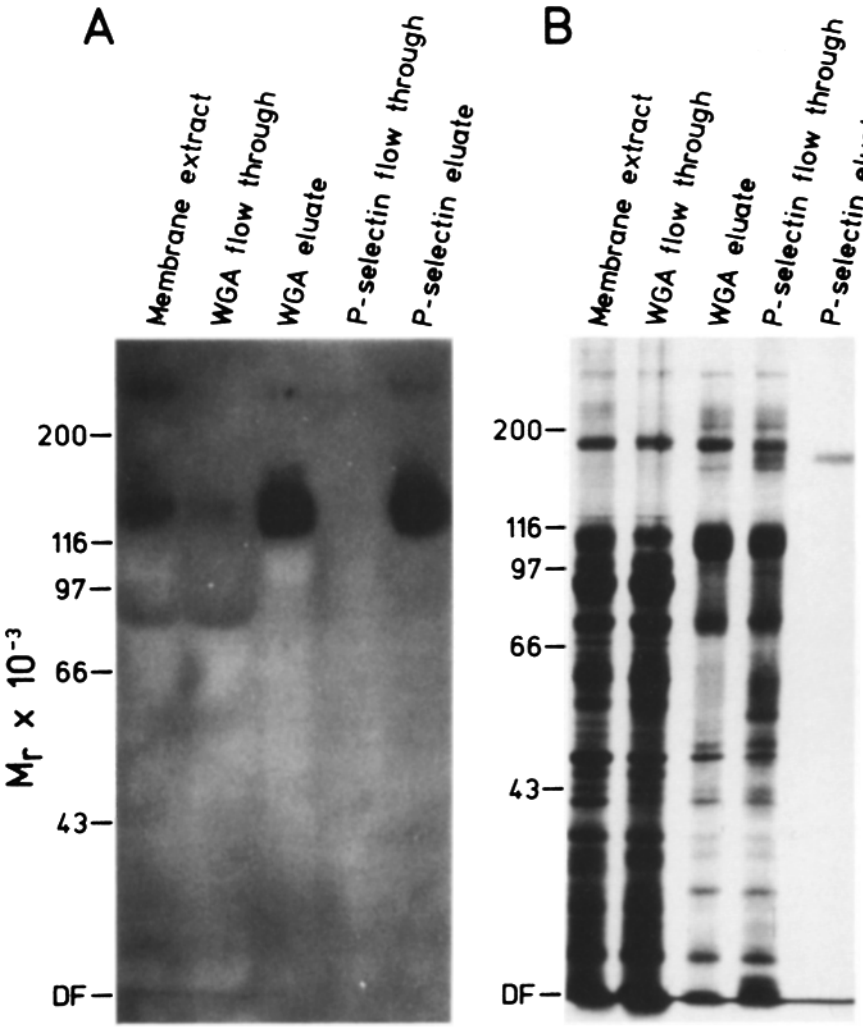

Figure 3. Partial purification of the P-selectin ligand from neutrophils by sequential WGA and P-selectin affinity chromatography. (*A*) Samples from the indicated steps of the isolation procedure were electrophoresed on 7.5% SDS–polyacrylamide gels under reducing conditions, transferred to Immobilon membranes, and probed with [^{125}I]P-selectin. The amounts of protein loaded onto the lanes were as follows: membrane extract and WGA flow through, 200 μg; WGA eluate and P-selectin flow through, 50 μg; P-selectin eluate, 2 μg. (*B*) The same samples (10 μg protein/lane) were also analyzed by SDS-PAGE under reducing conditions followed by silver staining.

tin affinity column. Approximately 2% of the protein in the WGA eluate bound to the P-selectin column and could be eluted with EDTA. Both the 250- and the 120-kD ligands bound quantitatively to the P-selectin column (Fig. 3 *a*). Quantitative analysis of the protein recovered from the P-selectin eluate indicated that the ligand(s) comprised <0.01% of the total protein in the neutrophil cavitate. Elution of bound proteins from the P-selectin column with EDTA demonstrated that the interaction of nondenatured neutrophil ligands with P-selectin was also Ca^{2+} dependent. Neither species was eluted from the Affigel-15 precolumn with EDTA (data not shown).

Fig. 3 *b* shows a silver-stained SDS–polyacrylamide gel of proteins from the various stages in the partial purification procedure run under reducing conditions. The major silver-stained band in the P-selectin eluate had an ≈150,000 M_r which is similar to that of P-selectin itself. To determine whether this protein represented P-selectin that had leeched off the P-selectin column, we analyzed the P-selectin eluate by SDS-PAGE under both reducing and nonreducing conditions, followed by silver staining, Western blotting with goat anti–P-selectin IgG, and P-selectin blotting. The major silver-stained protein in the P-selectin eluate was indeed P-selectin. Purified P-selectin migrates with an ≈120,000 M_r under nonreducing conditions; a minor component migrates with an ≈250,000 M_r (McEver and Martin, 1984). After reduction the protein migrates more slowly with an ≈150,000 M_r (McEver and Martin, 1984). The two nonreduced bands and the one reduced band detected by silver staining of the P-selectin eluate (Fig. 4 *b*) co-migrated with purified P-selectin and were recognized by anti-P-selectin IgG (data not shown). The P-selectin ligand identified in the blotting assay (Fig. 4 *a*) was not detected by silver staining and migrated differently than P-selectin under both reducing and nonreducing conditions. When the P-selectin eluate was electrophoresed without reduction, P-selectin did not bind to proteins at the stacking gel interface (Fig. 4 *a*). Therefore, the P-selectin–binding proteins at the stacking gel interface, observed in extracts of neutrophil membranes (Fig. 1), were probably an artifact due to the relatively high amount of protein loaded on the gel.

Characterization of the P-selectin Ligand

The ligand(s) on intact target cells requires sialic acids to interact with P-selectin (Zhou et al., 1991; Corral et al., 1990; Polley et al., 1991; Moore et al., 1991). To determine whether the ligand detected by blotting of neutrophil membranes contained sialic acids that were essential for recognition by P-selectin, neutrophil membrane glycoproteins which bound to WGA were treated with sialidase (200 mU/ml) for varying

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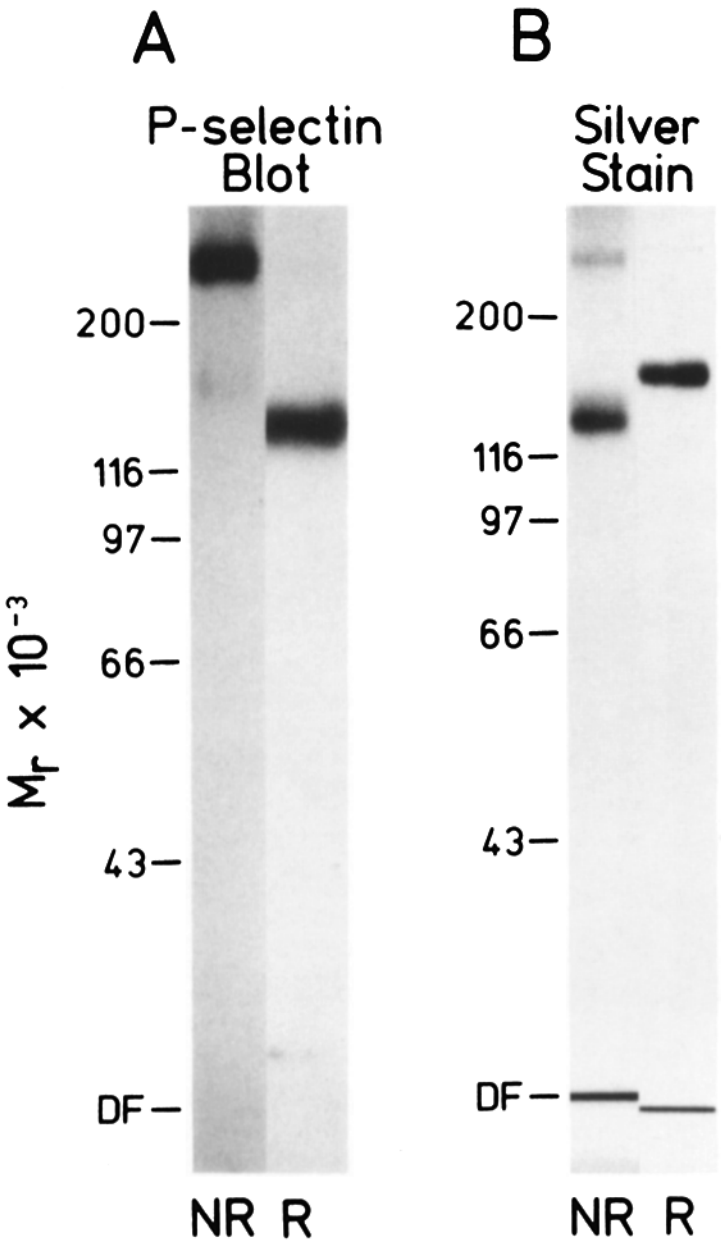

Figure 4. Analysis of P-selectin eluate. (*A*) P-selectin eluate (2 μg protein/lane) was electrophoresed under reducing (*R*) and nonreducing (*NR*) conditions, transferred to Immobilon membranes, and probed with [^{125}I]P-selectin. (*B*) Eluate from the P-selectin column (10 μg protein/lane) was electrophoresed on 7.5% SDS–polyacrylamide gels under reducing (*R*) and nonreducing (*NR*) conditions and stained with silver.

times before SDS-PAGE under reducing conditions and then analyzed for their ability to bind P-selectin (Fig. 5 *a*). Sialidase digestion for 30 min increased the apparent molecular weight of the major 120-kD ligand, a shift characteristic of heavily sialylated glycoproteins (Carlsson and Fukuda, 1986; Segrest et al., 1971; Cummings et al., 1983). Longer sialidase digestion did not further alter the electrophoretic mobility of the ligand but did abolish its ability to bind [^{125}I]P-selectin. Sialidase treatment had a similar effect on the minor 250-kD ligand. These results demonstrate that the ligand(s) contains sialic acid residues that are critical for recognition by P-selectin, but suggest that only a portion of the sialic acid residues are required for binding.

To examine whether the ligand contained N-linked glycans, neutrophil membrane glycoproteins which bound to WGA were digested with PNGaseF. This treatment did not affect [^{125}I]P-selectin binding but did decrease the apparent molecular weight of the ligand, consistent with the enzymatic removal of one or two N-linked glycan chains (Fig. 5 *b*). This demonstrates that the ligand contains at least one N-linked oligosaccharide chain that is not required for P-selectin binding. Although we cannot directly assess whether N-linked glycans were quantitatively removed from the ligand, we did use conditions that normally cleave such glycans from most proteins.

Prolonged treatment of neutrophil membrane extracts with endo-α-*N*-acetylgalactosaminidase (O-glycanase) abolished binding of [^{125}I]P-selectin in the blotting assay, whereas sham digestion was without effect (data not shown). This was a surprising result, since only nonsialylated Galβ1-3GalNAc disaccharides O-linked to serine or threonine residues are known substrates for the enzyme (Umemoto et al., 1977). We therefore considered the possibility that the commercial enzyme was contaminated with a sialidase. Assays using a

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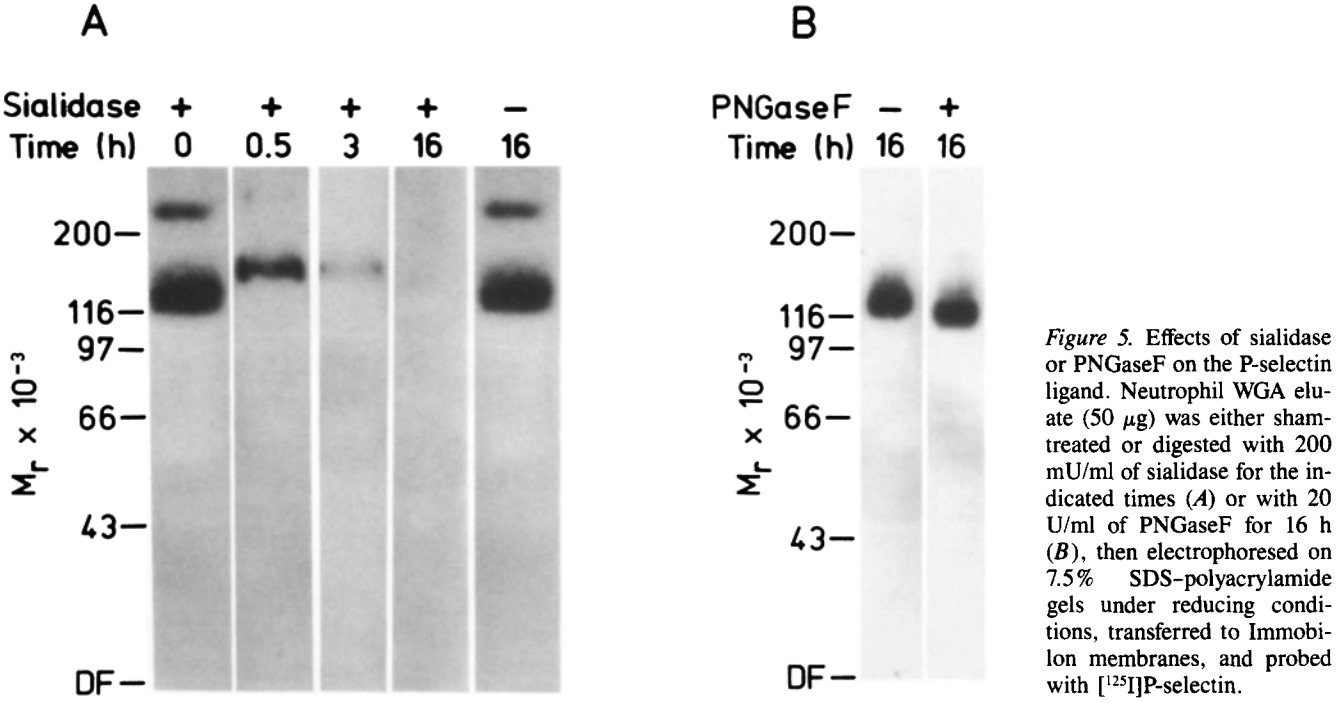

Figure 5. Effects of sialidase or PNGaseF on the P-selectin ligand. Neutrophil WGA eluate (50 μg) was either sham-treated or digested with 200 mU/ml of sialidase for the indicated times (*A*) or with 20 U/ml of PNGaseF for 16 h (*B*), then electrophoresed on 7.5% SDS-polyacrylamide gels under reducing conditions, transferred to Immobilon membranes, and probed with [^{125}I]P-selectin.

synthetic sialidase substrate confirmed the presence of a small amount of sialidase (0.01 mU/mU O-glycanase). Although the level of activity was small, it was stable to prolonged incubations under the conditions recommended by the manufacturer for use of the O-glycanase preparation. To prove that the contaminating sialidase was responsible for the loss of P-selectin binding, the digestions were repeated in the presence of a competitive sialidase inhibitor, Neu2en5Ac. Under these conditions endo-α-*N*-acetylgalactosaminidase digestion had no effect on [^{125}I]P-selectin binding to the ligand or the apparent molecular weight of the ligand (data not shown). Because the ligand requires sialic acid to interact with P-selectin, we could not use the blotting assay to assess the role of O-linked glycans in recognition by P-selectin.

Isolation of a P-selectin Ligand from Metabolically Labeled HL-60 Cells

P-selectin blotting of denatured membrane proteins from myeloid cells may not detect molecules whose ability to bind P-selectin is dependent on secondary and/or tertiary structure. As an independent approach to identify ligands for P-selectin, HL-60 cells were metabolically labeled with [^{3}H]glucosamine, solubilized with nonionic detergent, and applied to a P-selectin affinity column. After extensive washing, bound material was eluted with EDTA and analyzed by SDS-PAGE followed by fluorography. Fig. 6 *a* shows that a single metabolically labeled species was eluted, which comigrated under both nonreducing and reducing conditions with the major species detected in neutrophil and HL-60 cell membranes by blotting with [^{125}I]P-selectin. Only 0.15–0.5% of the total [^{3}H]glucosamine-labeled HL-60 glycoproteins bound to the P-selectin column, indicating that the ligand is not abundant. Sialidase treatment of the [^{3}H]glucosamine-labeled P-selectin ligand from HL-60 cells produced the same increase in apparent molecular weight that was observed for the major neutrophil ligand identified by the P-selectin blotting assay (Fig. 6 *b*). In addition, PNGaseF treatment caused the same decrease in the apparent molecular weight of the HL-60 cell ligand that was observed for the neutrophil ligand (Fig. 6 *c*).

Comparison of the P-selectin Ligand with Known Neutrophil Membrane Proteins

We compared the properties of the major 120-kD P-selectin ligand with those of three well-characterized neutrophil membrane proteins with similar apparent molecular weight. The first two molecules, lamp-1 and lamp-2, are abundant neutrophil proteins that are predominantly localized in lysosomal membranes but are also expressed in small amounts on the cell surface. These proteins have a large number of complex N-linked glycan chains (Fukuda et al., 1988; Carlsson et al., 1988; Carlsson and Fukuda, 1990), many of which carry the sialyl Le^x tetrasaccharide (Lee et al., 1990). Polyclonal antisera (1:5 dilution) and mAbs (40 μg/ml) to lamp-1 (CD3) and lamp-2 (BB6) had no effect on binding of P-selectin to neutrophils as assessed by flow cytometry (data not shown). Western blot analysis of neutrophil membranes with mAbs to lamp-1 and lamp-2 showed that the electrophoretic mobilities of these proteins under nonreducing conditions were distinct from that of the P-selectin ligand (Fig. 7 and Carlsson et al., 1988). In contrast to the P-selectin ligand, the electrophoretic mobilities of lamp-1 and lamp-2 are not affected by sialidase treatment (Carlsson et al., 1988). Although lamp-1 and lamp-2 from myeloid cells are rich in lactosaminoglycans sensitive to endo-β-galactosidase (Carlsson et al., 1988; Carlsson and Fukuda, 1990), treatment of intact neutrophils with the enzyme did not affect binding of [^{125}I]P-selectin (Moore et al., 1991). Pretreatment of crude neutrophil membrane extracts or WGA column eluate with endo-β-galactosidase (200 mU/ml, 1–2 h, 37°C) also did not affect the apparent molecular weight of the ligand or its ability to

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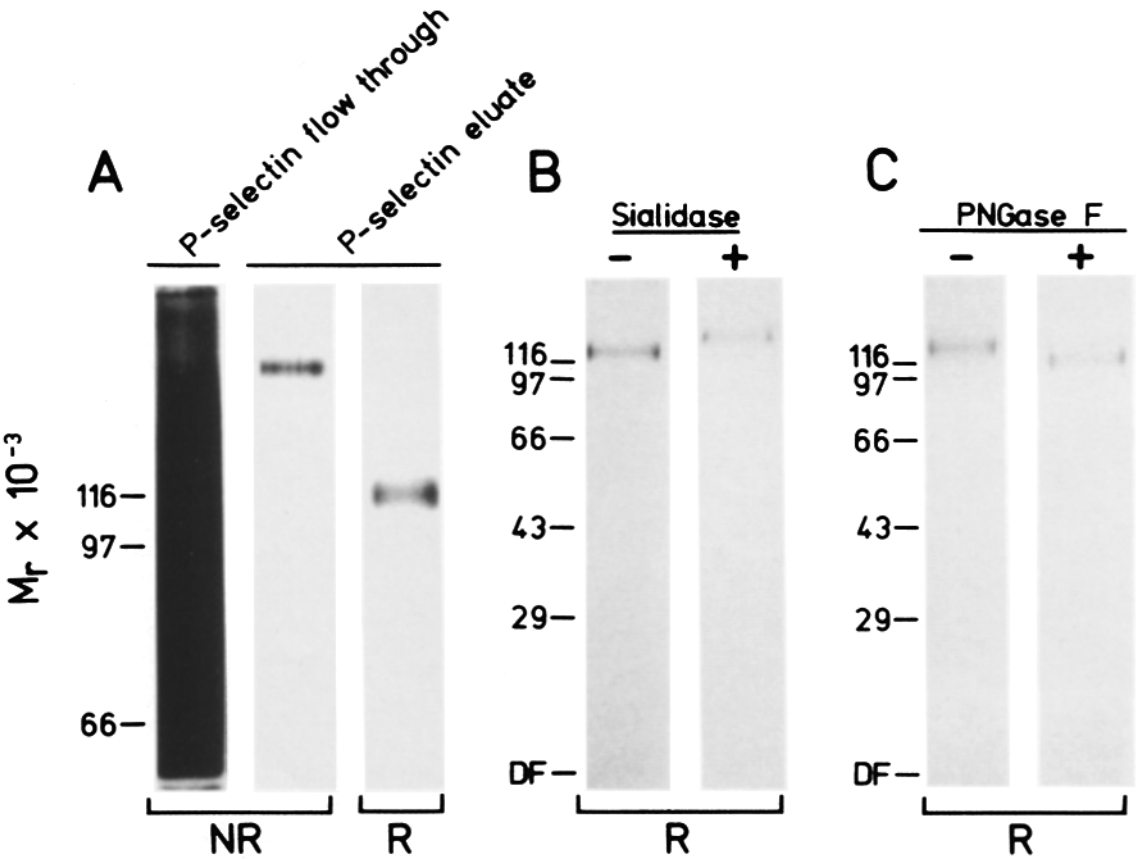

Figure 6. Analysis of the P-selectin ligand purified from [^{3}H]glucosamine-labeled HL-60 cells by P-selectin affinity chromatography. (*A*) Samples were electrophoresed on 10% SDS-polyacrylamide gels under both nonreducing (*NR*) and reducing (*R*) conditions and analyzed by fluorography. Other samples were either sham treated or digested with 1 U/ml of sialidase for 24 h (*B*) or with 3.3 U/ml of PNGaseF for 24 h (*C*), and then electrophoresed on 10% SDS-polyacrylamide gels under reducing conditions and analyzed by fluorography.

bind [^{125}I]P-selectin (data not shown). These data argue that lamp-1 and lamp-2 are not ligands for P-selectin even though they carry many sialyl Le^x structures.

The third molecule whose apparent molecular weight is similar to the 120-kD P-selectin ligand is CD43 (leukosialin, sialophorin), a heavily sialylated membrane protein present on platelets and all leukocytes (Remold-O'Donnell et al., 1986; Carlsson and Fukuda, 1986; Pallant et al., 1989; Shelley et al., 1989). It carries numerous O-linked sugar chains and is differentially glycosylated by cells of various hematopoietic lineages (Carlsson et al., 1986). Like the P-selectin ligand, treatment of leukosialin with sialidase increases its apparent molecular weight (Carlsson and Fukuda, 1986). However, in contrast to the P-selectin ligand, the electrophoretic mobility of leukosialin was unaffected by reduction (Fig. 7; Carlsson and Fukuda, 1986; Carlsson et al., 1988). Monospecific polyclonal anti-human leukosialin antisera (1:5 dilution) did not inhibit P-selectin binding to neutrophils as assessed by flow cytometry (data not shown). Furthermore, immunodepletion of leukosialin from neutrophil membrane extracts did not deplete P-selectin ligand as assessed by the blotting assay (data not shown). Finally, leukosialin purified from HL-60 cells did not bind P-selectin (Fig. 8).

Based on studies in which an antibody to L-selectin (DREG-56) partially inhibited neutrophil adhesion to P-selectin-transfected cells, it was suggested that L-selectin is an important glycoprotein ligand on myeloid cells for P-selectin (Picker et al., 1991*b*). Although L-selectin is present in membrane extracts and WGA eluates of neutrophil membranes, as detected by Western blotting (Fig. 7), [^{125}I]P-selectin did not bind to L-selectin in the blotting assay. In addition, the anti-L-selectin mAb DREG-56 (100 μg/ml) had no effect on the binding of purified P-selectin to quiescent neutrophils as assessed by flow cytometry (Fig. 9 *a*). Parallel control assays showed that the neutrophils expressed high levels of L-selectin detectable by DREG-56 (Fig. 9 *b*). Identical results were obtained with the anti-L-selectin mAbs DREG-55 and DREG-200 (data not shown). Thus, interactions with L-selectin do not appear to contribute to the binding of fluid-phase P-selectin to intact neutrophils or to immobilized proteins from neutrophil membrane extracts.

Discussion

We have used two independent and complementary approaches to identify ligands for P-selectin on myeloid cells, blotting of neutrophil and HL-60 cell membranes and affinity chromatography of metabolically labeled HL-60 cell extracts. The major ligand identified by both methods has an ≈250,000 and ≈120,000 M_r under nonreducing and reducing conditions, respectively. The requirements for interaction of P-selectin with this ligand are identical to those required for interaction of P-selectin with intact cells (Hamburger and

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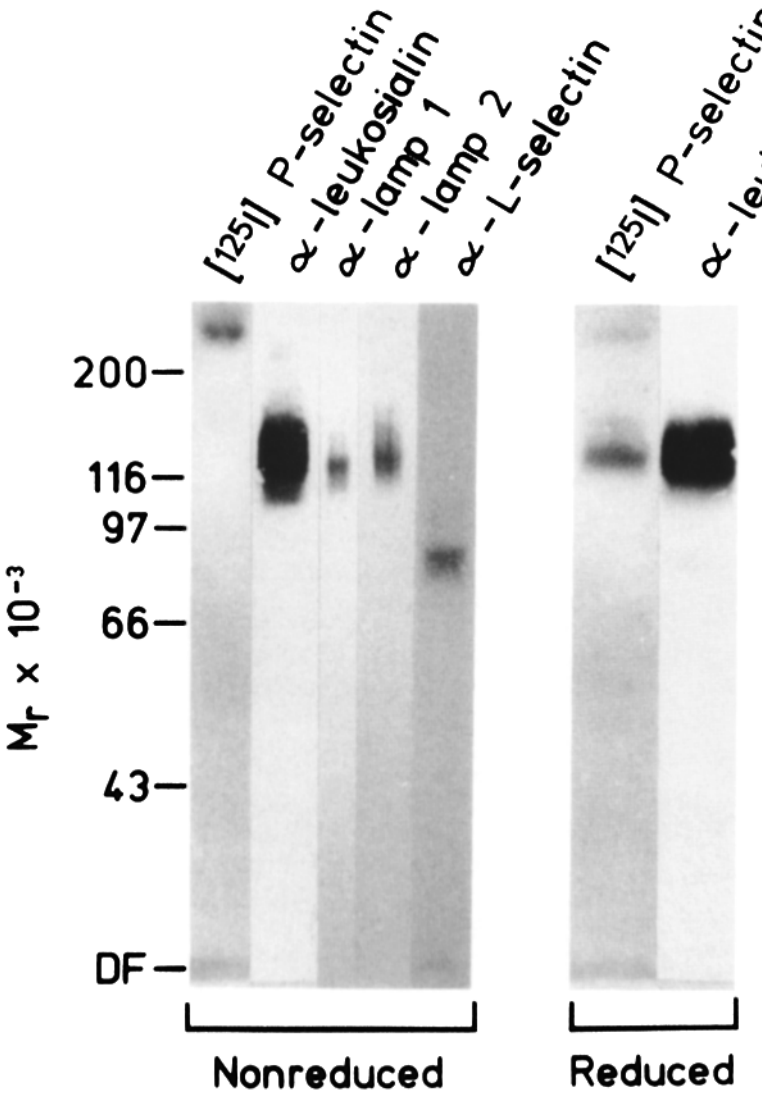

Figure 7. Western blotting and P-selectin blotting of neutrophil membrane proteins. Membrane extracts (200 μg protein/lane) were electrophoresed on 7.5% SDS-polyacrylamide gels under nonreducing or reducing conditions, transferred to Immobilon membranes, and probed with [^{125}I]P-selectin or murine monoclonal antibodies directed against human lamp-1 (*CR3*), human lamp-2 (*BB6*), human L-selectin (*DREG-200*), or human leukosialin (*Leu22*).

McEver, 1990; Geng et al., 1990; Moore et al., 1991; Corral et al., 1990; Zhou et al., 1991). Binding of P-selectin is Ca^{2+} dependent, is blocked by mAbs to P-selectin that inhibit the interaction between P-selectin and myeloid cells, and requires the presence of sialic acid residues on the ligand.

Sialidase treatment of both ligand-enriched neutrophil membrane proteins and metabolically labeled, affinity-isolated ligand from HL-60 cells results in a similar increase in apparent molecular weight, suggesting that the ligand is a heavily sialylated glycoprotein. Initial loss of sialic acids without loss of P-selectin binding could mean that the number of sialic acids may need to be reduced below a threshold level before loss of P-selectin binding is detectable in the blotting assay. Alternatively, sialic acid residues critical for P-selectin recognition may be relatively inaccessible to sialidase because of steric hindrance to enzyme action or because of substitutions that render them relatively resistant to digestion (Varki and Diaz, 1983).

Because the ligand is likely to be heavily sialylated, it may contain numerous N-and/or O-linked oligosaccharide chains terminated by sialic acid. PNGaseF digestion of both ligand-enriched neutrophil membrane proteins and metabolically labeled, affinity-isolated ligand from HL-60 cells causes a minor decrease in the apparent molecular mass of the ligand, but does not affect P-selectin binding. This demonstrates that the 120-kD ligand contains at least one N-linked chain that is not required for interaction with P-selectin. The glycans recognized by P-selectin may not be removed by PNGaseF because they reside on O-linked oligosaccharides. Alternatively, they might be N-linked structures that are resistant to cleavage by PNGaseF.

The differential mobility of the major ligand during SDS-PAGE in the presence and absence of reducing agents suggests that the native ligand is a disulfide-linked homodimer or that a 120-kD subunit is disulfide linked to a distinct subunit that is not directly involved in P-selectin binding. Since only a 120-kD band was detected after electrophoresis of reduced P-selectin eluate from metabolically labeled HL-60 cells, a heterodimer would have to consist of nonidentical subunits with the same apparent molecular weight and which undergo the same change in electrophoretic mobility after sialidase and PNGaseF digestion. Alternatively, the 120-kD–labeled subunit would have to be disulfide-linked to a subunit of similar apparent molecular weight that is not labeled with [^{3}H]glucosamine. A homodimeric ligand with

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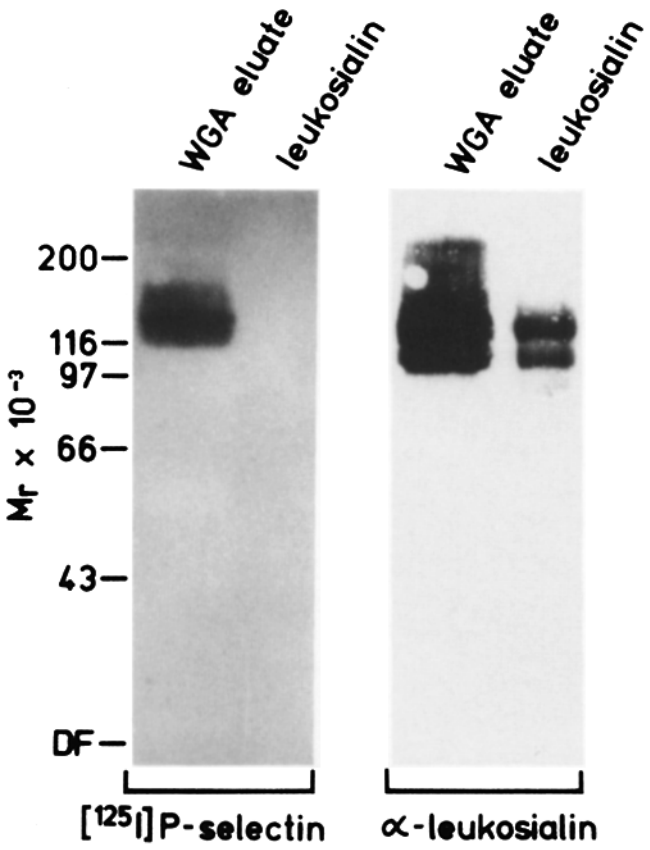

Figure 8. Western blotting and P-selectin blotting of leukosialin (CD43). Neutrophil WGA eluate (50 μg) and leukosialin purified from HL-60 cells (0.5 μg) were electrophoresed under reducing conditions on 7.5% SDS-polyacrylamide gels, transferred to Immobilon, and probed with [^{125}I]P-selectin. The same membrane was then probed with the monoclonal anti-human leukosialin antibody Leu22.

two equivalent binding sites might enhance the avidity of the interaction with P-selectin. The ability of [^{125}I]P-selectin to bind to the ligand after reduction and denaturation with SDS suggests that higher order structural features of the protein are not critical for recognition.

The blotting assay also detected two minor ligands. The first has an ≈250,000 M_r under reducing conditions. Because its mobility is identical to that of the major ligand under nonreducing conditions, it may represent a subpopulation of the major ligand that is resistant to reduction. The second has an ≈160,000 M_r under nonreducing conditions. Binding of P-selectin to both minor ligands was Ca^{2+} dependent and blocked by the mAb G1.

The isolation of a single glycoprotein from metabolically labeled HL-60 cells suggests that P-selectin has a marked preference for a particular ligand structure. It is noteworthy that L-selectin, which is expressed on leukocytes and binds to sialylated structures on endothelial cells (True et al., 1990), interacts preferentially with 50- and 90-kD sulfated, fucosylated glycoproteins from murine peripheral lymph nodes (Imai et al., 1991). Thus, both P-selectin and L-selectin appear to interact with a small subset of glycoprotein ligands.

It has been demonstrated that L-selectin on neutrophils carries the sialyl Le^x epitope and that a mAb to L-selectin partially blocks neutrophil adhesion to cells transfected with P-selectin cDNA (Picker et al., 1991b). Based on these observations, it was proposed that L-selectin on neutrophils is a predominant ligand for P-selectin. However, no direct interaction of L-selectin with P-selectin was demonstrated (Picker et al., 1991*b*). In the present study we have been unable to detect binding of P-selectin to L-selectin in neutrophil membrane extracts. Furthermore, the binding of P-selectin to intact neutrophils is unaltered by antibodies to L-selectin (this study) or by neutrophil activation that causes shedding of L-selectin from the cell surface (Moore et al., 1991). Although it is conceivable that L-selectin has weak affinity for P-selectin, the significance of this potential interaction remains to be established.

A recombinant P-selectin IgG chimera was shown to bind to myeloid cells and to sulfatide, Gal(3-SO_4) β1-Ceramide (Aruffo et al., 1991). Sulfatide also inhibited interaction of the chimera with monocytoid U937 cells (Aruffo et al., 1991). It was not demonstrated whether binding of the P-selectin chimera to the cells or to sulfatide was Ca^{2+} dependent, a fundamental characteristic of selectin-dependent cellular interactions (McEver, 1991). Protease digestion of intact cells should increase the accessibility of P-selectin to potential glycolipid ligands such as sulfatides (Watanabe and Hakomori, 1976; Stein et al., 1978). However, protease treatment abolishes binding of P-selectin to neutrophils (Moore et al., 1991) and HL-60 cells (Zhou et al., 1991; Aruffo et al., 1992) as well as adhesion of neutrophils to immobilized P-selectin (K.L. Moore and R.P. McEver, unpublished observations). In addition, although erythrocytes and platelets express sulfatides (Roberts et al., 1985; Hansson et al., 1978), they do not specifically interact with P-selectin (Moore et al., 1991; Larsen et al., 1989). Thus, it seems unlikely that sulfatides are the principal mediators of adhesion of myeloid cells to P-selectin. It remains to be determined whether sulfatides inhibit binding of P-selectin to myeloid cells by specific competition with a glycoprotein ligand or by indirect effects. It is possible that the P-selectin ligand we have described is sulfated or contains other structural features that are mimicked by sulfatides.

Previous studies have shown that P-selectin interacts with α(2-3) sialylated, α(1-3)fucosylated lactosaminoglycans, of which one is the sialyl Le^x tetrasaccharide (Zhou et al., 1991; Polley et al., 1991). However, several observations suggest that the sialyl Le^x tetrasaccharide per se does not bind with high affinity to P-selectin. First, some (Polley et al., 1991) but not all investigators (Moore et al., 1991; Aruffo et al., 1991) have found that sialyl Le^x inhibits interactions of myeloid cells with P-selectin. Second, CHO cells transfected with a fucosyltransferase express sialyl Le^x yet bind P-selectin with significantly lower affinity than do myeloid cells (Zhou et al., 1991). Third, HT-29 cells, which also express sialyl Le^x, do not interact at all with P-selectin (Zhou et al., 1991). Finally, several neutrophil membrane proteins known to carry the sialyl Le^x structure (Lee et al., 1990; Picker et al., 1991b; Asada et al., 1991; Fukuda et al., 1984), are distinct from the major glycoprotein ligand we have identified and do not bind P-selectin in the assays described here. These observations suggest that the ligand we have identified contains structural features in addition to the sialyl Le^x tetrasaccharide that enhance the affinity and/or specificity of its interaction with P-selectin.

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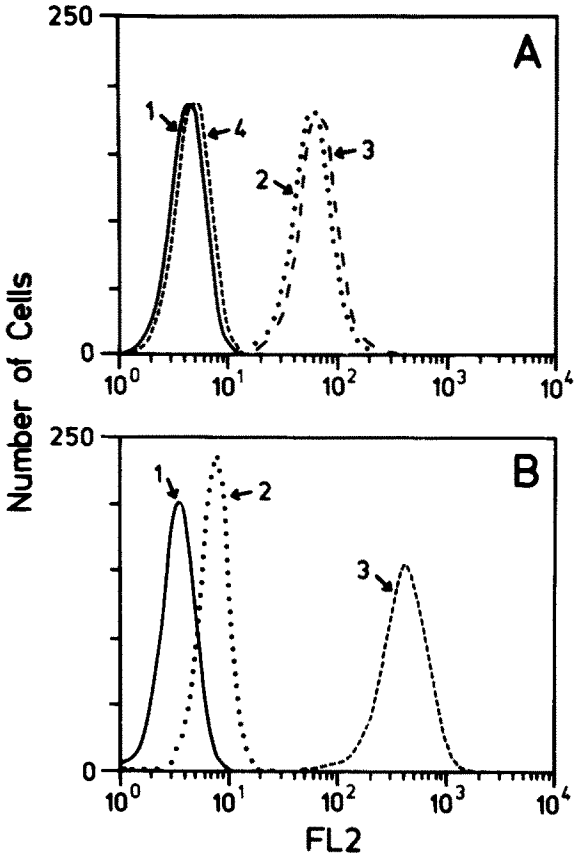

	mAb	P-selectin
1	None	–
2	None	+
3	DREG–56	+
4	G1	+

	DREG-56	PE-αIgG$_1$
1	–	–
2	–	+
3	+	+

Figure 9. Effect of anti-L-selectin mAbs on P-selectin binding to human neutrophils. (*A*) Neutrophils were preincubated for 15 min with buffer alone (*1* and *2*), 100 μg/ml of the anti-L-selectin monoclonal antibody (*mAb*) DREG-56 (*3*), or 100 μg/ml of the anti-P-selectin mAb G1 (*4*) before addition of buffer (*1*) or P-selectin (*2–4*). P-selectin binding was then detected by sequential incubation of the cells with biotinylated S12 (a noninhibitory monoclonal antibody to P-selectin) and phycoerythrin–streptavidin as described in Materials and Methods. Histograms 1 and 4 and histograms 2 and 3 were superimposable but were offset slightly for the purpose of clarity. (*B*) In parallel experiments binding of the anti-L-selectin mAb DREG-56 to the neutrophils was assessed by indirect immunofluorescence using a phycoerythrin-conjugated anti-murine IgG$_1$ antibody (*PE-αIgG$_1$*).

The methods used in this study to identify P-selectin ligands have certain limitations. The blotting procedure will not detect ligands whose function is destroyed by denaturation with SDS, nor will studies using metabolically-labeled HL-60 cells detect ligands which do not label with [^{3}H]glucosamine. Therefore we cannot exclude the possibility that other relevant P-selectin ligands exist. Nevertheless, affinity chromatography has the major advantage that it should identify the ligand(s) with relatively high affinity for P-selectin, which are likely to be physiologically relevant. Further studies are required to determine whether the ligand characterized here accounts for the limited number of binding sites for [^{125}I]P-selectin on intact neutrophils (Moore et al., 1991) and plays a role in adhesion of myeloid cells to P-selectin expressed on activated platelets and endothelial cells.

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