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

Kevin L. Moore,* Nancy L. Stults,† Sandra Diaz,‡ David F. Smith,‡ Richard D. Cummings,‡ Ajit Varki,§ and Rodger P. McEver‡

*Departments of Medicine and Biochemistry, St. Francis Medical Research Institute, University of Oklahoma Health Sciences Center and Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104; †Department of Biochemistry, University of Georgia, Athens, Georgia 30602, and §Department of Medicine and Cancer Center, University of California at San Diego, La Jolla, California 92093

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[^25I]P-selectin and affinity chromatography of[^3H]glucosamine-labeled HL-60 cell extracts were used to identify P-selectin ligands. A major ligand was identified with an =250,000 Mr 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^a, 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.

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., 1991a; 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^a 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-
hancing ligand affinity is supported by studies of CHO cells transplanted with a specific fusocysltransferase (Zhou et al., 1991). These cells express higher amounts of the sialyl Lea 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 modify 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 [3H]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)-agarose, pepstatin, aprotinin, N-acetylglucosamine, leupeptin, antipain, benzamidine, MOPS, Pipes, BSA, EDTA, IgG, and Ponceau S were purchased from Sigma Chemical Co. (St. Louis, MO). Difco, Difco Laboratories, Detroit, MI. Protein grade), and sialidase (neuraminidase) from Boehringer Mannheim Biochemicals (Indianapolis, IN). Peptide-N-glycosidase F (PNGaseF) from Flavobacterium meningosepticum (EC 3.2.2.18, N-glycanase) and endo-a-N-acetylgalactosaminidase from Diplodocus pneumoniae (EC 3.2.1.18), and rat liver (protein grade) were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Micro BCA protein assay kits and Lubrol PX (Surfact Amp Protein grade), and sialidase (neuraminidase) from Boehringer Mannheim Biochemicals (Indianapolis, IN). Peptide-N-glycosidase F (PNGaseF) from Flavobacterium meningosepticum (EC 3.2.2.18, N-glycanase) and endo-a-N-acetylgalactosaminidase from Diplodocus pneumoniae (EC 3.2.1.18), and rat liver (protein grade) were obtained from Calbiochem-Behring Corp. (La Jolla, CA).

**Antibodies and Proteins**

The anti-P-selectin murine mAbs SI2 and G1, and goat anti-human P-selectin IgG were prepared and characterized as previously described (McEvers and Martin, 1984; Geng et al., 1990; Lorsø et al., 1991). Rabbit polyclonal antiserum and murine mAbs to human lamp-1 (CD1) (Carlsson et al., 1988) and lamp-2 (BBB) (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 antibody.

1. Abbreviations used in this paper: Lamp, lysosomal-associated membrane protein; Nε2ε5εεε, 2.3-dehydro-2.3-dideoxy-N-acetylgalactosaminic acid; PNGaseF, Peptide-N-glycosidase F; WGA, wheat germ agglutinin.

**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 x 10^9 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 H2O for 10 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. Disosipropylfluorophosphate 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 Na2ATP, 3.5 mM MgCl2, 10 mM Pipes, pH 7.3 (relaxation buffer). To this suspension the following protease inhibitors were added at the indicated final concentrations: 2 mM disosipropylfluorophosphate, 20 µM leupeptin, 30 µM antipain, and 1 mM benzamidine. The cell suspension was pressured with N2 at 350 psi in a nitrogen bomb (model 4635; Parr Instrument Company, Moline, IL) for 40 min at 4°C with constant stirring as described previously (Borrengaard et al., 1983). The cavitate was collected into EGTA (2 mM final concentration) and nuclei and undispunited cells were pelleted at 500 g for 10 min at 4°C. The cavitate was fractionated as previously described (Eldund and Gabig, 1997). 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 r20) for 45 min at 4°C in a rotor (model SW28; Beckman Instruments, Inc., Palo Alto, CA). The top layer (FX1), the 40% sucrose layer (FX2), and the granule pellet (FX3) 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 (Borrengaard et al., 1983; Geng et al., 1990). Table I shows the distribution of marker enzymes in the various fractions. FX2 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 r20) for 60 min at 4°C in a rotor (model 50.2 Ti; Beckman Instruments, Inc.). The supernatant was collected as a P-selectin-Affigel 15 column (0.6 x 13 cm, 2 mg protein/ml resin). The column was then eluted with the above buffer containing 100 mM 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 x 20 cm, 76 µg 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 volume volumes of equilibration buffer, followed by two column volumes of 0.1 M NaCl, 10 mM MOPS, pH 7.5, 0.02% sodium azide, 0.1% 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, 0.1% Lubrol PX. The dialyzed WGA column eluate was made 1 mM in CaCl2 and MgCl2 and applied to a human serum albumin Affigel-15 precolumn (0.9 x 11 cm, 25 mg protein/ml resin) hooked in series to a P-selectin-Affigel 15 column (0.6 x 13 cm, 2 mg protein/ml resin). The columns were equilibrated with 0.1 M NaCl, 10 mM MOPS, pH 7.5, 1 mM MgCl2, 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

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Table 1. Distribution of Marker Enzymes from Subcellular Fractions of Nitrogen-cavitated Human Neutrophils

| Lactate dehydrogenase | Myeloperoxidase | Alkaline phosphatase |
|-----------------------|-----------------|---------------------|
| FX₁ (cytosol)         | 95.6 ± 0.5      | 0                   | 29.0 ± 2.7  |
| FX₂ (membrane)        | 4.1 ± 0.5       | 2.6 ± 1.0           | 58.6 ± 8.7  |
| FX₃ (granule)         | 0               | 97.4 ± 1.0          | 14.1 ± 5.5  |

Results are expressed as the percentage of the total enzyme activity in the cavi-

tate (mean ± SD, n = 3).

Flow Cytometry

Human neutrophils, isolated as previously described (Hamburger and McEver, 1990), were suspended (10⁶/ml) in HBSS containing 1% FCS and 0.1% sodium azide (HBSS/FCS/Az). 1 ml of neutrophil suspension was under-

laid 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 per-

formed at 4°C. After the last wash, the cells were fixed with 1 ml of 1% paraformaldehyde in HBSS and analyzed in a FACSscan 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 Ca²⁺-dependent, was blocked by G1, and was abolished by pre-

treatment of the cells with trypsin or sialidase (data not shown).

Immunoprecipitations

WGA eluate was incubated with 10 μg of anti-leukosialin (Leu22) or an iso-

type 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, 0.6 M Tris, pH 6.8, and 5% β-mercaptoethanol. Immunoprecipitates and immunosuperna-

tants 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. sialidase was assayed by incubation of dilutions of the enzy-

me with 50 ml of 4-methylumbelliferone-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 of 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 electro-

phoresed on 7.5% SDS-polyacrylamide gels, transferred to Immobion membranes, and probed with [3H]P-selectin. When samples were analyzed without reduction, P-selectin ligand was blocked by G1, and was abolished by pre-

treatment of the cells with trypsin or sialidase (data not shown).
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.

$\approx 250,000 \, M_r$ 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 $\approx 160,000 \, M_r$. When samples were analyzed after reduction, P-selectin preferentially bound to a glycoprotein with an $\approx 120,000 \, M_r$ (Fig. 1). Minor bands were observed at $\approx 250,000$ and $\approx 90,000$. 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 $\approx 5-7\% \, (n > 10)$ 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$_0$) or not effectively solubilized by nonionic detergent.

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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 pg; 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.

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 <0.1% 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 $\approx 150,000 \text{ M}_r$, which is similar to that of P-selectin itself. To determine whether this protein represented P-selectin that had leached 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 $\approx 120,000 \text{ M}_r$ under nonreducing conditions; a minor component migrates with an $\approx 250,000 \text{ M}_r$ (McEver and Martin, 1984). After reduction the protein migrates more slowly with an $\approx 150,000 \text{ M}_r$ (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; Policy 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
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}\text{I}]\text{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}\text{I}]\text{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-\(\alpha\)-\(N\)-acetylhexosaminidase (O-glycanase) abolished binding of \[^{125}\text{I}]\text{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
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 [125I]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 [3H]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 co-migrated under both nonreducing and reducing conditions with the major species detected in neutrophil and HL-60 cell membranes by blotting with [125I]P-selectin. Only 0.15–0.5% of the total [3H]glucosamine-labeled HL-60 glycoproteins bound to the P-selectin column, indicating that the ligand is not abundant. Sialidase treatment of the [3H]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 Leα 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 [125I]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 bind P-selectin.
bind \[^{125}\text{I}]\text{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\(^\alpha\) 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., 1991b). Although L-selectin is present in membrane extracts and WGA eluates of neutrophil membranes, as detected by Western blotting (Fig. 7), \[^{125}\text{I}]\text{P-selectin}\ did not bind to L-selectin in the blotting assay. In addition, the anti-L-selectin mAb DREG-56 (100 \mu g/ml) had no effect on the binding of purified P-selectin to quiescent neutrophils as assessed by flow cytometry (Fig. 9a). Parallel control assays showed that the neutrophils expressed high levels of L-selectin detectable by DREG-56 (Fig. 9b). 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 \(\approx 250,000\) and \(\approx 120,000\ M,\) 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
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 [3H]glucosamine. A homodimeric ligand with
two equivalent binding sites might enhance the avidity of the interaction with P-selectin. The ability of [\textsuperscript{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 \( \approx 250,000 \) Mr 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 \( \approx 160,000 \) Mr under nonreducing conditions. Binding of P-selectin to both minor ligands was Ca\(^{2+}\) dependent and blocked by the mAb Gl.

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 glycopolypeptides 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\(^{a}\) 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., 1991b). 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\(_4\)\(_3\)) \( \beta \)-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 Hako-mori, 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 \( \alpha(2-3) \) sialylated, \( \alpha(1-3) \) fucosylated lactosaminoglycans, of which one is the sialyl Le\(^{a}\) tetrasaccharide (Zhou et al., 1991; Polley et al., 1991). However, several observations suggest that the sialyl Le\(^{a}\) 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\(^{a}\) inhibits interactions of myeloid cells with P-selectin. Second, CHO cells transfected with a fucosyltransferase express sialyl Le\(^{a}\) 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\(^{a}\), do not interact at all with P-selectin (Zhou et al., 1991). Finally, several neutrophil membrane proteins known to carry the sialyl Le\(^{a}\) 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\(^{a}\) tetrasaccharide that enhance the affinity and/or specificity of its interaction with P-selectin.

Figure 8. Western blotting and P-selectin blotting of leukosialin (CD43). Neutrophil WGA eluate (50 \( \mu \)g) and leukosialin purified from HL-60 cells (0.5 \( \mu \)g) were electrophoresed under reducing conditions on 7.5\% SDS-polyacrylamide gels, transferred to Immobilon, and probed with [\textsuperscript{125}I]P-selectin. The same membrane was then probed with the monoclonal anti-human leukosialin antibody Leu22.
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 [3H]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 [125I]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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Representative authors and titles are cited here for context and to highlight key contributions to the field of selectin research. The full list of references is provided for further reading and exploration of the implications of these studies in the broader context of leukocyte adhesion and interactions with the vascular endothelium.