P-Selectin Mediates Adhesion of the Human Melanoma Cell Line NKI-4: Identification of Glycoprotein Ligands

Paul S. Kaytes and Jian-Guo Geng*

From Cell Biology and Inflammation Research, Pharmacia & Upjohn, Inc., Kalamazoo, Michigan 49001

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ABSTRACT: Activated endothelial cells and stimulated platelets express the cell adhesion molecule P-selectin (CD62P), which mediates adhesion to various leukocytes and certain types of cancer cells. In this study, we show Ca^{2+}-dependent binding of P-selectin to NKI-4 cells, a cell line derived from a human melanoma. The binding is inhibited by P7 (a leukocyte adhesion blocking mAb against P-selectin), but not by PL5 (a leukocyte adhesion blocking mAb against P-selectin glycoprotein ligand-1; PSGL-1). Further, expression of PSGL-1 could not be detected on NKI-4 cells by either PL5 mAb or an Ab against a synthetic peptide corresponding to a portion of the PSGL-1 sequence. P-selectin affinity chromatography of lysates from in vivo [^{3}H]-glucosamine-labeled NKI-4 cells resulted in the isolation of three glycoproteins, with apparent molecular masses of ∼250, ∼110, and ∼100 kDa under reducing conditions and ∼230, ∼105, and ∼85 kDa under nonreducing conditions. These molecules could be precipitated by P-selectin, but not by E-selectin. EDTA and the P7 mAb, but not the PL5 mAb, inhibited the binding of P-selectin to the purified ligands. Surprisingly, we found that sodium chlorate, a sulfation inhibitor, did not inhibit the binding of P-selectin to NKI-4 cells and that [^{35}S]-sulfate did not label the NKI-4 cell ligands. We conclude that P-selectin-dependent adhesion of the human melanoma cell line NKI-4 is mediated by a novel class of glycoprotein ligands.

P-selectin (CD62P) is a member of the selectin family of cell adhesion molecules. It is a presynthesized protein stored in the α granules of platelets and the Weibel-Palade bodies of endothelial cells. Upon thrombogenic and inflammatory challenges, P-selectin translocates (within minutes) to the cell surface by exocytosis, where it mediates rolling of leukocytes on stimulated endothelial cells and heterotypic aggregation of activated platelets to leukocytes. In addition, P-selectin can be abundantly expressed on stimulated endothelial cells by de novo synthesis (within hours) to further strengthen adhesive cellular interactions (*1*−*10*).

P-selectin interacts with PSGL-1,[1] a homodimeric protein composed of two disulfide bond linked subunits (the molecular mass of each unit is ∼120 kDa). PSGL-1 is expressed in a functional, selectin-binding form on a variety of human leukocytes, including neutrophils, monocytes, certain subsets of T-lymphocytes, and eosinophils (*11*−*15*). Molecular cloning of the molecule from a human promyeloid HL-60 cell library revealed that PSGL-1 is a type 1 membrane protein composed of ∼412 residues (*12*). The extracellular portion of the molecule contains three potential N-linked glycosylation sites (*12*), fifty-three predicted O-linked glycosylation sites (*16*), and three tyrosine sulfation sites (*17*, *18*). The high-affinity binding of P-selectin to PSGL-1 requires sulfation of at least one of the three tyrosine residues and an O-linked glycan on threonine-57. Both modifications are located in an anionic polypeptide region at the amino terminus of the mature, processed PSGL-1 molecule (*19*).

A recent increase in in vivo animal and clinical evidence indicates important roles of cell adhesion molecules, including P- and E-selectin, in cancer invasion and metastasis (*20*−*26*). In vitro, P-selectin has been shown to specifically bind to a variety of human cancers and human cancer-derived cell lines, such as colon cancer, lung cancer, including small cell lung cancer, breast cancer, gastric cancer, and neuroblastoma (*27*−*32*). E-selectin, another member of the selectin family, has also been reported to interact with a number of human cancers and human cancer-derived cell lines, such as colon cancer, breast cancer, gastric cancer, lung cancer, melanoma, and squamous cell cancer (*30*, *31*, *33*−*41*). However, the specific ligands for P- and/or E-selectin on these cancer cells have not been identified and characterized.

In the present study, we show that P-selectin specifically mediates adhesion to NKI-4 cells, a cell line derived from a human melanoma. We have also identified and partially characterized glycoprotein ligands for P-selectin on NKI-4 cells. Our results suggest that the P-selectin ligands on NKI-4 cells are functionally and structurally distinct from leukocyte PSGL-1. Thus, we conclude that they represent a novel class of glycoprotein ligands for P-selectin that are expressed on human cancer cells.

* To whom correspondence and reprint requests should be addressed: Cell Biology and Inflammation Research, 7239-267-307, Pharmacia & Upjohn, Inc., 301 Henrietta Street, Kalamazoo, MI 49001.

[1] Abbreviations: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; PSGL-1, P-selectin glycoprotein ligand-1; Rg, receptor globulin; SLe^x, sialyl Lewis x.

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EXPERIMENTAL PROCEDURES

Materials. An affinity-purified FITC-conjugated goat antibody against human IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Human IgG, mouse IgM, and Sephadex G-50 (medium) were purchased from Sigma (St. Louis, MO). Versine, Lipofectin, Geneticin (G418), dialyzed fetal calf serum, and sulfate-free RPMI medium 1640 (custom-made) were purchased from GIBCO BRL (Grand Island, NY). Protein A Sepharose-4 Fast Flow and Protein A-Sepharose CL-4B were purchased from Pharmacia Biotech (Uppsala, Sweden). The 48-well tissue culture plates (MULTIWELL) were purchased from Becton Dickinson Labware (Franklin Lakes, NJ). Sodium chlorate was purchased from Aldrich Chemical Co. (Milwaukee, WI). [^{35}S]-Sulfate was purchased from Amersham Life Science Inc. (Arlington Heights, IL).

Cell Lines. A cell line of human melanoma (NKI-4) was kindly provided by Dr. Ulrich Kunzendorf (Universitätsklinikum Steglitz, Berlin, Germany; *35*). A human promyeloid cell line (HL-60) was purchased from American Tissue Culture Collection (CCL 240; Rockville, MD). Both cell lines were cultured in a RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS; v/v), 4 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in the presence of 5% CO_2.

For establishment of a stable cell line expressing the full-length human PSGL-1, the entire coding sequence of PSGL-1 was subcloned into a mammalian expression vector, p3CLNeo (kindly provided by Dr. Michael J. Bienkowski, Cell Biology and Inflammation Research, Pharmacia & Upjohn; *42*). CHO-K1 cells were transfected with the plasmid of PSGL-1/p3CLNeo (10 μg/mL) using Lipofectin (*43*, *44*). The transfected CHO cells were selected and maintained in DME (high glucose) medium containing 10% FCS, 4 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.4 mg/mL of active Geneticin (w/v) at 37 °C in the presence of 5% CO_2 (44).

Proteins. P-Selectin was purified from out-dated human platelets using P23 mAb affinity chromatography (*13*). E-Selectin receptor globulin (Rg) was prepared as previously described (*15*).

P-Selectin Rg (lectin-EGF-repeat 1-repeat 2 domains fused with the F_c portion of human IgG_1) was constructed as previously described (*15*). The cDNA fragment amplified by polymerase chain reaction as well as its 5′ and 3′ junctions was verified by DNA sequencing. A stable CHO-K1 cell line secreting P-selectin Rg was established by cotransfection of the P-selectin Rg plasmid (10 μg/mL) with pcDNA3 (1 μg/mL; Invitrogen, San Diego, CA) using Lipofectin (*43*, *44*). The transfected cells were selected in 0.4 mg/mL active concentration of Geneticin and screened by a sandwich ELISA assay using an affinity-purified rabbit antibody to human IgG (Pierce, Rockford, IL) and a biotinylated P7 mAb to P-selectin (*14*). The cell line secreting the highest amount of P-selectin Rg was cultured in a serum-free medium (a modification of Ham's F12K medium supplemented with 2 μg/mL of bovine insulin, 2 μg/mL of phosphatidyl choline liposomes, and an additional gram of glucose per liter of medium). The harvested medium was loaded on a Protein A column (25 mL of Protein A Sepharose-4 Fast Flow) through a Sephadex G-50 guard column at 4 °C. The Protein A column was washed sequentially with (a) 50 mL of phosphate-buffered saline (PBS), pH 7.4, containing 1 M NaCl and 1% Triton X-100 (v/v), (b) 50 mL of PBS containing 1% Triton X-100 (v/v), and (c) 500 mL of PBS. The bound protein was eluted with 50 mM sodium acetate, pH 3.0. The eluted protein fractions were dialyzed into 27.3 mM Tris-phosphoric acid, pH 6.3, concentrated on a Mono-Q ion-exchange column (FPLC; Pharmacia Biotech, Uppsala, Sweden) with step elution by the same buffer containing 2 M NaCl. The purified P-selectin Rg was dialyzed against PBS and stored at −20 °C until use. NH_2-Terminal amino acid sequencing of P-selectin Rg revealed the following sequence: *X*TYHYSTKAY SW*X*ISRKY*X*Q NRYTDLVAI (*X*: ambiguous residue), fully consistent with the protein sequence of human P-selectin (*45*). The calculated $E_{280}^{1\%}$ for P-selectin Rg was 14.8, on the basis of the results from amino acid compositional analysis ($n = 3$).

Antibodies. Monoclonal IgG antibodies against P-selectin (P7, a leukocyte adhesion blocking mAb, and P23, a leukocyte adhesion non-blocking mAb) or E-selectin (E3, a leukocyte adhesion blocking mAb, and E2, a leukocyte adhesion non-blocking mAb), and monoclonal IgM antibodies against PSGL-1 (PL5, a leukocyte adhesion blocking mAb) or SLe^x (CSLEX), were prepared and characterized, as described previously (*13*, *15*, *41*, *44*).

Rabbit preimmune IgG and a PSGL-1 Ab against a synthetic peptide corresponding to residues 41−55 of the amino acid sequence of PSGL-1 were prepared as described (*44*). The PSGL-1 peptide Ab was initially isolated by Protein A chromatography and then further affinity purified on the immobilized synthetic peptide. The affinity-purified Ab was used in this study.

Flow Cytometric Assays. Confluent monolayers of NKI-4 cells were rinsed once with calcium and magnesium-free PBS and mechanically detached using cell scrapers (Nunc, Rochester, NY) in the presence of Versine. The detached cells were washed twice and resuspended in HBSS/FCS (Hanks' balanced salt solution with 1.26 mM $CaCl_2$ and 0.81 mM $MgCl_2$ containing 1% heat-inactivated FCS; 2×10^6 cells/mL). For experiments involving chlorate treatment, NKI-4 cells and HL-60 cells were cultured in the presence of 10 mM sodium chlorate in sulfate-free RPMI medium 1640 containing 10% dialyzed FCS for 16 h.

The washed cells (0.5 mL/aliquot) were incubated with 5 μg of human IgG, rabbit IgG, and mouse IgM (used as negative control), or P-selectin Rg, PSGL-1 peptide Ab, and PL5 mAb followed by 5 μg of a FITC-conjugated Ab against human IgG, rabbit IgG, or mouse IgM at 22 °C for 1 h with end-to-end rotation. Cells were sedimented (1000 rpm for 5 min on a tabletop centrifuge) and supernates were discarded. Each aliquot was then resuspended in 0.5 mL of HBSS/FCS for immediate flow cytometric analysis (FACScan; Becton Dickinson & Co., Mountain View, CA). The dose and time courses required to achieve "saturation" or "plateau" binding of P-selectin Rg were titrated in preliminary experiments. We found that, for one million cells, the maximal binding signal was obtained in less than 30 min using 3 μg of P-selectin Rg, for both HL-60 cells and NKI-4 cells (data not shown).

Cell Adhesion Assay. NKI-4 cells and HL-60 cells were labeled with 2 μM of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxy-

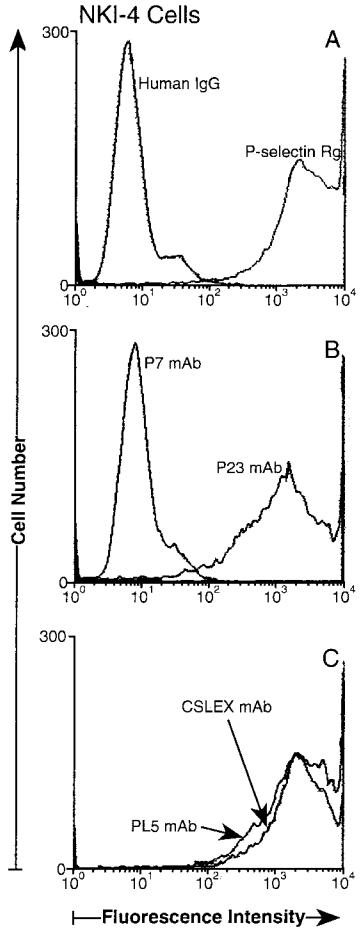

FIGURE 1: P-selectin binding to NKI-4 cells. NKI-4 cells were incubated with human IgG or P-selectin Rg and a FITC-conjugated Ab against human IgG. In antibody inhibition experiments, P-selectin Rg was preincubated with 30 μg of P23 (a leukocyte adhesion non-blocking mAb to P-selectin) or P7 (a leukocyte adhesion blocking mAb to P-selectin), and NKI-4 cells were preincubated with 30 μg of CSLEX (a mAb to SLe^x) or PL5 (a leukocyte adhesion blocking mAb to PSGL-1), at 22 °C for 30 min. The binding events were analyzed by flow cytometry (FACScan; Becton Dickinson & Co., Mountain View, CA). Results were presented as histograms of the log of fluorescence intensities from 10^4 cells. (A) Human IgG or P-selectin Rg binding to NKI-4 cells. (B) P-selectin Rg binding to NKI-4 cells in the presence of P7 mAb or P23 mAb. (C) P-selectin Rg binding to NKI-4 cells in the presence of PL5 mAb or CSLEX mAb.

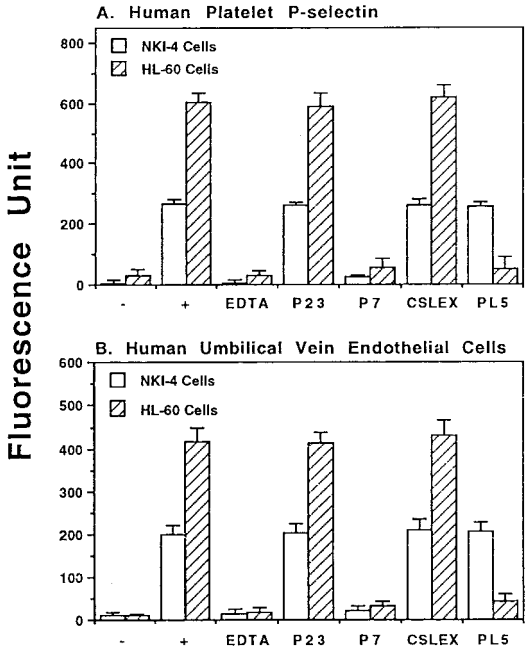

FIGURE 2: Adhesion of NKI-4 cells to P-selectin. Cell adhesion assays were carried out as previously described (*13*, *15*, *46*), using fluorescent dye labeled NKI-4 cells and HL-60 cells. Control wells containing immobilized FCS (panel A) or monolayers of 4α-PMA-stimulated human umbilical vein endothelial cells (panel B) were designated (−). Testing wells containing immobilized P-selectin (panel A) or monolayers of PMA-stimulated human umbilical vein endothelial cells (panel B) were designated (+). In chelating experiments, calcium- and magnesium-free HBSS/FCS in the presence of EDTA was used throughout the assay. In antibody inhibition experiments, the testing wells were preincubated with 30 μg of P23 mAb or P7 mAb and the fluorescent dye labeled NKI-4 cells and HL-60 cells were preincubated with 30 μg of CSLEX mAb or PL5 mAb, at 22 °C for 30 min. Results are expressed as the mean ± SD of fluorescence units from adherent cells determined in triplicate measurements of three separate assays.

fluorescein acetoxymethyl ester (BCECF-AM), as described previously (*13*). After being washed twice, the cells were resuspended in HBSS/FCS (2×10^6 cells/mL) and a 100-μL aliquot of cells was added into each well of 48-well tissue culture plates containing immobilized FCS or human platelet P-selectin (5 μg/mL of HBSS, 100 μL/well at 4 °C overnight) for 20 min at 22 °C. Alternatively, the BCECF-AM-labeled cells were added into 24-well tissue culture plates containing the confluent monolayers of human umbilical vein endothelial cells pretreated with PMA (phorbol 12-myristate 13-acetate) or its inactive analogue, 4α-PMA (*13*, *46*). Adhesion assays were performed as described previously (*13*, *46*). The cell numbers (~80 000 cells/well for maximal cell adhesion) and incubation times (less than 15 min for maximal cell adhesion) were titrated for both assays to ensure "saturation" or "plateau" conditions (data not shown).

Isolation of P-Selectin Ligands. Confluent NKI-4 cells were metabolically labeled with [^{3}H]-glucosamine (50 μCi/mL) in RPMI medium 1640 containing 10% FCS for 48−72 h. P-Selectin ligands were purified by P-selectin affinity chromatography, as previously described (*13*, *15*). The eluted fractions were pooled and dialyzed in dialysis tubing with a 12−14-kDa molecular mass cutoff (Spectra-Pore; Spectrum Medical Industries, Inc., Los Angeles, CA), which had been pretreated with 0.1% bovine serum albumin (w/v) to reduce nonspecific binding; samples were dialyzed against H_2O at 4 °C overnight. After dialysis, the samples were frozen in a dry ice/acetone bath and lyophilized to dryness in glass tubes. Samples were then redissolved in a small volume of PBS containing 0.01% Brij-35 (v/v) and 0.02% NaN_3 (v/w) and radioactivities were counted using a liquid scintillation counter (low-level detection; Packard Instrument Co., Mauritian, CT). Alternatively, the eluted fractions were dialyzed in 27.3 mM Tris−phosphoric acid, pH 6.3, 0.01% Brij-35 (v/v), and 0.02% NaN_3 (v/w) at 4 °C overnight. The fractions were then concentrated on a Mono-Q ion-exchange column (Smart System; Pharmacia Biotech, Uppsala, Sweden), equilibrated with the dialysis buffer. The bound molecules were step-eluted using equilibration buffer containing 2 M NaCl. Radioactive peaks were pooled and dialyzed against PBS containing 0.01% Brij-35 (v/v) and 0.02% NaN_3 (v/w) at 4 °C overnight.

Affinity Precipitation and Binding Assays. Aliquots of Protein A-Sepharose beads (1.5 g of protein A-Sepharose

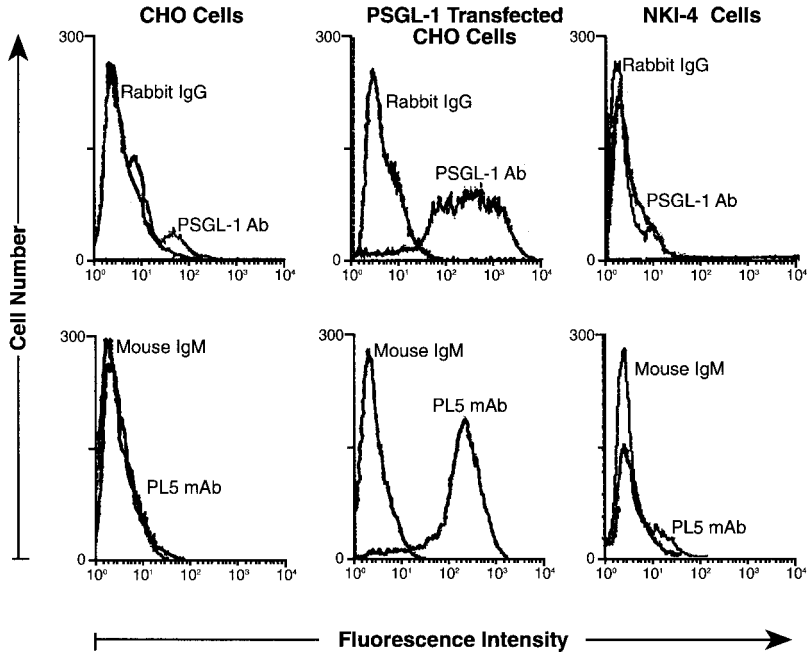

FIGURE 3: Staining of NKI-4 cells with PSGL-1 Abs. Parental CHO cells, PSGL-1 expressing CHO cells, and NKI-4 cells were incubated with rabbit IgG, PSGL-1 peptide Ab, mouse IgM, or PL5 mAb followed by a FITC-conjugated Ab against rabbit IgG or mouse IgM. The binding events were analyzed by flow cytometry, as described above. The upper panel shows the binding of rabbit IgG and PSGL-1 Ab, and the lower panel shows the binding of mouse IgM and PL5 mAb.

CL-4B in 30 mL of PBS and 0.02% NaN_3; 100 μL/aliquot) were incubated with 30 μg of human IgG, P-selectin Rg, or E-selectin Rg at 22 °C for 2 h. After washing three times with PBS, the beads were incubated with P-selectin ligands (100 000 cpm/tube for the precipitation assay and 5000 cpm/tube for the binding assay) in 200 μL of PBS containing 1 mM $CaCl_2$, 0.01% Brij-35 (v/v), and 0.02% NaN_3 (v/w), at 4 °C overnight with end-to-end rotation. After incubation, the beads were washed five times with the same buffer. P-Selectin ligands were isolated, from [^{3}H]-glucosamine-labeled NKI-4 cells, by P-selectin affinity chromatography and concentrated by Mono-Q ion-exchange chromatography, as described above. In the precipitation assay, samples were supplemented with SDS sample buffer in the presence of 5% β-mercaptoethanol (v/v), boiled for 5 min, and subjected to 7% SDS−PAGE followed by fluorography. In the binding assay, samples were resuspended in 200 μL of 50 mM glycine−HCl, pH 2.5, and boiled for 5 min. Following centrifugation, the supernates were collected. These steps were repeated once for complete recovery of the bound ligands. The two supernates for each sample were then combined and counted for radioactivity.

[^{35}S]-Sulfate Labeling of P-Selectin Ligands. Confluent NKI-4 cells were metabolically labeled with [^{35}S]-sulfate (50 μCi/mL), in a sulfate-free RPMI medium 1640 containing 10% dialyzed FCS for 16 h. NKI-4 cells metabolically labeled with [^{3}H]-glucosamine (50 μCi/mL) were used as a positive control for this experiment (see above). P-Selectin ligands from the [^{35}S]-sulfate- or the [^{3}H]-glucosamine-labeled NKI-4 cells were isolated by P-selectin affinity chromatography, as described above. The eluted fractions were pooled, dialyzed against H_2O, and lyophilized to dryness. Samples were then supplemented with SDS sample buffer containing 5% β-mercaptoethanol (v/v), boiled for 5 min, and subjected to 7% SDS−PAGE followed by fluorography.

In the [^{35}S]-sulfate and [^{3}H]-glucosamine incorporation experiments, confluent monolayers of NKI-4 cells in 96-well tissue culture plates were rinsed twice with PBS and labeled with [^{35}S]-sulfate and [^{3}H]-glucosamine (both at 50 μCi/mL, 0.2 mL/well) for 16 h, in either regular sulfate-containing RPMI medium 1640 containing 10% dialyzed FCS, or sulfate-free RPMI medium 1640 containing 10% dialyzed FCS and 10 mM sodium chlorate. Following washing five times with PBS, the cells were lysed with 1 N NH_4OH (0.2 mL/well) and radioactivities were counted in both the [^{35}S] and [^{3}H] channels.

RESULTS

Binding of P-Selectin to NKI-4 Cells. It has been reported that E-selectin binds to NKI-4 cells, a cell line derived from human melanoma (*35*). To investigate whether P-selectin is capable of binding to this cell line, a flow cytometric assay was used to measure the binding of P-selectin Rg. In this assay, the binding events were reported by a FITC-conjugated antibody to human IgG. Figure 1 shows that, in contrast to human IgG, P-selectin Rg bound avidly to NKI-4 cells (panel A). The binding specificity was verified by antibody inhibition experiments. As shown in Figure 1, P7 (a leukocyte adhesion non-blocking mAb to P-selectin) inhibited the binding of P-selectin Rg to NKI-4 cells, but P23 (a leukocyte adhesion not blocking mAb to P-selectin) or CSLEX (a mAb to SLe^x) did not (panels B and C). Interestingly, although PL5 (a leukocyte adhesion blocking mAb to PSGL-1) is capable of neutralizing the binding of P-selectin Rg to HL-60 cells (data not shown; *13*, *15*), it did not neutralize the binding of P-selectin Rg to NKI-4 cells (Figure 1C).

Adhesion of NKI-4 Cells to P-Selectin. Adhesion of NKI-4 cells and HL-60 cells to either isolated human platelet P-selectin, or PMA-stimulated human umbilical vein endothelial cells, was examined using cell adhesion assays.

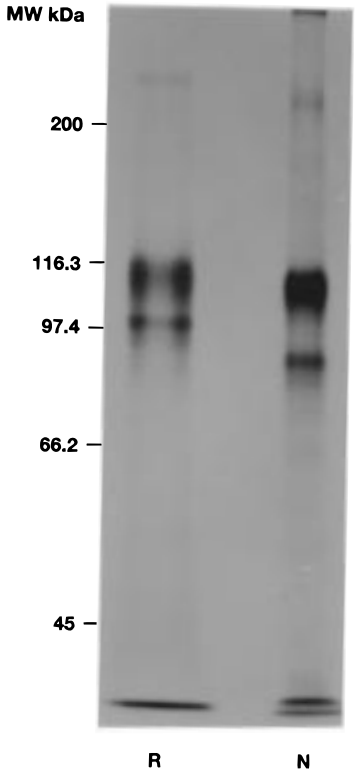

FIGURE 4: Isolation of P-selectin ligands from NKI-4 cells. NKI-4 cells were metabolically labeled with [^{3}H]-glucosamine, and P-selectin ligands were isolated by P-selectin affinity chromatography, with EDTA elution (*13*, *15*). The affinity-purified ligands were supplemented with SDS sample buffer, boiled for 5 min in the presence (R) or absence (N) of 5% β-mercaptoethanol (v/v), and subjected to 7% SDS−PAGE. The glycoproteins were detected by fluorography (*13*, *15*).

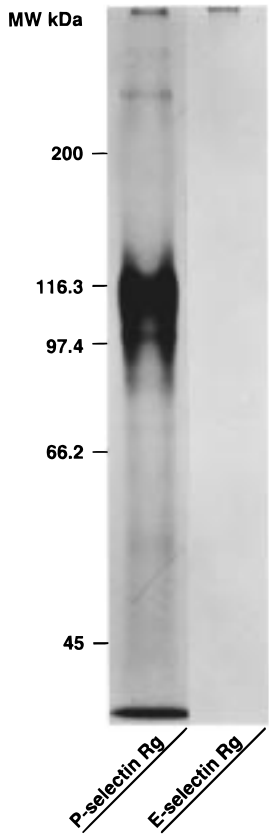

FIGURE 5: Precipitation of isolated P-selectin ligands. P-selectin ligands were purified from [^{3}H]-glucosamine-labeled NKI-4 cells by P-selectin affinity chromatography, as described in the legend to Figure 4. The isolated ligands were precipitated by P-selectin Rg or E-selectin Rg, previously bound to Protein A beads. After being washed, the beads were boiled for 5 min in SDS sample buffer in the presence of 5% β-mercaptoethanol (v/v) and subjected to 7% SDS−PAGE, and the glycoproteins were detected by fluorography.

Figure 2 shows that while NKI-4 cells and HL-60 cells both adhered to wells containing immobilized P-selectin (panel A) or to monolayers of human umbilical vein endothelial cells stimulated by PMA (panel B), they did not adhere to wells containing immobilized FCS or monolayers of human umbilical vein endothelial cells stimulated by 4α-PMA (designated−in panels A and B). Notably, if compared to HL-60 cells, less NKI-4 cells were apparently bound to both immobilized P-selectin (panel A) and PMA-stimulated human umbilical endothelial cells (panel B), under identical experimental conditions. This difference probably reflects variations in avidity and/or copy number of ligands for P-selectin between these two cell lines (*4*).

The adhesion of NKI-4 cells to P-selectin was inhibited by P7 (a leukocyte adhesion blocking mAb to P-selectin), but not by P23 (a leukocyte adhesion non-blocking mAb to P-selectin) or CSLEX (a mAb to SLe^x). Although PL5 (a leukocyte adhesion blocking mAb to PSGL-1) inhibited the adhesion of HL-60 cells to immobilized P-selectin (panel A) or PMA-stimulated human umbilical vein endothelial cells (panel B), it did not inhibit the adhesion of NKI-4 cells. Taken together, these results suggest an interaction between P-selectin and NKI-4 cells that is specific but distinct from the interaction between P-selectin and PSGL-1.

Absence of PSGL-1 on NKI-4 Cells. To corroborate these findings, we investigated whether NKI-4 cells expressed PSGL-1 molecules on their surface. Figure 3 shows that, in contrast to rabbit IgG and mouse IgM, both the PSGL-1 peptide Ab and the PL5 mAb bound avidly to CHO cells expressing PSGL-1, but did not bind to parental CHO cells. However, neither antibody appeared to recognize NKI-4 cells. These results confirm the specificity of these antibodies (*13*, *15*) and, more importantly, indicate the absence of PSGL-1 on the surface of NKI-4 cells. Thus, this finding provides independent, but convergent evidence that PSGL-1 is not the counter-ligand for P-selectin on NKI-4 cells.

Identification of P-Selectin Ligands on NKI-4 Cells. To isolate the ligand(s) for P-selectin on NKI-4 cells, we metabolically labeled cells with [^{3}H]-glucosamine and loaded detergent lysates on a P-selectin affinity column in the presence of calcium. After extensive washing in a Ca^{2+}-containing buffer, the bound protein(s) was(were) eluted from the P-selectin affinity column by EDTA (*13*, *15*). Figure 4 shows that the P-selectin column bound three glycoproteins, with apparent molecular masses of ~250, ~110, and ~100 kDa under reducing conditions and ~230, ~105, and ~85 kDa under nonreducing conditions, from the [^{3}H]-glucosamine-labeled NKI-4 cells. The ~110- and ~100-kDa bands appeared more intense than the ~250-kDa band.

These findings suggest a Ca^{2+}-dependent interaction between P-selectin and the ~250-, ~110-, and ~100-kDa molecules from NKI-4 cells. The slower mobilities under reducing conditions are an indication of intramolecular disulfide bonds in these ligands. The ~250-, ~110-, and ~100-kDa ligands on NKI-4 cells may represent three

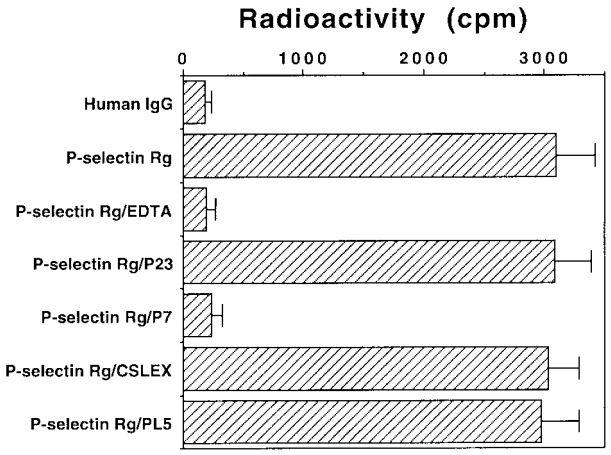

FIGURE 6: The binding specificity of the NKI-4 cell ligands. P-selectin ligands were isolated from [^{3}H]-glucosamine-labeled NKI-4 cells by P-selectin affinity chromatography, as described in the legend to Figure 4. The isolated molecules were precipitated with human IgG or P-selectin Rg, previously bound to Protein A beads. In chelation experiments (designated EDTA), EDTA, instead of $CaCl_2$, was used throughout the assay, including all incubation and washing steps. In antibody inhibition experiments, the P-selectin Rg bound beads were preincubated with 30 μg of P23 mAb or P7 mAb and the P-selectin ligands were preincubated with 30 μg of CSLEX mAb or PL5 mAb, at 22 °C for 30 min. After being extensively washed, the bound proteins were eluted by boiling the beads in 50 mM glycine−HCl, pH 2.5, and the amount of radioactivity in the supernates was determined. All results were expressed as the mean ± SD of radioactivities determined in triplicate measurements of three separate experiments.

distinct molecules. Alternatively, the three bands on SDS−PAGE could also originate from the same molecule. The ~250-kDa molecule could be a precursor for the ~110- and ~100-kDa molecules, which might be generated by proteolytic cleavage during post-translational processing. Further experiments are required to clarify these possibilities.

The specificity of the interaction between P-selectin and the NKI-4 cell ligands was confirmed by affinity precipitation and binding experiments. The affinity precipitation experiment showed that P-selectin Rg, but not E-selectin Rg, could precipitate P-selectin ligands isolated from NKI-4 cells (Figure 5). In the affinity binding assay, we found that P-selectin Rg, but not human IgG, bound to these ligands (Figure 6). EDTA abolished this binding, confirming that the interaction of P-selectin with these molecules was divalent cation-dependent (presumably Ca^{2+}-dependent). Furthermore, P7 (a leukocyte adhesion blocking mAb to P-selectin) inhibited the binding, but P23 (a leukocyte adhesion non-blocking mAb to P-selectin), CSLEX (a mAb to SLe^x), and PL5 (a leukocyte adhesion blocking mAb to PSGL-1) did not. These results suggest that P-selectin specifically interacts with the NKI-4 cell ligands. Again, the PL5 mAb does not inhibit the interaction between P-selectin and the purified NKI-4 cell ligands, a finding which is consistent with the previous results (Figures 1 and 2).

Absence of Sulfate on P-Selectin Ligands. Since tyrosine sulfation of PSGL-1 reportedly is required for high-affinity binding to P-selectin (*17*, *18*), we investigated whether sodium chlorate, a metabolic inhibitor of ATP sulfurylase activity, had any effect on the binding of P-selectin to NKI-4 cells. Figure 7 shows that, in contrast to human IgG (part A1), P-selectin Rg bound to HL-60 cells cultured in a regular

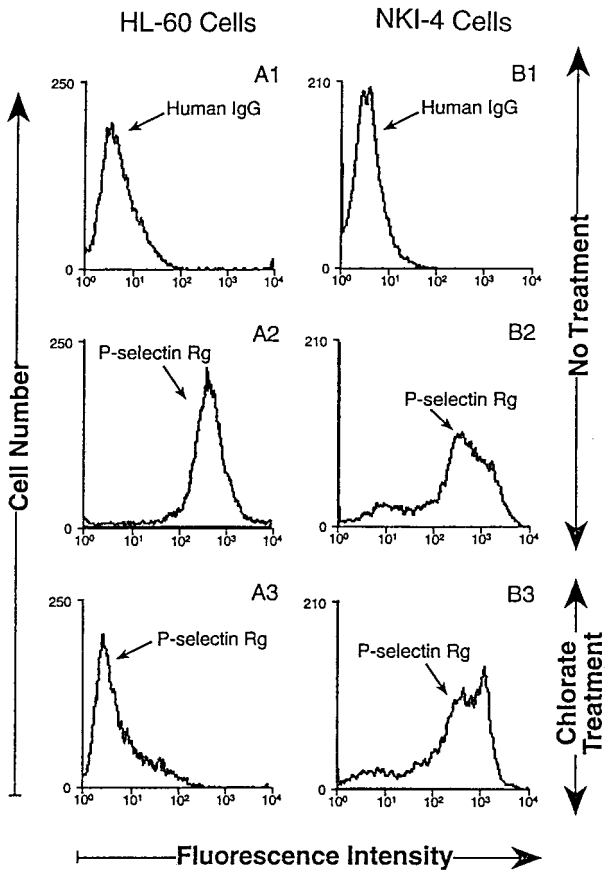

FIGURE 7: Effects of sodium chlorate on P-selectin binding to NKI-4 cells. NKI-4 cells were cultured either in a regular sulfate-containing medium or in a sulfate-free medium in the presence of sodium chlorate. Cells were then incubated with human IgG or P-selectin Rg and a FITC-conjugated Ab against human IgG. The binding events were examined by flow cytometry, as described in the legend to Figure 1.

sulfate-containing medium (part A2), and as expected, sodium chlorate inhibited the binding of P-selectin Rg to HL-60 cells cultured in a sulfate-free medium (part A3). Likewise, in contrast to human IgG (part B1), P-selectin Rg bound avidly to NKI-4 cells (part B2). However, sodium chlorate did not abolish the binding of P-selectin Rg to NKI-4 cells (part B3).

To ensure that, under the experimental conditions used, sodium chlorate did in fact inhibit the incorporation of sulfate into these cells, we metabolically labeled NKI-4 cells with both [^{35}S]-sulfate and [^{3}H]-glucosamine, either in regular sulfate-containing medium or in sulfate-free medium in the presence of sodium chlorate. We found that there were virtually no differences for the incorporation of [^{3}H]-glucosamine between the cells cultured either in the regular medium (529 471 ± 18 362 cpm/well, $n = 6$) or in the sulfate-free medium in the presence of sodium chlorate (530 139 ± 20 127 cpm/well, $n = 6$). By contrast, we observed significant differences in incorporation of [^{35}S]-sulfate between cells cultured in the regular medium (181 754 ± 9613 cpm/well, $n = 6$) and in the sulfate-free medium in the presence of sodium chlorate (9158 ± 417 cpm/well, $n = 6$). These results demonstrate that sodium chlorate inhibited ~95% of the incorporation of [^{35}S]-sulfate into the NKI-4 cells.

To corroborate the above findings, we investigated whether the NKI-4 cell ligands were modified post-translationally

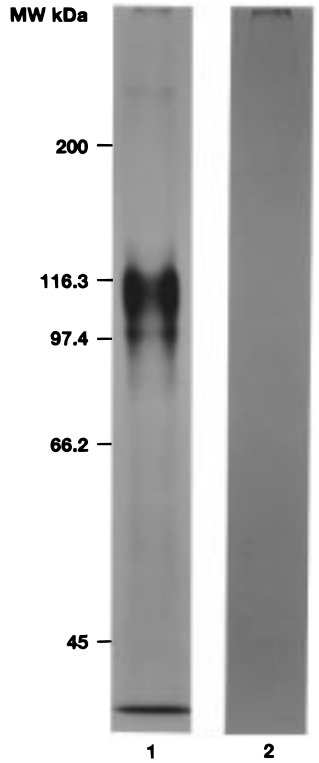

FIGURE 8: Isolation of P-selectin ligands from [^{35}S]-sulfate-labeled NKI-4 cells. P-selectin ligands were isolated, by P-selectin affinity chromatography, from NKI-4 cells labeled with [^{3}H]-glucosamine (lane 1) or [^{35}S]-sulfate (lane 2). The purified ligand molecules were supplemented with SDS sample buffer, boiled for 5 min in the presence of 5% β-mercaptoethanol (v/v), and subjected to 7% SDS−PAGE. The proteins were visualized by fluorography.

with sulfate. In these experiments, NKI-4 cells were metabolically labeled with either [^{35}S]-sulfate or [^{3}H]-glucosamine and the ligand molecules were isolated by P-selectin affinity chromatography, as described above. Figure 8 shows that P-selectin affinity chromatography yielded the expected ligands from the [^{3}H]-glucosamine-labeled NKI-4 cells (lane 1; compare Figure 4). However, the column failed to bind any molecules from the [^{35}S]-sulfate-labeled NKI-4 cells (lane 2). These results, together with the findings from the chlorate treatment, indicate the absence of sulfate modifications on the NKI-4 cell ligands.

DISCUSSION

In the present study, we have shown that P-selectin specifically binds to and mediates adhesion of NKI-4 cells, a cell line derived from a human melanoma. We also found that PL5, a leukocyte adhesion blocking mAb to PSGL-1, did not neutralize the binding of P-selectin to NKI-4 cells (Figures 1 and 2) and that neither the PSGL-1 peptide Ab nor the PL5 mAb stained NKI-4 cells (Figure 3). These findings suggest that NKI-4 cells do not express PSGL-1 and that they instead express other molecule(s) as the counter-ligand(s) for P-selectin.

Using P-selectin affinity chromatography, we isolated three glycoproteins from [^{3}H]-glucosamine-labeled NKI-4 cells (Figure 4). The specificity for the interaction of P-selectin with the ligands isolated from the NKI-4 cells was confirmed by affinity precipitation and binding assays (Figures 5 and 6). In contrast to the human leukocyte ligand PSGL-1, the NKI-4 cell ligands do not appear to be modified by sulfate (Figures 7 and 8). A detailed comparison of the functional and structural characteristics of P-selectin binding to NKI-4 cells and HL-60 cells is listed in Table 1. The data clearly indicate that the NKI-4 cell ligands are functionally and structurally distinct from leukocyte PSGL-1. Thus, we conclude that the human melanoma cell line NKI-4 expresses a novel class of glycoprotein ligands for P-selectin.

Tyrosine sulfation on the amino terminus of the mature, processed PSGL-1 molecule reportedly is required for high-affinity binding to P-selectin (*17*, *18*). However, during the functional and structural characterization of the NKI-4 cell ligands, we found that although sodium chlorate, a metabolic inhibitor of ATP sulfurylase activity, abolished ~95% of the incorporation of [^{35}S]-sulfate, it had no inhibitory effect on the binding of P-selectin to the cells. We were also unable to label the P-selectin ligands from NKI-4 cells with [^{35}S]-sulfate. These results suggest that a distinct mechanism may be involved in the interaction of P-selectin with the NKI-4 cell ligands. Additional studies on the structural and functional details of this mechanism are required to fully understand the differences in the interaction of P-selectin with PSGL-1 and with the NKI-4 cell ligands.

In conclusion, our experimental results suggest that P-selectin specifically mediates cell adhesion of the human melanoma cell line NKI-4 and that NKI-4 cells express a novel class of glycoprotein ligands for P-selectin. We believe that an extension of this line of investigation should provide insights in (a) the protein and carbohydrate structures of the NKI-4 cell ligands recognized by P-selectin and (b) the role(s) of these cell adhesion molecules in the invasion and metastasis of human melanoma.

Table 1: A Comparison between NKI-4 Cells and HL-60 Cells for P-Selectin Recognition

NKI-4 cells	HL-60 cells	references
	Functional	
PSGL-1 mAb (PL5) does not inhibit P-selectin binding	PSGL-1 mAb (PL5) inhibits P-selectin binding	*13*, *15*
P-selectin, but not E-selectin, interacts with the NKI-4 cell ligands for P-selectin	P- and E-selectin both interact with PSGL-1	*12*, *14*, *15*
sodium chlorate does not inhibit P-selectin binding	sodium chlorate inhibits P-selectin binding	*17*, *18*
	Structural	
~250, ~110, and ~100 kDa under reducing conditions and ~230, ~105, and ~85 kDa under nonreducing conditions	~120 kDa under reducing conditions and ~240 kDa under nonreducing conditions	*11*−*13*, *15*
absence of sulfate modification	tyrosine sulfation	*17*, *18*
	Cell Surface	
absence of PSGL-1	presence of PSGL-1	*11*−*15*

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