Mouse P-Selectin Glycoprotein Ligand-1: Molecular Cloning, Chromosomal Localization, and Expression of a Functional P-Selectin Receptor

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A mouse homolog of P-selectin glycoprotein ligand-1 (PSGL-1), a P-selectin receptor on myeloid cells, has been cloned using the human cDNA sequence to probe a cDNA library prepared from the mouse WEHI-3 monocytic cell line and a genomic DNA library prepared from 129/SvJ mouse tissue. The gene flanking the entire open reading frame of 397 amino acids is composed of a single exon. Mouse and human PSGL-1 show an overall similarity of 67% and an identity of 50% and contain a similar domain organization. However, there are 10 threonine-serine-rich decameric repeats in human PSGL-1. When the mouse PSGL-1 cDNA is coexpressed with an α1,3/1,4 fucosyltransferase cDNA in COS cells, a functional protein is expressed on the COS cell surface mediating binding to human P-selectin. The mouse PSGL-1 gene, Selpl, was mapped to a position on mouse chromosome 5 (Chr 5). Northern blot analyses of mouse tissues showed moderate expression of a PSGL-1 mRNA species in most tissues including heart, kidney, liver, muscle, ovary, and stomach and high levels of expression in blood, bone marrow, brain, adipose tissue, spleen, and thymus. Whereas certain mouse myeloid cell lines including PU5-1.8, WEHI-3B, and 32DC13 express high levels of PSGL-1 mRNA, only WEHI-3B and 32DC13 bind to P-selectin; this interaction is blocked by anti-PSGL-1 antibody. WEHI-3B cells bind significantly better to P-selectin than to E-selectin. Although comparable P-selectin binding is observed in 32DC13 cells, these cells bind better to E-selectin. Binding of 32DC13 cells to E-selectin is not blocked by anti-PSGL-1 antibody. Treatment of WEHI-3B cells with trypsin or neuraminidase abolished their ability to interact with P-selectin. These results indicate that mouse PSGL-1 has structural and functional homology to human PSGL-1 but is characterized by differences in the composition and number of the decameric repeats. PSGL-1 on mouse myeloid cells is critical for high-affinity binding to P-selectin but not to E-selectin.

The selectin family of adhesion molecules is crucial for the initial interaction of circulating leukocytes with the vascular endothelium at the site of injury. The three members of the selectin family include P-selectin, L-selectin, and E-selectin. L-selectin is expressed on a subset of lymphocytes and leukocytes. P-selectin and E-selectin are expressed on activated endothelial cells. In addition, P-selectin is expressed on activated platelets. All selectin molecules contain an N-terminal domain homologous to Ca++ dependent lectins that recognize carbohydrate determinants related to sialyl Lewisx and sialyl Lewisx structures. Binding of selectins to leukocytes or endothelium is mediated through a restricted number of glycoprotein ligands. Three mucin-like molecules including GlyCAM-1, CD34, and MadCAM-1 have been identified as L-selectin ligands of potential physiological significance. Similar to other mucin-like molecules, these L-selectin ligands contain long repetitive sequences rich in serine and threonine that provide attachment sites for extensive O-glycosylation. Oligosaccharide modifications of sialylation, sulfation, and, probably, fucosylation are required for L-selectin binding. Although sialyl Lewisx itself provides stable interaction with E-selectin, a 150-kD glycoprotein that binds to E-selectin has been identified. The cDNA sequence of this protein, E-selectin ligand-1 (ESL-1), shows strong homology to the chicken fibroblast growth factor receptor.

The P-selectin glycoprotein ligand-1 (PSGL-1), another mucin-like molecule, was identified in human neutrophils and HL-60 cells. The cDNA for this protein derived from HL-60 cells has been coexpressed with a cDNA encoding an α1,3/1,4 fucosyltransferase (FT-III). PSGL-1 shows a large amount of sialylated, fucosylated O-linked carbohydrates, and it was shown to mediate in vitro rolling of neutrophils on P-selectin. The human PSGL-1 purified from neutrophils and the recombinant protein expressed from the COS cells binds to E-selectin. A distinct function of PSGL-1 for P-selectin binding has been suggested in an in vitro T-lymphocyte rolling assay. Polyclonal antibodies against PSGL-1 interfered with rolling of T lymphocytes on P-selectin but showed no effect on the attachment of T cells to E-selectin.

To determine the physiological role of PSGL-1 in vivo and to learn if PSGL-1 is the biologically important P-selectin ligand on myeloid cells, we have begun to investigate the biology of PSGL-1 in the mouse. In this study, we have cloned the cDNA and the gene encoding the mouse homolog of PSGL-1, determined the chromosomal location of the mouse PSGL-1 gene and the tissue distribution of PSGL-1 mRNA, and characterized the functional properties of PSGL-1 expressed on myeloid and heterologous cells. Mouse PSGL-1 shares structural and functional homology with the human PSGL-1, but is also characterized by significant differences.

Materials and Methods

Materials. Restriction enzymes, DNA-modifying enzymes, and DNA molecular weight markers were from Gibco-BRL (Gaithersburg, MD) or Boehringer Mannheim (Mannheim, Germany). [32P]Deoxyctydine triphosphate (6,000 Ci/mmol), [35S]Deoxyadenosine triphosphate (1,000 Ci/mmol), and [32P]Adenosine triphosphate were from Amersham. [3H]Deoxyadenosine triphosphate (1,000 Ci/mmol), and [3H]Deoxythymidine triphosphate (6,000 Ci/mmol), [32P]Deoxyctydine triphosphate (6,000 Ci/mmol), [35S]Deoxyadenosine triphosphate (1,000 Ci/mmol), and [32P]Adenosine triphosphate were from Amersham.
(6,000 Ci/mmol) were from Dupont-New England Nuclear (Boston, MA). The deoxyribonucleotide triphosphate was from Pharmacia (Uppsala, Sweden). Synthetic oligonucleotide probes were prepared on an Applied Biosystems DNA Synthesizer (Applied Biosystems, Foster City, CA). WEHI-3 cells and PU-5 cells were obtained from American Tissue Culture Collection (ATCC; Rockville, MD). WEHI-3B cells and 32DC13 cells were a gift from Dr. G. Rovera (Wistar Institute, Philadelphia, PA). The plasmids expressing α1,3/1,4 fucosyltransferase (pEAPFTIII) and human PSGL-1 were a gift from Dr. D. Sako (Genetics Institute, Cambridge, MA). The CD43 expressing anti-peptide derived from the first 19 residues after the putative propeptide cleavage site of mouse PSGL-1 (Fig 1A) and against the soluble protein of human PSGL-1 were gifts from Genetics Institute. Rabbit antihuman P-selectin antibodies (SA-1) were raised in this laboratory against the purified protein.

Radioisotope labeling of DNA. DNA fragments were labeled with [32P]deoxyctydine triphosphate to a specific activity of greater than 10⁶ cpm/µg DNA by the random oligonucleotide primer method. Buffer and free nucleotides were removed by passing through a Push Column (Stratagene, La Jolla, CA).

Screening of genomic and cDNA libraries. Approximately 1.5 × 10⁶ plaques from an adult 129/SvJ mouse liver genomic DNA library cloned into 1 FIX II (#946305; Stratagene) was screened using a probe derived from a 1.6-kb insert of the full length human PSGL-1 cDNA (Genetics Institute). Hybridization was performed at 65°C overnight in a sodium chloride, sodium phosphate, and EDTA (SSPE) hybridization buffer containing 5× SSPE, 0.15% sodium dodecyl sulfate (SDS), and 10 µg/ml salmon sperm DNA. The filters were washed in 1× sodium chloride and sodium citrate (SSC) containing 0.2% SDS at room temperature for 30 minutes. Phage DNA samples from five positive clones were isolated and analyzed by restriction digestion followed by Southern blot hybridization using the full length probe and a probe derived from the cytoplasmic tail. Restriction fragments of interests were subcloned into the pBlue-script II vector (Stratagene).

A mouse macrophage (WEHI-3) cDNA library in the Uni-ZAP vector (#937306; Stratagene) was screened. The probe was prepared by polymerase chain reaction (PCR) amplification of the cytoplasmic tail of PSGL-1 using the human PSGL-1 cDNA or the mouse genomic DNA clones and primer pairs P7 (5’-AAGGG CCCGA TGGTC TCCCGT G3’) and P8 (5’-CTAAG GGAGG AAGGT GTGCA G3’) derived from the human PSGL-1 cDNA sequence. The filters were finally washed with 0.2× SSC containing 0.2% SDS at 45°C for 15 minutes. Positive clones were present in a frequency of about 1 in every 5 × 10⁶ plaques. Positive clones were purified and subjected to in vitro excision. Plasmid DNA from five of these clones was further purified and analyzed by restriction digestion and sequencing. A cDNA artifact was found in two of these clones because they contained a chimeric molecule containing a 5’ sequence of an unknown species.

DNA sequencing. Sequencing of plasmid DNA was performed using the Sequenase Version 2.0 kit from US Biochemicals. Overlapping sequences were obtained by using oligonucleotide primers from both strands or from more than one independent primer in the same orientation. The final sequence was assembled and analyzed using the programs of the Genetics Computer Group (Madison, WI).

Reverse transcription-PCR (RT-PCR) analysis. Poly(A)+ RNA was prepared from WEHI-3 cells using the FastTrack mRNA isolation method (Invitrogen, San Diego, CA). The RT reaction was performed using 1 µg of random hexamers and poly(A)+ RNA using the cDNA Cycle kit from Invitrogen. Subsequent PCRs were performed in standard buffer (Boehringer Mannheim) containing 1.75 mM MgCl₂. About 5% of the first-strand cDNA products were amplified using 50 pmol of primer P1 and P3 (Fig 1A) in the presence of 0.1 mM of each deoxyribonucleotide triphosphate and 2.5 U Taq DNA polymerase (Boehringer Mannheim). The reactions were subjected to 25 cycles of denaturing at 94°C for 1 minute, annealing at 63°C for 0.5 minutes, and extension at 72°C for 1 minute.

Construction of a mouse PSGL-1 expression vector. The P1:P3 PCR fragment obtained by RT-PCR was purified from agarose gel using the QIAEX Gel Extraction kit (Qiagen, Chatsworth, CA) and was subcloned into the pCR II vector using the TA Cloning kit from Invitrogen. The PCR product was sequenced using synthetic oligonucleotides. An approximately 0.8-kb BamHI/Acc I insert containing the complete 5’ PSGL-1 sequence was isolated and recombined into the BamHI/Acc I sites of a 1.3-kb partial cDNA clone. The vector was linearized with Xho I and blunt-ended using T4 DNA polymerase. The full length cDNA insert was released by digestion with Xba I, purified from agarose gel and cloned into the Xba I/Sma I sites of the pED expression vector (Genetics Institute).

Southern blot analysis. Mouse lung genomic DNA (10 to 15 µg) from C57BL/6J mice was extracted using SDS and proteinase K, was exhaustively digested with restriction enzymes, and was electrophoresed in a 0.8% agarose gel. The gel was transferred to Duralon-UV membrane (Stratagene), bonded in a Stratalinker (Stratagene), and hybridized in the SSPE hybridization buffer as described above. The probe derived from the repeat region of the mouse PSGL-1 cDNA was PCR-amplified using the longest partial cDNA clone and primer T3 (5’TAAACC CTCTAA GAAGG A3’) generated from the vector and primer P2 generated from the cDNA insert (Fig 1A). The probe derived from the cytoplasmic tail of PSGL-1 was obtained as previously described.

Genetic mapping. The progeny of two sets of genetic crosses were typed for inheritance of the mouse PSGL-1 gene: (NFSN/N FS/norC58J/Mus musculus musculus) × M musculusmusculus and (NFSN/Mus spretus) × M spretus C58J. The progeny of these crosses have been typed for over 850 markers including the chromosome 5 (Chr 5) markers Pd6b (phosphodiesterase beta), Mea2 (male enhanced antigen 2), and Tcf1 (hepatic transcription factor 1) as described previously. Data were stored and analyzed using the program LOCUS developed by C.E. Buckler (National Institutes of Allergy and Infectious Diseases, Bethesda, MD). Recombinational distances were calculated according to Green.

Northern blot analysis. Total RNA samples (10 to 20 µg) were prepared from mouse myeloid cells, WW6 embryonic stem cells (a gift from Dr. R. Stanley, Albert Einstein College of Medicine, Bronx, NY) and adult tissues from C57Bl/6J mice using Trizol reagent (GIBCO-BRL) were loaded onto a 1.2% to 1.5% agarose-formaldehyde gel. After electrophoresis in 1× 3-[N-morpholino]propanesulfonic acid buffer, the RNA was transferred to Biotrane membrane (ICN Biomedicals, Plainview, NY) in 20× SSC and was bonded in a Stratalinker (Stratagene). Prehybridization or hybridization was performed at 65°C overnight in a PIPES hybridization buffer containing 50 mM/L Piperazine-N,N’-bis(2-ethanesulfonic acid), pH 6.5; 100 mM/L sodium phosphate, pH 7.0; 1 mM/L EDTA; 5% SDS; and 60 µg/mL salmon sperm DNA. The probe was a 1.7-kb full length cDNA of mouse PSGL-1.

Cell adhesion assay. COS cells, WEHI-3B cells, and PU.5-18 cells were grown as monolayers in complete Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL) containing 10% fetal calf serum (GIBCO-BRL). 32DC13 cells were grown in suspension in the same medium supplemented with 10% conditioned medium from confluent WEHI-3B cells. HL-60 cells, Chinese hamster ovary (CHO) cells, P-selectin—expressing CHO cells (CHO-P), and E-selectin—expressing CHO cells (CHO-E) were maintained as described previously.

CHO, CHO-P, or CHO-E cells were seeded at 1 × 10⁴ cells/well in 96-well U-bottom plates (Costar, Cambridge, MA) and were
Fig 1.
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The amino acid sequence of the transmembrane domain is underlined, the putative mucin-like domain including 10 decameric repeats is in bold and letters is underlined, asparagine residues for the potential N-glycosylation sites are shown in shadowed letters, and the nucleotide sequence of the polyadenylation sites is in bold. The arrow represents the beginning of the nucleotide sequence derived from the longest cDNA clone. The putative cleavage sites for the signal peptide and for the propeptide recognized by the furin/PACE enzyme are indicated by arrow heads. Oligonucleotide primers used for PCR and for DNA sequencing are underlined. The numbers on the right indicate the position of the repeat unit in the mouse PSGL-1 sequence. The consensus sequences of mouse and human repeats are presented at the bottom of the diagram.

incubated at 37°C and 5% CO₂ for 24 hours to 90% confluency. Before the cell adhesion assay, wells were blocked with Dulbecco’s modified Eagle’s medium and 1% bovine serum albumin for 4 to 6 hours at 37°C and 5% CO₂. COS or mouse myeloid cells were harvested, resuspended at 1 x 10⁶ cells/mL of 5 mmol/L EDTA/phosphate-buffered saline (PBS), and fluorescently labeled with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes, Eugene, OR) at a final concentration of 0.0025% (wt/vol) at 37°C and 5% CO₂ for 30 minutes. Cells were washed and resuspended in Tris-buffered saline (TBS; Tris 10 mmol/L, pH 7.451, 130 mmol/L NaCl, 1 mmol/L CaCl₂, and 1 mmol/L MgCl₂), and 1 to 2 x 10⁶ labeled cells were added per well and incubated at room temperature for 15 minutes. The fluorescence signal was measured using a Cytofluor 2300 fluorescence measurement system (Millipore Corp, Bedford, MA) set at 485 nm (excitation) and 530 nm (emission) both before and after rinsing wells 3 to 5 times with TBS. The percentage of initial signal was expressed as percentage of cell binding. Each data point represents the results of identical residues in each repeat unit. The numbers on the right indicate the position of the repeat unit in the mouse PSGL-1 sequence. The consensus sequences of mouse and human repeats are presented at the bottom of the diagram.

To prepare trypsin-treated cells, fluorescently labeled WEHI-3B cells were resuspended at 1 x 10⁶ cells/mL in TBS containing bovine trypsin (1 µg/mL; Sigma, St Louis, MO) and were incubated for 60 minutes. To prepare neuraminidase-treated cells, fluorescently labeled WEHI-3B cells were resuspended at 1 x 10⁶ cells/mL in 5 mmol/L EDTA/PBS containing neuraminidase from Vibrio cholerae (200 µU/mL, type II; Sigma) and incubated for 90 minutes. Cells were washed 2 to 3 times with TBS before the binding assay.

RESULTS

Isolation of genomic DNA and cDNA encoding mouse PSGL-1. Using the human cDNA as a probe, the mouse PSGL-1 gene was isolated from a genomic DNA library prepared from the 129/SvJ mouse tissue. Because of the apparent low homology, low stringency conditions were used. Four overlapping genomic DNA clones contained two EcoRI fragments of 4 kb and 5 kb. The 4-kb fragment contained the upstream portion of the open reading frame, while the 4-kb fragment contained the downstream portion of the open reading frame. The complete open reading frame encoded a polypeptide of 397 residues with homology to that of human PSGL-1. An AGGT intron/exon junction site is present 7 nucleotides 5' of the ATG initiation codon. The sequence upstream of this site is likely to be in an intron because it showed no homology to the human cDNA counterpart. An additional clone may contain a 5' untranslated exon because it hybridized only to the 5' untranslated region of the human PSGL-1 cDNA.

cDNA clones encoding the mouse PSGL-1 homolog were isolated from a cDNA library prepared from the mouse myelomonocytic cell line WEHI-3. The longest cDNA of 1.3 kb contained a partial open reading frame starting at residue 120. The cDNA sequence included a complete 3' untranslated region, having two consecutive AATAAA poly(A) addition sites followed by a poly(A) tail (Fig 1A). The 3' untranslated region is likely to be included in the coding exon as evidenced by partial sequencing of the 4-kb EcoRI genomic DNA fragment using primers P4, P5, and P6 (Fig IA). This is also supported by PCR analysis of the four genomic DNA clones using primers P4 and P6 (data not shown).

To obtain a full length cDNA sequence, the 5' region of the mouse PSGL-1 cDNA was obtained from WEHI-3 poly(A)⁺ RNA by RT-PCR. Primer pairs P1 and P3 (Fig 1A) derived from the genomic DNA sequence were used. Sequence analysis of the PCR product showed complete agreement with the corresponding genomic DNA sequence (Fig 1A). The full length cDNA encoding mouse PSGL-1 predicts a protein of 397 amino acids. The N-terminus of the protein contains 18 residues of a hydrophobic signal sequence followed by a putative propeptide sequence of 23 residues. A cleavage site (RERR) for the paired basic amino acid-converting enzyme (furin/PACE) defines the end of the propeptide region. The sequence of the putative mature protein suggests the following structural features: a large extracellular domain of 265 residues, a transmembrane domain of 24 residues of hydrophobic core, and a cytoplasmic tail of 67 residues. A single extracellular cysteine resides at the N-terminus of the transmembrane domain. In the extracellular region, there are 10 decameric repeats and two po-
tential N-glycosylation sites located at position 66 and 261. This repeat region is rich in serine, threonine, and proline, although the striking repeat pattern of the decameric repeats is not observed. Of the 74 amino acids in this region, 23 are either serine or threonine, and 8 are proline. This would suggest the possibility of important O-linked glycosylation sites on a peptide backbone that is conformationally constrained by proline residues.

Sequence comparison between mouse and human PSGL-1. The mouse PSGL-1 polypeptide is five residues shorter than its human counterpart. The mouse and human PSGL-1 proteins show an overall similarity of 67% and an identity of 50% (Fig 2). Similar domain structures, including a signal peptide, a propeptide, the decameric repeat units, a transmembrane domain, and a cytoplasmic tail, are present in both species (Fig 2). The highest degree of similarity is present in the transmembrane domain (91% similarity and 83% identity) and the cytoplasmic tail (86% similarity and 76% identity). The mucin-like repeat regions of mouse and human PSGL-1 contain a similar number of serine/threonine residues although the striking repeat pattern of the decameric repeats is not observed. Of the 74 amino acids in this region, 23 are either serine or threonine, and 8 are proline. This would suggest the possibility of important O-linked glycosylation sites on a peptide backbone that is conformationally constrained by proline residues.

**Fig 2.** Sequence comparison between mouse and human PSGL-1. The amino acid sequences of mouse PSGL-1 and human PSGL-1 were aligned using the Bestfit program from the Genetics Computer Group, with a gap weight of 3.000 and a length weight of 0.1000. The decameric repeats in the mouse and human sequences are presented in bold and are underlined.
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5.0 Kb
4.5 Kb
4.0 Kb
2.7 Kb

Fig 3. Southern blot analysis of the mouse PSGL-1 gene. Mouse lung genomic DNA digested with BamHI (B), EcoRI (E), and HindIII (H) were electrophoresed, transferred to nylon membrane, bonded, and hybridized overnight at 65°C using 32P-labeled probe derived from the mucin-like repeat region (left panel) or from the cytoplasmic tail (right panel) of PSGL-1 cDNA. The blots were prewashed with 1 × SSC/0.2% SDS at room temperature for 2 hours, exposed to film overnight at −70°C, and then washed with 0.2 × SSC/0.2% SDS at 65°C for 4 hours. The size of each band is given.

linkage was established to markers on Chr 5, with the closest linkage observed to Tcf1. This map location places Selpl in a region of mouse Chr 5 with homology to human chromosomes 12 and 22.6 This is consistent with the localization of the human homolog to 12q24.57

Expression of the mouse PSGL-1 cDNA in COS cells.
To show the binding activity of P-selectin to heterologously expressed mouse PSGL-1, a full length PSGL-1 cDNA was cloned into pED, a eukaryotic expression vector, by recombining the 5′ region obtained by RT-PCR with a partial cDNA clone at a unique Acc I site. The mouse PSGL-1 cDNA was cotransfected in COS cells with an α1,3/1,4 fucosyltransferase (Fuc-TIII) cDNA vector, as previously described with human PSGL-1.25 The transfected COS cells, in contrast to untransfected COS cells, bound to human P-selectin expressed on CHO cells (CHO-P; Fig 5). Comparable binding was obtained in COS cells cotransfected with the Fuc-TIII cDNA and the human PSGL-1 cDNA but not with the CD43 cDNA, a mucin-like protein. Transfection with PSGL-1 alone had no effect. The results show that mouse PSGL-1 is a functional receptor for human P-selectin.

PSGL-1 mRNA in mouse tissues. Expression of PSGL-1 mRNA in mouse myeloid cell lines and in mouse tissues was analyzed by Northern blot experiments using the full length cDNA of mouse PSGL-1 as a probe. As indicated in Fig 6A, a major mRNA species of \( \approx 1.8 \) kb and a minor species of \( \approx 3.4 \) kb were expressed in 32DC13 neutrophil progenitor cells, PU5-1.8 monocyte-macrophage cells, and WEHI-3B myelomonocytic cells. However, both mRNA species were expressed at a low level in WW6 embryonic stem cells. The signal from the \( \approx 1.8 \)-kb mRNA was not caused by nonspecific hybridization of the 18S ribosomal RNA because no significant background was detected under the same condition when probes unrelated to PSGL-1 were used. The presence of PSGL-1 mRNA in mouse tissues were also tested. The \( \approx 1.8 \)-kb species of PSGL-1 mRNA was expressed in most tissues including blood, bone marrow, brain, adipose tissue, heart, kidney, liver, muscle, ovary, spleen, and thymus (Fig 6B). High levels of mRNA expression were found not only in hematopoietic cells derived from blood, bone marrow, spleen, and thymus, but also in nonhematopoietic tissues including brain and adipose tissue (Fig 6B).

Fig 4. Genetic map location of the mouse PSGL-1 gene, Selpl. To the right of the map are given recombination fractions between adjacent loci with the fraction from the \( M m \) musculus cross given first. Mea2 was not typed in the \( M s p r e t u s \) cross. Recombinational distances and standard errors are given in parentheses. To the left of the map are given the human map locations for the underlined genes.
Binding properties of mouse myeloid cells to human P-selectin and E-selectin. The abilities of PSGL-1-expressing cells, including WEHI-3B, 32DC13, and PU5-1.8, to recognize human P-selectin and E-selectin were determined using a cell-binding assay. WEHI-3B and 32DC13 cells bound to both P-selectin and E-selectin bearing CHO cells (CHO-P and CHO-E), in parallel to HL60 cells. However, PU5-1.8 cells did not bind to either CHO-P or CHO-E cells (Fig 7A). As shown above, all these myeloid cells synthesize PSGL-1 mRNA. Therefore, the failure of PU5-1.8 cells to bind P-selectin probably reflects defects in the posttranslational modification of PSGL-1 in the cells. WEHI-3B cells showed a significant preference for CHO-P over CHO-E, but 32DC13 cells and HL-60 cells showed a preference for CHO-E over CHO-P (Fig 7A). The interaction of 32DC13 cells (Fig 7B) or WEHI-3B cells (data not shown) with CHO-P cells was inhibited by polyclonal anti-P-selectin antibodies and by polyclonal anti-PSGL-1 antibodies. Anti-human PSGL-1 antibody, which does not bind to mouse PSGL-1, had no effect on cell interaction. The importance of PSGL-1 as a P-selectin receptor on 32DC13 cells was shown by blocking 32DC13 cell interaction with CHO-P cells using anti-PSGL-1 antibodies (Fig 7B). In contrast, the interaction of these cells with CHO-E cells was not affected by the anti-PSGL-1 antibodies.

To further characterize the properties of mouse PSGL-1, the effects of trypsin and neuraminidase on P-selectin binding of WEHI-3B cells were determined. The interaction of WEHI-3B cells with CHO-P cells was completely abolished by treatment of WEHI-3B cells with 1 μg/mL of bovine trypsin (Fig 8). Similarly, treatment of WEHI-3B cells with 200 mU/mL of neuraminidase from V cholerae abolished the ability of these cells to interact with CHO-P cells (Fig 8). The results showed that the protein backbone and sialic acid are required for mouse PSGL-1 to bind to P-selectin.
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Fig 7. Binding properties of mouse myeloid cells to human P-selectin and E-selectin. (A) Binding of mouse and human myeloid cells to P-selectin and E-selectin. Cells were harvested, fluorescently labeled, washed, and used in a binding assay as described in the Materials and Methods. The percentages of cells bound to CHO (□), human CHO-P (■), human CHO-E (■) are shown. (B) The effect of anti-PSGL-1 or anti-P-selectin antibodies on 32DC13 cell binding to P-selectin and E-selectin. Before the binding assay, 32DC13 cells were incubated with rabbit serum or with rabbit antibodies against mouse PSGL-1 (mPSGL-1), against human PSGL-1 (hPSGL-1), and against human P-selectin. The percentages of 32DC13 bound to CHO, CHO-P, or CHO-E cells in the presence of antibodies are presented.

Fig 8. P-selectin binding of WEHI-3B cells treated with trypsin and neuraminidase. Fluorescently labeled WEHI-3B cells were incubated with buffer, with bovine trypsin (1 μg/mL), or with neuraminidase from V. cholerae (200 mU/mL) and were washed and used in an adhesion assay to CHO (□) and CHO-P (■) cells as described in the Materials and Methods. The percentage of bound WEHI-3B cells is shown.

DISCUSSION

A number of glycoproteins including PSGL-1 and carbohydrates have been identified as counterreceptors or components of counterreceptors for P-selectin. Together with the expression cloning of a mucin-like glycoprotein from a cDNA library derived from HL-60 cells, PSGL-1 has emerged as an important candidate for the physiological P-selectin receptor. To explore the biology of PSGL-1 in vivo using a mouse model, we have cloned and characterized the gene of the mouse homolog of PSGL-1, established a system for expressing the functional receptor in a heterologous expression system, and determined the properties of this protein that are the same and that are distinct from human PSGL-1. The overall sequence of PSGL-1, except in the putative transmembrane domain and the putative cytoplasmic tail, is not highly conserved between human and mouse. The cytoplasmic tail of PSGL-1 may play an important role for signal transduction since Celi et al have shown that binding of P-selectin to monocytes resulted in upregulation of tissue factor gene expression. In addition, Zimmerman’s group has reported roles of P-selectin for upregulating the synthesis of monocyte chemotactic factor-1 and tumor necrosis factor-α and for regulating platelet-activating factor synthesis and phagocytosis by monocytes.

The repeat regions of mouse and human PSGL-1 share the general structural features of mucin-like molecules but are less conserved at the primary sequence level. Variation in the number of repeat units exists in the human PSGL-1
gene isolated from HL-60 and U-937 cell lines (15 units) as compared with that of the polymorphonuclear leukocytes, monocytes, and several other cell lines (16 units). It is not known whether similar polymorphism exists in different mouse strains. The repeat region may be important for displaying the carbohydrate recognition site for P-selectin. This is consistent with the carbohydrate features carried by the purified PSGL-1 protein. PSGL-1 possesses highly clustered O-linked sialyl Lewis\(^x\) tetrasaccharide structures. Although the nature of the N-linked carbohydrate moieties is not known, the human PSGL-1 is N-glycosylated. N-linked carbohydrates on PSGL-1 may also contribute to P-selectin binding, because HL-60 cells treated with the N-glycosylation inhibitor tunicamycin lost binding activity. Despite variations in primary sequence between human PSGL-1 and mouse PSGL-1, COS cells transfected with mouse PSGL-1 cDNA are capable of binding to the human P-selectin-bearing CHO cells. Mouse PSGL-1-mediated binding to human P-selectin has also been observed with the WEHI-3B and 32DC13 cell lines, and this interaction can be blocked by an anti-PSGL-1 antibody raised against a highly conserved peptide N-terminal of the repeat units of mouse PSGL-1. These results would suggest that the highly conserved residues in this peptide sequence may be important for P-selectin binding. Alternatively, the proximity of the epitope for this antibody to the carbohydrate bearing repeat units may interfere with the binding of the lectin domain of P-selectin to these sugars. The protein backbone of the mouse repeat units, although less conserved between mouse and human, may provide an adequate scaffold for presenting the necessary carbohydrate ligands.

The interaction between endothelial cells or platelets and leukocytes is believed to be controlled by regulated cell surface expression of P-selectin. P-selectin, stored in \(a\) granules of platelets and the Weibel-Palade bodies of endothelial cells, is redistributed to the cell surface on stimulation with agonists such as thrombin and histamine. De novo P-selectin mRNA synthesis can also be induced on endotoxin stimulation. In contrast, PSGL-1 is constitutively expressed on the target cells of P-selectin. In fact, Northern blot analyses showed a major PSGL-1 mRNA species of \(\approx 1.8\) kb in several mouse myeloid cell lines including PU-5, 32DC13, and WEHI-3B and in hematopoietic tissues including liver, spleen, thymus, and bone marrow. The highest level of PSGL-1 mRNA is present in bone marrow, which is consistent with recent reports that PSGL-1 is expressed in CD34\(^+\) hematopoietic progenitor cells, in myeloid cells, and in all the lymphocytes. PSGL-1 is also expressed in embryonic stem cells and in all the tissues examined including nonhematopoietic tissues such as brain, adipose tissue, stomach, muscle, and ovary. Interestingly, the expression levels of PSGL-1 in certain nonhematopoietic tissues such as brain and adipose tissue are apparently higher than those in liver and heart. Immunohistochemical staining of mouse tissues also showed PSGL-1 expression in smooth muscle cells of the vessel wall and in connective tissues (M.L. Palomba, personal communication, November 1995). These results suggest additional functions of PSGL-1, perhaps with a spectrum of different posttranslational modifications.

Similar to the human PSGL-1 gene that expresses two transcripts of \(\approx 2.5\) kb and \(\approx 4\) kb, the mouse PSGL-1 gene also transcribes two types of mRNAs, a major species of \(\approx 1.8\) kb and a minor species of \(\approx 3.4\) kb. Recently, Veldman et al\(^{17}\) showed that differential utilization of two polyadenylation sites is involved in regulating the \(\approx 2.5\) -kb and the \(\approx 4\) -kb human PSGL-1 transcripts. A different mechanism may be used to regulate the two mouse PSGL-1 transcripts because the \(3'\) untranslated region of PSGL-1 contains two adjacent polyadenylation signals that would not be able to account for the differences between the \(\approx 1.8\) -kb and the \(\approx 3.4\) -kb mRNAs. Furthermore, the \(3'\) untranslated regions of the two species have little homology, and analyses of three independent cDNA clones of mouse PSGL-1 showed an identical sequence at the \(3'\) end. Alternatively, the two transcripts may vary by an infrequent use of a more downstream polyadenylation signal.

PSGL-1 from the mouse and human \(^{17}\) belongs to the class of mucins encoded by a single coding exon. Leukosialin (CD43)\(^{53,54}\) falls into this class. The other classes of mucins are encoded by multiple exons. These include glycoporphin,\(^{55}\) the carcinoma-associated mucin MUC-1,\(^{56}\) and the L-selectin ligands CD34\(^{57}\) and GlyCAM-1.\(^{58}\)

The mouse PSGL-1 showed distinct binding specificity for P-selectin. WEHI-3B and 32DC13 cells have similar binding affinity to P-selectin. However, 32DC13 cells, from which the ESL-1\(^{19}\) was identified, bind markedly better to E-selectin than do WEHI-3B cells. Binding of 32DC13 cells to E-selectin was not affected by an anti-PSGL-1 antibody raised against a peptide derived from mouse PSGL-1. This result suggests that PSGL-1 is a specific ligand for P-selectin but not for E-selectin. This is contrary to a previous report by Asa et al.\(^{21}\) This group purified PSGL-1 by affinity chromatography using an E-selectin-globulin chimera and showed binding of the purified protein to both E-selectin and P-selectin.\(^{22,59}\) A monoclonal antibody, PL5, raised against the purified PSGL-1 protein, blocked binding of HL-60 cells or human neutrophils to E-selectin. Their monoclonal antibody may recognize an oligosaccharide antigen carried by PSGL-1. The carbohydrate determinants for E-selectin and P-selectin share overlapping structures,\(^{59,60}\) and the presence of a high concentration of sialyl Lewis\(^x\) is sufficient to confer E-selectin binding. In our study, the low binding activity of WEHI-3B cells for E-selectin may be because of the nature of the oligosaccharides displayed on WEHI-3B cells, carbohydrates that favor P-selectin but not E-selectin recognition. High concentrations of recombinant PSGL-1 or purified PSGL-1 may bind to E-selectin in an interaction that is nonphysiological.\(^{21,23}\) A distinct function of PSGL-1 was suggested in a T-lymphocyte rolling system using polyclonal antibodies against PSGL-1\(^{20}\); PSGL-1 mediated T-lymphocyte rolling on P-selectin but not on E-selectin. Alternatively, PSGL-1 can mediate low-affinity binding to E-selectin, and the recognition site for E-selectin is distinct from that for P-selectin.

In summary, we have cloned the mouse PSGL-1 cDNA to compare the structure of mouse PSGL-1 with that of the human PSGL-1. Although the general domain structure is preserved, mouse PSGL-1 shows only about 50% conserva-
tion of the overall amino acid sequence. Although mouse PSGL-1 binds to human P-selectin, the extracellular domain of PSGL-1 that represents the P-selectin receptor shows lower overall sequence similarity. Furthermore, we provide evidence that the mouse myeloid WEHI-3B cell line (which expresses PSGL-1) binds significantly better to P-selectin than to E-selectin. Blocking anti-PSGL-1 antibodies inhibit P-selectin but not E-selectin binding of mouse myeloid 32DC13 cell line. These results provide additional insights into the structure and function of PSGL-1 and will facilitate interpretation of further in vivo studies in the PSGL-1 null mouse.

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Mouse P-selectin glycoprotein ligand-1: molecular cloning, chromosomal localization, and expression of a functional P-selectin receptor

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