P-selectin glycoprotein ligand-1 (PSGL-1) is a homodimeric, transmembrane mucin on leukocytes. During inflammation, reversible interactions of PSGL-1 with selectins mediate leukocyte rolling on vascular surfaces. The transmembrane domain of PSGL-1 is required for dimerization, and the cytoplasmic domain propagates signals that activate $\beta_2$ integrins to slow rolling on integrin ligands. Leukocytes from knockin $\Delta$CD” mice express a truncated PSGL-1 that lacks the cytoplasmic domain. Unexpectedly, they have 10-fold less PSGL-1 on their surfaces than WT leukocytes. Using glycosidases, proteases, Western blotting, confocal microscopy, cell-surface crosslinking, FRET, and pulse-chase metabolic labeling, we demonstrate that deleting the cytoplasmic domain impaired dimerization and delayed export of PSGL-1 from the endoplasmic reticulum (ER), markedly increasing a monomeric precursor in the ER and decreasing mature PSGL-1 on the cell surface. A monomeric, full-length PSGL-1 made by substituting the transmembrane domain with that of CD43 exited the ER normally, revealing that dimerization was not required for ER export. Thus, the transmembrane and cytoplasmic domains cooperate to promote dimerization of PSGL-1. Furthermore, the cytoplasmic domain provides a key signal to export precursors of PSGL-1 from the ER to the Golgi apparatus en route to the cell surface.

During inflammation, reversible interactions of selectins with their glycosylated ligands mediate leukocyte rolling on vascular surfaces (1). Cooperative signaling upon engagement of selectin ligands and chemokine receptors causes rolling leukocytes to decelerate and arrest through interactions of integrin $\alpha_4\beta_2$ on leukocytes with intercellular adhesion molecule-1 (ICAM-1) on endothelial cells. Leukocytes then use integrins to migrate across blood vessels in response to chemokine gradients or other signals (2).

P-selectin is expressed on activated endothelial cells and platelets, E-selectin is expressed on activated endothelial cells, and L-selectin is expressed on leukocytes (1). Each selectin mediates leukocyte rolling by interacting with a subset of membrane glycoproteins that must be appropriately glycosylated and, for ligands binding to P- and L-selectin, sulfated. P-selectin glycoprotein ligand-1 (PSGL-1) is a homodimeric, type I transmembrane mucin (1,3,4). It is the dominant ligand for P- and L-selectin on leukocytes (5-8), and it cooperates with other ligands to mediate rolling on E-selectin (9-11). Dimerization of PSGL-1 enhances rolling on P-selectin (12). The subunits of the PSGL-1 homodimer are linked by a disulfide bond just outside the plasma membrane (13). Each extracellular domain has multiple Ser/Thr-linked O-glycans that cause it to adopt a highly extended conformation (14). PSGL-1 is localized on the tips of leukocyte microvilli (5) and has affinity for cholesterol-rich membrane domains, or lipid rafts (15). It moves to the uropods as cells polarize after chemokine stimulation (16,17).

The sequences of the transmembrane and cytoplasmic domains of PSGL-1 are highly conserved across species, implying important
functions for each domain. Mutating the extracellular cysteine does not prevent crosslinking of PSGL-1 by the short, membrane-impermeable compound bis(sulfosuccinimidyl) suberate (BS3). Substituting the transmembrane domain of PSGL-1 with the corresponding domain of CD43 creates a monomeric form of PSGL-1 that cannot be crosslinked (18). Therefore, PSGL-1 requires noncovalent interactions between transmembrane domains to form a dimer, which is stabilized by formation of the disulfide bond. Whether interactions between cytoplasmic domains contribute to dimerization has not been studied.

In vitro, the cytoplasmic domain of PSGL-1 binds to ezrin/radixin/moesin adaptors that, in turn, bind to actin filaments (19). Knockin “ΔCD” mice express a form of PSGL-1 that lacks the cytoplasmic domain. Despite the loss of the ezrin/radixin/moesin-binding site, the truncated PSGL-1 molecules on the plasma membrane localize on microvilli, partition into lipid rafts, and redistribute to the uropods of polarized cells (20). Therefore, PSGL-1 does not require direct interactions of its cytoplasmic domain with the cytoskeleton to localize in membrane domains. When normalized for PSGL-1 surface density, neutrophils from WT and ΔCD mice roll equivalently on P-selectin (20). Therefore, PSGL-1 does not require direct interactions of the cytoplasmic domain with the cytoskeleton to mediate rolling. Engagement of PSGL-1 as WT neutrophils roll on P- or E-selectin activates a Syk-dependent signaling pathway that converts integrin αLβ2 to a conformation that slows rolling through reversible interactions with ICAM-1 (21). However, ΔCD neutrophils rolling on P- or E-selectin do not activate integrin αLβ2 to slow rolling on ICAM-1 (20,22). Therefore, PSGL-1 requires its cytoplasmic domain to initiate the signals for integrin-mediated slow rolling.

Unexpectedly, ΔCD leukocytes express 10-fold less PSGL-1 on their surfaces than WT leukocytes (20). Western blots of ΔCD leukocyte lysates with anti-PSGL-1 antibodies confirm expression of a mature disulfide-linked homodimer with ~105-kDa subunits. These subunits migrate slightly faster than the ~115-kDa subunits of WT PSGL-1, consistent with deletion of the cytoplasmic domain. However, the blots also reveal an immunoreactive 65-kDa protein in lysates of ΔCD leukocytes that is not observed in lysates of WT leukocytes. This putative precursor of PSGL-1 does not form disulfide-linked dimers (20). The results suggest that the cytoplasmic domain of PSGL-1 facilitates surface expression of disulfide-linked homodimers but do not provide a mechanism for these effects. Here we demonstrate that interactions between cytoplasmic domains cooperate with interactions between transmembrane domains to promote dimerization of PSGL-1. Furthermore, the cytoplasmic domain provides a key export signal to move precursors of PSGL-1 from the endoplasmic reticulum (ER) to the Golgi apparatus en route to the cell surface.

**Experimental procedures**

**Antibodies.** Rabbit polyclonal IgG antibody against a 19-residue peptide corresponding to the N-terminal sequence of mature murine PSGL-1 (20) was a gift from Richard Cummings (Emory University School of Medicine, Atlanta, GA). All other antibodies were from BD Biosciences (San Diego, CA) unless noted otherwise.

**Mice.** Knockin “ΔCD” mice expressing PSGL-1 without the cytoplasmic domain (20) and knockout mice lacking T-synthase in endothelial and hematopoietic cells (EHC T-syn-/-) have been described (11,23). Mice of both genotypes were backcrossed at least 10 generations into the C57BL/6J background. WT C57BL/6J mice were used as controls. ΔCD mice were crossed with C57BL/6J mice to generate heterozygotes bearing one allele encoding WT PSGL-1 and one allele encoding ΔCD PSGL-1. All experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

**Leukocytes.** Leukocytes from murine bone marrow or peripheral blood were isolated as described (20). CFU-GM colonies from ΔCD bone marrow cells were cultured in MethoCult™ medium GF M3434 (Stem Cell Technologies, Vancouver, BC, Canada). Splenocytes were isolated by mincing murine spleens and flushing cells through a 100-µm strainer (BD Biosciences) with Hanks’ balanced salt solution (HBSS).
without Ca²⁺ or Mg²⁺. Red blood cells were lysed as described (20).

**Transfected CHO cells.** Stably transfected CHO cells expressing matched surface densities of human WT or ΔCD PSGL-1 have been described (20). The cDNA encoding murine WT or ΔCD PSGL-1 was used as template to mutate Cys-307 to Ser (C307S). All constructs were confirmed by DNA sequencing. Amplified products were inserted into the expression vector pZeoSV (Invitrogen, Carlsbad, CA) and transfected into CHO-K1 cells with Fugene 6 (Roche Applied Science, Indianapolis, IN). Stably transfected clones were selected and sorted for matched PSGL-1 surface expression. C307S and ΔCD C307S constructs were cloned into the pDsRed-monomer-N1 and pEGFP-N1 vectors (Clontech, Mountain View, CA) to fuse DsRed or GFP to the C terminus of each PSGL-1 protein. These constructs were transiently transfected into CHO-K1 cells.

**Cell-surface crosslinking.** Stably transfected CHO cells expressing matched surface densities of murine C307S or ΔCD C307S were incubated with BS⁳ as described (18). The cells were then lysed and subjected to Western blotting.

**Flow cytometry.** Leukocytes from peripheral blood or bone marrow or CFU-GM colonies were suspended in HBSS containing 0.5% human serum albumin. Cells were incubated with phycoerythrin-labeled rat anti-murine PSGL-1 mAb 2PH1 (PE-2PH1). To identify neutrophils, CFU-GM colonies were also incubated with FITC-labeled rat RG6-8C5 mAb to the murine myeloid differentiation antigen Gr-1 (FITC-Gr-1). In some experiments, 5 x 10⁶ bone marrow leukocytes in 0.5 ml HBSS were incubated for 30 min at 37°C with or without 250 µg/ml O-sialoglycoprotein endopeptidase (OSGE, Cedarlane Laboratories Ltd., Hornby, ON, Canada). The cells were then fixed in 3.5% paraformaldehyde for 10 min. In some experiments, fixed cells were permeabilized with 0.05% Triton X-100 in HBSS for 10 min. After blocking with 1% BSA in HBSS for 1 h, cells were incubated with 15 µg/ml biotinylated anti-murine PSGL-1 mAb 2PH1 (rat IgG1) in HBSS plus 0.5% BSA for 45 min, followed by streptavidin-Alexa 488 (Molecular Probes, Eugene, OR) for 45 min. Biotinylated rat IgG1 was used as an isotype control. Immunofluorescence was detected with a Leica TCS NT confocal microscope (Leica Microsystems, Deerfield, IL) equipped with a krypton/argon laser. For colocalization studies,
leukocytes were stained with 15 µg/ml rabbit anti-calnexin IgG (Santa Cruz, Santa Cruz, CA) followed by 1:500 dilution Alexa 546-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR). As controls for specificity of staining, cells were stained with rabbit anti-calnexin antibody and streptavidin-Alexa 488, or with 2PH1 and goat anti-rabbit IgG. Irrelevant rat and rabbit IgG were also used as controls. Immunofluorescence was detected with the Leica TCS NT confocal microscope. A series of 0.5-µm optical sections in the z axis was analyzed with Confocal Assistant software (University of Minnesota, Minneapolis, MN).

**Western blots.** Bone marrow leukocytes (5 x 10⁶) were lysed in 50 µl of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS. Before lysis, some leukocytes were treated with Pronase (Calbiochem, San Diego, CA) as described (26) or with 10 µg/ml brefeldin A (Sigma, St. Louis, MO) for 90 min. In some experiments, 5 x 10⁶ splenocytes were incubated with 100 µg/ml cycloheximide (Sigma), 8 µM MG132 (Sigma), or an equivalent volume of DMSO in Iscove’s modified Dulbecco’s medium containing 10% FBS. After various intervals, the splenocytes were lysed in the same buffer used for bone marrow leukocytes. Lysates were centrifuged at 12,000 x g for 10 min to remove insoluble material. Some supernatants (50 µl) were incubated at 37°C with 20 µU endoglycosidase H (New England Biolabs, Beverly, MA) for 45 min at 37°C or 250 µg/ml OSGE for 30 min. All supernatants were alkylated in 20 mM iodoacetamide or 30 mM N-ethylmaleimide, boiled in Laemmli sample buffer with or without 3% β-mercaptoethanol, and fractionated by SDS-PAGE. All samples were electrophoresed under reducing conditions except for the data shown in Fig. 7E, where non-reducing conditions were employed. Gels were blotted onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA) using a Trans-Blot SD system (Bio-Rad Laboratories). After blocking in 5% non-fat dry milk, the blots were incubated with rabbit anti-murine PSGL-1 IgG or control rabbit IgG. Peroxidase-labeled anti-rabbit IgG (1:5000, Pierce) and ECL reagents (GE Healthcare, Piscataway, NJ) were used to visualize protein bands.

**Non-denaturing PAGE.** Blue native PAGE was performed according to a protocol supplied by the manufacturer (Invitrogen). Briefly, cells were lysed with 1% n-dodecyl-β-D-maltoside on ice. The lysate was centrifuged at 15,000 x g for 30 min at 4°C. The supernatant was mixed with Native PAGE sample buffer and Coomassie G-250 at a final concentration of 0.125% w/v. Electrophoresis was performed with running buffer containing Coomassie G-250. Gels were transferred to a PVDF membrane. The membranes were incubated in 8% acetic acid to fix the proteins, blocked with 5% milk, and incubated with anti-murine PSGL-1 mAb 4RA10 (27). Peroxidase-labeled anti-murine IgG (1:5000, Pierce) and ECL reagents (GE Healthcare) were used to visualize protein bands.

**Pulse-chase.** Pulse-chase experiments were performed as described (28), with slight modifications. After starvation in methionine/cysteine-free Dulbecco’s modified Eagle’s medium for 30 min, murine splenocytes were metabolically labeled with 250 µCi/ml of [35S]methionine/cysteine (1,200 Ci/mmol, PerkinElmer) at 37°C for 30 min. After labeling, cells were washed three times and incubated in medium containing non-radiolabeled cysteine (500 µg/ml) and methionine (100 µg/ml). Cells were lysed at the indicated time in 1 ml 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS. After preclearing with protein A/G agarose (Santa Cruz), lysates were incubated overnight at 4°C with polyclonal anti-murine PSGL-1 antibody and then with protein A/G agarose for 2 h. The immunoprecipitates were washed seven times in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS. They were then analyzed by SDS-PAGE under reducing conditions, followed by fluorography.

**FRET.** CHO-K1 cells were transiently transfected with constructs encoding C307S-PSGL-1-GFP and C307S-PSGL-1-DsRed or with constructs encoding ΔCD-C307S-PSGL-1-GFP and ΔCD-C307S-PSGL-1-DsRed. After 48 h,
FRET between GFP and DsRed was measured by the sensitized acceptor emission method using a Zeiss LSM 510 confocal microscope. Excitation and emission wavelengths were 488 nm and 505-530 nm, respectively, for GFP, and 543 nm and 560-615 nm, respectively, for DsRed. For the FRET channel, excitation and emission were 488 nm and 560-615 nm, respectively. Cells expressing either GFP alone or DsRed alone were imaged to determine the bleed-through in the FRET, donor, and acceptor channels. For all samples, images were acquired in three separate channels: acceptor channel (488 excitation / 505-530 emission), donor channel (543 excitation / 560-615 emission), and FRET channel (488 excitation / 560-615 emission). FRET efficiency (E) was calculated as described (29), using fluorescence intensities measured in the FRET, donor, and acceptor channels.

**Results**

Deleting the cytoplasmic domain impairs expression of PSGL-1 on surfaces of leukocytes and transfected cells. ACD mice express a truncated form of PSGL-1 that lacks the entire 67-residue cytoplasmic domain, except for two arginines that anchor the protein in the membrane (20) (Fig. 1A). Peripheral blood neutrophils from ACD mice express 10-fold less PSGL-1 on their surfaces than peripheral blood neutrophils from WT mice (20). We observed a similar 10-fold reduction in surface expression of ACD PSGL-1 on neutrophils from bone marrow (Fig. 1B) and from CFU-GM grown in methylcellulose (Fig. 1C). Other leukocyte subsets from both peripheral blood and bone marrow exhibited a comparable reduction in surface expression of ACD PSGL-1 (data not shown). These data demonstrate that extraction and/or shedding of ACD PSGL-1 from circulating cells is not a major contributor to lower surface expression. Northern blots revealed equivalent levels of mRNA for PSGL-1 from both WT and ACD leukocytes (Fig. 1D). We also prepared constructs encoding human WT and ACD PSGL-1 (Fig. 1E) and selected stably transfected CHO cell clones expressing matched surface densities of each protein (Fig. 1F). Quantitative RT-PCR revealed nearly 10-fold-higher transcripts for PSGL-1 in cells expressing ACD PSGL-1 than in cells expressing WT PSGL-1 (Fig. 1G). Thus, much higher mRNA levels were required to match ACD PSGL-1 surface densities to those of WT PSGL-1 on transfected cells.

Deleting the cytoplasmic domain causes PSGL-1 to accumulate in an intracellular compartment. We reasoned that redistribution of ACD PSGL-1 to an intracellular compartment might account for its reduced surface expression. To test this hypothesis, we incubated leukocytes with or without OSGE, a protease that cleaves proteins with clustered, sialylated O-glycans such as PSGL-1 (30). The cells were then fixed, washed, incubated with or without buffer that permeabilized the plasma membrane, stained with fluorescent mAb to an N-terminal epitope on PSGL-1, and analyzed by flow cytometry. Almost all of the PSGL-1 was expressed on the surface of WT murine leukocytes (Fig. 2A), consistent with the predominant surface expression of PSGL-1 on human leukocytes (17). Much less PSGL-1 was expressed on the surfaces of ACD leukocytes (Fig. 2A). This surface pool was quantitatively removed by treating cells with OSGE. After permeabilization, comparable total levels of PSGL-1 were detected in WT and ACD leukocytes. This was due to a large intracellular pool of PSGL-1 in ACD cells that was not removed by treating the cell surface with OSGE. After permeabilization, comparable total levels of PSGL-1 were detected in WT and ACD leukocytes. This was due to a large intracellular pool of PSGL-1 in ACD cells that was not removed by treating the cell surface with OSGE. Immunofluorescence microscopy confirmed that most PSGL-1 was on the surface of WT leukocytes, whereas most PSGL-1 was inside ACD leukocytes (Fig. 2B). These data demonstrate that deleting the cytoplasmic domain of PSGL-1 causes accumulation of intracellular PSGL-1.

Deleting the cytoplasmic domain causes retention of a PSGL-1 precursor in the ER of leukocytes. As observed previously (20), Western blotting with a polyclonal antibody to the N terminus of PSGL-1 detected 115- and 105-kDa forms of PSGL-1 in lysates from WT and ACD PSGL-1 leukocytes, respectively, following electrophoresis under reducing conditions (Fig. 3A). The faster migration of the ACD PSGL-1 species is consistent with deletion of its cytoplasmic domain. The anti-PSGL-1 antibody detected the 65-kDa species in ACD but not in WT leukocytes (Fig. 3A). Treating cell lysates with OSGE eliminated the 115- and 105-kDa bands but did not affect the 65-kDa band (Fig. 3A). These
data suggest that the 65-kDa species is a precursor without the clustered, sialylated O-glycans required for recognition and cleavage by OSGE. Treating intact leukocytes with Pronase, a mixture of proteases, eliminated most of the 105-kDa form of ΔCD PSGL-1 (Fig. 3B). In contrast, Pronase did not decrease the 65-kDa form, suggesting an intracellular location that the proteases could not access.

Ser/Thr-linked core 1-derived O-glycans are major post-translational modifications of PSGL-1 (11,31-33). The 65-kDa species in ΔCD leukocytes lacked at least some of the O-glycan structures required for recognition by OSGE. Many polypeptide GalNAc transferases add the initial GalNAc to Ser/Thr residues (34), whereas a single core 1 β1-3-galactosyltransferase (T-synthase) adds Gal to GalNAc to form the core 1 structure, Galβ1-3-GalNAcα1-Ser/Thr, also known as the T antigen (35). The core 1 backbone is required for further extension or branching of this major class of O-glycans (36). Leukocytes from mice lacking T-synthase in endothelial and hematopoietic cells (EHC T-syn-/- mice) express truncated O-glycans with the structure GalNAcα1-Ser/Thr, also known as the Tn antigen (11,23). These cells expressed a 105-kDa form of PSGL-1 that was recognized by the plant lectin, Helix pomatia agglutinin, which binds to terminal GalNAc residues (Fig. 3C). In contrast, Helix pomatia agglutinin did not bind to the 65-kDa species, suggesting that it lacked even the initial Ser/Thr-linked GalNAc residues. Because this modification and all subsequent O-glycosylation steps occur in the Golgi apparatus (34), we reasoned that the 65-kDa protein remained in the ER.

High-mannose N-glycans are attached to asparagines in the ER and then modified into complex forms after transport to the Golgi apparatus (37). The enzyme endoglycosidase H removes high mannose but not complex N-glycans from proteins. Murine PSGL-1 has two potential sites for attaching N-glycans (38). Treating lysates of ΔCD leukocytes with endoglycosidase H increased the mobility of the 65-kDa species, consistent with conversion of its high-mannose glycans to enzyme-resistant complex forms in the Golgi apparatus. Confocal immunofluorescence microscopy of ΔCD leukocytes demonstrated that PSGL-1 colocalized with the ER marker, calnexin (Fig. 3E). These combined data indicate that the 65-kDa form of ΔCD PSGL-1 is a precursor that accumulates in the ER.

Deleting the cytoplasmic domain delays export of PSGL-1 from the ER to the Golgi apparatus. We hypothesized that the PSGL-1 precursor in ΔCD leukocytes accumulated in the ER because it moved very slowly to the Golgi, whereas the precursor in WT leukocytes moved rapidly to the Golgi. We detected a putative 75-kDa precursor in Western blots of WT leukocytes only after prolonged exposure of the blots (Fig. 4A). The 75-kDa species increased markedly after treating cells with brefeldin A, which collapses the cis-Golgi and causes biosynthetic intermediates to accumulate in the ER (39) (Fig. 4A). The 75-kDa species increased markedly after treating cells with brefeldin A, which collapses the cis-Golgi and causes biosynthetic intermediates to accumulate in the ER (39) (Fig. 4A). This observation provides strong evidence that the 75-kDa species is an ER-based PSGL-1 precursor. The 75-kDa precursor in WT cells was larger than the 65-kDa precursor in ΔCD cells, consistent with retention of its cytoplasmic domain. To measure the kinetics of conversion of precursor to mature forms of PSGL-1, we pulsed WT or ΔCD splenocytes with [35S]methionine/cysteine and chased them in unlabeled medium for 2 or 4 h. Cell lysates were immunoprecipitated with polyclonal anti-PSGL-1 antibody. The immunoprecipitates were subjected to SDS-PAGE under reducing conditions and analyzed by fluorography. In WT splenocytes, most of the 75-kDa precursor was converted to the 115-kDa mature form after a 2-h chase (Fig. 4B). We did not detect other biosynthetic intermediates, suggesting that ER-to-Golgi transport is a rate-limiting step. In sharp contrast, very little of the 65-kDa precursor in ΔCD splenocytes was converted to the 105-kDa mature form after a 4-h chase (Fig. 4C). These data demonstrate that impaired ER-to-Golgi transport is a primary cause of the low surface expression of PSGL-1 in ΔCD leukocytes.

Delayed ER export of ΔCD PSGL-1 could result loss of an export signal in the cytoplasmic domain. Alternatively, loss of the cytoplasmic domain might cause the luminal domain of PSGL-
1 to misfold. In the latter case, ER chaperones could initiate transport of ΔCD PSGL-1 from the ER to proteasomes, resulting in its rapid degradation. The pulse-chase data in Fig. 4C argue against rapid diminution of the ΔCD PSGL-1 precursor in the ER. To explore this issue further, we incubated splenocytes with cycloheximide for various intervals to block new protein synthesis. We then lysed the cells and measured turnover of PSGL-1 by Western blotting. The amount of ΔCD PSGL-1 precursor declined very slowly. Indeed, its decline was slower than that of mature WT or ΔCD PSGL-1 (Fig. 4D). Furthermore, incubating splenocytes with the proteasome inhibitor MG132 did not increase the amount of ΔCD PSGL-1 precursor (data not shown). These data indicate that the ΔCD PSGL-1 precursor is not prematurely degraded in proteasomes.

Deleting the cytoplasmic domain impairs dimerization of PSGL-1. When analyzed by SDS-PAGE under non-reducing conditions, very little of the accumulated 65-kDa precursor in ΔCD leukocytes forms disulfide-linked dimers (20). This suggests that lack of the cytoplasmic domain impairs the noncovalent interactions that are required to form the disulfide bond in the ER. To test this hypothesis, we prepared constructs of WT and ΔCD PSGL-1 that retained the single extracellular cysteine (residue 307 in the murine sequence) or replaced it with serine to prevent formation of disulfide bonds (Fig. 5A). These constructs were expressed at matched surface densities on stably transfected CHO cells. Western blots revealed that both the ΔCD and the ΔCD C307S cells expressed large amounts of the 65-kDa precursor (Fig. 5B). This result indicates that chaperone recognition of an unpaired cysteine in the lumen of the ER is not responsible for retention of the precursor. To test whether C307S or ΔCD C307S expressed mature noncovalent dimers on the cell surface by FRET. By measuring sensitized emission of DsRed following excitation of GFP, we detected significantly more FRET for C307S than for ΔCD C307S donor/acceptor pairs (Fig. 5E). The complementary crosslinking and FRET experiments demonstrate that the cytoplasmic domain contributes significantly to noncovalent dimerization of PSGL-1.

Dimerization of PSGL-1 is not required to exit the ER. Deleting the cytoplasmic domain impaired dimerization of PSGL-1 and caused precursors to accumulate in the ER. One interpretation of these data is that PSGL-1 must dimerize to efficiently move from the ER to the Golgi. The transmembrane domain also mediates noncovalent dimerization of PSGL-1. A human PSGL-1 construct containing the transmembrane domain of CD43 with no juxtamembrane cysteine is expressed on CHO cells as a monomer that BS³ does not crosslink (18). Accumulation of a 75-kDa precursor of this monomeric PSGL-1 was not observed. To readdress whether PSGL-1 must dimerize to exit the ER, we designed a murine PSGL-1 construct with the transmembrane domain of murine CD43 and an alanine replacing the extracellular cysteine, called CD43 TMD PSGL-1 (Fig. 6A). We also swapped the transmembrane domain of PSGL-1 with the transmembrane domain of glycophorin A (GPA), which is known to dimerize (40). Finally, we made ΔCD versions of CD43 TMD, GPA TMD, and WT murine PSGL-1 by deleting the cytoplasmic domain. All constructs were expressed at matched surface densities on stably transfected CHO cells. Western blots of lysates resolved by SDS-PAGE under reducing conditions revealed that 65-kDa precursors of PSGL-1 accumulated only in constructs lacking the cytoplasmic domain (Fig. 6C). Thus, the cytoplasmic domain promotes ER export of PSGL-1 even when it contains a heterologous transmembrane domain. Furthermore, dimerization of PSGL-1 is not required to exit the ER. A single cytoplasmic...
domain was sufficient for ER export of the monomeric CD43 TMD PSGL-1 chimera.

We asked whether a single cytoplasmic domain on a dimer pairing a WT subunit with a ΔCD subunit could “rescue” the ΔCD subunit from the ER and transport the heterodimer to the cell surface. We compared expression of PSGL-1 on leukocytes from WT mice, ΔCD mice, and heterozygous mice containing one allele for WT PSGL-1 and one allele for ΔCD PSGL-1. If interactions between cytoplasmic domains were irrelevant for dimerization, then ~50% of the surface PSGL-1 on heterozygous leukocytes should be WT/ΔCD heterodimers and another 25% should be WT/WT homodimers, with a total expression of ~75% of that on WT leukocytes given the very low surface expression of ΔCD/ΔCD homodimers. The export of WT/ΔCD heterodimers from the ER to the cell surface should decrease the 65-kDa ΔCD precursor. However, flow cytometry revealed that surface expression of PSGL-1 on heterozygous leukocytes reached only ~50% of that on WT leukocytes (Fig. 7, A and B). Furthermore, Western blots showed that heterozygous leukocytes still expressed high levels of the 65-kDa precursor (Fig. 7C). To clearly resolve the mobilities of mature PSGL-1 with or without the cytoplasmic domain, we fractionated cell lysates by SDS-PAGE under reducing conditions for a longer period in a gel with a low polyacrylamide concentration. The mature PSGL-1 in heterozygous leukocytes co-migrated with PSGL-1 in WT leukocytes and migrated slower than mature PSGL-1 in ΔCD leukocytes (Fig. 7D). Thus, very little ΔCD PSGL-1 reached the surface of heterozygous leukocytes. These data confirm that interactions between cytoplasmic domains promote dimerization of PSGL-1. Furthermore, dimerization of two WT subunits takes precedence over dimerization of a WT and ΔCD subunit. Almost all of the mature WT PSGL-1 in WT or heterozygous leukocytes migrated as disulfide-linked dimers under non-reducing conditions (Fig. 7E). This could occur if dimerization of two WT subunits in the ER is faster than export of a monomeric WT protein, if two cytoplasmic domains in a WT homodimer cause faster ER export than one cytoplasmic domain in a WT monomer, and/or if some WT monomers form disulfide-linked dimers after they reach the cell surface.

Discussion

Here we documented that the cytoplasmic domain was essential for efficient transport of PSGL-1 from the ER to the Golgi complex. The cytoplasmic domain also cooperated with the transmembrane domain to mediate noncovalent dimerization of PSGL-1. However, dimerization of PSGL-1 was not required for transport (Fig. 8). Our data provide mechanistic insights into how PSGL-1 is organized on cell surfaces and have implications for how this organization affects its functions.

Dimerization of two newly synthesized PSGL-1 subunits is initiated in the ER through noncovalent interactions between the transmembrane domains and between the cytoplasmic domains. These interactions juxtapose the cysteine in the ectodomain of each subunit so that a disulfide bond forms in the oxidizing environment of the ER. When the cysteine is mutated to prevent disulfide bonds, the noncovalent interactions are strong enough to maintain stable dimers on the cell surface (18). Substituting the transmembrane domain of PSGL-1 with that of CD43 prevents dimerization. Here we showed that deleting the cytoplasmic domain impaired dimerization. Thus, neither transmembrane domain interactions nor cytoplasmic domain interactions are sufficient to maintain noncovalent PSGL-1 dimers. Although greatly reduced in number, disulfide-linked homodimers of ΔCD PSGL-1 did reach the cell surface. Interactions between transmembrane domains likely form transient ΔCD homodimers in the ER, some of which are stabilized by disulfide bonds before they dissociate.

Most glycoproteins must properly fold and, in some cases, dimerize or oligomerize before they exit the ER (41–43). Chaperones retain these proteins in the ER until they fold; if folding is unsuccessful, the proteins are targeted to proteasomes for degradation (44). However, we observed that deleting the cytoplasmic domain did not cause rapid degradation of PSGL-1 in proteasomes. We excluded dimerization as a transport mechanism because a single cytoplasmic domain could transport a monomeric form of
PSGL-1 containing the transmembrane domain of CD43. The ectodomains of mucins such as PSGL-1 probably have little secondary structure before they enter the Golgi, where the attachment of O-glycans causes them to adopt a highly extended conformation (45,46). Some extremely large mucins are retained in the ER because of defective subunit assembly and/or disulfide bond formation (47). However, these retention mechanisms do not apply to PSGL-1, which can exit the ER as a monomer with no extracellular cysteine if it retains the cytoplasmic domain.

Anterograde trafficking signals are important mechanisms to facilitate protein movement from the ER to the Golgi apparatus (48,49). Luminal signals are bound by transmembrane receptors in the ER. The cytoplasmic domains of these receptors interact with the coat protein II (COPII) complex surrounding nascent ER-to-Golgi transport vesicles. In addition to sorting soluble proteins, these receptors probably identify luminal export signals on most transmembrane proteins to accelerate ER exit. However, the cytoplasmic domains of a few membrane proteins serve as export signals by interacting directly with COPII proteins. Some of these signals comprise short peptide sequences related to those used for endocytosis and other trafficking events (50-55). Given its critical role for ER exit, the cytoplasmic domain of PSGL-1 likely interacts directly with COPII proteins. Serial truncations suggested that multiple regions of the cytoplasmic domain are required for PSGL-1 to exit the ER efficiently (unpublished data). The cytoplasmic domain might present several discrete export signals, each comprising a short peptide sequence, or a combinatorial signal by juxtaposing different regions of the sequence. Because the cytoplasmic domain exports either dimers or monomers of PSGL-1, the signal(s) must be accessible on either single or dimeric proteins. The small numbers of disulfide-linked ΔCD PSGL-1 dimers that reached the cell surface probably exited the ER by random diffusion into budding COPII vesicles. Although dimerization of ΔCD PSGL-1 was slow, it was faster than ER export since disulfide-linked dimers comprised virtually all ΔCD PSGL-1 molecules on the cell surface.

The ER-export role of the cytoplasmic domain is essential for the cell adhesion and signaling functions of PSGL-1, which require its expression on the cell surface. It will be useful to determine whether other transmembrane mucins in hematopoietic cells carry ER export signals in their cytoplasmic domains. Remarkably, after it reaches the cell surface PSGL-1 does not need its cytoplasmic domain to mediate leukocyte rolling on P-selectin or to localize in microvilli or lipid rafts (20). However, PSGL-1 on neutrophils requires its cytoplasmic domain to propagate signals as it engages P- or E-selectin during rolling (20,22). This signaling pathway needs intact lipid rafts and involves the serial activation of Src family kinases, the adaptors DAP12 and FcRγ, Syk, Bruton’s tyrosine kinase, phospholipase Cy2, and p38 MAPK (20-22,56-58). These signals ultimately activate integrin αLβ2 to a conformation that slows rolling velocities on ICAM-1, and they cooperate with chemokine-mediated signals to maximize neutrophil recruitment to sites of inflammation. How the cytoplasmic domain initiates activation of Src family kinases and their downstream mediators is not known. It has no catalytic domain or other known signaling motifs. It will be important to determine whether the cytoplasmic domain of PSGL-1 must dimerize to propagate signals. Interactions of transmembrane domains mediate dimerization of the erythropoietin receptor (59). Engagement of this receptor alters the conformations of the cytoplasmic domains, triggering signaling (60). If dimerization of PSGL-1 is required for signaling, determining the respective structures of monomers and dimers will become an important task.

Acknowledgements

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Abbreviations

The abbreviations used are: BS3, bis(sulfosuccinimidyl) suberate; COPII, coat protein II; ER, endoplasmic reticulum; ICAM-1, intercellular adhesion molecule-1; OSGE, O-sialoglycoprotein endopeptidase; PSGL-1, P-selectin glycoprotein ligand-1.

References

Figure legends

Figure 1. **Deleting the cytoplasmic domain impairs expression of PSGL-1 on surfaces of leukocytes and transfected cells.** (A) Schematic of the extracellular domain, transmembrane domain (TMD), and cytoplasmic domain of murine PSGL-1. The amino acid sequence of the WT cytoplasmic domain is depicted. The two arginines in the ΔCD cytoplasmic domain are shown. (B and C) Flow cytometric analysis of PSGL-1 expression on neutrophils from bone marrow (B) or from CFU-GM colonies (C) from WT and ΔCD mice. Cells were incubated with PE-conjugated rat anti-murine PSGL-1 mAb. Neutrophils were identified by scatter properties and by staining with FITC-conjugated anti-Gr-1 mAb. The mean fluorescence intensity (MFI) of anti-PSGL-1 staining is shown. (D) Northern blot showing PSGL-1 mRNA levels in bone marrow leukocytes from WT and ΔCD mice (arrow). Ethidium bromide-stained 18S RNA was used as a loading control. (E) Schematic of the extracellular domain, TMD, and cytoplasmic domain of human PSGL-1. The amino acid sequence of the WT cytoplasmic domain is depicted. The single arginine in the ΔCD cytoplasmic domain is shown. (F) Flow cytometric analysis of human WT or ΔCD PSGL-1 expression on stably transfected CHO cells. (G) PSGL-1 mRNA levels from CHO cells with equivalent cell-surface expression of human WT or ΔCD PSGL-1. The mRNA levels were quantified by real-time RT-PCR and are depicted as relative expression levels, with that of WT PSGL-1 normalized to 1. The data in B, C, F, and G represent the mean ± SD of at least three experiments. The data in D are representative of three experiments.

Figure 2. **Deleting the cytoplasmic domain causes PSGL-1 to accumulate in an intracellular compartment.** (A) Bone marrow leukocytes were incubated with or without OSGE as indicated, fixed, permeabilized with Triton X-100 (TX-100) as indicated, and stained with PE-conjugated rat anti-murine PSGL-1 mAb. Neutrophils were identified by scatter properties and by staining with FITC-conjugated anti-Gr-1 mAb. The MFI of anti-PSGL-1 staining is shown. The data represent the mean ± SD of three experiments. (B) Fixed bone marrow leukocytes, permeabilized as indicated with Triton X-100 (TX-100) were serially incubated with biotinylated anti-murine PSGL-1 mAb and streptavidin-Alexa 488. The cells were visualized with a fluorescence microscope. The data are representative of at least 30 cells from three experiments. Bar, 5 μm.

Figure 3. **Deleting the cytoplasmic domain causes retention of a PSGL-1 precursor in the ER of leukocytes.** (A) Western blot of leukocyte lysates treated with or without OSGE. The blot was probed with rabbit anti-PSGL-1 antibody. The arrow marks the 65-kDa PSGL-1 species. (B) Intact ΔCD leukocytes were incubated with or without Pronase, washed, lysed, and analyzed by Western blotting with rabbit anti-PSGL-1 antibody. The arrow marks the 65-kDa PSGL-1 species. (C) Western blots of WT, ΔCD, and T-syn−/− leukocyte lysates probed with rabbit anti-PSGL-1 antibody or Helix pomatia agglutinin (HPA). (D) ΔCD leukocyte lysate was treated with or without endoglycosidase H and analyzed by Western blotting with rabbit anti-PSGL-1 antibody. The arrows marks the change in mobility of the 65-kDa PSGL-1 species after treatment with endoglycosidase H. (E) Fixed and permeabilized ΔCD leukocytes were stained with antibodies to calnexin (red) and PSGL-1 (green). Nuclei were stained with DAPI (blue). Fluorescence was measured with a confocal microscope. The data are representative of at least three experiments. Bar, 5 μm.

Figure 4. **Deleting the cytoplasmic domain delays export of PSGL-1 from the ER to the Golgi apparatus.** (A) WT leukocytes were treated with or without brefeldin A and then lysed. Lysates were analyzed by Western blotting with rabbit anti-PSGL-1 antibody. (B and C) Splenocytes from WT or ΔCD mice were incubated in medium containing [35S]methionine/cysteine and chased in medium containing...
unlabeled methionine/cysteine for the indicated time. Cell lysates were immunoprecipitated with rabbit anti-PSGL-1 antibody. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. (D) Splenocytes from WT or ΔCD mice were incubated in medium containing cycloheximide for the indicated time and then lysed. Lysates were analyzed by Western blotting with rabbit anti-PSGL-1 antibody. The data are representative of two to three experiments.

Figure 5. Deleting the cytoplasmic domain impairs dimerization of PSGL-1. (A) Schematic of murine PSGL-1 constructs. (B) Western blot of lysates from stably transfected CHO cells expressing matched surface densities of each PSGL-1 construct. The blot was probed with rabbit anti-PSGL-1 antibody. The data are representative of three experiments. (C) Stably transfected CHO cells expressing ΔCD C307S or C307S PSGL-1 were incubated with or without the crosslinker BS3. The cells were lysed and analyzed by Western blotting with rabbit anti-PSGL-1 antibody. The data are representative of three experiments. (D) Schematic of PSGL-1 constructs fused to GFP or DsRed. CD, cytoplasmic domain. (E) CHO cells were transiently transfected with constructs encoding C307S-PSGL-1-GFP and C307S-PSGL-1-DsRed or with constructs encoding ΔCD-C307S-PSGL-1-GFP and ΔCD-C307S-PSGL-1-DsRed. FRET between GFP and DsRed on the plasma membrane was measured by sensitized acceptor emission with a confocal microscope. FRET efficiency is plotted as the mean ± SD from measurements of at least 35 cells.

Figure 6. Dimerization of PSGL-1 is not required to exit the ER. (A) Schematic of murine PSGL-1 constructs. (B) Lysates from stably transfected CHO cells expressing matched surface densities of the indicated PSGL-1 construct were resolved by non-denaturing gel electrophoresis and transferred to a PDVF membrane. The blot was probed with rabbit anti-PSGL-1 antibody. (C) Western blot of lysates from stably transfected CHO cells expressing matched surface densities of each PSGL-1 construct. The blot was probed with anti-murine PSGL-1 mAb. The data in B and C are representative of three experiments.

Figure 7. Leukocytes from heterozygous mice expressing WT and ΔCD PSGL-1 do not transport WT/ΔCD heterodimers to cell surfaces. (A) Flow cytometric analysis of PSGL-1 expression on neutrophils from WT mice, ΔCD mice, and heterozygous mice containing one allele for WT PSGL-1 and one allele for ΔCD PSGL-1 (Het). Leukocytes from the indicated genotype were incubated with PE-conjugated anti-murine PSGL-1 mAb. WT leukocytes were also incubated with PE-conjugated isotype control mAb (Control). Neutrophils were identified by scatter properties and by staining with FITC-conjugated anti-Gr-1 mAb. (B) MF1 of PE-conjugated anti-PSGL-1 binding to neutrophils of the indicated genotype. The data represent the mean ± SD of three experiments. (C-E) Western blots of leukocyte lysates of the indicated genotype. SDS-PAGE was performed under reducing conditions in C and D and under non-reducing conditions in E. The blots were probed with rabbit anti-PSGL-1 antibody. The data in A and C-E are representative of three experiments.

Figure 8. Model for dimerization and ER export of PSGL-1. Cooperative interactions between cytoplasmic domains and between transmembrane domains form PSGL-1 homodimers. A disulfide bond added in the ER further stabilizes each dimer. The cytoplasmic domain contributes a key signal for export of PSGL-1 from the ER to the Golgi complex. Dimerization of PSGL-1 is not required for export. Swapping the transmembrane domain and mutating the extracellular cysteine generates a monomeric form of PSGL-1 that is exported efficiently from the ER. Deleting the cytoplasmic domain impairs dimerization and delays export.
Figure 1

A. Murine PSGL-1

WT: RLLRKTMYVPRNYSTEMICISSLPEGGDGAPVTANGKLKTEPSGDRDDDLTLHSFLP
ΔCD: RR

Human PSGL-1

WT: RLSKRGHMYPVNYSTEMVCISLLPDGEGSPATANGKLKAKSPGLEPREDREDDDLTLHSFLP
ΔCD: RR

B. Anti-PSGL-1 (MFI x 10^2)

C. Anti-PSGL-1 (MFI x 10^2)

D. Relative mRNA expression

E. Human PSGL-1

F. Anti-PSGL-1 (MFI x 10^2)

G. Relative mRNA expression
Figure 2

Anti-PSGL-1 (MFI x 10^2)

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Surface

Surface after OSGE

Inside

Figure 2
Figure 3
Figure 4
A Murine PSGL-1

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Figure 5
### Figure 6

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**B**

Non-denaturing PAGE

**C**

- **WT**
- **ΔCD**
- **GPA TMD**
- **ΔCD GPA TMD**
- **CD43 TMD**
- **ΔCD CD43 TMD**

- Mature
- Precursor

Figure 6
Figure 7

A. Flow cytometry analysis showing relative cell number against Anti-PSGL-1-PE for WT, Het, and ΔCD.

B. Bar graph displaying Anti-PSGL-1 (MFI x10^5) for WT, Het, and ΔCD.

C. Western blot showing WT, Het, and ΔCD bands for mature and precursor proteins.

D. Western blot showing WT and ΔCD bands for mature proteins.

E. Western blot showing WT, Het, and ΔCD bands for mature dimer and monomer, with non-reducing conditions.
Model for dimerization of PSGL-1 in the ER

- **WT**: Extended ectodomain with no predicted secondary structure. Efficient dimerization and ER export.
- **Monomeric CD43TMD**: Transmembrane domain interactions eliminated by substituting CD43 transmembrane domain. Cytoplasmic domain interactions are not sufficient for dimerization. No dimerization, but efficient ER export.
- **ΔCD**: Transmembrane domain interactions alone allow delayed dimerization. No ER export signal. Delayed dimerization and ER export.

Figure 8
Cytoplasmic domain of P-selectin glycoprotein ligand-1 facilitates dimerization and export from the endoplasmic reticulum


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