Human PSGL-1 Interacts with the Skin-Associated Chemokine CCL27 Via Sulfated Tyrosines at the PSGL-1 Amino Terminus

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Running title: PSGL-1 Interaction with CCL27
SUMMARY

P-selectin glycoprotein ligand-1 (PSGL-1), a sialomucin expressed on leukocytes, is a major ligand for P-selectin and mediates leukocyte rolling on the endothelium. Here we show that human PSGL-1 interacts with CCL27 (CTACK/ILC/ESkine), a skin-associated chemokine that attracts skin-homing T lymphocytes. A recombinant soluble form of PSGL-1 (rPSGL-Ig) preferentially bound CCL27 among several chemokines tested. This interaction was abrogated by arylsulfatase treatment of rPSGL-Ig, suggesting that sulfated tyrosines play a critical role. In contrast, removal of either N-glycans or O-glycans by glycosidase treatment of rPSGL-Ig did not affect the interaction. The binding of CCL27 to a recombinant PSGL-1 synthesized in the presence of a sulfation inhibitor was lower than that produced in normal medium. Moreover, mutation of the tyrosines at the amino terminus of PSGL-1 to phenylalanine abolished the binding, further supporting the role of sulfated tyrosines in the CCL27-PSGL-1 interaction.

Functionally, rPSGL-Ig reduced the chemotaxis of L1.2 cells expressing CCR10, the receptor for CCL27. In addition, the expression of human PSGL-1 on CCR10-expressing L1.2 cells resulted in reduced chemotaxis to CCL27. These findings suggest a role for PSGL-1 in regulating chemokine-mediated responses, in addition to its role as a selectin ligand.
INTRODUCTION

Leukocyte migration from the blood to tissues is initiated by transient and reversible interactions that capture leukocytes from flowing blood and allow them to roll on the surface of endothelial cells under blood flow. These interactions are primarily mediated by selectins, a family consisting of three cell-adhesion molecules: L-selectin (CD62L), which is expressed on most leukocytes, and E-selectin (CD62E) and P-selectin (CD62P), which are expressed on activated vascular endothelium (1, 2). The rolling cells sense activating factors such as chemokines presented on the endothelium, which induce the activation of leukocyte integrins, leading to stable cell attachment and the subsequent transmigration of leukocytes into tissues (3).

P-selectin glycoprotein-1 (PSGL-1; CD162) has been identified as the major ligand for P-selectin on myeloid cells and subsets of activated T cells (4). PSGL-1 mediates P-selectin-mediated neutrophil rolling and migration in vivo (5) as well as T-cell migration into inflamed skin (6, 7). Besides being a P-selectin ligand, PSGL-1 functions as a ligand for E-selectin and L-selectin in vivo (7-9). To bind selectins, PSGL-1 must be modified by specific core-2-type O-glycans containing the sialyl Lewis\(^x\) (sLe\(^x\)) moiety, which is synthesized by multiple glycosyltransferases, including core 2 -1,6-N-acetylglucosaminyltransferase I (C2GlcNAcT-I) and -1,3-fucosyltransferase VII (FucT-VII) (10). In human skin-homing T cells, the PSGL-1 glycans carry the cutaneous lymphocyte-associated antigen (CLA), a sLe\(^x\)-related carbohydrate epitope defined by the monoclonal antibody (mAb) HECA-452 (11). In addition to O-glycans, PSGL-1 also requires sulfated tyrosines at its amino terminus to bind P-selectin and L-selectin (12-15).

A recombinant PSGL-1 in a selectin-binding glycoform (rPSGL-Ig), consisting of the amino-terminal region of human PSGL-1 fused to the Fc portion of human IgG1, has been
developed (12), and it has been shown to have anti-inflammatory effects in various models of inflammation (16-19). Although the effects of rPSGL-Ig have been attributed to its ability to bind selectins, a recent report suggests that it has additional functions, including the ability to bind the murine chemokine KC (20).

Chemokines regulate leukocyte traffic throughout the body during immune surveillance and inflammation. Chemokines presented on the endothelium induce the firm adhesion of rolling leukocytes through the activation of integrins. Chemokines also direct the migration of leukocytes into specific microenvironments within tissues. Recent data reveal that chemokines and their receptors play an important role in controlling the specificity of lymphocyte subsets for certain sites (21). In inflamed skin, the expression of several chemokines, including CCL17 (TARC) and CCL27 (CTACK/ILC/ESkine) is up-regulated (22, 23). CCL27 is expressed exclusively in the skin and attract CLA⁺ T cells (24), which express CCR10, the receptor for CCL27 (25, 26), indicating that CCL27 is a skin-associated chemokine that mediates T-cell migration into the skin.

Several chemokine receptors, such as CCR5, CCR2b, CX3CR1, and CXCR4, are modified in the amino-terminal region by tyrosine sulfation (27-30). In addition, CCR5 is modified by O-glycosylation in the amino-terminal region, which, together with tyrosine sulfation, contributes to its high-affinity binding to chemokines (31). Chemokines also bind sulfated glycosaminoglycans such as heparan sulfate, which are thought to anchor the chemokine for recognition by a receptor-bearing cell, further suggesting the role of sulfation in chemokine binding (32, 33). It is well established that tyrosine sulfation and O-glycosylation are the posttranslational modifications of PSGL-1 required for selectin binding.
Based on the similar structural requirements of PSGL-1 and some chemokine receptors to bind their ligands with high affinity, we investigated the interaction of rPSGL-Ig with chemokines. Here we show that rPSGL-Ig preferentially bound CCL27, among the various chemokines examined. Our results show that sulfated tyrosines were critical for PSGL-1 binding to CCL27, whereas neither O-glycans nor N-glycans contributed to the binding. Moreover, rPSGL-Ig and cell-surface-expressed PSGL-1 partially inhibited the chemotaxis of L1.2 cells expressing CCR10. Thus, in addition to its well-characterized role as a selectin ligand, these results support a role for PSGL-1 in binding certain chemokines and thereby regulating cell responses to those chemokines.
EXPERIMENTAL PROCEDURES

Reagents—The rPSGL-Ig was a kind gift from Wyeth Research, Cambridge, MA. It was developed by linking a truncated human PSGL-1 to the Fc portion of human IgG1, and produced in Chinese hamster ovary cells that had been engineered to express FucT-VII and C2GlcNAcT-I (19). Two hinge-proximal amino acids at positions 234 and 237 within the IgG1 Fc portion are mutated to alanine to reduce complement activation and Fc receptor binding. The chemokine-Fc chimeric protein CCL27-Fc and control Fc were prepared as described previously (34). Human chemokines CCL1 (I-309), CCL2 (MCP-1), CCL3 (MIP-1\(^{\alpha}\)), CCL4 (MIP-1\(^{\beta}\)), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CXCL1 (GRO-\(\alpha\)), CXCL4 (PF-4), CXCL5 (ENA-78), CXCL8 (IL-8), CXCL10 (IP-10), and CXCL12 (SDF-1\(^{\beta}\)) were purchased from Peprotech, CCL21 (SLC) from DakoCytomation, and CCL27 and CCL28 (MEC) from R&D Systems. CCL17, CCL18 (PARC), CCL19 (ELC), CCL20 (LARC), XCL1 (SCM-1\(^{\alpha}\)), and XCL2 (SCM-1\(^{\beta}\)) were kindly provided by Shionogi (Osaka, Japan). Recombinant human P-selectin, E-selectin, biotinylated anti-P-selectin, biotinylated anti-CCL27, and biotinylated anti-CCL28 were purchased from R&D Systems, biotinylated anti-CCL21 from DakoCytomation, peroxidase-conjugated anti-human IgG, peroxidase-conjugated anti-rabbit IgG, and peroxidase-conjugated anti-mouse IgG\(+M\) from American Qualex, and peroxidase-conjugated streptavidin from Zymed. Polyclonal anti-PSGL-1 antibodies prepared using a synthetic peptide (QATEYEYLDYDFLPETEPP) based on residues 42-60 of human PSGL-1 were kindly provided by Dr. Bruce Furie (Harvard Medical School, Boston, MA). The anti-PSGL-1 mAbs PL1 and KPL1 were purchased from Immunotech and BD Biosciences, respectively.
Cells—COS-7 cells were maintained in DMEM containing 10% FCS. A mouse pre-B cell line L1.2 was kindly provided by Dr. Eugene Butcher (Stanford University, Stanford, CA) and maintained in RPMI 1640 containing 10% FCS. L1.2 cells expressing human CCR2b, CCR7, and CCR10 were maintained in RPMI 1640 containing 10% FCS and 0.8 mg/ml G418 (Sigma) (34). L1.2 cells coexpressing chemokine receptor and human PSGL-1 were maintained in RPMI 1640 containing 10% FCS, 0.8 mg/ml G418, and 20 μg/ml blasticidin (Invitrogen).

Generation of wild-type and mutant hPSGL-Fc proteins—To generate the hPSGL-Fc and hPSGL-long-Fc constructs, a fragment corresponding to the first 47 amino acids or the entire extracellular domain of mature human PSGL-1 was amplified by PCR using the 5’ primer CGGGAGCTAGCACAGGCCACCGAATATGAG and the 3’ primer GTAGGATCCCTTGCAGCAGGCTCCACAGTG or CAGCGGATCCTGCTTCACAGAGATGTGGTC. The amplified fragment was digested with NheI and BamHI and ligated into the CD5 leader-IgG1 vector, provided by Dr. Brian Seed (Massachusetts General Hospital, Boston, MA), at the NheI and BamHI sites. To generate the hPSGL-Fc constructs with mutations in tyrosines, the following 5’ primers (with the mutated nucleotides underlined) were used. FFF mutant: CGGGAGCTAGCACAGGCCACCGAATTTGAGTT
CCTAGATTATGATTTCCTG;
FYY mutant: CGGGAGCTAGCACAGGCCACCGAATTTGAGTACCT AGATTATGATTTCCTG;
YFY mutant: CGGGAGCTAGCACAGGCCACCGAATATGAGTTCCT AGATTATGATTTCCTG;
YYF mutant:
CGGGAGCTAGCACAGGCCACCGAATATGAGTACCTAGATTGTGATTTCCTG;

FFY mutant:
CGGGAGCTAGCACAGGCCACCGAATTTGAGTTCCCTAGATTATGATTTCCTG;

FYF mutant:
CGGGAGCTAGCACAGGCCACCGAATTTGAGTACCTAGATTGTGATTTCCTG;

YFF mutant:
CGGGAGCTAGCACAGGCCACCGAATATGAGTTCCTAGATTGTGATTTCCTG.

COS-7 cells were transfected with the constructs using DEAE dextran. In some experiments, COS-7 cells were cotransfected with hPSGL-Fc constructs and expression plasmids for FucT-VII and N-acetylglucosamine-6-O-sulfotransferase GST-3 (also called LSST or HEC-GlcNAc6ST)(35, 36). The culture supernatants were harvested 6 days after transfection and applied to a Protein A Sepharose column. The Chimeric proteins were eluted with 4 M imidazole (pH 8.0) and dialyzed against PBS (37). The protein concentration was measured by BCA protein assay kit (Pierce).

To generate unsulfated hPSGL-Fc protein, COS cells transfected with the hPSGL-Fc construct were cultured for 5 days in sulfate-free medium (Gibco) containing 10 mM sodium chlorate. The chimeric protein was purified as above.

**Enzyme treatment of rPSGL-Ig**—To remove sialic acids, rPSGL-Ig was incubated with 1 U/ml neuraminidase from *Clostridium perfringens* (Sigma) in 50 mM sodium phosphate (pH 5.0) at 37°C for 1 h. To remove O-glycan chains, the rPSGL-Ig was treated with a mixture of enzymes consisting of 1 U/ml neuraminidase, 0.15 U/ml -1,4-galactosidase (Prozyme), 2 U/ml -N-acetylglucosaminidase (Prozyme), and 50 mU/ml endo- -N-acetylgalactosaminidase
(O-Glycanase; Glyko) in 50 mM sodium phosphate (pH 7.0) at 37°C overnight. To remove N-glycan chains, the rPSGL-Ig was treated with 250 U/ml N-Glycosidase F (Calbiochem) in 50 mM sodium phosphate (pH 7.5) at 37°C overnight. For sulfatase treatment, the rPSGL-Ig was incubated with 1 U/ml sulfatase from abalone entrails (Sigma) in 100 mM sodium acetate (pH 5.0) at 37°C for 1 h. Control samples were incubated in the same buffer under the same conditions without enzyme.

**Dot blot assays**—To study the binding of PSGL-1 to chemokines, various chemokines and selectins were spotted onto nitrocellulose membranes (Hybond-C; Amersham). The membrane was blocked in PBS containing 3% BSA and incubated with 4 µg/ml rPSGL-Ig or control human IgG (Sigma) for 1 h. The membrane was then washed and incubated with a 1:50,000 dilution of peroxidase-conjugated anti-human IgG. The membrane was washed, exposed to ECL reagents (Amersham). In some experiments, rPSGL-Ig and control human IgG, untreated or treated with glycosidases or sulfatases, and various preparations of hPSGL-Fc were spotted onto a membrane. The membrane was blocked as described above and incubated with 0.3 µg/ml CCL27 for 1 h. The bound chemokine was detected using a biotinylated anti-CCL27 antibody followed by peroxidase-conjugated streptavidin.

**ELISA-like binding assays**—rPSGL-Ig, control human IgG, or various preparations of hPSGL-Fc (10 µg/ml) were immobilized on Sumilon H plates (10 µg/ml, 50 µl/well) at 37°C for 2 h, and the plates were blocked with 3% BSA in PBS at 4°C overnight. The plates were washed and incubated with CCL27, P-selectin, or a polyclonal anti-PSGL-1 antibody (0~3 µg/ml) for 1 h at room temperature, then washed again and incubated with 0.1 µg/ml biotinylated anti-CCL27,
biotinylated anti-P-selectin, or biotinylated anti-rabbit IgG for 1 h at room temperature. The plates were further incubated with a 1:5000 dilution of peroxidase-labeled streptavidin for 1 h at room temperature. To quantify the reaction, o-phenylenediamine was added, and the optical density was read at 490 nm on an Immuno Mini NJ-2300 microplate reader (InterMed).

**ELISA**—Various preparations of hPSGL-Fc (10 µg/ml) were immobilized on Costar 3690 plates (10 µg/ml, 25 µl/well) at 37°C for 2 h and the plates were blocked with 3% BSA in PBS at 4°C overnight. The plates were washed and incubated with a polyclonal anti-PSGL-1 antibody, CSLEX-1 (BD Biosciences), G72 (38), or 2H5 (39) for 1 h at room temperature. The plates were washed and then incubated with peroxidase-labeled anti-rabbit IgG or anti-mouse IgG+M for 1 h at room temperature.

**Flow cytometry**—To examine the PSGL-1 expression on transfected L1.2 cells, the cells were stained with PE-labeled anti-PSGL-1 KPL-1 (BD Biosciences) or non-labeled PL-2 (Immunotech) followed by FITC-labeled anti-mouse IgG. For CCR10 staining, the cells were incubated with goat anti-CCR10 (ImmunoDetect) followed by Alexa Fluor 488-labeled anti-goat IgG (Molecular Probes). Stained cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter). To assess the chemokine binding by flow cytometry, the cells were incubated with CCL27-Fc or control Fc for 30 min at 4°C, then washed, incubated with biotinylated goat anti-human IgG (Cappel), and stained with streptavidin-PE (BD Biosciences). In some experiments, the cells were treated with 120 µg/ml O-sialoglycoprotein endopeptidase (OSGE; Cedarlane) at 37°C for 1 h and washed before staining. To examine whether anti-PSGL-1 mAbs affect CCL27 binding, the cells were preincubated with 20 µg/ml PL1, KPL1, or control
mouse IgG before staining. CCL27-Fc binding was detected using biotinylated goat anti-human IgG adsorbed against mouse IgG using mouse IgG-Agarose (Sigma) and streptavidin-PE.

**Generation of L1.2 or L1.2-CCR10 cells expressing human PSGL-1**—To generate the human PSGL-1 expression plasmid, the entire coding region was amplified by PCR from HL-60 cDNA using the following primers: GCTGGATCCGGTGGTGCCATGCCTCTGCAAC and AGTGAATTCCAGGGAGGAAGCTGTGCAGGGTG. The amplified product was digested with BamHI and EcoRI and ligated into pcDNA/myc-His (Invitrogen) at the BamHI and EcoRI sites. Stable L1.2 or L1.2-CCR10 cell lines expressing human PSGL-1 were made by the transfection of L1.2 or L1.2-CCR10 cells with the PSGL-1 plasmid and selection with 20 µg/ml blasticidin.

**Chemotaxis assay**—Chemotaxis assays were performed using the ChemoTx system (NeuroProbe). Cells were suspended at 5 x 10^6 cells/ml in RPMI 1640 containing 0.5% BSA and 10 mM HEPES. Chemokine at the indicated concentrations were placed in the lower chamber, and a filter with a 5 µm pore size was placed on top. In some experiments, rPSGL-Ig was also added to the lower chamber. Aliquots of 1.25 x 10^5 cells/well were applied to the filter's top surface, and the plates were incubated at 37°C in 5% CO₂ for 4 h. The cells that migrated to the bottom were collected and counted on a FACSCalibur (Beckton Dickenson).
RESULTS

PSGL-1 binds CCL27—To investigate the interaction between chemokines and PSGL-1, we first examined the binding of rPSGL-Ig, a recombinant soluble form of PSGL-1, to chemokines immobilized on a nitrocellulose membrane. rPSGL-Ig consists of the 47 amino-terminal amino acids of mature human PSGL-1, fused to a mutated hinge region of human IgG1 that has reduced complement binding and Fc receptor binding (19). rPSGL-Ig is produced in Chinese hamster ovary cells that have been engineered to express FucT-VII and C2GlcNAcT-I, so it carries the glycans required for binding to P-selectin. In agreement with previous results (12), rPSGL-Ig, which contains only the first 47 amino acids of PSGL-1, bound to P-selectin but not detectably to E-selectin immobilized on the membrane (Fig. 1A). Among the human chemokines tested (CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL17, CCL18, CCL19, CCL20, CCL21, CCL27, CCL28, CXCL1, CXCL4, CXCL5, CXCL8, CXCL10, CXCL12, XCL1, and XCL2), rPSGL-Ig preferentially bound to CCL21, CCL27, and CCL28, whereas control human IgG did not bind to these chemokines (Fig. 1A). The binding of rPSGL-Ig to the other chemokines was either undetectable or very weak. We next examined the binding of chemokines to rPSGL-Ig and control human IgG immobilized on the membrane. CCL27 bound to rPSGL-Ig but not to control IgG (Fig. 1B). Incubation of the membrane with secondary reagents without prior incubation with CCL27 did not result in any binding (data not shown), confirming that the signals represented CCL27 binding. Although rPSGL-Ig had bound to CCL21 and CCL28 when these chemokines were immobilized on the membrane, specific binding of CCL21 and CCL28 to immobilized rPSGL-Ig was difficult to detect due to high background binding of these chemokines to the membrane, and thus PSGL-1 interaction with these chemokines was not investigated further in
this study. CCL27 also bound to rPSGL-Ig immobilized on a microtiter plate in a dose-dependent manner (Fig. 1C). These results indicate that the amino-terminal region of PSGL-1 binds CCL27.

*PSGL-1 glycans are not involved in CCL27 binding*—PSGL-1 is modified by sialylated and fucosylated glycans, which are required for its binding to selectins. To study the role of sialylated glycans in PSGL-1 binding to CCL27, we examined the effect of removing the sialic acids from the rPSGL-Ig glycans on the CCL27 binding. Treatment of rPSGL-Ig with sialidase from *Clostridium perfringens*, which hydrolyzes the -2,3, -2,6, and -2,8 glycosidic linkages of terminal sialic residues, did not affect the CCL27 binding, whereas it completely abrogated the P-selectin binding (Fig. 2A). We next investigated the role of the *O*-glycan chains in rPSGL-Ig binding to CCL27. rPSGL-Ig was treated with a combination of glycosidases that removed *O*-glycan chains. As expected, no binding of P-selectin to rPSGL-Ig was observed after this treatment. In contrast, CCL27 bound to the *O*-deglycosylated rPSGL-1-Ig, suggesting that *O*-glycans are not required for the CCL27 binding (Fig. 2A). Since PSGL-1 is also *N*-glycosylated, the role of *N*-glycan chains was also examined, by treating rPSGL-Ig with *N*-glycosidase. P-selectin bound to the *N*-deglycosylated rPSGL-Ig, confirming the previous results that *N*-glycans are not required for P-selectin binding (40). CCL27 also bound to the *N*-deglycosylated rPSGL-Ig (Fig. 2A). In these experiments, each treatment of rPSGL-Ig led to a shift in its migration on SDS-PAGE (Fig. 2B), confirming the effectiveness of the enzymes. The treatment of rPSGL-Ig with a combination of enzymes that removed both the *O*-glycan and *N*-glycan chains did not abrogate the CCL27 binding (data not shown). These results suggest that neither the *O*-glycan nor the *N*-glycan chains on rPSGL-Ig are required for CCL27 binding.
rPSGL-Ig contains only the amino-terminal region of PSGL-1. The remaining extracellular region of PSGL-1 contains a mucin domain with many putative O-glycosylation sites. To examine the role of glycans attached to this region, we generated constructs that contained either the same amino-terminal region as rPSGL-Ig, here termed hPSGL-Fc, or the entire extracellular domain of mature human PSGL-1, termed hPSGL-long-Fc; both were fused to the hinge region of human IgG1. These constructs were introduced into COS-7 cells that endogenously express C2GlcNAcT but not FucT-VII. As shown in Fig. 2C, CCL27 bound similarly to the hPSGL-long-Fc protein and the hPSGL-Fc protein, suggesting that the mucin domain does not play a role in CCL27 binding to PSGL-1. The cotransfection of COS-7 cells with a plasmid expressing FucT-VII, which enables the chimeric proteins to bind P-selectin, did not significantly affect the CCL27 binding (data not shown). Together, these results suggest that no glycosylation of PSGL-1 is required for its binding to CCL27.

_Sulfated tyrosines are required for CCL27 binding to PSGL-1_—It has been shown that in addition to O-glycosylation, tyrosine sulfation of the amino-terminal region of PSGL-1 is required for its high-affinity binding to P-selectin. Treatment of rPSGL-Ig with sulfatase from abalone entrails, which removes sulfates from tyrosines, not only abrogated the P-selectin binding but also the CCL27 binding (Fig. 3A). Sulfatase treatment did not alter the efficiency of immobilization of rPSGL-Ig onto the membrane as shown by an anti-IgG blot (Fig. 3A). This treatment did not alter the migration pattern of rPSGL-Ig on SDS-PAGE (Fig. 3B). In an ELISA-like binding assay where plate-immobilized rPSGL-Ig was treated with sulfatase or left untreated, the binding of both CCL27 and P-selectin to the sulfatase-treated rPSGL-Ig was reduced, whereas a polyclonal antibody that recognizes the amino-terminal region of PSGL-1 bound similarly to
the untreated and sulfatase-treated rPSGL-Ig proteins (Fig. 3C). These results suggest that the tyrosine sulfation of PSGL-1 plays an important role in its binding to CCL27.

To further define the role of tyrosine sulfation in the CCL27-PSGL-1 interaction, we examined the effect of sodium chlorate, which is an inhibitor of sulfation. COS-7 cells were transfected with the hPSGL-Fc construct and incubated in sulfate-free medium containing sodium chlorate. The unsulfated hPSGL-Fc protein prepared in the presence of sodium chlorate and the hPSGL-Fc prepared from cells grown in regular medium did not differ significantly in the amount secreted into the culture medium or in their migration pattern on SDS-PAGE (data not shown). Although CCL27 bound to hPSGL-Fc prepared in the presence of sulfate, it did not detectably bind to unsulfated hPSGL-Fc in a dot blot assay (Fig. 4A). In this experiment, these hPSGL-Fc preparations were similarly immobilized on the membrane (Fig. 4A). The reduced binding of CCL27 to unsulfated hPSGL-Fc was also observed in an ELISA-like binding assay (Fig. 4B). In contrast, a polyclonal anti-PSGL-1 antibody similarly bound to the sulfated and unsulfated hPSGL-Fc proteins (Fig. 4B). These results, together with the effect of sulfatase treatment, strongly indicate that sulfation on tyrosines is critical for PSGL-1 binding to CCL27.

*Sulfation of PSGL-1 glycans does not enhance CCL27 binding*—Sulfation can occur not only on tyrosines but also on glycan chains. To investigate whether sulfation on the PSGL-1 glycans would enhance the CCL27 binding, we transfected COS-7 cells with the hPSGL-Fc construct together with a plasmid for N-acetylglucosamine-6-O-sulfotransferase GST-3, which can sulfate the N-acetylglucosamines in glycans. To compare the CCL27 binding with P-selectin binding, the COS-7 cells were cotransfected with the FucT-VII-expressing plasmid and the hPSGL-Fc and GST-3 constructs. As shown in Fig. 5A, CCL27 binding to the hPSGL-Fc protein
was not enhanced when GST-3 was cotransfected. Similar results were obtained when the hPSGL-long-Fc construct was used for transfection instead of the hPSGL-Fc construct (data not shown). The hPSGL-Fc protein produced in the presence of GST-3 was shown to react with the mAb G72, which recognizes sialyl 6-sulfo LacNAc, confirming that the hPSGL-Fc glycan was sulfated (Fig. 5B). In contrast, P-selectin binding to the hPSGL-Fc protein prepared in the presence of GST-3 and FucT-VII appeared to be slightly enhanced, compared with that produced in the presence of FucT-VII alone (Fig. 5A). The generation of the fucosylated glycan was confirmed by reactivity with the mAb CSLEX-1, which recognizes sLe^x, and the mAb 2H5, which recognizes sLe^x as well as 6-sulfo sLe^x (Fig. 5B). All of the hPSGL-Fc preparations were similarly recognized by an anti-PSGL-1 antibody (Fig. 5B). Thus, the sulfation of glycans on PSGL-1 by GST-3 did not play a significant role in the CCL27 binding.

All three tyrosines in the amino-terminal region of PSGL-1 are important for CCL27 binding—There are three tyrosine residues in the amino-terminal region of human PSGL-1 that can be sulfated. To examine which tyrosine residue is important for CCL27 binding, hPSGL-1-Fc mutants with alterations in one or more of the tyrosines at positions 46, 48, and 51 were generated (Fig. 6A). To evaluate the P-selectin binding, wild-type and mutant hPSGL-Fc proteins were also prepared from COS-7 cells cotransfected with the FucT-VII-expressing plasmid. As shown in Fig. 6B, CCL27 binding to the hPSGL-Fc protein was dramatically reduced when even one tyrosine was mutated to phenylalanine. In contrast, P-selectin bound to the FYY, YYF, and FYF mutants. Thus, although tyrosine sulfation plays an important role in both the PSGL-1-P-selectin and PSGL-1-CCL27 interactions, the relative contribution of each tyrosine to the binding
PSGL-1 affects CCL27-induced chemotaxis—To investigate whether PSGL-1 affected CCL27 function, we examined the effect of rPSGL-Ig on the chemotaxis of L1.2 cells expressing human CCR10. rPSGL-Ig reduced the CCL27-induced chemotaxis in a dose-dependent manner, while control human IgG1 Fc did not (Fig. 7A). The inhibition was observed when rPSGL-Ig was added at 30-100 µg/ml (a molar ratio of 1:3-10). These results suggest that PSGL-1 binding to CCL27 may regulate the chemokine-mediated responses of cells.

We next examined whether cell-surface-expressed PSGL-1 would bind CCL27 and affect cell responses to CCL27. The interaction of CCL27 with cell-surface-expressed PSGL-1 was studied by examining the binding of a CCL27-Fc chimeric protein to L1.2 cells expressing human PSGL-1. CCL27-Fc bound to L1.2 clones expressing human PSGL-1 more strongly than to the parental L1.2 cells that do not express human PSGL-1 (Fig. 7B). The binding of CCL27-Fc to these clones was reduced by treating the cells with OSGE, which cleaves O-sialoglycoproteins such as PSGL-1 (Fig. 7B). A decrease in CCL27-Fc binding to parental L1.2 cells by treatment with OSGE was smaller compared to L1.2 clones expressing human PSGL-1. There was no alteration in the control Fc binding to any of these cells by this treatment. These results suggest that human PSGL-1 expressed on the cell surface may bind CCL27. We next tested whether anti-PSGL-1 mAbs that inhibit P-selectin binding would affect CCL27 binding. Neither PL1 nor KPL1 affected the binding of CCL27-Fc to L1.2 clones expressing human PSGL-1 (Fig. 7C). PL1 and KPL1 also failed to inhibit the CCL27 binding to rPSGL-Ig immobilized on an ELISA plate.
(data not shown). These results suggest that the CCL27 binding site on PSGL-1 may not exactly overlap with the epitopes recognized by these antibodies.

We next prepared L1.2 cells expressing both human CCR10 and human PSGL-1 and assayed these cells for the CCL27-induced chemotaxis. The chemotaxis of L1.2 clones expressing both human CCR10 and PSGL-1 was significantly reduced compared with that of L1.2 cells expressing CCR10 alone (Fig. 7D). The level of CCR10 expression was similar for all these clones (data not shown). Together, these results support the hypothesis that cell-surface-expressed human PSGL-1 binds CCL27 and thereby regulates cell responses to CCL27.
DISCUSSION

In the present study, we investigated the interaction of PSGL-1 with chemokines. We demonstrated that PSGL-1 can bind several chemokines including CCL27. Our data showed that the PSGL-1 interaction with CCL27 is dependent on tyrosine sulfation, but not the glycosylation of PSGL-1. All three tyrosines positioned at the amino terminus of PSGL-1 are required for the CCL27 binding. In addition, we showed that PSGL-1 is able to modulate the CCL27-induced response of CCR10-bearing cells.

Tyrosine sulfation is a late post-translational modification that occurs in the trans-Golgi network and is found in a number of secreted and membrane proteins (41). It plays an important role in various protein-protein interactions. Tyrosine sulfation of the amino terminus of PSGL-1 plays an essential role in P-selectin binding (12-14). Recently, sulfation of amino-terminal tyrosine residues of several G-protein-coupled receptors, including chemokine and chemoattractant receptors as well as glycoprotein hormone receptors, has been demonstrated (27-30, 42). In these receptors, tyrosine sulfation contributes to the high-affinity binding of their ligands. Our data showed that mutation in any one of the tyrosines in the amino terminus of PSGL-1 abrogated the CCL27 binding, indicating that all three tyrosines are required for this binding. Electrostatic interactions are likely to play a role in the binding of the negatively charged region of PSGL-1 generated by sulfated tyrosines to CCL27, which has several basic residues. The requirement of all three tyrosines for CCL27 binding is in contrast to the binding of P-selectin, which was retained to some extent even when tyrosine-46 or tyrosine-51 was mutated. These findings are in agreement with the results of crystallographic studies showing tyrosine-48 as an important component of the high-affinity interaction of PSGL-1 with P-selectin (43).
requirement of all three tyrosines for CCL27 binding might explain why mouse PSGL-1, which has only two tyrosines at its amino terminus, does not bind CCL27 in our assays (data not shown).

Although CCL27 interacts with tyrosine-sulfated PSGL-1, it is unclear whether the binding of CCL27 to its receptor CCR10 requires sulfation of the receptor. Human CCR10 has three tyrosine residues at positions 14, 22, and 32 from the amino terminus, but the Sulfinator, a software tool that predicts tyrosine sulfation sites in protein sequences (44), does not predict the sulfation of any of these tyrosines. The Sulfinator also does not predict tyrosine sulfation in mouse CCR10, which has four tyrosine residues at positions 14, 17, 22, and 32 in the amino-terminal region. It should be experimentally verified whether these tyrosines located in the amino-terminal region of CCR10 are sulfated and contribute to CCL27 binding to CCR10.

Our data also showed that the CCL27 binding to PSGL-1 is not dependent on the glycosylation of PSGL-1. This is in contrast to the selectin binding, which requires sialylated and fucosylated O-glycans of PSGL-1. Sialylated O-glycans of CCR5 were also shown to contribute to high-affinity binding of CCR5 ligands (31). Whether O-glycans are attached to CCR10 and contribute to the binding of CCL27 has not yet been shown.

rPSGL-Ig is a recombinant, soluble, and chimeric form of PSGL-1 that was developed as an antagonist to P-selectin. rPSGL-Ig has been shown to exert anti-inflammatory effects in many models of inflammation; it reduces hepatic ischemia/reperfusion injury in rats (45), accelerates thrombolysis and prevents reocclusion in a porcine model (19), ameliorates acute traumatic shock in rats (16), and protects against myocardial ischemic reperfusion injury in cats (17). A recent report showed that rPSGL-Ig binds the murine chemokine KC, which may in part account for its
anti-inflammatory effect (20). Our results also show that rPSGL-Ig binds to several chemokines, supporting the idea that its effect in various inflammatory conditions is not exclusively due to its inhibition of selectin function, but also of chemokine function. The mechanisms by which rPSGL-Ig inhibits chemokine function may involve the inhibition of chemokine binding to its receptor or the inhibition of chemokine-induced signal transduction.

Although the in vivo relevance of the interaction of PSGL-1 with CCL27 is not yet clear, the modulation of CCL27-induced cell responses by PSGL-1 may confer an additional level of regulation on the trafficking of skin-homing T cells. CCR10 is expressed on a subset of skin-homing T cells, which also express PSGL-1. PSGL-1 on the surface of T cells that have migrated into the skin may bind CCL27 and regulate its function in the local microenvironment. Alternatively, soluble PSGL-1 in the vicinity of these cells may bind CCL27 and affect cell responses to CCL27. A previous study showed that the phorbol ester PMA induces the ectodomain shedding and secretion of PSGL-1 from human neutrophils (46). Several proteases such as BACE1 have been implicated in mediating PSGL-1 shedding (47). In support of this idea, soluble PSGL-1 is found in human bronchoalveolar lavage fluids and in serum (46, 48). A raised concentration of soluble PSGL-1 in serum is associated with a lower frequency and severity of pulmonary fibrosis in systemic sclerosis (48), suggesting that soluble PSGL-1 could function as a protective factor by binding to certain chemokines that promote the progression of the disease.

Since CCL27 is critically involved in various T-cell-mediated inflammatory diseases of the skin such as atopic dermatitis and contact dermatitis (23), the identification of molecules that regulate the function of CCL27 should lead to the development of treatments to control these human skin diseases.
Acknowledgments—We thank Mahiru Kamiya for preparing the hPSGL-Fc construct and Dr. Myoung-Ho Jang and Tomoharu Kikuchi for preparing the CCL27-Fc protein. We also thank Drs. Toshiyuki Tanaka, Toshiyuki Murai, and Haruko Hayasaka for valuable comments, and Ms. Shinobu Yamashita and Ms. Miyuki Komine for secretarial assistance.
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FOOTNOTES

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1 The abbreviations used are: PSGL-1, P-selectin glycoprotein ligand-1; sLe\(^x\), sialyl Lewis\(^x\); C2GlcNAcT-I, core 2 1,6-N-acetylglucosaminyltransferase I; FucT-VII, 1,3-fucosyltransferase VII; CLA, cutaneous lymphocyte-associated antigen; mAb, monoclonal antibody; OSGE, O-sialoglycoprotein endopeptidase.
FIGURES LEGENDS

FIG. 1. **PSGL-1 binds to CCL27.** A, Binding of rPSGL-Ig to chemokines immobilized on a membrane. Selectins, chemokines, and BSA were spotted onto a nitrocellulose membrane. The membrane was incubated with rPSGL-Ig or control human IgG followed by peroxidase-conjugated goat anti-human IgG. Representative blots are shown. B, Binding of CCL27 to rPSGL-Ig immobilized on a membrane. rPSGL-Ig, control human IgG, and BSA were spotted onto a membrane. The membrane was incubated with CCL27 followed by biotinylated anti-CCL27 and then peroxidase-conjugated streptavidin. A representative blot is shown. C, Binding of CCL27 to rPSGL-Ig or control human IgG immobilized on a microtiter plate. rPSGL-Ig or human IgG was immobilized on the wells of a microtiter plate. The plate was incubated with the indicated concentrations of CCL27. The bound CCL27 was detected by biotinylated anti-CCL27 and peroxidase-conjugated streptavidin. Data points are the means from duplicate wells. One of three similar experiments is shown.

FIG. 2. **Glycosidase treatment of rPSGL-Ig does not abolish CCL27 binding.** A, Binding of CCL27 and P-selectin to glycosidase-treated rPSGL-Ig. rPSGL-Ig or control human IgG was treated with sialidase, a mixture of enzymes that together cleave O-glycans, or N-glycosidase. Untreated samples were incubated in the same buffer without the enzyme. These samples were spotted onto a membrane and CCL27 binding and P-selectin binding were examined. B, Effect of glycosidase treatment on migration on SDS-PAGE. rPSGL-Ig, untreated or treated with the enzymes, was separated by SDS-PAGE, and the gel was subjected to silver staining. C, Binding
of CCL27 to hPSGL-Fc and hPSGL-long-Fc proteins. Samples were spotted onto a membrane and CCL27 binding was examined.

**FIG. 3. Arylsulfatase treatment of rPSGL-Ig abrogates CCL27 binding.**  
*A*, Binding of CCL27 and P-selectin to sulfatase-treated rPSGL-Ig. rPSGL-Ig or control human IgG was treated with arylsulfatase. Untreated samples were incubated in the same buffer without the enzyme. Samples were spotted on a membrane and CCL27 binding and P-selectin binding were examined. The membrane was also blotted with anti-IgG to show equivalent binding of the unsulfated and sulfatase-treated rPSGL-Ig to the membrane.  
*B*, Migration of sulfatase-treated rPSGL-Ig on SDS-PAGE. rPSGL-Ig, untreated or treated with the enzyme was separated by SDS-PAGE and the gel was subjected to silver staining.  
*C*, Binding of CCL27, P-selectin, and anti-PSGL-1 antibodies to sulfatase-treated rPSGL-Ig immobilized on a microtiter plate. Microtiter plates were coated with sulfatase-treated or untreated rPSGL-Ig, and incubated with CCL27, P-selectin, or polyclonal anti-PSGL-1 antibodies. Values are the means from duplicate wells. The results represent one of three similar experiments.

**FIG. 4. Treatment of COS-7 cells with sodium chlorate reduces CCL27 binding to PSGL-1.**  
*A*, hPSGL-Fc was produced in COS-7 cells in the regular medium or sulfate-free medium containing sodium chlorate. Purified samples were spotted onto a membrane and CCL27 binding was examined. The membrane was also blotted with anti-IgG to show equivalent binding of the samples.  
*B*, hPSGL-Fc preparations were immobilized on a microtiter plate and CCL27 binding
and anti-PSGL-1 antibody binding were examined. Values are the means from duplicate wells.

The results represent one of three similar experiments.

FIG. 5. **Sulfation of the PSGL-1 glycan does not affect CCL27 binding.** *A*, Binding of CCL27 and P-selectin to hPSGL-Fc modified by GST-3. COS-7 cells were transfected with the hPSGL-Fc construct with or without the constructs for FucT-VII and GST-3, and the proteins were purified from the culture supernatants. Purified samples were spotted onto a membrane and CCL27 binding and P-selectin binding were examined. *B*, Reactivity of hPSGL-Fc preparations with antibodies against sLe^x^ related epitopes. hPSGL-Fc preparations were immobilized on a microtiter plate and assayed for the binding of anti-PSGL-1, G72 (anti-sialyl 6-sulfo LacNAc), CSLEX-1 (anti-sLe^x^), or 2H5 (anti-sLe^x^/6-sulfo sLe^x^). Values are the means from duplicate wells. The results represent one of three similar experiments.

FIG. 6. **CCL27 does not bind to hPSGL-1-Fc with tyrosine mutations.** *A*, Sequences of the tyrosine-mutated hPSGL-Fc constructs. Mutant hPSGL-Fc constructs were generated in which one or more tyrosine residues in the amino-terminal region of PSGL-1 were mutated to phenylalanine. *B*, Binding of CCL27 and P-selectin to the tyrosine-mutated hPSGL-Fc proteins. Tyrosine-mutated hPSGL-Fc proteins were spotted onto a membrane and CCL27 binding and P-selectin binding were examined. The blots shown are from one of three similar experiments.

FIG. 7. **Effect of PSGL-1 on CCL27-induced chemotaxis of L1.2 cells expressing CCR10.** *A*, Effect of rPSGL-Ig on the CCL27-induced chemotaxis of CCR10-expressing L1.2 cells. CCL27 at
100 nM together with rPSGL-Ig or human IgG1 Fc was placed in the lower chamber and CCR10-expressing L1.2 cells were placed in the upper chamber of a ChemoTx apparatus. Three hours later, the number of cells that had migrated into the lower chamber was counted. Migrated cells are expressed as a percentage of the cells that migrated in response to CCL27 in the absence of rPSGL-Ig and human IgG. Data are the means ± SEM from triplicate wells. One of three similar experiments is shown. B, CCL27 binding to PSGL-1 expressed on the cell surface. L1.2 cells and L1.2 clones expressing human PSGL-1 (clones #8 and #19) were treated or not with OSGE, stained with anti-PSGL-1 mAb KPL-1 (left panels) and CCL27-Fc or control Fc (right panels), and analyzed by flow cytometry. Unstained cells (left panels) or cells stained with control Fc (right panels) are shown in filled histograms, which do not show any shift by OSGE treatment. One of three similar experiments is shown. C, Effect of anti-PSGL-1 mAbs on CCL27-Fc binding. L1.2 clones expressing human PSGL-1 (clones #8 and #19) were preincubated or not with mouse IgG, PL1, or KPL1, then stained with PE-labeled KPL-1 (left panel) and CCL27-Fc (right panel), and analyzed by flow cytometry. Mean fluorescence intensity of the cells preincubated in the absence of antibodies is expressed as 100%. D, Effect of the cell-surface expression of PSGL-1 on CCL27-induced chemotaxis. L1.2 cells expressing CCR10 and L1.2 clones expressing both CCR10 and human PSGL-1 (clones #53, #13, and #65) were assayed for CCL27-induced chemotaxis. Data are the means ± SEM from triplicate wells. One of three similar experiments is shown.
Hirata et al. Figure 1

A

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<tr>
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Blot: CCL27

C

Absorbance at 480 nm

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

0.01 0.1 1 10

CCL27 (µg/ml)

- rPSGL-Ig
- Human IgG

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A

hPSGL-Fc + Chlorate
Blot: CCL27  Blot: Anti-IgG
200 100 50 25 12.5 (ng/spot)

B

Absorbance at 490 nm

0 1 2 3 CCL27 (µg/ml)
0 0.05 0.1 0.15 0.2 0.25 0.3

0 0.6 1.2 1.5 1.8 Anti-PSGL-1 (µg/ml)
0 1 2 3

hPSGL-Fc + Chlorate
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B

- BSA
- Human IgG
- Wild type
- FYY
- YFY
- YYF
- FFY
- FYF
- YFF
- FFF

Blot: CCL27
Blot: P-selectin
Human PSGL-1 interacts with the skin-associated chemokine CCL27 via sulfated tyrosines at the PSGL-1 amino terminus
Takako Hirata, Yuko Furukawa, Bo-Gie Yang, Kunio Hieshima, Minoru Fukuda, Reiji Kannagi, Osamu Yoshie and Masayuki Miyasaka

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