Structurally Distinct Requirements for Binding of P-selectin Glycoprotein Ligand-1 and Sialyl Lewis x to *Anaplasma phagocytophilum* and P-selectin*

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Tadayuki Yago‡, Anne Leppänen§, Jason A. Carlyon¶, Mustafa Akkoyunlu**, Sougata Karmakar‡, Erol Fikrig¶, Richard D. Cummings§, and Rodger P. McEver***

From the ‡Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation and ¶Department of Biochemistry and Molecular Biology and Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 and **Section of Rheumatology, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520

Colonization of neutrophils by the bacterium *Anaplasma phagocytophilum* causes the disease human granulocytic ehrlichiosis. The pathogen also infects mice, its natural host. Like binding of P-selectin, binding of *A. phagocytophilum* to human neutrophils requires expression of P-selectin glycoprotein ligand-1 (PSGL-1) and α1–3-fucosyltransferases that construct the glycan determinant sialyl Lewis x (sLeα). Binding of *A. phagocytophilum* to murine neutrophils, however, requires expression of α1–3-fucosyltransferases but not PSGL-1. To further characterize the molecular features that *A. phagocytophilum* recognizes, we measured bacterial binding to microspheres bearing specific glycoconjugates or to cells expressing human PSGL-1 and particular glycosyltransferases. Like P-selectin, *A. phagocytophilum* bound to purified human PSGL-1 and to glycopeptides modeled after the N terminus of human PSGL-1 that presented sLeα on a properly positioned core-2 O-glycan. Unlike P-selectin, *A. phagocytophilum* bound to glycopeptides that contained sLeα but lacked tyrosine sulfation or a specific core-2 orientation of sLeα on the O-glycan. *A. phagocytophilum* bound only to glycopeptides that contained a short amino acid sequence found in the N-terminal region of human but not murine PSGL-1. Unlike P-selectin, *A. phagocytophilum* bound to cells expressing PSGL-1 in cooperation with sLeα on both N- and O-glycans. Moreover, bacteria bound to microspheres coupled independently with glycopeptide lacking sLeα and with sLeα lacking peptide. These results demonstrate that, unlike P-selectin, *A. phagocytophilum* binds cooperatively to a nonsulfated N-terminal peptide in human PSGL-1 and to sLeα expressed on PSGL-1 or other glycoproteins. Distinct bacterial adhesins may mediate these cooperative interactions.

Human granulocytic ehrlichiosis is a tick-transmitted disease caused by a bacterium recently named *Anaplasma phagocytophilum* (1–3). Clinical manifestations include fever, headache, myalgia, leukopenia, thrombocytopenia, and impaired host defenses that occasionally lead to fatal complications (4–7). *A. phagocytophilum* is maintained in a zoonotic cycle between the arthropod vector, *Ixodes scapularis*, and mice that serve as reservoir hosts (8–10). Human infection is inadvertent and is not an obligate component of the life cycle of the bacterium.

A hallmark of infection in both mice and humans is the colonization of neutrophils (1, 2). This tropism for a particular cell type suggests that the bacteria recognize specific molecular determinants on the neutrophil surface. Current information suggests that these determinants are related to the cell-surface ligands for selectins, a family of cell adhesion molecules that mediate tethering and rolling of leukocytes on vascular surfaces during the earliest steps of inflammation (11, 12). P-, E-, and L-selectin bind to α2–3-sialylated and α1–3-fucosylated glycans such as sialyl Lewis x (sLeα),¹ which are expressed on most leukocytes. Targeted disruption of the genes encoding FTVII and FTIV, the α1–3-fucosyltransferases expressed in murine leukocytes, eliminates selectin-dependent interactions in vivo (13, 14). The major ligand for P- and L-selectin on leukocytes is P-selectin glycoprotein ligand-1 (PSGL-1), a homodimeric mucin (12, 15, 16). Monoclonal antibodies to N-terminal epitopes on human and murine PSGL-1 inhibit leukocyte interactions with P- and L-selectin (17–19). P-selectin binds in a stereospecific manner to the N terminus of human PSGL-1 through recognition of tyrosine sulfate residues, adjacent peptide determinants, and fucose and sialic acid residues on a properly positioned core-2 O-glycan (20–23). Murine PSGL-1 appears to use a related group of modifications to bind to P-selectin (24).

Binding of *A. phagocytophilum* to human myeloid HL-60 cells requires cell surface sialylation and correlates with expression of FTVII (25). Moreover, mAbs to N-terminal epitopes on human PSGL-1 inhibit binding of *A. phagocytophilum* to human neutrophils or HL-60 cells (26). Expression of both PSGL-1 and FTVII in heterologous cells renders them susceptible to binding and infection by *A. phagocytophilum*. These data were interpreted to indicate that *A. phagocytophilum* specifically recognizes fucosylated PSGL-1 (26). However, ¹ The abbreviations used are: sLeα, sialyl Lewis x; core2GlcNAcT, core-2 β1–6-N-acetylgalactosaminyltransferase; FT, α1–3-fucosyltransferase; GP, glycopeptide; GSP, glycosulfopeptide; mAb, monoclonal antibody; PHA-L, *P. vulgaris* leukoagglutinin; PSGL-1, P-selectin glycoprotein ligand-1; sPSGL-1, soluble PSGL-1; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary.
measurements of bacterial interactions with intact leukocytes did not demonstrate direct binding of *A. phagocytophilum* to PSGL-1. Studies in mice suggest that the molecular determinants required for *A. phagocytophilum* binding might be more complex than those originally proposed (27). Bacterial binding to and infection of leukocytes from mice lacking both FTVII and FTIV is markedly diminished in *vitro* and *in vivo*, consistent with the observed requirement for α1–3-fucosylation of bacterial ligands on human leukocytes. However, *A. phagocytophilum* binds to and infects leukocytes from PSGL-1-deficient mice much like leukocytes from wild-type mice (27). Thus, *A. phagocytophilum* infection in both humans and mice requires sialylation and α1–3-fucosylation of neutrophils, but PSGL-1 is only required in human neutrophils. These disparities raise the possibility that *A. phagocytophilum* expresses distinct adhesins, which bind cooperatively to distinct ligands on leukocytes. Some of these ligands might be specific for humans or mice.

In this study, we used purified molecularly defined glycoconjugates coupled to microspheres, in conjunction with experiments with transfected cells, to more precisely determine the structural features required for binding to *A. phagocytophilum*. Our data reveal clear differences in how P-selectin and *A. phagocytophilum* bind to PSGL-1. They further suggest that *A. phagocytophilum* binds cooperatively to distinct ligands on leukocyte surfaces.

**MATERIALS AND METHODS**

Preparation of Labeled, Fixed *A. phagocytophilum*—Cell-free *A. phagocytophilum* were prepared as described (27, 28). Bacteria were washed once with Iscove’s modified Dulbecco’s modified Eagle’s media (Invitrogen) and incubated with 10 μM CellTracker Green (Molecular Probes) for 45 min at 37 °C. Labeled bacteria were centrifuged twice to eliminate unbound probe before fixing with 1% paraformaldehyde (27). In some experiments, bacteria were fixed under identical conditions with Saccamanno fluid (Stablab Medical Products), which does not cross-link proteins. Unless noted otherwise, data from paraformaldehyde-fixed bacteria are presented in this paper.

Reagents—Anti-human PSGL-1 mAbs PL1 and PL2 (murine IgG1) were described previously (17). Anti-sLeX mAb HECA-452 (rat IgM) (29) was purified from hybridoma cells obtained from American Type Culture Collection. Monovalent sLeX coupled to biotin by a linker group was purchased from Glyotech. Anti-mouse PSGL-1 mAb 4RA10 (rat IgG1) (30) was a kind gift from Dr. Dietmar Vetewebster, University of Muenster, Muenster, Germany. CHO-131, a murine mAb that recognizes sLeX only on core-2 O-glycans (31), was a kind gift from Dr. Bruce Walcheck, University of Minnesota. FITC-conjugated goat anti-rat IgG mAb, FITC-conjugated goat anti-rat IgM, FITC-conjugated goat anti-mouse IgG (H + L), and FITC-conjugated goat anti-mouse IgM were purchased from Caltag. Isotype control rat IgM and mouse IgM were purchased from BD Biosciences.

The glyco(sulfo)peptides, 1-GP-1, 1-GP-6, 1-GP-6′, 2-GSP-1, 2-GP-6, 2-GSP-6, 4-GP-5, mouse GP-6, human-murine GP-6, and murine-human GP-6 were synthesized as described (20–22). Soluble recombinant human PSGL-1 (sPSGL-1) was purified as described (32).

**Biotinylation of Glycoconjugates and Proteins**—2-GSP-1, 2-GP-6, 2-GSP-6, and sPSGL-1 (20 μg) were biotinylated at their C-terminal cysteine residues by incubation with 4-mx N-6-(biotinamido)hexyl-3′(2-pyridyldithio)propionamide (Pierce) as described (29). Biotinylated sPSGL-1 was dialyzed against PBS to remove free biotin (32).

**Coupling Glycoconjugates or sPSGL-1 to Microspheres**—Streptavidin-coated, polystyrene microspheres (6-μm diameter, Polysciences; 2 × 10⁶ microspheres) were incubated with 5 ng of biotinylated sPSGL-1, 2-GSP-1, 2-GP-6, and 2-GSP-6, 500 ng of biotinylated sPSGL-1, or 50, 500, or 5,000 ng of biotinylated sLeX in 100 μl of PBS at 4 °C for 1 h and then washed three times with PBS (32). In some experiments, the N terminus of 1-GP-1, 1-GP-6, 1-GP-6′, 2-GSP-6, 4-GP-5, murine-human GP-6, or human-murine GP-6 was directly coupled to carboxylated microspheres (6-μm diameter; Polysciences) as described (33). Briefly, carboxylated microspheres (10⁸) were washed sequentially with carbonate and phosphate buffers. The microspheres were then gently mixed with 2% carbodiimide (Polysciences) in phosphate buffer for 4 h at room temperature. After incubation and washing with borate buffer, the microspheres were incubated with 50 ng of glyco(sulfo)peptides in 200 μl of borate buffer with gentle mixing overnight at 4 °C. The microspheres were then incubated with 0.2 μl ethanolamine for 30 min at room temperature to block unreacted carboxylate sites. After washing with PBS, the microspheres were stored in PBS containing 0.5% bovine serum albumin with 0.1% sodium azide.

**Cells**—Transfected Chinese hamster ovary (CHO) cells coexpressing human PSGL-1 with FTVII or with FTVII and core-2 β-1–6-N-acetylglucosaminyltransferase 1 (core2GlcNAcT-1) were prepared as described (34–36). Transfected CHO cells expressing human PSGL-1 in which Thr-16 was substituted with alanine (T16A, also known as T57A in an earlier numbering system) were prepared as described (35). HL-60 cells were maintained in RPMI 1640 containing 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Measurement of Binding of mAbs or A. phagocytophilum to Microspheres or Cells by Flow Cytometry**—Microspheres, PSGL-1-transfected CHO cells, or HL-60 cells (10⁵) in 200 μl of Hanks’ balanced salt solution with 0.5% human serum albumin were incubated with 10 μg/ml PL1, PL2, HECA-452, 4RA10, CHO-131, or the respective iso-type-matched control murine IgG1, rat IgM, rat IgG1, or murine IgM at 4 °C for 30 min. After washing, they were incubated with FITC-conjugated goat anti-mouse IgG (H + L), FITC-conjugated goat anti-rat IgG, FITC-conjugated goat anti-rat IgM, or FITC-conjugated goat anti-mouse IgM at 4 °C for 30 min.

**Binding of *A. phagocytophilum* to microspheres or cells was meas-
ured as described (27). Briefly, 10^6 microspheres or CHO cells were incubated with fixed, CellTracker Green-labeled A. phagocytophilum at room temperature for 30 min in 200 μl of Hanks’ balanced salt solution with 0.5% human serum albumin. After washing, microspheres or cells were fixed with 1% paraformaldehyde. In some experiments before binding assays, microspheres or cells were pretreated with a mixture of sialidases (0.6 units/ml sialidase from Arthrobacter ureafaciens and 5 μg/ml sialidase from Vibrio cholera) in Hanks’ balanced salt solution with 1% human serum albumin at 37°C for 1 h. In other experiments, binding assays were performed in the presence of 10 μg/ml anti-human FIG.2. A. phagocytophilum binds to a glycopeptide modeled after the N-terminal region of human PSGL-1 independently of tyrosine sulfation. A, microspheres bearing sPSGL-1 or the indicated glycopeptide were incubated with anti-PSGL-1 mAb or with an isotype-matched control mAb. Bound antibody was detected with FITC-conjugated goat anti-mouse IgG. B, microspheres bearing sPSGL-1 or the indicated glycopeptide were incubated with the anti-α-Le^a mAb HECA-452 or with an isotype-matched control mAb. Bound mAb was detected with FITC-conjugated goat anti-rat IgM. C–F, control microspheres or microspheres bearing sPSGL-1 or the indicated glycopeptide were incubated with fixed, CellTracker Green-labeled A. phagocytophilum in the presence or absence of EDTA or the anti-PSGL-1 mAbs PL1 or PL2. Bound bacteria were measured by flow cytometry. These data are representative of three experiments.

FIG.3. A. phagocytophilum binding to a PSGL-1-derived glycopeptide requires α1–3-linked fucose on the O-glycan. A, structures of 2-GP-6, which has an α1–3-linked fucose, and of 4-GP-5, which lacks an α1–3-linked fucose. Each peptide was covalently coupled through its free N-terminal amino group to carboxylated microspheres. B, binding of mAb PL1 to microspheres bearing the indicated glycopeptide was measured as in Fig. 2. C, binding of labeled A. phagocytophilum to control microspheres or to microspheres bearing the indicated glycopeptide was measured as in Fig. 2. These data are representative of three experiments.
Fig. 4. *A. phagocytophilum* binding to a PSGL-1-derived glycopeptide does not require a specific orientation of sLeα on the O-glycan. A, structures of 1-GP-6a, which presents sLeα on a core-2 O-glycan, and of 1-GP-6b, which presents sLeα on an extended core-1 O-glycan. B, binding of mAb PL1 to microspheres bearing the indicated glycopeptide was measured as in Fig. 2. C, control microspheres or microspheres bearing the indicated glycopeptide were pretreated in the presence or absence of sialidase. Binding of labeled *A. phagocytophilum* was measured as in Fig. 2. These data are representative of three experiments.

**RESULTS**

*A. phagocytophilum* Binds to a Glycopeptide Modeled after the N-terminal Region of Human PSGL-1 Independently of Tyrosine Sulfation—For *A. phagocytophilum* binding studies, we initially used a group of well defined ligands with varying affinities for P-selectin (Fig. 1). Recombinant sPSGL-1 was coexpressed in CHO cells with FTVII and core2GlcNAcT-I to confer the glycosylation required for optimal binding to selectins (34). P-selectin binds to a small N-terminal region that includes three sulfated tyrosines and a core-2 O-glycan capped with sLex at Thr-16. This region contains the epitope for PL1, a mAb that blocks binding of PSGL-1 to P-selectin (37). 2-GSP-6, a glycosulfopptide modeled after this region, binds with equivalent affinity to P-selectin (20). 2-GP-6, which lacks sulfate on the tyrosines, binds with much lower affinity to P-selectin. 2-GP-1 and 2-GSP-1, which have only a single N-acetylgalactosamine residue attached to Thr-16, do not detectably bind to P-selectin. sLeα, a simple tetrasaccharide that lacks all peptide determinants and the core-2 O-glycan presentation, binds with very low affinity to P-selectin (21).

sPSGL-1 and each glyco(sulfo)peptide contained a C-terminal cysteine, which allowed specific attachment of biotin to the sulfhydryl group. sLex was attached to biotin through a linker. Each biotinylated ligand was coupled to streptavidin coated on 6-μm microspheres. Microspheres were prepared with equivalent densities of sPSGL-1, 2-GSP-6, 2-GSP-1, and 2-GP-6, as measured by flow cytometry with PL1 (Fig. 2A). The anti-sLeα mAb HECA-452 bound equivalently to microspheres coated with 2-GSP-6 or GP-6 as expected, because each peptide contains a single core-2 O-glycan that is capped with sLeα at Thr-16. These data are representative of three experiments.

**Fig. 5.** *A. phagocytophilum* binds weakly to sLeα lacking peptide. A, biotinylated sLeα was bound to streptavidin-coated microspheres at low, medium, and high densities as measured by binding of mAb HECA-452. B, control microspheres or microspheres bearing sLeα at the indicated density were pretreated in the presence or absence of sialidase. Binding of labeled *A. phagocytophilum* was measured as in Fig. 2. These data are representative of three experiments.

### Structural Requirements for Binding to A. phagocytophilum

**RESULTS**

*A. phagocytophilum* binding to the N-terminal region of human PSGL-1 is independent of tyrosine sulfation. For *A. phagocytophilum* binding studies, we initially used a group of well-defined ligands with varying affinities for P-selectin (Fig. 1). Recombinant sPSGL-1 was coexpressed in CHO cells with FTVII and core2GlcNAcT-I to confer the glycosylation required for optimal binding to selectins (34). P-selectin binds to a small N-terminal region that includes three sulfated tyrosines and a core-2 O-glycan capped with sLex at Thr-16. This region contains the epitope for PL1, a mAb that blocks binding of PSGL-1 to P-selectin (37). 2-GSP-6, a glycosulfopptide modeled after this region, binds with equivalent affinity to P-selectin (20). 2-GP-6, which lacks sulfate on the tyrosines, binds with much lower affinity to P-selectin. 2-GP-1 and 2-GSP-1, which have only a single N-acetylgalactosamine residue attached to Thr-16, do not detectably bind to P-selectin. sLeα, a simple tetrasaccharide that lacks all peptide determinants and the core-2 O-glycan presentation, binds with very low affinity to P-selectin (21).

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**Fig. 5.** *A. phagocytophilum* binds weakly to sLeα lacking peptide. A, biotinylated sLeα was bound to streptavidin-coated microspheres at low, medium, and high densities as measured by binding of mAb HECA-452. B, control microspheres or microspheres bearing sLeα at the indicated density were pretreated in the presence or absence of sialidase. Binding of labeled *A. phagocytophilum* was measured as in Fig. 2. These data are representative of three experiments.
crospheres (Fig. 2C). The anti-PSGL-1 mAb PL1 blocked binding, whereas PL2, an mAb that recognizes a more membrane-proximal epitope on PSGL-1, had no effect on binding. This result demonstrates that *A. phagocytophilum* adheres to a PSGL-1-coated surface lacking other glycoproteins. Furthermore, the bacteria specifically bound to microspheres coated with 2-GSP-6, demonstrating that the P-selectin binding region of PSGL-1 was sufficient for productive binding (Fig. 2D). Chelation of divalent cations with EDTA had no effect on binding, as observed previously (25) for bacterial binding to HL-60 cells. This contrasts with the Ca$^{2+}$/H$^{+}$-dependent binding of selectins to PSGL-1 and other sLe$^{x}$-capped glycoconjugates (11, 12). Labeled *A. phagocytophilum* also bound specifically to microspheres coated with 2-GP-6 (Fig. 2E). This closely resembled binding to 2-GP-6 (see Fig. 2F), indicating that the orientation of the 2-GP-6 on the microspheres did not affect bacterial recognition. In contrast, *A. phagocytophilum* did not bind to 4-GP-5-coupled microspheres (Fig. 2F). These data demonstrate that an a1–3-linked fucose residue is an essential recognition determinant for *A. phagocytophilum*.

FIG. 6. *A. phagocytophilum* binding to a PSGL-1-derived glycopeptide requires an amino acid sequence present in the N terminus of human but not murine PSGL-1. A–D, four glycopeptides modeled after the N-terminal sequence of human and/or murine PSGL-1 were synthesized. Human 2-GP-6 contained the N-terminal sequence of human PSGL-1. Murine GP-6 contained the corresponding N-terminal sequence of murine PSGL-1. Murine-human GP-6 replaced five unique amino acids in human 2-GP-6 with the corresponding single asparagine found in murine GP-6. Human-murine GP-6 replaced the single asparagine in murine GP-6 with the corresponding five amino acids found in human 2-GP-6. Each glycopeptide had the same core-2 O-glycan capped with sLe$^{x}$ (C2-O-sLe$^{x}$) attached to the threonine. Binding of anti-human PSGL-1 PL1 was measured as in Fig. 2. Binding of anti-murine PSGL-1 mAb 4RA10 was measured identically, except that binding was detected with FITC-conjugated goat anti-rat IgG. E and F, binding of labeled *A. phagocytophilum* to control microspheres or to microspheres bearing the indicated glycopeptide was measured as in Fig. 2. These data are representative of three experiments.
A. phagocytophilum Binding to a PSGL-1-derived Glycopeptide Does Not Require a Specific Orientation of sLe\(^e\) on the O-glycan—P-selectin binds in a stereospecific manner to the N-terminal region of human PSGL-1, as dramatically demonstrated by its inability to bind to an isomeric glycosylolopeptide that presents sLe\(^e\) on an extended core-1 O-glycan rather than on a branched core-2 O-glycan (21). We measured A. phagocytophilum binding to two isomeric PSGL-1-derived glycopeptides, 1-GP-6, bearing a core-2 O-glycan, and 1-GP-6\(^\ast\), bearing an extended core-1 O-glycan (Fig. 4A). The glycopeptides were N-terminally coupled to microspheres at matched densities (Fig. 4B). Labeled A. phagocytophilum bound equivalently to both microspheres (Fig. 4C). The anti-PSGL-1 mAb PL1 blocked binding to both microspheres, confirming the specificity of binding (data not shown). Furthermore, sialidase treatment of microspheres eliminated A. phagocytophilum binding (Fig. 4C). These data confirm that binding requires sialylation, as well as fucosylation of the glycan. They further demonstrate that bacterial binding, unlike P-selectin binding, does not require a specific orientation of the glycan on the PSGL-1-derived peptide.

A. phagocytophilum Binds Weakly to sLe\(^e\) Lacking Peptide—The above results demonstrate that A. phagocytophilum binds readily to sLe\(^e\) presented on a PSGL-1-derived peptide independently of tyrosine sulfation or a specific alignment of the O-glycan. To determine whether the peptide itself contributes to bacterial recognition, we measured A. phagocytophilum binding to sLe\(^e\) lacking peptide, which was coupled to microspheres at three different densities as measured by mAb HECA-452 (Fig. 5A). Sialidase treatment eliminated HECA-452 binding to all three groups of microspheres (data not shown). Labeled A. phagocytophilum bound weakly to sLe\(^e\) in a sialidase-sensitive manner (Fig. 5B) but only when sLe\(^e\) was coupled to microspheres at an ∼100-fold greater density than the densities of glycopeptides (compare Fig. 5A with Fig. 2B). These data demonstrate that the peptide component of human PSGL-1 makes a major contribution to bacterial binding.

A. phagocytophilum Binding to a PSGL-1-derived Glycopeptide Requires an Amino Acid Sequence Present in the N Terminal of Human but Not Murine PSGL-1—A. phagocytophilum binds to both wild-type and PSGL-1-deficient murine neutrophils (27). The pathogen might have evolved a PSGL-1-independent mechanism to bind murine neutrophils, because it could not bind to murine PSGL-1. Consistent with this hypothesis, A. phagocytophilum bound only weakly to transfected CHO cells expressing murine PSGL-1 and FTVII (27). The N-terminal sequence of murine PSGL-1 sequence differs significantly from that of human PSGL-1 (41). Mutational analysis of the N-terminal region suggests that murine PSGL-1 requires sulfation of one of two tyrosines and O-glycosylation of a closely situated threonine to bind P-selectin (24). We compared A. phagocytophilum binding to biotinylated human and murine PSGL-1-derived glycopeptides that were coupled to streptavidin-coated microspheres. Each glycopeptide contained a core-2 O-glycan capped with sLe\(^e\) at the respective threonine thought to be important for P-selectin binding. Anti-human PSGL-1 mAb PL1 bound to microspheres coupled with human 2-GP-6 but not with murine GP-6, whereas anti-murine PSGL-1 mAb 4RA10 bound to microspheres coupled with murine GP-6 but not with human 2-GP-6 (Fig. 6, A and B). We also coupled two chimeric glycopeptides through their C-terminal cysteines to microspheres. Murine-human GP-6 substituted a five-residue peptide segment that separates the tyrosine closest to the O-linked threonine in the human sequence with a
single asparagine residue that separates the tyrosine closest to the O-linked threonine in the murine sequence (Fig. 6C). Human-murine GP-6 substituted this single asparagine in the murine sequence with the five-residue human peptide (Fig. 6D). The substitution in murine-human GP-6 eliminated binding of anti-human PSGL-1 mAb but introduced binding of anti-murine PSGL-1 mAb 4RA10. Conversely, the substitution in human-murine GP-6 nearly eliminated binding of 4RA10 but introduced binding of PL1. These data confirm the previously determined epitope for PL1 (37) and further localize the 4RA10 epitope (30).

A. phagocytophilum Binds to Cells Expressing Human PSGL-1 and FTVII in Cooperation with N- and O-glycans—Bacterial binding to glycopeptide-coated microspheres required cooperative interactions with both the human PSGL-1-derived peptide and a sLe\(^a\)-capped O-glycan attached to Thr-16. Because cooperative binding did not require a specific orientation of the O-glycan at Thr-16, we asked whether A. phagocytophilum might bind to PSGL-1-expressing cells through interactions with sLe\(^a\) presented on glycans at other locations. For this purpose we used transfected CHO cells expressing wild-type human PSGL-1 or PSGL-1 in which Thr-16 was replaced with an alanine (T16A). The cells coexpressed FTVII and core2GlcNAcT-I, which enables construction of sLe\(^a\) on both N-glycans and core-2 O-glycans. Other cells coexpressed wild-type human PSGL-1 with FTVII but not with core2GlcNAcT-I. These cells express only short core-1 O-glycans that cannot be α1–3-fucosylated (34, 42). PL1 (10 μg/ml) bound slightly less to
cells expressing T16A than wild-type PSGL-1 (Fig. 7A), but 50 μg/ml PL1 bound comparably to both cell lines (data not shown). The reduced affinity of PL1 for T16A is consistent with the mutation introduced in the center of the PL1 epitope. Anti-PSGL-1 mAb PL2 bound equivalently to cells expressing both wild-type and T16A PSGL-1 (Fig. 7B), confirming that the cells expressed comparable levels of PSGL-1. All three cell lines were equivalently fucosylated as measured by binding of anti-sLeα mAb HECA-452 (Fig. 7C). In contrast, CHO-131, a mAb that recognizes sLeα only on core-2 O-glycans (31), bound strongly to CHO cells expressing both FTVII and core2GlcNAcT-I but very weakly to cells expressing only FTVII (Fig. 7D). Labeled A. phagocytophilum bound to all three cell lines (Fig. 7E). PL1 prevented binding, confirming the requirement for PSGL-1 (Fig. 7F). Bacteria bound slightly less to cells expressing T16A than to cells expressing wild-type PSGL-1, suggesting that an O-glycan at Thr-16 partially contributes to binding (Fig. 7E), yet A. phagocytophilum bound equally well to cells expressing wild-type PSGL-1 in the presence or absence of core2GlcNAcT-I. These results demonstrate that A. phagocytophilum binds readily to PSGL-1-expressing cells in the absence of an O-glycan at Thr-16. They further suggest that the bacteria can use fucosylated N-glycans, as well as O-glycans, to cooperatively interact with PSGL-1.

To further explore the relative contributions of N- and O-glycans to binding to A. phagocytophilum, we treated transfected CHO cells with benzyl-GalNAc or kifunensine. Benzyl-GalNAc competitively inhibits extension and modification of O-glycans, resulting in accumulation of short Galβ1-3GalNaC and GalNaC O-glycans linked to serine or threonine (43). Kifunensine, a mannosidase I inhibitor that prevents trimming of mannosyl branches from high mannose N-glycans, prevents synthesis of complex N-glycans that are acceptors for α1-3-fucosyltransferases (44). Kifunensine treatment was effective, as documented by its ability to markedly reduce binding of PHA-L, a lectin that recognizes tri- and tetraantennary complex N-glycans containing outer galactose residues and an α-linked mannoside residue substituted at the C-2 and C-6 positions (45) (Fig. 8, A and B). Benzyl-GalNAc treatment was effective, as demonstrated by its ability to markedly reduce binding of mAb CHO-131 to CHO cells expressing both FTVII and core2GlcNAcT-I (Fig. 8C). It had no further effect on the low level binding of CHO-131 to CHO cells expressing only PSGL-1 and FTVII, suggesting that this background binding is not to fucosylated core-2 O-glycans (Fig. 8D).

Benzyl-GalNAc and kifunensine each partially reduced binding of HECA-452 to cells coexpressing PSGL-1, FTVII, and core2GlcNAcT-I, indicating that these cells express sLeα on both N- and O-glycans (Fig. 8E). In contrast, kifunensine virtually eliminated HECA-452 binding to cells coexpressing only PSGL-1 and FTVII, whereas benzyl-GalNAc had no effect (Fig. 8F). This further documents that these cells express sLeα primarily on N-glycans. Benzyl-GalNAc and kifunensine each partially inhibited binding of A. phagocytophilum to cells expressing PSGL-1, FTVII, and core2GlcNAcT-I (Fig. 8G). In cells expressing only PSGL-1 and FTVII, kifunensine eliminated bacterial binding, whereas benzyl-GalNAc had no effect (Fig. 8H). These data demonstrate that A. phagocytophilum can bind to PSGL-1 in cooperation with fucosylated N- and O-glycans. To confirm that this phenomenon was not specific to CHO cells, we studied human myelocytic HL-60 cells. Because A. phagocytophilum infects HL-60 cells as readily as human neutrophils, HL-60 cells are used to propagate the pathogen in vitro (27, 28). Kifunensine and benzyl-GalNAc inhibited, respectively, binding of PHA-L and CHO-131 to HL-60 cells (Fig. 9, A and B), and each partially inhibited binding of HECA-452 (Fig. 9C) and A. phagocytophilum (Fig. 9D). Thus, A. phagocytophilum uses fucosylated N- and O-glycans to interact with human myeloid cells or transfected cells expressing human PSGL-1.

A. phagocytophilum Binds to Microspheres Coupled Independently with PSGL-1-derived Glycopeptide Lacking sLeα and with sLeα Lacking Peptide—Human PSGL-1 has many O-glycans and up to three N-glycans (46–49). A. phagocytophilum binds to intact cells expressing PSGL-1 without an O-glycan at Thr-16. However, this observation does not distinguish whether the pathogen must bind to N- or O-glycans on PSGL-1 or whether it might bind to the peptide component of PSGL-1 in cooperation with N- or O-glycans on other cell surface proteins. To determine whether bacterial recognition requires that sLeα be presented on the PSGL-1 N-terminal peptide we coupled 2-GP-6, 2-GP-1, sLeα, or both sLeα and 2-GP-1 to microspheres at low and high densities. Peptide and sLeα densities were
matched as measured by PL1 binding (Fig. 10, A and E) and HECA-452 binding (Fig. 10, B and F), respectively. At the densities studied, *A. phagocytophilum* did not bind to microspheres bearing either 2-GP-1 or sLeX. In contrast, the bacteria bound in a PL1-inhibitable manner to microspheres bearing 2-GP-6 or 2-GP-1 plus sLeX (Fig. 10, C, D, G, and H). At high densities, the degree of bacterial binding to microspheres coupled with 2-GP-1 plus sLeX approached that to microspheres coupled with 2-GP-6 (Fig. 10G). These results demonstrate that *A. phagocytophilum* binds cooperatively to PSGL-1 peptide lacking sLeX and to peptide lacking sLeX that are independently coupled to the same microsphere. This suggests that the pathogen might bind to cells through cooperative interactions with the N terminus of PSGL-1 and with sLeX-capped N- or O-glycans on other cell-surface proteins.

**DISCUSSION**

The pathogen *A. phagocytophilum* employs some features that P-selectin uses to bind to neutrophils (25). The dependence of bacterial binding on expression of both human PSGL-1 and an α1-3-fucosyltransferase has been interpreted to indicate that *A. phagocytophilum* binds to fucosylated PSGL-1 (26). The ability of mAbs to the N-terminal region of human PSGL-1 to inhibit bacterial binding is consistent with a model in which *A. phagocytophilum* binds to the same N-terminal region of PSGL-1 recognized by P-selectin. This model is also supported by the dependence of bacterial binding on expression of α1-3-fucosyltransferases in murine neutrophils (27). However, *A. phagocytophilum* binds readily to PSGL-1-deficient murine neutrophils, suggesting more complex models for bacterial binding to neutrophils (27). Here we have further defined the molecular features that *A. phagocytophilum* recognizes. These features overlap but are clearly distinct from those that P-selectin uses to bind to human and murine neutrophils.

All published studies of *A. phagocytophilum* binding or infection employ intact bacteria. We measured binding of fixed bacteria to ligand-coupled microspheres or to cells. This allowed determination of requirements for binding independent of signaling or other events that might regulate bacterial in-
ternalization or viability within cells. It is important to note that this assay involves multivalent adhesion of one particle (fixed bacteria) to another particle (microsphere or cell). We coupled molecularly defined ligands to the microspheres or used cells with at least partially characterized cell surface structures. However, the adhesion or adhesins on A. phagocytophilum that mediate adhesion are unknown. Thus, interpretations of these binding data and previous binding data must include the possibility that A. phagocytophilum uses more than one adhesion to interact with the target particle and that target cells display more than one ligand. The bacterial binding assay differs from assays that measure binding of purified P-selectin to defined glycoconjugates, as well as to cell surfaces. Studies with microspheres demonstrated that A. phagocytophilum bound to purified human PSGL-1 or to a small glycosulfopetide modeled after the extreme N-terminal region of mature human PSGL-1 that binds to P-selectin. Therefore adhesion does not need additional cell surface molecules, and initial examination suggests that the same region of PSGL-1 that binds to P-selectin also binds to A. phagocytophilum. Consistent with this notion, bacterial adhesion required sialylation and 1–3-fucosylation of the O-glycan at Thr-16 of the glycopeptide. Unlike P-selectin binding, however, bacterial adhesion required neither tyrosine sulfation of the glycopeptide nor a specific core-2 alignment of sLeα on the O-glycan at Thr-16. Indeed, significant bacterial binding was observed to microspheres coupled with both sLeα lacking peptide and with a PSGL-1-derived peptide lacking an sLeα-capped O-glycan at Thr-16. Bacterial adhesion required cooperative interactions with sLeα lacking peptide and with peptide lacking sLeα, because neither alone supported significant adhesion even at high densities. Bacterial interaction with the peptide required a five-residue segment found in human but not murine PSGL-1. The peptide interaction region may be larger, because the amino acid sequence C-terminal to this five-residue segment is nearly identical in humans and mice. This species-dependent difference in bacterial binding to human and murine PSGL-1 may explain why A. phagocytophilum requires a PSGL-1-independent mechanism to bind to murine neutrophils (27).

The adhesion of A. phagocytophilum to microspheres that presented sLeα and a human PSGL-1-derived peptide in different orientations suggests that the bacteria might also recognize sLeα presented at different positions on PSGL-1-expressing cells. Our studies with HL-60 cells and transfected CHO cells confirm this interpretation. A. phagocytophilum bound to transfected cells expressing T16A PSGL-1, demonstrating that bacterial adhesion, unlike P-selectin binding, does not require an O-glycan at Thr-16 of PSGL-1. Inhibitors of N- or O-glycosylation revealed that A. phagocytophilum bound to PSGL-1 in cooperation with sLeα presented on both N- and O-glycans. It seems likely that bacterial adhesion to neutrophils or transfected cells involves cooperative interactions with the N-terminal peptide sequence of human PSGL-1 and with N- and/or O-glycans on both PSGL-1 and other glycoproteins. The moderately diminished bacterial adhesion to cells expressing T16A PSGL-1 suggests that sLeα presented on an O-glycan at this residue may be a favored but not exclusive interaction site.

It is conceivable that A. phagocytophilum expresses a single adhesion with separate binding sites for the N-terminal peptide of human PSGL-1 and for sLeα or related glycans. This adhesion might simultaneously bind to peptide and sLeα on the same PSGL-1 molecule. However, the ability of bacteria to adhere to microspheres or cells presenting PSGL-1 and sLeα in different orientations suggests that even a single adhesion might bind to peptide on one PSGL-1 molecule and to sLeα on another PSGL-1 molecule or on a different glycoprotein. It is equally conceivable that one adhesion binds to the PSGL-1 peptide and that a molecularly distinct adhesion binds to sLeα. Indeed, the available data do not exclude the possibility that different adhesins recognize, respectively, sialic acid and α1-3-linked fucose. Other bacteria use multiple adhesins to facilitate infection. A striking example is Helicobacter pylori, which expresses one adhesin that binds to the fucosylated glycan Lewis b and another adhesin that binds to sLeα-related glycans on gastric mucosa (51). The PSGL-1-independent binding of A. phagocytophilum to murine neutrophils strengthens the concept of multiple adhesins. Here binding might require a specific adhesion that recognizes an uncharacterized molecule on murine but not human neutrophils. This putative interaction might cooperate with binding of another adhesion(s) to sLeα or related glycans, allowing the bacterium to infect murine neutrophils as effectively as human neutrophils. Further definition of A. phagocytophilum interactions with neutrophils will require isolation and characterization of the bacterial adhesion or adhesins that recognize protein or carbohydrate structures on the neutrophil surface.

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