Functional Analysis of the Combined Role of the O-Linked Branching Enzyme Core 2 β1-6-N-Glucosaminyltransferase and Dimerization of P-selectin Glycoprotein Ligand-1 in Rolling on P-selectin*

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Leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) is expressed as a homodimer and mediates leukocyte rolling through interactions with endothelial P-selectin. Previous studies have shown that PSGL-1 must be properly modified by specific glycosyltransferases including α1,3-fucosyltransferase-VII, core 2 β1–6–N-glucosaminyltransferase (C2GlcNAcT-I), one or more α2,3-sialyltransferases, and a tyrosulfotransferase. In addition, dimerization of PSGL-1 through its sole extracellular cysteine (Cys280) is essential for rolling on P-selectin under shear conditions. In this report, we measured the contributions of both C2GlcNAcT-I glycosylation and dimerization of PSGL-1 to adhesive bonds formed during tethering and rolling of transfected cell lines on purified P-selectin. Tethering to P-selectin under flow involving dimerization compared with cells expressing monomeric PSGL-1 (referred to as C320A). The rolling defects (decreased cellular accumulation, PSGL-1/P-selectin bond strengths and tethering rates, and increased velocities and skip distance) demonstrated by transfectants expressing monomeric PSGL-1 could be overcome by increasing the substrate P-selectin site density and by overexpressing C2GlcNAcT-I in C320A transfectants. Two molecular weight variants of PSGL-1 were isolated from cell lines transfected with PSGL-1, C320A, and/or C2GlcNAcT-I cDNAs, and these differences in electrophoretic mobility appeared to correlate with C2GlcNAcT-I expression. C320A transfectants expressing low molecular weight PSGL-1 had lower cysteine (Cys320) is essential for rolling on P-selectin (17, 18). Cell lines expressing recombinant monomeric PSGL-1 had a severely compromised rolling on P-selectin in vitro rolling assays and reduced binding of soluble PSGL-1. Whereas these cell-based observations strongly indicate that monomeric PSGL-1 displays a complete set of P-selectin binding epitopes (19, 20). Furthermore, the binding affinity of the P-selectin-PSGL-3 complex is essentially identical to that of P-selectin binding to soluble, Lselectin, P-selectin glycoprotein ligand-1; FucT-VII, 1–3-linked fucosyltransferase; C2GlcNAcT-I, core 2 β1–6-N-glucosaminyltransferase; sLex, sialyl Lewis x; mAb, monoclonal antibody; CHO, Chinese hamster ovary.

Endothelial P-selectin mediates a critical component of leukocyte rolling during the early stages of the inflammatory response (1–3). The binding of P-selectin to leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) tethers the leukocyte to the vessel wall, allowing it to slowly roll along the vessel’s luminal surface and initiate the leukocyte adhesion cascade. PSGL-1 is a 240-kDa disulfide-linked homodimer (4, 5), whose 19-aminocysteine (Cys)280 is essential for rolling on P-selectin under shear conditions. In this report, we measured the contributions of both C2GlcNAcT-I glycosylation and dimerization of PSGL-1 to adhesive bonds formed during tethering and rolling of transfected cell lines on purified P-selectin. Tethering to P-selectin under flow involving dimerization compared with cells expressing monomeric PSGL-1 (referred to as C320A). The rolling defects (decreased cellular accumulation, PSGL-1/P-selectin bond strengths and tethering rates, and increased velocities and skip distance) demonstrated by transfectants expressing monomeric PSGL-1 could be overcome by increasing the substrate P-selectin site density and by overexpressing C2GlcNAcT-I in C320A transfectants. Two molecular weight variants of PSGL-1 were isolated from cell lines transfected with PSGL-1, C320A, and/or C2GlcNAcT-I cDNAs, and these differences in electrophoretic mobility appeared to correlate with C2GlcNAcT-I expression. C320A transfectants expressing low molecular weight PSGL-1 had lower cysteine (Cys320) is essential for rolling on P-selectin (17, 18). Cell lines expressing recombinant monomeric PSGL-1 had a severely compromised rolling on P-selectin in vitro rolling assays and reduced binding of soluble PSGL-1. Whereas these cell-based observations strongly indicate that monomeric PSGL-1 displays a complete set of P-selectin binding epitopes (19, 20). Furthermore, the binding affinity of the P-selectin-PSGL-3 complex is essentially identical to that of P-selectin binding to soluble, Lselectin, P-selectin glycoprotein ligand-1; FucT-VII, 1–3-linked fucosyltransferase; C2GlcNAcT-I, core 2 β1–6-N-glucosaminyltransferase; sLex, sialyl Lewis x; mAb, monoclonal antibody; CHO, Chinese hamster ovary.
recombinant PSGL-1 consisting of the entire dimeric extracellular domain (19). Although the similarities in these binding affinities argue that dimerization of PSGL-1 is not essential for interactions with P-selectin, it is not known how static measures of molecular binding affinities correlate with the ability of PSGL-1 to mediate rolling on P-selectin in the presence of fluid shear forces.

In this study, we have evaluated the contribution of both dimerization and C2GlcNAcT-I modification of PSGL-1 on P-selectin-mediated cell rolling. Using high speed digital video analysis, cell rolling was separated into the component steps of accumulation, tethering, and distance traveled between adhesive events. Based on the analysis of the duration and frequency of transient adhesive events during rolling, C2GlcNAcT-I expression makes a major contribution to the mechanical compliance of PSGL-1/P-selectin bonds. High speed video analysis of the frequency of cell tethering further demonstrated that covalent dimerization of PSGL-1 significantly enhanced the ability of cells to initiate rolling interactions via P-selectin while having a minimal affect on PSGL-1/P-selectin bond biomechanical properties.

EXPERIMENTAL PROCEDURES

Generation of Stable Transfectants in K562 and BJAB Cells—The generation of both K562 and BJAB cells stably expressing α3-fucosyltransferase-VII (FucT-VII) has been described (21), (22). Both cell lines (K562/FT7 and BJAB/FT7) were transfected by electroporation with wild type PSGL-1 or nonenzyming PSGL-1 (referred to as C2A20) in which cysteine at position 320 was mutated to alanine (17). Bulk transfectants were selected in medium containing 2.5 μg/ml puromycin, screened by flow cytometry with the anti-PSGL-1 mAb KPL1 (23), and cloned by limiting dilution. Transfectants were selected that had equal levels of KPL1 and HECA-452, which recognizes a reporter epitope associated with FucT-VII enzymatic activity. Clones with equivalent staining for these two mAbs were tested by semiquantitative reverse transcriptase-PCR analysis (17) for both FucT-VII and C2GlcNAcT-I mRNA expression. This analysis revealed that BJAB cells expressed higher endogenous levels of C2GlcNAcT-I. Thus, K562/FT7 cells expressing wild type dimeric PSGL-1 or nonenzymic C2A20 were transfected with C2GlcNAcT-I, drug-selected, and screened, and clones were obtained that expressed a level of mRNA for C2GlcNAcT-I similar to that in BJAB cells. The nomenclature for the transfectants is as follows: K562-FT7 (where FT7 refers to PSGL-1), BJAB-FT7, K562-C2A20 (where C2A20 refers to the dimerization mutant), BJAB-C2A20, K562-C2A20 (where C2 refers to C2GlcNAcT-I), and K562-C2A20. All cell lines expressed FucT-VII and were reactive to HECA-452. Additionally, all cells were tested for reactivity to CHO-131 (kindly supplied by Dr. Bruce Walcheck, University of Minnesota, St. Paul, MN), a mAb that is specific for the C2GlcNAcT-I-dependent linkages that are critical components of the PSGL-1 binding pocket (24). For each sample, ~10,000 antibody-labeled cells were analyzed on a FACScanIIl flow cytometer (Becton Dickinson) using CellQuest software.

Rolling Substrates and Flow Assay—A microscope slide coated with recombinant P-selectin-human IgG fusion protein (referred to as PLLgG; BD Pharmingen) was assembled as the lower wall of a parallel plate flow chamber (GlycoTech, Rockville, MD) and mounted on an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Garden City, NY) equipped with a Kodak MotionCorder Analyzer, model 1000 camera (Eastman Kodak Co., Motion Analysis System Division, San Diego, CA) for high temporal resolution of adhesive and rolling events. Transfectants were perfused (0.5–2.0 dyn/cm2 and viewed at a frame rate of 125 frames/s to measure tether lifetimes and distances between adhesive events. A standard CCD camera (Vicon V2410; Vicon Industries Inc., Melville, NY) recorded cell rolling velocities and cell accumulation events. In some experiments, transfectants were pretreated with either KPL1 (blocking anti-PSGL-1 mAb), KPL2 (nonblocking anti-PSGL-1 mAb), G1 (blocking anti-P-selectin mAb), or EDTA (5 mM) for 20 min in phosphate-buffered saline (pH 7.4) and incubated on an orbital shaker for 30 min at 4 °C. Extracts were clarified at 14,000 × g for 30 min at 4 °C, and supernatants were transferred to new tubes. Samples were boiled for 5 min in β-mercaptoethanol reducing buffer, electrophoresed on an 8% polyacrylamide gel (SDS-PAGE), and transferred to nitrocellulose. Membranes were blocked with 5% nonfat milk in phosphate-buffered saline (pH 7.4) and incubated with KPL1, washed three times with phosphate-buffered saline, and incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (Pierce). Membranes were washed six times, developed with enzyme chemiluminescence (Pierce), and exposed to CL-X Posure x-ray film (Pierce).

Image Acquisition and Processing—Adhesive events and distances between adhesive events were determined using a computer tracking program coded in MATLAB 5 (The MathWorks, Natick, MA), which used a sum of absolute difference algorithm and spline interpolation that allowed for subpixel resolution of changes in position to identify the cell in consecutive image frames. Video memory from the high speed camera (125 frames/s) was played back at standard video rates for archiving on VHS tapes. Images of rolling cells were captured from video to computer using Scion Image version 1.62 (Scion Corp., Frederick, MD) and a Macintosh G4 (Apple, Inc., Cupertino, CA) equipped with a Scion LG-3 frame grabber. The elapsed time for each digitized movie used to calculate rolling velocity was 2 s.

The signal/noise ratio for a typical phase-contrast image was calculated by determining the average grayscale signal intensity for a given cell. The value 0 was assigned to pure white and 255 was assigned to pure black, resulting in an average of 73 for the brightest regions of the cell to an average of 180 for the dark background. The signal intensity was the difference between these values, whereas the noise was the S.D. value of the intensity for the pixels representing the cell, which was 22, resulting in a signal/noise ratio of 5 for a 5–10-μm diameter object. The low bias (~0 pixels) and low S.D. of distance (~0.1 pixels or 37 μm for 2.0× magnification using the Kodak camera) indicated that the observed tracking of cells was very close to the actual movement of the cells.

Determination of Rolling Cell Accumulation and Adhesive Event Duration Analysis—A cell was considered rolling if it displayed at least three adhesive contacts across the field of view (640 μm in length) in a 2-s time period. Cell rolling interactions varied between a highly transient interaction to a very stable rolling, depending on PSGL-1 structural modifications. The tether bond lifetime (duration of an adhesive event at low site densities of P-selectin) was determined by counting high speed camera image frames starting with a drop in velocity of at least 25% relative to the hydrodynamic velocity of a cell near the wall and ending with an increase in velocity of 12.5 μm/s over three frames (24 μm). The increase in velocity of 12.5 μm/s, used to define the end of an adhesive event, was also 5 times higher than the highest fluctuation in apparent velocity of a stationary cell in our system. The dissociation rate constant was taken as the negative slope of the natural log of the number of events remaining versus the duration of the events at each wall shear stress.

The force on the tether was found through a force and torque balance using the geometry of a tethered cell and comparing the dissociation constants from our cellular system to the dissociation constants from a cell-free bead system (26). The K562, K562C2, and BJAB cell lines used in the adhesive event duration experiments had diameters of ~17 μm, which is significantly larger than a neutrophil (~8.5 μm), and this information was used to calculate the force on the tether of 475 piconewtons/dyn/cm2 that was imposed at a low flow rate, the average lever arm length and angle between the tether and substrate (lower wall of the chamber) were calculated from the relationship F = F_cost. This resulted in an average lever arm angle (θ of 60.7°), a lever arm length of 4.3 μm, a microvillus tether arm length of 0.8 μm, a shear force of 232 piconewtons/dyn/cm2, and a force on the tether of 475 piconewtons/dyn/cm2 that was used to scale the force on the tether for the K562 and BJAB transfectants (26).
RESULTS

Generation and Characterization of Transfectants Expressing PSGL-1, FucT-VII and C2GlcNAcT-I expression—Both the BJAB/FucT-VII and K562/FucT-VII cell lines lack the gene for PSGL-1 but express all glycosyltransferases required for appropriate enzymatic modification of PSGL-1. Stable transfectants expressing either wild type PSGL-1 (K562-WT or BJAB-WT) or monomeric PSGL-1 (K562-C320A or BJAB-C320A) were generated in these cell lines. Wild type PSGL-1 and C320A were expressed at similar levels on transfected cells, as was a FucT-VII reporter epitope recognized by HECA-452 (Fig. 1A). Semiquantitative reverse transcriptase-PCR using PCR cycles titrated to below plateau phase followed by Southern blotting (17, 27) revealed that BJAB/FucT-VII cells endogenously expressed higher levels of C2GlcNAcT-I compared with K562 cells (Fig. 1B). Because of these differences, K562-WT and K562-C320A cells were transfected with additional C2GlcNAcT-I (Fig. 1B). Transfectants with similar levels of C2GlcNAcT-I were used to evaluate the rolling phenotype associated with dimeric or monomeric PSGL-1.

To establish the functionality and specificity of transfected PSGL-1, all cell lines were flowed over purified P-selectin at a wall shear stress of 0.5 dyn/cm², and the number of cell interactions was quantified from analysis of videotape (see “Experimental Procedures”) and compared with the number of interactions with the appropriate specificity controls (Fig. 2). It was observed that the tethering and rolling of PSGL-1 or C320A transfectants was highly specific for P-selectin, since blocking mAbs to PSGL-1 (KPL1) or P-selectin (G1) significantly inhibited rolling. Inhibition experiments required a high density of P-selectin substrate (200 sites/μm²), because rolling of C320A expressing cells was extremely compromised at substrate densities below 100 sites/μm² (17). When no antibody or control antibody (KPL2) was present, all WT transfectants rolled with a steady, almost peeling-like motion much like neutrophil rolling on either P-selectin or E-selectin in vivo (1, 28, 29). In contrast, C320A rolling interactions were distinctly briefer and much less stable, suggesting an adhesive functional deficit (17). As expected, rolling interactions were completely eliminated in the presence of EDTA (Fig. 2). There were no adhesive interactions with untransfected K562 or BJAB cells on P-selectin (data not shown). It should be noted that the cell adhesion measure reported in Fig. 2 is equivalent to a flux, in that the total number of interacting cells per unit time was counted.

Influence of P-selectin Density and PSGL-1 Dimerization Status on Total Rolling Cell Accumulation—Total accumulation of rolling cells measures both bond formation frequency and its duration under stress. To investigate the influence of
PSGL-1 dimerization on this parameter, a substrate expressing P-selectin at a density of 200 sites/μm² was used. At a wall shear stress of 0.5 dyn/cm², cells expressing dimerized PSGL-1 bound more readily than cells expressing nondimerized PSGL-1, as indicated by the increased number of rolling cells after 2 min of steady flow (Fig. 3A). Observed differences were statistically significant at time points as early as 1 min after the start of flow (t test, p < 0.01, data not shown). K562, BJAB, and K562C2 transfectants expressing either dimerized PSGL-1 or monomeric C320A demonstrated an increase in total numbers of accumulated cells with time, although the increase in cellular accumulation was always significantly greater for wild type PSGL-1 compared with monomeric C320A (Fig. 3A). This suggests that PSGL-1 covalent dimerization increased the probability of P-selectin-mediated cell capture. Whereas BJAB-C320A cells rolled steadily, K562-C320A cells interacted much more transiently with the P-selectin substrate. The correlation between long duration rolling and C2GlcNAcT-I expression suggested that increased levels of C2GlcNAcT-I might partially compensate for the rolling defects associated with monomeric PSGL-1. Qualitatively similar patterns of enhanced binding of cells expressing dimerized PSGL-1 were observed at 1 dyn/cm² wall shear stress save for a lower overall level of binding (data not shown).

The rolling potential of both monomeric and dimeric PSGL-1 at a P-selectin density of 20 sites/μm² (Fig. 3B) was also investigated to probe the effect of limiting P-selectin site density on cell interactions under shear. At this lower substrate density, C320A transfectants had a much lower rolling potential than observed at 200 sites/μm² (Fig. 3, B versus A). Using a cell rolling criterion of a minimum of three adhesive events across the microscope field of view in a 2-s interval, the K562-C320A mutation was essentially unable to roll on P-selectin at 20 sites/μm². The cellular accumulation data were obtained after 2 min of perfusion for BJAB and K562C2 transfectants and after 6 min for K562 transfectants. The virtually complete abrogation of K562-C320A rolling, even after the increased perfusion period, was consistent with previous observations of impaired rolling on P-selectin-expressing CHO cells (17). In contrast, the BJAB-C320A transfectants had a less severe adhesive defect, with binding inhibited ~40% compared with BJAB WT (Fig. 3B).

The differential defect in the accumulation of rolling cells between the same PSGL-1 mutation in BJAB and K562 cells suggested that higher levels of C2GlcNAcT-I expression in BJAB transfectants might result in greater rolling potential on P-selectin compared with K562 transfectants. To test the effect of C2GlcNAcT-I levels on cell accumulation rates, the K562C2-WT and K562-C320A lines were flowed at 0.5 dyn/cm² P-selectin density. K562-C320A after 2 min of flow (p < 0.01, A and B). *, both K562-WT and K562-C320A were perfused for 6 min over the 20 sites/μm² P-selectin substrate to enhance the chance of a K562-C320A transfectant being able to form a rolling interaction (B).
Influence of PSGL-1 Dimerization and C2GlcNAcT-I Glycosylation on Distance between Tethering Events at High and Low P-selectin Site Density—The similarity in rolling velocity of all PSGL-1 and C320A transfectants expressing high levels of C2GlcNAcT-I suggested that dimerization might not enhance the formation of tethers when cells were rolling on a sufficiently high site density P-selectin substrate (Fig. 4, B and C). This is in contrast to the consistent and reproducible difference in rolling cell accumulation that was detected when comparing PSGL-1 and C320A transfectants on high site density P-selectin (Fig. 3A). In an attempt to reconcile these differences, we analyzed the discrete components of a selectin-mediated cell-adhesive interaction under shear: formation of the initial contact (cell tethering) and the initiation of the subsequent rolling interaction. At sufficiently low site densities of a selectin or selectin ligand, a leukocyte will skip along in a series of transient interactions that appear to be mediated by a discrete unit of adhesive potential, the tether bond (30–32). At such low substrate densities, the spatial gaps between discrete tether bonds may be reasonably inferred to correlate with a selectin’s intrinsic on rate, given the caveat of similar host cell membrane topographies, site densities, and spatial distribution in the plane of the membrane. Consequently, the distance between cell tethering events (step distance) was used to assess the relative probability of cell reattachment under flow conditions.

An instantaneous (per frame) cell velocity profile was determined with a resolution of 125 frames/s (4× standard video) to measure step size. A representative velocity trajectory was plotted for a K562C2-WT cell rolling on P-selectin at a density of 100 sites/μm² that indicates eight jumps (Fig. 5A). When the instantaneous velocity of a cell is greater than 40 μm/s, the cell has completely detached from the substrate, and a step was defined to begin. When the cell velocity decreases to less than 40 μm/s, the step is defined as ending, and the cell has tethered and begun to roll. The increase in velocity above 40 μm/s correlates with the visual detachment of the cell from the substrate (33–36). Distances traveled while the cell is moving faster than 40 μm/s represent single steps or, alternatively, the distance traveled between successive tethering events (Fig. 5A). P-selectin site densities of 100 sites/μm² (Fig. 5B) and 10 sites/μm² (Fig. 5C) were used to determine the distance between cell tethering events, since these concentrations span the range of densities that support steady rolling and those that predominantly support discrete tethering (26).

At a wall shear stress of 0.5 dyn/cm² and a P-selectin site density of 100 sites/μm², the distance traveled between adhesive events was relatively large for the K562-C320A cells compared with the K562-WT cells (Fig. 5B). Under the same experimental conditions, there were no significant differences between the BJAB-WT and BJAB-C320A or the K562C2-WT and K562C2-C320A skip distances, with all transfectants having a step distance of ~1 μm (Fig. 5B).

The contribution of dimerization and C2GlcNAcT-I glycosylation on step size was also investigated under the more stringent conditions of a low site density P-selectin substrate. Lowering the P-selectin density 10-fold (10 sites/μm²) led to an increase in the distance between tethering events for all transfectants (Fig. 5C). Rolling under these conditions was dominated by discrete binding events separated by skips ranging from 2 to 8 μm. Consistent with Fig. 5B, an 8-fold difference in skip distance was observed between K562-WT and the K562C2-WT and K562C2-C320A mutants on low site density P-selectin, suggesting a significant deficiency in tethering ability (p < 0.01) (Fig. 5C).

BJAB-WT cells traveled ~2 μm between adhesive events, whereas BJAB-C320A cells traveled ~4 μm. Similarly, the skip distance for K562C2-WT cells was ~4 μm, whereas K562C2-WT cells were almost 1 order of magnitude faster than K562-C320A transfectants (Fig. 4A) (t test, p < 0.01). In contrast, no significant differences in rolling velocity were noted between BJAB-WT and BJAB-C320A transfectants (Fig. 4B), suggesting that higher levels of C2GlcNAcT-I might influence rolling velocities in cells expressing monomeric PSGL-1. To test the effect of high C2GlcNAcT-I activity, since the K562-C320A transfectants (A; white circles) rolled at much higher velocities (~40 μm/s) (~ K562-C320A significantly different (p < 0.01) from all other transfectants at each wall shear stress was tested.

velocities were determined for K562 and BJAB transfectants over wall shear stresses ranging from 0.4 to 1.8 dyn/cm² on a high density P-selectin substrate (200 sites/μm²). Velocities of K562-C320A transfectants were almost 1 order of magnitude faster than K562-WT transfectants (~40 versus ~5 μm/s) (Fig. 4A) (t test, p < 0.01). In contrast, no significant differences in rolling velocity were noted between BJAB-WT and BJAB-C320A transfectants (Fig. 4B), suggesting that higher levels of C2GlcNAcT-I might influence rolling velocities in cells expressing monomeric PSGL-1. To test the effect of high C2GlcNAcT-I activity, since the K562-C320A transfectants (Fig. 4C) (Fig. 4C) compared with K562-C320A (~40 μm/s) (Fig. 4A) and was similar to that of K562C2-WT cells (8 μm/s) (Fig. 4C). As was observed with the BJAB cell lines, average rolling velocities were virtually identical for WT and C320A forms of PSGL-1 in K562 cells overexpressing C2GlcNAcT-I. These observations suggested that low endogenous levels of C2GlcNAcT-I expression combined with the C320A mutation resulted in increased rolling velocities of K562-C320A transfectants (Fig. 4A) and that high C2GlcNAcT-I levels in BJAB or K562C2 cells compensated for the rolling defect associated with monomeric PSGL-1 for the wall shear stresses tested.
C320A cells traveled ~8 μm between tethering events. Rolling velocities for K562C2 and BJAB transfectants correlated closely with the skip distance. At the lowest P-selectin site densities tested, dimerized PSGL-1 decreased the distance between tethering events (a P-selectin site density of 10 sites/μm²). A step begins when velocity exceeds 40.0 μm/s and ends when velocity is less than 40.0 μm/s. Distances traveled between consecutive frames where velocity is greater than 40.0 μm/s are added to give a single step. The brackets indicate the occurrence of a step. A shows eight interactions or steps at a P-selectin site density of 10 sites/μm². For transfectants with high C2GlcNAcT-I (BJAB and K562C2), the distance traveled between tethering events was similar for both the WT (black bar) and the C320A (white bar) at the higher site density (B). At low P-selectin site density, a significant difference (p < 0.01) was observed in the step size between monomeric and dimeric PSGL-1 K562 transfectants (C). The K562 C320A mean step size was significantly different (p < 0.01) from all other cell lines at both high and low site densities (B and C).

Alterations in PSGL-1 Molecular Weight Are Associated with C2GlcNAcT-I Levels—Probing whole cell lysates from the PSGL-1 or C320A transfectants with KPL1 revealed that high levels of C2GlcNAcT-I correlated with a slight decrease in PSGL-1 electrophoretic mobility (Fig. 7). Under reducing conditions, K562 cells transfected with PSGL-1 primarily expressed the ~120-kDa form of PSGL-1, whereas BJAB and K562C2 cells expressed a form of PSGL-1 with a molecular mass of ~135 kDa. Similar shifts in PSGL-1 electrophoretic mobility have been observed in C2GlcNAcT-I-deficient mice (11, 15). These differences have been attributed to the absence of branching structures associated with the C2GlcNAcT-I link- age of GlcNAc to GalNAc that have been deleted in the C2GlcNAcT-I-deficient mice (11). Based on these observations, the increased mobility of PSGL-1 in K562 transfectants might be due to low C2GlcNAcT-I activity due to the relatively low endogenous expression levels of C2GlcNAcT-I.

Although the major form of PSGL-1 detected from K562-WT lysates was ~120 kDa, a faint band corresponding to 135 kDa was also detected (Fig. 7, lane 5). There was no evidence of a similar band in the K562-C320A lanes (Fig. 7, lane 4) even at 5-fold greater loading densities (data not shown). This suggests that a small fraction of the PSGL-1 expressed by K562-WT cells as force was increased in cells expressing lower levels of C2GlcNAcT-I, k_{off} increased 3.5 times more rapidly for K562-C320A than K562C2-C320A transfectants, suggesting that tether bond strength was significantly dependent on C2GlcNAcT-I levels. The extrapolated untrapped k_{off} (26) for K562-C320A cells was severalfold higher than the other cells examined, further suggesting a less stable bond. Interestingly, the dimerization status of PSGL-1 did not significantly influence tether bond lifetime, suggesting that monomeric PSGL-1/P-selectin bonds were indeed being detected. Previous reports of differences between dimerized and monomeric PSGL-1 tether bond lifetimes may have been a consequence of spatial aliasing due to low image acquisition rates and incomplete sampling of data (38).
may have levels of C2GlcNAcT-I similar to levels found in K562C2 and BJAB transfectants. It is possible that the small subpopulation of K562WT cells that expressed PSGL-1 with lower electrophoretic mobility dominated tether bond analysis due to their longer lifetimes (Fig. 7).

Surface expression of sialylated core 2 O-glycans on K562 and BJAB transfectants was confirmed using the mAb CHO-131. CHO-131 binds to sialyl Lewis x extended from core 2 branched O-glycans and is specific for oligosaccharides modified by α,2,3-sialyltransferase, FucT-VII and C2GlcNAcT-1 (24).

After incubation with CHO-131, the mean fluorescent intensity of K562 cell lines transfected with C2GlcNAcT-I was greater than the K562 cell line expressing endogenous levels of the glycosyltransferase (Table I). Thus, a correlation exists between C2GlcNAcT-I levels and slow and fast rolling, high and low rates of electrophoretic mobility, and the degree of reactivity to CHO-131. These data support the conclusion that bond strength between PSGL-1 and P-selectin is increased by core 2 carbohydrate branching.

**DISCUSSION**

In this study, both PSGL-1 dimerization and C2GlcNAcT-I glycosylation were observed to make distinct contributions to cell rolling on P-selectin. High resolution video microscopy revealed that both association kinetics as measured by the frequency of cell tethering in flow and bond strength as represented by the lifetime of stressed bonds were differentially influenced by these two modifications of PSGL-1. Furthermore, we were able to demonstrate a major biomechanical role for C2GlcNAcT-I-dependent carbohydrate branching that was manifested as an increase in stressed PSGL-1-P-selectin tether bond lifetime. In contrast, dimerization of PSGL-1 significantly enhanced the ability of flowing cells to initiate rolling on P-selectin without having a significant effect on tether bond lifetime. Thus, these two structural features of PSGL-1 are important in cell tethering, bond strength and lifetime, and cellular accumulation under flow conditions.

Cell tethering on P-selectin mediated by both forms of PSGL-1 was enhanced by increasing the rolling substrate P-selectin site density and by increasing the level of C2GlcNAcT-I, although rolling by dimeric PSGL-1 was increased to a greater degree. Decreasing the P-selectin site density made conditions for initial cell attachment more stringent, a condition that could be overcome by K562WT but not by K562-C320A transfectants that displayed extreme deficits in binding to low site density P-selectin. It is possible that the P-selectin site density associated with CHO cells transfected with P-selectin and utilized in our initial report describing the loss of rolling associated with the C320A mutation (17) correlates with the low site densities used in this current report. In contrast, the higher C2GlcNAcT-I levels in both K562C2 and BJAB cells transfected with C320A appeared to modulate the rolling defect associated with K562-C320A transfectants at low P-selectin site densities. However, sufficiently high P-selectin site density allowed the K562-C320A mutant to tether under flow conditions, although the interactions were very transient and suggests that the higher site densities of P-selectin might compensate for the lower avidity interactions mediated by monomeric PSGL-1.

Cell rolling deficits attributable to monomeric PSGL-1 were evident in some but not all three C320A-expressing cell lines. The K562-C320A transfectants rolled very rapidly compared with the K562WT cells, but the C320A mutation had only modest effects on rolling velocity in transfectants expressing high levels of C2GlcNAcT-I (BJAB and K562C2), suggesting that C2GlcNAcT-I-dependent carbohydrate branching on PSGL-1 strengthens the molecular bond strength with P-selectin regardless of dimerization status.

In selectin-mediated rolling, two distinct adhesive phenotypes can be characterized that are dependent on substrate site density. The phenotype characteristic of rolling at “high” selectin site densities consists of a steady, peeling-like motion. Once rolling on a high density selectin substrate, a cell has considerably more time for bond formation than when initially tethering in flow. However, at lower densities, rolling becomes progressively more transient, and cell velocities between successive adhesive bonds typically increase (26). Increasing the P-selectin site density therefore allowed us to influence the probability of new bond formation as represented by the distance between sequential cell tethering events, or “step size.”

As shown by transfectants expressing high levels of C2GlcNAcT-I and rolling on high site density P-selectin (100 sites/μm²), step size between tether bond events was largely independent of dimerization status of PSGL-1. In contrast, at low P-selectin site densities (10 sites/μm²), monomeric PSGL-1, regardless of C2GlcNAcT-I expression levels, demonstrated increased step size relative to wild type PSGL-1. Under these conditions (Fig. 5C), PSGL-1 dimerization enhanced tethering frequency by a factor of 2, similar to the effect of L-selectin dimerization on cell tethering in flow (39).

Biochemical differences between the different forms of PSGL-1 appeared to correlate with host cell, suggesting that differences in post-translational modifications may underlie some of the observed differences in cell-adhesive dynamics. Wild-
type PSGL-1 or C320A isolated from K562 transfectants demonstrated an increase in electrophoretic mobility compared with high C2GlcNAcT-I expressing transfectants. PSGL-1 isolated from C2GlcNAcT-I−/− mice has a similar shift in electrophoretic mobility, suggesting that the differences in molecular weight may be due to alterations in C2GlcNAcT-I-dependent branching. Interestingly, neutrophils isolated from C2GlcNAcT-I−/− mice have rolling defects similar to those observed with the K562-C320A cell line. Although not formally proven, the generation of the high molecular weight forms of PSGL-1 by transfection of K562 cells with additional C2GlcNAcT-I implies that part of the adhesive defect observed with K562-C320A cells arises from insufficient C2GlcNAcT-I-dependent branching of O-glycans.

Transfectants with low levels of C2GlcNAcT-I (K562-C320A and K562-WT) (Fig. 1B and Table 1) expressed the low molecular weight form of PSGL-1 (Fig. 7), and this correlated well with K562-C320A rolling deficits but not with K562-WT bond strength, tethering rates, or rolling characteristics that were similar to those of transfectants expressing high levels of C2GlcNAcT-I. Thus, the lower levels of C2GlcNAcT-I did not seem to dramatically affect rolling on P-selectin in K562 cells expressing dimeric PSGL-1. However, a faint but reproducible band of high molecular weight PSGL-1 was evident in whole cell lysates probed from K562-WT transfectants that might account for this apparent discrepancy (Fig. 7, lane 5). It is possible that even the low level of high molecular weight PSGL-1 expressed by K562-WT was sufficient to dominate adhesive interactions with P-selectin that otherwise would have been mediated by weaker and slower forming bonds between P-selectin and PSGL-1 lacking C2GlcNAcT-I-dependent branching. Transfection of additional C2GlcNAcT-I into K562 cells dramatically increased cell tethering rates, consistent with the hypothesis that a small subpopulation of high expressing C2GlcNAcT-I K562-WT dominated rolling on P-selectin.

Based on the analysis of the distributions of tether bond lifetimes, it appeared that C2GlcNAcT-I-dependent branching had a significant impact on PSGL-1 bond strength with P-selectin. The reduction of C2GlcNAcT-I-dependent branching and bond lifetime under flow conditions suggests a more complex contribution to the bond's mechanical strength (40). It is possible that cells expressing lower levels of C2GlcNAcT-I have some form of carbohydrate modification at critical sites (such as threonine 16) and thus contribute to ligand recognition but results in suboptimal rolling and briefer stressed bond lifetimes (41). In conclusion, the use of video microscopy to analyze the movement of PSGL-1 and C320A transfectants over P-selectin substrates allowed the cellular adhesion and rolling process to be broken down into two key components: the initial cell tethering event and the lifetime of the tether bond. These two selectin binding parameters were then used to identify the contributions of dimerization and C2GlcNAcT-I-dependent branching of PSGL-1 for recognition of P-selectin under flow stress. Dimerization of PSGL-1, by clustering the receptor on a nanoscale, appears to facilitate recognition of P-selectin most significantly under flow conditions. Once rolling is initiated, the requirement for dimerization is less significant especially for rolling on high P-selectin site density. Additionally, C2GlcNAcT-I-dependent branching of O-linked glycans contributes to PSGL-1/P-selectin bond strength under mechanical tension, an observation not obvious from previous in vitro and in vivo cell adhesion studies or from the crystal structure of P-selectin complexed with PSGL-1.