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L-selectin Dimerization Enhances Tether Formation to Properly Spaced Ligand*

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Selectin counterreceptors are glycoprotein scaffolds bearing multiple carbohydrate ligands with exceptional ability to tether flowing cells under disruptive shear forces. Bond clusters may facilitate formation and stabilization of selectin tethers. L-selectin ligation has been shown to enhance L-selectin rolling on endothelial surfaces. We now report that monoclonal antibodies-induced L-selectin dimerization enhances L-selectin leukocyte tethering to purified physiological L-selectin ligands and glycopeptides. Microkinetic analysis of individual tethers suggests that leukocyte rolling is enhanced through the dimerization-induced increase in tether formation, rather than by tether stabilization. Notably, L-selectin dimerization failed to augment L-selectin-mediated adhesion below a threshold ligand density, suggesting that L-selectin dimerization enhanced adhesiveness only to properly clustered ligand. In contrast, an epidermal growth factor domain substitution of L-selectin enhanced tether formation to L-selectin ligands irrespective of ligand density, suggesting that this domain controls intrinsic ligand binding properties of L-selectin without inducing L-selectin dimerization. Strikingly, at low ligand densities, where L-selectin tethering was not responsive to dimerization, elevated shear stress restored sensitivity of tethering to selectin dimerization. This is the first indication that shear stress augments effective selectin ligand density at local contact sites by promoting L-selectin encounter of immobilized ligand.

Selectins are specialized C-type lectins that mediate the reversible capture (tethering) of circulating cell subsets to specific vessel walls and its subsequent rolling tethers in the direction of flow (1, 2). The biophysical basis for the exceptional ability of selectins to mediate cell capture (tethering) and rolling adhesions under highly disruptive forces is still obscure (3–6). This ability has been primarily attributed to fast formation rates of selectin bonds and to the ability of selectin tethers to tolerate rupture by elevated shear forces (7–10). A major unresolved question regarding selectin function is the high effective formation rate of selectin tethers to their physiological counterreceptors. Efficient tether formation, in particular by L-selectin, contrasts with the fairly low k_{on} of intrinsic L-selectin bonds (11). This k_{on} falls in a range shared by other molecular pairs with poor tethering capacity under shear flow (12, 13). It has thus become increasingly evident that selectin tethering to ligand is controlled by mechanical properties in addition to the biochemical properties measured under shear-free conditions (14, 15).

In addition to the fast effective kinetics of bond formation and intrinsically high mechanical stability of individual bonds (5), three other mechanisms have been proposed to account for the exceptionally high capacity of selectins to form tethers under physiological flow. Association of L-selectin with the actin cytoskeleton through its cytoplasmic domain was recently shown to reduce tether dissociation rates under flow and to enhance the mechanical stability of selectin tethers, independent of the intrinsic stability of the selectin:ligand bond (16). This stabilization was suggested to involve tail-mediated restriction of the selectin's mobility at the membrane, which facilitates its rebinding to a recently dissociated ligand on a countersurface (16). In addition, a regulatory role for the EGF[1] domain has been recently suggested to explain the efficiency by which the lectin domain of cell-based or cell-free L-selectin recognizes surface-bound ligand (15). Recent studies also demonstrated that dimerization of L-selectin or P-selectin, as well as the selectin ligand, PSGL-1, augments selectin-mediated rolling in different cellular systems (10, 17–19). The generation of multimeric binding by receptor oligomerization may provide a rapid means for dispersion of highly disruptive shear forces over multiple bonds. A role for selectin or ligand clustering in promoting cell adhesion under flow is feasible because all known physiological selectin ligands present multiple carbohydrate ligands on mucin-like scaffolds or on complex multivalent glycans (2, 20, 21). Selectins are also often found clustered on leukocyte surfaces (18, 22) although the contribution of spontaneous L-selectin clustering to the selectin adhesiveness under physiological shear flow has not been established.

In the present study, we assessed the effect of L-selectin dimer-

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[1] The abbreviations used are: EGF, epidermal growth factor; mAb, monoclonal antibody; SCR, short consensus repeats; PBS, phosphate-buffered saline; HSA, human serum albumin; sLe^x, sialyl Lewis^x; PNAd, peripheral node addressin; GlyCAM-1, glycoprotein cell adhesion molecule 1; PSGL-1, P-selectin glycoprotein ligand; WT, wild type.

ization on its adhesiveness to various purified L-selectin ligands and under various conditions of shear flow. The extensive data previously obtained on both the biochemical nature and clustering properties of PSGL-1 and its individual selectin-binding carbohydrates and backbone residues (19, 23, 24) render this ligand an ideal model to assess how L-selectin dimerization and ligand presentation affect specific kinetic properties of selectin tethers under defined flow conditions. We therefore focused our study on native neutrophil-derived PSGL-1 as well as on synthetically generated PSGL-1-derived glycopeptides (25). Unexpectedly, dimerization of L-selectin was found to alter entirely different properties of L-selectin tethers to PSGL-1 than those previously reported for P-selectin dimerization (10). L-selectin dimerization enhanced the tether formation rate without altering tether stability and did so only on ligands presented on an adhesive surface at densities above a critical threshold. Notably, increased shear stresses endowed dimerized L-selectin with the ability to increase its avidity even to highly diluted ligands. Thus, selectin dimerization results in enhanced avidity and tether formation conditional to proper ligand spacing and adequate conditions of shear flow. In contrast to dimerization, EGF domain substitution on L-selectin with a P-selectin EGF domain enhances tether formation regardless of ligand density or shear flow, suggesting that this domain controls intrinsic rather than dimerization properties of L-selectin.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, and Selectin Transfectants

The function-blocking anti-L-selectin mAb, DREG-200 (26), the anti L-selectin mAbs, LAM1–101 and LAM1–118 (both directed against the SCR domain of L-selectin) (27), and the anti-PSGL-1 mAb, PL-1 (28), were used as purified Ig. Full-length PSGL-1, purified from human neutrophils as previously described (29), a generous gift from Dr. R. P. McEver (University of Oklahoma, Oklahoma City, OK), was stored at 4 °C in 1% n-octyl β-D-glucopyranoside/PBS (octyl glucoside) solution until use. GlyCAM-1, purified from mouse serum by immunoaffinity chromatography (30), a generous gift from Dr. S. D. Rosen (University of California, San Francisco, CA), was stored in PBS. Biotin-labeled PSGL-1-derived sLe^x-decorated glycopeptide and a non-fucosylated control peptide, both corresponding to the 19-residue N′ terminus of human PSGL-1, and each containing a single biotin group at its C′ terminus, were synthesized as previously described (25). Neutralite avidin (31) was a gift from Dr. E. A. Bayer, (Weizmann Institute of Science, Rehovot, Israel). Fucoidin, a plant-derived sulfated polyfucan that saturably blocks the lectin domains of L-selectin and P-selectin (32), bovine serum albumin (fraction V), and Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, were obtained from Sigma. Human serum albumin, HSA (Fraction V) was obtained from Calbiochem (La Jolla, CA).

The stable expression of cDNA-encoding native human L-selectin or the L-selectin chimera, LPL, in which the EGF-like domain of L-selectin was replaced with the homologous domain from P-selectin in the mouse pre-B cell line 300.19 has been described elsewhere (33). Clones expressing similar levels of native L-selectin and LPL were maintained in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM glutamine, 0.1 μM 2-mercaptoethanol and antibiotics.

Preparation of Ligand-containing Substrates

PSGL-1 was diluted to concentrations of 0.001–0.2 μg/ml in coating medium (PBS supplemented with 20 mM bicarbonate, pH 8.5) and adsorbed onto a polystyrene plate for 15 h at 4 °C. Stock solutions of GlyCAM-1, fucoidin, or DREG-200 were diluted in coating medium and adsorbed onto the plates at 37 °C for 2 h. All substrates were washed five times with PBS and blocked with PBS supplemented with 2% HSA for 2 h at 4 °C. The site density of the L-selectin recognition sites on immobilized PSGL-1 was determined by radioimmunoassay using ^{125}I-labeled PL1 mAb performed as described (15). GlyCAM-1 site densities were assessed using ^{125}I-labeled CAM02 as described (15). Site densities were determined for PSGL-1 and GlyCAM-1 coated at input concentrations >0.05 μg/ml. Below this value, the site densities of both ligands were estimated from a linear regression of mAb binding to substrates coated with these mucins at concentrations between 0.05 and 0.2 μg/ml. PSGL-1-derived glycopeptides were immobilized on substrates coated with a nonglycosylated form of avidin, neutralite avidin (31). Neutralite avidin was diluted in PBS supplemented with 40 mM bicarbonate, pH 9.0, and immediately adsorbed onto a polystyrene plate for 15 h at 4 °C, then washed five times with PBS, and blocked with PBS supplemented with 2% HSA for 2 h at 4 °C. The PSGL-1-derived peptides monobiotinylated in their C′ termini, were each diluted in cell binding medium (see below) and adsorbed for 4 h on the avidin-coated plate.

Laminar Flow Assays

Cell Treatments—For cell inhibition studies, selectin transfectants were incubated in H/H medium (Hank's balanced salt solution/10 mM HEPES, pH = 7.4, supplemented with 2 mg/ml bovine serum albumin) in the presence of 10 μg/ml of the L-selectin-blocking mAb, DREG-200, 50 μg/ml fucoidin, or 5 mM EGTA. To induce L-selectin dimerization, cells (2×10^6/ml) were preincubated for 15 min at 25 °C in cell binding medium (H/H medium supplemented with 2 mM Ca^{2+}) containing 10 μg/ml of either the L-selectin-dimerizing mAb, LAM1–118, or the control mAb, LAM1–101 (27). Cells were perfused unwashed over the test substrates. These conditions were found to induce optimal L-selectin dimerization on various ligand systems; further cross-linking of the dimerizing mAb had no additional augmenting effects (data not shown).

Cell Tethering and Rolling Measurements—The polystyrene plate, on which purified ligand was adsorbed, was assembled in a parallel plate laminar flow chamber (260-μm gap) as previously described (15, 34). Transfected cells were washed in H/H medium at 10^7 cells/ml, resuspended in binding medium at 2×10^6 cells/ml, and perfused at room temperature through the flow chamber at desired flow rates, generated using an automated syringe pump (Harvard Apparatus, Natick, MA). Cellular interactions were visualized at two different fields of view (each 0.17 mm^2 in area) using the 10× objective of an inverted phase contrast microscope (Diaphot 300, Nikon Inc.). Cell images were videotaped as previously described (15, 34). When not otherwise indicated, cell images were manually quantitated by analysis of images directly from the monitor screen. Two types of initial cell tethering to the substrate were determined: transient tethers, in which cells attached briefly to the substrate without initiating rolling motions, or stable tethers, in which tethered cells established rolling on the substrate for at least 3 s after initial tethering. The number of either transient or rolling-associated tethers was divided by the flux of freely flowing cells moving through the same field (15). Cell accumulation assays were performed on cells allowed to settle onto the substrate for 30 s. The wall shear stress was then increased stepwise every 5 s. The number of cells accumulated at the end of each 5 s interval of increased shear and their rolling velocities were determined by computerized image analysis (see below).

Computerized Microkinetic Analysis

The imaging system developed for quantitative analysis of instantaneous velocities of cell movement, WSCAN-Array-3 (Galai, Migdal-Ha'emek, Israel), was described elsewhere (15). In summary, individual transfectants rolling on a PSGL-1 coated substrate were tracked during their movement through the field of view for 3 s at 0.02 s resolution, and the mean displacement velocities of total cells were derived. Two categories of cells were identified based on their mean velocity above or below a critical velocity value: cells moving at a mean velocity <0.25 that of the hydrodynamic velocity (*i.e.* of freely moving cells) but above a defined velocity threshold were referred to as jerkily rolling cells; cells moving below the velocity threshold were defined as smoothly rolling cells. Microkinetics of individual cells exhibiting jerky rolling was analyzed on video segments recorded with a high speed camera (500 frames/sec; Kodak Motion Corder Analyzer, FASTCAM-SUPER 500, Eastman Kodak Co.). Cell position analysis was preformed at 0.002-s intervals with the WSCAN-Array-3 software as described (16). At a shear stress of 1 dyn/cm^2, specific pauses were defined as cell displacements of <0.6 μm during a period of 0.006 s separated by cell displacements of at least 3 μm during 0.004-s periods before and after each pause. The number of L-selectin-specific pauses per jerky rolling cell was derived, and the mean number of pauses per cell was calculated for cell populations of at least 45 cells. The natural log of the number of pauses with a given duration after pause initiation was plotted against pause duration (16). A first-order dissociation plot yielded a straight line with the slope = $-k_{off}$.

RESULTS

Dimerization of L-selectin and EGF Domain Substitution Augments Adhesiveness to PSGL-1 by Different Mechanisms—To dimerize L-selectin while retaining its native cy-

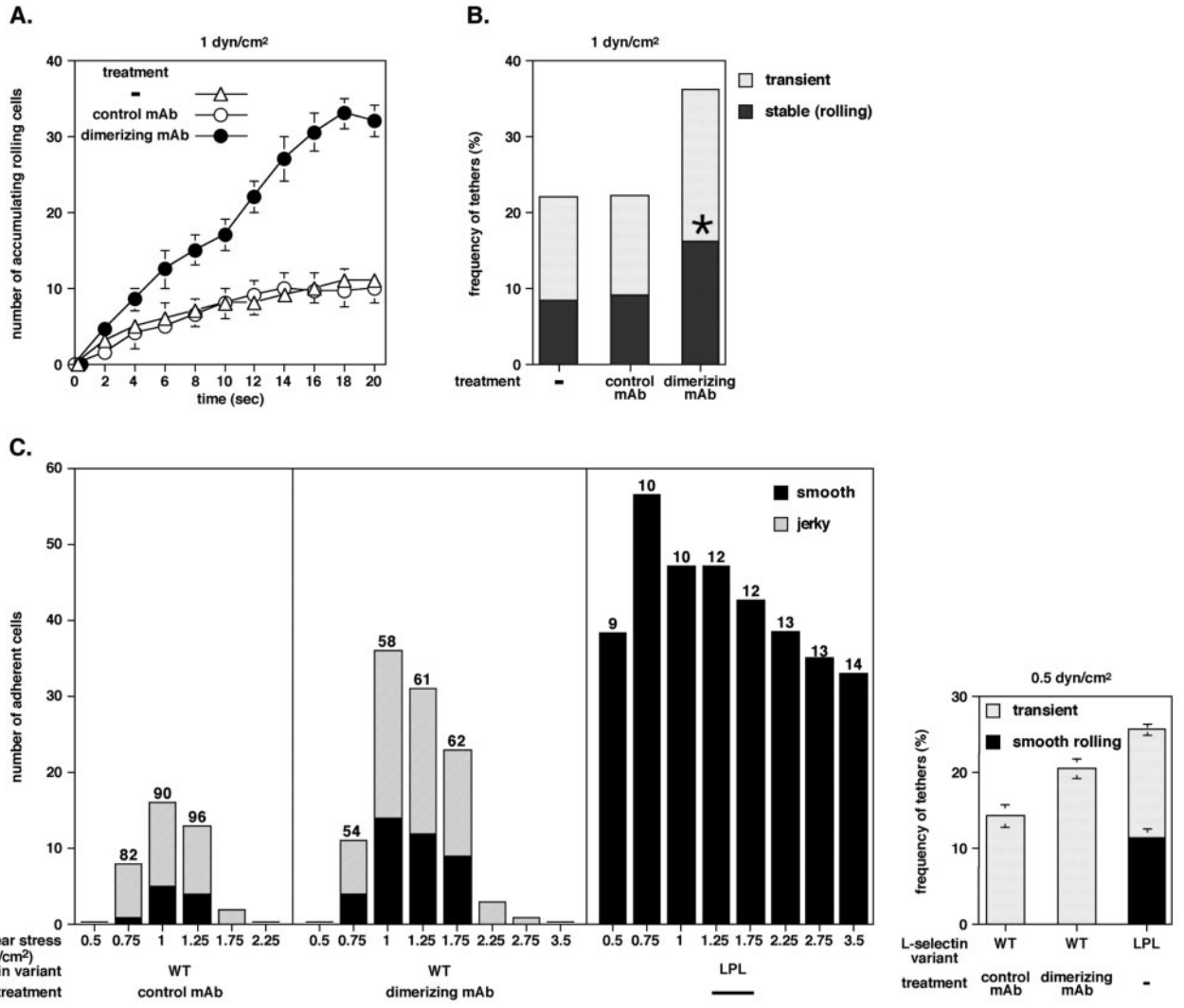

FIG. 1. **Dimerization of L-selectin increases the stability of rolling adhesions on PSGL-1.** *A*, effect of a dimerizing L-selectin mAb on accumulation at 1 dyn/cm^2 of rolling 300.19 murine pre-B cells transfected with human L-selectin on purified PSGL-1 coated at 110 sites/μm^2. Cells were either left intact or pretreated with control or dimerizing L-selectin-specific mAbs (LAM 1–101 and 1–118, respectively). *B*, frequency of transient tethering and stable tethering (*rolling*) of L-selectin transfectants pretreated with either dimerizing mAb or control mAb at 1 dyn/cm^2 on the same PSGL-1-coated substrate tested in *A*. Values are the mean ± range of two test fields. *, $p < 0.0015$ compared with control-mAb treated cells using a paired two-tailed student's *t* test. *C*, accumulation of rolling cells expressing comparable levels of either L-selectin (*WT*) or EGF-domain mutant (*LPL*) on PSGL-1 (110 sites/μm^2) under continuously incremented shear stresses. L-selectin transfectants were pretreated with either dimerizing or control mAb. Categories of rolling (*smooth* or *jerky*) are shown as fractions of the cells accumulated on the substrate at each shear stress. Cells were allowed to settle onto the substrate for 30 s and then were subjected every 5 s to the indicated stepwise increments of shear stress. The number of cells remaining adherent in the field of view at each shear stress was determined by computerized analysis. At each indicated shear stress, the mean velocity of smoothly rolling cells (with a mean velocity <100 μm/sec) is indicated on top of bars. *Inset*, frequency of tethering and rolling mediated by cells expressing LPL mutant or WT L-selectin pretreated with dimerizing or control mAb at low shear stress of 0.5 dyn/cm^2 on the PSGL-1-coated substrate tested in *C*. Data shown in *A–C* are representative of five independent experiments.

toskeletal associations, dimerization of L-selectin was induced by ligation of a site on the SCR remote from the ligand recognition site (17) using the L-selectin-dimerizing mAb, LAM-1–118. This mAb was recently shown to augment L-selectin-mediated rolling to a similar degree as that induced by cytoplasmic tail-mediated dimerization (17). This study demonstrated the augmented adhesiveness of mAb-dimerized L-selectin toward endothelial surfaces expressing L-selectin ligands (17). We therefore first characterized the effect of L-selectin dimerization on L-selectin adhesiveness to a purified PSGL-1 homodimer. Consistent with the previous study, artificial L-selectin dimerization by the dimerizing mAb augmented L-selectin-dependent capture and rolling of L-selectin transfected 300.19 pre-B cells on purified high density PSGL-1 under optimal dimerizing conditions (Fig. 1*A*), whereas a non-dimerizing control L-selectin-specific mAb did not augment capture or rolling (Fig. 1*A*). Cross-linking of the dimerizing mAb with a secondary antibody did not further enhance L-selectin adhesiveness over that induced by optimal levels of dimerizing mAb (data not shown). Similar to pre-B cells, L-selectin expressed on other types of leukocytes, including human neutrophils, peripheral blood lymphocytes, and the T cell line Jurkat, also responded to the dimerizing mAb by a similar augmentation of capture and rolling on PSGL-1 as well as on other ligands including GlyCAM-1 and PNAd (data not shown). Dissection of the augmenting effects of the dimerizing mAb revealed that both the ability of L-selectin-expressing cells to initiate tethers to high density PSGL-1 (110 sites/μm^2) and the conversion of these initial tethers into subsequent rolling were enhanced by L-selectin dimerization (Fig. 1, *A* and *B*). L-selectin dimerization also significantly increased the number of cells accumulated on a PSGL-1-coated substrate and the smoothness of their rolling, concomitantly with a reduction in mean rolling velocities (Fig. 1*C*). In contrast, L-selectin dimerization

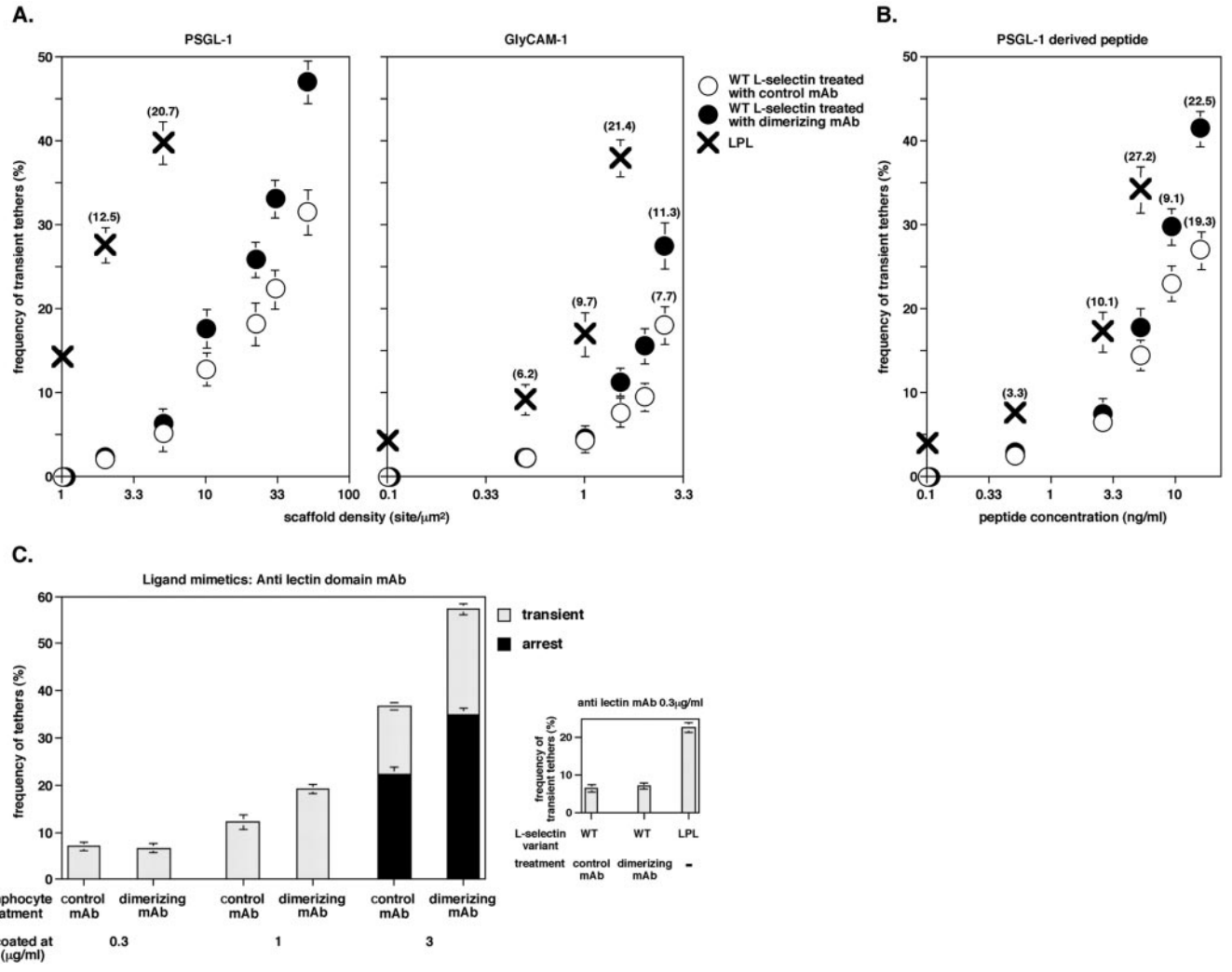

FIG. 2. **Tethering of L-selectin-expressing cells pretreated with dimerizing mAb to PSGL-1 and GlyCAM-1 coated at different densities.** *A*, tethering frequencies of WT L-selectin-expressing pre-B cells pretreated with dimerizing or control mAb on substrates coated with different densities of PSGL-1 or GlyCAM-1. Net tethering frequency at each ligand density was derived by subtracting background tethering measured in the presence of 5 mM EGTA. For comparison, net tethering of pre-B cells expressing the EGF domain mutant (LPL) is shown at the lowest ligand density range. Values are the mean ± range of two fields. At ligand densities, where tethered cells established persistent rolling, the fraction of these cells within initially tethered cells is indicated in *parenthesis*. *B*, tethering frequency of L-selectin-expressing cells pretreated with either dimerizing or control mAb to substrate coated at different site densities of sulfated sLe^x-bearing glycopeptide derived of the N′ terminus of PSGL-1. The peptide coated onto the surface via an avidin anchor as described in "Experimental Procedures." For comparison, tethering and rolling of pre-B cells expressing the LPL mutant is shown. *C*, tethering frequency (transient or followed by immediate arrest) of WT L-selectin-expressing cells pretreated with either dimerizing or control mAb to substrates coated at different site densities of the anti-L-selectin mAb, DREG-200. Neither the control nor dimerizing L-selectin-specific mAbs interfered with DREG-200 binding to L-selectin (data not shown). *Inset*, frequency of tethers initiated by cells expressing the LPL mutant on low density DREG-200. All measurements were performed at a shear stress of 1 dyn/cm^2. Data in *A–C* are representative of three experiments.

did not reduce the shear threshold required for lymphocytes to initiate L-selectin-mediated rolling on PSGL-1 (Fig. 1, *B* and *C*). Below 0.75 dyn/cm^2 neither control mAb-treated transfectants nor cells treated with the dimerizing mAb could roll on PSGL-1, although they could still transiently tether to the ligand (Fig. 1*C*, *inset*).

An EGF domain mutant of L-selectin, termed LPL, derived by a substitution of the L-selectin EGF domain with the homologous domain from P-selectin, although not altering the overall length of the mutated L-selectin, has been recently demonstrated by us to exhibit enhanced recognition of immobilized ligands under shear flow, even in a cell-free state (15). Remarkably, LPL expressed on 300.19 pre-B cells exhibited far superior rolling activity *versus* that of dimerized L-selectin (Fig. 1*C*). The LPL-transfected pre-B cells exhibited much higher resistance to detachment by elevated shear stresses than dimerized L-selectin and dramatically reduced rolling velocities compared with cells treated with the L-selectin dimerizing mAb (Fig. 1*C*). Furthermore, although L-selectin dimerization did not reduce the threshold shear for rolling, the EGF domain substitution lowered that shear threshold (Fig. 1*C* and *inset*). These results indicate that the EGF domain mutation of L-selectin induces more robust augmentation of cell capture and rolling than L-selectin dimerization. The data also suggest that the dependence of the selectin adhesion on a shear stress threshold is not altered by its dimerization state but, rather, by alteration of intrinsic kinetic properties of selectin binding to immobilized ligand (15).

Ligand Density and Spacing Dictates the Extent of Adhesion Augmented by L-selectin Dimerization—L-selectin dimerization is expected to augment avidity only to properly clustered ligand. Indeed, dimerization of L-selectin increased rates of cell capture and the extent of initial tethering followed by rolling on high densities of L-selectin ligands more than on low densities

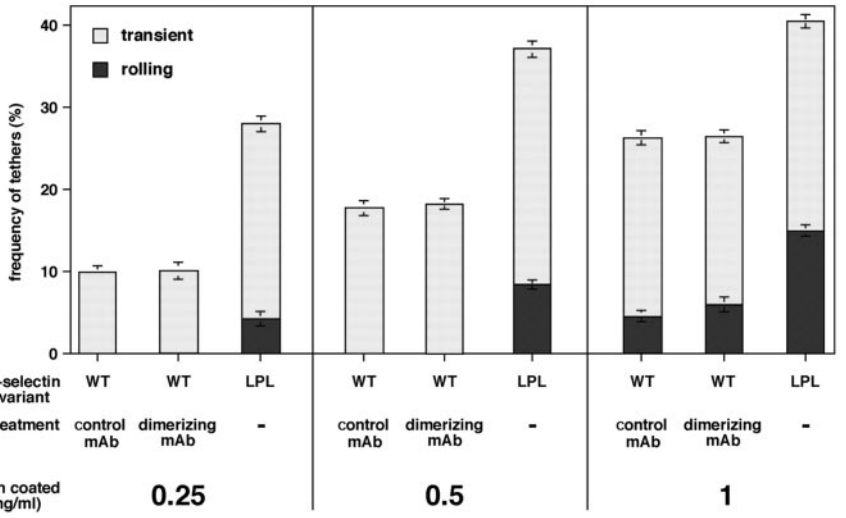

FIG. 3. **Dimerization of L-selectin does not augment lymphocyte adhesion to the polysaccharide ligand, fucoidin.** Frequencies of tethering and rolling mediated by WT L-selectin-expressing cells pretreated with either dimerizing mAb or control mAb, interacting with different densities of immobilized fucoidin at a shear stress of 1 dyn/cm^2 are shown. For comparison, tethering and rolling of cells expressing the EGF-domain mutant (*LPL*) was tested on identical fucoidin substrates. All tethers could be blocked in the presence of the lectin function-blocking mAb, DREG-200. Results are mean ± range of two test fields. A representative experiment of four is shown.

of coated ligands (Fig. 2*A*). Given that PSGL-1 is dimeric and that GlyCAM-1 is decorated with multiple L-selectin-binding carbohydrates (11), dimerization of L-selectin on the leukocyte surface should augment its avidity to these ligands at any site density if individual ligand moieties on these scaffolds are properly spaced. However, below a critical value of coating density, the augmented tethering induced by dimerization was abrogated on both PSGL-1 and on GlyCAM-1 (Fig. 2*A*). Similar dependence of dimerization-enhanced L-selectin avidity on critical site density and proper spacing of ligand was also observed with a PSGL-1-derived glycopeptide corresponding to the first 19 residues of PSGL-1, which contains the major P- and L-selectin recognition sequences of native PSGL-1 (Fig. 2*B*). This suggests that only above this density can the peptide, anchored to the substrate via avidin, form effectively spaced dimers recognized by mAb-dimerized L-selectin. Interestingly, the coating density below which L-selectin dimerization no longer augmented tether formation was significantly lower for GlyCAM-1 than for PSGL-1 (Fig. 2*A*). This suggested a higher proportion of properly spaced L-selectin-binding moieties on GlyCAM-1 than on PSGL-1 coated at similar protein scaffold densities. In support of this possibility, GlyCAM-1 mediated more frequent rolling than PSGL-1 (Fig. 2*A*), and the velocities of rolling mediated by GlyCAM-1 were far lower than rolling mediated on equivalent site densities of PSGL-1 (data not shown). Thus, although multivalent, when coated at 1 and 5 sites/μm^2 on the test substrates, L-selectin-binding moieties on the GlyCAM-1 and PSGL-1 scaffolds, respectively, could be spaced too far to form dimers that could be recognized by dimerized L-selectin on tethered lymphocytes. Consistent with this assumption, L-selectin dimerization augmented L-selectin-mediated B cell tethering to immobilized mAb directed against the lectin domain of the selectin only above a critical mAb coating density (Fig. 2*C*). Because the mAb binds L-selectin at much higher affinity than natural L-selectin glycoprotein (11, 15), these results indicate that L-selectin dimerization enhances L-selectin-mediated adhesion to a properly clustered ligand irrespective of its binding affinity to L-selectin. In contrast and consistent with its inherently higher adhesiveness, the EGF domain L-selectin mutant, LPL, readily formed tethers even to diluted ligands at up to 10–20-fold higher frequencies than native L-selectin (Fig. 2*A*). Notably, the extent of tether enhancement introduced by the EGF domain substitution was similar on both GlyCAM-1 and PSGL-1, excluding the possibility that a specific association between the P-selectin-derived EGF domain with a specific region on PSGL-1 accounted for the enhanced adhesiveness of LPL to PSGL-1. Indeed, the mutant also interacted much more readily than native L-selectin with the anti L-selectin mAb (Fig. 2*C*, *inset*, and data not shown). Thus, LPL retains an inherent capacity to form functional tethers on any ligand tested independent of its structure, density, or binding affinity to L-selectin.

Inspite of augmenting L-selectin adhesion to different ligands, L-selectin dimerization failed to augment L-selectin adhesiveness to a non glycoprotein ligand, fucoidin, a multivalent polysaccharide that consists of closely spaced L-selectin-binding sulfated carbohydrates (Fig. 3). Similar observations (data not shown) were also found on substrates coated with another highly clustered carbohydrate ligand, a sLe^x-decorated neoglycolipid (16). Nevertheless and consistent with its higher inherent adhesiveness, LPL-expressing cells tethered and rolled at higher efficiencies than L-selectin on fucoidin (Ref. 15 and Fig. 3). Thus, although dimerization of L-selectin augments its adhesiveness only to properly clustered ligands, hyperclustering of ligand as in the case of fucoidin masks the proadhesive effects of L-selectin dimerization. The EGF domain substitution of L-selectin, in contrast, augments the selectin adhesiveness even to this highly clustered ligand, consistent with an intrinsically higher adhesive capacity of the mutant to any ligand tested irrespective of its spacing on the substrate.

The EGF Domain Mutant Responds to Dimerization at Much Lower PSGL-1 Densities than L-selectin—The increased intrinsic reactivity of the LPL mutant toward surface-immobilized L-selectin ligands could result in loss of the mutant's responsiveness to dimerization. Therefore, we next tested the effect of the dimerizing mAb on LPL-mediated tethering to PSGL-1. mAb-induced dimerization of LPL was found to augment tethering to low density PSGL-1 (Fig. 4*A*), indicating that the EGF domain selectin mutant did respond to dimerization. Reminiscent of L-selectin, mAb-induced dimerization of LPL augmented its tethering capacity to PSGL-1 proportionally to PSGL-1 density, and below a critical PSGL-1 density dimerization no longer augmented tethering (Fig. 4*A* and data not shown). Strikingly, however, the responsiveness of the mutant to the dimerizing mAb was retained at PSGL-1 densities 10–20-fold lower than those required for dimerization of L-selectin to augment tether formation (Fig. 4*A* *versus* Fig. 2*A*). Thus, the EGF-domain mutant not only tethered to individual PSGL-1 scaffolds at much higher efficiency than L-selectin (Figs. 1, *C* and *C inset* and 2, *A* and *B*) but appeared to recognize, upon dimerization, functional ligands on PSGL-1 not recognized by dimerized L-selectin. We therefore speculated that LPL might

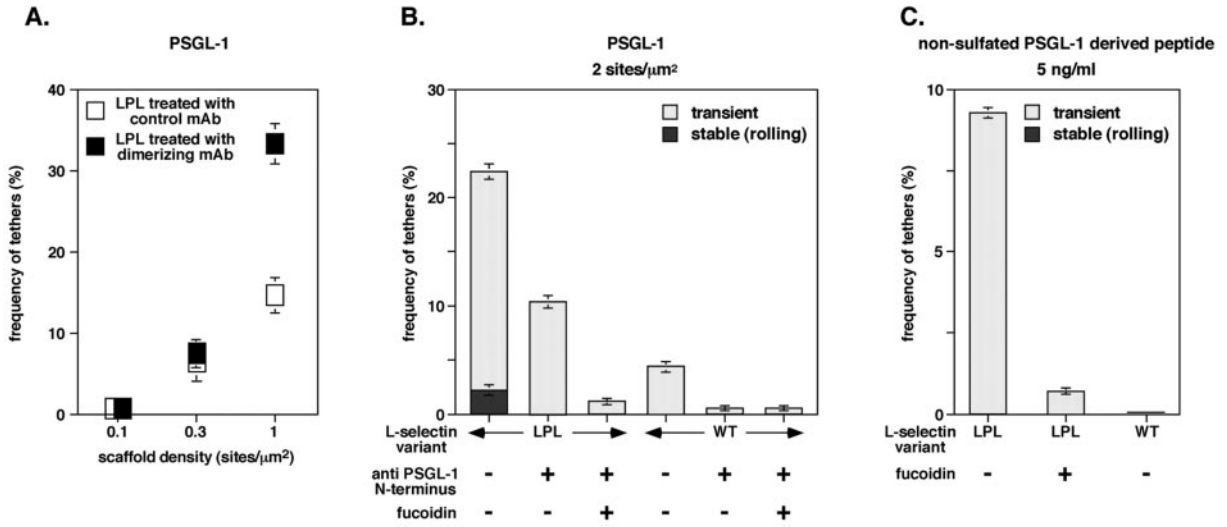

FIG. 4. **The EGF-domain mutant recognizes non-sulfated PSGL-1 moieties and responds to dimerization at lower PSGL-1 densities than L-selectin.** *A*, frequency of transient tethering mediated by LPL mutant-expressing cells pretreated with either dimerizing or control mAb on different site densities of PSGL-1 at shear stress of 1 dyn/cm^2. Results are shown as mean ± range of two test fields. *B*, residual cell tethering activity of PSGL-1 preblocked with mAb directed to the sulfotyrosine motif on the N′ terminus of PSGL-1. Tethering frequency of cells expressing either LPL mutant or WT L-selectin interacting with intact or mAb-blocked PSGL-1 is shown. The majority of tethers were eliminated in the presence of soluble fucoidin. Results are mean ± range of two test fields. *C*, frequency of tethering mediated by cells expressing either LPL mutant or WT L-selectin to immobilized nonsulfated PSGL-1-derived N′ terminal peptide bearing sLe^x glycans. Data in *A–C* are representative of three experiments.

recognize closely spaced L-selectin-binding carbohydrates on the PSGL-1 scaffold that are not recognized by native L-selectin. The high affinity P-selectin and L-selectin-binding moiety on PSGL-1, comprised of an N′-sulfotyrosine motif, necessary for the glycoprotein to support rolling of P- or L-selectin-expressing cells (35, 36). However, PSGL-1 is also decorated with multiple sLe^x moieties, potential low affinity ligands for E-selectin and L-selectin (29, 37, 38). mAb blocking of the N′ sulfotyrosine motif on PSGL-1 indeed abolished L-selectin-mediated tethering to PSGL-1 but, nevertheless, retained significant selectin-dependent tethering activity of LPL (Fig. 4*B*). Furthermore, the LPL mutant could efficiently form cellular tethers to a nonsulfated sLe^x-decorated PSGL-1-derived peptide, whereas L-selectin failed to form detectable tethers to this low affinity ligand (Fig. 4*C*). Thus, LPL appears to recognize multiple sLe^x moieties on the PSGL-1 scaffold, which are not recognized by native L-selectin under shear flow conditions. The effective sLe^x density recognized by the LPL mutant on each PSGL-1 scaffold is therefore higher than the density of the N′ sulfotyrosine motif recognized by L-selectin, rendering LPL more responsive to dimerization than native L-selectin.

Dimerization Augments L-selectin and LPL Tether Formation without Altering Tether Lifetime—Displacement motions of leukocytes rolling on glycoprotein selectin ligands are comprised of discrete steps separated by transient reversible pauses with characteristic duration, reflective of bond stability at microvillar contact zones (15, 16, 39). Notably, the duration of these tethers becomes progressively shorter with reduced bond number within each tether (39) and thus can serve as an indicator of L-selectin avidity. To gain further insights into a possible modulation of tether duration rather than formation by L-selectin dimerization, the microkinetics of rolling motions on different low densities of PSGL-1 was next analyzed at high temporal resolution (Fig. 5). Such measurements demonstrated that L-selectin dimerization did not prolong the dissociation rate constant of L-selectin tethers to PSGL-1, even at high PSGL-1 density (Fig. 5*A*). The duration of the vast majority of tethers mediated by both control mAb-treated and dimerized L-selectin could be fit into an homogenous group with a single first order dissociation rate constant, independent of PSGL-1 density (Fig. 5, *A* and *B*). Thus, the high temporal resolution analysis confirmed that the sole effect of L-selectin dimerization on quantal adhesive tethers is an enhancement of tether formation rate on properly clustered PSGL-1 (Fig. 5*A*). Furthermore, when PSGL-1 density was too low to allow dimerization-induced enhancement of tether formation, no effect on the duration of tethers could be detected (Fig. 5*C*). Thus, neither the formation nor the stability of L-selectin tethers were modulated by L-selectin dimerization when the ligand density approached a critical density value ($\leq$7 sites/μm^2). Similar to its effects on L-selectin, the dimerizing mAb augmented LPL mutant-mediated tether formation without altering tether duration (Fig. 6*A*). Thus, dimerization of both L-selectin and the LPL mutant resulted in augmented tethering, but conserved duration of quantal adhesive tethers. Notably, at low PSGL-1 site densities that supported equivalent amount of tethers of either L-selectin or LPL, *i.e.* 7 and 0.3 sites/μm^2, respectively, the dissociation rate constant of the majority of LPL mutant-mediated tethers was comparable with that measured for L-selectin tethers (Fig. 6*B versus* Fig. 5*B*). These results were consistent with previous kinetic measurements of dissociation of transient tethers of LPL from GlyCAM-1, which revealed that EGF domain substitution augments tether formation of L-selectin without altering the kinetic stability of tethers (15).

Shear Stress Reduces the Ligand Density Threshold Required for Tether Enhancement by L-selectin Dimerization—The cumulative results of this study suggested that to translate L-selectin dimerization into augmented tether formation, L-selectin must recognize properly spaced L-selectin-binding moieties on its counterreceptor scaffolds. Shear flow has been proposed to increase the bond number between L-selectin and ligand at local microvillar contacts based on the observation that tethers formed at elevated shear stresses dissociated with slower kinetics than tethers formed at low shear stresses (39). To test the effect of shear stress on the responsiveness of L-selectin to mAb-induced dimerization, we compared the frequency of tethering to PSGL-1 present at a density too low for mAb-induced dimerization to augment tether formation. Strikingly, the frequency of L-selectin tethering to low density PSGL-1 was rendered more sensitive to L-selectin dimerization

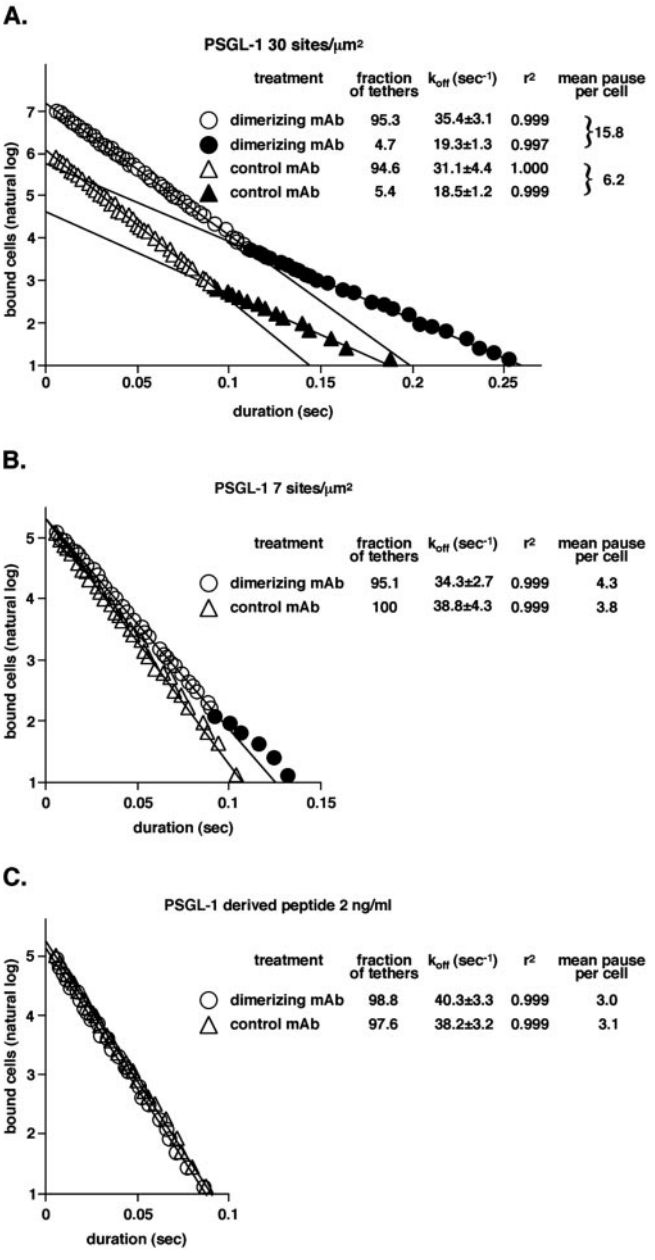

FIG. 5. **Effect of dimerization on tether formation and dissociation from low density PSGL-1 coated substrates.** Dissociation kinetics of tethers mediated by WT L-selectin-expressing cells pretreated with either control or dimerizing mAb and perfused at a shear stress of 1 dyn/cm^2 over PSGL-1 coated at 30 sites/μm^2 (*A*) or 7 sites/μm^2 (*B*). Cells were recorded at 500 frames/s. The k_{off} values were determined from the slope of the natural log of number of tethers plotted *versus* the duration of each tether. Tethers ≤0.004 s were not considered adhesive and were excluded from analysis. Data points that fit a first order dissociation curve (*open symbols*) are connected by a straight line with a slope equaling $-k_{off}$. Data points of L-selectin-mediated tethers that did not fit the first order dissociation approximation are indicated by *filled symbols*. These slower dissociating tethers are connected by a second line yielding a second $-k_{off}$ value (*A*). The fraction of each group of tethers of the total tethers is indicated. At low density ligand (*B*, *C*), these events were too rare to yield a second $-k_{off}$ value. The mean pause number per cells tethered to the substrate is also indicated. *C*, dissociation kinetics of tethers formed by control or dimerizing mAb-treated L-selectin-expressing cells interacting with sulfated PSGL-1-derived glycopeptide at 1 dyn/cm^2. *r*, coefficient of correlation. Data are representative of three experiments.

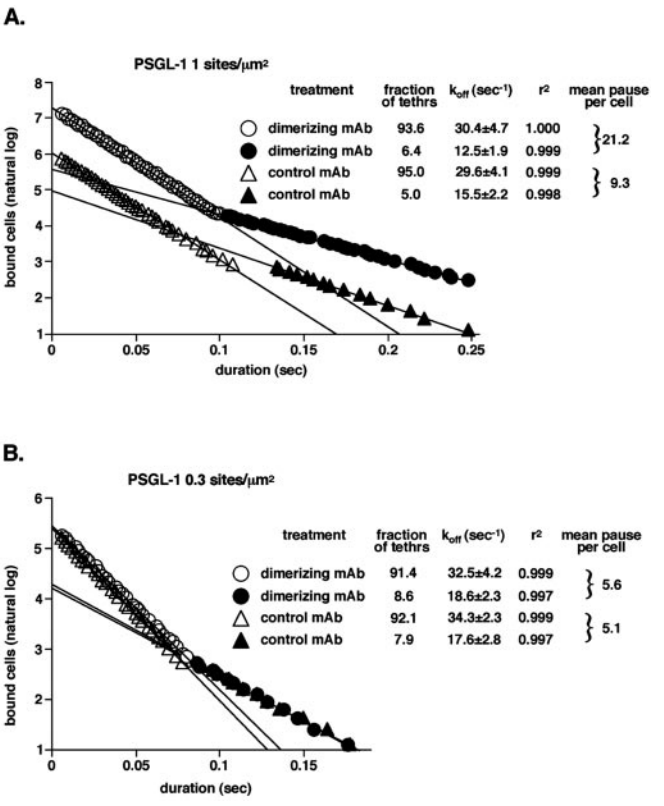

FIG. 6. **Effect of dimerization of the EGF-domain mutant on tether formation and dissociation from low density PSGL-1-coated substrates.** Dissociation kinetics of tethers mediated by LPL mutant expressing cells pretreated with either dimerizing or control mAb on PSGL-1 substrates coated at 1 sites/μm^2 (*A*) or 0.3 sites/μm^2 (*B*). The various k_{off} values were determined at 1 dyn/cm^2 as described in Fig. 5. Data are representative of three experiments.

when measured at high shear stress (1.75 dyn/cm^2) as compared with lower shear stress (1 dyn/cm^2) (Fig. 7, PSGL-1 2 or 22 sites/μm^2). Conversely, at high PSGL-1 density, where the frequency of L-selectin tethering at 1–1.75 dyn/cm^2 was sensitive to L-selectin dimerization, L-selectin dimerization failed to augment any tether formation at a shear stress of 0.5 dyn/cm^2 (Fig. 7, PSGL-1, 22 sites/μm^2). Similar increased sensitivity of tethering to L-selectin dimerization at increased shear stresses was observed on low density GlyCAM-1 as well as on the sulfated PSGL-1-derived glycopeptide (data not shown). Shear flow proportionally increases the velocity of flowing cells over the adhesive surface and thereby may enhance bond formation via cellular transport (40). To rule out the possibility that increased sensitivity of tether formation to L-selectin dimerization may be transport-dependent, the rate of transient tethering under each shear stress condition was divided by the corresponding shear rate to derive the transport-independent frequency of cellular tethers (Fig. 7*B*). Indeed, even after such normalization of tether frequency, the sensitivity of tether formation to L-selectin dimerization was significantly higher at elevated shear stresses, in particular at lower ligand densities. This is a first demonstration that the effective availability of immobilized L-selectin ligands, recognized by dimerized L-selectin, is increased by elevated shear stress. This increase appears independent of the transport rate of L-selectin expressing cells over the ligand-coated substrate.

DISCUSSION

In the present study, the role of L-selectin dimerization in conferring selectin adhesiveness to different selectin-binding scaffolds was investigated under various flow conditions. Our results provide the first evidence that L-selectin clustering up-regulates rolling adhesions as a result of an increased rate of tether formation to ligand clusters under shear flow. Nota-

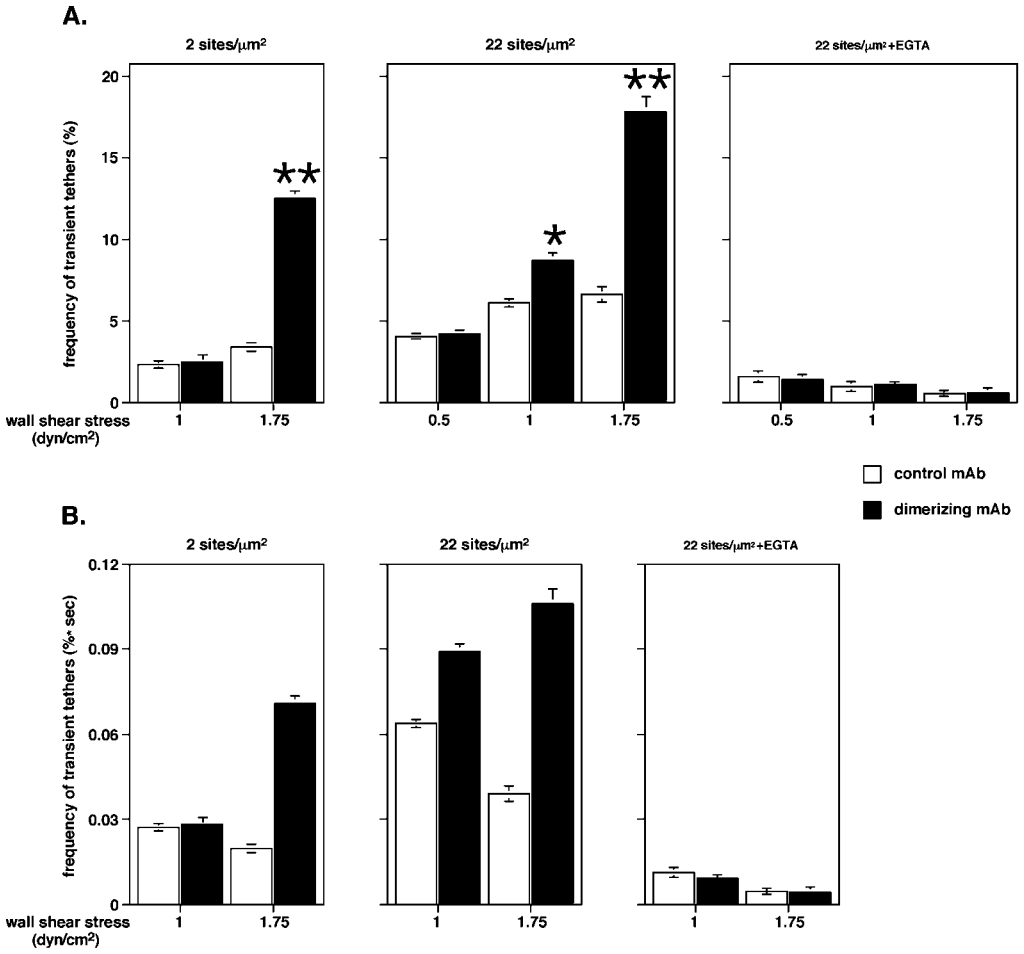

FIG. 7. **Dimerization-enhanced L-selectin tethering is shear force dependent.** *A*, frequency of tethering mediated by WT L-selectin-expressing cells pretreated with either dimerizing mAb or control mAb interacting with the indicated densities of immobilized PSGL-1. Tethering frequencies were measured at different shear stresses in separate experiments. Data are mean ± S.E. of four fields of view. *, $p < 0.01$ and **, $p < 0.00015$, are compared with control-mAb-treated cells. Tethers were effectively blocked in the presence of 5 mM EGTA (*right panel*) or saturating levels of soluble fucoidin (not shown). Results are representative of three independent experiments. *B*, same data as in *A*, but the frequency results in *A* were divided by the shear rate to derive the transport-independent frequency of cellular tethers. Data are representative of four experiments.

bly, dimerization augments L-selectin avidity solely to properly spaced ligand moieties. In other cases, *i.e.* below a critical density of selectin counterreceptor like PSGL-1 or GlyCAM-1 or when ligand moieties are highly clustered L-selectin dimerization does not augment adhesion. L-selectin dimerization can therefore serve as a readout for the presence of properly spaced ligand on the countersurface. Several other important conclusions have been derived. 1) Dimerization of L-selectin alters different dynamic properties of tethers than those altered by P-selectin dimerization even when the same scaffold protein, *i.e.* PSGL-1, serves as the exclusive ligand for the two selectins (10). Whereas L-selectin dimerization enhances tether formation without altering tether lifetime, P-selectin dimerization prolongs tether lifetime and increases the mechanical strength of P-selectin tethers to PSGL-1 but does not enhance tether formation rates (10). In fact, none of the structural or clustering modifications of L-selectin investigated here were found to alter the apparent k_{off} of L-selectin-mediated tethers measured at different densities or shear stresses. Rather, these modifications enhanced only the rate of tether formation. 2) In contrast to dimerization, EGF domain substitution enhances tether formation by L-selectin and does so regardless of the state of ligand density or spacing and independent of the shear stress tested.

One of the most intriguing observations in this study is that dimerization enhanced L-selectin avidity to ligand only under proper shear stress conditions. Thus, even when the ligand density was high enough to be recognized by dimerized L-selectin (Fig. 7), reduced shear stress eliminated the contribution of dimerization to tether formation. Conversely, when ligand density was low, such that dimerization of L-selectin did not contribute to tethering, elevation of shear stress introduced an augmenting effect for L-selectin dimerization (Fig. 7). Because dimerization of L-selectin augmented tethering only to properly clustered ligand, this result suggests that dimers of L-selectin:ligand pairs are more readily formed at elevated physiological shear stresses. As mAb-induced L-selectin dimers may mimic naturally occurring L-selectin dimers on the surface of tethered leukocytes, this result also suggests that such natural L-selectin dimers could form dimers with physiological selectin counterreceptors more readily at elevated shear stresses than at subphysiological stresses.

How can enhanced shear stress increase the productive formation of tethers between dimerized L-selectin and ligand clusters? Two nonmutually exclusive mechanisms have been proposed to explain the shear threshold requirement of L-selectin adhesion. The first suggests that cellular transport along the adhesive substrate, which is increased with applied shear, may increase the forward rate of binding between tethered reactants on interacting countersurfaces (40, 41). However, if only transport was involved, then at low density ligand, the local density of ligand clusters as seen by the dimerized L-selectin would remain con-

stant regardless of the cell transport and the global contact area formed between the leukocyte and the substrate (40). Indeed, even after correcting for contribution of cellular transport relative to the substrate (Fig. 7*B*), the fold increase of L-selectin tethering frequency induced by L-selectin dimerization steadily increased at elevated shear stress (Fig. 7*B*), suggesting that enhanced cellular transport could not account for the increased tethering capacity of dimerized L-selectin. The second mechanism proposed for the shear requirement of L-selectin suggests that shear stress directly activates L-selectin recognition of immobilized ligand. Because PSGL-1 was immobilized on the substrate and the effect of shear stress on dimerization-augmented tethering to PSGL-1 is fully reversible upon shear reduction (data not shown), it is unlikely that elevated shear stresses could have redistributed PSGL-1 to become more favorably recognized by L-selectin dimers. Shear stress could have, however, directly increased intrinsic L-selectin recognition of ligand, as it was reported to enhance the adhesive capacity of both cell-based and cell-free L-selectin toward multiple types of ligands (42, 43). However, shear stress failed to increase L-selectin tethering to L-selectin lectin-specific mAbs or to an artificial highly clustered ligand, such as fucoidin (15, 44). It therefore appears that shear stress facilitates the recognition by L-selectin of native low affinity carbohydrate ligand moieties. One way to achieve this facilitated recognition of ligand could involve a shear-dependent reduction in repulsive barriers between the negatively charged sialylated and sulfated L-selectin ligands and the L-selectin-bearing cell surface. Consistent with such a possibility, CD43, a highly sialylated mucin-like glycoprotein, has been reported to exert anti-adhesive effects on lymphocyte L-selectin adhesiveness under physiological shear flow (45). Shear flow may also generate local torque forces (46) impinging tethered leukocytes onto the substrate and thereby enhancing encounters between L-selectin dimers and properly spaced selectin ligand moieties on the countersurface.

L-selectin dimerization was found to selectively augment tether formation without affecting tether duration. Transient L-selectin tethers, quantal adhesive units formed to distinct L-selectin counterreceptors including PSGL-1, GlyCAM-1, and PNAd, share similar lifetimes even though these scaffolds have entirely different composition and spatial distribution of L-selectin carbohydrate units.[2] Interestingly, increased bond density, although dramatically prolonging tether lifetime of P-selectin (7, 10), results in only modest changes of L-selectin tether lifetime (8, 42). These observations collectively suggest that bond clustering at singular contact sites contributes relatively little to L-selectin tether stabilization as opposed to P-selectin tether stabilization. High temporal resolution analysis of L-selectin tethers to PSGL-1, performed here for the first time, further confirmed that kinetic stability of L-selectin tethers to highly diluted ligands is practically insensitive to selectin dimerization, although it increases tether formation to properly spaced ligand moieties. Thus, increased rebinding within a cluster of L-selectin occupied by properly spaced ligands increases tether formation, but once formed, the tether fails to undergo further stabilization. In contrast, stabilization of quantal L-selectin tethers is highly sensitive to perturbation of cytoskeletal associations of L-selectin, and dimerization does not rescue this perturbation (16). It is possible that rebinding of L-selectin to the same ligand, from which it has dissociated, depends on cytoskeletal anchorage and restricted mobility of the selectin (16). This autonomous rebinding increases tether stabilization, whereas rebinding of L-selectin within a cluster of bonds, facilitated by selectin dimerization, may increase tether formation. The distinct kinetic outcomes of cytoskeletal anchorage of L-selectin *versus* selectin dimerization raise the interesting possibility that distinct selectin rebinding events may involve different time scales that contribute to either tether formation or stabilization.

Oligomerization of L-selectin (11) or of its ligands (47, 48) has been shown to elevate L-selectin avidity by several orders of magnitude in cell-free shear-less systems, suggesting that rebinding of L-selectin at closely spaced ligand matrices can indeed compensate for the extremely low affinity with which it binds to monovalent ligand (11). Although the physiological occurrence and implications of L-selectin dimers on leukocyte microvilli are still obscure (17), L-selectin clustering could be up-regulated during inflammatory processes. Interestingly, exposure of human peripheral blood lymphocytes or murine pre-B transfectants to fever-range hyperthermia markedly increases L-selectin clustering and association with the cytoskeleton with concomitantly enhanced L-selectin-mediated adhesion (49). Our results predict that such clustering would bear physiological outcome only if the L-selectin ligand at the endothelial target site is also properly clustered. The site density and distribution of glycoprotein ligands, *i.e.* their homodimerization states, the spacing between their ligand decorated *O*-glycans, and possibly the dimerization of adjacent carbohydrate ligands on biantennary *O*-glycans (21, 32), could each affect the degree by which L-selectin dimerization on leukocytes would contribute to capture and rolling adhesions under various shear flow conditions. In conclusion, our studies suggest that L-selectin:ligand clusters facilitate selectin tether formation and consequently cell capture and rolling under physiological shear forces. The resolution of how spacing between selectin molecules and their particular counterreceptors contribute to selectin avidity at subsecond contact sites under shear flow should unravel the molecular basis of selectin function in distinct vascular beds and dynamic environments. Elucidation of this standing question will help in the future design of specific selectin or ligand antagonists functional at diverse pathological contexts implicating L-selectin.

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