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Direct Real-Time Observation of E- and P-Selectin-Mediated Rolling on Cutaneous Lymphocyte-Associated Antigen Immobilized on Western Blots

Robert C. Fuhlbrigge,* Sandra L. King,* Charles J. Dimitroff,* Thomas S. Kupper,* and Robert Sackstein2#†

Human memory T cells associated with cutaneous inflammatory responses are characterized by their expression of cutaneous lymphocyte-associated Ag (CLA), a carbohydrate determinant differentially expressed on P-selectin glycoprotein ligand-1 (PSGL-1). Although expression of the CLA epitope on PSGL-1 (CLA+ PSGL-1) by memory T cells is associated with acquisition of E-selectin ligand activity, it is not known whether CLA+ PSGL-1, itself, is a ligand for E-selectin on human T cells or whether other glycoproteins, with or without CLA modification, support E-selectin-dependent rolling in shear flow. To address this issue, we developed a method for real-time analysis of functional adhesive interactions between selectin-bearing cells in shear flow with leukocyte ligands resolved by SDS-PAGE and immobilized on standard Western blots. The results of these studies provide direct evidence that CLA+ PSGL-1 is a functional ligand for both E- and P-selectin, confirm that the P-selectin ligand activity of PSGL-1 is independent of CLA modification, and identify a distinct, non-PSGL-1 E-selectin ligand on CLA-positive human memory T cells. The Journal of Immunology, 2002, 168: 5645–5651.

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3 Abbreviations used in this paper: CLA, cutaneous lymphocyte-associated Ag; PSGL-1, P-selectin glycoprotein ligand-1; CHO, Chinese hamster ovary; CHO-E, CHO cell transfected with human E-selectin; CHO-P, CHO cell transfected with human P-selectin; CHO-mock, mock-transfected CHO cell; H/H, HBSS supplemented with HEPES, H/H/Ca2+; H/H and CaCl2; PVDF, polyvinylidene difluoride.

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ligand activity. Methods for in vitro study of leukocyte-endothelial interactions in shear flow, such as Stamper-Woodruff and parallel plate flow chamber assays, have provided valuable insights into physiologic adhesive interactions (23, 24). However, use of these methods to study the role of individual cellular components is largely limited by the character and availability of purified sub- strate materials that can be affixed to glass or plastic. Although purification of specific cell membrane components can be attained via the use of specific immunofluorescence reagents or with sufficient prior knowledge regarding the structure of the molecule of interest, the applicability of these assay methods to identify or study the function of ligands of unknown structure is inherently limited. As mentioned above, the production of high affinity selectin binding domains on leukocyte glycoproteins also requires one or more posttranslational modifications (e.g., tyrosine sulfation, production of core-2 (O-linked glycans, and sialylation and fucosylation of O-linked and N-linked glycans) that can vary between species, among cell populations, and across stages of cellular development (8, 12, 25). Thus, the use of synthetic or recombinant materials, or material from other cell lineages, may not reflect the true nature of the ligand of interest, further limiting the availability of purified ligand materials for study.

To address these issues, we developed a method for direct real-time observation of adhesive interactions between cells in shear flow and cellular components separated by SDS-PAGE and immobilized on blotting membranes using standard Western blot techniques. This method allows for the rapid and reproducible assess- sment of individual components, both known and unknown, within a complex mixture without the need for prior isolation or enrichment beyond standard SDS-PAGE. In this report, we use this method to survey lysates of CLA-positive and -negative hu- man T cells for molecules able to serve as E- and P-selectin ligands. These studies show that CLA$^+$ PSGL-1 is, unequivocally, a ligand for both E- and P-selectin in physiologic shear flow, in- dicate that the P-selectin ligand activity of T cell PSGL-1 is inde- pendent of modification with CLA, and provide preliminary evi- dence for an additional E-selectin ligand on CLA-positive T cells that is distinct from known ligands.

Materials and Methods

Antibodies

Anti-CLA mAb (HECA-452, rat IgM) was purchased from BD PharMingen (San Diego, CA). Anti-PSGL-1 (CD162) reagents included mAb PL-2 (MulgG1, nonblocking; Beckman Coulter, Miami, FL) (26), used for Western blotting; mAb PL-1 (MulgG1, blocks P-selectin binding; Beckman Coulter) (26), used for functional blocking studies; mAb PSL-275 (MulgG1, blocks P-selectin binding; Genetics Institute, Cambridge, MA), used for FACS staining; and 4H10 (MulgG1, blocks P-selectin binding; Genetics Institute), used for immunoprecipitation studies. Isotype-matched control Abs were purchased from Zymed Laboratories (South San Fran- cisco, CA). FITC-conjugated anti-iat IgM and anti-mouse IgG were pur- chased from Southern Biotechnology Associates (Birmingham, AL). Al- kaline phosphatase conjugated anti-iat IgM and anti-mouse IgG were from Zymed Laboratories. Anti-E-selectin mAb (CD62E, clone 68-5H11) and P-selectin (CD62 P, clone AK-4) were purchased from BD PharMingen.

Cells

PBMC were prepared by density gradient separation (Ficoll-Histopaque 1.077; Sigma-Aldrich, St. Louis, MO) of peripheral blood or of cells col- lected during platelet pheresis of normal donors. CLA-positive and -neg- ative T cell preparations were generated essentially as previously described (11, 16). Normal human PBMC were cultured in XVIVO15 medium (Bio- Whittaker, Walkersville, MD) or RPMI 1640 medium (Mediatech, Her- don, VA) with 10% FBS (Sigma-Aldrich; RPMI-10% FBS). Both media were supplemented with penicillin (100 U/ml) and streptomycin (100 $\mu$g/ml; Life Technologies, Grand Island, NY), HEPES (5 mM [pH 7.4]) and glutamine (2 mM; Mediatech), and human rIL-2 (100 U/ml; PeproTech, Rocky Hill, NJ). Cells for this study were used between 10 and 14 days after stimulation with immobilized anti-CD3 as described (11). Expression of relevant surface Ags was confirmed by indirect immunofluorescence flow cytometry performed on a FACSkan IV (BD Biosciences, Mountain View, CA) using CellQuest software (version 3.1). XVIVO-cultured T cells were typically 70–80% positive for CLA while RPMI-cultured T cells were typically 3–5% positive for CLA (data not shown). Staining for PSGL-1 revealed Ag present at approximately equal levels on each pop- ulation (e.g., 100% of cells positive and similar mean fluorescence inten- sities; data not shown). Cultured cells showed undetectable levels of CD16 positive. Membrane expression indicating lack of 10,000$\times$10$^6$ cell/ml; Life Technologies, Grand Island, NY) and glutamine (50 mM Tris (pH 7.5), 1 mM PMSF, 10% FBS, 1% nonessential amino acids, penicillin-streptomycin, and glutamine. CHO-mock were maintained in Ham’s F-12 medium (Mediatech) supplemented with 1% FBS,

Western blots

Cells of interest were collected by centrifugation, washed in protein free buffer (PBS or HBSS), and the pellet was resuspended at 4 × 10$^6$ cell equivalents/ml in lysis buffer consisting of 150 mM NaCl, 1 mM MgCl$_2$, 50 mM Tris (pH 7.5), 1 mM PMSF, 10 µg/ml aprotinin, and 1% N-octyl- glucoside (Roche, Indianapolis, IN). Lysates were incubated 2 h on ice and centrifuged for 30 min at 10,000 × g. Supernatants were collected and stored at −20°C until use. Protein concentrations were determined using Bradford’s reagent (Bio-Rad, Hercules, CA). Samples representing 25–100 µg of protein were electrophoresed through 4–20% acrylamide gradient SDS-PAGE gels (Criterion; Bio-Rad) under standard reducing conditions (5% 2-ME sample buffer). Prestained molecular mass standards (Amer- sham Biosciences, Piscataway, NJ) were included in adjacent lanes in all gel preparations. SDS-PAGE blots were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in 100 mM Tris, 75 mM glycine, 9% methanol using standard transfer techniques. Blots were subsequently blocked with newborn calf serum (Sigma-Aldrich) for 1 h and probed with anti-CLA (HECA-452) or anti-PSGL-1 (PL-2) Abs, as indicated. Isotype control blots were performed in parallel for each condi- tion and did not identify 240 or 140 kDa species in any samples tested. Bound Ab was visualized with species specific alkaline phosphatase cou- pled anti-Ig and Western Blue alkaline phosphatase substrate (Promega, Madison, WI). Following staining, the membranes were again blocked by incubation in newborn calf serum (Sigma-Aldrich) for at least 1 h at 4°C before use in flow studies.

Blot rolling assay

Before analysis, blots were equilibrated in Ca$^{2+}$-free and Mg$^{2+}$-free HBSS (Life Technologies) supplemented with 10 mM (pH 7.4); H/H) or HEPES and CaCl$_2$ (2 mM; H/H/Ca$^{2+}$). Where indicated, glyceral (Ameri- can BioAnalytical, Natick, MA) was added to a final concentration of 10% (v/v) (H/H/Ca$^{2+}$-10% glyceral). For rolling studies, blots were placed in a Petri dish filled with H/H/Ca$^{2+}$-10% glyceral and a circular parallel plate flow chamber apparatus (Cox Laboratory for Biomedical Engineering, Rice University, Houston, TX) (internal flow chamber dimensions 2 cm × 0.5 cm, height 0.25 cm) was mounted over the area of interest (Fig. 1). This parallel plate flow chamber is similar to that previously described for use with substances coated on glass or plastic (24, 28). No special conditions were required for attachment or use of this chamber on blotting membranes other than the addition of glyceral to the buffers. CHO cells were harvested by rinsing the culture flasks with H/H with 5 mM EDTA, washed, and resuspended at 1–2 × 10$^6$ cells/ml in H/H and maintained on ice. Imme- diately before use, cells were diluted at least 1/10 in binding medium (H/H/Ca$^{2+}$-10% glyceral) at room temperature, resulting in a final concentra- tion of 1–2 × 10$^6$ cells/ml. Cells were drawn into the chamber under defined flow conditions by a precision syringe pump (Harvard Apparatus, Cambridge, MA) and observed in real-time for interaction with the blot surface under stable shear force conditions. Tethering was observed at low physiologic wall shear stresses (0.5–1.5 dyn/cm$^2$) for short periods (one to several minutes). Bound cells were then subjected to timed stepwise in- creases in wall shear stress. Tethering was defined as reduction of forward motion below the hydrodynamic velocity lasting a minimum of two video
frames (0.07 s), and rolling was defined as >5 cell diameters of lateral translation below the hydrodynamic velocity. The majority of tethered cells were observed to roll smoothly across the entire field of view. Nonspecific interactions (i.e., cellular collisions with the substrate that did not lead to tethering and/or rolling) were defined as interactions lasting <0.07 s and were not included in the analysis. Calcium-dependent binding typical of selectin-mediated interactions was confirmed by perfusion of H/H with 5 mM EDTA which resulted in >95% release of bound cells for all samples studied. Nonspecifically bound cells (not rolling and/or not released by perfusion with EDTA) were also discounted from analysis. The frequency of nonspecific events observed was not different in experiments performed in medium with or without 5 mM EDTA, suggesting that these were not selectin-mediated events. There was a time-dependent increase in nonspecific (nonrolling) attachments in the absence of flow, though very few cells formed firm (nonrolling) attachments to the blot in continuous shear flow. All experiments were observed in real time and videotaped for analysis. Tethering rate was calculated as the number of cells that tethered per field per time (usually 30–60 s) at a defined shear stress and adjusted to per minute values. Wall shear stress (T) values were calculated according to the formula $T = \frac{6 \mu Q}{2b}$ (where $\mu$ is the coefficient of viscosity of the solution in the chamber (poise), $Q$ is the volumetric flow rate (cm$^3$/s), $b$ is the channel width (0.5 cm), and $a$ is the half-channel height (0.012 cm)) (29). A value of 0.009 poise was used for the viscosity ($\eta$) of water at 25°C (30) and a value of 0.0123 poise was used for the viscosity ($\eta$) of 10% glycerol at 25°C (31). Flow rates used in this study correspond to shear stresses ranging from 0.5 to 3.5 dynes/cm$^2$. Wall shear stress in 10% glycerol at 25°C is ~1.37 fold greater than at the same temperature.

For blot scanning analysis, 1 ml of CHO cells at 2 x 10$^6$ cells/ml were loaded into the chamber at 7.62 dyne/cm$^2$. Flow was reduced to 0.53 dyne/cm$^2$ for 1 min to promote tethering, then increased to 1.75 dyne/cm$^2$ and maintained at this level throughout the observation period. Sequential overlapping visual fields covering the entire available viewing area (~250–40 kDa) were observed for brief periods (2–10 s) and videotaped for subsequent analysis. Initiation of viewing at low vs high molecular mass ends of the channel did not reveal significantly different results. Observation of other molecular mass ranges was achieved by moving the chamber and repeating the study. Multiple observations of unmanipulated cell lysates did not reveal significant attachments below 40 kDa, including the gel dye front, or above 240 kDa apparent molecular mass (data not shown).

Ab inhibition studies

For anti-PSGL-1 inhibition studies, identical blots of 50 µg CLA$^-$ T cell lysates stained with anti-PSGL-1 (nonfunction-blocking mAb PL-2) were prepared and assembled into the flow apparatus. Each blot was assessed for normal binding function with both CHO-E and CHO-P cells using the method stated above. The chamber was then filled with H/H/Ca$^{2+}$-10% glycerol medium containing mAb (anti-PSGL-1 function-blocking mAb (PL-1) or mouse IgG1 control mAb) at 50 µg/ml in H/H/Ca$^{2+}$-10% glycerol and perfused with this material at 0.01 ml/min at 0°C. The chamber was flushed with H/H/Ca$^{2+}$-10% glycerol and cell binding with both CHO-E and CHO-P was repeated. All observations for blocking studies were made at the midpoint of the 140 kDa band identified by anti-PSGL-1 mAb.

Immunoprecipitations

For immunoprecipitation studies, aliquots of lysate from CLA-bearing T cells were incubated with 5 µg anti-PSGL-1 mAb 4H10, or isotype control mAb, for at least 1 h at 4°C. Reaction mixtures were transferred to fresh tubes containing 20 µl washed recombinant protein G-agarose beads that had been blocked with BSA (binding capacity 18 mg IgG/ml; Invitrogen, Carlsbad, CA) and incubated with agitation for at least 1 h at 4°C. Agarose bead immune complexes were collected by centrifugation and washed repeatedly with 1% N-octylglucoside lysis buffer. For some studies, the primary supernatant was subjected to serial immunoprecipitation by adding additional Ab and repeating the process as above. Each sample was brought to 1x SDS-sample buffer and incubated for 5 min in a boiling water bath before SDS-PAGE and Western blotting under standard conditions as outlined above.

Results

To characterize and identify selectin ligands on human T cells, we established a unique functional assay that allows real-time observation of selectin-mediated interactions with cellular glycoproteins immobilized on Western blots (Fig. 1). Lysates of CLA-positive and CLA-negative T cells were subjected to SDS-PAGE under standard reducing conditions and then blotted onto a PVDF membrane and immunostained using standard methods. Under reducing conditions, CLA/PSGL-1 stained with anti-CL (HECA-452) mAb typically appears as a single band with a relative molecular mass of 140 kDa (PSGL-1 monomer) (Fig. 2A). Differences in relative molecular mass attributable to differential glycosylation resulting in decoration with the CLA epitope appear to be beneath the resolution limit of this separation technique. Immunostained blots were placed in buffer containing 10% glycerol for use in flow analysis. Equilibration in dilute glycerol alters the opacity of the membrane sufficiently to allow transmission of light and the direct visualization of cells interacting with the surface of the blot by standard light microscopy. A parallel plate laminar flow chamber, used routinely for observing real-time interactions under controlled shear conditions of cells with substrates immobilized on glass or plastic, was mounted directly on the blot via a low-pressure vacuum seal and the entire apparatus was placed on the stage of an inverted microscope (see Fig. 1). Cells were introduced into the chamber through an inlet port under controlled aspiration conditions and monitored for interaction with the blot surface by direct observation. All experiments were videotaped for offline analysis. For this study, CHO-mock, CHO-E, or CHO-P cells were introduced into the chamber under a variety of wall shear stress conditions and observed for interaction with the blot surface. Under continuous flow conditions equivalent to physiologic shear in postcapillary venules, both CHO-E and CHO-P cells were observed to attach primarily to a single discrete band, corresponding to the 140 kDa band of monomer CLA/PSGL-1, on blots of CLA-positive T cell lysates (Fig. 2B). Cells could be observed, in real-time, tethering to the upstream edge of the stained area, rolling across the band, and releasing from the downstream edge (a digital movie showing this activity is included as supplemental material). Although this assay is nonphysiologic by design, using vascular selectins on nonadherent cells and immobilizing the leukocyte glycoproteins, the rolling behavior of bound cells was qualitatively similar to that observed in traditional binding assays using leukocyte binding to glass or plastic immobilized selectins. Maintaining

4 The on-line version of this article contains supplemental material.
cells in continuous shear flow substantially reduces or eliminates the background binding of CHO cells to the blotting membrane seen in static assays (data not shown). Scanning of the entire blot area visible in the laminar flow chamber showed smaller, but reproducible, numbers of CHO-E cells tethering and rolling on an area of the blot at ~95 kDa and a few cells between 140 and 90 kDa (Fig. 2B). This lower molecular mass band did not stain with anti-CLA or anti-PSGL-1 mAb (Figs. 2B and 4A). Observations made after relocating the chamber over higher and lower molecular masses did not reveal other bands supporting selectin-mediated attachment and rolling. Blots of CHO-negative T cell lysates, in contrast, supported binding of CHO-P cells at levels equivalent to those seen on the CLA-positive blot, but did not support significant binding of CHO-E cells (Fig. 2C). CHO-mock cells did not form rolling adhesions on either blot (data not shown).

Tethering rates and shear resistance profiles of cells binding the 140 kDa area of CLA-positive and -negative T cell lysate blots were nearly identical for CHO-P cells (Figs. 3, A and C), but markedly different for CHO-E cells (Figs. 3, A and B), indicating that the P-selectin ligand activity of the two preparations was similar while effective E-selectin ligand was present only in the CLA-positive T cell lysate. Blots prepared from gels loaded with decreasing amounts of CLA-positive cell lysate showed proportionally reduced binding of both CHO-E and CHO-P cells and did not identify a minimum threshold for supporting selectin-mediated attachment on blots (Fig. 3D). Bound CHO-E (Fig. 3E) and CHO-P (Fig. 3F) cells showed similar shear resistance profiles, with substantial rolling activity noted on blots loaded with as little as 25 μg of total cell protein from CLA-positive T cells. Duplicate blots of CLA-positive T cell lysate stained for CHO (mAb HECA-452) or PSGL-1 (nonblocking mAb PL-2) showed staining of a single band each at 140 kDa (Fig. 4A) and supported equivalent levels of CHO-E and CHO-P cell tethering and rolling activity on the respective labeled bands (Fig. 4B). Control CHO cells transfected with vector only (CHO-mock; Figs. 3, A and D, and 4B) and CHO-P cells in medium with 5 mM EDTA (data not shown), did not form rolling interactions on any areas of the blots examined.

To determine whether CHO-E cells bind to the same site on CLA-positive PSGL-1 as CHO-P cells, we assessed binding after blocking the PSGL-1 P-selectin binding function with mAb PL-1 (Fig. 4C). Observations were performed on identical blots before and after blocking with control IgG1 or mAb PL-1 (P-selectin blocking anti-PSGL-1 mAb). Treatment of CLA-positive T cell blots with excess anti-PSGL-1 Ab (mAb PL-1) resulted in complete inhibition of CHO-P cell binding and had no effect on CHO-E cell binding relative to control IgG-treated blots. Control IgG1 had no effect on CHO-E or CHO-P cell binding relative to untreated blots (data not shown). A similar experiment using HECA-452 showed only partial inhibition of CHO-E binding relative to control IgM Ab, with no effect on CHO-P binding (data not shown). The relative inability of HECA-452 to block E-selectin-mediated adhesion to CLA-positive cells has been reported by several other investigators. Knibbs et al. (20) showed that HECA-452 had no effect on the ability of T lymphoblasts to roll on E-selectin in a conventional flow chamber assay, and Wagvers et al. (21, 22) have described cell lines that lack detectable staining with HECA-452 yet bind avidly to E-selectin. Preincubation of CHO-E and CHO-P cells with function-blocking anti-E- or P-selectin mAb, respectively, resulted in complete abrogation of tethering and rolling on both CLA-positive and -negative substrates, confirming that the activity observed was mediated by selectins (data not shown).

Although both P- and E-selectin binding activity were coincident on these Western blots, it is possible that two distinct proteins that comigrate in SDS-PAGE under these conditions could serve as separate ligands for E- and P-selectin. To address this possibility, PSGL-1 was immunoprecipitated from lysates of CLA-positive T cells, subjected to SDS-PAGE, and transferred to PVDF membranes. Staining with Abs to either CLA (HECA-452) or PSGL-1 (PL-2), revealed a single band at ~140 kDa molecular mass (Fig. 5A; data not shown). Assessment of selectin ligand function confirmed that CLA-positive PSGL-1 immunopurified from human T cells has both functional E- and P-selectin ligand activity (Fig. 5B).

Discussion

This study provides direct evidence that human T cell CLA+ PSGL-1 is a functional ligand for both E- and P-selectin capable of
lysate protein. Both CHO-E and -P were observed to tether and roll on the 140 kDa HECA-452 reactive band from CLA-positive T cell lysate. Both CHO-E and -P were observed to tether and roll on the 140 kDa HECA-452 reactive band from CLA-positive T cell lysate (■). CHO-P, but not CHO-E, cells bound in a similar fashion to the corresponding area on a blot from CLA-negative cells (□). CHO-mock cells did not form rolling attachments on either blot. B and C. Cells remaining bound to CLA-positive or -negative substrate blots during timed stepwise increases in wall shear stress. CHO-E cells bound to CLA-positive, but not to CLA-negative, substrates and were able to remain attached and roll throughout the shear range tested (B). CHO-P cells, in contrast, showed nearly identical resistance to detachment by increasing shear on CLA-positive as on CLA-negative substrate (C). Western blots of SDS-PAGE loaded with decreasing amounts of CLA-positive T cell total cell lysate protein were also assessed for E- and P-selectin ligand activity. The number of cells observed rolling on the 140 kDa band per visual field (10X objective) centered on the 140 kDa area of blots containing 50 µg CLA-positive or -negative T cell lysate protein. Both CHO-E and -P were observed to tether and roll on the 140 kDa HECA-452 reactive band from CLA-positive T cell lysate (■). CHO-P, but not CHO-E, cells bound in a similar fashion to the corresponding area on a blot from CLA-negative cells (□). CHO-mock cells did not form rolling attachments on either blot. B and C. Cells remaining bound to CLA-positive or -negative substrate blots during timed stepwise increases in wall shear stress. CHO-E cells bound to CLA-positive, but not to CLA-negative, substrates and were able to remain attached and roll throughout the shear range tested (B). CHO-P cells, in contrast, showed nearly identical resistance to detachment by increasing shear on CLA-positive as on CLA-negative substrate (C). Western blots of SDS-PAGE loaded with decreasing amounts of CLA-positive T cell total cell lysate protein were also assessed for E- and P-selectin ligand activity. D. The number of cells observed rolling on the 140 kDa band per visual field (10X objective) at 1.06 dynes/cm² wall shear stress. The number of CHO-E (■) and CHO-P (□) cells observed to attach and roll on the 140 kDa band from CLA-positive T cells diminished in proportion to the amount of cell lysate protein loaded. There were no rolling attachments noted for CHO-mock cells under any conditions. E and F. The number of cells observed rolling per visual field (10X objective) during timed stepwise increases in wall shear stress. Bound CHO-E (E) and CHO-P (F) cells showed similar resistance to detachment profiles over the shear range tested. Values shown are the mean and the range of two independent determinations on the same substrate. Results are representative of observations on multiple blots of CLA-positive and -negative T cell lysates.

Supporting rolling adhesions under shear flow conditions. These observations support and expand prior studies linking CLA and E-selectin ligand activity by showing, unequivocally, E-selectin-mediated binding and rolling on T cell PSGL-1 and coexpression of functional E- and P-selectin ligand activity on this single T cell surface glycoprotein. As noted above, the absence of HECA-452 mAb did not appear to block the binding of CHO-E-bearing cells to CLA⁺ PSGL-1 (see Fig. 4). This is in agreement with a report by Knibbs et al. (20) showing that incubation with both mAb HECA-452 and mAb CSLEX1 (anti-sLex) did not significantly inhibit the binding of CLA-positive T lymphoblasts to E-selectin in a conventional flow chamber assay. Although the current study does not identify the epitope recognized by HECA-452 mAb as the E-selectin binding site, it establishes a direct correlation between expression of CLA on PSGL-1 and the acquisition of functional E-selectin ligand activity by PSGL-1 that was not possible with prior methods. Moreover, these data suggest that the E-selectin binding domain on CLA⁺ PSGL-1 is distinct from both the P-selectin binding domain and the HECA-452 epitope.

The findings in this report represent an attempt at global assessment of individual T cell glycoproteins that function as E- and P-selectin ligands in physiologic shear flow. Although this survey is limited to those ligands able to function after reducing SDS-PAGE, both the E- and P-selectin binding functions of CLA⁺ PSGL-1 were preserved and a potential novel E-selectin ligand was identified. We observed E-selectin ligand activity on a structure migrating at ~95 kDa apparent molecular mass in lysates of human CLA-positive T cells separated by SDS-PAGE under reducing conditions. This area did not stain with HECA-452, anti-PSGL-1 (PL-1 or PL-2), or anti-CD62L (L-selectin) mAb and supported E⁺, but not P⁺, selectin-mediated binding. Although this may be a processing or degradation product of PSGL-1, the lack of reactivity with two mAbs directed to separate domains, membrane-proximal in the case of PL-2 and membrane-distal in the case of PL-1, suggests that this is a distinct structure. The presence of an E-selectin ligand activity on human T cells separate from PSGL-1 and CLA has not been evident in prior studies using conventional leukocyte rolling assays. The structure of this ligand and its role in T cell homing is currently under investigation.

This report also describes and demonstrates the utility of a relatively simple method for the rapid and reproducible characterization of both known and unknown cell adhesion molecules within a complex mixture without the need for purification or enrichment before immobilization. This method allows for direct real-time observation of interaction parameters (e.g. rolling vs firm attachment, specificity, and reversibility with inhibitors) in both physiologic...
FIGURE 4. T cell CLA and PSGL-1 show identical E- and P-selectin binding activity, and attachment of CHO-E cells to CLA/PSGL-1 is not affected by mAb blocking PSGL-1 P-selectin binding function. A, Western blots of 100 µg CLA-positive T cell lysates stained with anti-CLA mAb (HECA-452; left panel) or a nonfunction-blocking anti-PSGL-1 mAb (PL-2; right panel) show a similar single reactive band at ~140 kDa. B, The number of CHO-E and -P cells observed to tether and roll across the stained area of the blot labeled with anti-CLA (■) at 0.53 dyn/cm² wall shear stress was similar to the number observed on the blot labeled with anti-PSGL-1 (□). CHO-mock cells were not observed to form rolling attachments on any areas of the blot observed. The field of view for each assessment was located at the darkest area of the band on each blot. Results represent the mean and range of three independent experiments. C, CHO-E, -P, and -mock cells were observed for formation of rolling attachments on the 140 kDa band on blots of CLA-positive T cell lysate stained with mAb PL-2 (nonfunction-blocking) and treated with control IgG1 (○) or anti-PSGL-1 mAb PL-1 (function-blocking; □). Pretreatment with mAb PL-1 blocked ≥95% of CHO-P binding to the 140 kDa PSGL-1 band but had no effect on CHO-E binding. CHO-mock cells did not form rolling attachments on either control or PL-1 blocked blots. Results represent the mean and the SD of at least three separate determinations.

FIGURE 5. Both CHO-E and -P cells bind and roll on immunopurified CLA-positive PSGL-1. A, Western blots of 50 µg unmanipulated CLA-positive T cell lysates (left panel) or PSGL-1 immunoprecipitate (right panel) immunostained with anti-CLA (mAb HECA 452), B, CHO-E (left panel) and -P (right panel) were observed for formation of rolling attachments at 0.53 dyn/cm² wall shear stress to the 140 kDa band of blots prepared from unmanipulated lysate (original), the product of the first round of immunoprecipitation (Pellet 1), and the product of a second round of immunoprecipitation (Pellet 2). CHO-mock cells did not form rolling attachments on either of the blots (not shown). Results shown are the mean and the SD of six separate determinations for each point from two independent immunoprecipitation experiments.

As protein components may lose function as a result of denaturation in SDS-PAGE, the method, as described in this report, is more suited to the study of carbohydrate components mediating binding to selectins or other cell surface lectins. In this study, however, CHO-E and -P cells showed comparable levels of binding to denatured and blot immobilized CLA/PSGL-1. E-selectin has been shown previously to bind carbohydrate alone, but P-selectin has been shown to require both a carbohydrate component and a protein component incorporating one or more sulfotyrosine residues near the N terminus. This suggests that either P-selectin binding does not require native conformation of the PSGL-1 protein backbone or that sufficient renaturation occurs after transfer to the blotting membrane to support selectin binding. Modification of this method to maintain function of more sensitive protein components (e.g., non-denaturing PAGE, direct adsorption to membrane) may be desirable to study other adhesion events (e.g., integrin binding to cell adhesion molecules or extracellular matrix proteins).

In summary, we report the direct visualization of both E- and P-selectin-mediated binding and support of rolling in shear flow to the single human T cell surface glycoprotein, CLA/PSGL-1, using a unique assay of cell binding to ligands immobilized on Western blots. These data definitively identify PSGL-1 as an E-selectin ligand on CLA-positive human T cells and provide preliminary evidence for a second HECA-452-nonreactive E-selectin ligand. The blot rolling method described will be particularly useful in the

and nonphysiologic shear conditions, thus permitting a unique user interface for the observation of adhesive events on membrane-immobilized materials. Ligands under investigation can be immobilized directly or segregated by gel electrophoresis (e.g., SDS-PAGE, isoelectric focusing) or other methods before transfer to the membrane, providing structural information that is not evident from previously available methods. This technique also allows for real-time manipulation of interaction conditions including wall shear stress, ion requirements, temperature, metabolic inhibitors, and the presence of activating agents or inhibitors of cell function. As shown in this study, blot-immobilized substrates can be used repeatedly, allowing direct comparison of different cell populations in shear flow or in situ manipulation of the substrate under continuous direct visualization. The capacity to observe sequential experimental and control conditions on a single substrate, and to observe physiologic behaviors and responses to manipulations in real-time, provides distinct advantages over conventional static and flow-based binding assays. As additional proof of principle, we have used this method to characterize, purify, and confirm the activity of CD44 as an E- and L-selectin ligand expressed on hematopoietic progenitor cells (32, 33).
pursuit of novel adhesion molecules, in that it allows direct real-time observation of interaction parameters (e.g., rolling vs firm attachments, specificity, and reversibility with inhibitors) on materials separated by standard electrophoretic methods (e.g., SDS-PAGE), but without requirements for further purification.

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References