Supporting Information

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SI Text

Mutant Design. The design module of Rosetta (1) was used to identify the best rotamer for mutations to each amino acid (except cysteine) at every position in the lectin and EGF domains of P-selectin. This was done for the unbound (PDB ID 1G1Q chain A) and ligand-bound (PDB ID 1G1S chain A) crystal structures (2). Before calculation, missing terminal side chain atoms, or terminal residues missing in chain A, but not other chains in the same coordinate file, were built by using the Swiss PDB Viewer defaults (3). For chain A of 1G1Q, side chain atoms of K17 and residues D159 and D160 were reconstructed. For chain A of 1G1S, side chain atoms of K32, N57, K58, N71, and D158 were reconstructed. These side chains are not near regions of interest in this study. The software defaults chose rotamers that were free of steric clashes. Residue-by-residue energy scores output from Rosetta for the two conformations of P-selectin were compared to identify mutations that would be expected to destabilize the bent, unbound conformation, while not destabilizing the extended conformation.

Plasmids, Cell Lines, and Protein Purification. The plasmid used for expression of soluble P-selectin described previously (4) was modified to include the second short consensus repeat and contains mature residues W1 to A281. This modification increased expression roughly 5-fold. We also included a high-affinity streptavidin-binding peptide tag (5). The resulting P-selectin construct in the pIRES2-EGFP vector encoded secretion into cell culture medium of the lectin and EGF domains and the first 2 short consensus repeats of P-selectin fused to a tobacco etch virus protease cleavage site, a 6xHis tag, and a streptavidin-binding peptide. A28H P-selectin is the same protein with the single amino acid change at position 28 from alanine to histidine. Calcium phosphate transient transfections using 50 μg of vector per 15-cm dish of HEK 293S GnTI$^-$ cells (6) were done in DMEM with 25 mM Hepes (Sigma Aldrich), supplemented with 10% FCS, pen/strep, L-glutamine, and nonessential amino acids. After transfection, media were changed to Opti-MEM serum-free media (Gibco BRL) supplemented with 2 mM L-glutamine. Supernatants were collected 7 days after transfection.

For protein purification, culture supernatant was adjusted to 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$, and 0.01% Tween 20 using concentrated stock solutions and filtered. Protein was loaded onto Ni-NTA resin (Qiagen), washed with 20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.01% Tween 20, pH 8.0, and eluted with 300 mM imidazole. Eluate was loaded onto Strep-tactin resin (IBA) equilibrated in 100 mM Tris, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 0.01% Tween 20, washed in the same buffer, and eluted with 2.5 mM *d*-desthiobiotin. The buffer was exchanged to 10 mM Hepes, pH 6.8, 1 mM $CaCl_2$. Protein was loaded onto SP-Sepharose and eluted with a gradient of 0–70% 1 M NaCl in the same buffer over 25 column volumes. The major peak was pooled and buffer exchanged to 10 mM Hepes, 150 mM NaCl, 1 mM $CaCl_2$, 0.01% Tween 20, pH 7.4. Concentration was determined by using the calculated extinction coefficient (7) of 81,280 M^{-1} for P-selectin (wild-type and A28H).

Yeast Display of P-Selectin. The yeast display system described by Boder and Wittrup (8) was used to display P-selectin on the yeast cell surface. Plasmid containing the lectin, EGF, and first 2 SCR domains of P-selectin fused to Aga2 was kindly provided by Prof. Dane Wittrup (Massachusetts Institute of Technology, Cambridge, MA).

Chemically competent yeast [strain EBY100 (8)] were prepared by using the S.c. EasyComp Transformation kit from Invitrogen as described by the manufacturer. Transformation was accomplished according to the manufacturer's protocol. Typically, 1 ml of media (20% glucose, 0.67% Yeast Nitrogen Base, 0.5% Casamino acids, 100 mM sodium phosphate, pH 6.0) was inoculated with a freshly transformed colony and grown at 30 °C with shaking for ≈24 h. Galactose was used in place of glucose in the above media to induce protein expression. Growth in galactose at room temperature for ≈24 h was performed immediately before use in flow chamber measurements and flow cytometry.

Flow Cytometry. Yeast were washed in PBS (Invitrogen; 1 mM KH_2PO_4, 3 mM Na_2HPO_4, 155 mM NaCl, pH 7.4) + 0.5% BSA, then incubated for 30 min at 30 °C with mouse anti-P-selectin antibodies G3 (anti-lectin domain) or AC1.2 (anti-SCR domains) at a dilution of 1:100 (9, 10). After washing, yeast was incubated for 10 min at 4 °C with phycoerythrin-conjugated goat anti-mouse IgG. Cells were washed a final time before analysis on a FACScan (Becton Dickinson).

Flow Chamber. PSGL-1-Fc (40-μl drop at 20 μg/ml in PBS) provided by Thios Pharmaceuticals was adsorbed onto a plastic Petri dish overnight in a humidified chamber at room temperature. PBS + 1% BSA was used to block the surface for 2 h at room temperature before data collection. Yeast displaying wild-type P-selectin or mutant P-selectin were diluted (100 μl of culture 24 h postinduction) into 1 ml of PBS + 1 mM $CaCl_2$ and subjected to several subsecond pulses from a needle-tip sonicator to disrupt clusters of yeast cells, resulting in a suspension of single yeast cells. These were further diluted to 10 ml with PBS + 1 mM $CaCl_2$ and used for flow assays. Cultures were at comparable cell densities postinduction.

An inverted microscope (Nikon Diaphot) with a Pixelink digital camera (model A662C, 1.9 megapixel CMOS) attached to the side C-mount was used with digital capture software from Norpix (Streampix version 3.26). Yeast cells were infused into the flow chamber by using a programmable syringe pump (Harvard Instruments) at 2 dyn/cm^2, flow was stopped for 30 s before each run, cells were brought into focus, then the shear was increased every 10 s to values of 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, and 18.0 dyn/cm^2. Images were captured at a rate of 15 frames per second with a 10× objective. Seconds 3 through 9 of each shear were used for data analysis. ImagePro 6.0 was used to process video and track cells. Custom macros were used to open segments of video at a given shear, run filters, and track cells using empirical parameters to identify single yeast cells. ImagePro parameters of roundness (between 1.0 and 1.1), area (between 9 and 85 pixels), and minimum diameter (between 1.9 and 15 pixels) were used to identify individual, nonbudding yeast cells. Track data for all cells were exported to Excel (Microsoft).

Custom Excel macros were used to determine average cell velocity from position over time data and summarize the data for all cells tracked at each shear. Cells traveling above the rate of fluid flow at the wall (determined empirically with uninduced yeast cultures and cultures containing no display plasmid) were excluded from analysis. This is only significant at the lower shear stresses (<0.5 dyn/cm^2). At higher wall shear stresses, fewer cells

were determined to be moving faster than the speed of fluid at the wall or were moving too fast to image. During the 6-s window of observation, data were included for partial tracks that persisted at least 8 frames (≈0.5 s). Thus, partial tracks were obtained for (*i*) cells that were adherent at the 3-s mark (either attached and displaying zero velocity, or rolling) and then detached and resumed flow at the fluid velocity during the 3.5- to 9-s period, and (*ii*) cells that tethered after the start of the observation period.

For all data, 3 runs through shear stress increases were performed for 3 separate yeast cultures, resulting in 9 trials for both wild-type and A28H P-selectin. Calculation of average velocity was derived from cumulative distance over time data. The slope of the best-fit line to the data were used. Cell tracks that did not fit a line with a correlation coefficient (r^2) >0.95 were excluded from the calculation. Cells exhibiting $r^2 > 0.95$ are referred to as stably rolling cells. Recalculating the average velocities including all cell tracks did not significantly alter the averages or trends.

Surface Plasmon Resonance. A BIACore 3000 was used to conduct SPR measurements as described previously (4). In brief, PSGL-1-Fc was coupled to a CM5 chip from BIACore using NHS-EDC chemistry as described in the amine coupling kit sold by BIACore. A reference chamber was reacted and blocked with ethanolamine. Concentrations of wild-type or A28H P-selectin ranging from 5 nM to 0.5 μM were injected over the chip surface. BIAEvaluation Software was used for data analysis and fitting. All data reported are the average of global fits to 3 sets of data.

Model of Ligand Binding Coupled to a Conformational Change. Microsoft Excel was used to simulate binding curves that would result from a model in which 2 distinct protein conformations are in equilibrium in solution. Both conformations are capable of binding to ligand with differing affinities. Changing the single parameter that describes the equilibrium between these two conformations can drastically alter the observed equilibrium binding profiles. These profiles will fit to a simple 1:1 binding isotherm with the observed binding constant resulting from the fit that reflects the underlying equilibria depicted in Fig. 5. If it was possible to detect the amounts of each conformation present, the observed binding affinity could be deconvoluted into the intrinsic affinities of ligand binding to each conformation as well as the equilibrium describing the ratio between the amounts of each conformation as follows:

$$K_{\text{obs}} = \frac{(K_B + K_E K_c)}{(1 + K_c)} \quad [1]$$

where K_B = {BL}/{B}{L}, K_E = {EL}/{E}{L}, K_c = {E}/{B}, B represents the bent selectin conformation, E is the extended selectin conformation, and L is the ligand.

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