Isolation of αL I domain mutants mediating firm cell adhesion using a novel flow-based sorting method

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The inserted (I) domain of α4β2 integrin (LFA-1) contains the entire binding site of the molecule. It mediates both rolling and firm adhesion of leukocytes at sites of inflammation depending on the activation state of the integrin. The affinity change of the entire integrin can be mimicked by the I domain alone through mutations that affect the conformation of the molecule. High-affinity mutants of the I domain have been discovered previously using both rational design and directed evolution. We have found that binding affinity fails to dictate the behavior of I domain adhesion under shear flow. In order to better understand I domain adhesion, we have developed a novel panning method to separate yeast expressing a library of I domain variants on the surface by adhesion under flow. Using conditions analogous to those experienced by cells interacting with the post-capillary vascular endothelium, we have identified mutations supporting firm adhesion that are not found using typical directed evolution techniques that select for tight binding to soluble ligands. Mutants isolated using this method do not cluster with those found by sorting with soluble ligand. Furthermore, these mutants mediate shear-driven cell rolling dynamics decorrelated from binding affinity, as previously observed for I domains bearing engineered disulfide bridges to stabilize activated conformational states. Characterization of these mutants supports a greater understanding of the structure–function relationship of the αL I domain, and of the relationship between applied force and bioadhesion in a broader context.

Keywords: αL integrin/conformational regulation/I domain/rolling adhesion/yeast display

Introduction

Recruitment of leukocytes to sites of localized inflammation involves alterations in adhesion to the endothelium. Leukocytes sample the state of the vascular endothelial wall by weakly adhering to endothelial cells primarily via selectin–carbohydrate ligand binding, which leads to dynamic adhesion under fluid shear forces, characterized by continuous formation and breakage of selectin–ligand bonds and cell rolling along the endothelium at velocities well below that of the free-flowing blood.

Lymphocyte function-associated antigen-1 (LFA-1, α4β2 integrin) mediates firm adhesion of leukocytes on inflamed endothelium through its interaction with intercellular adhesion molecule-1 (ICAM-1) in the activated state of the integrin, as well as rolling adhesion in the unactivated state (Lawrence and Springer, 1991; Springer, 1994; Salas et al., 2004; Jin et al., 2006; Salas et al., 2006). This molecule undergoes both inside-out and outside-in signaling, where force-driven rearrangements mediate conformational change of the molecule (Diamond and Springer, 1994; Salas et al., 2006). In response to this signaling, the LFA-1 molecule shows a 10 000-fold increase in affinity of for ICAM-1 upon activation (Labadia et al., 1998).

The inserted (I) domain of the α chain contains the entire ligand-binding portion of LFA-1. Rearrangements in the molecule during activation cause an intramolecular binding site to be revealed in the I-like domain region of the β chain that binds to the α7 helix of the I domain. The pulley-like motion of this helix causes rearrangements in the metal ion-dependant adhesion side (MIDAS), leading to the dramatic increase in affinity for ICAM-1. While the α7 I domain has not been crystallized in the ‘open,’ high affinity conformation, I domain mutants have been engineered to mimic this state (Shimaoka et al., 2003; Kang et al., 2012). One method used two cysteine mutations to lock the I domain in the open, high-affinity conformation by regulating the position of the α7 helix. Additionally, a number of other double cysteine mutants were created to lock the I domain in a range of intermediate affinity conformations (Shimaoka et al., 2003). More recent work takes advantage of a mutation isolated from a library of I domain mutants sorted for high affinity using soluble ligand binding to impart the high-affinity state (Jin et al., 2006; Kang et al., 2012).

We have previously demonstrated that soluble binding affinity for ICAM-1 is not the only factor that influences I domain-mediated rolling behavior on ICAM-1 surfaces. Using mutants engineered to display a range of affinities for ICAM-1 (Shimaoka et al., 2003), we showed that rolling behavior is decorrelated from soluble binding affinity in the intermediate affinity range (Pepper et al., 2006). This result indicates a complicated structure-function relationship exists for LFA-1 binding under force.

While the mutants developed by Shimaoka, et al. yield information toward an understanding LFA-1 binding, comparing clones locked into a conformation by disulfide bonds between cysteine residues is not ideal. These cysteine substitutions vary in bond distances between cysteine residues, and therefore could generate a mixture of high-affinity (HA) and low-affinity I domain conformations, complicating interpretation of dynamic rolling data. Those mutants isolated using affinity for
soluble ligand provide important insight into the allosteric regulation of binding affinity but do not take into account the impact of force on binding. Here, we describe a method to identify clones with a range of binding affinities under shear flow from a library of I domain mutants. Previous work in our lab has shown that cell-like microspheres can be separated by selectin-mediated rolling adhesion (Greenberg and Hammer, 2001). These microspheres have properties similar to adult bone marrow and fetal liver cells, and populations were designed to behave as such on a selectin-coated surface under shear flow. Separation by differential average rolling velocity on a substrate under shear flow was successful in this system, and the method was adapted for the library screening detailed here after testing with mock libraries of yeast displaying HA I domain mutant and wild-type I domain. Others have applied static cell adhesion-based selections to yeast-displayed libraries (Wang and Shusta, 2005; Richman et al., 2006; Wang et al., 2007); in one example, this approach was used to isolate firmly adherent αM I domain mutants from a yeast display library by incubating the library with HeLa cells that express ligands for the Mac-1 integrin and collecting those yeast that remain adherent after washing (Hu et al., 2010). Here we describe the isolation of firmly adherent mutants from a library of αI domain variants and the analysis of their properties. I domain mutants recovered by adhesive selection include both mutations previously observed in screens for tight binding to soluble ligand (Jin et al., 2006) and novel mutations not previously observed, highlighting the complexity of receptor-ligand binding properties governing dynamic adhesion. These mutants will provide information to better understand the relationship between binding affinity and behavior under shear flow.

Materials and Methods

Fluorescent yeast

The gene encoding enhanced green fluorescent protein (eGFP) was amplified out of pEGFP-Actin (Clontech) using polymerase chain reaction (PCR) and cloned into an integrating yeast vector based on pITy (Parekh et al., 1996). The plasmid was amplified and digested with Mfe I (New England Biolabs, Ipswich, MA, USA) to linearize the plasmid for recombination into the yeast genome. The yeast strain EBY100 was transformed with pITy-GFP plasmid using the lithium acetate method (Gietz and Woods, 2002), and resulting yeast were plated on selective G418 (Invitrogen) plates. Individual clones formed with pITy-GFP plasmid using the lithium acetate protocol for low mutation rate. PCR to amplify DNA, transformation of yeast using the gene and linearized vector, and analysis of yeast was performed out of pCT-I domain (Pepper et al., 1996; Richman et al., 1996). The I domain gene of pCT-I domain library construction

I domain cloning and expression

As previously described (Pepper et al., 2006), wild-type I domain (residues G128-Y307) were cloned into the modified yeast surface display vector pCT302 (Boder and Wittrup, 2006). The high-affinity double-cysteine mutant K287C/K294C (HA) identified from previous work (Shimaoka et al., 2003) was made using the Quick Change Site Directed Mutagenesis Kit (Stratagene).

Laminar flow assay

The laminar flow assay is conducted using a parallel plate flow chamber. A silanlated glass slide (Sigma) was coated with ICAM-1-Fc at a concentration of 10 μg/ml in carbonate buffer, pH 9.0 at room temperature for 2 h. The slide was washed and blocked with PBS + 2% bovine serum albumin (BSA) for 1 h at room temperature. The slide was washed with PBS with 1 mM Ca and Mg and assembled under water in a parallel plate flow chamber. The channel for the chamber was cut from 0.01 inch Duralastic sheeting (Allied Biomedical) as previously described (Eniola et al., 2005). DPBS+ buffer was degassed for 20 min under vacuum. The assembled chamber was mounted on a Nikon Diaphot inverted microscope, and DBPS + buffer was run through the chamber to remove bubbles. Experiments were monitored using a Cohu black and white CCD camera and recorded on a VHS recorder. Wall shear stress ($\tau_w$) was calculated using the equation:

$$\tau_w = \frac{6 \mu Q}{h w}$$

where $Q$ is the volumetric flow rate, $\mu$ is the fluid viscosity, $h$ is the channel height and $w$ is the channel width. Shear rate ($\gamma$) is given by the equation:

$$\gamma = \frac{\tau_w}{\mu}$$

Yeast were suspended in 1% BSA-DPBS + 1 mM CaCl2 and 2 mM MgCl2 at a concentration of 2 × 10⁶ cells/ml and sonicated for 3 s at approximately 10 W (RMS) using a tip sonicator. Samples were connected to the chamber inlet using rubber tubing and an infusion/withdrawal syringe pump (Harvard Apparatus) was used to draw yeast through the chamber. When analyzing single clones, each sample was analyzed at different points along the slide for 1 min each for a total of four minutes.

Separation of mock libraries

To validate the rolling separation method, mock libraries of eGFP-labeled yeast expressing the HA I domain mutant (K287C/K294C) and unlabeled wild-type I domain were sorted using rolling adhesion using the laminar flow setup described above. To enrich for yeast with a high rolling velocity, a mixed population of these yeast were allowed to settle in the inlet of the parallel plate chamber for 10 min at room temperature. Flow was initiated at a shear rate of 360 s⁻¹, and fractions of cells were collected. Enrichment for firmly adherent yeast was accomplished under steady flow. A mock library of yeast was perfused into the chamber, followed by perfusion of buffer at 450 s⁻¹. Progress was monitored under the microscope and the firmly adherent population was collected by perfusing buffer in the chamber at maximum shear rate by hand. Populations collected using both of these methods were analyzed using flow cytometry.

I domain library construction

The I domain gene of pCT-I domain (Pepper et al., 2006) was mutated using the GeneMorph II mutagenesis kit (Stratagene) to perform error prone PCR following the manufacturer’s protocol for low mutation rate. PCR to amplify DNA, transformation of yeast using the gene and linearized vector, and
further library manipulations were performed following the protocol of Chao et al. (2006). The final library size was estimated at approximately $2.5 \times 10^6$ clones.

**Isolation of firmly adherent clones**

One round of fluorescence-activated cell sorting was used to reduce the non-expressing portion of the library. Yeast were grown overnight in SD-CAA media (0.67% yeast nitrogen base without amino acids (Difco), 0.5% casamino acids (Difco) and 2% sugar (dextrose or galactose), pH 6.0, with 2% agar included in plates) at 30°C, subcultured at an OD$_{600}$ of 1, and grown overnight in SG-CAA media at 20°C to induce expression. After overnight induction, yeast were incubated with anti-c-myc antibody 9E10 in DPBS+ at room temperature for 30 min. ICAM-1-Fc was pre-complexed with Cy5-labeled anti-c-myc antibody 9E10 in DPBS (Invitrogen) for 1 h at room temperature (Fig. 2a). The labeled cells were sorted once on a FACSVantage instrument (BD Biosciences) for positive ICAM-1 binding (Fig. 2b). The remaining library was then regrown, induced and sorted three times in the parallel plate flow chamber using the method described above to isolate firmly adherent cells (Fig. 2c). Yeast were either regrown in SD-CAA pH 4.5 or plated on solid SD-CAA media to test individual clones.

**Characterization of individual clones**

Individual clones were tested for rolling adhesion in the method described previously (Pepper et al., 2006) and above. Clones were analyzed at a shear rate of 240 s$^{-1}$. Yeast moving at less than 0.1% of hydrodynamic velocity were labeled as firmly adherent. Soluble ICAM-Fc binding of I domain mutants was also determined at a range of concentrations as previously described (Pepper et al., 2006). Microscopy data recorded on video was transferred to DVD and compressed into 1 min avi files. These files were input into a particle tracking program based in the MATLAB software suite, using the image processing toolbox. Cells are identified in each frame by thresholding to create a binary image and then using intrinsic MATLAB functions to count and determine properties for each object (i.e. diameter, eccentricity and solidity). Budding yeast are eliminated during thresholding based on particle circularity. A particle in frame $n$ is then compared with itself in frame $n+1$ to construct trajectories based on the particle size and free stream velocity at its centroid, a procedure repeated for all particles. After particle tracking is complete, broken trajectories are reconstructed and noise is filtered by eliminating any particle that interacts for less than 4 s or deviates more than 20° from horizontal while traveling more than 4 μm. Particles are tracked at four locations on each slide for 1 min, and rolling velocities are based on total distance traveled and total time tracked for each particle. Rolling velocities must be equal to or less than 0.1% of the fluid velocity one cell radius (2 μm) from the chamber wall for cells to be classified as firmly adherent.

Individual clones were also labeled with anti-c-myc antibody 9E10 as described above to assess I domain expression level.

**Expression and purification of soluble I domain**

The gene encoding the I domain with a C-terminal 6xHis tag was cloned using conventional techniques into the pET23-based vector pBamUK (gift from Dr. Greg Vanduyne, University of Pennsylvania). Protein was expressed in shake-flask cultures of E. Coli strain BL21(DE3) (Invitrogen) after induction with 1 mM IPTG at approximately OD$_600$ = 0.4 and further growth for 3 h. Pelleted cells were frozen and lysed in B-Per (Thermo Scientific) following the manufacturer’s directions. Inclusion bodies were isolated and dissolved in 50 mM phosphate, 8 M urea, pH 7.5 (Buffer A). A reducing agent (β-mercaptoethanol) was present at this and subsequent steps for the K287C/K294C mutant. Protein refolding was accomplished using an on-bead method (McCusker and Robinson, 2008). Briefly, extracted inclusion bodies were spun at 15 000 × $G$ and the supernatant was shaken overnight with nickel-nitrilotriacetic acid beads (Fisher). The beads were washed twice with Buffer A supplemented with 20 mM imidazole and then gently stirred overnight at 4°C in 50 mM sodium phosphate (pH 7.5), 1 mM MgSO$_4$ and 5% (v/v) glycerol. The beads were collected in a column and protein was eluted using 50 mM sodium phosphate, 100 mM NaCl, 1 mM MgSO$_4$ and 300 mM imidazole, pH 7.5. Fractions containing I domain were dialyzed with Slide-A-Lyzer cassettes (Thermo Fisher) against 50 mM sodium phosphate, 100 mM NaCl, 1 mM MgSO$_4$, pH 7 and concentrated to ~0.5 mg/ml using a centrifugal filter device (Millipore).

**Analysis of I domain mutant binding affinities**

Surface plasmon resonance (SPR) measurements were conducted at the Center for Biomolecular Interaction Analysis at the University of Utah. ICAM-Fc was captured onto an anti-Human mAb chip surface (CM5) in a Biacore 2000 at three different surface densities (900, 330 and 230 RU). Each ligand was tested for binding in 20 mM Tris (pH 8.0), 150 mM NaCl, 10 mM MgCl$_2$, 0.005% tween-20 and 0.1 mg/ml BSA. Assays were run at 25°C at a flow rate of 50 μl/min. No regeneration was required. Each analyte was tested in a five sample concentration series except for the high-affinity LO mutant, which was tested at 7 concentrations. Each analyte concentration series was tested in duplicate. Responses from the reference surface as well as buffer injections were subtracted from the data. The data from the 330 and 900 RU surfaces were most reliable and used to determine the average and standard deviation of the $K_D$. 

**Results**

**Mock library selection based on dynamic cell adhesion**

In order to develop a method to separate yeast by adhesive properties under shear flow, we utilized mock libraries of HA I domain-expressing yeast mixed with wild-type I domain yeast. HA I domain was expressed on the surface of yeast engineered to express eGFP cytoplasmically to facilitate real-time monitoring of the selection. To sort for firmly adherent populations, yeast were perfused into a parallel port flow chamber and allowed to flow through the chamber; subsequently, buffer was perfused to eliminate non-adherent and low affinity yeast. Yeast remaining in the chamber were collected and analyzed by flow cytometry. After one round of selecting for firmly
adherent yeast, a population of 50% HA/eGFP yeast and 50% wild-type unlabeled yeast was enriched to 76% HA/eGFP yeast (Fig. 1a). In a similar manner, yeast were also separated by rolling adhesion but selecting for high average rolling velocity. Yeast were allowed to settle on the ICAM-1 surface at the entrance of the flow chamber, and buffer was perfused. Fractions of yeast exiting the chamber were collected and analyzed by flow cytometry. A population of 80% wild-type yeast was enriched to 97% wild-type in one round of sorting for higher rolling velocity (Fig. 1b).

**Selection of I domain mutants based on adhesion under flow**

A library of \( \sim 2.5 \times 10^6 \) I domain mutants was created using error-prone PCR and homologous recombination in yeast. About 3% of the library cells expressed full length I domain and bound labeled ICAM-1-Fc, as determined using flow cytometry (Fig. 2a). To reduce the number of non-expressing clones, the library was sorted once using fluorescence-activated cell sorting (FACS) for clones that both expressed the c-myc epitope tag and bound with any detectable affinity to labeled ICAM-1. One round of FACS enriched the library approximately 5-fold for these clones (Fig. 2b). The sorted library was subsequently amplified by culturing and subjected to three rounds of selection for firm adhesion in the rolling chamber, with amplification of the recovered clones between each round (Fig. 2c). From this population, individual clones were tested for rolling dynamics, and those that showed a rolling velocity significantly decreased compared with wild-type I domain were further analyzed.

**Characterization of firmly adherent I domain mutants**

Yeast displaying isolated I domain clones were labeled with anti-c-myc antibody and analyzed by flow cytometry. Expression of mutant I domain variants was found to be equivalent (data not shown). Rolling velocities of single, non-budded yeast expressing I domain clones were determined using particle tracking by video microscopy. The average rolling velocity and percentage of firmly adherent cells were calculated (Table I), and soluble ICAM-1 binding was confirmed by flow cytometry (data not shown); binding of yeast-displayed I domain to soluble ICAM-1 oligomers, although not as accurate as SPR, shows that all of these clones have binding affinity for ICAM-1 roughly similar to the locked-open HA I domain mutant and significantly higher than wild-type I domain. Clones were sequenced to identify mutations. The data indicate that the rolling selection was able to recover clones with mutations found by FACS screening of a similar library labeled with soluble ICAM-1 \((\text{Jin et al., 2006})\), as well as novel clones not found using this soluble binding method (Fig. 3).

Clones isolated displayed a range of average rolling velocities significantly reduced from wild type. Wild-type I domain showed an average rolling velocity of 11.8 \(\mu\)m/s, while the firmly adherent mutants isolated using this method show average rolling velocities ranging from 0.104 to 1.55 \(\mu\)m/s.

![Fig. 1. Sorting of mock libraries by rolling adhesion. Mock libraries were created using an eGFP-expressing yeast strain surface displaying the HA I domain variant and nonfluorescent yeast expressing the wild-type I domain variant were mixed at ratios of \( \sim 50 : 50 \) and \( 20 : 80 \) (HA : WT). The resulting populations, seen on the right, after one round of sorting for the firmly adherent phenotype (a), and rolling phenotype (b) using the flow-based adhesion assay in the rolling chamber.](image-url)
Several of the mutants identified using rolling selection were expressed in *E. coli*, purified and analyzed using SPR on ICAM-1-coated chips (Table I). The concentration of the wild-type I domain sample (600 μg/ml or 24 μM) was not sufficient to provide enough signal to calculate binding affinity, in agreement with the binding constant of 1.6 mM determined previously (Shimaoka et al., 2001). Dissociation constants ranged from 10 to 181 μM, significantly lower than wild-type I domain but slightly higher than the engineered high-affinity I domain mutant. Interestingly, a decorrelation of rolling velocity and binding affinity was found, similar to previous work using engineered I domain clones. The A242V mutant showed a predominantly firmly adherent phenotype and very low average rolling velocity, but a higher $K_D$, while the I288N mutant showed slightly less cells firmly attached, a slightly higher average rolling velocity, with a much lower $K_D$. The I288N mutant was also found in the soluble ligand sort by Jin et al. (2006) and is in the region of the I domain identified as the switch allostery region, while the A242V mutation is part of the ICAM-1 binding epitope of the I domain (Shimaoka et al., 2003).

### Discussion

Previous work has shown that soluble binding affinity alone does not determine the LFA-1-mediated rolling behavior of...
particles on ICAM-1 surfaces (Chang et al., 2000; Pepper et al., 2006); thus, other biophysical parameters of the receptor-ligand bond, such as reactive compliance, appear to play an important role in this phenomenon. To help better understand this interaction at the molecular level, we sought an approach for identifying amino acid mutations in LFA-1 I domain that impact dynamic particle adhesion in a defined manner. A method was developed to separate yeast by rolling velocity using a parallel plate flow chamber. While it is possible to separate HA I domain mutants from a library of yeast displaying I domain variants using FACS, an adhesion-based functional screen helps to understand the connection between soluble I domain binding and the binding of this molecule under force. Because soluble binding affinity and rolling velocity do not appear to be directly correlated, this method allows for the isolation of clones based on the dynamic ligand-recognition properties of the molecule under fluid shear-induced external force.

The method developed here was based on earlier work to separate hematopoetic stem and progenitor cells by differential rolling adhesion (Greenberg and Hammer, 2001). Our results show, initially using mock libraries, that a similar method can be applied to separate yeast displaying I domain variants with different affinities for ICAM-1. Separation by this method is efficient, and can be easily applied to other situations where a population of cells differs in rolling velocity.

I domain variants that exhibit firm binding to ICAM-1 surfaces under shear flow contain mutations found both within and outside of the ‘switch allostery’ region proposed by Jin et al. (2006) which were identified based on increased equilibrium binding to ICAM-1 in solution. Screening of these two similar I domain mutant libraries by different methods yielded significantly different results, highlighting the important role that force plays in the I domain/ICAM-1 interaction. Mutants isolated using soluble binding that showed increased affinity for ICAM-1 clustered exclusively in one region, while mutations isolated with a functional screen were seen throughout the I domain (Fig. 3). We found that the ligand binding site point mutation A242V induces a dramatic change in average rolling velocity of yeast under flow, primarily via an increase in the non-rolling fraction, while also yielding a 5-fold increase in I domain-ICAM-1 binding affinity. However, this mutation was not found using soluble ligand screening methods, suggesting this mutant can productively bind to ICAM-1 under external force despite a zero-force binding affinity too low to enable recovery by soluble ligand screening.

Residues 241 and 243 of the I domain, on either side of Ala-242, have long been seen as important in mediating LFA-1-ICAM-1 binding (Shimaoka et al., 2003). Glu-241 forms a critical salt bridge with Lys-39 of ICAM-1, and Thr-243 participates in hydrophobic interactions with ICAM-1 in the ring that surrounds the MIDAS site on the I domain. The slightly bulkier Val-242 substitution may allow this residue to participate in hydrophobic interactions to further stabilize the binding site. Alternatively, this substitution to a bulkier residue may shift the Glu-241 residue into a slightly different conformation. Glu-241 is not available to form a salt bridge in the wild-type I domain conformation, but is shifted into position in the open, high-affinity conformation to mediate the higher affinity interaction (Shimaoka et al., 2003). This mutation may partially mimic the effect of this conformational change in the active site.

Other mutations found using this method do not cluster as did those found by sorting with soluble ligand, but are distributed throughout the molecule. Residues I235 and I259 fall in the β sheets that make up the core of the molecule. Mutating these residues to the larger phenylalanine may disrupt interactions stabilizing this core, allowing more structural flexibility around the binding site. The T179I mutation also lies within a β sheet (β3), and the loss of polar interactions may similarly destabilize the central β sheet structure to allow for more flexibility at the active site. Thr-179 appears to have a polar interaction with Tyr-214 as a 2.6 Å distance separates the oxygen molecules, in line with a hydrogen bond.
Adhesive dynamics simulations based on the Bell model for force-induced bond dissociation (Bell, 1978) predict that the most important parameters that govern cell adhesion in flow are unstressed off-rate and reactive compliance (Chang et al., 2000). Reactive compliance is a functional property that describes the bond interaction length and therefore characterizes the acceleration of bond dissociation in the presence of an externally applied load. In the case of I domain/ICAM-1, we hypothesize that mutations altering the stressed conformation increase the flexibility of the binding interface, which allows for increased bond strength under force. However, they do not necessarily affect soluble binding affinity to the same extent as mutations that alter the unstressed conformation of the I domain. This can be readily seen when comparing affinities determined by SPR to the adhesion under shear flow of the same mutants when bound to the yeast cell surface (Table I). While A242V binds ICAM-1 approximately 6-18-fold weaker than three other mutants isolated using the adhesive sorting technique (D193N/I235F, I288N and I259F), the A242V mutant exhibits more robust firm adhesion and adhesive sorting technique (D193N/I235F, I288N and I259F), which are 6-18-fold weaker than three other mutants isolated using the adhesive sorting technique (D193N/I235F, I288N and I259F), the A242V mutant exhibits more robust firm adhesion and slower rolling under force. The S165L mutation also mediated adhesive behavior similar to these mutants, but the binding affinity could not be determined by SPR under our conditions (∼50 μM concentration), suggesting the possibility of an affinity similar to wild-type I-domain in the mM range (and thus representing a dramatic example of the impact of properties other than affinity on dynamic adhesion).

Although these mutants were sorted for a firmly adherent phenotype, two clusters of mutations appear in a plot of average rolling velocity vs. the percentage of firmly adherent yeast (Fig. 4). One group includes those with an average rolling velocity <0.5 μm/s and shows a strong correlation between rolling velocity and the firmly adherent fraction. The second group comprises those with a slightly higher average rolling velocity and shows less of a correlation with the firmly adherent phenotype. The LFA-1–ICAM-1 interaction is not only important in leukocyte trafficking to sites of inflammation, but it is also a key event in the binding of T cells to antigen-presenting cells. An HA I domain molecule could interfere with these interactions or bind to the ICAM-1 expressed at normal levels throughout the body. Therefore, other applications may necessitate binding at only those sites where ICAM-1 is up-regulated and displayed at a relatively higher cell surface concentration. In this case, enhanced selectivity may be accomplished through the combination of a slightly lower I domain affinity and increased cell surface density. Increased endothelial expression can allow an I domain clone with a slightly lower affinity for ICAM-1, such as those seen in this second group, to bind strongly only in these regions, which could provide an important function in drug and imaging agent delivery. Thus, used as a targeting domain in a therapeutic drug or imaging agent delivery application, the first group could potentially be used to completely block ICAM-1 binding sites, while the second could prove most useful for ICAM-1 targeting.

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