Interaction between Platelet Glycoprotein Ibα and Filamin-1 Is Essential for Glycoprotein Ib/IX Receptor Anchorage at High Shear*

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The interaction of the glycoprotein (GP) Ib-V-IX receptor complex with the membrane skeleton of platelets is dependent on a specific interaction between the cytoplasmic tail of GPIbα and filamin-1. This interaction has been proposed to regulate key aspects of platelet function, including the ligand binding of GPIb-V-IX and the ability of the cells to sustain adhesion to von Willebrand factor (vWF) under high shear. In this study we have examined sequences in the GPIbα intracellular domain necessary for interaction of the receptor with filamin-1. We have identified two adjacent sequences involving amino acids 557–568 and 569–579 of the GPIbα cytoplasmic domain that are critical for normal association between the receptor complex and filamin-1. Under flow conditions, Chinese hamster ovary (CHO) cells expressing these two mutant receptors exhibited an increase in translocation velocity that was associated with increased cell detachment from the vWF matrix at high shear. The shear-dependent acceleration in velocity of mutant Δ557–568 and Δ569–579 CHO cells was associated with a critical defect in receptor anchorage, evident from significant extraction of GPIb-IX from the CHO cell membrane at high shear. These studies define a critical role for amino acids within the 557–579 sequence of GPIbα for interaction with filamin-1.

Platelets play a critical role in the arrest of bleeding by adhering to vascular matrix proteins and to other activated platelets at sites of vessel wall injury. These adhesion processes involve the coordinated interaction of various platelet receptor-ligand interactions. Foremost among these is the binding of the vascular adhesive protein, von Willebrand factor (vWF), to the GPIb-V-IX interaction site. This interaction is associated with a rapid increase in cell mobility and spreading, and thrombin generation, resulting in irreversible platelet adhesion, spreading, and thrombus growth (1)

The GPIb-V-IX receptor complex consists of four individual subunits, GPIbα, GPIbβ, GPV, and GPIX (reviewed in Ref. 7). GPIbα is the largest and functionally most significant subunit of the complex. It has a globular extracellular domain containing the vWF-binding site, a short transmembrane region, and a C-terminal cytoplasmic domain of 96 amino acids which extends from residues 515 to 610 (7). The cytoplasmic domain of GPIbα is known to interact with two intracellular proteins, filamin-1 (previously referred to as actin-binding protein-280) (8, 9, 16) and the signaling adaptor protein 14-3-3 (10, 12). The functional significance of the interaction with 14-3-3 remains unclear, although it has recently been proposed to be important for the ability of GPIbα to transduce signals necessary for αIβ3 activation (11).

There is mounting evidence that the interaction of GPIbα with filamin-1 has a significant influence on various aspects of platelet morphology and GPIb-V-IX receptor function. The abnormal cytoskeletal architecture of platelets and megakaryocytes associated with the inherited deficiency of GPIbα (Bernard Soulier syndrome) has been proposed to arise from the absence of the linkage between GPIbα and filamin-1 (12). In addition, there is evidence that the GPIb-filamin-1 association is necessary for the entire receptor complex to the membrane skeleton (9) and for the ability of GPIb-IX-transfected cells to maintain adhesion to vWF under conditions of high shear (13).

Recently, there is evidence supporting a potentially important role for the GPIbα-filamin-1 linkage in regulating the adhesive function of the GPIb-V-IX receptor (14, 15). Dong et al. (22) suggested that the GPIbα tail is important for regulating mobility of the receptor complex in the plane of the cell membrane. Mistry et al. (14) demonstrated in platelets and GPIb-IX-transfected CHO cells that inhibiting actin polymerization dramatically enhanced the ability of vWF to induce cell aggregation. Analysis of the adhesive properties of CHO cells expressing mutant forms of GPIbα demonstrated that the effects of the cytoskeleton on the adhesive function of GPIb-V-IX are dependent on the physical association between GPIbα and filamin-1 (14). A subsequent study by Englund et al. (15) has demonstrated increased vWF binding to GPIbα on the surface of CHO cells, and enhanced adhesion to vWF under both static...
and flow conditions, when the GPIb-filamin interaction is severed in these cells. These studies have suggested a potentially important role for the receptors intracellular domains in regulating the adhesive function of the GPIb-V-IX complex and emphasize the need to examine more precisely the interactions between the GPIb cytoplasmic domain and filamin-1.

Previous studies have defined two large contiguous regions in the GPIb cytoplasmic tail potentially involved in the binding to filamin-1 (9, 16). Peptide binding studies demonstrated the involvement of amino acid residues between positions 535 and 569 and the GPIb tail in this interaction (16). Our previous studies in GPIb-IX expressing CHO cells confirmed a critical role for residues within this region in the association of the receptor complex with filamin-1 and the membrane skeleton (13). In a study by Cunningham et al. (9) using cells expressing truncated forms of GPIb a second region of the cytoplasmic tail, residues 570–590, was shown to be essential for binding filamin-1 (9). The relationship between these two regions has not been established. One of the possibilities is that the major recognition site for filamin-1 involves an amino acid sequence overlapping these two large regions, part of which is disrupted when either of these domains is deleted. We have therefore undertaken further studies to identify critical sequences in the GPIb cytoplasmic domain that are required for the binding of filamin-1. We report the identification of a highly conserved region, encompassing amino acids 557–579, that contributes amino acid interactions that are essential for the association of GPIb with filamin-1. We also demonstrate that these residues are indispensable for membrane anchorage of the receptor complex under conditions of high shear stress.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine vWF (BvWF) was purified as previously described (17, 18). Full-length complementary DNA (cDNA) for GPIbα, GPIbβ, and GPIX cloned into the mammalian expression vector pDx, and CHO cells coexpressing GPIbβ and GPIX (CHOβX) were kindly provided by Dr. J. Lopez (Houston, TX). Anti-CD44 antibody was purchased from Immunotech (Beckman Coulter Co., Marseille, France). Protein G-Sepharose was from Sigma and enhanced chemiluminescence reagent (ECL) was from Amersham Biosciences, Inc. (Sydney, Australia). Membrane-permeable EZ-link-NHS-Biotin was purchased from Pierce, and both Complete™ protease inhibitor mixture and calpain inhibitor 1 were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Laemmli Sample Buffer was purchased from Bio-Rad. All other reagents and antibodies were from sources described previously (19, 20).

**Generation of GPIbα Deletion Mutants and Expression of GPIb Complex on the Surface of CHO Cells**—The GPIbα Δ535–568 deletion mutant was generated as described previously (13). All other mutants were generated using a similar inverse PCR procedure, with the pDX-GPIbα plasmid as template and primers flanking the desired deletion region. Specific primer sequences are given in Table I. All deletions were confirmed by automated DNA sequence analysis. Transfection of CHO cells with cDNAs for GPIbα (CHO-WT), GPIbα Δ535–545, GPIbα Δ546–556 (CHO-Δ546–556), GPIbα Δ557–568 (CHO-Δ557–568), GPIbα Δ569–579 (CHO-Δ569–579), GPIbα Δ580–590 (CHO-Δ580–590), and GPIbα Δ591–610 (CHO-Δ591–610), and their subsequent characterization by fluorescence-activated cell sorter analysis were performed as described previously (13, 19, 20).

**Biointeraction and Immunoprecipitation of GPIbα and Filamin-1 from Transfected CHO Cells**—Adherent cells were detached with PBS containing 0.5 mM EDTA, and washed in PBS. Samples containing 8 × 10⁸ cells/ml were incubated for 15 min at room temperature with the membrane-permeable EZ-link-NHS-biotin (500 μg/ml). Cells were then washed three times with 10 mM NH₄Cl, and lysed by 20 min incubation on ice in 1% Triton X-100 in PBS containing 1 × Complete™ protease inhibitor mixture and 2 μl/ml calpain inhibitor 1. Samples were centrifuged at 150 (g) for 10 min at 4 °C. Supernatants were incubated with 50% (v/v) protein G-Sepharose beads in lysis buffer containing 1% Triton X-100 in PBS for 1 h at 4 °C. After centrifugation, 50 μl of the cleared lysates were incubated with 5 μg/ml mAb ALMA 12 (anti-GPIbα), Ram 1 (anti-GPIbβ), or an isotype-matched control plus 50 μl of protein G-Sepharose beads in lysis buffer containing 1% Triton X-100 in PBS at 1 °C for 15 min. After centrifugation, 50 μl of the cleared lysates were incubated with 5 μg/ml mAb ALMA 12 (anti-GPIbα), Ram 1 (anti-GPIbβ), or an isotype-matched control plus 50 μl of protein G-Sepharose beads in lysis buffer containing 1% Triton X-100 in PBS at 1 °C for 15 min. After centrifugation, 50 μl of the cleared lysates were incubated with 5 μg/ml mAb ALMA 12 (anti-GPIbα), Ram 1 (anti-GPIbβ), or an isotype-matched control plus 50 μl of protein G-Sepharose beads in lysis buffer containing 1% Triton X-100 in PBS at 1 °C for 15 min. After centrifugation, 50 μl of the cleared lysates were incubated with 5 μg/ml mAb ALMA 12 (anti-GPIbα), Ram 1 (anti-GPIbβ), or an isotype-matched control plus 50 μl of protein G-Sepharose beads in lysis buffer containing 1% Triton X-100 in PBS at 1 °C for 15 min. After centrifugation, 50 μl of the cleared lysates were incubated with 5 μg/ml mAb ALMA 12 (anti-GPIbα), Ram 1 (anti-GPIbβ), or an isotype-matched control plus 50 μl of protein G-Sepharose beads in lysis buffer containing 1% Triton X-100 in PBS at 1 °C for 15 min.

**Flow-based CHO Cell Adhesion Assays**—Flow assays were performed as described previously (13). Briefly, CHO cells (1 × 10⁶ cells/ml) were perfused through glass microcapillary tubes (Vitro Dynamics Inc., Mountain Lakes, NJ) coated with purified BvWF at 10 μg/ml and residual glass was blocked by incubation with 25% human serum in PBS. Cells were initially perfused at 1 dyne/cm² for 5 min followed by perfusion of cell-free buffer at the same shear stress. Following this, the shear stress was increased stepwise to 5, 20, 40, and 60 dyn/cm². For high shear stress tested, 5 representative fields of the microcapillary tube were visualized by video microscopy and recorded for subsequent analysis of cell tethering, rolling velocity, and detachment. For analysis of CHO cell tethering and rolling at high shear stress, CHO cells were perfused through microcapillary tubes in a concentrated red blood cell suspension. We have previously reported that CHO-Ib/IX cells resuspended in buffer alone tether poorly to vWF-coated microcapillary tubes at high shear (13), however, in the presence of human red blood cells, CHO-Ib/IX cells tether efficiently to vWF at high shear (50 dyn/cm²) (15). To investigate the red blood cell requirement for CHO cell tethering in our experimental flow system, red blood cells were isolated from fresh whole blood as described previously (21). Preliminary analysis of red cell concentrations required for optimal cell tethering demonstrated minimal CHO cell tethering at hematocrits 40 to 80% (data not shown), however, significant levels of CHO cell tethering and rolling was observed at 90% hematocrit (see data in Fig. 7). Analysis of the viscosity of the cell suspension at 90% hematocrit using a cone-and-plate viscometer (Carrimed rheometer, CSL100, Carr-Med, Dorking, United Kingdom) revealed a viscosity of 10 centipoise (0.01 pascal-sec) that remained constant over shear rates between 300 and 10,000 s⁻¹. The shear stress was calculated using the formula: shear stress (dyne/cm²) = μ × shear rate (s⁻¹) × viscosity (pascal-sec) × 10. For high shear experiments, the cell suspension was perfused through BvWF-coated microcapillary tubes (10 μg/ml) at 50 dyn/cm² for 5 min at room temperature and tethering cells were video recorded in random fields for 5 min. CHO cell tethering and rolling velocity was analyzed off-line as previously described (13).

**Analysis of CHO Cell Rolling Velocity Prior to Detachment**—CHO cells with cDNAs for GPIbα (CHO-WT), GPIbα Δ553–545, GPIbα Δ546–556 (CHO-Δ546–556), GPIbα Δ557–568 (CHO-Δ557–568), GPIbα Δ569–579 (CHO-Δ569–579), GPIbα Δ580–590 (CHO-Δ580–590), and GPIbα Δ591–610 (CHO-Δ591–610), and their subsequent characterization by fluorescence-activated cell sorter analysis were performed as described previously (13).
cells (1 x 10^6 cells/ml) were perfused through BvWf-coated microcapillary tubes for 5 min at a shear stress of 1 dyne/cm^2. The shear stress was then increased to 40 dyne/cm^2 and a single field was video recorded for 5 min. Cells which were marked as having detached during the video-recorded interval were examined for their rolling velocity prior to detachment by measuring distance traveled over 5-s intervals for the minute immediately prior to their detachment. The rolling velocity of between 9 and 16 detached cells was compared directly to that of cells which remained adherent to the matrix under the same experimental conditions.

**Studies of CHO Cell Receptor Extraction**—To study receptor extraction, CHO cells (1 x 10^6 cells/ml) were perfused through BvWf-coated microcapillary tubes at a shear stress of 1 dyne/cm^2 for 10 min. Following this, cells were exposed to shear stresses >60 dyne/cm^2 to detach the majority of cells from the matrix. Cells and receptors remaining on the BvWf matrix were fixed with 500 l of 4% paraformaldehyde in PBS. The remaining adherent CHO cells and matrix were labeled for 1 h at room temperature with WM23 (anti-Ib) for 30 min, followed by a 30-min incubation with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody. Analysis of surface expression (solid black histograms) was performed by flow cytometry. Gray histograms represent the negative controls, performed with an irrelevant isotype-matched mAb.

**RESULTS**

*Expression of GPIbα Deletion Mutants in CHO Cells and Their Interaction with Filamin-1*—To investigate more precisely the specific amino acid sequences of the GPIbα cytoplasmic domain required for its association with filamin-1, we generated a series of deletion mutants of GPIbα. These are neighboring deletions of 11–12 residues (Δ535–545, Δ546–556, Δ557–568, Δ569–579, and Δ580–590) within the amino acid 535–590 region of the cytoplasmic domain, and a final mutant with a deletion of the remaining 19 amino acids (Δ591–610) which removes the C-terminal 14-3-3-binding site (Fig. 1A). The various GPIbα constructs in the pDX mammalian expression vector were used to transfet CHO cells already expressing the GPIbα and GPIX receptor subunits (13). GPIbα expression was demonstrated by Western blot analysis of CHO cell lysates (data not shown) and surface expression of the GPIbα-IX receptor complex was confirmed and expression levels monitored by flow cytometry (Fig. 1B). In this way receptor expression levels were matched as closely as possible between the various cell
lines used for aggregation and flow studies. The cell line expressing the Δ591–610 GPIba consistently had slightly lower expression levels than the other cell lines, however, this proved not to be a significant problem in the studies involving this cell line.

We investigated the ability of the GPIb-IX receptor complex to associate with filamin-1 in CHO cells expressing either wild-type or mutant forms of GPIba. This was performed by immunoprecipitation of the GPIb-IX complex from cell lysates by anti-GPIba mAb (Ram.1), as described under “Experimental Procedures.” Immunoprecipitated proteins were separated by 4–15% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and the immunoprecipitated bands detected using ECL. Deletion of the residues 557–568 or 569–579 resulted in the complete loss of interaction between GPIba and filamin-1.

The addition of a blocking antibody against GPIba (data not shown). Furthermore, the aggregation response of CHO-WT cells was significantly enhanced when actin polymerization was inhibited using cytochalasin D (CD). Microscopic examination confirmed the presence of considerably larger cell aggregates in the CD-treated cells (Fig. 3A). We conducted similar studies with the CHO cells expressing mutant GPIbas, IbΔ535–568. Compared with the CHO-WT cells, these cells exhibited an increased basal aggregation response to vWF (Fig. 3B), despite having similar receptor expression levels. Moreover, pretreatment of the cells with CD did not result in any further enhancement of aggregation, but instead resulted in a decreased aggregation response (Fig. 3, B and C). Again, these findings were confirmed by the size of aggregates upon microscopic examination (Fig. 3B). Overall, the aggregation characteristics of the CHO-Δ535–568 cells were similar to our previous findings with CHO-Δ569–610 cells (14).

We then compared the aggregation properties of the various CHO cell lines expressing smaller GPIba deletion mutants with CHO-WT and CHO-Δ535–568 cells. As with the CHO-WT and CHO-Δ535–568 cells, each of the mutant cell lines aggregated to BvWF in a dose-dependent manner (data not shown). At a submaximal dose of BvWF of 5 μg/ml, the aggregation responses of the CHO-Δ557–568 and CHO-Δ569–579 cells were significantly greater than with any of the other deletion mutants (Fig. 4A). Furthermore, pretreating these cells with CD resulted in a reduction in cell aggregation (Fig. 4A). The behavior of these cells was therefore similar to the CHO-Δ535–568 cells. In contrast, the cells expressing the Δ535–545, Δ546–556, Δ558–590, and Δ591–610 GPIba mutants exhibited enhanced aggregation following CD treatment, similar to that seen with CHO-WT cells. Quantitation of the effects of CD on BvWF-induced aggregation for each of the cell lines is summarized in Fig. 4B and clearly illustrates the similarity between the two smaller deletions, Δ557–568 and Δ569–579, and the larger Δ535–568 deletion (Fig. 3C). These findings provide further evidence that deletion of residues 557–568 and 569–579 disrupts the linkage between GPIba and filamin-1.

**Effect of Deletions in the Amino Acid Δ557–579 Region on CHO Cell Tethering, Rolling, and Detachment under Flow**—There have been conflicting reports regarding the importance of the GPIba cytoplasmic tail in regulating the adhesive function of GPIba-IX, with some studies suggesting decreased vWF binding (22), others suggesting no alteration in ligand binding (9, 13) while a recent report suggested enhanced vWF binding (15). We therefore conducted a series of experiments under flow conditions to compare the adhesive function of CHO-WT cells with the cell lines expressing mutant GPIba receptors incapable of binding filamin-1 (CHO-Δ557–568 and CHO-Δ569–579). Cells were perfused through BvWF-coated microcapillary tubes and allowed to adhere to the matrix under low shear conditions (1 dyne/cm²), then subsequently exposed to progressively higher shear stresses of 5, 20, 40, and 60 dyne/cm². In preliminary studies, we did not find any significant difference in the ability of CHO-WT, CHO-Δ557–568, and CHO-Δ569–579 to tether to the vWF matrix at low shear (1 dyne/cm²) (data not shown). Furthermore, when we analyzed the rolling velocities of the CHO cells (Fig. 5) under these conditions there was no significant difference in their rolling velocity. In contrast, at elevated shear stress (60 dyne/cm²), there was a significant difference in translocation velocity between the individual cell lines, such that the CHO-WT and CHO-Δ557–568 and CHO-Δ569–579 to that of the CHO-WT at a mean velocity of 5 μm/s, whereas CHO-Δ557–568 and CHO-Δ569–579 exhibited at 17 and 14 μm/s, respectively (p < 0.05). We believe this difference is most likely a direct result of disrupting the GPIba-filamin linkage, rather than a nonspecific effect of tail short-
ening, as CHO cells expressing a similar length deletion mutant (CHO-Δ535–545) behaved similarly to the WT-expressing cells (data not shown).

Further analysis of individual cell lines at high shear demonstrated that mutant cell lines lacking the GPIb-filamin-1 interaction detached more readily from the vWF surface. In the case of CHO-WT cells, significant detachment from the matrix was only observed at shear stresses of 40 and 60 dyne/cm² (Fig. 6). By comparison, ~20% of CHO-Δ535–546 and 10% of CHO-Δ569–579 cells detached at 20 dyne/cm². The differences between the CHO-WT and mutant cell lines became more apparent at high shear, with ~80% of both mutant cell lines detaching at 60 dyne/cm² compared with 40% of the CHO-WT (p < 0.001) (Fig. 6). These studies support our previous findings.
to incremental increases in shear stress to 5, 20, 40, and 60 dyne/cm² for 2 min at each shear. Rolling velocity of 25 individual cells (5 cells over 569 fields) and CHO-/H9004A7, both CHO-WT (CHO cell tethering under high shear. As demonstrated in Fig. 5, significant changes in GPIb-IX adhesive function we established a flow assay, as described in “Experimental Procedures.” The receptors are more prone to extraction by high shear forces. There are a number of potential explanations for the increased rolling velocity and detachment of mutant CHO cell lines. One possibility is that disrupting the link between GPIb and filamin-1 selectively alters the kinetic properties of the vWF-GPIb interaction under high shear. Alternatively, there may be a reduction in the tensile strength of the vWF-GPIb bond leading to shorter bond lifetimes at high shear. A third possibility is that the receptors are more prone to extraction by high shear forces. To gain further insight into the effects of shear on GPIb-IX receptor function we established a flow assay, as described in “Experimental Procedures,” that enabled analysis of CHO cell tethering under high shear. As demonstrated in Fig. 7, both CHO-WT (A) and CHO-Δ557–569 (B) tethered to a similar extent on the vWF matrix under high shear conditions (50 dyne/cm²) and rolled at a similar velocity within the first 30 s of interaction with the matrix (Fig. 7, C and D). These results, combined with our adhesion studies under low shear, suggest that disrupting the linkage between GPIb and filamin-1 does not have a major effect on the ligand binding characteristics of the GPIb-IX receptor. To investigate potential time-dependent changes in receptor function, we performed analysis of CHO-WT and CHO-Δ569–579 adhesion prior to cell detachment from the vWF matrix. In these studies, a number of detaching cells were identified and the rate of translocation plotted for each cell over a 50–60-s time period at 5-s intervals prior to their detachment (Fig. 8). This analysis revealed a critical difference between the wild-type and mutant receptors in that for CHO-WT there was a constant rate of translocation velocity up to the point of detachment (Fig. 8, A and C). In contrast, all CHO-Δ569–579 cells exhibited a significant increase in their rate of translocation prior to detachment (Fig. 8, B and D). Interestingly, at early time points CHO-Δ569–579 cells exhibited constant rolling velocities similar to the CHO-WT cells, with the greatest change in rolling velocity observed in the translocation phase preceding their detachment (Fig. 8, A and B). Furthermore, the rolling velocity of nondetaching CHO-WT cells and nondetaching CHO-Δ569–579 cells were not significantly different over a 50-s observation period. These latter observations, combined with the findings that nondetaching WT and Δ569–579 cells rolled slower than their detaching counterparts (Fig. 8, C and D), raised the possibility that the CHO-Δ569–579 cells that detached from the matrix formed progressively fewer adhesion contacts with the vWF matrix during surface translocation.

Effects of Cytoplasmic Domain Deletions on Membrane Anchorage of the Receptor Complex—To investigate the possibility that loss of the GPIb-filamin-1 interaction leads to a critical defect in receptor anchorage leading to progressive receptor extraction, immunofluorescence analysis of the vWF matrix was performed to detect the presence of extracted receptor subunits after detachment of CHO cells at high shear. A low level of GPIbα staining was detectable on the matrix after detaching CHO-WT cells from the vWF matrix by high shear (>60 dyne/cm²) (Fig. 9). Similar levels of staining were seen in the case of cells expressing the Δ535–545, Δ546–556, and Δ580–590 deletion mutants, all of which retained their ability to associate with filamin-1. In contrast, high levels of fluorescent staining were seen following detachment of the CHO-Δ557–568 and CHO-Δ569–579 cells (Fig. 9A). Furthermore, in the case of these mutants, there were obvious tracks of fluorescence visible on the matrix, suggesting a progressive extrac-

**Fig. 5.** Effect of increasing shear stress on the rolling velocity of WT and mutant Δ557–568 and Δ569–579 CHO cells. CHO-WT and mutant cells (1 × 10⁶/ml) were perfused through bovine vWF-coated (10 µg/ml) microcapillary tubes at 1 dyne/cm² for 5 min. Cells were subjected to incremental increases in shear stress to 5, 20, 40, and 60 dyne/cm² for 2 min at each shear. Rolling velocity of 25 individual cells (5 cells over 5 fields) was determined at each shear as described under “Experimental Procedures.” The results presented represent the mean ± S.E. from three independent experiments. The rolling velocity of CHO-Δ557–568 and Δ569–579 was significantly higher than CHO-WT at 60 dyne/cm² (p < 0.05).

**Fig. 6.** Effect of shear on the detachment of CHO cells expressing WT and Δ557–568 and Δ569–579 mutant GPIbα. CHO-WT and mutant cells (1 × 10⁶/ml) were perfused through vWF-coated microcapillary tubes (10 µg/ml) at 1 dyne/cm² for 5 min. The shear stress was increased incrementally to 5, 20, 40, and 60 dyne/cm² for 2 min and 5 random fields recorded at each shear for off-line analysis. Cell adhesion was quantified at all shear stresses as a percentage relative to the number of adherent cells at 5 dyne/cm². The results presented represent the mean ± S.E. (n = 6). For CHO-Δ557–568 there was a significant increase in detachment relative to CHO-WT at shear stresses of 20, 40, and 60 dyne/cm² (p < 0.01, p < 0.01, and p < 0.001, respectively). For CHO-Δ569–579, a significant difference was observed at 40 and 60 dyne/cm² (p < 0.05 and p < 0.001, respectively).

(13) for an important role for the GPIbα-filamin-1 linkage in enabling GPIb-V-IX expressing CHO cells to sustain adhesion under high shear.

Disruption of the GPIbα-Filamin-1 Linkage Results in Time-dependent Changes in GPIb-IX Adhesive Function—There are a number of potential explanations for the increased rolling velocity and detachment of mutant CHO cell lines. One possibility is that disrupting the link between GPIb and filamin-1 selectively alters the kinetic properties of the vWF-GPIb interaction under high shear. Alternatively, there may be a reduction in the tensile strength of the vWF-GPIb bond leading to shorter bond lifetimes at high shear. A third possibility is that the receptors are more prone to extraction by high shear forces. To gain further insight into the effects of shear on GPIb-IX receptor function we established a flow assay, as described in “Experimental Procedures,” that enabled analysis of CHO cell tethering under high shear. As demonstrated in Fig. 7, both CHO-WT (A) and CHO-Δ557–569 (B) tethered to a similar extent on the vWF matrix under high shear conditions (50 dyne/cm²) and rolled at a similar velocity within the first 30 s of interaction with the matrix (Fig. 7, C and D). These results, combined with our adhesion studies under low shear, suggest that disrupting the linkage between GPIb and filamin-1 does not have a major effect on the ligand binding characteristics of the GPIb-IX receptor. To investigate potential time-dependent changes in receptor function, we performed analysis of CHO-WT and CHO-Δ569–579 adhesion prior to cell detachment from the vWF matrix. In these studies, a number of detaching cells were identified and the rate of translocation plotted for each cell over a 50–60-s time period at 5-s intervals prior to their detachment (Fig. 8). This analysis revealed a critical difference between the wild-type and mutant receptors in that for CHO-WT there was a constant rate of translocation velocity up to the point of detachment (Fig. 8, A and C). In contrast, all CHO-Δ569–579 cells exhibited a significant increase in their rate of translocation prior to detachment (Fig. 8, B and D). Interestingly, at early time points CHO-Δ569–579 cells exhibited constant rolling velocities similar to the CHO-WT cells, with the greatest change in rolling velocity observed in the translocation phase preceding their detachment (Fig. 8, A and B). Furthermore, the rolling velocity of nondetaching CHO-WT cells and nondetaching CHO-Δ569–579 cells were not significantly different over a 50-s observation period. These latter observations, combined with the findings that nondetaching WT and Δ569–579 cells rolled slower than their detaching counterparts (Fig. 8, C and D), raised the possibility that the CHO-Δ569–579 cells that detached from the matrix formed progressively fewer adhesion contacts with the vWF matrix during surface translocation.

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tion of receptor as the cells translocated at high shear before detaching completely from the vWf matrix. The presence of occasional cells with “tails” of receptor fluorescence confirmed the direct association of the receptor tracks with translocating cells (Fig. 9C).

To determine whether the entire GPIb-IX complex was extracted from the cell membrane under these conditions, we perfused CHO-WT and CHO-Δ569–579 cells on separate BvWf-coated microcapillary tubes and compared the matrix staining using antibodies against GPIbα, GPIX, and a control antibody against the unrelated membrane spanning CD44 receptor (anti-CD44). The anti-GPIX monoclonal Ab, similar to the anti-GPIbα, showed a low level of fluorescence after detachment of CHO-WT cells, but a high level of fluorescence and the
GPIb lines were perfused through BvWF-coated microcapillary tubes at 1 dyne/cm², then shear was increased above 60 dyne/cm² to induce detachment of and interestingly the longest of these lies within the 557 sequence. There are several stretches of sequence that show complete identity among man, canine, and murine species (reviewed in Ref. 23). There are sequences between residues 557–568 and 570–590. They demonstrate that two neighboring sequences (557–568 and 569–570) are essential for GPIb-filamin-1 interaction. The deletion abolished filamin-1 binding in CHO cells. Our conclusion that sequences between 557 and 579 are critical for GPIb-filamin-1 association in CHO cells. They also demonstrate that deletion of either of these regions is essential for the GPIb-filamin-1 association in CHO cells. They interpret these findings to mean that each of these regions is essential for the GPIb-filamin-1 association in CHO cells. They also demonstrate that deletion of either of these sequences results in a critical defect in the ability of the GPIb-V-IX receptor complex to remain anchored to the surface membrane under high shear. This defect in receptor anchorage leads to a time-dependent increase in cell translocation velocity and an inability of the cells to remain adherent to vWF under high shear.

The intracellular domain of GPIbα is increasingly recognized as contributing an important role toward a number of aspects of platelet morphology and adhesive function. At the structural level, the regions of the GPIbα cytoplasmic domain that interact with filamin-1 have been broadly defined as involving sequences between amino acids 535 and 590 (9, 13, 16). The present studies clarify the relationship between the two adjoining GPIbα regions implicated in filamin-1 binding, 535–568 and 570–590. They demonstrate that two neighboring sequences (557–568 and 569–579) overlapping these larger regions are essential for the GPIb-filamin-1 association in CHO cells. They also demonstrate that deletion of either of these sequences results in a critical defect in the ability of the GPIb-V-IX receptor complex to remain anchored to the surface membrane under high shear. This defect in receptor anchorage leads to a time-dependent increase in cell translocation velocity and an inability of the cells to remain adherent to vWF under high shear.

The GPIbα cytoplasmic tail is highly conserved between human, canine, and murine species (reviewed in Ref. 23). There are several stretches of sequence that show complete identity and interestingly the longest of these lies within the 557–579 sequence. Our finding that the major filamin-1 recognition site within GPIbα lies within the sequence 557–579 reconciles several apparently conflicting observations from earlier studies. For example, Cunningham et al. (9) studying various GPIbα truncation mutations concluded that sequences between residues 570 and 590 are essential for GPIb-filamin-1 interaction. However, these findings appeared to contradict earlier peptide binding studies (16) demonstrating that the major high affinity binding sites for filamin-1 involved sequences 536–554 and 550–568. The findings presented in the current study agree, in part, with the earlier peptide binding studies (16) in which a peptide of sequence corresponding to residues 550–568 was reported to have the strongest interaction with filamin-1. However, our studies do not support a key role for sequences between 536 and 554, since neither the 535–545 nor 546–556 deletion abolished filamin-1 binding in CHO cells. Our conclusion that sequences between 557 and 579 are critical for GPIbα-filamin-1 interaction are based on our combined findings from co-immunoprecipitation studies and functional characteristics of transfected CHO cells expressing the mutant receptors. These conclusions are also consistent with the recent studies of Englund et al. (15), who demonstrated that deletion of 20 amino acids between residues 551 and 570 abolished association of the GPIb-V-IX complex with the membrane skeleton.

Of the series of deletion mutants generated in our study, only the Δ557–568 and Δ569–579 deletions resulted in complete abolition of the filamin-1 interaction in CHO cells, a conclusion supported by the aggregation and adhesive properties of the cell lines. We interpret these findings to mean that each of these stretches of sequence contains one or more critical amino acid residues that contribute essential interactions for effective filamin-1 binding. We do not conclude that these sequences constitute a “binding site” for filamin-1 as such, since clearly such a site would be likely to involve the contribution of residues outside of these sequences. Indeed, the co-immunoprecipitation results on the Δ580–590 CHO cells suggest that there may be some contribution of residues in this region to filamin-1 binding.

Our studies do not support previous findings that suggested an important role for the GPIb-filamin-1 linkage in regulating the intrinsic binding characteristics of the GPIb-V-IX complex. Dong et al. (22) had previously suggested that the GPIbα tail is important for enabling GPIb-V-IX to bind vWF, possibly as a result of changes in receptor mobility. In contrast, a recent study by Englund et al. (15) has lead to opposite conclusions, in
which it is proposed that linkage of GPIb-V-IX to the membrane skeleton imposes constraints on the receptor complex that limits vWF binding. The studies presented here do not support either conclusion. We have demonstrated that under low and high shear conditions, wild-type and mutant receptors engage vWF in a similar manner. Furthermore, the initial translocation velocity of the various cell lines was similar, suggesting the binding characteristics of the mutant receptors have not been significantly altered. In support of this conclusion are previous studies by Cunningham et al. (9), who demonstrated normal vWF binding to mutant GPIb-V-IX complexes that do not associate with the membrane skeleton. The reason for these discrepant results between studies remains unclear, but presumably reflects technical differences. A clear advantage of the series of deletion mutants generated in the present study is the preservation of the overall length of the GPIb that do not associate with the membrane skeleton. This was unclear. Two important factors that need to be considered when interpreting the binding characteristics of mutant receptors are: (a) the effect of the mutation on the intrinsic binding kinetics, i.e. bond kinetics in the absence of an applied force; and (b) the effect of the mutation on the receptors reactive compliance i.e. the susceptibility of bond kinetics to applied force. As stated above, our studies do not support a critical role for the GPIb-filamin-1 interaction in regulating the intrinsic binding properties or the reactive compliance of the receptor complex under low or high shear. Rather, several lines of evidence suggest that the critical defect in receptor function is primarily due to receptor extraction from the surface membrane. First, there was no difference in the initial adhesive properties of CHO cells expressing wild-type or mutant receptors over a broad range of shear conditions. Second, in contrast to CHO-WT, which exhibited a uniform translocation velocity prior to detachment, CHO-Delta569–579 cells demonstrated a time-dependent acceleration in translocation velocity. We interpret this to mean that for the wild-type receptors, cell detachment is due to breakage of receptor-ligand bonds, whereas with mutant receptors the acceleration in translocation is due to a progressive loss of receptors from the cell surface. Third, consistent with the loss of receptors from the surface membrane was the appearance of receptor “tracks” on the matrix surface. These tracks were only apparent when adherent CHO cells were exposed to levels of shear stress high enough to induce cell detachment, confirming an essential force requirement for receptor extraction.

The demonstration that the linkage between GPIb-V-IX and filamin-1 is important for preventing receptor extraction under high shear has potentially important implications for platelet adhesion and thrombus growth. For example, it has recently been demonstrated that platelets translocate extensively on the injured vessel wall and also on the surface of forming thrombi in vivo (25). Sustained platelet contact with immobilized vWF during surface translocation requires receptor-ligand interactions with sufficient bond strength to resist the detachment effects of high shear. This is achieved in part as a result of the unique biomechanical properties of the vWF-GPIb bond and the multivalency of the adhesive interaction (reviewed in Ref. 1). Our studies suggest that these factors can only sustain cell interaction under high shear if the receptor complex is adequately anchored to the membrane skeleton. This may become critically important during thrombus growth whereby narrowing of the vessel lumen by the developing thrombus results in a progressive increase in the level of shear at the thrombus surface. With recent developments in transgenic mouse models (26), it will be possible to address this issue through imaging of arterial thrombi formed in mice expressing mutant forms of GPIbα.

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S. L. Cranmer, unpublished observations.
I. Pikovski, unpublished observations.
Interaction between Platelet Glycoprotein Ibα and Filamin-1 Is Essential for Glycoprotein Ib/IX Receptor Anchorage at High Shear

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