Glycoprotein (GP) Ib-IX-transfected Cells Roll on a von Willebrand Factor Matrix under Flow

IMPORTANCE OF THE GPIb/ACTIN-BINDING PROTEIN (ABP-280) INTERACTION IN MAINTAINING ADHESION UNDER HIGH SHEAR*

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Adhesion of platelets to sites of vascular injury is critical for hemostasis and thrombosis and is dependent on the binding of the vascular adhesive protein von Willebrand factor (vWf) to the glycoprotein (GP) Ib-V-IX complex on the platelet surface. A unique but poorly defined characteristic of this receptor/ligand interaction is its ability to support platelet adhesion under conditions of high shear stress. To examine the structural domains of the GPIb-V-IX complex involved in mediating cell adhesion under flow, we have expressed partial (GPIb-IX), complete (GPIb-V-IX), and mutant (GPIb cytoplasmic tail mutants) receptor complexes on the surface of Chinese hamster ovary (CHO) cells and examined their ability to adhere to a vWf matrix in flow-based adhesion assays. Our studies demonstrate that the partial receptor complex (GPIb-IX) supports CHO cell tethering and rolling on a bovine or human vWf matrix under flow. The adhesion was specifically inhibited by an anti-GPIbα blocking antibody (AK2) and was not observed with CHO cells expressing GPIbβ and GPIX alone. The velocity of rolling was dependent on the level of shear stress, receptor density, and matrix concentration and was not altered by the presence of GPV. In contrast to selectins, which mediate cell rolling under conditions of low shear (20–200 s⁻¹), GPIb-IX was able to support cell rolling at both venous (150 s⁻¹) and arterial (1500–10,500 s⁻¹) shear rates. Studies with a mutant GPIbα receptor subunit lacking the binding domain for actin-binding protein demonstrated that the association of the receptor complex with the membrane skeleton is not essential for cell tethering or rolling under low shear conditions, but is critical for maintaining adhesion at high shear rates (3000–6000 s⁻¹). These studies demonstrate that the GPIb-IX complex is sufficient to mediate cell rolling on a vWf matrix at both venous and arterial levels of shear independent of other platelet adhesion receptors. Furthermore, our results suggest that the association between GPIbα and actin-binding protein plays an important role in enabling cells to remain tethered to a vWf matrix under conditions of high shear stress.

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1 The abbreviations used are: vWf, von Willebrand factor; BvWf, bovine von Willebrand factor; HvWf, human von Willebrand factor; GP, glycoprotein; ABP, actin-binding protein; CHO, Chinese hamster ovary; mAb, monoclonal antibody; PBS, phosphate-buffered saline.
shear. Third, integrin engagement of the adhesive surface must occur rapidly to retain platelets at the site of vessel wall injury. Despite its fundamental importance, the unique biomechanical characteristics of the vWF/GPIb interaction enabling platelet tethering under high shear remain poorly understood. The high tensile strength of the vWF/GPIb interaction is likely to reflect the formation of a large number of active bonds between vWF and GPIb, a process favored by the high GPIb density on the platelet surface and the multivalency of vWF. In this regard, GPV may play an important role, as it has been postulated to cross-link two GPIb molecules on the cell surface, thereby facilitating multivalent interactions. Anchorage of GPIb to the membrane skeleton may also be important for regulating the vWF/GPIb interaction by regulating the receptor distribution on the cell surface. Previous studies examining the role of the cytoplasmic tail of GPIb in regulating receptor function have yielded conflicting results. Cunningham et al. (7) have reported normal binding of vWF to mutant GPIbα receptors with C-terminal deletions of up to 52 amino acids. In contrast, Dong et al. (8) have recently reported that the removal of as few as six C-terminal amino acids from GPIbα has a profound inhibitory effect on the ability of the receptor to bind vWF. The reason for this discrepancy is unclear, but may reflect methodological differences between the two studies.

Studies of the purified GPIb-V-IX complex have demonstrated that ~70% of GPIb is complexed to actin-binding protein in vivo (9). This linkage appears to play an important role in maintaining the normal cytoskeletal architecture of resting platelets and in limiting mobility of the receptor complex within the plane of the plasma membrane (8). Mutagenesis studies of the cytoplasmic tail of GPIbα have demonstrated that the interaction between GPIbα and ABP is essential for anchoring the entire receptor complex to the membrane skeleton (7). Peptide binding and antibody inhibition studies have demonstrated that sequences contained within the central portion of the GPIbα cytoplasmic tail (amino acids 535–568 and 570–590) interact with purified ABP (10). Although mutagenesis studies have confirmed an important role for residues 570–590 in this interaction in vivo (7), the contribution of amino acids between positions 535 and 568 in regulating the GPIb/ABP interaction in vivo remains to be established.

In an attempt to investigate the structural domains of the GPIb-V-IX complex involved in regulating cell adhesion, we have expressed partial (GPIb-IX), complete (GPIb-V-IX), and mutant (GPIbα cytoplasmic tail mutants) receptor complexes on the surface of Chinese hamster ovary (CHO) cells and examined the ability of these receptors to support cell adhesion under flow conditions. Our studies demonstrate that the GPIb-IX complex is pivotal in mediating cell rolling at both venous and arterial levels of shear in the absence of other platelet adhesion receptors. Interestingly, GPV does not appear to significantly alter GPIb-mediated cell rolling or the strength of cell attachment to a vWF matrix. Studies of GPIbα truncation mutants have also demonstrated that the cytoplasmic tail of GPIbα does not regulate the initial tethering or rolling of CHO cells on vWF at low shear stress, but plays an important role in maintaining cell attachment to the matrix under high shear.

EXPERIMENTAL PROCEDURES

Materials—The monoclonal antibodies (mAbs) WM23, AK2 (anti-GPIbα), and PMC25 (anti-GPIX) were generous donations from Prof. Michael Berndt (Baker Medical Research Institute, Melbourne, Australia). mAb SW16 (anti-GPV) was from The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). All other materials were from sources we have described previously (11–13).

Transfection of CHO Cells—CHO cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium supplemented with 10% fetal bovine serum. CHO cells were transfected with a set of three expression plasmids, which individually contained the cDNAs encoding for GPIbα, GPIbβ, and GPIX. These plasmids were a kind gift from Dr. José López and were constructed using the pDX expression vector as described previously (14). The selection plasmid pPNTα (a gift from Dr. Phil Bird, Monash Medical School, Box Hill Hospital) was cotransfected to confer resistance to G418. Transfections were performed using the calcium phosphate precipitation technique, and cells were put under selection pressure after 48 h using 800 μg/ml G418. Control CHO cells were transfected with the expression vector pDX (containing no GPIbα cDNA) and pPNTα and selected under the same conditions. Individual G418-resistant clones were isolated with glass cloning cylinders and screened for their surface expression of GPIbα by immunofluorescence microscopy using mAb WM23 and a fluorescein isothiocyanate-conjugated secondary antibody. Positive clones were analyzed for their expression level of GPIbα and GPIX by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson). CHO cells expressing GPIb-IX (CHO-IX) were subsequently cotransfected with pDX containing GPV cDNA and a selection plasmid, pPB-3 (a gift from Dr. Phil Bird), to confer hygromycin resistance. Resistant CHO cells were selected using 400 μg/ml hygromycin, analyzed for expression of GPV by fluorescence-activated cell sorter analysis and subjected to two rounds of cell sorting using magnetic beads conjugated to sheep anti-mouse IgG (Dynal, Inc.) according to the manufacturer’s instructions, after having labeled CHO cells with the anti-GPV mAb SW16.

Mutagenesis and Transfection of GPIbα Mutants—Deletions were introduced into double-stranded GPIbα cDNA in pDX by polymerase chain reaction using the ExSite™ polymerase chain reaction-based site-directed mutagenesis kit (Stratagene). Amplification was performed using two primers: P1, 5′-TTG TTT GTG GTG TTG GGTT AGC AGC-3′ (nucleotides 4718 to 4698, numbering according to Wenger et al. (15)); and P2, 5′-GCT TGG GTA CCG CCT AAT GGC-3′ (nucleotides 4821–4841). CHO-β-IX cells (kindly donated by Dr. José López) were transfected with either wild-type or deleted forms of GPIbα cloned in pDX and the resistance plasmid pZeoS using FuGene TM6 transfection reagent (Boehringer Mannheim). Selection was initiated using 200 μg/ml zeocin (Invitrogen), and resistant clones were cultured for 2 weeks and then analyzed by immunofluorescence microscopy.
Biotinylation and Immunoprecipitation of Proteins from Transfected CHO Cells—Samples containing 10^6 CHO cells/100 μl were permeabilized using 0.3% saponin in PBS (containing Complete™ anti-protease mixture (Boehringer Mannheim) and 130 mM sucrose) for 6 min at 37°C, after which the reaction was stopped on ice. The cells were washed and then incubated for 15 min on ice with 0.05 mg/ml NHS-Biotin (ImmuNo-Pure® NHS-SS-Biotin, Pierce) in PBS. After two further washes, the cells were lysed and incubated with 50 μl of protein G beads (Sigma) for 1 h at 4°C. After centrifugation, the cleared lysates were incubated with 20 μg/ml mAb (ALMA 12 (anti-GPIbα), ALMA 16 (anti-GPIX), or an isotype-matched control) plus 50 μl protein G for 2 h on ice and incubated thoroughly. Immunoprecipitated proteins were removed from the beads by boiling in 1% SDS buffer for 5 min, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After incubation with horseradish peroxidase-conjugated streptavidin for 1 h, the immunoprecipitated bands were detected using an ECL kit (Amersham Pharmacia Biotech).

Static Adhesion Assays—Glass coverslips were coated with 10 μg/ml bovine vWF (BvWf), human vWF (HvWf), or fibrinogen at 4°C overnight and then blocked with 0.1% bovine serum albumin at room temperature for 1 h. CHO-Ib-IX, CHO-β-IX, and CHO-pDX cells were harvested with 0.5 mM EDTA, washed twice with PBS, resuspended in Tyrode's buffer containing 2 mM EDTA to a final concentration of 1×10^6 cells/ml, and then applied to the vWf-coated coverslips for 30 min at room temperature. For some assays, a suspension of CHO-Ib-IX cells was preincubated with the anti-GPIbα mAb AK2 (5 μg/ml) prior to application to coverslips. Non-adherent cells were aspirated, and the coverslips were washed four times with PBS. Adherent cells were visualized using an inverted phase-contrast microscope (Olympus IX-70).

Flow-based Adhesion Assays—Flow assays were performed as described by Cooke et al. (16). Glass microcapillary tubes (Microslides, Vitro Dynamics Inc., Mountain Lakes, NJ; 100 mm long with a rectangular cross-section of 0.3×3.0 mm (height × width)) were coated with the indicated concentration (10–100 μg/ml) of BvWf or HvWf at 4°C overnight, washed three times with PBS, and then blocked with either 0.1% bovine serum albumin or 25% heat-inactivated human serum at room temperature for 1 h. In the majority of studies, CHO cells (1×10^6 cells/ml) were perfused through the microcapillary tubes at a shear rate of 150 s⁻¹ for 5 min and visualized using video microscopy. At the end of the cell perfusion period, the microcapillary tube was perfused with cell-free buffer at the same shear stress, and one representative field was videotaped, and the number of adherent cells/field was quantitated off line.

Calculation of Rolling Velocity and Image Analysis—Video recordings were analyzed off line, and the velocity of rolling was calculated as follows. All adherent cells present within one field (or 25 cells/field in some experiments) were marked, and the distance traveled per unit time was recorded. The monitor was calibrated using a 0.01-mm stage micrometer (Olympus), and the rolling velocity was expressed in μm/s.

Protein Purification and Analysis—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (17). Protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as a standard. Bovine and human vWF were purified from plasma cryoprecipitate by size-exclusion chromatography according to the method of Montgomery and Zimmerman (18). Fibrinogen was purified from fresh frozen plasma as described by Jakobsen and Kierulf (19). All proteins were demonstrated to be >95% pure by Coomassie Brilliant Blue staining of electrophoresed proteins.

Statistical Analysis—Significant differences were detected using one-way analysis of variance and Student's t test using the Prism software package (GraphPAD Software for Science, San Diego, CA).

RESULTS

It is well established that GPIb-IX expressed on the surface of heterologous cells is competent to bind human vWF in the presence of cationic modulators, such as ristocetin or botrocetin (7, 14). Although these modulators provide a convenient method to investigate the vWF/GPIb-IX interaction in vitro, their regulatory effects on vWF or GPIb-IX do not necessarily mimic those that occur in vivo. An alternative approach involves using bovine vWF, which spontaneously binds GPIb-IX in the absence of cationic modulators (20, 21). In preliminary studies, we examined the ability of immobilized BvWf to support adhesion of GPIb-IX-transfected CHO cells. Under static conditions CHO-Ib-IX, but not CHO-pDX or CHO-β-IX, adhered to purified BvWf (Fig. 1). This adhesion was specific to the vWF/GPIb-IX interaction in that it did not occur on a fibrinogen matrix and was abolished by the anti-GPIbα mAb AK2 (Fig. 1). Consistent with previous reports, GPIb-IX-transfected CHO cells did not form stable adhesion contacts with a human vWF matrix in the absence of a cationic modulator (7).

To investigate the effects of shear stress on the BvWf/GPIb-IX interaction, we performed in vitro flow-based assays using BvWf-coated microcapillary tubes as described under “Experimental Procedures.” In initial studies, CHO-Ib-IX cells

![Fig. 2](http://www.jbc.org/)

**Fig. 2. Effect of shear rate on the rolling velocity of CHO-Ib-IX cells.** A, shown are video micrographs depicting CHO-Ib-IX cell rolling on a BvWf matrix (5 μg/ml) at three different shear rates (150, 750, and 3000 s⁻¹). Each panel represents four superimposed images captured at 5-s intervals (a representative rolling cell is highlighted by arrows). Note that the phase-dark cells (marked by an asterisk in the middle panel) represent occasional nonviable CHO cells that nonspecifically adhere and do not roll. B, the rolling velocity of CHO-Ib-IX cells was measured at five different shear rates as described under “Experimental Procedures.” The mean ± S.E. is from one experiment representative of four. The number of cells analyzed was 54–80 at 150–3000 s⁻¹ and 25 at 6000 s⁻¹. There were significant increases in rolling velocity between consecutive increases in wall shear rate (p < 0.001).

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were preadhered to the BvWf matrix for 5 min prior to the initiation of flow. Applying shear (150 s⁻¹) to adherent CHO-Ib-IX cells converted their adhesion from stationary to rolling adhesion (Fig. 2A, upper panel). High power magnification of the CHO cells using differential interference contrast microscopy clearly demonstrated a rotational motion of the cells, indicative of cell rolling. The majority of cells appeared to roll at a uniform velocity, although occasional cells would halt periodically before returning to their previous rolling velocity. Prolonged cell contact with the BvWf matrix prior to the initiation of flow did not lead to irreversible cell adhesion or influence subsequent rolling velocity. Perfusion of CHO-Ib-IX cells in the presence of EDTA (2 mM) did not influence the rolling phenomenon, excluding a major role for endogenous CHO cell integrins in this process.

Exposure of CHO-Ib-IX cells or the vWf matrix to a broad range of shear rates (150–12,000 s⁻¹) did not appear to induce significant alterations in the interaction between cells and matrix. For example, prolonged exposure of the matrix to high shear stress did not alter subsequent CHO-Ib-IX cell tethering or rolling. Furthermore, utilizing the same matrix for multiple experiments at different shear stresses yielded identical results to experiments performed on virgin matrices. All cells appeared to roll at a similar velocity over the entire surface of the microcapillary tube, and the rolling velocity of individual cells did not appear to change over a 10-min observation period. These findings are consistent with the hypothesis that cell rolling is due to the reversible characteristics of the vWf/GPIb interaction rather than to loss of molecules from the cell surface or the matrix.

We examined the effect of several independent parameters, including shear rate, receptor density, and matrix concentration, on the velocity of CHO-Ib-IX cell rolling. In all experiments, rolling velocities were determined under equilibrium binding conditions as described under “Experimental Procedures.” As demonstrated in Fig. 2 (A and B), incremental increases in shear rate resulted in a corresponding increase in the velocity of cell rolling, from 2 μm/s at 150 s⁻¹ up to 8.5 μm/s at 6000 s⁻¹. Under similar experimental conditions, clones with 2–3-fold higher surface expression of GPIb-IX exhibited an ~50% reduction in rolling velocity (Fig. 3, A and B). Matrix concentration also had a profound effect on CHO cell rolling. As demonstrated in Fig. 3C, increasing the coating concentration of BvWf from 10 to 50 μg/ml resulted in a 5.5-fold reduction in the rate of cell rolling at 150 s⁻¹.

To investigate whether human vWf could also support rolling of GPIb-IX-transfected CHO cells, CHO-Ib-IX cells were perfused through HvWf-coated microcapillary tubes as described under “Experimental Procedures.” As with the bovine protein, CHO-Ib-IX cells were able to tether and roll on a HvWf matrix in the absence of cationic modulators. The ability of CHO-Ib-IX cells to tether was greatly influenced by the matrix concentration and wall shear rate. At a low HvWf-coating concentration (10 μg/ml), CHO-Ib-IX cells tethered poorly to the matrix at all shear rates tested, whereas at a high matrix concentration (100 μg/ml), cells preferentially tethered at low shear rates (Fig. 4). The bovine protein was more effective at supporting CHO-Ib-IX cell tethering at both low (10 μg/ml) and high (100 μg/ml) matrix concentrations; however, as with the human matrix, cell tethering was apparent only at relatively low shear rates (<300 s⁻¹). Similar to our findings with BvWf, the velocity of CHO-Ib-IX cell rolling on HvWf was dependent on the shear rate (Table 1). Overall, the velocity of CHO-Ib-IX cell rolling on a human matrix was 2–3-fold faster than that observed with the bovine protein.

As demonstrated in Fig. 5, at a high matrix concentration (100 μg/ml HvWf), >85% of CHO-Ib-IX cells remained attached to BvWf at shear rates as high as 3000 s⁻¹. Higher shear resulted in a progressive decrease in the number of attached cells, although even at the highest shear rate tested (10,500 s⁻¹), ~15% of cells were still able to adhere and roll on the BvWf matrix. This contrasted significantly with the HvWf matrix, from which ~30% of CHO-Ib-IX cells detached at a shear rate of 750 s⁻¹, ~65% at 1500 s⁻¹, and ~95% at 3000 s⁻¹.
Treating the cells with EDTA (2 mM) had no inhibitory effect on their resistance to detachment by high shear forces (data not shown). As with the rolling velocity, the resistance of CHO-Ib-IX cell detachment was greatly influenced by the matrix concentration (see Fig. 7) and receptor density (data not shown).

To investigate a potential role for GPV in regulating the rolling phenomena, we transfected CHO-Ib-IX cells with GPV as described under “Experimental Procedures.” As demonstrated in Fig. 6A, high levels of GPV were expressed on the surface of CHO-Ib-V-IX cells when analyzed by fluorescence-activated cell sorter analysis. Both CHO-Ib-V-IX and CHO-Ib-IX cells expressed comparable levels of GPIbα. Cells expressing GPV appeared to roll in a similar manner to cells expressing GPIb-IX alone. Interestingly, no difference was detected in the velocity of cell rolling between CHO-Ib-V-IX and CHO-Ib-IX cells when compared at both low (150 s⁻¹) and high (3000 s⁻¹) shear rates (Fig. 6B) and when examined over a broad range of different BvWf matrix concentrations (data not shown). The effect of GPV on cell detachment by increasing shear was also examined. As demonstrated in Fig. 7, the presence of GPV had no effect on the rate of cell detachment at various matrix concentrations (10, 25, and 50 µg/ml) and shear rates (150–6000 s⁻¹).

In addition to receptor density and matrix concentration regulating the strength of adhesion of CHO-Ib-IX cells to immobilized vWF, we investigated the possibility that the association of GPIbα with ABP may also play an important role in regulating resistance to detachment by high shear forces. For these studies, we generated a GPIbα cytoplasmic tail mutant that lacked the major putative binding domain for ABP (amino acid residues 535–568) and expressed this mutant on the surface of CHO cells as described under “Experimental Procedures.” To determine the effect this mutation has on the ability of GPIbα to associate with ABP, we performed immunoprecipitation studies as described under “Experimental Procedures.” The results of these experiments are shown in Fig. 8. In CHO-Ib-IX cells, ALMA 12 and ALMA 16 co-immunoprecipitated GPIbα, GPIbβ, GPIX, and ABP (with apparent molecular masses of 135, 28, 22, and 280 kDa, respectively). In CHOΔ535–568 cells, ALMA 12 and ALMA 16 co-immunopre-
GPIb/ABP-280 Interaction and Cell Rolling

FIG. 6. Effect of GPV expression on the velocity of CHO cell rolling. A, expression of GPV and GPIbα on the surface of CHO-Ib-IX and CHO-Ib-V-IX cells was analyzed by flow cytometry using two different primary mAbs, SW16 (anti-GPV) and WM23 (anti-GPIbα). Panel i demonstrates that CHO-Ib-V-IX cells express a high level of GPV on the cell surface compared with CHO-Ib-IX cells (which have a fluorescence peak identical to that of the isotype-matched control primary mAb). Panel ii demonstrates that expression of GPIbα is similar in both CHO-Ib-V-IX and CHO-Ib-IX cells. B, the effect of GPV on the velocity of CHO cell rolling was determined. The rolling velocities of CHO-Ib-IX (solid bars) and CHO-Ib-V-IX (hatched bars) cells on BvWF (25 μg/ml) were compared at shear rates of 150 and 3000 s⁻¹ as described under “Experimental Procedures.” The results presented are from one experiment representative of three in which 25 cells from each cell line were analyzed. The mean rolling velocities for CHO-Ib-IX and CHO-Ib-V-IX cells at 150 s⁻¹ were 4.34 and 3.93 μm/s, respectively. The mean velocities at 3000 s⁻¹ were 10.13 and 10.81 μm/s for CHO-Ib-IX and CHO-Ib-V-IX cells, respectively.

To investigate the effect of this mutation on the ability of GPIbα to interact with vWF, we initially compared adhesion of CHOΔ535–568 and CHO-Ib-IX cells on a HvWF matrix. These cell lines were matched for equivalent levels of GPIb-iX surface expression, and as demonstrated in Fig. 9, there was no significant difference in the ability of the mutant or wild-type receptor to mediate cell tethering at a wall shear rate of 75, 150, or 225 s⁻¹. Analysis of the rolling velocity and stability of adhesion at 150, 750, and 1500 s⁻¹ also revealed no difference between CHOΔ535–568 and CHO-Ib-IX cells. Interestingly, at a shear rate of 3000 s⁻¹, CHOΔ535–568 cells rolled ~50% faster than CHO-Ib-IX cells (p < 0.005) and appeared to be more easily detached from the matrix, such that at a shear rate of 3000 s⁻¹, ~5% of CHOΔ535–568 cells remained attached to the matrix compared with ~15% of CHO-Ib-IX cells. To investigate further the ability of the mutant receptor to support cell adhesion under high shear conditions, we performed flow studies on a BvWF matrix. With this matrix, it was possible to examine differences in adhesive characteristics of a larger number of CHOΔ535–568 and CHO-Ib-IX cells at higher shear rates. Consistent with the studies on human vWF, there was no significant difference in the ability of the mutant and wild-type receptors to support cell adhesion under static conditions or tethering under low shear conditions. Similar to the human protein, at high shear rates (3000 s⁻¹), CHOΔ535–568 cells rolled faster than CHO-Ib-IX cells and were more easily detached from the adhesive surface, such that at 3000 s⁻¹, <10% of CHOΔ535–568 cells remained tethered to the matrix compared with ~40% of CHO-Ib-IX cells (p < 0.05) (Fig. 10).

DISCUSSION

Much of the current understanding of the vWF/GPIb interaction has been derived from studies employing cationic modulators, such as ristocetin and botrocetin, to induce binding of soluble vWF to GPIb-V-IX under low shear conditions. Although these modulators have been invaluable in defining the functionally important domains on both vWF and GPIb, they do not mimic the binding conditions experienced by platelets in vivo, in which shear stress plays an important role in promoting the vWF/GPIb interaction. The experimental system described in this report represents a powerful new method of investigating the structural domains of the GPIb-V-IX complex involved in regulating cell adhesion under flow. Using CHO cells transfected with partial (GPIb-IX) or complete (GPIb-V-IX) receptor complexes, we have demonstrated that GPIb-IX is both necessary and sufficient to mediate cell tethering and rolling on a vWF matrix under flow. Furthermore, this receptor/ligand interaction has sufficient tensile strength to sustain cell adhesion.
Our studies examining the effect of receptor density and matrix concentration on the ability of CHO-Ib-IX cells to interact with immobilized vWF have emphasized the importance of the multivalent vWF/GPIb interaction in regulating cell tethering, rolling velocity, and the overall strength of the adhesive interaction. To date, there is limited information on the cellular factors that regulate the number of active bonds formed between vWF and GPIb. A candidate regulatory protein is GPV, which non-covalently associates with GPIb and has been postulated to cross-link adjacent GPIb molecules on the surface of platelets (22) and on GPIb-IX-transfected CHO cells (23). However, we could find no evidence for a role for GPV in regulating the vWF/GPIb interaction under flow. Comparison of CHO-Ib-IX and CHO-Ib-V-IX cells over a wide range of shear rates and matrix concentrations failed to identify a role for GPV in regulating the efficiency of cell tethering at low shear, velocity of rolling, or the resistance of cells to detachment by increasing shear. A recent study published during the preparation of this manuscript (24) has reported similar results with respect to the role of GPV in regulating cell adhesion on a human vWF matrix under flow when examining cells expressing high levels of GPIb. However, the authors suggested a potentially important role for GPV in regulating the vWF/GPIb interaction in cells expressing low levels of GPIb. Although potentially interesting, the physiological relevance of such findings remains to be established as such low levels of receptor expression are unlikely to be operative in normal platelets. Although our studies have not established an important role for GPV in cell adhesion, it remains possible that GPV may influence the tethering of platelets under high shear. Future studies using platelets deficient in GPV will be required to resolve this issue.

Our studies with CHOΔ535–568 cells have established a critical role for peptide sequences within the central region of the GPIbα cytoplasmic tail, independent of residues 570–590, in mediating association of the entire receptor complex with ABP and the membrane skeleton. These findings are consistent with previous reports demonstrating that 18-mer overlapping synthetic peptides spanning residues 534–568 bind ABP with high affinity in vitro (10). It is of interest that residues 570–590 also appear to be indispensable for GPIbα binding to ABP in vivo. These conclusions were based on the findings that removal of the C-terminal 41 amino acids of GPIbα (residues 569–610) completely eliminates its interaction with ABP and the membrane skeleton, whereas a shorter truncation mutant (residues 590–610) retains its ability to bind ABP. The relationship between residues 555–568 and 570–590 in mediating GPIbα binding to ABP is an important issue for future investigation. It is possible that the major recognition site for ABP is contained in an overlapping sequence between these peptide domains and that disruption of either end of this recognition site abolishes the GPIbα/ABP interaction. It is also possible that these adjacent sequences recognize distinct binding sites on ABP or that they interact with each other in the native protein to facilitate the GPIbα/ABP interaction. We also cannot exclude the possibility that the length of the GPIbα tail plays an important role in regulating the GPIbα/ABP interaction. It is clear that removal of 20 amino acids from the tail does not abolish binding; however, it remains to be formally excluded that shortening the tail further (33 amino acids for residues 535–568 and 41 amino acids for residues 569–610) does not influence the GPIbα/ABP interaction in vivo.

Our studies suggest for the first time a potentially important role for the GPIbα/ABP interaction in enabling platelets to re-

**Fig. 7. Effect of GPV on shear-induced cell detachment from bovine vWF.** CHO-Ib-IX and CHO-Ib-V-IX cells were detached from 50 μg/ml (panel i), 25 μg/ml (panel ii), and 10 μg/ml (panel iii) BvWF over a wide range of shear rates (150–6000 s⁻¹). The shear rate was increased incrementally every 60 s to a maximum of 6000 s⁻¹. At each shear rate, the same field was videotaped, and the number of adherent cells was counted off line. Note that CHO-Ib-V-IX cells were perfused through the same microcapillary tube as CHO-Ib-IX cells at a cell concentration of 1 × 10⁶ cells/ml.

at both venous and arterial levels of shear independent of other platelet adhesion receptors. The presence of GPV did not influence the efficiency of cell tethering, the velocity of cell rolling, or the resistance of the cells to detachment by high shear. Studies with a cytoplasmic tail mutant of GPIbα demonstrated that the interaction between GPIbα and ABP is not essential for cell tethering or rolling under low shear conditions, but is important for enabling cells to remain adherent to a vWF matrix under high shear.

Our studies with CHO-D35–568 cells have established a critical role for peptide sequences within the central region of the GPIbα cytoplasmic tail, independent of residues 570–590, in mediating association of the entire receptor complex with ABP and the membrane skeleton. These findings are consistent with previous reports demonstrating that 18-mer overlapping synthetic peptides spanning residues 534–568 bind ABP with high affinity in vitro (10). It is of interest that residues 570–590 also appear to be indispensable for GPIbα binding to ABP in vivo. These conclusions were based on the findings that removal of the C-terminal 41 amino acids of GPIbα (residues 569–610) completely eliminates its interaction with ABP and the membrane skeleton, whereas a shorter truncation mutant (residues 590–610) retains its ability to bind ABP. The relationship between residues 555–568 and 570–590 in mediating GPIbα binding to ABP is an important issue for future investigation. It is possible that the major recognition site for ABP is contained in an overlapping sequence between these peptide domains and that disruption of either end of this recognition site abolishes the GPIbα/ABP interaction. It is also possible that these adjacent sequences recognize distinct binding sites on ABP or that they interact with each other in the native protein to facilitate the GPIbα/ABP interaction. We also cannot exclude the possibility that the length of the GPIbα tail plays an important role in regulating the GPIbα/ABP interaction. It is clear that removal of 20 amino acids from the tail does not abolish binding; however, it remains to be formally excluded that shortening the tail further (33 amino acids for residues 535–568 and 41 amino acids for residues 569–610) does not influence the GPIbα/ABP interaction in vivo.

Our studies suggest for the first time a potentially important role for the GPIbα/ABP interaction in enabling platelets to re-
main adherent to a vWF matrix at high shear rates. It is important to emphasize that disrupting the cytoskeletal association of GPIb does not appear to adversely affect the ability of this receptor to bind vWF under static (10) or low shear conditions. Furthermore, our studies highlight the importance of examining the vWF/GPIb interaction over a wide range of shear conditions and the need to compare clones with a similar level of receptor expression to allow meaningful interpretation of differences between wild-type and mutant receptors. For example, the performance of rolling studies on CHO\(\text{D}_{535-568}\) cells at a single shear rate of 3000 s\(^{-1}\) would have demonstrated a clear difference in the number of cells adherent to human vWF and their rolling velocity, findings consistent with the cytoplasmic tail regulating the dynamics of the vWF/GPIb interaction under flow. However, by performing studies over a wide range of physiologically relevant shear conditions, we were able to clearly show that differences in numbers of adherent cells and rolling velocities are secondary to a reduced ability of the mutant receptors to withstand the detaching effects of high shear, rather than a reduced ability of the mutant receptors to engage vWF.

The experimental model described in this report is a powerful new means of investigating the structure/function relationships of the vWF/GPIb interaction. This limitation may in part reflect the large dimensions of CHO cells compared with platelets, although washed platelets also tether poorly to a vWF matrix compared with platelets in whole blood, highlighting the importance of red blood cells in promoting platelet/vessel wall interactions (25). A further limitation of using CHO-Ib-IX cells on a human vWF matrix is that these cells are far more sensitive to the detaching effects of high shear compared with platelets. This difference is likely to reflect differences in receptor density and cell dimensions. The receptor density on the CHO cell surface is likely to be at most only 5–10% of that on the platelet, and the force \(F = 32\pi^2\tau\) (where \(\tau\) is the wall shear stress) (26). We have been able to overcome this limitation by performing studies on a purified bovine vWF matrix. With this adhesive substrate, CHO-Ib-IX cells tether more effectively at low shear, roll slower, and are more resistant to the detaching effects of high shear. These observations presumably reflect different kinetic properties of the BvWF/GPIb interaction, such as an increased association rate, a slower dissociation rate, and higher tensile bond strength. It is also possible that these differences reflect the formation of a larger number of active bonds between GPIba and BvWF. Consistent with this possibility, our studies using cells with different receptor number and different matrix concentrations demonstrated that increasing the number of bonds enhanced tethering, slowed rolling velocity, and increased the ability of cells to

![Fig. 8. Association of wild-type (GPIb-IX) and mutant (GPIb\(\Delta_{535-568}\)) GPIba with actin-binding protein. A, shown is a schematic representation of wild-type (CHO-Ib-IX cells) and mutant (CHO\(\Delta_{535-568}\) cells) GPIba indicating the putative ABP-binding region within the cytoplasmic domain of GPIba (deleted in CHO\(\Delta_{535-568}\) cells). The 14-3-3-binding and transmembrane (TM) domains of GPIba are also indicated. B, Triton X-100 lysates of permeabilized and biotinylated CHO-Ib-IX and CHO\(\Delta_{535-568}\) cells were subjected to immunoprecipitation with mAbs: isotype-matched control (lanes 1), ALMA 12 (anti-GPIba) (lanes 2), and ALMA 16 (anti-GPIX) (lanes 3). Immunoprecipitations were performed as described under "Experimental Procedures."](http://www.jbc.org/DownloadedFrom HTTP://WWW.JBC.ORG/ BY GUEST ON AUGUST 31, 2017)
withstand high shear.

A key issue for future investigation will be to determine whether disrupting the link between GPIb and ABP alters the kinetics of the vWf/GPIb interaction under high shear. Although we could find no difference in cell tethering or rolling velocity at low shear, it is possible that anchorage of GPIb to the membrane skeleton may preferentially influence the on/off rate of the vWf/GPIb interaction under high shear, a possibility we have not been able to address with our current assay. It is also possible that disrupting the link between GPIb and ABP reduces the tensile strength of the vWf–GPIb bond; alters the topography of the GPIb receptors on the cell surface, thereby undermining the efficiency of bond formation under high shear; or results in extraction of the receptor complex from the cell membrane. These possibilities are currently under investigation and are critical in interpreting the role of the GPIb/ABP interaction in sustaining cell tethering under high shear.

Acknowledgments—We thank Dr. José López for generously donating the GPIb-IX receptor constructs and for supplying the CHO-b-IX cell line. We also thank Prof. Michael Berndt for monoclonal antibodies FIG. 9. Role of the GPIbα/ABP interaction in regulating cell adhesion under flow on human vWF. Shown are the effects of the GPIbα/ABP interaction on tethering (A), rolling velocity (B), and shear-dependent cell detachment (C) on 100 μg/ml human vWF. A, deletion of the major ABP-binding domain of GPIbα did not affect the ability of the vWF/GPIbα interaction to form at 75, 150, or 225 s⁻¹. The number of adherent CHO-Ib-IX and CHOΔ535–568 cells over 10 fields was counted at 60- and 180-s time points at 75, 150, and 225 s⁻¹ as described under “Experimental Procedures.” B, rolling velocity was unaffected by the GPIbα/ABP interaction at shear rates between 150 and 1500 s⁻¹, but was significantly faster for CHOΔ535–568 cells at 3000 s⁻¹ (p < 0.005). C, the stability of the vWF/GPIbα interaction at shear rates between 150 and 1500 s⁻¹ was similar for CHOΔ535–568 and CHO-Ib-IX cells, as assessed by the number of cells remaining adherent with increasing shear rates, whereas at 3000 s⁻¹, CHOΔ535–568 cells were more easily detached from the matrix. The data presented are from one experiment, representative of three.

FIG. 10. Role of the GPIbα/ABP interaction in regulating cell adhesion under flow on bovine vWF. Adhesion assays were performed as described under “Experimental Procedures,” and the number of adherent cells/field was counted over five fields. A, the number of adherent cells (mean ± S.E.) under static and low shear (150 s⁻¹) conditions was similar for both CHO-Ib-IX and CHOΔ535–568 cells. B, deletion of the major ABP-binding domain of GPIbα increased the rolling velocity of transfected CHO cells at high shear rates. CHO-Ib-IX (●) or CHOΔ535–568 (○) cells were allowed to adhere to immobilized BvWF (10 μg/ml) at 150 s⁻¹. The shear rate was incrementally increased, and rolling cells were videotaped for 60 s at each shear rate tested. The rolling velocity of 25 cells for each cell line was determined off-line, and the data shown are the mean ± S.E. from four experiments. C, deletion of the ABP-binding domain of GPIbα reduced the resistance of CHOΔ535–568 cells to detachment by high shear forces. In the same experiments as described for B, cells were exposed to increasing shear rates (60 s at each), and the number of cells remaining adherent at the end of each 60-s analysis was counted off line. Note that CHO-Ib-IX and CHOΔ535–568 cells were perfused through the same Microslide and that the same field was analyzed for both cell lines. The order in which the two cell lines were perfused was alternated between experiments.
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Glycoprotein (GP) Ib-IX-transfected Cells Roll on a von Willebrand Factor Matrix under Flow: IMPORTANCE OF THE GPIb/ACTIN-BINDING PROTEIN (ABP-280) INTERACTION IN MAINTAINING ADHESION UNDER HIGH SHEAR

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