The Cytoplasmic Domain of Glycoprotein (GP) Ibα Constrains the Lateral Diffusion of the GP Ib−IX Complex and Modulates von Willebrand Factor Binding†

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ABSTRACT: To study the role of the glycoprotein (GP) Ibα cytoplasmic domain in the mobility of the GP Ib−IX complex within the plasma membrane and in its ability to bind vWf, we established eight cell lines expressing GP Ib−IX complexes (these complexes lack GP V but function normally as receptors for vWf) that contain either wild-type GP Ibα or one of a series of GP Ibα truncation mutants missing different lengths of the cytoplasmic domain. To test the mobility of these complexes within the plasma membrane, we used the technique of fluorescence recovery after photobleaching after labeling them with a fluorescein-conjugated anti-GP Ibα monoclonal antibody. Fluorescence recovery within a bleached area on the cell surface was evaluated by scanning the cell surface with a low-intensity laser for 3 min after bleaching and then extrapolating the recovery values to infinite time. Fluorescence recovery in cells expressing wild-type GP Ibα was negligible. However, when only six amino acids were removed from the GP Ibα carboxyl terminus (t604 mutant, polypeptide length of 604 vs 610 residues for wild-type GP Ibα), complex mobility increased greatly, as judged by a more rapid recovery of fluorescence in the bleached area (48% recovery). The mobility increased further in the t594 mutant and remained approximately the same through the t534 mutant (55−67% recovery). A further increase in mobility was observed with the t518 mutant (>80% recovery), which lacks almost all of the GP Ibα cytoplasmic domain. The ristocetin-dependent binding of the mutant cell lines was also evaluated. Binding of vWf to cells expressing any of the mutant complexes was markedly lower than that to cells expressing the wild-type complex. These studies demonstrate that the cytoplasmic domain of GP Ibα fixes the position of the GP Ib−IX complex on the platelet surface and that this orientation is an important determinant of the complex's ability to bind vWf.

To arrest blood loss in the arterial circulation, platelets must adhere tightly to damaged blood vessel walls under conditions in which the forces of the rapidly flowing blood oppose this tendency of the platelets to adhere. Platelets are able to accomplish this difficult task through an interaction between a receptor on their surfaces, the glycoprotein (GP)[1] Ib−IX−V complex, and an adhesive ligand, von Willebrand factor (vWf), which is exposed in the subendothelium by the arterial injury. The important role played by the GP Ib−IX−V complex in this process is demonstrated by the often severe and potentially life-threatening bleeding suffered by patients with the hereditary disorder Bernard-Soulier syndrome, in whom the disorder is caused by an absent or dysfunctional complex (George & Nurden, 1994). The complex is a specialized and structurally novel adhesive receptor that is composed of four homologous polypeptide subunits, GP Ibα, GP Ibβ, GP IX, and GP V (López, 1994), each encoded by a unique gene (Lopez et al., 1987, 1988; Hickey et al., 1989; Lanza et al., 1993) and belonging to a large family of leucine-rich proteins (López, 1994).

Several aspects of the interaction of the complex with vWf have been elucidated. The sequences within the receptor that bind vWf reside in a 45 kDa region at the amino terminus of its largest polypeptide constituent, GP Ibα. This region contains the motif that identifies the polypeptide as a member of the leucine-rich motif family, and also contains a region with a high density of negative charges that is involved in binding vWf (Murata et al., 1991) and requires posttranslational sulfation of tyrosines to carry out this function (Dong et al., 1994; Ward et al., 1996). In addition to sequences directly involved in ligand binding, GP Ibα has another structural feature that may be important for its ability to bind vWf. Of the four GP Ib−IX−V complex polypeptides, it has by far the longest cytoplasmic domain (Lopez et al., 1987). This region of approximately 100 amino acids was shown to mediate association of the

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[1] Abbreviations: α-MEM, α-minimal essential medium; BSA, bovine serum albumin; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FRAP, fluorescence recovery after photobleaching; PBS, phosphate-buffered saline; GP, glycoprotein; vWf, von Willebrand factor.

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Table 1: Summary of Cell Lines Expressing GP Ibα Truncations

cell lines	amino acid changes	base changes
CHO βIX/α518	Q519 → stop	CAG → TAG
CHO βIX/α533	Q534 → stop	CAA → TAA
CHO βIX/α544	Q545 → stop	CAA → TAA
CHO βIX/α556	R557 → stop	CGA → TGA
CHO βIX/α569	W570 → stop	TGG → TAG
CHO βIX/α582	G583 → stop	GGA → TGA
CHO βIX/α594	Q595 → stop	CAG → TAG
CHO βIX/α604	Y605 → stop	TAC → TAG

complex with the cortical cytoskeleton of the platelet by directly binding actin-binding protein (ABP) (Fox, 1985; Andrews & Fox, 1991), and it has been suggested that this association may modulate ligand binding.

One possible mechanism by which the cytoskeletal association of the complex could affect ligand binding is through regulation of its ability to diffuse laterally, which, in addition to restricting movement, may maintain an ordered arrangement of complexes on the platelet surface. Precedents for such a mechanism influencing membrane protein function are numerous; many other proteins are indeed affected by their diffusion within the plasma membrane. Some of these functions require free diffusion (Jacobson et al., 1983, 1987; Benveniste et al., 1988) so that the membrane protein may encounter a cofactor, and some functions require that diffusion be limited (Golan & Veatch, 1980; Dragsten et al., 1981; Stühmer & Almers, 1982; Axelrod, 1983; Cherksey et al., 1985) so that the proteins can be present on the cell surface in the appropriate geometry to optimally bind an extracellular ligand.

In this study, we investigated the role of the GP Ibα cytoplasmic domain in the biosynthesis and membrane expression of the GP Ib−IX complex and its effect in regulating the mobility of the complex within the plasma membrane and its ability to bind vWf. We report that deletion of the GP Ibα cytoplasmic domain has no effect on expression of the complex on the cell surface but increases the mobility of the complex within the plasma membrane and decreases its ability to bind vWf.

MATERIALS AND METHODS

Glycoprotein Ibα Mutagenesis. Mutagenesis was carried out directly on the GP Ibα cDNA ligated into the *Eco*RI site of the expression vector pDX (López et al., 1992) using a series of gel-purified oligonucleotide primers containing base changes that converted selected amino acid codons to stop codons within the region encoding the GP Ibα cytoplasmic domain (Lopez et al., 1987) (Table 1). The technique for mutagenesis was that of Deng and Nickoloff (1992), with reagents from a commercially available kit (Transformer, Clontech, Palo Alto, CA). The mutations were verified by DNA sequencing; the sequencing reactions were performed using the ABI Dye terminator kit and were analyzed on an ABI model 373A automated sequencer (ABI, San Leandro, CA).

Cell Lines and Transfections. Studies of the effect of GP Ibα cytoplasmic truncation on GP Ib−IX surface expression were carried out on cells transiently expressing the truncated mutants. For these transfections, 1 μg of Zem228R plasmid containing a cDNA encoding either wild-type or truncated GP Ibα was transiently expressed in CHO βIX cells (Chinese hamster ovary cells that stably express GP Ibβ and GP IX) using liposomes as DNA carriers (LipofectAmine, Life Technologies Inc., Grand Island, NY) (López et al., 1994a; Li et al., 1995). CHO βIX cells have been described previously (López et al., 1992, 1994b). We selected these cells for expression of the two polypeptides by including methotrexate and G418 resistance markers in the expression plasmids for GP Ibβ and GP IX, respectively.

The experiments were performed in two parts. To examine the surface expression of mutants t518, t533, t544, and t556, four sets of identically prepared CHO βIX cells were transfected simultaneously with plasmids containing these mutants and expression of the mutant polypeptides on the cell surface was evaluated by flow cytometry 72 h later. Surface expression of the other mutants was evaluated similarly in separate sets of experiments. For both sets of experiments, cell surface expression of the mutants was compared to that of transiently expressed wild-type GP Ibα as a positive control; cells transfected with vector only served as a negative control. Each set of transfections was performed at least four times; the transfection efficiency was typically about 40%.

For studies of receptor mobility and ligand binding, stable cell lines were established that expressed GP Ib−IX complexes containing either wild-type GP Ibα or one of the truncated mutants. CHO βIX cells were transfected using the same technique that was used for the transient transfections. Transfectants were selected on the basis of their surface phenotype and sorted for high levels of GP Ibα surface expression by several rounds of fluorescence-activated cell sorting using a FACSsort cell sorter (Becton Dickinson, San Jose, CA) after staining them with the fluorescein isothiocyanate (FITC)-conjugated GP Ibα antibody, AN51 (DAKO, Carpinteria, CA). No drug selection was used to select for GP Ibα transfectants as expression of this polypeptide is very efficient in the presence of GP Ibβ and GP IX (López et al., 1992). The cells were grown in α-minimal essential medium (α-MEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 400 μg/mL G418, and 80 μM methotrexate (Sigma Chemical Co., St. Louis, MO) and maintained at 37 °C with 5% CO_2. Nine cell lines were thus established and named as in Table 1.

Flow Cytometry. Cells transiently expressing truncated or wild-type GP Ibα were harvested with 0.54 mM EDTA 72 h after transfection. They were then washed with phosphate-buffered saline (PBS), incubated in culture medium containing 1% bovine serum albumin (BSA, fraction V, Sigma) for 30 min to block nonspecific antibody binding, and then incubated with FITC-conjugated AN51 at a concentration of 3 μg/mL for 1 h at room temperature. After the cells were washed again twice with PBS, cell surface expression of wild-type or truncated GP Ibα was determined by flow cytometry on a FACScan flow cytometer (Becton Dickinson) after stimulating the fluorescent dye with an argon ion laser at 488 nm and collecting the light emitted above 530 nm. Nonspecific binding was determined by the background fluorescence from sham-transfected CHO βIX cells stained with the same antibody. The data were analyzed using Cellquest software from Becton Dickinson.

Immunoprecipitation and Western Blotting. Cells expressing wild-type or mutant GP Ibα polypeptides were lysed in digitonin lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 μg/mL leupeptin, 1.6 μg/mL benzamidine, 0.1 mg/mL soybean trypsin inhibitor, 1 mM phenylmethanesulfonyl

fluoride, and 1% digitonin] and centrifuged at 10000*g* for 5 min to remove debris. The lysate was incubated overnight at 4 °C with fixed *Staphylococcus aureas* cells (Pansorbin, Calbiochem, La Jolla, CA) to remove proteins that bind the dead bacteria nonspecifically. To immunoprecipitate GP IX, the monoclonal antibody SZ1 (1 μg/mL, AMAC, Westbrook, ME) was added to the lysate and the mixture was incubated for 4 h at 4 °C. Pansorbin beads that had been preequilibrated with rabbit anti-mouse IgG (5 μg/mL, 4 h) were then added to the lysate, and the mixture was incubated for 1 h. The bound protein was removed from the lysate by centrifugation at 10000*g* for 5 min. The bead pellet was then washed several times in lysis buffer by resuspension and centrifugation. Immunoprecipitated proteins were released from the beads by boiling for 5 min in SDS sample buffer containing 2% β-mercaptoethanol (final concentration). The proteins were then resolved on the basis of molecular mass by electrophoresis on 7.5% SDS−polyacrylamide gels and transferred electrophoretically to a nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the membrane in 5% nonfat milk in Tris-buffered saline [TBS, 50 mM Tris HCl (pH 7.4) and 150 mM NaCl] for 1 h at room temperature. The membrane was then probed for the presence of GP Ibα using the monoclonal antibody WM-23 (50 ng/mL in TBS, 1% nonfat milk, and 0.2% Tween-20). (WM-23 was a gift from M. C. Berndt of the Baker Medical Research Institute, Prahran, Victoria, Australia.) The antibody was detected using horseradish peroxidase-conjugated sheep anti-mouse Ig and luminol substrate with the ECL detection kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Fluorescence Recovery after Photobleaching (FRAP). Lateral diffusion of GP Ib−IX complexes containing wild-type or truncated GP Ibα was measured on a laser-based image cytometer (ACAS 570, Meridian Instruments, Okemos, MI) by observing fluorescence redistribution of FITC-conjugated AN51 after photobleaching. CHO βIX cells stably expressing wild-type or truncated GP Ibα were first placed into chamber slides (Titer-tek, Nunc Inc., Naperville, IL) at an initial confluence of 50−60%. Five hours after seeding the cells, the medium was replaced with α-MEM containing 1% BSA and no FBS to eliminate any potential effect on ligand binding from bovine vWf that might be present in the FBS. The cells were then grown in this medium overnight. For the FRAP measurement, the cells were stained with FITC−AN51 for 20 min at room temperature and then placed in an incubated chamber at 37 °C on the image cytometer. All experiments were run using the 488 nm line of the argon ion laser (Coherent, San Jose, CA), with the resulting FITC emission detected through a 530/30 band-pass filter. An initial fluorescent image of each cell was made to determine the optimal region for bleaching and to set the location of the interrogation line to monitor recovery. The interrogation line represents a plane across the cell, perpendicular to its surface, across which the monitoring laser scans at specified time intervals to detect fluorescence. On the cell surface, the interrogation line spans a straight line from one edge of the cell to the other, crossing through the center of the bleached spot. Ten prebleach line scans were made to determine a baseline fluorescence intensity profile, and scanning conditions were evaluated for nonspecific bleaching effects due to the monitoring scans. To bleach a spot on the cell surface, 100 mW of laser power was applied for 50 μs, which resulted in a bleached area 2−3 μm in diameter. The recovery of fluorescence in this spot was monitored over 3 min by scanning at 1 s intervals. With the scanning parameters employed in these experiments, fluorescence could be maintained for up to 20 min before the dye faded. We did not observe any morphological changes of the cells during or after photobleaching. This is consistent with previous reports showing that similar spot photobleaching caused no detectable damage to the cell membrane as determined by electron microscopy and trypan blue permeability tests (Jacobson et al., 1983).

For each mutant complex, 37−58 cells were scanned and analyzed. The analysis was carried out with the flat-cell method of Wade et al. (1986), where recovery curves were plotted by first subtracting background and then normalizing on a pixel-by-pixel basis to the intensity of fluorescence before bleaching. The resulting analysis curve for each cell was then plotted to determine the percent recovery of the fluorescence.

Iodination of vWf and Assay of Ligand Binding. Purified human vWf (kindly provided by M. C. Berndt) was iodinated with $Na^{125}I$ by the Iodogen method as previously described (Dong et al., 1994) and purified on a Sephadex G25 column (Pharmacia Laboratories, Piscataway, NJ). The specific activity of ^{125}I-labeled vWf was 0.31 mCi/(mg of protein). The binding of the radiolabeled ligand was assessed as previously described (Dong et al., 1994). Briefly, CHO βIX cells stably expressing wild-type or truncated GP Ibα were detached with EDTA and resuspended in Tyrode's buffer to a final concentration of 4×10^7 cells/mL. Twenty-five microliters of the cell suspension was placed into each of 10 Eppendorf tubes. ^{125}I-labeled vWf was added to duplicate sets of 5 tubes at final concentrations of 0, 1.25, 5, 10, and 15 μg/mL. A 36-fold excess of unlabeled vWf was added to one set of tubes, and the equivalent volume of Tyrode's buffer was added to the other set. Ristocetin was then added to both sets of tubes to a final concentration of 1.4 mg/mL. The final reaction volume was adjusted to 100 μL with Tyrode's buffer. The mixture was incubated at room temperature for 30 min and then layered onto a column containing a 30% sucrose solution. Membrane-bound vWf was separated from free vWf by centrifugation at 10000*g* for 5 min. The capillary tips of the column (containing the cell pellet) were cut off, and the radioactivity was counted in a γ counter. Nonspecific binding was determined from the cells incubated in excess unlabeled vWf. The binding specificity was also determined by incubating cells with AN51 (14 μg/mL) for 30 min prior to adding vWf and by incubating CHO βIX cells with ^{125}I-labeled vWf and ristocetin. All direct binding data were corrected for nonspecific binding and then normalized to correct for differences in surface levels of the complex. Typically, the cells used for binding studies were sorted for GP Ibα surface expression by FACS within 1 week of the study. Because only the top 5% of fluorescent cells were sorted, essentially the entire population was positive (always >95% of cells) for GP Ibα, as defined by gating using similarly stained parental CHO cells as negative controls. At the beginning of each binding study, one aliquot of cells was stained with FITC−AN51 to determine surface levels of GP Ibα. Mean fluorescence values for the entire cell population were compared between cells expressing the different mutants. The greatest variation between the highest and lowest values detected in all the

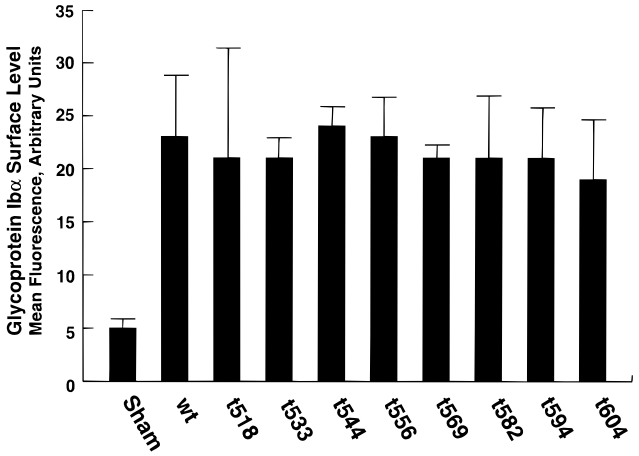

FIGURE 1: Surface membrane expression of GP Ibα mutants transiently expressed in CHO βIX cells. Plasmids containing cDNAs encoding wild-type or mutant GP Ibα polypeptides were transfected into CHO βIX cells, and the expression of the polypeptides on the cell membrane was evaluated 72 h later by flow cytometry using the monoclonal antibody AN51. Surface levels are expressed in arbitrary fluorescence units. Four sets of identical experiments were averaged to yield these results. Statistical analysis revealed no significant differences in surface levels of GP Ibα between any of the mutants and wild-type GP Ibα.

experiments was 20%. To correct for the effect of different GP Ibα levels on vWf binding, the cell line with the greatest fluorescence was arbitrarily designated as 100% and the binding values from the other cell lines were normalized to 100% on the basis of their relative surface levels.

Statistics. Statistical analysis used in this study included Student's *t* test and linear regression analysis.

RESULTS

Effect of GP Ibα Cytoplasmic Truncation on Cell Surface Expression. We evaluated the effect of truncating the GP Ibα cytoplasmic domain on plasma membrane expression of the GP Ib−IX complex by transiently expressing the mutant polypeptides in cells that constitutively express GP Ibβ and GP IX (CHO βIX cells). Surface expression was assessed by flow cytometry after labeling the cells with the GP Ibα monoclonal antibody AN51. The surface levels of the GP Ibα mutants were similar to each other and to that of wild-type GP Ibα, indicating that truncation of its cytoplasmic domain did not impair the ability of GP Ibα to reach the cell surface (Figure 1). Because we earlier had shown that efficient surface expression of the complex required the three GP Ib−IX complex subunits (López et al., 1992), we expected that the normal surface levels of mutant complexes were an indication that the truncated GP Ibα polypeptides complexed normally with GP Ibβ and GP IX. This was confirmed by immunoprecipitation studies of cells expressing the mutant complexes. When the GP IX antibody SZ1 was used to immunoprecipitate GP IX from lysates of cells expressing GP Ibα mutants t604, t556, and t518, the mutant GP Ibα polypeptides coprecipitated, as detected by Western blotting with the GP Ibα antibody WM-23 (Figure 2).

Effect of GP Ibα Cytoplasmic Truncation on the Lateral Diffusion of the GP Ib−IX Complex. Because of the known association of the GP Ib−IX complex with the platelet cortical cytoskeleton (Fox, 1985; Andrews & Fox, 1991, 1992; Aakhus et al., 1992), we used the technique of

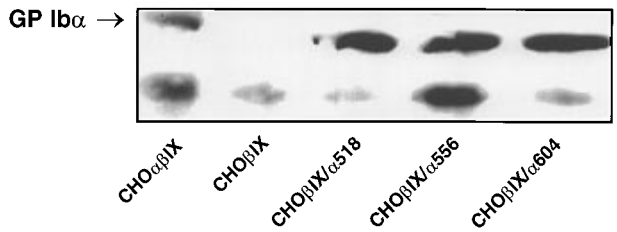

FIGURE 2: Truncated GP Ibα species associate with GP Ibβ and GP IX. Immunoprecipitations were performed with the monoclonal GP IX antibody SZ1 on lysates from cells expressing the GP Ib−IX complex with wild-type GP Ibα ($\alpha\beta$IX) or with one of three truncation mutants, t518, t556, or t604 (cell lines CHO βIX/α518, CHO βIX/α556, and CHO βIX/α604, respectively), or from control CHO βIX cells without GP Ibα. After electrophoresis of the immunoprecipitates on SDS−polyacrylamide gels, the proteins were transferred to nitrocellulose membranes and detected with the anti-GP Ibα antibody WM-23. All lanes contain a second band, which represents the IgG heavy chain. The different intensities likely represent different amounts of secondary antibody eluted from the beads by the boiling procedure.

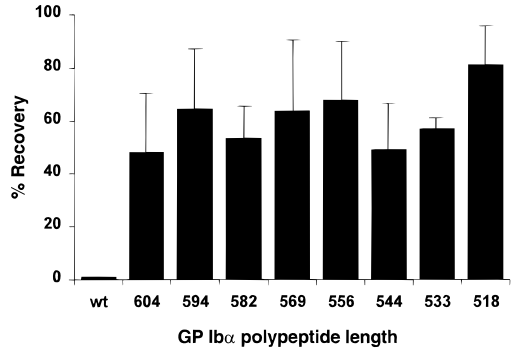

FIGURE 3: Fluorescence recovery after photobleaching in cells expressing wild-type GP Ibα or mutants with truncated cytoplasmic domains. The cells were labeled with FITC−AN51 and bleached with a 100 mW laser, and recovery of fluorescence within the bleached area was determined by repeated scanning with a low-intensity laser. The percentage recovery indicates the extent to which the fluorescence has recovered compared to prebleach fluorescence values for the area bleached. The data represent means and standard deviations from 37−58 cells in each group.

fluorescence recovery after photobleaching (FRAP) to determine if this association affects the complex's lateral mobility within the plasma membrane. CHO βIX cells expressing wild-type or mutant GP Ibα were grown on chamber slides and stained with FITC−AN51. Individual fluorescent cells were then chosen on the basis of their staining characteristics, and a 2−3 μm diameter spot was bleached on the surface of each cell with 488 nm laser light. Using 100 mW of laser energy, the efficiency of bleaching ranged from 37 to 86%, but only those cells bleached to 50−60% were analyzed further, to avoid artifacts induced by overheating in cells bleached to a greater extent. The fluorescence of the bleached spot recovered to various extents in all of the mutants, whereas no recovery was observed in cells expressing wild-type GP Ibα (Figure 3). Linear regression analysis revealed an inverse correlation between the extent of recovery and the length of the GP Ibα cytoplasmic tail ($r = -0.7143$, $n = 8$, and $p < 0.001$). Analysis of the individual cell lines revealed two regions of mobility. Fluorescence recovery increased from 0% in cells expressing wild-type GP Ibα to between 48 and 69% in the cells lines with GP Ibα truncations of 6−67 amino acids (CHO βIX/α604 − CHO βIX/α533 cells). Fluorescence recovery increased further in CHO βIX/α518 cells which,

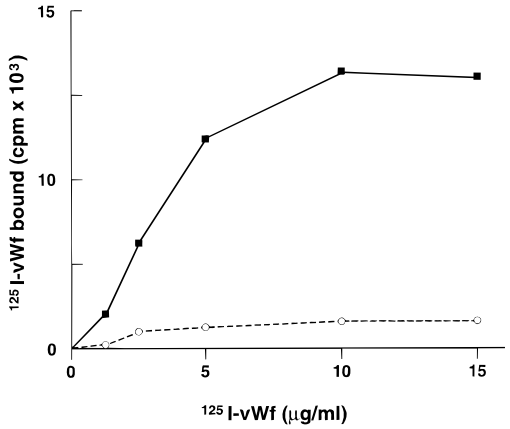

FIGURE 4: Binding of ^{125}I-labeled vWf to cells expressing wild-type GP Ib−IX or mutant complexes. ^{125}I-labeled vWf [0.31 mCi/(mg of protein)] was incubated with cells at room temperature for 30 min in the presence of ristocetin (1.4 mg/mL). Membrane-bound radioactivity was counted after the cells were spun through a sucrose cushion, and specific binding was determined by subtracting the counts from [^{125}I]vWf nonspecifically bound in the presence of excess unlabeled vWf: (■) values for wild-type cells and (○) average values for all of the cell lines expressing truncated receptors.

on average, recovered 81% of their initial fluorescence intensity (Figure 3).

Cytoplasmic Truncation and Ristocetin-Induced vWf Binding. The effect of GP Ibα cytoplasmic truncation on the ligand-binding function of the GP Ib−IX complex was determined by comparing the ristocetin-induced vWf binding of cells expressing truncated GP Ibα to that of cells expressing wild-type GP Ibα. Aliquots of 1×10^6 cells were incubated with ^{125}I-labeled vWf at concentrations ranging from 1.25 to 15 μg/mL in the presence of 1.4 mg/mL ristocetin. The incubations were carried out in the presence or absence of excess unlabeled vWf. After the background counts were subtracted, the specific binding for each cell line was normalized on the basis of the mean surface level of GP Ibα in that population as determined by flow cytometry. The binding reached a plateau at a vWf concentration of 10 μg/mL in cells expressing wild-type GP Ibα (Figure 4). In contrast, while the vWf binding of the mutant cell lines was above background, each mutant cell line bound far less vWf than did the wild type. The differences in binding between the individual mutant lines were not statistically significant.

DISCUSSION

In this study, we evaluated the role of the GP Ibα cytoplasmic domain in several aspects of GP Ib−IX complex biology: its synthesis and surface expression, its mobility within the plasma membrane, and its ability to bind its major ligand, vWf. These studies were carried out on heterologous cells that express the GP Ib−IX complex, containing either wild-type GP Ibα or mutant GP Ibα polypeptides missing different amounts of the cytoplasmic domain. The longest mutant lacked only six amino acids from the carboxyl terminus; the shortest was missing almost the entire cytoplasmic domain (Table 1). Because our planned experiments required mutant polypeptides to be expressed on the cell surface, we first examined the effect of these truncations on surface expression of the complex. Even those truncations that removed virtually the entire cytoplasmic domain failed to reduce surface expression, unlike what has been found with other membrane proteins (Rose & Bergmann, 1983; Wills et al., 1984; Lehrman et al., 1985; Doyle et al., 1985; Griffith et al., 1988).

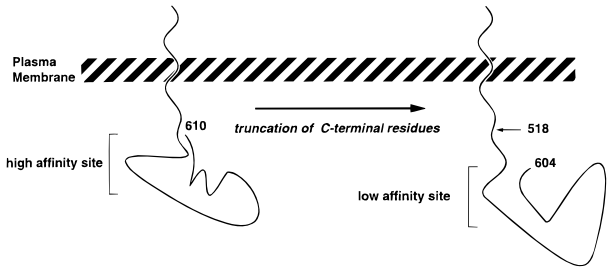

FIGURE 5: One-site model for explaining the mobility data. A mechanism such as the one depicted could account for the molecular mobility data if one site in the GP Ibα cytoplasmic domain is bound to the cytoskeleton but its affinity is regulated by distant sites. In this model, truncation of the six carboxyl-terminal amino acids would change the affinity of the cytoskeleton-binding site and increase the lateral mobility of the complex. Truncations that remove the actual binding site would remove all constraints to diffusion and increase mobility further. Alternative explanations appear in the text.

A delay in the synthesis or intracellular transit of GP Ibα would have affected formation of the complex and its expression on the cell surface. Earlier studies from our laboratory indicate that the three GP Ib−IX subunits are required for efficient surface expression of the complex (López et al., 1992), with GP Ibβ serving as a link between GP Ibα and GP IX (López et al., 1994a). Thus, because they appeared on the cell surface at normal levels, we expected that the mutant GP Ibα polypeptides associated normally with GP Ibβ and GP IX. This association was clearly demonstrated by immunoprecipitation studies in which truncated GP Ibα polypeptides were co-immunoprecipitated with a GP IX antibody.

In contrast to their lack of effect on membrane expression of the GP Ib−IX complex, truncations of the GP Ibα cytoplasmic domain dramatically increased the mobility of the complex within the plasma membrane. FRAP studies identified two regions of the GP Ibα cytoplasmic domain that regulate complex mobility. The first region is disrupted by truncation of the six carboxyl-terminal residues, resulting in the increased mobility of the complex. The mobilities of complexes with polypeptide lengths between 604 and 533 residues were similar; mobility increased further when the polypeptide was shortened to 518 amino acids (Figure 3).

What accounts for this somewhat complex mobility pattern? One possible explanation is depicted in Figure 5. In this model, the cytoplasmic domain of GP Ibα interacts with only one cytoskeletal element that constrains complex mobility (presumably ABP) through sequences within the central region of the cytoplasmic domain (Andrews & Fox, 1992) and the affinity of the interaction is modulated by sequences at the carboxyl terminus. Truncation of the carboxyl terminus might propagate a conformational change along the polypeptide that decreases the affinity of the binding site but fails to abolish binding. This would be reflected in the FRAP studies as an increase in mobility. A truncation that removes the binding region, such as the one that produces mutant t518, would completely remove the binding site and further increase mobility.

An alternative explanation for our findings is that the cytoplasmic domain interacts with more than one cytoplasmic

element capable of regulating mobility, a scenario that might explain why the interaction of purified ABP with purified GP Ib−IX on beads displayed a much lower affinity than expected on the basis of observations of the tight association of GP Ib−IX with the cytoskeleton in detergent lysates (Andrews & Fox, 1991). The 14-3-3 ζ isoform was recently shown to bind to the cytoplasmic domain of GP Ibα, within the 15 C-terminal amino acids (Du et al., 1994, 1996). This protein could also limit complex mobility, by cross-linking complexes or by connecting the complex to a scaffold of other molecules. Consistent with this possibility, recent data suggest that 14-3-3 ζ can act as an adapter capable of serving as an intermediary between two or more proteins (Vincenz & Dixit, 1996).

The third conclusion from our studies was that the GP Ibα cytoplasmic domain plays a major role in determining the ability of the receptor to bind vWf. Despite expressing equivalent levels of the complex on their surfaces, cells expressing the mutant complexes bound markedly less vWf than did cells expressing the wild-type complex at vWf concentrations at which binding to the wild-type receptor approached saturation (Figure 4). Because vWf exists as a multimer of 250 kDa subunits and each subunit contains an A1 domain (the region through which vWf binds the GP Ib−IX complex), these findings suggest that the affinity of the interaction may be related to the arrangement of the complex in a fixed array on the cell surface where one multimeric vWf molecule might interact with several GP Ib−IX complexes.

Alternatively, cytoplasmic truncation may also decrease vWf binding by preventing the GP Ib−IX complex from forming higher-order complexes, which appear to be the functional units on the cell surface (López, 1994; Li et al., 1995; Dong et al., 1997). A complex containing two or more GP Ibα polypeptides would be able to bind to vWf better because the disulfide bonds involved in vWf multimer formation juxtapose two A1 domains (Lyons & Ginsburg, 1994). Consistent with this explanation is the recent finding that a snake-venom protein, 50 kDa alboaggregin, with two binding sites for GP Ibα was able to activate platelets while similar monovalent venom proteins bound GP Ibα but did not activate the platelets (Andrews et al., 1996).

We should note that, in a recent report, Cunningham et al. (1996) reported finding no differences in botrocetin-induced vWf binding between a GP Ibα truncation mutant (truncated after 544) expressed in CHO cells and wild-type GP Ibα expressed in melanoma cells. The reasons for the differences in the two studies are not clear, but several possibilities come to mind. The most obvious is the fact that different modulators were used to induce vWf binding, ristocetin in one study and botrocetin in the other. The exact mechanisms by which these modulators induce vWf binding have not been worked out, although it is clear that certain blocking reagents and mutations can differentiate between the two (Murata et al., 1991; Ward & Berndt, 1995). A second possible source of the discrepancy is the cell lines used. Their study compared binding of the wild-type and mutant polypeptides expressed in different cell lines, which could have several effects on binding unrelated to properties of the polypeptides themselves.

These differences notwithstanding, it is clear that the cytoplasmic domain of GP Ibα, for which no homologous domains have been found, is vital for anchoring the GP Ib−IX−V complex so that its mobility is restricted on the cell membrane and it is in the optimal conformation to bind vWf. Further studies of this region, and of the cytoplasmic domains of the other polypeptides of the complex, are sure to lead to a better understanding of complex synthesis and function.

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