The glycoprotein (Gp) Ib-IX-V complex is essential for platelet-mediated hemostasis and thrombosis. The cytoplasmic domain of its largest polypeptide subunit GpIbα possesses a binding region for filamin A, which links GpIb-IX-V to the platelet cytoskeleton. There is evidence that filamin A binding to GpIbα directs the surface expression of GpIb-IX. To investigate the mechanism of this effect, we examined GpIbα biosynthesis in Chinese hamster ovary (CHO) cells stably co-expressing wild-type or mutant GpIbα with GpIbβ, GpIX with and without filamin A. We observed that surface GpIb expression is enhanced in CHO cells co-expressing human filamin A. In comparison with cells expressing only GpIbα, GpIbβ, and GpIX (CHO-GpIbβ/GpIX), lysates from CHO-GpIbα/GpIX + filamin A-expressing cells showed greater amounts of immature, incompletely O-glycosylated and fully mature GpIbα, but lesser amounts of the ~15-kDa C-terminal peptide released when the extracellular domain of GpIbα is cleaved by proteases. When filamin A binding is eliminated by truncation of GpIbα at C-terminal residue 557 or by a deletion between amino acids 560–570, the decreased synthesis of mature GpIbα is accompanied by decreased immature GpIbα and by an increased immunodetectable C-terminal peptide. The synthesis of mature GpIbα in CHO-GpIbα/GpIX cells is eliminated by brefeldin A (which inhibits transport out of the endoplasmic reticulum (ER)) and restored by lactacystin (which inhibits proteasomal degradation). These results suggest that GpIbα binds to filamin A within the ER and that filamin A binding directs post-ER trafficking of GpIbα to the cell surface.

The platelet glycoprotein (Gp) Ib-IX-V complex is essential for von Willebrand factor-dependent platelet adherence and aggregation in the microvasculature and under conditions of pathologically elevated arterial wall shear rates (1–3). It is aggregation in the microvasculature and under conditions of pathologically elevated shear stresses. In addition, the 96 amino acid cytoplasmic domain of GpIbα forms non-covalent bonds with the protein filamin A, and the GpIbα/filamin A interaction links the GpIb-IX-V complex to the platelet cytoskeleton.

There is human genetic evidence that an abnormality in the expression of GpIbα, GpIbβ or GpIX, but not GpV, eliminates complex formation, resulting in the human bleeding disorder Bernard-Soulier syndrome (5). In vitro analyses establish that complex assembly occurs in the endoplasmic reticulum (ER) and that proper assembly serves to optimize GpIb-IX-V complex trafficking, including glycosylation (6, 7). Further, in addition, it appears that intact complex assembly and proper trafficking and glycosylation through the ER and Golgi compartments are required for rendering the juxtamembranous extracellular region of GpIbα less susceptible to proteolysis, possibly because its covalent interaction with GpIbβ or its mucin-rich macroglycopeptide domain offers protection from degradation by extracellular proteases such as trypsin and calpain (8).

During the course of experiments using genetically engineered Chinese hamster ovary (CHO) cells co-expressing wild-type and mutant human GpIbα with wild-type human GpIbβ, GpIX, and filamin A, we observed that perturbations of the interaction between the cytoplasmic domain of GpIbα and its cytoskeletal partner filamin A affected surface expression of the GpIb-IX complex (9). This observation was similar to that made by Meyer et al. (10) using paired recombinant wild-type GpIb-IX/mutant filamin A or mutant GpIb-IX/full-length filamin A in filamin-deficient cells. In considering the mechanism of this effect, we hypothesized that filamin A binding to GpIbα regulates post-translational complex assembly and trafficking. Such a hypothesis is consistent with the results of two studies examining heterologous cell synthesis of recombinant GpIbα carrying a dinucleotide deletion in its transmembranous domain that, in humans, causes Bernard-Soulier syndrome (BSS) (11, 12). This mutation, which results in shortening of the C-terminal cytoplasmic domain of GpIbα from 96 to 58 amino acids and eliminates its filamin A binding domain, prevents GpIbα from being expressed on the cell surface. Deficient “anchoring” of GpIbα to the cytoskeleton is considered to be a mechanism for this unique BSS phenotype. To investigate how the anchoring of GpIbα to filamin A regulates GpIb-IX complex assembly and surface expression, we examined the biosynthesis of wild-type and mutant human GpIbα in CHO cells co-expressing recombinant human GpIbβ, GpIX, and filamin A.
MATERIALS AND METHODS

Antibodies and Reagents—The antibody AN51, a monoclonal mouse IgG2a anti-human platelet GpIb/H9251 antibody that recognizes the ligand-binding domain was from DAKO (Carpinteria, CA). WM23, a mouse monoclonal antibody that recognizes the macroglycopeptide domain of GpIb/H9251 was kindly provided by Dr. Michael C. Berndt (Monash University, Victoria, Australia). Anti-GpIb-C, a rabbit anti-peptide antibody corresponding to the C-terminal 15 residues of GpIb/H9251 was kindly provided by Dr. Xiaoping Du (University of Illinois, Chicago, IL). Recombinant human filamin A cDNA in PREP4 vector was a gift from Dr. John Hartwig (Brigham and Women’s Hospital, Boston, MA). Recombinant human GpIb, GpIbβ, and GpIX were kindly provided by Dr. J. A. López (Baylor College of Medicine, Houston, TX). Brefeldin A, NH4Cl, leupeptin, chloroquine, aprotinin, phenylmethylsulfonyl fluoride, pepstatin A, EDTA, endoglycosidase H, and dimethyl sulfoxide were purchased from Sigma. Lactacystin and endoglycosidase D were from Calbiochem. The silver staining kit, ECL reagents, anti-mouse or anti-rabbit IgG peroxidase-conjugated antibodies, protein A-Sepharose CL-4B, and rainbow molecular weight standards were from Amersham Biosciences. Minimum essential medium α, fetal bovine serum, penicillin-streptomycin, HT supplement, hygromycin B, Zeocin™, pcDNA™3.1/Zeo, and PCR superMix were from Invitrogen.

Cell Culture and Transfections—CHO cells were maintained in minimum essential medium α containing 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 1× HT supplement at 37 °C with 5% CO2. Cells were transfected with the cDNA coding for GpIbβ, GpIX, GpIbα, or filamin A using Lipofectamine. Stable clonal cell lines (CHO-GpIbβIX, CHO-GpIbα/βIX, CHO-GpIbα/βIX + filamin A) were established by selection in hygromycin B, Zeocin™, and G418, followed by limiting dilution. Levels of recombinant protein expression in the cell lines were measured by flow cytometry and Western blotting. The C-terminal mutations of GpIbα (the truncation at residue 594 or 557, the deletion between residues 560–570, and site-directed mutagenesis from residues RGS 557–559 to AGA 557–559) were constructed as described previously (9).

Protein Immunoprecipitation, Separation, and Western Blot—Transfected CHO cells were washed twice with ice-cold PBS, detached in PBS containing 0.5% EDTA, and lysed during a 20-min incubation at 4 °C in lysis buffer containing protease inhibitors (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, pH 8.0, 1 mM Na3VO4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin). Samples were then sonicated briefly (~5 s), cleared of insoluble debris by centrifugation at 14,000 rpm for 5 min at 4 °C, and diluted with the same volume of ice-cold PBS to bring the final Triton X-100 concentration to 0.5%. GpIbα was immunoprecipitated by incubating the samples with 4 μg of mAb AN51 and 40 μl of Sepharose-conjugated protein A overnight at 4 °C. Following three washes with ice-cold PBS containing protease inhibitors, precipitated proteins were used for treatment of endoglycosidases or resuspended in 50 μl of sample buffer, heated for 5 min at 95 °C. Equal amounts of proteins were loaded and separated by 7 or 12% SDS-PAGE under reducing conditions, and visualized by either

FIG. 1. Recombinant filamin A enhances the expression of GpIbα in CHO cells stably transfected with GpIb-IX. A, shows that filamin A co-immunoprecipitated with GpIbα is detectable by silver staining only in CHO-GpIbα/βIX cells transfected with the filamin A cDNA. B, shows that surface GpIbα expression is enhanced in CHO-GpIbα/βIX cells co-expressing filamin A. The data are representative of results from two separate experiments.
silver staining or Western blotting. For Western blotting, proteins were transferred onto nitrocellulose membranes, blocked with 5% milk in Tris-buffered saline with Tween 20 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), (TBST) for 1 h, incubated with primary antibodies WM23 or anti-GpIb/H9251-C overnight at 4 °C, washed in TBST, and then followed by a 1-h incubation at room temperature with the appropriate peroxidase-conjugated secondary antibodies. Reactive bands were visualized by chemiluminescence.

Endoglycosidase Treatment—GpIb/H9251/H9252 IX-transfected CHO cells were lysed and immunoprecipitated with antibody AN51. The precipitated proteins were resuspended in 50 mM sodium citrate, pH 5.5, and heated for 5 min at 95 °C. The samples were then incubated with endoglycosidase H or endoglycosidase D at a final concentration of 0.4 unit/ml enzyme for 12 h at 37 °C. Following the treatment, proteins were boiled again in sample buffer, separated by 7% SDS-PAGE, and blotted with antibody WM23.

Flow Cytometry—The expression of the GpIbα/βIX complex on the cell surface was analyzed by flow cytometry. To identify GpIbα, cells were washed with PBS, detached in EDTA, and incubated with 1 g/ml fluorescein isothiocyanate-conjugated AN51.

Data Analysis—The quantitation of Western blot signals was made using a LKB Bromma Ultrascan XL enhanced laser densitometer (Amersham Biosciences). Paired statistical analyses of data were performed using Sigma Plot software.

RESULTS

Hamsters express filamin, but its primary structure has not been elucidated, and it does not cross-react with the antibody we use to detect human filamin A (9). To optimize our heterologous system for studying the biology of platelet GpIbα binding to filamin A, we engineered CHO cells to stably co-express human GpIbα, GpIbβ, GpIX, and filamin A. Fig. 1A shows that silver staining reports recombinant filamin A co-precipitating with GpIbα in CHO cells expressing the four polypeptides.
(CHO-GpIbα/βIX + filamin A) but that there is no detectable endogenous filamin band that co-immunoprecipitates with GpIbα in equal volumes of lysate from cells not expressing recombinant human filamin A (CHO-GpIbα/βIX). Fig. 1B shows that the surface expression of GpIbα, reported as fluorescence by flow cytometry, is nearly 1 log-order increased when filamin A is co-expressed with GpIbα, GpIbβ, and GpIX in CHO cells. The magnitude of the filamin A effect shown in Fig. 1B is almost identical to that previously reported by Meyer et al. (10) using GpIb-IX co-transfected with human filamin in a filamin-deficient melanoma cell line.

The mechanism of the effect of filamin A on GpIbα is not understood, although it has been attributed to an indirect effect not requiring its binding to GpIbα (10). An alternative explanation, that filamin A binding regulates GpIb-IX complex trafficking, is also plausible, however, as published work has demonstrated an effect of filamin on the trafficking of furin (13), dopamine receptors (14), and opioid receptors (15). To explore this possibility, we examined lysates from CHO-GpIbα/βIX cells without and with co-expressed filamin A for differences in the electrophoretic mobility of GpIbα detected by immunoblotting. Fig. 2A shows that lysates from CHO cells expressing filamin A with GpIb-IX have four bands that blot with the monoclonal antibody WM23 that recognizes the macroglycopeptide region of GpIbα. Based on previous work on GpIb-IX biosynthesis using pulse-chase methods, we deduce that the bands represent the following: 65 kDa, "immature" GpIbα (partially N-glycosylated but without any O-glycosylation); 85 kDa, partially O-glycosylated; 105 kDa, nearly fully O-glycosylated; 135 kDa, fully glycosylated mature GpIbα (6–8). To confirm the validity of these deductions, CHO-GpIbα/βIX lysates were subjected to endoglycosidase treatment before separations and immunoblotting. Fig. 2B shows that recombinant GpIb-IX from CHO cells without human filamin A is susceptible to digestion with endoglycosidase H, which cleaves high mannose-containing N-glycosidic bonds from polypeptides residing in the ER or ER/pre-cis Golgi compartment. In contrast, there was no cleavage of GpIbα from lysates digested with endoglycosidase D, which cleaves maturing N-glycosylated polypeptides containing five mannose residues after processing is the cis- or trans-Golgi apparatus (data not shown). These results prove that GpIbα from CHO cells without co-expressed filamin A resides mainly in the ER and that the 65 kDa band prominently displayed in the immunoblots from CHO-GpIbα/βIX cells + filamin A reports an immature form of GpIbα that is partially N-glycosylated (7).

To prove that results in Fig. 2 represent changes in GpIbα, rather than changes in antibody binding separate from GpIbα maturation, we examined lysates from CHO-GpIbα/βIX without and with co-expressed filamin A using an antibody that specifically recognizes the C-terminal 15 amino acids in the cytoplasmic domain of GpIbα (16). Fig. 3A shows that this anti-GpIbα C terminus antibody weakly reports mature and immature GpIbα and strongly reports a band of ~15 kDa. Fig. 3B shows that the 15-kDa band is relatively increased in lysates from CHO-GpIbα/βIX cells without human filamin A. Based on this molecular weight, which is calculated to approximately equal the molecular weight of the 125 amino acid fragment, containing the cytoplasmic, transmembranous and a short extracellular stub, of GpIbα released by proteolytic digestion of reduced GpIbα (17), we conclude that this 15-kDa band reports a C-terminal fragment of GpIbα produced by the degradation of GpIbα.

To buttress results in Figs. 1–3 suggesting that filamin A binding to GpIbα affects surface expression by directing trafficking to the Golgi and away from a degradative pathway, we...
turned to additional genetic and pharmacological approaches. Fig. 4 shows the results of experiments using the genetic approach, in which the processing of GpIb/H9251 was examined by low exposure immunoblotting in CHO cells co-expressing filamin A and GpIb/H9251/H9252 IX rendered incapable of binding filamin because of either a truncation or a deletion in the cytoplasmic domain of GpIb/H9251. Fig. 4A shows that a truncation at residue 557 (which eliminates filamin binding) but not at residue 594 (which has no effect on filamin binding) decreases the expression of mature and immature GpIb/H9251 in CHO-GpIb/H9251/H9252 IX filamin cell lysates. Of note, the quantity of the immature polypeptide is decreased further than the quantity of the mature GpIb/H9251, and the quantity of both polypeptides begins to approach that observed in CHO-GpIb/H9251/H9252 IX cells without co-expressed filamin A.

Because the truncation mutants do not bind the C-terminus antibody used to identify the 15-kDa degradation fragment shown in Fig. 3, we used two alternative mutants to corroborate data in Fig. 4A and to examine the theory that filamin A binding to GpIb/H9251 directs its trafficking away from a degradative pathway. Fig. 4B shows normal processing when CHO-GpIb/H9251 + filamin A cells express substitutions in the primary filamin binding domain of GpIb/H9251 that do not affect filamin A binding (m557–559). In contrast, when filamin A binding is eliminated by deleting C-terminal residues 560–570 (d560–570), there is decreased mature and immature GpIb/H9251 and an increase in its 15-kDa C-terminal fragment.

Results of Figs. 1–4 support the hypothesis that filamin A binding to GpIb/H9251 occurs in the ER and stabilizes nascent GpIb/H9251 trafficking to the cell surface. To cross-examine this hypothesis from an alternative perspective, we performed a series of experiments using pharmacological probes for ER to Golgi transit (brefeldin A, Ref. 18), post-ER lysosomal degradation (leupeptin/\(\text{NH}_4\text{Cl}\), Refs. 6 and 7), and ER-directed proteasomal degradation (lactacystin, Refs. 19 and 20) using cells in which trafficking is compromised because of absent human filamin A co-expression (CHO-GpIb/H9251/H9252 IX cells). In these experiments, cells deprived of serum for 24 h were treated simultaneously for 6 h with serum plus the pharmacological probe. Fig. 5 shows that brefeldin A eliminates mature GpIb/H9251 synthesis and enhances its breakdown to the 15-kDa C-terminal fragment. The proteasome inhibitor lactacystin enhances mature GpIb/H9251 synthesis but has no measurable effect
on the amount of the immunodetectable C-terminal 15-kDa fragment. Consistent with previously reported data using pulse-chase methodology, the combination of leupeptin/NH\(_4\)Cl has little to no effect on the maturation or degradation of GpIb\(_\alpha\) (6). These results are consistent with the conclusion that the proteasome is the major route of degradation for uncomplexed GpIb\(_\alpha\) (19, 20).

**DISCUSSION**

The biosynthesis of megakaryocyte GpIb-IX-V is required for the production of functional platelets. BSS is caused by a genetic defect leading to decreased production of GpIb\(_\alpha\), GpIb\(_\beta\), or GpIX. Although BSS is rarely encountered, research on persons affected by BSS has provided important insights into the biology of the GpIb-IX-V complex. Among the ~100 families worldwide with members suffering from BSS, there are two families with unique mutations affecting the capacity of the cytoplasmic domain of GpIb\(_\alpha\) to anchor to filamin connected to the cortical skeleton and cytoskeleton (11, 12). These mutations cause frame shifts that affect the hydrophobicity of the transmembranous domain and introduce a stop codon that truncates the cytoplasmic domain by 38 residues, thereby eliminating filamin A binding. This results in greatly decreased surface expression of GpIb\(_\alpha\). Although it has been reasonably hypothesized that decreased surface expression results from either unstable plasma membrane insertion, poor cytoskeletal anchoring, and/or increased susceptibility to proteolysis, none of these effects adequately explains why there was strikingly decreased total synthesis of GpIb-IX when these mutant polypeptides were co-expressed with GpIb\(_\beta\) and GpIX in CHO cells (11, 12). We now present data indicating that one explanation for the decreased synthesis of GpIb\(_\alpha\) in these BSS variants is that they lack an essential component for normal post-translational trafficking, filamin A binding. Our data support the hypothesis that GpIb-IX is directed toward a productive biosynthetic pathway by filamin A binding to the cytoplasmic domain of GpIb\(_\alpha\) in the endoplasmic reticulum.

These results are consistent with our (9) observations and the observations of others (10) that the co-expression of recombinant filamin A with recombinant GpIb\(_\alpha\), GpIb\(_\beta\), and GpIX increases the quantity of immunodetectable GpIb-IX in heterologous cells. Our results, however, are in disagreement with the conclusion suggested by Meyer et al. (10) that the effect of filamin A “does not appear to involve . . . direct interaction with receptors.” Data presented in Fig. 4 indicate that only mutations that specifically affect filamin A/GpIb\(_\alpha\) interactions, without gross disturbances in secondary structure or disrupting the binding of a 14-3-3\(\zeta\) adapter protein to GpIb\(_\alpha\), prevent proper trafficking of nascent GpIb\(_\alpha\) through the ER and Golgi. The effect is not absolute, however, and it is likely that measurements of mature GpIb\(_\alpha\) using flow cytometry and immunoblotting, such as were employed by Meyer et al. (10), could overlook the relative decrease in GpIb\(_\alpha\) biosynthesis resulting in the mature form.
from disrupting the filamin A/GpIbα interaction. The persistent biosynthesis of mature GpIbα in the absence of GpIbα/filamin A interactions is evidence that there are redundant pathways and multiple chaperones involved in the trafficking of nascent GpIbα from the ER to the Golgi to the plasma membrane, only one of which is modulated by filamin A binding.

In contrast to that observed with mature GpIbα, the expression of immature 65-kDa GpIbα is greatly decreased in cells expressing mutant GpIbα incapable of binding to filamin A (Figs. 2–4). This observation provides an important clue about where the filamin A/GpIbα interaction is established and how it directs trafficking. Fig. 2B demonstrates that the 65-kDa immature polypeptide appears identical to the 65-kDa protein created when mature GpIbα is subjected to deglycosylation with endoglycosidase H, which removes N-linked high mannose sugar residues from the ER compartment. The absence of this band from CHO cell lysates in which GpIbα/filamin A interactions are disrupted supports the conclusion that filamin A binds GpIbα in the ER and stabilizes it such that it undergoes normal N-linked glycosylation. Not all GpIbα is “held” in the ER by filamin A binding, and some normal processing occurs, but the majority of immature GpIbα molecules not bound to filamin A are either rapidly degraded and/or moved centrifugally into the Golgi and then onto the plasma membrane.

The degradation of uncomplexed GpIbα is not very well understood, although there is pharmacologic evidence for a minor lysosomal-mediated pathway (6) and immunofluorescence evidence for a major ER-mediated pathway (7). Because our experimental system shows increased amounts of a 15-kDa C-terminal GpIbα peptide from cells in which the filamin A/GpIbα interaction is perturbed, we considered it to be a marker useful for exploring the routes by which uncomplexed GpIbα is degraded. Not surprisingly, we observed that brefeldin A mimics (and exaggerates) the effects of disrupting filamin A/GpIbα interactions, leading to almost no synthesis of mature GpIbα and to a nearly 2-fold increase in the quantity of the 15-kDa C-terminal peptide in CHO-GpIbα/βIX cells (Fig. 5). These results are consistent with the hypothesis that GpIbα retained by the ER is shunted to a degradative pathway. To identify this degradative pathway, we examined how specific inhibitors of lysosomal proteases or proteasomal proteases affect the quantities of immunoreactive mature GpIbα and its C-terminal peptide fragment. We observed that only proteasomal inhibition restored the productive synthesis of GpIbα in CHO-GpIbα/βIX cells without filamin A. Somewhat surprisingly, however, we found that proteasomal inhibition had no measurable effect on the amount of C-terminal peptide (Fig. 5). This result indicates that the majority of the 15-kDa C-terminal peptide is generated by GpIbα degradation outside of the lysosomal and proteasomal compartments. It is likely that it derives from degradation of cell surface-expressed GpIbα rendered more susceptible to extracellular proteases because it has lost its cytoskeletal tether. Such an effect accounts, in part, for the decreased expression of GpIbα in BSS variants caused by the 38 amino acid GpIbα C-terminal truncation (11, 12) and by a single amino acid substitution (Asn-64→Thr) in the extracellular domain of GpIbβ (8).

In conclusion, we have provided evidence that the biosynthesis of GpIb-IX is enhanced by GpIbα binding to filamin A. We have also provided evidence that uncomplexed GpIbα is retained by the ER and undergoes ER-directed proteasomal degradation. Finally, we have provided additional evidence that some GpIbα not bound to filamin is directed to the cell surface but that GpIb-IX without its cytoskeletal connections may be more susceptible to extracellular proteolysis. Although novel, these results are not entirely unexpected, as there are data that other proteins are chaperoned toward productive biosynthesis by filamin binding (13–15), other unprocessed glycoproteins undergo ER-directed proteasomal degradation (21, 22), and uncomplexed GpIbα expressed on the cell surface is particularly vulnerable to extracellular proteases (8). Nonetheless, these results are useful not only because they elucidate mechanisms underlying the cell biology of megakaryocytes or the pathophysiology of Bernard-Soulier syndrome, but because they may someday facilitate organizing and optimizing an approach to cell engineering or gene therapy aimed at manipulating or restoring platelet function for therapeutic purposes (23, 24).

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