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from the affected kindred, the ultimate proof of their role in the disorder requires that the biosynthetic defect apparent in the platelets of these patients be reconstituted by expression of the mutant in vitro. This was the goal of the studies reported here.

MATERIALS AND METHODS

Cell lines. Two stable cell lines were used in the current studies; both have been described previously. CHO βIX cells are Chinese hamster ovary cells in which expression of GP Ibβ and GP IX has been amplified by growth in methotrexate-containing media. CHO αβn cells express GP Iba and GP Ibβ and resistance to G418; polypeptide expression in these cells has not been amplified. CHO DUK" cells (ATCC CRL 9096; American Type Culture Collection, Rockville, MD) were used for transient expression. All cells were grown in α-minimal essential medium (α-MEM; GIBCO BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). The media for CHO αβn cells were supplemented with G418 (400 μg/mL; Sigma Chemical Co, St Louis, MO) and those for CHO βIX cells were supplemented with 400 μg/mL of G418 and 80 μM methotrexate.

Antibodies. Polyclonal antisera to GP Ibβ has been described. CHO αβn cells were supplemented with G418 (400 μg/mL; Sigma Chemical Co, St Louis, MO) and those for CHO βIX cells were supplemented with 400 μg/mL of G418 and 80 μM methotrexate.

Transfections. CHO αβn cells were transiently transfected with GP IX plasmids, and CHO DUK" cells were transiently transfected with either the three GP Ib-IX complex polypeptides (GP Iba, GP Ibβ, and wild-type or mutant GP IX) or only plasmids for GP Ibβ or GP IX (wild-type or mutant). Transfections were by lipofection, using a commercially available kit (LipofectAMINE; GIBCO BRL). Cells were passed on the day before the transfections so as to reach approximately 80% confluence at the time of transfection. Eight microliters of liposome suspension and 1 μg of plasmid DNA were separately mixed in 100 μL of serum-free medium; the two suspensions were then combined, mixed gently, and allowed to incubate for 30 minutes at room temperature to form DNA-liposome complexes. The mixture was diluted in 0.8 mL of serum-free medium and added to cells in a 35-mm² culture dish that had been washed twice in the same medium. The cells were exposed to the DNA/liposome mixture for 5 hours under standard culture conditions (37°C, 5% CO₂), after which time an additional 1 mL of medium containing 10% FBS was added. The medium was changed 24 hours later. After 48 hours, the cells were detached from the dishes with 0.54 mM/L of EDTA and were analyzed further.

Flow cytometry. Detached cells were labeled either with FITC-conjugated monoclonal antibodies or with unlabeled primary antibodies followed by FITC-conjugated rabbit anti-mouse secondary antibodies. When a two-antibody sandwich was used to label the cells, they were first incubated in the primary antibody (5 μg/mL) for 30 minutes at room temperature, washed twice by resuspension in phosphate-buffered saline (PBS), incubated for 30 minutes in 5 μg/mL of FITC-labeled secondary antibody, and washed twice again with PBS. To determine viability, the cells were then resuspended in PBS containing propidium iodide (PI; 20 ng/mL) before analysis.

To analyze the population of live cells, PI-positive cells were first gated out by stimulating the fluorophor at 488 nm and detecting at greater than 620 nm. Cells with fluorescence emission at greater than 620 nm that was 200-fold over background were excluded from further analysis. The cells were also gated based on the forward- and side-scatter characteristics of a control parent CH0 cell population. At least 10,000 gated cells from each transfection were then analyzed by exciting the FITC label at 480 nm using an argon ion laser and evaluating emission at 520 nm. The analyses were performed on a Becton Dickinson (San Jose, CA) FACStar flow cytometer.

Fluorescence microscopy. For these experiments, transfections were performed directly on two-well chamber slides (Titertek; Nunc, Naperville, IL), with the amount of DNA and liposome suspension adjusted based on the surface area of the chamber. At 48 to 72 hours after transfection, the cells were washed with PBS, fixed with 4% paraformaldehyde (Sigma) for 15 minutes, and permeabilized with 0.1% Triton X-100 (Sigma) for 15 minutes. The fluorescence of the fixative was quenched with several washes in 0.15 mM/L ammonium acetate. The cells were then incubated in 5 μg/mL of monoclonal antibody (FMC25) for 30 minutes, washed several times with PBS, and incubated in a 1:1,000 dilution of FITC-conjugated rabbit anti-mouse secondary antibody for 30 minutes. After several washes with PBS, the cells were examined on a Zeiss fluorescence microscope (Carl Zeiss, Inc, Sanheandro, CA). Immunofluorescence studies were also performed using polyclonal anti-GP IX. In this case,
the secondary antibody used was FITC-conjugated goat anti-rabbit antiserum.

**Mutagenesis.** The GP IX cDNA was cloned into the mammalian expression vector pDX,\(^{10}\) in which all the mutagenesis reactions were performed. A commercially available kit (Transformer; Clontech, Palo Alto, CA) was used to perform mutagenesis using the method of Deng and Nickoloff.\(^{11}\) To mutate codon 21 from an Asp to a Gly codon, the following mutagenic primer corresponding to nucleotides 322 to 344 of the published sequences\(^{12}\) was annealed to the plasmid containing the GP IX cDNA: CTG TGG GTG GgC TGC AGG GCC CA. To mutate codon 45 from Asp to Ser, the following oligonucleotide was used: CTG TGG GCC AAC AgC AGC CTT CAG TCC G. This primer, with the exception of the single nucleotide change, corresponds to nucleotides 391 to 418 of the GP IX cDNA. The cDNA insert of each of the mutant plasmids was sequenced in its entirety to verify the mutation and rule out unwanted mutations. The ABI Dye terminator kit was used for sequencing, and the sequencing reactions were analyzed on an ABI (Foster City, CA) model 373A automated sequencer.

**Metabolic labeling and immunoprecipitation.** CHO DUK+ cells transiently transfected with a GP Ibβ plasmid and wild-type or mutant GP IX plasmids in 50-cm\(^2\) cell-culture dishes were washed twice in cysteine-free α-MEM and then incubated in this medium for 1 hour before the addition of 0.1 mCi \(^{35}\)S-cysteine (ICN, Irvine, CA). The cells were incubated in the radioactive medium for 4 hours, the medium was then removed, and the cells were washed twice in PBS. Radiolabeled cells were lysed in digitonin lysis buffer (100 mmol/L Tris pH 7.4, 1 mg/mL leupeptin, 1.6 mg/mL benzamidine, 0.1 μg/mL soybean trypsin inhibitor, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% digitonin) and centrifuged at 14,000 g for 5 minutes to remove debris. The lysate was incubated overnight at 4°C with fixed Staphylococcus aureus cells (Pansorbin; Calbiochem, La Jolla, CA) to remove proteins that bind the dead bacteria nonspecifically. To immunoprecipitate GP IX, FMC25 (10 μg/mL) was added to the lysate, and the mixture was incubated for 4 hours. Pansorbin beads that had been preequilibrated with rabbit anti-mouse IgG (5 μg/mL, 4 hours) were then added to the lysate and incubated for 4 hours.

To precipitate GP Ibβ, the cleared lysate was incubated with polyclonal GP Ibβ antiserum (10 μg/mL) for 4 hours, and the Pansorbin beads were then added directly to the lysate and incubated for an additional 4 hours. In both cases, bound protein was removed from the lysate by centrifugation at 14,000 g for 5 minutes. The bead pellet was then washed several times in lysis buffer by resuspension and centrifugation. Immunoprecipitated proteins were removed from the beads by boiling in sodium dodecyl sulfate (SDS)-sample buffer\(^{13}\) for 5 minutes. The proteins were then resolved on the basis of molecular mass by electrophoresis on 12.5% SDS-polyacrylamide gels. The radioactivity on the dried gels was determined by autoradiography using a Fuji phosphorimager (Model BAS100), and the results were analyzed with the Fuji (Stamford, CT) MACBAS software package.

**RESULTS**

Our previous studies have demonstrated that supertransfection of GP IX into cells that stably express GP Ib (α and β) can significantly increase the level of GP Ib on the cell surface, presumably by forming a complex that stabilizes GP Ib.\(^{a}\) We examined whether either mutant GP IX (GP IX Asp\(^{21}\)→ Gly and GP IX Asn\(^{45}\)→ Ser) or a combination of both could do the same. We chose to use a cell line in which expression of GP Ib was not amplified (CHO αβ8); only a small population of these cells actually express GP Ib on their surfaces (Fig 2). CHO αβ8 cells were transfected with plasmid (pDX) only (sham-transfected), with wild-type GP IX, or with the two GP IX mutants, alone or together. When labeled with the anti-GP Ibα antibody AN51, sham-transfected CHO αβ8 cells displayed two cell populations (Fig 2A): a large negative population with surface fluorescence equivalent to that of untransfected CHO cells (not shown) and a smaller population positive for GP Ib. The two populations were gated based on fluorescence, as shown in Fig 2A. The numbers of sham-transfected cells and those

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**Fig 2.** Transfection of wild-type and mutant GP IX plasmids into CHO cells expressing GP Ib (α and β). Expression of GP Ib on the cell surface was measured with the FITC-conjugated GP Ibα monoclonal antibody AN51. (A) CHOαβ8 cells sham-transfected with the expression plasmid pDX displayed a small GP Ib-positive population, which was gated as gate 2 (bar) for further analysis. (B) Average fluorescence values from three transfection experiments. In these experiments, CHOαβ8 cells were transfected with either control plasmid alone (sham) or with plasmids for wild-type (wt) or mutant GP IX, as indicated. The mean fluorescence values for cells in gate 2 (evaluated 48 hours after transfection) were averaged, and the values are expressed as a percentage of the fluorescence of the sham-transfected control cells. *Fluorescence value was significantly greater than that of sham-transfected controls (P < .01, Student’s t test; t = 7.6, n = 3).
As another means of evaluating the role of the mutant GP IX polypeptides on surface expression of GP Ib, all three GP Ib-IX complex polypeptides were transiently expressed by cotransfection into CHO DUK- cells. Expression of GP Ib on the cell surface was again evaluated with ANS1. Once again, GP Ib expression on the cell surface was greater when plasmids for GP Ibα and GP Ibβ were cotransfected with wild-type GP IX than when they were cotransfected with either of the mutant GP IX plasmids or with both together (Fig 3). The mean fluorescence of the cells containing wild-type GP IX (average from three experiments) was approximately twice that of the cells containing any of the mutants.

The results shown in Figs 2 and 3 indicate that the mutant GP IX polypeptides do not function like wild-type GP IX in increasing plasma membrane expression of GP Ibα, possibly because they do not associate with GP Ib. We had demonstrated earlier that GP IX associates with GP Ib through a noncovalent association with GP Ibβ and demonstrated that the association of GP IX with GP Ibβ increases GP IX expression on the cell surface. We therefore also tested whether the mutant GP IX polypeptide increased on the cell surface when transfected with GP Ibβ. In these experiments, expression of GP IX on the cell surface after transfection of the wild-type or mutant plasmid with GP Ibβ was compared with expression after transfection of wild-type GP IX by itself (Fig 4). Expression of wild-type GP IX on the cell surface was considerably greater when it was cotransfected with GP Ibβ than when it was transfected alone (Fig 4A, upper left panel). In marked contrast, neither of the mutant GP IX polypeptides, either when each was transfected alone or when they were cotransfected with GP Ibβ, were found on the cell surface at levels significantly higher than those of sham-transfected cells. Mean GP IX fluorescence in cells from these cotransfections did not differ from that of cells transfected with wild-type GP IX alone (Fig 4B).

One possibility for the observed dysfunction of the GP IX mutants is that both mutations might result in synthetic defects of the mutant polypeptides. Alternatively, the mutants could be sequestered within intracellular compartments and not reach the cell surface or be targeted for early degradation. We investigated these possibilities by immunofluorescence microscopy of cells transiently cotransfected with GP IX (wild-type or mutant) and GP Ibβ after the cells were rendered permeable with 0.1% Triton X-100. A polyclonal antiserum was used to detect GP IX. The wild-type polypeptide was detected at high levels within the intracellular compartments (Fig 5B). Each of the mutant polypeptides was also detected intracellularly (Fig 5C and D), but at lower levels than wild-type GP IX. The GP IX Asp/ - Gly mutant was affected more severely (Fig 5C). In cells transfected with this mutant, only rare fluorescent cells were found, and those had much lower GP IX levels than did cells transfected with wild-type GP IX (Fig 5C).

This result clearly indicated that the most probable explanation for the failure of either GP IX mutant to augment GP Ib expression on the plasma membrane was its failure to associate noncovalently with GP Ibβ. Our previous studies have shown that antibodies against either polypeptide are capable of precipitating both polypeptides. We therefore
tested directly the GP IX mutants associated with GP Ibβ by immunoprecipitating GP Ibβ from cells transiently coexpressing both polypeptides. In the cotransfections with either mutant polypeptide, the amount of GP IX associated with GP Ibβ was greatly diminished when compared with the amount of wild-type GP IX associated (Fig 6). The relative ratios of GP IX to GP Ibβ were only 23% and 7% for GP IX Asnα → Ser and GP IX Aspα → Gly, respectively, as compared with wild-type GP IX. The amounts precipitated paralleled the levels found by microscopy in intracellular compartments.

**DISCUSSION**

BSS is an autosomal recessive bleeding disorder resulting from an absent or abnormal receptor for vWF on the surfaces of the platelets of affected patients. Because four polypeptides (GP Ibα, GP Ibβ, GP IX, and GP V) have been described as diminished or absent in platelets from different BSS patients, and because each of the missing polypeptides is encoded by its own gene, the disorder could potentially be caused by homozygous or compound heterozygous mutations in any one of these genes. Thus far, cases of BSS with decreased levels of the GP Ibα-Ib-IX-V polypeptide arising from mutations of GP Ibα14,15 and GP IX7 have been described, but not cases arising from mutations of the genes encoding either of the other two polypeptides. Mutations of GP Ibβ also are likely to cause the disorder, particularly given the role of this polypeptide in synthesis of the complex.6 Mutations of GP V might also cause BSS, but they appear less likely to do so, given that a functional receptor can be reconstituted in heterologous cells by transfecting only cDNAs for GP Ibα, GP Ibβ, and GP IX.5

Mutations that diminish GP Ibα synthesis cause BSS for obvious reasons: this polypeptide contains the regions that mediate complex binding to both vWF and thrombin. Neither of these functions apparently requires participation from either of the other polypeptides of the complex; interpretation of the functional defect is, therefore, straightforward. In cases of BSS due to mutations of GP IX, however, interpretation of the mechanism by which the mutation causes this disorder is not as obvious. Our studies in heterologous cells indicate that any mutation that interferes with synthesis of either GP Ibβ or GP IX might cause the disorder if these mutations decrease synthesis of the mutant polypeptide or if they lead to its instability or failure to associate with its counterpart.59 This seems to be the mechanism by which the mutations studied in this report (originally described by Wright et al1 in three affected members of one family) cause BSS. We found that both mutant polypeptides (GP IX Aspα → Gly and GP IX Aspα → Ser) associated poorly with GP Ibβ.

Failure to associate with GP Ibβ prevents the mutants from performing what may be one of the more vital functions of GP IX—to stabilize GP Ib on the cell surface. This hypothesis is fully compatible with the observations of the platelet phenotype made by Wright et al.7 They found no expression of GP IX in the platelets of the patients, either on the cell surface by flow cytometry or within the cells by immunoblotting of platelet lysates. (One caveat about these studies, which also applies to our studies shown in Fig 4, is that the FMC25 epitope may be destroyed by the mutations and, thus, not detect mutant polypeptide on the cell surface.) They did find, however, a subset of platelets with reduced but detectable levels of GP Ibα on their surfaces, as determined by immunostaining with a monoclonal antibody against GP Ibα.

This platelet population may have represented younger platelets that were more recently released from the bone marrow. This observation from patient platelets clearly parallels observations in our laboratory, in this and a previous study, where although GP Ib alone appears on the surface and is completely processed, it is much less stable there than it is in the presence of GP IX.5

The finding that two GP IX molecules with mutations in different regions of the conserved leucine-rich motif both do
not associate with GP Ibβ suggests a role for this conserved motif in the biology of the GP Ib-IX-V complex: this may be the region through which these two polypeptides associate with each other. The leucine-rich motif is present in a very large number of proteins and has been implicated in protein-protein interactions.3 Particularly interesting among those interactions are the adhesion reactions mediated by three developmentally important Drosophila proteins: Toll, chaoptin, and connectin. Chaoptin and connectin appear to mediate homophilic aggregation of cells in which they are expressed by binding to identical receptors on other cells16,17; Toll is able to induce aggregation through a heterophilic mechanism.18 GP Ibβ and GP IX, whose extracellular regions are very similar,3 may interact with each other in an analogous manner. In this case, however, rather than recognizing an identical protein on the surface of another cell, they may bind to a very similar protein on the same cell. This hypothesis remains to be proved directly.

At least two possible mechanisms could account for failure of GP IX to associate with GP Ibβ, one of which implies
nothing about the mechanism by which the polypeptides associate. First, the conformational alteration in GP IX could lead to its rapid degradation in the endoplasmic reticulum, or possibly such a change in conformation would first lead to its retention (possibly by binding BiP)90 and then its subsequent degradation. In this situation, the mutants might not associate with GP Ibβ because they are degraded before the association can occur. That small fraction that does associate may be stabilized by the association. Alternatively, excess GP IX molecules (in excess of those needed to bind GP Ibβ) under normal circumstances might be degraded fairly rapidly, and failure of the mutants to bind GP Ibβ could commit them to the same default degradation pathway. In this case, the failure to associate with GP Ibβ is the direct cause of the instability.

In either situation, the biosynthetic defects described in this report ascribe a very important function to the leucine-rich motif of GP IX in maintaining the stability of the polypeptide. As more mutations from patients with BSS are described, more will be learned about the roles of the motif in the functions of all of the polypeptides of the GP Ib-IX-V complex.

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