Effect of Mutagenesis of GPIIb Amino Acid 273 on the Expression and Conformation of the Platelet Integrin GPIIb-IIIa†

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ABSTRACT: A G273D mutation immediately proximal to the first calcium binding domain of platelet GPIIb impairs the export of GPIIb-IIIa heterodimers to the platelet surface. To examine how this mutation might alter the structure of GPIIb, G273 was replaced by other amino acids and the resulting mutants were coexpressed with GPIIIa in COS-1 cells. Although replacement with Ala or Val had no effect on GPIIb-IIIa expression, replacement with Glu, Lys, Pro, or Asn caused intracellular retention of GPIIb-IIIa. Concurrently, the consequences of these replacements were examined by comparative modeling by introducing them into the analogous position of the first helix−loop−helix (HLH) motif of calmodulin, based on homology between the calcium binding domains of GPIIb and the calcium binding loops of HLH-containing proteins. The modeling revealed that as the side chain of the introduced amino acid increased in size, it progressively interfered with hydrophobic interactions between the incoming and outgoing helices of the motif. To test whether this observation also applies to GPIIb, V286, located immediately distal to the first GPIIb calcium binding domain, was replaced by Asp and Phe. Expression of these mutants in COS-1 cells also resulted in the intracellular retention of GPIIb-IIIa, suggesting that interactions between sequences that flank the first calcium binding domain of GPIIb affect its folding. Finally, the endoplasmic reticulum chaperone BiP was detected in immunoprecipitates of GPIIb-IIIa containing GPIIb with Ala, Val, Lys, or Pro, but not Gly, at position 273. This suggests that although BiP binding is a sensitive indication of the fidelity of GPIIb-IIIa folding, it is not sufficient to account for the intracellular retention of the heterodimer.

The platelet integrin GPIIb-IIIa (αIIbβ3) is a calcium-dependent heterodimer whose binding site for ligands such as fibrinogen and von Willebrand factor is exposed by platelet activation (Phillips et al., 1988). GPIIb-IIIa heterodimers are assembled from nascent GPIIb and GPIIIa monomers in the calcium-rich environment of the endoplasmic reticulum (ER)[1] (Bennett, 1996). Correctly folded GPIIb-IIIa heterodimers, but not GPIIb or GPIIIa monomers, are then exported from the ER to the Golgi apparatus where they undergo further processing before they are expressed on the cell surface (Bennett, 1996). Mutations that impair the synthesis of either GPIIb or GPIIIa prevent the export of sufficient numbers of GPIIb-IIIa heterodimers on the platelet surface to support primary hemostasis, resulting in the bleeding disorder Glanzmann thrombasthenia (George et al., 1990). However, GPIIb or GPIIIa mutations that have no apparent effect on monomer synthesis can also give rise to thrombasthenia, either by impairing GPIIb-IIIa function (Loftus et al., 1990; Chen et al., 1992) or by perturbing the conformation of assembled heterodimer so they fail to be exported from the ER (Poncz et al., 1994).

We have reported that a point mutation in GPIIb, Gly273[2] → Asp (G273D), detected in a patient with thrombasthenia whose platelets expressed little or no GPIIb-IIIa on their surface (Poncz et al., 1994), caused the intracellular retention of GPIIb-IIIa. Although the mutation did not prevent the assembly of GPIIb-IIIa heterodimers, it did impair recognition of the heterodimer by heterodimer-specific monoclonal antibodies, indicating that it had altered the conformation of GPIIb-IIIa. G273 is located in the extracellular portion of GPIIb proximal to the first of four domains that are homologous to the calcium-binding loops (helix−loop−helix, HLH, or EF-hand motifs) of proteins such as calmodulin and parvalbumin (Poncz et al., 1987) (Figure 1, Table 1). Although there is no direct evidence that calcium actually binds to this region of intact GPIIb, a bacterial fusion protein containing the four GPIIb sequences was found by equilibrium dialysis to contain four sites that could be occupied by

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[1] Abbreviations: ER, endoplasmic reticulum; HLH, helix−loop−helix; PCR, polymerase chain reaction; MPRC, medium subunit of the photosynthetic reaction center of *Rhodopseudomonas viridis*; AαA, acid α-amylase from *Aspergillus niger*.

[2] Numbering of the amino acids in GPIIb is based on that of Poncz et al. (1987).

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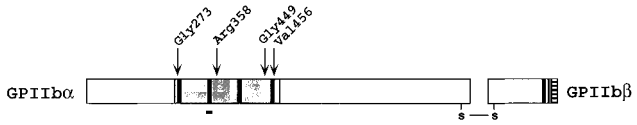

FIGURE 1: Schematic diagram of GPIIb indicating the location of the G273D mutation and three other mutations in the putative calcium binding region of GPIIb that produce Glanzmann thrombasthenia. GPIIbα, GPIIb heavy chain; GPIIb*β*, GPIIb light chain; black areas, putative calcium binding sequence; gray area, GPIIb region containing four putative calcium binding sequences; vertical striping, transmembrane domain; horizontal striping, cytoplasmic tail; **S**−**S**, disulfide bond. The vertical arrows indicate the positions of known point mutations in the extracellular domain of GPIIb that produce Glanzmann thrombasthenia (Bennett,1996). The horizontal line beneath the second calcium binding domain indicates the location of a possible ligand binding site.

Table 1: Comparison of the Amino Acid Sequences of the Calcium Binding Domains in GPIIb with Those of Selected HLH-Motifs

	-2-1 1 3 5 7 8 12 13 16
GPIIb-1 [272-289]	V *G* E F D G D L N T T E Y V *V* G A P
GPIIb-2 [326-342]	V T D V N G D G R H D L L V G A P L
GPIIb-3 [393-410]	L G D L D R D G Y N D I A V A A P Y
GPIIb-4 [455-472]	A V D I D D N G Y P D L I V G A Y G
Calmodulin EF1 [18-35]	L F D K D G D G T I T T K E L G T V
Calmodulin EF2 [54-71]	E V D A D G N G T I D F P E F L T M
Calmodulin EF3 [91-108]	V F D K D G N G Y I S A A E L R H V
Calmodulin EF4 [128-144]	E A N I D G D G Q V N Y E E F V Q M
Parvalbumin [83-100]	D G D K D G D G M I G V D E F A A M
Parvalbumin [49-66]	V I D Q D K S G F I E E D E L K L F
Troponin [20-37]	M F D A D G G G D I S V K E L G T V

[a] Numbers above the amino acid sequences correspond to the numbering of a generic EF-hand motif (Strynadka & James, 1989). The numbers in brackets indicate the position of each calcium binding domain in bovine calmodulin (Babu et al., 1985) and GPIIb (Poncz et al., 1987), respectively. The italicized and underlined residues in the sequence of GPIIb-1 indicate the locations of G273 and V286.

calcium (Gulino et al., 1992). Nevertheless, calcium binding to GPIIb appears to play a role in maintaining the folded configuration of GPIIb because calcium chelators enhance the susceptibility of GPIIb to hydrolysis by thrombin (Fujimura & Phillips, 1983). Moreover, the region of GPIIb containing its calcium binding domains appears to make an important contribution to the overall conformation of GPIIb-IIIa because three naturally occurring point mutations involving the domains, in addition to G273D, also produce thrombasthenia by causing the intracellular retention of malfolded heterodimers (Wilcox et al., 1994, 1995; Basani et al., 1996). In the work reported in this paper, we have studied how the G273D mutation affects the conformation of GPIIb-IIIa by replacing G273 with selected amino acids using site-directed mutagenesis. The biological consequences of the mutations were then examined using transfected COS-1 cells and their possible structural consequences using homology modeling.

EXPERIMENTAL PROCEDURES

Production of GPIIb Mutants by Oligonucleotide-Mediated Mutagenesis. Aside from the substitution of Asn for Gly273, all of the mutations in GPIIb were produced as described previously (Poncz et al., 1994). Briefly, a full-length cDNA for GPIIb was ligated into double-stranded M13mp18 phage and transfected into *Escherichia coli* JM107 to prepare single-stranded template DNA. A commercial kit (Amersham) employing the method of Taylor et al. (1985) was used for the mutagenesis. Phosphorylated 18−30-base antisense oligonucleotides containing the desired mutations were annealed to the single-stranded template DNA and mutagenesis was performed according to the manufacturer's instructions. *E. coli* TG1 cells were transformed with the resulting double-stranded mutant DNA, and single-stranded DNA was isolated and sequenced to identify clones containing the desired mutations. Phage DNA recovered from the mutant clones was shuttled into the plasmid pMT2ADA, kindly provided by R. J. Kaufman (Genetics Institute, Cambridge, MA), using the restriction enzyme *Eco*RI as previously described (Kolodziej et al., 1991b). A GPIIb cDNA encoding Asn273 was prepared by overlap extension PCR using Vent polymerase (Loh et al., 1995). The resulting DNA fragment, corresponding to nucleotides 338−1620 of the GPIIb sequence (Poncz et al., 1987), was sequenced to confirm that only the desired mutation had been introduced by the Vent polymerase and then subcloned into unique *Not*I and *Cla*I sites in GPIIb.

Transfection of COS Cells with GPIIb- and GPIIIa-Containing Plasmids. The effect of amino acid substitutions in GPIIb on GPIIb-IIIa expression was examined using COS-1 cells as previously described (Kolodziej et al., 1991b). Briefly, COS-1 cells obtained from the American Type Culture Collection, Rockville, MD, were grown in a medium consisting of Dulbecco's modified Eagle's medium (high glucose) (Gibco Laboratories, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.3 mg/mL L-glutamine. Cotransfection of 1×10^6 cells plated in 75-cm^2 flasks (Corning Glass Works, Corning, NY) with plasmids containing cDNAs for GPIIb and GPIIIa was performed using 1 μg of each plasmid and DEAE-dextran, followed by incubation with 100 μM chloroquine diphosphate. After transfection, the cells were rinsed twice with Dulbecco's phosphate-buffered saline, complete medium was added, and incubation was continued for 48−72 h.

Identification of GPIIb-IIIa Heterodimers Synthesized by Transfected COS Cells. Recombinant GPIIb-IIIa was detected by immunoprecipitation as previously described (Kolodziej et al., 1991b). Briefly, transfected cells were incubated sequentially with methionine-free medium for 60 min, with medium containing ^{35}S-methionine at 200−400 μCi/mL for 60 min, and with medium containing unlabeled methionine for 2−4 h. Cells were then harvested using 0.02% (w/v) EDTA (Flow Laboratories, Inc, McLean, VA), rinsed once with Dulbecco's phosphate-buffered saline, and extracted with 0.02 M Tris-HCl buffer, pH 7.2, containing 1% Triton X-100 (Sigma Chemical Co., St. Louis) for 60 min at 4 °C. After sedimentation at 12000*g* to remove particulate debris, 500-μL aliquots of the extracts were incubated sequentially with 4 μL of nonimmune serum for 15 min at 4 °C, with 100 μL of a 10% suspension of fixed staphylococci (Pansorbin, Calbiochem) for 30 min at 4 °C, and with either the GPIIb-IIIa-specific monoclonal antibody (mAb) A2A9 (Bennett et al., 1983), the GPIIIa-specific mAb SSA6 (Silver et al., 1987), or rabbit polyclonal anti-GPIIb antisera at 4 °C for 1 h. Immune complexes were collected with 100 μL of a 10% suspension of Affi-Gel A (Bio-Rad Laboratories, Richmond, CA) for 1 h at 4 °C. The resin was washed six times: three times with 50 mM Tris buffer, pH 7.5, containing 0.01 M NaCl, 0.1% (w/v) SDS, 1% Triton X-100, and 0.5% (w/v) deoxycholic acid and three times with the same buffer containing 0.3 M NaCl. Immune complexes were then eluted by heating at 100 °C for 5 min in a 0.01 M

Tris-HCl buffer, pH 6.8, containing 3% SDS and 0.2 M dithiothreitol and electrophoresed on 0.1% SDS−7.5% polyacrylamide slab gels. Gels were fixed with 10% (v/v) acetic acid−30% (v/v) methanol, incubated with Autofluor (National Diagnostics, Manville, NJ) according to the manufacturer's instructions, dried, and exposed to Kodak X-Omat AR film at −70 °C. GPIIb-IIIa complexes were detected on the surface of cotransfected cells by immunoprecipitation after the surface of the cells had been labeled with ^{125}I using lactoperoxidase/H_2O_2. Positive controls for the immunoprecipitations were extracts of human platelets surface-labeled with ^{125}I using lactoperoxidase/H_2O_2.

To examine the interaction of the GPIIb mutants with the chaperone BiP (GRP78), GPIIb-IIIa immunoprecipitates from unlabeled cells were immunoblotted with an anti-BiP mAb as previously described (Bennett et al., 1993). Briefly, immunoprecipitated GPIIb-IIIa obtained from cells extracted in the presence of apyrase (Sigma) was electrophoresed on 0.1% SDS−7.5% polyacrylamide slab gels and transferred to nitrocellulose paper (0.45 μm, Schleicher & Schuell, Keene, NH). The paper was then incubated sequentially with the supernatant from the culture of a hybridoma producing a nonprecipitating monoclonal antibody to BiP, a gift from Dr. Linda Hendershot at St. Jude's Research Hospital, Memphis, TN, and goat anti-rat antisera (Organon Teknika Corp., West Chester, PA). Sites of goat antibody binding were detected with ^{125}I-labeled staphylococcal protein A (Boehringer Mannheim, Indianapolis, IN). The labeled blots were dried and exposed to Kodak X-Omat AR film at −70 °C.

Molecular Modeling of the First Calcium Binding Domain of GPIIb. We initially examined the extent to which the first calcium binding domain in GPIIb (GPIIb-1) adopts a conformation similar to that of the helix−loop−helix (HLH) motif present in calcium binding proteins like calmodulin and parvalbumin. We used the program Blast (Altschul et al., 1990) to identify sequences in the Brookhaven crystallographic data base similar to the first calcium binding domain of GPIIb (Table 1). This analysis was used as a guide to identify the proximal and distal borders of the calcium binding loop in GPIIb-1 and to establish the position of residue 273 in the motif. Next, using the energy-optimized conformation of the first HLH motif (EF1) of calmodulin as the starting geometry, we examined the changes in local conformation that would be introduced by replacing the amino acid analogous to GPIIb amino acid 273 with the amino acids described above. To examine the folding of the EF1 in the absence of restraints imposed by the tertiary environment of calmodulin, residues 11−37 were extracted from the calmodulin crystal structure using the program Insight II (version 3.1, Biosym Technologies). Side chains and amino- and carboxyl-terminal groups were considered to be in their charged state. Side-chain angles of the substituted amino acids were set according to angles identified in the data base of side chains present in the program. A combination of molecular dynamics simulations and energy minimization was used to identify structural features displayed by the mutated sequences. Energy minimization to convergence was performed using the program Discover (Biosym Technologies, version 2.95). Calculations were performed in vacuo employing a dielectric of 80. Typical convergence criteria were assumed for complete optimization. Following a molecular dynamics protocol for analysis of HLH domains (Tuckwell et al., 1992), simulations were commenced for the individual residue-substituted domains at 0 K and structures were heated to the desired temperature at a rate of approximately 20 K/ps up to 100 K. After equilibration, a production run over 100 ps at 100 K with coordinates sampled every 1 ps was recorded. The time step was 1 fs.

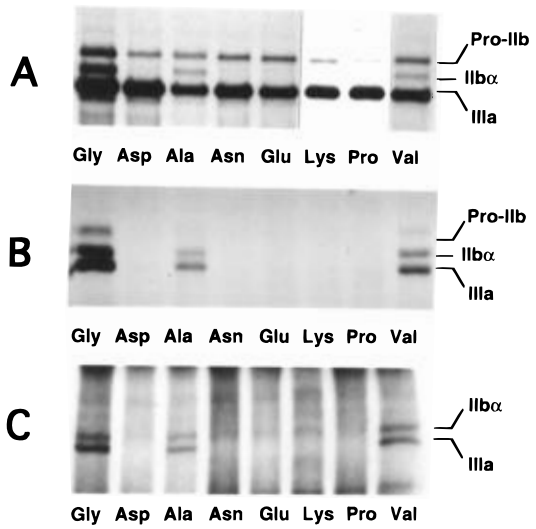

FIGURE 2: Effect of replacing G273 in GPIIb with various amino acids on the expression of GPIIb-IIIa heterodimers. The indicated amino acids were substituted for G273 by site-directed mutagenesis as described under Experimental Procedures. Wild-type GPIIb (Gly) and the GPIIb mutants were then coexpressed with GPIIIa in COS-1 cells and the resulting GPIIb-IIIa heterodimers were immunoprecipitated from cells metabolically labeled with ^{35}S-methionine or surface-labeled with ^{125}I. (**A**) GPIIb-IIIa immunoprecipitated from cells labeled metabolically with ^{35}S-methionine using anti-GPIIIa mAb SSA6; (**B**) GPIIb-IIIa immunoprecipitated from cells labeled metabolically with ^{35}S-methionine using the anti-GPIIb-IIIa mAb A2A9; (**C**) GPIIb-IIIa immunoprecipitated from cells surface labeled with ^{125}I using polyclonal rabbit antisera against GPIIb. Pro-IIb is the single chain glycoprotein IIb precursor. IIbα is the GPIIb heavy chain. IIIa is glycoprotein IIIa.

RESULTS

Expression of Site-Directed Mutants of GPIIb Amino Acid 273 in COS-1 Cells. The naturally occurring mutation G273D, located proximal to the first of four putative calcium binding domains in GPIIb, prevents the export of GPIIb-IIIa heterodimers to the platelet surface by altering the conformation of GPIIb-IIIa (Poncz et al., 1994). Reasoning that replacement of G273 with other amino acids might provide insight into the structural changes in GPIIb-IIIa induced by G273D, we used site-directed mutagenesis to substitute Ala, Val, Gln, Pro, Glu, and Lys for G273. cDNAs for wild-type GPIIb and for each of the GPIIb mutants were then cotransfected with a cDNA for GPIIIa into COS-1 cells and the resulting GPIIb-IIIa heterodimers were immunoprecipitated from lysates of the transfected cells using antibodies specific for GPIIIa, GPIIb-IIIa, and GPIIb.

As seen in Figure 2A, GPIIb-IIIa heterodimers were immunoprecipitated from lysates of each of the transfected cells with the GPIIIa-specific mAb SSA6. Thus, each of the mutant forms of GPIIb was able to associate with GPIIIa. However, the immunoprecipitates from cells expressing GPIIb with Gly, Ala, or Val at position 273 also contained a band corresponding to the GPIIb heavy chain (GPIIbα), whereas immunoprecipitates from cells expressing

GPIIb with Asp, Asn, Glu, Lys, or Pro at this position did not. Because GPIIbα is generated by the endoproteolytic cleavage of pro-GPIIb in the Golgi complex (Kolodziej et al., 1991a), these data suggest that the former set of heterodimers had been exported from the ER to the Golgi and the latter set had not.

To determine which of the GPIIb mutations, like G273D, perturbed the conformation of GPIIb-IIIa, immunoprecipitations were also performed using the GPIIb-IIIa-specific mAb A2A9. As seen in Figure 2B, GPIIb-IIIa could only be immunoprecipitated from the lysates of cells expressing GPIIb with Gly, Ala, or Val at position 273 with this antibody but not from the lysates of cells expressing GPIIb with Asp, Asn, Glu, Lys, or Pro at this position. Thus, despite the ability of GPIIb containing Asp, Asn, Glu, Lys, or Pro at position 273 to interact with GPIIIa, the resulting heterodimers were sufficiently malfolded to either conceal or eliminate the epitope recognized by A2A9.

Endoproteolysis of GPIIb into heavy and light chains is not required for the expression of GPIIb-IIIa on the cell surface (Kolodziej et al., 1991a). To determine whether the GPIIb mutants that failed to undergo endoproteolysis also failed to reach the cell surface, we labeled the surface of the transfected cells with ^{125}I and immunoprecipitated GPIIb-IIIa using polyclonal anti-GPIIb antisera. As seen in Figure 2C, only heterodimers containing Gly, Ala, or Val at GPIIb position 273 were exported to the COS cell surface, whereas heterodimers containing Asp, Asn, Glu, Lys, or Pro at this position were not. Thus, the experiments described above demonstrated that the presence of Pro or a charged or polar amino acid immediately proximal to the first calcium binding domain of GPIIb alters the conformation of assembled GPIIb-IIIa heterodimers, preventing the export of these heterodimers to the cell surface, likely because they are retained in a pre-Golgi compartment.

Homology Modeling of the First Calcium Binding Domain of GPIIb. To study how mutations at GPIIb position 273 might alter the conformation of GPIIb-IIIa, we took a comparative modeling approach because X-ray or solution structures of integrin α and β subunits are not available. Because of the lack of structural information, our modeling could only directly address the effect of mutations on the local conformation of GPIIb. Based on the report of Tuckwell et al. (1992) that the calcium binding domains in integrin α subunits generally resemble those of standard HLH-containing proteins, our initial plan was to replace the first calcium binding loop in calmodulin with the sequence of the first calcium binding domain in GPIIb (GPIIb-1) and examine the consequences of the various residue 273 substitutions. However, as shown in Table 1, the GPIIb-1 sequence deviates substantially from that of the standard HLH-motif, precluding direct homology modeling. In particular, invariant amino acids in the calcium binding loops of calmodulin and parvalbumin—Asp at position 1, Gly at position 6, and Glu at position 12—are replaced by Glu, Leu, and Val, respectively (Strynadka & James, 1989). In the standard HLH motif, the carboxylates of Asp at position 1 and Glu at position 12 provide one and two oxygen atoms, respectively, for coordination with calcium and the Gly at position 6 permits the calcium binding loop to bend so that its calcium ligands are in proper position. Furthermore, the residues flanking the calcium binding loops in standard HLH motifs assume helical configurations, but there is no evidence

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          |   helix   |  loop  |  helix  |
MPRC   29 AVWSWWGRTYLRAQ                          42
          ..  .:: ...
GPIIb 261 YFDGYWGYSVAVGEFDGDLNTTEYVVGAPTWSWTLGAV 298
                          :::::: .: .  . :
AaA   175                 DLNTTETAVRTIWYDW        190
```

FIGURE 3: Alignment of amino acids 261−290 of GPIIb with homologous sequences from the medium subunit of the photosynthetic reaction center of *Rps. viridis* (MPRC) and the acid α-amylase from *A. niger* (AαA) obtained by searching the crystallographic database. Underlined sequences are α helices. **:** indicates amino acid identity; · indicates a conservative amino acid substitution.

that this is the case in GPIIb. Consequently, several different alignments of the GPIIb-1 sequence with the sequences of various HLH motifs are possible, based on differing predictions of the relative length of the GPIIb-1 helices and loop. Accordingly, we sought secondary structure templates that would identify the beginning and end of helices in the GPIIb-1 sequence by searching the Brookhaven crystallographic data base for proteins containing sequences homologous to GPIIb amino acids 261−298.

Two sequences with the desired homology were identified in the crystallographic data base by a Blast search. The first sequence was a 14-amino-acid stretch (residues 124−137) in the M (medium) subunit of the photosynthetic reaction center of *Rhodopseudomonas viridis* (MPRC) (Deisenhofer & Michel, 1989) that displayed 59% homology to residues 261−274 in GPIIb (Figure 3). The secondary structure of the MPRC sequence is an α-helix whose carboxyl-terminal boundary, A136, aligns with G273 in GPIIb. Thus, it is possible for residues GPIIb 261−273 to assume a helical configuration with a carboxyl-terminal boundary at G273, an assumption in keeping with previous alignments of GPIIb-1 with HLH motifs (Tuckwell et al., 1992) and the extensive crystallographic data indicating that the carboxyl-terminal boundary of the incoming helix of HLH motifs is the −1 position (Falke et al., 1994). The second sequence was a 16-amino-acid stretch (residues 175−190) in the acid α-amylase from *Aspergillus niger* (AαA) (Boel et al., 1990) that displayed 69% homology to GPIIb residues 278−293 (Figure 3). Residues 180−190 of AαA display an α-helical conformation whose amino-terminal boundary corresponds to GPIIb residue E283. In the aligned sequences shown in Table 1, GPIIb E283 corresponds to position 10 of the calcium binding loop. Position 10 is the first residue of the outgoing helix of the standard HLH motif (Strynadka & James, 1989). Thus, it is also possible for residues 283−293 in GPIIb to assume an α-helical structure.

Extrapolating from the above analysis, it is possible that GPIIb-1 assumes an HLH conformation in the folded GPIIb molecule. To predict how the amino acid substitutions in GPIIb described above might perturb the secondary structure of GPIIb-1, each of the amino acids was introduced into the −1 position of the first HLH motif (EF1) of calmodulin (Table 1), followed by molecular dynamics simulations using either the full calmodulin structure or a 27-residue stretch of calmodulin containing EF1. The average conformation of EF1 from 100 sampled structures was then compared to the structure of the native motif as determined by X-ray crystallography. Simulations were performed at 100 K because this temperature was shown previously to minimize thermal disruption of calcium binding to HLH motifs (Tuckwell et al., 1992).

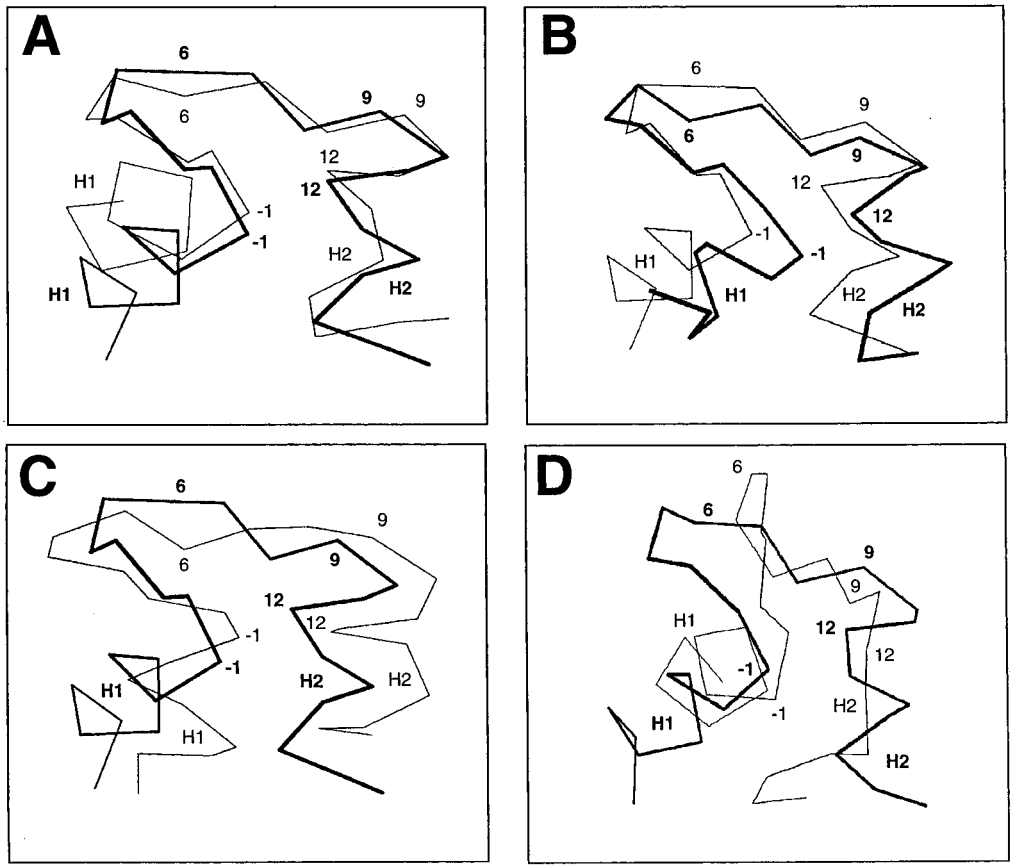

FIGURE 4: Molecular dynamics simulations of the secondary structure assumed by residues 11−37 of calmodulin. This stretch of calmodulin contains its first HLH motif (EF1). The numbers in the figure correspond to the position of amino acids in the calcium binding loop of the standard HLH motif (Strynadka & James, 1989). In calmodulin, −1 corresponds to F19; 6 to G25; 9 to T28; and 12 to E31. H1 corresponds to the incoming helix; H2 corresponds to the outgoing helix. The heavy lines in the figure represent slightly different projections of the native three-dimensional conformation of the calmodulin sequence as determined by X-ray crystallography using coordinates obtained from the Brookhaven crystallographic data base. The thin lines resulted from molecular dynamics simulations performed at 100 K of 100 sampled structures with various amino acids at the −1 position: (**A**) Phe; (**B**) Gly; (**C**) Val; (**D**) Asp.

When the native amino acid Phe was present at the −1 position in the isolated EF1 motif, the structures of EF1 determined by calculation and X-ray crystallography could be readily superimposed by least-squares fitting with rms differences of 0.68 Å for the incoming and outgoing helices and 0.93 Å for the calcium binding loop (Figure 4A). The 14.79-Å distance between the Cαs of Asp at position 3 and Thr at position 9 in the averaged structure was comparable to 15.29 Å in the native motif. Thus, the average structure of native isolated EF1 determined by calculation is an accurate representation of the structure of EF1 determined by crystallography.

Replacing Phe with Gly in the isolated motif had a minimal effect on the topography of the calcium binding loop of EF1 with a displacement of 0.88 Å and an Asp to Thr distance of 15.44 Å. Although the amino-terminal helix (H1) and carboxyl-terminal helix (H2) in the isolated motif were displaced relative to each other, with H1 rotated from an orientation normal to the loop to a more perpendicular position (Figure 4B), there was only minimal displacement of the helices when the complete molecule was modeled (data not shown). When Val was present at the −1 position in the isolated motif, the Asp to Thr distance increased to 19.83 Å, and superimposing the calcium binding loop of the isolated motif on the native structure resulted in an rms deviation of 2.65 Å. The effect on the relative positions of H1 and H2 was similar to the Gly replacement (rms of 2.8 Å) and was due to a hydrophobic interaction between the substituted Val and the Leu at position 13 (Figure 4C). Again, there was only minimal displacement of the helices when their positions were constrained by the remainder of the molecule (data not shown).

By contrast, the presence of Asp at the −1 position resulted in a marked change in the conformation of EF1 in both the isolated motif and the complete molecule. Although there was little distortion of H1 in the isolated motif (rms 0.41Å), there was a marked change in the conformation of the calcium binding loop and a loss of helicity in H2 (rms of 3.42 and 2.48 Å, respectively) (Figure 4D). The observed Asp to Thr distance of 9.14 Å indicated that the topography of the loop was substantially contracted and the change in the conformation of the H2 helix suggested that the side chain of the Asp at position 9 now competed with a backbone hydrogen bond between the Leu at position 13 and the Met at position 17 (Met 36). Similar changes were noted when the entire molecule was studied (data not shown). Further, replacement of Phe with either Lys or Pro at the −1 position had the same effect (data not shown). Thus, it appears that the presence of Asp, Lys, or Pro at the −1 position substantially alters the conformation of EF1, impairing the hydrophobic interactions that normally occur between the ends of the H1 and H2 helices (Strynadka & James, 1989).

Expression of Site-Directed Mutants of GPIIb Amino Acid 286 in COS-1 Cells. The modeling described above suggests

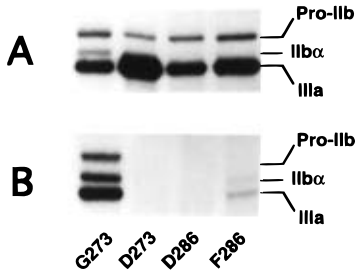

FIGURE 5: Effect of replacing G286 in GPIIb with Asp or Phe on the expression of GPIIb-IIIa heterodimers. G286 was replaced by Asp or Phe by site-directed mutagenesis as described under Experimental Procedures. The resulting GPIIb mutants, wild-type GPIIb, and GPIIb G273D were then coexpressed with GPIIIa in COS-1 cells and GPIIb-IIIa heterodimers were immunoprecipitated from cells metabolically labeled with ^{35}S-methionine using either the anti-GPIIIa mAb SSA6 or the anti-GPIIb-IIIa mAb A2A9. (**A**) Immunoprecipitations using SSA6; (**B**) immunoprecipitations using A2A9.

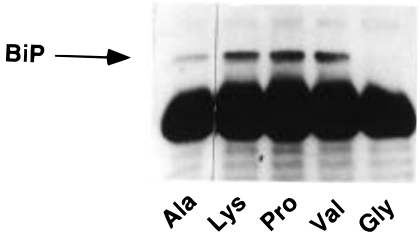

FIGURE 6: Association of GPIIb 273 mutants with the ER chaperone BiP**.** GPIIb G273 was replaced by Ala, Val, Lys, and Pro by site-directed mutagenesis. Following the coexpression of wild-type GPIIb and the GPIIb mutants with GPIIIa in COS-1 cells, GPIIb-IIIa heterodimers were immunoprecipitated using the anti-GPIIIa mAb SSA6. The immunoprecipitates were then transferred to nitrocellulose paper and blotted sequentially with an anti-BiP mAb, goat anti-rat IgG, and ^{125}I-labeled staph protein A as described under Experimental Procedures. The location of BiP on the immunoblots is indicated by the arrow. The intensely stained band below BiP is IgG heavy chain.

that mutations located at the −1 position in the incoming helix of EF1 of calmodulin can impair interactions between its incoming and outgoing helices. Consequently, mutations in the outgoing helix might have the same effect. To test whether this could apply to GPIIb, we used site-directed mutagenesis to replace V286 of GPIIb (Table 1). V286 is the first amino acid distal to GPIIb-1, although it would be located at position +13, the fourth residue of the outgoing helix, in the standard HLH motif (Strynadka & James, 1989). V286 was replaced with Asp by analogy with the G273D mutation and with Phe because this amino acid is present at position +13 in two of the four outgoing helices of calmodulin and one the two outgoing helices of parvalbumin (Table 1). Each GPIIb mutant was then coexpressed with GPIIIa into COS-1 cells, as were wild-type GPIIb (G273) and GPIIb G273D. As seen in Figure 5A, immunoprecipitations using the GPIIIb-specific mAb SSA6 revealed that wild-type GPIIb and each of the GPIIb mutants could associate with GPIIIa. However, only the immunoprecipitate containing wild-type GPIIb also contained a band corresponding the GPIIb heavy chain, suggesting that only wild-type GPIIb-IIIa had been exported from the ER to the Golgi apparatus. Moreover, when the immunoprecipitations were performed using the GPIIb-IIIa-specific mAb A2A9 (Figure 5B), GPIIb-IIIa could be immunoprecipitated from lysates of cells transfected with wild-type GPIIb but not from cells transfected with the G273D and V286D mutants, demonstrating that these mutations had perturbed the A2A9 epitope. However, faint bands corresponding to GPIIbα and GPIIIa were present in the immunoprecipitate from cells transfected with the G286F mutant, suggesting that the presence of this hydrophobic, although bulky, amino acid had a less deleterious effect on the conformation of GPIIb-IIIa than either G273D or V286D.

Association of the GPIIb Mutants with the ER Chaperone BiP. The intracellular retention of malfolded proteins occurs in the ER (Gething & Sambrook, 1992), but the mechanism is not entirely clear. Many nascent proteins associate transiently with the soluble ER chaperone BiP that binds to exposed hydrophobic surfaces as the proteins fold (Blond Elguindi et al., 1993). The association with BiP is more stable when malfolding occurs. To determine if BiP recognizes GPIIb containing Gly, Ala, Val, Lys, or Pro at residue 273, GPIIb-IIIa was immunoprecipitated from extracts of transfected COS-1 cells using the anti-GPIIb-IIIa mAb SSA6 and the immunoprecipitates were immunoblotted with anti-BiP mAb. Unfortunately, the number of GPIIb mutants that could be studied was limited by the amount of anti-BiP mAb available. We found that BiP was present in each immunoprecipitate, except that from cells expressing wild-type (Gly) GPIIb (Figure 6). This result suggests that the presence of an amino acid other than Gly at position 273, even when the presence of this amino acid does not preclude the export of GPIIb-IIIa from the ER, results in a change in the conformation of GPIIb that can be recognized by BiP. Moreover, these results are consistent with the results of the homology modeling in which each of the amino acid replacements resulted in a change in the conformation of the EF1 motif. Nevertheless, because GPIIb-IIIa containing Ala and Val at residue 273 can be transported to the cell surface, the association of BiP with the other GPIIb mutants cannot account for their intracellular retention.

DISCUSSION

To determine how the G273D mutation in GPIIb causes the intracellular retention of GPIIb-IIIa heterodimers, we replaced G273 with selected amino acids by in vitro mutagenesis and examined the expression of the GPIIb-IIIa containing the mutations in COS-1 cells. Although each of the GPIIb mutants associated with GPIIIa, only heterodimers containing the nonpolar amino acids Ala or Val at position 273 were recognized by the GPIIb-IIIa-specific mAb A2A9 and were exported to the cell surface, whereas heterodimers containing negatively charged Glu or positively charged Lys at this position were not recognized by the antibody and were retained intracellularly. These data, taken alone, suggested that simple charge-repulsion interactions due to the presence of an additional charged side chain was sufficient to explain GPIIb-IIIa retention. However, we found that replacing G273 with the uncharged amino acids Asn and Pro also resulted in GPIIb-IIIa retention, suggesting that the explanation was more complex.

Because no structural information about GPIIb-IIIa is available that could be used to explain the results of our mutagenesis, we turned to homology modeling, based on the similarity of the four calcium binding domains in GPIIb to the calcium binding loops of HLH-containing proteins such

as calmodulin and parvalbumin for which X-ray crystal structures are available (Strynadka & James, 1989). A similar approach was taken by Tuckwell et al. (1992) to study calcium binding by integrin EF-hand motifs. These investigators replaced the amino acids of the third calcium binding loop of calmodulin (EF3) with the homologous domains of various integrin α subunits. Using molecular dynamics simulations in vacuo, they found that most of the hybrids retained calcium at temperatures between 100 and 200 K but lost calcium when the structures were heated to 300 K and above to disrupt the local minima.

However, these studies relied on calmodulin to provide critical helices flanking the integrin calcium binding loops. The secondary structure of the sequences that flank the calcium binding loops in GPIIb, or in any integrin α subunits for that matter, is not known and it may not be appropriate to extrapolate from the effects of mutations in the flanking regions of the standard HLH motif to GPIIb. Accordingly, we searched the crystallographic data base to ascertain the secondary structure of sequences homologous to the sequences that flank the calcium binding loop of GPIIb-1. Two homologous sequences were identified by the search: a 14-amino-acid stretch in the M subunit of the photosynthetic reaction center of *Rps. viridis* and a 16-amino-acid stretch in the acid α-amylase from *A. niger*. Each sequence contains a helix whose boundaries corresponded exactly to those of the proximal and distal helices of the standard HLH motif when the sequences were aligned. On the basis of this structural homology, we investigated the effect of amino acid substitutions at the −1 position of GPIIb-1 on the conformation of GPIIb by introducing these amino acids at the analogous position of EF1 in calmodulin. Molecular dynamics simulations then revealed that the side chains of the substituted amino acids projected into a hydrophobic core formed by amino acids at the −1, 8, 13, and 16 positions of the HLH motif (Strynadka & James, 1989). Moreover, as the size of the side chains increased, the conformation of the calcium binding loop was progressively distorted. Although such steric clashes might also have occurred in the GPIIb mutants, it seems unlikely that a change in the conformation of the calcium binding loop alone could account for the intracellular retention of GPIIb-IIIa. As a case in point, missense mutations located in a possible calcium binding region of GPIIIa do not impair the expression of GPIIb-IIIa on the cell surface, although they impair the interaction of GPIIb-IIIa with ligands (Loftus et al., 1990; Bajt & Loftus, 1994).

The HLH motifs in calcium binding proteins occur in pairs and the four helices of each pair of motifs pack against one another in a highly conserved fashion, a result of hydrophobic interactions between the ends of the incoming and outgoing helices of individual motifs and between the adjacent helices of neighboring motifs (Strynadka & James, 1989; Falke et al., 1994). Interfering with these interactions has been shown previously to exert a deleterious effect on both HLH motif structure and function. For example, although substitution of hydrophobic residues in the flanking regions of synthetic peptides corresponding to the third calcium binding site of troponin C had little effect on α helix formation, the resulting impairment of intermolecular interactions decreased the affinity of the peptides for calcium by 3 orders of magnitude and decreased their conformational stability (Monera et al., 1992). Further, the increased calcium affinity observed when the length of peptides corresponding to troponin C site III was increased from 21 to 34 residues was associated with both increased α helix formation (Reid et al., 1981) and the formation of dimers involving hydrophobic interactions between residues in opposing helices (Shaw et al., 1990). Extrapolating from these observations to GPIIb-IIIa, it is possible that disruption of interactions between the sequences flanking the first calcium binding domain of GPIIb may have been responsible for the effect of the G273D mutation. Clearly, in the absence of structural data, there is no way to be certain that such changes could explain the effect of G273D. Nevertheless, there is indirect evidence that sequences located in region of GPIIb containing its calcium binding domains do interact. For example, the K_{Ca}s of 14-residue peptides corresponding to each of the GPIIb calcium binding domains, measured by the ability of calcium to displace terbium, ranged from 0.73×10^2 to 6.14×10^2 M^{-1} (Cierniewski et al., 1994), whereas measurements of calcium binding to a bacterial fusion protein containing the four calcium binding domains indicated that the fusion protein contained four calcium binding sites, two with a $K_{d_{Ca}}$ of ≈30 μM and two with a $K_{d_{Ca}}$ of ≈120 μM (Gulino et al., 1992). Moreover, we found that amino acid replacements both proximal and distal to the first calcium binding domain had identical effects on GPIIb-IIIa expression. Thus, it is conceivable that interactions outside of the GPIIb calcium binding domains themselves, perhaps involving the sequences that flank the domains as our modeling and experimental data suggest, modulate the conformation of this region of GPIIb, accounting for the magnitude of its affinity for calcium and its ability to be exported from the ER.

Nevertheless, how megakaryocytes, or the COS-1 cells used in this study, recognize the conformational change induced by amino acid substitutions at residue 273 is not completely understood. Resident ER proteins termed chaperones assist in the folding of nascent proteins by transiently binding to hydrophobic surfaces (Gething & Sambrook, 1992) or to proteins containing appropriately trimmed Asn-linked carbohydrates (Hammond et al., 1994). Binding of these chaperones to misfolded proteins is more stable and likely contributes to their retention and degradation in the ER. We found that BiP, a chaperone present in the lumen of the ER that binds to hydrophobic surfaces on folding intermediates (Blond Elguindi et al., 1993), was associated with GPIIb when G273 was replaced by Ala, Val, Pro, or Lys. It is noteworthy that the sequences that flank GPIIb-1 contain three 7-amino-acid stretches (residues 254−260, FDSSNP; residues 262−268, FDGYWGY; and residues 289−295, PTWSWTL) that would be predicted to interact with BiP (Blond Elguindi et al., 1993; Poncz et al., 1987; M. J. Gething, personal communication). Thus, it is possible that the conformational change in GPIIb induced by the presence of amino acids other than Gly at position 273 results in the inappropriate exposure of one or more of these sequences. However, because GPIIb-IIIa containing Pro or Lys is retained by the ER, whereas heterodimers containing Ala or Val at this position are exported to the cell surface, BiP binding appears to be a sensitive indicator of the fidelity of GPIIb-IIIa folding but cannot explain the retention of specific GPIIb-IIIa mutants.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403.

Bajt, M., & Loftus, J. (1994) *J. Biol. Chem. 269*, 20913.

Basani, R. B., Vilaire, G., Shattil, S. J., Kolodziej, M. A., Bennett, J. S., & Poncz, M. (1996) *Blood* (in press).

Bennett, J. S. (1996) *Trends Cardiovasc. Med. 16*, 31.

Bennett, J. S., Hoxie, J. A., Leitman, S. F., Vilaire, G., & Cines, D. B. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2417.

Bennett, J. S., Kolodziej, M. A., Vilaire, G., & Poncz, M. (1993) *J. Biol. Chem. 268*, 3580.

Boel, E., Brady, L., Brzozowski, A. M., Derewenda, Z., Dodson, G. G., Jensen, V. J., Petersen, S. B., Swift, H., Thim, L., & Woldlike, H. F. (1990) *Biochemistry 29*, 6244.

Blond Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F., & Gething, M. J. (1993) *Cell 75*, 717.

Chen, Y., Djaffar, I., Pidard, D., Steiner, B., Cieutat, A.-M., Caen, J. P., & Rosa, J.-P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10169.

Cierniewski, C., Haas, T., Smith, J., & Plow, E. (1994) *Biochemistry 33*, 12238.

Deisenhofer, J., & Michel, H. (1989) *Science 245*, 1463.

Falke, J. J., Drake, S. K., Hazard, A. L., & Peersen, O. B. (1994) *Q. Rev. Biophys. 27*, 219.

Fujimura, K., & Phillips, D. R. (1983) *J. Biol. Chem. 258*, 10247.

George, J. N., Caen, J. P., & Nurden, A. T. (1990) *Blood 75*, 1383.

Gething, M.-J., & Sambrook, J. (1992) *Nature 355*, 33.

Gulino, D., Boudignon, C., Zhang, L., Concord, E., Rabiet, M.-J., & Marguerie, G. (1992) *J. Biol. Chem. 267*, 1001.

Hammond, C., Braakman, I., & Helenius, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 913.

Kolodziej, M. A., Vilaire, G., Gonder, D., Poncz, M., & Bennett, J. S. (1991a) *J. Biol. Chem. 266*, 23499.

Kolodziej, M. A., Vilaire, G., Rifat, S., Poncz, M., & Bennett, J. S. (1991b) *Blood 78*, 2344.

Loftus, J. C., O'Toole, T. E., Plow, E. F., Glass, A.., Frelinger, A. L., III, & Ginsberg, M. H. (1990) *Science 249*, 915.

Loh, E., Beaverson, K., Vilaire, G., Qi, W., Poncz, M., & Bennett, J. S. (1995) *J. Biol. Chem. 270*, 18631.

Monera, O. D., Shaw, G. S., Zhu, B.-Y., Sykes, B. D., Kay, C. M., & Hodges, R. S. (1992) *Protein Sci. 1*, 945.

Phillips, D. R., Charo, I. F., Parise, L. V., & Fitzgerald, L. A. (1988) *Blood 71*, 831.

Poncz, M., Eisman, R., Heidenreich, R., Silver, S. M., Vilaire, G., Surrey, S., Schwartz, E., & Bennett, J. S. (1987) *J. Biol. Chem. 262*, 8476.

Poncz, M., Salahandrin, R., Coller, B. S., Newman, P. J., Shattil, S. J., Parrella, T., Fortina, P., & Bennett, J. S. (1994) *J. Clin. Invest. 93*, 172.

Reid, R. E., Gariepy, J., Saund, A. K., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2742.

Shaw, G. S., Hodges, R. S., & Sykes, B. D. (1990) *Science 249*, 280.

Silver, S. M., McDonough, M. M., Vilaire, G., & Bennett, J. S. (1987) *J. Clin. Invest. 69*, 1031.

Strynadka, N. C. J., & James, M. N. G. (1989) *Annu. Rev. Biochem. 58*, 951.

Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8765.

Tuckwell, D. S., Brass, A., & Humphries, M. J. (1992) *Biochem. J. 285*, 325.

Wilcox, D. A., Wautier, J. L., Pidard, D., & Newman, P. J. (1994) *J. Biol. Chem. 269*, 4450.

Wilcox, D. A., Paddock, C. M., Lyman, S., Gill, J. C., & Newman, P. J. (1995) *J. Clin. Invest. 95*, 1553.

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