Crystal Structure of the Platelet Glycoprotein Ibα N-terminal Domain Reveals an Unmasking Mechanism for Receptor Activation*

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Sarah Uff‡, Jeannine M. Clementson§, Tim Harrison‡, Kenneth J. Clementson¶, and Jonas Emsley‖

From the ‡Department of Biochemistry, University of Leicester, Leicester LE1 7RH, United Kingdom and ¶Theodor Kocher Institute, University of Berne, Freiestrasse 1, CH-3012 Berne, Switzerland

Glycoprotein Ib (GPIb) is a platelet receptor with a critical role in mediating the arrest of platelets at sites of vascular damage. GPIb binds to the A1 domain of von Willebrand factor (vWF-A1) at high blood shear, initiating platelet adhesion and contributing to the formation of a thrombus. To investigate the molecular basis of GPIb regulation and ligand binding, we have determined the structure of the N-terminal domain of the GPIbα chain (residues 1–279). This structure is the first determined from the cell adhesion/signaling class of leucine-rich repeat (LRR) proteins and reveals the topology of the characteristic disulfide-bonded flanking regions. The fold consists of an N-terminal β-hairpin, eight leucine-rich repeats, a disulfide-bonded loop, and a C-terminal anionic region. The structure also demonstrates a novel LRR motif in the form of an M-shaped arrangement of three tandem β-turns. Negatively charged binding surfaces on the LRR concave face and anionic region indicate two-step binding kinetics to vWF-A1, which can be regulated by an unmasking mechanism involving conformational change of a key loop. Using molecular docking of the GPIb and vWF-A1 crystal structures, we were also able to model the GPIb-vWF-A1 complex.

The glycoprotein (GP)1 Ib-V-IX complex is a platelet membrane receptor complex with a critical role in adhesion to the damaged vessel wall under conditions of high shear stress (1). Platelets are gradually slowed, and subsequently, integrins are activated by repeated interactions of GPIb-V-IX with von Willebrand factor (vWF) bound to the subendothelium. The GPIb-V-IX complex contains four subunits, GPIbα, GPIbβ, GPIX, and GPV, each with an extracellular domain, a single transmembrane helix, and a short cytoplasmic tail. The most important component of the GP Ib-V-IX complex in terms of mass and functional sites is the 150-kDa GPIbα chain with binding sites for the vWF-A1 domain and thrombin in its extracelluar N-terminal domain and binding sites for filamin and 14–3-3ζ in its cytoplasmic domain. Mutations in GPIb result in the congenital bleeding disorders, Bernard Soulier syndrome (BSS), and platelet-type von Willebrand disease (Pt-vWD). Several informative BSS point mutations result in an expressed GPIb receptor deficient in vWF binding, whereas the Pt-vWD point mutations produce a hyperactivated receptor. In addition, von Willebrand disease type Ib point mutations in the vWF-A1 domain also produce high affinity binding by inducing conformational changes in the A1 domain.

Although the principal ligand for GPIbα is vWF (1, 2), it has also been shown to bind a growing number of other proteins including thrombin (3, 4), kininogens (5), Factor XI (6), Factor XII (7), P-selectin (8), and Mac-1 (9). Proteolysis of GPIbα yields a 40–45-kDa N-terminal domain containing binding sites for all the principal ligands (4). Studies on the mechanism of GPIbα-vWF interactions are complicated by the fact that the two molecules do not normally interact under static conditions but only under shear stress (10, 11). Several techniques have been used to induce interactions between these proteins including ristocetin, an aminoglycoside antibiotic (12), or botrocetin, a snake C-type lectin (13).

GPIbα is a member of the leucine-rich repeat (LRR) family (14, 15), which includes a large number of proteins that are principally involved in mediating protein-protein interactions (16). LRRs are typically 22–28 amino acids long and occur in tandem repeats that are commonly flanked by conserved disulfide loop structures (17). The GPIbα N-terminal domain, LRRs, and C-terminal flanking anionic region have all been implicated in contributing to the vWF binding site (18, 19), whereas GPIbα binding to thrombin is localized exclusively to the area of the anionic region. The anionic region includes three tyrosine residues (Tyr-276, Tyr-278, and Tyr-279) that are post-translationally sulfated in the native receptor, and this modification has been shown to be essential for binding to vWF and thrombin (20, 21). We have previously determined the crystal structure of the A1 domain of vWF containing the binding site for GPIbα (22, 23). To investigate further the molecular basis of the GPIbα-vWF interaction, we now report the crystal structure of the N-terminal domain of the GPIbα chain.

**EXPERIMENTAL PROCEDURES**

Crystalization and Data Collection—The GPIbα N-terminal domain was prepared from platelets and in recombinant form. Glycocalcin, the GPIbα extracellular domain, was isolated and purified from platelets as described previously (24). This was cleaved to generate the N-terminal 45-kDa region (amino acids 1–288) and macroglycopeptide using 1:250 w/w Lys-C endoprotease treatment for 24 h at 4 °C. The N-terminal domain was separated from the macroglycopeptide by linear salt gradient (0.2–1 M) on a Mono Q column equilibrated with 25 mM Tris 7.5, 0.2 M NaCl. DNA corresponding to the GPIbα signal peptide and resi-
Structure of Platelet GPIbα N-terminal Domain

Table I

| Dataset          | Native | PIP | PtCl4 | K2OsO4 | PtI6 |
|------------------|--------|-----|-------|--------|------|
| Resolution (Å)   | 20–3.5 | 20–2.8 | 20–3.5 | 20–4.0 | 20–3.6 |
| Rwork (%)        | 0.071 (0.359) | 0.110 (0.471) | 0.099 (0.435) | 0.145 (0.450) | 0.116 (0.425) |
| Completeness (%  | 99.3 (98.9) | 99.8 (99.8) | 98.9 (99.1) | 99.9 (100.0) | 99.5 (97.3) |
| Redundancy       | 5.4 (4.0) | 11.1 (8.9) | 4.3 (3.3) | 10.2 (9.8) | 9.7 (7.5) |
| Sigf1            | 12.7 (2.8) | 16.1 (2.1) | 9.1 (2.0) | 11.4 (3.8) | 12.8 (2.3) |
| Phasing Power    | 0.93    | 0.72 | 0.79  | 0.84   |
| Rfree (%)        | 0.213  | 0.246 | 0.097 | 0.341  |
| No. of sites     | 5      | 5    | 7    |
| Refinement       | 0.245  | 0.275 | r.m.s. deviations bond lengths 0.0083 Å bond angles 1.84° mean positional error° 0.4 |
| Real space CC,Rfactor^{c} | 0.912, 0.086 |


^{a} Rwork = \sum ||Fo|-|Fc||/\sum |Fo| where |Fo| > |Fc|.

^{b} Phasing power = F/Fo where Fo is the lack of closure.

^{c} Denotes real space correlation coefficient (CC) for the atomic model against a sigma weighted 2Fo – Fc electron density map (calculated using program CNS).

^{d} Mean positional error calculated from the Luzzati plot using CNS.

RESULTS

The GPIba Structure—We expressed residues 1–288 from human GPIb in simian cv-1 cells and the resulting purified protein crystalized in space group p6_22. Isomorphous crystals grow from the same GPIbα fragment generated by proteolysis of glyocalcicin isolated from platelets. The structure was determined to 2.8 Å using four heavy atom derivatives (Table I) and refined to an R-factor of 24.5% (R_{work} 27.6%) with two molecules in the asymmetric unit. The GPIbα-fold consists of an N-terminal 6-hairpin (residues 2–18), eight LRRs (19–204), a disulfide knot structure (amino acids 205–264), and a C-terminal anionic region (amino acids 265–280) (Table I). The N-terminal 6-hairpin has two anti-parallel strands with a disulfide (C4–C17) bridge at the base. The second \( \beta \)-strand joins the convex parallel \( \beta \)-sheet in a configuration very similar to the spliceosomal complex subunit U2A*, which has only five LRRs (27). The two \( \beta \)-strands present on the concave face of the GPIbα LRRs 3 and 4 are also present in U2A*. The eight LRRs fold into a characteristic arc shape with a parallel \( \beta \)-sheet on the concave face. Previous sequence comparisons and/or models suggested that GPIbα contains seven LRRs and not the eight found here. This discrepancy is the result of the earlier definition of the sequence of an LRR, which left an overhanging sequence at each end as “flanking sequences.” By changing the start and end sites of the LRR, these sequences are incorporated into the structure as an additional repeat.

The concave face of LRRs 5, 6, 7, and 8 each has a novel LRR motif consisting of a tandem arrangement of three \( \beta \)-turns. They form a flattened amphipathic structure with the main chain hydrogen bonds in a linear arrangement (Fig. 2). The consensus for this motif is PXGLLXGL with leucine side chains projecting into the hydrophobic core. Adjacent \( \beta \)-turn repeat motifs form a regular parallel packing arrangement with the core leucine...
residues pointing alternately in and out of the plane of the structure interlocking with the corresponding leucines from the adjacent repeat. The LRR8 β-turn repeat motif has two phenylalanine residues in place of leucines in the central β-turn. These bulkier side chains serve to fill the gap between the end of the LRRs and the core of the C-terminal flank by packing against Phe-232 and Trp-235 from helix α1. It is unclear why the up and down repeating β-turn motif found here is not a more common secondary structural element. It is possible that the flattened structure is appropriate for packing in the LRR-fold but would be less stable in other contexts.

The GPIba C-terminal Flanking Region—The GPIba C-terminal flanking sequence contains two disulfide bonds, C209–C248 and C211–C264, an α-helix (residues 214–223), and a loop (residues 227–242), which extends over the interior of the GPIba LRR concave face (termed the regulatory or R-loop in Fig 3, a and b). Initially, the structure continues to follow a circular coil similar to the LRRs with helix α1 lying parallel to the β-turn repeats of the concave face. Following helix α1 comes the triangular-shaped R-loop, two short 3₁₀ helices set at right angles, and a further loop (residues 251–264) extending to Cys-264. The structure of residues 269–279 from the C-terminal anionic region is illustrated in Fig. 3c. These residues are well defined in the electron density of one GPIba molecule but are disordered in the second molecule. The anionic region extends outwards from Cys-264 with Asp-269 to Asp-272 resembling a hinge that turns through 180° and is followed by residues Asp-274 to Asp-277, which form a single α-helical turn. The carboxyl group of Asp-274 forms helix-capping hydrogen bonds to Tyr-276 and Asp-274 main chain nitrogen atoms, and the Asp-277 carboxyl forms a hydrogen bond to the Tyr-274 main chain. Tyrosine residues 276, 278, and 279 are post-translationally sulfated. Interactions with the convex face of LRR8 and C-terminal flank occur through the Tyr-278 and Tyr-279 sulfate groups respectively. The Tyr-278 sulfate moiety forms a hydrogen bond to the main chain nitrogen of Gly-190, and similarly, the Lys-189 side chain nitrogen makes a hydrogen bond to the Tyr-278 main chain carbonyl. The Tyr-279 sulfate forms salt bridges with the Arg-217 and Arg-218 guanidinium groups from helix α1. The Tyr-276 sulfate forms adventitious hydrogen bonding interactions with Asn-59 and Asn-61 from a symmetry-related molecule.

The interaction of the anionic region with the main body of GPIba structure buries a small surface area, and in solution, this structure may be in equilibrium with a more extended conformation through flexibility around the Asp-269 to Asp-272 hinge. The alignment of the human, canine, and murine GPIba sequences (Fig. 4) shows poor conservation within the anionic region. Only Asp-272, Asp-274, Tyr-276, and Asp-277 are absolutely conserved with deletions occurring on either side of this sequence in the alignment. This is consistent with the Asp-272 to Asp-277 helix representing a conserved structure with short sequences on either side, possibly acting as flexible hinges connecting to the GPIba LRRs and the membrane proximal macroglycopeptide.

vWF Binding to GPIba—The GPIba structure reveals a large patch of negatively charged amino acids (Glu-14, Asp-18, Glu-40, Asp-63, Asp-83, Asp-106, Glu-128, Glu-151, and Asp-175) across the concave surface formed by the LRRs and the contiguous β-strands of the N-terminal hairpin, which indicates a possible binding surface for interaction with vWF-A1. Although only a few examples of structures of the LRR domain complexes with ligands have been determined (18, 27), the binding site involves the concave face β-sheet of the LRRs that is either positively or negatively charged. The view of the GPIba structure shown in Fig. 5 resembles the profile of an open hand with the R-loop forming the thumb, which extends over the “palm” region of the LRR concave face, leaving a 20-Å aperture to the tips of the fingers. The 45 × 40 × 30-Å dimensions of the vWF-A1 structure (11) indicates that this globular domain cannot contact the ligand binding surface on the palm of GPIba.
because of steric hindrance from the thumb. We propose that the GPIbα crystal structure represents the low affinity or "closed" form of the receptor and that a conformational change in the thumb is required to unmask the A1 domain binding site. The structure of the high affinity or "open" form could either involve the thumb folding back partially and participating in an extended concave-shaped vWF-A1 binding site or, alternatively, a simple removal of the steric constraint. If the R-loop is removed from the GPIbα structure, the A1 domain can be satisfactorily docked against the concave surface.

BSS point mutations causing loss of vWF binding localize to the general area around the concave face β-strands from LRRs 5, 6, and 7 (L129P, A156V, and L179del) and also the sides of LRR2 (C65R and L57F) (Fig. 1). These mutations are all buried hydrophobic core residues with the exception of Ala-156, which lies on the surface of the concave face partially buried by surrounding side chains and the R-loop. Pt-vWD mutations G233V (28) and M239V (29), which activate the receptor, lie on the outer tips of the triangular R-loop and presumably act by favoring a more open conformation of the receptor (Fig. 3, a and b). Further "activating" mutations D235V and K237V (30) have been identified by site-directed mutagenesis studies in the region of the R-loop. Further support for an unmasking mechanism comes from antibody 24G10 that inhibits vWF binding.

**FIG. 3.** Fine structure of the GPIbα R-loop and anionic region. a and b, the R-loop and β-strands from the concave face viewed from two orientations related by 90° rotation are shown. Key residues are labeled including platelet-type von Willebrand disease mutations G233V and M239V and the Bernard-Soulier syndrome mutation A156V. c, fine structure of the GPIbα anionic region. Key residues in the anionic region (residues 569–279) are indicated showing the interactions with the disulfide loop and β-turn repeat motif from LRR8. The backbone of the anionic region is colored red, the disulfide loop is colored dark green, and the LRR8 is colored blue. Key hydrogen bonding/electrostatic interactions are indicated as dotted blue lines.

**FIG. 4.** Sequence alignment of the GPIbα N-terminal domains. Alignment of human, canine, and murine sequences (ClustalW) is shown. Secondary structure assignments made from the human GPIbα structure are superimposed on the alignment. Concave face LRR β-strands are colored gray, convex face β-strands are colored green, α-helices are colored dark blue, and $\beta_1$ helices are colored light blue. The R-loop is colored orange, and the anionic region is colored red. Asparagine residues forming consensus LRR-buried hydrogen bonds are underlined (at residue 65, this position is occupied by cysteine). Mutations from Bernard-Soulier syndrome and platelet-type von Willebrand disease are shown as black and orange balls, respectively (note that the Leu-179 mutation is a single residue deletion).
and maps to GPIbα residues 1–81 (20). This antibody binds to the Pt-vWd GPIbα mutants with increased affinity, indicating that it overlaps with a vWF-A1 binding site that is regulated by the conformation of the R-loop.

A Model of the GPIbα-vWF Complex—Coordinates from the GPIbα structure (R-loop removed) and the vWF-A1 von Willebrand disease type IIb mutation structure (31) (residues 499–701) were docked together using the automated search program FTDock (32). This model was manually adjusted to optimize side chain interactions and remove steric conflicts using XtalView (33). The R-loop was then modeled back into the complex structure lying to one side of the A1 interface. In this model, loops from the top of the A1 domain-fold are oriented toward the GPIbα N-terminal β-hairpin with further extensive interactions formed down the full-length of the GPIbα LRR concave face β-strands (Fig. 6, a and b). Loops from the top of the A1 domain contribute basic residues Arg-524, Arg-629, Arg-632, which form salt bridges with Glu-14, Glu-40, and Asp-83, respectively. His-656 of A1 also forms a hydrogen bond to the main chain carbonyl from Lys-8. Gln-590 hydrogen bonds to Tyr-58/Gln-59, and Gln-628 forms a hydrogen bond to Glu-40. At the center of the interface, residues Asp-560 and Gln-604 and Ser-242 GPIbα side chain, respectively.

In the GPIbα structure, the C-terminal anionic region is located ~50 Å from the center of the LRR concave face, indicating that this forms a distinct binding site for vWF-A1. The model in Fig. 6, a and c, illustrates that an extended form of the anionic peptide with the Asp-274 to Asp-277 helix intact can act in concert with the GPIbα concave face to engage a single vWF-A1 domain. In this model the helical arrangement of three sulfate groups from GPIbα residues, Tyr-276, Tyr-278, and Tyr-279, form interactions with four basic residues, Lys-569, Arg-571, Lys-572, and Arg-573 from A1. The overall model is consistent with the reported 1:1 stoichiometry for the GPIbα-vWF-A1 interaction. All A1 domain residues described in this model have been implicated by mutagenesis in binding to GPIbα with the exception of the GPIbα anionic region (red) and R-loop (orange), which are assumed to have backbone flexibility. b, stereo diagram of the GPIbα LRR/A1 domain interface in close-up view with interacting residues labeled black (GPIbα) and green (A1), and A1 helices and loops are colored blue. c, Stereo diagram close-up showing the interaction between GPIbα-sulfated tyrosines, Tyr-276, Tyr-277, and Tyr-278, and basic residues from the A1 domain loop βC-α3 at the bottom of the A1-fold.

The highest scoring complex with an RPscore of 5.4 was used (32).
interactions with vWF-A1. The model has a similar architecture to the crystal structure of the spliceosomal U1A‘U2B’ protein complex in that the αβ-fold of the U2B’ domain binds the concave face of the U1A’ LRRs.

The model of the complex provides a rationale for the affinity differences observed for BSS and Pt-vWD GPIbα mutants and also between the wild type and activated type IIb mutant vWF-A1. In the latter case, as residue Asp-560 A1 is buried at the center of the complex interface, the small conformational differences in this residue observed between the wild type and type IIb mutant crystal structures (31) can exert a disproportionately large effect on binding affinity. In the wild type vWF-A1 structure, the Asp-60 peptide bond is rotated through ~180°, which results in the loss of a hydrogen bond between the Asp-560 main chain carbonyl and Lys-152 GPIbα NZ nitrogen. Perhaps more significantly, the Asp-560 side chain moves into a position creating a steric conflict with Tyr-130 GpIbα and is also closer to Glu-128 carboxylate than its predicted binding partner Lys-152. Because of the dense side chain packing on the surface of the LRR concave β-strands, it is difficult to alleviate these steric clashes through rotation of GPIbα side chain torsional angles. A similar argument may explain the complete loss of vWF binding caused by the subtle GPIbα A156V mutation in Bolzano variant BSS patients. Ala-156 is not predicted to interact with the A1 domain as it is partially buried by surrounding GPIbα side chains Lys-132, Glu-180, Leu-178, and Asn-157. However, the mutation to the bulkier valine would push the neighboring side chains out of their wild type conformation, and in particular, this would be predicted to disrupt the Lys-132 salt bridge to Glu-596 from the A1 domain.

**DISCUSSION**

The GPIbα structure shows how the N- and C-terminal flanking regions are intimately related with the LRRs and reveals a mechanism for regulating the affinity of the receptor via the conformation of the R-loop by unmasking the LRR binding site for vWF-A1. The role of the LRR concave face binding site is fundamental for binding the A1 domain as the mutations here or binding of antibodies with epitopes in this region block A1 binding in response to all reagents (20). Thus, the A1 domain interaction with the GPIbα anionic peptide probably precedes binding to the concave face in multistep-binding kinetics that are reminiscent of the thrombin-hirudin interaction (Fig. 7) (39). It is also conceivable that the GPIbα anionic peptide binds and stabilizes a high affinity conformation of the A1 domain. Heparin is able to activate vWF binding to GPIb, which can be explained if heparin binds to the A1 domain and stabilizes a high affinity conformation. This notion is further supported by the observation that a monoclonal antibody to the A1 domain that blocked heparin binding (mAb 724) was also able to activate vWF binding to GPIb (40). There is also the possibility that GPIbα anionic region could activate the A1 domain as the putative interaction site on A1 lies in the same region as the von Willebrand disease type IIB mutations (40).

The GPIbα-unmasking mechanism may provide a general pathway whereby other members of the family of LRR proteins can regulate ligand binding by utilizing their flanking sequences in a manner similar to the GPIbα R-loop. How the in vivo high shear stress conditions required to stimulate GPIbα-vWF binding affect the two vWF binding sites and the conformation of the R-loop will require further study. Platelet-sized latex beads coated with recombinant GPIbα N-terminal domain show the same elevated binding to immobilized vWF at increasing shear stress as platelets (41). This indicates that the shear-dependent activation is inherent within the N-terminal domain and is not induced by changes in the cytoskeleton or receptor clustering effects. It is possible that fluid shear forces act directly on the GPIbα N-terminal domain structure, affecting the equilibrium between open and closed conformations of the R-loop.

Binding between GPIbα and vWF is a central element in the regulation of hemostasis and progression to the pathological condition thrombosis. Restriction of vessel size by arteriosclerosis enhances shear and is a major factor in propagating cardiovascular disease. Recent studies have also implicated the abnormal expression of the GPIb complex on breast cancer cells in malignancy and metastasis (42, 43). The GPIbα-vWF axis is an important target for anti-thrombotic strategies and drugs, capable of inhibiting GPIbα-vWF binding, and would be important tools for prophylaxis and treatment of such diseases.

**Thrombin and P-selectin Binding to GPIbα—GPIbα binds constitutively to α-thrombin (3, 4), P-selectin (8), and integrin Mac-1 (9) through interactions with its C-terminal flanking sequences. Of these interactions, the 0.1 μM Kᵦ α-thrombin binding to GPIbα is the best characterized. In particular, sulfated tyrosines 276, 278, and 279 of the GPIbα anionic region and the electropositve heparin binding site of thrombin were shown to be essential components for binding (23). There is an interesting similarity between the GPIbα Asp-274 to Asp-277 α-helix and the α-helical region in the anionic peptide from the thrombin inhibitor hirudin. Here, the sulfated Tyr-276 in GPIbα occupies the same position on the helical turn as the sulfated Tyr-63 in hirudin. However, the structure of the thrombin/hirudin complex showed that the anionic peptide of hirudin binds to a different region of thrombin (exosite I) than GPIbα (44). A recombinant fragment of vWF blocked thrombin binding to platelets (45), indicating that these binding sites in the anionic region overlap. Although the role of the thrombin binding site on GPIbα in platelet physiology has been controversial in the past, evidence for a critical function is growing (46).

The GPIbα anionic region is also strongly implicated in mediating the GPIbα P-selectin interaction, which is thought to play a role in the adhesion of platelets to endothelial cells lining the vessel wall. A crystal structure exists for P-selectin in complex with the anionic peptide from the PSGL-1 leukocyte receptor (47). In this case, 10 amino acid residues of the peptide were involved in binding with the sulfate groups from two modified tyrosine residues contributing through hydrogen bonding and electrostatic interactions. Unlike the interaction with GPIbα, the P-selectin/PSGL-1 anionic peptide binding requires calcium and carbohydrate core-2 branching or ω-(1,3)-
fucosylation. In conclusion, these cell-adhesive anionic peptides are inherently partially disordered in the unbound state. Short elements of secondary structure and a variety of positions of charged residues and sulfated tyrosines allow for diverse specificities.

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