Leucine-rich Repeats 2–4 (Leu$^{60}$–Glu$^{128}$) of Platelet Glycoprotein Ibα Regulate Shear-dependent Cell Adhesion to von Willebrand Factor*

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Glycoprotein (GP) Ib-IX-V binds von Willebrand factor (VWF), initiating thrombosis at high shear stress. The VWF-A1 domain binds the N-terminal domain of GPIbα (His1–Glu$^{282}$); this region contains seven leucine-rich repeats (LRR) plus N- and C-terminal flanking sequences and an anionic sequence containing three sulfated tyrosines. Our previous analysis of canine/human and human/canine chimeras of GPIbα expressed on Chinese hamster ovary (CHO) cells demonstrated that LRR2–4 (Leu$^{60}$–Glu$^{128}$) were crucial for GPIbα-dependent adhesion to VWF. Paradoxically, co-crystal structures of the GP Ibα N-terminal domain and GPIbα-binding VWF-A1 under static conditions revealed that the LRR2–4 sequence made minimal contact with VWF-A1. To resolve the specific functional role of LRR2–4, we compared wild-type human GPIbα with human GPIbα containing a homology domain swap of canine for human sequence within Leu$^{60}$–Glu$^{128}$ and a reverse swap (canine GPIbα with human Leu$^{60}$–Glu$^{128}$) for the ability to support adhesion to VWF under flow. Binding of conformation-specific anti-GPIbα antibodies and VWF binding in the presence of botrocetin (which does not discriminate between species) confirmed equivalent expression of wild-type and mutant receptors in a functional form competent to bind ligand. Compared with CHO cells expressing wild-type GPIbα, cells expressing GPIbα, where human Leu$^{60}$–Glu$^{128}$ sequence was replaced by canine sequence, supported adhesion to VWF at low shear rates but became increasingly ineffective as shear increased from 50 to 2000 s$^{-1}$. Together, these data demonstrate that LRR2–4, encompassing a pronounced negative charge patch on human GPIbα, is essential for GPIbα-VWF-dependent adhesion as hydrodynamic shear increases.

Binding of platelet glycoprotein (GP)$^2$ Ib-IX-V to von Willebrand factor (VWF) in plasma, subendothelial matrix, or on endothelium initiates thrombus formation at high shear stress in normal hemostasis and thrombotic diseases, such as heart attack or stroke (1–5). GPIb-IX-V consists of four members of the leucine-rich repeat (LRR) family: GPIbα disulfide-linked to GPIβ and associated with GPIIX and GPV (2,2:2,1) (1). VWF binds to the N-terminal domain of GPIbα (His$^1$–Glu$^{282}$), consisting of seven leucine-rich repeats (Leu$^{36}$–Ala$^{209}$), N- and C-terminal flanking sequences (His$^1$–Ile$^{35}$ and Phe$^{201}$–Gly$^{268}$), and an anionic sequence (Asp$^{209}$–Glu$^{282}$) containing three sulfotyrosines at positions 276, 278, and 279. We previously expressed a series of human/canine and canine/human chimeras of GPIbα and mapped binding sites for VWF and a panel of inhibitory anti-GPIbα antibodies to precise structural domains (6, 7). This approach is based on the specificity of human VWF and murine antibodies for human (not canine) GPIbα (6). Chimeras consisted of human sequence incrementally replaced by canine sequence from the N terminus at domain boundaries and canine sequence His$^1$–Glu$^{282}$ rehumanized from the N terminus (human replacing canine sequence). LRR2–4, spanning residues Leu$^{60}$–Glu$^{128}$, was identified as crucial for GPIbα-dependent adhesion to VWF under shear conditions. Paradoxically, co-crystal structures subsequently reported for GPIbα N-terminal domain and VWF-A1 domain fragments (8–10) revealed major contact sites clustered N- and C-terminally to LRR2–4, whereas the Leu$^{60}$–Glu$^{128}$ sequence made minimal contact with VWF-A1 (with the exception of a single water-mediated contact between Asp$^{63}$ of GPIbα and Arg$^{571}$ of VWF-A1) (10). This discrepancy between structure and function requires resolution to understand the molecular basis for shear-dependent platelet adhesion, especially if the GPIbα-VWF interaction is considered as an anti-thrombotic target (4, 5). Notably, LRR2–4 in human but not canine GPIbα has a pronounced negative charge patch at the concave surface of the repeats, complementary to a positive patch on VWF, implying electrostatic interactions are critical for GPIbα-mediated adhesion to VWF (1, 11), even though this...
sequence makes minimal contact with VWF-A1 under static conditions used for crystallography (8–10).

Here we analyzed GPIbα human/canine homology domain swaps designed to maintain N- and C-terminal contact sites (based on crystal structures) (9, 10) but altered the intervening electrostatic region (6), to establish the specific functional role of Leu60–Glu128. Comparing wild-type (WT) human GPIbα and a homology swap with canine instead of human sequence within Leu60–Glu128 (HUMCAN60–128) expressed on CHO cells shows that LRR2–4 of GPIbα is essential for GPIbα/VWF-dependent adhesion as hydrodynamic shear increases.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Murine anti-GPIbα monoclonal antibodies have been characterized elsewhere: AN51 maps to His1–Ile35, 6D1 to Leu105–Glu128 (LRR4), VM16d to Val226–Gly268, and WM23 to the macroglycopeptide region downstream of Glu282 (6, 7). CR1 against VWF-A1 (12) was used as a negative control.

**Molecular Modeling**—Models of canine GPIbα N-terminal domain and human/canine homology swaps, based on the GPIbα crystal structure, were built as described previously (6, 11).

**GPIb-IX-transfected CHO Cells**—Human/canine GPIbα Leu60–Glu128 homology domain swap constructs were gen-
RESULTS

To establish the functional role of Leu60–Glu128 of GPIbα, especially the prominent negative charge patch that this region contributes to the concave face of the repeats (complementary to a positive charge patch on the interacting face of VWF-A1) (1, 9–11), we expressed human/canine homology swaps of GPIbα (Fig. 1A). This approach enables functional analysis of the N-terminal domain of GPIbα that is conformationally sensitive and not amenable to analysis by short peptides or scanning mutagenesis (6, 7). Furthermore, expressing mutants on CHO cells enables antibody binding and adhesion to VWF under flow to be evaluated. Several lines of evidence confirm that GPIbα homology swaps are expressed in a functional form without significant conformational disruption. First, modeling of constructs as described previously (6, 11) suggests no significant structural disorder because all core-structural residues are conserved across the species. The models also illustrate conspicuous electrostatic differences in surface charge between human and canine GPIbα (Fig. 1B). A negative patch on human GPIbα is predominantly centered on Asp63 and other residues comprising this surface. In canine GPIbα, Asp63 is substituted by Arg, which together with His61 results in loss of the negative patch. There is no direct contact between the human GPIbα sequence, Leu60–Glu128 (LRR2–4), and VWF-A1 (Fig. 1B), although the co-crystal structure (10) reveals a single water-mediated contact between Asp63 and Arg571 of VWF-A1 (an interaction that is predicted to be abolished in the HUMCAN60–128 swap). Second, GPIbα homology swaps expressed as a GPIb-IX complex on CHO cells are recognized by conformation-sensitive antibodies (6, 7, 11): epitopes for AN51 mapping to the N-terminal sequence; His1–Ile35 and VM16d mapping to the C-terminal flank sequence; Val126–Gly128 are both present in WT-GPIbα and HUMCAN60–128 and 6D1 maps to Leu104–Glu128, present in WT-GPIbα and CAN_HUM60–128 (Fig. 2, cf. Fig. 1A). WM23, with an epitope C-terminal of Glu128, recognizes WT-GPIbα, HUMCAN60–128, and CAN_HUM60–128 confirming equivalent expression on cells used for functional analysis. Third, all of the mutants bind human VWF in the presence of the modulator botrocetin, which does not discriminate between species (6), suggesting that the recep-
Electrostatic Interactions of GPIbα and VWF

Adhesion of GPIbα-expressing CHO cells to VWF. A, adhesion of WT-GPIbα-expressing CHO cells or CHO cells expressing HUM60–128 or HUM60–128 mutant GPIbα to immobilized human VWF with increasing shear rate (s⁻¹). B, rolling velocity of adherent cells from A. C, ratio of the number of adherent HUM60–128 and WT-GPIbα cells from A. D, ratio of rolling velocities of HUM60–128 and WT-GPIbα cells from B.

To establish the functional role of Leu⁶⁰–Glu¹²⁸, we expressed human/canine homology swaps of GPIbα on CHO cells. Three lines of evidence (molecular modeling, conformation-specific antibody binding, and botrocetin-dependent VWF binding) support the correct folding of the mutant receptor as published previously (6, 7); in addition, the HUM60–128 mutant still supports adhesion to VWF at low shear rates (see above). These functional data, on adhesion of GPIbα-expressing cells to VWF in a flow chamber mimicking pathophysiological shear (14–16), however, show how the relative functional importance of elements within Leu⁶⁰–Glu¹²⁸ increases as the shear force increases.

DISCUSSION

The aim of this study was to reconcile discrepancies between structural and functional analyses of binding of platelet GPIbα (the major ligand-binding subunit of GPIb-IX-V) to VWF, an interaction that initiates pathophysiological thrombus formation under shear stress (1–5). Our previous studies using human/canine chimeras of GPIbα suggested that the LRR2–4 sequence, Leu⁶⁰–Glu¹²⁸, is required for GPIbα-dependent adhesion to VWF under flow conditions (6), whereas co-crystal structures of GPIbα and VWF fragments under static conditions reveal interactive sites, predominantly N- and C-terminal to LRR2–4, and a bidentate mode of GPIbα binding to ligand (8–10). All of the chimeras showing impaired VWF binding, however, not only lack human Leu⁶⁰–Glu¹²⁸ sequence (6) but also lack either N- or C-terminal contact sites (8, 9).

HUM60–128 cells that did roll on VWF rolled 2.5–6-fold faster than WT-GPIbα; this difference became more pronounced with increasing shear rate (Fig. 4, C and D). The lack of binding of CANHUM60–128 (Fig. 4A) suggests human Leu⁶⁰–Glu¹²⁸ alone is not sufficient to support adhesion to VWF. In this regard, the co-crystal structures of GPIbα N-terminal domain and VWF-A1 fragments (9, 10) demonstrate that elements flanking LRR2–4 make contact under the static conditions used for crystallography, and these regions are evidently required for optimal VWF recognition at low or high shear. However, the combined functional data show that the relative functional importance of specific structural elements within Leu⁶⁰–Glu¹²⁸ increases as the shear force increases.
This is not to imply that regions outside LRR2–4 are not important for the interaction of GPIbα with VWF but rather that the elements within LRR2–4 become increasingly critical for binding as shear rate increases. The lack of binding of CAN_{HUM60–128} to VWF suggests that human Leu^{60–128} alone is not sufficient to support adhesion to VWF at low or high shear.

The structure of the N-terminal domain of GPIbα has been described as a cupped human hand with LRR domains as the palm and fingers, the extended loop of the C-terminal flanking sequence as the thumb, and the anionic/sulfated sequence as the wrist (17). The dimensions of VWF-A1 preclude contact with a ligand-binding surface at the palm of GPIbα because of steric hindrance from the thumb, but Uff et al. (17) propose that “the GPIbα crystal structure [may represent] the low affinity or “closed” form of the receptor and that a conformational change in the thumb [may be] required to unmask the A1 domain binding site.” In contrast, the GPIbα-VWF-A1 complex crystal structure, with only the N- and C-terminal projections (but not the concave surface) in direct contact (8), would appear more consistent with a low-affinity structure postulated by Uff et al. (17). However, the VWF-A1 domain could be satisfactorily docked against the concave surface of GPIbα if the “thumb” is moved (17). In the absence of VWF modulators ristocetin and botrocetin, shear stress may provide the necessary impetus to open the structure sufficiently to allow interaction between VWF-A1 and the concave surface of the LRR domain, mediated predominantly by complementary electrostatic patches on GPIbα and VWF-A1 (9–11). This model would predict that although the contact sites may confer specificity, there is an increasing requirement for the electronegative surface encompassing Leu^{60–128} as the shear rate increases, consistent with the functional data (6) (Fig. 4). Definitive resolution of the contact surface between receptor and ligand will undoubtedly require further structural information. However, in the context of shear this will be almost impossible to achieve using current technologies. Instead, the functional analysis of homology domain swaps of GPIbα identifies shear-dependent regions of the receptor involved in binding VWF.

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