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Human platelet glycoprotein V: Characterization of the polypeptide and the related Ib–V–IX receptor system of adhesive, leucine-rich glycoproteins

(arterial platelet adhesion/von Willebrand factor/genomic and cDNA cloning/Bernard–Soulier syndrome/megakaryocytes)

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ABSTRACT **Human platelet glycoprotein (GP) V (M_r 83,300), whose primary structure is reported here, is a part of the Ib–V–IX system of surface glycoproteins (GPs Ibα, Ibβ, V, IX) that constitute the receptor for von Willebrand factor (vWf) and mediate the adhesion of platelets to injured vascular surfaces in the arterial circulation, a critical initiating event in hemostasis. System members share physical associations, leucine-rich glycoprotein (LRG) structures, and a congenital deficiency state, Bernard–Soulier syndrome. With PCR techniques and platelet cDNA templates, 1.4 kb of GP V cDNA sequence was obtained that encodes 469 GP V amino acids. A genomic 3.5-kb *Bam*HI fragment was then isolated that includes 3.46 kb of GPV cDNA sequence: the 1.7-kb open reading frame plus 2 bases of the 5′ and 1.8 kb of the 3′ untranslated regions. Northern blot analysis reveals three GP V platelet transcripts of 3.8, 4.2, and 5.2 kb. A 16-amino acid signal peptide is present. Mature GP V is a 544-amino acid transmembrane protein with a 504-amino acid extracellular domain that encompasses a set of 15 tandem LRG repeats in a "flank-LRG center-flank" array [Roth, G. J. (1991) *Blood* 77, 5–19] along with eight putative N-linked glycosylation sites and cleavage sites for thrombin and calpain. GP V is a transmembrane, adhesive LRG protein that plays an undefined, but potentially critical, role in the expression and/or function of the Ib–V–IX receptor for vWf/shear-dependent platelet adhesion in arteries.**

Glycoprotein (GP) V (M_r 83,300) is a surface element of human platelets involved in adhesion to arterial blood vessels. First detected by surface-labeling techniques and distinguished by its susceptibility to proteolytic cleavage by thrombin (1–3), GP V was subsequently purified and characterized according to its isoelectric point, carbohydrate content, and partial amino acid (aa) sequence (4–7). The protein is a part of the Ib–V–IX receptor system that mediates platelet adhesion in arteries, an initial event in hemostasis and thrombosis (8–10).

Hemostasis begins with vascular injury, followed by the exposure of the subendothelium to circulating platelets and subsequent adhesion of platelets to the injured site (11). In the arterial circulation, a single receptor–ligand pair mediates adhesion, utilizing a specific, shear-dependent interaction between the plasma/subendothelial ligand, von Willebrand factor (vWf), and the platelet surface receptor, GP Ib–V–IX (12–16). The receptor consists of four distinct polypeptides, GPs Ibα (M_r 143,000), Ibβ (M_r 22,000), V (M_r 83,000), and IX (M_r 20,000), in 1:1:0.5:1 stoichiometry with 25,000 copies per platelet (9, 10, 17–19). Individual members are encoded by separate genes (20, 21). A discrete binding site for vWf is located in the GP Ibα chain (22), but all four proteins appear to contribute to the surface receptor (23). The receptor is referred to as the "Ib–V–IX system" because of three common features: (*i*) physical associations within the surface receptor (8–10); (*ii*) a prominent 24-aa structure of unknown function, termed the leucine-rich glycoprotein (LRG) segment (16–19); and (*iii*) a congenital deficiency state marked by deficient platelet adhesion, large circulating platelets, and thrombocytopenia, termed Bernard–Soulier syndrome (B–Ss) (8, 12, 24). The molecular basis of the classic form of B–Ss is unknown.

In the current study, we set out to complete the characterization of the primary structures of the polypeptides of the Ib–V–IX system (17–19). The cDNA sequence encoding GP V was determined and translated, and the predicted primary structure of GP V was analyzed, revealing a transmembrane protein with 15 tandem LRG repeats in a "flank-LRG center-flank" array (16, 19). In regard to structural features, GP V possesses a unique thrombin cleavage site, not found in Ib–IX, along with transmembrane, calpain, and LRG domains that are present in the other members. The interactive nature of the platelet Ib–V–IX system suggests that each member, including GP V, plays a critical role in the expression and/or function of the surface receptor for vWf.

MATERIALS AND METHODS

PCR Methods. Template cDNA was synthesized [GIBCO/BRL kit, random/oligo(dT) primers] from total platelet RNA (25) and amplified with primer pairs (Table 1) designed on the basis of known GP V peptide sequences (5–7). The expected product sizes are 122 bp for pair "A" between codons 104 and 144, 1080 for "B" between codons 120 and 479, and 750 for "C" between codons 11 and 261. Codons are numbered in Fig. 1. Primer sequence degeneracies are noted by I, M, N, R, Y (26), and peptide sequences are given in single-letter code. An initial 30-cycle amplification (94°C, 1 min/42°C first 3 cycles and 55°C next 27 cycles, 1 min/72°C, 1.5 min, pH 8.3; 2 mM $MgCl_2$, 0.2 mM dNTPs, 25 pmol of primer, 2.5 units of Taq polymerase per 100 μl) was repeated with 1/20th of the original reaction as a template under identical conditions (27). The A primers were synthesized with 5′ "Prime" sequences, and their product was detected by Southern blotting with end-labeled internal probe, isolated, cut back with T4 polymerase, ligated into pVEGT′, and sequenced (27). With the B and C primers, *Eco*RI or *Xho* I cloning sites were added at

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Abbreviations: GP, glycoprotein; vWf, von Willebrand factor; LRG, leucine-rich glycoprotein; B–Ss, Bernard–Soulier syndrome; HEL, human erythroleukemia; PMA, phorbol 12-myristate 13-acetate; ORF, open reading frame; UTR, untranslated region.

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Table 1. PCR primers

Primer pair		Nucleotide sequence	Related peptide
"A"	Sense:	AARATGGTIYTIYTNGARCAGYT	KMVLLEQL
	Antisense:	AARTCNARYTGRTTYTGRTT	NQNQLDF
internal probe:		GAYCARAAYATGTTYCARAA	DQNMFQK
"B"	Sense:	GGCATTGACCAAAACATGTTTCAG	GIDQNMFQ
	Antisense:	GCNGGRTGNACNGGNGCYTC	EAPVHPA
"C"	Sense:	TGYGTNTTYMGNGAYGCNGCNCARTG	CVFRDAAQC
	Antisense:	GGTTCTCGAACAGAGTCAACAGAG	TLLTLFENP

the 5′ ends, and products were subcloned into pBluescript SK+ (Stratagene) and M13mp18 for mapping and sequencing, respectively (19, 21, 28).

Genomic DNA Cloning and Southern/Northern Blotting. A 425-bp PCR fragment, produced from a B sense/C antisense primer pair, was labeled by random-priming and used to screen 1×10^6 phage from a human genomic library (lung fibroblast; Stratagene). One positive clone was plaque-purified, and the 19-kb insert was confirmed to contain GP V genomic DNA by restriction mapping and Southern blotting as described (21, 29). One *Bam*HI fragment of 3.53 kb that hybridized to the probe was sequenced in its entirety (28). Northern blot analysis of human RNA was performed with the GP V probe noted above and with a described GP IX probe (21).

Ribonuclease (RNase) Protection. A genomic fragment extending from the *Pst* I site at nucleotide 300 of the published sequence to an *Eco*RI site 39 bp 5′ of the *Bam*HI site at the first base was subcloned into Bluescript SK+ and used as the template for preparation of radiolabeled RNA probes with [α-^{32}P]UTP (NEN). The plasmid was linearized with *Eco*RI and transcribed with T3 polymerase for antisense transcripts or with *Pst* I and T7 polymerase for sense transcripts (MAXIscript kit; Ambion, Austin, TX). RNase protection assays were performed using a kit (Ambion RPA II) under conditions recommended by the manufacturer. Sizes of RNase-resistant products were calculated by comparison with a sequencing ladder of M13mp18.

Analysis of Nucleotide and Amino Acid Sequences. The 3533-base nucleotide sequence of the *Bam*HI fragment of GP V genomic DNA (Fig. 1) has been deposited in the GenBank data base.§ Nucleotide and protein homology searches (30) were performed on EMBL release 33 and SwissProt 24 data bases using PCGENE (IntelliGenetics). The GP V cDNA sequence was analyzed with the same software, and the signal cleavage site was predicted as described (31).

RESULTS AND DISCUSSION

Initial screenings of a described λgt11 human erythroleukemia (HEL) cell cDNA library (17–19) for GP V inserts were unsuccessful with oligonucleotide and anti-GP V antibody probes. As discussed below, later work suggested that HEL cells do not transcribe the GP V gene.

An initial PCR amplification of platelet cDNA with degenerate primers (pair A) produced a 122-bp GP V cDNA, identified by the internal probe (27). From the unique 122-base sequence, nondegenerate PCR primers were constructed and paired (B and C) with degenerate primers, based on other GP V peptide sequences, to provide overlapping unique cDNA sequences of 1080 and 750 bases, respectively. The resultant composite 1406-base cDNA sequence encodes a continuous internal GP V peptide of 469 amino acids (11–479, Fig. 1) that includes all of the published GP V peptides (≈200 residues; refs. 5–7). Therefore, the PCR-derived sequence represents a major part of the coding region of transcripts for GP V.

Screening of a human genomic library with a GP V cDNA probe led to the isolation of a 19-kb insert that includes part, and perhaps all, of the GP V gene. Southern blot analysis (Fig. 2*A*) of human genomic DNA detects 5.2-kb *Eco*RI, 3.6-kb *Bam*HI, 3.0-kb *Eco*RI/*Hin*dIII, and 2.8-kb *Bam*HI/*Hin*dIII fragments with the probe hybridizing between the 5′ *Bam*HI and *Hin*dIII sites. Fragment analysis gives the limited map of restriction sites shown below:

5′ *Eco*RI 0.2-kb *Bam*HI 2.8-kb *Hin*dIII 0.8-kb *Bam*HI 1.4-kb *Eco*RI 3′

The order of sites was confirmed by analysis of the insert alone. The sequence of the 3.53-kb *Bam*HI fragment (Fig. 1) encompasses the entire 1.4-kb GP V cDNA sequence determined by PCR methods.

The same probe was used for Northern blot analysis of platelet and HEL cell RNA (Fig. 2*B*). The three discrete GP V transcripts found in platelet RNA, estimated to be 3.8, 4.2, and 5.2 kb in size, are not detected in HEL cell RNA even after PMA induction (Fig. 2*B*) (32). Unlike megakaryocytes, HEL cells apparently do not synthesize GP V (32–34). Since GP V may enhance surface receptor content, its absence in HEL cells may explain their relatively small amount of surface GP Ib–IX (32–34). As a control, the same blot was probed with GP IX cDNA and showed that HEL cells increase their content of GP IX transcripts in response to phorbol (19, 21).

RNase protection assays confirm the absence of GP V transcripts in HEL cells and indicate the presence of an intron at the 5′ end of the open reading frame (ORF). The fragment protected by platelet and Dami RNA (35) and the 339-base probe comigrate with 206-base and 309-base DNAs, respectively (Fig. 2*C*). The calculated hybrid size of 226 bp is consistent with an intron/exon boundary 2 bases to the 5′ side of the ATG start codon shown in Fig. 1. Therefore, the first 69 bases at the 5′ end of the GP V gene sequence are part of an intron.

The ATG start codon for GP V was identified as follows. (*i*) Described genes for LRG proteins, including GPs Ibα, V, and IX and oligodendrocyte myelin glycoprotein, have introns located within a few bases of their ATG start codons. The genes also contain an exon that includes the entire ORF and 3′ untranslated region (UTR) of the transcript (20, 29, 36–38). (*ii*) The identified methionine in GP V begins a putative hydrophobic signal peptide (−16 to −1, Fig. 1) that terminates in a typical cleavage site for signal peptidase (. . . GLLRA/Q . . .) (31). (*iii*) The predicted NH_2 terminus (QPFPCPPACKC . . . C . . .) of mature GP V resembles the cysteine-rich consensus (. . . CPXXCXC . . . C . . .) NH_2 terminus of GPs Ibα/Ibβ/IX and related LRG proteins (17–19, 36–38), and glutamine accounts for the "blocked" NH_2 terminus of GP V (5–7). The final cysteine in the conserved sequence, residue 19 in GP V, overlaps with the NH_2-terminal "flank" that borders the LRG domains in these proteins as discussed below.

Two additional lines of evidence indicate that the intron found by RNase protection is the only one present in the available genomic sequence (Fig. 1). First, the entire 1.4-kb GP V cDNA sequence determined by PCR is present, without interruption, in the GP V gene sequence. Second, three overlapping sets of PCR primers give identically sized products from oligo(dT)-primed platelet cDNA and from *Bam*HI genomic fragment templates (data not shown). The amplifi-

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L11238).

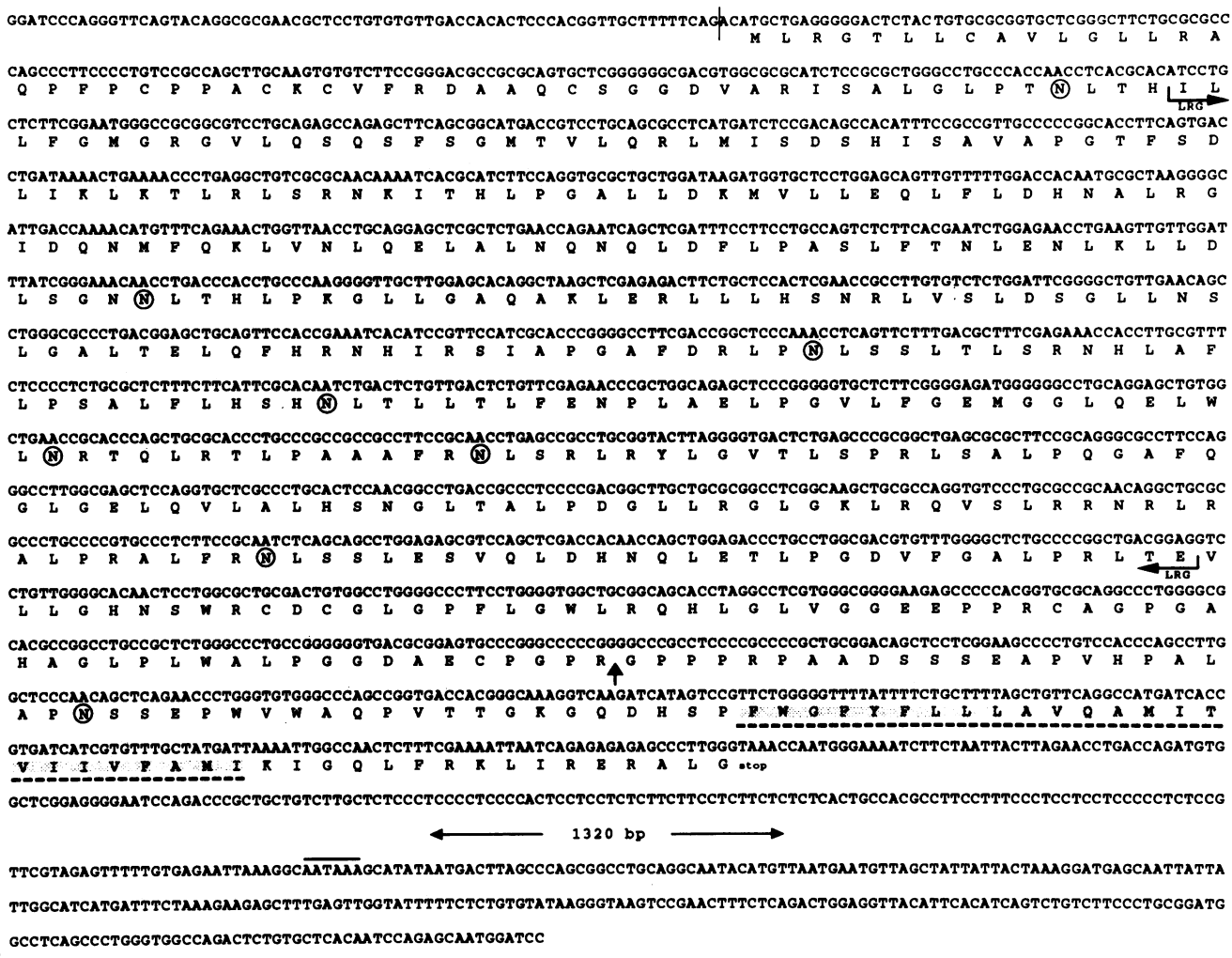

FIG. 1. Genomic DNA encoding human platelet glycoprotein V. The nucleotide sequence of a 3533-bp *Bam*HI fragment of the GP V gene is shown with 1320 bp from the 3′ end omitted (bottom arrows: ←→). The GP V open reading frame (ORF), translated into a 560-aa sequence (single-letter code, residue numbers on left), begins with an ATG start codon (−16, Met) and ends with a TAA stop codon (after 544, Gly). Preceded by a 16-aa signal peptide (−16 to −1), the 544-aa mature protein includes a 24-aa transmembrane domain (505–528, dashed underline), 15 LRG repeats (39–399: I L... →, ...T E ←), a thrombin cleavage site (residue 460/461: .R ↑ G.), and eight N-linked glycosylation sites (Ⓝ). The intron/exon boundary is marked, 2 bases 5′ of the ATG start codon (..AG|AC..), and a poly(A) signal is overlined (..AATAAA..).

cations covered the entire 3′ end of the genomic sequence from codon 467, through the TAA stop codon, to a point 33 bases 3′ of the AATAAA poly(A) signal (Fig. 1).

Since the RNase protection and PCR experiments document the absence of introns in the GP V ORF and 3′ UTR, one can read the cDNA sequence for this region directly from the gene sequence. This accounts for ≈3.5 kb of the GP V transcript, from 2 bases upstream of the ATG start codon through a poly(A) site. Upstream of the known cDNA sequence, the transcriptional start site and the 5′ UTR of the transcript remain undefined. Additional 5′ transcriptional start sites, splice sites, and/or poly(A) signals may account for other GP V transcripts. Further definition of the GP V cDNA/gene awaits additional sequencing and the localization of a transcriptional start site(s).

Mature human platelet GP V is predicted to contain 544 amino acids (M_r 59,276) composed of 44 Ala, 36 Arg, 26 Asn, 18 Asp, 8 Cys, 26 Gln, 19 Glu, 49 Gly, 18 His, 15 Ile, 111 Leu, 13 Lys, 8 Met, 25 Phe, 39 Pro, 37 Ser, 21 Thr, 7 Trp, 2 Tyr, and 22 Val. The paired/unpaired status of the 8 cysteines is unknown. Added carbohydrate would lead to a glycoprotein of M_r 83,300 if all eight putative N-linked glycosylation sites are utilized, consistent with the 82- to 89-kDa size estimated by SDS/PAGE (4–7). The amount of O-linked carbohydrate in GP V is unknown, and the mucin-related repeats of GP Ibα are not found in GP V (39).

Hydropathy analysis of the entire translated sequence reveals 16-aa signal (−16 to −1) and 24-aa transmembrane (505 to 528) domains (40), indicating that GP V is a transmembrane protein with 504-aa extracellular and 16-aa intracellular regions (Fig. 1). The earlier isolation of a hydrophilic form of GP V (M_r 82,000) reflects calpain cleavage of the GP V extracellular domain, similar to the release of glycocalicin from GP Ibα (4, 41). The exact site of calpain cleavage of GP V is unclear as it is in GP Ibα. However, the site is located near the transmembrane region in both proteins.

GP V contains a thrombin cleavage site (. . . CPGPR/GPPPR . . .) between residues 460/461, consistent with the size of GP Vfl, the 69-kDa peptide produced by thrombin (1, 3–7). The thrombin and calpain sites in GP V lie within ≈40 residues of each other, and cleavage by both proteases results in a 13-kDa peptide that appears to include an N-linked carbohydrate chain at residue 483 (7). The GP V thrombin cleavage site resembles that found in other thrombin substrates such as fibrinogen and the thrombin receptor (42, 43). However, GP V lacks a second domain found in other thrombin-binding proteins—namely, the anionic site of GP Ibα (DEGDTDLYDYYPEEDTEGD), the thrombin receptor

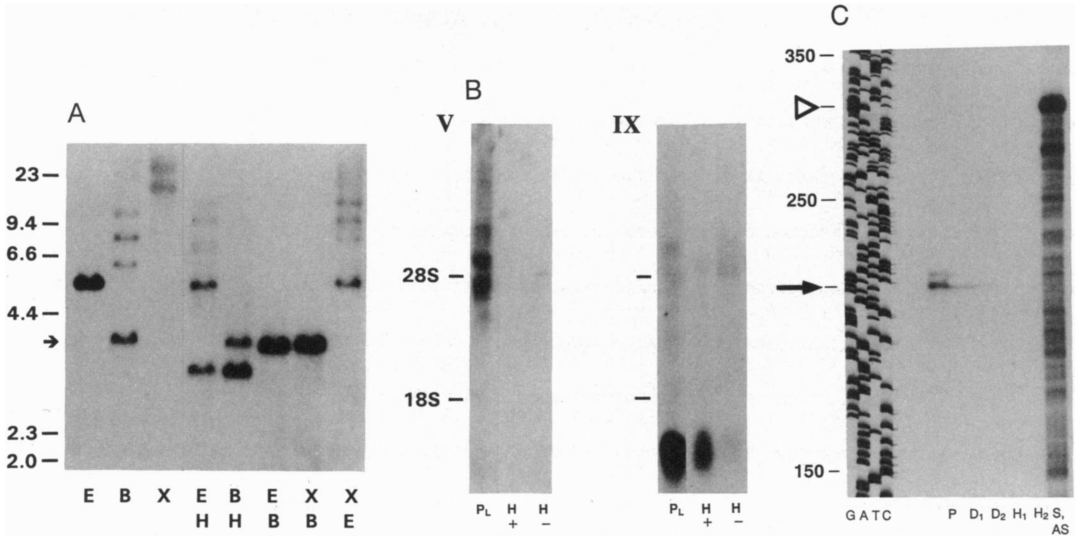

FIG. 2. Analyses of nucleic acids by blotting. (*A*) Southern blot analysis of human genomic DNA. Single (lanes: E, *Eco*RI; B, *Bam*HI; X, *Xba* I) or double (lanes H, *Hin*dIII: E/H, B/H, E/B, X/B, X/E) restriction digests of human genomic DNA were analyzed by Southern blotting with a labeled GP V cDNA probe. Major fragments, detected by autoradiography, are E (5.2 kb), B (3.6 kb: indicated by →), X (13.5 kb), E/H (3.0 kb), and B/H (2.8 kb). Additional sequencing located the 5′ *Eco*RI site 39 bases upstream of the 5′ *Bam*HI site. Sizes are indicated (kb). (*B*) Northern blot analysis of human platelet and HEL cell RNA. Total RNA samples (10 μg) from human platelets (lanes P_L) or HEL cells, grown with (lanes H +) or without (lanes H −) phorbol 12-myristate 13-acetate (PMA) (32), were analyzed by Northern blotting with GP V (V) or GP IX (IX) cDNA probes. GP V (3.8, 4.2, 5.2 kb) and GP IX (1.0 kb) transcripts are detected in platelet RNA. HEL cells contain GP IX transcripts, inducible with PMA, but appear to lack GP V mRNA. Size markers are rRNAs (28S, 4.1 kb; 18S, 1.9 kb). (*C*) RNase protection analysis of the 5′ intron/exon boundary. Radiolabeled 339-base antisense probe was hybridized with full-length sense transcript (lane S, AS, ▷-) or with RNA from platelets (lane P), Dami cells grown in bovine serum albumin (BSA) (lane D_1) or fetal calf serum (lane D_2), and HEL cells grown in BSA (lane H_1) or PMA (lane H_2) (32). The antisense probe forms digestion-resistant hybrids with platelet (→, prominent band in lane P) and Dami RNA (faint bands in lanes $D_1 > D_2$), indicating an intron/exon boundary 2 bases upstream of the ATG start codon of the GP V ORF (vertical line, Fig. 1). HEL cell RNA formed no hybrids (lanes H_1 and H_2). Sizes are deduced by comparison with a DNA sequence ladder (GATC) of M13mp18, marked at 100-base intervals (350, 250, 150).

(YEEPFWEDEE), and hirudin (DFEEIPEEYLQ) (17, 43, 44). GP V does not provide a thrombin receptor function to the platelet (45, 46), and the functional consequence of thrombin cleavage of GP V is unknown. The presence of thrombin-related domains in GP V and Ibα may reflect the evolution of a common precursor. For example, a thrombin receptor domain added to a precursor may have later diverged, leading to a reciprocal retention/loss of one aspect of the receptor in each product, the cleavage site residing in GP V and the hirudin tail in GP Ibα.

A flank-LRG center-flank domain dominates the primary structure of GP V (16, 19). As shown (Figs. 1, 3, and 4), 15 tandem LRG repeats constitute the bulk of the molecule, extending from residue 39 to residue 399 of the mature protein. All of the repeats contain 24 aa, except the 12th repeat with 25 aa, and they include six of the eight putative N-linked glycosylation sites. A consensus LRG sequence for GP V is given in Fig. 3*A* along with both consensus and single sequences from the other members of the system. In Ib–V–IX, the "central" LRG repeats are bordered on both NH_2- and -COOH terminal sides by conserved "flanking" sequences of ≈22 aa as shown in Fig. 3 *B* and *C*. The flanking sequences lack a known function, but their conservation suggests a significant role in the Ib–V–IX system.

A sketch of the Ib–V–IX system (Fig. 4) shows the presence of transmembrane and LRG domains in each protein along with their covalent and noncovalent associations. Additional structural features are shown such as the vWf and thrombin binding sites and the O-carbohydrate (O-CHO) domain in Ibα, the phosphorylation site in Ibβ (47), plus cleavage sites for thrombin in V and for calpain in Ibα and V. In addition, the cytoplasmic domain in Ibα is noted that interacts with actin binding protein, providing a likely route for transmembrane signaling following the Ib-vWf binding event (48). Potential evolutionary relationships among the members of the Ib–V–IX system can be appreciated from the diagram. As the smallest and least complex member of the system (19, 29), GP IX may be a prototypic molecule that gives rise to the other members of the system, consistent with the structural similarity between the GP IX and GP Ibα genes

A LRG sequences, consensus and single

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Ib α  L X L S X N X L T T L P X G L L X X L P X L X X
Ib β  L V L T G N N L T A L P P G L L D A L P A L R T
V     L X L X X N X L A X L P X X L F X X L X X L X X
IX    L L L A N N S L Q S V P P G A F D H L P Q L Q T
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B NH_2-terminal flank

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Ib α  V N C - 4 - L T - A - L P P D L P K D T T I
Ib β  V D C - 4 - L T W A S L P T A F P V D T T E
V     A Q C - 7 - I S - A - L - G - L P T N L T H
IX    V D C - 4 - L T - A - L P A - L P A R T R H
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C COOH-terminal flank

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Ib α  A F L H G N P W L C N C E I L Y F R R W L
Ib β  A H L G A N P W R C D C R L V P L R A W L
V     V L L G H N S W R C D C G L G P F L G W L
IX    L D V T Q N P W H C D C S L T Y L R L W L
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FIG. 3. Flank-LRG center-flank sequences. (*A*) Consensus and single LRG sequences in Ib–V–IX. The single LRG sequences of GP Ibβ and GP IX are shown along with consensus sequences for the tandem 24-aa LRG repeats of GP Ibα and GP V (7 and 15, respectively). Conserved residues are boxed. (*B*) NH_2-terminal flanks in Ib–V–IX. Sequences flanking LRG repeats on their NH_2- side are shown with gaps (-) and inserts (-4-/-7-). Conserved residues are boxed. (*C*) COOH-terminal flanks in Ib–V–IX. Sequences flanking LRG repeats on their COOH- side are shown with conserved residues boxed.

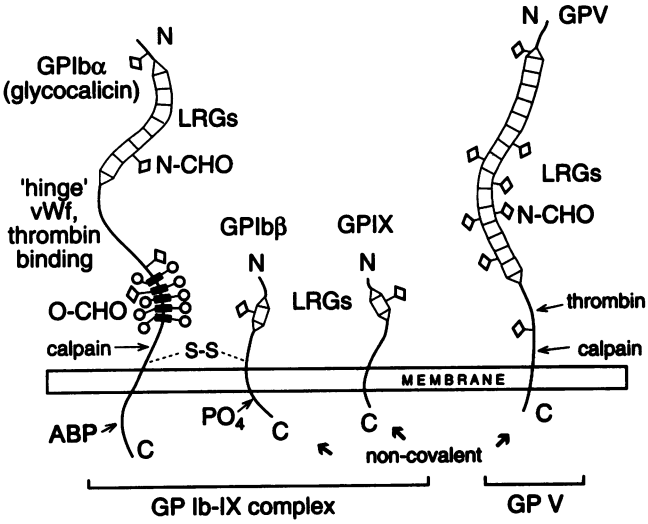

FIG. 4. Sketch of the GP Ib–V–IX system. Large extracellular NH_2-terminal (N) domains extend above the membrane with small COOH-terminal domains (C) below. Single (Ibβ/IX, □) and multiple (Ibα/V, ..□□□..) LRG repeats are shown with flanks (◁ ▷). Tandem repeats (▬) carrying O-linked carbohydrate (O-CHO, –○) in Ibα are noted along with an intervening "hinge" region that binds thrombin and vWf. Thrombin and calpain cleavage sites are shown (→) as are N-linked CHO sites (–◇), a disulfide bond (S–S) linking Ibα and Ibβ, the actin-binding protein (ABP) site of Ibα, and noncovalent associations (↑) that link the GP Ib–IX complex together with GP V.

(20, 29). The first branch from the GP IX prototype may produce GP Ibβ with its single LRG domain and novel intracellular phosphorylation site (47). The remaining common precursor for GPs V and Ibα may develop features found in both proteins: multiple LRGs, a calpain-cleavage site, and a thrombin receptor domain as discussed above. Following the divergence of GP V, the final elaboration of the receptor occurs in Ibα with its O-carbohydrate domain and binding sites for vWf and actin-binding protein.

In summary, GP V is a prominent element of the platelet surface and a member of the Ib–V–IX system that mediates platelet adhesion in the arterial circulation. In view of the multiple associations among GPs Ibα–Ibβ–V–IX, GP V is likely to make critical contributions to the surface expression and the receptor function of the Ib–V–IX system. The current study describes the primary structure of GP V and provides the cDNA sequence with which to pursue further work on the structural and functional relationships within the GP V polypeptide and gene.

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