Human Platelet Glycoproteins V and IX: Mapping of Two Leucine-Rich Glycoprotein Genes to Chromosome 3 and Analysis of Structures†

Mayumi Yagi,‡ Susanne Edelhoff,§ Christine M. Disteche,§ and Gerald J. Roth*,‡

Hematology Section, Medical and Research Services, Seattle Veterans Administration Medical Center, Seattle, Washington 98108, and Departments of Medicine and Pathology, University of Washington, Seattle, Washington 98195

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ABSTRACT: Human platelet glycoproteins Ibα, Ibβ, V, and IX comprise an interrelated set of molecules (the Ib-V-IX system) that together form a surface adhesion receptor for the ligand, von Willebrand factor. To complete the primary structural characterization of the genes involved in this system, we have analyzed cosmid clones for both the glycoprotein V and IX genes and used these clones to localize the two genes by fluorescence *in situ* hybridization. Both genes were found on the long arm of chromosome 3, but at distinct sites, the GPV gene on 3 band q29 and the GP IX gene on 3 band q21. The transcriptional start site of the GPV gene was defined by "anchored" PCR and primer extension. The GPV gene contains two exons, the first consisting of approximately 37 bases and the second of approximately 3500 bases, interrupted by a single 958 base intron. The GPV transcript has multiple start sites spread over a twenty base region. The 5′ flanking region of the GPV gene has a series of potential consensus regulatory elements including GATA, *ets,* and Sp-1 sites, similar to those found in other described megakaryocyte/platelet genes, including those of the Ib-V-IX system. In assessing the four Ib-V-IX genes as a group, all four have a simple, "intron-depleted" structure with the entire open reading frame of the mature polypeptide located within a single exon. The Ib-V-IX genes may have evolved from a single site on the long arm of chromosome 3, related to the GP IX gene; and the system of genes may have developed through a sequential acquisition of structural domains to produce the activation-independent, shear-dependent, multisubunit receptor for von Willebrand factor.

Human platelet glycoproteins (GP)[1] V and IX, in association with the GPIb α/β heterodimer, constitute the surface receptor for von Willebrand factor (vWf), a large multimeric plasma protein (Clemetson *et al.*, 1982; Phillips & Agin, 1977). The interaction of platelets with vWf on the arterial subendothelial matrix mediates platelet adhesion and initiates hemostasis and thrombosis (Weiss *et al.*, 1974; Roth, 1991). Referred to as GPIb-V-IX, the platelet vWf receptor is composed of GPIbα (M_r 143 000) linked by disulfide bonds to GPIbβ (M_r 22 000) and noncovalently associated with GPIX (M_r 20 000) and GPV (M_r 83 300) (Lopez *et al.*, 1987, 1988; Hickey *et al.*, 1989, 1993). The vWf binding site has been localized within GPIbα, but the other three polypeptides appear to contribute to receptor function as well (Vicente *et al.*, 1990; Lopez *et al.*, 1992b). GPIbβ and IX have been shown to contribute to the expression of GPIbα on cell surfaces (Lopez *et al.*, 1994). The function of GPV in the receptor is less well-understood although its close association with the GPIb-IX complex suggests that it plays a role in receptor expression and/or function (Modderman *et al.*, 1992).

The members of the Ib-V-IX system share several features. All four polypeptides contain a 24 amino acid leucine-rich segment that is found in a variety of proteins and participates in cellular adhesion and protein−protein interactions (Takehashi *et al.*, 1985; Kobe & Deisenhofer, 1994). The expression of all four proteins is decreased in a congenital bleeding disorder, Bernard−Soulier syndrome (Bernard & Soulier, 1948; Nurden & Caen, 1975). However, the described mutations responsible for this disorder affect the gene for only a single member (Ware *et al.*, 1990; Wright *et al.*, 1993). Three of the genes have been mapped to different chromosomes (GPIbα: 17-p12-ter, GPIbβ: 22-q11.2, GPIX to chromosome 3) (Wenger *et al.*, 1989; Kelly *et al.*, 1994; Yagi *et al.*, 1994; Hickey *et al.*, 1990). The gene structures are similar, with introns located only at the 5′ end of the sequence and with long open reading frames for the entire mature polypeptide sequence encompassed within a single exon (Rajagopalan & Konkle, 1992; Hickey & Roth, 1993; Lanza *et al.*, 1993).

An earlier report suggested that the promoter for the GPV gene utilized a canonical TATA box(es) which would distinguish this promoter from those of the other members of the Ib-V-IX system (Lanza *et al.*, 1993). In view of this finding, we focused first on the transcriptional start site for the GPV gene in order to compare its conserved consensus promoter sequences with those present in the GPIbα, Ibβ, and IX genes. Coupling this data with the subchromosomal locations of the genes for GPV and IX, both of which proved to be located on human chromosome 3, we report that all

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* Corresponding author, at the Seattle VAMC, (111-Med), 1660 S. Columbian Way, Seattle, WA 98108. Phone: (206) 764-2475; fax: (206) 764-2689.

‡ Seattle Veterans Administration Medial Center.

§ University of Washington.

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[1] Abbreviations: glycoprotein, GP; polyadenylated, poly(A+); untranslated region, UTR; von Willebrand factor, vWf.

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Table 1: GPV-Specific Oligonucleotides[a]

name	sequence	bases
αSV	GGTTCTCGAACAGAGTCAACAGAG	complement of 878−901
2AS	TCATGAGGCGCTGCAGGACGGTC	complement of 291−313
VSEQ1	GGAAGACACACTTGCAAGCTGGC	complement of 138−160
PE4	CAGTAGAGTCCCCCTCAG	complement of 76−93
VUTR	AGTCTTTCAGTTACTTTGGA	1425−1444[b]

[a] Except for VUTR, base numbering refers to the sequence presented in Hickey *et al.* (1993). [b] Base numbers of the sequence presented in Lanza *et al.* (1993).

four genes of this system are highly interrelated with similar putative promoter sequences and that these genes appear to stem from a common progenitor sequence.

MATERIALS AND METHODS

Isolation and Characterization of the GPV and GPIX Genes. The isolation of the λ phage clone containing the GPV gene has been described previously (Hickey *et al.*, 1993). For *in situ* hybridization experiments, a human cosmid library (Stratagene, WI38) was sequentially screened with probes specific for the GPV and GPIX genes. The GPV probe was that used to screen the λ library, while the GPIX-specific probe was a *Hin*dIII−*Sac*I fragment isolated from the promoter region of GPIX (GPV, Hickey *et al.*, 1993; GPIX, Hickey & Roth, 1993). Both probes were labeled with [α-^{32}P]dCTP by random priming. The identity of the GPV cosmid was confirmed by restriction mapping and hybridization to specific oligonucleotides, while that of the GPIX cosmid was verified by direct sequencing of cosmid DNA. Sequencing reactions were performed using the Sequenase 2.0 kit (USB) and [α-^{35}S]dATP (NEN). Sequences were compiled and analyzed using PCGENE (Intelligenetics).

Amplification of mRNA 5′ Ends. Table 1 lists the oligonucleotides used in this study. The 5′ ends of GPV mRNAs were amplified using the protocols and reagents of the Clontech AmpliFINDER kit. In the initial experiments, 2 μg of polyadenylated (poly(A)+) RNA prepared from platelets (Roth *et al.*, 1989) was used to synthesize cDNA with the GPV-specific primer αSV. After hydrolysis of the RNA template and purification of single-stranded cDNA, a short oligonucleotide ("anchor") was ligated to the cDNA using T4 RNA ligase. The GPV cDNAs were amplified by PCR using 2AS as the antisense primer, and an oligonucleotide complementary to the "anchor" sequence as the sense primer. Both the anchor sequence and its complementary oligonucleotide were provided in the kit. For subsequent experiments, cDNA synthesis was primed with either αSV or 2AS and amplified with VSEQ1 (antisense) and either the anchor primer or VUTR (corresponding to the 5′ exon identified in the initial experiment). Amplification of the ligated cDNAs was performed in a Perkin-Elmer thermal cycler using 40 cycles of 96 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min, followed by a final incubation at 72 °C for 7 min. Amplified products were analyzed by electrophoresis on a 4% NuSieve (3:1) agarose gel, confirmed as GPV-specific by Southern blot hybridization to the oligonucleotide PE4 (Table 1), and then cloned into the pCRII vector (InVitrogen) for sequence analysis.

Primer Extension Analysis of GPV RNAs. Total cellular or poly(A)+ RNAs were mixed with 10 pmol of PE4 in 20 μL 150 mM KCl, 10 mM Tris (pH 7.4), and 1 mM EDTA. Tubes were heated to 80 °C for 10 min, cooled slowly to 48 °C, incubated for 3 h, and precipitated overnight with ethanol. The precipitated nucleic acids were resuspended in a 20 μL reaction mixture containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dATP, dGTP, and dTTP, 0.05 mM dCTP, 4 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), and 44 units of SuperScript reverse transcriptase (GibcoBRL Life Technologies). The mixtures were incubated 10 min at room temperature, and 90 min at 42 °C. A mixture of RNase A (Boehringer-Mannheim) and RNase I (Promega) (1 μL/tube) was added and incubated at 37 °C for 30 min. The reaction mixtures were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. The precipitates were resuspended in 80% formamide/bromphenol blue/xylene cyanol, heated to 75 °C for 2 min, and analyzed by electrophoresis on a 7 M urea−8% polyacrylamide gel using an M13mp18 sequence as a size marker.

Chromosomal Localization by Fluorescence in Situ Hybridization. Human cosmid clones (approximately 40 kb) containing the GPV or GPIX genes were labeled with biotin-11-dATP by nick translation (GibcoBRL Life Technologies). The size of the product was determined to be between 200 and 400 bp. As previously described (Yagi *et al.*, 1994; Edelhoff *et al.*, 1994), metaphase chromosome preparations of methotrexate-synchronized lymphocyte cultures from a normal human male were obtained and hybridized to the probe. Bound probe was detected with primary (anti-biotin) and secondary (fluoresceinated) antibodies (Vector, Burlingame, CA). Alternatively, the probe was labeled directly utilizing a fluorescence labeling kit from Stratagene that does not require antibody detection. Chromosomes were banded by Hoechst 33258−actinomycin D staining. Signals were visualized by fluorescence microscopy.

RESULTS AND DISCUSSION

Previous studies show the open reading frame (ORF) of the GPV gene residing in one exon that includes 2 bases of the 5′ untranslated region (UTR). To understand the regulation of the GPV gene, adjoining restriction fragments were first subcloned and sequenced, confirming earlier reports (Hickey *et al.*, 1993; Lanza *et al.*, 1993). To identify the transcriptional start site(s) for GPV, we first used "anchored" PCR. cDNA synthesized from platelet mRNA using a GPV-specific primer (αSV, Table 1) was ligated to a single-stranded oligonucleotide and amplified with an internal GPV-specific primer (2AS) and a primer complementary to the ligated oligonucleotide. Products were cloned and sequenced, indicating cDNA with a 39 base 5′UTR consisting of the 37 bases of exon 1 and 2 bases of exon 2 (Figure 1A). The putative exon 1 sequence corresponds to bases 1425−1461 of a GPV sequence, and comparison to

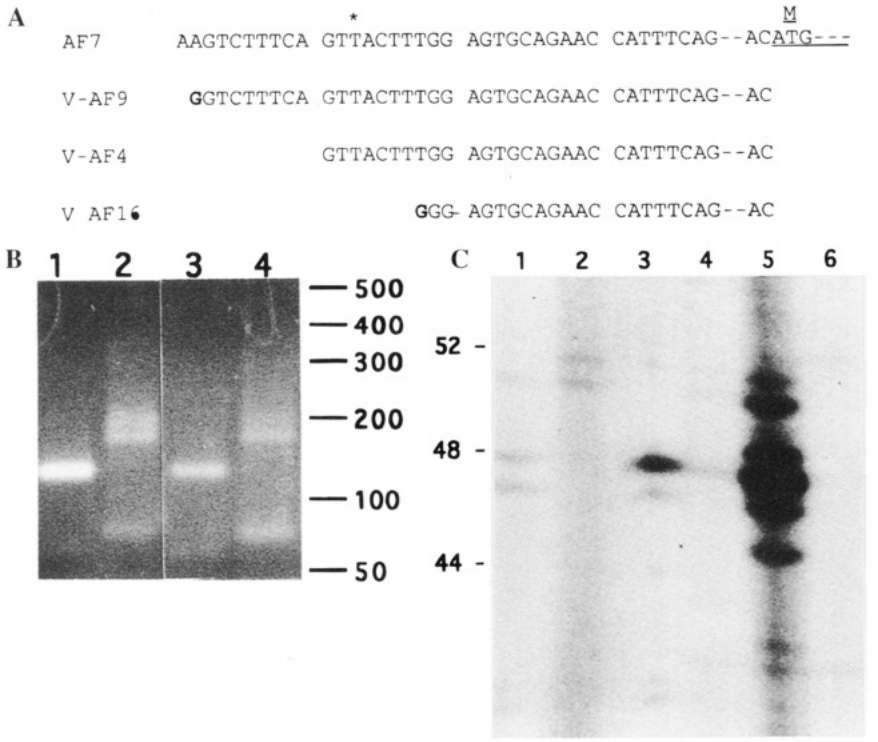

FIGURE 1: Location of the transcriptional start site of the GPV gene. (A) Sequences of GPV 5′ UTRs. Anchor PCR products were subcloned into pCRII and sequenced. Clone AF7 was isolated in the initial anchor PCR experiment, and V-AF9, 4, and 16 were isolated from a subsequent experiment (panel B). G residues not present in the genomic sequence are indicated in boldface (G). Hyphens indicate the position of the 958 base intron, and the initial residues of the open reading frame are underlined. The underlined M indicates the initiator methionine. The asterisk indicates the major start site identified by primer extension analyses. (B) GPV-specific anchored PCR. Single-stranded cDNA was synthesized from poly(A)+ platelet RNA using the primers αSV (lanes 1 and 2) or 2AS (lanes 3 and 4), ligated to a short single-stranded oligonucleotide anchor, and amplified using the primer VSEQ1 and either VUTR (lanes 1 and 3) or an oligonucleotide complementary to the anchor (lanes 2 and 4). Sequences of the oligonucleotide primers are noted in Table 1. Sequences of several clones are presented in panel A of the figure. (C) Primer extension analysis of GPV transcripts. RNA from human platelets, Dami, and K562 cells was annealed to primer PE4 and extended with reverse transcriptase in the presence of [^{32}P]dCTP. Radiolabeled products were analyzed by electrophoresis on a 7 M urea−8% polyacrylamide gel and autoradiographed. Products from different RNA templates are shown: 200 ng of platelet poly(A)+ (lane 1); 100 ng of DAMI poly(A)+ (lane 2); 5 μg platelet total (lane 3); 9 μg DAMI total (lane 4); 5 μg PMA-treated DAMI total (lane 5); and 20 μg K562 total (lane 6).

the genomic sequence shows exon 1 separated from exon 2 by a 958 base intron (Lanza *et al.*, 1993).

A second anchor PCR experiment further characterized the 5′ exon. cDNAs synthesized from platelet poly(A)+ RNA with GPV-specific primers (αSV or 2AS) were ligated to the anchor oligonucleotide and amplified with an internal GPV-specific primer (VSEQ1) and an anchor-specific or a 5′UTR-specific primer (discussed above: VUTR). Results in Figure 1B show the PCR products with the 5′UTR primer (lanes 1 and 3) and the anchor-specific primer (lanes 2 and 4). VUTR and VSEQ1 give the expected 128 bp products (lanes 1 and 3) while those obtained with the anchor primer and VSEQ1 (lanes 2 and 4) range from 175 to 195 bp, with a fainter smear extending to approximately 225 bp. The observed size difference between the two sets of products is consistent with the additional length contributed by the anchor and its primer (48 bases). The smaller band observed in lanes 2 and 4 appears to be a PCR artifact for two reasons: (1) it is not consistently seen and appears in some control reactions that lack cDNA, and (2) it does not hybridize to GPV-specific oligonucleotides. Sequencing of the GPV products shown in Figure 1A indicates multiple 5′ ends, as expected from the size heterogeneity observed on the agarose gel. Exon 1 ranges in size from 20 bases (corresponding to a 22 base 5′UTR) up to the previously identified 37 bases. Many clones possess a G residue not present in the genomic sequence at the 5′ end, suggesting that these clones are derived from capped mRNAs and thus represent the extreme 5′ ends of authentic transcripts.

Transcriptional start site(s) were confirmed by primer extension using RNA from platelets and two hematopoietic tumor cell lines, Dami and K562 (Figure 1C). DAMI cells express several platelet genes, including GPV, and expression is increased by phorbol ester. The K562 cells used in our experiments do not express GPIbα, Ibβ, or V genes (Hickey *et al.*, 1993). The major start site was assigned to a 48 base product, sized by an M13 sequencing ladder. This corresponds to a 25 base exon 1. Other bands, both larger and smaller, are present at lower intensity, again reflecting a heterogeneity in start sites. The results of the primer extension experiments confirm that the bulk of platelet GPV mRNA is initiated near the start sites identified by anchored PCR. These results indicate that the 5′UTR for GPV is heterogeneous and may be both longer and shorter than that reported initially (Lanza *et al.*, 1993). The locations of start sites extend over approximately twenty bases, characteristic of RNAs transcribed from promoters that lack a TATA box (Sehgal *et al.*, 1988).

The data imply that expression of the GPV gene is regulated in the same way as the other Ib-V-IX genes since the same potential regulatory sequences are present in the 5′ flanking region of the GPV gene as are found in the

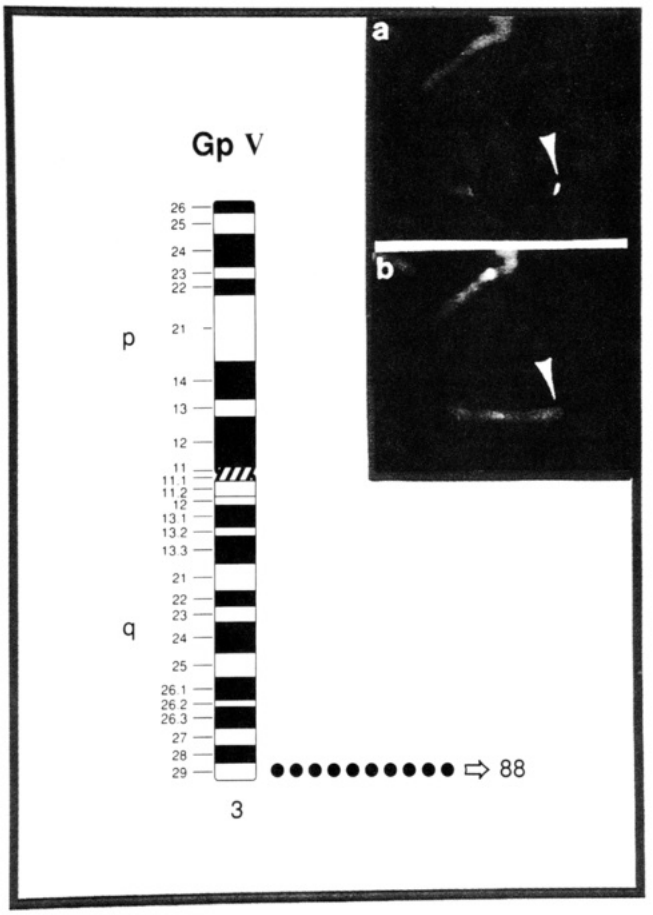

FIGURE 2: Assignment of the GPV gene to human chromosome 3q29 by FISH. The biotin-labeled GPV cosmid was hybridized to fixed metaphase chromosomal spreads and detected with fluoresceinated antibody. Chromosomes were stained with Hoechst 33258−actinomycin D and counterstained with propidium iodide. The distribution of 88 hybridization signals is shown in the diagram of human chromosome 3. (a) Hybridization pattern, and (b) chromosomal banding. The arrows indicate fluorescence signals on both chromatids of chromosome 3.

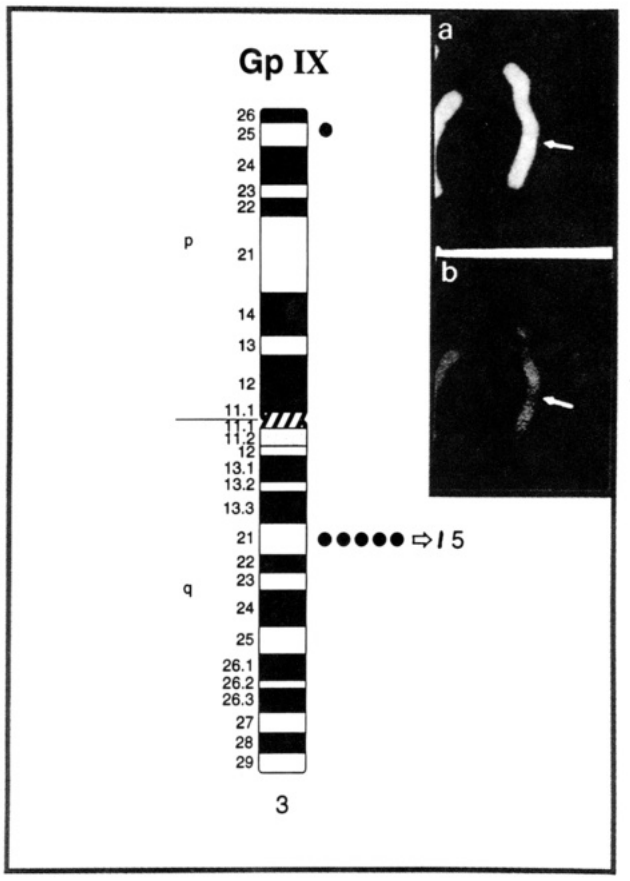

FIGURE 3: Assignment of the GP IX gene to chromosome 3q21 by FISH. An identical experiment to that described in Figure 3 was performed with the GP IX cosmid probe. The distribution of 15 hybridization signals is shown in the diagram of chromosome 3. The probe hybridizes to chromosome 3 band q21 as shown by the arrows (a) and banding pattern (b).

transcripts for Ibα, IX, and the platelet form of Ibβ. An alternative form of GPIbβ, expressed in endothelium appears to utilize a different promoter (currently uncharacterized) than that of platelets (Kelly *et al.*, 1994). Like the promoters for other platelet-specific genes (Hickey & Roth, 1993), the GPV promoter possesses binding sequences for GATA (at −50, −104, and −140 relative to the 5′ end of the longest identified transcript), Sp-1 (at −285 and −347), and *ets* factors (at −60 and −306, and in the opposite orientation at −247) (Lanza *et al.*, 1993). GATA, *ets*, and Sp-1 sites regulate activity of other megakaryocyte/platelet genes (GPIIb, PF-4; Doi *et al.*, 1987; Lemarchandel *et al.*, 1993; Visvader & Adams, 1993), but such work is not yet available for the Ib-V-IX genes. Potential TATA sequences at −162 and −227 were noted earlier (Lanza *et al.*, 1993), but these may not be utilized, judging from the results of the studies to identify the transcriptional start site(s). mRNAs initiating near these upstream TATA sequences would yield products considerably larger than those arising from the identified exon 1. Such products were searched for but not found in our experiments.

Fluorescence *in situ* hybridization (FISH) with a 40 kb cosmid clone was used to map the chromosomal location of the GPV gene (Figure 2). Of 119 metaphase chromosomal spreads examined, 88 (74%) showed signals on both chromatids of one or both chromosomes 3 at band q29, and 11 (9.25%) showed signals on one chromatid of one or both chromosomes 3 band q29 without significant hybridization to other chromosomes. Previous work mapped the GPIX gene to chromosome 3 (Hickey *et al.*, 1990), and to determine whether these two genes were physically associated as seen with the platelet GPIIb-IIIa genes (Bray *et al.*, 1988), a cosmid containing the GPIX gene was used for FISH analysis (Figure 3). Of 82 cells examined, 15 (18%) showed signals on both chromatids of one or both chromosomes 3 band q21, while 2 (2%) showed signals on one chromatid. A second site of hybridization to the centromere of chromosome 2 was observed in 13% of the cells. Since earlier work using flow-sorted chromosomes and cDNA probes did not indicate significant hybridization to chromosome 2 (Hickey *et al.*, 1990), we conclude that chromosome 3 band q21 contains the GPIX gene. The hybridization signals on chromosome 2 probably reflect the presence of related sequences in the cosmid clone or dimerization of the clone. The locations of GPIX at 3q21 and GPV at 3q29 show that the genes, although present on the same chromosome, are not linked within a cluster of genes.

The structures of the Ib-V-IX genes are diagrammed in Figure 4, showing their "intron-depleted" natures and elongated, continuous, uninterrupted open reading frames. Three of the four genes (Ibα, Ibβ, and V) possess one intron while that for GPIX has two. In all four cases, the introns

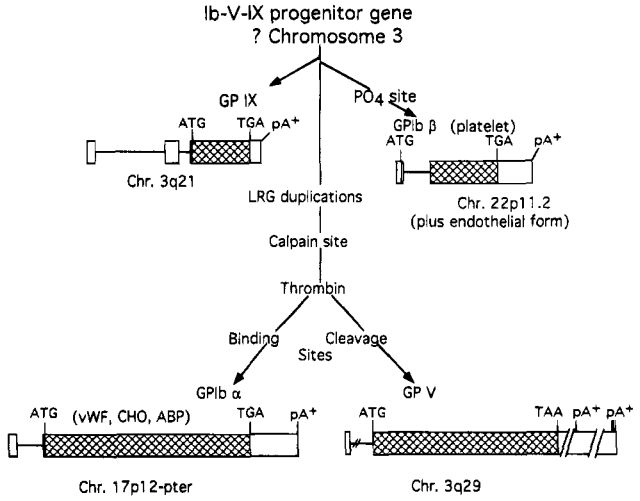

FIGURE 4: Structures of the human platelet GP Ibα, Ibβ, IX and V genes. Exons are indicated by boxes, either open boxes for 5′/3′ untranslated regions (5′/3′UTRs) or hatched boxes for open reading frames (ORFs). Start (ATG) and stop (TGA or TAA) codons are indicated along with polyadenylation sequences (pA^+). The intron and 3′UTR sequences in the GPV gene are interrupted and therefore are not drawn to the scale of the other three genes. Three of the four genes (Ibα, IX, and V) have their entire ORFs within a single continuous exon with intron/exon boundaries located just upstream (6:Ibα, 12:IX, or 2:V bases 5′) of their ATG start codons. The GPIbβ gene is different with a 274 base intron inserted just downstream (10 bases 3′) of the ATG start codon, along with a different mode of expression in endothelial cells. All four transcriptional start sites have been localized along with polyadenylation (pA^+) signals and sites of polyadenylation (single sites are found in the Ibα, Ibβ, and IX genes while several such sites are present in the GPV gene). A progenitor gene, perhaps located on chromosome 3, may have evolved to produce the described GPIX gene on chromosome 3q21, followed by divergence to produce the GPIbβ gene on chromosome 22p11,2 with its phosphorylation site. The genes encoding GPIbα and GPV share three features not seen in the GPIX and Ibβ genes, namely, multiple tandem LRG repeats, cleavage sites for calpain, and aspects of the thrombin receptor (a binding site in Ibα and a cleavage site in V). GPIbα possesses additional functional domains, including binding sites for von Willebrand factor (vWf) and actin binding protein (ABP) along with a polymorphic domain that is rich in O-linked carbohydrate (CHO).

are located exclusively within the 5′ region of the gene (Roth, 1995). Another gene encoding a "leucine-rich glycoprotein (LRG)" protein has a similar intron-depleted structure (Mikol *et al.*, 1990), but the small number of characterizations of such related genes does not permit a generalization about structural similarities among them.

The structural similarity of the Ib-V-IX genes (Figure 4) suggests that they may have evolved from a common ancestral gene. One can speculate that a progenitor or precursor genomic sequence may have been duplicated on more than one occasion, accompanied by dispersal. GPIX is the smallest and least complex member of the group, and the GPIX gene may be most closely related to a hypothetical precursor/progenitor sequence. Dispersal of the four genes to different chromosomal locations may have been accompanied, during evolution, with the addition of the different functional elements present in the individual gene products. For example, a gene with a single LRG segment and a transmembrane domain, the postulated "progenitor" GPIX-related sequence (Hickey *et al.*, 1989), may have been elaborated to provide for (1) an intracellular phosphorylation site (GPIbβ, Lopez *et al.*, 1988; Wardell *et al.*, 1989), (2) calpain cleavage sites, multiple LRG repeats, and elements of the thrombin receptor (GPV and GPIbα, Lopez *et al.*, 1987; Hickey *et al.*, 1993), and (3) a mucin-related, carbohydrate-rich polymorphic region and binding sites for actin binding protein and von Willebrand factor (GPIbα, Andrews & Fox, 1992; Lopez *et al.*, 1992a). In conclusion, the genes of the Ib-V-IX system possess both relatedness and diversity that combine to produce the multicomponent receptor for von Willebrand factor with its unique physiologic properties of activation independence and shear dependence.

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