Human Platelet Glycoprotein V: Its Role in Enhancing Expression of the Glycoprotein Ib Receptor

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Platelet adhesion to an injured blood vessel wall is a critical initiating step in hemostasis mediated by a four member receptor complex (glycoprotein Ib/IX) interacting with plasma von Willebrand factor (vWF). The function of the GPV subunit within this complex is presently undefined. To study the role of glycoprotein (GP) V within the GPIb receptor complex, we transfected the GPV subunit gene into a hematopoietic cell line that constitutively expresses the other three subunits (human erythroleukemia [HEL] cells). Using flow cytometry, we found transfected GPV was surface expressed in HEL cells; this, in turn, led to increased surface expression of the ligand-binding GP Ib and GPIX subunits. Radioligand binding assays showed that GPV-transfected HEL cells bound more vWF than their non- or mock-transfected counterparts. We employed confocal microscopy of GPV-transfected HEL cells to show that GPV colocalizes with GPIb on the cell surface. These findings suggest that the GPV subunit plays a role within the GPIb receptor complex by enhancing Ib surface expression.

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Materials and Methods

Plasmid construct. A 3.5-kb BamHI fragment of genomic DNA including the entire open reading frame for platelet GP V was subcloned into mammalian expression vector zem228R (kindly provided by Dr. Linda Sandell, Seattle VAMC, Seattle, WA). The latter two are noncovalently associated with GPIbα and β. The GPIbα contains the vWF binding site as well as a thrombin cleavage site. The four subunits of the GPIb/V/IX complex can be considered members of a distinct glycoprotein family on the basis of three common features: physical association, amino acid sequences (leucine-rich glycoprotein [LRG] segments), and a congenital deficiency state, Bernard-Soulier syndrome.

The role of the GPV subunit (Mr 83,000) within GPIb/V/IX is uncertain. This subunit is present in about half the quantity of its Ibα counterpart on the platelet surface and is not required for expression of the Ib/IX components on the surface of transfected mammalian cells. However, the presence of multiple LRG's in GPIb and a unique thrombin cleavage site along with an association with GPIb suggest that GPV likely plays a role in vWF receptor function.

In this work, we set out to determine if expression of GPV affects surface expression of the GPIbα component of the GPIb receptor complex. The approach involved transfecting human erythroleukemia (HEL) cells with a plasmid containing the GPV gene and assessing changes in surface expression of the resulting GPIbα chain.

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line were treated with 0.53 mmol/L EDTA for 5 minutes after washing in Dulbecco’s phosphate buffered saline (DPBS; BioWhittaker Inc, Walkersville, MD). The cells were suspended at a concentration of 10^7/mL in DPBS containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide, incubated with an anti-Iba, anti-IX, anti-V, or control antibody (1:10 to 1:100) in DPBS (4°C, 30 minutes), then centrifuged (130g for 10 minutes) and washed twice in DPBS. The cells were then incubated with the appropriate fluochrome-conjugated antibody (4°C for 30 minutes), washed twice in DPBS and fixed in 1% methanol-free formaldehyde in DPBS (Polysciences Inc, Warrington, PA).

HEL cells assessed for GPIba and V coexpression were incubated (4°C, 30 minutes) at 10^7 cells/mL with a 1:10 to 1:100 dilution of both mouse anti-GPb and rabbit anti-Iba antibodies. After centrifugation (130g, 10 minutes) and washing, they were colabeled with 1:10 to 1:100 dilutions of FITC-conjugated goat antimouse and PE-conjugated goat antirabbit antibodies (4°C for 30 minutes). The cells were then washed and suspended in 1% methanol-free formaldehyde before performing two-color flow cytometry.

Flow cytometry of all samples was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) with an argon-ion laser at an excitation wavelength of 488 nm. Cells colabeled with FITC and PE antibodies were assessed after fluorescent compensation was performed for spectral overlap of the two. Gains adjustments for FITC and PE were set with an unstained control in the case of colabeled samples.

Serial cell sorting was performed on a FACStar flow cytometer (Becton Dickinson). The 2% to 5% of cells with the highest fluorescence intensity were collected under sterile conditions and replated in selective media at 1.8 to 5.0 × 10^5 cells/mL.

Iodination of vWF and vWF binding studies. vWF^126 in 100 mmol/L NaPO_4, pH6.5 (0.1 mL, 1 mg/mL) was labeled with 1 mCi of ^125I using iodobeads (Pierce, Rockford, IL). The radiolabeled protein was isolated with chromatography using a PD-10 Sephadex G-25M column (Pharmacia, Piscataway, NJ).

HEL cells were treated with 80 μmol/L zinc sulfate and harvested as described above before resuspension at a final concentration of 2.2 × 10^7/mL in a buffer solution of 150 mmol/L NaCl, 20 mmol/L Tris, 5 mmol/L dextrose, 1 mg/mL BSA, 1 mmol/L EDTA, pH 7.5. A range of approximately 7 to 53 pg/mL of iodinated vWF

![Flow cytometry of HEL cells.](image)
was added to the cells in a final volume of 300 µL in the presence or absence of 1.4 mg/mL of ristocetin. After incubation at room temperature (30 minutes), 185 µL was layered over 80 µL of a silicone oil solution consisting of 80% Dow Corning 550 fluid and 20% 200 fluid (Nye Inc, New Bedford, MA). After centrifugation (10,000g for 2 minutes, at 22°C), the supernatant was removed with a Pasteur pipette, the tube was inverted, and the pellet clipped. Radioactivity associated with the pellet (bound ligand) of each sample was determined. Parallel incubations in the absence of ristocetin were performed to assess the degree of nonspecific binding between ligand and receptor, and the values for each point were subtracted from those obtained in the presence of ristocetin to derive specific binding activity.

Confocal microscopy. Transfected HEL cells were colabeled with two different primary antibodies and their respective fluoro-

chrome-conjugated secondary antibodies as noted above. This was followed by mounting 20 µL on a glass slide onto which a glass coverslip was applied separated by an adhesive tape spacer to prevent crush artifact. The slide was visualized using an ACAS Ultima Laser Cytometer (Meridian Instruments, Okemos, MI). Cells were scanned in dual color confocal mode (pinhole = 400 µm) using a 100 x oil immersion objective (numerical aperture = 1.3) with a step size of 0.3 µm/pixel and a field size of either 180 or 270 pixels squared. FITC fluorescence was detected in PMT1 with a 530/15 nm band pass filter, and PE fluorescence was detected after separation with a dichroic mirror (575 short pass filter) in PMT2 with a 605 long pass filter. Dual-color digital images were displayed on a DASY 9000 Image analysis workstation (Meridian) using the manufactur-

![Confocal microscopy](image)

**Fig 2.** Contour graphs comparing GPV and Iba surface expression. Nontransfected (A) and GPV-transfected (B) HEL cell populations were colabeled with anti-V/FITC (FL1) and anti-Iba/PE (FL2). Nontransfected and mock-transfected cells had similar results. Numbers in each quadrant indicate percentage of total cells. Overlay histograms of transfected (----) and nontransfected (-----) populations colabeled with anti-

V/FITC (C) and anti-Iba/PE (D) confirm the increased surface expression of both V and Iba subunits in the transfectants.
er's software and saved as tag interchange file format (TIFF) files. Similarly, after thresholding for background fluorescence, two-color pixel histograms were generated from these images and displayed using the manufacturer's software and printed with a Sony Mavigraph video printer (Sony Corp, Tokyo, Japan).

RESULTS

Flow cytometry studies. HEL cells are a tumor cell line that manifests several surface megakaryocyte and platelet antigens including GPIb and IX. While cloning the GPV gene, our laboratory established through reverse transcriptase-polymerase chain reaction (RT-PCR) amplification, Northern blot analysis, and RNase protection studies that GPV mRNA was absent from the HEL cells used in the present study. To determine if expression of GPV leads to enhanced surface expression of other GPIb subunits, cells were electroporated with zm228R containing GPV in the sense or antisense orientation and selected in (3418. Approximately 28 days later the cells were harvested and labeled with the following antibodies: SW16 (anti-GPV); Bebl (anti-GPIX); rabbit polyclonal, HIPt-5, GS296, ES85 (anti-GP Ibα); anti-type I collagen (negative control), or antibody B79.7 (anti-GPIIIa, positive control).

Transfected cells were analyzed for GPV, GPIX, and GPIbα surface expression (Fig 1). After stimulation of the mettallothionein I promoter with 80 μm zinc sulfate, cells were selected by serial cell sorting. Control experiments included cells transfected with whole genomic DNA or metallothionein I promoter with 80 μm zinc sulfate, and those showing increased GPIbα expression were the same as those with increased GPV expression. Averaged results of four radiolabeled ligand binding assays are shown in Fig 3. When GPV-transfected, non-transfected, or mock-transfected HEL cells were exposed to progressively increasing amounts of iodinated vWF in the presence of ristocetin, after correcting for nonspecific binding (see Materials and Methods), vWF binding was consistently increased in cells transfected with the GPV gene. The average amount of vWF bound to GPV-transfected cells was 1.5 times that bound to nontransfected cells at saturating concentrations.

Confocal microscopy. After demonstrating the presence of transfected GPV and increased amounts of GPIbα and GPIX on the HEL cell surface, we proceeded to colabel transfected cells with mouse anti-GPV/goat antimouse FITC and rabbit anti-GPIbα/goat antirabbit PE for visualization by confocal microscopy (Fig 4C and F). Our objective was to look for evidence that these two proteins colocalized and associated with each other on the cell surface to within the limits of resolution of a single pixel (approximately 300 nm). Cell samples were also colabeled with mouse anti-GPIIIa/ goat-antimouse FITC and rabbit anti-Ibα/goat antirabbit PE (negative control, Fig 4B and E), or mouse anti-GPIX/goat antimouse FITC and rabbit anti-Ibα/goat antirabbit PE (positive control, Fig 4A and D). Colocalization of FITC (green) and PE (red) antibodies is evidenced by a yellow appearance (Fig 4A and C) and by corresponding pixel histograms that demonstrate a linear correlation in antibody expression for each fluorescence intensity level (Fig 4D and F). Absence of colocalization is suggested by areas of red and green on cell surfaces (Fig 4B) and by a pixel histogram exhibiting a diffuse pattern (Fig 4E).
Fig 4. Colocalization of transfected GPV and GPIbα on the surface of HEL cells. Cells were labeled with either (A) anti-GPIX/FITC (green) and anti-GPIIbα/PE (red) (positive control, field size 81 μm x 81 μm), (B) anti-GPIIla/FITC and anti-GPIIbα/PE (negative control, field size 81 μm x 81 μm) or (C) anti-GPV/FITC and anti-GPIIbα/PE (field size 54 μm x 54 μm). Insets: Detector 1 = FITC fluorescence intensity, detector 2 = PE fluorescence intensity. Yellow fluorescence suggests the two antibodies colocalize to within approximately 300 nm. Pixel histograms for each of the photomicrographs above reflect colocalization of GPIX/Ibα (D) and GPV/Ibα (F): note approximately equal contributions from each fluorochrome-conjugated antibody at different fluorescence intensities in D and F versus GPIIla/Ibα (E) in which a diffuse pattern reflecting noncolocalization is seen.
To provide statistical evidence for the above conclusions, individual cell ratios of red to green pixel values were calculated and converted into coefficients of variation (CV) (standard deviation divided by mean) for each of the images shown in Fig 4A-C. These CV's were averaged for each image and analyzed for differences using a two-sample, one-tailed t-test assuming unequal variances. Thus, the colocalization between GPIX/Iba and GPV/Iba was found not to significantly differ (P = .128), while that between GPIIa/Iba and GPV/Iba did (P = .021). Hence, these images, pixel histograms, and statistical analysis suggest that surface GPV and GPIIba associate with each other to the same degree as surface GPIX and GPIIba.

DISCUSSION

Using a hematopoietic cell line that lacks GPV but normally expresses GPIb, we find that transfection of GPV DNA and GPV surface expression are associated with increased GPIIba and GPIX surface expression. Radioligand binding studies show increased vWF binding to HEL cells transfected with GPV compared with non- and mock-transfected controls. This latter result is a reflection of increased surface GPIIba receptor number in GPV-transfected cells demonstrated by the flow cytometry studies. Any conclusion about GPV's influence on binding affinity between GPIIba and vWF cannot be reached in the present study. Thus, the binding study data support the conclusion that GPV enhances GPIIba surface expression. We have also used confocal microscopy to provide suggestive evidence that transfected GPV associates with GPIIba on the HEL cell surface.

Preliminary work reported in abstract form by our group suggested that the presence of GPV did not influence GPIIba expression in stably-transfected HEL cells. Subsequent to this work, these cells underwent serial cell sorting which, along with ZnSO4 stimulation of the metallothionin promoter upstream from GPV, led to enhanced GPV expression in these cells. With this increased GPV expression, we have been able to document enhanced GPIIba expression in the present work.

Our findings support the idea that subunits of the GPIb receptor are expressed on the cell surface in a coordinated fashion and that maximal expression of the complex probably requires all its constitutive subunits. Transferring GPIX cDNA into nonhematopoietic cells previously transfected with GPIIa and β cDNA has been shown to significantly increase GPIIa surface expression and function. The present work shows that GPIIa expression is likewise enhanced when subunits Iba, Ibb, and IX are complemented by the presence of GPV. Both GPIX and V are noncovalently associated with GPIIa; both may play similar roles in imparting membrane targeting and stability to the Iba subunit carrying the ligand binding site.

Data obtained from patients with Bernard-Soulier syndrome have been useful with respect to elucidating function and assembly of the GPIIb/IIIa complex. Patients with this bleeding disorder have documented point mutations involving either of two of the four subunits (GPIIa and GPIX). Although these individuals carry mutations affecting a single subunit gene, most of them have a phenotype marked by a significant reduction of all four subunits on the platelet surface, and hence, nearly absent interaction with vWF ligand in ristocetin-dependent platelet agglutination assays. The significant reduction of GPIIa expression in platelets of Bernard-Soulier individuals with GPIX point mutations suggests GPIIa may be dependent on the presence of other subunits for its optimal surface expression. Our data showing enhanced expression of GPIIa in HEL cells transfected with GPV support this hypothesis.

GPV function with respect to the Ia complex remains somewhat unclear. Although containing a thrombin cleavage recognition site, it appears GPV is not the platelet activation receptor for thrombin as a thrombin receptor distinct from the Ia/V/IX complex has been cloned. GPV may play a role in the interaction of each GPIb complex with vWF or may increase the number of GPIb receptors available for vWF on the platelet surface. Our flow cytometry data suggest GPV is likely capable of increasing GPIIIa-vWF binding through the latter mechanism (Fig 1). Anti-GPV antibodies present in a Bernard-Soulier patient have been reported to inhibit ristocetin-dependent platelet agglutination.

There is evidence suggesting GPIIIa is capable of binding its ligand without the participation of neighboring subunits. Several studies demonstrate that soluble fragments of GPIIa and GPIIa bind vWF in the presence of ristocetin. We observed the saturability of GPIIa receptors with the vWF ligand both when GPV was present and absent (Fig 3), showing that GPV can increase, but is not required for receptor-ligand interaction. In conclusion, we have transfected HEL cells with GPV DNA and have shown the presence of GPV leads to increased GPIIa and GPIX surface expression. Therefore, GPV appears to play a role in the GPIIa complex by increasing GPIIa surface expression.

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Human platelet glycoprotein V: its role in enhancing expression of the glycoprotein Ib receptor

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