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Identification of Three Tyrosine Residues of Glycoprotein Ibα with Distinct Roles in von Willebrand Factor and α-Thrombin Binding*

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The interaction between von Willebrand factor (vWF) and the platelet membrane glycoprotein (GP) Ib-IX-V complex is essential for platelet adhesion at sites of vascular injury under high shear stress flow conditions. Moreover, GP Ib-IX-V may contribute to the mechanisms of platelet activation through its high affinity binding of α-thrombin. There are two distinct but partially overlapping regions of GP Ibα thought to be involved in interacting with vWF (residues 251–279) and α-thrombin (residues 271–284); they share three tyrosine residues (positions 276, 278, and 279) that have recently been shown to be sulfated (Dong, J., Li, C. Q., and Lopez, J. A. (1994) *Biochemistry* 33, 13946–13953). To define the functional role of these three residues, we have introduced selected mutations in a soluble recombinant GP Ibα fragment (corresponding to the sequence 1–302 of the mature protein) that binds vWF and α-thrombin with the same attributes as intact GP Ib-IX-V complex. Fragments containing a single Tyr → Phe substitution either at position 276 or 278 or 279 exhibited normal interaction with vWF but markedly reduced or absent binding of α-thrombin. GP Ibα fragment with normal sequence but synthesized under sulfate-free conditions also failed to bind α-thrombin and, in addition, had markedly reduced interaction with vWF. The simultaneous substitution of three neighboring Asp residues with Asn at positions 272, 274, and 277, a multiple mutation that may impair Tyr sulfation, also resulted in loss of binding of both ligands. These results define distinct structural features of GP Ibα selectively involved in supporting the interaction with vWF or α-thrombin.

Glycoprotein (GP)[1] Ib, composed of the disulfide-linked α and β chains, associates with GP IX (1) and GP V (2) to form a noncovalent hetero-oligomeric complex expressed on the platelet membrane. The amino-terminal extracytoplasmic domain of GP Ibα contains binding sites for the adhesive protein, von Willebrand factor (vWF) (3), and the platelet agonist, α-thrombin (4), thus supporting two interactions potentially relevant for normal hemostasis as well as the development of pathological thrombosis. The amino-terminal domain of GP Ibα has been expressed in Chinese hamster ovary (CHO) cells as an isolated soluble fragment and, with regard to the interaction with vWF, is known to possess the same binding specificity and affinity of the intact platelet receptor (5).

Previous studies with synthetic peptides (3) have led to the conclusion that GP Ibα residues within the sequence 251–279 are involved in vWF binding, a fact confirmed subsequently by site-directed mutagenesis experiments highlighting the activity of a cluster of negatively charged residues located between Asp^{252} and Asp^{287} (5). Moreover, residues within the sequence 271–284 have been shown to participate in the binding of α-thrombin (6). These results suggest that the GP Ibα sites interacting with vWF and α-thrombin may overlap, but additional experimental evidence indicates that each has unique features. Indeed, anti-GP Ibα monoclonal antibodies can selectively inhibit binding of one or the other ligand (4, 7); and a single GP Ibα point mutation identified in a patient with Bernard-Soulier syndrome impairs vWF but not α-thrombin binding (8, 9).

With this information as a background, we have employed site-directed mutagenesis to verify the effect of selected amino acid substitutions on the ligand binding activity of the recombinant GP Ibα amino-terminal domain. In particular, we have focused on three contiguous Tyr residues (positions 276, 278, 279) that, as mentioned above, are shared by synthetic peptides that inhibit either vWF or α-thrombin binding to platelets. These Tyr residues meet the consensus criteria for the occurrence of post-translational sulfation (10, 11) and, indeed, have recently been shown to be sulfated when expressed in eukaryotic cells (12). The results presented here provide initial information on the distinctive structural features that characterize the two GP Ibα sites interacting with vWF and α-thrombin.

EXPERIMENTAL PROCEDURES

Recombinant Expression of the Amino-terminal Domain of GP Ibα—The characterization of a recombinant plasmid directing the synthesis of the amino-terminal domain of GP Ibα has been described in detail elsewhere (5, 9). Briefly, a mammalian cell expression plasmid was constructed from a fragment of the GP Ibα gene (coding for the signal peptide and mature residues His^1–Ala^{302}) synthesized in a polymerase chain reaction that added *Bam*HI restriction sites on the ends of the amplified fragment. This was cloned into M13mp19 as a *Bam*HI fragment and sequenced to verify that no spontaneous mutations had arisen during the polymerase chain reaction. When indicated, mutations within the expression plasmid were constructed using the appropriate oligonucleotides and site-directed mutagenesis on uracil-containing templates of the original M13 construct (13). The DNA sequence of

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[1] The abbreviations used are: GP, platelet membrane glycoprotein; vWF, von Willebrand factor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium.

all mutants was verified to ensure that it corresponded to the predicted one. The GP Ibα insert was removed from the M13 construct by digestion with *Eco*RI and *Xba*I and cloned into the corresponding restriction sites of the polylinker region of pBS/KS$^-$ (Stratagene). As a result of this, restriction sites for *Xho*I (5′ to the GP Ibα-initiating Met codon) and *Not*I (3′ to the Ala^{302} codon) were acquired from the vector and were used to clone the fragment into the mammalian cell expression plasmid pCDM8^{neo}. The latter is identical to pCDM8 (14) except that it contains a neomycin gene for conferring resistance to the aminoglycoside antibiotic, G418, or Geneticin (Sigma). CHO-K1 cells were grown in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5 mM non-essential amino acids, 2 mM L-glutamine (Whittaker Bioproducts) and 10% fetal calf serum (10%-DMEM). DNA (10 μg/dish) was introduced into cells (subcultured at a density of 1.5×10^5 per 60-mm dish 24 h prior to transfection) using a calcium phosphate-mediated transfection procedure (15); transfected cells were then maintained in 10%-DMEM. Medium to be used as a source of soluble recombinant GP Ibα fragment was collected from confluent cultures of cells grown for 24 h in the absence of fetal calf serum. A sulfate-free recombinant GP Ibα fragment was obtained from cells grown for 24 h in minimum essential medium (MEM) prepared from stock solutions devoid of sulfate ions (2.5 mM Hepes, 1.35 mM $CaCl_2$, 5.4 mM KCl, 116 mM NaCl, 1 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 26 mM $NaHCO_3$, 5 mM D-glucose, 20 mM sodium pyruvate, pH 7.4); the medium also contained MEM amino acid solution (Life Technologies, Inc.) supplemented with 4 mM L-glutamine, non-essential amino acids (Whittaker), MEM vitamin solution (Life Technologies, Inc.), and 10 mg/ml phenol red solution as an indicator of pH. Moreover, the sulfate-free medium contained a final concentration of 5 mM $NaClO_3$, an inhibitor of protein sulfation (16).

Monoclonal Anti-GP Ibα Antibodies—The production and characterization of anti-GP Ibα monoclonal antibodies have been described elsewhere (7, 17, 18). As demonstrated previously using proteolytic fragments of GP Ibα generated by trypsin or *Serratia marcescens* protease (7), antibodies LJ-Ib1 and LJ-P3 interact with distinct epitopes present only in native GP Ibα and located between residues 1 and 290; in contrast, antibody LJ-Ib10 interacts with an epitope not affected by denaturation with SDS nor reduction of disulfide bonds and located between residues 238 and 290. Using the same methods, we have also found[2] that antibody LJ-Ibα1 recognizes an epitope not affected by denaturation with SDS nor reduction of disulfide bonds and located within residues 1–237, and antibody LJ-P19 reacts with an epitope present only in native GP Ibα and located within residues 1–290. Monoclonal antibodies (all IgG_1k) were purified by binding to protein A-Sepharose, as described previously (19). When indicated, purified IgG were radiolabeled with ^{125}I using the IODO-GEN procedure (20).

Evaluation of Immunochemical Reactivity—The nondenaturing dot-blot technique employed for analysis of antibody binding to different recombinant GP Ibα fragments has been described in detail in a previous publication (5). In brief, recombinant fragment was immobilized onto nitrocellulose membrane (0.45 μm pore size; Bio-Rad) by filtering culture medium through the membrane with a peristaltic pump applied to a device (ELIFA, Pierce) that delimits a circular area of application (5); filtration time was 5 min for each 200 μl of medium applied. The membrane was then removed from the apparatus, and its protein binding capacity was blocked with Blotto solution (21), prepared with 50 g/liter fat-free dry milk and 0.5 ml/liter of Antifoam A emulsion (Sigma); the membrane was then incubated with the appropriate specific antibody, washed with Blotto solution, and finally incubated with ^{125}I-labeled rabbit anti-mouse IgG. Positive reactivity was detected by autoradiography; a quantitative estimate of bound antibody was obtained by counting in a γ-scintillation spectrometer the radioactivity associated with each dot cut out from the nitrocellulose membrane.

vWF Binding to Immobilized Recombinant GP Ibα Fragments—vWF was purified and characterized as reported previously (22); it was radiolabeled with ^{125}I using the IODO-GEN procedure (20). Serum-free culture medium from cell lines to be tested was used as such or diluted with the appropriate amount of serum-free medium from non transfected CHO-K1 cells to obtain comparable concentrations of different recombinant fragments, as judged by immunochemical dot-blot analysis with antibody LJ-Ibα1. Proteins in culture medium were immobilized onto nitrocellulose membrane by filtering 100–800 μl through the ELIFA apparatus, as described above; this and all subsequent steps of the assay were performed by filtering reagents through the same device at room temperature (22–25 °C). Filtration time was 5 min for each 200 μl of medium applied, with variation between different samples not greater than 30 s. It should be noted that the immunoadsorption procedure used to measure the interaction of α-thrombin with recombinant GP Ibα fragments (see below) was not used for vWF, because it gave lower levels of specific binding, presumably as a result of steric hindrance caused by the immobilizing antibody. Following insolubilization of the proteins contained in the culture media, the membrane was

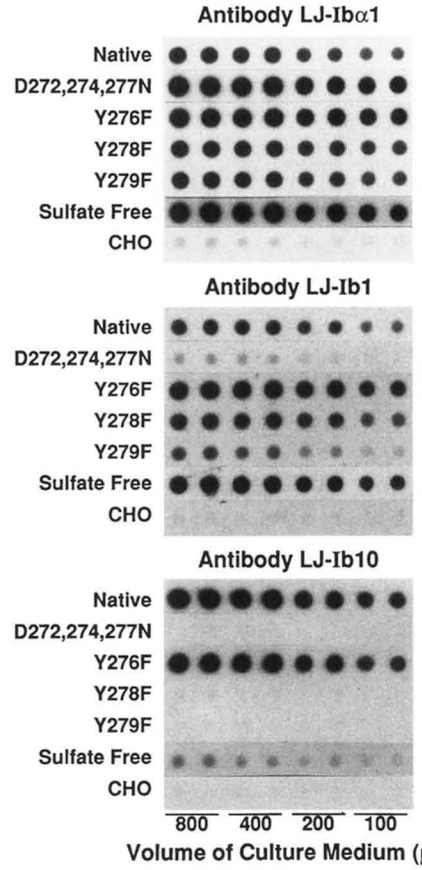

FIG. 1. **Immunochemical analysis of native and modified GP Ibα fragments using three different monoclonal antibodies.** Volumes of 100–800 μl of culture medium containing different types of recombinant GP Ibα fragment, precleared with a 0.45-μm filter, were aspirated through nitrocellulose membrane in a circular application area, in duplicate as indicated. For testing with antibodies LJ-Ibα1 and LJ-Ib10 (see below), disulfide bond reduction in the sample was achieved by treatment with 60 mM dithiothreitol at 37 °C for 1 h before filtration. All steps of the assay procedure were performed at 22–25 °C. *Native* indicates the fragment with normal GP Ibα sequence (residues 1–302) derived from cells grown under standard conditions; *D272,274,277N* indicates the fragment with three Asp → Asn mutations at positions 272, 274, and 277; *Y276F* indicates the fragment with a Tyr → Phe mutation at residue 276; *Y278F* indicates the fragment with a Tyr → Phe mutation at residue 278; *Y279F* indicates the fragment with a Tyr → Phe mutation at residue 279; sulfate-free indicates the fragment with normal sequence but derived from cells grown under sulfate-free conditions; CHO indicates the medium of nontransfected cells used as control. The membrane with bound recombinant fragments was soaked for 1 h in Blotto solution, followed by a 2–3-h incubation with a 1:1000 dilution (in Blotto) of ascitic fluid containing the monoclonal antibody to be tested, as indicated (all mouse IgG). At the end of the incubation, the membrane was washed three times (10 min each) with fresh Blotto solution and then incubated for 1 h in Blotto solution containing ^{125}I-labeled rabbit anti-mouse IgG (0.05 mCi of total radioactivity). The membrane was again washed three times with fresh Blotto solution and one last time with 20 mM Hepes buffer, pH 7.4, containing 150 mM NaCl and 500 μl/liter of Tween 20 (Sigma). At this point the membrane was dried and an autoradiograph was obtained by exposure for 12–18 h to a Kodak X-Omat RP XRP-1 film with a Dupont Cronex Quanta III intensifying screen. Although not all fragments were tested at the same time, the results from different experiments are readily comparable, since the native GP Ibα fragment used as control on each membrane always gave reactivity similar to that shown in this figure.

[2] M. Murata, J. Ware, and Z. M. Ruggeri, unpublished results.

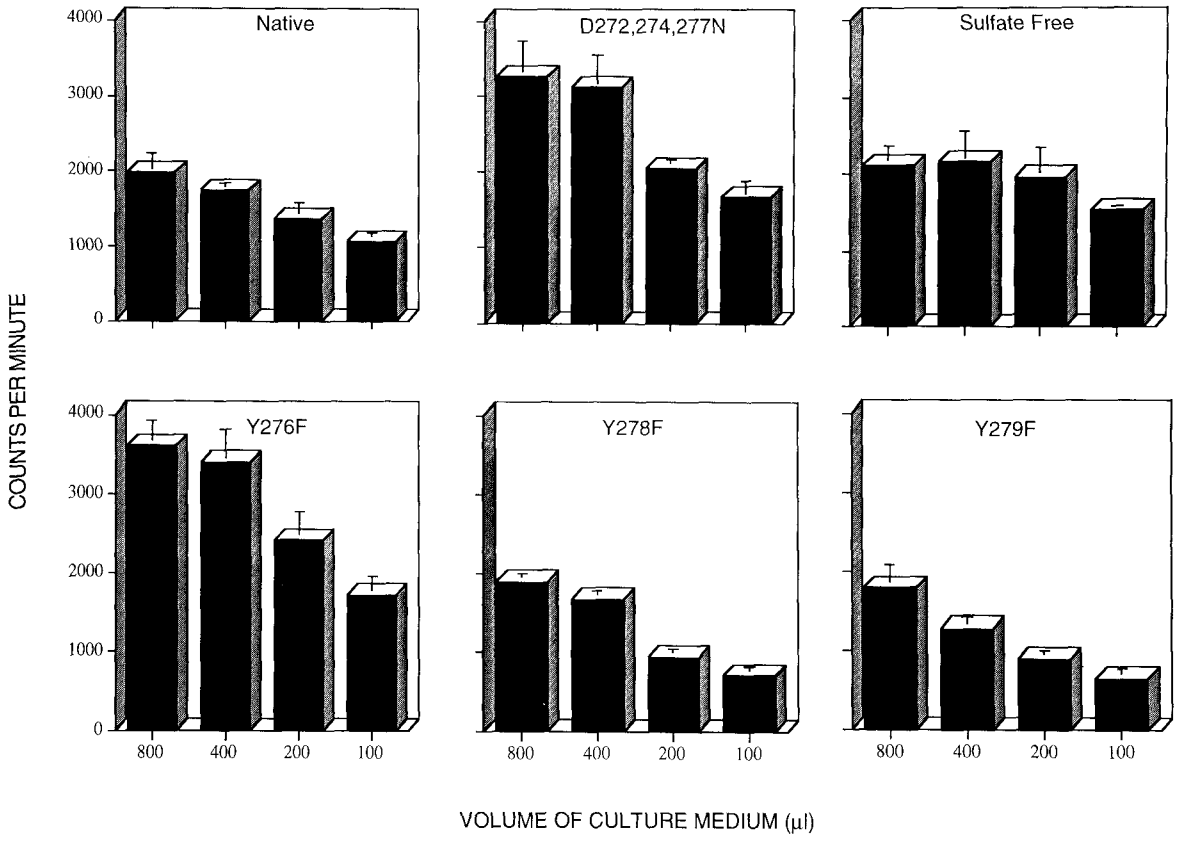

FIG. 2. **Binding of the monoclonal antibody LJ-Ibα1 to different GP Ibα fragments.** This assay was performed as described in the legend to Fig. 1 (see also for the nomenclature used for the different fragments) except that, at the end of the procedure, each circular area corresponding to the sample application point was cut out and the associated radioactivity was measured in a γ-scintillation spectrometer to obtain a quantitative estimate of antibody bound. The results shown are the mean (±S.E.) of four distinct experimental observations. In interpreting these values it should be noted that the level of maximum antibody binding may be influenced by the concentration of other proteins in each culture medium relative to that of GP Ibα fragment, since all proteins contribute to saturation of the membrane; regardless of this problem, however, the data allow us to compare the amount of immunoreactive material (*i.e.* GP Ibα fragment) bound to the nitrocellulose with each medium.

blocked with three washes (200 μl each; filtration time: 3 min) of Hepes buffer containing 1% bovine serum albumin and 1% bovine γ-globulin (Sigma), the latter added to reduce nonspecific binding. After blocking, 50 μl of ^{125}I-labeled vWF, preincubated for 20 min with botrocetin or immediately mixed with ristocetin as modulators of binding (5), was filtered through the nitrocellulose membrane in 5 min. When indicated, appropriate anti-GP Ibα monoclonal antibodies or unlabeled vWF were also present in these mixtures. Ristocetin (Sigma) was used at the final concentration of 1 mg/ml; botrocetin (two-chain form) was purified as described previously in detail (23, 24) from the crude venom of *Bothrops jararaca* (Sigma) and was used at the final concentration of 4 μg/ml. The membrane was then washed two additional times with blocking solution (200 μl each wash; filtration time: 3 min); in the assays employing the modulator ristocetin, the latter was also present during this step at the same concentration used with vWF. Finally, the membrane was dried, the spots corresponding to each application well were cut out, and the bound radioactivity was measured in a γ-scintillation spectrometer.

α-Thrombin Binding to Immobilized Recombinant GP Ibα Fragments—α-Thrombin, a generous gift of Dr. John W. Fenton II (Wadsworth Center for Laboratory and Research and Departments of Physiology and Biochemistry, Albany Medical College, Union University, Albany, NY), was purified and characterized as described previously (25); it was radiolabeled with ^{125}I using the IODO-GEN technique (20), as reported in detail in a previous publication (4). The binding assay was performed using recombinant GP Ibα fragment insolubilized onto Sepharose beads by binding to the anti-GP Ibα monoclonal antibody, LJ-P3, covalently coupled to the beads; the antibody was selected for this application, because it has no inhibitory effect on α-thrombin binding to platelets. As determined in preliminary experiments not shown here, this method gave considerably lower nonspecific binding of α-thrombin than the one, described above for vWF, performed with recombinant fragment immobilized directly onto nitrocellulose. Purified IgG of LJ-P3 was coupled to cyanogen bromide-activated Sepharose CL-4B at a density of 4 mg/ml of packed beads. The LJ-P3/Sepharose beads were washed twice with a buffer composed of 0.1 M Tris-HCl, pH 7.3, containing 0.5 M LiCl and 1 mM EDTA, and then incubated with culture medium containing recombinant GP Ibα fragment (or medium of non transfected cells as control) at a ratio of 800 μl of packed beads/2 ml of medium. After 1 h at 22–25 °C with constant mixing, the beads were again washed twice and finally resuspended into 25 mM Tris-HCl, 136 mM CH_3CO_2Na, pH 7.3, containing 0.6% polyethylene glycol 6000 and 4.1% bovine serum albumin (binding buffer) at a ratio of 1 volume of packed beads and 6 volumes of buffer. To ensure that different fragments bound to LJ-P3 beads with equivalent efficiency, the procedure was monitored by measuring the binding to immobilized fragments of ^{125}I-labeled IgG of another anti-GP Ibα monoclonal antibody, LJ-P19, that does not cross-react with LJ-P3. The interaction of α-thrombin with recombinant GP Ibα fragments immobilized onto LJ-P3-Sepharose beads was measured by mixing 20 μl of bead suspension with 45 μl of binding buffer, 20 μl of a 10 mg/ml solution of bovine IgG (to reduce nonspecific binding), and 40 μl of the desired concentration of ^{125}I-labeled α-thrombin. When indicated, appropriate anti-GP Ibα monoclonal antibodies or unlabeled α-thrombin were also present in the mixtures. After a 30-min incubation at 22–25 °C, the whole volume of suspension was layered onto 250 μl of a 20% sucrose solution (in binding buffer) placed in microcentrifuge tubes; radiolabeled ligand bound to the beads was separated from free ligand by centrifugation at 12,000 × *g* for 4 min. The quantitative parameters of binding were calculated on the basis of the specific activity of the radiolabeled ligand; binding isotherms were analyzed using the computer-assisted program LIGAND (26, 27).

RESULTS

Effect of Different Mutations and Sulfate Depletion on the Immunochemical Reactivity of the Recombinant GP Ibα Amino-terminal Fragment—The molecules tested in these experi-

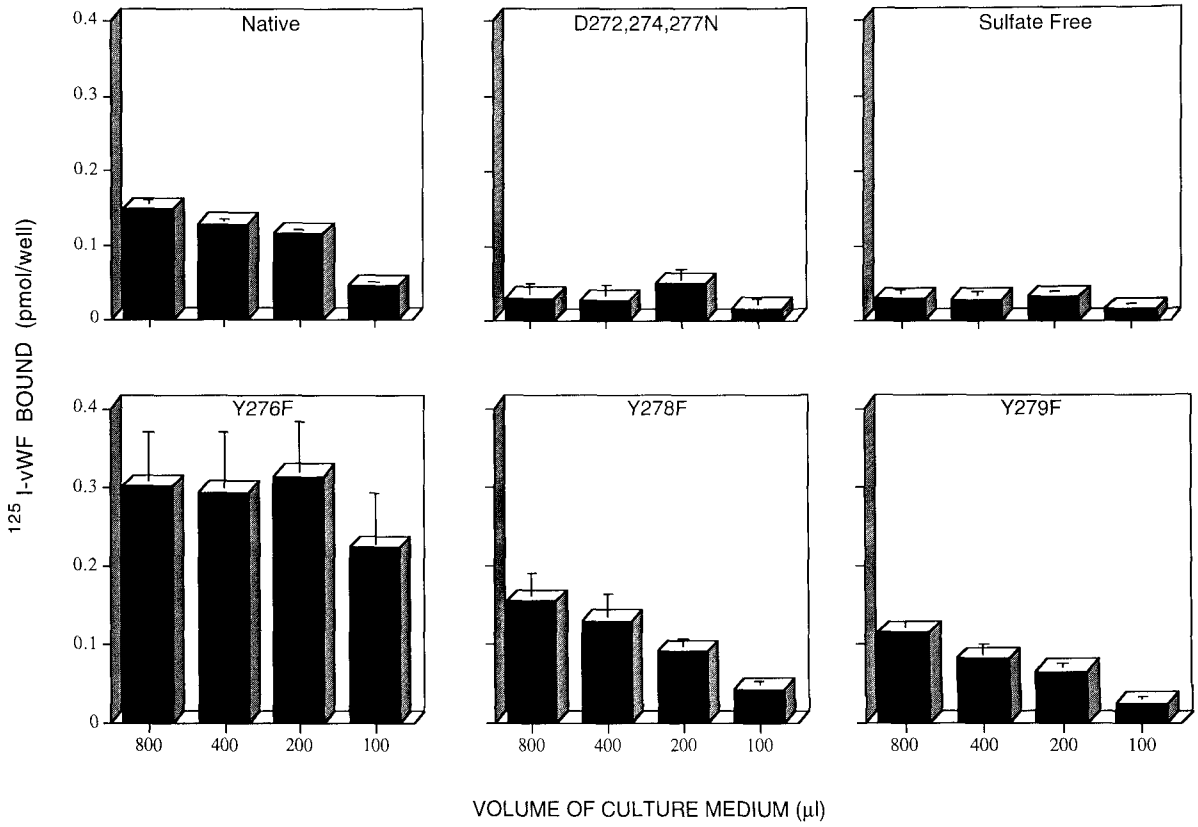

FIG. 3. **Ristocetin-mediated binding of ^{125}I-labeled vWF to different GP Ibα fragments.** Culture medium containing native or modified GP Ibα fragments was immobilized onto nitrocellulose membrane, in duplicate application points and in variable volume, as described in the legend to Fig. 1 (see also for the nomenclature used for the different fragments). The membrane was then saturated and washed by filtering through it 200 μl of Hepes buffer containing 1% bovine serum albumin and 1% bovine γ-globulin; the procedure was repeated three times. At this point, 50 μl of ^{125}I-labeled vWF (4 μg/ml final concentration, corresponding to 0.7 pmol/well) mixed with ristocetin (1 mg/ml final concentration) was filtered through each well. The membrane was then washed twice by filtering through each well 200 μl of Hepes/bovine serum albumin/γ-globulin solution containing 1 mg/ml of ristocetin. All steps of this assay were performed at 22–25 °C. Each circular area corresponding to the application wells was then cut out from the membrane, and the associated radioactivity was measured in a γ-scintillation spectrometer. Bound vWF was calculated based on its specific radioactivity. The results shown are the mean (±S.E.) of four distinct experimental observations.

ments included normal GP Ibα fragment (comprising residues 1–302 of the mature protein and secreted by transfected CHO cells grown under standard conditions) and four different mutants, three containing single Tyr → Phe substitutions either at residue 276 (Y276F) or 278 (Y278F) or 279 (Y279F) and one containing three Asp → Asn substitutions at positions 272, 274, and 277 (D272N,D274N,D277N); the latter mutations may affect tyrosine sulfation (see "Discussion"). Moreover, we tested the GP Ibα fragment with native sequence but synthesized by transfected CHO cells grown under sulfate-free conditions. Three different monoclonal antibodies were used to evaluate the immunochemical reactivity of the GP Ibα fragments; two of the antibodies, LJ-Ib1 and LJ-Ib10, were chosen because of their selective inhibitory effect on vWF or α-thrombin binding to GP Ib, respectively (4, 9); the third, LJ-Ibα1, a non-inhibitory antibody, because of its ability to interact with fully denatured GP Ibα and because the corresponding epitope, is located between residues 1–237, thus at a distance from the area targeted for mutagenesis.

Antibody LJ-Ibα1 reacted in a similar manner with the native GP Ibα fragment and all the mutant molecules tested, as well as with the sulfate-free fragment; thus, it was assumed to report on the amount of recombinant protein present in each culture medium (Fig. 1, *top panel*). In contrast, different mutant GP Ibα fragments exhibited distinct reactivity with antibodies LJ-Ib1 and LJ-Ib10. In particular, LJ-Ib1, the selective inhibitor of vWF binding to GP Ib, showed markedly reduced interaction with the mutant D272N,D274N,D277N, but only a modestly reduced reactivity with the mutant Y279F and normal reactivity with all the other mutant molecules as well as with the sulfate-free fragment (Fig. 1, *middle panel*). Antibody LJ-Ib10, the selective inhibitor of α-thrombin binding to GP Ib, reacted well with the mutant Y276F but exhibited markedly reduced interaction with all the other mutant molecules and with the sulfate-free fragment (Fig. 1, *bottom panel*).

Effect of Different Mutations and Sulfate Depletion on vWF Binding to Recombinant GP Ibα Fragment—The interaction of vWF with immobilized recombinant GP Ibα fragment, like with intact GP Ib-IX-V receptor complex on platelets, requires exogenous modulators such as ristocetin or botrocetin (5); both were used to evaluate the vWF-binding function of the mutant molecules produced. Binding of antibody LJ-Ibα1 was measured in parallel to monitor the content of expressed recombinant GP Ibα amino-terminal fragment in the culture media used; expression levels were found to be within a 2-fold limit of variation for all the molecules tested (Fig. 2). The three single Tyr → Phe mutants bound vWF in the presence of ristocetin at levels equal to or greater than those observed with the native fragment (Fig. 3); their binding capacity was in accordance with the levels of recombinant protein expressed, as measured by interaction with antibody LJ-Ibα1 (compare Fig. 3 with Fig. 2). In contrast, both the mutant D272N,D274N,D277N and the sulfate-free fragment exhibited a marked reduction of ristocetin-mediated vWF binding (Fig. 3), in spite of expression levels of recombinant protein equal to or greater than those of the normal control fragment (Fig. 2). Seven different preparations of

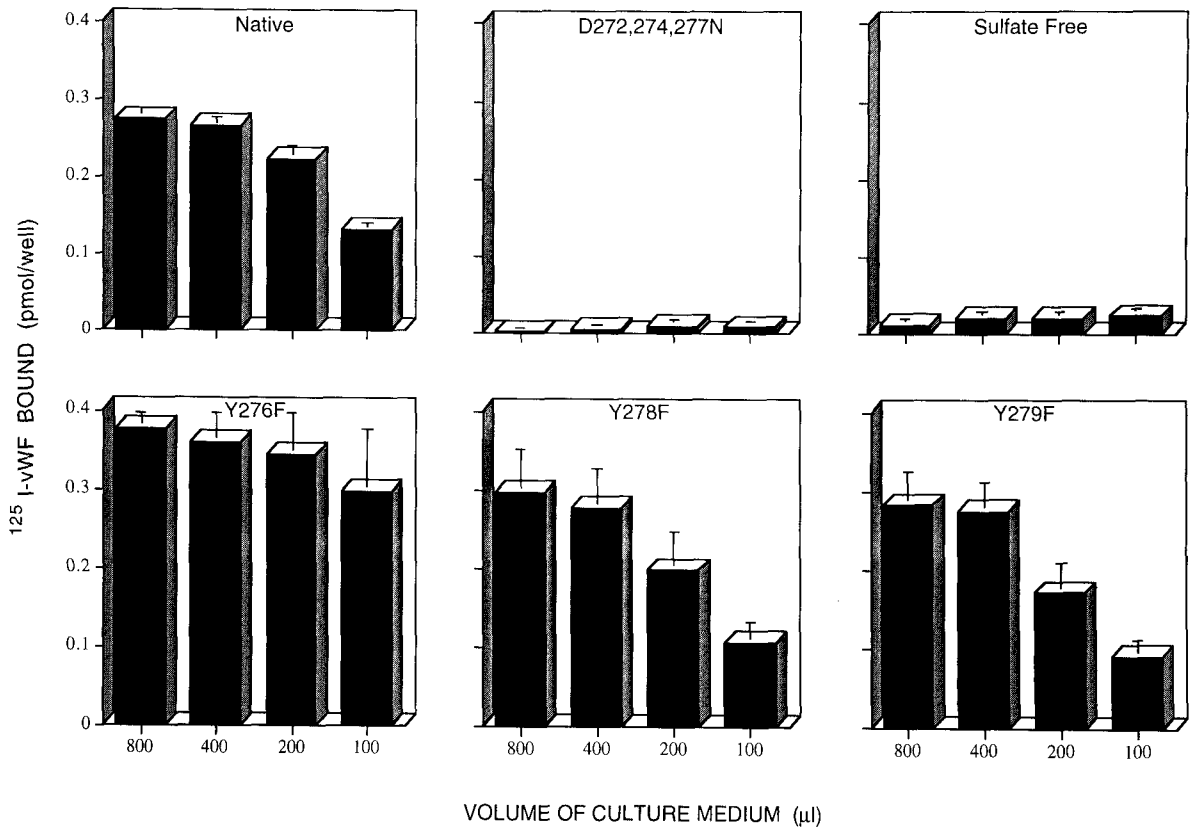

FIG. 4. **Botrocetin-mediated binding of ^{125}I-labeled vWF to different GP Ibα fragments.** This assay was performed as described in the legend to Fig. 3, except that ristocetin was omitted from all steps and vWF was preincubated with botrocetin (4 μg/ml final concentration, 20 min) before filtration through the membrane. The results shown are the mean (±S.E.) of four distinct experimental observations.

sulfate-free fragment were tested for ristocetin-mediated vWF binding using a single ligand concentration of either 2 or 4 μg/ml: the average binding, relative to normal GP Ibα fragment tested in parallel, was 64% (range from 23 to 86%). Measurement of botrocetin-mediated vWF binding confirmed the normal activity of the three single Tyr → Phe mutants as well as the markedly reduced function of the mutant D272N,D274N,D277N and of the sulfate-free fragment (Fig. 4). Six different preparations of sulfate-free fragment were tested for botrocetin-mediated vWF binding using a single ligand concentration of either 2 or 4 μg/ml: the average binding, relative to normal GP Ibα fragment tested in parallel, was 13% (range from 7 to 19%). As verified with normal GP Ibα fragment, vWF binding was inhibited at least 80% by the antibody LJ-Ib1, regardless of the modulator used. Moreover, the binding was saturable, as demonstrated by the fact that addition of 100-fold excess unlabeled vWF together with the labeled ligand resulted in 85% inhibition of ristocetin-mediated binding and 98% inhibition of botrocetin-mediated binding.

Binding isotherms obtained in the presence of increasing concentrations of added vWF confirmed the reduced binding activity of the sulfate-free GP Ibα fragment, both in the presence of ristocetin and botrocetin, as well as the more marked abnormality with the latter modulator (Fig. 5). The interaction of vWF with GP Ibα, whether measured with intact platelets or under the experimental conditions described here, is not reversible in the presence of exogenous modulators; thus, these data were not subjected to Scatchard-type analysis. At the highest concentration of ligand added, maximal binding in the presence of ristocetin was (mean ± S.E. of two separate experiments): 4.71 ± 0.19 pmol of vWF subunit/well coated with normal fragment, 2.12 ± 0.03 pmol of vWF subunit/well coated with sulfate-free fragment, and 1.37 ± 0.14 pmol of vWF subunit/well coated with nontransfected CHO cell control medium. The corresponding values in the presence of botrocetin were: 5.37 ± 0.30 pmol of vWF subunit/well coated with normal fragment, 1.05 ± 0.02 pmol of vWF subunit/well coated with sulfate-free fragment, and 0.58 ± 0.02 pmol of vWF subunit/well coated with nontransfected CHO cell control medium. The binding to nontransfected CHO cell control medium was assumed to be nonspecific and was subtracted from each point shown in Fig. 5; such value was approximately twice as high in the presence of ristocetin than botrocetin.

Effect of Different Mutations and Sulfate Depletion on the Binding of α-Thrombin to Recombinant GP Ibα Fragment— Recombinant fragments were immobilized by binding to the anti-GP Ibα monoclonal antibody, LJ-P3, covalently coupled to Sepharose beads. Coupling efficiency was comparable for the native and all but one of the modified recombinant GP Ibα fragments tested, as shown by binding of another anti-GP Ibα monoclonal antibody, LJ-P19, labeled with ^{125}I (results not shown); only the mutant D272N,D274N,D277N failed to interact with the antibody coupled to Sepharose beads and could not be tested in this assay. The normal GP Ibα fragment bound α-thrombin in a saturable manner, as demonstrated by the fact that addition of 100-fold excess unlabeled α-thrombin to a reaction mixture containing labeled ligand resulted in >85% inhibition of binding. Moreover, greater than 80% of the ^{125}I-labeled α-thrombin bound to normal GP Ibα fragment was displaced within 5 min after addition of a 100-fold excess of unlabeled ligand, a result indicative of equilibrium binding. Scatchard-type analysis of binding isotherms revealed one class of binding sites and demonstrated that nonspecific binding calculated as a fitted parameter corresponded closely to that measured with control beads prepared with the culture medium of nontransfected CHO cells (Fig. 6). Analysis of the

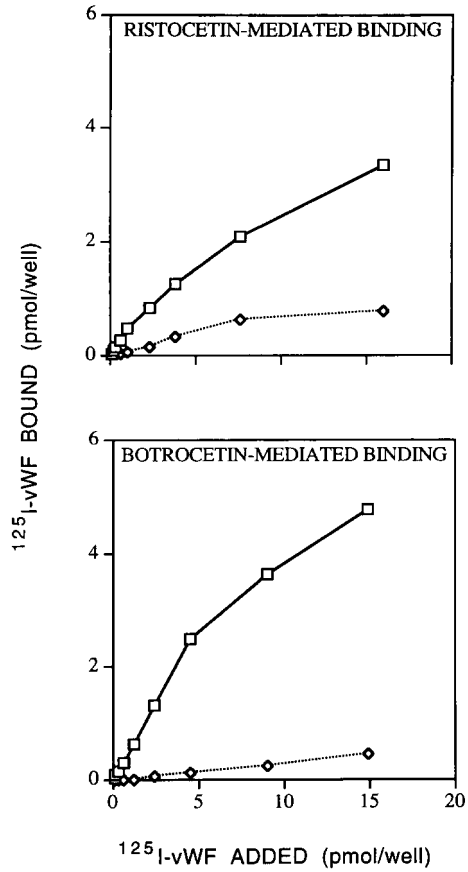

FIG. 5. **Dose-dependent binding of ^{125}I-labeled vWF to normal or sulfate-free GP Ibα fragment.** These experiments were performed as described in the legend to Figs. 3 and 4, with the exception that labeled vWF was used in increasing concentrations, as indicated on the *abscissa*, and each culture medium was used at the fixed amount of 800 μl/well. Different symbols indicate vWF binding to GP Ibα fragment synthesized under normal culture conditions (□) or under sulfate-free conditions (◇), either in the presence of ristocetin (*upper panel*) or botrocetin (*lower panel*).

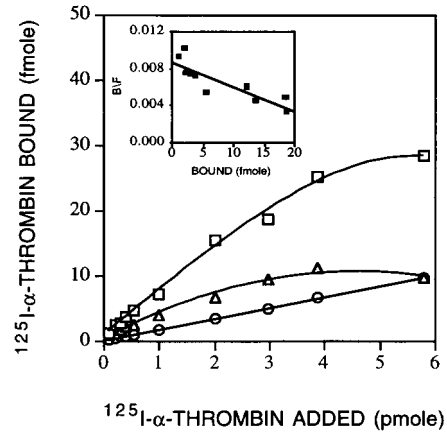

FIG. 6. **Binding isotherm and Scatchard-type analysis of ^{125}I-labeled α-thrombin binding to normal GP Ibα fragment immobilized onto Sepharose beads.** Purified IgG of the anti-GP Ibα antibody LJ-P3 was coupled to cyanogen bromide-activated Sepharose CL-4B at a density of 4 mg/ml of packed beads. The LJ-P3/Sepharose beads were washed twice with a buffer composed of 0.1 M Tris-HCl, pH 7.3, containing 0.5 M LiCl and 1 mM EDTA, and then incubated with culture medium containing native recombinant GP Ibα fragment (or medium of nontransfected cells as control) at a ratio of 800 μl of packed beads/2 ml of medium. After 1 h at 22–25 °C with constant mixing, the beads were washed twice with the above mentioned buffer and then resuspended into a binding buffer composed of 25 mM Tris-HCl, 136 mM CH_3CO_2Na, pH 7.3, containing 0.6% polyethylene glycol 6000 and 4.1% bovine serum albumin (1 volume of packed beads and 6 volumes of buffer). Each experimental mixture contained 20 μl of bead suspension (corresponding to 3 μl of packed beads), 45 μl of binding buffer, 20 μl of a 10 mg/ml solution of bovine IgG (to reduce nonspecific binding), and 40 μl of ^{125}I-labeled α-thrombin (in increasing concentrations, as indicated). After a 30-min incubation at 22–25 °C, the whole mixture was layered onto 250 μl of a 20% sucrose solution in binding buffer placed in microcentrifuge tubes; bound and free ligand were separated by centrifugation of the beads at 12,000 × *g* for 4 min. Bound thrombin was calculated based on its specific activity. The values shown represent binding to native GP Ibα fragment (□), binding to control medium of nontransfected CHO cells (△), nonspecific (nonsaturable) binding calculated as fitted parameter from the total binding to native GP Ibα fragment (○). The *inset* shows the Scatchard plot (bound/free *versus* bound) of binding to native GP Ibα fragment after subtraction of calculated nonspecific binding.

binding data obtained with normal GP Ibα fragment in four separate experiments (23 points, in duplicate, corrected by subtracting the nonspecific binding measured with culture medium of nontransfected CHO cells; Fig. 7) showed a mean binding at saturation of 50 ± 7 (S.E.) fmol of α-thrombin per 3 μl of packed beads carrying GP Ibα fragment (*i.e.* the volume of beads present in each experimental mixture); the corresponding k_a value of 5.64 ± 1.15 (S.E.) × 10^7 M^{-1} is similar to that (2.17 × 10^7 M^{-1}) obtained for α-thrombin binding to glycocalicin (*i.e.* the purified extracytoplasmic domain of GP Ibα derived from platelets; not shown). In contrast to their normal ability to interact with vWF, the three mutant molecules containing a single Tyr → Phe substitution exhibited either markedly reduced (Y278F) or undetectable (Y276F and Y279F) α-thrombin binding, as did the sulfate-free fragment (Fig. 7). Only the Y278F mutant allowed calculation of binding parameters at saturation: B_{max} was 12.7 ± 2.4 (S.E.) fmol/3 μl of packed beads, *i.e.* approximately 25% of the value obtained with normal control fragment, and the corresponding K_a was 3 ± 0.88 (S.E.) × 10^7 M^{-1} (eight points, in duplicate, in two separate experiments; Fig. 7). In the case of the other two mutants tested (Y276F and Y279F) and of the sulfate-free fragment, binding at saturation could not be calculated; the experimental data indicated that specific binding was less <5% of that measured with the normal control fragment (Fig. 7).

DISCUSSION

The results presented here demonstrate that the three tyrosine residues at position 276, 278, and 279 of mature GP Ibα, known to be the only sites of sulfation in the GP Ib-IX complex (12), are concurrently necessary for expression of α-thrombin binding activity; however, each one of them can be mutated individually to phenylalanine, abolishing the possibility of sulfation, without loss of vWF binding activity. In contrast, nonselective blockage of sulfation on all three Tyr residues causes severe impairment of both vWF and α-thrombin binding. Thus, two sulfotyrosine residues are sufficient for normal GP Ib interaction with vWF, whereas all three are needed for normal interaction with α-thrombin. As a corollary observation, it is of note that the mutation of either Tyr^{278} or Tyr^{279}, but not of Tyr^{276}, resulted in markedly reduced binding of the monoclonal antibody LJ-Ib10, previously shown to be a specific inhibitor of α-thrombin binding to GP Ib (4). This finding supports the hypothesis that the antibody epitope overlaps with, but does not exactly correspond to, the α-thrombin binding site.

The mutation of Tyr residues to Phe is not likely to cause any major rearrangement in the polypeptide backbone, since the two amino acids, apart from the presence of the hydroxyl group in the Tyr side chain, have essentially identical structure. In fact, normal reactivity with the conformation sensitive antibody, LJ-Ib1, which fails to react with denatured GP Ibα (7), is a good indication that each of the mutant molecules containing

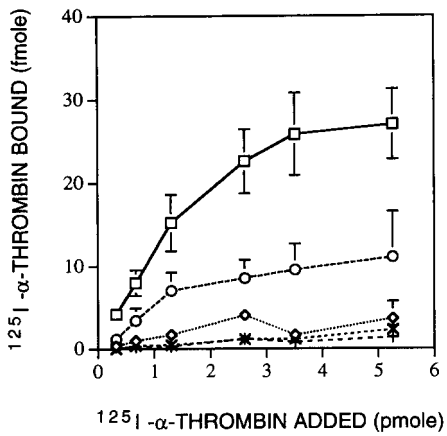

FIG. 7. **Dose-dependent binding of ^{125}I-labeled α-thrombin to recombinant GP Ibα fragments immobilized onto Sepharose beads.** The assays were performed as described in the legend to Fig. 6. The results shown are the mean (with S.E.) of four separate experiments for the native fragment (□) and two separate experiments each for the mutant molecules Y276F (×), Y278F (○), Y279F (+), and for the sulfate-free fragment (◇); *error bars* are not shown when the corresponding values are too small for effective graphic representation.

Tyr → Phe substitutions retained an overall conformation similar to that of the native GP Ibα fragment. The Tyr hydroxyl group, however, may become involved in hydrogen bonding and, thus, influence the three-dimensional molecular structure. Moreover, the hydroxyl group may undergo sulfation, a post-translational modification occurring in at least 1% of tyrosine residues in eukaryotic organisms (28); in this case, the added negative charge may become engaged in electrostatic interactions. Both these important structural properties of tyrosine may be necessary for expression of specific molecular activities.

The potential functional relevance of Tyr sulfation in GP Ibα has been recently highlighted by the demonstration that ristocetin-dependent vWF binding to heterologous cells expressing the GP Ib-IX complex is partially decreased when tyrosine sulfation is inhibited (12). We have confirmed and extended that finding by showing that the sulfate-free recombinant GP Ibα fragment exhibits a more pronounced reduction of vWF binding capacity supported by botrocetin as compared with ristocetin. Although unexplained at present, this result reflects the fact that the mechanisms of ristocetin- and botrocetin-dependent vWF binding to GP Ib are not the same (3, 18, 29). Regardless of the modulator used, however, it is clear that the vWF binding abnormality exhibited by the sulfate-free GP Ibα fragment is not shared by any of the single Tyr → Phe mutants, suggesting that the presence of any two of the three sulfate groups located at positions 276, 278, and 279 is sufficient to mediate a normal interaction with vWF. The alternative possibility that lack of sulfation may cause conformational aberrations in the GP Ibα fragment, and thus explain the loss of function, seems to be excluded by the observation that the sulfate-free fragment, like the normal functioning molecules containing single Tyr → Phe mutations, exhibited normal reactivity with the conformation-sensitive antibody LJ-Ib1. Also consistent with this conclusion is the fact that sulfation takes place in the trans-Golgi network at a relatively late stage after protein synthesis and folding (30) and that sulfate-free GP Ibα is normally expressed on the cell surface in complex with GP Ibβ and GP IX (12).

In addition to decreased vWF binding, the recombinant GP Ibα fragment secreted by cells grown under sulfate-free conditions also failed to express α-thrombin binding activity. This finding is consistent with the hypothesis that one reason, if not the only one, why single Tyr → Phe mutations resulted in a similar functional abnormality is the lack of necessary sulfate groups (Phe residues cannot be modified by sulfation). In fact, the presence of sulfotyrosine appears to be a recurrent structural motif in α-thrombin-binding proteins, like the specific inhibitor hirudin (31, 32). Thus, our results with the recombinant GP Ibα fragment are consistent with the notion that sulfated tyrosine residues may influence in a significant manner the affinity of interaction with α-thrombin (32). By analogy with the well established three-dimensional model of the hirudin-thrombin complex (33, 34), it seems reasonable to hypothesize that the GP Ibα domain containing the sulfated tyrosine residues interacts with the anion-binding exosite of α-thrombin and that electrostatic forces play a key role in supporting the binding.

Pronounced loss of vWF binding function resulted from the concurrent substitution of three negatively charged Asp residues with noncharged Asn at positions 272, 274, and 277 of mature GP Ibα. The functional abnormality associated with this mutation may simply reflect the abolition of sulfation on the neighboring Tyr residues at positions 276, 278, and 279; in fact, clusters of acidic amino acids appear to be essential for the action of tyrosylprotein sulfotransferase, the enzyme that catalyzes this post-translational modification (28). The D272N,D274N,D277N mutant, however, unlike the sulfate-free fragment or any of the Tyr → Phe mutants, exhibited markedly reduced reactivity with the conformation-sensitive antibody LJ-Ib1. This finding may indicate that the Asp → Asn mutations cause a substantial conformational change in the GP Ibα fragment or, alternatively, that the mutated Asp residues are directly part of the antibody combining site and, perhaps, or the vWF binding site. The latter hypothesis is supported by the knowledge that antibody LJ-Ib1 is a competitive inhibitor of vWF binding to GP Ib (35, 36), and the corresponding epitope is thought to overlap, at least in part, with the vWF-binding site in GP Ibα; and by the known precedent of a single amino acid substitution (Ala^{156} → Val), identified in a variant form of Bernard-Soulier syndrome (9), which results in loss of vWF binding function as well as of the LJ-Ib1 epitope. It remains to be established whether the lack of reactivity with antibody LJ-Ib1 is the reflection of a unique structural requirement that, in alternative or in addition to sulfation of Tyr residues, is necessary to support vWF binding. Answering this question may require a more detailed structural knowledge of the amino-terminal domain of GP Ibα.

The conclusions discussed here are based on data obtained with a recombinant fragment comprising residues 1–302 of mature GP Ibα; however, they are likely to apply to the intact GP Ib-IX-V complex expressed on platelets. Indeed, we have shown in previous studies that the isolated fragment interacts with vWF in a manner similar to intact GP Ib (5) and that mutations in the fragment reproduce functional aberrations of the receptor observed in congenital diseases, resulting both in decreased (8, 9) or paradoxically increased affinity for soluble vWF (37). Of note, the demonstration that each of the sulfated residues Tyr^{276}, Tyr^{278}, and Tyr^{279} have a concurrent functional role in mediating α-thrombin binding to GP Ibα, whereas none of them is individually necessary to support vWF binding, provides a potential target for either selective or combined inhibition of α-thrombin and vWF binding to platelets.

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