Type IIB von Willebrand Factor with Normal Sialic Acid Content Induces Platelet Aggregation in the Absence of Ristocetin

Role of Platelet Activation, Fibrinogen, and Two Distinct Membrane Receptors

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Abstract

Three preparations of purified von Willebrand factor (vWF), obtained from unrelated patients affected by type IIB von Willebrand disease, were found to have normal sialic acid content (between 129 and 170 nmol/mg of vWF, as compared with 158±17 nmol/mg in four normal preparations) and to induce platelet aggregation in the presence of physiologic levels of divalent cations and without addition of ristocetin. A monoclonal antibody that blocks the vWF binding domain of the platelet glycoprotein (GP)Ib caused complete inhibition of IIB vWF-induced aggregation. In contrast, a monoclonal antibody that blocks the receptor for adhesive proteins on the platelet GPIIb/IIIa complex failed to inhibit the initial response of platelets to high concentrations of IIB vWF. Moreover, IIB vWF caused agglutination of formalin-fixed platelets that was blocked only by the anti-GPIb antibody, suggesting that the binding of vWF to GPIb, even in the absence of ristocetin, results in platelet-platelet interaction that is followed by exposure of the GPIIb/IIIa receptors for adhesive proteins. Endogenous ADP, normally active platelet metabolism and fibrinogen binding to GPIIb/IIIa were necessary for maximal and irreversible platelet aggregation. In the absence of fibrinogen, however, aggregation was mediated by vWF binding to GPIIb/IIIa. A 52/48-kD tryptic fragment containing the GPIb binding domain of normal vWF completely blocked the aggregation induced by all three IIB vWF preparations. The present study defines in detail the mechanisms involved in IIB vWF-induced platelet aggregation. Moreover, it establishes that the GPIb binding domain of normal and IIB vWF are closely related and that desialylation is not required for the direct interaction of IIB vWF with GPIb.

Introduction

Type IIB is a variant form of von Willebrand disease (vWD)[1] characterized by responsiveness of platelet-rich plasma to concentrations of ristocetin lower than those necessary to induce aggregation in normal (1, 2). This finding is thought to be the consequence of enhanced interaction of the abnormal IIB von Willebrand factor (vWF) molecule with platelets (3), a situation different from that seen in pseudo- (4) or platelet-type vWD (5) in which abnormal platelet receptors show enhanced interaction with normal vWF (6). More recently, purified IIB vWF has been shown to induce platelet aggregation in vitro without the need of any other agonist (7, 8). Binding of IIB vWF, and particularly the larger multimers, to platelets in vivo provides a reasonable explanation for three findings typical of IIB vWD, namely: the lack of large vWF multimers in the plasma of these patients, but their presence in the cellular compartment (2, 9); the accelerated removal of large vWF multimers following their release into the circulation after infusion of 1-desamino-8-D-arginine vasopressin (DDAVP) (3, 9); and the thrombocytopenia observed in these patients after administration of DDAVP (10).

The molecular abnormality responsible for the direct binding of IIB vWF to platelets is still undefined. Gralnick and co-workers have found that the sialic acid and galactose content was decreased in the vWF isolated from one IIB vWD patient (8). This observation led them to suggest that IIB vWF might function in a manner similar to normal desialylated vWF (11–13). Against this hypothesis is the finding that normal and IIB vWF have a similar isoelectric point that is lower than that of asialo vWF (14).

We report here our findings in patients from three unrelated families with type IIB vWD. In one family, spontaneous platelet aggregation and thrombocytopenia were observed in the affected members. The sialic acid content of vWF purified from all these patients was normal. Nevertheless, IIB vWF bound to platelet membrane glycoprotein (GP)Ib in the absence of ristocetin and caused platelets to interact with each other. Optimal aggregation required endogenous ADP, a normally active platelet metabolism and exposure of the receptor for adhesive proteins on the GPIIb/IIIa complex. Fibrinogen binding to this receptor resulted in maximal aggregation. In the absence of fibrinogen, however, vWF could partly substitute for its function. Moreover, these studies demonstrate that the binding of IIB vWF to GPIb involves a molecular domain closely related to the one mediating the ristocetin-dependent binding of normal vWF.

Methods

All blood samples were obtained from patients and normal volunteers with their informed consent and in accordance with the Declaration of Helsinki. They had taken no medications for at least 2 wk. The afibrinogenemic patient (V.M.) has been described in a previous publication (15).

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1. *Abbreviations used in this paper:* DDAVP, 1-desamino-8-D-arginine vasopressin; GP, platelet membrane glycoprotein; vWD, von Willebrand disease; vWF, von Willebrand factor.

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Purification of proteins. The purification of vWF was performed as reported (7, 11). Starting material was citrated plasma obtained by plasmapheresis. Three different preparations of IIB vWF were obtained from (*a*) 1,700 ml of plasma from one patient (D.Z.) previously described in detail (reference 1, patient 19, Table II); (*b*) 2,000 ml of plasma obtained from two patients from the same family (P.). These patients fulfilled the criteria for IIB vWD and, in addition, presented spontaneous platelet aggregation in platelet-rich plasma and had transient episodes of thrombocytopenia. They have not been previously reported; and (*c*) 1,300 ml of pooled plasma from three patients belonging to another family (C.) previously described (reference 1, patients 12, 13, 16, Table II). The isoelectric point of plasma vWF was measured in patients from two of these families (D.Z. and C.) and was similar to normal (14). In addition, normal vWF was purified from 1 liter of plasma obtained either from single volunteers or pooled from four different donors. Purified vWF was contained in the first protein peak (detected by light absorbance at 280 nm) eluting at the void volume of the gel filtration column (7, 11). The peak was smaller in the patients as compared with normal, because of the lower content of vWF, and its ascending part was less represented, because of the relative decrease of larger multimers, but no difference in elution position was detected. The final yields (based on recovery of ristocetin cofactor activity) were: 17–25% for normal; 3.2% for patient D.Z.; 5.2% for family P.; and 3.7% for family C. The lower yield of patient vWF is due to the lower efficiency of the cryoprecipitation step, due to the decreased concentration of larger multimers. Analysis of the purified vWF on reduced polyacrylamide gels (16, 17) showed a major band with apparent mobility of 220 kD in both normal and patients and some smaller bands, due to proteolytic degradation, which were increased in the patients (16). All the bands stained by Coomassie Blue were identified as vWF by immunoblotting (17). Purified normal and IIB vWF were stored in 0.02 M Tris·HCl, 0.15 M NaCl, pH 7.3 (Tris buffer), and kept at −70°C. Ristocetin cofactor activity and multimeric composition were tested as previously reported (7, 18–20). 1 U of ristocetin cofactor activity corresponds to the amount present in 1 ml of pooled normal plasma.

Fibrinogen was purified and characterized as reported (21). It was stored in 0.02 M Tris·HCl, 0.15 M NaCl, pH 7.3, at −70°C. Over 95% of the protein clotted when thrombin was added.

The reduced and alkylated 52/48 kD tryptic fragment of vWF was prepared and characterized as recently described (22). It was also stored in 0.02 M Tris·HCl, 0.15 M NaCl, pH 7.3, at −70°C.

Hirudin (1,000 U/mg) from *Hirudo medicinalis* was obtained commercially (Sigma Chemical Co., St. Louis, MO) and further purified by reverse-phase high performance liquid chromatography (HPLC) on a wide pore C-4 column (Vydac-The SEP/A/RA/TIONS Group, Hesperia, CA) with a 0–60% gradient of acetonitrile in 0.1% trifluoroacetic acid. Several protein peaks were observed, but most of the anticoagulant activity corresponded to an area representing 5% of the total protein content.

Protein concentration was measured either by the method of Lowry (23) using bovine serum albumin as a standard; or by light absorbance at 280 nm corrected for light scattering at 320 nm; or by the method of Bradford (24). The concentration of vWF was measured with the method of Lowry which, for both normal and patient vWF, gave results ~ 20% higher than the other assays. It should be noted that, if the protein concentration were overestimated as a consequence of this choice, the result would be a lower specific content of sialic acid than actually true. Nevertheless, the patients studied here showed a normal content as opposed to the lower content reported previously in another patient (8). Fibrinogen and IgG concentration was measured with either the method of Bradford or by light absorbance, and the two assays gave similar results. In this case the choice was based on ease of execution.

Desialylation of vWF. Normal purified vWF was treated with protease-free neuraminidase from *Vibrio cholerae* (Calbiochem-Behring Corp., La Jolla, CA) as previously described (11). Over 90% of the sialic acid content of vWF was removed by this treatment.

Sialic acid measurement. The sialic acid content of vWF was measured according to the method of Warren (25) after incubating purified vWF with 0.1 N H_2SO_4 for 60 min at 80°C. Samples were tested in duplicate or triplicate. *N*-acetyl neuraminic acid was used as a standard.

Radioiodination of asialo vWF. This was performed with ^{125}I (Amersham Corp., Arlington Heights, IL) using Iodogen (Pierce Chemical Co., Rockford, IL) according to the method of Fraker and Speck (26). The specific activity of ^{125}I-asialo vWF was between 0.7 and 0.9 mCi/mg.

Monoclonal antibodies. Monoclonal antibodies against GPIb and the GPIIb/IIIa complex were prepared and characterized as described (27, 28). The three antibodies used were: LJ-Ib1, specific for GPIb (28); LJ-P5 and LJ-CP8, both directed against the GPIIb/IIIa complex (27). LJ-Ib1 inhibits vWF binding to GPIb (28); LJ-P5 inhibits vWF but not fibrinogen or fibronectin binding to GPIIb/IIIa (27, 29); and LJ-CP8 completely inhibits the binding of both fibrinogen and vWF, as well as fibronectin, to GPIIb/IIIa. Purified IgG and monovalent Fab′ fragments were prepared as reported (27, 30–32) and kept in a buffer composed of 0.02 M Tris·HCl, 0.15 M NaCl, pH 7.3.

Aggregation studies. Citrated platelet-rich plasma from normal individuals or the afibrinogenemic patient was prepared as described (27) from blood containing 0.011 mol/liter of trisodium citrate. Platelet-rich plasma was also prepared from blood anticoagulated with 200 U/ml of purified hirudin (1 U of hirudin neutralizes the activity of 1 National Institutes of Health [NIH] unit of α-thrombin).

Washed platelets were prepared according to a modification of the albumin density gradient technique (33), as reported elsewhere (13). All aggregation studies were performed with a Chrono-log aggregometer (Chrono-log Corp., Havertown, PA) in siliconized glass cuvettes, at 37°C, with constant stirring at 1,200 rpm and with a platelet count between 2 and 3×10^8/ml. In experiments where apyrase (grade III; Sigma Chemical Co.) was used, this was added at a final concentration of 5 ATP′-ase U/ml. When indomethacin (Sigma Chemical Co.) was used, this was added at 10 μM final concentration. Formalin-fixed platelets were prepared according to a procedure previously reported (34). These platelets were kept in a buffer composed of 0.02 M Tris·HCl, 0.15 M NaCl, pH 7.3.

Binding experiments. The binding of ^{125}I-asialo vWF to stimulated platelets was measured using either citrated platelet-rich plasma or washed platelets. When indicated, appropriate concentrations of EDTA, $CaCl_2$ and/or apyrase were incubated with the platelets for 10 min at room temperature before addition of the radiolabeled ligand. This was followed by incubation for 30 min at 37°C without stirring, after which separation of platelet-bound from free ligand was achieved by centrifugation through 20% sucrose in Tyrode buffer, as previously described, followed by measurement of the bound radioactivity (20). Nonspecific binding was evaluated for selected points by measuring the binding in the presence of a 40-fold excess of unlabeled asialo vWF. Binding isotherms were evaluated by Scatchard-type analysis to determine binding parameters (including the estimate of nonspecific binding) using the computer-assisted program Ligand (35).

Results

Characterization of purified IIB vWF. Purified vWF from the three families with IIB vWD lacked the larger multimeric forms seen in normal vWF (Fig. 1). The ristocetin cofactor activity of the purified preparations (measured with a final ristocetin concentration of 1.2 mg/ml) was 55 U/mg for D.Z., 48 U/mg for C., and 68 U/mg for P. These values were lower than normal (153±24 U/mg; mean±SD of three normal preparations). All three preparations of IIB vWF mediated aggregation of normal washed platelets at lower concentrations of ristocetin than necessary with normal vWF (not shown here), as previously demonstrated with another preparation of IIB vWF from patient D.Z. (7). All three preparations, when used at higher concentrations, induced aggregation of citrated platelet-rich plasma in the absence of ristocetin or any other agonist (Fig. 2). In this regard, IIB vWF isolated from the patients who showed spontaneous platelet aggregation (P.) was effective at lower concentrations (1–3 μg/ml) than IIB vWF from the other two families (5–7 μg/ml for D.Z. and 10–14 μg/ml for C.) (Fig. 2). Lower

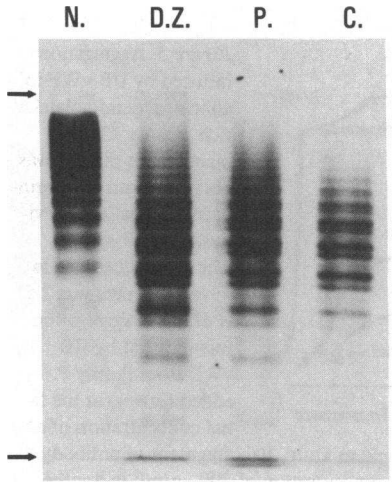

Figure 1. Multimeric composition of purified vWF. Each lane contains 5–10 ng of purified vWF from a normal control (*N*) and from the three families (patient D.Z., family P., and family C.) with von Willebrand disease, type IIB. Electrophoresis was run in an agarose gel containing sodium dodecyl sulfate; vWF multimers were detected by autoradiography after reaction with a radiolabeled antibody, following a procedure described elsewhere (2, 19). The arrow at the top indicates the interface between stacking and running gel, the one at the bottom indicates the dye front. Cathode at the top. Note the lack of largest vWF multimers in the patient samples.

concentrations of patients vWF than used in the experiment described in Fig. 2 gave aggregation after a longer lag phase and to a lesser extent; higher concentrations gave more rapid aggregation. The aggregation, therefore, was dose dependent. It should be noted that the response of different individual platelet-rich plasmas to the same concentration of IIB vWF varied to a considerable extent, particularly when submaximal aggregating doses were used, as is true for any other platelet agonist.

Sialic acid content of purified IIB vWF. The content of sialic acid was normal in the three IIB vWF preparations (Table I),

Table I. Sialic Acid Content of Purified IIB von Willebrand Factor

Source	Sialic acid
	nmol/mg of von Willebrand factor
Patient D.Z.	170
Family P.	164
Family C.	129
Normal	158±17 (4)

Results in normal represent the mean±SD of four different preparations.

including that from the patients of family P., in whom spontaneous platelet aggregation and episodes of thrombocytopenia had been observed.

Effect of physiological concentrations of divalent cations on aggregation induced by IIB vWF. To evaluate the effect of divalent cations on the aggregation induced by IIB vWF, platelet-rich plasma was prepared from blood in which clotting had been prevented either with trisodium citrate (a chelating agent causing lower than normal divalent cation concentration) or hirudin (an antithrombin substance that has no effect on divalent cation concentration). Aggregation was observed in the platelet-rich plasma anticoagulated with hirudin, but a higher concentration of IIB vWF was necessary to elicit a response and the extent of aggregation was less than in citrated platelet-rich plasma (Fig. 3).

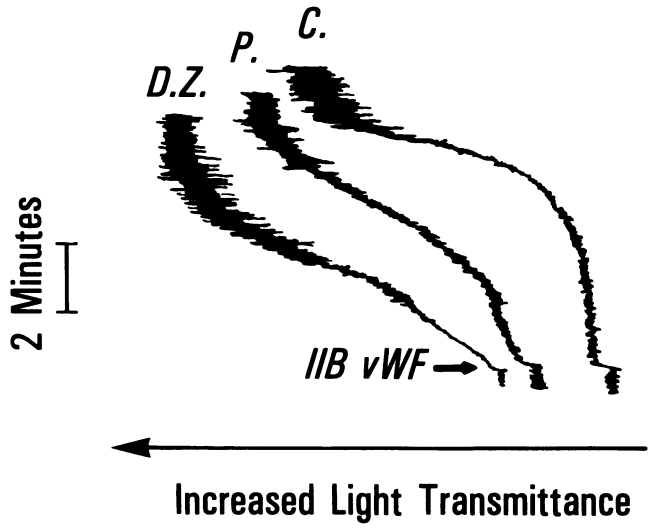

Figure 2. Platelet aggregation induced by purified IIB vWF. Citrated platelet-rich plasma from a normal donor (final count 2.5×10^8 platelets/ml) was stirred (1,100 rpm) in the aggregometer cuvette at 37°C. After establishment of a straight baseline, purified IIB vWF was added (*arrow*) and aggregation was recorded as increase in light transmittance. Final concentration of IIB vWF was 10 μg/ml for patient D.Z.; 1.1 μg/ml for family P.; and 14 μg/ml for family C. Note the lower concentration of vWF from family P. required to induce aggregation. No aggregation was observed with normal vWF at concentrations up to 100 μg/ml (not shown).

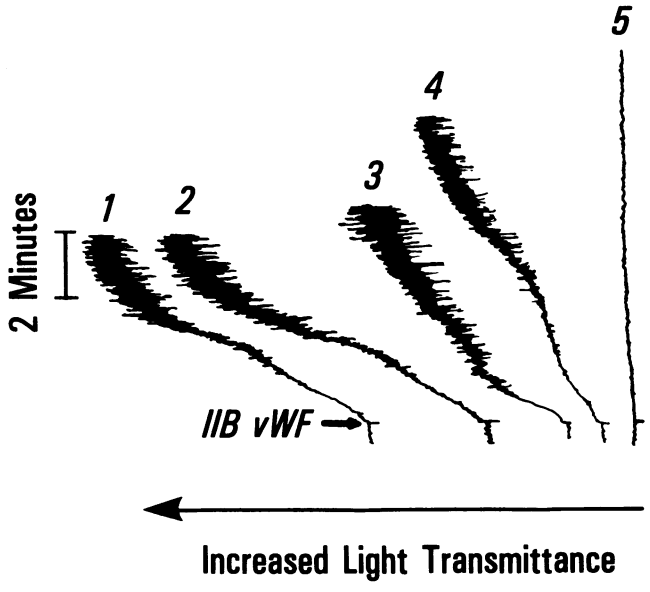

Figure 3. Effect of divalent cations on platelet aggregation. The experimental conditions are like those described in Fig. 2, except that two different preparations of normal platelet-rich plasma were used, one anticoagulated with trisodium citrate (traces *1* and *2*) and the other with hirudin (traces *3, 4,* and *5*). Aggregation was induced by the addition of IIB vWF (*arrow*). Traces *1* and *3:* IIB vWF from family P., final concentration of 10 μg/ml and 17 μg/ml, respectively. Traces *2* and *4:* IIB vWF from patient D.Z., final concentration of 10 and 17 μg/ml, respectively. Trace *5,* normal vWF, final concentration of 100 μg/ml.

Role of fibrinogen, GPIIb/IIIa, and platelet activation in IIB vWF-induced aggregation. Aggregation of normal platelet-rich plasma induced by IIB vWF was blocked completely by an anti-GPIb monoclonal antibody (LJ-Ib1) that inhibits the binding of vWF to this receptor (Fig. 4). An anti-GPIIb/IIIa monoclonal antibody (LJ-CP8) that blocks platelet binding of adhesive proteins (fibrinogen, vWF, and fibronectin) inhibited aggregation markedly, although an initial response of platelets was still visible when higher concentrations of IIB vWF were used (Fig. 4). A similar response was observed when high concentrations of IIB vWF from the other two families were used. As discussed later, this platelet response may be mediated by vWF binding to GPIb and, therefore, may be considered agglutination rather than aggregation. In contrast, an anti-GPIIb/IIIa monoclonal antibody (LJ-P5) that blocks only vWF, but not fibrinogen nor fibronectin binding, had no effect on IIB vWF-induced aggregation of normal platelet-rich plasma (Fig. 4). Antibody LJ-P5, however, completely inhibited IIB vWF-induced aggregation in afibrinogenemic platelet-rich plasma, but its effect could be overcome by the addition of purified fibrinogen (Fig. 5). It is also noteworthy that IIB vWF-induced aggregation in afibrinogenemic platelet-rich plasma occurred after a longer lag phase, but proceeded to the same maximal extent as seen in the presence of fibrinogen (Fig. 5). As with normal platelet-rich plasma, the extent of aggregation was dose dependent. The addition of purified IIB vWF to formalin-fixed platelets resulted in agglutination that was blocked by the anti-GPIb antibody, but minimally affected by the anti-GPIIb/IIIa antibody (LJ-CP8) that inhibited aggregation of metabolically active platelets (Fig. 6). As expected, the antibody LJ-P5 (which, like LJ-CP8, inhibits vWF binding to GPIIb/IIIa) had no effect on the agglutination of formalin-fixed platelets.

The ADP scavenger, apyrase, could block completely platelet aggregation induced by low concentrations of IIB vWF isolated from the patients of family P. In contrast, the initial aggregation induced by higher concentrations of the same IIB vWF was not noticeably affected by the presence of apyrase, but late disaggregation occurred when the ADP scavenger was present (Fig. 7). Similar results were obtained with vWF from patient D.Z., whereas the aggregation induced by all tested concentrations (up to 15 μg/ml) of vWF from family C was always blocked by apyrase. In the presence of indomethacin, a nonsteroidal anti-inflammatory compound that acts as an antiaggregating agent by interfering with the platelet prostaglandin metabolism, the second wave of aggregation induced by high concentrations of IIB vWF (family P) was inhibited. Similar results, with an additional slight decrease of the initial platelet response, were observed when indomethacin and apyrase were used together (Fig. 7).

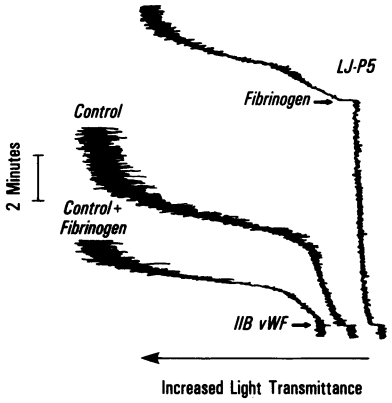

Figure 5. Aggregation induced by IIB vWF in afibrinogenemic platelet-rich plasma. Citrated platelet-rich plasma was obtained from an afibrinogenemic patient previously described (15). The final platelet count in the mixtures was 2.5 × 10^8/ml. Aggregation was induced by IIB vWF from family P. added (*arrow*) at the final concentration of 15 μg/ml (control mixture). In one mixture, Fab' fragment of antibody LJ-P5 was added at the final concentration of 200 μg/ml; in another, fibrinogen was added at the final concentration of 600 μg/ml. Note that the addition of fibrinogen (600 μg/ml) to the mixture containing antibody LJ-P5 restores the aggregation previously blocked by the antibody. See Fig. 2 for additional technical details.

Effect of the 52/48 kD tryptic fragment of vWF on IIB vWF-induced aggregation. A 52/48-kD tryptic fragment of normal vWF that contains the domain interacting with GPIb has recently been characterized (22). This reduced and *S*-carboxymethylated fragment inhibits ristocetin-induced aggregation as well as asialo vWF-induced aggregation of normal platelet-rich plasma, and blocks the binding of normal vWF to GPIb in the presence of ristocetin. The 52/48-kD fragment of normal vWF completely inhibited aggregation induced by all three purified IIB vWF preparations (Fig. 8). More fragment was necessary to block the effect of IIB vWF from family P. than from patient D.Z. and

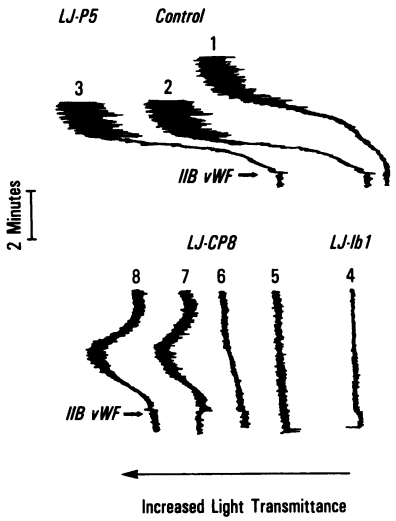

Figure 4. Effect of antiplatelet monoclonal antibodies on IIB vWF-induced aggregation. Normal citrated platelet-rich plasma was incubated for 1 min, in the aggregometer, with Fab' fragment of three different monoclonal antibodies, as indicated. LJ-P5 (final concentration 200 μg/ml; trace *3*) binds to GPIIb/IIIa and blocks vWF, but not fibrinogen nor fibronectin binding; LJ-CP8 (final concentration 83 μg/ml; traces *5–8*) binds to GPIIb/IIIa and blocks binding of vWF, fibrinogen and fibronectin; LJ-Ib1 (final concentration 95 μg/ml; trace *4*) binds to GPIb and blocks binding of vWF to this receptor. The two control traces (*1* and *2*) contained Tris buffer instead of antibody. Aggregation was induced by IIB vWF from family P. added (*arrow*) at the following final concentrations: trace 1, 1.8 μg/ml; traces *2–4*, 9.3 μg/ml; trace *5*, 1.8 μg/ml; trace *6*, 3.7 μg/ml; trace *7*, 7.4 μg/ml; trace *8*, 9.3 μg/ml. See Fig. 2 for additional technical details.

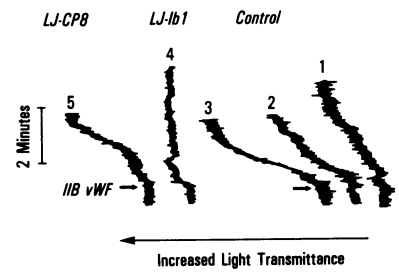

Figure 6. Agglutination of formalin-fixed platelets. This was evaluated in the aggregometer (see Fig. 2 for additional technical details) by adding (*arrow*) IIB vWF from family P. to platelets at a final count of 3 × 10^8/ml. The effect of Fab' fragment of two monoclonal antibodies, LJ-Ib1 (92 μg/ml, final concentration) and LJ-CP8 (98 μg/ml, final concentration), was tested in comparison to control mixtures containing Tris buffer instead of antibody. Final concentration of IIB vWF was: trace *1*, 3.5 μg/ml; trace *2*, 4.6 μg/ml; traces *3–5*, 7 μg/ml.

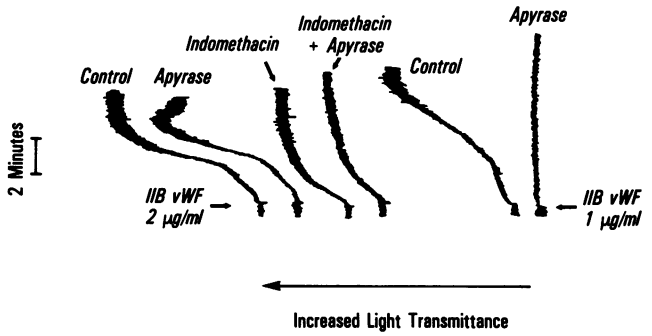

Figure 7. Effect of apyrase and indomethacin on IIB vWF-induced aggregation. Apyrase (final concentration 5 ATP'-ase U/ml) or indomethacin (10 μM) were added to normal citrated platelet-rich plasma before the addition (*arrow*) of IIB vWF from family P. at the final concentrations indicated on the graph (2 μg/ml for the four traces on the left and 1 μg/ml for the two traces on the right). The control mixtures contained Tris buffer instead of apyrase or indomethacin. See Fig. 2 for additional technical details.

Table II. Effect of EDTA and Apyrase on the Binding of ^{125}I-Asialo vWF to Nonstimulated Platelets in Platelet-rich Plasma

	K_d	Number of sites
	$\times 10^7$ M	molecules/platelets
Citrated platelet-rich plasma	2.40	22637
+EDTA (5 mM)	1.87	66448
+Apyrase (5 ATP'-ase U/ml)	1.77	18665

Citrated platelet-rich plasma was prepared as described in Methods and incubated (final platelet count = 10^8/ml) with increasing concentrations of ^{125}I-asialo vWF (between 8 and 56 μg/ml), in the presence or absence of EDTA or apyrase at the indicated concentrations. After 30 min at 37°C, without agitation, the platelets were separated from the binding mixture and the associated radioactivity was determined as described in Methods. The binding parameters were calculated using the computer assisted program Ligand (35) and assuming a molecular mass for vWF of 275 kD. Nonspecific binding was calculated as a fitted parameter and was found to be 0 for all curves.

family C. (Fig. 8). This is in agreement with the observation that IIB vWF from family P. was a more potent aggregating agent than the other two preparations.

Effect of divalent cations and apyrase on asialo vWF binding to nonstimulated platelets. Desialylated vWF binds to GPIb in the absence of ristocetin and the binding can be demonstrated directly in platelet-rich plasma (11, 13). In the present studies we found that removal of divalent cations with EDTA resulted in increased binding as compared with that observed in citrated platelet-rich plasma, without relevant changes in the dissociation constant of the interaction (Table II). Under all circumstances, the binding was inhibited > 90% in the presence of the anti-GPIb monoclonal antibody LJ-Ib1. The effect of Ca^{2+} on the binding of asialo-vWF was evaluated using washed, nonstimulated platelets. The free Ca^{2+} concentration in the platelet suspension was < 10 μM as determined with a calcium electrode. Maximal binding was observed in the presence of 5 mM EDTA, whereas the binding decreased as the concentration of added Ca^{2+} increased (Fig. 9). When the Ca^{2+} concentration was between 0.5–2 mM, the binding was only 30% of that observed in the presence of EDTA (Fig. 9).

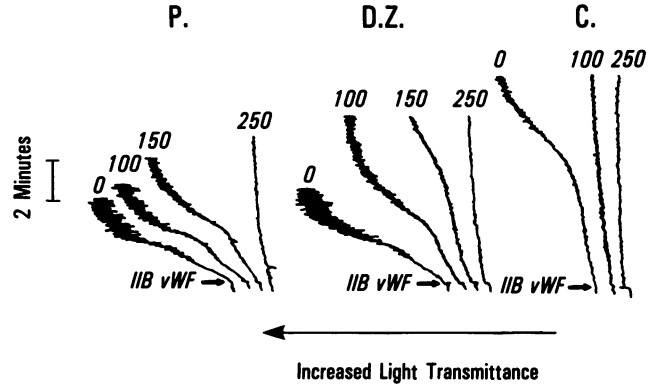

Figure 8. Effect of the 52/48 kD fragment of normal vWF on IIB vWF-induced aggregation. Normal citrated platelet-rich plasma was mixed with varying final concentrations (indicated on the graph in μg/ml) of the 52/48-kD tryptic fragment of normal vWF (22). Immediately after this, IIB vWF was added (*arrow*) and aggregation recorded. Control mixtures (0) contained Tris buffer instead of fragment. Final concentration of IIB vWF was 10 μg/ml (family P.); 10 μg/ml (patient D.Z.); and 14.5 μg/ml (family C.). See Fig. 2 for additional technical details.

Discussion

Three distinct preparations of purified IIB vWF, isolated from the plasma of patients belonging to three unrelated families, acted as a direct platelet agonist and induced aggregation. Clinical reports on cases of type IIB vWD demonstrate that the laboratory features of the disease are heterogeneous, as some patients present with thrombocytopenia and in vitro evidence of spontaneous platelet aggregation (8, 36, 37). One of the families described in the present report had such characteristics and, in fact, the corresponding vWF induced aggregation of normal platelets in lower concentrations than were necessary with vWF purified from the other two families. This finding suggests the possibility that a similar abnormality of IIB vWF is expressed to varying degrees in different families or, alternatively, that there are intrinsically

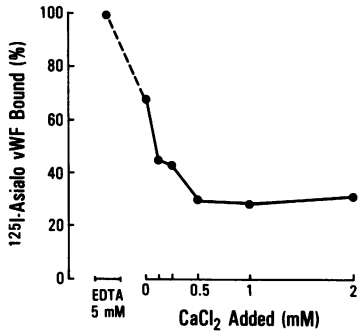

Figure 9. Effect of EDTA and Ca^{2+} on binding of ^{125}I-asialo vWF to platelets. Washed platelets (10^8/ml) were incubated for 10 min at room temperature with 5 mM EDTA or various concentrations of $CaCl_2$, followed by the addition of ^{125}I-asialo vWF to give a final concentration of 12 μg/ml. After incubation for an additional 30 min at 37°C, the platelet bound radioactivity was determined as described in Methods. The amount of radioactivity that was bound to platelets in the presence of EDTA was assigned the value of 100%. This corresponded to 0.49 μg of ^{125}I-asialo vWF/10^8 platelets. The free Ca^{2+} concentration in the washed platelet suspension used was < 10 μM, and Mg^{2+} was omitted from the modified Tyrode buffer (13) used in these experiments. Results similar to these were observed at concentrations of added ^{125}I-asialo vWF as low as 3 μg/ml.

distinct abnormalities in unrelated patients resulting in vWF molecules with different affinity for the GPIb platelet receptor.

In no case did we find a reduced content of sialic acid in IIB vWF. This is in agreement with a previous study showing that IIB vWF has a normal isoelectric point, whereas that of desialylated vWF is markedly higher than normal (14), but it is in contrast with the 50% decrease of sialic acid and galactose reported in one patient by others (8). Even considering the possibility of heterogeneity in this regard, it is clear that desialylation is not the general pathogenetic mechanism by which IIB vWF acquires the property of directly aggregating platelets, as proposed by some authors. In fact, these studies demonstrate that fully sialylated vWF can bind to GPIb in the absence of any other modifying substance.

Recently, it has been suggested that platelet aggregation induced by asialo vWF cannot occur in the presence of physiological concentrations of calcium (38). Nevertheless, we demonstrate in this report that platelet aggregation induced by IIB vWF occurs in platelet-rich plasma containing normal levels of divalent cations, in agreement with the indirect evidence suggesting that the IIB vWF-platelet interaction can occur in vivo (10). In studies not reported here we have found that desialylated normal vWF behaves like IIB vWF in this regard, i.e., it induces aggregation in platelet-rich plasma anticoagulated with hirudin but to a lesser extent than in citrated platelet-rich plasma with a decreased content of divalent cations. We have performed binding studies with ^{125}I-labeled asialo vWF and found that divalent cations, and Ca^{2+} in particular, decrease the direct binding of vWF to GPIb in the absence of ristocetin. Although we have no direct evidence that the same applies to type IIB vWF, as binding studies could not be performed with this material due to limited availability, the findings with asialo vWF provide a reasonable explanation for the similar results observed with both normal desialylated vWF and type IIB vWF in aggregation studies. The mechanism(s) of this effect of divalent cations remains obscure at present.

Endogenous ADP appears to be an absolute requirement for platelet aggregation only when IIB vWF acts as a weak agonist, as in the case of the molecule isolated from family C. or with low concentrations of the one from family P. This finding is not surprising, as it is common to other platelet agonists (39) very different in nature from IIB vWF, and can explain discrepant results reported in the literature and based on the study of different patients (7, 8, 40). Apyrase, however, was found to have no relevant effect on the binding of asialo-vWF to GPIb, suggesting that it is likely to have no effect on the binding of IIB vWF to GPIb as well. Therefore, endogenous ADP is likely to mediate events that follow the initial binding of vWF to the GPIb receptor. These events clearly require a normally active platelet metabolism, as shown by the inhibitory effect of indomethacin, a compound that interferes with the platelet prostaglandin metabolism (39, 41), on the extent of aggregation induced by IIB vWF.

The binding of IIB vWF to GPIb is the event triggering subsequent platelet aggregation, as shown by the complete inhibitory effect of a specific anti-GPIb monoclonal antibody. Moreover, we have obtained evidence that the molecular domain of IIB vWF involved in binding to GPIb is closely related to the GPIb binding domain of normal vWF. In fact, platelet aggregation induced by all three purified IIB vWF molecules was blocked by a denatured, fully reduced and *S*-carboxymethylated proteolytic fragment derived from normal vWF and extending from Val_{449} to Lys_{728} of the constituent subunit (22). It is of interest that this 52/48-kD fragment isolated from normal vWF binds to platelets in the absence of ristocetin, thus suggesting that other regions of the molecule, or specific conformations lost in the isolated fragment, are responsible for modulating the binding of whole vWF to GPIb. It is also relevant that the carbohydrate contained in the 52/48 kD fragment does not play a role in its binding to GPIb nor prevents it (22). These observations should help direct the efforts aimed at defining the molecular abnormality of type IIB vWF.

We and others have previously demonstrated that platelet aggregation induced by IIB vWF proceeds through exposure of the GPIIb/IIIa common receptor for adhesive proteins (7, 8). Our initial interpretation was that fibrinogen binding to this receptor was necessary for aggregation to occur. The present studies, however, clearly demonstrate that vWF binding to GPIIb/IIIa can substitute for fibrinogen binding in mediating platelet aggregation induced by IIB vWF. This can only be shown in the absence of fibrinogen and, in this regard, the findings with IIB vWF are similar to those obtained with other platelet agonists (29).

Whereas maximal platelet aggregation in response to IIB vWF occurs only through the function of the GPIIb/IIIa common receptor for adhesive proteins, we found that platelet aggregation induced by higher concentrations of IIB vWF is not completely blocked by an anti-GPIIb/IIIa monoclonal antibody that totally inhibits the binding of fibrinogen and other adhesive molecules. Thus, the direct binding of IIB vWF to GPIb, in the absence of ristocetin, mediates platelet-platelet interaction even when the GPIIb/IIIa receptor is blocked. This was confirmed by the demonstration that IIB vWF agglutinates formalin-fixed platelets, and that such agglutination is blocked by the anti-GPIb but not the anti-GPIIb/IIIa antibody. GPIb-mediated platelet agglutination, independent of GPIIb/IIIa receptor function, was particularly evident with IIB vWF isolated from the family exhibiting spontaneous platelet aggregation in vitro and episodes of thrombocytopenia. This abnormal molecule may have higher affinity for GPIb than other IIB vWF molecules and seems to mimic more closely some of the ristocetin-dependent functions of normal vWF, like platelet agglutination independent of GPIIb/IIIa binding activity. In this regard, the disaggregation observed following the initial aggregation of metabolically active platelets in the presence of the anti-GPIIb/IIIa antibody may resemble the disaggregation of thrombasthenic platelets after ristocetin-induced aggregation (42).

In conclusion, the present studies demonstrate that the GPIb binding domain of IIB vWF is closely related to the one in normal vWF. Binding of fully sialylated IIB vWF to GPIb in the absence of ristocetin results in initial platelet-platelet interaction that is followed, with normal platelets, by exposure of the GPIIb/IIIa common receptor for adhesive proteins, a process dependent on endogenous ADP and active platelet metabolism. As with other agonists, fibrinogen binds preferentially to the exposed sites, but in the absence of fibrinogen vWF can bind to GPIIb/IIIa and mediate platelet aggregation.

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