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Interaction of purified type IIB von Willebrand factor with the platelet membrane glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb/IIIa complex and initiates aggregation

(von Willebrand disease/bleeding disorder/platelet receptors)

LUIGI DE MARCO*, ANTONIO GIROLAMI†, THEODORE S. ZIMMERMAN, AND ZAVERIO M. RUGGERI‡

Departments of Basic and Clinical Research and of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037

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ABSTRACT **von Willebrand factor (vWF) was purified from the plasma of a patient with type IIB von Willebrand disease (vWF from such a patient, IIB vWF) who had a normal platelet count and showed no evidence of spontaneous platelet aggregation. Large multimers of IIB vWF were absent from purified preparations and from plasma. Ristocetin-induced platelet aggregation was enhanced by purified IIB vWF. The aggregation of washed normal platelets mixed with IIB vWF (0.4 μg/ml) required lower amounts of ristocetin than the aggregation of normal platelets mixed with the same concentrations of normal vWF. Moreover, purified IIB vWF alone induced aggregation of platelet-rich plasma at concentrations as low as 10 μg of IIB vWF/ml in the absence of any other agonist. Aggregation was blocked by a monoclonal antibody against the platelet membrane glycoprotein, GPIb, as well as by an anti-GPIIb/IIIa antibody. Washed platelet suspensions were promptly aggregated by IIB vWF only when fibrinogen and $CaCl_2$ were added to the mixture. Purified IIB vWF induces the binding of fibrinogen to platelets. Such binding was blocked by the anti-GPIb monoclonal antibody as well as by the anti-GPIIb/IIIa monoclonal antibody that inhibited aggregation. A second anti-GPIIb/IIIa antibody, which has the property of blocking vWF but not fibrinogen binding to platelets, blocked neither aggregation nor fibrinogen binding induced by IIB vWF. These studies demonstrate that platelet aggregation is triggered by the initial interaction of IIB vWF with GPIb which is followed by exposure of fibrinogen binding sites on GPIIb/IIIa. Fibrinogen binds to these sites and acts as a necessary cofactor for the aggregation response.**

Type IIB is a variant form of von Willebrand disease (vWD) characterized by enhanced responsiveness of platelet-rich plasma to ristocetin (1). Typically, aggregation of patient platelet-rich plasma is observed at concentrations of ristocetin that are significantly lower than those necessary to induce aggregation in normal platelet-rich plasma (1). Such hyper-responsiveness is thought to reflect the enhanced interaction between an abnormal von Willebrand factor (vWF) molecule and platelets. This may be responsible for the disappearance of the large multimeric forms of vWF from the circulation (2, 3). Another disease, called pseudo- or platelet-type vWD (4, 5), is also characterized by enhanced responsiveness of platelet-rich plasma to ristocetin, but this abnormality is related to dysfunctional receptors on the platelet membrane (4–6).

Recently, Holmberg *et al.* (7) have shown that, following the infusion of the vasopressin analog des-1-amino[8-D-arginine]vasopressin (des-1-amino[D-arg^8]VP), thrombocytopenia develops in patients with type IIB vWD. Normally, this drug causes release of vWF from storage sites, with consequent increase of plasma levels and appearance of multimeric forms larger than those present in basal conditions (3). The thrombocytopenia following des-1-amino[D-arg^8]VP infusion in type IIB vWD has been considered to be related to increased plasma levels of the abnormal vWF molecule (7), but the majority of patients with type IIB vWD have a normal platelet count under basal conditions (1).

We have purified the vWF from the plasma of an untreated patient with type IIB vWD (hereafter this factor will be referred to as IIB vWF). We report here that purified IIB vWF causes platelet aggregation in the absence of any other agonist. Such aggregation requires availability of receptors on the platelet membrane glycoprotein, GPIb, as well as on the GPIIb/IIIa complex. Moreover, we found that IIB vWF induces fibrinogen binding to GPIIb/IIIa. This latter interaction plays an important role in the aggregation of platelets induced by IIB vWF.

MATERIALS AND METHODS

All blood samples were obtained from consenting volunteers, according to the Declaration of Helsinki. They had taken no medication for at least 2 weeks.

Purification of vWF and Fibrinogen. The method used for the purification of vWF from plasma has been previously published in detail (8, 9). IIB vWF was isolated from 900 ml of citrate-treated plasma obtained by plasmapheresis from a patient described in detail (ref. 1, patient 19, Table 2). Normal vWF was routinely isolated from pooled normal plasma from several individuals. In addition, 1 liter of citrate-treated plasma, obtained by plasmapheresis from a single volunteer, was processed exactly like the IIB plasma. The purified vWF was stored in 0.02 M Tris·HCl/0.15 M NaCl, pH 7.3. The ristocetin cofactor activity, multimeric composition, and purity of the vWF preparations were tested as described (10–12). The method used for the purification of fibrinogen was that of Kazal *et al.* (13). The purified fibrinogen was at a concentration of 11–34 mg/ml in 0.02 M Tris·HCl/0.15 M NaCl, pH 7.3. Its ability to clot was >93%. $NaDodSO_4$/PAGE (14) (10% acrylamide with 5% cross-linking) of fibrinogen showed the typical αA, βB, and γ chains. Fibrinogen and vWF were labeled with ^{125}I by the procedure described by Fraker and Speck (15).

Monoclonal Antibodies. Monoclonal antibodies were prepared following standard hybridoma techniques (16). Four different monoclonal antibodies were used. RG46 (IgG1) reacts with native as well as reduced and *S*-carboxymethyl-

Abbreviations: vWD, von Willebrand disease; vWF, von Willebrand factor; des-1-amino[D-arg^8]VP, des-1-amino[8-arginine]vasopressin.

*Permanent address: Centro Immunotrasfusionale, Ospedale Civile, Pordenone, Italy.
†Permanent address: Istituto di Semeiotica Medica, Università degli Studi, Padova, Italy.
‡To whom reprint requests should be addressed.

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ated vWF and inhibits ristocetin-induced binding of vWF to platelets as well as ristocetin-induced aggregation. LJIb1 (IgG1) is specific for GPIb, as determined by crossed-immunoelectrophoresis against platelet membrane components (kindly performed by Tom Kunicki, The Blood Center of Southeastern Wisconsin, Milwaukee, WI) (17); it inhibits vWF binding to GPIb in the presence of ristocetin as well as ristocetin-induced platelet aggregation. LJP5 (IgG1) and LJP9 (IgG2b) are both directed against the GPIIb/IIIa complex, as determined by crossed-immunoelectrophoresis of platelet membrane components (17). LJP5 inhibits vWF binding to GPIIb/IIIa on platelets stimulated by either thrombin or ADP but does not block fibrinogen binding under the same conditions (18). LJP9 blocks both vWF and fibrinogen binding to GPIIb/IIIa (18). LJP9 blocks platelet aggregation induced by ADP, collagen, or thrombin, whereas LJP5 has no effect. In studies performed with ^{125}I-labeled IgG or monovalent Fab fragment we have determined that LJP5 and LJP9 bind to distinct epitopes of GPIIb/IIIa, although they compete with each other for binding to platelets (18). In fact, in the presence of a 10-fold excess of LJP5, the inhibitory effect of LJP9 on platelet aggregation and fibrinogen binding to GPIIb/IIIa is blocked (18). Purified IgG was obtained from ascitic fluid using affinity chromatography with either protein A-Sepharose (Sigma) (19) or DEAE Affi-Gel Blue (Bio-Rad Laboratories) (20). The latter method was used only for LJP5 because of its low affinity for staphylococcal protein A. LJP5 and LJP9 were further processed to obtain the monovalent Fab fragment of the IgG molecule (21). This was necessary because LJP5 Fab had no effect on fibrinogen binding to GPIIb/IIIa, whereas the corresponding IgG inhibited it, although only minimally (10–20%).

Aggregation Studies. Platelets were washed free of plasma constituents following the method of Walsh *et al.* (22) with modifications described (23). Aggregation studies were performed using either platelet-rich plasma, prepared as described (12), or washed platelet suspensions. Reaction mixtures were prepared in siliconized glass cuvettes and then placed in the aggregometer (Chrono-Log, Havertown, PA) at 37°C. The platelet suspension was constantly stirred at 1200 rpm. Ristocetin (sulfate salt, >90% ristocetin A) and apyrase (grade III) were from Sigma.

Fibrinogen Binding Studies. The measurement of ^{125}I-labeled fibrinogen binding to platelets in the presence of purified IIB vWF was performed following the method recently described for measuring fibrinogen binding induced by asialo-vWF (23). Washed platelets (4×10^8 cells per ml final concentration) were mixed with vWF and calcium chloride (1 mM final concentration) and stirred in the aggregometer cuvette for 5 min. In some experiments, monoclonal antibodies or apyrase were also added to this mixture. After this, the mixture was removed from the aggregometer, and appropriate aliquots were mixed with ^{125}I-labeled fibrinogen in the presence or absence of excess unlabeled fibrinogen. The platelet count at this point was 1×10^8 cells per ml. Binding was measured after incubation at 22–25°C for 30 min without agitation as described (12, 23).

RESULTS

Three hundred micrograms of purified vWF was isolated from 900 ml of citrate-treated plasma obtained from the type IIB vWD patient. The purified IIB vWF lacked the largest molecular forms, which were present in normal vWF purified with the same procedure (Fig. 1). The ristocetin cofactor activity of IIB vWF was 65 units/mg as compared to 126 units/mg for normal vWF. Therefore, as seen in plasma, the lack of large multimers correlates with a decreased specific-ristocetin-cofactor activity. The subunit composition was identical in normal and IIB vWF, and a predominant band of

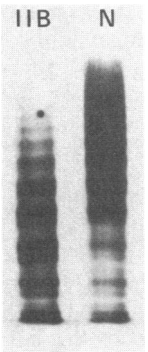

FIG. 1. Multimeric composition of purified IIB vWF. Ten nanograms of purified IIB vWF (IIB) and purified normal vWF (N) were electrophoresed in a $NaDodSO_4$-containing agarose gel, as described (11). At the end of the run, the gel was fixed, and the vWF detected by means of ^{125}I-labeled affinity-purified polyclonal rabbit anti-human vWF IgG (2, 11). Cathode at the top. Note that the largest, slowest moving multimers present in the normal vWF (top of the gel) are absent in the IIB vWF.

M_r 230,000 was observed in both preparations after reduction.

When IIB vWF was added to a suspension of washed normal platelets, aggregation was induced by lower concentrations of ristocetin than that necessary to induce aggregation with equivalent amounts of normal vWF (Fig. 2). Using ristocetin (final concentration, 0.5 mg/ml) rapid aggregation was observed with IIB vWF (0.4 μg/ml) but essentially no aggregation was observed when the same amount of normal vWF was added to the mixture (Fig. 2). Ristocetin-induced aggregation was completely blocked by the anti-GPIb mono-

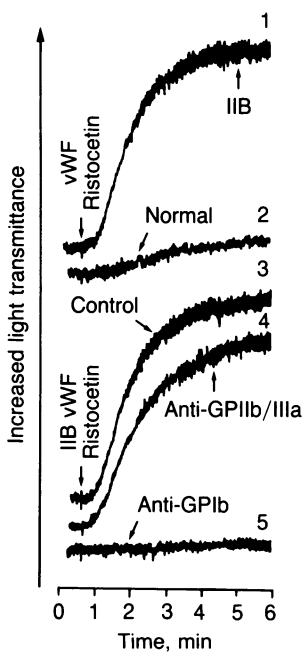

FIG. 2. Effect of purified IIB vWF on platelet aggregation induced by low-dose ristocetin. Washed platelets were prepared from normal blood and used at a final count of 2.5×10^8 cells per ml in the experimental mixtures. Traces 1 and 2 show the aggregation induced by a final ristocetin concentration of 0.5 mg/ml in the presence of either IIB or normal vWF (0.4 μg/ml), as indicated. Note the rapid aggregation with IIB vWF and the lack of response with normal vWF. Traces 3–5 show that platelet aggregation induced by ristocetin in the presence of IIB vWF was blocked completely by an anti-GPIb monoclonal antibody (LJIb1; 10 μg/ml, final concentration) but was unaffected by an anti-GPIIb/IIIa monoclonal antibody (LJP9; 20 μg/ml, final concentration).

clonal antibody, LJIb1. It was not affected, however, by the anti-GPIIb/IIIa monoclonal antibody, LJP9 (Fig. 2), which blocks fibrinogen and vWF binding as well as platelet aggregation induced by ADP or thrombin (18).

Purified IIB vWF induced aggregation when added to platelet-rich plasma in the absence of any other agonist with the maximal response at a final concentration of 20–30 μg of IIB vWF/ml (Fig. 3). The threshold dose for aggregation induced by IIB vWF alone was 5–10 μg of IIB vWF/ml. Purified normal vWF did not elicit any aggregation response in the absence of ristocetin (Fig. 3).

Platelet aggregation induced by IIB vWF was blocked by the same anti-GPIb antibody that inhibited ristocetin-induced aggregation (compare Figs. 2 and 4). Moreover, the anti-vWF monoclonal antibody (RG46) that blocked the ristocetin-cofactor activity of normal vWF also completely inhibited IIB vWF-induced aggregation (Fig. 4). In contrast to results in the presence of ristocetin, platelet aggregation induced by IIB vWF was markedly inhibited by the anti-GPIIb/IIIa antibody, LJP9 (compare Figs. 2 and 4).

Since both vWF and fibrinogen bind to GPIIb/IIIa, we performed experiments to determine whether binding of both was required for aggregation induced by IIB vWF. For this purpose, the anti-GPIIb/IIIa monoclonal antibody, LJP5, was used. As previously reported (18), this antibody blocks vWF binding to GPIIb/IIIa but has no effect on fibrinogen binding. Moreover, when present in sufficient excess, LJP5 interferes with the binding to platelets and the inhibitory effects of the other anti-GPIIb/IIIa antibody used in these studies, LJP9. In the presence of a 10-fold excess of LJP5, platelet aggregation induced by IIB vWF occurred as in the control mixture even in the presence of concentrations of LJP9 that could block aggregation completely (Fig. 4). Thus, blocking vWF binding to GPIIb/IIIa had no effect on aggregation, whereas blocking fibrinogen binding prevented it.

Further evidence for the role of fibrinogen in IIB vWF-induced platelet aggregation was derived from experiments with washed platelets. If platelets were resuspended in buffer or afibrinogenemic citrate-treated plasma, there was no aggregation by IIB vWF unless fibrinogen was added to the mixture (Fig. 5). Also, aggregation was completely inhibited in the presence of the ADP-scavenger apyrase (Fig. 5).

Purified IIB vWF induced binding of ^{125}I-labeled fibrinogen to platelets (Fig. 6). Such binding was blocked by the anti-GPIIb/IIIa antibody, LJP9, which also inhibited the binding induced by thrombin or ADP (18). On the contrary, fibrinogen binding induced by IIB vWF was increased in the presence of the anti-GPIIb/IIIa antibody, LJP5 (Fig. 6). This antibody, as mentioned above and reported (18), inhibits vWF binding but not fibrinogen binding to platelets stimulated with thrombin or ADP. The anti-GPIb antibody also inhibited fibrinogen binding induced by IIB vWF (Fig. 6), although this antibody has no effect on the binding induced by thrombin or ADP. Finally, as seen with other stimuli, fibrinogen binding induced by IIB vWF was blocked by the ADP-scavenger apyrase (Fig. 6).

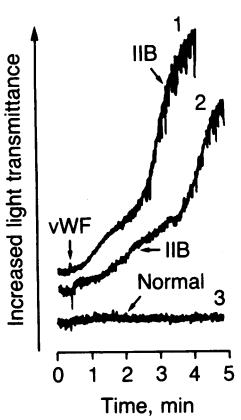

FIG. 3. Platelet aggregation induced by purified IIB vWF. In this experiment platelet-rich plasma was used at a final platelet count of 2.5×10^8 cells per ml. Aggregation was induced by the addition of purified IIB vWF at 28 (trace 1) and 14 (trace 2) μg of IIB vWF/ml, in the absence of any other agonist. No aggregation was observed upon addition of purified normal vWF (60 μg/ml, trace 3).

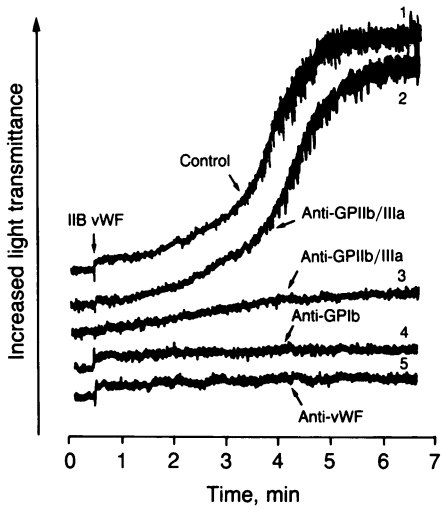

FIG. 4. Effect of monoclonal antibodies on platelet aggregation induced by purified IIB vWF. Aggregation of normal platelet-rich plasma (final platelet count 2.5×10^8 cells per ml) was elicited by the addition of purified IIB vWF (20 μg/ml; trace 1). The effect of various monoclonal antibodies on such aggregation was tested by incubating them with the platelet-rich plasma for one minute, before adding the IIB vWF. Trace 2, anti-GPIIb/IIIa (LJP5 at 200 μg/ml and LJP9 at 20 μg/ml); trace 3, anti-GPIIb/IIIa (LJP9 at 20 μg/ml); trace 4, anti-GPIb (LJIb1 at 10 μg/ml); trace 5, anti-vWF (RG46 at 60 μg/ml). Note that when present at a 10-fold excess, antibody LJP5 blocks the inhibitory effect of LJP9. Both antibodies are directed against GPIIb/IIIa, and both block the vWF binding site, but only LJP9 blocks the fibrinogen binding site. The two antibodies compete for binding to platelets (18). Therefore, very little LJP9 binds to GPIIb/IIIa in the presence of a 10-fold excess of LJP5.

DISCUSSION

This study demonstrates that purified IIB vWF acts as a direct platelet agonist and induces aggregation of platelet-rich plasma in the absence of ristocetin or any other exogenous stimulus. This requires higher concentrations of IIB vWF than those sufficient to demonstrate the increased responsiveness to ristocetin, but these concentrations can be attained *in vivo* (20–30 μg of purified IIB vWF/ml caused maximal platelet aggregation.) This finding can be correlated with the clinical observation that the majority of patients with type IIB vWD have a normal platelet count (1), but thrombocytopenia develops after administration of des-1-amino[D-arg^8]VP (7). This drug causes release of vWF from tissue storage sites (3) and may elevate the blood concentration of the abnormal vWF molecule to the levels at which, according to our *in vitro* findings, platelet aggregation is to be expected. It is noteworthy that the studies reported here have been performed with IIB vWF isolated from the plasma of an untreated patient. The purified molecule exhibited the absence of the largest multimers seen in the plasma of typical type IIB vWD patients (1, 11). Therefore, direct interaction between IIB vWF and platelets may occur without administration of des-1-amino[D-arg^8]VP and the consequent transient appearance of larger multimers in the circulation (3).

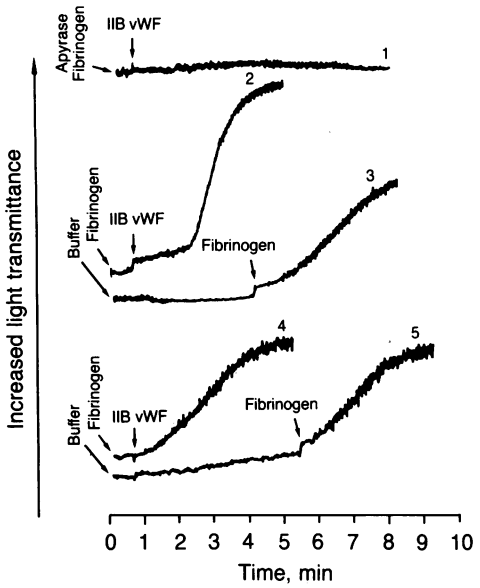

FIG. 5. Aggregation of washed platelets induced by purified IIB vWF. Washed platelets were used at a final count of 2.5×10^8 cells per ml. For the experiment represented in traces 4 and 5, washed platelets were resuspended in modified Tyrode's buffer (12), whereas they were resuspended in afibrinogenemic citrate-treated platelet-poor plasma (fibrinogen concentration <5 μg/ml) for the experiment represented in the three upper curves. In each case, either Tyrode's buffer or fibrinogen (final concentration, 1 mg/ml) was added to the mixtures, as indicated by arrows. The top curve shows an experiment where apyrase (final activity, 5 units of ATPase/ml) was also added. The addition of IIB vWF (final concentration, 20 μg/ml) resulted in aggregation of washed platelets only when fibrinogen was present in the mixture, either added before or after the IIB vWF. Aggregation was completely blocked in the presence of apyrase.

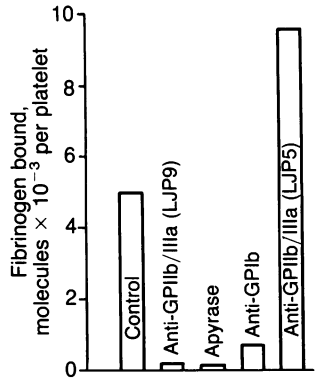

FIG. 6. Binding of ^{125}I-labeled fibrinogen to platelets induced by purified IIB vWF. Washed platelets were mixed at a count of 4×10^8 cells/ml with 20 μg of IIB vWF/ml and 1 mM $CaCl_2$. In some mixtures, specific monoclonal antibodies or apyrase were also added, as indicated, using the same concentrations reported in the legends to Figs. 4 and 5. The mixtures were kept at 37°C for five min, with constant stirring at 1200 rpm. After this, ^{125}I-labeled fibrinogen was added (330 μg/ml) with or without a 20-fold excess of unlabeled fibrinogen, and the incubation was continued for 30 min at 22–25°C without agitation. The platelet count at this point was 1×10^8 cells per ml. Duplicate aliquots of each mixture were then applied onto a layer of 20% sucrose in Tyrode's buffer (12), and the platelets were sedimented at 13,000 × *g* for four min. The radioactivity associated with the platelet pellet was then counted. Nonspecific binding, measured in the presence of excess unlabeled fibrinogen, was subtracted from total measured binding (the value obtained in the absence of unlabeled fibrinogen) to give the results shown. Note that the binding measured in the presence of LJP5, a monoclonal anti-GPIIb/IIIa antibody that blocks vWF but not fibrinogen binding sites, was increased over that seen in the control mixture.

In this report we provide clues as to the mechanisms involved in platelet aggregation induced by IIB vWF in the absence of any other agonist. The process appears to be initiated by the interaction of the abnormal vWF molecule with the platelet membrane GPIb. When such interaction is blocked by either a monoclonal antibody against vWF or one against GPIb, platelet aggregation induced by IIB vWF is abolished. These findings also suggest that the GPIb binding site for normal and IIB vWF, as well as the binding domain on the normal and IIB vWF molecules, are closely related, if not identical, since they are blocked by the same monoclonal antibodies.

In addition to requiring interaction of IIB vWF with GPIb, however, platelet aggregation induced by this abnormal vWF molecule also requires fibrinogen binding to GPIIb/IIIa. This was demonstrated by showing that an anti-GPIIb/IIIa monoclonal antibody that blocks the fibrinogen and vWF binding sites but not one that blocks only vWF binding (18) effectively inhibited IIB vWF-induced platelet aggregation. Moreover, the experiments performed with washed platelets also point to the crucial role of plasma fibrinogen in such aggregation. Experiments with congenitally deficient plasma showed that fibrinogen was essential even when all other plasma proteins were present in the mixture. On the contrary, interaction of IIB vWF with GPIb appears necessary and sufficient to mediate platelet aggregation induced by low-dose ristocetin. In fact, such aggregation is inhibited by an anti-GPIb antibody, whereas blocking of the GPIIb/IIIa complex with a monoclonal antibody, which inhibits both vWF and fibrinogen binding, has no appreciable effect.

Interaction of IIB vWF with GPIb is essential to trigger the exposure of GPIIb/IIIa-related binding sites, as shown by the fact that the binding of fibrinogen induced by IIB vWF is inhibited by the anti-GPIb antibody. Antibodies like this have no blocking effect on the fibrinogen binding site *per se* (12) and must act by blocking the initial interaction of IIB vWF with GPIb. Abrogation of aggregation with apyrase indicates that the exposure of GPIIb/IIIa binding sites induced by IIB vWF is likely to be mediated by released ADP, as is the case for other agonists (12, 24).

When the anti-GPIIb/IIIa antibody that blocks only vWF binding was used, fibrinogen binding induced by IIB vWF was nearly doubled. This demonstrates that IIB vWF, like the normal molecule, can compete with fibrinogen for binding to sites on GPIIb/IIIa (25). Interaction of vWF with GPIIb/IIIa, however, does not seem to be sufficient to fully sustain platelet aggregation in the absence of fibrinogen.

There are striking analogies in the mechanisms involved in platelet aggregation induced by IIB vWF, as reported here, and those involved in aggregation induced by normal desialylated vWF (23, 26, and 27). However, isoelectric focusing of the plasma of several IIB vWD patients, including the one reported here, has suggested that IIB vWF is not significantly desialylated. In fact, the isoelectric points of normal and IIB vWF are very similar, whereas the isoelectric point of the normal molecule is significantly increased when sialic acid is removed (28). Therefore, it is likely that the molecular abnormality responsible for the direct interaction of IIB vWF with GPIb is not related to the lack of sialic acid residues.

In conclusion, this report demonstrates that fibrinogen-mediated platelet aggregation is initiated by the interaction of a naturally occurring variant form of vWF with GPIb. This mechanism is probably responsible for the *in vivo* platelet aggregation and thrombocytopenia seen in some patients with type IIB vWD. Moreover, these studies suggest that vWF not only promotes platelet adhesion to exposed subendothelium but it also functions in the hemostatic process as an inducer of platelet aggregation.

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