von Willebrand Factor Bound to Glycoprotein Ib Is Cleared From the Platelet Surface After Platelet Activation by Thrombin

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We recently reported that after activation of human platelets by thrombin, glycoprotein (GP) Ib-IX complexes are translocated to the surface-connected canalicular system (SCCS) (Blood 76:1503, 1990). As GPIIb is a major receptor for von Willebrand factor (vWF) in platelet adhesion, we have now examined the consequences of thrombin activation on the organization of vWF bound to GPIb on the platelet surface. Studies were performed using monoclonal or polyclonal antibodies in either immunogold staining and electron microscopy (Au-EM) or in flow cytometry. When unstimulated platelet-rich plasma was incubated with ristocetin, bound vWF was located by Au-EM as discrete masses regularly distributed over the cell surface. Platelets from a patient with Glanzmann’s thrombasthenia, lacking GPIIIa-IIIa complexes, gave a similar pattern, confirming that this represented binding to GPIb. That ristocetin was not precipitating vWF before their binding to the platelets was shown by the detection of similar masses on the surface of platelets of a patient with type IIB von Willebrand disease. Experiments were continued using washed normal platelets incubated in Tyrode-EDTA, the purpose of the EDTA being to limit the surface expression of endogenous vWF after platelet stimulation. Under these conditions, platelets were treated with ristocetin for 5 minutes at 37°C in the presence of increasing amounts of purified vWF. This was followed by incubation with thrombin (0.5 U/mL) for periods of up to 10 minutes. Flow cytometry showed a time-dependent loss in the surface expression of vWF bound to GPIb and these changes were confirmed by Au-EM. In particular, immunogold staining performed on ultrathin sections showed that the bulk of the vWF was being cleared to internal membrane systems. Surface clearance of vWF during thrombin-induced platelet activation is a potential mechanism for regulating platelet adhesivity.

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to examine the distribution of vWF on the surface of platelets incubated sequentially with ristocetin and α-thrombin. We report that vWF bound to GPIb is cleared to the SCCS after platelet stimulation by thrombin. This phenomenon, which may resemble the internalization of ligand-receptor complexes originally observed with latex particles in plasma, is a potential mechanism for regulating platelet adhesivity.

**MATERIALS AND METHODS**

*Appyrase and prostaglandin E1 (PGE1)* were purchased from Sigma Chemical Co (St. Louis, MO); glutaraldehyde from Fluka AG (Buchs, Switzerland); ristocetin from Lundbeck (Copenhagen, Denmark). Human α-thrombin (Fibrinex) was from Ortho Diagnostic Systems (Raritan, NJ); Lowicryl K4M and Epon 812 from Taab (Aldermaston, UK). Affinity-purified IgG of goat antisera to rabbit or mouse IgG, coupled to Amonobeads (Taab) (Aldermaston, UK). Affinity-purified IgG of goat antisera to vWF containing factor XI only from Silenus (Victoria, Australia). Protein A-Sepharose 4B was purchased from Pharmacia-LKB (Stockholm, Sweden). Low-temperature gelling agarose was from Litex (Glostrup, Denmark).

**Blood Sampling and Preparation of Washed Platelets**

Venous blood was taken with informed consent from healthy volunteers and anticoagulated with acid-citrate-dextrose (ACD; NIH formula A) (1 part anticoagulant to 9 parts blood). Blood from a patient with type I Glanzmann's thrombasthenia was similarly obtained. Platelet-rich plasma (PRP) was prepared by centrifugation at 120g for 10 minutes. Washed platelets were prepared using ACD-A anticoagulated blood. Here, PGE1 (100 nmol/L), 25 μg/mL appyrase, and ACD-A (1 vol:9 vol PRP) were immediately added to the PRP. Platelets were sedimented by centrifugation at 1,200g for 15 minutes and washed three times, as previously described by us. Unless stated otherwise, washed platelets were resuspended in a modified Tyrode buffer consisting of 137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L NaHCO3, 0.3 mmol/L NaH2PO4, 5.5 mmol/L glucose, 5 mmol/L HEPES, 5 mmol/L EDTA, 0.35% (wt/vol) bovine serum albumin (BSA), pH 7.4 (Tyrode-EDTA). In some experiments, EDTA was replaced by 2 mmol/L of CaCl2 and 1 mmol/L MgCl2 (Tyrode-Ca2+).

**Incubation of Platelets With Ristocetin or α-Thrombin**

Initial studies with ristocetin were performed using citrated PRP. Platelet counts were adjusted to 2 x 10^9/mL using platelet-poor plasma, prepared by further centrifuging the residual red blood cells and buffy coat for 10 minutes at 2500g. Most other studies were performed using washed platelets resuspended in Tyrode-EDTA at 10^9/mL (unless stated otherwise) in the presence of purified vWF added to give final concentrations between 1 and 16 μg/mL. Ristocetin was usually used at 1.0 or 1.5 mg/mL but was also tested at lower concentrations. Incubations were for up to 30 minutes. Human α-thrombin (0.5 U/mL) was incubated with washed platelets at 10^9/mL in Tyrode-EDTA or Tyrode-Ca2+ for periods ranging from 1 to 10 minutes at 37°C without stirring, except for an initial mixing. In other experiments, platelet suspensions in Tyrode-EDTA were first incubated with ristocetin in the presence of purified vWF for 5 minutes, then 0.5 U/mL of thrombin was added and the incubations continued for periods up to 10 minutes at 37°C without stirring. Alternatively, samples were incubated first with thrombin for 5 or 10 minutes at 37°C and then ristocetin was added in a 10-fold excess whereupon ristocetin was added and the incubation continued for 5 minutes. Incubations were finally stopped by the addition of the fixative medium (see below).

**Fixation Procedures**

For analysis by flow cytometry, samples were fixed with an equal volume of 2% (wt/vol) paraformaldehyde (PFA) followed by incubation for 30 minutes at room temperature. For standard immunogold staining and electron microscopy, citrated PRP or washed platelet suspensions were fixed in 1.25% (vol/vol) glutaraldehyde (diluted in 0.1 mol/L phosphate buffer, pH 7.2) for 10 minutes at 37°C as previously described by us. Patient with Type IIB von Willebrand Disease

The patient (C. . .) has previously been described and contains a duplication of a methionine within the GPIb binding domain of vWF. She shows the typical characteristics of type IIB von Willebrand disease in that her vWF has an increased reactivity with platelets and this is shown by a lack of large multimers from her plasma. Here, citrated PRP was added to 1.25% glutaraldehyde fixative without addition of ristocetin.

**Antibodies and Purified vWF**

The rabbit anti-vWF serum used in most of the studies was obtained from Dakopatts (Copenhagen, Denmark). Isolated IgG from a rabbit anti-vWF antibody were also generously provided by Dr Dominique Meyer (Paris, France). Concentrations used were 25 μg/mL for the antibody of Meyer and, unless otherwise stated, a dilution of 1:50 for the commercial antibody, AP-1, an MoAb directed against the α-chain of GPIb. It was used as ascites fluid at a dilution of 1:2,000. Bx-1 is a murine MoAb prepared in our laboratory that reacts with GPIIb in immunoblotting experiments. With this antibody, IgG were isolated from ascites fluid by chromatography on protein-A Sepharose according to standard procedures and used at a concentration of 23 μg/mL. Rabbit antilyoglycoprotein antibody was kindly provided by Dr Kenneth Clemenson (Berne, Switzerland). IgG were isolated from the serum by affinity chromatography on protein-A Sepharose and were used at a concentration of 15 μg/mL when immunogold labeling was performed on ultrathin sections. Lyophilized preparations of vWF containing factor VIII were purchased from the Centre National de Transfusion Sanguine (CNTS, Paris, France). vWF was also purified in the laboratory of one of us (H.R.G.) according to previously published procedures, packed at 4°C, and transported by air to Bordeaux, where it was used within 72 hours of arrival. The commercial preparation was obtained from cryoprecipitate by treatment with TNBP-Tween 80 followed by two cycles of ion exchange chromatography. Affinity chromatography on gelatin-Sepharose 4B removed trace amounts of fibronectin. Purified factor VIII was added before freeze drying. According to the manufacturer, the preparation contained 45% to 55% multimers of molecular weight greater than 5 x 10^6 d. The final preparation was reconstituted at 0.40 to 0.70 mg/mL and contained 40 to 65 ristocetin cofactor U/mL.

**Transmission Electron Microscopy**

Immunogold staining pre-embedding. In the standard procedure, glutaraldehyde-fixed platelets were twice washed and resuspended at 10^9/mL in 0.1 mol/L phosphate buffer containing 1.15 mol/L NaCl and 0.1% wt/vol BSA, pH 7.2 (PBS-alb). All incuba-
tions with primary antibodies were performed at the concentration given above. The platelets were further washed in 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 8.2, containing 0.1% (wt/vol) BSA. They were then incubated with a 1/10 dilution of goat antirabbit or antimouse IgG coupled to 5 or 10 nm gold particles for 4 hours at room temperature followed by incubation overnight at 4°C, as previously detailed by us. Controls were performed by omitting the first antibody or by using an equivalent concentration of nonimmune mouse or rabbit IgG. Platelets were postfixed in 1% (wt/vol) osmic acid, dehydrated with graded alcohols and propylene oxide, and finally embedded in Epon. Ultrathin sections were obtained using an Ultracut E ultramicrotome (Reichert-Jung Optische Werke AG, Vienna, Austria) and stained with uranyl acetate and lead citrate before being observed in a Philips EM 201 electron microscope (Eindhoven, Holland) at 80 kV.

Immunogold staining performed postembedding. Procedures previously used by us were modified as described by Berryman and Rodewald. Here, the initial fixation was for 2 hours at room temperature in 0.1 mol/L phosphate buffer containing 4% (wt/vol) paraformaldehyde, 1% (wt/vol) glutaraldehyde, 0.2% (wt/vol) picric acid, 0.5 mmol/L CaCl₂, pH 7.4. Fixed platelets were washed three times in 0.1 mol/L phosphate buffer containing 3.5% (wt/vol) sucrose, 0.5 mmol/L CaCl₂, pH 7.4 (sucrose-phosphate buffer). Free aldehydes were quenched with 50 mmol/L glycine in sucrose-phosphate buffer, pH 7.4, for 1 hour at room temperature. Platelets were then washed four times in cold 0.1 mol/L maleate buffer containing 3.5% (vol/vol) sucrose, pH 6.5 (sucrose-maleate buffer). They were postfixed for 2 hours in ice with 2% (wt/vol) uranyl acetate in sucrose-maleate buffer, pH 6.5. Platelets were then pre-embedded in 1% (wt/vol) low-temperature gelling agarose in 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4, dehydrated through a series of graded acetone solutions at −20°C before being embedded in Lowicryl K4M at this temperature. The resin was photopolymerized at 4°C for 48 hours. Ultrathin sections were mounted on collodium-coated nickel grids as previously described by us. Incubations with antiglycocalicin or anti-vWF antibodies (used at a 1/300 dilution) were continued overnight in a moist chamber, after which the sections were rinsed by floating onto five changes of PBS-alb. They were then transferred to a solution containing a 1/70 dilution of goat antirabbit IgG conjugated to 5-nm gold particles. After 2 hours at room temperature, the grids were rinsed and stained for 15 minutes in 2% (wt/vol) osmium tetroxide. After further rinsing and counterstaining with lead citrate, the sections were examined at 50 kV in the electron microscope.

Immunofluorescence and Flow Cytometry

PFA-fixed unstimulated platelets, or those incubated with ristocetin and/or thrombin, were washed three times in PBS-alb. They were then resuspended at 5 × 10⁶ platelets/mL and incubated for 1 hour with either rabbit antibody to vWF or MoAb to the α-chain of GPIb. Concentrations were identical to those used in immunogold staining and given above. After the incubation with the primary antibody, the samples were washed and incubated for 1 hour at room temperature with a 1:80 dilution (vol/vol) of FITC-conjugated F(ab’), fragments of sheep antibody monospecific for mouse or rabbit IgG (dilution in PBS-alb buffer). Platelets were washed three times and resuspended in PBS-alb at a concentration of 50,000 platelets/µL. Controls were again the absence of primary antibody or the corresponding amounts of nonimmune mouse or rabbit IgG. Samples were analyzed with a Spectrum III flow cytometer (Ortho) using a 70-µm aperture and a laser argon-ion lamp (emission 488 nm). The samples were first analyzed by forward and wide angled light scatter and the gates set so as to include the majority of the platelets and to exclude larger particles that may be platelet aggregates or other blood cells. Green fluorescence was measured after passage through a 530-nm long pass interference filter. The fluorescence was analyzed using a logarithmic scale. A fluorescence histogram was obtained for 10,000 cells.

RESULTS

Ultrastructural Studies of the Binding of vWF to GPIb of Platelets

In whole blood or PRP. Immunogold staining and electron microscopy was used to visualize vWF bound to the surface of human platelets. Figure 1a illustrates unstirred normal platelets incubated for 5 minutes with 1.5 mg/mL ristocetin in citrated PRP. The platelet membrane is seen to be covered by discrete masses identified as vWF through the presence on their periphery of gold particles identifying attached anti-vWF antibodies. The masses were frequently separated by membrane domains free of immunogold staining. Reducing the amount of ristocetin decreased the number of masses but not their size. Only the occasional labeling was seen on platelets incubated in PRP in the absence of ristocetin, and here the gold particles were restricted to a small population of platelets and to a few points on the membrane (Fig 1b). In Fig 1c, the incubation with ristocetin has been allowed to continue for 30 minutes. Here, some platelet to platelet attachment has occurred and protein bridges cross-linking platelets can be distinguished. These bridges have been recognized by the anti-vWF antibody. It should be noted that even in the agglutinates platelet shape change and granule centralization are minimal. This suggests that under our experimental conditions (i.e., unstirred suspensions), binding of vWF does not lead to granule secretion. Similar experiments were performed using platelets from a patient with Glanzmann’s thrombasthenia. Figure 1d shows an identical distribution of vWF on the surface of platelets of this patient, proving that GPIIb-IIIa complexes were not contributing significantly to the binding of vWF under these conditions. All of the above experiments were performed with ristocetin. To see if similar structures were observed when vWF bound to GPIb of platelets independently of this agonist, PRP of a patient with type IIB von Willebrand disease was added directly to glutaraldehyde fixative. Figure 1e confirms that anti-vWF antibody detected similar masses on the platelet surface. The illustrated platelet was typical of the majority of those obtained from the patient.

To washed platelets. Washed platelets were incubated with ristocetin in the presence of purified vWF added in concentrations ranging between 1 and 16 µg/mL. Immunogold staining suggested a dose-dependent binding of vWF and this was confirmed by flow cytometry (not illustrated). Figure 2a and b shows the patterns obtained when incubations were performed in the presence of 2 and 16 µg/mL vWF, respectively. Again, the staining of the platelet surface was not continuous, and was composed of separate masses (identical structures were seen with both sources of purified vWF used in this study). Interestingly, the frequency of the masses, but not their size, was
dependent on vWF concentration. Control studies in which washed platelets were incubated with ristocetin in the absence of exogenous protein showed only a limited surface staining for vWF and, as for the PRP (see Fig 1b), that which was detected was confined to the occasional platelet (not illustrated). The distribution of bound vWF was then compared with that of GPIb-IX complexes, which were detected using Bx-1 and AP-1, two MoAbs specific for the GPIba chain. As previously described by us\(^2\) and others (see Discussion), GPIb-IX complexes were regularly distributed on the surface of unstimulated platelets (not illustrated). Somewhat surprisingly, a similar distribution was seen using platelets that had been incubated with ristocetin (Fig 2c). Signs of widespread receptor clustering were not detected and the presence of bound vWF had only a small effect on Bx-1 binding (see also flow cytometry experiments). The same result was obtained for AP-1 (data not given). Interestingly, Fig 2c also illustrates that some elements of the SCCS were stained for GPIb, showing that the surface network of GPIb-IX complexes also extends to this membrane system.

\(\text{vWF on Platelets Incubated With } \alpha\text{-Thrombin}\)

Washed platelets were incubated with \(\alpha\)-thrombin and the surface expression of endogenous vWF observed using immunogold staining. As shown in Fig 3, the platelets were now highly activated and exhibited pseudopodia and an electron dense central mass typical of platelets having undergone secretion. Platelets stimulated in Tyrode-EDTA possessed little or no surface-bound vWF (Fig 3a). This is to be expected, for vWF binding to GPIIb-IIIa is inhibited by the presence of EDTA (see Discussion). Similarly, platelets from the Glanzmann’s thrombasthenia patient expressed little or no vWF even when stimulated in Tyrode-Ca\(^{2+}\) (Fig 3b), confirming the importance of GPIIb-IIIa complexes in the expression of secreted vWF. When normal platelets were stimulated with thrombin in Tyrode-Ca\(^{2+}\), vWF was
irregularly spaced on the platelet surface (Fig 3c). As these were washed platelets, the source of the vWF is the α-granule pool. As expected, the staining was much less dense than was seen with ristocetin, with masses most often located in invaginations, some of which appear to lead from the platelet interior (see arrowheads on Fig 3c). It is tempting to speculate that some of these may represent openings of the SCCS. When platelets in Tyrode-Ca²⁺ were stimulated with α-thrombin in the presence of purified vWF, staining was more abundant and the surface structures typical of bound vWF were again seen (Fig 3d). However, the staining remained irregular in comparison with that seen with ristocetin. Again, little or no staining was obtained when platelets of the thrombasthenic patient were stimulated in the presence of added vWF (data not shown). Overall, these studies suggest that ultrastructural studies alone do not permit a distinction between vWF bound to GPIb or GPIIb-IIIα.

Flow Cytometry

Platelets in Tyrode-EDTA were treated as described above, but instead of being processed for immunogold staining, the cells were fixed with paraformaldehyde and the surface expression of vWF and GPIb assessed using immunofluorescence procedures with bound antibody quantitated by flow cytometry. Unstimulated platelets showed a small but consistent labeling for vWF (Fig 4a). The nature of the fluorescence profile suggested that this concerned a small fraction of the total platelet population (see Discus-
Fig 3. Expression of vWF on platelets stimulated with α-thrombin. Washed platelets (10^9/mL) were incubated with 0.5 U/mL α-thrombin for 10 minutes at 37°C before fixation and immunogold staining for vWF as described in the legend to Fig 1. Results are for (a) normal platelets in Tyrode-EDTA, (b) platelets from a Glanzmann’s thrombasthenia patient in Tyrode-Ca++, (c) normal platelets in Tyrode-Ca++, and (d) normal platelets in Tyrode-Ca++ with 16 μg/mL purified vWF added before the α-thrombin. Note the divalent cation-dependent presence of surface-bound endogenous vWF (arrows) on normal platelets activated in Tyrode-Ca++ and the increased staining when the platelets were treated with α-thrombin in the presence of added vWF. Bar = 0.5 μm.

Ristocetin-induced binding of vWF was readily quantifiable in flow cytometry and a fairly sharp peak was seen to the right of the profile (Fig 4c). Over 95% of the platelets had bound vWF and, from the shape of the histogram, most expressed appreciable quantities. In confirmation of the immunocytochemical studies, preincubation of the platelets with ristocetin and vWF had little effect on their binding of the anti-GPIb MoAb, Bx-1 (compare Fig 4b and d). Similar results were obtained with AP-1 (not illustrated). In contrast, and in confirmation of our previous results, the surface expression of GPIb-IX complexes was much decreased on thrombin-stimulated cells (Fig 4f). In agreement with George and Torres, these platelets showed a much decreased vWF binding and ristocetin-induced agglutination (not illustrated).

When platelets were incubated first with ristocetin and then with thrombin, flow cytometry showed that the thrombin treatment resulted in a marked reduction in the amounts of vWF present on the platelet surface (Fig 4g). The loss was time dependent and reached a maximum after 10 minutes (see ultrastructural studies below). Nonetheless, the flattened shape of the histogram suggested heterogeneity in the distribution of the residual surface-bound vWF within the total platelet population. Parallel studies with Bx-1 showed that the surface expression of GPIb-IX complexes was again markedly reduced (Fig 4h), but that the surface clearance was less complete than for platelets stimulated by thrombin alone (Fig 4f). Interestingly, we noted that the clearance of bound vWF was greatest at low added vWF concentrations (such as at 4 μg/mL vWF, as illustrated in Fig 4), but was less apparent at 16 μg/mL vWF (not illustrated). This might suggest that at high density the amount of bound vWF becomes limiting, perhaps through saturation of the translocating mechanism.

Ultrastructural Studies on Ultrathin Sections

The above results showed that vWF bound to GPIb was cleared from the platelet surface after the action of thrombin. Immunogold staining on ultrathin sections of pre-embedded samples was then performed to establish whether the vWF was accumulating in the SCCS. Washed platelets were incubated with ristocetin and exogenous vWF. Thrombin was added and samples were fixed at different times after the addition of the thrombin. With unstimulated platelets, both the surface-bound and α-granule pools of vWF were located (not illustrated). Figure 5a shows that 1 minute after the addition of the thrombin, platelet activation was well advanced. Although surface-bound vWF was still present, its distribution was irregular. Already, there was appreciable labeling within the outer channels of the SCCS. Note that the internal staining also appeared to include the residual α-granule pool, for secretion was incomplete at this stage. As previously described by others, staining of the residual α-granules was rarely homogeneous. Three minutes after addition of the thrombin, staining within the channels of the SCCS was increased and
Fig 4. Expression of vWF and of GPIb on platelets as assessed by flow cytometry. Here, all incubations were performed at 37°C using washed normal platelets (10^9/mL) resuspended in Tyrode-EDTA. Platelets were incubated (a and b) for 5 minutes without agonist or exogenous vWF; (c and d) for 5 minutes with 1.5 mg/mL ristocetin and 4 pg/mL vWF; (e and f) for 10 minutes with 0.5 U α-thrombin in the absence of exogenous vWF; and (g and h) 5 minutes with 1.5 mg/mL ristocetin and 4 μg/mL vWF followed by 10 minutes with 0.5 U α-thrombin. Incubations were terminated by the addition of paraformaldehyde. Fixed cells were washed and incubated with rabbit anti-vWF antibody (a, c, e, and g) or Ex-1, an MoAb to GPIb (b, d, f, and h). Bound IgG were detected using FITC-conjugated species-specific anti-IgG. A maximum of 10,000 cells were analyzed in a Spectrum flow cytometer (Ortho) and platelet-bound immunofluorescence expressed on a logarithmic scale. In (a) and (b) the broken lines delimit the maximum fluorescence obtained in control experiments where platelets were incubated in the absence of primary antibody. The illustrated results are typical of three separate experiments.

seen throughout the platelet (Fig 5b). Little or no surface staining for vWF was now detected. After 10 minutes, staining continued to be seen within the SCCS (Fig 5c) and the staining was now more central in nature. Clearly, some of the outer channels were free of particles. Parallel studies with a rabbit antibody to glycocalcin showed that the bound vWF colocalized with “internalized” GPIb-IX complexes (not illustrated). Control studies, where platelets in Tyrode-EDTA were stimulated with thrombin in the absence of prebinding of vWF to GPIb, and where detected vWF must come from the α-granule pool, showed a much reduced staining for vWF in the SCCS (Fig 5d).

DISCUSSION

We have used immunogold staining and electron microscopy to locate vWF bound to the surface of human platelets. Initial experiments were performed using platelets incubated with ristocetin in PRP. Results showed that the anti-vWF antibody located discrete masses that were regularly distributed over the entire platelet surface. It has been reported that ristocetin in plasma can cause fibrinogen and perhaps other adhesive proteins to precipitate and that these interact with vWF. However, we obtained an identical staining when washed platelets were incubated with ristocetin and either of two preparations of purified vWF. This makes unlikely a major contribution from coprecipitating adhesive proteins to the structures identified by the anti-vWF antibody. Furthermore, a similar pattern of labeling was seen when the platelets of a patient with type IIB von Willebrand disease were examined. Here, platelets in plasma were fixed in the absence of ristocetin. In this disorder, an abnormality within the vWF molecule causes the high molecular weight multimers to fix directly onto GPIb of circulating platelets. In our patient, the abnormality is related to the duplication of a methionine within the GPIb binding domain of vWF. In the studies with ristocetin, further evidence that we were observing binding to GPIb was the identical pattern obtained using the PRP of a patient with Glanzmann’s thrombasthenia. The platelets of this patient were almost totally lacking GPIb-IIIa complexes, thus ruling out the participation of this receptor in the formation of the masses initially observed.

The presence of a surface layer of “protein” on the surface of platelets incubated with ristocetin in PRP was first reported by Escolar et al, who performed tannic acid staining during fixation. These investigators reported that the staining ranged from homogeneous to irregular with a nodular appearance. In another study, Rand et al, used peroxidase- and ferritin-labeled antibodies to locate vWF on the surface of platelets incubated with ADP or ristocetin. These investigators also reported large “precipitates” stained by the anti-vWF antibody. Our studies confirm these early results. However, immunogold staining permits a much greater resolution of the recognized protein. A feature of our study was that, whereas the vWF bound regularly over the platelet surface, it did so discretely. Individual masses were clearly separated on the platelet surface, even at high vWF concentrations (see Fig 2b). Previous studies performed with ristocetin and purified vWF (discussed below) or, indeed, with the platelets from patients with type IIB von Willebrand disease suggest that it is the high molecular weight multimers that bind preferen-
tially to GPIb. This may explain the large size of the structures that we were visualizing. In the absence of stirring, some agglutinates were formed during the longer incubations (see Fig 1c). Even allowing for the fact that fixation and dehydration procedures may modify protein structure, immunogold staining clearly showed that the bound vWF was directly cross-linking platelets. Thus, clearly, surface-bound vWF directly mediates the ristocetin-induced agglutination of platelets.

When normal platelets are activated with physiologic agonists, exogenous vWF is able to bind to GPIIb-IIIa complexes. However, this binding, which is mediated by the RGD sequence in vWF, is not only inhibited after divalent cation chelation, but it has also been shown to be dependent on the concentration of Ca$^{2+}$ when divalent cations are present. Secreted vWF also binds to GPIIb-IIIa by a mechanism that is divalent cation dependent. Not surprisingly, therefore, we detected little or no surface labeling when washed platelets were stimulated with thrombin in the presence of EDTA; and while vWF was detected on the surface of platelets stimulated in Tyrode-Ca$^{2+}$, the labeling intensity was much less than that observed when platelets were incubated with ristocetin and an exogenous source of vWF. This may also be related to the relatively low amount of vWF in α-granules. These experiments established that it was possible to follow the effect of thrombin stimulation on vWF prebound to GPIb, especially if the platelets were resuspended in the presence of EDTA. Large masses were again stained with anti-vWF when platelets were stimulated with thrombin in the presence of divalent cations. However, staining was less regular than was seen with ristocetin. As the goal of our study was to investigate the effect of thrombin on vWF bound to GPIb, this interaction was not investigated further.

Immunogold staining confirmed previous studies and showed that GPIb-IX complexes were evenly distributed over the platelet surface. Somewhat surprisingly, ristocetin-induced binding of vWF had little effect on either the
The intensity of staining or the organization of the gold particles. This finding was observed both with Bx-1 and with AP-1, two MoAbs reacting with different epitopes on the GPIbα subunit. Although Bx-1 has no effect on ristocetin-induced platelet aggregation (A.T. Nurden and D. Pintigny, unpublished observations, June 1991), AP-1 has been shown to inhibit both ristocetin-induced agglutination and the ristocetin-induced binding of vWF to platelets. Explanations for the lack of effect of vWF binding on the density of staining of the anti-GPIb monoclonals include the possibility that vWF binding involves a minority of GPIb-IX complexes. Alternatively, the Bx-1 and AP-1 determinants may remain accessible after vWF binding to the complexes. At present, our results do not allow us to distinguish between these possibilities. It is of interest that the maximum density of immunogold staining for vWF after ristocetin-induced binding of purified vWF occurred at vWF concentrations of ≈ 16 μg/mL (see Fig 2b), a concentration that has been shown to saturate GPIb receptors in direct binding studies with 125I-vWF. In the cited reference, ristocetin induced the binding of the equivalent of ≈ 25,000 vWF monomers at saturation. This would represent a much smaller number of vWF multimers. Recent data have confirmed that each vWF molecule contains one or more binding sites for GPIb, so it is probable that multimers composed of several repeating units bind to adjacent molecules of GPIb-IX, a hypothesis suggested by Chopek et al and by Berndt et al. Such an interaction could contribute to the morphology of the discrete masses that we have visualized.

There is abundant evidence to suggest that vWF circulates in the blood in the form of high molecular weight multimers that vary between 0.5 × 10^6 and 20 × 10^6 Kd in size. It has been shown that the high molecular weight multimers bind with increased affinity to the platelet membranes in the presence of ristocetin. Although we have not made a specific attempt to characterize the size of the vWF molecules that have bound to the platelet surface in our study, it is reasonable to assume that high molecular weight multimers are being located. Interestingly, vWF molecules occur in normal plasma as long flexible strands, yet appear to be present on the platelet surface as discrete masses. Although the effect of fixation and dehydration procedures on vWF is unknown, conformation changes associated with the binding of the multimers to platelet surface receptors cannot be excluded. It has been shown that ristocetin lowers the negative charge of the platelets and it has been proposed that this reduces the natural electrostatic repulsion between vWF and the platelets. However, others have suggested that ristocetin also interacts directly with the vWF multimers. In the circulation, it is now known that modifications of vWF conformation resulting from its binding to the subendothelium are responsible for the exposure (and therefore increased accessibility) of the determinant(s) responsible for its interaction with GPIb in platelet adhesion. The type of image seen in Fig 1c, where vWF can be clearly seen to be cross-linking agglutinating platelets, may represent an analogous situation to that in the vessel wall, where the alternative surface is provided by the subendothelium.

While the binding of vWF to GPIb leads to platelet agglutination, studies with asialo-vWF or purified type IIB vWF suggest that this binding can also lead to platelet activation and the association of vWF with other adhesive proteins to GPIb-IIIa. This process, which leads to platelet aggregation, is apparently dependent on platelet-platelet contact. In our studies, where incubations were performed without stirring, vWF binding to GPIb per se did not lead to detectable platelet activation. This was apparent from the morphology of the platelets that lacked pseudopods or signs of granule secretion (see Fig 1). Studies on the state of activation of the platelets of the patient with type IIB von Willebrand disease will be presented elsewhere (P. Hourdilé, A. Derlon, manuscript in preparation). It should also be noted that our studies using washed platelets were performed at 37°C in a medium containing EDTA, conditions in which GPIb-IIIa complexes dissociate and are unable to bind adhesive proteins. Thus, any feedback mechanism of platelet activation involving adhesive protein binding to GPIIb-IIIa cannot occur. With unstimulated platelets in Tyrode-EDTA, immunogold staining for vWF on the platelet surface was low, although not absent, and flow cytometry consistently showed a small population of platelets that reacted positively with the rabbit anti-vWF antibody. The presence or absence of basal levels of vWF on unstimulated platelets is controversial. We do not know the source of the vWF that we were locating. One possibility is that this represented vWF that bound during bleeding and/or the preparation of washed platelets. Another possibility is that this represents a small proportion of vWF that leaked from α-granules during the incubation of platelets at 37°C. Whatever its source, the amount of vWF located is too small to affect the major thrust of our studies.

We have recently described how thrombin induces the translocation of GPIb-IX complexes from the platelet surface to the membranes of the SCCS. This process occurred in both Tyrode-Ca2+ and Tyrode-EDTA, although it was more readily apparent in the latter buffer. Our observation explained previous findings of how the binding of different MoAbs to GPIb-IX is reduced when platelets have been pretreated with thrombin. Studies by George and Torres have indicated that this process may have a physiologic significance for thrombin-treated platelets were shown to have a diminished vWF-dependent agglutinability with ristocetin. While confirming the studies of George and Torres, we have now described how vWF bound to GPIb is also cleared from the platelet surface when platelets are stimulated with thrombin. Binding of vWF to GPIb in the presence of ristocetin did not modify the internal organization of the platelets. However, when these platelets were then stimulated with α-thrombin, the α-granules disappeared as secretory vesicles formed and secretion occurred. However, in contrast with platelets incubated with thrombin alone, staining for vWF within the SCMS increased with time. This result resembled very closely the result we have reported for GPIb-IX complexes.
pears, therefore, that ligands bound to GPIb-IX are translocated into the SCCS together with the free complexes.

The SCCS represents a complex system of channels that penetrate and form a network within the interior of the platelet.\textsuperscript{15,49} While it is generally accepted that this network serves as a secretory pathway for granule contents after activation, it has also been shown that foreign material fixed to the platelet membrane is cleared to the SCCS. This has been shown, for example, for both latex particles and cationized ferritin.\textsuperscript{58-60} In our experiments, vWF bound to GPIb was not seen to be spontaneously removed from the periphery during the 30-minute incubation period of this study. Thus, vWF binding to GPIb did not in itself lead to clearance. The phenomenon was seen only after platelets were activated by thrombin. Immunogold staining of Lowicryl-fixed sections suggested that the process was progressive. After 1 minute, the staining was most abundant in the peripheral SCCS channels, while after 10 minutes staining extended well into the central part of the platelet. The exact mechanism involved in the clearance of surface-bound vWF remains to be elucidated. However, an involvement of the cytoskeleton is thought probable as the movement of GPIb-IX complexes is inhibited by cytochalasins that disrupt actin filaments.\textsuperscript{13,15} It should be emphasized that the process appears to differ from that observed for fibrinogen-bound to GPIb-IIIa complexes,\textsuperscript{51,52} where only occupied complexes appear to be cleared to the SCCS. In our studies, both free and vWF-bound GPIb-IX complexes behaved similarly. However, the situation remains puzzling, for platelet stimulation by thrombin is followed by an augmentation of the number of GPIb-IIIa complexes after secretion and the externalization of the internal pools of this receptor.\textsuperscript{53,54} Movements of membrane glycoproteins seem to be occurring in opposite directions.

Thrombin-induced internalization of GPIb-IX complexes and of vWF bound to such complexes represents a potential regulatory mechanism of platelet adhesivity. It may also represent a mechanism whereby platelets that have been stimulated with thrombin, and as a result discharged their α-granule contents, no longer react with the subendothelium in platelet adhesion reactions. Further studies are planned to determine to what extent such a mechanism occurs in vivo and whether the phenomenon is reversible.

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von Willebrand factor bound to glycoprotein Ib is cleared from the platelet surface after platelet activation by thrombin

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