Assembly and GPIIIa Content of Cytoskeletal Core in Platelets Agglutinated With Bovine von Willebrand Factor

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The association between occupancy of the von Willebrand factor (vWF) receptor glycoprotein (GP) Ib, agglutination, and the assembly and composition of the cytoskeletal core was studied in 125I-surface-labeled aspirin-treated washed platelets. Binding of ligands to GPIIb-IIIa and platelet aggregation were abolished by addition of EDTA. Platelet agglutination induced by bovine vWF generated a complete cytoskeletal core (Triton-insoluble residue), shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to be composed of actin-binding protein (ABP) (260 Kd), 235-Kd protein, myosin heavy chain (200 Kd), α-actinin (100 Kd), and actin (43 Kd). In addition, autoradiography of the gels showed a 125I-105-Kd GP, identified by immunoblot as GPIIIa, as well as GPIIb, GPIb, and another band at 87 Kd, probably GPV. Neither cytoskeletal assembly nor GPIIIa incorporation was altered if calpain was inhibited with leupeptin. Platelet suspensions exposed to bovine vWF without stirring (ie, nonagglutinated) or platelets in which agglutination was inhibited with ADP showed smaller cytoskeletons with little ABP, 235 Kd protein, and α-actinin. Autoradiographs showed mainly GPIb. Cytochalasin D (CD) and monobromobimane (MB) enhanced agglutination and prevented the inhibitory action of ADP on bovine vWF-induced platelet agglutination. CD markedly inhibited the assembly of the cytoskeletal core as well as GPIIIa retention, whereas MB resulted in a large Triton-insoluble residue which contained GPIIIa. Thus, development of a platelet cytoskeletal core is apparently not required for agglutination, but when a cytoskeletal core is assembled in agglutinated platelets, GPIIIa is retained.

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plastic tubing to a syringe containing one-ninth volume of 0.109 mol/L sodium citrate. Platelet-rich plasma (PRP) was separated and then incubated for 30 minutes with one-tenth volume of 1 mmol/L ASA. Platelets were collected by centrifugation and washed as previously described except that the final suspension medium was buffered to pH 7.4 with 10 mmol/L HEPES (N-2-hydroxyethylpiperazine-N'2 ethane sulfonic acid) and contained 0.1 mg/mL apyrase, 2 mg/mL bovine serum albumin, and no added Ca++. For some studies, a portion of the suspension at pH 6.5 containing 1 x 10^9 platelets was spun, resuspended in 1 mL buffer without apyrase or albumin, and labeled with 125I using lactoperoxidase. The labeled platelets were then diluted in HEPES-Tyrode solution with albumin, and 5 mmol/L EDTA, added to the unlabeled once washed platelets, centrifuged, and washed twice with this solution. Labeled platelets represented approximately 5% of the total number.

Platelet agglutination studies and preparation of the cytoskeletal core. Platelet agglutination in 0.6 mL ASA-treated washed platelet suspension (0.6 x 10^9/mL) was recorded with 5 mmol/L EDTA added at 37°C with an aggregometer (Payton Associates, Buffalo, NY, or Chrono-Log, Haverton, PA). The platelets were incubated with either 10 pmol/L ADP or saline for 1 minute before addition of 140 μL of the fibrinogen solution (9 μg bovine vWF). Inhibition was expressed as the percentage of decrease in the steepest slope of the agglutination curve. In experiments in which MB (250 μmol/L) or CD (10 μmol/L) was used to prevent ADP-induced inhibition, the reagents were added 1 minute before ADP was added. Two minutes after vWF was added, the suspension was mixed and vortexed with an equal volume of 2% Triton X-100, 100 mmol/L Tris, 10 mmol/L EGTA, and, in some experiments, 20 μmol/L leupeptin and 0.5 mmol/L PMSF, and allowed to stand in ice for 10 minutes. The suspension was spun in an Eppendorf centrifuge (12,000 x g) for 3 to 5 minutes with or without 50 mmol/L dithiothreitol and frozen.

SDS-PAGE. Samples were electrophoresed according to the method of Laemmli on 7.5% polyacrylamide gels with 4.5% polyacrylamide in the stacking gel, cast on GelBond film, and stained with Coomasie brilliant blue. Gels containing 125I-labeled platelets were dried and exposed for autoradiography. Each gel represents results in the Triton-insoluble residues from a single experiment, and equal volumes of each sample were applied to each lane. Each experiment was performed at least five times. Scanning densitometry (EC Densitometer, EC Apparatus, St Petersburg, FL, attached to a Hewlett Packard HP3396A Integrator, Purchase, NY) was used to estimate the proportions of cytoskeletal proteins in each lane of the gel.

Immunoblotting. Immunoblots were performed in the laboratory of Dr Simon Karpatkin. Unreduced proteins were separated by 10% SDS-PAGE, transferred electrophoretically to unmodified nitrocellulose paper, and were detected first with a human MoAb to Plaα, an antigen located on GPIIIa, and then with an alkaline phosphatase-labeled γ-chain-specific anti-human IgG antibody.

RESULTS

Formation of a cytoskeletal core in washed 125I-labeled platelets agglutinated with vWF. Association of GPIIIa with the core. Agglutination was induced in these studies with bovine vWF instead of human vWF plus ristocetin because addition of ristocetin after Triton X-100 resulted in nonspecific precipitation of cytoskeletal proteins.

ASA-treated platelets agglutinated when stirred with bovine vWF and 5 mmol/L EDTA (Fig 1A). The presence of leupeptin (260 μmol/L) did not influence the slope of agglutination.

Cytoskeletons made from platelets agglutinated with bovine vWF alone or with leupeptin added contained a well-developed cytoskeletal core consisting of ABP (260 Kd), a small band at 235 Kd (presumably talin), myosin heavy chain (200 Kd), a 100-Kd protein probably representing α-actinin, and actin (43 Kd) (Fig 2A, lanes c and d). Comparison of the amount of protein in cytoskeletons made from 7.2 x 10^9 platelets (Fig 2, lane c) with the total amount of protein in 5.4 x 10^7 platelets (Fig 2, lanes a and b) indicates that the cytoskeleton consists of only a small portion of the total platelet protein. The proportion of each cytoskeletal protein in vWF-agglutinated samples was estimated from scans of the gels (Fig 3). The total area of integration (measured as counts; one count equals 0.125 μV-s) was 5.2 x 10^6 ± 0.33 (±SE) (n = 9). Myosin and actin were the most abundant proteins, representing 10.3% ± 1.5% (±SE) and 21.7% ± 4.2% (±SE), respectively. ABP and α-actinin were present in approximately equal amounts [6.94% ± 0.65 (±SE) and 7.1% ± 0.99 (±SE)], and 235-Kd protein formed an even smaller portion of the Triton-insoluble residue [1.35% ± 0.08 (±SE)]. The three bands

![Fig 1. Effects of monobromobimane (MB) (250 μmol/L), ADP (10 μmol/L), and MoAb 328 (11 μg) on the response of stirred ASA-treated washed platelets to bovine vWF (B-vWF). EDTA (6 mmol/L) was added to 0.6 mL platelets 30 seconds before the first reagent, B-vWF (9 μg) added after EDTA caused agglutination (A); ADP 1 minute before B-vWF inhibited agglutination (B); MoAb 328 2 minutes after B-vWF reversed agglutination (C); MB before ADP prevented ADP-induced inhibition and enhanced agglutination (D).](www.bloodjournal.org)
observed below 68 Kd in Fig 2, lanes c through f and h through j are not fibrinogen because their migration differs from that of authentic fibrinogen (data not shown).

Triton-insoluble residues made from unstirred EDTA-treated platelets with bovine vWF added were about half the size of those made from stirred samples. The major constituents again were the heavy chain of myosin and actin (Fig 2, lane h). The percentage of ABP was only about 35% of that in the cores of agglutinated platelets, and 235-Kd protein and α-actinin were not evident.

Four labeled membrane GPs were selectively associated with the cytoskeletal core of agglutinated platelets (Fig 2B, lanes c and d). Their molecular weights (mol wt), estimated from scans of the autoradiographs, were 143, 132, 105, and 87 Kd, under reduced conditions, and 170, 143, 114, and 87 Kd in unreduced samples (not shown). These values correspond to the reported mol wt of GPlbI, IIb, IIIa, and IV, respectively. The identity of GPlIla was confirmed by immunoblotting (Fig 4, lane d). The proportion of these GPs varied in different experiments. When calculated from the total area of integration of autoradiographs in five experiments, it was 19.5% ± 4.26 (±SE) for GPlb; 11.5% ± 2.46 (±SE) for GPIIb; 33.2% ± 5.6 (±SE) for GPlIla; and 11.8% ± 1.5 (±SE) for GPIV. The cytoskeleton of unstirred vWF-treated platelets showed mainly GPlb (Fig 2B, lane h).

Agglutination induced by bovine vWF was completely reversed on addition at 2 min of 11 μg MoAb 328 (Fig 1C), a MoAb raised against the epitope on human vWF that binds to GPlb. SDS-PAGE showed that the cytoskeletal core was disassembled at 4 minutes (Fig 5, lane d) as compared with
The amount of ABP incorporated into the cytoskeletal core correlated with the presence or absence of GPIIIa and with the degree of ADP-induced inhibition; e.g., in an experiment in which ADP inhibited agglutination by 65%, ABP was decreased 25% and GPIIIa was reduced 33%. When agglutination was inhibited by 85%, ABP was decreased 76%, with almost zero retention of GPIIIa in the Triton-insoluble residue.

Preincubation of platelets for 1 minute with 250 μmol/L MB or 10 μmol/L CD enhanced bovine vWF-induced agglutination (not shown) and prevented the inhibitory effect of ADP (Fig 1D). MB increased the amount of cytoskeletal protein and GPIIIa in platelets agglutinated after addition of ADP (Fig 6A and B, lanes f and g) or without it (Fig 7A and B, lanes c and d). In contrast, CD substantially decreased the amount of cytoskeletal protein and GPIIIa in platelets agglutinated with ADP (Fig 5A and B, lane h) or without it (Fig 5A and B, lane c and Fig 7A and B, lane b).

DISCUSSION

We and other researchers have shown that unstimulated platelets have a small Triton-insoluble residue composed mainly of actin. Aggregating agents induce the formation of a larger cytoskeleton and cause incorporation of the fibrinogen receptor GPIIb-IIIa into the cytoskeleton of an areaated platelet. Little attention has been paid to determining whether occupancy of the platelet vWF receptor GPIb or agglutination results in the assembly of cytoskeleton, however.
Bovine vWF binds to GPIb on human platelets in the absence of ristocetin.\textsuperscript{20,22} Our present studies show that the Triton-insoluble residue or cytoskeleton of unstirred samples prepared with bovine vWF is composed mainly of myosin and actin. The amount of ABP is minimal and the 235-Kd protein and \( \alpha \)-actinin are not evident. The large amount of myosin is attributable to the presence of EDTA, which causes rigor bonds to form between myosin and actin.\textsuperscript{12} The only GP incorporated into the cytoskeletal core of unstirred samples is GPIb. Other researchers showed that ABP is complexed with GPIb in both Triton-soluble and Triton-insoluble fractions of resting platelets.\textsuperscript{5,13}

Stirring labeled platelets with bovine vWF induces agglutination and the assembly of a larger cytoskeleton. The amount of actin and myosin in Triton-insoluble residues is about the same as in unstirred samples, but the amounts of ABP, 235-Kd protein, and \( \alpha \)-actinin are markedly increased. Addition of MoAb 328 reverses agglutination as well as assembly of the cytoskeleton. Although MoAb 328 is made against the GPIb-binding domain of human vWF,\textsuperscript{31} it inhibits agglutination induced by bovine vWF, suggesting that this portion of the vWF molecule is very similar in both species.

The cytoskeleton of agglutinated platelets contained not only GPIb but also the GPIb-IIIa complex and a 87-Kd GP, probably GPIV. Thus, GPIIIa is incorporated into the cytoskeleton during agglutination, even though it presumably has no role in this response. Because in these experi-
Plates, EDTA prevented occupancy of GPIIb/IIIa by vWF or fibrinogen, occupancy of that receptor is not necessary for its incorporation into the cytoskeleton.

The association between GPIIb and the cytoskeleton is accompanied by an increase in cytoskeletal ABP, 235-Kd protein, and a-actinin. Because only 20% of the total ABP pool is associated with GPIb, a portion of the remaining ABP pool, either as such or modified, is available to react with other GPs. An association between ABP and GPIIb-IIIa has been noted previously, but the underlying mechanism is not known. ABP phosphorylation was proposed as a mechanism for modulating its affinity for actin, and a similar mechanism may operate to bind it to GPIIb-IIIa.

The effects of the fungal metabolite CD and the thiol reagent MB were studied. The cytochalasins inhibit actin polymerization in platelets and decrease cytoskeletal size, whereas MB, which binds to the thiol group of cytoskeletal proteins, increases the amount of the cytoskeleton. Both CD and MB increased vWF-induced platelet agglutination, but had different effects on the size of the Triton-insoluble...
residue and retention of GPIIIa: CD disassembled the cytoskeletal core with loss of GPIIIa retention, whereas MB increased the amount of cytoskeleton, especially ABP and 235-Kd protein, and enhanced retention of GPIIIa. Wheeler et al. observed that cytochalasin E decreased the size of the cytoskeleton and the incorporation of GPIIIa in platelets aggregated with phorbol myristate acetate. Because CD enhances agglutination and does not inhibit aggregation, it appears that formation of a large cytoskeleton containing GPIIIa is not necessarily associated with either agglutination or aggregation. Furthermore, the absence of Triton-insoluble GPIIIa despite the marked agglutination indicates that the association of GPIIIa with the cytoskeleton of agglutinated platelets is not an artifactual result of trapping of unlysed membranes in the large clumps.

Pretreatment of platelets with ADP inhibits agglutination induced by bovine vWf or human vWf plus ristocetin. The mechanism of this inhibition is unclear, as ADP does not decrease the amount of multimer size of platelet-bound vWf. Inhibition is accompanied by decreased incorporation into the cytoskeleton of ABP, 235-Kd protein, and α-actinin. This decrease does not result from proteolysis of these calpain-sensitive proteins, because it was not prevented by leupeptin. Both MB and CD reverse or prevent ADP-induced inhibition of platelet agglutination, but, as in the absence of ADP, MB enhances cytoskeletal assembly and GPIIIa retention, whereas CD has the opposite effect.

The present studies establish that platelet agglutination induced by vWf is not a passive event because it is usually accompanied by changes in the organization and assembly of the cytoskeletal components, as well as in their association with GPIIIa and other GPs.

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