RAPID COMMUNICATION

Differential Redistribution of Platelet Glycoproteins Ib and IIb-IIIa After Plasmin Stimulation

By Elisabeth M. Cramer, He Lu, Jacques P. Caen, Claudine Soria, Michael C. Berndt, and Danièle Tenza

The subcellular localization of the platelet membrane receptors glycoproteins (GP) Ib and IIb/IIIa has been studied within resting platelets by a combination of biochemical and cytochemical techniques. While both GPIb and GPIIb/IIIa are localized within the plasma membrane and surface-connected canalicular system (SCCS) membranes, only GPIb/IIIa is present within the internal face of α-granular membranes. Previous studies demonstrated that plasmin can induce platelet stimulation and also decrease ristocetin-induced platelet aggregation; it was suggested that this was because of GPIb degradation by plasmin. In this study, the respective localizations of both GPIb and GPIIb/IIIa were visualized during in vitro plasmin stimulation of platelets. Generally, plasmin induced shape change, pseudopod formation, organelle centralization either with or without α-granule release depending on the conditions of stimulation. Plasmin treatment of platelets at 37°C resulted in the disappearance of GPIb from the cell surface and its subsequent redistribution into the channels and vesicles of the SCCS with no significant modification of GPIIb/IIIa remaining on the plasma membrane. Within degranulated platelets, GPIb/IIIa was expressed on the plasma membrane and within membranes of large vacuoles containing the α-granule proteins. GPIb was virtually absent from these structures and mainly restricted to the SCCS. Addition of cytochalasin D inhibited the migration of GPIb to the SCCS. Biochemical measurements confirmed that no important hydrolysis of GPIb had occurred because only very little amounts of glycocalcin were generated during the reaction. In conclusion, in plasmin-treated platelets GPIb/IIIa is externalized to the plasma membrane while GPIb is internalized into the SCCS. Although previous studies have suggested that plasmin degrades GPIb, the reduction in ristocetin-induced aggregation may be explained by its apparent redistribution within the membranes of the SCCS.

© 1991 by The American Society of Hematology.

MATERIALS AND METHODS

Platelets

Venous blood was collected into plastic tubes containing ACD buffer (6.8 mmol/L citric acid, 11.2 mmol/L trisodium citrate, 24 mmol/L glucose). The platelet-rich plasma (PRP) was obtained by centrifugation at 180g for 10 minutes at room temperature. The isolated platelets were obtained by further centrifugation of PRP at 1,100g for 10 minutes at room temperature and washed three times with the washing buffer (36 mmol/L citric acid, 5 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 103 mmol/L NaCl, 5 mmol/L glucose, pH 7.4) containing 3.5 mg/mL bovine albumin (Sigma Chemical Co, St Louis, MO), 7 ng/mL prostaglandin E₁ (Sigma), and 25 µg/mL apyrase (Sigma). The washed platelets were resuspended in Tyrode’s buffer containing 2.9 mmol/L CaCl₂. Washed platelets were exposed to 0.2 CU/mL plasmin (Kabi Laboratory, Stockholm, Sweden) or buffer for control at 37°C or at 22°C for 5 minutes, conditions that were associated to a decrease in ristocetin-induced platelet agglutination. The reaction was performed with or without cytochalasin D 25 mmol/L (Sigma).

Electron Microscopy

The reaction was stopped and the platelets fixed by 1% glutaraldehyde in Tyrode buffer, washed three times with Phosphate buffer 0.1 mol/L pH 7.4 and embedded in glycol methacrylate for immunoelectron microscopy. Immunostaining for GPIb and GPIIb/IIIa was performed on thin sections as described previously. The anti-GPIb antibody was raised against GPIb IX complex and affinity purified against glycocalcin to render it α chain specific. The preparation of the anti-GPIb/IIIa antibody was described.
previously. The electron dense probe was goat antirabbit IgG coupled to 10 nm colloidal gold (Janssen Laboratories, Beerse, Belgium). The sections were observed in an electron microscope.

Biochemical Study

The amount of platelet glycocalicin was measured before and after plasmin treatment, both in cell lysates and supernatants. The release of platelet glycocalicin was measured in the supernatant of plasmin-treated platelets. Glycocalicin was expressed in percent of total glycocalicin platelet content of control autologous platelet lysate.

Preparation of Platelets Lysate

Washed platelets were incubated for 30 minutes at 37°C with 1% Triton X100 in 0.05 mol/L Tris, 0.1 mol/L glycine and 5 mmol/L calcium chloride, pH 8.7, and then centrifuged at 100,000g for 45 minutes. As suggested by George and Torres, solubilization in the presence of calcium at 37°C was performed to allow the activation of the endogenous calcium-dependent protease, which hydrolyses actin-binding protein to release GPIb from the triton-insoluble cytoskeleton.

Preparation of the Plasmin-Treated Platelets Supernatants

Washed platelets, 5 x 10^8/mL were incubated at 37°C for 6 minutes with 0.2 CU/mL plasmin. After incubation plasmin action was stopped by aprotinin addition (50 μmol/L) (Bayer Pharma, Puteaux, France), and then immediately centrifuged as above.

Glycocalicin was also determined in the plasmin-treated platelets. After incubation with plasmin the platelet pellets were washed twice to remove trapped released GPIb and then the pellets were solubilized as described above. The results are expressed as a percentage of solubilized untreated platelets.

Glycocalicin Enzyme-Linked Immunosorbent Assay (ELISA) Assay

ELISA microplates were firstly coated with 200 μL of 10 μg/mL of IgG isolated from rabbit antiserum antihuman glycocalicin in 0.05 mol/L bicarbonate buffer, pH 9.5 for 2 hours at 37°C. Two hundred microliters of diluted samples in dilution buffer (1:5) (Tyrode buffer containing 0.05% tween and 1% bovine serum albumin) were incubated with coated wells for 2 hours at 37°C. Immunoadsorbed glycocalicin was assayed with biotinylated SZ-2 monoclonal antibody (MoAb) against GPIb (Immunotech, Marseille, France) used at 2 μg/mL in dilution buffer. After 2 hours of incubation with biotinylated SZ-2, the plates were washed three times and then incubated for another 2 hours with peroxidase-streptavidin (Amersham, UK) diluted 1/500 in dilution buffer. After three washes, peroxidase bound to the plates was quantified with 0-phenylene diamine.

In these conditions a linear standard curve was established from control untreated platelet lysate between 5 x 10^6 platelets/mL. The ELISA method used for this study had a sensitivity that allowed the detection of the amount of glycocalicin contained in 5 x 10^6 platelets/mL glycocalicin from samples containing 5 x 10^8 platelets/mL. Thus, the threshold of detection is 0.1% of total glycocalicin.

Electrophoretic Blotting

The supernatants and platelet lysates from 5 x 10^8 platelets/mL prepared as described for ELISA test were electrophoresed under nonreduced condition on a 6.5% acrylamide gel and transferred electrically to a nitrocellulose sheet. The binding to the blot of the primary polyclonal antiglycocalicin antibody was detected with 125I-labeled protein A.

RESULTS

Immunoelectron Microscopy

Resting Platelets

GPIb. On resting platelets, GPIb immunolabeling was mainly found on the outside face of the plasma membrane where the larger part of the molecule is localized. The channels of the surface-connected canalicular system (SCCS) appeared to be more weakly labeled, gold particles being often distributed as clusters. Other platelet organelles displayed no labeling, including α-granules and mitochondria (Fig 1).

GPIb/IIa. GPIb/IIa had the same distribution on resting platelet membranes as GPIb, and was also present on the α-granule membrane, outlining their inner wall, as described earlier (Fig 2).

Plasmin-Stimulated Platelets

GPIb. After treatment with plasmin at 37°C, the platelets changed shape, with emission of pseudopods and organelle centralization. GPIb immunolabeling disappeared from the cell surface but was found in the prominent channels of the SCCS (Fig 3).

When degranulation had occurred, mainly when platelets were treated by plasmin at low temperature (22°C) as described previously, GPIb was also found to be absent from the plasma membrane and to be redistributed into the channels from the SCCS. It was virtually absent from the limiting membrane in the area of granule fusion, formed mainly by the α-granule membrane (Fig 4).

GPIb/IIa. As a control for the immunogold staining technique, after platelet stimulation by plasmin at 37°C
GPIIb/IIIa immunolabeling remained unchanged on the plasma membrane. It was still scant in the SCCS and unchanged on the α-granule membrane. However, platelets had undergone shape change and organelle centralization (Fig 5).

When α-granule release had occurred the expression of GPIIb/IIIa on the plasma membrane was much increased in contrast to the one of GPIb and was also intense on the limiting wall of degranulation ponds, where α-granules had fused. Superficial channels of SCCS were still poorly labeled (Fig 6).

**Plasmin and Cytochalasin D**

Finally, when cytochalasin D, which is known to disrupt the polymerization of actin filaments, was applied to platelets before plasmin stimulation (either at 37°C or 22°C) migration of GPIb was impaired and no internalisation of GPIb was observed; the labelling for GPIb remained

---

**Fig. 2.** Section of a resting platelet labeled for GPIIb/IIIa by immunogold. Labeling outlines the plasma membrane (arrowheads) and SCCS as well as the innerface of α-granule wall (A). (Original magnification ×26,150)

**Fig. 4.** When plasmin stimulation of platelets is associated with granule release, GPIb is mainly cleared from the plasma membrane (arrowheads) and is redistributed to the superficial channels of the SCCS. Degranulation ponds limiting membranes (D) do not display labeling for GPIb. α-granules have disappeared. (Original magnification ×26,150)

**Fig. 3.** Plasmin stimulation of platelets performed at 37°C induced shape change and pseudopod formation as well as granule centralization. Immunolabeling for GPIb shows that it is virtually cleared from the plasma membrane (arrowheads) and that it is redistributed into the channels of the SCCS. α-granules (A) are negative. (Original magnification ×26,150)

**Fig. 5.** After plasmin treatment of platelets, GPIIb/IIIa immunolabeling remains localized on the plasma membrane (arrowheads). The SCCS is faintly labeled. α-granules (A) have strong immunolabeling. (Original magnification ×26,150)
REDISTRIBUTION OF GPIb AND IIb-IIIa BY PLASMIN

Table 1. ELISA Assay of Platelet Glycocalicin Before and After Plasmin Treatment

<table>
<thead>
<tr>
<th>Agents added</th>
<th>Supernatants % (mean ± SD)</th>
<th>Platelet Lysates % (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmin 0.2 CU/mL</td>
<td>2.47 ± 0.03</td>
<td>104.31 ± 5.42</td>
</tr>
<tr>
<td>Control buffer</td>
<td>2.22 ± 0.05</td>
<td>100</td>
</tr>
</tbody>
</table>

The amount of glycocalicin released in the medium after plasmin action on platelets was only minimal (less than 3% of total) and not different from control samples, confirming that no degradation of GPIb had occurred before the bulk of GPIb being moved to the open canalicular system. These percentages are expressed in comparison with the initial total glycocalicin content of untreated patients. N = 4.

**DISCUSSION**

GPIb binding to vWF associated with exposed subendothelium is a major step in platelet adhesion. This function is reflected in vitro by vWF-mediated platelet agglutination. GPIb has previously been reported to be sensitive to degradation by several proteinases.8,11 Indeed, in vivo studies indicated that GPIb function was impaired during thrombolytic therapy and that this impairment was caused by degradation of GPIb by generation of plasmin.11 In this report, we present evidence that the defective agglutination of plasmin-treated platelets in the presence of vWF is not related to GPIb cleavage, because less than 3% of total glycocalicin was found in the supernatant of plasmin treated platelets, but to a redistribution of GPIb, which moves from the external membrane into the SCCS, preventing its interaction with plasma vWF. Indeed, platelet agglutination is mediated by surface-associated GPIb, but the intracellular pool would be ineffective for cellular interaction. This phenomenon is related to platelet structural

**Biochemical Study**

Elisa assay showed only a small amount of released glycocalicin when platelets were exposed to plasmin for a short time (Table 1). Most of the GPIb remained associated with platelets. Similar result was obtained with immunoblotting method. A time-dependent release of glycocalicin in the supernatants was observed. However, this release was not severe enough to cause a substantial decrease in the amount of platelet-associated GPIb (Fig 8).

![Fig. 6 Using the same conditions as in Fig 4, immunolabeling for GPIIIa increases on the plasma membrane (arrowheads) and in the channels of SCCS were degranulation has taken place (D). Scant labeling only is seen in superficial channels of SCCS. (Original magnification ×26,150)](image)

virtually unchanged at the surface of the plasma membrane. Platelet shape change was also blocked by cytochalasin D (Fig 7).

This last experiment led to the conclusion that only minimal degradation of GPIb was enhanced by plasmin.

![Fig. 7. When platelets are treated with cytochalasin D before plasmin, shape change and GPIIIa redistribution are prevented; plasma membrane immunolabeling for GPIb is virtually identical to the one of resting platelets, demonstrating that no GPIb degradation has occurred (A = α-granule). (Original magnification ×26,150)](image)

![Fig. 8. Immunoblot of GPIb in platelets lysates and in supernatants before and after plasmin treatment. The autoradiogram was exposed for 7 hours. Thirty microliters of platelet lysate or supernatant from 5 × 10^6 platelets/mL were electrophoresed, and detected with polyclonal anti-glycocalicin antibody. Lane 1, control platelet lysate. Lanes 2 through 4, lysates of platelets treated with 0.2 CU/mL plasmin for (lane 2) 6 minutes, (lane 3) 15 minutes, and (lane 4) 30 minutes. Lane 5, supernatant of control platelets. Lanes 6 through 8, supernatants of the platelets treated with plasmin for (lane 6) 6 minutes (lane 7) 15 minutes, and (lane 8) 30 minutes. Arrow = GPIb. Arrowhead = glycocalicin.)](image)
change characterized by the invagination of the cell wall and fenestration of SCCS, which remains patent.  

GPIb is a transmembrane GP attached to actin-binding protein that connects GPIb to submembrane actin filaments. The polymerization of actin filaments has been found to regulate the platelet shape changes and platelet glycoprotein functions in response to stimuli. We have shown that the translocation of GPIb from the plasma membrane into the channels of the SCCS is related to actin polymerization. Cytochalasin D, a specific inhibitor of actin polymerization, inhibits ultrastructural modification induced by plasmin, such as shape change and centralization of granules. We have now demonstrated that cytochalasin D inhibits the migration of GPIb after plasmin stimulation. This redistribution of GPIb can be compared with the redistribution of the protein, talin, after platelet stimulation. Indeed, in our observation, in the presence of cytochalasin D immunolabeling for GPIb remains unchanged on the plasma membrane after plasmin treatment. In the work of Adelman et al degradation of GPIb was observed after longer incubation with plasmin (up to 90 minutes), where endogenous proteases could be responsible for proteolysis. Indeed, in our work we only studied a short time of stimulation, i.e., 6 minutes, when vWF-mediated agglutination of platelets is initially decreased. Under these conditions, platelet lysis is unlikely, as demonstrated by morphologic examination. These authors described the release of a glycoepicin-like glycopeptide. However, for our immunocytochemical study, we used an antibody affinity-purified against glycoepicin, and, indeed, we could demonstrate the apparition of an intracellular pool of GPIb (glycoepicin) after plasmin stimulation. A reduction in external GPIb was also observed after stimulation of platelets by thrombin. In this case, we (personal observation) and others could observe that the reduction in GPIb expression of platelet surface after thrombin stimulation was also mainly caused by intracellular translocation. In parallel, no removal of GPIIb/IIIa from the plasma membrane to the SCCS was observed on spread and suspended platelets.  

ACKNOWLEDGMENT

The authors acknowledge A. Higgs for editing the English, Dr G.F. Savidge for valuable discussion, J.M. Mass for photographic assistance, and R. Quintel for typing the manuscript.

REFERENCES

19. Stenberg PE, Shuman MA, Levine SP, Bainton DF: Redis-


Differential redistribution of platelet glycoproteins Ib and IIb-IIIa after plasmin stimulation [published erratum appears in Blood 1991 Jul 15;78(2):545]

EM Cramer, H Lu, JP Caen, C Soria, MC Berndt and D Tenza