Glycoprotein V Is Not the Thrombin Activation Receptor on Human Blood Platelets

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Thrombin activation of platelets involves two receptors: glycoprotein Ib (GPIb), which affects the kinetics of the response; and, as a strong candidate for the second, essential receptor, GPV, a hydrophobic, 82-kd glycoprotein with an isoelectric point (pI) of pH 5.85 to 6.55. Whole platelets were treated with endogenous platelet calcium-activated proteases, yielding a major fragment, GPV, with molecular weight (mol wt) of 79 kilodaltons (kd). The fragment was purified by affinity chromatography on wheat germ agglutinin followed by ion exchange chromatography on DEAE-Sephacel using first a 0 to 0.7-mol/L NaCl gradient. A rabbit was immunized with the purified GPV, for preparation of polyclonal antibodies. Crossed immunoelectrophoresis and two-dimensional polyacrylamide gel electrophoresis (PAGE) electrophoretic blotting with the separate phases of a Triton X-114 phase partition of human platelets showed the characteristic pattern of GPV in the hydrophobic phase. During thrombin-induced platelet aggregation GPV is hydrolysed, releasing a fragment, 

where GPV hydrolysis is normal. Most of GPV (plus several other glycoproteins) can be removed with chymotrypsin or endogenous platelet calcium-dependent proteases without affecting the extent of platelet activation, only the rate of activation is altered. These results cast doubt on the role of GPV as the essential thrombin receptor on platelets but it might still participate in postreceptor signal processing.

To clarify the function of GPV in platelet activation by thrombin, we have purified GPV, a fragment of GPV produced by cleavage with platelet calcium-activated proteases, and produced antibodies against it. We have examined the influence of the antibodies bound to GPV on proteolysis of GPV and activation of the platelets. Our results lead to the conclusion that proteolysis of GPV by thrombin is not essential for platelet activation and that therefore GPV is not the thrombin activation receptor on human platelets.

MATERIALS AND METHODS

Isolation of human blood platelets. Platelets were isolated from citrate-treated blood collected for the Central Laboratory of the Swiss Red Cross, within 20 hours after collection. The buffy coats were transferred into a buffered glucose solution to yield platelet-rich plasma containing about 20 mmol/L glucose, 12 mmol/L sodium phosphate buffer, and about 4×10

The platelets were isolated by centrifugation and were washed twice with 12 mmol/L sodium citrate, 30 mmol/L glucose, 120 mmol/L NaCl, and 10 mmol/L EDTA buffer, pH 6.5, and once with 10 mmol/L Tris/HCl, 150 mmol/L NaCl, and 10 mmol/L EDTA buffer, pH 7.4.

Isolation of glycoprotein V fragment (GPV). Washed platelets were suspended in 0.14 mol/L NaCl, 5 mmol/L CaCl,

and 10 mmol/L Mes/NaOH, pH 6.5. One quarter of this suspension was cooled to 4°C and sonicated with a B-30 sonifier (Branson Sonic Power, Danbury, Conn) for two minutes (output control, 7; 50% duty cycle, pulsed mode). The sonicated platelets as a crude source of calcium-activated proteases were added back to the rest of the platelet suspension which was left for 20 minutes at 37°C. The calcium concentration was previously adjusted to 20 mmol/L with CaCl solution. To prevent further degradation, phenylmethylsulfonyl fluoride (PMSF) in methanol and N-ethylmaleimide (both 2 mmol/L final concentration) were added and the suspension centrifuged at 100,000g for one hour. The supernatant, to which EDTA (5 mmol/L final concentration) was added, was applied to a wheat
germ agglutinin-Sepharose 4B column (Medac, Hamburg, FRG) and the bound material eluted with 2.5% N-acetylglucosamine.\(^1\)

Bound GPV was separated from glycolacticin by chromatography on DEAE-Sephacel (Pharmacia, Uppsala, Sweden) using a 0 to 0.7-mol/L NaCl gradient. Further purification was achieved by a second chromatography on DEAE-Sephacel using a 0 to 0.3-mol/L NaCl gradient.

**Preparation of rabbit antibodies. IgG and F(ab')\(_2\) fragments.** A rabbit was immunized at two-week intervals for six weeks with GPV (0.25 mg/mL) in physiological saline, mixed 1:1 with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich). After five weeks of rest, a booster immunization was given. The antisera titer was regularly checked by Ouchterlony double diffusion in agarose and later by Laurell immunoelectrophoresis, and high-titer bleedings were pooled. IgG was prepared from the rabbit antisera by treatment\(^1\) with DEAE-Sephacl.

F(ab')\(_2\) fragments of rabbit IgG were prepared\(^1\) by digestion with papain followed by ion exchange chromatography on CM-cellulose (Whatman CM52, W. and R. Balston, Kent, England) and gel filtration on Ultrogel AcA 34 (LKB-Producter AB, Bromma, Sweden).

**Triton X-114 phase separation.** Washed platelets were solubilized and separated into hydrophilic and hydrophobic phases by Triton X-114 (Sigma, St Louis) phase partitioning as previously described.\(^2\)

**Polyacrylamide gel electrophoresis.** Two-dimensional polyacrylamide gel electrophoresis (isoelectric focusing/5 to 15% gradient polyacrylamide gel electrophoresis) was performed as described earlier.\(^3\) One-dimensional gel electrophoresis was done with 1.5-mm thick, 5% to 15% polyacrylamide gradient gels, using combs with 4.5-mm slots. The samples (10 to 50 \(\mu\)g) contained 1% sodium dodecyl sulfate (SDS) and 1% dithiothreitol for reducing conditions and were heated to 100 °C for two minutes before electrophoresis. The gels were silver-stained by the method of Morrissey\(^2\) or were prepared for fluorography.\(^4\)

**Electrophoretic blotting.** Glycoproteins were transferred from gels to nitrocellulose sheets (Schleicher and Schüll, Dassel, FRG) by the method of Towbin.\(^5\) The binding to the blot of the primary rabbit antibody against GPV, was detected with peroxidase-labeled goat antirabbit IgG (Bio-Science Products AG, Emmenbrücke, Switzerland) using 4-chloro-1-naphthol (Sigma) as substrate.

**Affinity chromatography on thrombin-Sepharose 4B.** 1 mg bovine thrombin (1,000 U/mL, Calbiochem, Zürich, Switzerland) was coupled to Sepharose 4B by the cyanogen bromide method.\(^6\) GPV, purified as described above, was loaded on the thrombin column and washed with 20 mmol/L Tris/HCl, pH 7.4 to remove any excess of GPV, over the capacity of the column. The bound material was eluted with heparin (40 U/mL; Sigma) in Tris buffer.

**Surface labeling by the periodate/\(^3\)H-NaBH\(_4\) method.** Platelets were isolated from platelet-rich plasma, washed and surface-labeled by the periodate/\(^3\)H-NaBH\(_4\) method\(^7\) as modified by Steiner.\(^8\)

**Thrombin treatment.** Washed platelets (5 \(\times\) 10\(^7\)/mL) were \(^3\)H-labeled and preincubated with anti-GPV antibody (antisera), preimmune serum, or platelet buffer (as control) for ten minutes. Thrombin (8 U/mL; Hoffmann-La Roche, Basle, Switzerland) was added and the suspension left for three minutes at 37 °C. Then the platelets were centrifuged at 110g for ten minutes. In one half of the supernatant, proteolysis was inhibited with \(\beta\)-amidinophenylmethylsulphonyl fluoride (APMSF, 1 mmol/L final concentration; Calbiochem, Zürich, Switzerland) and leupeptin (100 \(\mu\)g/mL final concentration; Fluka AG, Buchs, Switzerland). The other half was treated again with thrombin (400 U/mL) for 30 minutes at 37 °C and then proteolysis was inhibited with APMSF and leupeptin. The thrombin-treated platelets were washed twice with platelet buffer and solubilized in 1% Triton X-114, 10 mmol/L Tris/HCl, 154 mmol/L NaCl, 20 mmol/L EDTA, 2 mmol/L PMSF, 100 \(\mu\)g/mL leupeptin for 30 minutes at 0 °C. The solution was then centrifuged for 30 minutes at 100,000g at 0 °C and the supernatant used for one-dimensional gel electrophoresis.

**Aggregation studies.** Platelet-rich plasma was washed twice in 5 mmol/L glucose, 3 mmol/L KCl, 3 mmol/L Mes (2-(N-morpholino)ethanesulfonic acid), pH 6.5 and suspended at a concentration of 3 \(\times\) 10\(^7\)/mL in the same buffer. Aliquots of 400 \(\mu\)L in aggregometer tubes were incubated either with anti-GPV antibody [F(ab')\(_2\) fragments] or with buffer as control before treatment with thrombin (0.05 U/\(\mu\)L), ristocetin (1.5 mg/mL, Lundbeck, Copenhagen, Denmark), human von Willebrand factor (100 \(\mu\)L plasma), adenine diphosphate (ADP) (1.2 \(\mu\)g/mL, Serva, Heidelberg, FRG) or collagen (5 \(\mu\)g/mL, Hormon Chemie GmbH, Munich, FRG).

**RESULTS**

**I solation of GPV fragment.** During earlier work on the isolation of platelet glycolacticin\(^9\) it was noted that during treatment of platelets with endogenous calcium-activated proteases a large fragment of GPV was released from the platelet as well as glycolacticin. The fragment, referred to here as GPV\(_5\), contains the thrombin cleavage site and coisolates with glycolacticin on affinity chromatography on wheat germ agglutinin. This provides a rapid isolation step from the platelet supernatant and as shown in Fig 1, GPV\(_5\), due to its more basic pl, can be separated from glycolacticin by ion exchange chromatography on a DEAE-Sephacel column with a 0 to 0.3-mol/L NaCl gradient. The pooled fractions after this step contained only GPV, when examined...
Fig 2. Two-dimensional polyacrylamide gel (isoelectric focusing/gel electrophoresis) of the isolated GPV, the fragment obtained by calcium-activated protease treatment. The gel was silver-stained by the method of Morrissey.29

by two-dimensional gel electrophoresis followed by silver staining (Fig 2). The mol wt and heterogeneous pl found for GPV, were similar to those reported earlier for GPV.29 As previously reported18 GPV was the only surface glycoprotein on platelets to be cleaved by thrombin. The cleavage fragment GPV, from intact platelets was identical to that obtained by treating GPV, with thrombin.

Preparation of antibody. The purified GPV, was used to immunize a rabbit for preparation of polyclonal antibodies. The specificity of the antiserum was determined by immunoblotting with two-dimensional polyacrylamide gel separations of platelet fractions. Figure 3 shows such an immuno-

Fig 3. Immunoblotting of a two-dimensional polyacrylamide gel (isoelectric focusing/gel electrophoresis) separation of the Triton X-114 phase from phase partition of platelet proteins with anti-GPV, antibody as first antibody. Faint staining in the higher molecular weight, more acidic region is nonspecific background.

blot of the Triton X-114 phase from a phase partition of platelets with the anti-GPV, serum. GPV was the only component that was stained. The Triton X-114 phase was used because it is enriched in most platelet membrane glycoproteins including GPV29 (Fig 4). Immunoblots with whole platelet preparations also showed that GPV, was the only component to react with the anti-GPV, serum although the staining was relatively weaker. The aqueous phase of the Triton X-114 phase separation did not contain any components which reacted with the anti-GPV, serum (data not shown) but contained all the GP Ib (Fig 4). On one-

Fig 4. Two-dimensional gel electrophoresis (isoelectric focusing/gel electrophoresis) separation of platelet proteins, silver-stained by the method of Morrissey.29 (A) Triton X-114 phase from phase partition of platelet proteins; (B) aqueous phase from phase partition of platelet proteins.
dimensional gels the component recognized by the anti-GPV, serum showed the characteristic behavior of GPV and moved to a lower mol wt of 69,500 d in the supernatant of platelets treated with thrombin (data not shown).

**Influence of the antibody on GPV cleavage by thrombin.** Since GPV is so far the only substrate for thrombin detected on the platelet surface, the effect of anti-GPV, IgG on the cleavage of GPV from platelets and on the activation of platelets by thrombin were studied. Cleavage of GPV from periodate/[3H]NaBH₄ labeled platelets by thrombin treatment gives rise to the readily identifiable fragment GPV₁ in the supernatant which provides a semiquantitative measurement of the extent of the cleavage reaction.³⁰

When surface-labeled platelets were treated with anti-
GPV, IgG before exposure to thrombin, cleavage of platelet GPV to GPV₁ was strongly inhibited in comparison to platelets treated with preimmune serum (Fig 5). To demonstrate that the GPV fragment in the supernatant really is GPV₁ and not GPV, the cleavage product with calcium-activated proteases, the supernatant was treated again with a large excess of thrombin. No further degradation of the GPV fragment was detectable (Fig 5C). The GPV₁ band in Fig 5C, lanes c and d, appears to run slightly lower because of the presence of an unlabeled protein band from the thrombin preparation running just above. This result indicates that the antibodies block thrombin binding to the cleavage site. The anti-GPV₁ IgG also blocked cleavage of GPV, to GPV₁ in the supernatant from labeled platelets, in the presence of a large excess of thrombin, confirming that the inhibition was due to binding to GPV and not to an indirect effect on the platelets (data not shown).

Platelets treated with F(ab')₂ fragments of anti-GPV, IgG were compared with untreated and with preimmune serum-treated platelets in aggregation with thrombin to see if the activation of platelets by thrombin is influenced when cleavage of GPV is blocked (Fig 6). Binding of F(ab')₂ fragments of anti-GPV, IgG had no effect on the platelet aggregation response to thrombin, ADP, collagen, or ristocetin/von Willebrand factor, nor did it delay the shape change response.

**Binding of GPV, to insolubilized thrombin.** Binding and cleavage of GPV₁ were studied using thrombin which was coupled to Sepharose 4B beads. A solution of GPV₁ was loaded on the column and eluted with buffer. Any excess of GPV₁ over the capacity of the column appeared in the flow-through (Fig 7, lane a) but the bulk of the GPV₁ bound to the thrombin. Attempted elution with a variety of buffers was unsuccessful but heparin gave an elution peak. Examination of this peak by one-dimensional SDS–polyacrylamide gel electrophoresis of two fractions from a thrombin–Sepharose 4B column; (a) Excess of GPV₁, over the capacity of the column recovered in the flow-through. (b) Bound material eluted with heparin. Proteins were silver-stained by the method of Morrissey.³⁹
gel electrophoresis showed a band at the same mol wt as the GPV₁ fragment (Fig 7, lane b). A smaller piece corresponding to the difference between GPV₁ and GPVᵢ was not detected in the flow-through or the eluate.

**DISCUSSION**

GPV is the only demonstrable substrate for thrombin on human platelets and for this reason has long been a prime candidate for the platelet thrombin receptor. However, in recent years several studies have cast doubt on the role of GPV in the activation of platelets by thrombin.⁵,¹⁰,²⁶,²⁷ The development of a simplified method for the purification of a major proteolytic fragment of GPV has provided the opportunity to prepare antibodies to GPV and to test their effects on platelet activation by thrombin. The preparation of a major proteolytic water-soluble fragment of GPV is based on treatment of platelets with a crude preparation containing calcium-activated proteases. The calcium-activated proteases remove not only glycolcalcin from GPIb but also most of GPV as the main membrane glycoprotein fragments found in the supernatant. This fragment of GPV, GPVᵢ, is probably what was originally isolated and described as GPV₂⁹ since it has similar properties. We have already shown,²¹ that the intact GPV molecule is slightly larger and hydrophobic. Here we demonstrate again that intact GPV is entirely found in the hydrophobic phase of a Triton X-114 phase partition. Antibodies prepared against GPV were shown to be specific for platelet GPV by immunoblotting. Since GPV contains the thrombin cleavage site and can be cleaved to GPVᵢ by thrombin, we examined the effect of these antibodies on thrombin proteolysis of membrane-bound intact GPV. As thrombin treatment causes platelet activation and the release of several proteins from storage granules it is easier to observe the effect on GPV by using surface-labeled platelets. Platelets that were pretreated with anti-GPV antibodies before adding thrombin showed a greatly reduced cleavage of GPV to GPVᵢ. Even a large excess of thrombin (8 U/mL) caused no detectable release of GPVᵢ, indicating that the antibodies had blocked the cleavage site. However, in aggregation studies, pretreatment of platelets with anti-GPV antibodies had no effect on platelet activation by thrombin. As little as 0.05 U/mL thrombin (160-fold less than in the GPVᵢ release study) caused normal aggregation. These findings and the dose-response and time-response relationship studies of McGowan et al.,¹⁰ indicate that the hydrolysis of GPV by thrombin is not essential for platelet activation and that GPV is therefore not the thrombin receptor on platelets. This result supports earlier work with Bernard-Soulier syndrome platelets where GPV as well as GPIb and GP17 (GPIX) are absent¹¹-¹⁻ but the platelets even so respond to thrombin.¹⁴ They also agree with the results of McGowan et al.¹⁰ and White and Knupp,¹⁰ who found no direct relationship between cleavage of GPV by thrombin and the extent of the platelet response. Since GPIb, although a thrombin receptor, only influences the rate of response to thrombin but not the extent and is also not necessary for platelet activation, the essential platelet component must be a thrombin-sensitive surface protein that has not yet been identified. Because surface labeling methods have so far been unsuccessful in finding a suitable candidate, it must be concluded either that the receptor is poorly labeled if at all or that only a very minor portion is cleaved during platelet activation.

The role of GPV in platelet function and in relation to thrombin remains obscure. Antibodies to GPV did not affect platelet activation by several stimulators including thrombin. However, the fact that GPV is cleaved by thrombin may imply that it has some role in the physiological activity of thrombin other than in activation of platelets. The lack of any apparent relationship between GPIb and GPV either through complex formation such as is found between GPIb and GP17 (GPIX)²¹,²² or through immunological cross-reactivity makes the absence of both glycoproteins in the recessive genetic disorder Bernard-Soulier syndrome even more puzzling. As we show here and in previous work,²¹ GPIb and GPV separate in different phases in Triton X-114 phase partition. Immunoprecipitation experiments with anti-GPIb and anti-GPV antibodies (data not shown) also failed to show coprecipitation of these glycoproteins. Immunoblotting experiments showed no cross-reactivity between GPIb and GPV (Fig 3). One possibility that remains to be explored is that both glycoproteins have similar glycosylation steps during synthesis and that the defect in Bernard-Soulier syndrome lies there. A solution to this problem will require the characterization of the genes for these glycoproteins in normal and in Bernard-Soulier syndrome patients.

We have investigated the binding of GPV to insolubilized thrombin and found that it bound strongly. On elution with heparin it was the cleavage fragment GPVᵢ that was recovered. This shows that GPVᵢ is still capable of binding thrombin and, when thrombin cleaves GPV, the thrombin remains associated with the soluble GPVᵢ that is removed from the platelet. The presence of GPVᵢ in plasma may be a good indicator of platelet exposure to thrombin under in vivo conditions and is under investigation. The availability of a specific antiserum to GPV should allow studies on its tissue distribution and on its suitability as a marker during mega-karyocyte development.

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