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Hydrolysis of human platelet membrane glycoproteins with a *Serratia marcescens* metalloprotease: Effect on response to thrombin and von Willebrand factor

(platelets/proteases/platelet aggregation/serotonin release)

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ABSTRACT **Metalloproteases from at least two Gram-negative organisms selectively hydrolyze the surface of human fixed washed platelets. The protease-treated platelets lose their ability to aggregate with bovine von Willebrand factor and human von Willebrand factor plus ristocetin. The present study reports the membrane glycoprotein alterations and loss of platelet function that occur after incubation of fresh washed human platelets with a purified protease produced by *Serratia marcescens*. The studies were undertaken in order to examine, using the *Serratia* protease as an enzymatic probe, the relationship between externally oriented glycoproteins and two known aggregating agents, bovine von Willebrand factor and thrombin. Platelet membrane glycoproteins were analyzed after discontinuous NaDodSO$_4$/polyacrylamide gel electrophoresis using both staining with periodic acid–Schiff reagent and autoradiography of ^{3}H-labeled platelets. Both methods of detection demonstrated that at *Serratia* protease concentrations above 0.6 μg/ml there was hydrolysis within 3 min of a membrane glycoprotein (M_r 185,000) corresponding to GPIb in control platelets. The loss of GPIb was accompanied by the appearance in the platelet supernatant of a glycopeptide (M_r 156,000). Under reducing conditions, the hydrolyzed membrane glycopeptide in the supernatant had a consistently faster migration (M_r 149,000). Platelets treated with *Serratia* protease at concentrations sufficient to give maximal cleavage of GPIb were unresponsive to bovine von Willebrand factor and did not aggregate or release [^{14}C]serotonin at doses of von Willebrand factor up to 0.33 unit/ml. On the other hand, the response to bovine α-thrombin was only minimally impaired by treatment with *Serratia* protease. These results implicate GPIb in the response by platelets to bovine von Willebrand factor but suggest that surface components other than GPIb play a major role in the response by platelets to thrombin.**

The platelet responds to environmental stimuli with a series of events mediated by surface components. Among these components are a number of externally oriented proteins and glycoproteins that could serve the role of specific binding proteins or receptors (1, 2). In certain platelet disorders there is a relationship between altered platelet response and qualitative or quantitative differences in one or more of the known membrane glycoproteins (3–6).

When washed human platelets are suspended in buffers containing no chelating agents and are treated with sonication, freezing and thawing, or prolonged storage, they readily lose a surface-associated glycoprotein called glycocalicin (7). Glycocalicin is soluble in aqueous buffers, highly glycosylated, rich in sialic acid, and composed of a single polypeptide chain with a molecular weight (M_r) of about 150,000. Initial reports indicated that this glycoprotein acted as both a potent inhibitor of thrombin-induced platelet aggregation and the platelet receptor for von Willebrand factor (vWF) (8–10).

Later studies, however, have presented a somewhat contradictory view. By using either a receptor bioassay (11) or immunochemical techniques (12), the vWF-binding protein was shown to be an integral membrane glycoprotein, probably GPIb.* This membrane component bound strongly to phenyl-Sepharose (15), demonstrating a hydrophobic region missing in glycocalicin (6). On the other hand, immunochemical techniques, lectin affinity, and determination of electrophoretic mobility (2, 5, 12, 16) have suggested that glycocalicin and the detergent-soluble membrane glycoprotein may share a partial identity. In addition, platelets from patients with Bernard–Soulier syndrome that lack or have an abnormal GPIb on their surface do not respond to or bind vWF (17) and have a reduced number of binding sites for thrombin (9).

All the data could be reconciled if soluble glycocalicin were proteolytically derived from the heavily glycosylated surface-oriented membrane glycoprotein GPIb. Because chelating agents, such as EDTA, interfere with the loss of glycocalicin from the surface of stored platelets, a Ca^{2+}-dependent protease normally found in platelets (18, 19) might be responsible for the cleavage. One objection to this hypothesis is a report that peptide maps of the GPIb material prepared from platelet membranes by lectin affinity chromatography in cation-containing buffers show no similarity to the peptide maps obtained by using purified glycocalicin (20). In a more recent study, however, in which buffers containing EDTA were used, great similarity was found between the tryptic peptide maps of glycocalicin and GPIb (21).

In the present study, we have utilized a highly selective bacterial protease to further examine the role of GPIb in the platelet response to thrombin and vWF. In previous studies, we showed that certain Gram-negative organisms, particularly *Serratia marcescens* and *Pseudomonas aeruginosa*, produce extracellular metalloproteases that selectively attack the surface of human fixed washed platelets (22). Incubation of such platelets with these proteases destroyed their ability to aggregate with bovine vWF and with human vWF plus ristocetin. The purified *Serratia* protease cleaved the fixed washed platelet surface more

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Abbreviations: vWF, vonWillebrand factor; PRP, platelet-rich plasma; PAS, periodic acid–Schiff reagent.

* The terms "GPIb" and "GPV" correspond to the nomenclature recommended by Phillips and Agin for platelet surface glycoproteins (13). Mosher and coworkers have described an alternative system of nomenclature based on molecular weight of reduced platelet glycoproteins determined by polyacrylamide gel electrophoresis. In their nomenclature GPIbα is GP140 and GPV is GP68 (14).

selectively than either chymotrypsin or trypsin did. However, it was difficult to take advantage of this selectivity because chemical crosslinking of surface components during formaldehyde fixation precluded precise identification by polyacrylamide gel electrophoresis of the hydrolyzed surface components. This, and the fact that fixed platelets do not aggregate with thrombin, prompted us to extend our previous studies to a system employing fresh washed platelets in order to identify the surface substrates for the *Serratia* protease and to examine the effect of selective proteolysis on platelet–thrombin interactions.

MATERIALS AND METHODS

Reagents. Water was deionized and distilled in glass. All chemicals were reagent grade unless otherwise specified. Tritiated sodium borohydride, 5–15 Ci/mol (1 Ci = 3.7×10^{10} becquerels), was obtained from Research Products International (Kankakee, IL), and was dissolved in 1 mM NaOH. Neuraminidase (*Vibrio cholerae*), devoid of proteolytic activity, was purchased from Schwarz/Mann. Galactose oxidase from Worthington was further purified by gel chromatography on Sepharose 6B (Pharmacia). Aquasol-2 and [^{14}C]serotonin were obtained from New England Nuclear. *Serratia* protease was a gift from Arnold Kreeger (Bowman Gray School of Medicine, Winston-Salem, NC). The highly purified protease was free of phospholipase C, deoxyribonuclease, lipase, elastase, hexapeptidase, collagenase, and alkaline phosphatase activities. Thrombin was bovine α-thrombin, 3000 NIH units/mg, kindly supplied by Roger L. Lundblad (University of North Carolina, Chapel Hill, NC).

Washed platelets were obtained from normal healthy male or female adult volunteer donors with no evidence of liver or hematological disease and no exposure to aspirin-like drugs or medications for at least 2 weeks prior to venipuncture. Informed consent was obtained in accordance with the Helsinki Declaration. The blood was drawn into citric acid/citrate/dextrose (ACD) by the two-syringe technique and platelet-rich plasma (PRP) was prepared by centrifugation at $100 \times g$ for 20 min at room temperature. The PRP was then pelleted and the platelet pellet was washed with citrate/saline/glucose buffer by differential centrifugation. In another procedure, the PRP was layered onto a plastic column containing Sepharose 2B (Pharmacia) previously equilibrated with divalent cation-deficient Tyrode's solution (pH 7.4) containing 1 mM EDTA. The column was developed by using the same buffer, and the gel-filtered platelets ($2–4 \times 10^8$ per ml) were collected.

Fresh Washed Platelet Aggregability. Aggregability was assayed at 37°C with constant stirring at 1100 rpm in a dual-channel aggregometer from Payton (Buffalo, NY). The slope of the aggregation response was calculated from the steepest part of the aggregation curve. In the test system, fresh, washed, [^{14}C]serotonin-labeled platelets were treated with *Serratia* protease as described. Appropriate concentrations of bovine vWF or thrombin were added and the slope of the aggregation response was calculated and compared to that obtained with the same initial platelet preparation treated in an identical fashion with buffer.

Preparation and Treatment of ^{3}H-Labeled Fresh Washed Platelets. Proteins on the surface of gel-filtered platelets were labeled by the galactose oxidase technique (13). ^{3}H-Labeled platelets were suspended in 2 ml of divalent cation-deficient Tyrode's solution ($2–4 \times 10^6$ per μl, 20.8 μg/μl, 0.004 cpm per platelet). In preparation for enzymatic digestion with *Serratia* protease, 75 μl of labeled platelets was added to 825 μl of divalent cation-deficient Tyrode's buffer warmed to 37°C. *Serratia* protease 100 μl, was added to give final concentrations of 0.6–20 μg/ml and the mixture was incubated for 3 min. The treated platelets were pelleted in a Beckman Microfuge B for 60 sec. The supernatant was carefully removed and prepared for polyacrylamide gel electrophoresis by the addition of an equal volume of sample buffer (4). The pellet was resuspended in 80 μl of Tyrode's buffer, sonicated, and mixed with an equal volume of sample buffer. Both sets of samples were boiled for 3 min after addition of sample buffer.

One-Dimensional NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in 7.5% polyacrylamide, according to the technique of Laemmli (23). Gels were stained sequentially with periodic acid–Schiff reagent (PAS) and 0.1% Coomassie brilliant blue in 50% trichloroacetic acid. The gels were subsequently equilibrated with 2,5-diphenyloxazole, dried, and exposed to x-ray film at −70°C (24). PAS-stained gels and autoradiographs were scanned with a laser densitometer.

Preparation and Treatment of [^{14}C]Serotonin-Labeled Fresh Washed Platelets. Whole blood was drawn as previously described and PRP was prepared by centrifugation at $460 \times g$ for 8 min at 23°C. Incorporation of label by the platelets was effected by adding 0.5 μl of [^{14}C]serotonin (specific activity, 47.2 mCi/mmol) per ml of PRP and incubating the mixture at 37°C for 30 min.

After incubation, the platelets were pelleted by centrifugation at $860 \times g$ for 8 min at 23°C. The platelet pellet was resuspended in citrate/saline buffer and washed three times. The labeled platelets were adjusted to a final count of 8×10^5 per μl using a Thrombocounter C (Coulter).

Serotonin release was quantitated by methods previously reported (4). Equal volumes of [^{14}C]serotonin-labeled fresh washed platelets were mixed with *Serratia* protease or buffer and incubated for 15 min at 23°C. After incubation, 0.4 ml of the mixture was added to a glass cuvette and aggregation was initiated by adding dilutions of bovine plasma or thrombin.

RESULTS

Effect of *Serratia* Protease on Platelet Surface Components. Fresh human platelets labeled with ^{3}H were incubated with increasing concentrations of *Serratia* protease and the digested platelets were pelleted. The *Serratia* protease-treated pellets and their corresponding supernatants were electrophoresed on NaDodSO$_4$/polyacrylamide gels under both reducing and nonreducing conditions. The gels were analyzed for proteins and glycoproteins by both conventional PAS and Coomassie staining and autoradiography. Fig. 1 shows representative densitometric scans of the PAS-stained unreduced gels of the platelet pellets obtained after incubation with *Serratia* protease at final concentrations ranging between 0.6 and 20 μg/ml. At 0.6 μg/ml, the residual glycoproteins gave a pattern indistinguishable from that obtained with the control platelets. However, above 0.6 μg/ml, the unreduced gels of the *Serratia* protease-treated platelet pellets showed a significant reduction in a PAS-positive band of approximately 185,000 molecular weight. This band corresponded to GPIb in the conventional gel pattern obtained with control platelets. Similarly, in the reduced gels of the pellets, there was a decrease in a single PAS-staining band with an estimated molecular weight of 155,000.

Similar results were obtained from the analysis of the autoradiograms. Again, increasing concentrations of *Serratia* protease resulted in the loss of a radiolabeled protein (Fig. 2) that comigrated with the PAS-positive band that was shown to disappear in the same gels stained for carbohydrate (Fig. 1). At 10–20 μg/ml of *Serratia* protease there was a slight but consistent reduction in at least one other labeled band (GPIa) as determined by autoradiography. There was no obvious reduction in GPV.

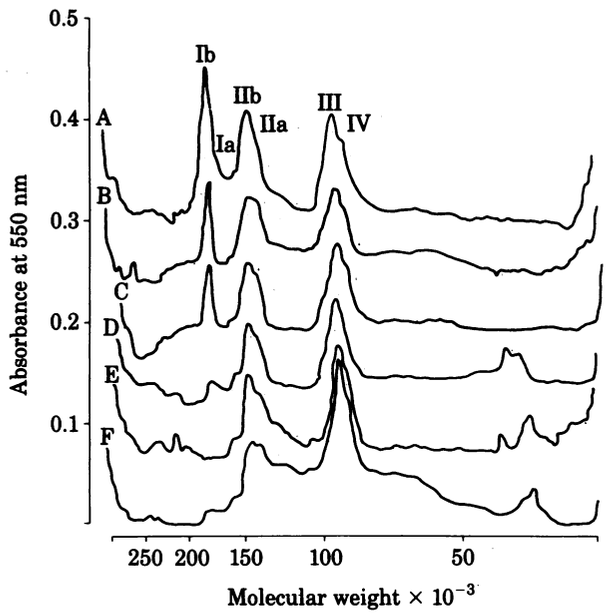

FIG. 1. Densitometric scans of samples of *Serratia* protease-treated platelet pellets electrophoresed unreduced on one-dimensional $NaDodSO_4$/7.5% polyacrylamide gels and stained for carbohydrate. Fresh washed gel-filtered platelets (75 μl at 2–4 × 10^6/μl), 825 μl of divalent cation-deficient Tyrode's buffer, and 100 μl of *Serratia* protease to give final μg/ml concentrations of 0.6 (A), 1.25 (B), 2.5 (C), 5.0 (D), 10.0 (E), and 20.0 (F) were mixed and incubated for 3 min at 37°C.

When the reduced and unreduced gels were stained for protein and densitometric scans of the stained gels were compared, no significant differences were observed.

The $NaDodSO_4$/polyacrylamide gels of the supernatants from these labeled platelet pellets demonstrated one band that was detected by both PAS staining and autoradiography. The band had a molecular weight on unreduced gels of approximately 156,000 and on reduced gels of approximately 149,000. The amount of this material in the supernatant, as determined by densitometric scans of the gels stained for PAS and then developed as autoradiographs, increased when increased concentrations of *Serratia* protease were used in the original incubation mixture.

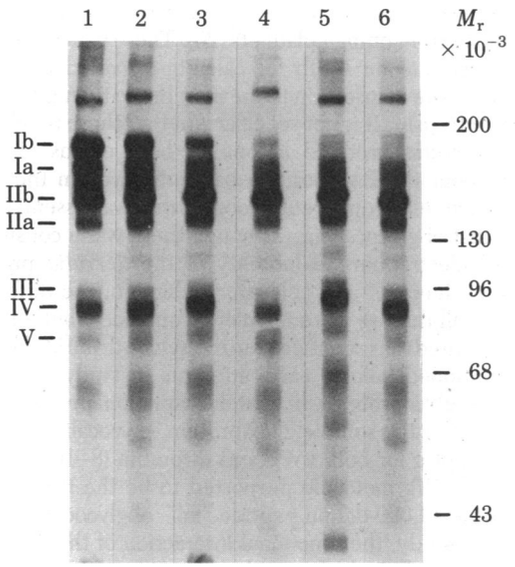

FIG. 2. Effect of *Serratia* protease on platelet surface proteins studied by autoradiography. Gel-filtered platelets were labeled by the neuraminidase–galactose oxidase–NaB^3H_4 method and incubated with *Serratia* protease for 3 min at 37°C. The platelets were then centrifuged and the pellets were prepared for electrophoresis in 7.5% acrylamide gels. The gels were prepared for autoradiography and then exposed to film for 72 hours at −70°C. The final concentrations of *Serratia* protease in μg/ml were 0.6 (lane 1), 1.2 (lane 2), 2.5 (lane 3), 5.0 (lane 4), 10 (lane 5), and 20 (lane 6).

Effect of *Serratia* Protease on Platelet Function. Fresh, washed, [^{14}C]serotonin-labeled platelets were incubated with *Serratia* protease at a final concentration of 5 μg/ml. At this concentration, with most of the GPIb cleaved from the surface (Fig. 1), *Serratia* protease did not cause platelet aggregation or the release of [^{14}C]serotonin. These *Serratia* protease-treated platelets were compared with untreated platelets for their response to bovine vWF and thrombin. Bovine vWF, even at 0.33 unit/ml, caused neither [^{14}C]serotonin release nor aggregation of *Serratia* protease-treated platelets (Fig. 3). In the control system, it can be noted that at low concentrations of vWF, 0.02 unit/ml, there was measurable platelet aggregation with no evidence of appreciable [^{14}C]serotonin release.

Similar experiments using thrombin are shown in Fig. 4. *Serratia* protease-treated platelets that were unresponsive to vWF would both release and aggregate with thrombin. However, the *Serratia* protease-treated platelets required about two times the concentration of thrombin to obtain the same degree of release and about four times the concentration of thrombin to obtain the same rate of aggregation as untreated platelets. At a thrombin concentration of 0.1 unit/ml, the *Serratia* protease-treated platelets demonstrated release with no measurable aggregation.

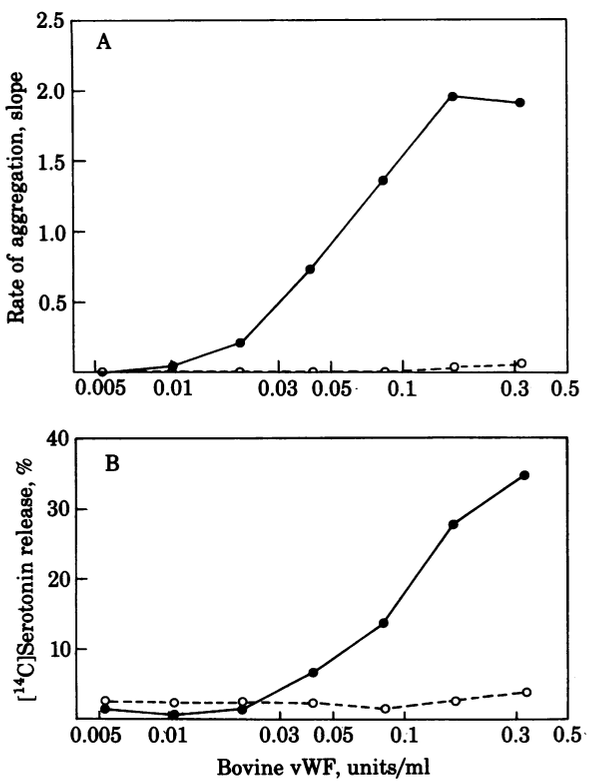

FIG. 3. Effect of various concentrations of bovine vWF on [^{14}C]-serotonin-labeled fresh washed platelets incubated with buffer (●) or with a final concentration of *Serratia* protease of 5 μg/ml (○) for 15 min at 23°C. Control or *Serratia* protease-treated labeled platelets were adjusted to 8 × 10^5 per μl and monitored for rate of aggregation (*A*) and [^{14}C]serotonin release (*B*).

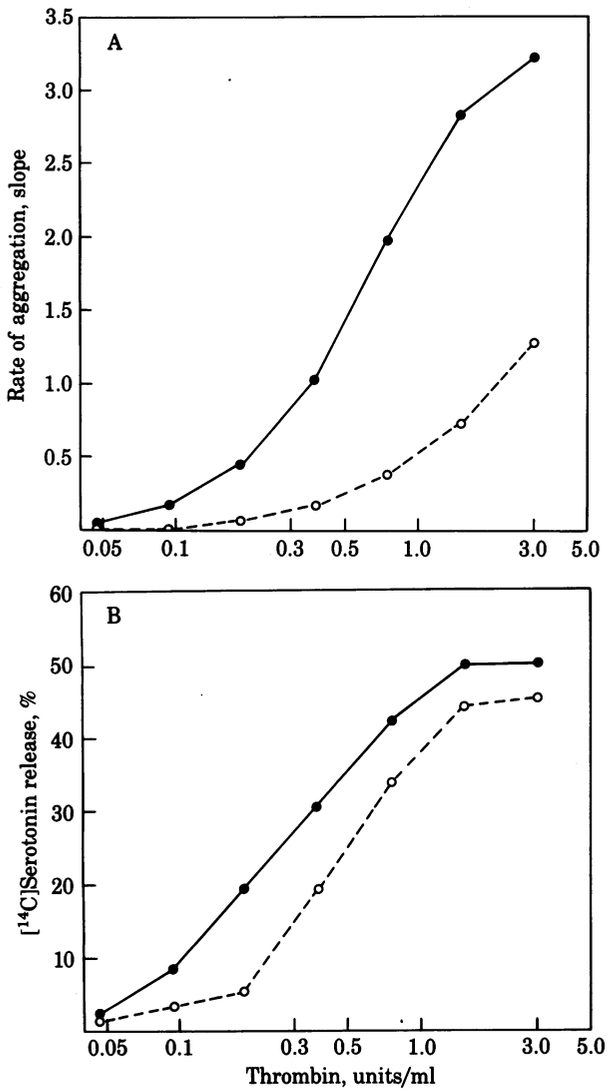

FIG. 4. Effect of various concentrations of bovine α-thrombin on [^{14}C]serotonin-labeled fresh washed platelets incubated with buffer (●) or with *Serratia* protease at a final concentration of 5 μg/ml (○) for 15 min at 23°C. Control or *Serratia* protease-treated platelets were adjusted to 8×10^5 per μl and monitored for rate of aggregation (*A*) and [^{14}C]serotonin release (*B*).

DISCUSSION

The present studies indicate that the interaction of a purified metalloprotease from *Serratia marcescens* with human platelet membranes is highly specific over a range of concentrations. This specificity has been used to further probe the relationship of bovine vWF and thrombin with receptor or binding components in the membrane of human platelets.

The membrane glycoprotein pattern obtained by NaDodSO$_4$/polyacrylamide gel electrophoresis of platelet pellets treated with *Serratia* protease showed a progressive decrease and eventual disappearance of the major membrane glycoprotein that in gels with nonreducing conditions had a molecular weight of 185,000 (Fig. 1) and in gels with reducing conditions had a molecular weight of 155,000. This was confirmed by using autoradiography, in which the major radiolabeled protein that was cleaved comigrated with the PAS-stained band, which in both systems exhibited mobility before and after reduction consistent with GPIb. The loss of this glycoprotein was almost complete after incubation of human platelets with a final *Serratia* protease concentration of 5.0 μg/ml (Fig. 1). The loss of GPIb from the surface was accompanied by the appearance in the corresponding platelet supernatant of a labeled 156,000-dalton glycopeptide (unreduced gels).

The reduction of GPIb on the platelet surface was associated with a complete loss of the ability of the platelet to release [^{14}C]serotonin or to aggregate in the presence of bovine vWF. These observations suggest that the portion of GPIb hydrolyzed by *Serratia* protease is intimately involved in the interaction of vWF with the platelet surface. Previous studies showed that the fragment cleaved by *Serratia* protease from fixed washed platelets (22) could not act as a competitive inhibitor in the aggregation of platelets with bovine vWF. Although failure to demonstrate blocking activity in the *Serratia* protease supernatant precludes the assignment of receptor or binding function to the fragment from fixed platelets, it does not prevent the assignment of these functions to GPIb. In addition, the interaction of vWF with a receptor on the platelet surface could require more than one GPIb molecule or GPIb and one or more additional membrane components (lipid or protein). The latter concept is consistent with our previous observations that receptor activity is altered after selective solubilization of platelet membranes with nonionic detergents (11). Alternatively, native GPIb, including the *Serratia* protease cleavage product, may have a different conformation on the platelet surface than it has when liberated in solution by detergent or enzyme and may therefore be less reactive toward vWF.

It is interesting to compare the effect of *Serratia* protease on GPIb with the known effects of other proteases on platelet membrane glycoproteins. GPIb is a two-chain molecule composed of a large molecular weight subunit, GPIbα, that contains most of the carbohydrate and a smaller subunit, GPIbβ. Trypsin cleaves a macroglycopeptide (M_r 120,000) from the surface of intact platelets (25) that is immunologically and analytically identical with a macroglycopeptide cleaved from glycocalicin by trypsin (6). The remaining peptide tail of the glycocalicin molecule (M_r 45,000) is practically devoid of carbohydrate and has no known hydrophobic domain. A calcium-activated protease has been shown to liberate the 148,000-dalton fragment, glycocalicin, from human platelets (26). These data suggest that the cleavage site on GPIb for calcium-activated protease is closer to the site of insertion in the membrane than is the cleavage site for trypsin. It is not yet clear whether there is a cleavage site for *Serratia* protease in one or both chains of GPIb. On reduction, the PAS-positive band observed in the supernatant had an electrophoretic mobility that was essentially the same as that of glycocalicin. Although there was a consistently increased electrophoretic mobility of the *Serratia* protease-cleaved product on 7.5% gels after reduction, the differences were so small that we were unable to conclude whether or not this represented the reduction of an interchain disulfide bond.

As mentioned before, early attempts to study the role of membrane glycoproteins in platelet function produced data suggesting that the soluble glycoprotein, glycocalicin, was the surface receptor for both vWF and thrombin (8–10). The portion of the GPIb molecule purported to be the thrombin receptor is the 45,000-dalton peptide "tail" of glycocalicin (6). In the present study, the functional interaction of thrombin with platelets was relatively intact despite enzymatic removal by *Serratia* protease of most of the exposed GPIb from the platelet surface, and it would appear that the 156,000-dalton fragment removed does not play an essential role in the response of platelets to thrombin. Nevertheless, the response of *Serratia* protease-treated platelets to thrombin was not entirely normal. Thus, GPIb may modulate the response of platelets to thrombin. Tam and coworkers previously showed that chymotrypsin, a protease that also cleaves GPIb, induced a lag phase in the

response by platelets to thrombin, suggesting that chymotrypsin-sensitive components on the platelet surface were involved in the postbinding receptor processing that leads to generation of a stimulatory signal (27). The specificity of *Serratia* protease suggests that the chymotrypsin-sensitive component may be GPIb. On the other hand, removal of this product abolishes the vWF–platelet interaction. This is also further indirect support for the role of GPIb as the receptor for vWF, because not only bovine vWF aggregation but also bovine vWF-induced platelet release is inhibited.

Recent observations have indicated a possible relationship between GPIb and another platelet membrane glycoprotein, GPV, which is a known substrate for thrombin on the platelet surface (13, 14). Like GPIb, GPV is eluted or released from the platelet surface under certain conditions (14) or by prolonged storage (28). In addition, GPIb and GPV are both reduced in isolated membrane preparations from normal platelets (28), and clinical studies in patients with Bernard–Soulier syndrome indicate that both GPIb and GPV are missing on the surface of whole platelets from patients with this disorder (29, 30). However, platelets treated with *Serratia* protease at concentrations that cleave GPIb retain GPV on the cell surface (Fig. 2), indicating that, at least under certain conditions, GPIb and GPV may not be associated on the platelet surface or that the association between GPIb and GPV is not mediated by the portion of GPIb cleaved by *Serratia* protease. Although it is not evident from our experiments, it is possible that some GPV is lost and that the small reduction in GPV might contribute to the decreased response of *Serratia* protease-treated platelets to thrombin.

It is of interest that the hydrolysis of some membrane components may stimulate platelets, whereas known proteases that appear to more or less selectively cleave GPIb alone (*Serratia* protease, the calcium-activated protease, and chymotrypsin) do not. Future studies utilizing enzymes with known differences in membrane substrate specificity should be helpful in further understanding the relationships among GPIb, GPV, and their functional ligands.

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