A Form of Protease Nexin I Is Expressed on the Platelet Surface During Platelet Activation

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A protein that has several similarities to protease nexin I, a fibroblast thrombin and urokinase inhibitor, has been detected on platelets (Gronke RS, Bergman BL, and Baker JB: J Biol Chem 262:3030, 1987). On incubation of platelets with $^{125}$I-thrombin, this platelet protein forms complexes with $^{125}$I-thrombin that are found both in the incubation medium and, as demonstrated here, associated with purified platelet plasma membranes. The present results indicate that interaction with the platelet surface may modulate the conformation and function of this platelet form of protease nexin I (PNI) because: (a) an antibody against protease nexin I inhibited released PNI, but not platelet-bound PNI, from complexing $^{125}$I-thrombin; and (b) whereas PNI extracted from platelets bound both thrombin and urokinase, platelet-bound PNI bound only thrombin. In experiments using several different platelet isolation methods, PNI accounted for a large fraction of the rapid high affinity binding of $^{125}$I-thrombin to platelets. However, platelets isolated and maintained in the presence of metabolic inhibitors failed to take added thrombin into $^{125}$I-thrombin-PNI complexes. This finding suggests that PNI is released from inside platelets during activation, and thus does not function to transmit the primary activating signal that is generated by thrombin binding to platelets.

The interactions of thrombin with platelets play a key role in blood coagulation. Thrombin is formed at the platelet surface where circulating prothrombin is cleaved via the prothrombinase complex. Once activated, thrombin is a potent platelet agonist, causing platelets to change shape, aggregate, and secrete the contents of their storage granules. Harmon and Jamieson recently demonstrated that thrombin activation of platelets is a receptor-mediated event and its binding to high affinity receptors (50 sites/platelet; $K = 0.3 \text{nmol/L}$) is essential for platelet activation. Evidence has been presented that this receptor is the glycoprotein IIb. Platelets possess a factor that rapidly forms a 77 kilodalton (Kd) sodium dodecyl sulfate (SDS)-resistant complex with $^{125}$I-thrombin. We have reported that this "platelet form of protease nexin I" (PNI) accounts for the majority of specific thrombin binding to platelets at $<0.3 \text{nmol/L}$. $^{125}$I-thrombin, and have proposed that PNI could function to impose a threshold thrombin concentration below which platelets do not respond to thrombin. Recently Lerea and Glomset reported that the formation of $^{125}$I-thrombin-PNI complexes on platelets is temporally closely coupled to the thrombin-stimulated formation of inositol trisphosphate, and have proposed that binding of thrombin to PNI may transduce thrombin's platelet-activating signal.

The present results show that (a) $^{125}$I-thrombin-PNI complexes form on platelets that have been isolated by several differernt procedures, but not on platelets that have been isolated in the presence of metabolic inhibitors, suggesting that PNI is released from intracellular granules during platelet activation; (b) PNI is immunoprecipitated with antibody against fibroblast PNI; (c) PNI is found in stable association with purified platelet plasma membranes; and (d) the extracted, but not the membrane-bound, form of PNI binds urokinase.

MATERIALS AND METHODS

Materials

Human a thrombin (~3,000 NIH U/mg) originally purified in the laboratory of Dr R. Lundblad (University of North Carolina) was kindly donated by Dr L. Houston (University of Kansas). Two-chain urokinase was donated by Dr G. Murano (Center for Drugs and Biologies of the Food and Drug Administration). PNI was purified as previously described. Phosphocreatine (disodium salt), creatine phosphokinase, EGTA, bovine serum albumin (fatty acid free), HEPES, diisopropylfluorophosphate, prostaglandin E, antymcin A, D-glucocic 1-lactone, deoxy-D-glucose, and Sepharose 2B-300 were all purchased from Sigma (St Louis). Seventy-micron nylon screen was a gift from Tekno Inc (Elmsford, NY). Dow Corning 710 silicon fluid was a gift from the Dow Corning Company (Midland, MI). Osmium tetroxide was purchased from Ladd Research Industries (Burlington, VT). Glutaraldehyde was purchased from Electron Microscopy Science (Fort Washington, PA). Osmium tetroxide was purchased from Stevens Metallurgical Corporation (New York).

Methods

Platelet preparation. Human platelets from normal healthy donors who denied aspirin ingestion were purified as previously described unless otherwise noted. Following purification, plate-
Platelets were washed with 10 mL of medium A (0.12 mol/L NaCl, 4.3 mmol/L KCl, 8.5 mmol/L MgCl₂, 3 mmol/L glucose, 10 mmol/L HEPES, pH 7.2). The platelets were then centrifuged at 3,000 g for 20 minutes and resuspended in 900 μL of medium A. Platelets were counted using a Coulter counter and used in experiments within six hours of isolation. The platelets were incubated 30 minutes before use in experiments in freshly prepared medium B (medium A containing 10 mmol/L creatine phosphate, 40 μg/mL creatine phosphokinase, and 0.5 mmol/L EGTA). 11,13,16

Radioiodinations of thrombin and urokinase were carried out as previously described except that benzamidine was omitted. 17 Specific activities of radioiodinated proteases varied between 0.5 to 2 x 10⁶ cpm/ng. Thrombin was inactivated using diisopropylphosphofluoridate as previously described. 17 ¹²⁵I-thrombin binding assays were carried out as previously described. 17 Rabbit polyclonal anti-PNI antibody was prepared as described by Howard and Knauer. 18 Partial purification of PNI was carried out by passing solubilized platelets over heparin-agarose as described by Baker and Gronke, 19 except the pH of the HEPES buffer was 7.2. ²⁵I PF4 release from platelets was measured with a commercial PF4 radioimmunoassay as described in the manufacturer's instructions.

Immunoprecipitation of PNI. Immunoprecipitation was carried out according to the method of Baker et al with modifications. 19 One x 10⁶ platelets were incubated with 0.4 nmol/L ¹²⁵I-thrombin for 30 seconds in medium B. Platelets were separated from the binding medium by centrifugation through oil and then solubilized in 0.5% Triton X-100 for ten minutes at 37°C. Anti-protease nexin IgG was added at 75 μg/mL and incubated for 12 hours at 4°C. Twenty microliters protein-A-Staphylococcus aureus was added per 5 μL of antiserum and incubated for 12 hours at 4°C on a rocking platform. The radioactivity specifically absorbed to the protein-A- S. aureus was determined by the method of Kaplan et al. ²⁰

Platelet plasma membrane preparation. Platelet plasma membranes were prepared according to the method of Barber and Jamieson with modifications. ²⁰ Briefly, platelets in medium B were layered over a 0% to 40% glycerol gradient made isotonic with medium A. All subsequent procedures were carried out at 4°C. The platelets were first centrifuged at 1,000 g for 30 minutes. The glycerol was carefully aspirated and the pellet was resuspended in 5 mL of cold 0.01 mol/L Tris, 0.25 mol/L sucrose, pH 7.5. Inspection with phase microscopy revealed many unbroken platelets, so the suspension was sonicated using a Biosonik III probe (Bronwell, Pittsburgh, PA) for five minutes at 11,000 rpm, washed with 50 mmol/L sodium phosphate buffer, pH 7.3. After one hour postfixation in 1% osmium tetroxide, the membranes were sedimented and resuspended in deionized water before en bloc staining for one hour with 1% uranyl acetate. Four minutes, washed with distilled water, and stained with Reynolds lead citrate, three minutes. ²¹ Membranes were viewed on a Philips 300 transmission electron microscope at an accelerating voltage of 60 kV.

¹²⁵I-thrombin binding. Binding assays were carried out as previously described. 19 Briefly, platelets (2 to 3 x 10⁶/mL unless noted otherwise) were incubated in medium B containing ¹²⁵I-thrombin and 0.25% fatty acid-free albumin. Binding incubations were terminated (after 30 seconds, unless noted otherwise) by layering the platelets over oil and centrifugation in a microfuge. ¹²⁵I-thrombin–PNI complexes were resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. These complexes migrate in a 77 kDa band, whereas thrombin that is reversibly bound to platelet receptors or binding sites migrates in a 36 kDa band. The latter band also contains ¹²⁵I-thrombin that is nonspecifically bound, defined as ¹²⁵I-thrombin that the platelets bind when 1 μmol/L diisopropyl phosphoryl-thrombin is present in the binding assay.

RESULTS

Similarities of PNI, to Fibroblast Protease Nexin I

Our previous work, carried out before high affinity antibodies against PNI were available, suggested that PNI was immunologically similar to fibroblast protease nexin I because PNI bound to agarose that had been derivatized with anti-protease nexin I antibody. 11 A rabbit polyclonal antibody has now been obtained that immunoprecipitates ¹²⁵I-thrombin–protease nexin I complexes ²² and that also inhibits complex formation between protease nexin I and ¹²⁵I-thrombin in fibroblast-conditioned medium. ²³ Figure 1
shows that complexes formed between platelet-bound PNI and $^{125}$I-thrombin were immunoprecipitated by this antibody, as were $^{125}$I-thrombin–PNI complexes formed by released PNI. Most or all of the 77 Kd complexes were precipitated because supernatants left after the immunoprecipitations were depleted of these complexes (data not presented).

To determine whether the antibody inhibited the protease-binding activity of PNI, platelets were incubated with the antibody or nonimmune IgG for one hour at 37°C before carrying out a binding incubation with $^{125}$I-thrombin. The autoradiograms in Fig 2 show $^{125}$I-thrombin–PNI complexes that formed on the platelets (panel A) and in the medium (panel B). Although the $^{125}$I-thrombin–PNI bands in the medium samples (Fig 2B, lanes 4 through 6) are considerably fainter than the $^{125}$I-thrombin–PNI bands in platelet samples (Fig 2A, lanes 2 through 6), much more PNI was in the binding medium than is suggested by the band intensities. For each reaction mixture, it was possible to load all of the platelets in a gel lane but only 12% of their binding medium. Figure 2 shows that the antibody prevented $^{125}$I-thrombin–PNI complexes from appearing in the platelet binding medium (panel B) but caused little or no inhibition of complex formation on platelets (panel A). Laser densitometry demonstrated that $^{125}$I-thrombin–PNI complexes accounted for 3% to 4% of the soluble radioactivity in platelet preparations incubated with 0.5 nmol/L $^{125}$I-thrombin and nonimmune IgG. Anti-PNI antibody lowered this value to 0.2%. In contrast, the platelet-associated $^{125}$I-thrombin–PNI accounted for approximately the same percentage of total radioactivity in the presence of nonimmune IgG and anti-PNI IgG (26.7% and 28.4%, respectively). Representative tracings are shown in Fig 3. These indicate that the reactive center of PNI is inaccessible to or unreactive with the antibody when PNI is bound to the platelet plasma membrane.

Earlier we made another finding that distinguished platelet-bound PNI from soluble protease nexin I, namely that platelet-bound PNI does not bind $^{125}$I-urokinase. This property indicates that either PNI is different from protease nexin I or that the interaction of PNI with the platelet surface modifies its protease specificity. To examine this question, soluble PNI was prepared by Triton X-100 extraction of 1.3 x 10$^9$ platelets and partial purification of the protein on heparin-agarose. This material, when incubated with $^{125}$I-urokinase for ten minutes at 37°C formed SDS-resistant complexes, and nonlabeled thrombin (5 nmol/L) prevented the formation of these complexes (Fig 4). Conversely, excess nonlabeled urokinase prevented the formation of $^{125}$I-thrombin–PNI complexes (data not shown).
relationship of PNI to protease nexin I is addressed in the Discussion section.

To ascertain whether active PNI, cofractionated with platelet plasma membranes, platelets were loaded with glycerol (4.3 mol/L) by gradient centrifugation, sonicated, and then fractionated by continuous sucrose density gradient centrifugation. The resulting plasma membrane preparation, examined using transmission electron microscopy, appeared similar to platelet plasma membrane preparations shown in the literature. Figure 5 indicates that 125I-thrombin, incubated with purified plasma membranes, was taken into 125I-thrombin–PNI complexes. Additionally, an experiment was carried out in which platelets were fractionated after incubation with 125I-thrombin. Approximately 55% of the platelet-associated 125I-thrombin–PNI complexes cofractionated with plasma membranes. The remainder sedimented in the fraction containing unbroken platelets, granules, mitochondria, and cellular debris.

Effect of Metabolic Poisons

Platelet granules contain several soluble serine protease inhibitors. Although the above experiments indicated that a fraction of the PNI, is associated with the plasma membrane, in fact, certain granule proteins become displayed on the plasma membrane. Thus PNI could be originally held in the granules and then released or exposed at the platelet surface during platelet isolation or during incubation with thrombin or other agonists. Therefore, platelets were isolated and continuously maintained in the presence of a combination of metabolic inhibitors (D-gluconic δ-lactone, antimycin A, and deoxy-D-glucose), which profoundly inhibit platelet degranulation. Strikingly, such platelets neither displayed nor released significant amounts of PNI when they were incubated with 125I-thrombin (Fig 6A).

When platelets were prepared in the absence of metabolic inhibitors, and then exposed to the inhibitors before addition of 125I-thrombin, we observed a 70% decrease in the number of 125I-thrombin–PNI complexes compared with platelets that were never exposed to the metabolic inhibitors (Fig 6B). These data suggest that most of the PNI presented on platelets is mobilized in response to thrombin. However, a significant percentage, ≈30% must be mobilized before exposure to thrombin, i.e., during platelet isolation.

The present data, together with our previous findings, indicate that PNI, is expressed in response to subnanomolar (0.3 nmol/L) doses of thrombin. To examine whether this property of PNI, is compatible with α granules being the origin of PNI, we examined the thrombin dose-dependence for release of an α granule marker protein, PF4. Consistent with findings reported by others, Table I shows that thrombin at 0.15 nmol/L stimulated release of a substantial amount of this protein: about one quarter of the amount released in response to 4.05 nmol/L thrombin. Thus, significant α granule release does occur in response to the low levels of thrombin at which PNI, expression occurs.

Influence of Platelet Isolation Methodology

All of our studies have been carried out with platelets that have been isolated by essentially the same method. Although we have previously reported that these platelets have not undergone aggregation nor released serotonin, it is nevertheless possible that this isolation method results in more PNI release than would occur with other isolation methods. Numerous other platelet isolation procedures have been used in studying thrombin-platelet interaction.
Platelet-associated PNl were assayed for their analysis of the interaction of 125I-thrombin with high affinity receptors. Blood was collected into CPDA-1 and the platelets isolated by centrifugation (see Table 2 legend). Results of two experiments, showing both the amounts of 125I-thrombin–PNl and of reversibly bound 125I-thrombin, are given in Table 1. In both experiments PNl accounted for the majority of 125I-thrombin binding at 0.2 nmol/L 125I-thrombin but only about one quarter of the 125I-thrombin binding at 1.0 nmol/L 125I-thrombin.

In the second platelet isolation method, prostaglandin E1 was added to the platelet-rich plasma, and platelets were purified by gel filtration. Results from two experiments are shown in Table 2. In both, PNl binding accounted for the majority of 125I-thrombin but only about one quarter of the 125I-thrombin binding at 0.2 nmol/L 125I-thrombin. Although the amounts of 125I-thrombin–PNl and reversibly bound 125I-thrombin varied over a several-fold range between the experiments in Table 1 and 2, the results have similarities to those obtained with the platelet isolation procedure that we have used previously: PNl accounted for a substantial percentage of the binding of 125I-thrombin to platelets and its percentage increased as 125I-thrombin doses decreased. It should be emphasized that the amounts of reversibly bound 125I-thrombin shown in Tables 2 and 3 include both specifically and nonspecifically bound ligand.

The contribution of PNl to specific 125I-thrombin binding will be greater than its contribution to total 125I-thrombin binding. Additionally, the experiments in Tables 2 and 3 suggest that binding of 125I-thrombin to PNl approaches saturation at ~1.0 nmol/L 125I-thrombin, consistent with the view that PNl has the binding properties of a high affinity, low abundance thrombin binding site.

Outdated platelets (two to three days old), obtained from a blood bank were also assayed for PNl and found to form 125I-thrombin–PNl complexes, although in somewhat lower quantity than when fresh platelets were used (data not shown).
Table 3. \(^{125}\)I-Thrombin Binding to Platelets That Were Prepared by Passage over a Sepharose-2B Column*

<table>
<thead>
<tr>
<th>(^{125})I-Thrombin (nmol/L)</th>
<th>(^{125})I-Thrombin–PNI, Complexes (cpm)</th>
<th>Reversibly Bound (^{125})I-Thrombin (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1 † ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>489</td>
<td>357</td>
</tr>
<tr>
<td>0.6</td>
<td>900</td>
<td>1,208</td>
</tr>
<tr>
<td>Experiment 2 † ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>846</td>
<td>268</td>
</tr>
<tr>
<td>0.6</td>
<td>867</td>
<td>370</td>
</tr>
</tbody>
</table>

*Platelets were purified by the method of Timmons and Hawiger with modifications. \(^{31}\) Briefly, blood (20 mL), drawn into 3.5 mL acid-citrate-dextrose anticoagulant, was centrifuged at 1,000 g for nine minutes. Prostaglandin E\(_1\) (1 \(\mu\)g/mL) was added to the platelet-rich plasma, which was then passed down a Sepharose-2B column that had been equilibrated with saline solution. Platelets were collected into polypropylene tubes, centrifuged at 3,000 g for 20 minutes, resuspended in 0.14 mol/L NaCl, 6.1 mmol/L KCl, 3.2 mmol/L Na\(_2\)HPO\(_4\), 6.4 mmol/L HEPES, 5.6 mmol/L glucose, 0.5% BSA (pH 7.2), and \(^{125}\)I-thrombin binding assays were carried out.

†These experiments used the same iodinated thrombin preparation and platelets from two different individuals.

DISCUSSION

Platelets both display on their surfaces and release a factor that forms 77 Kd SDS-resistant complexes with \(^{125}\)I-thrombin.\(^{12,32,33}\) The present work extends the previous evidence for immunologic similarity between this platelet thrombin-complexing factor and the thrombin inhibitor PNI, by showing that the platelet factor is immunoprecipitated by an anti-PNI antibody. The sole known property of this platelet form of PNI that may distinguish it from fibroblast PNI is that the platelet-bound form of this platelet protein does not bind urokinase. Here we have shown that PNI\(_p\) that has been purified from platelets by detergent extraction and heparin-agarose chromatography does bind urokinase. This suggests that PNI\(_p\) may be identical to fibroblast PNI, but that its protease specificity may be altered when it is bound to the platelet surface. Recent unpublished experiments by S. Wagner and D. Cunningham provide evidence in favor of this conclusion, by showing that the binding of PNI to fibroblasts restricts its protease specificity such that it still binds thrombin but no longer binds urokinase (personal communication; September 1987). Analogous experiments with platelets are not possible, at least with resting platelets, because added PNI does not bind to these cells.\(^{11}\) The failure of exogenous PNI to bind to platelets is consistent with the possibility that the membrane binding site for PNI\(_p\), like PNI\(_p\) itself, is not displayed on the platelet surface until platelet activation occurs.

It seems likely that PNI\(_p\) is presented on the platelet surface only after or concomitant with platelet activation, because metabolic inhibitors, preincubated with the platelets before incubating the cells with \(^{125}\)I-thrombin, caused a dramatic decrease in the amount of \(^{125}\)I-thrombin–PNI\(_p\) that formed. Maintaining the platelets in the presence of the inhibitors from the time of blood collection resulted in the virtual elimination of PNI\(_p\). Because the cocktail of inhibitors used profoundly inhibits release of platelet granules,\(^{29}\) it seems likely that PNI\(_p\) originates in intracellular granules, although the possibility that it comes from the surface-connected canalicular system cannot be ruled out.\(^{24}\) It is well established that release of \(\alpha\) granules results in both release of soluble granule proteins and incorporation of certain granule proteins on the platelet surface.\(^{22,27,28}\) Membrane-associated proteins of the granules may give rise to the latter.\(^{27,28}\) We have recently shown that released, soluble \(^{125}\)I-thrombin–PNI\(_p\) does not bind to platelets and thus cannot give rise to the platelet associated \(^{125}\)I-thrombin–PNI\(_p\).\(^{10}\) Recent findings by Miller et al\(^{35}\) also indicate that PNI\(_p\) is expressed during platelet activation. Their data shows that prostacyclin, an inhibitor of platelet activation, prevents formation of a 77 Kd complex between \(^{125}\)I-thrombin and a platelet factor (presumably PNI\(_p\)).

PNI\(_p\) is found on platelets incubated with very low (eg, 0.15 nmol/L) concentrations of \(^{125}\)I-thrombin. The present data indicates that this PNI\(_p\) could be expressed in a discharge of granules that takes place in response to these low levels of \(^{125}\)I-thrombin because 0.15 nmol/L thrombin caused a significant release of granules, as indicated by measurement of released PF4. Additionally, the experiments with metabolic inhibitors suggest that isolated platelets have some PNI\(_p\) on their surfaces before exposure to thrombin, possibly as a consequence of a small amount of granule release that occurs during platelet isolation.

The present experiments, using platelets prepared by several different isolation procedures, all indicate that PNI\(_p\) accounts for a major fraction the \(^{125}\)I-thrombin that binds to platelets under conditions of brief incubation time (30 seconds) and low (subnanomolar) ligand dose. Several functions have been suggested for PNI\(_p\). We have proposed that it acts strictly as a thrombin inhibitor, and therefore could impose a thrombin concentration threshold below which platelets do not respond to thrombin.\(^{11}\) Recently, Lerea and Glomset have suggested that this protein may participate in the development of the platelet-activating signal transmitted by thrombin.\(^{12}\) The formation of \(^{125}\)I-thrombin–PNI\(_p\) complexes is temporally closely linked to the production of inositol trisphosphate.\(^{11}\) The significance of the formation of this complex is currently not understood. The present finding that little or no PNI\(_p\) is displayed on unactivated platelets suggests that PNI\(_p\) does not serve as the primary signal-transducing thrombin receptor in this activation pathway. However, the possibility that PNI\(_p\) functions in another capacity in this pathway cannot be excluded. Interestingly, Miller et al\(^{35}\) have shown that formation of the \(^{125}\)I-thrombin–PNI\(_p\) complex is necessary for formation of the disulfide-bridged complex between thrombin and platelet thrombospondin. The significance of the formation of this complex is currently not understood.

ACKNOWLEDGMENT

We thank R. Gould, A. Schmaier, and G.A. Jamieson for their advice. We gratefully thank Lorraine Hammer for her assistance with the electron microscopy and the laboratory staff at Watkins Memorial Hospital, Lawrence Kansas for their technical assistance in drawing blood.
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