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Formation of Protease Nexin-Thrombin Complexes on the Platelet Surface

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We have recently described a platelet factor that is similar to the fibroblast thrombin inhibitor protease nexin I (PNI) [12]. The present manuscript shows that this platelet form of PN (PN_p) does not complex [^{125}I]-thrombin that has been blocked at its active site, consistent with the conclusion that it is a thrombin inhibitor. When platelets are incubated with [^{125}I]-thrombin, PN_p-[^{125}I]-thrombin complexers accumulate both in the medium and on the platelet surface. In the case of fibroblasts, PNI-[^{125}I]-thrombin complexes that form in solution bind to the cells as a consequence of a receptor-mediated clearance process [Low et al, Proc Natl Acad Sci USA 78:2340, 1981]. We show here that the PN_p-[^{125}I]-thrombin complexes that accumulate in platelet-binding incubation medium do not bind to platelets. Thus, the platelet-associated complexes must form by [^{125}I]-thrombin binding to PN_p that is associated with the platelet surface. Pretreatment of platelets with heparin markedly increases the number of PN_p-[^{125}I]-thrombin complexes that form on platelets. The basis for this increase in unclear. This effect seems incompatible with a heparinlike factor acting as the surface binding site for PN_p.

Key words: protease nexin, thrombin, platelets, protease inhibitor

A fraction of the thrombin that binds to platelets is taken into an SDS-resistant complex with a platelet factor [1,2] that is similar to PNI [3], a 43-kD thrombin inhibitor produced by fibroblasts [4,5]. Complexes between [^{125}I]-thrombin and the platelet factor are electrophoretically indistinguishable from thrombin-PNI complexes and bind to Sepharose that has been derivatized with anti-PNI antibody [3]. We have provisionally termed this platelet factor "platelet protease nexin" (PN_p).

The physiological function of PN_p is not certain, but it is intriguing that at low levels of thrombin, PN_p captures most of the thrombin that binds to platelets. The dose-dependence curve for thrombin binding to platelet receptors is sigmoidal, sug-

Abbreviations used: EGTA, ethylene glycol bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; DIP-thrombin, diisopropyl phosphoryl-thrombin; DFP, diisopropylfluorophosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PNI, protease nexin I; PN_p, platelet protease nexin.

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gesting that PN_p may damp the response of platelets to thrombin below a certain thrombin concentration/exposure time.

The purpose of the work described here was to determine the origin of the platelet-associated PN_p-thrombin complexes. In the case of fibroblasts, PNI is first secreted, and PNI-thrombin complexes that form in the culture medium then bind to cell surface receptors [6]. Following incubation of platelets with [^{125}I]-thrombin, PN_p-[^{125}I]-thrombin complexes are found in the binding incubation medium [3], compatible with the possibility that platelet-associated complexes form away from the platelet surface. Alternatively, they might form by [^{125}I]-thrombin binding to PN_p that is already associated with the platelet surface. The results suggest that PN_p-thrombin complexes that are bound to platelets form by the latter mechanism. This conclusion is consistent with the possibility that PN_p functions to limit thrombin interaction with platelet receptors.

EXPERIMENTAL PROCEDURES

Materials

Human α-thrombin (~3,000 NIH units/mg) originally purified in the laboratory of Dr R. Lundblad (University of North Carolina) was kindly donated by Dr L. Houston (University of Kansas). PNI was purified as previously described [7]. Heparin from porcine intestinal mucosa (170 units/mg) was purchased from Cal-Biochem (LaJolla, CA). Hirudin (1,000 units/mg), phosphocreatine (disodium salt), creatine phosphokinase, EGTA, bovine serum albumin (fatty acid-free), HEPES, and diisopropylfluorophosphate were all purchased from Sigma Chemical Company (St. Louis, MO). Dow Corning 710 silicon fluid was a gift from Dow Corning Company (Midland, MI). Apiezon A (specific gravity 0.8788), originally purchased from J. Biddle Company (Bluebell, PA), was a gift from Dr T. Detweiler. Na[^{125}I] and penicillin-streptomycin were purchased from Amersham (Arlington Heights, IL) and K.C. Biological (Lenexa, KS), respectively.

Methods

Human platelets were purified as previously described [3,8]. Radioiodination of α-thrombin was carried out as described by Baker et al [6]. Specific activity of [^{125}I]-thrombin varied from 1.1 to 1.4×10^4 cpm/ng. [^{125}I]-Thrombin was inactivated with diisopropylphosphofluoridate (DFP) as previously described [3]. Medium A contains 0.12 M NaCl, 4.3 mM KCl, 8.5 mM $MgCl_2$, 3 mM glucose, 10 mM HEPES, pH 6.5 [9]. Medium B was prepared by adding 10 mM creatine phosphate, 40 μg/ml creatine phosphokinase, and 0.5 mM EGTA to medium A [10].

RESULTS

As previously reported [1–3], incubation of platelets with [^{125}I]-thrombin results in the formation of platelet-associated SDS-resistant 77-kD complexes between [^{125}I]-thrombin and PN_p (Fig. 1). These complexes are electrophoretically indistinguishable from complexes formed between [^{125}I]-thrombin and purified fibroblast PNI. [^{125}I]-thrombin that is blocked at its active site with a diisopropylphosphoryl group is not complexed by PN_p (Fig. 1). Formation of SDS-resistant complexes with active serine

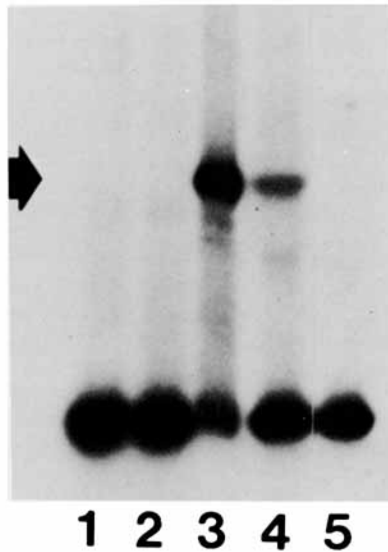

Fig. 1. Inability of PN_p to complex DIP-thrombin. Complexes were formed between [^{125}I]-thrombin and PN_p by incubating platelets (2×10^8/ml) in medium B with 3.3 nM [^{125}I]-thrombin for 2 min at 23°C. The binding incubation was terminated by layering the platelet mixture over a 0.5-ml silicon-oil mixture (2.5:1, Dow 710: Apiezon A) and sedimenting the mixture in a microfuge (Fisher model 235A) for 2 min at 11,000 rpm [13]. Platelet pellets were solubilized in SDS-PAGE sample buffer containing 2% β-mercaptoethanol and resolved by SDS-PAGE using 5–20% gradient gels as described by Laemmli [14]. Lane 1, [^{125}I]-thrombin marker; lane 2, DFP-treated [^{125}I]-thrombin marker; lane 3, [^{125}I]-thrombin-PNI complexes; lane 4, [^{125}I]-thrombin bound to platelets; lane 5, DFP-treated [^{125}I]-thrombin bound to platelets. Arrow denotes position of 77-kD complexes.

proteases but not with serine proteases that are blocked at their active sites is a characteristic of most mammalian serine protease inhibitors.

PN_p-[^{125}I]-thrombin complexes are found both associated with the platelets and free in the [^{125}I]-thrombin incubation buffer [3]. To check whether the platelet-associated complexes actually form in the soluble phase and then bind to the platelet surface, platelets were incubated with 1 nM [^{125}I]-thrombin. The resulting "conditioned" [^{125}I]-thrombin incubation buffer was separated from the platelets by centrifugation and saved for incubation with a second set of platelets. Table I shows, consistent with previous results [3], that the recovered buffer contained PN_p-[^{125}I]-thrombin complexes. In the present experiment the PN_p-[^{125}I]-thrombin complexes were partitioned roughly equally between the platelets and the buffer (Table I). Before incubation with the second set of platelets, the thrombin inhibitor hirudin (at 40 μg/ml) was added to the conditioned [^{125}I]-thrombin incubation buffer. The hirudin prevented uncomplexed [^{125}I]-thrombin from being complexed to the PN_p provided by the second set of platelets (see Table I). We have previously shown that PNI-[^{125}I]-thrombin complexes bind to fibroblasts in the presence of hirudin [11]. As shown in Table I, little of the soluble PN_p-[^{125}I]-thrombin bound to the second set of platelets. The small amount that did bind may have been nonspecifically bound. This result suggests that the complexes found in association with platelets form by [^{125}I]-thrombin binding to platelet-associated PN_p.

TABLE I. Location of [^{125}I] Thrombin-PN$_p$ Complexes

Complexes associated with platelet pellet (cpm)	Total complexes in supernatant (cpm)	Complexes that bound to platelets (cpm)	Total complexes remaining in supernatant (cpm)
Experiment I[a]			
1,432	1,290	59	1,200
Experiment II			
1,042	1,605	31	840

2.5 × 10^8 platelets/ml in medium B were incubated for 30 sec with 1 nM [^{125}I] thrombin. Following sedimentation through oil, platelet pellets (column 1) and a fraction of the supernatants (column 2) were assayed for PN$_p$^{125}I-thrombin complexes by SDS-PAGE and autoradiography. The remaining supernatants (column 2) were incubated with 10 μg/ml hirudin for 5 min at 23°C to inhibit uncomplexed [^{125}I] thrombin. The hirudin mixtures were incubated with fresh platelets (2.5 × 10^8/ml) for 30 sec at 23°C and sedimented through oil. Pellets (column 3) and supernatants (column 4) were assayed for complexes.
[a]These experiments utilized the same iodinated thrombin preparation and platelets from two different individuals.

A modified version of the above experiment was also carried out. DFP (5 mM) instead of hirudin was used to inhibit uncomplexed [^{125}I]-thrombin in the conditioned binding incubation medium. The soluble PN$_p$-[^{125}I]-thrombin complexes in this mixture again failed to bind to platelets (data not shown).

The above results suggest that a fraction of PN$_p$ is immobilized on platelets. This material might arrive on the platelet surface by first being released from platelets and then being recaptured by sites on the platelet surface. If so, it might be possible to load platelets with PN$_p$. Medium preincubated with platelets for several hr contains PN$_p$, but in trace quantities, and this material has not been purified. In view of the similarity of PNI to PN$_p$ and the availability of PNI in purified form, we checked whether platelets bound PNI. Platelets were incubated for 30 min at 37°C in medium A that contained 0.05% BSA (carrier) either without PNI or with PNI at 10, 1, or 0.1 μg/ml. The platelets were rinsed twice by sedimentation and resuspension in medium B and then incubated for 2 min at 23°C with [^{125}I]-thrombin. The 77-kD radioactive material was resolved and detected by SDS-PAGE/autoradiography and quantitated by counting gel slices in a gamma counter. Platelets incubated with PNI did not contain a detectably elevated level of this material (data not shown).

The quantity of PN$_p$-[^{125}I]-thrombin found on platelets is substantially reduced when incubations of platelets and [^{125}I]-thrombin are carried out in the presence of 3.4 U/ml heparin [3]. Possible explanations for this inhibitory action are that heparin releases PN$_p$ from its binding sites on the platelet surface or that heparin inhibits binding of [^{125}I]-thrombin to platelet-bound PN$_p$. In the former case *pretreatment* of the platelets with heparin would be expected to be sufficient to cause the inhibition. Platelets were incubated with heparin for 15 min. The heparin was removed by twice sedimenting and resuspending the platelets in medium B, and the platelets were incubated with [^{125}I]-thrombin. As shown in Figure 2, the quantity of PN$_p$-[^{125}I]-thrombin found in association with platelets was substantially increased as a result of preexposure to heparin. At the highest heparin concentration tested (0.5 mg/ml), this increase was approximately threefold, as indicated by quantitation of the 77-kD band shown in Figure 2.

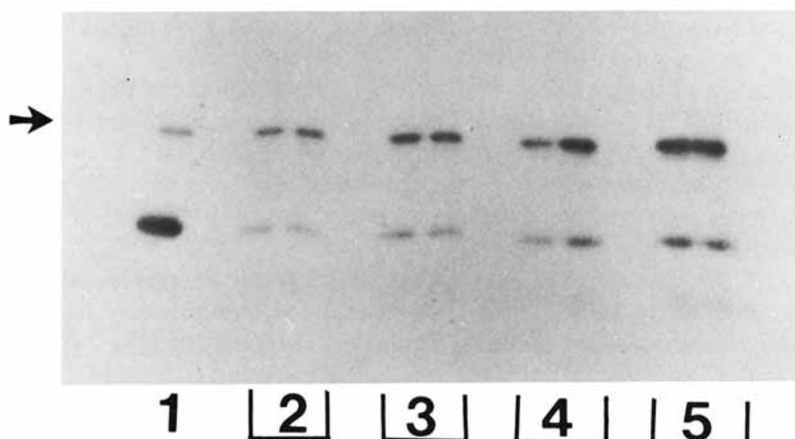

Fig. 2. Effect of heparin pretreatment on [^{125}I]-thrombin binding to platelets. Platelets (3 × 10^8/ml) in medium B were preincubated for 15 min at 23°C with no addition (lanes numbered 2) or with heparin at 3.2 units/ml (lanes numbered 3), 17 units/ml (lanes numbered 4), or 85 units/ml (lanes numbered 5). Heparin was removed by centrifugation at 3,000g for 20 min and subsequent resuspension in 1 ml of medium B. The centrifugation and resuspension were repeated. [^{125}I]-Thrombin (0.8 nM) was incubated with pretreated platelets for 2 min at 23°C, and SDS-PAGE and autoradiography were carried out as described in the legend to Figure 1. [^{125}I]-Thrombin-PNI marker is shown in lane 1 (denoted by →).

DISCUSSION

The PN_p-[^{125}I]-thrombin complexes that are found associated with platelets must arise by [^{125}I]-thrombin binding to PN_p that is immobilized on the platelet surface, because complexes between [^{125}I]-thrombin and PN_p that accumulate in thrombin-binding buffer do not bind to platelets. This simple mechanism for formation of the surface-bound complexes is compatible with the kinetics of appearance of these complexes on platelets. We have previously shown that when platelets are incubated with 1 nM [^{125}I]-thrombin, maximum formation of platelet-bound complexes occurs in less than 30 sec at 23°C [3]. The association of PN_p with the platelet surface could profoundly influence its function. Its protease specificity and reactivity could be dictated in part by interaction with certain platelet surface components. Also, because the distribution of PN_p on the platelet surface could be nonrandom, PN_p could preferentially protect certain surface proteins. Based on the amount of PN_p on platelets, we have calculated that PN_p cannot significantly protect platelet thrombin receptors from thrombin unless PN_p is distributed close to or in association with the receptors [3].

The finding that complexes between PN_p and [^{125}I]-thrombin do not bind to platelets contrasts with findings that complexes between fibroblast PNI and [^{125}I]-thrombin undergo high-affinity binding to fibroblasts. The latter interaction can be largely accounted for by PNI-[^{125}I]-thrombin binding to receptors that mediate the endocytosis and degradation of the complexes [6]. It is not unreasonable that platelets do not actively carry out clearance of polypeptide ligands. It will be worthwhile to determine whether fibroblasts can bind and internalize PN_p-[^{125}I]-thrombin complexes. PN_p and PNI are similar but may not be identical. Recent experiments suggest that PN_p may have a much lower affinity for urokinase [3], but this is uncertain

because interaction with platelet surface components might alter the protease specificity of PNI.

In view of its similarity to PNI, PN_p is likely a peripheral rather than an integral membrane protein. PNI is a member of the serpin family of proteins (based on gene sequencing information; M. McGrogan et al, unpublished data), all known members of which are globular, water-soluble proteins. Because peripheral membrane proteins are frequently sloughed, this could explain the appearance of PN_p-[^{125}I]-thrombin in the binding medium.

Like PNI, PN_p has a high affinity for heparin [12]. It is anticipated that heparin at high concentration would dissociate PN_p from possible heparinlike material on platelets. The present results show that platelets pretreated with heparin did not have a reduced capacity to form surface-bound PN_p-[^{125}I]-thrombin complexes. Thus, the platelet surface-binding site for PN_p is probably not heparinlike. In fact, platelets pretreated with heparin had an increased capacity to form platelet-associated complexes. It is possible that surface-bound PN_p is cryptic and is unmasked by heparin. Alternatively, heparin may stabilize PN_p on the platelet surface and thus reduce dissociation of PN_p-[^{125}I]-thrombin complexes from platelets.

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