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COMPLEXES OF THROMBIN WITH SECRETED PLATELET PROTEINS

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SUMMARY: A labeled 77-kDa complex formed when ^{125}I-thrombin was added to platelet suspensions or to the supernatant solution of ionophore-activated platelets. Prostacyclin inhibited complex formation with whole platelets but not with the supernatant solution of ionophore-activated platelets. This is evidence that the complex formed with a factor secreted from activated platelets. Smaller complexes of 70 and 58 kDa formed between labeled thrombin and lysed platelets. The 77-kDa complex was necessary for the formation of a thrombin-thrombospondin complex.

There have been several reports of the formation of a labeled, SDS-stable, 77-kDa complex formed after addition of labeled thrombin to suspensions of washed platelets (1-4). Bennett and Glenn (1) suggested that the complex included thrombin and a thrombin receptor, presumably on the platelet surface. Gronke et al. (3) presented evidence that it was indeed a surface protein, and Gronke et al. (4) concluded that the protein was similar, but not identical, to protease nexin I. They proposed that it was a membrane protein that dissociated from the platelet surface and that it served to protect the platelet from activation by very low levels of thrombin. Lerea and Glomset (5) found that agents that elevate platelet cAMP inhibited formation of the complex, and they proposed that it played a role in thrombin stimulus-response coupling.

A smaller complex (58 kDa) was reported by Shuman et al. (2), and similar complexes have been inconsistently observed by others (5,6). A disulfide-linked complex (>450 kDa) of thrombin with thrombospondin (TSP), a secreted platelet protein, has also been reported (7).

We have investigated the SDS-stable complexes formed by reaction of

ABBREVIATIONS:
SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; APMSF, amidinophenylmethylsulfonyl fluoride; PGI_2, prostacyclin; TSP, thrombospondin; PADGEM, platelet activation-dependent granule-external membrane.

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labeled thrombin with platelets. We report here that the 77-kDa complex forms with a protein that is secreted by activated platelets and thus is unlikely to act as a protective inhibitor and cannot act as an agent in stimulus-response coupling. This complex seems to be necessary for the formation of the larger thrombin-TSP complex. Smaller complexes form with platelet proteins that are made available only by lysis of the cells.

MATERIALS AND METHODS

Platelet preparation--Blood was drawn by venipuncture from healthy volunteers into 0.15 volume anticoagulant (120 mM citrate, 5 mM EDTA, pH 7.4) and then centrifuged at 300 x g for 20 min. Platelets were separated from the platelet-rich plasma by centrifugation at 1000 x g for 20 min, and were washed twice in HEPES buffered saline (HBS) (150 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.4). Washed platelets were resuspended in HBS at 1 - 3 x 10^9/ml. Platelets were maintained at 4°C and were handled with polypropylene tubes and pipets.

Supernatant solution--Washed platelets were activated with the calcium ionophore A23187 (Calbiochem, San Diego, CA). For activation, platelets were warmed to 37°C, mixed with 0.01 volume A23187 (500 µM) in dimethyl sulfoxide, and allowed to react for 2 min. Supernatant solution was separated from the platelet pellet by centrifugation at 12,000 x g for 15 s. Prior to fractionation on the heparin-agarose column, supernatant solution was centrifuged an additional 30 min at 12,000 x g at 4°C.

Heparin-agarose fractionation--Prepared heparin-agarose columns were obtained from Pierce Chemical Co., Rockford, IL. Columns were washed with 2 M NaCl and equilibrated with 0.15 M NaCl prior to each use. Supernatant solution was applied to the column and was eluted with nine 1 ml fractions of NaCl solution of increasing concentration from 0.15 to 0.9 molar. A short length of tubing was added to the column outlet to adjust the void volume to 1 ml. All NaCl solutions were buffered with 10 mM HEPES, pH 7.4. TSP eluted with the 0.5 M fraction, and the 77-kDa-complex-forming activity eluted in the 0.8 M fraction.

Thrombin--Highly purified thrombin was generously supplied by Dr. John W. Fenton, II, Division of Laboratories and Research, New York State Department of Health. Thrombin was iodinated to a specific activity of 50 - 100 Ci/mmole with Iodo-gen (Pierce Chemical Co.). Five µl $Na^{125}I$ solution (0.5 mCi, Amersham Corp., Arlington Heights, IL) was placed in a microtube coated with 100 µg Iodo-gen. 100 µl thrombin (60 µM) was added, and the mixture was allowed to react 2 min on ice. ^{125}I-thrombin was separated from free $Na^{125}I$ on a P-6 desalting column (Bio-Rad, Rockville Centre, NY) and assayed for activity with S-2238 (KabiVitrum, Stockholm, Sweden). Labeled thrombin was inactivated by reaction with 100-fold molar excess APMSF (Calbiochem, San Diego, CA). APMSF was dissolved in dry dimethyl sulfoxide, then twice 0.025 volume was reacted with thrombin for 2 min at room temperature.

Polyacrylamide gel electrophoresis--Gels were prepared essentially as described by Laemmli (8), with the following modifications. AcrylAide cross-linker and GelBond support film from FMC Corp., Rockland, ME, were used as recommended by the manufacturer. Gels were electrophoresed at high current (100 mA per gel) in approximately 90 min using the Hoefer SE 6160 heat exchanger.

RESULTS

Figure 1 summarizes on a single gel the key results from many experiments. Reaction of ^{125}I-thrombin with a platelet suspension resulted in formation of a complex with an apparent mass of approximately 77 kDa (Fig. 1, lane 2), consistent with previous reports (1-4). Addition of labeled thrombin to the

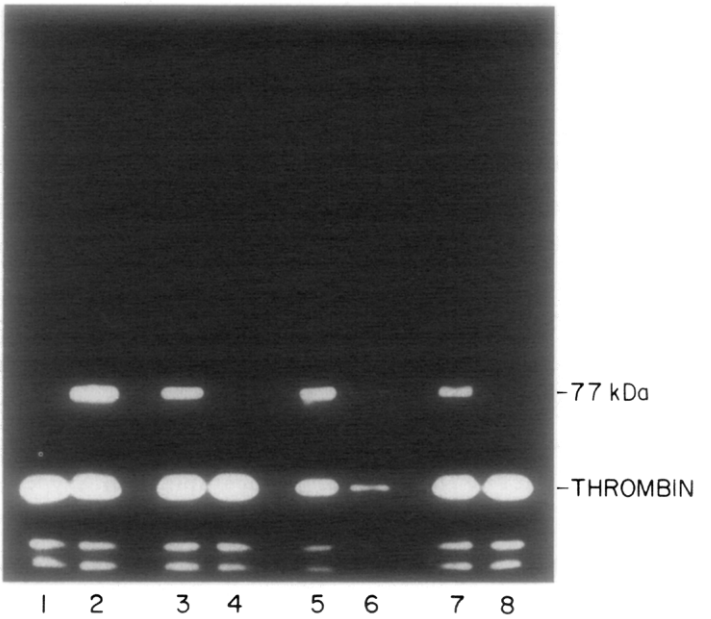

FIG 1. **Formation of Supernatant Complex with ^{125}I-Thrombin.** Each reaction included the whole suspension, the supernatant solution, or the pellet from 100 µl washed platelets (3.0 x 10^9/ml). Five µl ^{125}I-thrombin (5 nM final concentration) was reacted with each of the following samples for 5 min at 37°C. lane 1, control (no platelets); lane 2, whole platelets; lane 3, whole platelets plus 5 µl PGI_2 buffer; lane 4, whole platelets plus 5 µl PGI_2 (500 nM final concentration, added 2 min before ^{125}I-thrombin); lane 7, A23187-activated supernatant solution; lane 8, A23187-activated supernatant solution plus APMSF-^{125}I-thrombin. Lanes 5 and 6 included 100 µl platelet suspension reacted with ^{125}I-thrombin and then separated by centrifugation into supernatant solution (lane 5) and pellet (lane 6). Reactions were stopped by addition of sample buffer with 10% mercaptoethanol. Samples were electrophoresed on a 4 to 16% gradient polyacrylamide gel. A contact print of an autoradiogram from the dried gel is shown.

supernatant solution of resting platelets resulted in no complex (not shown), but addition to the supernatant solution of platelets activated with the ionophore A23187 resulted in 77-kDa complex (Fig. 1, lane 7). Prostacyclin (PGI_2), which inhibits platelet activation, prevented formation of this complex when thrombin was added to the suspension (Fig. 1, lane 4), but not when it was added to the A23187-activated supernatant solution (not shown). This indicates that platelet activation was required for the protein to become reactive with thrombin. Lanes 5 and 6 show the relative distribution of the complex between the platelet pellet and supernatant solution after addition of labeled thrombin to the suspension. Bands from these two lanes were cut out and counted, revealing that 75% of the complex was in the supernatant solution, only slightly more than found by Lerea and Glomset (5). The complex was not formed when the supernatant solution was reacted with active-site inhibited thrombin (lane 8). We conclude from these data that the complex-forming protein was not accessible on the surface of resting

platelets, but that it was released into the supernatant solution by activated platelets.

Intact platelets reacted with ^{125}I-thrombin formed only the 77-kDA complex, but additional complexes of approximately 70 and 58-kDa were formed with sonicated platelets (Fig. 2). When the platelet suspension was sonicated prior to reaction, all three complexes were formed (Fig. 2, lane 3). The resuspended pellet of ionophore-activated platelets formed virtually no complex with ^{125}I-thrombin (lane 4), but after sonication the 70-kDa and 58-kDa complexes were formed (lane 5). The supernatant solution from the same platelets formed only the 77-kDa complex. We initially hypothesized that the smaller complexes arose from the action of platelet proteases on the 77-kDa complex. There was, however, no change in the relative amounts of the three complexes when we varied the time of incubation with any combination of supernatant solution, sonicate or whole platelets with or without thrombin present (not shown). This suggests that the smaller complex-forming proteins are not proteolytic fragments of the larger complex-forming protein.

Kinetic evidence[1] suggested that the 77-kDa complex is an intermediate in the formation of a 450-kDa complex which includes thrombin and TSP (7). We

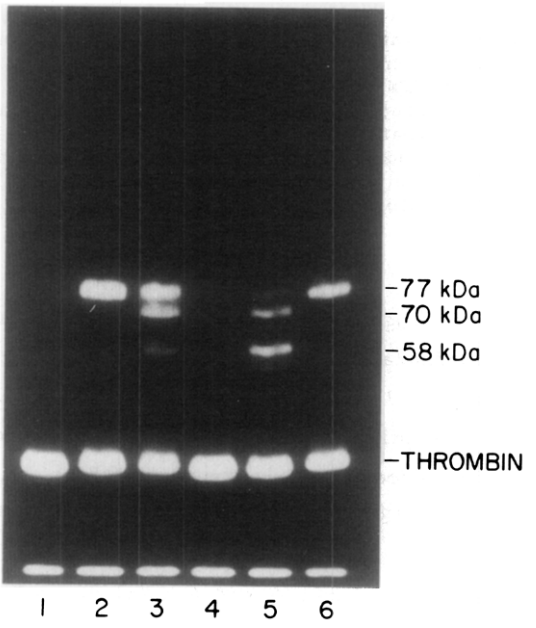

FIG 2. **Formation of Pellet Complex with ^{125}I-Thrombin.** ^{125}I-labeled thrombin was reacted as in Figure 1 with the following samples. lane 1, control (no platelets); lane 2, intact platelets; lane 3, sonicated platelets; lane 4, resuspended pellet from A23187-activated platelets; lane 5, sonicated resuspended pellet; lane 6, supernatant solution from sample 4. Samples were electrophoresed on a 10% polyacrylamide gel under reducing conditions. A contact print of an autoradiogram from the dried gel is shown.

[1]P. C. Browne, J. J. Miller, and T. C. Detwiler, manuscript in preparation.

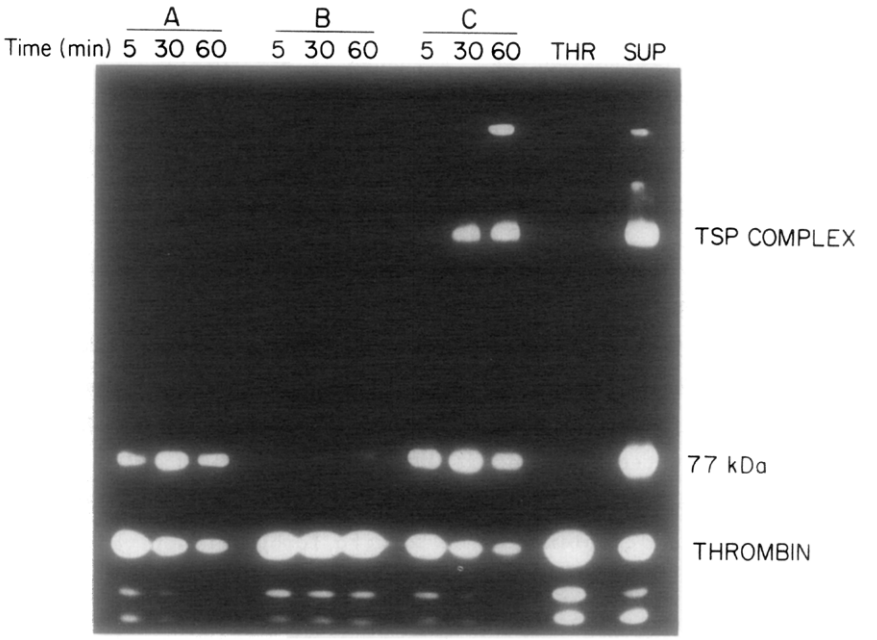

FIG 3. **77-kDa Complex is an Intermediate for Formation of TSP Complex.** Supernatant solution from A23187-activated platelets was fractionated on a heparin-agarose column as described in Materials and Methods. Fractions containing partially purified 77-kDa-complex-forming protein (A), TSP (B), or fractions A and B together (C), were reacted with 2 nM ^{125}I-thrombin. Aliquots from each reaction were mixed with sample buffer at 5, 30, and 60 min after the start of each reaction. The lane labeled "SUP" includes unfractionated supernatant solution reacted with ^{125}I-thrombin for 60 min. The lane labeled "THR" contains ^{125}I-thrombin with platelet buffer. Samples were electrophoresed on a 4-16% gradient gel under non-reducing conditions. A contact print of an autoradiogram from the dried gel is shown.

obtained partially purified TSP and 77-kDa-complex-forming fractions by heparin-agarose affinity chromatography of the supernatant solution of A23187-activated platelets. These two fractions were reacted separately or together with ^{125}I-thrombin (Fig. 3). The 77-kDa complex formed within 5 min (Fig. 3A), but partially purified TSP formed virtually no complex with ^{125}I-thrombin, even after 1 hr (Fig. 3B). Only when the 77-kDa-complex-forming fraction was mixed with the TSP fraction was the 450-kDa complex seen (Fig. 3C). We conclude that the fraction containing the 77-kDa-complex-forming protein was necessary for the formation of the thrombin-TSP complex.

DISCUSSION

There is confusing literature on the nature and the functions of the complexes formed when thrombin is added to platelet suspensions. The 77-kDa complex has been considered to be a surface protein that either mediates platelet activation by thrombin (1,5) or that protects the platelet from low levels of thrombin (4). We demonstrate here, however, that the protein that forms the 77-kDa complex is not reactive on resting platelets but is released

from activated platelets. Thus its reaction with thrombin is a consequence, not a cause, of platelet activation. Although it may function as a feed-back inhibitor of thrombin activation, we consider it more likely to have a function unrelated to mediation or inhibition of platelet activation.

We assume that it is released from alpha granules along with other proteins, but that has not been proved and will be difficult to test rigorously. Berman et al. (9) presented evidence that a fraction of an alpha granule protein (referred to as PADGEM protein) becomes integrated into the platelet membrane upon platelet activation. This type of granule-membrane translocation may well explain why some of the 77-kDa complex remains associated with activated platelet pellets. Gronke et al. (3) showed that the 77-kDa complex does not re-bind to resting platelets. We confirmed this and also found that the complex did not bind to ionophore-activated platelets (not shown).

A 58-kDa complex of thrombin and a platelet protein has been reported by others (2,5). We find that this complex forms only with disrupted platelets, which explains its inconsistent appearance. We have also observed a 70-kDa complex formed on addition of thrombin to lysed platelets. The proteins that form these complexes are not released on platelet activation, and no precursor-product relationship between these and the 77-kDa complex could be demonstrated.

While the function of the 77-kDa complex is not clear, it may be significant that it is essential for the incorporation of labeled thrombin into a complex with TSP. TSP is a protein that is secreted from platelet alpha granules and that is believed to be an "adhesive" protein that binds to other proteins and to cells (10,11).

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