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Effect of thrombin on the platelet membrane

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SUMMARY

A surface-exposed membrane glycopeptide has been identified in human platelets which is sensitive to the action of thrombin.

It is well recognized that the physiological role of thrombin is not restricted to its action on plasma fibrinogen but includes also its effect on blood platelets. While thrombin is not unique in its ability to aggregate platelets its relatively high specificity as a proteolytic enzyme has made it an interesting tool for studying the molecular changes of platelet aggregation. In searching for a specific substrate of thrombin a number of platelet proteins have been identified which show lability towards this enzyme. Platelet fibrinogen, similar in most respects to that in plasma, has been claimed to be the primary target for thrombin's action on platelets[1,2]. While firm evidence to substantiate this hypothesis is lacking, the thrombin-induced release of a thrombosthenin-like protein from intact platelets[3] and the sensitivity of adenyl cyclase[4] and fibrin-stabilizing factor[5,6] to this enzyme has been established more conclusively. Although some or all of these thrombin-labile proteins may be located in the platelet membrane, so far no definite direct proof for the existence of a thrombin-sensitive membrane protein has been brought forward. In this report a platelet membrane protein is identified which appears to be highly sensitive to the action of this enzyme both in intact platelets and their isolated membrane vesicles.

Exposed tyrosine residues on the platelet surface were iodinated by a modification of the lactoperoxidase method of Phillips and Morrison[7]. Human platelets isolated by standard methods were washed twice with Tris–HCl buffered (pH 7.4) 0.15 M NaCl (Tris-buffered saline) containing 1 mM EDTA, and were then suspended in a 0.05 M phosphate buffered (pH 7.2) 0.15 M NaCl at a final concentration of approximately $15 \cdot 10^9$ platelets

per ml. Lactoperoxidase (10^{-6} M) and 80 μCi carrier-free $Na^{125}I$ were added per ml platelet suspension. Hydrogen peroxide was generated by a glucose (1 mg/ml--glucose oxidase (2.5 μg/ml) system. Enzymic iodination was continued for 30 min at 30 °C. After washing the platelets twice with the above buffer the cells were suspended in Tris-buffered saline ($4 \cdot 10^9$ platelets/ml) and their plasma membranes isolated by the method of Barber and Jamieson[8]. In other experiments platelet membranes were first isolated and then iodinated essentially according to the procedure outlined above. The membranes were then solubilized in sodium dodecylsulfate (0.1%)-containing phosphate (0.01 M) buffer, pH 7.2

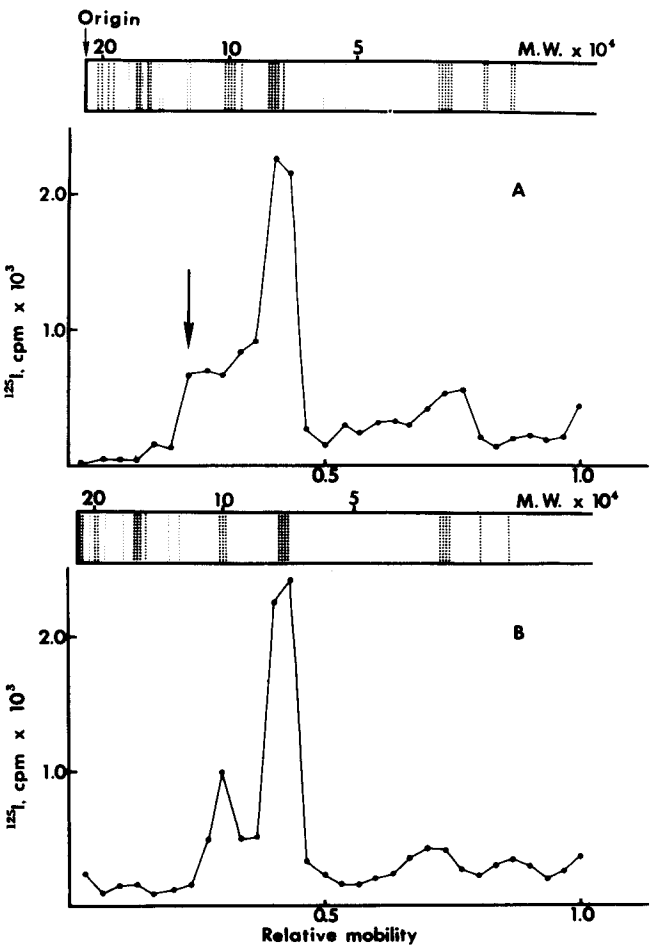

Fig. 1. Distribution of ^{125}I-iodinated platelet membrane polypeptides. Intact platelets were iodinated by the lactoperoxidase method. Plasma membranes were isolated and solubilized either in the presence (A) or absence (B) of 1% 2-mercaptoethanol. The solubilized membranes were subjected to polyacrylamide gel (5% cross-linked) electrophoresis in 0.1% sodium dodecylsulfate. A schematic drawing of the distribution of Coomassie blue stainable polypeptide bands is shown at the top of each panel. The arrow points to the glycopeptide appearing in mercaptoethanol-treated membranes. Gels were sliced into 1.5-mm sections and counted. The gel was calibrated for molecular weight by the use of standard proteins.

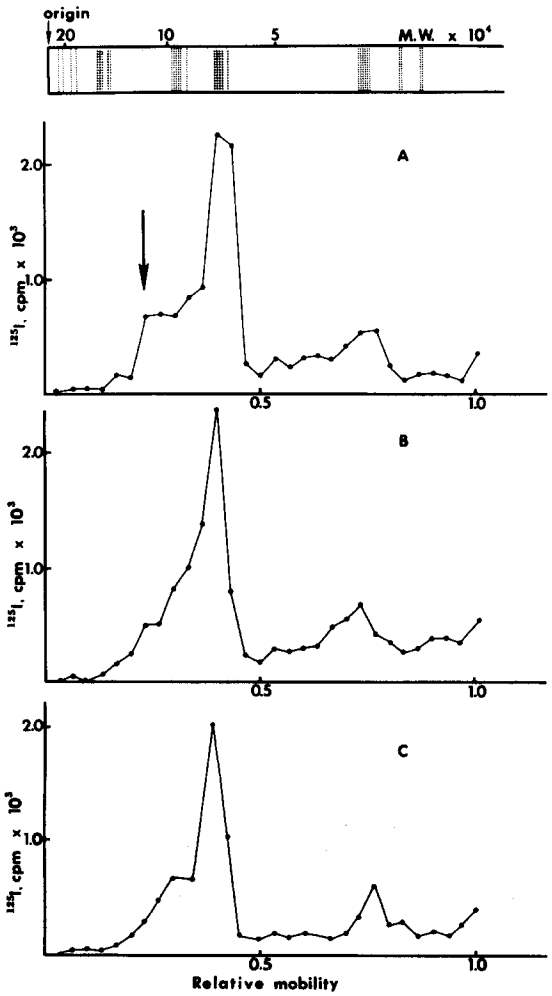

Fig. 2. Effect of thrombin on iodinated platelets. Intact platelets were incubated without thrombin (A) or with thrombin 0.1 unit/ml (B) and 1 unit/ml (C). Platelet membranes were isolated, solubilized with 1% 2-mercaptoethanol and subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis. A schematic drawing of the distribution of Coomassie blue stainable polypeptide bands is shown at the top of the figure. The arrow points to the thrombin-sensitive glycopeptide.

and membrane proteins were separated by sodium dodecylsulfate–polyacrylamide gel (5% cross-linked) electrophoresis.

The distribution of radioactivity along the gel showed one major peak corresponding to a polypeptide of approximate molecular weight 70 000 and 2 smaller peaks one representing a polypeptide of molecular weight 34 000, the other a polypeptide of molecular weight 100 000 (Fig. 1). The profile of radioactive counts was virtually identical whether intact platelets or isolated membrane vesicles were iodinated. This finding attests to the fact that the surface topography of membrane proteins was preserved when

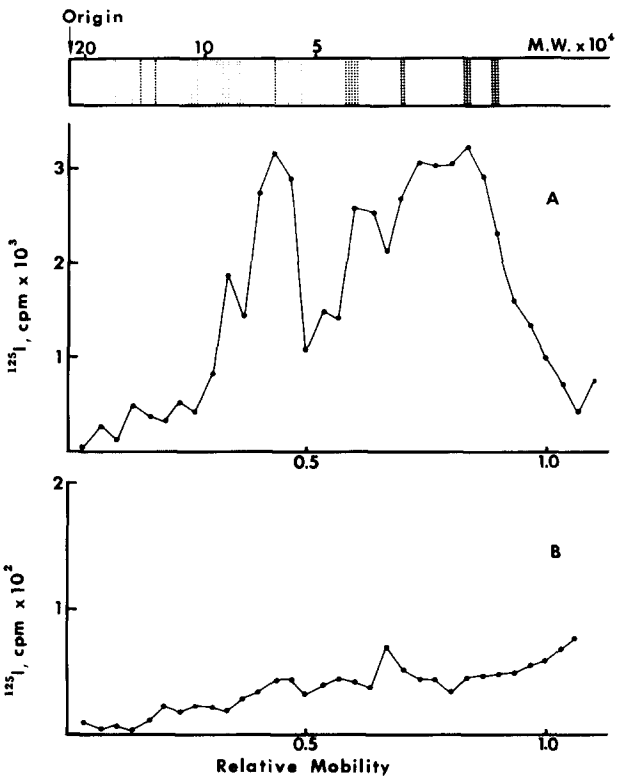

Fig. 3. Effect of trypsin on platelet membrane polypeptides. Intact platelets were either treated with trypsin (A) and then iodinated or first iodinated and then subjected to trypsin (B). Platelet membranes were then isolated, solubilized in the presence of 1% 2-mercaptoethanol and subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis. The schematic drawing at the top of the figure denotes the pattern of Coomassie blue stainable membrane polypeptides.

plasma membranes were isolated from platelets disrupted by the glycerol-lysis technique. The addition of 2-mercaptoethanol to the solubilizing medium resulted in minor changes in the pattern of polypeptide bands stainable with Coomassie blue. Analysis of ^{125}I-iodinated platelet membranes solubilized in the presence of this disulfide-reducing agent revealed iodination of an additional polypeptide (approximate molecular weight 121 000) besides those which were found to be labeled when membrane proteins were solubilized without mercaptoethanol. The labeling pattern of the mercaptoethanol-treated membrane polypeptides remained essentially unchanged whether intact platelets or isolated membrane vesicles were iodinated.

Intact platelets or isolated platelet membrane vesicles iodinated by the lactoperoxidase method were incubated for 10 min at 37 °C with thrombin in concentrations ranging from 0.1 to 10 units/ml. Aggregation of platelets was prevented by addition of 1 mM EDTA to the suspending medium. The reaction was stopped by addition of proteolytic inhibitors, *i.e.* toluenesulfonyl fluoride and *N*-carbobenzoxy-α-L-glutamyl-L-

tyrosine and sedimentation of platelets or platelet membrane vesicles by centrifugation. Both platelets and platelet membrane vesicles were washed twice with Tris-buffered saline containing EDTA. The platelets were then lysed, their plasma membranes separated and both the platelet membrane vesicles as well as the plasma membranes of the intact platelets were solubilized as described above. Membrane polypeptides were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis and the distribution of ^{125}I among the identifiable polypeptide bands was determined.

There was virtually no difference in the profile of radioactivity whether platelets or platelet membrane vesicles were incubated with thrombin. A general reduction in radioactive counts was observed when thrombin was used in concentrations exceeding 0.5 units/ml but none of the surface-exposed iodinated membrane polypeptides showed a specific decrease or disappearance.

The inclusion of mercaptoethanol in the membrane solubilizing buffer which in control platelets resulted in the appearance of an additional iodinated glycopeptide led to the disappearance of this ^{125}I peak in thrombin-incubated platelets (Fig. 2). The staining intensity with Coomassie blue of the band corresponding to this polypeptide was only slightly decreased by thrombin; A concentration related decrease in the ^{125}I counts of this glycopeptide could be demonstrated. As little as 0.1 unit/ml of thrombin was sufficient to produce a specific change in the ^{125}I profile.

The specificity of the action of thrombin on platelet membrane proteins or polypeptides was further substantiated by comparing it to the effect of trypsin on platelet membrane proteins. The plasma membranes of intact platelets and isolated membrane vesicles were initially iodinated by the lactoperoxidase method and after repeated washing these structures were incubated for 10 min at 37 °C with trypsin at a concentration of 0.5–1.0 nmoles/ml. Aggregation of platelets or membrane vesicles was prevented by addition of 2 mM EDTA. The incubation was stopped and membrane polypeptides were solubilized as described above. Trypsin-treated membranes revealed a profound change in the distribution of polypeptides. Generally speaking there was a drastic decrease in the staining intensity of high molecular weight polypeptide bands while at the same time the apparent concentration of lower molecular weight polypeptides increased (Fig. 3). Bands at the anodal end of the gel which usually are barely visible when stained with Coomassie blue became more prominent and other polypeptides ranging in molecular weight between 70 000 and 34 000 made their appearance. There was a sharp reduction in the ^{125}I associated with the surface exposed membrane proteins but none of the labeled polypeptides disappeared completely. Isolated membrane vesicles and the plasma membranes of trypsin-treated intact platelets exhibited a similar pattern. While the temporal sequence of iodination and incubation of platelets or platelet membrane vesicles with thrombin did not affect significantly the distribution of ^{125}I among membrane polypeptides, trypsin treatment did so. When the exposure of platelet membranes to trypsin preceded enzymic iodination the labeling with ^{125}I was altered both quantitatively and qualitatively. There was a much greater ^{125}I-labeling intensity of surface exposed membrane proteins and a shift in the iodination pattern towards predominance of low molecular weight polypeptides

indicating that through the action of trypsin a large number of tyrosine residues had become exposed. This finding is in general agreement with that of Phillips and Morrison[9] on red cells.

These results strongly indicate that thrombin exerts a specific effect on the platelet surface. As the thrombin-sensitive glycopeptide did not completely disappear this proteolytic enzyme apparently removes only the surface-exposed portion of the protein. The nature of this membrane glycopeptide remains to be determined. None of the known thrombin-labile platelet proteins appears to be identical to this membrane polypeptide. The observation that a reducing agent was necessary in the solubilizing medium to demonstrate this polypeptide suggests that in the native state this polypeptide is linked through a disulfide bridge(s) to other polypeptide(s). Although this may be completely fortuitous, the fact that the individual polypeptide subunits of plasma fibrinogen are covalently linked by disulfide bridges cannot be ignored. Our results represent direct evidence of the existence of a specific thrombin-sensitive membrane protein in platelets.

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