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THROMBIN SUBSTRATES AND THE PROTEOLYTIC SITE OF THROMBIN ACTION ON HUMAN-PLATELET PLASMA MEMBRANES*

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SUMMARY

Intact or isolated plasma membranes of human platelets were treated with thrombin (EC 3.4.4.13), and the membrane proteins hydrolyzed by this enzyme were identified by acrylamide gel electrophoresis and lactoperoxidase-catalyzed iodination. In isolated membranes, a high-molecular-weight polypeptide (220 000) and three glycoproteins with molecular weights of 150 000, 118 000 and 93 000 were reduced in concentration and/or molecular weight after thrombin treatment. In intact membranes, however, only the 118 000-mol. wt glycoprotein was reduced in concentration. Although two other thrombin substrates (the 150 000- and 93 000-mol. wt glycoproteins) were exposed on the surface of intact membranes, they were not hydrolyzed. Thus, the plasma membrane of human platelets appears to contain at least two thrombin substrates which, because of their orientation in the membrane, are resistant to hydrolysis on intact platelets. The 118 000-mol. wt glycoprotein, the only protein hydrolyzed by thrombin on intact platelets, may be the proteolytic site of thrombin action on the membrane surface.

INTRODUCTION

The platelet plasma membrane is the primary site of action of many agents that affect platelet morphology and hence hemostatic and thrombotic processes involving the platelet. Among these biologically important agents are collagen, ADP, particulate matter, and thrombin [1–4]. Thrombin (EC 3.4.4.13) is of particular interest as it is a potent inducer of platelet aggregation and the platelet release reaction. Because of its proteolytic activity, thrombin is assumed to hydrolyze protein on the surface of the platelet membrane as an early step in thrombin-induced reactions of the platelet. Although many platelet proteins can serve as substrates for this enzyme [5–8], it is not known which proteins of the platelet membrane are thrombin substrates and which, if any, of these substrates are hydrolyzed by thrombin on the

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intact platelet. Acquiring this information is an essential step in identifying the proteolytic site of thrombin action on the platelet.

We recently showed, using the lactoperoxidase iodination technique, that three glycoproteins and many non-glycosylated polypeptides are exposed on the surface of human-platelet plasma membranes [9]. We now demonstrate that these three glycoproteins and one membrane polypeptide are substrates for thrombin, but that only the 118 000-mol. wt glycoprotein is hydrolyzed on the intact membrane. This protein may be the proteolytic site of thrombin action on the membrane surface.

MATERIALS AND METHODS

Radioisotopes, ^{125}I and ^{131}I, were purchased from New England Nuclear. Lactoperoxidase was isolated from bovine milk by the method of Morrison and Hultquist [10]. Thrombin (bovine), purified by affinity chromatography [11], was a gift of Dr E. Davie. Apyrase (potato) was purchased from Sigma Chemical Co. The remaining chemicals were of reagent grade.

Fresh human platelets were collected and washed as previously reported [9]. The isolation media contained 0.0965 M NaCl, 0.0857 M glucose, 0.0011 M Na_2 EDTA, and 0.00858 M Tris (pH 7.4). Platelet plasma membranes were isolated by the glycerol-lysis method [12]. Washed, intact platelets were iodinated as described previously [9]. Protein concentrations were determined by the method of Lowry et al. [13].

Thrombin treatment of platelet plasma membranes

Immediately after isolation, platelet plasma membranes were suspended in 0.15 M sucrose, 10 mM Tris, pH 7.4, to a concentration of 0.25 mg/ml, divided into two aliquots, and equilibrated at 37 °C. To one aliquot we added thrombin to a final concentration of 10 NIH units/ml; to the other we added an equal volume of saline. After these additions, the membrane suspension was incubated an additional 15 min, cooled to 0–4 °C in an ice bath, and centrifuged at 105 000 $\times g$ for 45 min. The membranes were dissolved in 2 % sodium dodecylsulphate and frozen.

Thrombin treatment of intact platelets

Washed platelets were suspended to a concentration of 10^9 platelets/ml in isolation media without EDTA but containing 50 μg apyrase/ml. We then added thrombin at a final concentration of 10 NIH units/ml and incubated the suspension at 37 °C for 30 min in a shaker bath. In a control experiment, the platelets were treated identically except that thrombin was not added. The solution was cooled to 0–4 °C in an ice bath and centrifuged at 1500 $\times g$ for 10 min. The platelets were washed by suspension in the original volume of isolation medium, followed by centrifugation at 1500 $\times g$. This procedure was repeated twice. The isolated cells were then solubilized in 2–3 % dodecylsulphate and frozen.

Gel electrophoresis

Proteins of solubilized platelets and platelet plasma membranes (50 μg samples) were separated by electrophoresis on 5 % acrylamide gels after reduction as previously described [9]. The gels were 13 cm in length and contained 0.1 % dodecylsulphate. They were stained for protein with Coomassie Brilliant Blue or for car-

bohydrate with fuschin sulphite. The radioactive iodine distribution in the gels of radioactive samples was determined by slicing unstained gels laterally into 1.5-mm sections and counting the radioactivity in each slice. Samples containing ^{125}I or ^{131}I were also subjected to electrophoresis on the same gel and counted. Separate electrophoresis of both samples showed that, at the concentrations employed, there was no overlap between the channels when the doubly labeled samples were co-electrophoresed.

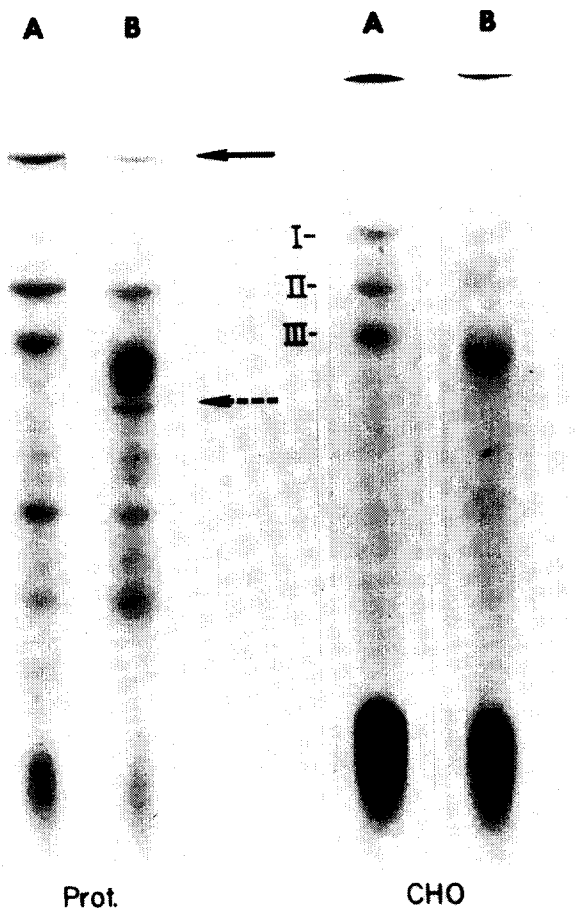

Fig. 1. Effect of thrombin on isolated plasma membranes. Plasma membranes were isolated by the glycerol-lysis method and suspended in 0.25 M sucrose and 10 mM Tris, pH 7.4. One-half of the membrane suspension was treated with thrombin (10 units/ml)(B), while the other half was untreated (A). Both samples were incubated at 37 °C for 15 min. The membranes were then collected by centrifugation at 105 000 × *g* for 15 min, dissolved in 2 % dodecylsulphate, and electrophoresed on 5 % acrylamide gels containing 0.1 % dodecylsulphate. Parallel gels were stained for carbohydrate (CHO) and for protein (Prot.). As shown by these gels, three membrane glycoproteins and a high-molecular-weight polypeptide (solid arrow) were affected by thrombin treatment. Also shown is a hydrolytic product (broken arrow) not containing carbohydrate.

RESULTS

Fig. 1 shows the susceptibility of membrane polypeptides and glycoproteins to thrombin hydrolysis on isolated membranes. The three glycoproteins, labeled I, II and III in the figure, have mol. wts of 150 000, 118 000 and 93 000, as determined from 5% gels. Glycoprotein I, which stained poorly for protein, as did the major glycoprotein in the human erythrocyte membrane [14], was reduced in concentration by thrombin treatment. Glycoproteins II and III, which stained for both protein and carbohydrate, were reduced not only in concentration, but also in molecular weight. The concentration of a high-molecular-weight polypeptide (220 000) was aiso reduced. One polypeptide band corresponding to a molecular weight of 80 000 appeared after hydrolysis (broken arrow). The 220 000-mol. wt polypeptide and its 80 000-mol. wt product corresponded to platelet myosin and its hydrolytic product, which have been described previously [15].

Plasma membranes isolated from cell types other than platelets are permeable to macromolecules unless resealed [16]. Similarly, electron micrographs of platelet plasma membranes isolated by glycerol lysis indicate that many of these membranes are open vesicles [12]. Thus thrombin treatment of isolated membranes could conceivably hydrolyze protein on both the inner and outer surfaces of the membrane. To determine which of the thrombin substrates were exposed to the outside of the intact platelet, we treated plasma membranes from iodinated platelets with thrombin. The iodination procedure employed lactoperoxidase, which has been shown to

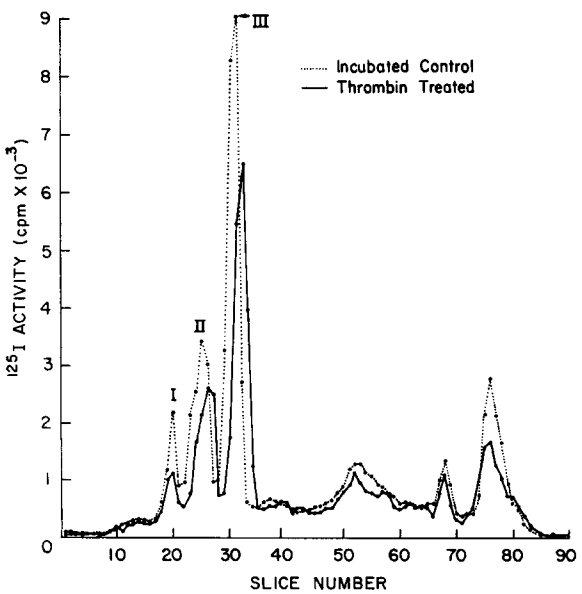

Fig. 2. Effect of thrombin on plasma membranes from iodinated intact platelets. Plasma membranes were isolated from iodinated platelets, treated with thrombin and electrophoresed as described in Fig. 1. The gels were sliced laterally into 1.5-mm sections and the radioactivity in each slice was determined. The reduced radioactivity in Glycoproteins I, II and III indicates that iodination did not alter the susceptibility of these proteins to thrombin.

specifically iodinate proteins on the outer membrane surface of intact cells [17]. As expected, the three glycoproteins were labeled (Fig. 2), demonstrating that these glycoproteins are exposed on the membrane surface. Moreover, in agreement with Fig. 1, Glycoprotein I was reduced in concentration after thrombin treatment, while Glycoproteins II and III were reduced not only in concentration but also in molecular weight. A polypeptide of low molecular weight was also reduced in concentration. Interestingly, the 220 000-mol. wt polypeptide visible in Fig. 1 was not iodinated on intact cells, although it is labeled readily when isolated membranes are subjected to lactoperoxidase-catalyzed iodination [18]. These data indicate that the polypeptide was hydrolyzed by thrombin on the inner surface of the membrane.

We next examined the effect of thrombin on the polypeptide composition of intact platelets. Comparison of acrylamide gels (Fig. 3) confirmed the observation of

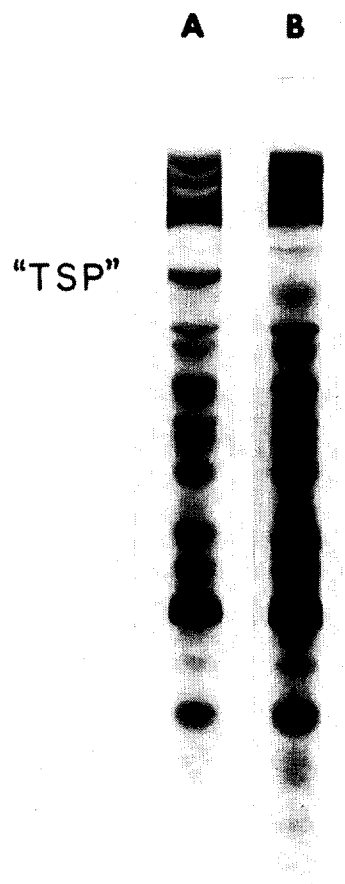

Fig. 3. Effect of thrombin on the polypeptide composition of intact platelets. Intact platelets were incubated in the presence (B) or absence (A) of thrombin (10 units/ml), washed and solubilized in 2% dodecylsulphate. The solubilized platelets were then electrophoresed as described in Fig. 1 and stained for protein. These gels show that the intracellular protein, termed thrombin-sensitive protein or "TSP", is the only detectable component that is reduced in concentration.

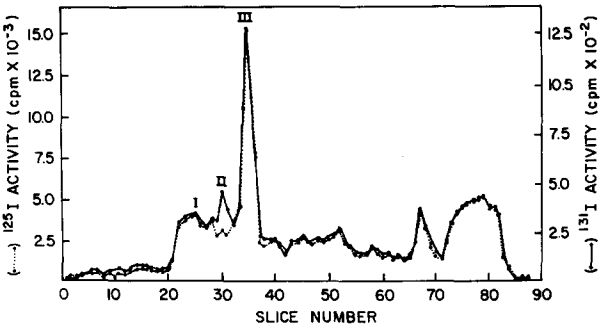

Fig. 4. Effect of thrombin on iodinated, intact platelets. Platelets were iodinated with either ^{131}I or ^{125}I by the lactoperoxidase procedure. Thrombin (10 units/ml) was then added to the ^{125}I-labeled platelets while an equal volume of buffer was added to the ^{131}I-labeled sample. Both solutions were then incubated at 37 °C for 15 min. The platelets were isolated, washed, and solubilized in 2 % dodecylsulphate. The two samples were then mixed, electrophoresed on one gel, sliced into 1.5-mm sections, and counted. These tracings show that the only thrombin substrate which is hydrolyzed by thrombin on the intact platelet is Glycoprotein II (mol. wt 118 000).

Baenziger et al. [19, 20] that only one major polypeptide is decreased in concentration as a result of thrombin hydrolysis. This polypeptide, designated in the figure as "TSP" or thrombin-sensitive protein [19], was not hydrolyzed from the surface of the intact platelet, but was released from within the platelet as a result of thrombin action on a plasma-membrane protein. This interpretation is based on the following observations. "TSP" is not isolated with the plasma membrane [19], but is released unaltered from the platelet by thrombin action [20]. Its release is inhibited by pre-incubation of platelets with prostaglandin E_1 [21], an inhibitor of the platelet release reaction [22], and it is not iodinated by lactoperoxidase on the intact cell [9].

To determine which protein on the membrane surface was hydrolyzed by thrombin, we first iodinated platelets by lactoperoxidase to label the proteins on the membrane surface; then we treated the membrane with thrombin and directly determined the concentrations of iodinated proteins. Preliminary experiments showed that differences between the thrombin-treated samples and incubated controls were very subtle, and difficult to assess by comparison of gels. In order to analyze more accurately the effect of thrombin, we labeled the platelets with either ^{125}I or ^{131}I. Cells labeled with ^{125}I were incubated with thrombin, while those labeled with ^{131}I were incubated in isolation media alone. The isolated, solubilized platelets were then mixed and electrophoresed on the same gel. This procedure allowed us to compare the two samples on the same gel (Fig. 4), thus eliminating the possibility of mobility differences between separate gels. As shown in Fig. 4, iodinated Glycoproteins I and III, although hydrolyzed on the isolated membrane, were unaffected by thrombin treatment of intact platelets. Glycoprotein II, however, was reduced in concentration. The amount of Glycoprotein II that could be removed by thrombin varied considerably from experiment to experiment with reductions of 10–50% observed.

DISCUSSION

The results reported here bear on two distinct but closely related aspects of

the human platelet plasma membrane: (1) the proteolytic site of thrombin on the membrane surface, and (2) the organization of platelet plasma-membrane proteins.

Proteolytic site of thrombin action

Many platelet proteins can serve as substrates for thrombin, and some have been suggested as the site of thrombin action. Jackson, Conley and co-workers [23, 24] concluded that fibrinogen was the initial target site for thrombin, but observations by others conflict with this interpretation. Davey and Luscher [4], for example, have shown that the ability of various proteolytic enzymes to induce aggregation is not related to their ability to convert fibrinogen to fibrin, and other workers [25, 26] have demonstrated that platelets from afibrinogenemic patients are aggregated by thrombin, although at a reduced rate. Moreover, inhibition of the thrombin–fibrinogen reaction has no effect on thrombin-induced aggregation [27]. Although Factor XIII and thrombosthenin M are substrates for thrombin and have been implicated as receptor sites for this enzyme, they have not been identified as surface components [5, 6, 8].

One explanation for these apparently conflicting results is that the profound morphological changes induced in the platelet by thrombin make it difficult to distinguish primary thrombin-catalyzed hydrolysis from secondary alterations. For example, much of the fibrinogen in washed platelets is in granules within the platelet [28]; and the contents of these granules, including fibrinogen, are released during the release reaction [29]. It is thus difficult to determine whether thrombin acts on fibrinogen, which is normally present on the membrane surface, or whether the fibrinogen is released from within the cell only after thrombin acts on another component on the membrane surface.

The present study demonstrates the difference between thrombin substrates and the site of thrombin proteolytic action on the membrane surface. The three glycoproteins in the plasma membrane are all substrates for thrombin, as they were hydrolyzed when this proteolytic enzyme was added to isolated membranes. When thrombin was added to iodinated intact platelets, however, only Glycoprotein II, which has an apparent molecular weight of 118 000, was reduced in concentration. This result strongly suggests that Glycoprotein II is the proteolytic site of thrombin action on intact platelets. The remaining substrates in the membrane, Glycoproteins I and III, and the high-molecular-weight polypeptide were resistant to hydrolysis on intact platelets, apparently because of their orientation in the membrane.

The high molecular weight of the thrombin-labile glycoprotein does not correspond to any previously characterized thrombin-sensitive component. Factor XIII (fibrin-stabilizing factor), which has a similar molecular weight to Glycoprotein II, contains no carbohydrate [5]. The molecular weights of thrombosthenin M [30], platelet fibrinogen [31] and thrombin-sensitive protein [20] are all different from that of Glycoprotein II.

In contrast to our results, Nachman et al. [32] have reported that thrombin did not affect the concentration of iodinated membrane proteins on intact platelets. The double-isotope technique used in our study, however, permitted a more precise analysis of concentration differences caused by the proteolytic action of thrombin.

It is interesting that thrombin hydrolyzed less than half of all the Glycoprotein II available on the membrane surface. This could indicate the presence of another

labeled component with an identical molecular weight, but this possibility seems unlikely because radioactivity extracted from the 118 000-mol. wt region of acrylamide gels was homogeneous by hydroxylapatite chromatography in dodecylsulphate and by isoelectric focusing in Triton X-100 (Phillips, D. R. and Agin, P. P., unpublished). A more reasonable explanation of the result is either (1) that Glycoprotein II has more than one orientation in the membrane and is thus only partially accessible to thrombin; or (2) that the initial hydrolysis of Glycoprotein II induces a conformational change in the membrane, hence reducing the availability of remaining molecules on the membrane surface. Contrary to the first hypothesis, we have shown that more than 70% of Glycoprotein II is susceptible to trypsin hydrolysis on intact cells, indicating that most of this macromolecule is exposed to the membrane surface [9]. It appears, therefore, that the membrane undergoes a conformational change after partial hydrolysis of Glycoprotein II. In support of this view, hydrolysis of platelet-membrane glycoproteins by another proteolytic enzyme, trypsin, has been shown to alter the conformation of glycopeptide fragments that remain with the membrane [9]. Moreover, trypsin hydrolysis changes the orientation of glycoproteins in the human erythrocyte membrane [33]. Thrombin, like trypsin, can induce reactions leading to conformational changes in the membrane. For example, thrombin treatment of platelets induces the release reaction [29], which allows expression of platelet factor 3 activity on the membrane surface [34]. Most important, the resulting membrane is now "sticky," which permits platelets to aggregate [35]. These observations indicate that the membrane surface has a different composition after thrombin has acted on the platelet. They suggest, further, that hydrolysis of only a small fraction of Glycoprotein II will produce a structural change in the platelet and that these changes could reduce the availability of this glycoprotein to further hydrolysis by thrombin. Recent studies from our laboratory have shown that thrombin also binds to platelets at a site different from this proteolytic site [32]. Further experiments are required to determine if hydrolysis of Glycoprotein II alone is sufficient for thrombin-induced reactions of the platelet.

Organization of platelet plasma-membrane glycoproteins

We have demonstrated that the three membrane glycoproteins are exposed to the outer surface of the platelet. However, two of these glycoproteins, labeled I and III, are hydrolyzed by thrombin only when the isolated membrane is treated. Two alternative explanations for this observation are plausible: (1) Glycoproteins I and III span the thickness of the membrane and the thrombin-labile bond is on the inner face of the membrane, accessible only after cell lysis; or (2) the plasma membrane is altered during isolation so that a thrombin-labile bond on these glycoproteins becomes available on the outer surface of the isolated membrane.

Similar studies on the major glycoprotein in the plasma membrane of human erythrocytes indicate that only one region of this macromolecule is labeled by non-penetrating reagents on the intact cell, whereas different parts of the molecule are labeled when isolated membranes are treated [36–38]. This glycoprotein is also subject to proteolysis on inside-out vesicles [39]. Thus, it has been concluded that the glycoprotein is accessible on both the inner and outer membrane faces and hence spans the membrane [36, 37].

The assignment of penetrating glycoproteins, however, depends on an isola-

tion technique that does not alter the outer surface of the membrane. If this surface is altered, agents can react with a region of the glycoprotein that is not available on intact cells. In this regard, several studies have shown that the erythrocyte plasma membrane undergoes a conformational change upon isolation, making the assignment of penetrating glycoproteins [40, 41] difficult. Similar studies have not been done with the human platelet plasma membrane, but Steiner et al. [42] have demonstrated at least a functional similarity between the membrane surfaces of intact platelets and isolated membranes, in that membranes isolated by the glycerol-lysis technique [12] were aggregated by ADP. However, the conclusion that the two platelet glycoproteins penetrate the thickness of the membrane must await studies showing that their availability on the outer face of intact platelets is similar to that on the outer face of isolated membranes.

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