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DEMONSTRATION OF ^{125}I-LABELLED THROMBIN BINDING PLATELET PROTEINS BY USE OF CROSSED IMMUNOELECTROPHORESIS AND AUTORADIOGRAPHY

INGER HAGEN, GEIR O. GOGSTAD, FRANK BROSSTAD and NILS OLAV SOLUM

Research Institute for Internal Medicine, Section on Hemostasis and Thrombosis, University of Oslo, and Hematological Laboratory, Department IX, Ullevål Sykehus, University of Oslo, Oslo (Norway)

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A possible receptor for thrombin on the platelet membrane has been identified. Whole platelets were treated with ^{125}I-labelled thrombin followed by washing of the platelets, solubilization in Triton X-100, crossed immunoelectrophoresis and autoradiography. A heavily labelled antigen which migrated slightly more slowly than albumin was observed. No corresponding arc was seen on the same immunoplate when stained with Coomassie brilliant blue, indicating that the antigen possessed weak antigenic properties and/or was present in very small amounts. When ^{125}I-labelled thrombin that had been inactivated by phenylmethylsulphonyl fluoride was used, no such labelled arc was seen. The radiolabelled immunoprecipitate does not represent any of the antigens identified hitherto in the immunoelectrophoretic patterns obtained with platelets or platelet material. The electrophoretic mobility of the antigen was influenced neither by neuraminidase treatment of the platelets prior to the ^{125}I-labelled thrombin exposure nor by inclusion of concanavalin A, wheat-germ lectin or lentil lectin in the gel during the first-dimension electrophoresis. This suggests that the antigen does not represent a glycoprotein. Upon subcellular fractionation the radioactively labelled arc was observed in the cytosol fraction following crossed immunoelectrophoresis and autoradiography. Analysis of the secreted proteins after induction of the release reaction with ^{125}I-labelled thrombin revealed labelling of immunoprecipitates representing thrombospondin, albumin and the 'line' form of platelet factor 4. This confirms that stable complexes of ^{125}I-labelled thrombin and platelet proteins can exist in the presence of Triton X-100 and during electrophoresis.

Introduction

Thrombin is a potent inducer of the blood platelet release reaction [1]. Binding of ^{125}I-labelled thrombin to the platelets has been demonstrated [2–4], and studies of whole platelets as well as isolated membranes have demonstrated the presence of one class of high-affinity binding sites (approx. 500 sites) and one class of low-affinity binding sites (approx. 50000 sites) for thrombin on the platelet membrane [4–6]. Alternatively, the platelets may possess one class of thrombin receptors exhibiting negative co-operativity [7].

Thrombin that has been blocked at the catalytic centre exhibits binding characteristics similar to those of the unmodified enzyme [2,4,5]. However, in this case the release reaction does not occur, indicating that the triggering of this process involves a proteolytic event. Several efforts have been made to identify thrombin receptors and proteolytic substrates for thrombin in the platelet membrane. The surface glycoprotein termed GP Ib has been shown to possess thrombin-binding properties [8–10], but is not degraded by the enzyme. The physiological significance of this binding is still obscure. Phillips and Agin [11] and

Mosher et al. [12] have demonstrated that a platelet surface glycoprotein, GP V, is proteolyzed during thrombin treatment of the platelets, and they suggested that the splitting of glycoprotein GP V may represent an early event in the triggering of the release reaction. The relation between glycoproteins GP Ib and GP V on the one hand and the low- and high-affinity binding sites on the other is unclear at the moment.

In the present investigation we wanted to identify possible complexes formed between platelet proteins and thrombin following incubation of the platelets with radioactively labelled enzyme. After solubilization in Triton X-100, the proteins were analyzed by crossed immunoelectrophoresis using anti-platelet antibodies [13], and the radioactive immunoprecipitates were identified by autoradiography. Furthermore, the presence of ^{125}I-labelled thrombin-protein complexes in the extracellular medium obtained after induction of the release reaction was investigated by this method.

Materials and Methods

Crude bovine thrombin (Topostasine, Hoffmann La-Roche, Basle, Switzerland) was purified by ion exchange chromatography as described by Brosstad [14]. The activity of the final product was 1800 NIH units per mg protein. Purified thrombin was labelled with ^{125}I according to the method of McFarlane [15]. ^{125}I-labelled thrombin was blocked at the active site by phenylmethylsulphonyl fluoride [16]. Platelets that had been loaded with ^{3}H-labelled serotonin released 80–90% of the radioactivity upon stimulation with ^{125}I-thrombin, whereas only 4–7% of the [^{3}H]serotonin was secreted after treatment of the platelets with phenylmethylsulphonyl fluoride-inactivated ^{125}I-labelled thrombin.

Concanavalin A, wheat-germ lectin and lentil lectin were from Pharmacia Fine Chemicals, Uppsala, Sweden, and antimycin A, 2-deoxyglucose and phenylmethylsulphonyl fluoride from Sigma Chemical Co., St. Louis, U.S.A. Neuraminidase, protease free (from *Vibrio comma*) was from Behringwerke AG, F.R.G.

Blood was obtained from registered blood donors, and the platelets were isolated as described previously [13]. The washed platelets were suspended in 1 ml 0.15 M NaCl/0.03 M Tris (pH 7.4)/1 mM EDTA (approx. 10^9 platelets per ml) and incubated with ^{125}I-labelled thrombin (1 NIH unit per ml, final concentration) for 5 min. at 37°C. After centrifugation (2500 × *g*, 10 min.) the platelets were washed once in Tris-buffered saline with 1 mM EDTA and solubilized in 0.1 M glycine/0.038 M Tris (pH 8.7), containing 1% Triton X-100 as described [13,17]. In some experiments the release reaction was prevented by incubation of the platelets with antimycin A (4 μg/ml) and 2-deoxyglucose (20 mM final concentration) prior to the thrombin treatment [18]. Platelet homogenization and separation into membranes, α-granules and cytosol were performed according to Gogstad [19].

Neuraminidase treatment was performed with platelets suspended in 0.05 M sodium acetate buffer (pH 5.5)/0.15 M NaCl/0.009 M $CaCl_2$. Neuraminidase (0.05 units) was added to 1 ml of the platelet suspension containing $1 \cdot 10^9$ platelets, followed by incubation for 30 min at 37°C.

Solubilized platelet proteins were examined by crossed immunoelectrophoresis using antibodies raised against whole platelets [13]. The radioactive immunoprecipitates were revealed by autoradiography. Crossed affinity immunoelectrophoresis using lectins in the gels (100 μg/cm^2) during the first-dimension electrophoresis was performed according to Bøg-Hansen [20] and Hagen et al. [13].

Results

In order to identify possible complexes of platelet surface proteins with thrombin, platelets were incubated with ^{125}I-labelled thrombin, washed and solubilized in Triton X-100. Crossed immunoelectrophoresis of the solubilized proteins against anti-platelet antibodies revealed several immunoprecipitates (Fig. 1A). The identification of the antigens corresponding to the various precipitates has been decribed in detail elsewhere [10,13]. After autoradiography of the immunoplate, one heavily labelled immunoprecipitate and two faint arcs were seen (Fig. 1B). The faint arcs were congruent with the immunoprecipitates representing glycoprotein Ib (No. 13) and the complex containing glycoprotein IIb/IIIa (No. 16),

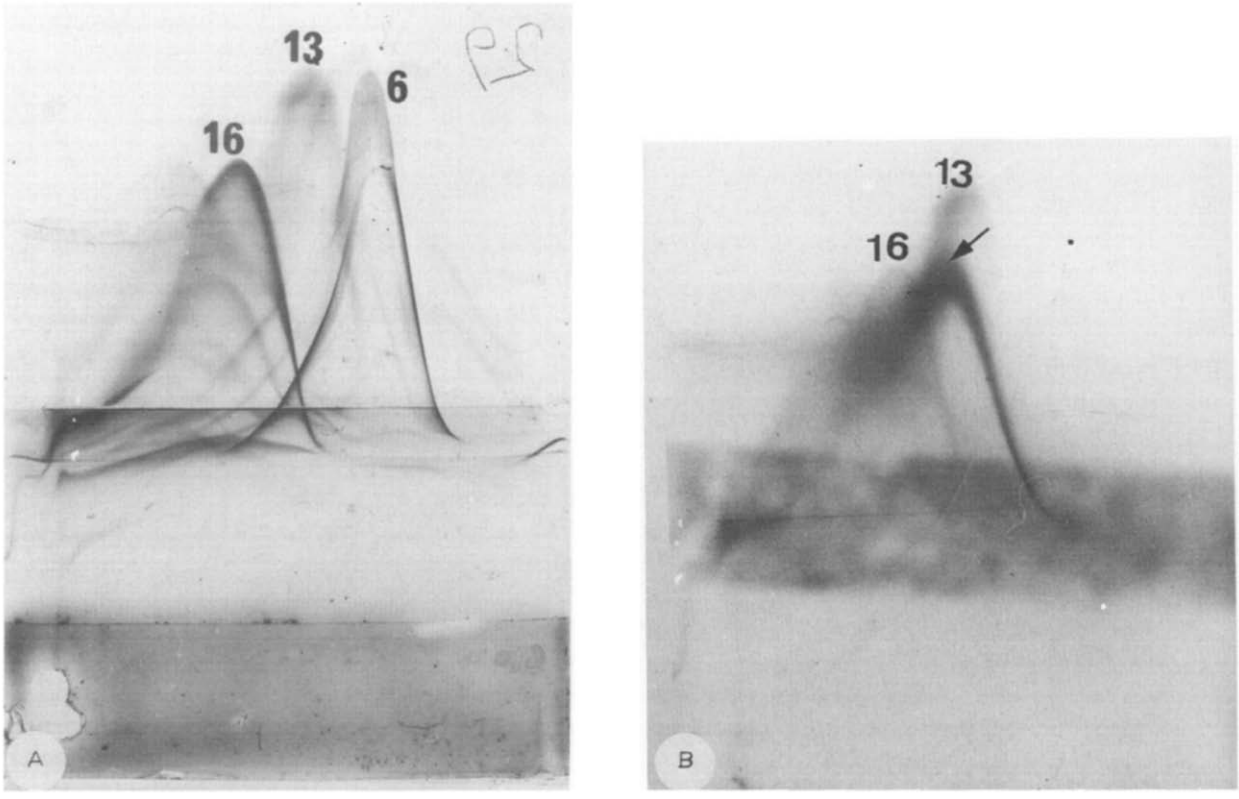

Fig. 1. Crossed immunoelectrophoresis of proteins (approx. 150 μg) solubilized from metabolically blocked platelets treated with ^{125}I-labelled thrombin. The antibodies used were raised against whole platelets. The first-dimension electrophoresis was performed at 10 V/cm for 45 min and the second dimension overnight at 1–2 V/cm. (A) Coomassie brilliant blue staining. Immunoprecipitate No. 6 represent albumin, No. 13 glycoprotein GP Ib and No. 16 consists of a complex of glycoprotein GP IIb and glycoprotein GP IIIa. (B) Autoradiography. Note the radiolabelling of glycoprotein GP Ib and an antigen with similar electrophoretic mobility (arrow). No corresponding immunoprecipitate was seen congruent with the most strongly labelled antigen.

respectively, whereas no immunoprecipitate congruent with the strongly labelled arc could be detected on the protein-stained immunoplate. In the above experiment the release reaction had been prevented by incubation of the platelets with antimycin A and 2-deoxyglucose prior to the addition of ^{125}I-labelled thrombin. However, the same pattern was observed in the absence of the inhibitors [21]. Inclusion of antibodies to glycocalicin, a proteolytic split product of glycoprotein Ib, in the intermediate gel revealed that the most heavily labelled antigen did not cross-react with glycoprotein Ib (not shown). No labelled arc was seen after crossed immunoelectrophoresis of the ^{125}I-labelled thrombin preparation alone (not shown).

Crossed immunoelectrophoresis was performed of platelets that had been treated with phenylmethylsulphonylfluoride-inactivated ^{125}I-labelled thrombin (corresponding to 10 NIH units/ml). In this case, no labelled arc was seen (not shown).

Analysis of subcellular fractions obtained from ^{125}I-labelled thrombin-treated platelets revealed that the most heavily labelled antigen was recovered in the soluble fraction, whereas only a faint arc, probably representing glycoprotein Ib, was seen in the membrane fraction. No labelled arcs were seen in the α-granule fraction (not shown). Homogenization of the platelets in the presence of various protease inhibitors, i.e., leupeptin, benzanidine, iodoacetamide, phenyl-

methylsulphonyl fluoride or *N*-ethylmaleimide, did not affect the distribution of the radioactively labelled antigens among the subcellular fractions. Incubation of the isolated fractions with ^{125}I-labelled thrombin did not result in the formation of complexes between thrombin and platelet proteins as revealed by crossed immunoelectrophoresis and autoradiography.

Antithrombin III and α_2-macroglobulin are plasma proteins with the ability to bind thrombin under similar electrophoretic conditions as described here [22]. The migration of the α_2-macroglobulin-^{125}I-labelled thrombin complex was almost identical to the heavily labelled immunoprecipitate observed in our experiments with solubilized platelet proteins. Crossed immunoelectrophoresis of solubilized ^{125}I-labelled thrombin-treated platelets against anti-platelet antibodies was performed using antiserum to α_2-macroglobulin in the intermediate gel. Inspection of the immunoplate after autoradiography revealed that the labelled protein was not precipitated by the α_2-macroglobulin antiserum and thus represents a different protein.

To examine the possibility that the heavily labelled antigen represented a platelet surface sialoglycoprotein platelets were incubated with neuraminidase prior to the incubation with ^{125}I-labelled thrombin. By this treatment, terminal sialic acid residues of the available sialoglycoproteins will be removed, resulting in reduced electrophoretic mobility of these glycoproteins as compared to the unmodified molecules. However, the position of the heavily labelled immunoprecipitate did not change following neuraminidase treatment, indicating that the antigen did not contain sialic acid residues available to neuraminidase. In contrast, the mobility of the glycoprotein IIb/IIIa complex was markedly reduced by this treatment (not shown).

In order to examine whether the heavily labelled immunoprecipitate represented a glycoprotein,

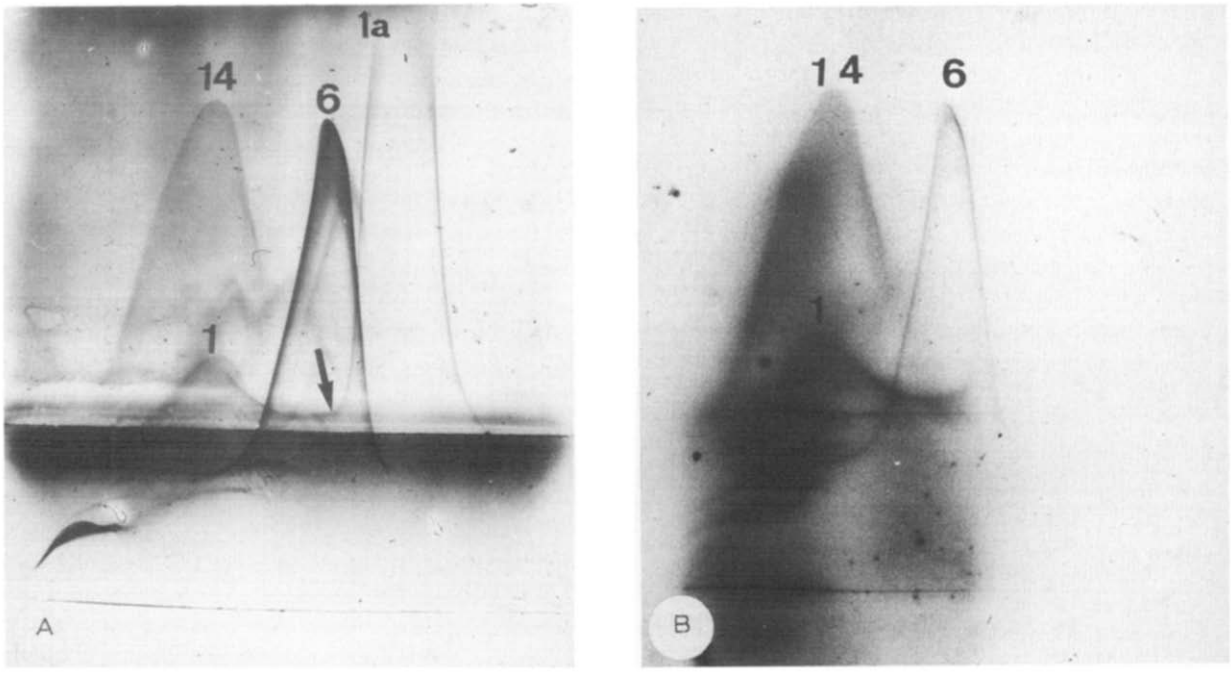

Fig. 2. Crossed immunoelectrophoresis of proteins secreted from platelets following treatment with ^{125}I-labelled thrombin. The antibodies used were raised against whole platelets. (A) Coomassie Brilliant blue staining. Immunoprecipitate 1a represents the peak form of platelet factor 4, No. 1 represents the line form, No. 6 albumin and No. 14 thrombospondin. Note the fusion between the line and the peak form of the platelet factor 4 immunoprecipitate (arrow). (B) Autoradiography. Note the labelling of the line form of platelet factor 4 (No. 1), albumin (No. 6) and thrombospondin (No. 14).

crossed affinity immunoelectrophoresis was performed using different lectins in the gel during the first-dimension electrophoresis. Glycoproteins that interact with the lectins will exhibit a reduced electrophoretic migration and/or a reduced area of their immunoprecipitates as compared to the control without lectin, Neither the position nor the area of the labelled immunoprecipitate did change significantly when wheat-germ lectin, lentil lectin or concanavalin A were included in the gel (not shown). Thus, apparently, the labelled antigen does not seem to represent a glycoprotein.

Thrombin induces secretion of intracellularly stored material from the platelets. Crossed immunoelectrophoresis of the released material obtained following incubation of the platelets with ^{125}I-labelled thrombin revealed the presence of immunoprecipitates containing platelet factor 4 (Nos. 1 and 1a), albumin (No. 6), thrombospondin (No. 14) and fibrinogen (No. 24) in addition to several unidentified antigens (Fig. 2A). Autoradiography of the immunoplate demonstrated radiolabelling of thrombospondin and albumin (Fig. 2B). Nor radioactivity was seen with the platelet factor 4 immunoprecipitate in the 'peak' form (No. 1a). This is the electrophoretic form of platelet factor 4 normally found on examination of released material [23]. In extracts of whole platelets the platelet factor 4 immunoprecipitate appears as a long, irregular line (No. 1). In some experiments traces of the 'line' immunoprecipitate is also seen on analysis of the released material (Fig. 2A). This line fuses with the left leg of the peak form immunoprecipitate (Fig. 2A, arrow). The line form of the platelet factor 4 immunoprecipitate was clearly labelled with ^{125}I-labelled thrombin, whereas no radioactivity could be detected with the peak form of the platelet factor 4 immunoprecipitate (Fig. 2B).

Discussion

In the present investigation we have demonstrated that complexes formed between certain platelet proteins and ^{125}I-labelled thrombin do not dissociate in Triton X-100 nor during electrophoresis in the presence of Triton X-100 at pH 8.7. Thus, autoradiography following crossed immunoelectrophoresis of solubilized proteins from ^{125}I-labelled thrombin-treated platelets confirmed binding of thrombin to glycoprotein Ib [8–10], thrombospondin [24] and the 'line' form of platelet factor 4 [10]. In addition we observed radioactive labelling of albumin and an hitherto unidentified immunoprecipitate. Since no labelled arcs were seen on analysis of ^{125}I-thrombin alone all the radiolabelled immunoprecipitates most probably represent true complexes of ^{125}I-labelled thrombin with platelet antigens.

The most heavily labelled immunoprecipitate could not be observed after staining of the immunoplate with Coomassie brilliant blue, indicating that the corresponding antigen was present in very small amounts and/or possessed weak antigenic properties. The strongly labelled complex between the platelet protein and ^{125}I-labelled thrombin was formed regardless of whether or not the release reaction had been prevented, but was not seen when ^{125}I-labelled thrombin had been blocked with phenylmethylsulphonyl fluoride. This may seem to be in contrast to other studies where a similar binding has been reported with active thrombin and with thrombin which had been blocked at the serine site [2,4,5]. However, the different techniques employed makes a direct comparison complicated. Also, the relation between thrombin receptors as identified by kinetic studies and the ^{125}I-labelled thrombin labelled antigens described in this paper is not yet clear.

The presence of the strongly labelled component in the cytosol fraction following homogenization and fractionation of the platelets suggests that thrombin either had been internalized and complexed with a cytosol protein, or complexed with a peripheral protein which was liberated from the membrane during platelet disruption. Alternatively, such a liberation was due to proteolysis of an integral membrane protein. Since homogenization of the platelets in the presence of different proteolytic inhibitors had no effect, this latter explanation seems less likely. No labelled arc could be detected after incubation of the cytosol fraction with ^{125}I-labelled thrombin, indicating that the strongly labelled immunoprecipitate seen on analysis of whole platelets did not represent a cytosol protein. The labelled arc might represent a contaminating plasma proteins. Grimmer and coworkers [22] have applied a similar technique in

their studies of thrombin binding plasma proteins, and binding of ^{125}I-labelled thrombin to antithrombin III and α_2-macroglobulin was observed. The mobility of the antithrombin III-^{125}I-labelled thrombin complex was markedly slower than the heavily labelled antigen seen in the present investigation, whereas α_2-macroglobulin appeared approximately at the same position as the thrombin-binding platelet protein. However, since the latter was not precipitated by antiserum to α_2-macroglobulin, the two proteins must represent different molecules This is further supported by the experiments with lectins, and the observation that ^{125}I-labelled thrombin treatment of platelet subcellular fractions did not result in the formation of the strongly labelled complex. This indicates that the antigen may represent a peripheral membrane protein that becomes solubilized during homogenization of the platelets. Steric relations or other factors related to the topographical position in the membrane may be essential for binding to occur.

Glycoprotein V is a surface glycoprotein that has been shown to be proteolyzed by thrombin [11,12]. The possibility exists that the heavily labelled immunoprecipitate represents a complex of ^{125}I-labelled thrombin and glycoprotein V. However, our experiments indicate that the platelet antigen does not represent a glycoprotein, since neither neuraminidase treatment nor the presence of various lectins in the first-dimension gel affected the electrophoretic mobility of the complex. However, the possibility that the labelled arc represents a complex of ^{125}I-thrombin and a non-glycosylated split product of glycoprotein V cannot be excluded.

Recently, Larsen and Simmons [25] have isolated a complex of thrombin and a platelet protein which might represent the high-affinity receptor. This protein is present in very small amounts, and it has not yet been possible to find out whether it represent a glycoprotein. However, its molecular weight of approx. 160 000 indicates that it is different from glycoprotein V. The possibility exists that the strongly labelled immunoprecipitate seen in our experiments represents the same protein as that isolated by Larsen and Simmons [25], but further studies are needed to elucidate this problem.

Analysis of the material secreted from the platelets upon ^{125}I-thrombin treatment revealed radiolabelling of thrombospondin, albumin and the line form immunoprecipitate of platelet factor 4. Thrombospondin is a granule glycoprotein [26] consisting of three polypeptide chains linked together by disulphide bonds [27]. Its function is still obscure, but Lawler and Slayter [24] have shown that thrombospondin is slowly proteolyzed by thrombin. The radiolabelling of the immunoprecipitate containing thrombospondin in the present experiments may reflect the presence of an enzyme-substrate complex between ^{125}I-labelled thrombin and thrombospondin.

In some experiments when the platelets were exposed to ^{125}I-labelled thrombin without inhibition of the release reaction a faint arc probably representing thrombospondin was seen on the autoradiogram following analysis of the platelet extract. This was most likely due to adsorption of thrombospondin to the platelet surface during the secretion process. George et al. [28] and Phillips and co-workers [29] have demonstrated that thrombospondin becomes associated with the platelet membrane after the release reaction has occurred.

Using a modified crossed immunoelectrophoretic technique with immobilized thrombin-Sepharose 2B we have previously demonstrated an interaction between thrombin and the line, but not the peak form of platelet factor 4 [10]. This observation is supported in the present investigation in that the line form, but not the peak form of the platelet factor 4 immunoprecipitate was radiolabelled.

In conclusion, complexes of ^{125}I-labelled thrombin and platelet proteins can be detected by crossed immunoelectrophoresis of solubilized platelet proteins after exposure of the platelets to ^{125}I-labelled thrombin. Further studies are needed to elucidate the nature of the most heavily labelled antigen.

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