A fibronectin-binding glycoprotein from human platelet membranes

Mogens Sandbjerg HANSEN and Inge CLEMMENSEN

Department of Clinical Chemistry, University of Copenhagen, Hvidovre Hospital, DK-2650 Hvidovre, Denmark

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Fibronectin ('cold-insoluble globulin') has been suggested as a possible mediator of platelet adhesion. A fibronectin-binding protein was partially purified from washed solubilized human platelet membranes by affinity chromatography on fibronectin–Sephacrose. The isolated protein migrated as a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an M_r (relative molecular mass) of approx. 125 000 under reducing conditions. The protein migrated as a dimer in non-reduced gels. The purified protein did not react with immunoglobulins against fibrinogen or fibronectin when tested in crossed immunoelectrophoresis or electro-immunoassay. The protein and purified fibronectin formed a complex that had a significantly faster mobility in crossed immunoelectrophoresis than did native fibronectin. The presence of heparin in the binding-protein–fibronectin mixture resulted in an even faster mobility of the complex, whereas the mobility of native fibronectin was unaffected. Crossed affinity-immunoelectrophoresis of the complex using different lectins suggested that the binding protein is a glycoprotein containing *N*-acetylglucosamine residues. The complex, but not purified fibronectin, bound to phenyl–Sephacrose on crossed hydrophobic-interaction immunoelectrophoresis. The results strongly suggest the presence of a fibronectin-binding glycoprotein in the platelet membrane.

Fibronectin is a glycoprotein with M_r 450 000 that is present in a soluble form in plasma and in an insoluble form in connective tissue. Fibronectin binds to collagen, is incorporated into fibrin, is a substrate for blood coagulation factor XIIIa and appears to be important in cellular adhesion (Vaheri & Mosher, 1978). Fibronectin also binds to various cells, e.g. *Staphylococcus aureus* (Kuusela, 1978). Human platelets contain fibronectin antigen (Zucker *et al.*, 1979; Plow *et al.*, 1979; Bensusan *et al.*, 1978), which may be of importance for the adhesive properties of this cell. It has recently been demonstrated that an increase in platelet adhesiveness induced by stimulation with thrombin is associated with an increase in the amount of fibronectin antigen bound to the platelet surface (Ginsberg *et al.*, 1980). Thus fibronectin may be an important modulator of the platelet–collagen interaction, although its precise role is unknown (Bensusan *et al.*, 1978; Sochynsky *et al.*, 1980). We have isolated a glycoprotein from human platelet membranes that binds fibronectin and thereby may participate in the reactions between platelets and connective tissue.

Experimental

Materials

Buffers for gel electrophoresis and chromatography were as follows: Tris, 0.1 M-Tris/HCl/0.05 M-NaCl, pH 7.6 (I 0.15); electrophoresis buffer: Tris/barbiturate, 4 mM-Tris/25 mM-diethylbarbituric acid/0.3 mM-calcium lactate/2 mM- NaN_3 , pH 8.6 (I 0.07).

Human blood was drawn from normal donors (with their informed consent) into 5.2 mM-citric acid/11.2 mM-sodium citrate/20.4 mM-glucose.

Agarose was obtained from L'Industrie Biologique Française, S.A., Genevilliers, Seine, France.

Phenyl-Sepharose CL-4B, *Helix pomatia* (Roman snail)-lectin–Sepharose 6 MB, wheat-germ-lectin–Sepharose 6 MB, concanavalin A–Sepharose and CNBr-activated Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. Gelatin was from Difco, Detroit, MI, U.S.A. L-Arginine hydrochloride from Sigma, St. Louis, MO, U.S.A. Sodium dodecyl sulphate and MgCl_2 were from Merck, Darmstadt, Germany. Acrylamide was from Eastman Kodak Co., Rochester, NY, U.S.A.

Methods

Ultrafiltration. This was done in a stirred cell (Amicon, Lexington, MA, U.S.A.) with a PM10 membrane, which retained molecules with M_r higher than 10000.

Determination of protein concentration. This was obtained from A_{280} , by using a specific absorbance coefficient of $10 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Müllertz & Clemmensen, 1976).

Immunochemical techniques. Electroimmunoassay was performed as described by Laurell (1972). Crossed immunoelectrophoresis was performed as described by Axelsen *et al.* (1973). Crossed affinity-immunoelectrophoresis was performed as described by Bjerrum (1978).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The technique was essentially as described by Weber & Osborn (1975) and Müllertz & Clemmensen (1976), relative-molecular-mass markers from Pharmacia being used.

Preparation of fibronectin. This was prepared from human plasma by affinity chromatography on Sepharose-linked gelatin (Engvall & Rouslahti, 1977). Approx. 10 mg of gelatin was coupled to 1 g of CNBr-activated Sepharose 4B (Axén *et al.*, 1967) as described in the manufacturer's instructions. The volume of plasma applied was 400 ml. Unadsorbed proteins were eluted by percolating Tris buffer through the column until A_{280} was less than 0.01. The adsorbed proteins were eluted with the same buffer containing 3 M-MgCl₂ or 3 M-arginine. This material was concentrated, dialysed at 20°C and coupled to CNBr-activated Sepharose 4B. The amount of fibronectin was estimated from A_{280} by using a specific absorbance coefficient of $12.8 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Approx. 3 mg of fibronectin was coupled to 1 g of Sepharose. Immunoglobulins against fibronectin were raised in rabbits and partially purified as described previously (Clemmensen & Andersen, 1981).

Preparation of platelet membranes. Human platelets were harvested and washed as described previously (Hansen & Clemmensen, 1980). The final platelet suspension was frozen in a solid-CO₂/acetone mixture and thawed. This was repeated six times. The membranes were isolated by the method of Barber & Jamieson (1970) and solubilized in Tris buffer containing 2 M-KBr (Cheng & Hawiger, 1979). The membrane proteins were separated from the insoluble matrix by centrifugation at 4°C for 1 h at $123000 g_{av}$ in a Damon IEC B-60 ultracentrifuge equipped with a SB283 rotor. KBr was removed by dialysis against Tris buffer.

Preparation of a complex between fibronectin and the platelet membrane protein. The partially purified membrane protein was incubated with dilutions of

purified fibronectin for 300 s at 20°C. This mixture was subjected to crossed immunoelectrophoresis.

Results

Isolation of a fibronectin-binding protein from human platelet membranes

Platelet membranes were isolated from fresh, washed human platelets as described under 'Methods'. The membranes were solubilized by incubation with 2 M-KBr. After dialysis to remove KBr, the extract contained 28 mg of protein. The extract was subjected to affinity chromatography on Sepharose-linked purified fibronectin. The column was washed with buffer until the A_{280} of the effluent was below 0.01. The bound material was eluted with 2 M-KBr in the same buffer. The two pools were dialysed and concentrated before further characterization. Approx. 4 mg of protein, corresponding to 0.15 of the total amount, was bound to the column.

Characterization of the membrane protein

The concentration of protein in the material eluted from the fibronectin-Sepharose column was about 4.3 g/litre. It was studied by crossed immunoelectrophoresis and by electroimmunoassay in agarose gel containing antibodies against total human serum, against fibrinogen or against fibro-

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis pattern of human platelet membrane material after solubilization and affinity chromatography on fibronectin-Sepharose

The patterns demonstrate material retained by the affinity column under non-reducing (a) and reducing (b) conditions. The gel consisted of 3.5% acrylamide. The samples were treated with 2% (w/v) sodium dodecyl sulphate for 3 min at 100°C. Reduction was done by the addition of dithiothreitol to 2% (w/v).

nectin. No precipitates were detectable in the gels. The sensitivities of the assays for fibrinogen and fibronectin were $0.1 \mu\text{M}$. This was done to ensure that the binding protein was a blood platelet protein and not a fibronectin-binding plasma protein.

The binding protein was studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions (Fig. 1). The solubilized membrane-protein material that was applied to the

affinity column contained multiple bands. The material that bound to the column formed one dominant band with an M_r of about 260 000 and very weak bands with M_r values of about 410 000, 125 000 and 105 000. After reduction a dominant band with an M_r of about 125 000 and weak bands with M_r values of about 260 000, 150 000 and 70 000 were seen. The dominant bands were stainable with glycoprotein dye (periodate/Schiff).

Demonstration of a complex between fibronectin and the membrane protein

The purified fibronectin and complexes between fibronectin and the membrane protein were studied by crossed immunoelectrophoresis (Fig. 2). The complex-formation increased the electrophoretic mobility of fibronectin. Because of the known interaction of fibronectin with heparin (Stathakis & Mosesson, 1977), we also performed crossed immunoelectrophoresis in the presence of heparin. This experiment demonstrated that the electrophoretic mobility of fibronectin was unaffected by

Fig. 2. Formation of a complex between purified fibronectin and the thrombocyte membrane protein demonstrated by crossed immunoelectrophoresis in agarose

(a) Purified fibronectin; (b) complex between purified fibronectin and the membrane protein preparation; the final concentrations were: fibronectin, $0.6 \mu\text{M}$; membrane protein preparation, approx. 4 g/litre ; (c) mobility of the complex in the presence of heparin (final concn. $2.5 \text{ k-units/litre}$). The application slits in the first dimensions of the three crossed immunoelectrophoreses are marked by a vertical bar. The second-dimension electrophoreses were run into agarose gel containing immunoglobulins against fibronectin. The anode is towards the right in the first dimension and towards the top in the second dimension. The first dimensions were run with a potential-difference gradient of 2.5 V/mm for 2 h, the second for 17 h at 0.7 V/mm .

Fig. 3. Crossed affinoimmunoelectrophoresis of purified fibronectin and of a complex between fibronectin and the thrombocyte membrane protein

(a) Purified fibronectin; (b) complex between purified fibronectin and the membrane protein preparation, prepared as in Fig. 2. The first dimension was run with a potential-difference gradient of 2.5 V/mm for 2 h, the second for 17 h at 0.7 V/mm . The second-dimension electrophoresis was run into agarose gel containing immobilized wheat-germ lectin in the intermediate gel and immunoglobulins against fibronectin in the uppermost gel.

the presence of heparin, whereas the mobility of the complex was increased (Fig. 2).

Crossed affinoimmuno-electrophoresis of the complex between fibronectin and the membrane protein

The complex was analysed by crossed immuno-electrophoresis with immunoglobulins against fibronectin in the second-dimension gel and by using an intermediate gel containing various immobilized lectins (Fig. 3). Wheat-germ lectin and *Helix pomatia* (Roman snail) lectin were chosen because of their known high specificity for *N*-acetylglucosamine residues and *N*-acetylgalactosamine residues respectively. Wheat-germ lectin in the intermediate gel did not stop purified fibronectin from forming an immunoprecipitate in the second-dimension gel. The complex was stopped in the intermediary gel, suggesting the presence of *N*-acetylglucosamine residues in the complex. With *Helix pomatia* lectin in the intermediary gel (results not shown) a precipitate was formed in the antibody-containing gel with the complex, but not with purified fibronectin, suggesting the obliteration of *N*-acetylgalactosamine residues as a result of the complex-formation. Concanavalin A, which is a relatively non-specific carbohydrate binder, stopped, as expected, both fibronectin and the complex in the intermediary gel.

Fig. 4. Crossed hydrophobic-interaction immuno-electrophoresis of purified fibronectin and of a complex between fibronectin and the thrombocyte membrane protein. Details are as for Fig. 3, except for the intermediate gel containing phenyl-Sepharose instead of wheat-germ lectin.

Crossed hydrophobic-interaction immuno-electrophoresis was performed by using phenyl-Sepharose in the intermediary gels (Fig. 4). This did not interfere with the formation of a precipitate by fibronectin, but inhibited the formation of a precipitate by the complex. This suggested the presence of hydrophobic areas in the membrane protein.

Discussion

Glycoproteins from the platelet membrane are involved in several aspects of platelet function, e.g. aggregation and adhesion. Thus it is well known that platelets from patients with Bernard-Soulier syndrome are deficient in a membrane glycoprotein designated 'glycoprotein I' (Nurden & Caen, 1975). This defect renders the Bernard-Soulier-syndrome platelets unable to bind coagulation factor VIII or a component of the factor VIII complex and may at least partially explain the lack of binding to the sub-endothelium of Bernard-Soulier-syndrome platelets.

The role of fibronectin in platelet adhesion is not yet clear. Non-activated platelets contain fibronectin, at least partially located in the intracellular secretory granules (Zucker *et al.*, 1979; Plow *et al.*, 1979; Bensusan *et al.*, 1978). An increase in the platelet adhesiveness caused by treatment with thrombin also increases the amount of fibronectin that is accessible from the outside by radiolabelled antibodies against fibronectin or by immunofluorescent techniques. This is not accompanied by the disintegration of the cell, since a cytoplasmic marker (^{51}Cr) used in these studies was retained after thrombin treatment (Ginsberg *et al.*, 1980).

The presence of a fibronectin-binding protein in the platelet membrane might be an important mediator for the binding of platelets to collagen or fibrin, which is probably an important physiological event *in vivo*. The view that this is a possible function of fibronectin is further strengthened by investigations using *Staphylococcus aureus*, which demonstrated that the site on the fibronectin molecule that was bound to the bacterial cell membrane was distinct from the collagen-binding site (Mosher & Proctor, 1980), thus making a bridging function possible. The binding of fibronectin to platelets is of potential physiological importance, as evidenced by the description of a patient with Ehlers-Danlos disease whose platelet dysfunction was corrected by fibronectin (Arneson *et al.*, 1980).

We also investigated the carbohydrate moieties of purified fibronectin and of a complex between fibronectin and the platelet membrane glycoprotein. This was done since carbohydrates are known to be of importance for some interactions between platelets and plasma proteins. Thus the von Willebrand activity of coagulation factor VIII is related to the carbohydrate content of the molecule; the pen-

ultimate galactose residues especially are of critical importance (Gralnick, 1978). The crossed affinity-immunoelectrophoresis demonstrated that complex-formation caused changes in the dominant carbohydrate residues. The appearance of *N*-acetyl-galactosamine residues after the formation of a complex between fibronectin and the membrane glycoprotein might cause profound changes in the reactivity of the complexed fibronectin compared with 'native' fibronectin. The results from the hydrophobic-interaction immunoelectrophoresis suggest the presence of hydrophobic areas in the surface of the complex, presumably from the membrane glycoprotein.

The binding of fibronectin to platelet membranes may be important for the platelet function in initial haemostasis and later for the removal of the cellular debris in the haemostatic plug. The formation of a complex between fibronectin and a glycoprotein in the platelet membrane is suggested by immunological methods. Another part of the same fibronectin molecule might be cross-linked, by coagulation factor XIIIa, to collagen or fibrin, and provide a mode of platelet attachment. This would ensure the presence of the coagulation-promoting activities of the platelet membrane (Majerus & Miletich, 1978; Zwaal, 1978) and the fast-acting thrombocyte plasmin inhibitor (Hansen & Clemmensen, 1980) at the nidus, thus providing a catalytic and protective surface for the coagulation enzymes and protecting the forming fibrin against premature degradation. Fibronectin has been thought to enhance the removal of high-molecular-weight soluble fibrin complexes into which it is incorporated (Bang *et al.*, 1973) and collagen by macrophages (Saba *et al.*, 1978; Jilek & Hörmann, 1978) and to opsonize *Staphylococcus aureus* for neutrophils (Proctor *et al.*, 1979). This might also be a mechanism for the removal of platelet membrane material during the degradation of a thrombus.

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