Monoclonal antibody against the *N*-terminal end of human plasma fibronectin

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Purified human plasma fibronectin was digested with cathepsin G and the degradation products were tested for reactivity towards a monoclonal antibody. In an immunoblotting assay, after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the digestion products, the 85 000- M_r and 72 000- M_r gelatin- and heparin-binding fragments as well as the *N*-terminal 30 000- M_r heparin-binding fragment reacted with the antibody, whereas the 64 000- M_r gelatin- and heparin-binding fragment did not. In enzyme immunoassay the antibody reacted with intact fibronectin and the 30 000- M_r fragment but not with a 40 000- M_r gelatin-binding fragment. The alignment of the binding domains in these fragments and in the intact molecule [Vartio (1982) *Eur. J. Biochem.* **123**, 223–233] localizes the antigenic determinant to the 21 000 Da *N*-terminal *Staphylococcus aureus*-binding region of fibronectin.

Fibronectin is a high- M_r glycoprotein [for references, see Mosher (1980), Vaheri *et al.* (1980), Ruoslahti *et al.* (1981) and Hynes & Yamada (1982)] found in association with basement membranes, in interstitial connective-tissue matrix (Stenman & Vaheri, 1978) and in soluble form in plasma (Mosesson & Umfleet, 1970) and other body fluids (Chen *et al.*, 1976; Kuusela *et al.*, 1978; Vartio *et al.*, 1981). The soluble form of fibronectin is known to be composed of two disulphide-bonded polypeptides of M_r 220 000–250 000 (Mosesson *et al.*, 1975; Mosher, 1975; Wagner & Hynes, 1979; Kurkinen *et al.*, 1980). Limited proteolysis has been widely used in structural and functional studies of the molecule. Recently, monoclonal anti-fibronectin antibodies have also been used in structural studies and in characterization of proteolytically derived fragments of the molecule (Pierschbacher *et al.*, 1981; Smith & Furcht, 1982; Smith *et al.*, 1982; Vartio *et al.*, 1982). In the present study we have used cathepsin G degradation products of human plasma fibronectin to define the antigenic determinant for a monoclonal antibody. The determinant

is localized to the *Staphylococcus aureus*-binding region that has not been previously amenable to immunochemical characterization by poly- or monoclonal antibodies. The results also confirm the previously reported (Vartio & Vaheri, 1981) alignment of the binding domains in the *N*-terminal end of fibronectin.

Materials and methods

Enzymes and fibronectin

Cathepsin G (EC 3.4.21.20), a gift from Dr. Jeremy Saklatvala, Strangeways Research Laboratory, Cambridge, U.K., had been purified from human neutrophil leucocytes as described previously (Baugh & Travis, 1976) and had an activity of 350 units per mg in the azocasein-hydrolysis assay described by Starkey (1977). Thrombin (3.4.21.5) from human plasma (Sigma, St. Louis, MO, U.S.A.) had an activity of 3000 N.I.H. (National Institutes of Health) units/mg.

Fibronectin was purified from human plasma by using gelatin–Sephrose (Engvall & Ruoslahti, 1977) as described previously (Vuento & Vaheri, 1979). The protein concentrations were assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Abbreviations used: NaDodSO₄, sodium dodecyl sulphate; PhCH₂SO₂F, phenylmethanesulphonyl fluoride; IgG, immunoglobulin G; PEG, poly(ethylene glycol).

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NaDodSO₄/polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of NaDodSO₄ was performed as described by Laemmli (1970), with vertical slab gels. The acrylamide concentration was 3.3% (w/v) in the spacer gel and 8 or 10% in the separating gel. The samples were analysed with 10% (v/v) β -mercaptoethanol in Laemmli's (1970) sample buffer. After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 (Fairbanks *et al.*, 1971). Commercially obtained low- M_r markers (Pharmacia, Uppsala, Sweden) were used.

Digestion of purified fibronectin and isolation of gelatin- and heparin-binding fragments

Digestion of purified plasma fibronectin (1.5–3.0 mg/ml) with cathepsin G was performed in 50 mM-Tris/HCl, pH 7.5, at 37°C at an enzyme/substrate ratio of 1:200 (w/w) for 10 s, and the digestion was terminated by the addition of PhCH₂SO₂F to give a final concentration of 5 mM. For binding experiments, 20–40 μ l (20–40 μ g of protein) of the PhCH₂SO₂F-treated digest was diluted with 50 mM-Tris/HCl, pH 7.5, to a final volume of 600 μ l and 50 μ l of 50% (v/v) gelatin- or heparin-Sepharose was added.

After slow rotation in an end-over-end mixer for 2–4 h at 22°C, the agarose conjugates with the bound fragments were centrifuged, washed, and the protein was eluted with Laemmli's (1970) sample buffer for polyacrylamide-slab-gel electrophoresis. Unconjugated Sepharose 4B and CL-Sepharose 6B were used in a similar manner to the conjugated Sepharoses in control experiments. In another set of experiments, purified fibronectin (5 mg) was digested by thrombin (50 units) for 20 h.

For enzyme immunoassay, the *N*-terminal 30 000- M_r heparin-binding and the 40 000- M_r gelatin-binding fragments were isolated by using affinity-chromatography procedures previously described (Vartio, 1982). Briefly, a total 30 s cathepsin G digest of fibronectin (Fig. 1, track 2) was treated with unconjugated Sepharose and then with gelatin-Sepharose, the bound material was eluted by 1 M-arginine, dialysed against 50 mM-Tris/HCl, pH 7.5, treated with heparin-Sepharose to remove all fragments except the 40 000- M_r gelatin-binding peptide. To isolate the 30 000- M_r fragment the material of the total digest not binding to gelatin-Sepharose was treated with spermine-Sepharose to remove all fragments except the *N*-terminal 30 000- M_r heparin-binding peptide. To complete the purification, the fragment was further bound to heparin-Sepharose, eluted with 1 M-NaCl and dialysed against 50 mM-Tris/HCl, pH 7.5. The purity of the isolated fragments was tested by using NaDodSO₄/polyacrylamide-gel electrophoresis (Fig. 1, tracks 5 and 8).

Fig. 1. Polyacrylamide-gel electrophoresis of the cathepsin G-cleaved fragments of fibronectin

Fibronectin was digested by cathepsin G as described in the Materials and methods section. The gel (acrylamide content 10%) was stained with Coomassie Brilliant Blue. Track 1, intact human plasma fibronectin (8 μ g); 2, cathepsin G digest of fibronectin; 3, gelatin-Sepharose-bound material of the digest in track 2; 4, fragments that bind to heparin-Sepharose from the material of track 3; 5, heparin-non-binding fragment (40 000- M_r gelatin-binding fragment) of the material of track 3; 6, gelatin-non-binding fragments of the total digest of track 2; 7, spermine-Sepharose-bound fragments (eluted with 1 M-NaCl) from the material of track 6; 8, spermine-non-bound fragment (= *N*-terminal 30 000- M_r heparin-binding fragment) material of track 6.

Preparation of the Sepharose conjugates

Heparin-CL-Sepharose 6B and CNBr-activated Sepharose 4B were obtained from Pharmacia. Gelatin (type I; porcine; Sigma) was coupled to activated Sepharose 4B according to the instructions of the manufacturer. Spermine-Sepharose was prepared as described previously (Vartio, 1982).

Monoclonal anti-fibronectin antibody and immunoblotting

A Balb c/J mouse was immunized with fragments of a 24 h plasmin digest of purified human plasma fibronectin (DePetro *et al.*, 1981) according to the 'hyperimmunization' schedule described by Stähli *et al.* (1980). Spleen cells were fused with the FO myeloma (Fazekas de St. Groth & Scheidegger, 1980), which does not produce its own immunoglobulin chains, by a modification (Stähli *et al.*, 1980) of the method of Köhler & Milstein (1975). The monoclonal antibody, f-42, used in the present study is an IgG₁ containing a κ light chain. The immunoblotting procedure described by Towbin *et*

al. (1979) was used as described in detail previously (Vartio & Vaheri, 1981; Vartio *et al.*, 1982).

Enzyme immunoassay

Disposable polystyrene microtitre wells (Nunc, Roskilde, Denmark) received serially diluted concentrations (up to 5 µg/ml) of fibronectin or its fragments diluted in 0.01 M-phosphate-buffered saline (0.01 M-sodium phosphate/0.14 M-NaCl, pH 7.4). The 75 µl volumes of dilutions represented an active immobilization area of 0.65 cm². The wells were covered with adhesive plastic sheet during the overnight incubation (16–20 h) at room temperature (22°C). The immobilization was terminated by a 10 min incubation with 200 µl of phosphate-buffered saline containing 0.02% Tween 20 [(Z-sorbitan monododecanoate poly(oxyethane-1,2-diyl) derivative; phosphate-buffered saline/Tween] at room temperature, and two brief washes with distilled water, 200 µl each, and air-dried.

Solid-phase-bound fibronectin or its fragments received 75 µl of monoclonal antibody (f-42) diluted 1:500 in phosphate-buffered saline/Tween containing 4% (w/v) PEG (M_r 6000–7500; Fluka, Buchs, Switzerland; PEG buffer) for 1 h at room temperature. The inclusion of PEG in diluent buffers has been shown to enhance the specific antigen–antibody reactions without the use of elevated temperatures (Salonen & Vaheri, 1981). The washes were repeated as described above and the wells received 75 µl of 1:100-diluted alkaline phosphatase-labelled rabbit anti-(mouse IgG) antibodies (Orion Diagnostica, Helsinki, Finland) in PEG buffer for 1 h at room temperature.

After repeated washes, 75 µl of 0.2% (w/v) disodium *p*-nitrophenyl phosphate in diethanolamine/MgCl₂ buffer (Orion Diagnostica) was added and the enzyme reaction was terminated after 30 min at room temperature by the addition of 75 µl of 1 M-NaOH. A_{405} was measured with a vertical-pathway spectrophotometer (Titertek; Multiskan, Eflab, Helsinki, Finland). No reaction occurred if the monoclonal antibody was omitted.

Results

Digestion of fibronectin by cathepsin G and thrombin

Purified human plasma fibronectin was digested by cathepsin G and the digestion products were analysed by NaDodSO₄/polyacrylamide-gel electrophoresis. After a 10 s digestion, fragments of $M_r \geq 120\,000$, 85 000, 72 000, 64 000, 40 000 and 30 000 were detected (Fig. 2a, track 1). Of the fragments, the high- M_r ($\geq 150\,000$) ones and the 85 000-, 72 000-, 64 000- and 40 000- M_r ones bound to gelatin–Sepharose and the $\geq 120\,000$ -, 85 000-, 72 000-, 64 000- and 30 000- M_r ones bound to

Fig. 2. Polyacrylamide-gel electrophoresis and immunoblotting of the cathepsin G-cleaved and thrombin-cleaved fragments of fibronectin

Fibronectin was digested by cathepsin G (a) and thrombin (b) as described in the Materials and methods section. The gel (acrylamide content 8%) was either stained with Coomassie Blue [tracks (a) 1–3 and (b) 1] or the proteins were electrophoretically transferred to nitrocellulose sheets [tracks (a) 4 and (b) 2]. The transferred proteins in the sheets were allowed to react with the monoclonal antibody and the protein–antibody complexes were stained with anti-mouse IgG–peroxidase. Track (a) 1, total digestion products of cathepsin G; (a) 2, gelatin–Sepharose-bound material of the digest in track 1; (a) 3, heparin–Sepharose-bound material; (a) 4, immunoblotting of the material in track (a) 1. Track (b) 1, thrombin digestion products; (b) 2, immunoblotting of the material shown in track (b) 1.

heparin–Sepharose (Fig. 2a, tracks 2 and 3). In control experiments no fragments were bound to unconjugated Sepharose 4B or CL-Sepharose 6B used in the same way as the conjugated Sepharoses.

When purified fibronectin was digested with 50 units of thrombin/5 mg of protein, a fragment of M_r 30 000 was generated, together with fragments of M_r 180 000, when analysed by NaDodSO₄/polyacrylamide-gel electrophoresis (Fig. 2b, track 1).

Localization of the antigenic determinant of the monoclonal antibody

On immunoblotting with the monoclonal antibody the non-reduced dimeric fibronectin was seen as a single polypeptide band and the reduced monomeric subunit polypeptides as a doublet of two equally stained closely spaced bands (result not shown).

The cathepsin G fragments of fibronectin shown in Fig. 2(a), track 1, were also tested for reactivity

by the antibody. Track 4 in Fig. 2(a) shows that the 85000-, 72000- and 30000- M_r fragments reacted with the monoclonal antibody, whereas the 64000- and 40000- M_r fragments did not. Of the thrombin-digested fragments of fibronectin, the 30000- M_r one stained intensively on immunoblotting (Fig. 2b, track 2).

In enzyme immunoassay the monoclonal antibody reacted both with intact fibronectin and with the 30000- M_r fragment, in a dose-dependent way (Fig. 3). The same antibody binding as with intact

Fig. 3. Solid-phase enzyme immunoassay of the purified fragments by the monoclonal antibody

Different concentrations of intact fibronectin (Δ), the 30000- M_r N-terminal fragment ($\bullet$) or the 40000- M_r gelatin-binding fragment ($\circ$) were used for immobilization. Solid-phase bound proteins received a constant amount of the monoclonal antibody. The bound antibody was detected by enzyme immunoassay.

fibronectin was obtained with half the concentration of the fragment. The minimum concentrations detected were 80 and 160 ng of the fragment and intact fibronectin respectively. No reaction was obtained with the gelatin-binding 40000- M_r fragment.

Discussion

In the present paper we report on a monoclonal antibody that reacts with the N-terminal end of human plasma fibronectin. The localization of the determinant (see Fig. 4) is based on the reactivity of the 85000- and 72000- M_r and the non-reactivity of the 64000- M_r gelatin- and heparin-binding fragments with the antibody. According to previous studies (Vartio, 1982) the 85000- and 72000- M_r fragments are located in the N-terminal end of fibronectin. Both these fragments contain the *Staphylococcus aureus*-, heparin- and gelatin-binding domains of fibronectin in this order from the N-terminal end (Vartio, 1982). The 64000- M_r fragment does not bind to *S. aureus* (Vartio, 1982). Thus the determinant of the monoclonal antibody must be in the *S. aureus*-binding N-terminal region of fibronectin, which is within the N-terminal 21000 Da section (85000–64000, see Fig. 4). The N-terminal location of the determinant is further confirmed by the reactivity of the 30000- M_r fragments produced by cathepsin G or thrombin. These fragments bind to *S. aureus* and heparin but not to gelatin (Furie & Rifkin, 1980; Mosher & Proctor, 1980; Vartio, 1982), and the one produced by thrombin digestion has been shown to be derived from the N-terminal end of fibronectin (Furie & Rifkin, 1980).

The monoclonal antibody also reacted with undenatured 30000- M_r fragment in an enzyme

Fig. 4. Schematic presentation of the domains of the N-terminal end of fibronectin

Alignment of the known domains in the subunit polypeptide of fibronectin: 1, *S. aureus*-binding site; 2, heparin-binding site; 3, gelatin-binding site. O indicates the determinant of the present monoclonal antibody.

immunoassay. If calculated on a molar basis, it appears that the antibody reacted roughly equally with the purified fragment and intact fibronectin (Fig. 3).

It is noteworthy that the present monoclonal antibody also reacts with the N-terminal region in the immunoblotting procedure, which involves treatment of antigen with NaDodSO₄ and β -mercaptoethanol. We are not aware of previous poly- or mono-clonal antibodies with this specificity (see, e.g., Vartio *et al.*, 1982). The reason for the weak antigenicity of this region is not clear; it may be due to the multiple disulphide bonds and homologous sequences observed in the corresponding fragment of bovine fibronectin (Petersen *et al.*, 1982).

The large size, domain structure and susceptibility to proteolytic fragmentation make monoclonal antibodies exceptionally rewarding and powerful tools for studies on structure-function relationships of fibronectin and its naturally occurring fragments. The N-terminal region of fibronectin carries several important domains, such as the sites binding to fibrin (Hörmann & Seidl, 1980), staphylococci (Mosher & Proctor, 1980), heparin (Sekiguchi & Hakomori, 1980), actin (Keski-Oja & Yamada, 1981) and transglutaminase cross-linking site (Mosher & Proctor, 1980). This is also the region most readily cleaved off from the molecule by proteinases, which makes it likely that this kind of fragment is found under physiopathological conditions. The applications of the monoclonal antibody specific for the N-terminal domain will undoubtedly be of interest, e.g. in studies on bacteria-fibronectin interactions and on detection and functions of the fragment.

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