Monoclonal antibodies used as probes for the structural organization of the central region of fibronectin

M. Dziadek, H. Richter, M. Schachner* and R. Timpl

Max-Planck-Institut für Biochemie, 8033 Martinsried bei München and **Institut für Neurobiologie der Universität Heidelberg, 6900 Heidelberg 1, FRG*

Received 24 March 1983

Two monoclonal mouse antibodies against human plasma fibronectin were compared in their reactivity for proteolytic fragments of the antigen by enzyme immunoassay and immunoblotting. These antibodies were shown to react with two different structures within a short segment (about 30 kDa) located about one-third away from the C-terminus of the fibronectin chains.

Fibronectin *Central region structure* *Monoclonal antibody* *Immunoblotting*

1. INTRODUCTION

Fibronectin is a large molecule composed of two quite similar polypeptide chains (220–250 kDa) which are joined at the C-terminus by interchain disulphide bonds. The binding properties of fibronectin to eukaryotic cell surfaces, bacteria, heparin, gelatin, fibrin and actin can be attributed to specific structural domains within the molecule [1–3]. These domains appear to be more rigid than the joining polypeptide sequences and possess considerable stability against proteases. Monoclonal antibodies which have been raised against native and denatured fibronectin appear in the majority of cases to react against these functional domains, suggesting that these sites are highly antigenic [4–11]. Such monoclonal antibodies are potential probes to study the importance of specific binding domains for the function of fibronectin in vivo. They may also allow a precise analysis of the alignment of these domains within the molecular structure of fibronectin. The fibronectin molecule has been well characterized at the N- and C-terminal regions, including comprehensive sequence data [12,13], but the large central region of each chain has been more difficult to analyze. Two biological functions have been assigned to this central region; chemotaxis and cell attachment [5,14,15].

Here, we localize the antigenic determinants for two monoclonal antibodies to the C-terminus of the central region, using enzyme immunoassays and immunoblotting techniques with well-defined isolated peptides of human fibronectin.

2. MATERIALS AND METHODS

2.1. *Preparation of monoclonal antibodies*

Monoclonal antibodies resulted from a fusion of NS1 myeloma cells with splenocytes from BALB/c mice which had been immunized with human fibronectin (Collab. Res., Waltham MA). BALB/c female mice, 4–6 weeks old, were injected subcutaneously with 20 μg antigen which was emulsified in complete Freund's adjuvant for the first injection, and with incomplete Freund's adjuvant for the second injection. The third injection was given intraperitoneally without adjuvant, and 3 days later spleens were removed for cell fusion, which was done as in [16]. Hybridoma supernatants were screened on living monolayer cell cultures of early postnatal mouse cerebellum by indirect immunofluorescence as in [17]. Monoclonal antibodies from two hybridoma clones 286 and 317, were affinity purified on fibronectin–Sepharose columns and used in these studies. The immunoglobulin subclass of these antibodies was

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

determined by gel diffusion using Ig subclass specific antibodies (anti-IgG1, IgA and IgM were obtained from Miles GmbH, Frankfurt; anti-IgG2a and IgG2b were a generous gift of Dr Gerisch, Martinsried). Goat anti-mouse IgG was kindly supplied by Dr H.P. Neubauer (Hoechst AG, Frankfurt).

2.2. *Purification of fibronectin and its fragments*

Purified human plasma fibronectin [18] was used for all assays and for the production of specific peptides. Completely unfolded fibronectin was prepared by reduction with 0.02 M dithioerythritol in 8 M urea followed by alkylation.

The large cathepsin D-derived fragments Ca-200 and Ca-140 (see fig.1) were prepared by mild digestion (enzyme:substrate, 1:300; 4 h) of human fibronectin as in [18]. Fragments Ca-95 and Ca-23 were prepared from a heparin non-binding fraction of a more processed digest (fraction I in [19]) by chromatography on Ultrogel AcA44. The chymotryptic peptide Ch-125 was obtained from a digest [20] dialyzed against 0.05 M Tris–HCl (pH 7.4), 1 mM EDTA by fractionation on heparin–Sepharose (column 2.5 × 17 cm) using stepwise elution with 0.05, 0.1 and 0.5 M NaCl. The 0.05 M eluate was passed over gelatin–Sepharose and the non-adsorbed fraction purified on Ultrogel AcA44. Fragment Ch-60 was prepared from the same digest as in [20].

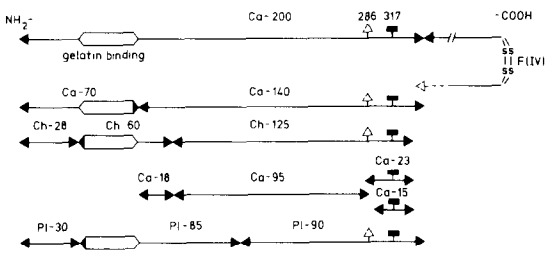

Fig.1. Scheme of a single chain of fibronectin and localization of fragments obtained by digestion with cathepsin D (Ca), chymotrypsin (Ch) and plasmin (Pl). The localization of antigenic determinants reacting with monoclonal antibodies 286 (△) and 317 (■) is based on data shown in fig.3 and 4. The large white box indicates the position of the gelatin-binding domain which was useful for affinity purification of fragments Ca-70, Ch-60 and Pl-85. Fragment F(IV) denotes a carboxy-terminal fragment including the interchain disulfide bonds [19]. Further details are in section 2.

Fragments Pl-85 (gelatin-binding) and Pl-90 (gelatin-non-binding) resulted from a prolonged plasmin digest of fragment Ca-200 (Forschungsplasmin KABI; enzyme:substrate, 1:20; 24 h, 37°C) and were isolated by successive chromatography on gelatin–Sepharose, heparin–Sepharose and Ultrogel AcA44 as will be described elsewhere. M_r-Values were determined by SDS gel electrophoresis [21] on 5–20% polyacrylamide gels, using globular proteins as M_r standards. Protein concentrations were estimated spectrophotometrically using $A_{280}^{1\%} = 12.8$ [18].

2.3. *Immunological assays*

Binding of monoclonal antibodies with fibronectin was determined by radioimmunoassay [22] and enzyme immunoassay [23]. Fibronectin was labelled with ^{125}I using the chloramine T procedure [22], and used in a double antibody assay. In the enzyme immunoassay microtiter plates (Greiner Labortechnik, Nurtingen) were coated with 25–50 μg/ml of fibronectin, and peroxidase-conjugated anti-mouse IgG (Miles GmbH, Frankfurt) was used in the second phase. 5-Amino-2-hydroxy-benzoate 0.8 mg/ml (Ega-Chemie, Steinheim/Albuch) was used as the substrate for the peroxidase reaction [23]. The bound product was solubilized with 1 N NaOH and measured at 492 nm.

In the inhibition assays peptide samples ranging from 1.6–50 nM were incubated overnight at 4°C with monoclonal antibodies (0.2–0.4 μg/ml) before application to the microtiter plates coated with fibronectin (25 μg/ml).

For immunoblotting, 5–10 μg peptide preparations were electrophoretically separated on 6–15% SDS–polyacrylamide gels, and then transferred to nitrocellulose paper (Bio-Rad) by Western blotting according to [24], at a constant 0.3 A overnight at 4°C. After transfer nitrocellulose sheets were washed in water and incubated in a blocking solution, 2.5% bovine serum albumin (Behringwerke AG, Marburg) in Tris-buffered saline (TBS) (pH 7.2) for 2 h at room temperature with constant shaking. Sheets were subsequently incubated in monoclonal antibodies at 2.0 μg/ml in blocking solution for 2 h, washed in several changes of TBS, and incubated in peroxidase-conjugated goat anti-mouse IgG (Miles) at a 1:400 dilution in blocking solution for 2 h. After further washing in

TBS, antigen–antibody complexes were visualized by incubating filters in a substrate solution containing 0.5 mg/ml catechol (Merck, Darmstadt) and 0.5 mg/ml 1,4-phenylenediamine (Merck) in 0.1 M cacodylate buffer (pH 6.0) for 2–5 min (H. Perry, Oxford, personal communication). Filters were washed in TBS and stored in water before photography.

3. RESULTS AND DISCUSSION

Both monoclonal antibodies 317 and 286 were shown to belong to the IgG1 subclass by gel diffusion. They showed an equal reactivity against native fibronectin in enzyme immunoassay (fig.2). The antibodies also showed comparable binding to ^{125}I-labelled fibronectin but precipitated $\leqslant 60\%$ of the antigen even in extreme antibody excess. Reaction with only a portion of the antigen has also been found with other monoclonal antibodies; for example, those against collagen type I [25]. Both reactions were totally inhibited by as little as 3 nM purified fibronectin (fig.3). The antibodies showed similar reactivity against reduced and alkylated fibronectin both in binding and inhibition assays (not shown) indicating that the antigenic determinants are in the primary amino acid sequence rather than the disulphide-linked tertiary structure.

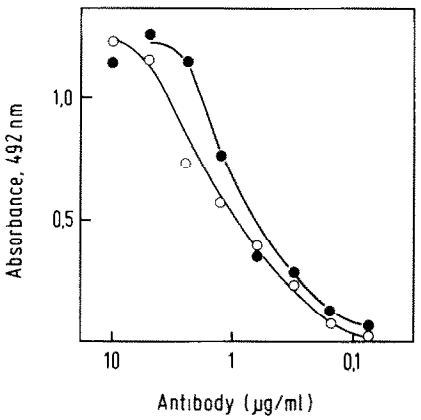

Fig.2. Reaction of monoclonal antibodies 286 (○) and 317 (●) with fibronectin in enzyme immunoassay. The microtiter wells were coated with fibronectin (50 µg/ml) and bound antibody detected by reaction with peroxidase-conjugated anti-mouse Ig antiserum followed by the enzymic reaction which was measured at 492 nm. Background values obtained with normal IgG were subtracted.

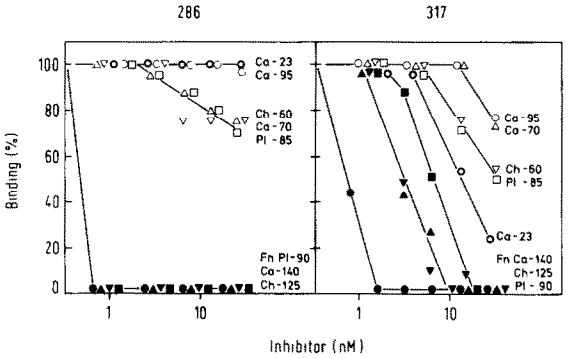

Fig.3. Inhibition of binding of monoclonal antibodies 286 and 317 to fibronectin in enzyme immunoassay by fibronectin fragments. Inhibitors of the reaction were fibronectin (●) and fragments Ca-140 (▲), Ca-95 (○), Ca-70 (△), Ca-23 (⊙), Ch-125 (▼), Ch-60 (▽), Pl-90 (■) and Pl-85 (□). These fragments are described in fig.1. Microtiter plates were coated with fibronectin (25 µg/ml). Results are expressed as reduction in binding of antibody compared to a non-inhibited control.

This has also been demonstrated with other monoclonal antibodies [7].

The proteolytic fragments which were tested for antibody binding are presented in diagrammatic form in fig.1, showing their positions in the intact fibronectin molecule. Antibodies 317 and 286 reacted only with fragments arising from the large central region, and not with N- or C-terminal fragments. The large central cathepsin D fragment, Ca-140, released in early digestion, and a similar chymotryptic fragment, Ch-125, were equivalent to the intact fibronectin molecule in inhibition assays with antibody 286 and only 5-fold less active with antibody 317 (fig.3). Both fragments also reacted very strongly in an immunoblot reaction (fig.4). Gelatin-binding fragments, Ch-60 and Ca-70, which are located at the N-terminal site of fibronectin, showed weak inhibiting activity (fig.3), and when these peptides were transferred to nitrocellulose it was evident that reactivity was confined to small amounts of contaminating higher-M_r fragments in these preparations (fig.4). Likewise, the C-terminal F(IV) fragment [19] did not contain antigenic determinants for these antibodies (not shown).

The different specificities of the two antibodies were only revealed with smaller fragments released upon further digestion of the central region. The

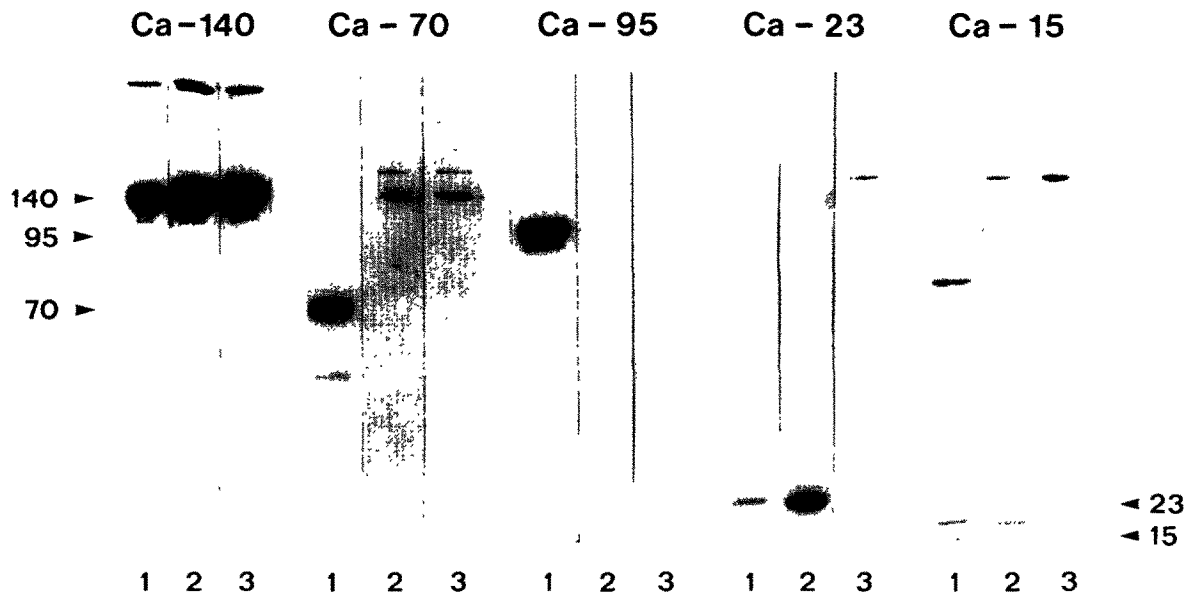

Fig.4. Immunoblot reaction of monoclonal antibodies with cathepsin D fragments of fibronectin. The various fragments (Ca-15 to Ca-140, see fig.1) were electrophoretically separated in SDS–polyacrylamide gels and visualised either by staining with Coomassie blue (lanes 1) or after transfer to nitrocellulose and reaction with monoclonal antibodies 317 (lanes 2) or 286 (lanes 3) by peroxidase reaction. The numbers on both sides indicate the kDa-values of major peptides.

Ca-95 fragment which does not bind to heparin–Sepharose did not react with either antibody in inhibition assays or by immunoblotting (fig.3,4). A smaller Ca-18 fragment which lies next to the gelatin-binding domain [20] was also negative by immunoblot with 286 and 317 antibodies. However, a Ca-23 peptide generated at an intermediate stage of cathepsin D digestion of intact fibronectin retained activity with antibody 317 but not 286. When this fragment was purified, a very strong reaction was observed in an immunoblot reaction with antibody 317 (fig.4), and distinct activity was demonstrated in inhibition assays, although not as strong as the Ca-140 or Ch-125 fragments (fig.3). Complete digestion of Ca-140 with cathepsin D revealed smaller peptides which retained binding activity for antibody 317, the smallest being fragment Ca-15 (15 kDa) (fig.4). The localization of these Ca-23 and Ca-15 peptides to the C-terminus of the central region was confirmed after plasmin digestion of the large Ca-200 fragment. Three peptides were generated, a P1-30 N-terminal fragment, a P1-85 gelatin-binding fragment, and a P1-90 non-gelatin-binding fragment (fig.1). Reactivity of both 286 and 317 antibodies was restricted to the P1-90 piece (fig.3), indicating that both determinants had to be placed to the C-terminus of fragment Ca-140. Loss of the 286 antigenic determinant in the later cathepsin D digests could indicate its position at the site of cleavage between the Ca-95 and Ca-23 fragments, or on a missing fragment (about 7 kDa) which could be positioned between these two. Further cathepsin D digestion of the P1-90 and Ch-125 pieces also generated fragments with electrophoretic mobility similar to Ca-15 and which also reacted only with antibody 317. This pattern indicates that the 317 determinant lies at the very end of the central region, with the 286 determinant presumably close to it at its N-terminus (fig.1).

We have demonstrated here how monoclonal antibodies can be used in conjunction with well-defined proteolytic fragments of fibronectin to accurately determine the position of antigenic determinants. The 286 and 317 determinants lie within a region which contains the chemotactic and cell-binding activities of fibronectin. When fibronectin fragments are assayed for these activities it is clear

that the Ca-140 fragment is highly active in promoting chemotaxis and cell attachment, while the Ca-95 fragment has no activity ([26], Aumailley, unpublished). The chemotactic site has been localized to the Ca-23 fragment [26]. In [5], cell attachment was inhibited by a monoclonal antibody which reacts with a 15 kDa peptide from the central region of fibronectin. This fragment corresponds to the 317 reacting Ca-15 fragment here. A synthetic peptide, consisting of only 30 amino acid residues, is sufficient to promote cell attachment [27]. It is not yet clear whether the same structural domain of fibronectin is responsible for both chemotactic and cell-binding activities. Since both antibodies react with completely reduced fibronectin the elucidation of the structure of antigenic determinants is feasible, and should allow a precise correlation between antigenic and biological activities.

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ti 95/5 and Ho 740). M.D. was in receipt of a fellowship from the European Molecular Biology Organization.

REFERENCES

[1] Ruoslahti, E., Engvall, E. and Hayman, E.G. (1981) Collagen Rel. Res. 1, 95–128.
[2] Hörmann, H. (1982) Klin. Wochenschr. 60, 1265–1277.
[3] Hynes, R.O. and Yamada, K.M. (1982) J. Cell Biol. 95, 369–377.
[4] Atherton, B.T., Taylor, D.M. and Hynes, R.O. (1981) J. Supramol. Struct. Cell. Biochem. 17, 153–161.
[5] Pierschbacher, M.D., Hayman, E.G. and Ruoslahti, E. (1981) Cell 26, 259–267.
[6] Smith, D.E. and Furcht, L.T. (1982) J. Biol. Chem. 257, 6518–6523.
[7] Kavinsky, C.J., Clark, W.A. and Garber, B.B. (1982) Biochim. Biophys. Acta 705, 330–334.
[8] Koteliansky, V.E., Arsenyeva, E.L., Bogacheva, G.T., Chernousov, M.A., Glukhova, M.A., Ibraghimov, A.R., Metsis, M.L., Petrosyah, M.N. and Rokhlin, O.V. (1982) FEBS Lett. 142, 199–202.
[9] Hasty, D.L. and Mainardi, C.L. (1982) Biochim. Biophys. Acta 709, 318–324.
[10] Vartio, T., Zardi, L., Balza, E., Towbin, H. and Vaheri, A. (1982) J. Immunol. Methods 55, 309–318.
[11] Sekiguchi, K., Patterson, C.M., Ishigami, F. and Hakomori, S. (1982) FEBS Lett. 142, 243–246.
[12] Skorstengaard, K., Thøgerson, H.C., Vibe-Pederson, K., Peterson, T.E. and Magnusson, S. (1982) Eur. J. Biochem. 128, 605–623.
[13] Peterson, T.E., Thøgerson, H.C., Skorstengaard, K., Vibe-Pederson, K., Sahl, P., Sottrup-Jensen, L. and Magnusson, S. (1983) Proc. Natl. Acad. Sci. USA 80, 137–141.
[14] Postlethwaite, A.E., Keski-Oja, J., Balian, G. and Kang, A.H. (1981) J. Exp. Med. 153, 494–499.
[15] Seppä, H.E.J., Yamada, K.M., Seppä, S.T., Silver, M.H., Kleinman, H.K. and Schiffman, E. (1981) Cell Biol. Int. Rep. 5, 813–819.
[16] Lagenaur, C., Sommer, I. and Schachner, M. (1980) Dev. Biol. 79, 367–378.
[17] Schnitzer, J. and Schachner, M. (1981) J. Neuroimmunol. 1, 429–456.
[18] Richter, H. and Hörmann, H. (1983) FEBS Lett. 155, 317–320.
[19] Richter, H., Seidl, M. and Hörmann, H. (1981) Hoppe Seyler's Z. Physiol. Chem. 362, 399–408.
[20] Seidl, M. and Hörmann, H. (1983) Hoppe Seyler's Z. Physiol. Chem. 364, 83–92.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Timpl, R. and Risteli, L. (1982) in: Immunochemistry of the Extracellular Matrix (Furthmayr, H. ed) vol.1, pp.199–235, CRC Press, Boca Raton FL.
[23] Adelmann-Grill, B.C. (1981) Collagen Rel. Res. 1, 367–375.
[24] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[25] Linsenmayer, T.F., Hendrix, M.J. and Little, C.D. (1979) Proc. Natl. Acad. Sci. USA 76, 3703–3707.
[26] Pontz, B.F. and Richter, H. (1982) Hoppe Seyler's Z. Physiol. Chem. 362, 905–906 abst.
[27] Pierschbacher, M., Hayman, E.G. and Ruoslahti, E. (1983) Proc. Natl. Acad. Sci. USA 80, 1224–1227.