Isolation of an actin-binding fragment of fibronectin

Jorma KESKI-OJA* and Kenneth M. YAMADA†‡

*Laboratory of Viral Carcinogenesis and †Laboratory of Molecular Biology,
National Cancer Institute, National Institutes of Health, Bethesda, MD 20205, U.S.A.

(Received 15 August 1980/Accepted 9 September 1980)

We have identified a specific actin-binding site in the adhesive glycoprotein fibronectin, isolated from chicken fibroblasts. Affinity chromatography of fragments, released from fibronectin by limited proteolysis with trypsin, chymotrypsin and subtilisin, on actin–Sephacrose and other protein–Sephacrose columns was used to locate the binding site. A 27000-mol.wt. subtilisin-digest fragment bound efficiently to actin. The results suggest that the actin-binding site is close to, but not identical with, the reported collagen-binding site.

Fibronectins are adhesive glycoproteins found in fibroblasts and several other cell types and in plasma (Yamada & Olden, 1978; Vaheri & Mosher, 1978). Cellular and plasma fibronectins are immunologically and structurally very similar (Ruoslahti & Vaheri, 1975; Mosesson & Umfleet, 1970; Keski-Oja *et al.*, 1977; Vuento *et al.*, 1977), although they differ in several properties (Yamada & Kennedy, 1979). Recent studies on the structure of fibronectin have shown that the molecule has different binding sites for macromolecules such as collagen (Ruoslahti *et al.*, 1979; Balian *et al.*, 1979; Hahn & Yamada, 1979a), heparin (Stathakis & Mosesson, 1977; Sekiguchi & Hakomori, 1980; Yamada *et al.*, 1980) and hyaluronic acid (Yamada *et al.*, 1980), as well as a site for binding to the cell surface. The molecule is a dimer or multimer of disulphide-bonded subunits (Mosesson & Umfleet, 1970; Hynes & Destree, 1977; Keski-Oja *et al.*, 1977), and studies with proteinases have revealed that the interchain disulphide bridges are only at one end of the molecule (Chen *et al.*, 1977; Jilek & Hörmann, 1978; Wagner & Hynes, 1979).

The gelatin-binding site (Engvall & Ruoslahti, 1977) of fibronectin has been localized to trypsin-digest fragments with mol.wts. 70000 and 30000 (Ruoslahti & Hayman, 1979; Ruoslahti *et al.*, 1979; Hahn & Yamada, 1979a), to a chymotrypsin-digest fragment with mol.wt. 40000 (Hahn & Yamada, 1979a,b), to a plasmin-digest fragment with mol.wt. 42000 (Balian *et al.*, 1979) and to cathepsin-

G-digest, chymase-digest and elastase-digest fragments with mol.wt. 40000 (Vartio *et al.*, 1980; McDonald & Kelley, 1980). Subtilisin-digestion products of human plasma fibronectin with mol.wts. 30000 and 50000 have also been shown to retain the ability to bind gelatin (Gold *et al.*, 1979). A corresponding binding site for the collagen $\alpha_1(I)$ -chain has been identified (Kleinman *et al.*, 1978; Dessau *et al.*, 1978). Fibronectin can also serve as a substrate for transglutaminases and can become cross-linked to collagen, fibrin or itself (Mosher *et al.*, 1979; Mosher, 1980). The cross-linking site of fibronectin to collagen is, however, distinct from the binding site (Mosher *et al.*, 1980).

We have recently demonstrated that fibronectins from human plasma and cultured human fibroblasts can bind efficiently to actin–agarose conjugates (Keski-Oja *et al.*, 1980). In addition, the binding of fibronectin to actin-coated micro-titre wells was inhibited by gelatin but not by heparin or bovine serum albumin (J. Keski-Oja, E. Ruoslahti & E. Engvall, unpublished work). In the present study, we examine the binding of proteinase-resistant fragments of purified chicken cellular fibronectin to actin. We find that fibronectin's binding site for actin is in the trypsin-digest fragment of fibronectin with mol.wt. 70000 and in a subtilisin-digest fragment with mol.wt. 27000, and that the actin-binding site is different from the collagen-binding site.

Materials and methods

Materials

α -Chymotrypsin (49.2 units/mg) and trypsin (1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated) (238 units/mg) were obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), subtilisin (5 units/mg) and soya-bean trypsin inhibitor

Abbreviations used: T70K and T30K, trypsin-digest fragments of fibronectin with mol.wts. 70000 and 30000 respectively; fragment Ch40K, chymotrypsin-digest fragment of fibronectin with mol.wt. 40000; fragments S50K, S40K, S30K and S27K, subtilisin-digest fragments of fibronectin with mol.wts. 50000, 40000, 30000 and 27000 respectively; SDS, sodium dodecyl sulphate.

‡ To whom requests for reprints should be addressed.

were from Boehringer Mannheim Corp. (New York, NY, U.S.A.), and [^{14}C]glycine (339 Ci/mol) was from ICN Corp. (Irvine, CA, U.S.A.).

Rabbit skeletal-muscle actin was purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.) and was greater than 95% pure in SDS/polyacrylamide-gel electrophoresis. Bovine serum albumin, carbonic anhydrase, ovalbumin, gelatin (type I), α -casein and aprotinin were obtained from Sigma. α -Actinin was generously given by Dr. K. Burridge (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.).

Chicken cellular fibronectin was purified as described previously (Yamada *et al.*, 1977) after radiolabelling *in vivo* with [^{14}C]glycine (5 $\mu\text{Ci/ml}$) in routine culture medium for 24 h.

Proteolytic digestion with trypsin, chymotrypsin and subtilisin

Radioactively labelled chicken cellular fibronectin was digested with trypsin (5 $\mu\text{g/ml}$), chymotrypsin (5 $\mu\text{g/ml}$) or subtilisin (0.5 $\mu\text{g/ml}$ or as shown) for 1 h at room temperature (22°C) at enzyme/substrate ratios (w/w) of 1:10 or 1:100 (subtilisin), then the digestion was terminated by adding soya-bean trypsin inhibitor (25 $\mu\text{g/ml}$), phenylmethanesulphonyl fluoride (2 mM) and Trasylol (2.5 units/ml) (Sigma) and the mixture was chilled on ice for 15 min before chromatography.

Coupling of proteins to Sepharose

Sepharose 2B (Pharmacia Chemicals, Piscataway, NJ, U.S.A.) was activated with CNBr at room temperature at pH 10.0–10.5 as described in detail by Cuatrecasas (1970). Actin was dissolved at a concentration of 2 mg/ml at 4°C by extensive dialysis in 10 mM-Tris/HCl buffer (pH 8.0)/50 mM-KCl/0.2 mM-ATP as described previously (Keski-Oja *et al.*, 1980). The solution was then clarified by centrifugation at 10000 *g* for 30 min. Gelatin type I, bovine serum albumin, ovalbumin, α -casein and α -actinin were dissolved in phosphate-buffered saline (0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) at a concentration of 2 mg/ml. The conjugation of the proteins to Sepharose beads was performed by mixing them at room temperature for 1 h followed by 4 h at 4°C. The conjugation was terminated as described by Cuatrecasas (1970). Sepharose conjugates were packed in 0.7 cm-diameter polypropylene columns (1.0 ml packed volume) and equilibrated with 20 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-KCl and 5 mM-MgCl₂.

Affinity chromatography

The columns were pre-washed extensively with 8 M-urea and 1 M-KBr in 20 mM-Tris/HCl buffer, pH 8.0, and then equilibrated with the column buffer

[20 mM-Tris/HCl buffer (pH 8.0)/50 mM-KCl/5 mM-MgCl₂]. To minimize non-specific binding, the columns were first washed with 2 column volumes of bovine serum albumin (1 mg/ml) in column buffer, followed by extensive washing with the column buffer. The radiolabelled fibronectin digests were applied on the columns in a small volume of the column buffer (500 μl per 1.0 ml of column), and the fragments were allowed to bind for 30–60 min at room temperature. The samples were recycled by adding 500 μl of column buffer and re-applying the unbound material to the columns for 10 min. The unbound radioactivity was removed by washing the columns with 5 column volumes of the column buffer, and the bound proteins were finally eluted with 2 column volumes of 1 M-KBr or 8 M-urea in the column buffer.

The bound and unbound fractions of the columns were precipitated with trichloroacetic acid (10%, w/v, final concentration) and analysed in SDS/polyacrylamide gels.

Fig. 1. *Proteolytic digests of chicken cellular fibronectin* (a) Chicken cellular fibronectin was digested with trypsin, with chymotrypsin and with subtilisin, and the reactions were terminated by adding proteinase inhibitors. The digests were then analysed in an 8% polyacrylamide resolving gel. A radioautogram of the gel is shown. Key: T, trypsin digestion; Ch, chymotrypsin digestion; S, subtilisin digestion. The molecular weights ($\times 10^{-3}$) of the major fragments of interest are indicated at the sides. (b) Gelatin-binding fragments of chicken cellular fibronectin were isolated from chymotrypsin and subtilisin digests of fibronectin by affinity chromatography and analysed in a 10% gel; a radioautogram is shown. Key: Ch, chymotrypsin-digest fragment; S, subtilisin-digest fragment.

Polyacrylamide-gel electrophoresis

Samples were reduced with 2-mercaptoethanol and heated at 100°C for 3 min before electrophoresis. Electrophoresis was performed in vertical slab polyacrylamide gels containing 0.1% SDS. A discontinuous buffer system with a 3.3% polyacrylamide stacking gel (pH 6.8) and a resolving gel (8–12% polyacrylamide, pH 8.8) was used (Laemmli, 1970).

Results

Digestion of chicken cellular fibronectin by trypsin, chymotrypsin and subtilisin

To characterize the proteolytic-digestion products of chicken cellular fibronectin, we incubated fibronectin with trypsin, chymotrypsin or subtilisin and analysed the resulting fragments in gels (Fig. 1a). Treatment with trypsin yielded fragment T70K

and several other fragments. Chicken cellular fibronectin was not cleaved to the T30K form (Ruoslahti *et al.*, 1979), in contrast with human plasma fibronectin. Chymotrypsin treatment yielded as major fragments one of mol.wt. 140 000–160 000 and fragment Ch40K. Subtilisin yielded fragments S40K and S27K in addition to higher-molecular-weight fragments.

Specificity of binding of trypsin-digest fragments of fibronectin to proteins

To test the binding and the specificity of the binding of fragments of radiolabelled cellular fibronectin obtained after treatment of fibronectin with trypsin, we incubated equal portions of the fragments in actin-agarose columns and in five other protein-agarose columns.

Radioautograms of the unbound fractions showed quantitative removal of a 70 000-mol.wt. fragment

Fig. 2. *Binding of trypsin-digest fragments of cellular fibronectin to different proteins*

Five different protein-Sepharose conjugates were washed with column buffer containing bovine serum albumin (1 mg/ml) and subsequently washed extensively with buffer to remove bovine serum albumin. Fibronectin was digested with trypsin (5 µg/ml) for 1 h at 22°C, then the digestion was terminated by adding phenylmethanesulphonyl fluoride (2 mM) and soya-bean trypsin inhibitor (25 µg/ml) and the reaction mixture was chilled on ice for 15 min. Approx. 75 µg of fibronectin was used per column. The fibronectin digest was then applied to the columns in 0.5 ml of loading buffer per 1.0 ml of column matrix. The fragments were incubated for 1 h in the column, and the binding was ensured by recycling the material twice through the column by adding 0.5 ml of loading buffer. The unbound material was removed by washing with 5 column volumes of loading buffer and eluted with 1 M-KBr. The radiolabelled polypeptides of bound and unbound fractions were analysed in gels after precipitation with trichloroacetic acid. (a) Unbound material; (b) bound and eluted proteins. Key: T, original trypsin digest; 1, with carbonic anhydrase-agarose; 2, with actin-agarose; 3, with bovine serum albumin-agarose; 4, with casein-agarose; 5, with ovalbumin-agarose; 6, with α-actinin-agarose.

from the tryptic digest by the actin-Sepharose and gelatin-Sepharose columns, whereas none of the other protein-Sepharose columns retained this fragment (Fig. 2). The T70K fragment could be eluted from the actin-agarose column with 1 M-KBr (Fig. 2). The affinity of fragment T70K for gelatin was higher, since even 4 M-KBr was unable to elute this fragment. We finally eluted the T70K fragment from gelatin columns by using 8 M-urea. The control columns bound only trace amounts of the fragments (Fig. 2). In some experiments there was partial retention of fragment T70K by casein-agarose; however, this binding was inhibited by preincubation of columns with bovine serum albumin before the assay, suggesting that this binding was non-specific. The binding of fragment T70K to actin therefore appears to be a specific interaction. Chicken fibroblast fibronectin was not degraded to the gelatin-binding fragment T30K described for human plasma fibronectin (Ruoslahti *et al.*, 1979), suggesting that there may be species-specific variation in the structure of fibronectin.

Binding of chymotrypsin-digest and subtilisin-digest fragments of fibronectin to gelatin

Since both actin and gelatin could bind the trypsin-digest fragment T70K of fibronectin, we tested whether actin could also bind the gelatin-binding fragment Ch40K obtained by digestion with chymotrypsin (Hahn & Yamada, 1979a). Radiolabelled cellular fibronectin was digested exhaustively with chymotrypsin or subtilisin, and was applied to parallel gelatin-Sepharose and actin-Sepharose columns. Analysis of the eluates in gels indicated that only gelatin could bind the 40 000-mol.wt. fragments that were obtained after digestion by either chymotrypsin or subtilisin (Fig. 1b). This finding suggests that the binding sites for gelatin and actin are in different parts of the T70K fragment. The cleavage of fibronectin by chymotrypsin greatly decreased the binding of fragments to actin, suggesting that the binding site for actin is destroyed by this proteinase.

Our attempts to digest fragment T70K bound to actin-Sepharose by subtilisin were unsuccessful, possibly because of the digestion of actin in the column. Gelatin-Sepharose is relatively resistant to proteinases (Hahn & Yamada, 1979b). Therefore we performed an experiment with fragment T70K bound to gelatin-Sepharose as substrate for subtilisin in columns. The unbound material from this column contained a faint band corresponding to mol.wt. 28 500 and a major band of mol.wt. 27 000. The column matrix retained a 40 000-mol.wt.-fragment (gel not shown). Digestion of the fragment T70K by subtilisin thus gave a 40 000-mol.wt. gelatin-binding fragment and a 27 000-mol.wt. fragment that did not bind to gelatin.

Fig. 3. Binding of subtilisin-digest fragments of fibronectin to actin

Fibronectin was digested with increasing concentrations of subtilisin, and the reaction was terminated by addition of phenylmethanesulphonyl fluoride and aprotinin (Trasyol). The digests were then chromatographed as described in the legend to Fig. 1. Bound and unbound fractions were analysed in a 10% polyacrylamide gel followed by radioautography. (a) Bound and eluted proteins; (b) unbound material. Key: 1, 8 ng of subtilisin/ml used; 2, 80 ng of subtilisin/ml used; 3, 0.8 μ g of subtilisin/ml used; 4, 8 μ g of subtilisin/ml used.

Binding of subtilisin-digest fragments to actin

When fibronectin was digested with increasing concentrations of subtilisin and the fragments were chromatographed on actin-agarose, a 28 500-mol.wt. fragment that was further cleaved to fragment S27K was efficiently retained (Fig. 3). At the lowest concentration of subtilisin, high-molecular-weight fragments of fibronectin (mol.wt. >200 000) were retained in the columns, whereas further cleavage of fibronectin yielded fragments of mol.wt. >150 000 that could no longer bind, plus the smaller fragments that remained bound. Thus it is likely that the cleavage of the actin-binding fragment by subtilisin takes place from one end of the molecule.

Discussion

It has been proposed that fibronectin consists of ordered domains of tertiary structure that are connected by flexible polypeptide regions (Alexander *et al.*, 1978). Fibronectins have been shown to possess several binding sites that are located in different parts of the molecule. The interchain disulphide bridges are in the C-terminal portion of

the molecule close to a proteinase-sensitive site (Wagner & Hynes, 1980). Binding sites for heparin, collagen and cell surfaces have been described (reviewed by Mosher, 1980; Yamada & Olden, 1978). The collagen-binding site is in the *N*-terminal third of the molecule (Balian *et al.*, 1980; Furie & Rifkin, 1980).

Fibronectins from plasma and cultured cells can also bind to purified actin molecules *in vitro* (Keski-Oja *et al.*, 1980). We have examined the binding of trypsin-digest, chymotrypsin-digest and subtilisin-digest fragments of radiolabelled chicken cellular fibronectin to actin coupled to agarose. The T70K fragment of fibronectin bound quantitatively to actin and gelatin, whereas the other protein-agarose conjugates bound only trace amounts of radiolabelled fragments, suggesting that the binding is specific. When fibronectin was digested with chymotrypsin or subtilisin, the collagen-binding fragments Ch40K and S40K did not bind to actin. Subtilisin digests of chicken cellular fibronectin also contained a 28500-mol.wt. actin-binding fragment that was further cleaved to yield fragment S27K, which also could bind to actin. Fragment S27K was thus different in binding activity from the gelatin-binding fragment S40K.

The similarity of the sizes of these collagen-binding fragments suggests that both chymotrypsin and subtilisin act on similar sites in this part of the molecule. However, there are differences elsewhere. We were unable to demonstrate efficient binding of any fragment from chymotryptic digests to actin, whereas subtilisin generated the actin-binding fragment S27K. Furie *et al.* (1980) showed that a 72000-mol.wt. cathepsin-D-digest fragment of human plasma fibronectin contained a 43000-mol.wt. gelatin-binding fragment and a 29000-mol.wt. fragment and showed that the latter fragment is located at the *N*-terminal end of fibronectin. Previously they had shown that human thrombin cleaved a 29000-mol.wt. fragment from the *N*-terminal end of the molecule (Furie & Rifkin, 1980). The actin-binding site might thus be located in the *N*-terminal domain of fibronectin, although other locations are also possible.

The binding site of fibronectin for actin is not in the proposed cell-binding chymotrypsin-digest fragment Ch160K (Hahn & Yamada, 1979a). Ruoslahti & Hayman (1979) also showed that fragment T70K did not promote cell adhesion. Whether the affinity between these conserved molecules plays a role in the interactions between fibronectin and the cytoskeleton is not clear. However, this affinity might be involved in phagocytosis of cellular debris. Fibronectin is also known as the opsonic α 2-surface-binding glycoprotein (Saba *et al.*, 1978), and the binding of fibronectin to exposed actin-containing targets might facilitate the uptake of these

targets by reticuloendothelial cells. Interestingly, the interaction of *Staphylococcus aureus* with plasma fibronectin is mediated via the trypsin-digest fragment T27K that also contains the cross-linking site (Mosher & Proctor, 1980). Plasma fibronectin might also play a role in wound healing and tissue repair by binding to exposed actin molecules and serving as a temporary matrix for proliferating fibroblasts.

The identification of the actin-binding fragment S27K comprising only about 10% of the fibronectin monomer should further facilitate studies on the structural and functional aspects of this protein.

We thank Ms. Dorothy Kennedy for valuable assistance, Dr. K. Burrige for the gift of α -actinin and Dr. G. J. Todaro for his support. This study was supported by the Virus Cancer Program of the National Institutes of Health.

References

- Alexander, S. S., Colonna, G., Yamada, K. M., Pastan, I. & Edelhoch, H. (1978) *J. Biol. Chem.* **253**, 5820–5824
- Balian, G., Click, E. M., Crouch, E., Davidson, J. M. & Bornstein, P. (1979) *J. Biol. Chem.* **254**, 1429–1432
- Balian, G., Click, E. M. & Bornstein, P. (1980) *J. Biol. Chem.* **255**, 3234–3236
- Chen, A. B., Amrami, D. L. & Mosesson, M. W. (1977) *Biochim. Biophys. Acta* **493**, 310–322
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065
- Dessau, W., Adelmann, B. C., Timpl, R. & Martin, G. R. (1978) *Biochem. J.* **169**, 55–59
- Engvall, E. & Ruoslahti, E. (1977) *Int. J. Cancer* **20**, 1–5
- Furie, M. B. & Rifkin, D. B. (1980) *J. Biol. Chem.* **255**, 3134–3140
- Furie, M. B., Frey, A. B. & Rifkin, D. B. (1980) *J. Biol. Chem.* **255**, 4391–4394
- Gold, L. I., Garcia-Pardo, A., Frangione, B., Franklin, E. C. & Pearlstein, E. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4803–4807
- Hahn, L.-H. E. & Yamada, K. M. (1979a) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1160–1163
- Hahn, L.-H. E. & Yamada, K. M. (1979b) *Cell* **18**, 1043–1051
- Hynes, R. O. & Destree, A. T. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2855–2859
- Jilek, F. & Hörmann, H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 247–250
- Keski-Oja, J., Mosher, D. F. & Vaheri, A. (1977) *Biochem. Biophys. Res. Commun.* **74**, 699–706
- Keski-Oja, J., Sen, A. & Todaro, G. J. (1980) *J. Cell Biol.* **85**, 527–533
- Kleinman, H. K., McGoodwin, E. B., Martin, G. R., Klebe, R. J., Fietzek, P. P. & Woolley, D. E. (1978) *J. Biol. Chem.* **253**, 5642–5646
- Laemmli, U. K. (1970) *Nature (London)* **227**, 261–269
- McDonald, J. A. & Kelley, D. G. (1980) *J. Biol. Chem.* **255**, in the press
- Mosesson, M. W. & Umfleet, R. A. (1970) *J. Biol. Chem.* **245**, 5728–5736
- Mosher, D. F. (1980) *Prog. Hemostasis Thromb.* **5**, 111–151

- Mosher, D. F. & Proctor, R. A. (1980) *Science* **209**, 927–929
- Mosher, D. F., Schad, P. A. & Kleinman, H. K. (1979) *J. Clin. Invest.* **64**, 781–787
- Mosher, D. F., Schad, P. A. & Vann, J. M. (1980) *J. Biol. Chem.* **255**, 1181–1188
- Ruoslahti, E. & Hayman, E. G. (1979) *FEBS Lett.* **97**, 221–224
- Ruoslahti, E. & Vaheri, A. (1975) *J. Exp. Med.* **141**, 497–501
- Ruoslahti, E., Hayman, E. G., Kuusela, P., Shively, J. E. & Engvall, E. (1979) *J. Biol. Chem.* **254**, 6054–6059
- Saba, T. M., Blumenstock, F. A., Weber, P. & Kaplan, J. E. (1978) *Ann. N.Y. Acad. Sci.* **312**, 43–55
- Sekiguchi, K. & Hakomori, S.-I. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2661–2665
- Stathakis, N. E. & Mosesson, M. W. (1977) *J. Clin. Invest.* **60**, 855–865
- Vaheri, A. & Mosher, D. F. (1978) *Biochim. Biophys. Acta* **517**, 1–25
- Vartio, T., Seppä, H. & Vaheri, A. (1980) *J. Biol. Chem.* **255**, in the press
- Vuoto, M., Wrann, M. & Ruoslahti, E. (1977) *FEBS Lett.* **82**, 227–231
- Wagner, D. & Hynes, R. O. (1979) *J. Biol. Chem.* **254**, 6746–6754
- Wagner, D. & Hynes, R. O. (1980) *J. Biol. Chem.* **255**, 4304–4312
- Yamada, K. M. & Kennedy, D. W. (1979) *J. Cell Biol.* **80**, 492–498
- Yamada, K. M. & Olden, K. (1978) *Nature (London)* **275**, 179–184
- Yamada, K. M., Schlesinger, D. H., Kennedy, D. W. & Pastan, I. (1977) *Biochemistry* **16**, 5552–5559
- Yamada, K. M., Kennedy, D. W., Kimata, K. & Pratt, R. M. (1980) *J. Biol. Chem.* **255**, 6055–6063