Characterization of sea-urchin fibronectin

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Sea-urchin fibronectin from the ovary of the sea urchin *Pseudocentrotus depressus* bound to gelatin, fibrin and fibrinogen. After mild digestion of the protein labelled with ^{125}I , a 195 000 Da domain was observed. Sea-urchin fibronectin was aggregated by spermine (1 mM) at neutral pH. When the concentration of spermine was decreased or increased, the aggregation was diminished. The addition of 1 M-NaCl or 4 M-urea inhibited the spermine-induced aggregation. Sea-urchin fibronectin mediated the spreading of baby-hamster kidney cells on the plastic surface.

Fibronectin is a transformation-sensitive glycoprotein isolated from various tissues of mammals and birds. A homologous, but not identical, protein designated 'sea-urchin fibronectin' has been isolated from the gonads of sea urchins (Iwata & Nakano, 1981). Fibronectin is known to exhibit a high affinity towards collagen and gelatin, a property that could be involved in some of its biological functions (Kleinman *et al.*, 1978; Engvall & Ruoslahti, 1977). It has also an affinity towards non-collagenous macromolecules, such as fibrin (Mosher, 1975) and heparin (Perkins *et al.*, 1979). These properties of fibronectin reside in a specific structural domain of the molecule (Sekiguchi & Hakomori, 1980; Sekiguchi *et al.*, 1981; Wager & Hynes, 1979). Fibronectin in plasma or cold-insoluble globulin was reported to be identical with opsonic $\alpha_2\text{SB}$ glycoprotein and to participate in host defence mechanisms (Molnar *et al.*, 1979).

Although fibronectin plays important roles *in vivo* and is believed to be distributed in a variety of animals, no fibronectin has been isolated from invertebrates except sea urchins (Iwata & Nakano, 1981). The present work describes some biochemical characteristics of sea-urchin fibronectin, which are similar to those of mammals and birds.

Materials and methods

Materials

Ovaries of the sea urchin *Pseudocentrotus depressus* were obtained by the method of Iwata & Nakano (1981).

Abbreviation used: SDS, sodium dodecyl sulphate.

Isolation and iodination

Sea-urchin fibronectin was isolated by gelatin-Sepharose 4B affinity chromatography as described previously (Iwata & Nakano, 1981). This protein was also isolated by fibrin- and fibrinogen-Sepharose 4B affinity chromatography by the method of Sekiguchi & Hakomori (1980).

Sea-urchin fibronectin isolated by gelatin-Sepharose 4B affinity chromatography was labelled with ^{125}I by the chloramine-T method. Free ^{125}I was removed by passage through a column of Sephadex G-50.

Trypsin digestion

^{125}I -labelled sea-urchin fibronectin was digested with trypsin (20 $\mu\text{g}/\text{ml}$; Sigma) at 37°C for various times (0–1 h). The reaction was stopped by adding soya-bean trypsin inhibitor (Sigma).

Spermine-induced aggregation

Sea-urchin fibronectin was aggregated with spermine by the method of Vuento *et al.* (1980). The isolated protein (0.8 mg/ml) was dialysed against 5 mM-Tris/HCl, pH 7.5. Spermine (Sigma) was added to the protein solutions and incubated at 25°C. The extent of aggregation was determined by measuring the increase in A_{330} .

Protein concentration

Protein concentration was determined by the method of Lowry *et al.* (1951).

SDS/polyacrylamide-gel electrophoresis

This was performed by the method of Laemmli (1970). After electrophoresis the gels were either stained with Coomassie Brilliant Blue, or fluoro-

graphed by the method of Laskey & Mills (1975) by using Kodak X-Omat film. The protein markers used were γ -globulin (M_r 50000; Boehringer), bovine serum albumin (M_r 68000; Sigma), phosphorylase *a* (M_r 94000; Boehringer), ferritin (M_r 220000; Boehringer) and thyroglobulin (M_r 330000; Boehringer).

Cell-spreading assay

Spreading of baby-hamster kidney cells on the tissue-culture dish (Nunc) was performed as described by Grinnell *et al.* (1977).

Results and discussion

Sea-urchin fibronectin binds to fibrin- and fibrinogen-Sepharose 4B affinity gels

Plasma fibronectin has been shown to be cross-linked to fibrin and fibrinogen (Mosher, 1975). More recently, several fibrin-binding domains of plasma fibronectin have been identified (Sekiguchi & Hakomori, 1980; Sekiguchi *et al.*, 1981).

Fig. 1 shows a typical chromatogram of sea-urchin fibronectin on a column of fibrin-Sepharose 4B. Bound materials were sequentially eluted with 0.5 M-NaCl, 1 M-NaCl and 8 M-urea in 50 mM-Tris/HCl, pH 7.5, containing 50 mM- ϵ -aminohexanoic acid and 0.02% NaN₃. Peak fractions were passed through a Millipore filter and analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 2). Bands corresponding to sea-urchin fibronectin (M_r 220000) were found in fractions eluted with 0.5 M-NaCl and 8 M-urea. These bands were identified as sea-urchin fibronectin by the Ouchterlony technique, by using anti-(sea-urchin fibronectin) serum ob-

tained as described by Iwata & Nakano (1981). Inasmuch as sea-urchin fibronectin does not bind to Sepharose 4B gel itself, these results indicate that it binds to the fibrin component of the column.

Sea-urchin fibronectin was also prepared by using fibrinogen-Sepharose 4B affinity gels. Bands of sea-urchin fibronectin appeared in fractions eluted with 0.5 M-NaCl and 8 M-urea. Larger amounts of sea-urchin fibronectin were bound to the fibrin-Sepharose 4B column than to fibrinogen-Sepharose 4B under the same conditions. This may suggest that sea-urchin fibronectin has a greater affinity to fibrin than to fibrinogen.

Trypsin digestion of sea-urchin fibronectin

To study the effect of tryptic digestion, sea-urchin fibronectin labelled with ¹²⁵I was incubated with a low concentration of trypsin (20 μ g/ml) for various periods of time (0–1 h). The reaction was stopped by adding soya-bean trypsin inhibitor. The trypsin-

Fig. 1. Affinity chromatography of sea-urchin fibronectin on fibrin-Sepharose 4B

The column (1.2 cm $\times$ 6.0 cm) was equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM- ϵ -aminohexanoic acid and 0.02% NaN₃. Bound proteins were sequentially eluted with 0.5 M-NaCl, 1 M-NaCl and 8 M-urea.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of sea-urchin fibronectin

Sea-urchin fibronectin was prepared by affinity chromatography on fibrin-Sepharose 4B, and 100 μ l of each sample was subjected to SDS/polyacrylamide-gel electrophoresis. The gel was stained with Coomassie Brilliant Blue to locate proteins. Gels 'Na' and 'U' show proteins eluted from a column of fibrin-Sepharose 4B with 0.5 M-NaCl and 8 M-urea respectively. Both fractions contain sea-urchin fibronectin (M_r 220000).

digested samples were subjected to SDS/polyacrylamide-gel electrophoresis, followed by fluorography. Fig. 3 shows the time course of trypsin digestion. Trypsin cleaved sea-urchin fibronectin to a lower-molecular-weight domain of 195 000 Da within 5 min at 37°C. The same 195 000-Da domain was produced under both reducing and non-reducing conditions. This means that the 195 000-Da domain may have no interchain disulphide bridges which bind it covalently to the remainder of the protein.

Mild trypsin digestion also converts human plasma fibronectin, consisting of two subunits of 230 000 and 210 000 Da, into fragments of 200 000 and 180 000 Da respectively (Sekiguchi *et al.*, 1981; Keski-Oja *et al.*, 1976).

Fig. 3. Trypsin digestion of ^{125}I -labelled sea-urchin fibronectin

Sea-urchin fibronectin purified by affinity chromatography on gelatin–Sephadex 4B was labelled with ^{125}I as described in the Materials and methods section. The labelled product was treated with trypsin (20 $\mu\text{g}/\text{ml}$) at 37°C, and 100 μl samples were taken from the reaction medium at various time intervals and analysed by SDS/polyacrylamide-gel electrophoresis. Radioactivity in the gel was detected by fluorography.

Spermine-induced precipitation of sea-urchin fibronectin

Sea-urchin fibronectin was aggregated by the addition of spermine of low ionic strength and neutral pH. Table 1 shows the aggregation of sea-urchin fibronectin at various concentrations of spermine. A maximal and plateau value of precipitation was reached within 5 min after the addition of spermine. The extent of aggregation was estimated from the protein concentration of supernatants after removal of the aggregates by centrifuging at 10 000g for 10 min. The values were expressed as percentages of controls in which sea-urchin fibronectin was incubated without spermine. The results show that the optimal concentration of spermine for aggregation was 1 mM. When the concentration of spermine was increased or decreased, the aggregation was diminished. Table 1 shows the extent of polyamine-induced aggregation of sea-urchin fibronectin in the presence of 1 M-NaCl or 4 M-urea. The aggregation was

Table 1. Effect of spermine on the aggregation of sea-urchin fibronectin

Sea-urchin fibronectin (0.8 mg/ml) was incubated with spermine for 30 min at room temperature.

Spermine (mM)	Additions	Aggregation (%)
0.1		10
1.0		52
5.0		15
10.0		14
50.0		9
1.0	Urea (4 M)	0
1.0	NaCl (1 M)	3

Fig. 4. Cell-spreading activity of purified sea-urchin fibronectin

For details see the text. ●, Sea-urchin fibronectin; ○, calf plasma fibronectin; △, bovine serum albumin (as a control).

inhibited by both increasing the ionic strength and adding a denaturing reagent.

The interaction of polyamines with human plasma fibronectin has been suggested by Vuento & Vaheri (1978). They used spermine to elute fibronectin from gelatin-agarose affinity gels and also have shown that human plasma fibronectin treated with polyamine has a filamentous structure (Vuento *et al.*, 1980). The results obtained in the present experiments were compatible with those reported with human plasma fibronectin.

Cell-spreading activity of sea-urchin fibronectin

Baby-hamster kidney cells were used to test the cell-spreading activity of sea-urchin fibronectin. After 1 h exposure to sea-urchin fibronectin (10 µg/ml), the cells significantly flattened and spread on the plastic surface as compared with control cultures. Fig. 4 shows the results of a typical experiment in which cell spreading was performed in the presence of fibronectin at different concentrations. Sea-urchin fibronectin mediated the spreading of baby-hamster kidney cells, but was less active than calf plasma fibronectin.

Immunocytochemical study has revealed that sea-urchin fibronectin is localized in the connective tissue and basement membranes of the sea-urchin ovary (Iwata & Nakano, 1981). The present results indicate that (1) sea-urchin fibronectin of 220 000 Da binds to macromolecules such as collagen, fibrin and fibrinogen, (2) the molecule is precipitated with spermine, (3) trypsin cleaves sea-urchin fibronectin to a 195 000 Da domain, and (4) sea-urchin fibronectin increases the spreading of baby-hamster kidney cells. These data suggest that sea-urchin

fibronectin may be homologous to mammalian fibronectins.

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