TWO ACTIVE SITES WITH DIFFERENT CHARACTERISTICS IN FIBRONECTIN

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Received 7 November 1978

1. Introduction

Fibronectin is a high molecular weight glycoprotein present on cell surfaces and in the blood [1,2]. It binds to collagen [3] and mediates adhesion of cells to collagen-coated surfaces [4–7]. The abundance of fibronectin in basement membrane structures [8] has led to the suggestion that its in vivo function may be to attach cells to the extracellular matrix.

To study the molecular interactions responsible for the effects of fibronectin on cell attachment, we have produced fragments of fibronectin from human plasma and studied their biological activity. We report here that the collagen-binding and cell attachment-promoting activities of fibronectin reside in different fragments. This suggests that fibronectin has 2 binding sites, one for collagen and another for cell surfaces.

2. Materials and methods

Fibronectin was isolated using affinity chromatography on gelatin–Sepharose as in [9]. The column was eluted with 4 M urea in 0.05 M Tris–HCl buffer (pH 7.5) and the fractions containing fibronectin were pooled, made 0.1 M with ammonium bicarbonate, and digested with trypsin (TPCK-trypsin, Worthington Biomedical Corp., Freehold, NJ). The details of the digestion are in [10]. The digests were made 10^{-4} M with phenylmethylsulphonylfluoride (PMSF, Sigma Chemical Co., St Louis) and fractionated on gelatin–Sepharose. The non-bound fraction was collected, and the bound material eluted with 4 M urea. These fractions were dialysed against water, lyophilized, and fractionated by gel filtration on columns of Sephadex G-200 equilibrated with phosphate-buffered saline (PBS) (pH 7.4). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed according to [11] using 10% gels.

Fibronectin and its various peptides were tested for promotion of cell adhesion using normal rat kidney cells [12] as follows: Wells of Linbro microtiter plates or Petri dishes (diam. 5 cm) were coated with the protein to be studied as in [3,9]; $\leqslant 10$ μg protein/ml were used for the coating; freshly trypsinized (2X crystalline, Worthington) cells were washed 3 times with Ca^{2+}- and Mg^{2+}-free PBS containing 0.5 mg/ml of soybean trypsin inhibitor (Sigma). After final wash, the cells were resuspended in Eagle's minimal essential medium and added to the coated plates in the same medium used to wash the cells. The microtiter plate wells received 10^4 cells in 0.2 ml, and the Petri dishes, 10^5 cells in 1 ml. After 1 h at 37°C, the plates were washed with PBS to remove the non-attached cells and inspected for attached and spread cells.

3. Results

In [10], we found that tryptic digests of fibronectin show 10–25% binding to gelatin–Sepharose, depending on the extent of digestion. A group of peptides with mol. wt ~70 000 and a homogeneous peptide with mol. wt 30 000, all of which retain the collagen-binding activity, can be isolated from such digests. Gel electrophoresis of these peptides is shown in fig.1 (lanes d,e). The remaining material, devoid of collagen-binding activity as evidenced by lack of binding to gelatin–Sepharose, consists of an array of peptides with mol. wt 15 000–80 000 in SDS–gels

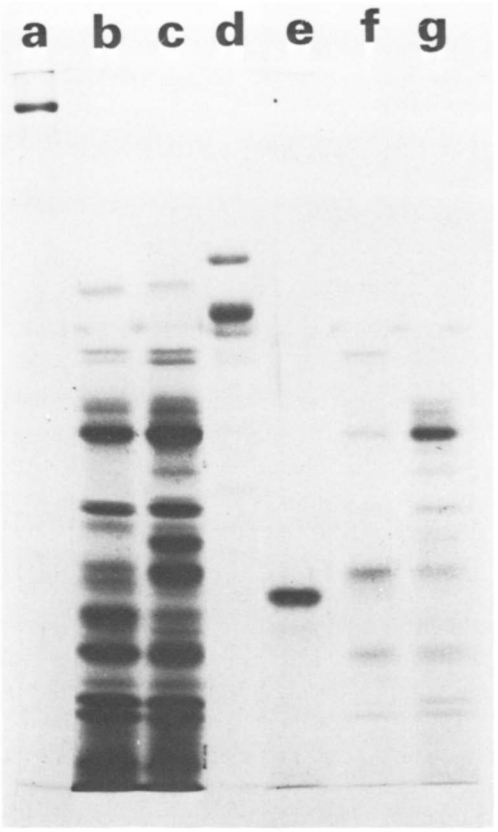

Fig.1. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate: (a) Human plasma fibronectin; (b) tryptic digest of fibronectin; (c) collagen non-binding fraction of the tryptic digest; (d) collagen-binding 70 000 mol. wt fragments; (e) collagen-binding 30 000 mol. wt fragment; (f,g) collagen non-binding Sephadex G-200 fractions of the digest (elution vol. 270 ml and 385 ml in fig.2, respectively).

under reducing conditions (fig.1, lane c). Initial experiments showed that this fraction had retained some of the cell attachment-promoting activity of fibronectin. Gel filtration of the non-collagen-binding peptide fraction resulted in the appearance of the attachment-promoting activity in mol. wt 40 000–150 000 fractions (fig.2). The fractions with the highest activity were found in the 150 000 mol. wt region. Only a few % of the protein was present in these fractions, giving them a high specific activity in the cell adhesion assay. Multiple bands of mol. wt 20 000 –70 000 were found when the

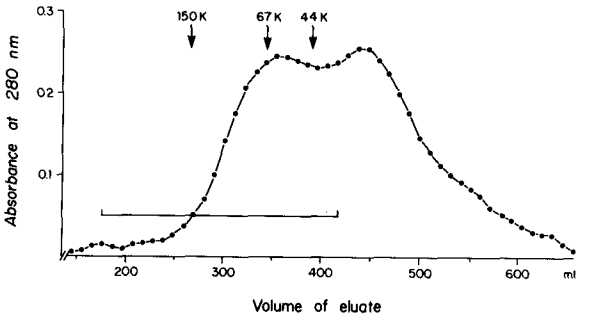

Fig.2. Gel filtration on Sephadex G-200. Collagen non-binding peptides (100 mg) from a tryptic digest of human plasma fibronectin were separated on a 2.5 × 100 cm column. The digest was obtained as in section 2. The fractions active in the cell attachment assay are indicated by brackets. The standards were human IgG (150 000 mol. wt), human serum albumin (67 000 mol. wt) and ovalbumin (44 000 mol. wt).

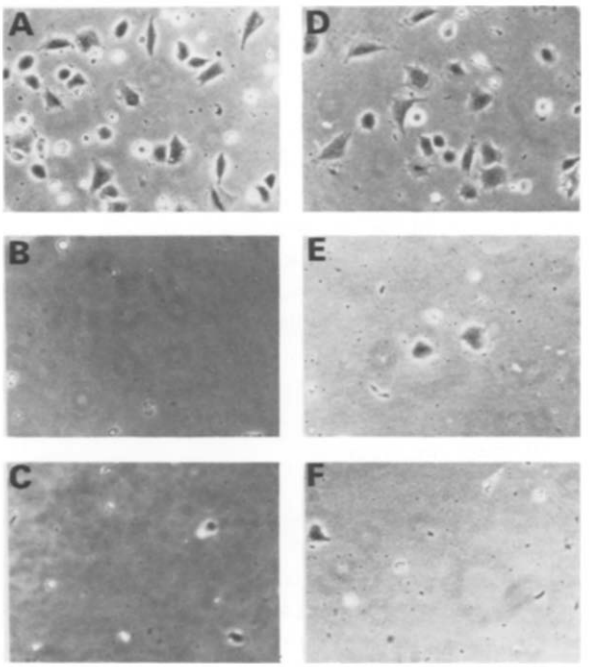

Fig.3. Cell attachment assay. Plastic culture dishes were coated with different protein (10 μg/ml) and assayed for their capacity to support attachment of trypsinized normal rat kidney cells. (A) Fibronectin. (B) 70 000 mol. wt peptides. (C) 30 000 mol. wt peptide. (D) Collagen non-binding peptides, Sephadex G-200 fraction (elution vol. 270 ml in fig.2). (E) Collagen non-binding peptides, Sephadex G-200 fraction (elution vol. 415 ml). (F) Ovalbumin.

Table 1
Activity of human plasma fibronectin and its fragments in cell adhesion assay

	Smallest protein concentration giving an active coating of microtiter plate wells (μg/ml)
Fibronectin	0.3
70 000 mol. wt	> 100
30 000 mol. wt	> 100
Collagen non-bound fibronectin peptides	10
Collagen non-bound fibronectin peptides Sephadex G-200 fraction with highest activity	0.1
Gelatin	10
Ovalbumin	> 100

highly-active (fig.1, lane f) and marginally-active (fig.1, lane g) fractions were analysed in gel electrophoresis.

Figure 3 shows typical results obtained in the cell attachment assay with whole fibronectin and its fragments. Attachment and spreading of the indicator cells were greatly enhanced by fibronectin and the Sephadex G-200 fractions of the collagen non-binding fragments, while the collagen-binding fragments were inactive at all concentrations tested. The lack of the attachment-promoting activity in the mol. wt 70 000 and 30 000 peptides is not due to lack of binding of these peptides to the plastic surface. We have shown elsewhere, using antibodies to these fragments, that they bind to the plastic surfaces used in this study (E. Engvall and E. R., unpublished).

Titration of fibronectin and the fragments with the highest activity showed that the fragments were on a weight basis more active than whole fibronectin in promoting cell adhesion (table 1). Antibodies to fibronectins abolished the attachment-promoting activity of fibronectin and the fragments when added to the coated wells prior to the cells or with them. Gelatin promoted the attachment of cells slightly but was 30–100-times less active than fibronectin and its fragments. That gelatin is slightly active may depend on mediation of attachment to gelatin by the cells' own surface fibronectin regenerated during the incubation. Ovalbumin was inactive.

4. Discussion

While the functional properties of fibronectin are beginning to be understood, little is known about the structural basis of these functions. Based on the different requirements for the binding of fibronectin to collagen [9] and for its functioning in cell attachment [5], we suggested [13] that fibronectin has 2 binding sites, one for collagen and another for cell surfaces. The present results are in agreement with that suggestion, since we found that the cell attachment-promoting activity resides in fragments different from those that bind to collagen. However, our results only establish the cell attachment-promoting activity in fragments which lack the collagen-binding site(s), they do not provide direct evidence for a binding site that would attach to a receptor. It is an intriguing possibility that such a receptor would exist since its expression or lack of it could greatly influence the attachment of cells to the extracellular matrix. Such interactions could be important in the behavior of malignant cells. Our results represent a step toward understanding of the fibronectin–cell surface interaction.

Acknowledgements

We thank Aulikki Pekkala for technical assistance. This work was supported by grants CA 22108 and CA 16434 from the National Cancer Institute.

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