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Synthetic peptide with cell attachment activity of fibronectin

(cell surface/cell culture/plastic surfaces/prosthetic materials)

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ABSTRACT **Four synthetic peptides that together constitute the cell attachment domain of fibronectin [Pierschbacher, M. D., Ruoslahti, E., Sundelin, J., Lind, P. & Peterson, P. (1982) *J. Biol. Chem.* 257, 9593–9597] were constructed and tested for their ability to induce cell attachment and spreading. One of these peptides, consisting of the 30 amino acid residues nearest the COOH terminus of the domain, contained all of the cell attachment activity of the whole domain. Under suitable conditions the peptide was approximately as active as intact fibronectin on a molar basis. The activity could be demonstrated by binding the peptide to polystyrene directly, or via albumin, or by coupling it to agarose beads. This synthetic peptide will be useful in the elucidation of the molecular details of the attachment of cells to fibronectin and could allow manipulation of the adhesive properties of cell culture surfaces and prosthetic materials.**

The extracellular adhesive glycoprotein, fibronectin, is composed of apparently independent globular domains with separate functional activities that can be placed into two categories: sites that interact with other extracellular molecules such as collagen (1, 2), proteoglycans (3–6), fibrin(ogen) (7–9), and actin (10), and sites that can be recognized by cells (11–15) or certain bacteria (16, 17). The interaction with the cell membrane leads to the attachment and spreading of cells (called attachment here for convenience) on a fibronectin surface. There appears to be one cell-binding site in each of the two polypeptides that make up the fibronectin molecule. This site is unique and separate from the recognition site for bacteria (13, 18) and the other binding sites (19). The cell-binding region of fibronectin is localized in an area of the molecule 170–200 kilodaltons (kDal) from the NH_2 terminus (14, 20) and is contained in an 11.5-kDal fragment (21). We have recently determined the primary structure of this fragment, which consists of 108 amino acids (21).

To define further the structure of the cell attachment site we have designed four synthetic peptides each containing sequences of 29 or 30 amino acids from the 11.5-kDal fragment and together constituting this entire region. Using these peptides, we demonstrate that the COOH terminal 30 amino acids account for the cell attachment activity of the 11.5-kDal cell adhesive fragment of fibronectin.

MATERIALS AND METHODS

Source of Peptides. Human fibronectin was obtained from freshly drawn plasma by using gelatin-Sepharose chromatography as described (1). The 11.5-kDal peptide was isolated from a peptic digest of fibronectin (19). Four peptides were synthesized chemically by using the Merrifield solid-phase procedure (22); the synthesis was performed according to our specifications at Peninsula Laboratories (San Carlos, CA). The design of the synthetic peptides was such that each sequence follows the amino acid sequence of the 11.5-kDal cell attachment fragment and has three or four amino acids in common with the adjacent peptide(s). Each peptide has a sequence of 29 or 30 amino acids from the 11.5-kDal fragment plus a cysteine residue at the COOH terminus to facilitate coupling of the peptide to solid phases. The composition of the peptides was verified by amino acid analysis. The peptides are numbered with Roman numerals, starting from the NH_2 terminus of the 11.5-kDal fragment.

Binding of Peptides to Polystyrene. The four synthetic peptides were tested for cell attachment activity in plastic culture dishes as follows. One milligram of each peptide was dissolved in 6 M urea at a concentration of 2 mg/ml at pH 8.0 and reduced by adding dithiothreitol to a final concentration of 45 mM. The peptide was then freed of these reactants by passing it through a 5-ml Sephadex G-25 column equilibrated with phosphate-buffered saline. The fractions containing the peptide were collected and pooled and used in the cell attachment assays. Wells in untreated polystyrene microtiter plates were either left uncoated or coated with bovine serum albumin (hereafter referred to as albumin) by incubating a 20 μg/ml solution in the wells for 2 hr at room temperature. After washing the wells to remove unattached protein, the albumin coating was first derivatized with 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (Sigma), a bifunctional crosslinker, at 10 μg/ml for 30 min at room temperature, and then a solution containing the reduced peptide was added to the wells at different concentrations and allowed to react for at least 1 hr. (The crosslinker reacts mainly with amino groups in the albumin and subsequently crosslinks the peptide to albumin through the cysteine residue in the peptide.) After repeated washing to remove unattached peptide the plates were used for cell attachment assays. Cell attachment in wells coated directly with the peptides was compared with that seen in wells containing albumin-linked peptides, albumin-linked fibronectin, fibronectin alone, or derivatized albumin alone.

Coupling of Peptides to Sepharose Beads. The four synthetic peptides as well as the 11.5-kDal fragment and a 200-kDal fragment (14) of fibronectin were coupled to cyanogen bromide-activated Sepharose-6MB beads (Pharmacia) according to the manufacturer's instructions. The peptides were first reduced as described above and used at 8 mg of peptide per ml of Sepharose. Albumin-coated beads and beads derivatized with ethanolamine were used as controls.

Cell Attachment Assay. The cell attachment assays were performed as described (23) with human fibroblasts or normal rat kidney (NRK) cells. Briefly, 100 μl of a single-cell suspension containing 10^4 cells was placed in a flat-bottom microtiter well that had been coated with one of the peptides or with fibronectin. After 1 hr at 37°C, unattached cells were washed away and attached cells were fixed and stained for counting. In some

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Abbreviations: peptides I–IV, synthetic 30- or 31-amino-acid peptides modeled after the amino acid sequence of the cell attachment site of fibronectin; NRK cells, normal rat kidney cells; kDal, kilodalton(s).

Peptide I

1 10 20

Ile-Gly-Gln-Gln-Ser-Thr-Val-Ser-Asp-Val-Pro-Arg-Asp-Leu-Glu-Val-Val-Ala-Ala-Thr-Pro-Thr-Ser-Leu-Leu-Ile-Ser-Trp-Asp-Cys

Peptide II

30 40 50

Ser-Trp-Asp-Ala-Pro-Ala-Val-Thr-Val-Arg-Tyr-Tyr-Arg-Ile-Thr-Tyr-Gly-Glu-Thr-Gly-Gly-Asn-Ser-Pro-Val-Gln-Glu-Phe-Thr-Val-Cys

Peptide III

60 70 80

Phe-Thr-Val-Pro-Gly-Ser-Lys-Ser-Thr-Ala-Thr-Ile-Ser-Gly-Leu-Lys-Pro-Gly-Val-Asp-Tyr-Thr-Ile-Thr-Val-Tyr-Ala-Val-Thr-Cys

Peptide IV

90 100

Tyr-Ala-Val-Thr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Ile-Ser-Ile-Asn-Tyr-Arg-Thr-Glu-Ile-Asp-Lys-Pro-Ser-Gln-Met-Cys

FIG. 1. Four synthetic peptides designed after the known sequence of the 11.5-kDal fragment of fibronectin that induces cell attachment. The numbers above the peptides correspond to the residue number in the natural fragment. The broken lines indicate amino acids common to two peptides.

experiments soluble peptide was added to the medium to determine if its presence during the attachment would have an inhibitory effect. Cell attachment to the agarose beads was performed on a layer of beads in the bottom of a round-bottom microtiter well exactly as described above.

Iodination of Peptides. The chloramine-T method (24) was used to label 50 μg of peptides II, III, and IV with 0.33 mCi (1 Ci = 3.7×10^{10} Bq) of ^{125}I. Uncoupled iodide was removed and quantitated by passage through a column of Sephadex G-25.

RESULTS

The four synthetic peptides studied for their effect on cell attachment are shown in Fig. 1. Some degree of overlap was included to avoid the possibility of splitting, and thereby losing, the cell recognition site. The peptides were allowed to adsorb directly to polystyrene microtiter wells or were attached to albumin-coated polystyrene via their COOH-terminal cysteine residue by using a bifunctional crosslinker. Surfaces derivatized in this manner were then tested for their ability to support the attachment of cells. Fig. 2 shows the results of such experiments.

Peptide IV, which consists of the COOH-terminal 30 amino acids of the 11.5-kDal cell attachment fragment of fibronectin (plus a cysteine residue), supported the attachment of both NRK cells and human fibroblasts whether coupled to albumin or adsorbed directly to the surface. Coupling to albumin, however, greatly increased the activity of this peptide. When coupled to albumin, as little as 20 ng of peptide IV could be detected per microtiter well (see Fig. 2). Peptides I, II, and III, on the other hand, had no activity in this assay. The activity of whole fibronectin was independent of the pretreatment of the substrate with the crosslinker-derivatized albumin. Because the binding curves for fibronectin were similar whether the protein was bound to plastic directly or via albumin, only the data obtained by using plastic-bound fibronectin are shown.

To confirm that the lack of activity of the inactive peptides was not due to a lack of binding to the polystyrene surface, peptides II, III, and IV were radioiodinated and incubated overnight in microtiter wells. Fig. 3 shows that all three peptides bind to the microtiter wells to similar extents. Peptide IV bound slightly less well than the others, possibly because, of the four peptides, it is the most hydrophilic (25). The binding of all three peptides to polystyrene could be inhibited by the corresponding unlabeled peptide, indicating that radioiodination had not altered their capacity to bind to this type of surface. Furthermore, cells attached to ^{125}I-labeled peptide IV in a dose-de-

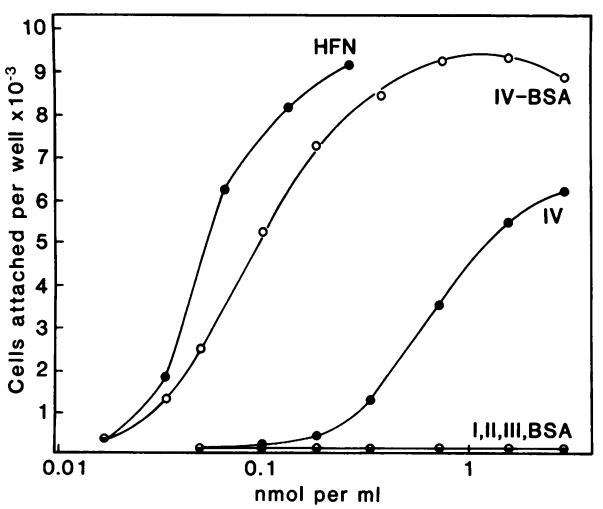

FIG. 2. Attachment of NRK cells to polystyrene microtiter wells coated with human fibronectin (HFN) or synthetic peptides. In each well 10^4 cells were cultured for 1 hr and those cells that attached were counted. Fibronectin and peptides I, II, III, and IV were assayed both when adsorbed directly to the polystyrene (●) and when coupled to albumin (BSA; ○). Albumin alone was also tested. The number of moles of fibronectin was calculated by using the molecular mass of one subunit (240 kDal). Cells attach to wells containing fibronectin and peptide IV, but not to wells containing the other peptides or albumin alone.

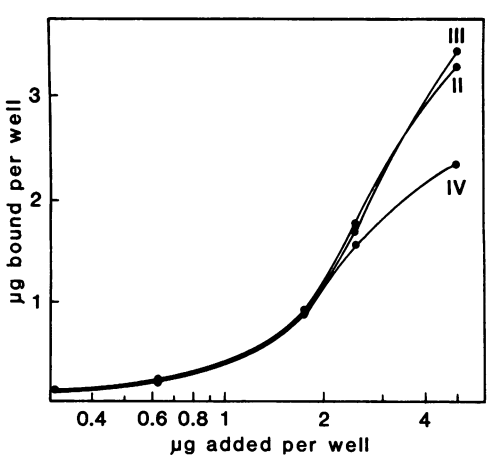

FIG. 3. Binding of ^{125}I-labeled peptides II, III, and IV to plastic microtiter wells. The amount of peptide bound in each well was calculated from the specific radioactivity of each peptide.

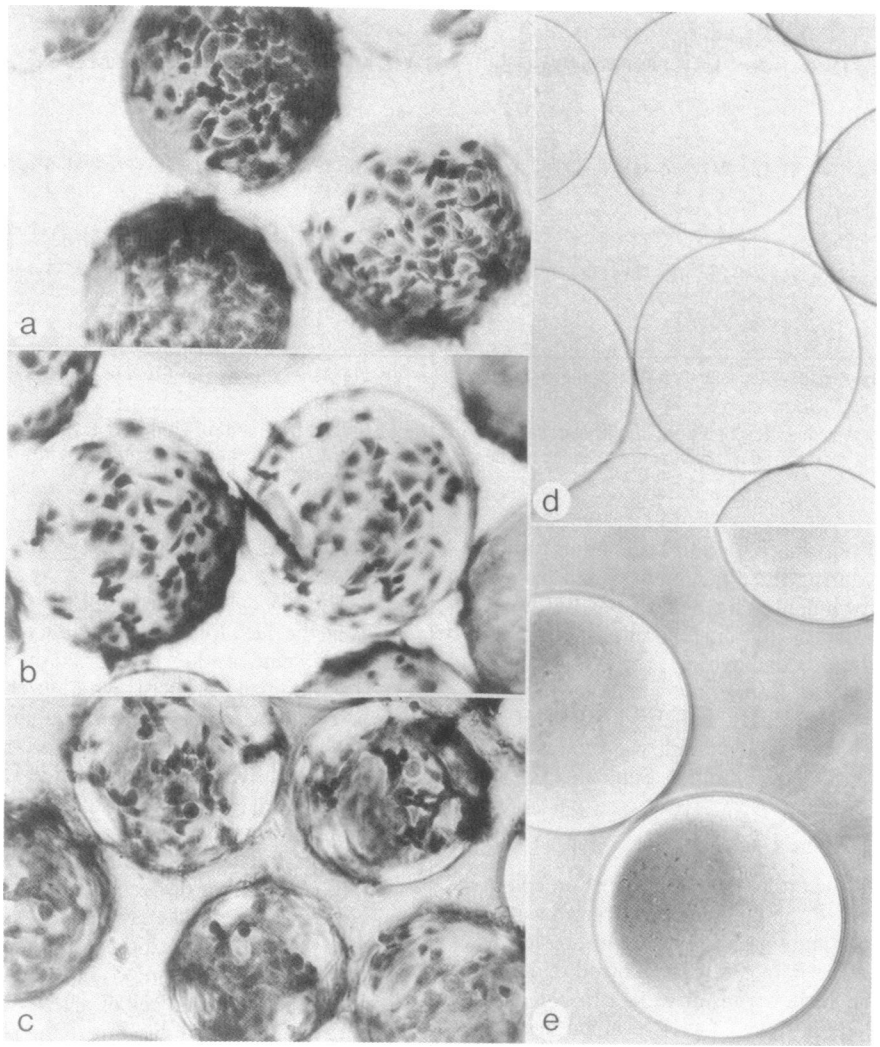

FIG. 4. Attachment of NRK cells to Sepharose 6MB beads carrying covalently linked 200-kDal peptide (*a*); 11.5-kDal peptide (*b*); or peptide IV (*c*). These can be compared with ethanolamine-derivatized beads (*d*) or beads coated with peptide III (*e*), which have no attached cells. Peptides I and II were also inactive, as were albumin-coated beads (not shown).

pendent manner identical to that seen with unlabeled peptide (data not shown). We also tested whether the presence of peptide IV in a soluble form would inhibit the adhesion of cells to microtiter wells coated with this peptide or with fibronectin. At concentrations up to 150 μg/ml, however, peptide IV had no effect on the attachment of cells to these surfaces.

To confirm the specificity of the activity found in peptide IV, all of the peptides were coupled to cyanogen bromide-activated Sepharose 6MB. The efficiency of coupling of all four peptides was greater than 90% as judged on the basis of UV absorbance. Beads to which peptide IV was coupled supported attachment of cells (Fig. 4), as did beads with the entire 11.5-kDal fragment or a 200-kDal fragment of fibronectin (14). The latter fragment was used instead of intact fibronectin because of the greater stability and higher biological activity of beads coated with fragments (26). Agarose beads derivatized with ethanolamine or with albumin were devoid of cell attachment activity, as were beads having peptides I, II, or III on their surfaces.

DISCUSSION

Our data show that most, if not all, of the cell attachment activity of fibronectin can be accounted for by 30 amino acid residues. Because this is only about 1% of the intact fibronectin polypeptide monomer, an extremely specific interaction between the cell surface and this portion of the fibronectin molecule can be inferred. The active peptide, peptide IV, is the most hydrophilic of the four synthetic peptides constituting the cell attachment domain of fibronectin, whereas peptides II and III are quite hydrophobic and peptide I has intermediate characteristics (25). This probably results in the 30 residues of peptide IV being exposed on the surface of the corresponding portion of the intact fibronectin molecule and is consistent with the presence of the activity in this part of the fragment.

It is important to note that peptide IV was active on three different types of surfaces, including the hydrophobic polystyrene surface and the hydrophilic Sepharose beads. This makes it unlikely that we would be dealing with some kind of nonspecific modification of the surface that would make it more attractive to cells. This argument is further strengthened by the fact that we could show that the binding of the different peptides to these surfaces was similar, yet only peptide IV was effective in cell attachment. Furthermore, this peptide had a high specific activity. When crosslinker-derivatized albumin was used to mediate the binding of the peptides to the polystyrene surface, peptide IV was nearly as active on a molar basis as intact fibronectin itself. When peptide IV was used to coat polystyrene microtiter wells directly, however, about 10 times the molar amount was required to obtain the same effect as was seen with intact fibronectin in inducing cell attachment. This could

be due to loss of function as a result of binding, or it may reflect a relative inefficiency or reversibility of the binding of this peptide to polystyrene compared to the larger fibronectin molecule (27).

The nature of the component(s) (receptor) at the cell surface that interacts with the cell attachment site of fibronectin is not known. The data presented here strongly suggest that a discrete cell surface receptor, or class of receptors, exists. The synthetic peptide, or shorter derivatives of it if active, could be helpful in identifying this receptor. From calculations using the method of Rose (25), one can predict a turn in the peptide chain in the hydrophilic area around the proline residue number 10 in peptide IV. This could result in a loop available for interaction with cells.

For reasons that are incompletely understood, expression of the cell attachment function of fibronectin requires that fibronectin is presented to cells bound to a solid phase such as a plastic surface or a collagen matrix, whereas soluble fibronectin does not bind detectably to cells (28, 29). Peptide IV behaves similarly, in that soluble peptide IV did not inhibit the attachment of cells to immobilized peptide IV or fibronectin. It is likely that a cooperative binding of the cell surface with several fibronectin molecules is required for a productive interaction. It may be possible to construct an analog(s) of this peptide that would bind more strongly to the cell surface. Not only would this facilitate the identification of the cell surface receptor for fibronectin, but also it might allow the modulation of cell attachment.

Practical applications such as the preparation of surfaces for optimal cell culture and derivatization of various prosthetic materials to promote bonding with surrounding tissues can also be envisioned. Since a peptide of 30 amino acids is unlikely to have more than one binding site, one question that can be addressed now is whether the interaction of all types of cells with fibronectin involves this same region of the molecule. Platelets, for example, may bind fibronectin on their surfaces by a different mechanism (30, 31). This would be an important detail in using this peptide to regulate cell attachment or in the design of prosthetic materials. It would also shed light on the role played by fibronectin *in vivo*.

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