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The Cell Attachment Determinant in Fibronectin

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Fibronectin possesses a domain that interacts with cell surfaces. The ability of fibronectin to promote cell attachment can be duplicated with a short amino acid sequence, glycyl-L-arginyl-glycyl-L-aspartyl-L-serine, taken from that domain. The tripeptide Arg-Gly-Asp appears to be irreplaceable for maintenance of the activity of this peptide, whereas the serine residue can be replaced with some, but apparently not all, possible residues. This recognition sequence, or a closely related sequence, is present in a number of proteins other than fibronectin that interact with cells. These proteins include collagens, fibrinogen, thrombin, a bacterial surface protein, and two viral proteins, as well as discoidin-I, a protein implicated in the aggregation of Dictyostelium discoideum. A similar sequence is also repeated in some, but not all, fibronectin molecules, making it possible that some fibronectin molecules have more than a single cell attachment site. Synthetic peptides constructed from sequences taken from several of these other proteins have also been shown to promote cell attachment. The tripeptide sequence may, therefore, constitute an ancient cellular recognition mechanism common to many proteins.

Key words: cell adhesion, fibronectin, peptides

Many important biological phenomena—including morphogenetic migration of cells, wound closure, and tumor metastasis—involve the ability of cells to establish and to break adhesive interactions with the extracellular substratum or with other cells. That many normal cells must adhere and spread to survive in vitro reflects the importance of these interactions.

The discovery of fibronectin as a major extracellular matrix protein and the demonstration that it interacts in vitro with other structural molecules [1–10] and promotes the attachment of suspended cells to substratum [11–15] has stimulated intense interest in this protein. The cell attachment-promoting activity suggests that fibronectin acts as an anchor for cells in the extracellular matrix and in blood clots [7,16]. The involvement of fibronectin in the control of cell division and cellular morphology has been inferred from the observation that it is absent from the cell surface in many transformed cell lines and tumors (for recent reviews, see Mosher [17], Hynes and Yamada [18], and Ruoslahti [19]).

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The fibronectin molecule consists of highly structured domains separated by flexible polypeptide segments [20–24]. These domains can be isolated as protease resistant fragments that retain one or more of the functional activities of fibronectin [14,25–30]. Fragments of fibronectin that bind to collagen [30–34], glycosaminoglycans [27,35], fibrinogen [36,37], Staphylococci [38], and actin [10] have been isolated and subsequently studied extensively.

The cell attachment site in fibronectin is unique and separate from the other binding sites [17,39,40]. It is localized in an area of the molecule 170–200 kilodaltons (kD) from the NH_2-terminus [41,42]. Using a monoclonal antibody that inhibits attachment of cells to immobilized fibronectin [39], we isolated an 11.5-kD fibronectin fragment that retains the capacity to promote cell attachment and determined its primary structure [43]. Using synthetic peptides modeled after this sequence, we have localized the cell attachment function of the fibronectin molecule to the peptide Arg-Gly-Asp-(Ser).

The purpose of this article is to summarize our current knowledge of the structure of the fibronectin cell attachment site.

METHODS

Cell Attachment Assays

Microtiter well assays. The cell attachment assay was performed as described by Ruoslahti et al [44]. In this assay 96-well polystyrene microtiter plates that had not been treated for tissue culture were coated with fibronectin. A single-cell suspension of normal rat kidney (NRK) cells was used as indicator cells.

The synthetic peptides were assayed for their ability to promote the attachment of NRK cells by first attaching the peptides via the heterobifunctional cross-linker SPDP (N-succinimidyl-3-(pyridyldithio)propionate; Sigma) to bovine serum albumin or to rabbit IgG that had been immobilized on polystyrene [45]. Coupling of the peptides using this method is more than 85% complete. The attachment assay was carried out as described above [44] with freshly trypsinized NRK cells. After incubation for 1 hr at 37°C, those cells that had attached were fixed, stained, and quantitated with either an Artek cell counter or a vertical pathway spectrophotometer. In all cases, maximum attachment was 80–90% of the cells plated.

Nitrocellulose filter assays. Proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred by diffusion to nitrocellulose and the filters were used for cell attachment assays as described elsewhere [46]. Briefly, a filter with transferred protein was rinsed in phosphate-buffered saline (PBS) and soaked for 16 hr in 500 ml of PBS containing 5 mg/ml bovine serum albumin to prevent nonspecific cell attachment to uncoated filter paper. The filter was then washed with PBS and placed in a flat-bottom dish, blotted side up. A single-cell suspension in DME (10^7 cells per 50 ml) of NRK cells, which had been prepared with crystalline trypsin (Sigma type III, Sigma Chemical Co., St. Louis) and washed with soybean trypsin inhibitor (Gibco Laboratories, Grand Island, NY) as described earlier [44], was added to the filter and incubated for 1 hr at 37°C. The filter was washed with PBS, fixed with 3% paraformaldehyde, stained for 1–2 min with 0.1% amido black (Sigma) in 45% methanol, 10% glacial acetic acid, 45% deionized water, and destained in 90% methanol, 2% acetic acid, 8% water. With this staining procedure cells that are attached to the protein bands give dark blue bands on a pale blue background.

Inhibition of Attachment

Effect of antibodies. To determine the effect of antifibronectin on cell attachment to fibronectin, we incubated the coated microtiter wells with mouse anti-human fibronectin, monoclonal antibody to fibronectin, or phosphate-buffered saline. The nonbound antibody was washed away with phosphate-buffered saline prior to performing the attachment assay as described above.

Effect of soluble synthetic peptides. The peptides were titrated in microtiter wells coated with 5 μg/ml fibronectin. NRK cells were incubated in these wells at 37°C for 45 min, and attached cells were fixed, stained, and counted. Incubation media for these experiments was Dulbecco's modified Eagle's medium (Flow) supplemented with glutamine-penicillin-streptomycin at 2 mM, 100 U, and 100 μg/ml, respectively (Irvine Scientific) and bovine serum albumin, 2 mg/ml (Sigma).

RESULTS

Preparation of a Monoclonal Antibody Reacting at the Cell Attachment Site of Fibronectin

The attachment of cells to immobilized fibronectin can be inhibited by polyclonal antisera to fibronectin [47,48]. It was of interest to see whether monoclonal antibodies reactive with different parts of the fibronectin molecule could inhibit attachment of cells to fibronectin. The reactivity of one monoclonal antibody, 3E3, correlated with the presence of cell attachment activity in various proteolytic fragments of fibronectin [39]. The 3E3 antibody reacted with both chains of fibronectin (Fig. 1) and the location of the binding site within each chain could be confirmed by electron microscopy (Fig. 2). This antibody inhibited cell attachment to fibronectin [39], but other monoclonal antifibronectin antibodies alone or in combination had no effect on cell attachment. These results showed that the 3E3 antibody reacts at or near the cell attachment site.

Isolation and Primary Structure Determination of a Cell Attachment-Promoting Fragment of Fibronectin

We next used the 3E3 antibody as well as other monoclonal antifibronectin antibodies to fractionate a pepsin digest of fibronectin. Of the fragments that were obtained, a small, approximately 11.5-kD fragment bound to 3E3-Sepharose. Cell attachment-promoting activity was found in this 11.5-kD peptide, but fragments binding to the other antibodies were inactive in this respect [39].

This cell attachment fragment of plasma fibronectin contained 108 amino acid residues (Fig. 3) and had a calculated molecular weight of 11,482. This fragment is contained within the region of type III homologous internal repeating sequences that are present in the fibronectin molecule [55–57].

Synthesis of the Cell Attachment Site of Fibronectin

The structure of successive beta turns depicted in Figure 3 for the cell attachment-promoting, peptic fragment was predicted from the primary structure by the methods of Chou and Fassman [58] and Kyte and Doolittle [59]. To localize the site recognized within the peptic fragment, the sequence was initially synthesized as four peptides as shown in Figure 3, and the effect of these peptides on cell attachment was studied [45]. The peptides were allowed to adsorb directly to polystyrene microtiter

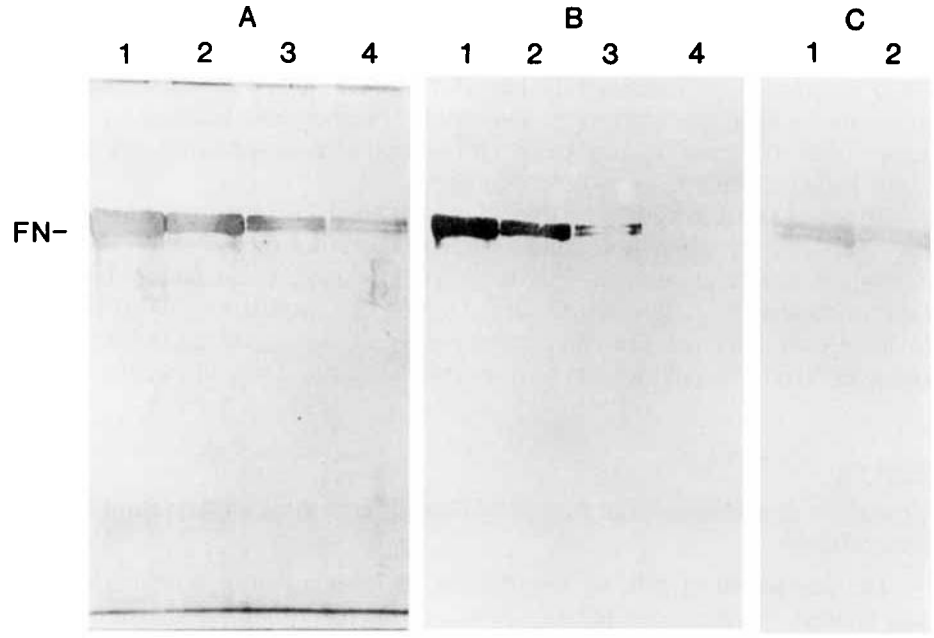

Fig. 1. Purified bovine fibronectin was run on a 5% SDS-PAGE and stained for proteins (A) or transferred to nitrocellulose for cell attachment (B), as described in Methods. Lanes 1, 2, 3, and 4 in A and B represent 4, 2, 1, and a 0.5 μg protein, respectively. In panel C, 5 μg (lane 1) or 2.5 μg (lane 2) of fibronectin are stained with the 3E3 antibody. Each chain contains both the cell attachment and 3E3 determinant.

wells or were attached to albumin-coated polystyrene via their COOH-terminal cysteine residue by means of a bifunctional cross-linker. Surfaces derived in this manner were then tested for their ability to support the attachment of cells.

Peptide IV, which consists of the COOH-terminal 30 amino acids of the 11.5-kD 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 (Fig. 3) [45]. 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. Peptides I, II, and III, on the other hand, had no activity in this assay.

Minimum Sequence Having Cell Attachment-Promoting Activity

The group of residues boxed in Figure 3 probably forms a hydrophilic loop at the surface of the molecule and is thus available to interact with the cell surface. To test whether these amino acids were involved in the cell attachment activity of the peptide, we obtained several shorter synthetic peptides (Fig. 4), coupled them to protein-coated plastic, and tested each for cell attachment-promoting activity [60]. All of the peptides that promoted the attachment of rat kidney fibroblasts contained the sequence Arg-Gly-Asp-Ser (or Arg-Gly-Asp-Cys) (Fig. 4). The activity of the peptides decreased somewhat with size. This may have been a result of a decrease in

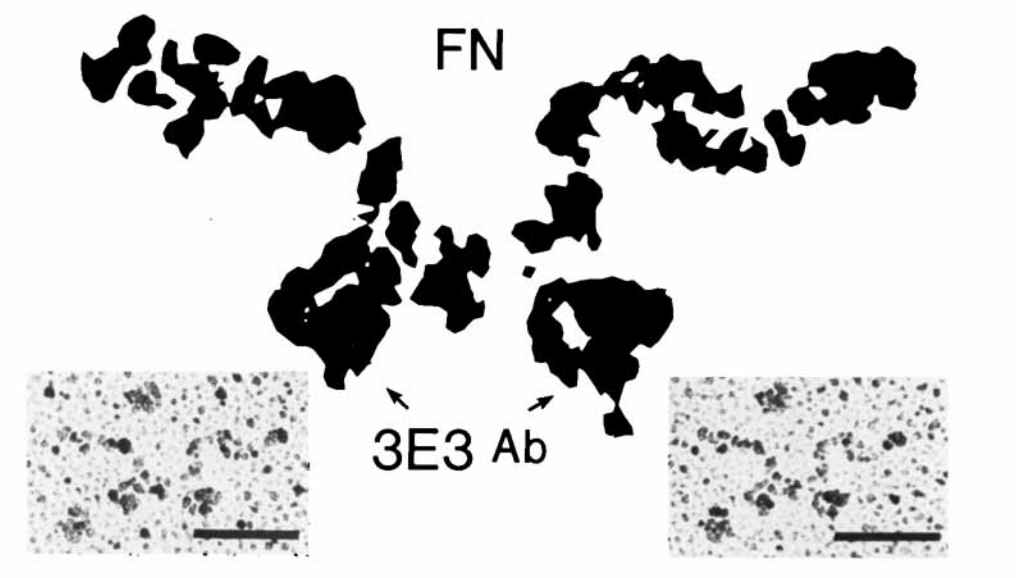

Fig. 2. Rotary shadowing image of fibronectin and bound 3E3 monoclonal antibody after computer enhancement (Slide Graphics, Phoenix, AZ). The original images (inset) were viewed at +20° or −20° from center and superimposed for composite. Fibronectin and 3E3 were mixed in a ratio of two antibody molecules for every polypeptide chain of fibronectin at a concentration of 2.5 mg/ml of fibronectin in 0.1 M ammonium acetate. After a 20-min incubation, the solution was diluted fivefold with 50% glycerol in 0.1 M ammonium acetate, sprayed onto a freshly cleaved mica sheet, shadowed with platinum/palladium, and viewed with a Hitachi 600 STEM electron microscope. The antibody consistently bound near the interchain disulfide bond in each chain. The bar represents 100 nm.

their stability or a relative inaccessibility on the substrate. The tetrapeptide Arg-Gly-Asp-Ser itself was active when immobilized on Sepharose beads at the end of a six-carbon-atom spacing arm [60].

Inhibition of Cell Attachment With Synthetic Peptides

High concentrations of soluble fibronectin are needed to overcome the cooperative interaction of many immobilized fibronectin molecules with cells [61–64]. Such concentrations are difficult to reach with fibronectin or its large fragments. In contrast, the small peptides are highly soluble, which makes it possible to test them at millimolar concentrations for inhibition of cell attachment. The Gly-Arg-Gly-Asp-Ser-Pro and Arg-Gly-Asp-Ser peptides, which were designed not to have a cysteine residue to avoid possible disulfide exchanges, did indeed interfere with the attachment of NRK cells to fibronectin-coated substrates (Fig. 5). The inhibition by the peptides occurred in a dose-dependent manner, and peptides not containing the Arg-Gly-Asp-Ser sequence showed no inhibition of cell attachment to fibronectin, indicating that the inhibition was specific and that these concentrations of peptides in the cell attachment assay had no noticeable adverse effects on the cells. Furthermore, the attachment of cells to concanavalin A remained unaffected by the Arg-Gly-Asp-Ser-containing peptides [60].

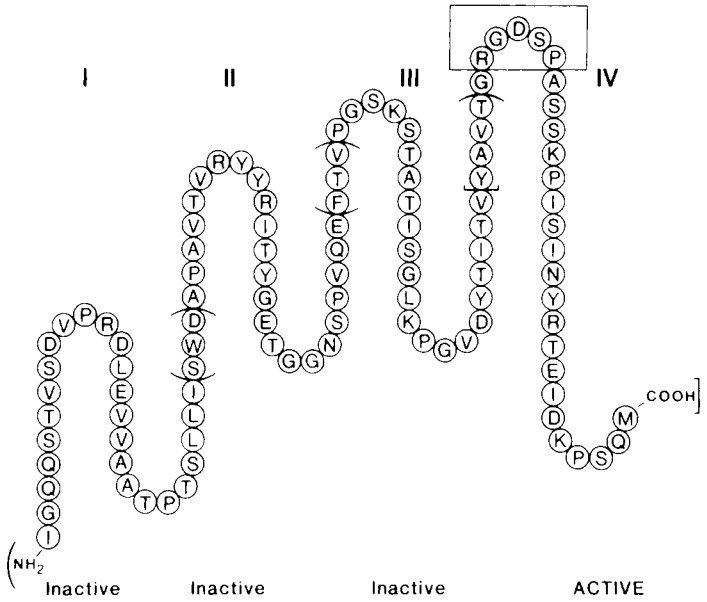

Fig. 3. The primary structure of the 11.5-kD peptic fragment of fibronectin. The secondary conformation depicted was predicted solely on the basis of the primary structure [58,59]. The peptide consists of a series of potential beta turns, the most hydrophilic of which (boxed) has been shown to contain the cell attachment site [60]. Peptides I-IV [45] (enclosed in brackets) are labeled active or inactive with respect to their ability to promote cell attachment. The one-letter code used to denote each amino acid is: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr.

CODE	SEQUENCE	CELL ATTACHMENT
FIBRONECTIN		
IV	Y A V T G R G D S P A S S K P I S I N Y R T E I D K P S Q M(C)	+ + + +
IVA	V T G R G D S P A S S K P I (C)	+ + +
IVB	S I N Y R T E I D K P S Q M(C)	-
IVA1	V T G R G D S P A(C)	+ + +
IVA2	S P A S S K P I S(C)	-
IVA1a	V T G R G D(C)	+ +
IVA1b	G R G D S(C)	+ + +
IVA1c	R G D S P A(C)	+ +
RVDS	R V D S P A(C)	-
TGRG	T G R G	-
RGDS	R G D S	+ +
GDSP	G D S P	-

Fig. 4. Peptides modeled after the cell attachment site of fibronectin were synthesized by Peninsula Laboratories (Belmont, CA) according to our specifications including, in most cases, a COOH-terminal cysteine. The peptides were assayed for their activity in promoting cell attachment as described in Methods. The tetrapeptides were coupled to Sepharose beads and the attachment of cells to these derivative beads was compared visually to beads carrying the active peptide IVA1b or fibronectin coupled in the same manner. The relative attachment to the active peptides is indicated by the number of pluses.

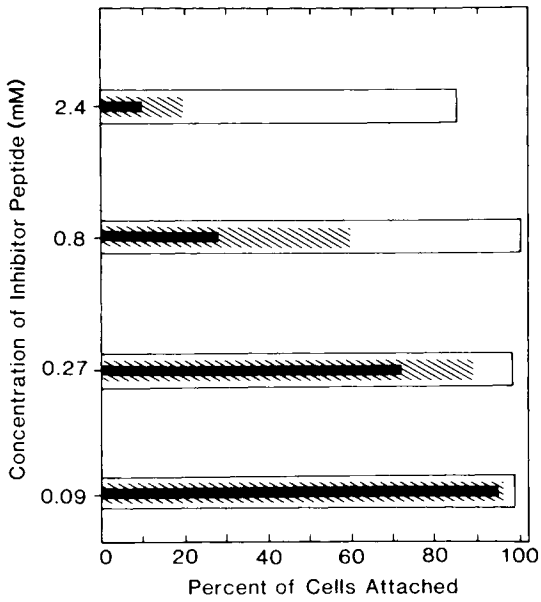

Fig. 5. The peptides Gly-Arg-Gly-Asp-Ser (solid bars), Arg-Gly-Asp-Ser (hatched bars) and Thr-Gly-Arg-Gly (open bars) were tested at the concentrations indicated for their ability to inhibit attachment of cells to plates coated with 5 μg/ml fibronectin as described in Methods.

The Cell Recognition Sequence in Other Proteins

A search through the library of protein sequences compiled by the National Biomedical Research Foundation (Georgetown University Medical Center, Washington, DC) revealed six proteins having the tetrapeptide (Table I) and a number of proteins having the Arg-Gly-Asp sequence [65].

Of the six proteins listed in Table I, fibrin(ogen) was of most immediate interest because of its demonstrated interaction with fibronectin [7] and the platelet surface [66,67]. A synthetic nonapeptide modeled after residues 568–580 of the α chain of fibrinogen was found to promote cell attachment (Fig. 6). We and others [68], however, have been unable to demonstrate the attachment of cells to intact fibrinogen or fibrin insolubilized on plastic.

It may be that the cell attachment site in fibrinogen is cryptic or destroyed during the assay procedures, or that the surrounding sequences in some way weaken the activity of this site so that it is not recognized by fibroblastic cells in the intact molecule. Platelets do, however, interact with fibrinogen [69,70] at more than one site within the molecule [71], and they also interact with fibronectin [72]. Recent work has shown that the Arg-Gly-Asp-Ser sequence is recognized in the fibronectin molecule by platelets [73]. Future work will show whether this sequence mediates the interaction of platelets with fibrinogen or fibrin.

We have also tested the activity of some peptides that resemble the Arg-Gly-Asp-Ser sequence and are present in proteins other than fibronectin. One such sequence, *Arg-Gly-Asp-Thr*-Gly-Ala-Thr-Gly-Arg (taken from type I collagen) [74], promotes cell attachment almost as well as the fibronectin peptides (Fig. 6); two other

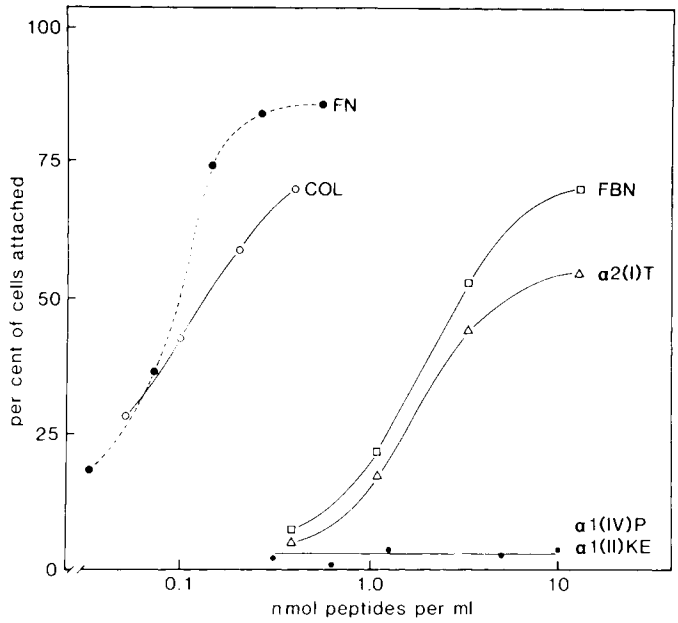

Fig. 6. Assays were done to detect attachment of NRK cells to various concentrations of immobilized synthetic peptides based on sequences in collagen and fibrinogen. The activity of fibronectin (FN) and type I collagen (COL) is included here for comparison. FBN is the synthetic fibrinogen peptide (Ser-Tyr-Asn-Arg-Gly-Asp-Ser-Thr-Phe), and T (Arg-Gly-Asp-*Thr*-Gly-Ala-Thr-Gly-Arg), P (Gly-Ser-Arg-Gly-Asp-*Hyp*-Gly-Thr-Hyp) and KE (*Lys*-Gly-*Glu*-Ser-Gly-Ser-Pro) come from collagen sequences [73–75]. The assay of attachment of cells to collagen was done in the presence of purified antifibronectin antibodies at a concentration that inhibited attachment to fibronectin to ensure that there would be no effect of newly synthesized fibronectin.

TABLE I. Proteins Other Than Fibronectin Containing the Cell Attachment-Promoting Sequence From Fibronectin

Sequence	Protein	Ref
Gly-Arg-Gly-Asp-Ser	α-Lytic protease—Myxobacter 495	[49]
Arg-Gly-Asp-Ser	Testis-specific basic protein—rat	[50]
Arg-Gly-Asp-Ser	Fibrinogen alpha chain—human	[51]
Gly-Arg-Gly-Asp-Ser	Lambda receptor protein—E coli	[52]
Gly-Arg-Gly-Asp-Ser	Coat and membrane polyprotein—Sindbis virus	[53]
Arg-Gly-Asp-Ser	VPI foot and mouth disease virus	[54]

sequences (taken from collagens of different genetic types [74-77]), Gln-Gly-*Ile-Arg-Gly-Asp-Lys*-Gly-Glu-Pro (not shown) and Gly-*Ser-Arg-Gly-Asp-Hyp*-Gly-Thr-Hyp (Fig. 6), when tested as synthetic peptides, had no cell attachment-promoting activity at all. Synthetic peptides modeled after sequences found in thrombin and an additional sequence present in some fibronectin molecules as a result of alternate RNA splicing [56] were also found to be active [65].

These results indicate that the serine residue in the Arg-Gly-Asp-Ser sequence is not essential for the activity. That the sequences flanking the Arg-Gly-Asp residues affect the cell attachment activity is illustrated by the fact that the Gly-Arg-Gly-Asp-Ser-Pro peptide inhibits cell attachment to fibronectin better than the Arg-Gly-Asp-Ser peptide [60]. Moreover, another sequence taken from collagen [72], Lys-Gly-Glu-Ser-Gly-Ser-Pro (Fig. 6), as a synthetic peptide had no cell attachment-promoting activity (data not shown), indicating that a mere conservation of the charge (Lys-Gly-Glu versus Arg-Gly-Asp) does not maintain the activity.

DISCUSSION

Our data show that the short amino acid sequence Arg-Gly-Asp-(Ser) in fibronectin is responsible for the binding of cells to fibronectin and that each of these amino acids contributes to the binding to a greater or lesser extent. That this sequence is common to a few other proteins that may interact with cells implies that cells may use a single mechanism (receptor?) for interacting with several proteins, although no structural basis for the surprising activity of such a small peptide is readily apparent at present. By means of the synthetic peptides, however, a cell surface receptor for fibronectin has been identified [78].

The tetrapeptide is found in a segment of fibronectin that would be very likely to form a hydrophilic loop at the surface of the molecule [58,59] and would thus be available to interact with cells. It is clear that binding is not due solely to any one residue since at least the arginine, glycine, and aspartate residues of the tetrapeptide are clearly important [60,65].

The data we have obtained by selective substitution within the recognition sequence [60,65] reveal some interesting possibilities about modification of the fibronectin activity in cells and tissues. Based on these results, one can infer that a single point mutation in the fibronectin gene could destroy the activity of the recognition sequence. Duplication of this sequence could create additional cell attachment sites. This raises the possibility that different fibronectins exist that are more effective or ineffective cell attachment promoters.

We have also discovered that the Arg-Gly-Asp-X sequence (where X is a residue compatible with activity) is present in some other proteins that may interact with cells, including fibrinogen, thrombin, a surface protein of Escherichia coli, and discoidin I from D discoideum [65].

These results raise the intriguing question of whether some prokaryotes and viruses use the fibronectin recognition sequence. The λ phage receptors on the surface of E coli and Sindbis virus coat protein share a five-amino acid sequence with the cell attachment region of fibronectin (Table I). It may be that some microorganisms use this sequence to interact with membranes of eukaryotic cells, and that the λ bacteriophage imitates the eukaryotic cell to gain entrance into E coli. Our recent finding that normal rat kidney cells bind to λ receptor purified from E coli [79] confirms the possibility that some proteins other than fibronectin that have the Arg-Gly-Asp sequence can also have the capacity to interact with the cell surface fibronectin receptor.

The possibilities for future study discussed above are, at present, speculative. These possibilities, however, can be tested by using the peptides that inhibit cell attachment to fibronectin and, therefore, are also likely to interfere with other

interactions that use this same recognition sequence. The peptides also provide us with the ability to inhibit the cell attachment function of fibronectin, making it possible to study this function independently of the other functions of the protein in a variety of systems such as differentiation and cell motility, and in models of tumorigenesis and metastasis, all of which appear to be influenced by fibronectin [17–19].

As research tools, the cell attachment peptides have been quite useful in the identification of the cell suface structure (receptor) that interacts with fibronectin [78]. It will be important to elucidate the nature of this receptor, because it could, for example, shed light on the abnormalities of cell adhesion that accompany malignant transformation. The peptides can also be used in experiments that will clarify the physiologic functions of fibronectin. The ability to inhibit the attachment of cells to fibronectin will make it possible to selectively prevent the cell attachment function of fibronectin, to see how this would affect a given experimental model.

Practical applications such as the preparation of surfaces for optimal cell culture and derivation of various prosthetic materials to promote bonding with surrounding tissues can also be envisioned. In other cases, it may be advantageous to interfere with cell attachment, eg to prevent the formation of adhesions in tissue. The soluble peptides that prevent attachment of cells to fibronectin will be useful in such situations.

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