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Variants of the cell recognition site of fibronectin that retain attachment-promoting activity

(cell adhesion/extracellular matrices/collagen/synthetic peptides)

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ABSTRACT **A tetrapeptide sequence, Arg-Gly-Asp-Ser, is the minimal structure recognized by cells in the large, adhesive glycoprotein fibronectin. We now have defined the structural requirements for this cell recognition site by testing several synthetic variants of the active tetrapeptide sequence. The conservative substitutions of lysine for arginine, alanine for glycine, or glutamic acid for aspartic acid each resulted in abrogation of the cell attachment-promoting activity characteristic of the natural sequence. However, in the position of the serine residue, some alterations were compatible with activity. Assay of peptides containing the structure Arg-Gly-Asp-X (where X = another amino acid residue) showed that an Arg-Gly-Asp-Val sequence predicted to be present in some, but not all, fibronectin molecules as a result of alternative RNA splicings could potentially create a second cell attachment site in those fibronectin polypeptide chains carrying that sequence. Other proteins with potentially active Arg-Gly-Asp-X sequences include several proteins that are known to interact with the cell surface. Among these are various types of collagens, thrombin, and discoidin, a slime-mold protein that may be involved in cell aggregation. The results presented here show that the arginine, glycine, and aspartic acid residues are absolutely required for the cell recognition, and that the surrounding amino acids may play a role in the expression of cell attachment activity in fibronectin and other proteins having this sequence. We suggest, based on these data, that this recognition mechanism may be common to a number of biological systems.**

Adhesion of cells is studied intensively because it is thought to be of prime importance for the control of cellular functions such as replication, motility, and differentiation. In recent years, rapid progress has been made in the analysis of the proteins that mediate the adhesion of eukaryotic cells to their extracellular matrices. Fibronectin has emerged as a prototype of such proteins.

Fibronectin interacts with other extracellular matrix macromolecules (1–3; for review, see ref. 4) and with a receptor(s) of an unknown nature at the surface of most eukaryotic cells. The interaction of fibronectin with cells results in the attachment and spreading of those cells on a surface covered with fibronectin (5–8; reviewed in ref. 4). We have determined the complete primary structure of the site in the fibronectin molecule that interacts with cell surfaces (9, 10) and have utilized this knowledge to synthesize peptides that reproduce the adhesive properties of fibronectin (11). Analysis of small synthetic peptides has shown that the recognition site for cells in fibronectin is carried by the sequence Arg-Gly-Asp-Ser (12). Peptides that contain this sequence promote cell attachment when insolubilized on a surface, and they also inhibit the attachment of cells to fibronectin when they are present in a soluble form (12). The Arg-Gly-Asp-Ser sequence is present in at least five other proteins, and at least one of these, the λ phage receptor protein of *Escherichia coli*, which has a Gly-Arg-Gly-Asp-Ser sequence in common with fibronectin, has cell attachment activity similar to that of fibronectin (unpublished data).

To define precisely the structural requirements for the recognition site, we assayed the cell attachment-promoting activities of a number of peptides with structures closely resembling the Arg-Gly-Asp-Ser peptide. We show here that the arginine, glycine, and aspartate residues cannot be replaced even with closely related amino acids, but that several amino acids can replace serine without loss of activity. The permutations in this fourth position add several proteins onto the list of proteins potentially capable of interacting with cell surfaces. The fact that some of these proteins are already known to interact with cell membranes may shed light on the nature of this interaction.

MATERIALS AND METHODS

Proteins and Peptides. Human fibronectin was isolated from freshly drawn plasma by using gelatin-Sepharose chromatography as described (1). Peptides were synthesized according to our specifications at Peninsula Laboratories (San Carlos, CA). When indicated, the peptides had 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.

Cell Attachment Assays. Normal rat kidney cells (designated NRK cells) (13) were used for the assays. The peptides were tested for cell attachment-promoting activity by coupling them to microtiter wells through a protein coating as described (11, 12). Inhibition assays were carried out by including the soluble peptide in an assay where cells were attaching to microtiter wells coated with fibronectin by using a slight modification of the assay described earlier (12), since inhibition by the peptides was found to be dependent upon cell number and time of incubation. Briefly, NRK cells were released from nearly confluent cultures with trypsin (Sigma), washed with a solution containing soybean trypsin inhibitor (Sigma), and plated at a density of 8×10^3 cells per well in fibronectin-coated wells (2 μg/ml) already containing peptide. After 30 min, nonbound cells were washed away, and attached cells were fixed, stained, and counted. Incubation media for these experiments consisted of Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with glutamine/penicillin/streptomycin at 2 mM, 100 units, and 100 μg/ml, respectively (Irvine Scientific) and bovine serum albumin at 2 mg/ml (Sigma).

Sequence Search. Computer searches through published protein sequences were conducted by the National Biomedical Research Foundation (Georgetown University, Washington, DC) using the program SEARCH.

RESULTS

Two assays were used to study the contribution of each of the amino acids in the Arg-Gly-Asp-Ser sequence toward the

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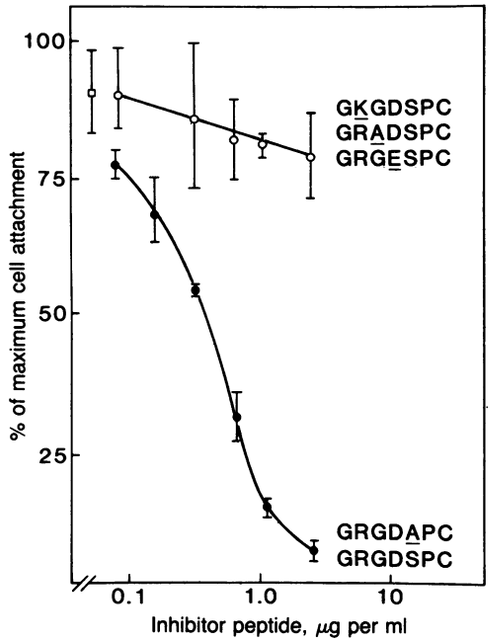

FIG. 1. Inhibition of attachment of NRK cells to immobilized fibronectin by synthetic fibronectin peptides. Microtiter wells were coated with human plasma fibronectin, and the attachment of NRK cells was assessed after incubation in the presence of various concentrations of soluble peptides. The attached cells were fixed, stained, and counted using an Artek cell counter. Data are represented as the mean of three experiments (± the range). Maximum attachment was about 50% of the cells plated.

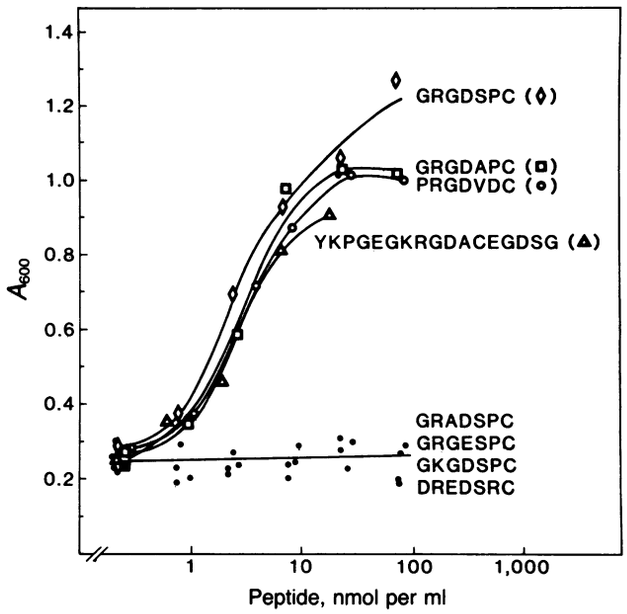

FIG. 2. Attachment of NRK cells to an immobilized synthetic fibronectin peptide and its variants. The synthetic peptides were assayed for their ability to promote the attachment of NRK cells by first attaching the peptides through the heterobifunctional crosslinker *N*-succinimidyl 3-[pyridyldithio]propionate (Sigma) to rabbit IgG that had been immobilized on polystyrene. The attachment assay was then carried out as described (14) with freshly trypsinized NRK cells. After a 1-hr incubation at 37°C, those cells that had attached were fixed, stained, and quantitated by using a vertical pathway spectrophotometer (Flow Laboratories). Maximum attachment was about 80–90% of the cells plated.

cell attachment-promoting activity. First, a set of five peptides, each differing by only one amino acid, were compared in terms of their capacity to inhibit attachment of cells to a substrate coated with intact fibronectin. In addition, the ability of each of the synthetic peptides described here to interact with cells was determined directly by using a cell attachment assay as described (11, 12). The results of these two experiments are shown in Figs. 1 and 2, respectively, and are summarized in Tables 1 and 2.

The two assays gave concordant results. Regarding the glycine in the active sequence, we previously had shown that substitution of this amino acid with the bulky valine residue abrogates the cell attachment activity (12). Results in Figs. 1 and 2 show that the introduction into this position of alanine, which has a relatively small side chain, or of glutamic acid also produces an inactive peptide. Similarly, either of the conservative substitutions of the charged amino acids arginine and aspartic acid with lysine and glutamic acid, respectively, resulted in a peptide that had no detectable cell attachment-promoting activity.

Earlier results also suggested that at least some variation in the position occupied by serine is compatible with activity because, when this residue was replaced with the closely related cysteine residue, full activity was retained. Therefore, we tested a number of peptides with the structure Arg-Gly-Asp-X, in which X stands for another amino acid residue. These peptides were designed either after sequences in other proteins that are capable of interacting with cells (see below), such as collagen and thrombin, or after sequences present elsewhere in the fibronectin molecule (see Table 2). The results presented in Fig. 2 (and compiled in Tables 1 and 2) show that, in addition to the previously reported cysteine, at least threonine, alanine, and valine can occupy this position with retention of activity. In contrast, using two collagen peptides in which lysine or hydroxyproline substitute for the serine, no activity could be demonstrated.

DISCUSSION

Each of the three amino acids in the sequence Arg-Gly-Asp appears to be integral to the site recognized by cells through the cell-surface structures (receptors) that bind fibronectin. This conclusion is based on the complete lack of activity that was observed with synthetic peptides in which substitutions were made by using the most closely related amino acid (lysine for arginine, alanine for glycine, and glutamic acid for aspartic acid) to replace one of the original three. Since these conservative substitutions give inactive peptides, it seems reasonable to conclude that other, less closely related amino acids would also render the sequence inactive, as is the case when asparagine replaces aspartic acid in the sequence (12) or when valine (12) or glutamic acid are substituted for the glycine residue.

The role played by the amino acids flanking the Arg-Gly-Asp sequence is less clear. We have shown earlier that the glycine residue, which in fibronectin is on the NH_2-terminal side of the arginine residue, imparts improved activity to those peptides containing it (12). On the other hand, fibrinogen, which has the Arg-Gly-Asp-Ser sequence in common with fibronectin, is not active in promoting the attachment of NRK cells, even though a nonapeptide modeled after the

Table 1. Activity of structural analogues of the cell attachment-recognition sequence of fibronectin in the cell attachment assays

Peptide	Interaction with cells
Gly-Arg-Gly-Asp-Ser-Pro-Cys	Active
Gly-Lys-Gly-Asp-Ser-Pro-Cys	Inactive
Gly-Arg-Ala-Asp-Ser-Pro-Cys	Inactive
Gly-Arg-Gly-Glu-Ser-Pro-Cys	Inactive
Gly-Arg-Gly-Asp-Ala-Pro-Cys	Active

Table 2. Proteins containing potentially active cell attachment sequences

Sequence*	Protein	Ref.
RGDS	α-Lytic protease, *Myxobacter* 495	16
	Testis-specific basic protein, rat	17
	Fibrinogen α chain, human	18
	λ receptor protein, *E. coli*	19
	Coat and membrane polyprotein, Sindbis virus	20
	Viral protein I, foot-and-mouth disease virus	21
RGDA	Collagen α1(I), bovine and human	22
	Thrombin, bovine and human	23
	Discoidin I, A chain, *Dictyostelium discoidium*	24
	Vitellogenin I precursor, fruit fly	25, 26
	3-Hydroxyacyl-CoA dehydrogenase, pig	27
	Collagen α2(I), chicken	22
RGDT	Collagen α2(I), human	28
	P1 protein, human influenza A virus (two strains)	29, 30

*The one-letter amino acid code (15) is used.

fibrinogen amino acid sequence around the Arg-Gly-Asp-Ser tetrapeptide in fibrinogen is active (12). These observations suggest that the surrounding sequences in an intact protein may either enhance or suppress the activity of the cell attachment sequence. Possible mechanisms for such modulation of activity include folding of the tetrapeptide in such a way as to make the Arg-Gly-Asp sequence more (or less) available for the cell-surface receptor or to cause steric hindrance of this interaction by a nearby amino acid. Both of these mechanisms may contribute to the effects we have observed when replacing the serine residue with other amino acids.

Several amino acids can occupy the position of serine in the fibronectin sequence without substantially changing the activity of the resulting peptide as long as the Arg-Gly-Asp sequence remains intact. We have so far tested six substitutions and found that the ones with threonine, alanine, valine, and cysteine are active. Two peptides, each modeled after sequences occurring in collagen chains (28, 31, 32) and containing a lysine or hydroxyproline residue at the serine position, were inactive, suggesting that there are restrictions concerning which amino acids can occupy this position. The latter peptides differ from the active peptides with regard to several other amino acids surrounding the Arg-Gly-Asp sequence. However, since all of the Arg-Gly-Asp-Ser-containing peptides that we have tested have been active in promoting cell attachment regardless of surrounding sequences, it is likely that it is the substitution of serine that prevents the expression of this activity in these peptides. A nonapeptide from thrombin (33) that contains the sequence Arg-Gly-Asp-Ala was active, as would be predicted from the results described above for the modified fibronectin peptides, suggesting that surrounding sequence can be significantly altered with retention of activity, at least within the peptides.

The new cell attachment-promoting peptide sequences described here raise several intriguing possibilities regarding the cell attachment-promoting activity in fibronectin variants and other proteins. The sequence Pro-Arg-Gly-Asp-Val-Asp, which is predicted to occur in some fibronectin polypeptides but not in others as a result of alternative RNA splicings (34), is active as a peptide, suggesting that some fibronectin polypeptides may possess an additional cell attachment site. At this point our results should be interpreted with caution, since we have not yet shown that this sequence is active when integrated into the protein. However, some of the differences that have been observed between the biological activities of fibronectins from different sources such as plasma and cultured cells (4, 35–37) could relate to the presence of a different number of cell attachment sites. On the other hand, the sequence from the extreme COOH terminus of the fibronectin molecule, Asp-Arg-Glu-Asp-Ser-Arg-, had, as was predicted from earlier results (12), no activity.

Various collagen chains contain Arg-Gly-Asp-X sequences (28, 31, 32), and in some of these, the X position is occupied by a residue that we have shown to be compatible with cell attachment activity. The presence of the potentially active sequences in collagens is of particular interest for two reasons. First, collagens have been shown to mediate cell attachment independent of fibronectin (38–40); second, variations of the tetrapeptide sequence are especially abundant in collagens. Such sequences are repeated at least three times along the α2 chain of type I collagen, and several copies are present in other collagen types that have been sequenced (see ref. 22). These observations suggest that the Arg-Gly-Asp sequence may be involved in the cell attachment-promoting activity of collagen. However, as yet we have no direct proof of this, and it may well be that the rigid triple helical structure of native collagen suppresses the activity of the Arg-Gly-Asp sequences. Cell lines have been described that attach to fibronectin but not to type I collagen (7, 41–43), suggesting also that other mechanisms may be involved in the attachment of cells to collagen. In any event,

Table 3. Selected list of sequences homologous to the cell attachment site of fibronectin

Peptides	Sequence*	Ref.	Interaction with cells
Fibronectin			
Cell attachment site, all fibronectins	A-V-T-G-R-G-D-S - P-A-S - S-K	3	Active†
Second cell attachment site (?), variant rat fibronectin	G-H-V-P-R-G-D-V - D-Y-H - L-Y	34	Active†
Phosphorylation site, bovine fibronectin	V-Q-A-D-R-E-D-S - R-E	44	Inactive
Collagen			
α2(I)	A-P-G-L-R-G-D-T - G-A-T - G-R	22	Active
α2(I)	P-Q-G-I-R-G-D-K - G-E-P - G-E	22	Inactive
α1(IV)	D-X-G-S-R-G-D-P‡-G-T-P‡-G-V	31	Inactive
Thrombin	G-E-G-K-R-G-D-A - C-E-G - D-S	33	Active†

*The one-letter amino acid code (15) is used. Sequences flanking the actual synthetic peptides tested have been included for comparison.
†The actual synthetic peptide tested and the results obtained with that peptide are shown in Fig. 2.
‡4-Hydroxyproline.

our results point to new ways of exploring the interaction of cells with collagens.

A variety of other proteins contain the Arg-Gly-Asp sequence and, therefore, could have the same capacity as fibronectin to interact with cell surfaces. A selected list of such proteins is presented in Table 3. We have found that one protein that we studied because it possesses this sequence, the λ phage receptor of *E. coli*, actively promotes the attachment of NRK cells (unpublished data). This confirms the possibility that some of the other proteins on this list could be active. A particularly interesting candidate is discoidin Ia (24, 45, 46), a slime-mold protein believed to be involved in cellular aggregation.

The nature of the cellular receptor for fibronectin is unknown. If indeed the fibronectin–cell interaction is a manifestation of a widely distributed recognition mechanism, the critical aspects of the structure that interacts with the Arg-Gly-Asp sequence also must be conserved. Thrombin, which has an Arg-Gly-Asp-Ala sequence, binds to many types of cells by interacting with apparent cell-surface receptors (47). The Arg-Gly-Asp-Ala sequence is near the catalytic serine in thrombin and could be involved in cell-surface binding (47). Perhaps these receptors are related to the fibronectin receptor, which has proven elusive in spite of much study devoted to its identification.

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