Journal of Cellular Biochemistry 28:99–104 (1985)
Extracellular Matrix: Structure and Function 219–224

Amino Acid Sequence Specificities of an Adhesive Recognition Signal

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Synthetic peptides derived from the cell-binding domain of fibronectin have previously been found to inhibit fibronectin-mediated adhesion in vitro competitively and reversibly, as well as inhibiting cell migratory events in vivo. The amino acid sequence specificity required for this inhibitory activity has been examined further using variations of the originally identified active peptide sequences. The most active small peptide was found to be the pentapeptide Gly-Arg-Gly-Asp-Ser. Although the tetrapeptide Arg-Gly-Asp-Ser was found to retain substantial activity, it was approximately threefold less active. An "inverted" peptide sequence with these same four amino acids arranged in the mirror symmetrical sequence Ser-Asp-Gly-Arg was found to be nearly as active as the forward sequence. However, the same inverted tetrapeptide sequence embedded in a synthetic decapeptide derived from a sequence of histocompatibility antigens has minimal activity, suggesting the importance of adjacent sequences in modifying the activity of such peptides. Neither substitution of amino acids of the same charge nor reversal of the positions of the two charged amino acids retains biological activity. Decreasing the spacing between the charged residues also causes a loss of activity. Our results suggest the hypothesis that this adhesive recognition signal consists of a specific arrangement of one acidic and one basic charged group and additional information provided by adjacent amino acids.

Key words: fibronectin, cell adhesion, synthetic peptides, fibroblasts

The multifunctional glycoprotein fibronectin has provided a valuable system for the analysis of cell adhesion [see accompanying review article, Yamada et al, this issue]. Synthetic peptides from the "cell binding" or "cell recognition" region of fibronectin can mimic the activity of intact fibronectin in cell attachment and in competitive inhibition of fibronectin-mediated adhesion if they are present in excess in solution [1–4]. We have examined the requirements for inhibition of cell adhesion to fibronectin in hopes of defining this recognition signal. Our results suggest that the recognition signal consists of a specific spacing of two charged groups in a peptide sequence.

MATERIALS AND METHODS

Synthetic peptides were synthesized to our specifications by Peninsula Laboratories (Belmont, CA), Vega (Tucson, AZ), and Biosearch (San Rafael, CA). All peptides were further purified and characterized as described previously [2]. The

Received December 5, 1984; accepted January 10, 1985.

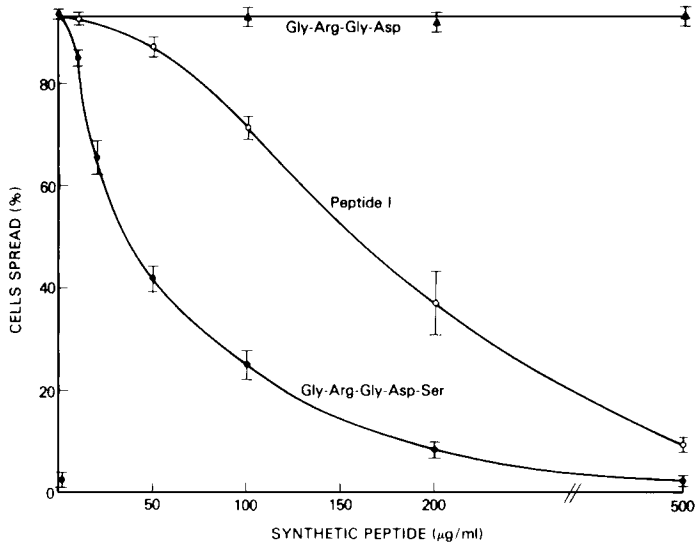

Fig. 1. Inhibition of BHK cell spreading by synthetic peptides derived from the fibronectin cell-binding domain. The percentage of BHK cells capable of spreading during 45 min incubation on a substrate coated with human plasma fibronectin at 3 μg/ml is indicated for varying concentrations of three purified synthetic peptides added to the incubation medium. Peptide I, Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro. Gly-Arg-Gly-Asp is a tetrapeptide lacking the COOH-terminal serine. The point at the lower left of the graph indicates the extent of background spreading in the absence of fibronectin. Points indicate mean ± standard error of five fields of 100 cells, for a total of 500 cells.

sequence of the peptide Ser-Asp-Gly-Arg (Peninsula Laboratories) was confirmed by amino acid sequencing by Sequemat, Inc. (Watertown, MA). Biological activity as quantitated by inhibition of baby hamster kidney (BHK) cell attachment and spreading was determined exactly as described previously [2].

RESULTS

As described previously [2,4], the decapeptide Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro can mimic the activity of fibronectin and its cell-binding peptide in competitive inhibition of cell adhesion to fibronectin (Fig. 1). In the same assay, we compared the activity of the pentapeptide sequence Gly-Arg-Gly-Asp-Ser, since previous results indicated that these amino acids were part of a hexapeptide sequence that retained maximal activity in synthetic peptides from this region [compare 1,2, 4]. This new peptide displays high activity (Fig. 1), and it appears to be as active as the previously described hexa- and heptapeptide sequences. Half-maximal inhibition in this assay occurred at 38 μg/ml (7.8×10^{-5} M), which is only fourfold less active than intact fibronectin (2×10^{-5} M). An identical synthetic peptide differing only by the absence of the serine residue (Gly-Arg-Gly-Asp) has no detectable biological activity, suggesting that the presence of this COOH-terminal residue is crucial for activity (Fig. 1).

Since Pierschbacher and Ruoslahti [1] had reported that the tetrapeptide sequence lacking the NH_2-terminal glycine residue was the minimal sequence required for activity in other biological assays using normal rat kidney (NRK) cells, we tested

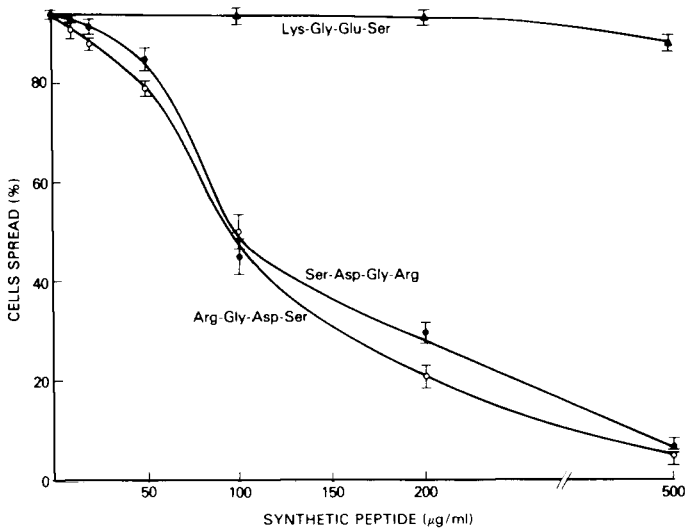

Fig. 2. Inhibition of BHK cell spreading by synthetic tetrapeptide variants. Percent spreading with 3 μg/ml fibronectin is indicated. Arg-Gly-Asp-Ser is based on the amino acid sequence of the cell-binding domain. Ser-Asp-Gly-Arg includes a reversal of the amino- to carboxy-terminal sequence. Lys-Gly-Glu-Ser contains substitutions at the charged amino acids. Points indicate mean ± standard error; N = five fields, for a total of 500 cells scored.

TABLE I. Activities of Synthetic Peptides

Peptides	Concentrations required for half-maximal inhibition	
	μg/ml	M
Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro	163	1.6×10^{-4}
Gly-Arg-Gly-Asp-Ser	38	7.8×10^{-5}
Gly-Arg-Gly-Asp	>>500	$>>1 \times 10^{-3}$
Arg-Gly-Asp-Ser	97	2.2×10^{-4}
Ser-Asp-Gly-Arg	101	2.3×10^{-4}
Lys-Gly-Glu-Ser	>>500	$>>1 \times 10^{-3}$
Gly-Arg-Gly-Glu-Ser	>>500	$>>1 \times 10^{-3}$
Gly-Asp-Gly-Arg-Ser	>500	$>1 \times 10^{-3}$
Asp-Val-Gly-Ser-Asp-Gly-Arg-Phe-Leu-Arg	>1,000	$>1 \times 10^{-3}$

this sequence in our assay and confirmed its retention of biological activity (Fig. 2). In direct comparison with the pentapeptide sequence described above, however, it was found to be three- to four fold less active than the pentapeptide (eg, half-maximal inhibition at 97 μg/ml or 2.2×10^{-4} M in Fig. 2). Remarkably, a synthetic peptide containing the same four amino acids arranged in the reverse, mirror-symmetric order, ie, Ser-Asp-Gly-Arg, displayed activity nearly identical to the original tetrapeptide sequence (Fig. 2; Table I). However, substituting lysine for arginine and glutamic for aspartic acid leads to a drastic loss of activity, indicating the importance of these specific amino acids (Fig. 2).

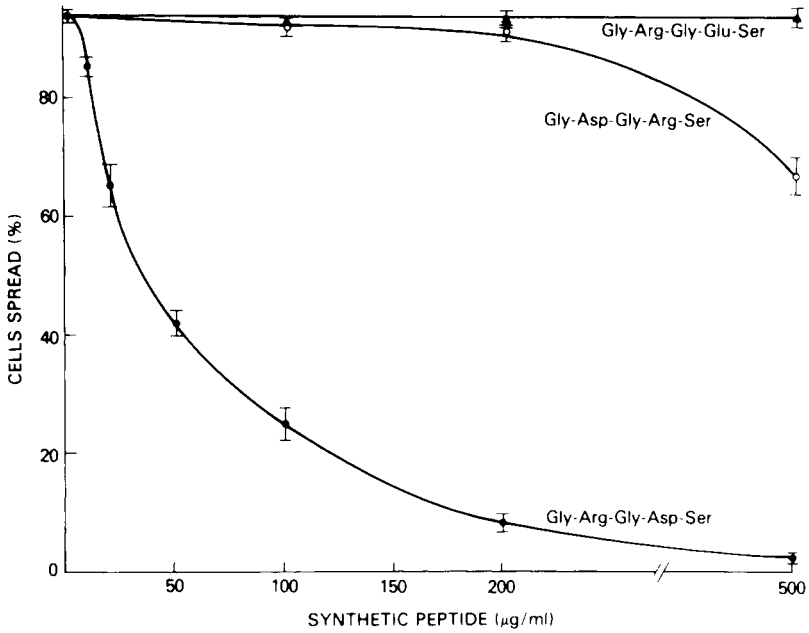

Fig. 3. Inhibition of BHK cell spreading by modified synthetic pentapeptides. Percent spreading with 3 μg/ml fibronectin is indicated. The highly active pentapeptide Gly-Arg-GLy-Asp-Ser is from the cell-binding domain sequence. Gly-Asp-Gly-Arg-Ser includes a reversal of the positions of the two charged amino acids. Gly-Arg-Gly-Glu-Ser includes a substitution of a glutamic acid residue for the aspartic acid. Points indicate mean ± standard error; 500 cells were scored.

To evaluate the contribution of adjacent sequences to activity, the synthetic peptide Asp-Val-Gly-Ser-Asp-Gly-Arg-Phe-Leu-Arg derived from a peptide sequence present in HLA and H-2 molecules [5] that contains the same inverted tetrapeptide sequence Ser-Asp-Gly-Arg was examined. This peptide showed minimal inhibitory activity (Table I; 500 μg/ml of this peptide showed 83.8 ± 2.2% cells spread compared to 90.0 ± 1.6% in controls on fibronectin without inhibitors; N = 5).

Further experiments were based on variations of the pentapeptide sequence, which was the most active small peptide. Substitution of a glutamic acid residue for the aspartic acid caused a loss of activity (Fig. 3; Table I). Even retention of the two charged amino acids, but reversing their positions without altering the location of the COOH-terminal serine residue, resulted in a substantial, though not complete, loss of activity (Fig. 3; Table I). Reversal of the third and fourth amino acids so that the aspartic acid was immediately adjacent to the arginine in the peptide Gly-Arg-Asp-Gly-Ser also resulted in a loss of activity (Table I; at 500 μg/ml, this peptide showed 90.0 ± 1.7% cells spread compared to 90.0 ± 1.6% in controls; N = 5).

DISCUSSION

These studies are a direct extension of previous investigations of synthetic peptide mediators and inhibitors of fibronectin adhesive function [1-4] to the analysis of a series of new synthetic peptide sequences. Our results are summarized below, in

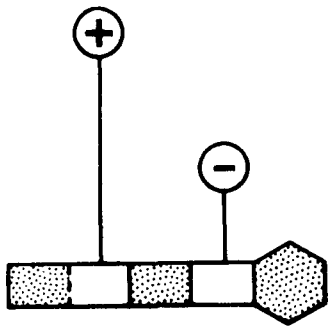

Fig. 4. Proposed possible characteristics of the fibronectin adhesive recognition signal. The recognition event appears to require one positive and one negative charge at a specific spatial orientation, flanked by modulating amino acid sequences that include a carboxy-terminal amino acid such as serine (hexagon).

Figure 11 of the accompanying review [Yamada et al, this issue] and here in Figure 4. The key findings appear to be that the location and type of charged amino acids are crucial and that they must be arranged in a specific configuration relative to each other and to adjacent amino acids. For example, substitution of glutamic acid for aspartic, or an additional substitution of lysine for arginine, causes a drastic loss of activity. Similar conclusions concerning these two amino acids have been reported by others very recently [3].

Surprisingly, there appears to be some flexibility in the tolerated order of the key amino acids, since a reversal of the order of the minimal sequence needed for activity still retains activity, ie, Ser-Asp-Gly-Arg is nearly as active as the normal sequence Arg-Gly-Asp-Ser. A strong modulation by adjacent amino acids is indicated by the substantial, though not complete, loss of activity if the two charged amino acids are reversed without altering the location of the serine residue in the peptide Gly-Asp-Gly-Arg-Ser, which is much less active than the original sequence Gly-Arg-Gly-Asp-Ser. Additional evidence for modulation by adjacent sequences is provided by a decapeptide derived from HLA and H-2 sequences, which contains the inverted tetrapeptide sequence but is inactive.

Finally, the requirement for spatial separation between the charged residues was examined with the peptide Gly-Arg-Asp-Gly-Ser, in which the order of the Arg and Gly residues was reversed to place the charged amino acids adjacent to one another. This rearranged peptide showed no activity, indicating that the spacing between the charged amino acids is crucial.

In a paper published after the completion of these studies, Pierschbacher and Ruoslahti [3] examined other aspects of the sequence specificity of this type of peptide. They found that each of the amino acids in the minimal tetrapeptide sequence is crucial for activity in the sense that substitution with certain other amino acids caused a loss of activity. Since certain variations in the sequence, especially in the COOH-terminal serine residue to match published sequences, still retain activity [3], they also suggest that this recognition sequence may be used by other adhesive systems.

What is the signal recognized by cells that is provided by these peptides? Figure 4 suggests a tentative model: Since the two charged amino acids appear to be crucial

and altering the length of the negative side group by only one carbon atom causes a loss of activity [3], the location of a single positive and a negative charge at specific distances from the peptide backbone may be crucial. Second, since the order in amino acids can be altered in only certain ways before activity is lost, it appears from our data that the two charges must be located a specific distance apart. Finally, adjacent amino acids appear to modulate activity, since the maintenance of the correct spacing is still not sufficient in the peptide in which the separation of the charged groups was maintained but another amino acid was present adjacent to the positive charge, and in another peptide in which the inverted, active tetrapeptide was embedded in a longer peptide sequence from another protein.

These results are therefore consistent with a simple model in which the specific spacing of one positive and one negative charge flanked by a certain amino acid may account for the activity of this recognition sequence, which can then be modulated by more distant sequences. A conceptually similar, but quite distinct, requirement for specific spatial relationships of charges and a hydrophobic residue has been described for the interaction of plasminogen with aminocarboxylic acid [6]. This model for the cell adhesion recognition signal can be tested further in the future by the use of a number of other possible variations in peptide sequence and comparisons with other possible active peptide sequences such as that recently implicated in slime mold morphogenesis [7].

ACKNOWLEDGMENTS

We thank Drs. Jean Paul Thiery and Charles Auffrey for valuable discussions concerning histocompatibility sequences.

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