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A Synthetic Peptide from the COOH-Terminal Heparin-binding Domain of Fibronectin Promotes Focal Adhesion Formation

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Cell adhesion to extracellular matrix molecules such as fibronectin involves complex transmembrane signaling processes. Attachment and spreading of primary fibroblasts can be promoted by interactions of cell surface integrins with RGD-containing fragments of fibronectin, but the further process of focal adhesion and stress fiber formation requires additional interactions. Heparin-binding fragments of fibronectin can provide this signal. The COOH-terminal heparin-binding domain of fibronectin contains five separate heparin-binding amino acid sequences. We show here that all five sequences, as synthetic peptides coupled to ovalbumin, can support cell attachment. Only three of these sequences can promote focal adhesion formation when presented as multicopy complexes, and only one of these (WQPPRARI) retains this activity as free peptide. The major activity of this peptide resides in the sequence PRARI. The biological response to this peptide and to the COOH-terminal fragment may be mediated through cell surface heparan sulfate proteoglycans because treatment of cells with heparinase II and III, or competition with heparin, reduces the response. Treatment with chondroitinase ABC or competition with chondroitin sulfate does not.

INTRODUCTION

Multidomain extracellular matrix molecules contain instructional information in discrete amino acid sequences within distinct domains. For example, the RGD sequence within the "cell-binding" domain (type III repeats 8–11) (Ruoslahti, 1988; Hynes, 1990) of fibronectin interacts with RGD-dependent integrins (e.g., $\alpha_5\beta_1$) at cell surfaces to promote attachment and spreading (reviewed in Ruoslahti, 1988; Yamada, 1989; Hynes, 1990). This interaction has been shown to promote the phosphorylation of two focal adhesion components: pp125^{FAK} and paxillin (Burridge *et al.*, 1992; Guan and Shalloway, 1992). However, these tyrosine phosphorylation events do not appear sufficient to promote focal adhesion and stress fiber formation (Woods *et al.*, 1986; Burridge *et al.*, 1992; Woods and Couchman, 1992a,b). A second "signal" may be needed for the further organization of internal cytoskeletal and membrane receptors into these structures (Izzard *et al.*, 1986; Woods *et al.*, 1986; Streeter and Rees, 1987; Burridge *et al.*, 1988, 1992; Woods and Couchman, 1988; Hynes, 1990). We previously showed that this reorganization can occur within 30 min of addition of low amounts of the 29-kDa amino- or 31-kDa COOH-terminal heparin-binding fibronectin fragment to cells prespread on substrates of 105-kDa cell-binding fragments or by coating the substrate with a mixture of these two types of fragment (Woods *et al.*, 1986). Reorganization did not require protein synthesis, did involve a contractile event, and possibly was due to activation of protein kinase C (Woods *et al.*, 1986; Woods and Couchman, 1992a,b). Previous studies implicated cell surface heparan sulfate proteoglycans (HSPG)[1] as the "receptors" that transduce

[1] Abbreviations used: FN-C/H, C-terminal heparin-binding fibronectin sequence; IRM, interference reflection microscopy; HSPG,

this signal (Couchman *et al.*, 1988; LeBaron *et al.*, 1988; Woods and Couchman, 1988), but the biologically active site(s) within the heparin-binding domains was not further investigated at that time.

Five heparin-binding amino acid sequences have been identified in the 31-kDa heparin-binding fibronectin domain. Substrates coated with these as synthetic peptides can support the attachment and spreading of melanoma, neural, and other cells (McCarthy *et al.*, 1988, 1990; Haugen *et al.*, 1990; Jalkanen and Jalkanen, 1992; Drake *et al.*, 1992; Iida *et al.*, 1992; Mooradian *et al.*, 1993). Furthermore, the binding of these peptides to cells is through cell surface proteoglycans (Jalkanen and Jalkanen, 1992; Drake *et al.*, 1992). Recent studies (Barkalow and Schwarzbauer, 1991) have confirmed that cationic sequences within the COOH-terminal domain of fibronectin are needed for its heparin-binding activity, but the active site for biological activity may have different, or enhanced, specificity of sequence to that needed for binding to heparin. We have tested here the ability of five peptides from within the COOH-terminal heparin-binding domain, which all bind heparin to a similar extent, to promote focal adhesion formation in cells prespread on substrates coated with the RGD-containing cell-binding fibronectin fragment. In addition, we have tested the effects of peptide CS1, which does not bind heparin but interacts with cells in an RGD-independent manner (Wayner *et al.*, 1989; Guan and Hynes, 1990; McCarthy *et al.*, 1990). We show that although most of these peptides have minor effects on cell behavior, one heparin-binding sequence (WQPPRARI) is able to promote focal adhesion formation in the majority of cells. Furthermore, we show that the major activity is through the sequence PRARI and that this peptide may act through cell surface HSPG.

MATERIALS AND METHODS

Supplies and Antibodies

All general chemicals, polylysine, and polyarginine (both molecular weight 70–150 kDa) were from Sigma Chemical (St. Louis, MO). Coverslips, 24-well plates, and cultureware were from Fisher (Atlanta, GA). All antibodies were diluted in phosphate-buffered saline$^-$ (PBS$^-$) (8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4 per liter, pH 7.2). Rabbit polyclonal antibodies against talin were a kind gift from Dr. K. Burridge (University of North Carolina, Chapel Hill) and were used at 1:100 dilution. Monoclonal antibodies against integrin β_1 subunits (Adeza Biomedical, Sunnyvale, CA) or vinculin (Sigma Chemical) were diluted 1:5 and 1:50, respectively, and fluorescein isothiocyanate-conjugated goat anti-mouse F(ab')$_2$ and anti-rabbit F(ab')$_2$ (Cappel, Organon Teknika, West Chester, PA) were diluted 1:50. Rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) was diluted 1:100.

Cells and Assay Conditions

Human embryo fibroblasts were used in all experiments between the second to fifth passage from frozen stocks. They were routinely cultured in alpha minimum essential medium (MEM) (Mediatech, Washington, DC) containing 10% fetal calf serum (Intergen, Purchase, NY) and shown by Hoechst 33257 staining to be free of mycoplasma contamination. Details of experimental procedures have been previously reported (Couchman *et al.*, 1983; Woods *et al.*, 1986; Woods and Couchman, 1992a). Briefly, cells used for focal adhesion or attachment assays were treated with 25 μg/ml cycloheximide for 2 h, suspended by trypsin/EDTA, resuspended in MEM without serum (MEM$^-$) but containing 100 μg/ml soybean trypsin inhibitor, and centrifuged. They were resuspended in MEM$^-$ for focal adhesion assays or phosphate-buffered saline (PBS) (PBS$^-$ plus 1×10^{-3} M $CaCl_2$ and $MgCl_2$) containing 1 mg/ml bovine serum albumin and glucose for attachment assays and plated onto substrates coated with fibronectin or its fragments, or ovalbumin-conjugated (OA-) peptides. Cycloheximide was present throughout all assays to prevent endogenous fibronectin secretion. Cells were plated onto coated glass coverslips for analysis of spreading by phase contrast and interference reflection microscopy (IRM), localization of F-actin, talin, vinculin, and β_1 integrin subunits, and into coated wells for attachment studies. To test the effects of synthetic peptides in focal adhesion and stress fiber promotion, cells were allowed to spread for 2.5 h on coverslips coated with the 105-kDa cell-binding fragment of fibronectin before the addition of coded peptides for 30 min and fixation with 3% glutaraldehyde in MEM$^-$ for IRM or 3.5% freshly prepared paraformaldehyde in PBS for other analysis (Woods and Couchman, 1992a). To examine which proteoglycans were involved, cells were treated in suspension for 30 min before plating with 20 U/ml heparinase II (Sigma Chemical) and 0.2 U/ml heparinase III (Seikagaku America, Rockville, MD) in PBS$^-$ containing 10^{-5} M $CaCl_2$ and $MgCl_2$, 1 g/l glucose, or 0.3 U/ml chondroitinase ABC (ICN Biomedicals, Costa Mesa, CA) in CB buffer (5.12 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 4.104 g Na acetate, 1 g glucose, per liter, pH 7.2). Treated cells (50 μl) were directly added to 450 μl appropriate buffer diluted 1:1 with MEM$^-$, thus diluting the enzymes 1:10 for the rest of the assay. Heparin from porcine intestinal mucosa, low molecular weight (Sigma) or chondroitin sulfate A from bovine trachea (Sigma) were mixed with the active peptide 30 min before addition to cells, giving a final concentration of 100 μg/ml glycosaminoglycan. Cells were viewed on a Nikon Optiphot (Garden City, NY) microscope equipped with phase contrast, IRM, and epifluorescence optics. The percentage of cells having focal adhesions by IRM were scored as previously (Woods and Couchman, 1992a) in blind assays. Human embryonic fibroblasts either lack focal adhesions totally or form numerous large focal adhesions with a characteristic pattern by IRM or by labeling for talin or integrin β_1 subunits (Woods and Couchman, 1986, 1992a,b). Typically, ~10–15% of cells escape the cycloheximide block, produce endogenous fibronectin, and form the normal number and characteristic pattern of focal adhesions on substrates composed of the 105-kDa cell-binding fragment (Woods *et al.*, 1986). At least 500 cells were scored in each assay, using a grid system to prevent recounting the same cells and in lots of 100 to facilitate statistical analysis (Student's *t* test) of the difference of the means between treated and untreated cells. Attachment assays were performed in triplicate as previously described (Couchman *et al.*, 1983) using ^{35}S-methionine-labeled cells and monitoring the percentage of added cells that attached in 30 min.

Substrate Preparation

Glass coverslips (Fisher) were routinely coated by drying down 70 μl of MEM$^-$ containing 5 μg of 105-kDa fibronectin fragment at room temperature (Woods and Couchman, 1992a). In attachment assays, 24-well plates (Costar, Cambridge, MA) were coated by drying down 30 μg/well of synthetic peptide conjugated to ovalbumin (OA-peptide) or ovalbumin cross-linked to itself, 5 μg of 31-kDa heparin-binding bovine fibronectin fragment, or 5 μg of bovine fibronectin, all diluted in MEM$^-$. Details of preparation of these peptides and OA conjugates (McCarthy *et al.*, 1988; Haugen *et al.*, 1990; Drake *et al.*, 1992) and of bovine fibronectin and its fragments (Austria and Couchman, 1989; Woods and Couchman, 1992a,b) have been previously published.

heparan sulfate proteoglycans; MEM, minimum essential medium; OA-, ovalbumin conjugated.

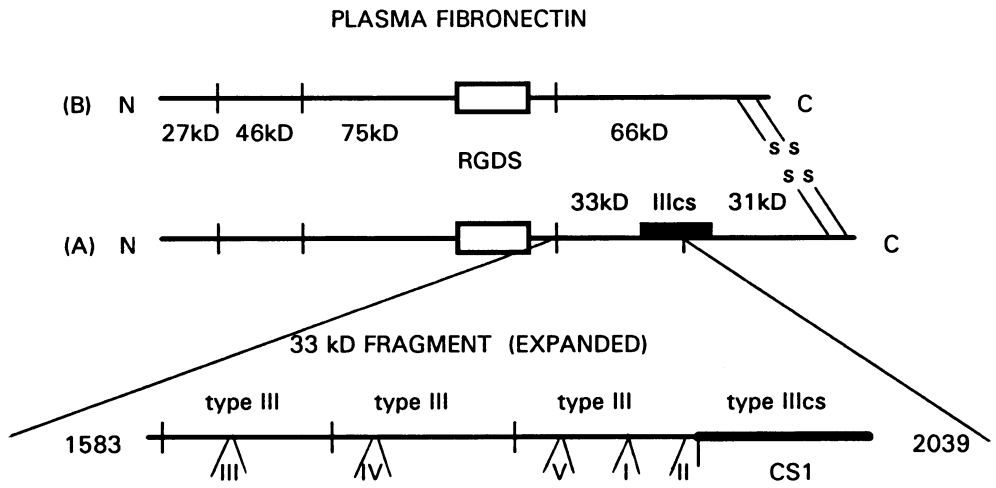

Figure 1. Diagram of the location within fibronectin of the peptides used in this study.

Coated substrates were rehydrated, blocked with bovine serum albumin, and washed as previously described. In some experiments, substrates coated with 105-kDa fragment and blocked with bovine serum albumin were then incubated for 30 min with 10 μg/ml OA-C-terminal heparin-binding fibronectin sequence (FN-C/H) V, 100 μg/ml free FN-C/H V, or 0.2 ng/ml 31-kDa heparin-binding fragment. These were washed 3 × 10 min with MEM^- before addition of cells. In other experiments, substrates were cocoated with 5 μg each of 105-kDa fragment and OA-FN-C/H V. The peptides and fragments used and their positions in the fibronectin dimer are shown in Figure 1, and the sequence of the synthetic peptides is given in Table 1.

RESULTS

When cells were plated onto substrates composed of the 105-kDa RGD-containing fibronectin fragment, they attached and spread, but only 11% of the population formed focal adhesions visible by IRM (Figures 2 and 3a) (Woods *et al.*, 1986; Woods and Couchman, 1992a). The majority of cells were adherent by close contacts (Izzard and Lochner, 1980) with a total lack of focal adhesions visible by IRM. Treatment with 0.2 ng/ml (~0.01 nM) soluble 31-kDa heparin-binding fibronectin fragment for 30 min increased the percentage of cells with focal adhesions to 76% (Figure 2), confirming previous results (Woods *et al.*, 1986). This occurred in the continuous absence of protein synthesis.

We next tested the effects on focal adhesion formation of adding soluble heparin-binding synthetic peptides to cells prespread on substrates of 105-kDa fragment. Focal adhesion formation was promoted by four of the five heparin-binding peptides, but of these, peptide FN-C/H V was by far the most potent (Figures 2 and 3b). OA-peptide FN-C/H V at 10 μg/ml (~0.2 μM peptide with

Table 1. Peptides used in assays

Code	Previous name	Sequence
26	FN-C/H I	YEKPGSPPREVVPRPRPGV
25	FN-C/H II	KNNQKSEPLIGRKKT
28	FN-C/H III	YRVRVTPKEKTGPMKE
30	FN-C/H IV	SPPRRARVT
19, 83	FN-C/H V	WQPPRARI
27	CS1	DELPQLVTLPHPNLHGPEILDVPST
84	—	WQPPR
85, 88	—	PRARI
89	—	RARI
90	—	ARI
94	—	RPQIPWAR

Some peptides have two codes because repeat syntheses were encoded differently for each set of experiments. Peptides FN-C/H I–V and CS1 were used either as free peptide or as ovalbumin conjugates.

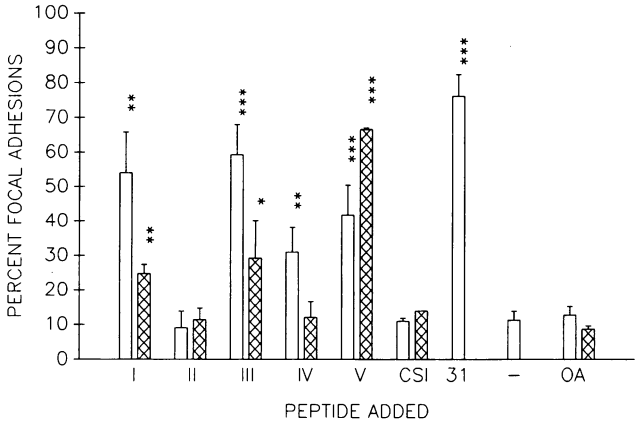

Figure 2. Effect of addition of soluble OA-peptides or 31-kDa fragment to cells prespread on substrates of 105-kDa fibronectin fragment. Open bars: 100 μg/ml OA-peptides FN-C/H I–V and CS1, and ovalbumin coupled to itself (OA); 0.2 ng/ml (≅0.01 nM) 31-kDa (31); or no addition (–). Hatched bars: 10 μg/ml OA-peptides FN-C/H I–V, CS1, and OA. SD are shown (n = 3–5). ***, **, and * denote significantly different from (–) at p = 0.001, 0.01, and 0.02, respectively. OA-FN-C/H V 10 μg/ml ≅ 0.2 μM.

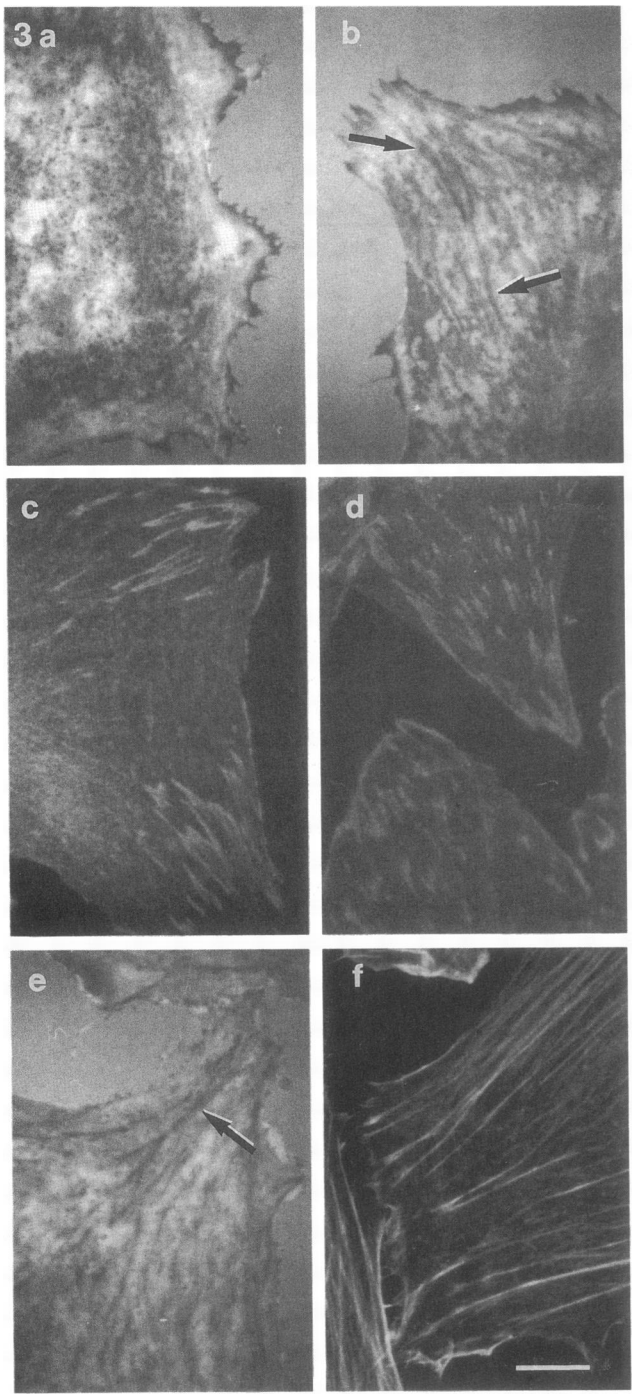

Figure 3. Effect of addition of soluble peptides to cells prespread on substrates of 105-kDa fragment. Cells lack focal adhesions by IRM (a) after addition of 10 μg/ml ovalbumin coupled to itself but form focal structures (arrows) visible by IRM (b) and labeling for talin (c) and integrin β_1 subunits (d) when 10 μg/ml ($\cong$0.2 μM) OA-peptide FN-C/H V is added. Cells form focal adhesions (arrows) visible by IRM (e) and stress fibers visible by rhodamine-phalloidin labeling (f) after addition of 100 μg/ml ($\cong$100 μM) free peptide FN-C/H V. Bar, 10 μm.

an average of 5 mol peptide/mol ovalbumin) gave 87% of the positive control response generated by 0.2 ng/ml 31-kDa heparin-binding fragment of fibronectin. At 100 μg/ml, peptide FN-C/H V caused cell rounding and a decrease in focal adhesion formation in comparison with 10 μg/ml, probably due to an exaggerated contraction response of the cells. This has been noted previously with excess doses of the whole 31-kDa fragment (Woods *et al.*, 1986). Peptide FN-C/H II and CS1 (which does not bind heparin) were without effect. The type of IRM image also differed with peptide. Peptides FN-C/H V and III appeared to induce the formation of large discrete focal adhesions characteristic of human embryonic fibroblasts (Woods *et al.*, 1986, 1992a,b), whereas those formed in response to the other peptides appeared smaller and thinner. The focal adhesions that formed in response to peptide FN-C/H V contained talin and integrin β_1 subunits (Figure 3, c and d), vinculin (not shown), and cellular F-actin was concomitantly reorganized into stress fibers (Figure 3f).

When peptides FN-C/H I–V and CS1 were tested by adding free peptides to cells prespread on 105-kDa fragment substrates, a marked specificity of response was noted with only peptide FN-C/H V promoting focal adhesion formation (Figures 3e and 4). Even when cells were incubated with concentrations of peptides FN-C/H I–IV and CS1 as high as 1 mg/ml, the percentage of cells possessing focal adhesions was not significantly different from that of untreated cells spread on substrates of 105-kDa fibronectin fragment (Figure 4). Free peptide FN-C/H V at 100 μg/ml (100 μM) exhibited activity that was 62% equivalent to 0.2 ng/ml of whole 31-kDa fibronectin fragment. The induction of focal adhesion formation by peptide FN-C/H V was not increased at 1 mg/ml but did show a dose response (Figure

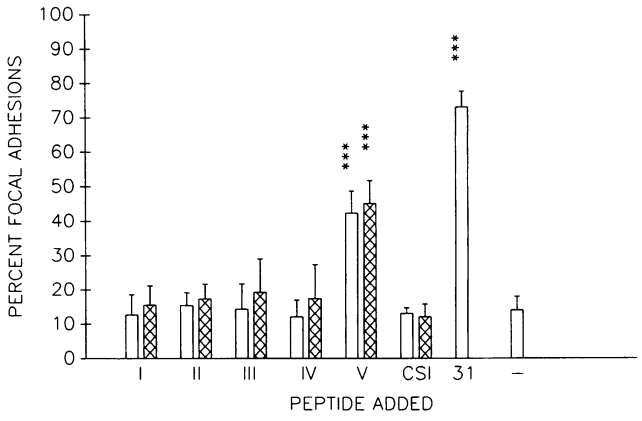

Figure 4. Promotion of focal adhesion formation in cells prespread on substrates of 105-kDa fragment by addition of soluble free peptides. One milligram per milliliter ($\cong$1 mM, open bars) or 100 μg/ml ($\cong$100 μM, hatched bars) peptide FN-C/H I–V and CS1 or 0.2 ng/ml ($\cong$0.01 nM) 31-kDa heparin-binding fragment were added. –, no addition. SD are shown (n = 3–21). ***, significantly different from –, p = 0.001.

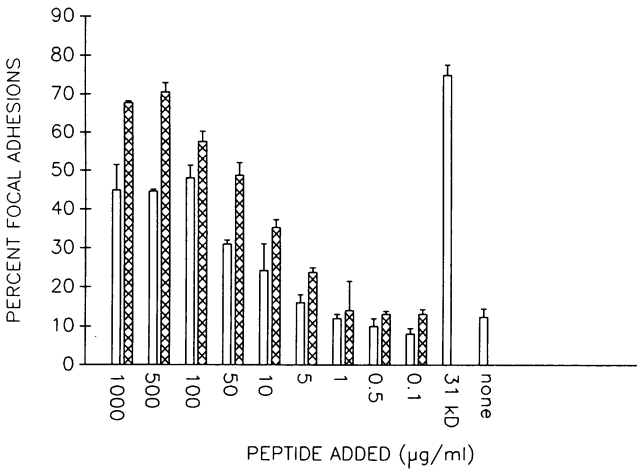

Figure 5. Dose response of focal adhesion induction. Soluble peptide FN-C/H V (WQPPRARI, open bars) or WQPPRARITGY (hatched bars) were added to cells prespread on substrates of 105-kDa fragment. SD are shown (n = 4–21).

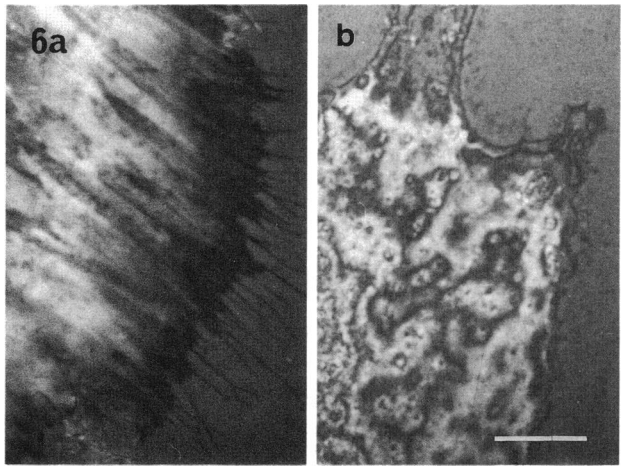

Figure 6. Effect of addition of combinations of peptides or polyargine to cells prespread on substrates of 105-kDa fragment. IRM image after addition of 100 μg/ml each of peptides FN-C/H I–V (a) or 100 μg/ml polyarginine (b). Bar, 10 μm.

5) below 100 μg/ml. WQPPRARI is the shortest of peptides FN-C/H I–V, and, unlike the other peptides, the basic amino acids are near the COOH-terminus, possibly affecting the activity. We therefore tested the activity of the longer fibronectin peptide WQPPRARITGY. This promoted focal adhesion formation in a higher percentage of cells than equivalent concentrations of WQPPRARI (Figure 5).

The percentage of cells prespread on 105-kDa fragment substrates that formed focal adhesions when WQPPRARI was added in solution was not increased by adding other active or inactive peptides nor did other peptides decrease the concentration of WQPPRARI needed. This was true for both the OA- and the free peptides (Table 2). It was apparent, however, that at high concentrations, particularly of free peptides, cellular contraction and distortion of shape occurred (Figure 6a). Peptides I–V are negatively charged and contain amine groups, which can promote cell attachment and spreading when used as substrates (Massia and Hubbell, 1992). Addition of arginines or lysines as soluble polyarginine, polylysine, or free arginine did not, however, promote focal adhesion formation in cells prespread on 105-kDa fragment substrates when tested at concentration ranges of 1 mg/ml to 0.1 μg/ml. In fact, high concentrations (10 μg/ml and above) caused considerable cellular shape changes and an abnormal IRM image (Figure 6b). The need for a specific amino acid sequence, rather than charge or a simple arginine requirement, is also emphasized by the fact that a synthetic peptide with the sequence RPQIPWAR (peptide FN-C/H V scrambled) was ineffective (Figure 7).

Table 2. Effect of addition of combinations of peptides

	I	II	III	IV	V	Focal adhesions (%)
OA conjugates	100		100		10	63 ± 1.0
	10		10		10	56 ± 1.6
	1		1		1	22 ± 5.6
	0.1		0.1		0.1	15 ± 1.6
	10	10	10	10	10	57 ± 2.9
	1	1	1	1	1	13 ± 0.5
Free peptides	100	100	100	100	100	26 ± 2.6

OA- or free peptides were added at the concentrations shown (100–0.1 μg/ml) to cells prespread on substrates of 105-kDa fragment. SD are shown (n = 3–5).

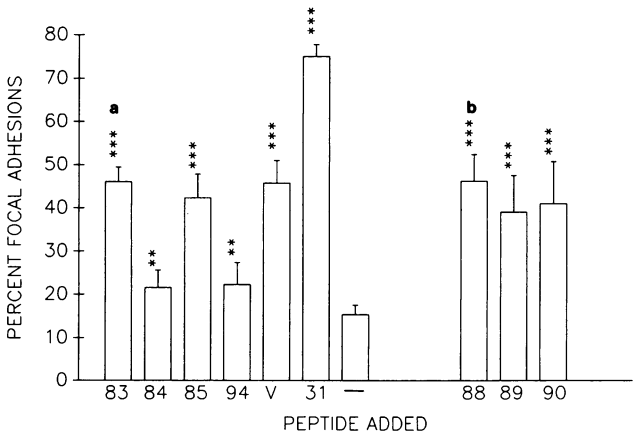

Figure 7. Sequence of FN-C/H V needed for focal adhesion induction. Cells prespread on substrates of 105-kDa fragment were treated with 100 μg/ml truncated, scrambled, or intact soluble peptide FN-C/H V (a) and truncated and intact PRARI (b). Codes are as in Table 1. SD are shown (n = 5–9). ***, **, and * denote significantly different from no addition at p = 0.001, 0.01, and 0.02, respectively.

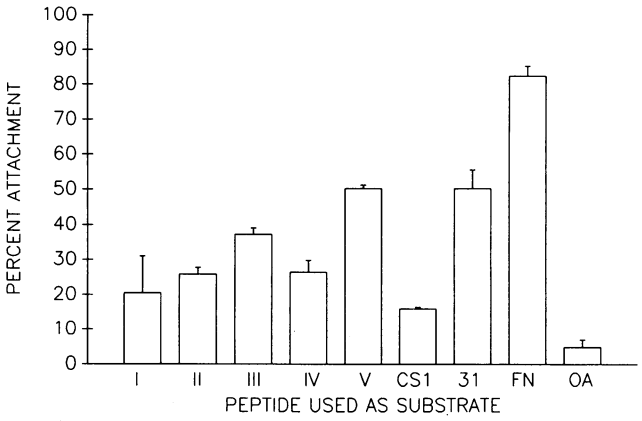

Figure 8. Attachment of cells to substrates coated with OA-peptides FN-C/H I–V or CS1, fibronectin, or 31-kDa heparin-binding fragment. SD are shown (n = 3).

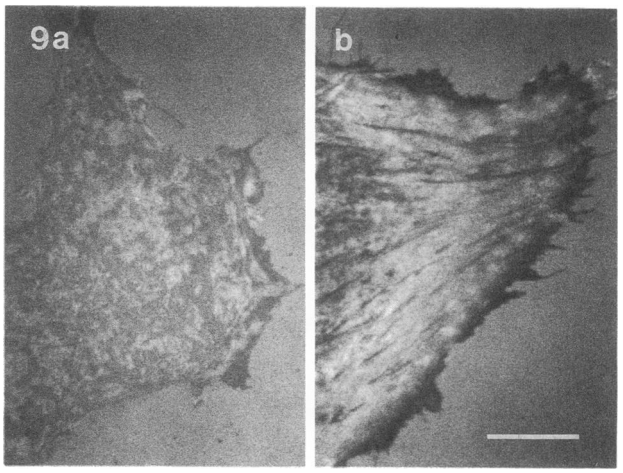

Figure 9. IRM image of cells spread on substrates coated with OA-peptide FN-C/H V (a) or a mixture of both OA-peptide FN-C/H V and 105-kDa fragment (b). Bar, 10 μm.

To define further the molecular basis of the biological activity of peptide FN-C/H V, overlapping synthetic peptides encompassing the front (WQPPR: coded 84) or back (PRARI: coded 85) halves of this peptide were synthesized and tested in blind assays. As a check on the original data showing that peptide FN-C/H V was active, a repeat synthesis of this peptide was encoded (83) and included in the assay. Figure 7a illustrates the activity of these peptides at 100 μg/ml to induce focal adhesion formation when added to cells prespread on 105-kDa fragment substrates. PRARI induced focal adhesion formation comparable with that induced by WQPPRARI, but WQPPR had little effect. Smaller peptides of sequences RARI (coded 89) and ARI (coded 90) gave more variable results; a range of 29–54% of cells formed focal adhesions when batches of 100 cells/coverslip were monitored (Figure 7b). When compared with the percentage of cells with focal adhesions on substrates of 105-kDa fragment but not further treated, however, a significant increase was seen with both peptides. Increasing the peptide concentrations to 500 μg/ml elicited no additional focal adhesion formation.

The cells used in the above assays had been pretreated with cycloheximide before suspension, and it is possible that this may result in a lack of cell surface receptors for peptides FN-C/H I–IV and CS1 in comparison with normal cells. To confirm that the cells retained receptors for peptides FN-C/H I–IV and CS1, their ability to attach to substrates coated with OA-peptides was monitored. Figure 8 shows that limited attachment occurred to all substrates except that of cross-linked ovalbumin alone. Maximum attachment was to fibronectin, as expected from previous studies (Woods *et al.*, 1986), with the 31-kDa fragment substrate promoting ~60% of this level. Peptide FN-C/H V appeared to be as effective as the whole 31-kDa fragment in promoting attachment. As seen in previous studies with the whole 31-kDa fragment (Woods *et al.*, 1986), only limited spreading was promoted by substrates composed solely of OA-peptide FN-C/H V (Figure 9a) or the other FN-C/H peptides, and the cells did not form focal adhesions. Focal adhesions were formed, however, by 53% of cells added to substrates coated by drying down a mixture of both OA-peptide FN-C/H V and 105-kDa fragment (Figure 9b). Peptide FN-C/H V and the 31-kDa fragment both appear to act directly on cells rather than by inducing changes in the 105-kDa fragment substrate. Only 12–16% cells formed focal adhesions when substrates of 105-kDa fragment were incubated with OA-peptide FN-C/H V, free peptide V, or the 31-kDa fragment before the addition of cells.

To examine the nature of the receptor involved in focal adhesion formation, cells were treated with chondroitinase ABC or heparinase II and III before plating onto substrates of 105-kDa fragment. Cells appeared to

Table 3. Effect of enzymes on focal adhesion formation

Additive	Enzyme	Focal adhesions (%)
None	None	13 ± 2.2
	Heparinase II + III	14 ± 2.5
	Chondroitinase ABC	23 ± 2.9
OA-FN-C/H V	None	70 ± 3.1
	Heparinase II + III	28 ± 3.2
	Chondroitinase ABC	64 ± 1.5
31-kDa fragment	None	75 ± 2.7
	Heparinase II + III	33 ± 4.2
	Chondroitinase ABC	72 ± 2.5

Cells adhering to substrates coated with 105-kDa fragment were treated with heparinase II + III or chondroitinase ABC before addition of 10 μg/ml OA-FN-CH V or 0.2 ng/ml 31-kDa heparin-binding fragment. SD are shown (n = 3–5).

spread normally, and 10 μg/ml OA-peptide FN-C/H V or 0.2 ng/ml 31-kDa heparin-binding fragment promoted focal adhesion formation in chondroitinase-treated cells (Table 3). Treatment with heparinase II and III dramatically reduced focal adhesion formation (Table 3). Similarly, addition of heparin at 100 μg/ml caused a 65 and 75% decrease in focal adhesion formation in response to 100 μg/ml free peptide FN-C/H V or 0.2 ng/ml 31-kDa fragment, respectively. Cells treated with chondroitin sulfate at 100 μg/ml formed focal adhesions in response to free peptide or 31-kDa fragment at 93 and 92% of control (untreated) levels.

DISCUSSION

Using a series of synthetic heparin-binding peptides with sequences from the COOH-terminal heparin-binding domain of fibronectin, we show that specific amino acid sequences, rather than positive charge, presence of amine groups, or the ability to bind to heparin, underlie the biological activity of regions of this domain in promoting focal adhesion assembly. Four separate sequences showed some biological activity. When added as soluble peptides, FN-C/H I, III, and V induced focal adhesion formation in the majority of cells (and peptide FN-C/H IV in a low percentage of cells) prespread on substrates of 105-kDa cell-binding fragment of fibronectin. Peptides I, III, and IV were, however, only effective at high concentrations (100 μg/ml) and when added as an OA-complex containing several copies of the peptide. None of these three peptides retained the ability to promote focal adhesion formation when added in the free form, even at 1 mg/ml. Peptide FN-C/H V, in contrast, had potent inductive effect at 10 μg/ml as an OA-complex and retained activity as a free peptide. In terms of relative activity, intact 31-kDa COOH-terminal heparin-binding fragments promoted maximal focal adhesion formation at 0.2 ng/ml (=0.01 nM), whereas OA-peptide FN-C/H V (~5 mol peptide/mol ovalbumin) was maximally active at 0.2 μM and the free peptide at 100 μM. This difference in activity of free, compared with coupled, peptide strongly implies that the relative lack of conformational constraints limit the effectiveness of free peptides. It has been seen before that isolated fibronectin peptides may not be as active as the parent molecule (e.g., in comparison of the effects of small RGD-containing peptides versus the whole cell-binding domain [Ruoslahti, 1988; Yamada, 1989; Hynes, 1990]). In addition, cellular responses to RGD-dependent interactions may be modulated by sequences within other type III repeats of the 105-kDa domain (Obara *et al.*, 1988; Akiyama *et al.*, 1990; Aota *et al.*, 1991; Nagai *et al.*, 1991). The 31-kDa fragment contains at least four sites that showed some activity, and these may act to enhance the biological effect of the whole fragment. Consideration of the recently described structures of the 10th fibronectin type III (Baron *et al.*, 1992) and the third tenascin type III (Leahy *et al.*, 1992) modules indicates that the amino acid sequence PRARI of peptide FN-C/H V may span a loop structure between two β strands and hence be exposed in the whole molecule. Induced conformational changes on binding of one sequence (e.g., as indicated when heparin binds vitronectin [Cardin and Weintraub, 1989] or fibronectin [Ankel *et al.*, 1986]) may, however, allow further interactions with different heparin-binding sequences within the whole fragment. Nonetheless, free peptide FN-C/H V (WQPPRARI) or the smaller peptide PRARI shows potent biological activity at concentrations similar to those needed for RGD-dependent events (reviewed in Ruoslahti, 1988; Hynes, 1990).

Barkalow and Schwarzbauer (1991) showed in cell-free assays that two type III repeats (III-13 and III-14) of the heparin-binding domain cooperate in the binding of the COOH-terminal half of fibronectin to heparin affinity matrices. These repeats contain peptides FN-C/H I, II, IV, and V. No activity was associated with repeat III-12, which contains peptide FN-C/H III. Deletion of regions containing either peptide FN-C/H IV or FN-C/H V markedly reduced this heparin-binding activity. In our assay of focal adhesion promotion, however, peptide FN-C/H V was the most active of the peptides, with peptide FN-C/H IV being only marginally active even in the conjugated form. It is, therefore, difficult to directly extrapolate from the ability of a peptide to interact with heparin to its ability to induce a particular biological effect. Specificity may lie in the glycosaminoglycan fine structure of the "receptor," as seen for antithrombin III and basic fibroblast growth factor (Kjellén and Lindahl, 1991; Turnbull *et al.*, 1992).

The dose response to free peptide FN-C/H V addition showed biological activity of this peptide down to 10 μg/ml. Further analysis of the ability of portions of peptide FN-C/H V to stimulate focal adhesion formation indicated that the sequence of PRARI was the most active part of WQPPRARI, retaining 93% of the activity of the whole peptide. However, the most effective peptide tested was the longer WQPPRARITGY, again indicating that conformation may be important. The focal adhesions formed had the characteristic pattern, size, and frequency/cell normally seen in cells responding to fibronectin (Woods *et al.*, 1986). Most cells failed to respond to WQPPR or the scrambled sequence (RPQIPWAR) with only 10–14% of cells showing a normal pattern of focal adhesions, a typical percentage that escapes the cycloheximide block and secretes endogenous fibronectin (Woods *et al.*, 1986). Direct comparison of the stimulatory effects of PRARI with RARI and ARI indicated very little difference between the smaller two peptides, but individual cells responded much more variably than with PRARI.

The specific structure formed by WQPPRARI or PRARI cannot be mimicked in biological action by peptide sequences with similar features or by polylysine,

polyarginine, or free arginine. For example, peptide FN-C/H IV (SPPRRARVT) bears some similarity to peptide FN-C/H V but was not effective in inducing focal adhesion formation. Analysis of the current Swissprot data bank indicated that the sequence PRARI is uncommon in proteins, being present in only two G1/S cyclins and the abutilon mosaic virus coat protein. However, PRARI is conserved in the bovine, human, and rat sequences of fibronectin.

Peptides FN-C/H I–V and CS1 all induced cell attachment indicative of binding to cell surface receptors. Of these peptides, however, only three, even when added as a multicopy OA-complex, could promote focal adhesion formation in cells prespread on substrates of 105-kDa RGD-containing cell-binding fragment. Thus, the capacity to induce attachment and some limited spreading does not correlate with the ability to induce focal adhesion formation. Indeed, cells are fully spread on substrates of 105-kDa fibronectin fragment (Woods *et al.*, 1986) but need an additional signal to form focal adhesions (Woods *et al.*, 1992a,b). Recent reports (Massia and Hubbell, 1992) have indicated that surfaces composed of arginine, lysine, and other diamines can induce cell attachment and spreading and that spreading is reduced by treatment with chondroitinase ABC and not heparinase. However, these substrates do not induce focal adhesions or the typical stress fiber networks seen on fibronectin substrates (Massia and Hubbell, 1992). In our experiments, the addition of polylysine, polyarginine, or arginine did not induce focal adhesion formation in cells prespread on 105-kDa fragment substrates. Interactions of cell surface chondroitin sulfate proteoglycans with amine-derivatized substrates, although sufficient to allow attachment and some spreading, may not generate a similar signal to that from interactions of peptide FN-C/H V or the 31-kDa heparin-binding fragment with a different cell surface proteoglycan. The ability of peptide FN-C/H V and the 31-kDa fragment to provide the signal to induce focal adhesion formation was markedly reduced by treatment of cells with heparinase II and III or by the presence of heparin. Treatment of cells with chondroitinase ABC or the presence of chondroitin sulfate did not prevent focal adhesion induction. Previous evidence also indicates that cell surface HSPG are the receptors for heparin-binding domains of fibronectin and are responsible for transducing this signal through the agency of protein kinase C (Woods *et al.*, 1992a,b). Cell surface HSPG are found in focal adhesions and aligned with the internal stress fiber system of cells (Woods *et al.*, 1984; Rapraeger *et al.*, 1986). A hydrophobic HSPG species is retained in detergent-resistant cytoskeleton-matrix preparations (Woods *et al.*, 1985), and mutant cells either lacking (LeBaron *et al.*, 1988) or having cell surface HSPG of reduced sulfation and binding to fibronectin and reduced half-life (Couchman *et al.*, 1988) do not form focal adhesions. Several species of cell surface proteoglycans may be present in fibroblasts (Bernfield *et al.*, 1992; Lories *et al.*, 1992), and we are currently determining which proteoglycan species bind to the 31-kDa heparin-binding domain and peptide FN-C/H V and may mediate focal adhesion formation.

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