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Dualistic Nature of Adhesive Protein Function: Fibronectin and Its Biologically Active Peptide Fragments Can Autoinhibit Fibronectin Function

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ABSTRACT Fibronectin and certain polypeptide regions of this adhesive glycoprotein mediate cell attachment and spreading on various substrates. We explored the theoretical prediction that this adhesive protein could become a competitive inhibitor of fibronectin-mediated processes if present in solution at appropriately high concentrations. Fibronectin function was inhibited by purified plasma fibronectin at 5–10 mg/ml, by a 75,000-dalton cell-interaction fragment of the protein at 0.5–1 mg/ml, and even by two synthetic peptides containing a conserved, hydrophilic amino acid sequence at 0.1–0.5 mg/ml. Inhibition of fibronectin-dependent cell spreading was dose dependent, noncytotoxic, and reversible. It was competitive in nature, since increased quantities of substrate-adsorbed fibronectin or longer incubation periods decreased the inhibition. A peptide inhibitory for fibronectin-mediated cell spreading also inhibited fibronectin-mediated attachment of cells to type I collagen, but it did not affect concanavalin A-mediated spreading.

These results demonstrate the potential of a cell adhesion molecule and its biologically active peptide fragments to act as competitive inhibitors, and they suggest that fibronectin may act by binding to a saturable cell surface receptor.

Cell adhesion to other cells or to substrates is a complex process which is still poorly understood at the molecular level (e.g., references 1 and 2). Adhesive events mediated by fibronectin and other extracellular attachment proteins provide experimental systems for analyzing polypeptide domains that mediate binding and adhesive functions (3–9). For example, a region of fibronectin termed the "cell-binding" region, which interacts with the cell surface to mediate cell attachment and spreading on substrates, has been identified and localized to polypeptide fragments (10, 11). Even a synthetic peptide from this region of fibronectin can mediate cell attachment to a plastic substrate (12).

Theoretically, a cell adhesion protein might become a specific inhibitor of its own function if it were bound in excess to a cellular receptor. Saturation of receptors by soluble adhesive molecules might prevent receptor interactions with substrate- or cell-attached adhesive molecules. A rough analogy might be the prozone effect in immunoprecipitation, in which an excess of antibody can saturate sites on antigens and prevent the formation of aggregates.

We tested this theoretical model with soluble plasma fibronectin and certain peptide fragments of this glycoprotein. These molecules were found to be competitive, reversible inhibitors of fibronectin function. Our results suggest that adhesive proteins can have a dualistic nature, mediating positive or negative effects depending on their concentrations and whether they are in solution or attached to a substrate.

MATERIALS AND METHODS

Fibronectin and Fibronectin Fragments: Plasma fibronectin was isolated from human plasma (National Institutes of Health Blood Bank), either by sequential gelatin-Sepharose and heparin-Sepharose affinity chromatography with elution and washing by 4 M urea as described (13, 14) or by elution under nondenaturing conditions at pH 5.5 from a gelatin-Sepharose column (15), and centrifuged at 25,000 *g* for 15 min; results with either type of preparation were similar. As shown previously (11), such fibronectin preparations migrated as a homogeneous doublet band in SDS polyacrylamide gels.

The 75,000-dalton cell-binding fragment was generated by tryptic digestion of human plasma fibronectin and purified exactly as described previously (11). A 60,000-dalton collagen-binding fragment was generated by digestion of human plasma fibronectin by 5 μg/ml α-chymotrypsin (Worthington Biochem-

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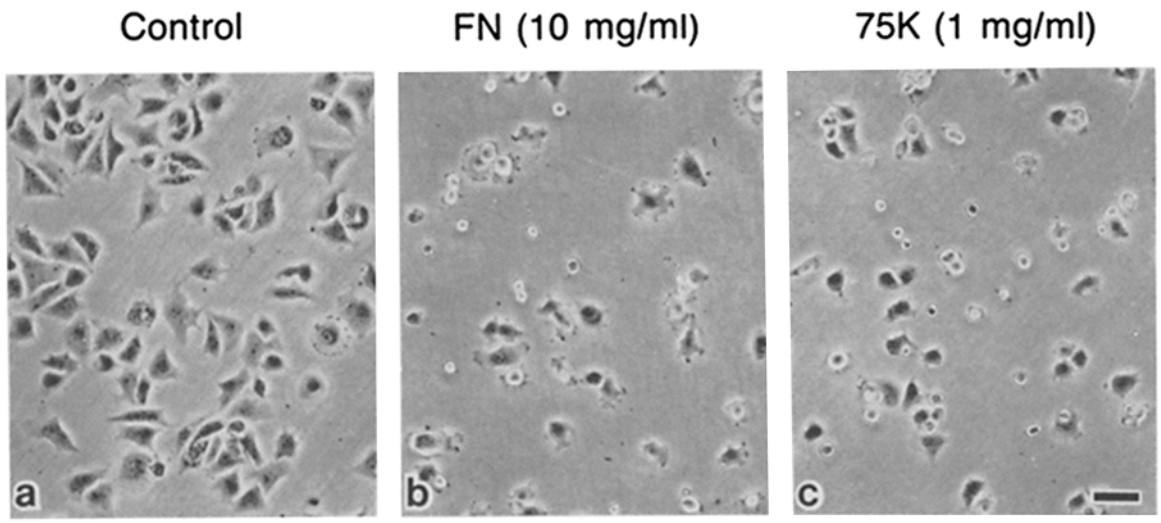

FIGURE 1 Autoinhibition of fibronectin-mediated cell spreading. (*a*) Control BHK cells spreading on a tissue culture dish precoated with 3 μg/ml plasma fibronectin, followed by BSA to block nonspecific interactions, as described in Materials and Methods. (*b*) BHK cells on a parallel, fibronectin-coated dish in the presence of 10 mg/ml soluble plasma fibronectin (*FN*). (*c*) BHK cells on a fibronectin-coated dish in the presence of 1.0 mg/ml 75,000-dalton cell-binding fragment (*75K*) of fibronectin. Phase-contrast microscopy; bar, 50 μm.

ical Corp., Freehold, NJ; 50 U/mg) in 150 mM NaCl, 1 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.4, for 30 min at room temperature. The reaction was terminated with soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) at 10 μg/ml, and the 60,000-dalton collagen-binding fragment was purified by sequential gelatin-Sepharose and Sephacryl S-200 chromatography essentially as described for a similar fragment from chicken cellular fibronectin, except that the fragment was eluted from the gelatin affinity column with 4 M rather than 8 M urea, and the gel permeation column was eluted with 150 mM NaCl, 10 mM Tris-HCl, pH 7.4 (16). The synthetic peptides Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro (peptide I), Gly-Arg-Gly-Asp-Ser-Pro-Cys (peptide II), and Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr (peptide III) were purchased from Peninsula Laboratories, Inc., Belmont, CA. They were purified further by chromatography on TSK HW-40(S) Fractogel columns (2.5 × 100 cm, flow rate 12–15 ml/h; Pierce Chemical Co., Rockford, IL), and their compositions were confirmed by quantitative amino acid analyses.

Peptide II was alkylated where indicated as follows: 20 μmol of cell-binding peptide II was incubated with 40 mM dithiothreitol (Calbiochem-Behring Corp., San Diego, CA) in 0.5 M Tris-HCl, 2 mM EDTA, pH 8.5, under a nitrogen atmosphere for 4 h at 37°C. The samples were alkylated by incubation with 0.1 M iodoacetic acid (Aldrich Chemical Co., Milwaukee, WI) for 30 min at room temperature in the dark. The reaction was terminated by the addition of excess dithiothreitol (final concentration 0.13 M). The alkylated peptide was isolated by rechromatography on a TSK HW-40(S) (Pierce Chemical Co.) column.

Biological Assays: Quantitation of fibronectin-mediated cell spreading of BHK (baby hamster kidney)[1] cells and CHO (Chinese hamster ovary) cell attachment to type I collagen was performed as described (16, 17). Based on quantitative fibronectin adsorption data published by Grinnell and Feld (18), the cell-spreading assay was modified as follows to prevent further adsorption of soluble fibronectin to the substrate during assays: 16-mm multiwell or 35-mm tissue culture dishes (Costar, Data Packaging, Cambridge, MA) were preincubated with 3 μg/ml plasma fibronectin in 0.3 or 1.0 ml adhesion medium, respectively, for 60 min at room temperature (23–24°C). All remaining protein adsorption sites were saturated with 10 mg/ml heat-denatured (80°C for 3 min) BSA for 30 min. Cells were incubated in adhesion medium (150 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 6 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.3; reference 17) with or without added soluble fibronectin or its fragments for 45 min at 37°C. After this attachment period, cells were fixed with 2.5% glutaraldehyde in Dulbecco's PBS for >1 h, and cell spreading was quantitated as described (16, 17).

Controls for the possible nonspecific effects of protein present during the cell-spreading assay included incubation with 10 mg/ml BSA, fibrinogen, or ovalbumin from Calbiochem-Behring, as well as fetuin (types III and IV), transferrin, soybean trypsin inhibitor, cytochrome *c*, hemoglobin, and myoglobin from Sigma Chemical Co.

[1] *Abbreviations used in this paper:* BHK, baby hamster kidney; CHO, Chinese hamster ovary.

Additional peptide controls were: fibrinopeptide A and luteinizing hormone purchased from Vega Biochemicals, Tucson, AZ; tuftsin obtained from Calbiochem-Behring; and adrenocorticotropin, angiotensin I, aprotinin, bradykinin, fibrinopeptide B, α-melanocyte-stimulating hormone, neurotensin, oxytocin, Arg-Gly-Pro-Phe-Pro-Ile, Gly-Pro-Arg-Pro, Arg-Asp, Gly-Asp, and mixtures of free amino acids corresponding to synthetic peptide I and synthetic peptide II, all purchased from Sigma. The pH of samples was adjusted to pH 7 with HCl or NaOH.

Reversibility and Amino Acid Incorporation Experiments: BHK cells were incubated for 45 min in the standard cell-spreading assay on substrates coated with 3 μg/ml plasma fibronectin with or without added 10 mg/ml human plasma fibronectin or 0.5 mg/ml peptide I or peptide II. Cultures were fixed with glutaraldehyde to determine the extent of spreading at 45 min, and parallel cultures were gently washed three times with adhesion medium lacking inhibitors and incubated further for 45 min at 37°C. Other parallel cultures were incubated for the additional 45 min with the inhibitors.

Amino acid incorporation was determined for BHK cells incubated in the standard spreading assay in Costar 35-mm dishes containing adhesion medium with 1 mM glucose and 1 μCi/ml ^{14}C-amino acids mixture (Amersham Corp.). After 45 min at 37°C, the cultures were rinsed with Dulbecco's PBS, solubilized with 1 N NaOH, and neutralized with HCl. After precipitation with 10% trichloroacetic acid at 4°C, collection, and washing on Millipore (Bedford, MA) HAWP filters, radioactivity was determined in a scintillation spectrometer.

Lectin-mediated Cell Spreading: Freshly prepared solutions of concanavalin A (Pharmacia Inc., Uppsala, Sweden) were preincubated with 24-well Costar tissue culture plates at 0.5–50 μg/ml for 60 min at room temperature, then rinsed well with adhesion medium. Cell-spreading assays were performed with or without the addition of 0.5 mg/ml synthetic peptide I exactly as for fibronectin-coated substrates.

RESULTS

Inhibition of Fibronectin-mediated Cell Spreading by Fibronectin

In a cell-spreading assay used routinely to quantitate fibronectin biological activity (11, 16, 17), BHK cells spread nearly completely on tissue culture substrates precoated with 3 μg/ml human plasma fibronectin. As shown in Fig. 1 and quantitated in Fig. 2, this fibronectin-mediated spreading was progressively inhibited by increased concentrations of plasma fibronectin added to the adhesion medium. Inhibition appeared maximal at ~10 mg/ml; various preparations displayed maximal inhibition at 6–12 mg/ml of soluble fibronectin. Although spreading was severely inhibited, some cells appeared to display abortive spreading, with increased phase

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density of cytoplasm according to phase-contrast microscopy, but with poor elaboration of peripheral lamellae (Fig. 1).

Nine other proteins and glycoproteins that were tested at 10 mg/ml as controls for nonspecific effects of protein added to the assay system did not affect fibronectin-mediated spreading of these cells (Table I). Proteins without inhibitory effects in this assay included fetuin and fibrinogen.

Inhibition of Fibronectin-mediated Cell Spreading by a Cell-binding Fragment

A purified 75,000-dalton fragment of fibronectin which displays nearly intact molar activity compared with native fibronectin for mediating cell spreading (11) became inhibitory when incubated in increased concentrations with BHK cells (Figs. 1 and 3). Inhibition by this polypeptide fragment was maximal at 0.5–1.0 mg/ml, which is equivalent to a concentration of $\sim 10^{-5}$ M peptide. Half-maximal inhibition occurred at 2.4×10^{-6} M 75,000-dalton fragment. Fig. 3 also indicates that another fragment of fibronectin of 60,000 daltons containing the collagen-binding domain had no effect

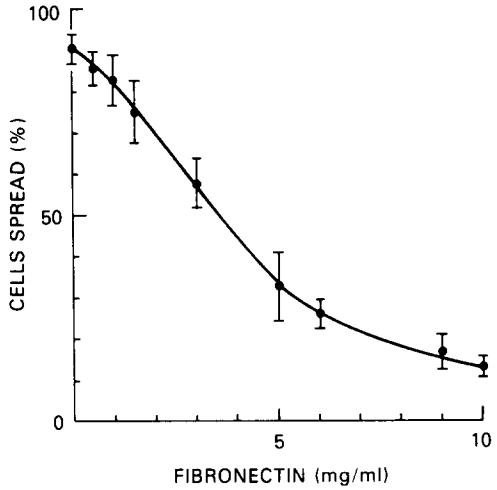

FIGURE 2 Inhibition of fibronectin-mediated spreading by increased concentrations of added plasma fibronectin. Cells were placed into dishes precoated with 3 μg/ml plasma fibronectin and albumin and incubated in the presence of the indicated concentrations of added plasma fibronectin. A total of 600 cells was scored for spreading for each point; bars indicate standard deviation (n = six fields).

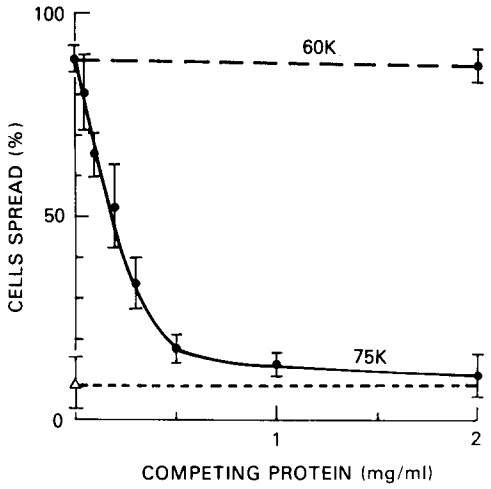

FIGURE 3 Inhibition of fibronectin-mediated spreading by a 75,000-dalton fragment of fibronectin containing the cell-binding domain. The percentages of BHK cells that exhibited a spread morphology on dishes coated with 3 μg/ml fibronectin in the presence of the indicated concentrations of 75,000-dalton cell-binding domain (*75K*) in solution or 60,000-dalton collagen-binding domain (*60K*) in solution are indicated. The triangle on the ordinate and the dashed line indicate the background level of cell spreading on dishes not coated with fibronectin. Each point represents data from 600 cells; bars indicate standard deviation for six random microscope fields.

TABLE I

Concentrations of Peptides Required for Half-maximal Inhibition of BHK Cell Spreading

Peptide	Amino acid sequence	Concentration for half-maximal inhibition
		μg/ml*
Synthetic peptide I	Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro	120
Synthetic peptide II	Gly-Arg-Gly-Asp-Ser-Pro-Cys	32
Synthetic peptide III	Cys-Gln-Asp-Ser-Glu-Thr-Arg-Thr-Phe-Tyr	>500
Adrenocorticotropin		>500
Aprotinin		>500
Insulin chain A		>500
Luteinizing hormone		>500
Fibrinopeptide A	Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg	>500
Fibrinopeptide B	pGlu-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg	>500
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	>500
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	>500
Melanocyte-stimulating hormone	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val	>500
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	>500
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly	>500
Tuftsin	Thr-Lys-Pro-Arg	>500
—	Arg-Gly-Pro-Phe-Pro-Ile	>500
—	Gly-Pro-Arg-Pro	>500
—	Arg-Asp	>500
—	Gly-Asp	>500

* Activities of peptides were determined for BHK cells on a substrate coated with 3 μg/ml plasma fibronectin in a 45-min cell-spreading assay performed as described in Materials and Methods. Also tested were mixtures of free amino acids at the same molar concentrations as the amino acids present in 500 μg/ml of either synthetic peptide I or synthetic peptide II; both mixtures were devoid of inhibitory activity.

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on fibronectin-mediated cell spreading. This fragment alone had no intrinsic activity in mediating cell spreading (data not shown).

Inhibition of Cell Spreading by Synthetic Peptides

A comparison of published (19–21) amino acid sequences from a limited portion of the cell-binding region of fibronectin is shown in Fig. 4. The sequences reveal a complete identity of amino acids between the three species, except for a conservative substitution of isoleucine for valine. We also focused on this region because it contains the point of greatest local hydrophilicity in the cell-binding domain, as determined for averages of decapeptide units based on the values of Hopp and Woods (reference 22; data not shown). These properties suggest that this region is evolutionarily conserved and particularly well exposed on the outer surface of the protein molecule because of its paucity of hydrophobic residues. Two synthetic peptides from this region were synthesized, one of which contained an additional carboxyl-terminal cysteine residue to permit coupling of the peptide to other proteins and its labeling by alkylation.

As shown in Figs. 5 and 6, a synthetic decapeptide (peptide I) from this region inhibited cell spreading. Inhibition was dose dependent, with half-maximal inhibition at 120 μg/ml. A conserved decapeptide region from the collagen-binding domain (peptide III) containing an identical amino acid sequence in comparisons of bovine and chicken fibronectin (23, 24) was not inhibitory (Fig. 6). A series of 15 other nonrelated peptides shown in Table I also did not produce such inhibition.

Figs. 5 and 7 show that another synthetic peptide (peptide II) containing five amino acids in common with the first peptide was also an effective inhibitor of fibronectin-mediated cell spreading. Half-maximal inhibition occurred at 32 μg/ml. Alkylation of this peptide with iodoacetic acid to block the free -SH group did not alter its inhibitory activity (Fig. 7).

The inhibition appeared to be competitive, since the inhibitory effect of synthetic peptide I was diminished in a dose-dependent fashion as the amount of fibronectin preadsorbed onto the substrate was increased by preincubation of the dishes with increased amounts of fibronectin (Fig. 8). As shown in Fig. 8, there was a 95% inhibition of spreading by 0.5 mg/ml synthetic decapeptide I at a low concentration of fibronectin in the adsorption step, but <25% inhibition at the highest concentration.

Fibronectin biological activity can also be quantitated by

Human	Ala	Val	Thr	Gly	Arg	Gly	Asp	Ser	Pro	Ala	Ser	Ser	Lys	Pro	Ile	Ser	Ile
Bovine	Ala	Val	Thr	Gly	Arg	Gly	Asp	Ser	Pro	Ala	Ser	Ser	Lys	Pro	Val	Ser	Ile
Rat	Ala	Val	Thr	Gly	Arg	Gly	Asp	Ser	Pro	Ala	Ser	Ser	Lys	Pro	Val	Ser	Ile

I ——————————
II ——————————- Cys

FIGURE 4 Conserved cell-binding region of fibronectin and two synthetic peptides. Partial amino acid sequences from published data on the cell-binding regions of human, bovine, and rat fibronectins are listed (references 19–21), with the amino terminus at the left. Synthetic peptide I is indicated by the upper bar, and it contains a relatively hydrophilic decapeptide sequence. Synthetic peptide II is marked by the lower bar, and it contains a nonoverlapping glycine residue, five amino acids in common with peptide I, and a COOH-terminal cysteine residue.

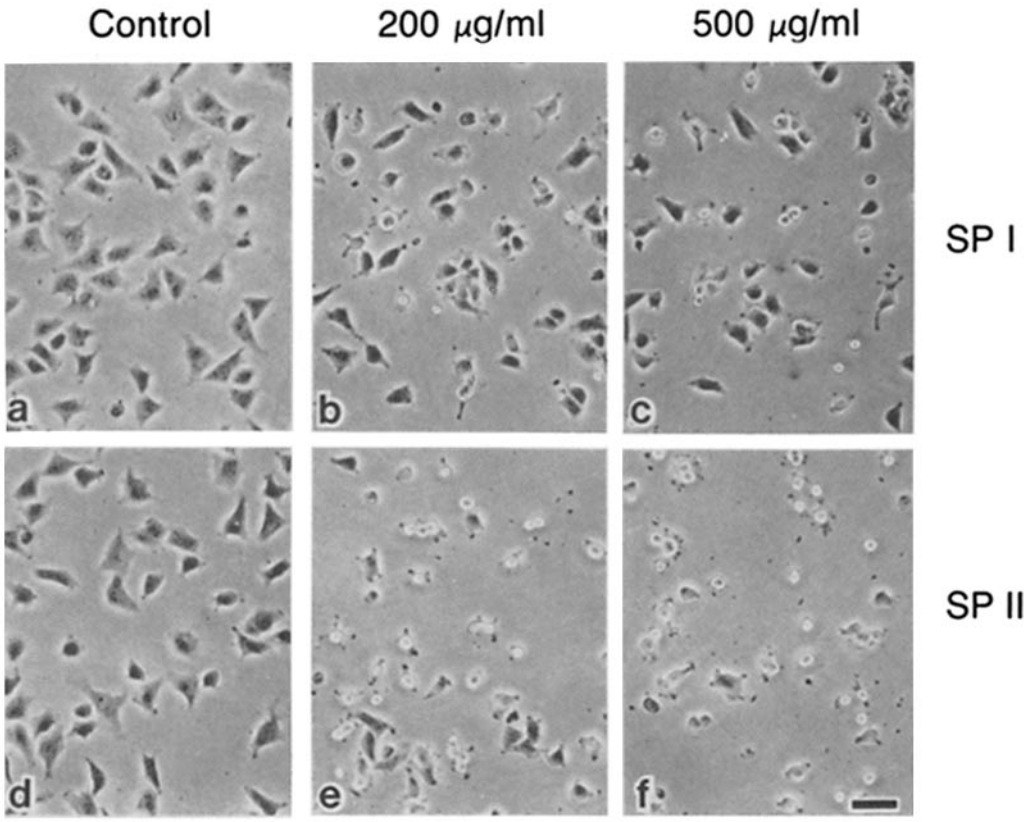

FIGURE 5 Inhibition of fibronectin-mediated cell spreading by synthetic peptides. BHK cells attached to fibronectin-coated plates with no additions (*a* and *d*), synthetic peptide I (*SP I*) in solution at concentrations of 200 (*b*) or 500 μg/ml (c), and synthetic peptide II (*SP II*) at concentrations of 200 (*e*) or 500 μg/ml (*f*). Phase-contrast microscopy; bar, 50 μm.

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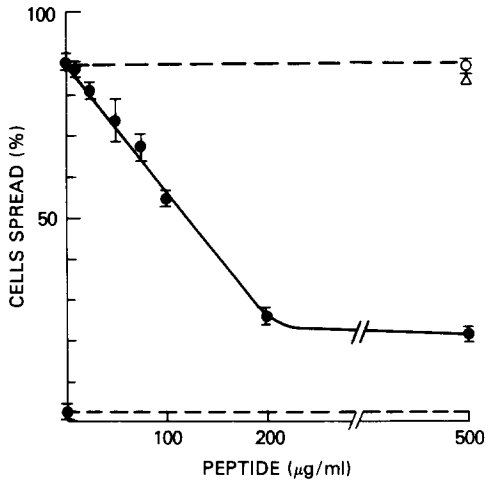

FIGURE 6 Quantitation of the inhibition of cell spreading by synthetic peptide I. BHK cells were incubated on tissue culture dishes exposed to 3 μg/ml plasma fibronectin in the presence of the indicated solution concentrations of the synthetic decapeptide I (●), an equal volume of buffer (○), or 500 μg/ml synthetic peptide III (△) from the collagen-binding domain. Points indicate mean ± SE of six random fields (600 cells each).

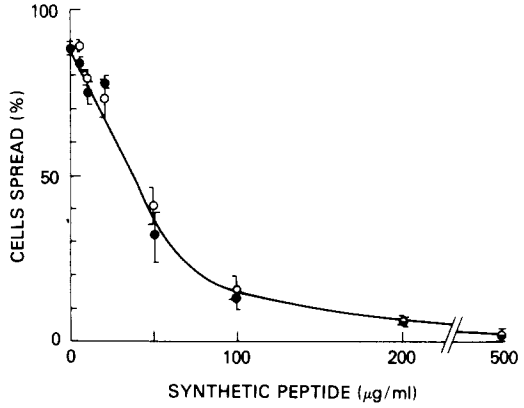

FIGURE 7 Quantitation of the inhibition of cell spreading by synthetic peptide II. The percentage of cells with a spread morphology on a substrate coated with 3 μg/ml plasma fibronectin is shown at the indicated solution concentrations of synthetic peptide II (○) or reduced and alkylated synthetic peptide II (●). Error bars facing to the left indicate the standard errors for synthetic peptide II and those facing to the right indicate standard errors for alkylated synthetic peptide II; each point represents 600 cells.

attachment assays by simply measuring the number of CHO cells that attach or cannot attach to a type I collagen substrate. A similar dose-dependent inhibition of fibronectin-dependent adhesion was observed (Fig. 9), indicating that the inhibitory effect is not unique to an assay based on cell spreading.

Controls for Cytotoxicity

None of the autogenous or synthetic inhibitors appeared to be cytotoxic by phase-contrast microscopy (Fig. 5). Viabilities as determined by trypan blue exclusion remained unaltered (e.g., 98.4 ± 0.7% for control cells, 98.0 ± 1.4% for cells with rounded morphology in the presence of 6 mg/ml plasma fibronectin, and 99.2 ± 0.7% for rounded cells in the presence of 0.3 mg/ml synthetic decapeptide I).

Protein synthesis was also not substantially inhibited: the incorporation of ^{14}C-labeled amino acids into BHK cells increased 33% in cells that had spread on fibronectin compared with parallel cultures in dishes with no fibronectin coating (uncoated = 28.2 ± 1.8 × 10^4 cpm per 35-mm dish, compared with 37.4 ± 6.6 × 10^4 cpm per dish on fibronectin-coated dishes; values indicate mean ± SD, n = 3). Cells that remained rounded in the presence of the highly inhibitory synthetic peptide II at a concentration of 0.5 mg/ml incorporated amounts of radiolabeled amino acids similar to those of the rounded control cells (24.2 ± 7.5 × 10^4 cpm per dish compared with 28.2 ± 1.8 × 10^4 cpm per dish in controls). Even a drastic inhibition of protein synthesis by treatment with cyclohexamide does not inhibit fibronectin-mediated spreading of BHK cells (reference 25; and data not shown).

Removal of the inhibitors resulted in a rapid re-initiation of spreading to final percentages approaching control values, further indicating the absence of cytotoxicity (Fig. 10). In fact, controls in which inhibitors remained present for a second 45-min assay period showed a modest recovery of the capacity to spread even in the continued presence of synthetic decapeptide (Fig. 10; showing 5.7 ± 0.9% spread cells at 45 min compared with 22.2 ± 5.8% after an additional 45 min). There is particularly striking recovery of spreading in the

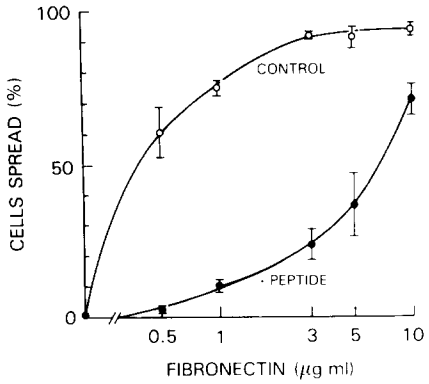

FIGURE 8 Competitive nature of the inhibition by synthetic peptide. The graph indicates the percent of total BHK cells with a spread morphology coated with the indicated concentrations of plasma fibronectin in the absence (○) or presence (●) of 0.5 mg/ml synthetic peptide I in solution.

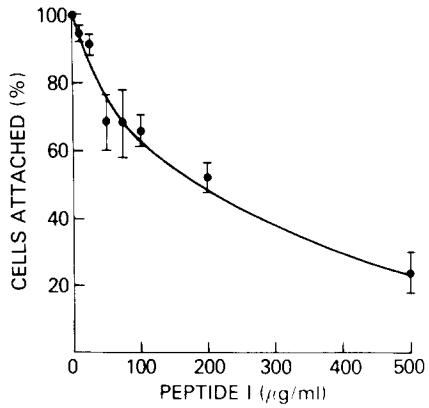

FIGURE 9 Inhibition of the adhesion of CHO cells to type I collagen. Percent of control cell attachment to dishes coated with type I collagen, preincubated with 3 μg/ml plasma fibronectin, then incubated with the cells in the presence of the indicated concentrations of synthetic peptide I. Bars indicate standard error of triplicate samples. Approximately 75% of the CHO cells initially added to the dishes attached in the controls (0 μg/ml peptide I).

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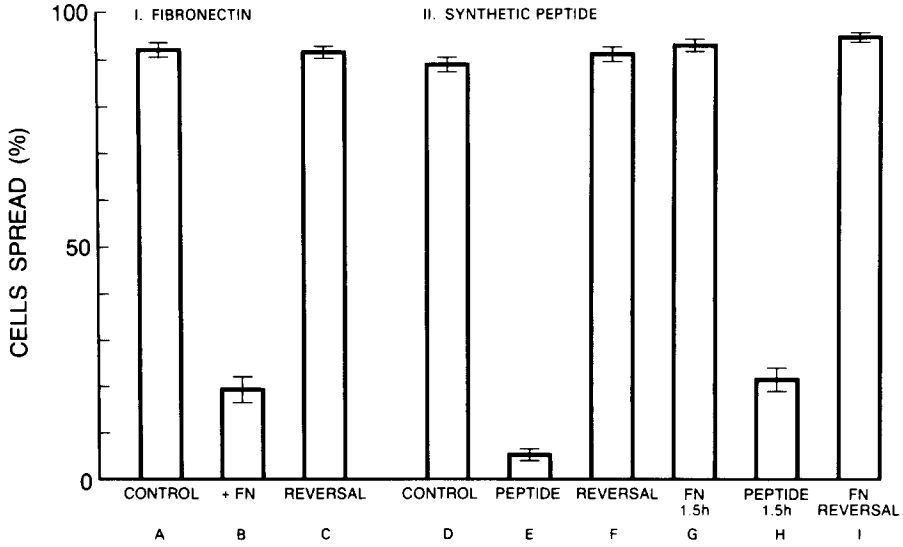

FIGURE 10 Reversibility of the autoinhibition by fibronectin (I) or by a synthetic peptide (II). Percentage of BHK cells with a flattened, spread morphology on a control fibronectin substrate after precoating with 3 μg/ml plasma fibronectin (*A*) or the same substrate plus 6 mg/ml soluble plasma fibronectin in adhesion medium (*B*), or after the same treatment with soluble fibronectin for 45 min, followed by three washes and a further incubation for 45 min in the absence of soluble fibronectin (*C*). Spreading of control BHK cells with 3 μg/ml plasma fibronectin (*D*), fibronectin plus 0.5 mg/ml synthetic peptide I (*E*), or after washing and incubation for a second 45-min period in the absence of inhibitory peptide (*F*). Control BHK cells were also incubated on fibronectin for two consecutive 45-min periods (*G*), plus the continuous presence of 0.5 mg/ml synthetic peptide I (*H*), or after three washes of the control cultures (no peptide) after 45 min and a second incubation for 45 min (*I*). Error bars indicate standard error, $n = 6$; 600 cells for each point.

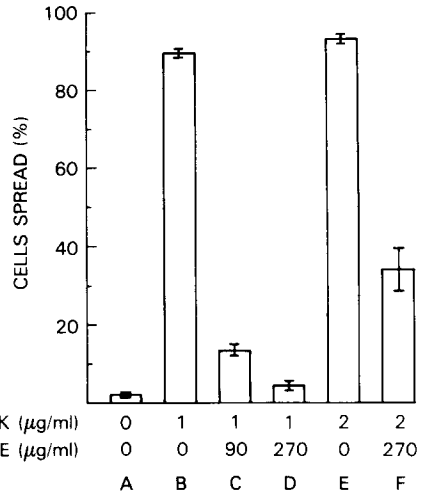

FIGURE 11 Inhibition by a synthetic peptide of cell spreading mediated by a purified cell-binding fragment of fibronectin. The percentage of BHK cells with a spread morphology is indicated for control cells with no additions (*A*), compared with cells spreading on dishes coated with 1 μg/ml 75,000-dalton cell-binding fragment (*75K*) (*B–D*) in the presence of no additional peptide (*B*), 90 μg/ml synthetic peptide I (*C*), or 270 μg/ml synthetic peptide I (*D*). Cells were also examined using dishes coated with 2 μg/ml 75,000-dalton cell-binding fragment (*E* and *F*) in the presence of no additional peptide (*E*) or 270 μg/ml synthetic peptide I (*F*). Note that the inhibition by synthetic peptide is less when the cell-binding peptide is present at twice the concentration.

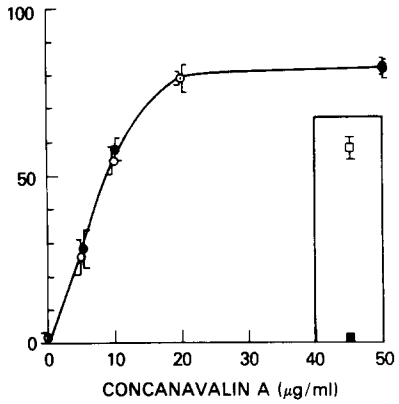

FIGURE 12 Absence of inhibition of concanavalin A-mediated cell spreading by synthetic peptide I. The percentage of BHK cells with a spread phenotype on substrates precoated with the indicated conditions of concanavalin A was determined as described in Materials and Methods in the absence (○) or presence (●) of 0.5 mg/ml synthetic peptide I. The rectangular inset indicates the degree of spreading of the BHK cells in this experiment when the substrate was coated instead with plasma fibronectin at 3 μg/ml in the absence (□) or in the presence (■) of the 0.5 mg/ml concentration of synthetic peptide I. All values represent mean and standard error ($n = 6$), based on counts of 600 cells per point.

presence of 10 mg/ml fibronectin (to 64.2 ± 3.1% after incubation for an additional 45-min period).

Effect on Spreading Mediated by a Fibronectin Fragment

It was possible that the inhibition occurred by interference with a binding site on fibronectin unrelated to the "cell-binding" site, e.g., with a heparin- or collagen-binding site. However, Fig. 11 shows that synthetic peptide I caused a similar dose-dependent inhibition of cell spreading that was mediated by the 75,000-dalton cell-binding fragment; this fragment is known to lack heparin-, collagen-, and fibrin-binding activities (11).

Effect of Concanavalin A-mediated Spreading

The lectin concanavalin A binds to cell surface glycoconjugates and mediates cell spreading (26, 27; Fig. 12). Such lectin-mediated spreading was not inhibited by synthetic decapeptide I in an experiment in which fibronectin-mediated spreading was inhibited completely (Fig. 12). It is important to note that no inhibition could be demonstrated even at low concentrations of adsorbed concanavalin A, where inhibition might be expected to be detected most easily.

DISCUSSION

Our major conclusions are (*a*) fibronectin-mediated adhesion as measured by a standard cell-spreading assay can be inhibited by fibronectin itself; (*b*) similar inhibition is produced by a purified cell-binding fragment of 75,000 daltons and two synthetic peptides from this region, but not by a series of other proteins and peptides; (*c*) the inhibition appears to be

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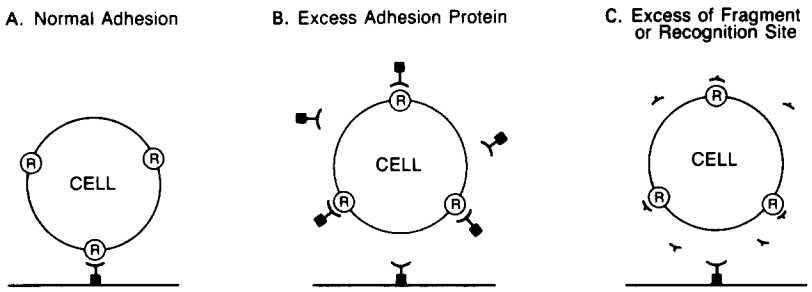

FIGURE 13 Theoretical interpretation of the autoinhibition of fibronectin function by fibronectin and its fragments. (*A*) Cell-to-substrate adhesion mediated by fibronectin adsorbed to a substrate. *R* indicates a postulated saturable receptor for fibronectin. The receptor binds to the cell-binding (recognition) site on fibronectin (curved appendage on the closed square). (*B*) Inhibition of fibronectin-mediated adhesion by incubation of cells with an excess of soluble fibronectin. A sufficiently high concentration of fibronectin would occupy most receptors, resulting in inability of these receptors to bind to substrate-adsorbed fibronectin. (*C*) Inhibition of fibronectin-mediated adhesion by fragments of fibronectin. Fragments that can still be recognized by the postulated receptor could also block the receptors. This model could be generalized to all cell adhesion proteins that function by binding to a specific, saturable receptor.

competitive, and can be overcome at least partially by higher concentrations of adsorbed fibronectin or by substantially longer incubation times; and (*d*) similar inhibition is found in an assay measuring fibronectin-mediated cell attachment to type I collagen, but not in an assay of fibronectin-independent cell spreading mediated by a lectin.

The simplest interpretation of our results is summarized in Fig. 13. Fibronectin is known to be highly effective as a cell-to-substrate adhesion molecule after it has been adsorbed to a substrate (3–9, 28, 29; Fig. 13*A*). However, our data indicate that the same molecule can become inhibitory if it is present in sufficient quantities in solution (Fig. 13*B*); certain fragments of this adhesive molecule can also be inhibitory (Fig. 13*C*). The most likely target for this specific inhibition would be a saturable, receptor-like moiety.

The concentrations of soluble fibronectin required for half-maximal inhibition may provide an estimate of the apparent affinity of soluble fibronectin for its "receptor" on the cell surface. The most effective inhibitor was the cell-binding fragment, which inhibited spreading maximally at a concentration of $\sim 10^{-5}$ M. If such inhibition requires saturation by a 10-fold excess of protein, the affinity (dissociation constant) would be 10^{-6} M. Recently, direct binding studies with tritiated plasma fibronectin yielded an apparent dissociation constant of 0.8×10^{-6} M (S. K. Akiyama and K. M. Yamada, submitted for publication).

The decrease in effectiveness of this autogenous inhibition when more fibronectin was adsorbed onto the substrate or when the incubation time was prolonged may be explained by the multivalency, and therefore higher expected effective affinity, of molecules adsorbed onto a substrate. In fact, it had been suggested previously that fibronectin requires aggregation or adsorption onto a surface such as a culture dish or a bead in sufficiently high concentrations in order to interact with a low-affinity receptor (2, 30). Substrate-attached, multivalent ligands would be expected to compete more effectively for cellular receptors than monovalent or divalent ligands such as intact fibronectin, its cell-binding fragment, and peptides. Increased quantities of substrate-adsorbed fibronectin or longer incubation times would be expected to favor the formation of adhesive bonds with fibronectin on the substrate, thus permitting cell spreading even in the presence of soluble autogenous inhibitors.

A previous study from our laboratory reported the inhibition of cell attachment to collagen after preincubation of cells with a cell-binding fragment of *cellular* fibronectin; the final concentrations of fragment were quite low (<14 μg/ml). The inability of our and other laboratories to repeat this observation with equal concentrations of soluble plasma fibronectin or its cell-binding fragment supports the hypothesis that the cellular form of fibronectin can bind to cells with high apparent affinity because it is aggregated; a requirement for fibronectin to be aggregated or adsorbed to a substrate to bind effectively to its receptor has been suggested (2, 4, 28–30). Our results, however, suggest the existence of direct interactions of soluble molecules with the putative fibronectin receptor, since soluble plasma fibronectin, its 75,000-dalton fragment, and highly soluble, hydrophilic synthetic peptides can all function effectively as inhibitors.

The efficacy of synthetic peptides as inhibitors suggests that they may be useful probes of fibronectin-related adhesion in vivo. The synthetic decapeptide was specific in that it did not inhibit another cell-spreading event, i.e., lectin-mediated spreading, but it is important to note that the specificity of such peptides for fibronectin as compared with other attachment factors such as laminin remains to be determined.

Inhibition of normal rat kidney cell attachment by related synthetic peptides has been reported recently by Pierschbacher and Ruoslahti in work accepted for publication after the completion of our study (31). They discovered that the sequence Arg-Gly-Asp-Ser was the minimal sequence necessary to mediate cell attachment, and they were able to estimate the affinity constants of peptides containing this sequence for binding to the cell surface as $3–6 \times 10^{-4}$ M. The inhibitory activities of synthetic peptides in their assay were less than for the synthetic peptides in our study, perhaps as a result of differences in assay conditions or cell types.

Our experiments indicate that adhesion proteins can display either positive or negative activities depending on their concentrations and locations, i.e., in solution or bound to a substrate. The concentration of fibronectin in human plasma is 0.3 mg/ml; it is interesting that some inhibition was detected even at 1.5 mg/ml (Fig. 2). It is possible that similar antagonistic relationships might occur in vivo, and that weakening of adhesive interactions might result from events that increase the local concentrations of soluble adhesive proteins, e.g., in certain disease states or possibly even at localized regions on the cell surface near the sites of secretion of such molecules.

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Note Added in Proof: Plasma fibronectin and a mixture of its proteolytic fragments were recently found to produce a partial, tran-

sient inhibition of hepatocyte attachment to fibronectin (Johansson, S., and M. Höök, 1984, *J. Cell Biol.*, 98:810–817).

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