Fibronectin's Cell-adhesive Domain and an Amino-terminal Matrix Assembly Domain Participate in Its Assembly into Fibroblast Pericellular Matrix*

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Fibroblasts organize the modular cell-adhesive glycoprotein fibronectin into a highly structured pericellular matrix by poorly understood mechanisms. Previous studies implicated an amino-terminal domain in matrix assembly and suggested that fibronectin's cell-adhesive domain and the corresponding fibroblast receptor were not involved in this process. To further elucidate the fibronectin region(s) involved in matrix assembly, we mapped a library of proteolytic fragments and antibodies to various fibronectin domains. The fragments and antibodies were used to probe the role of fibronectin's amino-terminal and cell-adhesive domains in a fibroblast matrix assembly assay. We found that fibronectin fragments including the first 25-kDa sequence of fibronectin and antibodies to amino-terminal domains inhibited pericellular matrix assembly. Polyclonal antibodies to the 40-kDa collagen binding domain following the 25-kDa amino-terminal domain also inhibited matrix assembly. However, collagen binding is not required for matrix assembly as neither monoclonals blocking collagen binding nor purified collagen binding domains themselves inhibited matrix assembly. Therefore, the amino-terminal region of fibronectin contains a site important in matrix assembly, and most activity is present in the first 25-kDa of fibronectin. Fibronectin's cell-adhesive domain and the fibroblast receptor binding to this domain also play an important role in fibronectin matrix assembly. Apart from a monoclonal antibody to the amino-terminal domain, only monoclonal antibodies binding to fibronectin's cell-adhesive domain and inhibiting cell adhesion also inhibited matrix assembly. In addition a 105-kDa fragment containing the cell-adhesive domain inhibited matrix assembly. We conclude that at least two discrete and widely separated sites in fibronectin with different binding properties—the carboxy-terminal fibronectin cell-adhesive domain and an amino-terminal fibronectin assembly domain localized primarily within the first 25 kDa—are required for fibronectin pericellular matrix assembly by fibroblasts. Fibronectin's cell-adhesive domain and its cell surface-receptor complex appear to be involved in the matrix assembly process prior to a step involving the amino-terminal domain. We believe that this step is likely to be the initiation of cell-associated fibronectin fibril formation by the fibronectin-adhesive-receptor complex.

Fibronectins are a family of plasma and pericellular matrix cell-adhesive glycoproteins with multiple binding domains for cells and other extracellular macromolecules (1–3). Cultured fibroblasts organize fibronectin into a highly structured, disulfide-cross-linked pericellular matrix containing other matrix components (4–7). A similar fibronectin-rich pericellular matrix has been implicated in the directed movement of embryonic cells and in the connective tissue deposition and cell migration characteristic of wound healing (8–12). Thus, elucidating the cellular and molecular details of fibronectin matrix assembly by fibroblasts should aid our understanding of events as fundamental as wounding and embryogenesis. However, the mechanism of fibronectin matrix assembly is less well understood than that of cell adhesion which involves the binding of a carboxyl-terminal site in fibronectin containing the sequence Arg-Gly-Asp-Ser to a specific polypeptide cell surface receptor complex (13–15).

The highly modular structure of fibronectin lends itself to structure-function studies using antibodies and proteolytic fragments to probe the role of domains (3). Using this approach, we found that antibodies to the amino-terminal collagen binding domain inhibited binding of fibronectin to collagen and collagen deposition in fibroblast cultures (16, 17). Unexpectedly, these antibodies also prevented the assembly of a normal fibronectin matrix by fibroblasts, providing the first evidence that an amino-terminal domain of fibronectin was somehow involved in matrix assembly (17). Subsequent studies confirmed that the incorporation of soluble plasma fibronectin into matrix, a potential step in matrix assembly (18–20), involves an amino-terminal site within the first 70 kDa of fibronectin (20). Similarly, a 70-kDa amino-terminal fibronectin fragment was found to inhibit endogenous matrix assembly by a variant of the ganglioside-deficient cell line NCTC 2071A (21).

Thus, antibody and fragment inhibition studies suggest that a site in the amino-terminal 70-kDa sequence of fibronectin is important in matrix assembly. However, neither the precise

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location nor the specific role of this so-called “matrix assembly domain” is clear. In particular, as the amino-terminal-directed antibodies and fragments inhibiting endogenous fibronectin assembly also competitively inhibit fibronectin-collagen binding (17, 21) the relationship between collagen binding and fibronectin matrix assembly remains unclear. Moreover, the involvement in fibronectin matrix assembly of other binding sites such as fibronectin’s well-characterized cell-adhesive domain has not been well explored.

To help answer these questions we assembled a library of fibronectin fragments and monoclonal and polyclonal antibodies to human plasma fibronectin. These fragments and antibodies were applied in a fibroblast matrix assembly assay to probe the fibronectin domains involved. Our results demonstrate that this process requires the participation of both the fibronectin cell-adhesive domain and its cellular receptor and that of a matrix assembly domain located primarily within the first 25 kDa of fibronectin. The incorporation of plasma fibronectin into an established fibroblast matrix is not inhibited by a large cell-adhesive fragment (20), whereas endogenous fibronectin matrix assembly is inhibited by this fragment and by antibodies blocking cell adhesion (this work). Therefore, we believe that the cell-adhesive domain and its receptor participate in an event either occurring before the involvement of the amino-terminal matrix assembly domain or an event unique to the assembly of endogenous (i.e. cellular) fibronectin. Because the fibronectin cell-adhesive receptor is co-distributed with the fibronectin matrix found on the fibroblast surface soon after matrix formation is initiated (14, 22, 23), we propose that the fibronectin cell-adhesive-receptor complex somehow nucleates fibronectin matrix assembly.

EXPERIMENTAL PROCEDURES

RESULTS

We constructed a library of fibronectin polypeptides using limited proteolysis and used immunoblotting to establish the binding sites of monoclonal antibodies (Table I). Certain monoclonals were then used to assist mapping by establishing the origin of smaller proteolytic fibronectin fragments. Finally, the fragments and antibodies were employed in a qualitative assay of fibroblast pericellular matrix assembly. The mapping data and results of binding experiments are presented in the accompanying Miniprint Supplement.

Effects of Antibodies and Fragments upon Pericellular Matrix Assembly Amino-terminal Domains—IMR-90 deposit an abundant fibronectin-rich pericellular matrix during 48 h in culture (Fig. 11A). This panel is from a well containing 100 μg/ml monoclonal antibody D9b, which inhibits fibronectin binding to collagen. The staining pattern was indistinguishable from cultures receiving no antibody or preimmune IgG (see Fig. 11, panels E or J). As previously described (17), polyclonal Fab’ to the 60-kDa collagen binding domain totally blocked pericellular matrix assembly (Fig. 11F). Approximately 100 μg/ml Fab’ were required for near-maximal activity. When affinity-purified Fab’ specific for the amino-terminal 40-kDa fragment of the 60-kDa collagen binding domain or to its 20-kDa carboxyl-terminal domain were tested in the matrix assembly assay, the anti-40-kDa Fab’ blocked matrix assembly at concentrations as low as 10 μg/ml (Fig. 11C), whereas at this and higher concentrations, Fab’ to the 20-kDa carboxyl-terminal domain were much less effective (Fig. 11D). Interestingly, IgG was less effective at inhibiting matrix assembly than Fab’, suggesting that anti-fibronectin IgG can cross-link fibronectin molecules possibly mimicking a physiologic fibronectin-fibronectin interaction, whereas univalent Fab’ blocks an amino-terminal binding site for a receptor, another fibronectin molecule, or other matrix molecules.

The pattern of inhibition of matrix assembly by fragments from the amino-terminus of fibronectin correlated well with the results of antibody studies. The 70-kDa amino-terminal fragment containing all nine type I homologous regions (28) inhibited matrix assembly (compare Fig. 11E, control, with 11F, containing the 70-kDa fragment). After cleavage of the 70-kDa fragment to 25- and 40-kDa subfragments, the mixture retained considerable matrix inhibitory activity (Fig. 11G). The 25-kDa amino-terminal fragment alone (Fig. 11H) inhibited matrix assembly, whereas purified 40- and 60-kDa collagen-binding fragments at concentrations as high as 2 mg/ml had no demonstrable effect (16) (not shown). Monoclonal 304 to the 25-kDa amino-terminal domain also inhibited matrix assembly (Fig. 11J), but monoclonal N-287 binding to the link region between the 25- and 60-kDa amino-terminal domain and the 40- and 60-kDa collagen binding domain had no effect (not shown).

We have not yet tested the effects of the 30/40-kDa carboxyl-terminal fragment containing type I repeats upon matrix assembly.

Cell-adhesive Domain—The cell-adhesive chymotryptic 105-kDa fragment inhibited matrix assembly (compare Fig. 11J, control, with 11K, containing the 105-kDa fragment). Contamination of 105-kDa fragment preparations with amino-terminal fragments could potentially account for inhibition of matrix assembly activity. However, a dose-response experiment with a 25-kDa fragment revealed that 0.25 mg/ml was only slightly inhibitory in this assay. Because the 105-kDa preparations gave single bands upon SDS-polyacrylamide gel electrophoresis when overloaded under conditions where a 5% contamination with the 25-kDa fragment could readily be detected, the 105-kDa preparation could not have contained more than 50 μg/ml 25-kDa fragment. Thus, contamination with amino-terminal fragments could not account for the observed inhibition with the 105-kDa fragment.

Similar to the effect of the 105-kDa cell-adhesive fragment, Fab’ of the anti-cell-adhesive monoclonal N-294 resulted in a marked decrease in the total numbers of matrix fibrils, absent short cell-associated fibrils, and the appearance of abnormally thick fibers (Fig. 11L). We previously found that Fab’ from this monoclonal did not appear to inhibit matrix assembly (44, 45). However, in three separate experiments, Fab’ containing less than 0.3% dimeric IgG purified by molecular exclusion high performance liquid chromatography reproducibly inhibited pericellular matrix assembly.

Because cell movement is likely to play an important role in structuring the fibronectin matrix, it was important to determine whether antibodies altered the normal parallel alignment of IMR-90. As shown in Fig. 12, A and B, normal

1 Portions of this paper (including Experimental Procedures,” Figs. 1-10, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3241, cite the authors, and include a check or money order for $8.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HAT, hypoxanthine, thymidine, amethopterin; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco’s modified Eagle’s medium; CHO, Chinese hamster ovary; TPCK, tosylphenylalanyl chloromethyl ketone.
parallel alignment of IMR-90 was not grossly altered by a presence of $[35S]$cysteine and $[35S]$methionine. The medium, cultures receiving concentrations of amino-terminal fragments consisting of a larger number of more rounded fibroblasts in cultures (not shown), but no gross alterations suggesting abnormalities of cell movement.

Inhibition of cell adhesion by antibodies to fibronec廷's cell-adhesive domain was correlated with their ability to inhibit matrix assembly. Compared with controls (Fig. 12C), N-294 inhibited matrix assembly (Fig. 12D), whereas N-295 had little effect upon matrix assembly (Fig. 12E). This agrees well with their differing ability to inhibit IMR-90 adhesion to fibronectin (Table II). The combination of N-294 and N-295 at 100 μg/ml each augmented the inhibition observed with N-294 alone (Fig. 12F). Interestingly, these antibodies appeared to give additive inhibition of Chinese hamster ovary cell adhesion (not shown). Monoclonal 333, a good inhibitor of cell attachment, was also an effective inhibitor of matrix assembly, at least in IgG form (Fig. 12G).

Biochemical Confirmation of the Qualitative Matrix Assembly Assay—Continuous (and pulse) metabolic labeling of IMR-90 cultured with fragments or antibodies inhibiting matrix assembly in the qualitative assay revealed total inhibition of fibronec廷 deposition in the disulfide-cross-linked sodium deoxycholate-insoluble matrix fraction. Fig. 13 displays the results of a dose-response experiment in which IMR-90 were cultured with 0–1 mg/ml 70-kDa fragment for 48 h in the presence of $[35S]$cysteine and $[35S]$methionine. The medium, total cell-associated, and sodium deoxycholate-insoluble fibronec廷 content was determined by immunoprecipitation and SDS-polyacrylamide gel electrophoresis and fluorography. The 70-kDa fragment at 0.25 mg/ml (3.5 $\times$ 10$^{-6}$ M) completely inhibited fibronec廷 incorporation into the deoxycholate-insoluble matrix pool. In addition, there was a dose-dependent increase in total fibronec廷 accumulation accompanying the inhibition of fibronec廷 deposition in the cell layer, reaching 2.3-fold at 1.0 mg/ml 70-kDa fragment (Table IV). The increased fibronec廷 accumulated exclusively in the culture medium. The profile of labeled polypeptides in medium and cell layer remained similar in control and experimental cultures (compare lanes 1 and 4 in the total cell category in Fig. 13), indicating that the 70-kDa fragment had no deleterious effects upon general protein synthesis. Although the results shown were obtained in an experiment using the 70-kDa fragment, similar results have been obtained with all fragments at the concentrations used in Fig. 11 as well as with anti-40-kDa Fab'.

**DISCUSSION**

Validity of the Qualitative Matrix Assembly Assay—We utilized immunofluorescence microscopy to monitor fibronec廷 matrix assembly by fibroblasts. In parallel metabolic labeling and immunoprecipitation experiments, inhibition of matrix assembly judged qualitatively was accompanied by near-total inhibition of fibronec廷 incorporation into the deoxycholate-insoluble matrix fraction and by 2–3-fold increases in fibronec廷 accumulation. Thus, this matrix assembly assay appears to reflect accurately the incorporation of endogenous fibronec廷 into a disulfide-cross-linked insoluble matrix (4–7). A quantitative assay is now being used to study the matrix assembly process further, but the qualitative assay proved quite useful for the rapid identification of important domains.

Fibronectin Possesses an Amino-terminal Matrix Assembly Domain Located Primarily within the First 25 kDa—Our results clearly confirm the presence of an amino-terminal matrix assembly domain in fibronec廷 initially suggested by the observation that antibodies to the amino-terminal collagen binding domain totally prevented matrix assembly (17) and confirmed by subsequent studies demonstrating that amino-terminal fragments inhibited plasma fibronec廷 incorporation into matrix (19, 20) and endogenous matrix assembly (21). Both a monoclonal antibody to the 25-kDa amino-terminal domain and the domain itself inhibited endogenous matrix assembly. A 70-kDa fragment extending beyond the first 25 kDa of fibronec廷 was also a potent inhibitor of matrix assembly, although fragments including the carboxyl-terminal portions of this domain (the 40- and 60-kDa collagen-binding fragments) were by themselves largely inactive in the absence of the 25-kDa domain.

We cannot exclude the possibility that the 25-kDa amino-terminal domain requires an additional carboxyl-terminal sequence for full activity, as the 70-kDa amino-terminal fragment is more inhibitory in the quantitative plasma fibronec廷 incorporation assay than the 25-kDa amino-terminal fragment (20). However, because neither an antibody binding to

**TABLE I**

Summary of fibronectin fragments and monoclonal antibodies used in this study

<table>
<thead>
<tr>
<th>Fragment name</th>
<th>Amino-terminal sequence</th>
<th>First residue</th>
<th>Binding activity</th>
<th>Protease used</th>
<th>Anti-40 kDa 20 kDa</th>
<th>Anti-40 kDa</th>
<th>Anti-20 kDa</th>
</tr>
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<tbody>
<tr>
<td>FN</td>
<td>QAQQ</td>
<td>1</td>
<td>S, G, C, H</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>25 kDa</td>
<td>QAQQ</td>
<td>1</td>
<td>S</td>
<td>N-elastase</td>
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<td>+</td>
</tr>
<tr>
<td>29 kDa</td>
<td>QAQQ</td>
<td>1</td>
<td>S</td>
<td>Trypsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>40 kDa</td>
<td>VYQPQPHPQP</td>
<td>263</td>
<td>G</td>
<td>P-elastase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>60 kDa</td>
<td>VYQPQPHPQP</td>
<td>263</td>
<td>G</td>
<td>N-elastase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>100 kDa</td>
<td>SPLVATSESE/</td>
<td>689/692</td>
<td>C, H</td>
<td>N-elastase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VATSSES</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140 kDa</td>
<td>SPLVATSESE/</td>
<td>689/692</td>
<td>C, H</td>
<td>N-elastase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VATSSES</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105 kDa</td>
<td>SPLVATSESE/</td>
<td>689/692</td>
<td>C, H</td>
<td>Chymotrypsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VATSSES</td>
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</tr>
<tr>
<td>95 kDa</td>
<td>ND</td>
<td>689/692</td>
<td>Pepsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>94 kDa</td>
<td>ND</td>
<td>689/692</td>
<td>Pronase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>111 kDa</td>
<td>IQQSTV</td>
<td>109</td>
<td>Pepsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>40 kDa-C</td>
<td>QTAVTIPAPDTDLK 1594</td>
<td>H</td>
<td>Chymotrypsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>30 kDa</td>
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<td>Chymotrypsin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* ND, not determined.
* Code for binding activity is S, Staphylococcus aureus; G, gelatin; C, cell; H, heparin.
* N-elastase, neutrophil elastase; P-elastase, pancreatic elastase.
FIG. 11. Effect of antibodies and fragments upon pericellular matrix assembly by cultured fibroblasts. IMR-90 were cultured with the indicated addition for 48 h, rinsed, fixed, and stained for fibronectin pericellular matrix using affinity-purified anti-fibronectin conjugated to Texas Red. A, 100 μg/ml monoclonal antibody D9b which inhibits fibronectin-collagen binding. This is a control for panels B–D (preimmune IgG and Fab' gave identical results, see panels E and J). B, polyclonal anti-60-kDa Fab', 100 μg/ml; C, anti-40-kDa Fab', 100 μg/ml; D, anti-20-kDa Fab', 100 μg/ml; E, control for F–I; F, 70-kDa amino-terminal fragment, 1.8 mg/ml; G,
FIGURE 12. Effect of antibodies to fibronectin's cell-adhesive domain upon IMR-90 morphology and matrix assembly. Panel A, control culture without antibody; panel B, phase photomicrograph of IMR-90 cultured in medium containing 100 μg/ml each N-294 and N-295 (see panel F); panel C, fibronectin matrix of IMR-90 cultured with no additions. Panels D-G are fluorescence micrographs of the fibronectin matrix of IMR-90 cultured with: 100 μg/ml N-294 Fab' (D); 100 μg/ml N-295 Fab' (E); 100 μg/ml each N-294 and N-295 Fab'(F); 100 μg/ml of 333 IgG (G). Scale bar in panel B represents 100 μm. Panel A is the same magnification. Scale bar in panel F is 50 μm and applies to panels C-G.

same concentration of 70-kDa fragment used in F cleaved to 25- and 40-kDa fragments with pancreatic elastase; H, purified 25-kDa amino-terminal fragment (2.1 mg/ml) alone; I, monoclonal 304 to the 25-kDa amino-terminal domain, 100 μg/ml; J, control for K and L; K, 105-kDa chymotryptic fragment, 1 mg/ml; L, monoclonal N-294 Fab' to the cell-adhesive domain, 100 μg/ml. Bar in L, 100 μm.
Fibronectin Domains Involved in Pericellular Matrix Assembly

**TABLE IV**

Effect of the 70-kDa amino-terminal fragment upon fibronectin accumulation in IMR-90 cultures

<table>
<thead>
<tr>
<th>70-kDa fragment</th>
<th>Medium</th>
<th>Cell</th>
<th>Deoxycholate-insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.25</td>
<td>210</td>
<td>17</td>
<td>&lt;10</td>
</tr>
<tr>
<td>0.50</td>
<td>218</td>
<td>9</td>
<td>&lt;10</td>
</tr>
<tr>
<td>1.0</td>
<td>232</td>
<td>6</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

These data are taken from the experiment depicted in Fig. 13. Fibronectin is expressed in arbitrary units based upon laser scanning densitometry of immunoprecipitated samples scanned within the linear response range. About 44% of the fibronectin in the cell layer was deoxycholate-insoluble in the cultures not receiving the 70-kDa amino-terminal fragment, whereas none was detectable in the cultures receiving the 70-kDa fragment. During the 48 h of culture, approximately 10% of the total fibronectin synthesized was deposited in the cell-associated fraction.

**FIG. 13. Effect of the 70-kDa amino-terminal fragment upon fibronectin accumulation and deposition in fibroblast pericellular matrix.** IMR-90 were cultured with none (lane 1), 0.25 (lane 2), 0.5 (lane 3), or 1.0 mg/ml (lane 4) 70-kDa fragment in Dulbecco’s modified Eagle’s medium containing 5% of the normal cysteine and methionine content and 25 μCi/ml each [35S]cysteine and [35S]methionine for 48 h. The cultures were separated into three fractions: the culture medium (MED), the sodium deoxycholate-insoluble fraction (DOC), and the total cell layer (CELL). The labeled polypeptides are displayed in the left panel (TOTAL) and samples immunoprecipitated with antifibronectin IgG in the right panel (IP). This is a fluorograph of a 7.5% SDS-polyacrylamide gel run reduced. The sodium deoxycholate-insoluble fraction was obtained by sequential extraction of the cell layer with: (a) 3% Triton X-100 in phosphate-buffered saline containing 2 mM PMSF and 2 mM EDTA. (b) DNasel (0.1 mg/ml in 1 M NaCl containing 2 mM PMSF and 2 mM EDTA). (c) Two extractions with 2% sodium deoxycholate containing 2 mM PMSF, 2 mM EDTA, and 2 mM N-ethylmaleimide. The remaining deoxycholate-insoluble matrix was dissolved in the same solution lacking N-ethylmaleimide and containing 5 mM dithiothreitol. Before immunoprecipitation, iodoacetamide was added to 20 mM to prevent inactivation of the IgG. The position of the fibronectin monomer is indicated by the arrowhead on the left.

The fluorograph is overexposed to allow visualization of the deoxycholate-insoluble and cell-associated fibronectin. IMR-90 have small pools of intracellular fibronectin. Most of the cell-associated fibronectin is extracellular as it is trypsin-sensitive (not shown). When correctly exposed for quantitative densitometry, medium fibronectin was increased 2.3-fold in the presence of 1.0 mg/ml 70-kDa, and total cellular fibronectin decreased 16-fold. Deoxycholate-insoluble fibronectin was undetectable in the cell cultures receiving the lowest concentration of the 70-kDa fragment. Results of quantitative densitometry are given in Table IV. In less exposed fluorographs, there were no quantitative or qualitative differences in the profile of total cellular polypeptides between the control culture and the cultures receiving the 70-kDa fragment. The two high molecular weight polypeptides immunoprecipitated out of the medium fractions containing 70-kDa fragment are the α-chains of procollagen type I which we have shown previously bind to fibronectin-antifibronectin immune complexes, in this case presumably formed by 70-kDa fragment and antibody (34).

The link sequence between the 25- and 40-kDa domains nor the carboxyl-terminal 40-kDa portion of the 70-kDa domain were inhibitory, we believe a major endogenous matrix assembly site is located primarily in the first 25 kDa of fibronectin. In addition, because the 25-kDa domain inhibits both exogenous plasma fibronectin (20) and endogenous cellular fibronectin assembly (this work) into matrix, cellular and plasma forms of fibronectin utilize the same amino-terminal domain and presumably the same mechanism for at least one phase of matrix assembly.

**Fibronectin Matrix Assembly Is Largely Independent of Collagen Binding**—Collagen binding does not appear to be required for fibronectin matrix assembly. Presumably, the polyclonal Fab' to fibronectin's collagen binding domain which inhibit matrix assembly (17, this work) sterically interfere with a binding event involving the amino-terminal domain. Only the Fab' directed to sites in the 40-kDa amino-terminal sequence adjacent to the 25-kDa amino-terminal domain effectively inhibited both matrix assembly and collagen binding. Although this suggests a link between collagen binding and matrix assembly, results with three monoclonal antibodies to the 40-kDa collagen binding domain suggest otherwise. These monoclonals inhibited collagen binding to fibronectin in vitro and decreased immunochemically detectable procollagen in the fibroblast cell layer. However, they had no detectable effect upon fibronectin matrix assembly even when combined. This result argues strongly against a common site mediating both collagen binding and fibronectin matrix assembly. Additional evidence against a role for collagen binding is given by the observation that neither the 40- nor the 60-kDa purified collagen binding domain inhibited matrix assembly. Other evidence against a role for collagen in fibronectin deposition has been summarized (17, 46).

**Mechanisms by Which Fibronectin's Amino-terminal Domain Participates in Matrix Assembly**—Three hypotheses have been advanced which could explain the involvement of fibronectin's amino-terminal domain in matrix assembly. Based upon results obtained from studying the binding of radiolabeled plasma fibronectin to fibroblast cultures, a specific matrix assembly receptor binding to the amino-terminal domain and distinct from the fibronectin cell-adhesive receptor has been proposed (19, 20). To date, no such receptor has been isolated, and we believe that the published results are compatible with alternative hypotheses such as fibronectin-fibronectin binding mediated by the amino-terminus as discussed below. Ganglioside binding to fibronectin has been implicated in matrix assembly mediated by the amino-terminal domain of fibronectin (21, 47, 48). Ganglioside-deficient NCTC 2071A cells lack matrix assembly activity yet regain this ability when supplemented with exogenous gangliosides (47). A revertant strain of the NCTC 2071A line assembled matrix spontaneously and had increased ganglioside content, and the newly acquired matrix assembly activity was inhibited by fibronectin's 70-kDa amino-terminal domain (21). Thus, it is particularly interesting that the 25-kDa amino-terminal domain binds to the oligosaccharide moiety of gangliosides (48). Obviously, the oligosaccharides of fibronectin-binding gangliosides should be investigated as potential inhibitors of
matrix assembly. However, evaluating the role of gangliosides in matrix assembly is complicated by their possible involvement in the interaction of fibronectin with its cellular adhesive receptor (49, 50), which we have now demonstrated to be also involved in matrix assembly. In addition to ganglioside binding, the amino-terminus of fibronectin contains a weak cell-adhesive site acting synergistically with the central cell-adhesive domain (51), although the mechanism for this effect is unknown.

The mechanism we favor, fibronectin binding to itself via the amino-terminus, stems from the finding that the amino- and carboxyl-terminal domains are homophilic or self-recognition sites for fibronectin (17, 52). Homandberg and co-workers (53, 54) have found that the amino-terminal and carboxyl-terminal domains containing sequences of type I homology contain fibronectin-fibronectin-binding sites. Ehrißmann et al. (55) also proposed that the amino-terminal 60-kDa sequence of fibronectin possessed homophilic properties. However, there is uncertainty about the precise origin of the 60-kDa fragment they implicated in fibronectin-fibronectin binding and it may have been similar to the 60-kDa collagen-binding fragment described here rather than the 70-kDa cathetic amino-terminal fragment.

Whatever the mechanism, fibronectin fragments lacking the amino-terminal domain are not incorporated into matrix in vivo (18) or in vitro (56). Thus, the amino-terminal domain is undoubtedly important in the matrix assembly process in vivo as well as in culture systems.

**Fibronectin's Cell-adhesive Domain Is Critical for Matrix Assembly**—A very interesting aspect of our results is confirmation of our previous observation that fibronectin's cell-adhesive domain participates in matrix assembly (44). Other than a monoclonal binding to the 25-kDa matrix assembly domain, only monoclonal antibodies binding close to the Arg-Gly-Asp-Ser cell-adhesive sequence and inhibiting fibroblast adhesion inhibited matrix assembly. Whereas previously we found that N-294 IgG inhibited matrix assembly, Fab' from monoclonal N-294 apparently did not. Accordingly, we attributed the effect of IgG to either steric hindrance or cross-linking of fibronectin molecules (45). However, in three separate experiments, N-294 Fab' convincingly inhibited matrix assembly (Figs. 11 and 12).

Antibody blocking experiments raise concerns over steric effects or the cross-linking of dimeric molecules such as fibronectin. However, the inhibition of matrix assembly by N-294 and 333 appears to result from inhibiting the binding of endogenous fibronectin to its cell-surface adhesive receptor. Neither cell adhesion nor their parallel alignment are inhibited by N-294 or by cell-adhesive fragments in the presence of serum; thus alternate explanations such as altered cell adhesion or cell movement seem less likely. Both antibodies effectively inhibit fibroblast adhesion (Table II), and inhibition of reticulocyte precursor adhesion to fibronectin by N-294 correlates directly with the expression of the fibronectin-receptor complex (57). The specificity of this inhibition is emphasized by monoclonal N-295 which binds to the 11-kDa pepsim-generated cell-adhesive fragment very close to the binding site of N-294 and 333. N-295 is a very weak inhibitor of IMR-90 fibroblast and reticulocyte precursor adhesion (57) and of matrix assembly. However, its effects were additive to the inhibition of both cell adhesion and matrix assembly by N-294.

Additional evidence linking the cell-adhesive site with matrix assembly was provided by the finding that the chymotryptic 105-kDa fragment containing a cell-adhesive site (26) and no other known binding domains also inhibited matrix assembly. This result is compatible with the 105-kDa fragment competing with endogenous fibronectin for the adhesive receptor (42, 54). This mechanism predicts that the 105-kDa fragment should be co-localized with the fibronectin-receptor complex, whereas little staining of the cells was observed using polyclonal antibodies which react with the 105-kDa fragment (Fig. 11K). However, the images depicted in Fig. 11 were obtained using fixed exposure times based upon the much brighter controls. Based upon our experience visualizing the fibronectin-receptor complex in IMR-90, we might not visualize small amounts of 105-kDa fragment bound to the receptor. Additional studies using anti-receptor antibodies and fragment-specific monoclonals are underway to clarify this apparent paradox.

Our observed inhibition of endogenous matrix assembly by the 105-kDa fragment contrasts sharply with the results obtained in studies of exogenous plasma fibronectin incorporation into matrix. McKeown-Longo and Mosher (20) demonstrated that a similar fingerless cell-adhesive fragment derived from the central portion of fibronectin had no inhibitory effect. This difference likely stems from a major difference between the assays. In our assay, fragments or antibodies are introduced shortly after plating the fibroblasts and thus potential inhibitors are present throughout the entire process of matrix assembly. In contrast, the plasma fibronectin addition assay is performed after cells have initiated matrix assembly (19, 20). Thus, the plasma fibronectin addition assay would be expected to be more sensitive to perturbation of later steps in matrix assembly and possibly might not detect critical early steps.

Based upon this logic, it seems reasonable that our results implicate the fibronectin cell-adhesive receptor in steps occurring before the event(s) involving the amino-terminal (and possibly the carboxyl-terminal (53, 54) domain. An alternative, but less likely possibility is that endogenous matrix assembly in part utilizes a different pathway than that of exogenous plasma fibronectin.

Two alternative models for participation of the fibronectin receptor complex in matrix assembly suggest themselves. First, every fibronectin molecule may have to bind to the fibronectin-adhesive-receptor complex before it can be inserted into a fibronectin fibril. In this case, blocking fibronectin receptor function will inevitably prevent subsequent matrix assembly regardless of the state of the pre-existing matrix. Alternatively, the fibronectin receptor could somehow initiate (e.g. by promoting the proper alignment of molecules for homophilic binding) fibril formation shortly after the fibroblasts are plated and spread. Once initiated, fibrils could then utilize a receptor-independent mechanism such as lateral and staggered homophilic association via the type I repeats to grow at sites distal to the receptor. This model predicts that blocking fibronectin receptor function will only inhibit matrix assembly shortly after fibroblasts are subcultured. Once initiated, matrix assembly should continue unimpeded. Studies are underway to distinguish between these two models and to determine the role of the amino-terminal domain in matrix assembly.

Apart from this study, several lines of evidence support a role for the fibronectin-receptor complex in matrix organization. The receptor complex is concentrated at sites of initial pericellular matrix formation on the fibroblast surface (15, 22, 23). Oncogenic transformation results in phosphorylation of the receptor accompanied by loss of surface fibronectin and redistribution of the receptor to a more diffuse organization (59, 60). Synthetic peptides competing with fibronectin for receptor occupancy as well as cytoskeletal disrupting agents
lead to loss of surface fibronectin fibrils (14, 60). It seems clear that, in addition to the fibronectin-receptor complex's role in matrix assembly, it also links the cytoskeleton with the matrix once assembled. Thus, the movement of fibroblasts coupled to the fibronectin matrix via the receptor complex may help structure the pericellular matrix.

Fibronectin-Receptor Interaction—Our results on monoclonal antibody inhibition of fibroblast adhesion and spreading support the hypothesis that fibronectin-mediated fibroblastic cell adhesion is localized to a small domain in the central portion of the molecule but that the receptor-binding surface of this domain is larger than the short sequence of amino acids which can inhibit receptor binding (13, 14, 42, 58, 61-63). The two monoclonals binding within approximately 10-kDa sequence (N-294 and N-295) of the cell-adhesive sequence differ dramatically in their biological activities (53). Further structural work would be required to determine why N-294 is a much better inhibitor of fibroblastic cell adhesion than N-295. However, additional peptide mapping suggests that N-294 binds to the ninth type III repeat in plasma fibronectin, the type III repeat amino-terminal to the tenth repeat which contains the Arg-Gly-Asp-Ser cell-adhesive epitope. Our results are compatible with the fibroblast receptor binding to a region of fibronectin comprised of more than one type III repeat as proposed from affinity isolation studies (61), or with a folded conformation of the type III repeats in this region which bring the N-294 epitope in proximity to the Arg-Gly-Asp-Ser cell-adhesive sequence (62). It will be informative to determine whether monoclonal antibody 333, which binds to the 11-kDa sequence, and strongly inhibiting fibroblast adhesion also interferes with the binding of N-294 to fibronectin (arguing that N-294 merely inhibits by sterically limiting access to the 11-kDa sequence) or whether the ninth type III repeat is actually involved in receptor binding.

The mechanism by which fibronectin-depleted serum reverses the inhibitory effect of N-294 on fibroblast spreading is unclear. Presumably non-fibronectin-derived factors such as serum-spreading factor (vitronectin) replace the requirement for fibronectin in adhesion and spreading (63). Regardless of the mechanism, this serum reversal allowed us to culture fibroblasts with N-294 (and the 105-kDa fragment) without affecting adhesion, yet perturbing matrix assembly.

Preventing Fibronectin Matrix Assembly Augments Its Accumulation in Fibroblast Culture—Fibroblasts prevented from assembling a fibronectin matrix accumulated about 2-fold more fibronectin over 48 h (Fig. 13), virtually all in the culture medium. Identical results were obtained when cultures were metabolically labeled for the last 10 h of the 48-h period or for the entire 48 h. There are two likely explanations for the increased total fibronectin. First, the absence of normal amounts of pericellular matrix likely communicated to the cell via unoccupied fibronectin receptors may be compensated for by increased fibronectin synthesis. Evidence for this potential regulatory mechanism has been obtained in smooth muscle cell culture, where synthesis of fibronectin and collagen types I and III is decreased when the cells are plated on fibronectin (64). A more trivial explanation is that fibronectin not incorporated into matrix could simply be less susceptible to proteolysis because of physiological antiproteases (α2-macroglobulin, α2-antiprotease) in the culture medium, whereas matrix fibronectin is likely subjected to continuous remodeling during cell movement and division. Thus, preventing fibronectin assembly into matrix could simply prevent its turnover.

REFERENCES

* J. A. McDonald, B. J. Quade, and T. J. Broekelmann, unpublished results.
Fibronectin Domains Involved in Pericellular Matrix Assembly


EXPERIMENTAL PROCEDURES

MATERIALS

Chemicals and Reagents: Fibronectin was purchased from Sigma Chemical Co., St. Louis, Mo. Anti-fibronectin monoclonal antibodies reacting with human, mouse, and rat fibronectins were purchased from Cappel Laboratories, West Chester, Pa. or from Sigma. Pronase-CP was obtained from Calbiochem-Behring. Heparin, bovine plasma fibronectin (29-31), porcine pancreatic elastase, and heparin-Sepharose were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.). Other organic and inorganic chemicals were obtained from Sigma unless otherwise stated.

Fibronectin purification: Human plasma fibronectin was purified by a minor modification of the method of Engvall and Ruoslahti (24) from pooled human plasma using anti-fibronectin-Sepharose, gelatin-Sepharose, and SDS-prefabs.

METHODS

Cell adhesion: Fibroblast adhesion assays were performed using two test cell lines, H929 diploid fetal human lung fibroblasts which secrete fibronectin in culture and A310T murine melanoma cells which do not.

Fibronectin Collagen Binding Assay: Binding of [125I]-labeled fibronectin to denatured collagen was measured by incubating 0.5 mg/ml of fibronectin in a 200 u1 assay volume at 4°C for 20 hrs. The assay was terminated by washing the cell layer.

Fibronectin Immunoblotting: Immunoblotting was performed using 4% SDS-polyacrylamide gel electrophoresis, blotting to glass filter paper, the polypeptide bands cut out and sequenced. Sequence information was compared with the published sequences for human plasma fibronectin deduced from cDNA clones (28) and for bovine plasma fibronectin (29-31).

RESULTS

Fibronectin Collagen Domains: The amount of various proteases used is expressed as a weight ratio to fibronectin. Typically, fibronectin (or other matrix protein) was incubated with pancreatic elastase using 1/5000 elastase for 3 hrs at 37°C and the 60 kD and 40 kD collagen binding fragments separated by gelatin-Sepharose and chromatography.

Elastase digestion: Non-overlapping fragments of the 25 kD aminoterminal binding domain with porcine pancreatic elastase. Fibronectin Polypeptides: The amount of various proteases used is expressed as a weight ratio to fibronectin. Typically, fibronectin (or other matrix protein) was incubated with pancreatic elastase using 1/5000 elastase for 3 hrs at 37°C and the 60 kD and 40 kD collagen binding fragments separated by gelatin-Sepharose and chromatography.
Fibronectin Domains Involved in Pericellular Matrix Assembly

Fibronectin domains involved in pericellular matrix assembly were characterized by immunoblotting with monoclonal antibodies to tryptic and chymotryptic fragments of fibronectin. Monoclonal antibodies N-288 and N-294 bound to the 60 kd fragment containing the central domain of fibronectin, which is involved in collagen binding. These antibodies were used to assess the binding of the central domain to collagen-binding domain (29). However, the antibodies did not bind to the 40 kd fragment containing the carboxy-terminal domain of the 105 kd fragment, which is involved in cell adhesion.

Monoclonal antibodies N-294 bound to the 95 kd fragment released by pronase digestion of the 105 kd fragment, which contains the epitope for N-294. This fragment also contains the epitope for N-295, which binds to the carboxy-terminal domain of the 105 kd fragment. Therefore, the binding of N-294 and N-295 to the same fragment suggests that 11-294 must bind near the carboxy-terminal domain of the 105 kd fragment.

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Antibodies to fibronectin's collagen binding domain: Monoclonal antibody N-294 bound to the 95 kd fragment released by pronase digestion of the 105 kd fragment, which contains the epitope for N-294. This fragment also contains the epitope for N-295, which binds to the carboxy-terminal domain of the 105 kd fragment. Therefore, the binding of N-294 and N-295 to the same fragment suggests that 11-294 must bind near the carboxy-terminal domain of the 105 kd fragment.

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Figure 1. Origin of major proteolytic fibronectin fragments. Binding sites for N-288, P-5288, and P-ELASSE were located by immunoblotting with monoclonal antibodies to tryptic and chymotryptic fragments of fibronectin. Monoclonal antibodies N-288 and N-294 bound to the 60 kd fragment containing the central domain of fibronectin, which is involved in collagen binding. These antibodies were used to assess the binding of the central domain to collagen-binding domain (29). However, the antibodies did not bind to the 40 kd fragment containing the carboxy-terminal domain of the 105 kd fragment, which is involved in cell adhesion.

Figure 2. Immunoblotting of monoclonal antibody N-288 on tryptic (lanes 1-3) or neutrophil elastase (lanes 4-6) digests of fibronectin. Lanes 1 and 2 are stained for polyclonal and lanes 1 and 4 are stained for monoclonal antibodies to fibronectin. Lanes 2 and 4 are stained for polyclonal and lanes 2 and 4 are stained for monoclonal antibodies to fibronectin. Lanes 2 and 4 are stained for polyclonal and lanes 2 and 4 are stained for monoclonal antibodies to fibronectin. Lanes 2 and 4 are stained for polyclonal and lanes 2 and 4 are stained for monoclonal antibodies to fibronectin. Lanes 2 and 4 are stained for polyclonal and lanes 2 and 4 are stained for monoclonal antibodies to fibronectin. Lanes 2 and 4 are stained for polyclonal and lanes 2 and 4 are stained for monoclonal antibodies to fibronectin.

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Fibronectin Domains Involved in Pericellular Matrix Assembly

Limited trypsin cleavage of the 105 kd fragment yielded an 80 kd polypeptide bound by mAbs 200 and 128, and a small portion of the 11 kd carboxyterminal sequence of the 105 kd fragment (Figure 8). This peptide was also bound by N-294 and N-295. Monoclonal N-294 bound fibronectin electrophoresed on a 4% polyacrylamide gel revealed that N-294 bound both the alpha and beta subunits of fibronectin. As noted, chymotryptic digestion of the 105 kd fragment purified a mixture of 160 and 140 kd carboxyterinal fragments from a neutrophil elastase digest. Although the 105 kd fragment released by the neutrophil elastase appeared to contain a fragment clearly visible in the immunoblot; the fragment was not detectable in the immunoprecipitate. The primary difference between the two enzymes appears to be the fragility of the 290 k fragment resulting in differential susceptibility to proteolysis (25,43).

Left panel. Antibodies include: 21, preimmune Fab', A-39, antibodies to the carboxyterminal 20 kd of the 60 kd collagen binding domain. A-40, antibodies to the amino-terminal 20 kd of the 60 kd collagen binding domain. Antibodies to the carboxyterminal 60 kd collagen binding domain which were immunospecificity purified to yield a single A-39, and anti A-40 antibodies. Note that Fab' to the carboxyterminal 20 kd of the 60 kd collagen binding domain have no affect upon collagen binding, whereas the other monoclonals all bind to the carboxyterminal 40 kd of the 60 kd collagen binding domain and inhibit collagen binding.

Table II. Effect of monoclonal antibodies upon human lung fibronectin 1F0-90 adhesion to a fibronectin coated substrate.

<table>
<thead>
<tr>
<th>Antibodya</th>
<th>N-294</th>
<th>N-295</th>
<th>322</th>
<th>125</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>8.9</td>
<td>22.5</td>
<td>19.5</td>
<td>22.7</td>
</tr>
<tr>
<td>50</td>
<td>6.8</td>
<td>19.4</td>
<td>9.5</td>
<td>9.9</td>
</tr>
<tr>
<td>125</td>
<td>2.8</td>
<td>9.3</td>
<td>3.9</td>
<td>21.3</td>
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<tr>
<td>250</td>
<td>1.8</td>
<td>6.1</td>
<td>3.2</td>
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<tr>
<td>1000</td>
<td>2.5</td>
<td>0.8</td>
<td>0.8</td>
<td>10.3</td>
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</table>

A 46 well tissue culture dish was incubated with 2.5 ug/ml of human plasma fibronectin in phosphate buffered saline with a solution of 1/1000 bovine serum albumin. The well was incubated for 1 hr with the immunoprecipitated antibodies to the 40 kd polypeptide of fibronectin. The culture dish was washed, inverted end-over-end 10 times to dislodge loosely attached cells, and the remaining cells lysed and heparinase activity determined colorimetrically (32).

In a quantitative ELISA competitive binding assay, N-294 and N-295 did not compete for fibronectin binding sites on neutrophils tested for collagen binding inhibition at 1F0-90 attachment and spreading. Thus, a pair of 1F0-90 antibodies were identified which are each able to inhibit the spreading of 1F0-90 with the exception of the four monoclonal antibodies above, which prevented the inhibitory effect of N-294 upon attachment and spreading of 1F0-90, allowing us to utilize this antibody in matrix assembly assays as described below.

Table III. Effect of monoclonal antibody N-294 or anti-fibronectin Fab' upon 1F0-90 spreading on fibronectin in the presence or absence of serum.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Antbody</th>
<th>Substrate</th>
<th>Mean cell area</th>
<th>Square microns</th>
</tr>
</thead>
</table>

Experiment A. Polyclonal anti-fibronectin and monoclonal antibody N-294 Fab' inhibited 1F0-90 spreading.

1. Preimmune Fab' - fibronectin 814 +/- 46
2. Anti-Fab' - fibronectin 301 +/- 18
3. N-294 - fibronectin 412 +/- 30
4. Preimmune Fab' - plastic 886 +/- 50
5. Anti-Fab' - plastic 478 +/- 18

Experiment B. Serum reverses anti-fibronectin inhibition of 1F0-90 spreading on plastic.

1. No antibody - plastic 1899 +/- 82
2. Preimmune Fab' - plastic 1792 +/- 94
3. Anti-Fab' - plastic 1209 +/- 12
4. Preimmune Fab' - plastic 1899 +/- 118
5. Anti-Fab' - plastic 1899 +/- 118

* Mean +/- standard error of the mean. b P < 0.05 when with preimmune control when compared by t test.

Results of two experiments are shown. In experiment A, 1F0-90 were plated at 10 cells/well and preimmune Fab' or anti-fibronectin Fab' were added with 10 ug/ml of human plasma fibronectin in pH 7.4 sodium carbonate buffer ("fibronectin") or buffer alone and rinsed. Cells were fixed and stained for 3 hrs, rinsed, fixed, and cell areas determined as described in Methods. Antibodies were added to the cell lines 1 hr before the cells were added.

Experiment B was carried out similarly, except that 1.1 x 105 1F0-90 were plated, and the cells were incubated (in basal medium) alone or containing 10% humid serum depleted of fibronectin for 2-2.5 hours before addition of antibodies. Polyvalent anti-Fab' and N-294 significantly inhibited 1F0-90 spreading on plastic and fibronectin coated substrate.

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