Mode of Action of Fibronectin in Promoting Chicken Myoblast Attachment

$M_r = 60,000$ GELATIN-BINDING FRAGMENT BINDS NATIVE FIBRONECTIN*

(Received for publication, June 11, 1980, and in revised form, December 12, 1980)

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Chicken myoblasts require fibronectin, a disulfide-linked dimeric glycoprotein, for their attachment to and elongation upon a gelatin-coated substrate. Reduced and alkylated horse serum fibronectin no longer binds to a gelatin-Sepharose column, but does promote myoblast attachment and elongation when used to precoat nongelatinized culture dishes, indicating that the attachment-promoting site is unaffected by this treatment. Limited plasmin digestion produces fragments in the range of $M_r = 200,000$ that lack the interchain S-S bridge(s). Such monovalent fragments both bind to gelatin and promote attachment to a gelatin-coated substrate. Digestion with a-chymotrypsin of fibronectin bound to gelatin-Sepharose leads to the release of two fragments of $M_r = 160,000$ and $135,000$; subsequent elution with 4 M urea yields a single fragment of $M_r = 60,000$ in the amount expected if each subunit possesses a gelatin-binding region. The larger chymotryptic fragments lack the ability to bind to gelatin but have essentially the same activity as intact fibronectin in promoting myoblast attachment and elongation when used to precoat nongelatinized dishes. Our results confirm, by direct assays for the respective functions, that the gelatin-binding and attachment-promoting activities are located in discrete regions of each fibronectin subunit and that these can be separated with preservation of the structural integrity required for each activity. Disks precoated with the $M_r = 60,000$ chymotryptic fragment do not promote cell attachment unless fibronectin is present in the culture medium, suggesting that the gelatin-binding fragment also contains a site involved in fibronectin-fibronectin binding. Strongly supporting this suggested assignment, specific binding of fibronectin to the $M_r = 60,000$ fragment was shown by affinity chromatography.

The fibronectins are high molecular weight glycoproteins of cell surfaces, connective tissue matrices, and plasma. They have been implicated in cell-substrate attachment (with attendant changes in cell shape), cell-cell aggregation, cell motility, and clearance of colloids from plasma (for review, see Ref. 1). Fibronectin is known to self-aggregate (2) and to possess binding sites for collagens of various types (both native and denatured; Refs. 3 to 5), for fibrin and fibrinogen (6–9), for heparin (8,10) and proteoglycans (11, 12), and possibly for gangliosides (13).

We have been particularly interested in studying the involvement of fibronectin in the various processes which contribute to skeletal myogenesis in the chicken. We developed a procedure for assaying cell-substrate attachment (14) using a population of terminally differentiating suspended myoblasts (15) that have not been exposed to fibronectin in vitro (16), that do not themselves synthesize fibronectin (17), and that are not significantly contaminated by cells (e.g., fibroblasts) that do (15). It is thus possible to study the effects of added fibronectin in an unambiguous way. In the absence of a source of fibronectin (such as serum), very few of the cells become attached to a gelatin-coated substrate; with added fibronectin, most cells attach and elongate (14).

We have attempted to exploit the advantages of our cell attachment assay in investigating the mechanism of fibronectin action. In this we have been able to rely on the many recent studies of the relationship of fibronectin structure to its functions in other systems. Among the more important findings to emerge from earlier work are these. 1) The sugar residues of fibronectin are not required for any of several biological activities tested (18,19). 2) Cell-associated and plasma forms of fibronectin are probably structurally distinct (20) and may differ in their macromolecular interactions and, hence, in their functions (21). 3) Limited proteolysis with a variety of proteases has allowed the isolation of different fragments of fibronectin containing the gelatin-binding site of the molecule (22–25); additional fragments have been identified which promote cell attachment or spreading to some extent (26–28) or which are able to inhibit cell binding to intact fibronectin (29).

We describe here the production and characterization of the following modifications of horse serum fibronectin: 1) reduced and alkylated fibronectin, which no longer binds to gelatin, but still provides a substrate for myoblast attachment; 2) large fragments from plasmin digestion that retain both gelatin-binding and attachment-promoting activities; 3) complementary chymotryptic fragments, the smaller of which binds to gelatin and does not mediate cell attachment and the larger of which promotes attachment although it does not bind to gelatin. By using the combination of the gelatin-binding fragment and intact fibronectin, we have been able to examine the possible importance of fibronectin-fibronectin interactions in promoting cell attachment.
Enzymatic Cleavage of Fibronectin with Plasmin and Chymotrypsin—Fibronectin was purified from horse serum (Gibco) by affinity chromatography on gelatin coupled to Sepharose 4B (Pharmacia) as described by Chiquet et al. (14). A 0.15% solution of fibronectin in 0.1 M Tris(hydroxymethyl)aminomethane-HCl containing 0.1 M NaCl, pH 7.5, was digested for 10 min at 37 °C with 0.5 unit/ml of porcine plasmin (Sigma, 0.4 unit/mg). The reaction was stopped by the addition of soybean trypsin inhibitor (Sigma) to 0.1 mg/ml. The mixture was then passed over a gelatin-Sepharose column and gelatin-binding material was isolated by elution with 4 M urea (14). For digestion with a-chymotrypsin (Worthington, 68 units/mg) at 22 °C, 9 to 12 mg of fibronectin were first bound to a gelatin-Sepharose column (bed volume: 5 ml) and then 20 ml of a 20 μg/ml solution of chymotrypsin in NaCl/P, were passed over the column (flow rate: approximately 3 ml/min). Fractions (2 ml) were collected in tubes containing soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (Serva Feinbiochemica, Heidelberg) sufficient to give final concentrations of 20 μg/ml and 2 mM, respectively. After extensive washing of the column with NaCl/P, the material that remained bound to gelatin was eluted with 4 M urea (ultrapure; Schwarz/Mann) in NaCl/P,.

Reduction and Alkylation of Fibronectin—Purified horse serum fibronectin was reduced with dithiothreitol (1.2 mg/mg of fibronectin; Sigma) in the presence of 8 M urea (ultrapure; Schwarz/Mann) as described by Konigsberg (29) and alkylated with iodoacetamide (Serva; 2.7 mg/mg of fibronectin, 20 min at 22 °C); the reaction mix was then dialyzed extensively against NaCl/P, at 4 °C.

Cell Cultures and Attachment Assay—Cells obtained by dissociation of 11-day embryonic chick breast muscle were cultured in a serum-free medium (15). This procedure allows myoblasts, which accumulate in suspension, to be separated from fibroblasts, which remain attached to the culture dish (14, 15). Details of this culture system are given elsewhere (15). To test both attachment-promoting and gelatin-binding activity of fibronectin and its derivatives, myoblast suspensions were plated on gelatinized dishes (14) and the respective reagents were added to the culture medium. To assay for attachment-promoting activity independently of gelatin-binding, non-gelatinized dishes were precoated with various concentrations of fibronectin or its modification products (for 1 h at 37 °C unless indicated to the contrary). After precoating, the dishes were rinsed with NaCl/P, and were not permitted to dry before plating the cells. Evaluation of the assay was performed as described previously (14).

Antifibronectin—Specific antisera against electrophoretically pure human plasma fibronectin isolated according to Chen and Mosesson (30) were raised in rabbits as described previously (14, 16). A crude IgG fraction of antiserum precipitated with 40% ammonium sulfate (Merck, Darmstadt) and then redissolved and dialyzed against NaCl/P, was used in the inhibition experiments described under "Results."

Gel Electrophoresis—Polyacrylamide gradient gel electrophoresis in 1.5-mm slabs containing 1% SDS was adapted from the Laemml procedure (31). Except where indicated to the contrary, the gradient of acrylamide (Serva) was from 3% to 15%; no stacking gel was used. Samples to be reduced were heated for 5 min at 35 °C in sample buffer (31) containing 5% β-mercaptoethanol, whereas samples not being reduced were heated in the same buffer without β-mercaptoethanol. Gels were stained with Coomassie brilliant blue R (Sigma). Protein markers were (M, kDa): chicken myosin heavy chain (200,000), rabbit muscle phosphorylase b (95,000), bovine serum albumin (68,000), rabbit muscle puruvate kinase (57,000), chicken M-creatine kinase (40,000), bovine chymotrypsinogen (24,000), and horse cytochrome c (12,000).

Protein Determination—Protein concentrations of fibronectin and its modification products were estimated spectrophotometrically at 280 nm using an absorption coefficient of ε280 = 12.8 (32).

Affinity Columns—Proteins were coupled to CNBr-activated Sepharose 4B (Pharmacia) using the procedures suggested by the supplier.

RESULTS

Fragments of Horse Serum Fibronectin Produced by Digestion with Plasmin and Chymotrypsin—Purified fibronectin was subjected to SDS-polyacrylamide gel electrophoresis both with and without prior reduction (Figs. 1 and 2). The unreduced disulfide-linked fibronectin dimers run as what appears to be a single high molecular weight band (Fig. 1, lane 3; Fig. 2, lane d). Reduction routinely yields two closely spaced subunit bands of approximately equal intensity (Fig. 2, lane a; Ref. 4); our molecular weight estimates for these bands are 235,000 and 222,000. Similar subunit doublets are regularly obtained upon reduction of other fibronectins as well (21, 33, 34).

The plasmin-digested fibronectin was passed over gelatin-Sepharose to isolate any fragments still able to bind to gelatin. The material retained on the column consisted of three bands (Fig. 1, lanes d and h; estimated M, values: 217,000, 203,000, and 189,000). When reduced, these fragments have electrophoretic mobilities very similar to those before reduction; if anything, the nonreduced fragments migrate more rapidly. Thus, all three bands are fragments that have lost that region of the fibronectin molecule which carries the interchain disulfide bond. Similar results have been obtained for fibronectins of other origins using limited proteolysis with plasmin or other proteolytic enzymes (35–37); the interchain disulfide-bearing regions have now been located with some certainty at the COOH termini of fibronectin subunits (38).

Treatment of fibronectin bound to gelatin-Sepharose (23) with chymotrypsin caused the release of two fragments with M, estimated as 135,000 and 160,000 under both reducing and nonreducing conditions (Fig. 2, lanes b and e). Lesser amounts of smaller fragments were released as well, including one prominent band (M, = approximately 31,000). Given the uncertainty of the molecular weight determinations, it is conceivable that this band is produced along with the M, =
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135,000 band by cleavage of the $M_r = 160,000$ fragment. Elution with 4 M urea of the material that had remained bound yielded a single, homogeneous fragment with an apparent $M_r$ of 60,000 when reduced and 51,000 when not reduced (Fig. 2, lanes c and f). That none of these fragments migrated faster when reduced demonstrates that chymotrypsin, like plasmin, cleaves off the terminal region containing the interchain disulfide(s). The markedly slower migration of the reduced gelatin-binding fragment resembles the electrophoretic behavior of a gelatin-binding fragment of human plasma fibronectin produced by digestion with cathepsin D (22). This behavior was interpreted as suggesting that the nonreduced fragment has a globular structure and that reduction of its intrachain disulfides results in an extended conformation that migrates less rapidly (22). Further evidence for the existence and functional importance of intrachain disulfides is presented in the following section.

The procedure whereby fibronectin is treated with chymotrypsin while bound to the gelatin-Sepharose column (23) allows us to estimate the recovery of the $M_r = 60,000$ gelatin-binding fragment. When 20 ml of horse serum were passed over the column and pure fibronectin then eluted with 4 M urea, the yield of fibronectin was approximately 12 mg. If fibronectin from the same amount of serum was digested on the column with chymotrypsin before elution with 4 M urea, the yield of protein was reduced to approximately 3 mg, i.e. to one-fourth. Thus, the yield of gelatin-binding fragment was that expected if both fibronectin subunits bind to gelatin. To our knowledge, this is the first direct evidence that the two fibronectin subunits possess equivalent gelatin-binding regions (cf. 23, 24).

Reduced and Alkylated Fibronectin Does Not Bind to Gelatin—Fibronectin was reduced and alkylated in the presence of 8 M urea under conditions which are expected to allow reduction and alkylation of all disulfides present, intrachain as well as interchain (29). After this treatment, fibronectin ran as a monomer (closely spaced doublet, $M_r = 235,000$ and 220,000) on electrophoresis under both reducing and nonreducing conditions. In contrast to the three plasminolysis fragments of fibronectin, which also lack interchain S-S bridges, reduced and alkylated fibronectin (after dialysis to remove the urea) did not bind to a gelatin-Sepharose column.

This result suggests, but does not prove, that reduction of intrachain disulfides abolishes the three-dimensional structure required for binding to gelatin. Conceivably, the failure of reduced and alkylated fibronectin to bind to gelatin might reflect not a requirement for disulfides in stabilizing the gelatin-binding site, but the inability of alkylated fibronectin to refold into the proper conformation after denaturation in urea. That disulfides are involved in stabilizing the gelatin-binding

| Supplement          | Nuclei in attached cells/dish fixed after 3 h | Nuclei in attached cells/dish fixed after 20 h |
|---------------------|---------------------------------------------|---------------------------------------------|
| Native fibronectin  | 3.2 $\times 10^{-3}$                        | 1.5 $\times 10^{-3}$                       |
| Plasminolysis fragments | 3.0 $\times 10^{-3}$                    | 1.5 $\times 10^{-3}$                       |
| 3% Horse serum      | 2.8 $\times 10^{-3}$                        | 3.0 $\times 10^{-3}$                       |
| None                | 0.3 $\times 10^{-3}$                        | 0.3 $\times 10^{-3}$                       |

Table I
Attachment-promoting activity of native fibronectin and of the fragments produced by plasminolysis tested by adding 40 µg/ml of protein together with the cells to gelatinized dishes.

FIG. 2. Electrophoretic analysis of the chymotryptic digest of fibronectin. a to c, Reduced samples; d to g, unreduced samples; a and d, intact fibronectin; b and e, material released from the gelatin column upon chymotrypsin treatment; c and f, fragment retained by the gelatin column; g, chymotryptic (ChT) and soybean trypsin inhibitor (TI).

FIG. 3. Electrophoretic analysis of the substrates of variously precoated culture dishes used in the attachment assays. Protein concentration of the pretreatment solutions was 0.1 mg/ml in each case and the incubation time was 1 h at 37 °C. The material from whole plates was scraped into 50 µl of sample buffer. a and b, Unreduced samples; e to g, reduced samples; a and c, intact fibronectin; b and d, reduced and alkylated fibronectin; e, plasmin fragments of $M_r \approx 200,000$; f, chymotryptic fragments of $M_r = 135,000$ and 160,000; g, chymotryptic fragments of $M_r = 60,000$. 

Reduced and alkylated fibronectin (after dialysis to remove the urea) did not bind to a gelatin-Sepharose column.

This result suggests, but does not prove, that reduction of intrachain disulfides abolishes the three-dimensional structure required for binding to gelatin. Conceivably, the failure of reduced and alkylated fibronectin to bind to gelatin might reflect not a requirement for disulfides in stabilizing the gelatin-binding site, but the inability of alkylated fibronectin to refold into the proper conformation after denaturation in urea. That disulfides are involved in stabilizing the gelatin-binding
region is, however, strongly suggested by a further experiment. When a gelatin-Sepharose column to which fibronectin had been bound was rinsed with (stepwise) increasing concentrations of dithiothreitol in NaCl/\(\pi\), the bulk of fibronectin which had been bound was rinsed with (stepwise) increasing concentrations of dithiothreitol in NaCl/\(\pi\), the bulk of fibronectin was eluted at a concentration of 100 mM dithiothreitol. In this case, reduction alone abolished affinity to gelatin. Taken together with the evidence suggesting that the \(M_1\) = 60,000 chymotryptic fragment is folded into a disulfide-stabilized conformation, this failure of reduced fibronectin to bind to gelatin indicates that intrachain disulfides are most likely required for maintaining the structural integrity of the gelatin-binding site (cf. 22). It will be shown in the next section that this is not true of the attachment-promoting site.

**Attachment Assays with Fibronectin and Its Modification Products**—The plasminolysis fragments purified over a gelatin-Sepharose column show the same attachment-promoting activity as does intact fibronectin when added together with suspended myoblasts to gelatinized culture dishes (Table I). Neither the dimeric structure *per se* nor that terminal part of the molecule which contains the interchain disulfide(s) is thus required to link the cells to gelatin.

The chymotryptic fragments and reduced and alkylated fibronectin were tested for ability to promote myoblast attachment independently of gelatin binding by using them to precat nongelatinized dishes. All of the these modified fibronectins bound to the dishes as judged by electrophoretic analysis of the material scraped with a rubber policeman from the variously precoated dishes into sample buffer (Fig. 3). Intact fibronectin, reduced and alkylated fibronectin, and the larger fragments produced by chymotryptic cleavage \(^2\) all promoted comparable attachment of the cells in assays with precoated dishes (Table II). By contrast, gelatin alone and the gelatin-binding chymotryptic fragment (\(M_1\) = 60,000) were both inactive (Table II). We conclude from these data that the attachment-promoting activity of fibronectin is not sensitive to reduction and alkylation. This finding was unexpected since reduced fibronectin no longer promotes cell spreading (41-43) and since it has been claimed that binding of fibronectin to cells, as a prerequisite for cell attachment and spreading, requires that the disulfides of fibronectin be intact (41-43). We also conclude (as have others for fibronectins, see introduction) that the gelatin-binding and cell attachment-promoting activities reside in different chymotryptic fragments. Not only was the attachment of cells to substrates precoated with reduced and alkylated fibronectin and with the larger chymotryptic fragments unimpaired, but the elongation of myoblasts occurred to the same extent upon all of the substrates that promoted attachment (Fig. 4). Even though monomeric fragments retain the full activity of native fibronectin, this does not allow us to claim that each of the fibronectin subunits possesses a domain with attachment-promoting activity since native fibronectin could conceivably be composed of one active and one inactive subunit.

The attachment of myoblasts to gelatinized dishes mediated by horse serum or by purified fibronectin can be blocked by antifibronectin (14). The results presented in Table III show that antifibronectin inhibits attachment of myoblasts to all of the active substrates. The inhibition observed in the earlier paper cannot have been due entirely to interference of antibodies with gelatin binding since the antibody preparation also inhibits the attachment mediated by fragments lacking this region.

To test the specific attachment-promoting activity of the active chymotryptic fragments in comparison to native fibronectin, plates were precoated with the fragments and with fibronectin, using solutions of different concentrations. Precoating with solutions containing about 10 \(\mu\)g of protein/ml

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### Table II

| Precoted plates* | Horse serum concentration in attachment assay | Nuclei in attached cells/dish |
|------------------|---------------------------------------------|-----------------------------|
|                  | Fixed at 3 h | Fixed at 20 h |
| Native fibronectin | 0 | 4.8 | 2.8 |
| Reduced and alkylated fibronectin | 0 | 3.9 | 2.4 |
| Chymotryptic fragments of \(M_1\) = 135,000 and 160,000 | 0 | 3.8 | 2.9 |
| Chymotryptic fragment of \(M_1\) = 60,000 | 0 | 0.4 | 0.1 |
| Gelatin | 0 | 0.5 | 0.2 |
| Gelatin, 3% | 3% | 3.7 | 3.8 |

* Precoating was with solutions containing 100 \(\mu\)g/ml of protein in each case.
Evidence that Fibronectin-Fibronectin Interactions are Important in Cell Attachment—The maximal level of attachment-promoting activity of fibronectin. Myoblast attachment was then tested with cell suspensions that had been supplemented with horse serum, intact fibronectin, or a modified fibronectin. Both horse serum and intact fibronectin gave attachment at 20 h comparable to that obtained with control plates that had been precoated with gelatin (Table IV). Both reduced and alkylated fibronectin and the larger chymotryptic fragments (Mₜ = 135,000 and 160,000) were much less effective in promoting myoblast attachment at 20 h; with both, the attachment to dishes precoated with the Mₜ = 60,000 fragment was actually

Table III

| Dishes pretreated with 15 µg/ml | Horse serum concentration in attachment assay | Nuclei in attached cells at 3 h | Without antifibronectin | With antifibronectin* |
|------------------------------|---------------------------------------------|--------------------------------|-------------------------|-----------------------|
|                              | %                                           | × 10⁻⁵                         |                         |                       |
| Native fibronectin           | 0                                           | 3.9                            | 0.7                     |                       |
| Reduced and alkylated fibronectin | 0                                           | 1.7                            | 0.4                     |                       |
| Plasminolysis fragments of Mₜ = 200,000 | 0                                           | 2.5                            | 0.8                     |                       |
| Chymotryptic fragments of Mₜ = 160,000 and 135,000 | 0                                           | 4.6                            | 0.5                     |                       |
| Gelatin                      | 0                                           | 0.4                            |                          |                       |
| Gelatin                      | 3                                           | 5.7                            |                          |                       |

* The precoated plates were incubated for 1 h at 37 °C with 500 µl of antifibronectin (1:5 diluted with NaCl/Pi) before plating the cells.

gave half-maximal activity for both intact fibronectin and for the fragments (Fig. 5). By this measure, therefore, chymotryptic cleavage did not appreciably diminish the specific attachment-promoting activity.

Fig. 5. Dependence of myoblast attachment (measured at 20 h) on the concentration of horse serum fibronectin (○) and of the chymotryptic fragments of Mₜ = 135,000 and 160,000 (A) used to pretreat the nongelatinized dishes. Similar curves were obtained when attachment was measured at 2 h.

Dishes were precoated with the Mₜ = 60,000 chymotryptic fragment of fibronectin. Myoblast attachment was then tested with cell suspensions that had been supplemented with horse serum, intact fibronectin, or a modified fibronectin. Both horse serum and intact fibronectin gave attachment at 20 h comparable to that obtained with control plates that had been precoated with gelatin (Table IV). Both reduced and alkylated fibronectin and the larger chymotryptic fragments (Mₜ = 135,000 and 160,000) were much less effective in promoting myoblast attachment at 20 h; with both, the attachment to dishes precoated with the Mₜ = 60,000 fragment was actually

Table IV

| Medium supplement* | Nuclei in attached cells/dish |
|--------------------|------------------------------|
|                    | 3 h  | 20 h | 3 h  | 20 h | 3 h  | 20 h |
| 3% horse serum     | 2.0  | 7.5  | 6.4  | 8.0  | 0.4  | 3.9  |
| Intact fibronectin (40 µg/ml) | 1.7  | 3.8  | 4.5  | 3.7  | 1.5  | 1.2  |
| Reduced and alkylated fibronectin (40 µg/ml) | 0.8  | 1.9  | 0.1  | 0.4  | N.D.* | 3.8  |
| Chymotryptic fragments of fibronectin Mₜ = 135,000 and 160,000 (40 µg/ml) | 1.1  | 1.8  | 0.1  | 0.5  | N.D.* | 3.3  |

* Without any supplement about 0.5 × 10⁵ nuclei/dish were counted on each of the substrates.

Fig. 6. Affinity chromatography of fibronectin on gelatin-binding fragment coupled to Sepharose. A, columns (1 ml) of CNBr-activated Sepharose 4B coupled with glycine (control, a), with gelatin (b and c), and with gelatin-binding chymotryptic fragment (d and e) were incubated for 2 h at 37 °C with a solution of fibronectin. In the first experiment (a, b and d), 0.8 mg of purified fibronectin was used and in the second (c and e), 0.4 mg of a different fibronectin preparation was used with new batches of coupled Sepharose. Each bar shows the fraction of recovered fibronectin as determined from the absorbance at 280 nm. Recovered fibronectin in each case comprises unbound material (eluted with 3 ml of NaCl/Pi, open part) and bound material (subsequently released with 4 M urea; filled part). B, affinity columns (1 ml) were incubated with 500 µl of horse serum for 3 h at room temperature. After washing the columns with 3 ml of NaCl/Pi, bound material was eluted with 4 M urea. Horse serum (0.5 µl) and aliquots (20 µl) of the eluates were electrophoresed on a 10% SDS-polyacrylamide gel in the presence of β-mercaptoethanol. a, Horse serum; b, eluate from the gelatin-Sepharose column; c, eluate from the column of Mₜ = 160,000 and 130,000 fragments bound to Sepharose. The arrow marks the fibronectin subunit bands.
considerably less than when uncoated dishes were used (Table IV). These results show that the smallest of the chymotryptic fragments, which is capable of coating the plastic surface yet does not by itself promote myoblast attachment, can bind sufficient fibronectin to give attachment at 20 h that is indistinguishable from that seen when dishes are precoated with gelatin. At 3 h, there was less attachment to plates precoated with the gelatin-binding fragment than to plates precoated with gelatin (Table IV). Since the fragment does give full fibronectin-mediated attachment at 20 h, it is likely that this reflects slower binding of fibronectin to the fragment than to gelatin. That there is no attachment to dishes precoated with the gelatin-binding fragment in the presence either of those chymotryptic fragments that lack a gelatin-binding region or of reduced and alkylated fibronectin can most readily be explained by a failure of these modified fibronectins to bind to the gelatin-binding fragments.

Direct Evidence for Specific Fibronectin Binding to the $M_r = 60,000$ Chymotryptic Fragment That Bears the Gelatin-Binding Site(s)—The $M_r = 60,000$ chymotryptic fragment was covalently coupled to Sepharose 4B. A column of this material retained fibronectin from solutions of the purified protein (Fig. 6A). Of the recovered fibronectin, 66% and 71% bound to the column in two separate experiments. This compares favorably with the fibronectin bound (88% and 85%) to control columns of gelatin-Sepharose in the same two experiments (Fig. 6A). Fibronectin was also retained when the column was incubated with horse serum (Fig. 6B). The eluate from the column of $M_r = 60,000$ fragment bound to Sepharose (Fig. 6B, lane c) was highly enriched in fibronectin, compared to horse serum, but did contain other proteins that usually contaminate fibronectin that has been affinity purified on gelatin-Sepharose. By contrast, when the complementary ($M_r = 160,000$ and 135,000) fragments were coupled to Sepharose 4B and tested, they did not "fish out" appreciable amounts of fibronectin from horse serum (Fig. 6B).

DISCUSSION

By studying the capability of horse serum fibronectin fragments and of reduced and alkylated horse serum fibronectin to promote myoblast attachment and bind to gelatin, we reached the following conclusions. 1) Both the gelatin-binding and attachment-promoting functions are inherent in the subunits; a dimeric structure linked by one or more interchain disulfides is required for neither activity. 2) The gelatin-binding domain can be isolated in a $M_r = 60,000$ fragment that lacks attachment-promoting activity when tested by itself. 3) Each of the subunits possesses a gelatin-binding domain; this has been widely assumed but never before demonstrated for any fibronectin. 4) The gelatin-binding domain very probably depends on the integrity of one or more intrachain disulfides for its activity. 5) The same $M_r = 60,000$ fragment which contains the gelatin-binding domain appears to carry the site(s) at which fibronectin-fibronectin binding occurs; like gelatin, this fragment of fibronectin can indirectly promote myoblast attachment by collecting fibronectin from the medium. 6) The attachment-promoting (cell-binding) domain can be isolated on fully active fragments of $M_r = 135,000$ and 160,000 that lack the gelatin-binding region. 7) Intrachain disulfides are not essential for the attachment-promoting activity of the cell-binding domain. In the general approach taken and in the principal conclusion reached (separable domains for distinct functions of fibronectin), our work resembles that recently reported elsewhere (see introduction). However, in certain details (see "Results"), our findings appear to conflict with those of others. Possible sources of some of these differences will be explored below as we consider the advantages and disadvantages of different cell attachment assays and the likely importance of fibronectin-fibronectin binding in different fibronectin-mediated processes.

The Modified Myoblast Attachment Assay—Myoblast attachment to gelatin-coated dishes is dependent on exogenous fibronectin (14). In our standard assay, the fibronectin can be added either as the purified molecule or as a component of a complex mixture (e.g. serum, conditioned medium) (14). Moreover, the fibronectin can either be added to the cell suspension (in which case the gelatin selectively binds enough fibronectin to mediate cell-substrate attachment) or used to precoat the gelatinized dishes (in which case cells subsequently attach and elongate even when the medium is devoid of fibronectin) (14). When nongelatinized dishes are used, however, the results are very different. If whole serum is used to precoat the dishes or is added to the medium, other proteins compete for substrate binding sites, reducing the amount of bound fibronectin below that required for myoblast attachment. Even purified fibronectin cannot be added directly to the myoblast suspension, for proteins present in the medium (from the high molecular weight fraction of embryo extract) compete effectively with the fibronectin for substrate binding sites. If, however, nongelatinized dishes are preincubated with solutions of purified fibronectin, myoblasts attach even if the cells are suspended in protein-rich medium. This modification of our attachment assay, precoating nongelatinized dishes, allowed direct measurement of the attachment-promoting activity of fibronectin and its derivatives independent of their ability to bind to gelatin. Similar assays, based on precoating plastic dishes with fibronectin derivatives, have been used by others (19, 26, 27).

An important advantage of the myoblast attachment assay is that the myoblasts do not themselves synthesize fibronectin (17). It has been hypothesized that cell strains, in contrast to established cell lines, normally produce their own fibronectin and thus do not require exogenous fibronectin for attachment (43, 44); chicken primary myoblasts and myotubes are an obvious exception. That endogenously synthesized fibronectin may complicate the measurement of cell attachment mediated by exogenous fibronectin has been suggested for several systems (26, 43-45).

The experiments presented in this paper confirm our finding (14) that myoblast attachment continues to increase between 2 and 20 h in the presence of horse serum, whereas it declines during the same period if purified fibronectin alone is added to the attachment assay. Possible explanations for this phenomenon were considered in our earlier paper (14).

The Functional Significance of Fibronectin-Fibronectin Interactions—We have presented evidence that fibronectin-fibronectin interactions, although not strictly necessary for myoblast attachment, may contribute to enhanced attachment. We have further shown that the region of fibronectin which interacts with other fibronectin molecules is likely to be located in the same fragment ($M_r = 60,000$) as the gelatin-binding domain. There is considerable evidence that fibronectin-fibronectin binding occurs in natural and artificial matrices (42, 46), in part via intermolecular disulfide bonds (41, 47). All of the fibronectin in extracellular matrices cannot be linked by disulfide or other covalent bonds, however, since extraction with urea yields appreciable amounts of dimeric fibronectin (48).

There are, moreover, observations on the functions of fibronectin that are difficult to explain unless fibronectin-fibronectin binding can be assumed. The most important of these is...
the finding that an excess of fibronectin does not inhibit the attachment of myoblasts (14) or other cells (43) to gelatinized dishes. If fibronectin-mediated attachment requires that each fibronectin molecule involved bind simultaneously to gelatin and to a cell surface receptor, excess fibronectin might be expected to bind to cells and, thus, by competing with gelatin-bound fibronectin to inhibit cell attachment. If soluble fibronectin is unable to bind to cells (43), that would explain the lack of inhibition. Indeed, it has been proposed that fibronectin is somehow "activated" by its binding to collagen or another substrate (43, 49). There is no direct evidence for this, however. Alternatively, fibronectin might have a much higher affinity for gelatin or collagen than for cells, implying that cells always have enough unoccupied fibronectin receptors able to attach to substrate-bound fibronectin.

The other explanation assumes that fibronectin can bind to itself. Occupation of receptor sites would then no longer be a problem. Excess fibronectin could simply "pile on" to fibronectin already bound to the cell or the substrate without impairing attachment. If fibronectin binds to itself more readily than it binds to the surface of a given cell, the attachment step would involve binding of surface components other than fibronectin to a thicker lawn of fibronectin. This may be the case with cell types which themselves do not accumulate fibronectin on their surfaces, such as transformed cells (42) or chicken myoblasts (17). If fibronectin binds to itself and to a given cell type with about the same avidity, the actual attachment step would involve fibronectin-fibronectin binding. This mechanism is supported by experiments showing that well spread cells which already have much fibronectin on their surfaces bind more soluble fibronectin than their transformed variants (12).

Mild proteolysis of cultured cells releases almost all of the fibronectin from the cell surface (39) and yet the fibronectin fragments so released are, like our plasminolysis fragments, only slightly smaller than intact fibronectin subunits (40). Moreover, in at least one case, such fragments were active in promoting cell attachment and spreading (28). We have now confirmed (see "Results") that large fragments lacking only that terminal part of the molecule bearing the the interchain disulfide(s) have both gelatin-binding and cell-binding regions intact. But why should clipping off a small region that carries fragments so released be, like our plasminolysis fragments, transformed variants intact. But why should clipping off a small region that carries

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