Demonstration of High Affinity Fibronectin Receptors on Rat Hepatocytes in Suspension*

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A cell-binding peptide ($M_r = 85,000$) which lacks the gelatin- and heparin-binding domains, was purified from trypsin-digested fibronectin. Preincubation of rat hepatocytes in suspension with the peptide, inhibited initial attachment of the cells to immobilized fibronectin while attachment to immobilized laminin and collagen was unaffected. $^{125}$I-labeled 85-kDa peptide bound to the cells in suspension at 4°C in a time-dependent, saturable, and partially reversible reaction. Scatchard analysis of the binding data indicated a single class of receptors with a $K_d$ of $1.7 \times 10^{-8}$ M. The number of binding sites was $2.8 \times 10^7$ cell. Unlabeled 85-kDa peptide inhibited the binding of $^{125}$I-labeled 85-kDa peptide 30-fold more effectively than intact fibronectin. These results provide direct evidence for the presence of a domain in the fibronectin molecule which has or may acquire a high affinity for receptors involved in adhesion of hepatocytes to immobilized fibronectin.

The glycoprotein fibronectin is a mediator of cell-matrix interactions (1). It has affinity for matrix components such as collagens (2), glycosaminoglycans (3), and fibrin (4). These interactions have been studied in some detail and domains in the fibronectin molecule, responsible for the interactions, have been identified and isolated after proteolytic fragmentation of fibronectin. A large number of different cell types, including fibroblasts (5), hepatocytes (6), and platelets (7) have been shown to adhere to and spread out on substrates of immobilized fibronectin. A cell-binding tetrapeptide in the fibronectin molecule has been identified by use of a monoclonal antibody which inhibits cell attachment to fibronectin substrates (8, 9). However, the cellular receptor for fibronectin has not been identified.

A problem in studies of fibronectin receptors is that soluble fibronectin binds very poorly to cells, making classical receptor-ligand studies difficult to perform. Furthermore, since fibronectin has binding sites for a number of different components it may be difficult to interpret binding data, particularly if binding to collagen- and proteoglycan-containing cell layers is studied. The explanations suggested for the lack of binding of soluble fibronectin to cells have been either (a) that cooperative interactions between immobilized fibronectin molecules and many cell surface receptors are required for stable binding (10) or (b) that fibronectin becomes activated by conformational changes during incorporation into extracellular matrices or by coating onto plastic surfaces (11). Recently, support for the latter theory was presented (12). The ability of soluble fibronectin to compete with immobilized fibronectin for binding to cellular receptors and thereby inhibit initial attachment of rat hepatocytes to fibronectin-coated plastic dishes, was markedly increased by a limited trypsin digestion of the soluble fibronectin. The interaction of soluble fibronectin with hepatocytes in suspension could also be stimulated by addition of collagen or heparan sulfate, as measured as increased inhibition of initial attachment to fibronectin dishes.

These results suggested that tryptic fibronectin fragments could be useful in studies of fibronectin receptors. In this investigation an 85-kDa fibronectin fragment, which lacks affinity for collagen and heparin, has been used to demonstrate the presence of a specific fibronectin receptor on rat hepatocytes. The binding of the fibronectin fragment to the cells in suspension has been characterized and compared with that of intact fibronectin.

MATERIALS AND METHODS

Trypsin (TPCK-treated, type XIII), bovine serum albumin, phenylmethylsulfonyl fluoride, EDTA, dithiothreitol, Bis-Tris, and gus nidinium chloride were purchased from Sigma. DEAE-Sepharose, gelatin-Sepharose, and Percoll were obtained from Pharmacia, Uppsala, Sweden. TSK 3000 columns were from LKB, Bromma, Sweden. Na$^{111}$I (carrier-free) was purchased from the Radiochemical Centre, Amersham, England and Iodo-Beads from Pierce Chemical Co. Fibronectin was purified from human plasma according to the method of Vuorio and Vaheri (13). Neutral salt-soluble collagen from rat skin (type I and III) and laminin were kind gifts from Dr. K. Rubin, University of Uppsala, Sweden, and Dr. R. Timpl, Max Planck Institut, West Germany, respectively.

Purification of the Cell-binding 85-kDa Fibronectin Fragment—Fibronectin (3 mg/ml) in 10 mM Tris-HCl buffer, pH 7.5, containing 0.14 M NaCl and 0.02% NaN$_3$ was digested with trypsin (7 μg/ml) for 90 min at 37°C. The digestion was terminated by addition of phenylmethylsulfonyl fluoride to a final concentration of 0.4 mM. The digest was dialyzed against 50 mM Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl, 10 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.02% NaN$_3$ and sequentially applied to columns of heparin-Sepharose (1 ml of gel/10 mg of fibronectin digest) and gelatin-Sepharose (1 ml of gel/2 mg of fibronectin digest) in the same buffer. During a second passage of the unbound fraction through the columns no further material was retained, demonstrating that no heparin- or gelatin-binding peptides remained in this fraction. The unbound material was dialyzed against 10 mM Bis-Tris buffer, pH 6.0, containing 50 mM NaCl and 0.02% NaN$_3$, and applied to a DEAE-Sepharose column (50 mg of protein/10 ml of gel). The DEAE column was eluted with a linear gradient (100 ml) of NaCl from 50 to 300 mM in this buffer (Fig. 1). The fractions which contained peptides that

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1 The abbreviations used are: TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; Bis-Tris, bis(2-hydroxyethyl)iminotri(hydroxymethyl)methane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
induced attachment and spreading of rat hepatocytes (see below for assay conditions) were pooled and concentrated to 20 mg/ml by vacuum dialysis. After dialysis against 4 M guanidinium chloride, and 2 mM dithiothreitol, the peptides were chromatographed on a TSK 3000 column (volume, 40 ml; length, 100 cm) in the same buffer (Fig. 2). The first peak contained the adhesion-promoting material which in SDS electrophoresis migrated as a single peptide with an apparent M, of 85,000 (Fig. 3). The 85-kDa fraction was pooled and dialyzed back to phosphate-buffered saline (10 mM PO4, 0.14 M NaCl, 4.7 mM KCl, 0.65 mM MgSO4, 7 H2O, 1.2 mM CaCl2 x 2 H2O, 10 mM HEPES) containing 1.5% bovine serum albumin, were seeded in each well and incubated at 37 °C in humidified air. After incubations for indicated times the microtiter plates were washed and the number of cells attached determined as described (17). In short, the cells were lysed in a solution of Triton X-100, and the activity in the lysate of the enzyme hexosaminidase was determined. Cell spreading was studied in the presence of cycloheximide (25 μg/ml) as described (18).

Assay for Binding of 125I-labeled 85-kDa Peptide to Hepatocytes—Cells (5 x 104) in buffer 3 containing 1.5% bovine serum albumin were incubated end over end with 125I-labeled 85-kDa peptide (106 cpm/well) for 30 min at 22 °C to block remaining protein binding sites on the plastic surface. This solution was removed before seeding of the cells.

Cell Attachment Assay—Hepatocytes were isolated from male Sprague-Dawley rats after perfusion of the liver in situ with collagenase as described (16). Cells (5 x 104) in 50 μl of buffer 3 (see Ref 16; 0.137 M NaCl, 4.7 mM KCl, 0.65 mM MgSO4 x 7 H2O, 1.2 mM CaCl2 x 2 H2O, 10 mM HEPES) containing 1.5% bovine serum albumin, were seeded in each well and incubated at 37 °C in humidified air. After incubations for indicated times the microtiter plates were washed and the number of cells attached determined as described (17). In short, the cells were lysed in a solution of Triton X-100, and the activity in the lysate of the enzyme hexosaminidase was determined. Cell spreading was studied in the presence of cycloheximide (25 μg/ml) as described (18).
partly cleaved 85-kDa peptide purified under nondenaturing conditions gave essentially identical results as shown in Figs. 6 and 7. Fibronectin 65,000 and 20,000 co-purify with the 85-kDa peptide. These peptides are slowly formed during trypsin digestion of purified 85-kDa peptide structures in fibronectin to which hepatocytes can attach.

Coating fibronectin dishes with intact fibronectin. Almost complete inhibition was achieved with the 85-kDa fragment. Preincubation of hepatocytes with the purified 85-kDa fragment 

RESULTS

Trypsin-digested fibronectin added to the medium has previously been shown to inhibit initial cell attachment to fibronectin-coated dishes more efficiently than intact fibronectin (12). Such a digest was fractionated as described under “Materials and Methods” to obtain a cell-binding peptide devoid of gelatin- and heparin-binding domains. Cell-binding activity was associated with an 85-kDa peptide (not shown) which was accumulating during the incubation of fibronectin with trypsin (Fig. 3, A and B). Since the 85-kDa peptide does not bind to gelatin or heparin it must originate from the central region of the fibronectin arms (70-160 kDa from the N-terminal) (19-21). Tryptic fibronectin fragments apparently identical to this 85-kDa fragment have been described by others (22, 23), but have not been obtained free from contaminating peptides. In the present study, denaturing conditions were used in order to purify the 85-kDa peptide.2

Preincubation of hepatocytes with the purified 85-kDa fragment in suspension, inhibited initial attachment to dishes coated with intact fibronectin. Almost complete inhibition (95%) of attachment to fibronectin dishes was obtained at a concentration of 50 µg/ml of the 85-kDa fragment (Fig. 4). These data indicate that the 85-kDa fragment contains all structures in fibronectin to which hepatocytes can attach.

![Inhibition of cell attachment by the 85-kDa fragment.](image)

The effect of the 85-kDa peptide in solution on cell attachment was specific in that only attachment to immobilized fibronectin was inhibited while attachment of hepatocytes to immobilized laminin or collagen was unaffected (Table I). The 85-kDa peptide immobilized on plastic dishes was as effective as intact fibronectin in promoting both attachment and spreading of hepatocytes (data not shown). This is in accordance with the results of Hayashi et al. (22) who studied the ability of fibroblasts to attach and spread out on dishes coated with the partly purified 85-kDa peptide.

125I-labeled 85-kDa fragment incubated with hepatocytes in suspension at 4°C bound to the cells in a time-dependent reaction which was completed after 1-2 h (Fig. 5). Previously bound 125I-labeled 85-kDa fragment was partially displaced (45%) by addition of excess unlabeled 85-kDa fragment (10 µg/ml) (Fig. 5). However, complete reversal of the binding of radioactivity could not be achieved even if the amount of unlabeled 85-kDa fragment was further increased (data not shown). The presence of unlabeled 85-kDa fragment or intact fibronectin from the start of the incubation, inhibited the binding of 125I-labeled 85-kDa peptide in a dose-dependent manner. On a molar basis ~30-fold more fibronectin (counted on number of monomers of M, = 220,000) than 85-kDa fragment was required to achieve 50% inhibition of the binding of 125I-labeled 85-kDa fragment to the hepatocytes (Fig. 6). When the cells were incubated with increasing amounts of 125I-labeled 85-kDa fragment a saturation curve of binding was obtained (Fig. 7A). Half-maximal binding occurred at 15-20 nM (1.3-1.7 µg/ml) and saturation was achieved at 60-100 nM. A Scatchard plot of the binding data, indicated a single class of receptors. The apparent Kd was 1.7 x 10^-8 M and the average number of binding sites was 2.8 x 10^6/cell.

When unfractionated peptides of trypsin-digested fibronectin was 125I-labeled and incubated with the hepatocytes, the 85-kDa peptide was selectively enriched in the cell-associated fraction (Fig. 8). This suggests that the 85-kDa peptide has the highest affinity for the fibronectin receptors among the trypsin-generated fibronectin fragments.

DISCUSSION

Recently several reports have indicated that the shape of fibronectin molecules may vary from globular to extended

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2 Under nondenaturing conditions peptides of molecular mass 65,000 and 20,000 co-purify with the 85-kDa peptide. These peptides are slowly formed during trypsin digestion of purified 85-kDa peptide or fibronectin (S. Johansson, unpublished results). Preparations of partly cleaved 85-kDa peptide purified under nondenaturing conditions gave essentially identical results as shown in Figs. 4-7 for intact, renatured 85-kDa peptide.
forms depending on ionic strength and pH of the solute (24-27). Also immobilized fibronectin attains different conformations, depending on the nature of the surface to which it is coated (24). Changes in absorption spectra induced by binding of collagen (27) or heparin (28) to fibronectin have previously been reported, and interpreted to reflect conformational changes in fibronectin. The increased binding of soluble fibronectin to hepatocytes in the presence of collagen or heparan sulfate/heparin or after limited trypsin digestion of the fibronectin suggests that such conformational changes in fibronectin are functionally important (12). Fibrillogenesis of fibronectin provides another example, since a conformational change must precede the disulphide bridging between thiold groups, as these are not exposed in native soluble fibronectin (29, 30).

In this study the increased affinity of fibronectin for cell surface receptors, induced by "activation" of fibronectin, has been investigated by a more direct approach than previously. The major cell-binding peptide of trypsin-digested fibronectin was identified and purified. It had an apparent Mr of 85,000 and did not contain binding sites for gelatin or heparin. In contrast to soluble 125I-labeled fibronectin, which did not detectably bind to hepatocytes (12), the 85-kDa tryptic cell-binding fragment of fibronectin bound to the cells in suspension at 4°C with high affinity (1.7 × 10^9 M) in a time-dependent, saturable, and specific manner. Furthermore, preincubation of the cells with the 85-kDa fragment in suspension inhibited the initial attachment of the cells to dishes coated with fibronectin. This result strongly suggests that the binding sites recognizing the 85-kDa peptide are identical with the receptors used by hepatocytes for adhesion to fibronectin matrices. In contrast, the cell-binding peptide isolated by Piersbacher et al. (31) did not detectably bind to cells in suspension. Neither did it affect cell attachment to fibronectin dishes at concentrations where the 85-kDa fragment is completely inhibitory (31). However, at millimolar concentrations of the tetra-peptide, cell attachment to fibronectin dishes was inhibited. From these data a Kd of 5 × 10^-5 M for the interaction of the tetra-peptide and its receptor was calculated (9). The difference in affinity between the tetra-peptide and the 85-kDa fragment, respectively, for the fibronectin receptor (>10^4-fold) may indicate that the 85-kDa fragment contains additional structures which are required for optimal binding strength. The tetra-peptide is also present in collagen and a few other proteins (9). However, cell attachment to collagen (as well as to laminin) was not affected by the presence of the 85-kDa peptide in the medium indicating that also collagen contains additional cell-binding structures, which are recognized by specific collagen receptors.

Intact fibronectin at high concentrations did compete with 125I-labeled 85-kDa peptide for binding to the hepatocytes although 30-fold less effectively than the 85-kDa peptide. However, it is not clear whether this competition is due to a lower affinity of intact fibronectin for the receptor or to a small fraction of the fibronectin molecules being "activated" to the high-affinity form. A recent paper by McKeown-Longo and Mosher (32) can be interpreted in favor of the idea that activation of fibronectin is a cellular event that precedes the actual binding of fibronectin to its receptor. They could detect a binding of 125I-labeled fibronectin at 37°C to cell layers of growing fibroblasts. A fraction of the bound fibronectin was found in a saturable, detergent-soluble pool with a calculated Kd of 3.6 × 10^-8 M. Thus it is quite possible that the hepatocyte receptor for the 85-kDa fragment and the detergent-soluble fibronectin receptor on the fibroblasts are homologous. In the study of McKeown-Longo and Mosher (32), transformed cells were shown to attach to fibronectin-coated dishes but failed to bind soluble 125I-labeled fibronectin. The transformed cells apparently had functional fibronectin receptors but may have been defective in the activation of fibronectin to a form which can bind to its receptor. A possible candidate as cellular "activator" of fibronectin would be membrane-bound heparan sulfate proteoglycan (33, 34). A close co-distribution of cell surface heparan sulfate proteoglycan, fibronectin, and actin has been demonstrated on fibroblasts early in their spreading process (35). Furthermore, exogenously added heparan sulfate has been shown to stimulate the binding of fibronectin to

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**Table I**

Specificity of inhibition of cell attachment by the 85-kDa fragment

Rat hepatocytes (1 × 10^5/ml) were preincubated at 4°C for 20 min in the absence and presence of purified 85-kDa fragment (50 μg/ml), and then seeded into wells coated with the indicated proteins as described under "Materials and Methods." After incubation for 12 min at 37°C the wells were washed and the number of cells attached determined. The number of cells attached in the absence of 85-kDa fragment to wells coated with fibronectin is set to 100%. The values shown represent averages of two parallel incubations.

| Amount 85-kDa fragment added to the medium | Number of cells attached to wells coated with |
|------------------------------------------|--------------------------------------------|
| 50 μg/ml                                 | Fibronectin | Laminin | Collagen |
| 0                                        | 100         | 69.0    | 50.6     |
| 50                                       | 20.7        | 72.4    | 50.2     |

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**Fig. 7:** Concentration dependence of binding of 125I-labeled 85-kDa fragment to hepatocytes in suspension. A, the cells were incubated with increasing amounts of 125I-labeled 85-kDa fragment (specific activity 2 × 10^6 cpm/μg) and the bound radioactivity determined as described under "Materials and Methods." The ranges obtained from duplicate incubations are shown. Specific binding (I) was calculated by subtracting nonspecific binding (Δ) from total binding (C). Nonspecific binding was determined in the presence of 100 μg of unlabeled 85-kDa fragment/ml (the dotted line denotes extrapolated values). B, Scatchard plot of the data presented in A. The curve was fitted by the least square method (regression coefficient = 0.97).
hepatocytes (12). In agreement with this hypothesis, tumor cells generally synthesize heparan sulfate with a low sulfate content (36–38) and reduced affinity for fibronectin (37). Cell attachment and spreading to a performed “matrix” would not be affected by reduced sulfation of heparan sulfate since a fibronectin-heparan sulfate interaction apparently is not required for these events. This was demonstrated in this study to be the case for hepatocytes which in the presence of cycloheximide spread out on dishes coated with the 85-kDa fragment. The migration distance of molecular mass standards (kDa) are indicated.

In conclusion, the results presented here support the concept that activation of fibronectin by conformational changes to be the case for hepatocytes which in the presence of fibronectin-heparan sulfate interaction apparently is not required for these events. This was demonstrated in this study to be the case for hepatocytes which in the presence of cycloheximide spread out on dishes coated with the 85-kDa fragment. The migration distance of molecular mass standards (kDa) are indicated.

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