Chemotaxis of 3T3 and SV3T3 Cells to Fibronectin Is Mediated through the Cell-Attachment Site in Fibronectin and a Fibronectin Cell Surface Receptor

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Abstract. Fibronectin (FN) is a multidomain extracellular matrix protein that induces attachment and chemotactic migration of fibroblastic cells. In this study we analyzed the molecular determinants involved in the FN-induced chemotactic migration of normal and SV40-transformed 3T3 cells. Two different monoclonal antibodies to the cell-binding site of FN blocked chemotaxis to a 140-kD FN fragment (Ca 140) containing the cell-binding domain. A monoclonal antibody to a determinant distant from the cell-binding site did not affect chemotaxis. A synthetic tetrapeptide, RGDS, which represents the major cell-attachment sequence, was able to compete with FN and the Ca 140 fragment in chemotaxis assays, but this peptide itself had no significant chemotactic activity. A larger peptide encompassing this sequence, GRGDSP, was chemotactic, while the peptide GRGESP, where a glutamic acid residue was substituted for aspartic acid, was inactive. Chemotactic migration could be prevented in a dose-dependent manner by a rabbit polyclonal antiserum to a 140-kD cell surface FN receptor. This antibody was more effective on normal than on transformed 3T3 cells. Neither the anti-FN receptor antiserum nor a monoclonal antibody to the cell-binding site of FN blocked migration induced by another potent chemoattractant, platelet-derived growth factor. These data indicate that FN-induced chemotaxis of 3T3 and SV3T3 cells is mediated via the RGDS cell-attachment site of FN and the 140-kD cell surface FN receptor. The interaction is specific and can be altered by transformation.

A number of studies indicate that fibronectin (FN)[1] and certain FN fragments are potent chemoattractants for fibroblastic cells (5, 17, 27, 34, 41, 44, 47). Presumably this activity is important in wound healing where fibroblasts from the surrounding tissues are recruited to the site of damage to produce the matrix molecules necessary for full repair (48). The rate of repair is believed to be related to the numbers of cells recruited to the wound site and thereby to the level of chemoattractants and the duration of their production. Chemotaxis to FN may also be involved in pathological situations including fibrosis and tumor cell metastasis (2, 22, 28, 30, 31, 44, 52–54). The chemotactic region of FN has been localized within a large cell-binding chymotryptic 160-kD peptide (47) and in a cathepsin D–derived 140-kD peptide which lacks gelatin-binding activity (3, 5, 41).

1. *Abbreviations used in this paper*: FN, fibronectin; PDGF, platelet-derived growth factor.

Studies with various chemoattractants in eukaryotic and prokaryotic cells show that the responding cells have receptors which bind the attractant and elicit the intracellular events that direct cell movement (6, 12, 22, 24, 36, 56). Here we have attempted to determine if the chemotactic response of 3T3 cells and their SV40-transformed counterpart to FN occurs through the RGD-attachment site of the molecule (37, 40) and an FN receptor (M_r 140,000) (18, 19, 51). Studies with antibodies to the cell-binding site in FN, with antibodies to the FN receptor, and with various synthetic peptides suggest that the cell-attachment site of FN also elicits chemotaxis.

Materials and Methods

Cellular Material

3T3 cells and SV3T3 were kindly provided by Dr. P. Prehm (Max-Planck-Institüt für Biochemie, Munich, FRG). Cells were cultivated in DME, supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (50 μg/ml),

and glutamine (300 μg/ml), and were harvested by trypsin treatment (0.05% trypsin, 0.02% EDTA in PBS, pH 7.4).

Chemoattractants

FN (Sigma, Munich, FRG) was diluted with DME to 30 μg/ml. Ca 140, a large cathepsin D fragment, was prepared from purified human plasma FN by mild digestion (45). The preparation, purification, and electrophoretic purity of the Ca 140 fragment has been previously described (16, 45). Ca 140 was shown previously to be a potent chemoattractant (3, 5) and was used here at 20 μg/ml. Platelet-derived growth factor (PDGF) was purchased from Collaborative Research, Inc. (Lexington, MA) and used as a chemoattractant at 5 ng/ml (46). The tetrapeptide RGDS was purchased from Nova Biochemical AG (Läwfelfingen, Switzerland). The hexapeptides GRGDSP and GRGESP were synthesized by Prof. P. Neri of Centro di Ricerca Interdipartimentale fer le Scienze Mediche Avantate (CRISMA, University of Siena, Siena, Italy), using an automated peptide synthesizer (model No. 430A; Applied Biosystems, Inc., Foster City, CA) equipped with the chemistry provided by the manufacturer. After cleavage from the resin with hydrogen fluoride the peptides were purified by two cycles of chromatography on Sephadex G10 in water, lyophilized, and stored at −20 C.

Antibodies

A317 and A286 are two distinct monoclonal antibodies reacting with determinants within the COOH-terminus of peptide Ca 140 (16, 45). These antibodies were kindly provided by M. Schachner (Institüt für Neurobiologie, Heidelberg, FRG). The monoclonal antibody 3E3, a gift of M. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA), reacts with the cell-attachment domain of FN (39). Goat anti-FN serum was a gift of Klaus von der Mark (Max-Planck-Institut, Martinsried, FRG). The anti-baby hamster kidney (BHK) serum reacting with the 140-kD FN receptor on BHK cells has been previously characterized (18, 19, 51). An IgG fraction from this antiserum was prepared by affinity chromatography on Protein A-Sepharose. Immunoprecipitation of the cell surface receptor proteins was performed as previously described (51). The 135-kD component of the FN receptor was purified from SR/BALB/c 3T3 cell membranes on lectin-Sepharose column followed by hydroxyapatite chromatography (20). For some experiments the anti-BHK antiserum was adsorbed on a receptor affinity column.

Chemotaxis Assays

These assays were carried out in Boyden chambers as described previously (42). Cells were harvested in trypsin (0.05%) and EDTA (0.01%) in PBS (pH 7.4). The cells were recovered by centrifugation, resuspended in DME, recentrifuged, and then resuspended in DME without serum. Cells (1×10^5) were added to the upper compartment (0.8 ml) of the Boyden chamber and the lower compartment (0.2 ml) was filled with media usually containing a chemoattractant, in the presence or absence of different antibodies.

Table I. Inhibition of Chemotactic Activity of 3T3 Cells by Antibodies to the Cell-binding Site of FN

Attractant	Antibody	Concentration	Maximal activity
		μg/ml	*%*
FN	–	0	100 ± 22
Ca 140	–	0	89 ± 18
Ca 140	A317	20	69 ± 16
Ca 140	A317	40	38 ± 3
Ca 140	A286	20	84 ± 5
Ca 140	A286	40	91 ± 6
Ca 140	3E3	20	38 ± 14
PDGF	–	0	100 ± 8
PDGF	3E3	20	100 ± 15

We analyzed the effect of different monoclonal antibodies recognizing Ca 140 (which contains the cell-binding domain) on the migration of 3T3 cells to Ca 140 itself (20 μg/ml) and to PDGF (5 ng/ml). A317 and 3E3 are antibodies directed to the cell-binding site in Ca 140, while A286 recognizes a determinant in a more NH_2-terminal region of the fragment. Data are expressed as percent of maximal migration as compared to the chemoattractant alone. Experiments were run in triplicate and repeated twice. SDs are indicated.

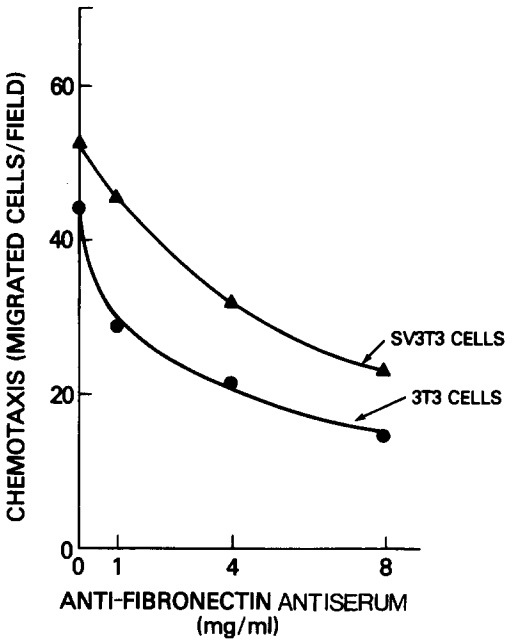

Figure 1. Inhibition of 3T3 and SV3T3 cell chemotaxis towards FN by anti-FN polyclonal antiserum. 30 μg/ml of FN was placed in the lower compartment of Boyden chambers with increasing amounts of goat anti-FN antiserum. 3T3 and SV3T3 cells were placed in the upper compartment and the assays run for 5 h. Data are expressed as migrated cells/field. Two separate experiments were carried out with each assay performed in triplicate and did not differ by more than 10%.

FN (30 μg/ml), Ca 140 (20 μg/ml), and PDGF (5 ng/ml) were used at the concentrations exerting maximal chemotactic activity (5, 34, 46) as confirmed in preliminary experiments. The two compartments were separated by a polycarbonate filter (8 μm pore size; Nuclepore, Concorezzo, Italy) coated with gelatin (5 μg/ml; Sigma) an attachment substrate for fibroblasts (42). Of the chemoattractants used, only FN has been reported to bind to gelatin, and Ca 140 lacks the gelatin-binding domain (45). In some experiments FN and FN peptides were placed either in the upper, in the lower, or in both compartments of the Boyden chamber, and the migratory activities were compared with those obtained when only the lower compartment contained chemoattractant (57). Cells were allowed to migrate for 5 h at 37°C in a humidified atmosphere containing 5% CO_2 (3). After this time, cells on the upper side of the filter were removed mechanically; the cells on the lower surface of the filter were fixed in ethanol, stained with toluidine blue, and 5 U fields per filter were counted at 160× with a microscope (Zeiss, Oberkochen, FRG). Filters were numbered at the onset of the assay and counted without knowledge of their identity. The fields to be counted were selected randomly from the surface of the migrated cells. One field represents ~1/160 of the total area of migrated cells. Each assay was carried out in triplicate and repeated at least twice.

Results

Inhibition of Fibroblast Migration by Antibodies to the Cell-binding Site of FN

It is well known that FN is a potent chemoattractant for fibroblastic cells and that the active region lies within a large fragment (Ca 140) which contains the major cell-binding domain of the molecule (38, 41, 47). The effect of three monoclonal antibodies which react with this fragment were tested (Table I). Monoclonals A317 and 3E3 react with the cell-binding site within Ca 140 while A286 recognizes a determinant in a more NH_2-terminal region of Ca 140 (16). Monoclonal antibodies A317 and 3E3 (Table I) as well as

Table II. Effect of Synthetic Peptides on Migration of 3T3 and SV3T3 Cells towards FN

Attractant		Migrated cells	
Lower compartment	Upper compartment	3T3	SV3T3
		%	%
–	–	7 ± 2	7 ± 2
FN	–	100 ± 1	100 ± 4
RGDS	–	10 ± 2	7 ± 1
GRGESP	–	6 ± 1	7 ± 1
–	FN	7 ± 2	7 ± 4
–	RGDS	10 ± 4	9 ± 4
FN	FN	41 ± 2	49 ± 6
RGDS	RGDS	14 ± 4	ND
FN	RGDS	75 ± 2	32 ± 2
FN	GRGESP	102 ± 7	95 ± 9

For this experiment, chemoattractants were placed either in the lower or in the upper compartment of chemotaxis chambers. FN was used at 30 μg/ml. RGDS was used at the concentration exerting maximal activity in preliminary experiments; i.e., 200 μg/ml. GRGESP was used at the same concentration; other concentrations tested were inactive as well. Data are expressed as percent of maximal migration as compared to the chemoattractant alone. Experiments were run in triplicate and repeated three times. SDs are indicated.

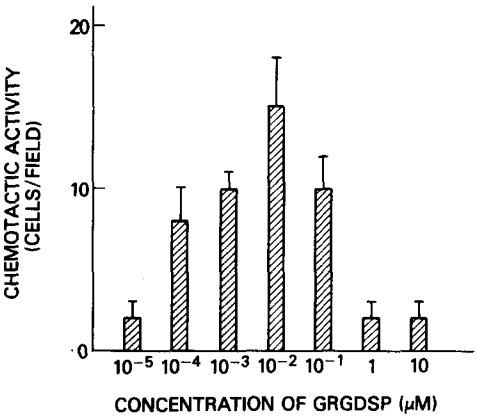

Figure 2. Chemotactic activity of 3T3 cells to GRGDSP synthetic peptide. Increasing concentrations of the peptide were used in the lower compartment of Boyden chambers. Data are expressed as migrated cells/field. GRGESP was not chemotactic for any of the concentrations tested.

polyclonal antiserum to the FN molecule (Fig. 1) all caused significant inhibition of chemotaxis to Ca 140 or FN, while monoclonal A286 did not. These antibodies did not inhibit chemotaxis of 3T3 cells to the chemoattractant PDGF (46) (only 3E3 shown), indicating that the inhibition of migration was specific to FN (Table I). These studies indicate that it is a portion of Ca 140 associated with the cell-binding site that is the principal active sequence on FN directing the chemotactic migration of 3T3 cells.

Effect of Synthetic Peptides on Cell Migration

The sequence Arg-Gly-Asp-Ser (RGDS) has been identified as the major cell-binding domain of FN (37, 38, 40). The possibility that synthetic peptides containing this sequence were chemotactic or could inhibit FN-induced migration was investigated. RGDS alone in the lower compartment of the Boyden chamber at levels up to 1,000 μg/ml did not stimulate cell migration (Table II). In contrast, GRGDSP was found to elicit a dose-dependent response (Fig. 2). The response was biphasic with a peak of maximal activity observed between 10^{-3} and 10^{-1} μM. A related peptide with glutamic acid substituted for aspartic acid was not chemotactic, nor did it exhibit competitive activity (Table II) at concentrations up to 1 mg/ml. When the RGDS peptide was added in the lower compartment of the Boyden chamber together with FN (Table II) or the Ca 140 peptide (Table III), a dose-dependent reduction of cell migration was observed. A reduction of FN-induced cell migration was also observed when RGDS was added to the upper compartment (Table II). In this case maximal activity was achieved with 200 μg/ml. SV3T3 cells were more responsive to the peptides than 3T3 cells (Table II). RGDS (Table II) stimulated random migration when added to both compartments. To see if the migration to GRGDSP was mostly chemotactic or chemokinetic, we ran a checkerboard analysis (57) using concentrations of the peptide active in stimulating migration. Table IV shows that the migration of cells in the presence of equimolar concentrations of the peptide (i.e., chemokinesis [values on the diagonal]) is lower than their migration when the peptide was present only in the lower compartment (chemotaxis), or only in the upper compartment. These experiments indicate that the RGDS cell-binding site is involved in 3T3 and SV3T3 cell chemotaxis to FN and in stimulated random migration.

Inhibition of Cell Migration by Antibodies to the FN Receptor

To investigate the role of cell surface proteins in the chemotactic response to FN, we used an antiserum (anti-BHK) that inhibits fibroblast attachment and spreading on FN-coated dishes (19). This antiserum was tested here for its ability to precipitate the FN receptor complex (18, 19). Under reducing conditions the precipitated material migrated as a diffuse band at M_r 140,000 (Fig. 3). In the absence of reducing agents, two major components are observed, with molecular masses of 150 and 135 kD, respectively (Fig. 3). Recent results (Tarone, G., and F. Giancotti, unpublished results) indicate that the two polypeptides are parts of a heterodimeric complex. When the anti-BHK antiserum was added to the lower compartment of the Boyden chamber, cell migration toward FN was inhibited in a dose-dependent manner (Fig. 4). The antibodies did not affect migration of cells towards PDGF, a potent chemoattractant (46) known to act

Table III. Effect of the RGDS Peptide on the Migration of 3T3 Cells towards Ca 140

Attractant	RGDS concentration	Migration
	μg/ml	%
–	0	5 ± 1
Ca 140	0	100 ± 20
Ca 140	20	81 ± 3
Ca 140	200	52 ± 5
Ca 140	1,000	19 ± 3

Ca 140 was used at concentrations of 20 μg/ml. Increasing concentrations of RGDS were used along with the chemotactic fragment in the lower compartment of Boyden chambers. Data are expressed as percent migration as compared to Ca 140. Experiments were run in triplicate and repeated twice. SDs are indicated.

Table IV. Checkerboard Analysis of GRGDSP Migration-inducing Activity for 3T3 Cells

	Upper compartment		
Lower compartment	0	10^{-2} μM	10^{-1} μM
0	3 ± 2	9 ± 1	2 ± 1
10^{-2} μM	16 ± 2	3 ± 1	2 ± 1
10^{-1} μM	10 ± 2	2 ± 1	1 ± 0

Concentrations of GRGDSP active in chemotactic stimulation (Fig. 2) were put in the lower and/or upper compartment. The first column on the left represents chemotaxis, the diagonal represents chemokinetic activity.

through a different receptor (8, 24). Furthermore, adsorption of the antiserum with partially purified FN receptor resulted in the virtually complete loss of inhibitory activity on cell migration (data not shown). The possibility that antibodies inhibited migration by preventing adhesion of the cells to the upper surface of the filter was ruled out since a concentration of 250 μg/ml of antibodies in the lower compartment significantly inhibited migration (50%) without affecting adhesion (data not shown). The antibodies were found to be more

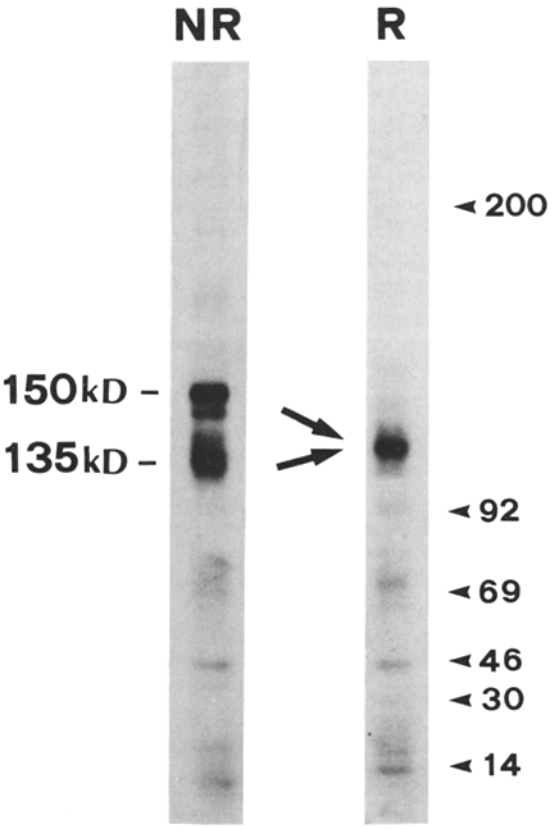

Figure 3. Immunoprecipitation of the cell surface FN receptor complex. Cell surface proteins were immunoprecipitated by incubating [^{35}S]methionine-labeled transformed mouse fibroblasts with the anti-BHK serum. Immunocomplexes were recovered by Protein A-Sepharose and radioactive proteins were detected by fluorography after electrophoretic separation in a 5–10% polyacrylamide gel in the presence of SDS. Two major bands with apparent molecular masses of 150 and 135 kD were detected in the nonreduced (*NR*) sample. These components migrate as a single diffuse band after reduction (*R*) of disulfide bonds with mercaptoethanol. Numbers on the right indicate the relative mobility of molecular mass markers.

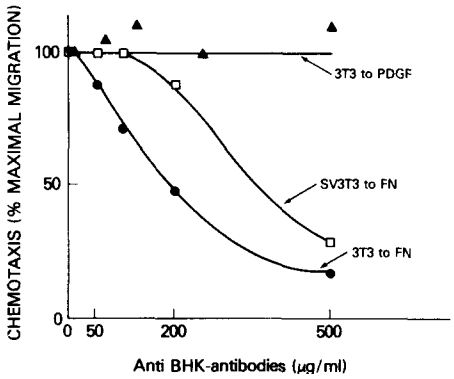

Figure 4. Inhibition of chemotaxis of 3T3 and SV3T3 cells to FN by anti-FN receptor antibodies. 30 μg/ml FN was put in the lower compartment of chemotaxis chambers with increasing amounts of anti-BHK antibodies. 3T3 and SV3T3 cells were used as target cells in the upper compartment. Migration to PDGF (5 ng/ml) under the same experimental conditions was used as control. Data are expressed as percent migration as compared with the chemoattractant alone. Three independent experiments were carried out. Each assay was done in triplicate and did not differ by more than 10%.

effective in preventing FN-induced directional migration of normal than of SV40-transformed cells. As shown in Fig. 4, in fact, while 200 μg/ml gave ~50% inhibition of migration of normal 3T3 cells, an approximately twofold higher concentration of the antibodies was necessary to obtain the same degree of inhibition with SV3T3 cells.

Discussion

FN is a potent chemoattractant, particularly for mesenchymal cells, and chemotactic activity resides in a cathepsin D-derived fragment (M_r 140,000) (3, 5, 41, 47). When coated onto surfaces, this peptide also mediates cell attachment by binding to FN receptors on cell surfaces (5, 47). Current concepts suggest that chemotaxis is also a receptor-mediated phenomenon, which allows the cells to detect and migrate along a gradient of a chemotactic factor (2, 6, 12, 22, 24, 36, 49, 56). Due to the coincidence of attachment and chemotactic activities within the Ca 140 fragment of FN, it seemed possible that they represent different functions mediated via the same recognition system.

In the present work, we show that RGDS-containing FN sequences (37, 38, 40) and the 140-kD cell surface receptor (18, 19, 51) known to be involved in cell–substratum adhesion also mediate the migratory response of 3T3 and SV3T3 cells to FN and an FN fragment.

Two monoclonal antibodies (A317 and 3E3), able to block cell attachment by reacting with the portion of Ca 140 close to the cell-attachment site (16, 39), also inhibit the chemotactic response of the cells to the Ca 140 peptide. Moreover, the synthetic hexapeptide GRGDSP, corresponding to the cell-attachment sequence in FN (37, 38, 40), was able to stimulate both random and chemotactic migration, although its chemotactic activity is lower (i.e., 30%) than that of FN. A shorter peptide, RGDS, was not significantly chemotactic itself, but did reduce chemotaxis to the Ca 140 cell-binding peptide indicating that it can compete in FN–cell recognition. GRGDSP is also a better substrate for adhesion than RGDS (37). The loss of chemotactic activity of RGDS could

be due to a less stable binding of this short peptide. Another possibility is a more rapid diffusion of this small molecule with disruption of the gradient across the filter.

FN-mediated cell adhesion appears to occur via cell receptors (1, 9, 11, 13, 15, 21, 23, 25, 35, 43, 50). Using antibodies to the FN receptor of mouse fibroblasts (18, 19, 51), we provided evidence for the involvement of this molecule in the chemotactic response to FN. These antibodies, in fact, strongly inhibited cell migration through the filter. Neither the antibodies to the cell-attachment site in FN nor the antibody to the FN receptor altered chemotaxis to PDGF under the same experimental conditions. This shows that the interactions with FN are specific and also that the general chemotactic ability of the cells to translocate on the filter and migrate directionally is not impaired by the anti-FN and anti-FN receptor antibodies. It is likely that the 3T3 cells can locomote via independent means to PDGF.

It is interesting to note that neither the anti-FN, the anti-FN receptor antibodies, nor the peptide completely inhibited the chemotactic response to FN. This suggests the involvement of other sites in FN which are active in the chemotactic stimulation of fibroblastic cells. The existence of adhesion-mediating sites on FN, different than the RGDS site, have been described (26, 31). Our Ca 140 peptide is localized 65–75 kD from the COOH-terminal end of the molecule. Therefore it contains the domains III and IV which have been investigated for adhesion and motility-promoting activities by McCarthy et al. (32), who suggest that an adhesion and motility-promoting site, in addition to RGDS (located in domain IV), are present in domain III.

Our results also indicate that both anti-FN and anti-FN receptor antibodies were less effective in preventing migration of SV3T3 cells than that of 3T3 cells. This is consistent with the fact that SV40-transformed cells display an enhanced chemotactic response to FN (3, 33). In contrast, SV3T3 were more sensitive than 3T3 cells to the inhibitory effect of the RGDS peptide. We conclude that, although the migration of 3T3 and SV40-transformed 3T3 cells involves the same molecular mechanisms, the response to FN, to anti-FN receptor antibody, and to an FN peptide is somewhat altered in SV40-transformed cells. Perhaps this is related to the differences in distribution of FN receptor, which in normal cells are clustered in the adhesion plaques and in transformed fibroblasts have a diffuse distribution (18, 55). It is possible that the lack of clustering of FN receptors within localized membrane domains may contribute to the higher motility of transformed cells.

Preliminary observations that either antibodies to the FN receptor or synthetic peptides inhibit translocation on a matrix substrate were recently presented (4, 7, 10, 14, 29). It is not surprising that antibodies or peptides able to interfere with cell adhesion also prevent migration on a matrix substrate. Our work presents evidence that the cell-binding domain of FN and its receptor are involved in the chemotactic response of fibroblasts to a gradient of FN in solution.

In summary, our data show that the chemotaxis of 3T3 and SV3T3 cells is mediated through the RGDS cell attachment site of FN and through the FN cell surface receptor. The interaction is specific and there is preliminary evidence for its alteration with transformation.

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