Analysis of Fibronectin Receptor Function with Monoclonal Antibodies: Roles in Cell Adhesion, Migration, Matrix Assembly, and Cytoskeletal Organization

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Abstract. We have developed two rat mAbs that recognize different subunits of the human fibroblast fibronectin receptor complex and have used them to probe the function of this cell surface heterodimer. mAb 13 recognizes the integrin class 1 beta polypeptide and mAb 16 recognizes the fibronectin receptor alpha polypeptide. We tested these mAbs for their inhibitory activities in cell adhesion, spreading, migration, and matrix assembly assays using WI38 human lung fibroblasts. mAb 13 inhibited the initial attachment as well as the spreading of WI38 cells on fibronectin and laminin substrates but not on vitronectin. Laminin-mediated adhesion was particularly sensitive to mAb 13. In contrast, mAb 16 inhibited initial cell attachment to fibronectin substrates but had no effect on attachment to either laminin or vitronectin substrates. When coated on plastic, both mAbs promoted WI38 cell spreading. However, mAb 13 (but not mAb 16) inhibited the radial outgrowth of cells from an explant on fibronectin substrates. mAb 16 also did not inhibit the motility of individual fibroblasts on fibronectin in low density culture and, in fact, substantially accelerated migration rates. In assays of the assembly of an extracellular fibronectin matrix by WI38 fibroblasts, both mAbs produced substantial inhibition in a concentration-dependent manner. The inhibition of matrix assembly resulted from impaired retention of fibronectin on the cell surface. Treatment of cells with mAb 16 also resulted in a striking redistribution of cell surface fibronectin receptors from a streak-like pattern to a relatively diffuse distribution. Concomitant morphological changes included decreases in thick microfilament bundle formation and reduced adhesive contacts of the streak-like and focal contact type. Our results indicate that the fibroblast fibronectin receptor (*a*) functions in initial fibroblast attachment and in certain types of adhesive contact, but not in the later steps of cell spreading; (*b*) is not required for fibroblast motility but instead retards migration; and (*c*) is critically involved in fibronectin retention and matrix assembly. These findings suggest a central role for the fibronectin receptor in regulating cell adhesion and migration.

THE interaction of cells with solid substrates is important for their anchorage, proliferation, migration, and differentiation. Cells can attach, spread, and migrate on a variety of extracellular glycoproteins including fibronectin, laminin, vitronectin, and collagen. These interactions occur through specific cell surface receptors. Many of these cell adhesion receptors belong to a large superfamily of related cell surface complexes called the integrins or the cytoadhesins (for reviews see 3, 11, 26, 30, 33, 37, 52). The integrins are noncovalent, heterodimeric glycoprotein complexes that consist of a 140,000–180,000-D alpha subunit and a 105,000–125,000-D beta subunit. The alpha subunit often consists of a small, transmembrane polypeptide of ~20,000 D disulfide bonded to a larger, extracellular polypeptide (3, 6, 35, 48).

The various integrin heterodimers have been divided into classes based on the different beta polypeptides (33). The class 1 integrins include the human T cell "very late antigen" (VLA)[1] heterodimers (30, 31), the avian 140k fibronectin receptor complex (5, 32), the mammalian fibronectin receptor (2, 9, 10, 49, 50, 60, 61), laminin receptors (25, 54, 58), and collagen receptors (31, 38, 55–57, 60, 61). Members of this family contain one of at least six unique, but related, alpha subunits noncovalently associated with the common beta subunit. There are at least three different class 2 integrin heterodimers characteristically present on leukocytes. There

1. *Abbreviations used in this paper*: DPBS$^+$, Dulbecco's phosphate-buffered saline with divalent cations; DPBS$^-$, Dulbecco's phosphate-buffered saline without divalent cations; VLA, very late antigen.

are three different alpha subunits that can associate with the class 3 beta subunit. Class 3 integrins include the platelet glycoprotein IIb–IIIa complex and the mammalian vitronectin receptor (17, 48).

Both the alpha and beta subunits of the human fibronectin receptor have been cloned, and their sequences have been deduced (6, 7, 23). Both subunits have putative membrane-spanning sequences, small carboxy-terminal intracellular segments of 28 residues (alpha) or 47 residues (beta), and large amino-terminal extracellular domains. The alpha subunits contain two to four putative calcium-binding sequences. The beta subunits contain four cysteine-rich homologous regions that presumably contain extensive intrachain disulfide bonding. Both subunits are glycosylated (2, 6, 7). mAbs have recently been produced against this receptor, which is thought to be involved in cell attachment to fibronectin and which is expressed in tissue-specific patterns (10, 61).

To elucidate the roles of this receptor in cell biological functions, we have used purified fibronectin receptor preparations to develop mAbs that bind to the fibronectin receptor (also known as VLA 5). Here, we describe two antibodies designated mAb 16 and mAb 13 which bind to the alpha and beta subunits, respectively, of the fibronectin receptor from cultured human fibroblasts. These antibodies define different functions of the fibronectin receptor in cell attachment or spreading, cell migration, and extracellular matrix assembly.

Materials and Methods

Cell Culture

WI38 fibroblasts (CCL 75; passages 18–24) were obtained from American Type Culture Collection (Rockville, MD). Human foreskin fibroblasts (passages 6–12) were used for some early mAb screening experiments and were a generous gift from Dr. Steve Alexander (Bethesda Research Laboratories, Gaithersburg, MD). Both cell lines were cultured in DME supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 1 mM glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin (culture medium). Hybridomas were routinely maintained as described (4).

Purification of Proteins and Immunological Reagents

Fibronectin and vitronectin were purified as described from fresh-frozen human plasma obtained from the Department of Transfusion Medicine (National Institutes of Health, Bethesda, MD) (1, 44, 64). The concentrations of fibronectin and vitronectin were determined by absorbance at 280 nm, assuming extinction coefficients of 1.28 and 1.38 ml/mg, respectively, for a 1-cm path length (19, 45). Laminin was obtained from Bethesda Research Laboratories.

Sprague-Dawley rats were immunized with fibronectin receptor purified from human placenta as described previously (47, 49, 50, 51). Hybridomas producing anti-fibronectin receptor mAbs were produced by the fusion of Y3 rat myeloma cells with spleen cells as described (24). Clones were first screened for those antibodies that recognized cell surface antigens in indirect immunofluorescence staining of cultured human foreskin fibroblasts. The second and third screens were for those antibodies that recognized integrin polypeptides in immunoblots of human placenta homogenates or that could immunoprecipitate radioactive integrin polypeptides from lysates of human fibroblasts grown in the presence of 50 μCi/ml [^{35}S]methionine. Useful clones were then subcloned twice by the method of limiting dilution. The mAbs were isotyped using a commercially available kit (ICN Immunobiologicals, Irvine, CA).

mAbs were purified from hybridoma-conditioned serum-free medium as described (4, 13). Fab fragments of mAbs were obtained by incubating purified IgG with immobilized papain (Pierce Chemical Co., Rockford, IL) for 5 h at 37°C following the manufacturer's instructions. The digestion products were examined by SDS-PAGE and were found to be free of intact IgG heavy chain. mAbs and Fab fragments were concentrated by vacuum dialysis and dialyzed against Dulbecco's PBS without divalent cations (DPBS$^-$). Polyclonal anti-human placental fibronectin receptor antibodies have been characterized previously (51).

Assays for Cell Attachment and Cell Spreading

Substrates for cell attachment and cell spreading assays were prepared as described (5, 63) using fibronectin, laminin, or vitronectin at the concentrations indicated in the figure legends. The prepared substrates were covered with 100 μl of serum-free DME which was removed just before use. All subsequent steps of both assays were performed at 37°C with reagents prewarmed to 37°C unless otherwise noted.

Cell attachment was assayed by a modification of the procedure of Nagata et al. (46). WI38 fibroblasts were labeled overnight with 4 μCi/ml [^{3}H]thymidine (21 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) followed by a 2-h chase to deplete unincorporated label. The cells were washed with calcium- and magnesium-free Hanks' balanced salt solution and incubated in 0.25% trypsin (Gibco Laboratories, Grand Island, NY) in calcium- and magnesium-free Hanks' balanced salt solution for 2 min. The cells were then dislodged by shaking, suspended in 5 ml of regular culture medium, centrifuged, resuspended in 5 ml of regular culture medium, allowed to recover from the trypsinization for 20 min at 37°C, and counted. The cells were then centrifuged and resuspended in serum-free DME at a concentration of 2×10^5 cells/ml. Aliquots (50 μl) of cells were mixed with 50-ul aliquots of various concentrations of antibodies in serum-free DME, added to substrates prepared with adhesion proteins, and incubated for 30 min at 37°C in a 95% air, 5% CO_2, humidified atmosphere. Unbound cells were removed by gentle aspiration. The wells were then gently washed twice with serum-free DME by swirling at 150 rpm for 30 s. Bound cells were solubilized in 150 μl 2% SDS in 0.01 N NaOH and quantitated by liquid scintillation spectrometry. Three 10-μl aliquots of the original cell suspension were mixed with 150 μl of 2% SDS, 0.01 N NaOH and counted to determine the amount of radioactivity added to each well.

Cell spreading assays were performed essentially as described (5) with the following modifications. Subconfluent WI38 fibroblasts were prepared as described for the cell attachment assays, but not radiolabeled. Cells were suspended in serum-free DME and allowed to spread on substrates prepared with adhesion proteins for 1 h at 37°C in a CO_2 incubator before being fixed and quantitated as described (28, 29).

Background levels in both the cell attachment and the cell spreading assays were determined by adding cells to wells that had not been exposed to adhesion proteins before being blocked with heat-denatured BSA. In additional control experiments, either purified nonimmune rat IgG (Calbiochem-Behring Corp., La Jolla, CA) or a class-matched, control rat mAb was used. Neither control antibody had significant inhibitory effects at concentrations equal to or greater than those used for mAbs 13 and 16. It should also be noted that wide ranges of antibody concentrations were chosen to rule out artifacts such as possible prozone effects.

Cell Migration Assays

Cell migration was quantitated by determining the extent of outward cell migration from an agarose droplet using a modification of the methods of Varani et al. (59). Cells were harvested and resuspended at a concentration of 3.3×10^7 cells/ml in growth medium prepared with bicarbonate-free DME buffered with 25 mM Hepes, pH 7.4 (Gibco Laboratories). After adjusting to 0.2% agarose using a stock of 2% low melting point agarose (Bethesda Research Laboratories) maintained at ≥38°C, the cells were plated in 1-μl droplets on Immulon I (Dynatech Laboratories, Chantilly, VA) 96-well plastic substrates precoated with 5 μg/ml human plasma fibronectin. After the agarose was gelled by incubation at 4°C for 5 min, regular growth medium containing the indicated quantities of mAb was added with care to avoid detachment of the droplets. Cells were cultured in a CO_2 incubator for 2 d and then fixed with 4% formaldehyde. After rinsing, cultures were stained with 1% crystal violet in 95% ethanol, destained in water, and air dried. Each well was photographed by transmitted light using a dissecting microscope. Total areas of cell outgrowth were digitized using a Sigma-Scan measurement system to measure the outer perimeter of the sheets of radially migrating cells (Jandel Scientific, Corte Madera, CA). Photographs of zero-time control agarose droplets were also digitized, and these areas were subtracted from total outgrowth to calculate net areas of outgrowth.

The migration of individual cells was quantitated by time-lapse video microscopy. Plastic 35-mm Petri dishes (Falcon Labware, Oxnard, CA) were

coated with 5 μg/ml human plasma fibronectin and then blocked with heat-denatured BSA as described (63). WI38 fibroblasts were plated in bicarbonate-free DME containing 10% heat-inactivated FCS and 25 mM Hepes at 10^4 cells/ml. The dishes were sealed and placed on the stage of an inverted microscope (Carl Zeiss, Inc., Thornwood, NY) enclosed within a chamber maintained at 37 ± 0.2°C. Cells were visualized with a video camera (2000; RCA, Lancaster, PA) connected to a time-lapse video cassette recorder (AG 6010; Panasonic, Secaucus, NJ). Migration of cells was traced at 1- or 3-h intervals over a 15-h period starting 6 h after plating. Cell paths marked at 1-h intervals were traced for illustrations. Alternatively, individual time segments were digitized from 3-h time points on the tracings. At least 40 time segments were digitized for each condition.

Assays for Fibronectin Matrix Accumulation and Assembly

WI38 fibroblasts were plated at 3 × 10^5 cells/ml in 8-well chambers on a glass substrate (Lab-Tek; Miles Scientific Div., Naperville, IL) in 0.4 ml/well regular culture medium containing the indicated concentrations of mAb, control rat IgG, or class-matched control rat mAb. After 24 h of culture, the medium was removed, and the monolayers were rinsed and fixed with 4% formaldehyde with 5% sucrose (to suppress blebbing) in Dulbecco's PBS with divalent cations ($DPBS^+$) for 30 min and then rinsed with fresh $DPBS^+$. Where indicated, cells were permeabilized with 0.4% Triton X-100 in $DPBS^-$. Intact or permeabilized cells were incubated with 25 μg/ml goat 1213 anti-human fibronectin that was affinity purified and characterized as described (62) in 1 mg/ml BSA followed by 1:100 FITC-labeled rabbit anti-goat IgG (Miles Scientific Div.). The mounting medium contained 1 mg/ml *p*-phenylenediamine in 10% $DPBS^+$, 90% glycerol to inhibit photobleaching (36). For comparative immunolocalization of fibronectin and its receptor on the same cell, fibronectin receptor was localized with 25 μg/ml rabbit anti-human fibronectin receptor IgG followed by rhodamine-labeled goat anti-rabbit IgG which had been previously depleted of reactivity to rat IgG by passage through an 0.5 × 7-cm column of nonimmune rat IgG-Sepharose. Fibronectin was visualized with a 1:100 dilution of goat anti-human fibronectin IgG directly labeled with FITC.

Immunofluorescence microscopy was performed using a Photomicroscope III (Carl Zeiss, Inc.) equipped for epifluorescence using 40× (NA 1) and 63× (NA 1.4) objectives. Photographs were taken with Tri-X pan film with speed increased to ASA 1600 using Diafine (Accufine, Inc., Chicago, IL) and printed under conditions of identical exposure and development to permit direct comparisons. Antibody binding to extracellular fibronectin was quantified by photometry as described previously (16) using a 25× objective and the Photomicroscope III photometer set to measure the entire field at 800 ASA.

Alternatively, total quantities of fibronectin in monolayers (including intracellular pools) were determined by dot immunoblotting. Antibody-treated and control cultures were rinsed twice with $DPBS^+$ and once with $DPBS^-$, scraped with a rubber policeman into 100 μl 2% SDS, 2 mM PMSF in 10 mM sodium phosphate, pH 7, and then incubated at 100°C for 3 min. Aliquots of homogenate (3 μl) were spotted onto nitrocellulose and quantitatively assayed for fibronectin as described (5), except that 5% nonfat milk solids were substituted for the BSA for initial blocking, and rabbit anti-human plasma fibronectin antiserum in 5% milk solids followed by 0.5 μCi/ml ^{125}I-labeled protein A in 5% BSA (8.5 μCi/μg; New England Nuclear, Boston, MA) were used. Quantities of fibronectin were calculated based on standards of purified human plasma fibronectin. Because only low final concentrations of fibronectin were secreted and because of the risk of antibody cross-reaction with bovine fibronectin, secretion or release of fibronectin into medium was determined after labeling for 24 h with 10 μCi/ml [^{35}S]methionine (Translabel; ICN Biomedicals, Inc., Irvine, CA) in 0.4 ml regular medium/well in 8-well chambers. Medium from each well was precipitated with 10% TCA, neutralized, and analyzed by SDS-PAGE, autoradiography, and densitometry. Radioactively labeled cell monolayers were also subjected to SDS-PAGE and autoradiography and revealed no differences in overall incorporation or in protein band patterns.

Other Procedures

Immunoprecipitations were carried out essentially as described (51). After centrifuging and preclearing, the cell extracts were diluted with an equal volume of $DPBS^+$ supplemented with 2.5 mg/ml crystalline BSA. Immunoprecipitations with the rat mAbs were performed with protein G-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) instead of protein A-Sepharose. mAb 13 was used at a final concentration of 20 μg/ml, and mAb 16 was used at a final concentration of 50 μg/ml.

SDS-PAGE was performed as described by Laemmli (39), using a 4% stacking gel and 7.5% resolving gel. Radioactive polypeptides were visualized as described (2). Nitrocellulose transfers and Western blotting were performed essentially as described (12, 15) with the following minor modifications. Nonfat milk solids (5% [wt/vol]) were substituted for the BSA for initial blocking. Blots were then incubated with either 10 μg/ml of mAb 13 or 50 μg/ml mAb 16 in 5% milk solids followed by 0.5 μCi/ml ^{125}I-anti-rat IgG (8 μCi/μg, Amersham Corp., Arlington Heights, IL) in 5% BSA.

Staining of cells for F-actin using rhodamine-labeled phalloidin (Molecular Probes, Inc., Junction City, OR) was performed as described (8). Characterization of adhesive contacts on the ventral cell surface by interference reflection microscopy was performed as described (15).

Results

Specific Inhibition of Cell Attachment by mAb 13 and mAb 16

Human fibronectin receptor (VLA 5) was purified from placenta by affinity chromatography and used to immunize rats for the production of mAbs. We previously used such preparations to produce monospecific polyclonal anti-fibronectin receptor antibodies (51). In the present study, two rat mAbs have been identified that specifically inhibit WI38 human lung fibroblast attachment to fibronectin. Both mAbs were found to be of the IgG_{2a} subtype by Ouchterlony mAb typing. As shown in Fig. 1, the WI38 fibroblasts could attach to substrates prepared with purified fibronectin, laminin, and

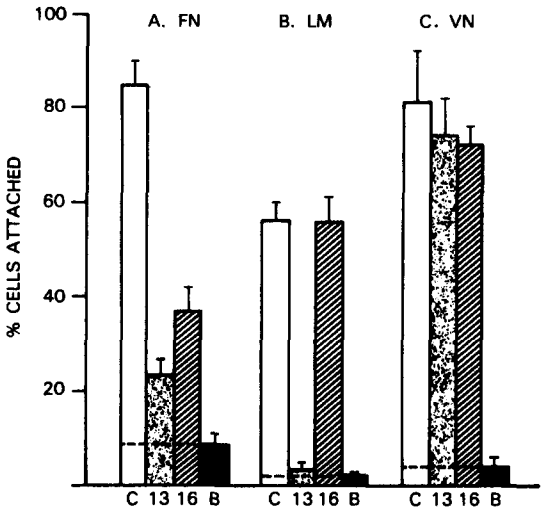

Figure 1. Effect of mAbs on WI38 fibroblast attachment to fibronectin, laminin, and vitronectin. Substrates were prepared by coating 96-well tissue culture clusters with 25 μg/ml of fibronectin (*A*), 25 μg/ml of laminin (*B*), or 40 μg/ml vitronectin (*C*). WI38 fibroblasts were either added alone (*open bars*) or in the presence of mAb 13 (*stippled bars*) or mAb 16 (*crosshatched bars*). Background levels of cell attachment in the absence of adhesive protein coating are also indicated (*solid bars*). In *A*, the concentrations of mAbs 13 and 16 were 50 μg/ml and 2 mg/ml, respectively. In repeated experiments using lower concentrations of mAb 16, an inhibitory effect, often only partial, was still observed. In *B*, the concentrations of mAbs 13 and 16 were 2 μg/ml and 1 mg/ml, respectively. In *C*, the concentrations of mAbs 13 and 16 were both 1 mg/ml. Each bar is the average of values determined from triplicate wells (±SEM).

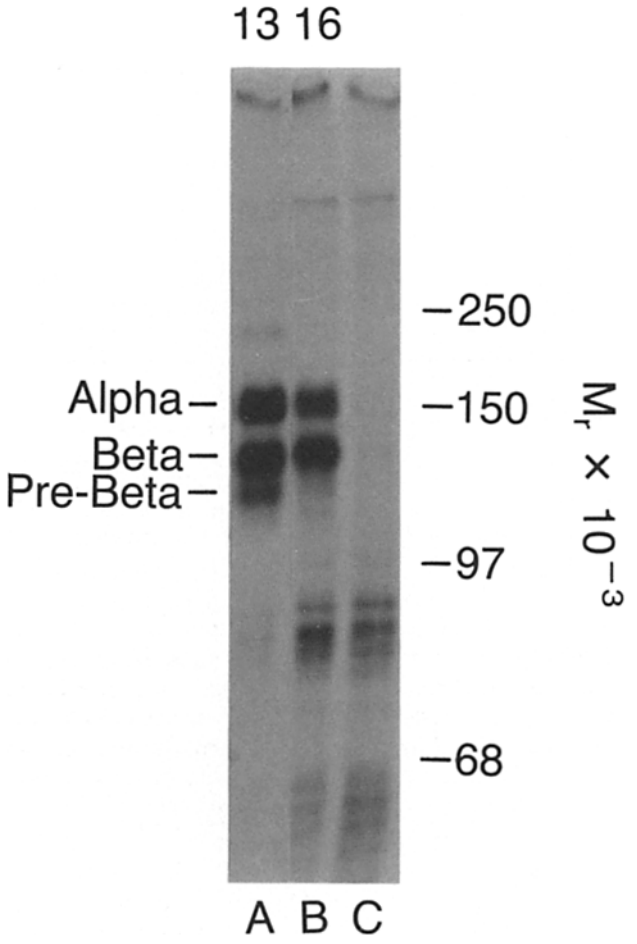

Figure 2. Recognition of fibronectin receptor polypeptides by mAbs. Immunoprecipitation of fibronectin receptor polypeptides from cell lysates metabolically labeled with [^{35}S]methionine using mAbs 13 (lane *A*) and 16 (lane *B*). In lane *C*, cell lysate was treated with protein G–Sepharose in the absence of mAb as a control. The positions of the alpha, beta, and pre-beta polypeptides are shown on the left, and molecular mass standards are shown on the right.

vitronectin (Fig. 1, *A–C*, respectively). mAb 13 significantly inhibited cell attachment to both fibronectin ($p < 0.001$) and laminin ($p < 0.001$). The inhibition of attachment to laminin occurred at low concentrations of mAb 13; e.g., complete inhibition was found at only 2 μg/ml (Fig. 1 *B*). These results are consistent with mAb 13 recognition of the integrin beta subunits shared by fibronectin and laminin receptors.

As also shown in Fig. 1, mAb 16 inhibited cell attachment on fibronectin substrates ($p < 0.005$), but had no significant effect on cell attachment to laminin ($p > 0.98$). Neither mAb 13 ($p > 0.6$) nor 16 ($p > 0.3$) significantly inhibited cell attachment on vitronectin substrates (Fig. 1 *C*). These results are consistent with mAb 16 recognition of the specific alpha subunit of the receptor. The ≥80% inhibition of cell attachment to fibronectin by mAb 13 shown in Fig. 1 is maximal. In repeated experiments in which the concentration of mAb 13 was varied over a range of 0.1–2 μg/ml, the inhibition of cell attachment consistently became maximal at ~10–50 μg/ml IgG (not shown).

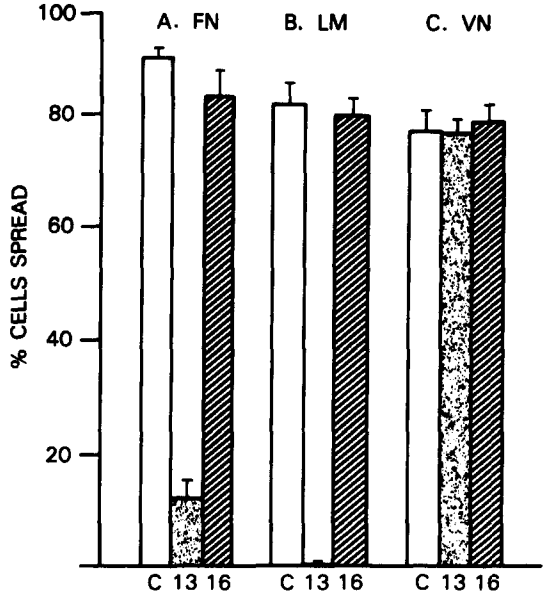

Figure 3. Effect of mAbs on WI38 fibroblast spreading on fibronectin, laminin, and vitronectin. Substrates were prepared by coating 96-well tissue culture clusters with 5 μg/ml of fibronectin (*A*), laminin (*B*), or vitronectin (*C*). WI38 fibroblasts were either added alone (*open bars*) or in the presence of mAb 13 (*stippled bars*) or mAb 16 (*crosshatched bars*). In all experiments, there was no cell spreading if the substrate was not coated with an adhesive protein. In *A*, the concentrations of mAbs 13 and 16 were both 500 μg/ml. In *B*, the concentrations of mAbs 13 and 16 were 1 μg/ml and 1 mg/ml, respectively. In *C*, the concentrations of mAbs 13 and 16 were both 1 mg/ml. Each bar is the average of five determinations from random fields of cells (±SEM). In control experiments, higher concentrations (up to 1 mg/ml) of mAbs 13 and 16 had no additional inhibitory effects on cell spreading on fibronectin substrates.

Immunoprecipitation of Fibronectin Receptor Polypeptides with mAbs 13 and 16

mAb 13 immunoprecipitated three radioactive polypeptides from lysates of cells that had been metabolically labeled with [^{35}S]methionine (Fig. 2, lane *A*). These polypeptides have been identified as the alpha, beta, and pre-beta polypeptides of the fibronectin receptor (2, 10, 34). As shown in Fig. 2, lane *B*, mAb 16 immunoprecipitated only the alpha and mature beta polypeptides and not the pre-beta polypeptide. Immunoblots showed binding of mAb 13 only to beta and not alpha subunits from placenta or fibroblasts, whereas mAb 16 could not be characterized due to drastic losses of epitope recognition, presumably because of the SDS denaturation. As in previous integrin characterizations (2, 10, 31; Fig. 2, lane *A*), the alpha polypeptide appears in the immunoprecipitate because it is bound to the beta subunit. Conversely, in Fig. 2, lane *B*, the beta polypeptide is present because it is bound to the alpha polypeptide. The pre-beta polypeptide does not appear in Fig. 2, lane *B*, because under normal circumstances it does not form a complex with the alpha polypeptide (2).

Inhibition of Cell Spreading by mAbs 13 and 16

WI38 fibroblasts spread on substrates prepared with fibronectin, laminin, and vitronectin (Fig. 3, *A–C*, respectively).

As shown in Fig. 3, mAb 13 significantly inhibited cell spreading on both fibronectin ($p < 0.001$) and laminin ($p < 0.001$). In contrast, mAb 16 had no significant inhibitory activity on fibronectin ($p > 0.7$), as well as on laminin ($p > 0.25$), at this standard assay termination time. As shown in Fig. 4, a time course analysis indicated that cell spreading on fibronectin was in fact inhibited substantially for 30 min, but that the cells were subsequently able to overcome the inhibition and were fully spread by the 60-min endpoint. This result can be explained by hypothesizing that the cells are eventually able to overcome the inhibitory effects of mAb 16 in this assay system by using a second adhesion receptor system.

As was the case for cell attachment, neither mAb 13 ($p > 0.97$) nor 16 ($p > 0.90$) inhibited cell spreading on vitronectin (Fig. 3 *C*). The inhibitory effect of mAb 13 on cell spreading on fibronectin was substantial but never entirely complete. In repeated experiments in which the concentration of mAb 13 was varied over a range of 10 μg/ml to 2 mg/ml, the inhibition of cell spreading consistently became maximal at ~200–500 μg/ml IgG (not shown). However, even at concentrations of 2 mg/ml, the small amount of residual cell spreading could never be decreased to background levels. Similarly, when higher concentrations of mAb 16 were used (up to 1 mg/ml), no inhibitory effect was observed on fibronectin substrates (not shown).

The lack of effects of both mAb 13 and 16 on cell spreading and attachment on vitronectin indicates that these antibodies are specific for class 1 integrins and do not recognize the class 3 vitronectin receptor.

Promotion of Cell Spreading by mAbs 13 and 16

Both mAb 13 and 16 could themselves serve as substrates for cell spreading. As shown in Fig. 5, the WI38 cells spread on substrates prepared with mAbs 13 and 16 in a concentration-dependent manner. Half-maximal spreading occurred at similar IgG concentrations for both mAbs (~70 μg/ml for mAb 13 and 80 μg/ml for mAb 16). At higher antibody concentrations, the maximum level of cell spreading activity exhibited by both antibodies was not significantly different ($p > 0.80$). The experiment shown in Fig. 5 further demonstrates that neither mAb 13 nor 16 is cytotoxic.

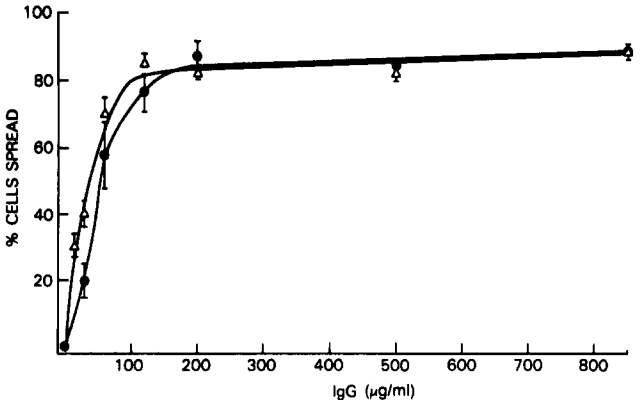

Figure 5. Spreading of WI38 fibroblasts on mAb substrates. Substrates were prepared by coating 96-well tissue culture clusters with the indicated concentrations of mAb 13 (●) or 16 (△). The percentage of WI38 fibroblasts scored as spread at each mAb concentration is indicated. Each point is the average of five determinations from random fields of cells (±SEM).

Effects on Cell Migration of mAbs 13 and 16

The roles of the fibronectin receptor, or of integrin class 1 beta receptors in general, in fibroblast migration were explored using mAbs 13 and 16. As quantitated in Fig. 6, WI38 fibroblasts migrated outward readily from agarose droplets onto a fibronectin substrate. This migration was inhibited by mAb 13. The inhibition observed with mAb 13 was highly significant ($p < 0.0001$). In contrast, no significant inhibition of outgrowth was observed after treatment with either mAb 16 ($p \geq 0.3$) or nonimmune IgG ($p \geq 0.4$). In other experiments, mAb 16 was confirmed to be noninhibitory at concen-

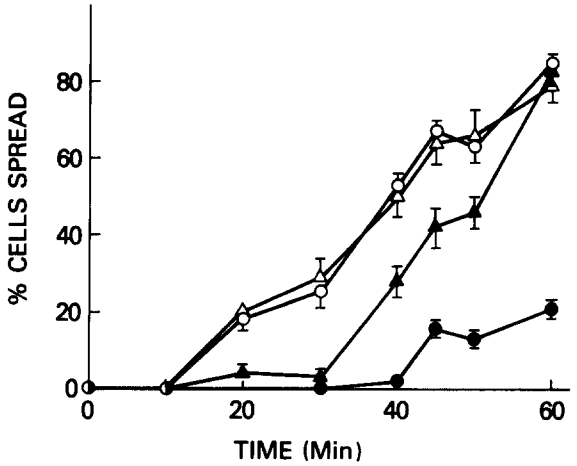

Figure 4. Time course of WI38 fibroblast spreading on fibronectin. Substrates were prepared by coating 96-well tissue clusters with 5 μg/ml fibronectin. WI38 fibroblasts were either added alone (○) or in the presence of 500 μg/ml mAb 13 (●), mAb 16 (▲), or nonimmune rat IgG (△). At the indicated times, the cells were fixed and scored for spreading. Each point is the average of five determinations from random fields of cells (±SEM).

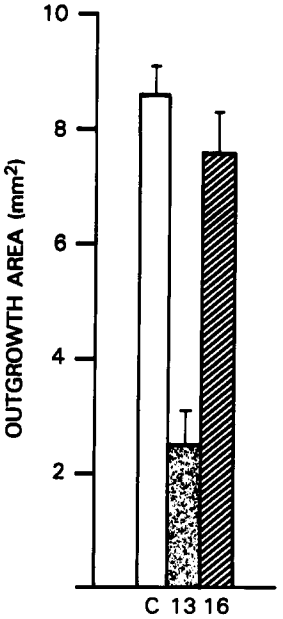

Figure 6. Effects of anti-receptor mAbs on WI38 fibroblast migration. WI38 fibroblasts were allowed to migrate from high density explants on fibronectin substrates as described under Materials and Methods. Outgrowth was quantitated by staining the explants with crystal violet and measuring the area covered by cells, subtracting the area at zero time. Cells were either added to fibronectin substrates alone (*open bar*) or in the presence of 100 μg/ml mAb 13 (*stippled bar*) or 16 (*crosshatched bar*). There was no inhibition by treatment of adjacent wells with equal amounts of nonimmune rat IgG (9.35 ± 0.72 mm²).

Table I. Effect of Anti-Fibronectin Receptor Antibodies on Migration of WI38 Fibroblasts

	Rate	*n*	Significance*
	μm/h		*p*
Control WI38 cells	16.0 ± 1.0	132	–
WI38 cells + mAb 13	12.1 ± 1.3	74	0.01
WI38 cells + mAb 16	25.7 ± 2.0	65	<0.001
WI38 cells + Fab 16	24.4 ± 2.5	42	<0.001

The migration of individual cells was recorded by time-lapse video microscopy, and the rates of cell migration in the presence or absence of mAbs or Fab fragments of mAb 16 (*Fab 16*) were measured as described under Materials and Methods. mAbs 13 and 16 were at 200 μg/ml and Fab 16 was at 500 μg/ml. In this experiment, 500 μg/ml mAb 13 produced only slightly more inhibition of migration than at 200 μg/ml (rate = 11.5 ± 2.1, n = 40). The values are expressed as mean ± SEM.

* Evaluation of statistical significance of difference from control using t test.

trations ranging from 20 to 500 μg/ml (not shown). A possible explanation for the lack of inhibition by mAb 16 was that these WI38 fibroblasts were able to migrate on collagen that they had secreted and which had bound to the fibronectin substrate: for example, a requirement for both fibronectin and collagen receptors for cell adhesion has been proposed for HT1080 fibrosarcoma cells by Wayner et al. (61). However, continuous treatment with 100 μg/ml collagenase added to the cultures had no effects on outward migration in the presence or absence of mAb 16 (not shown).

Stimulation of Single Cell Migration of mAb 16

The effects of the mAbs on cell migration were examined further by video time-lapse microscopy of single cell migration in sparse cultures. mAb 13 was moderately inhibitory (Table I). In extensive analyses, no inhibition by mAb 16 could be demonstrated by this independent method. Unexpectedly, a stimulation of migration rates was observed in the presence of mAb 16 (Fig. 7) that averaged 60% and was significant statistically (Table I). The increased rate of migration was not due to microaggregation of cell surface fibronectin receptor by bivalent mAb. As shown in Fig. 7 *C* and Table I, monovalent Fab fragments of mAb 16 also stimulated the motility of individual fibroblasts in this assay.

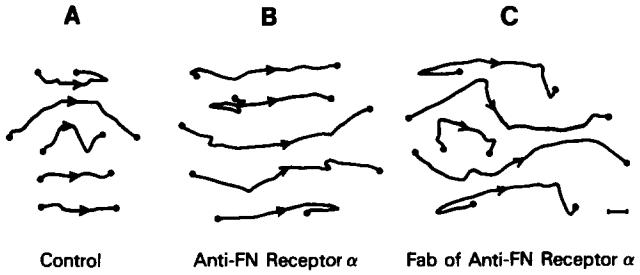

Figure 7. Effect of mAb 16 on the motility of individual WI38 fibroblasts in sparse culture. Migrating WI38 fibroblasts were followed by video microscopy for a 15-h period. Tracings of the paths of five randomly selected cells in a control culture (*A*) and in the presence of intact mAb 16 (200 μg/ml; *B*) and monovalent Fab fragments of mAb 16 (500 μg/ml; *C*) are shown. The tracings were aligned in parallel for this figure for clarity; no overall direction of the migration paths of these cells was noted. Bar, 50 μm.

Inhibition of Extracellular Matrix Assembly by mAb 16

Besides possible roles in adhesion and migration, obvious potential functions of fibronectin receptors could be in the retention and organization of secreted fibronectin into fibrils. Effects of the anti-fibronectin alpha subunit antibody on this matrix assembly process were examined by immunofluorescence and radiolabeling approaches. In Fig. 8, immunofluorescence microscopy for fibronectin shows that mAb 16 treatment resulted in a substantial decrease in fibronectin fibril accumulation. There were striking decreases in the numbers and thickness of fibronectin fibrils (Fig. 8 *C* and *D*). Similar inhibition was observed after treatment with monovalent Fab fragments of mAb 16 (Fig. 8 *E*). After permeabilization of cells, intracellular pools of fibronectin became visible, which appeared similar to those of control cells (Fig. 9). No additional fibronectin fibrils were visualized after permeabilization. In addition, a subpopulation of cells displayed unusual, dot-like strings or aggregates of fibronectin accumulating on the substrate under the ventral cell surface (e.g., Fig. 9 *D*). These structures were most frequent under cells with a free edge not in contact with other cells; ~20% of the cells displayed such clustered spots (22 ± 5%, counting 600 cells). A similar striking inhibition of fibronectin fibril formation was found with mAb 13 treatment (Fig. 9 *C*). With mAb 13, 21 ± 3% of the cells showed clusters of ventral dots of fibronectin. These clusters appeared to be present in larger numbers per cell.

The effects of mAb 16 on levels of extracellular fibronectin were concentration dependent, as shown quantitatively in Fig. 10. There was significant inhibition when even 1 μg/ml of the antibody was used (≤40% inhibition, $p < 0.001$), increasing gradually to a level of 85% inhibition at 200 μg/ml mAb 16. Total fibronectin in WI38 monolayers treated with mAb 16 was also quantitated by dot immunoblotting. These measurements included both extracellular and biosynthetic intracellular pools of fibronectin. As shown in the legend to Fig. 11, there were also substantially decreased amounts of fibronectin in mAb 16–treated cultures by this criterion as well.

Fibronectin released into culture medium from control and mAb 16–treated cultures was evaluated directly by electrophoretic analysis of radiolabeled cultures. The decreased levels of fibronectin in the cell monolayers described above were accompanied by a corresponding increase of approximately fourfold the fibronectin released into culture medium as shown in Fig. 11. These results all suggest that the defect in matrix assembly was due to impaired retention on the cell surface and not to inhibition of fibronectin synthesis. In repeated experiments, a clear inhibition of matrix assembly by mAb 16 was found in all experiments, with the inhibition of fibronectin accumulation varying from 60 to 85% in different experiments. A similar substantial inhibition of matrix assembly was observed after mAb 16 treatment of human foreskin fibroblasts (not shown).

We examined the distribution of fibronectin receptors after such mAb treatment using polyclonal, monospecific anti-fibronectin receptor antibodies characterized previously (51). As shown in Fig. 8 *F*, controls showed a characteristic organization of integrin receptors into streaks, previously shown to correlate with extracellular fibronectin fibrils as well as with certain intracellular cytoskeletal elements (15, 16, 20).

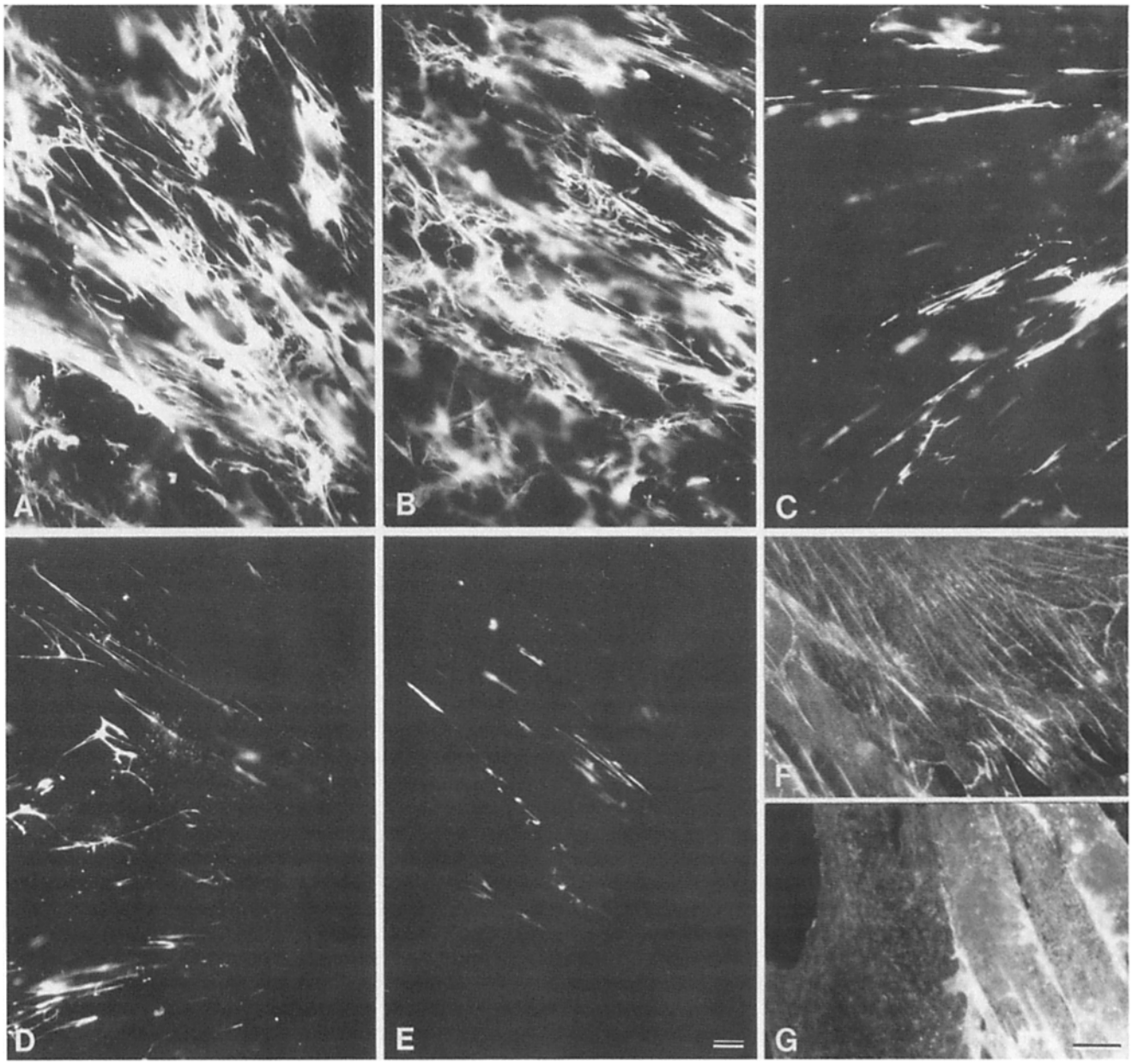

Figure 8. Single-antibody immunofluorescence analysis of matrix assembly by WI38 fibroblasts. WI38 fibroblasts were grown in regular medium alone (*A*) or in the presence of 200 μg/ml control IgG (*B*), 50 μg/ml mAb 16 (*C*), 200 μg/ml mAb 16 (*D*), or 200 μg/ml Fab fragments of mAb 16 (*E*). The formation of fibronectin fibrils was visualized by fixing the cells and staining them with affinity-purified goat anti-fibronectin antibodies followed by FITC-conjugated rabbit anti-goat IgG antibodies. For comparison of receptor distribution, cells were stained with polyclonal rabbit anti-human fibronectin receptor after growth in regular medium either alone (*F*) or in the presence of 200 μg/ml mAb 16 (*G*). Bars: (*A–E*) 10 μm; (*F* and *G*) 10 μm.

In contrast, cells treated with mAb 16 revealed a marked loss of this receptor clustering; instead, the receptor became relatively diffusely distributed on the cell surface (Fig. 8 *G*). Few, if any, streaks of receptor staining could be detected. However, local increases in immunofluorescence staining were seen where cell margins overlapped (Fig. 8 *G*). In control experiments, Fab fragments of mAb 16 produced a similar diffuse pattern of receptor. A similar diffuse organization of receptor on the cell surface was seen by direct staining of bound mAb 16 anti-alpha subunit antibodies themselves with rabbit anti-rat IgG (not shown).

The relative distribution of fibronectin and receptors was examined by comparative double immunofluorescence labeling of the same cells (Fig. 9). As noted in the previous literature, controls showed a striking, though not absolute, concordance of staining with anti-fibronectin and anti-fibronectin receptor antibodies. After mAb 16 treatment, the distribution became diffuse, and no clustering of receptor could be detected even over the mAb 16–induced spots of fibronectin found under the ventral surfaces of certain cells (Fig. 9). Thus, treatment with mAb 16 resulted in receptor redistribution as well as in a substantial inhibition of fibronectin reten-

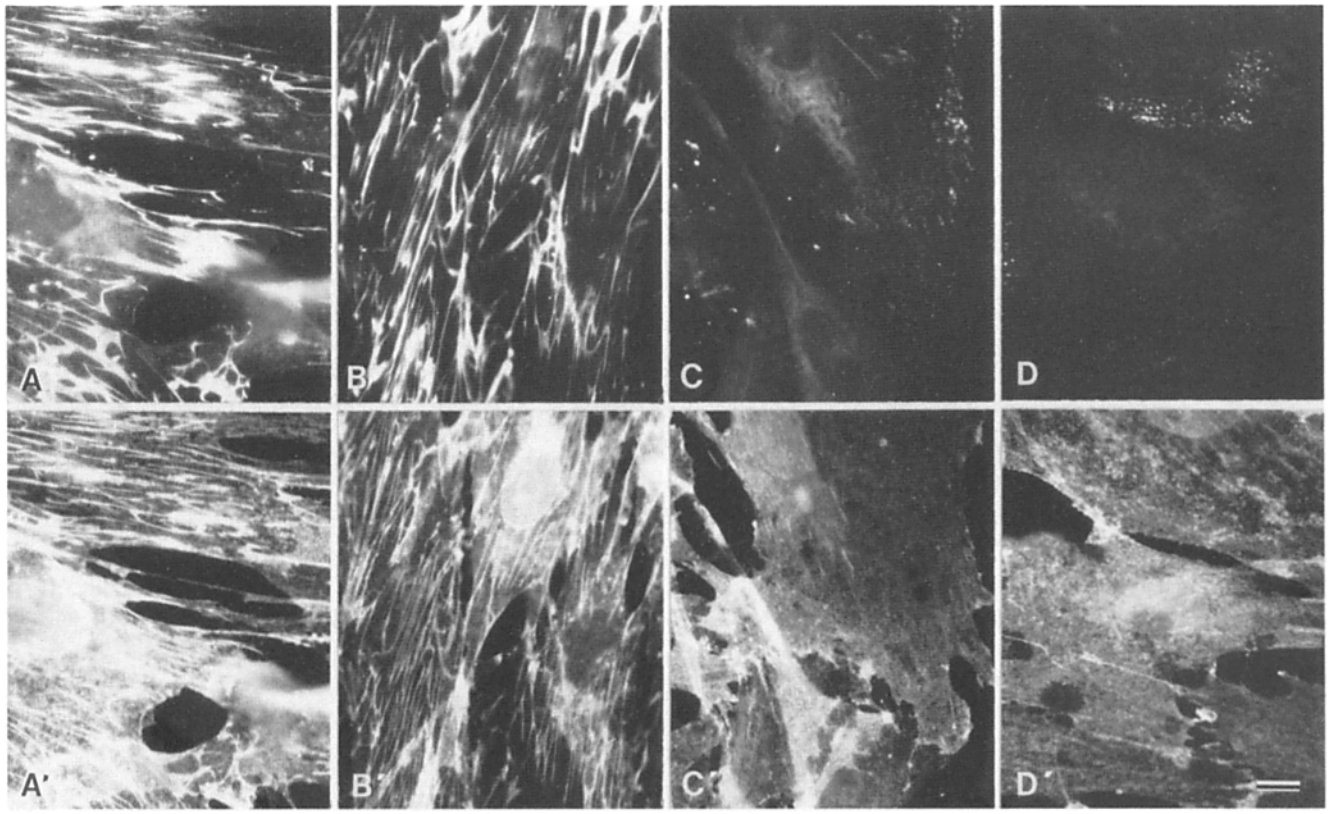

Figure 9. Redistribution of fibronectin receptor after treatment with mAbs and comparison by double-antibody immunofluorescence analysis with fibronectin matrix assembly by WI38 fibroblasts. WI38 fibroblasts were grown in regular culture medium with control nonimmune IgG at 200 μg/ml (*A* and *B*) or in the presence of 200 μg/ml mAb 13 IgG (*C*) or 200 μg/ml mAb 16 (*D*). The localization of the fibronectin receptor (*A'–D'*) was visualized by fixing the cells and staining with rabbit polyclonal anti-receptor antibodies followed by rhodamine-conjugated goat anti-rabbit IgG antibodies unable to bind rat IgG. Fibronectin (*A–D*) was visualized in the same cells by incubating with FITC-labeled goat anti-fibronectin antibodies along with the goat anti-rabbit IgG. Bar, 10 μm.

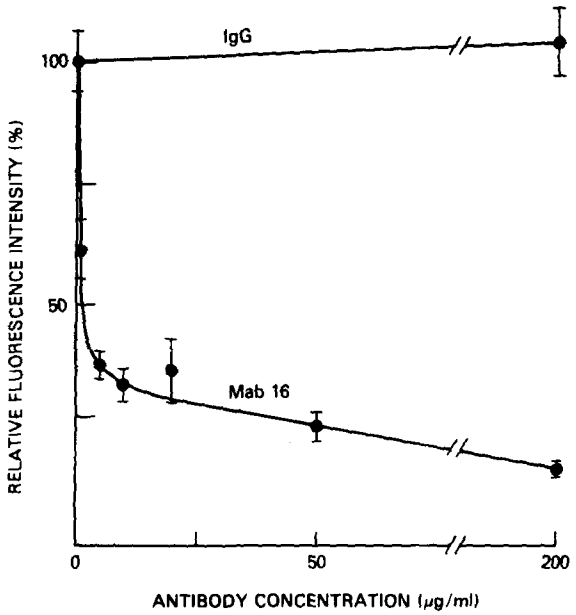

Figure 10. Quantitation of matrix formation by WI38 fibroblasts. Cultures of WI38 fibroblasts were treated with the indicated concentrations of mAb 16 or nonimmune IgG. The formation of fibronectin fibrils was visualized by fixing the cells and staining with affinity-purified goat anti-human fibronectin antibodies followed by FITC-conjugated rabbit anti-goat IgG antibodies. Immunofluorescence in the presence of phenylenediamine was quantitated as described in Materials and Methods. Background determined in the absence of the first antibody was subtracted.

tion and matrix assembly, whereas neither cell spreading nor migration of these normal human fibroblasts on fibronectin were inhibited.

Effects of mAb 16 on Cytoskeletal Organization and Adhesions

Since microfilament bundles often show partial colocalization with fibronectin fibrils, we examined for effects of mAb 16 treatment on actin-containing microfilaments. As shown in Fig. 12, *A* and *C*, control cells showed strong rhodamine-phalloidin staining of F-actin in thick microfilament bundles (stress fibers) that often extended most of the length of cells. Cells treated for 24 h with mAb 16 appeared to have significantly fewer thick bundles of F-actin containing microfilaments, but numerous fine strands of microfilaments were still retained (Fig. 12, *B* and *D*). Similar effects were found with Fab fragments of mAb 16, but not with control IgG, and on substrates that had been precoated with purified fibronectin (not shown).

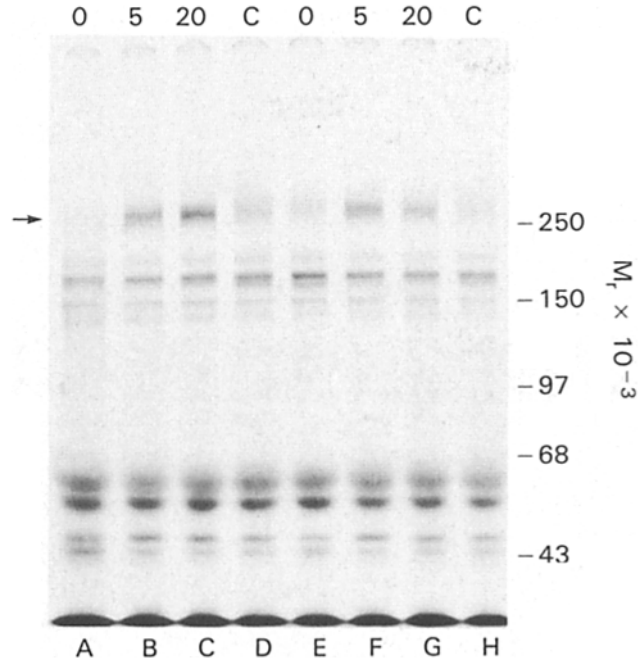

Figure 11. Autoradiographic analysis of fibronectin secreted by WI38 fibroblasts in the presence of mAb 16. WI38 fibroblasts were grown in the presence of 50 μCi/ml [^{35}S]methionine alone (lanes *A* and *E*), 5 μg/ml mAb 16 (lanes *B* and *F*), 20 μg/ml mAb 16 (lanes *C* or *G*), or equal amounts of rat IgG (lanes *D* and *H*). After 24 h, a 50-μl aliquot of conditioned culture medium was resolved by SDS-gel electrophoresis and analyzed by autoradiography. The fibronectin band (*arrow*) was confirmed by immunoprecipitation with rabbit anti-human fibronectin antibody. Scanning densitometry of fibronectin in relative absorbance units, setting untreated controls = 1.00, showed 5 μg/ml mAb 16 = 3.97; 20 μg/ml mAb 16 = 4.04; and IgG control = 1.31. Dot immunoblot quantitation of fibronectin in the remaining cell monolayers was also performed directly using anti-fibronectin and ^{125}I as described under Materials and Methods; radioactivity was determined in a gamma counter to obtain data independent of the ^{35}S labeling. Relative amounts of fibronectin, with controls = 1.00, were 5 μg/ml mAb 16 = 0.45; 20 μg/ml mAb 16 = 0.34; and control IgG = 0.78. In a separate experiment, 200 μg/ml mAb 16 did not produce a significantly larger decrease (32 vs. 34% in this experiment).

Concomitant with this decreased formation of thick microfilament bundles, we observed striking alterations in cell–substratum contacts as characterized by interference reflection microscopy (Fig. 13, *A* and *B*). Untreated WI38 fibroblasts had numerous dark contact regions indicative of close approach to the culture substrate in adhesion plaques and prominent streak-like contacts corresponding, respectively, to specialized adhesion sites termed focal contacts and extracellular matrix contacts (e.g., see 15). As shown in Fig. 13, treatment with mAb 16 resulted in decreased numbers of these darkest contacts, with especially striking losses of the streak-like extracellular matrix contacts. The ventral surface instead appeared as a relatively uniform series of greys, characteristic of the "close" contacts found in migrating cells (Fig. 13 *B*). Fab fragments of mAb 16 produced similar effects, but control nonimmune rat IgG had no detectable effects (not shown). Inhibition by mAb 16 was also observed on substrates precoated with fibronectin (not shown).

Discussion

We have developed two mAbs that bind to different subunits of the fibronectin receptor heterodimer complex and used them to dissect the function of this receptor in cell biological functions in vitro. The related, but sometimes distinct, effects of these antibodies on a variety of biological assay systems are summarized in Table II which evaluates cell adhesions, morphology, cell migration, extracellular matrix assembly, and microfilament bundle formation.

Blocking the function of the beta subunit had multiple effects, including inhibiting cell adhesion to both fibronectin and laminin but not to vitronectin. These results were anticipated because a single beta polypeptide can associate with one of several different alphas to form receptors for fibronectin, collagen, or laminin (25, 54–58). In contrast, adhesion to vitronectin is mediated by distinct class 3 integrins (14, 17, 27, 33, 48). The anti-alpha subunit antibody mAb 16 inhibited cell attachment to fibronectin but not to laminin or vitronectin. However, it had no effect in a standard cell spreading assay on fibronectin, laminin, or vitronectin substrates. There was thus a discrepancy between attachment and spreading assays, which showed inhibition or no inhibi-

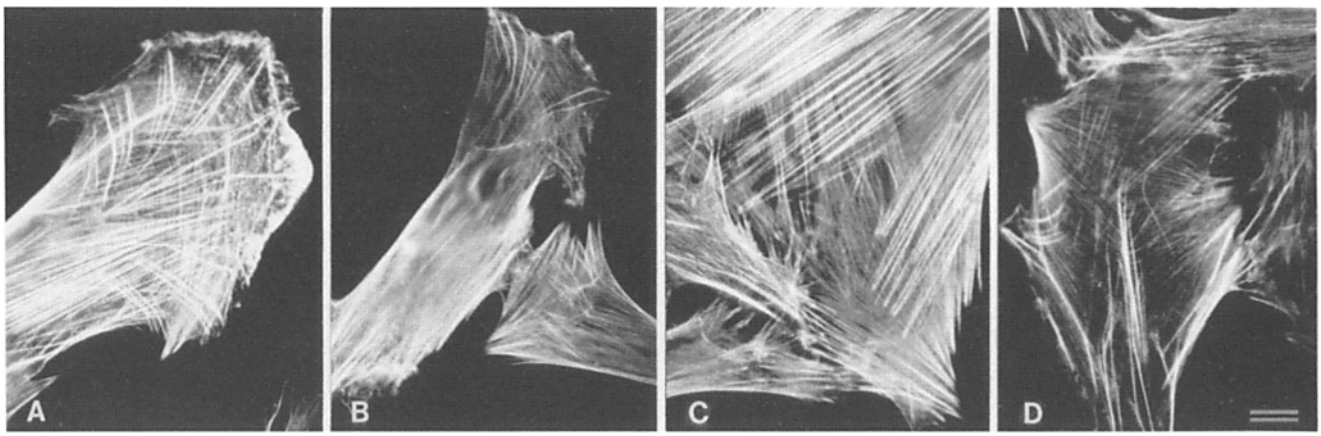

Figure 12. Alterations in microfilament bundle organization. WI38 fibroblasts were cultured for 24 h in regular culture medium in the presence or absence of 200 μg/ml mAb 16. For visualization of F-actin, cells were fixed and extracted with acetone and then stained with rhodamine-labeled phalloidin to compare control cells (*A* and *C*) with mAb 16–treated cells (*B* and *D*). Bar, 10 μm.

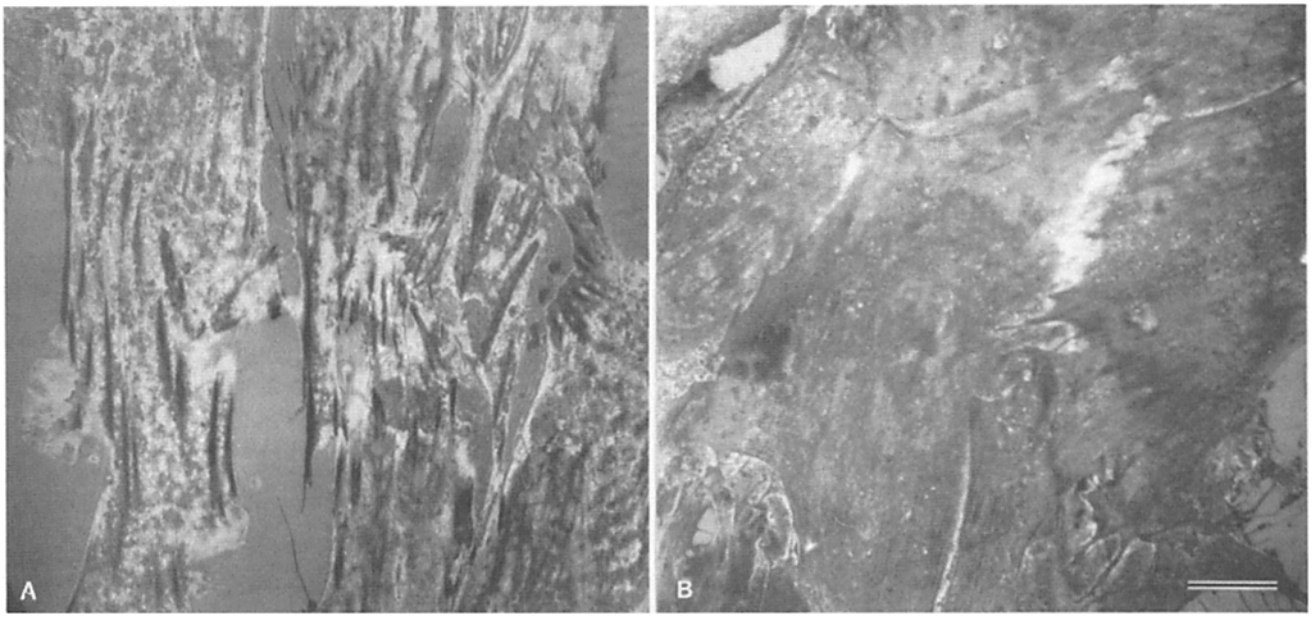

Figure 13. Alterations in cell substratum contacts. WI38 fibroblasts were cultured for 24 h in regular culture medium alone (*A*) or in the presence of 200 μg/ml mAb 16 (*B*). For visualization of adhesive contacts at the ventral cell surface, cells were fixed with glutaraldehyde/paraformaldehyde and examined immediately by interference reflection microscopy. Bar, 10 μm.

tion, respectively, by mAb 16 of interactions with fibronectin. This apparent contradiction was resolved by examining a time course of cell spreading that showed substantial initial inhibition of cell spreading for the period of time covered by the attachment assay, but then eventually showed successful cell spreading by the time of termination of routine spreading assays. The simplest explanation of these results is that attachment and early phases of spreading are dependent on fibronectin receptor function, but subsequent spreading uses another as yet undefined class 1 integrin receptor system.

The alpha subunit appears to play an important role in the assembly of the fibronectin-containing extracellular matrix. Blocking either alpha or beta subunits inhibited cellular retention of endogenous fibronectin and extracellular matrix formation. Instead of forming a matrix, newly synthesized fibronectin was secreted into the culture medium. mAb 16 thus appears to inhibit matrix formation by inhibiting the cell surface retention rather than the biosynthesis of fibronectin.

Table II. Summary of Inhibitory Activities of Anti-Fibronectin Receptor Antibodies

Assay	Anti-alpha mAb 16	Anti-beta mAb 13
Cell attachment		
Fibronectin	Inhibits	Inhibits
Laminin	No effect	Inhibits
Vitronectin	No effect	No effect
Cell spreading		
Fibronectin	No effect	Inhibits
Laminin	No effect	Inhibits
Vitronectin	No effect	No effect
Cell migration from explant	No effect	Inhibits
Single cell migration	Enhances	Partially inhibits
Matrix assembly	Inhibits	Inhibits
Actin microfilament bundles	Inhibits	Inhibits
Focal and streak-like extracellular matrix contacts	Inhibits	Inhibits

In previous studies, two different assay systems have been used to analyze matrix assembly by cultured human fibroblasts. In one series of studies, the incorporation of isolated, exogenous plasma fibronectin into the extracellular matrix was examined (41–43). Because this assay system was particularly sensitive to an exogenously added amino-terminal fragment of fibronectin that lacks the cell adhesive domain, these investigators proposed the existence of a matrix assembly fibronectin receptor that is distinct from the VLA 5 cell attachment fibronectin receptor (43). The matrix assembly assay used in the present study directly examines the incorporation of endogenous fibroblast fibronectin into fibrils, an assay similar to that of McDonald et al. (40). The assembly of endogenous fibroblast-derived fibronectin into an extracellular matrix has been shown to require the cell adhesive domain of fibronectin (40). The results presented here also indicate that both subunits of the fibronectin adhesion receptor itself are also required for the assembly of endogenous fibroblast cellular fibronectin into an extracellular matrix.

Even after treatment with high concentrations of the mAbs, there are some residual short fibrils or circular aggregates (dot- or spot-like patterns) of fibronectin visible on or under the fibroblasts. The origin of the dot-like patterns of fibronectin is not known. The two most likely possibilities are that they represent fibroblast cellular fibronectin that is aggregated or precipitated abnormally on the ventral (lower) cell surface–substrate interface after secretion, or that they represent natural precursors of fibronectin fibrils.

The precise roles of the alpha and beta subunits in fibroblast migration are less clear. Both the outward migration of human fibroblasts from cell explants and the random migration of single cells in sparse cultures on fibronectin were in-

hibited by the antibody against the class 1 beta subunit (mAb 13) but not by the antibody against the alpha subunit (mAb 16). This finding, like the lack of inhibition of cell spreading on fibronectin, suggests the possible existence of a second mechanism of cell–fibronectin interaction. This second mechanism does not appear to involve collagen, since high levels of collagenase did not interfere with cell migration.

Rather than inhibiting migration, the anti-alpha mAb unexpectedly stimulated significantly the migration of single cells as followed by time-lapse microscopy. This stimulation may be explained by our findings that mAb 16 caused a decrease in cell–substrate adhesive contacts associated with immobility (i.e., in "focal" contacts and especially in streak-like extracellular matrix contacts) and also converted integrin receptor patterns from a clustered to a diffuse distribution. Previous studies have suggested that a diffuse distribution of the receptor is characteristic of rapidly migrating and malignant cells (16, 21, 22, 53). The absence of the streak-like receptor patterns associated with decreases in ventral cell adhesions and transmembrane linkages (16) would permit accelerated cell migration.

In a previous report, it was suggested that the interaction of fibronectin fibrils with cells regulates the distribution of the fibronectin receptor (16). The present study suggests that this putative regulatory interaction can be blocked by either anti-alpha or -beta subunit antibodies.

The disruption of receptor clustering on human fibroblasts is reminiscent of similar effects on avian integrin receptor clustering by an mAb against the shared class 1 beta subunits of avian cells. Duband et al. (22) observed that treatment of avian cells with a wide-spectrum anti-integrin mAb also resulted in a more diffuse receptor distribution and high mobility of that receptor. In the present study, however, the diffuse pattern could be induced even by antibodies that bind only to the alpha subunit of a single integrin receptor, pointing to VLA 5 as the crucial integrin in these redistribution effects.

Previous studies (18, 22) described correlations between the slowing of cell migration, formation of adhesive contacts with clusters of integrin receptors, organization of the actin microfilament cytoskeleton, and increased accumulation of a fibronectin matrix. It is noteworthy that mAb 16 generally produces the opposite effects. The broader-specificity mAb 13 also interferes with two other processes, cell spreading and migration, presumably because it also inhibits other integrins including VLA 2 and VLA 3 (e.g., see 57, 60, 61).

Fig. 14 is a summary of the findings from this and other papers suggesting how the fibronectin receptor mediates its effects on cell behavior and morphology. If the streak-like clusters of receptor on the dorsal (upper) cell surface are functionally equivalent to the receptor clusters at adhesive sites on the ventral cell surface, the key element in both these adhesive structures and in matrix assembly would be the presence of multivalent clusters of fibronectin receptor that bind to fibrils of fibronectin. We suggest that multivalency then results in high net affinity (e.g., see Discussion in 22), which holds cells more effectively in place and thus slows cell migration if located on the ventral surface. Alternatively, multivalent receptor clusters located on the dorsal surface contribute to the assembly of fibronectin fibrils. Thus, clustered fibronectin receptors may play a central role in establishing the stationary cell phenotype—e.g., when certain embryonic cells cease migration and establish a surrounding extracellular matrix.

Treatment of cells with mAb 16 appears to disrupt fibronectin receptor clustering and function without interfering with other cell adhesion receptors, resulting in a loss of fibronectin due to poor matrix assembly and accelerated migration due to lack of certain ventral adhesions. However, there are only transient effects on cell adhesion that can be rapidly overcome by other adhesive mechanisms that permit cell spreading and migration. In addition, actin microfilament bundle organization is partially inhibited, probably due to the disruption of transmembrane linkages with fibronectin fibrils. In conclusion, receptor clustering, fibronectin fibril formation, microfilament bundle formation, firm adhesion, and migration are apparently interdependent processes regulated by the state of this single cell surface receptor.

We thank Hieu Nguyen for technical assistance in quantitating single cell migration.

This work was partially supported by National Institutes of Health grants CA 45515 and CA 14718 to S. K. Akiyama and CA 39077 and HL 33711 to W.-T. Chen.

Received for publication 7 March 1989 and in revised form 19 April 1989.

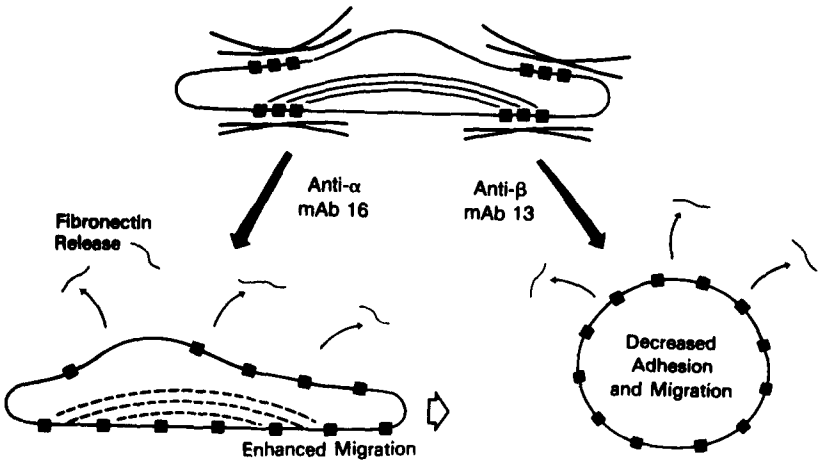

Figure 14. Diagrammatic summary of key findings of this study. Black squares represent the VLA-5 fibronectin receptor associated with intracellular microfilament bundles. The thicker strands on the cell surface represent fibronectin fibrils and the thinner wavy lines represent secreted fibronectin. Untreated WI38 fibroblasts (*top*) are compared with cells treated with mAb 16 directed against the alpha subunit of the fibronectin receptor (*bottom left*) or with mAb 13 against the beta subunit. See text for discussion.

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