Inhibition of Binding of Fibronectin to Matrix Assembly Sites by Anti-Integrin (α5β1) Antibodies

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Abstract. Fibroblasts have cell surface sites that mediate assembly of plasma and cellular fibronectin into the extracellular matrix. Cell adhesion to fibronectin can be mediated by the interaction of an integrin (α5β1) with the Arg-Gly-Asp-Ser (RGDS)-containing cell adhesion region of fibronectin. We have attempted to elucidate the role of the α5β1 fibronectin receptor in assembly of fibronectin in matrices. Rat monoclonal antibody mAb 13, which recognizes the integrin β1 subunit, completely blocked binding and matrix assembly of 125I-fibronectin as well as binding of the 125I-70-kD amino-terminal fragment of fibronectin (70 kD) to fibroblast cell layers. Fab fragments of the anti-β1 antibody were also inhibitory. Antibody mAb 16, which recognizes the integrin α5 subunit, partially blocked binding of 125I-fibronectin and 125I-70-kD.

Fibronectin is a dimeric glycoprotein consisting of two similar 250-kD disulfide-bonded subunits. Fibronectin exists in soluble and insoluble forms. Soluble proteolytic fibronectin is found at high concentrations in plasma and other bodily fluids and in the conditioned media of cultured cells. Insoluble fibronectin consists of high molecular weight disulfide-bonded multimers organized into an extracellular fibrillar matrix (Mosher, 1980, 1984; Hynes, 1981; Mosher and McKeown-Longo, 1985; Hedman and Váheri, 1989; Yamada, 1989).

Insoluble fibronectin mediates cell attachment, provides a substrate for cell migration during embryogenesis and wound healing, and thus is considered the primary functional form of the protein (Ruoslahti, 1988; Thiery et al., 1989; Colvin, 1989). A crucial cell attachment site has been localized to a specific tetrapeptide sequence, Arg-Gly-Asp-Ser (RGDS), found in the central region of each fibronectin subunit (Pierschbacher and Ruoslahti, 1984; Yamada, 1989).

Cell attachment to fibronectin is mediated in part by the interaction of the RGDS site with a specific cell surface fibronectin receptor (α5β1), a member of the noncovalent heterodimer integrin receptor group (Pytel et al., 1985; Hemler et al., 1987; Hynes, 1987; Takeda et al., 1987; Ruoslahti, 1988).

When cell layers were coincubated with fluorescein-labeled fibronectin and either anti-β1 or anti-α5, anti-β1 was a more effective inhibitor than anti-α5 of binding of labeled fibronectin to the cell layer. Inhibition of 125I-fibronectin binding by anti-β1 IgG occurred within 20 min. Inhibition of 125I-fibronectin binding by anti-β1 Fab fragments or IgG could not be overcome with increasing concentrations of fibronectin, suggesting that anti-β1 and exogenous fibronectin may not compete for the same binding site. No β1-containing integrin bound to immobilized 70 kD. These data indicate that the β1 subunit plays an important role in binding and assembly of exogenous fibronectin, perhaps by participation in the organization, regeneration, or cycling of the assembly site rather than by a direct interaction with fibronectin.

The mechanism by which fibronectin is incorporated into the extracellular matrix is unclear. One proposed mechanism involves a disulfide exchange reaction between the amino-terminal portion of different fibronectin molecules (McKeown-Longo and Mosher, 1984). Transglutaminase-mediated cross-linking also promotes insolubilization (Barry and Mosher, 1988, 1989). Fibronectin insolubilization and assembly appear to occur at the cell surface. Dimeric and multimeric fibronectin have been localized at the cell surface using metabolic and cell surface-labeling techniques (Hynes and Destree, 1977; Keski-Oja et al., 1977; Choi and Hynes, 1979). In vitro and in vivo studies have shown that cell surface-associated fibronectin consists of both endogenously synthesized cellular fibronectin and circulating plasma fibronectin (Oh et al., 1981; Sekiguchi et al., 1986).

Fibroblast monolayers bind soluble iodinated plasma and cellular fibronectin in a specific and saturable manner (McKeown-Longo and Mosher, 1983; Ailio and McKeown-Longo, 1988). Initially, fibronectin is reversibly bound and soluble in 1% deoxycholate but with longer incubation times fibronectin forms deoxycholate-insoluble disulfide-bonded multimers. Pulse-chase and saturation kinetic experiments indicate that the deoxycholate soluble pool is the precursor of the insoluble pool (McKeown-Longo and Mosher, 1983;
Barry and Mosher, 1988). The binding data are further supported by microscopic studies which show that fluorescent and gold-labeled fibronectin localize initially in clusters along the edge of the cell that at later times become rearranged into long fibrillar structures along the cell surface (McKeown-Longo and Mosher, 1983; Pesciotta-Peters and Mosher, 1987). $^{125}$I-fibronectin does not bind specifically to isolated matrices and does not form disulfide-bonded or transglutaminase cross-linked multimers in the absence of cells (McKeown-Longo and Mosher, 1983; Barry and Mosher, 1988).

The importance of cells in mediation of fibronectin matrix assembly is supported by the observation that transforming growth factor–beta and agents which modulate intracellular cAMP levels influence fibronectin matrix assembly activity (Allen-Hoffmann and Mosher, 1987; Allen-Hoffmann et al., 1988). HT1080 fibrosarcoma cells bind and assemble exogenous fibronectin into a matrix only after treatment with dexamethasone (McKeown-Longo and Etzler, 1987).

Three experimental results suggest that exogenous soluble fibronectin binding to cell monolayers is mediated by the fibronectin amino-terminal region rather than the RGDS cell attachment site. First, 70- and 27-kD amino-terminal fragments, lacking the RGDS cell attachment site, bind to fibroblast monolayers and compete with exogenous fibronectin for binding (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988). Second, the 70-kD amino-terminal fragment (70 kD) and a monoclonal antibody to the fibronectin amino-terminal region block endogenously synthesized fibronectin matrix assembly (McDonald et al., 1987). Third, 3T3 cells, infected with a retroviral expression vector containing cDNA that encodes for fibronectin lacking the amino-terminal half, synthesize and secrete recombinant truncated fibronectins referred to as deminectins. Deminectin homodimers are not incorporated into the matrix (Schwarzbauer et al., 1987). Exogenous fibronectin binding and matrix assembly mediated by the amino-terminal region rather than the RGDS-containing cell adhesion domain led to the proposal of a distinct “matrix assembly receptor” on substrate attached cells (McKeown-Longo and Mosher, 1983, 1985, 1989).

There are also data that suggest involvement of both the fibronectin cell adhesion domain and the $\alpha_\beta_1$ fibronectin cell adhesion receptor in fibronectin matrix assembly. A 105-kD RGDS containing cell binding fragment and a monoclonal antibody that recognizes the fibronectin cell attachment site block matrix assembly of endogenously synthesized fibronectin (McDonald et al., 1987). Rat monoclonal antibodies mAb 16 and mAb 13, which bind to the $\alpha_\beta$ and $\beta$, subunits, respectively, of the human fibroblast fibronectin receptor complex, inhibit fibronectin matrix formation when incubated with freshly seeded human WI38 fibroblast cultures (Akiyama et al., 1989).

In this paper, we have examined the effect of monoclonal antibodies specific for the integrin $\alpha_\beta_1$ subunit (mAb 16) and the integrin $\beta_1$ subunit (mAb 13) on exogenous fibronectin and 70-kD binding to fibroblast monolayers in short-term assays. We wished to learn if these antibodies block binding and assembly of exogenous fibronectin, about the kinetics of the inhibition, and especially whether the antibodies block binding of the 70-kD fragment. These studies indicate that the $\beta_1$ protein plays an important although unclear role in matrix assembly.

### Materials and Methods

#### Materials

Human neonatal foreskin fibroblasts were from a locally established strain (Dr. Lynn Allen-Hoffman, University of Wisconsin) and were studied between passages 10 and 20. Human osteosarcoma cells (MG-63) were from the American Type Culture Collection (Rockville, MD). Cathepsin D, cycloheximide, soybean trypsin inhibitor, chymotrypsinogen (type II), PMSF, BSA (fraction V), lactoperoxidase (from bovine milk), deoxycholate, cytochrome c, gelatin (from swine skin), and Sepharose CL-4B-200 were from Sigma Chemical Co. (St. Louis, MO). Trypsin was from ICN Biomedicals, Inc. (Irvine, CA). Oxyt-1-6-thioglycoscarosyl and protein A–agarose were purchased from Calbiochem-Behring Corp. (La Jolla, CA). Heparin-Sepharose was from Pharmacia (Piscataway, NJ). Carrier-free $^{125}$I-labeled and Na$^{125}$I-Cr$^{57}$CrO$_4$ were from DuPont (Hoffmann–Estate, IL). Gelatin-Sepharose was prepared in this lab following a published procedure (Cuatrecasas, 1970). Acrylamide/bis-Acrylamide (37.5:1 solution) was purchased from American Research Products Corp. (Solon, OH). Reagents for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA).

#### Antibodies

MAB 16 and MAB 13 have been previously described (Akiyama et al., 1989; Matsuyama et al., 1989). Rabbit polyclonal anti- $\alpha_\beta_1$ was a generous gift from Dr. Erkki Ruosjoki (La Jolla Cancer Research Foundation, La Jolla, CA). Mouse monoclonal antibody J143 was a gift from Dr. Lloyd J. Old (Memorial Sloan-Kettering Cancer Center, New York) and is specific for the integrin $\alpha_\beta_1$ subunit (Hemler et al., 1987; Kantor et al., 1987). Mouse monoclonal antibody PIB5 was a gift from Dr. Elizabeth Wannier and William Carter (Fred Hutchinson Cancer Research Center, Seattle, WA). Mouse monoclonal antibody LM609 was specific for integrins $\alpha_\beta_1$ and was previously shown to block $\alpha_\beta_1$ function (Wannier and Carter, 1987; Takada et al., 1988). Mouse monoclonal antibodies LM142 and LM609 were gifts from Dr. David A. Cheresh (Research Institute of Scripps Clinic, La Jolla, CA). Monoclonal antibody LM142 is directed against the vitronectin receptor $\alpha_\beta_1$ subunit (Cheresh and Harper, 1987). Monoclonal antibody LM609 is $\alpha_\beta_1$ complex specific and was previously shown to block $\alpha_\beta_1$ function (Cheresh and Sprio, 1987).

#### Experimental Procedures

**Cell Culture.** Human foreskin fibroblasts were cultured in 50% Ham's F-12 (Gibco Laboratories, Grand Island, NY), 50% Dulbecco's modified Eagle's medium (Gibco Laboratories), supplemented with 10% fetal calf serum (Hazelton, Denver, PA). Human osteosarcoma MG-63 cells were cultured in Eagle's minimum essential media (Gibco Laboratories) supplemented with 5% fetal calf serum.

**Fibronectin and Fibronectin Fragments.** Human plasma fibronectin was purified as previously described from a fibronectin- and fibrogen-rich by-product of Factor VIII production (Mosher and Johnson, 1983). The 70-kD amino-terminal, gelatin-binding fragment of fibronectin (70 kD) was purified from cathepsin D digests of fibronectin as previously described (McKeown-Longo and Mosher, 1985) with the following modifications. Digestion was allowed to proceed for 3 h at 37°C and an additional 2 h at 22°C. The pH of the digest was immediately raised to 7.4 with the addition of 0.5 M Tris, and the neutralized digest was directly applied to a gelatin-Sepharose column. After elution with 3 M guanidine hydrochloride, 70-kD fragment was separated from a contaminating 40-kD gelatin-binding breakdown product on a G-100 gel filtration column run in TBS (150 mM NaCl/10 mM Tris, pH 7.4). Purity of fragments was assessed by SDS-PAGE (Laemmli, 1970). Fragment aliquots were stored at −70°C.

**Iodination of Fibronectin and 70 kD.** Iodination of fibronectin and 70 kD was performed using the chloramine T method as previously described (McKeown-Longo and Mosher, 1983, 1985). The specific activities of $^{125}$I-fibronectin and $^{125}$I-70 kD were typically ~350 $\mu$Ci/mg. Purity of labeled fibronectin and 70-kD fibronectin were determined using a Bio-Rad Biovert 3100 gel filtration system. Purity of labeled fibronectin and 70-kD fibronectin were determined using a Bio-Rad Biovert 3100 gel filtration system. Purity of labeled fibronectin and 70-kD fibronectin were determined using a Bio-Rad Biovert 3100 gel filtration system.
proteins was assessed by SDS-PAGE with and without reduction followed by autoradiography. 125I-Fibronectin and 125I-70 kD Binding Assays. Human foreskin fibroblasts were seeded 6–8 x 10^4 cells per 2-cm^2 well (24-well plates; Becton Dickinson Labware, Lincoln Park, NJ) and were used in binding assays 2–4 d after seeding when the cells reached confluence as assessed by phase microscopy. Medium (0.5 ml) containing 0.2% BSA, radioligand, and sometimes unlabeled ligand and/or antibody was added to the cell layers and incubated at 37°C for the indicated times. Free ligand was separated from bound by removing the binding mix and washing the cell layers thrice with cold PBS (150 mM NaCl/20 mM phosphate, pH 7.4). Then cell layers were either solubilized in 1 N NaOH to determine the total amount of labeled ligand associated with the cell layer or separated into fractions soluble and insoluble in 1% deoxycholate (Pool II assay) as previously described (McKeown-Longo and Mosher, 1985). In long-term (6 h) binding assays, cycloheximide (10 μg/ml), was added to eliminate possible effects of endogenously synthesized fibronectin.

Cell Adhesion Assay. Cell adhesion assays were performed using a method similar to that of Wayner and Carter, (1987). Microtiter 96-well polystyrene plates (model 3596; Costar, Cambridge, MA) were coated with fibronectin or heat-denatured BSA (200 μl protein solution in PBS per well, 5 μg protein/ml). Plates were coated for 1.5–4.5 h at 37°C and then blocked with 200 μl of 1% heat denatured BSA in PBS for 1–2 h at 37°C. Plates were washed with PBS several times after blocking in BSA. Fibroblasts in 75-cm^2 dishes (Becton Dickinson Labware) were labeled with Na_232CrO_4 (50 μCi/ml) for 2–4 h at 37°C in medium. Cells were released by incubating for 5 min at 37°C in a mixture of 2 ml trypsin/PBS (0.25% w/v) and 2 ml EDTA/PBS (0.02% w/v). Trypsinization was stopped with the addition of 4 ml medium containing 10% FBS. Cells were pelleted and then resuspended in Ham's F-12 containing 0.2% BSA. Suspended cells (0.8–1.3 x 10^5 cells/ml) were incubated 30 min at room temperature with antibody or media alone. Aliquots (0.2 ml) of each incubation mixture (~1.5–2.5 x 10^5 cells) were pipetted into coated microtiter wells, and cells were allowed to adhere to the fibronectin or BSA coated surface for 15–30 min at 37°C. Nonadherent cells were removed by washing with PBS. Adherent cells were dissolved in 1 N NaOH and quantitated in a gamma counter.

Incorporation of Fluoresceinlabeled Fibronectin into Cell Layers and Localization of Anti-α1 (mAb 13) and Anti-α5 (mAb 16) IgG. Fluorescin-labeled fibronectin (FITC-fibronectin) was prepared as previously described (McKeown-Longo and Mosher, 1983). Fibroblasts were seeded onto 13-mm glass coverslips (No. 2; Ernst Fullam, Inc., Schenectady, NY) placed in 2-cm^2 wells at 1 x 10^5 cells per well, and allowed to attach overnight. The next day, serum-containing media was replaced with 0.5 ml of Ham's F-12 medium containing 0.2% BSA and 20 μg/ml FITC-fibronectin either alone or supplemented with 5 μg/ml anti-α1 or anti-α5 IgG. Cell layers were incubated for 60 min at 37°C, then rinsed three times with PBS, and fixed with 10% formaldehyde from temperature in 1 mM phosphate buffer (pH 7.4) containing 3.5% paraformaldehyde. The distribution of integrin α1 and β1 subunits in cells incubated with anti-α1 and/or β1, IgGs was visualized by immunofluorescence. After fixation, cells that had been incubated with FITC-fibronectin plus antibody were rinsed and then incubated 45 min at room temperature with a 1:16 dilution of rhodamine conjugated rabbit anti-rat IgG. Coverslips were rinsed with PBS and mounted on glass slides in glycerol-gelatin (Sigma Chemical Co.). Slides were viewed and photographed on a Nikon microscope equipped with epifluorescence and phase contrast. Cells were photographed using Ektachrome 400 film (Kodak, Rochester, NY).

Surface Labelling and Extraction of Cells. Fibroblasts grown to confluence in 75-cm^2 dishes were detached with 1 mM EDTA in PBS for 30–60 min and collected by centrifugation. Cells were washed once with PBS and resuspended to 1.0 x 10^6 cells/ml in PBS with 1 mM MgCl_2. All subsequent steps were done at 4°C. The suspended cells were radioiodinated according to the procedure of Lebien et al. (1982) using 1 μCi 125I-sodium iodide and 0.2 mg/ml of lactoperoxidase per 10^6 cells. Cells were lysed by adding 1 ml lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 50 mM ocyl-β-thioglycoloyanoside, 1 mM MgCl_2, 1 mM CaCl_2, 1 mM MnCl_2, 3 mM EDTA) and incubating 30 min on ice. Detergent-insoluble material was removed by centrifugation at 27,000 g for 30 min.

Affinity Chromatography. The α1β1 fibronectin cell adhesion receptor was isolated following the methods of Pytell et al. (1985, 1987). The 120-kD chymotryptic cell binding fragment of fibronectin was prepared as previously described (Pierschbacher et al., 1981). Briefly, 50 μg of fibronectin was diluted to a final concentration of 2 mg/ml in 50 mM Tris, pH 7.4, containing 1 mM CaCl_2. Fibronectin was digested 75 min at 37°C with chymotryptsin (type II; Sigma Chemical Co.) at a final concentration of 20 μg/ml. The reaction was stopped with the addition of PMSF (final concentration of 2 mM) and soybean trypsin inhibitor (final concentration of 200 μg/ml). The 120-kD fragment was purified by gelatin-Sepharose and heparin-Sepharose chromatography. Approximately 9 mg of the 120-kD fragment was coupled to 25 ml of CNBr-activated Sepharose (Cautrecasas, 1970). 70 kD was prepared as described above, and ~25 mg was coupled to 60 ml of CNBr-activated Sepharose. Polyclonal rabbit antibodies to 70 kD were passed over the column and recovered with 0.2 M glycine, pH 2.3, to verify that 70 kD had coupled to Sepharose.

Batch adsorption was carried out by mixing 1 ml of the iodinated cell lysate with 3 ml of 120-kD-activated or 2.5 ml of 70-kD Sepharose overnight at 4°C. The slurries were then poured into columns and allowed to settle and equilibrate to room temperature. The unbound material was collected, and the column beds were then washed with 25 mM octyl-β-thioglycoloyanoside in 150 mM NaCl/50 mM Tris, pH 7.4. Bound material was eluted with 10 mM EDTA in 25 mM octyl-β-thioglycoloyanoside/150 mM NaCl/50 mM Tris, pH 7.4. 70-kD Sepharose was further eluted with 0.2 M glycine, pH 2.3. Unbound and bound fractions were subjected to immunoprecipitation and analyzed by SDS-PAGE and autoradiography.

Immunoprecipitations. Immunoprecipitations were carried out as described (Kessler, 1981; Harlow and Lane, 1988). Samples were preclared by incubating with 25 μg/ml preimmune rabbit IgG for 60 min at 4°C followed by 20-min incubation with protein A–agarose. The supernatant was then removed to a new tube and incubated overnight with 50–100 μg/ml rabbit anti-as/βt IgG. The next day, antigen–antibody complexes were precipitated by incubating 60 min with protein A–agarose. The resin was allowed to settle, supernatant was removed, and resin was washed five times with 1 ml of 1% Triton X-100/PBS. Antigen–antibody complexes were eluted with 10% SDS in 9 M urea and subjected to SDS-PAGE.

Gel Electrophoresis. SDS-PAGE was performed using 8% separating and 3.3% stacking gels (Laemmli, 1970). 14C-methylated protein standards (Amersham Corp., Arlington Heights, IL) were myosin (Mr 200,000), phosphorylase-b (Mr 92,500), bovine serum albumin (Mr 69,000), ovalbumin (Mr 46,000), carbonic anhydrase (Mr 30,000). Dried gels were exposed to Kodak XAR-2 film for 4 d.

Results

Effect of Monoclonal Antibodies Specific for the Human Fibroblast Fibronectin Receptor α1 Subunit and β1 Subunit on Binding of Exogenous Fibronectin, Binding of the Amino-terminal 70-kD Fragment, and Initial Attachment of Suspended Fibroblasts to a Fibronectin Substratum

Anti-α5 partially blocked 125I-fibronectin and 125I-70 kD binding to fibroblast cell layers without gross effects on cell morphology (Fig. 1 A). Approximately 40–60% of the 125I-fibronectin and 125I-70 kD specific binding was blocked with 2.0 μg/ml IgG. Higher concentrations, up to 20 μg/ml, did not inhibit more. Anti-α5 inhibited initial fibroblast attachment to fibronectin with a dose response similar to that observed for inhibition of binding (Fig. 1 A).

In contrast, anti-β1 completely blocked binding of both 125I-fibronectin and 125I-70 kD to cell layers and blocked initial cell attachment to a fibronectin substratum (Fig. 1 B). Similar inhibition of 125I-fibronectin and 125I-70 kD binding by anti-β1 and anti-α5 was seen with MG-63 osteosarcoma cells (data not shown).

We examined what effect these antibodies had on exogenous FITC-fibronectin binding to cultured fibroblast monolayers (Fig. 2). The fluorescence microscopic data corroborated the binding study results. Coincubation of fibroblast
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Figure 1. Effect of anti-α5 and -β1 antibodies on binding of fibronectin and 70-kD fragment and on cell attachment to a fibronectin substrate. Confluent monolayers of human foreskin fibroblasts (~2.0 × 10⁶ cells) grown in 2-cm² wells were incubated in 0.5 ml of Ham's F-12 media containing 0.2% bovine albumin and either ¹²⁵I-fibronectin (300,000 cpm, 246 ng) or ¹²⁵I-70 kD (380,000 cpm, 200 ng) alone or in the presence of increasing concentrations of anti-α5 (A) or anti-β1 (B) for 60 min at 37°C. Cell layers were then solubilized in 1 N NaOH, and the radioactivity was counted.

Antibodies. Nonspecific binding, measured in the presence of 5 μg/ml of anti-β1, IgG, the β1 subunit was arranged in linear streaks whereas β1 appeared more clustered in cells that were fixed before anti-β1 incubation (compare Fig. 2 G [anti-β1, IgG staining of live cells] and 2 H [anti-β1, IgG staining of fixed cells]). Anti-α5 IgG slightly reduced FITC-fibronectin incorporation into the matrix in a 60-min incubation (compare Fig. 2 A [anti-α5, IgG + FITC-fibronectin] with 2 B [FITC-fibronectin alone]). The α5 subunit also appeared to be less clustered in cells that had been incubated 60 min with 5 μg/ml anti-α5 before fixation (compare Fig. 2 E [anti-α5, IgG staining of live cells] with 2 F [anti-α5, IgG staining of fixed cells]).

Staining of anti-β1, on fixed cells after a 1-h incubation with FITC-fibronectin indicated that β1 distribution closely paralleled exogenous fibronectin-fibronectin distribution (compare Fig. 2 D [FITC-fibronectin only] and 2 H [localization of β1 on same cell after fixation]). However, colocalization was not absolute (see arrows, Fig. 2, D and H) since FITC-fibronectin could be found in places where there was no β1 staining. There was no obvious relationship between the distribution of FITC-fibronectin and α5.

In the phase-contrast micrographs and in routine observation of cells by phase microscopy during the various binding assays, anti-β1 and anti-α5 IgGs did not cause gross changes in cell morphology during a 60-min incubation.

Two monoclonal antibodies specific for α5β1, J143 and P1B5, failed to block binding of fibronectin or 70-kD fragment to cell layers (data not shown). Monoclonal antibodies to α5 (LM142) and α5β1 (LM609) also were not inhibitory (data not shown). These results suggested that neither the α5β1 nor the α5β1 integrins were involved in binding the amino-terminal region of fibronectin and provided further proof that the observed inhibition of binding by anti-α5 and anti-β1 was due to specific interactions.

Time Course of Inhibition of Binding of ¹²⁵I-Fibronectin by Anti-β1, IgG

To learn whether inhibition of exogenous fibronectin binding by anti-β1, is immediate, fibroblast monolayers were incubated with ¹²⁵I-fibronectin in the presence or absence of anti-β1 or excess fibronectin for varying times (Fig. 3). Inhibition of binding was demonstrable 20 min after the addition of radioligand and IgG. In contrast, inhibition by excess fibronectin occurred at 3 min.

in the absence of antibody. Nonspecific background cell adhesion to albumin-coated wells was negligible (<1.0%). Each point represents the average of triplicate determinations, and error bars indicate the standard deviation.
**Figure 2.** Effect of anti-α5 and -β1 IgGs on binding of FITC-fibronectin to cell layers and on the distribution of the fibronectin receptor subunits. The effect of anti-α5 IgG on the binding of FITC-fibronectin was visualized by fluorescence microscopy after a 60-min incubation of cell layers with 20 μg/ml FITC-fibronectin alone (B) or in the presence of 5 μg/ml anti-α5 IgG (A). The cell layers were fixed and the distribution of α5 on cells exposed to anti-α5 during the 1-h incubation (E) or on cells reacted with anti-α5 after fixation (F) was determined with a rhodamine-labeled second antibody. Corresponding phase fields are shown in (I) and (J). The effect of anti-β1 IgG on the binding of FITC-fibronectin was visualized after a 60-min incubation of cell layers with 20 μg/ml FITC-fibronectin alone (D) or in the presence of 5 μg/ml anti-β1 IgG (C). The cell layers were fixed and distribution of β1 on cells exposed to anti-β1 IgG during the 1-h incubation (G) or on cells reacted with anti-β1 IgG after fixation (H) was determined with a rhodamine-labeled second antibody. Arrowheads mark places that contain FITC-fibronectin (D) but lack β1 staining (H). Bar, 1 μm.

**Effect of Anti-β1 Fab Fragments on Binding of 125I-Fibronectin and 125I-70 kD to Fibroblast Cell Layers and on Fibroblast Attachment to a Fibronectin Substratum**

Monovalent Fab fragments were tested to rule out the possibility that inhibition of fibronectin and 70 kD binding at 37°C by anti-β1 IgG was the indirect result of changes in membrane organization associated with receptor clustering induced by the bivalent IgG. A longer incubation time (6 h) was used so that inhibition of 125I-fibronectin incorporation into deoxycholate-insoluble matrix (pool II) could be quantified. Anti-β1 Fabs blocked 125I-fibronectin and 125I-70 kD binding to fibroblast cell layers and 125I-fibronectin accumulation in Pool II (Fig. 4). Anti-β1 Fabs also inhibited initial cell attachment to a fibronectin substratum (Fig. 4). No changes in cell morphology were detected by phase-contrast microscopy after a 6-h incubation with anti-β1 Fabs.

**Increasing Concentrations of Exogenous Fibronectin Do Not Decrease the Inhibitory Effect of Anti-β1 Fab Fragments**

If anti-β1 Fabs and exogenous fibronectin were competing for the same binding site, then one might expect increasing concentrations of fibronectin to eventually overcome the anti-β1 Fab inhibitory effect. Fig. 5 compares binding of fibronectin in Pool I and accumulation of fibronectin in Pool II measured in the absence or presence of anti-β1 Fabs. Increasing concentrations of exogenous fibronectin up to 30 μg/ml, a concentration previously shown to occupy >50% of matrix assembly sites (McKeown-Longo and Mosher, 1983), failed to overcome the inhibitory effect of 5 μg/ml anti-β1 Fabs. This was true for both fibronectin binding in Pool I and its accumulation in Pool II. Similar results were obtained with anti-β1 IgG (data not shown).

**Affinity Chromatography of Extracts of Surface-labeled Cells on Immobilized Fibronectin 120-kD Cell Attachment Fragment and on Immobilized Fibronectin 70-kD Amino-terminal Fragment**

The fact that monoclonal antibodies specific for β1 and, to a lesser extent, α5 subunits of the fibronectin cell adhesion receptor inhibited 70 kD binding prompted us to examine whether or not α5β1 or another β1-containing integrin interacts with 70 kD. Detergent extracts of surface-labeled fibroblasts were subjected to affinity chromatography on either immobilized 120-kD cell attachment fragment or immobilized 70 kD. Bound β1-containing integrin was detected by immunoprecipitation using purified human placental α5β1 polyclonal antibodies. Immunoblotting studies showed that these antibodies react primarily with the β1 subunit (data not shown).
plasma fibronectin to monolayers of fibroblasts and its incorporation into extracellular matrix. The ability of both IgGs to inhibit fibronectin and 70 kD binding paralleled their ability to inhibit the initial attachment of suspended fibroblasts to a fibronectin substratum. Monovalent Fab fragments of anti-β, also inhibited fibronectin and 70 kD binding suggesting that inhibition of ligand binding is not a nonspecific effect due to receptor clustering induced by bivalent IgG.

**Discussion**

Antibodies to the β, and, to a lesser extent, α5 subunits of the fibronectin receptor blocked binding of the 70-kD aminoterminal fragment of fibronectin and binding of exogenous plasma fibronectin to monolayers of fibroblasts and its incorporation into extracellular matrix. The ability of both IgGs to inhibit fibronectin and 70 kD binding paralleled their ability to inhibit the initial attachment of suspended fibroblasts to a fibronectin substratum. Monovalent Fab fragments of anti-β, also inhibited fibronectin and 70 kD binding suggesting that inhibition of ligand binding is not a nonspecific effect due to receptor clustering induced by bivalent IgG.

Anti-β, IgG (5 μg/ml) blocked FITC-fibronectin binding to cell layers whereas anti-α5 IgG was much less effective. In contrast, Akiyama et al. (1989) found significant inhibition of extracellular fibronectin matrix accumulation with as little as 1 μg/ml of anti-α5 IgG when human embryonic lung fibroblasts (WI38 cells) were plated in the presence of the antibody and incubated an additional 24 h. The discrepancy may be related to differences in the assay. In the assay described in this paper, we examined the ability of anti-α5 to block binding of exogenously supplied FITC-fibronectin during a 60-min incubation by using human foreskin fibroblasts that have been allowed to attach and spread in the absence of antibody. Most of the assembly is probably at the ends of already established fibrils (Barry and Mosher, 1989).

The finding that anti-β, more effectively inhibited fibro-

**Figure 3.** Time course of inhibition with anti-β, IgG of 125I-fibronectin binding to cell layers. Confluent monolayers of human fibroblasts were washed once with 50% DME/50% Ham's F-12 containing 0.2% BSA and 25 mM Hepes, pH 7.4 (binding medium). Cell layers were then incubated at 37°C for indicated lengths of time in 0.5 ml binding medium containing 125I-fibronectin (800 ng, 300,000 cpm) alone (solid triangles), plus 500 μg/ml unlabeled fibronectin (open circles), or 5 μg/ml anti-β, IgG (open squares). Binding was stopped by removing the binding medium, and cell layers were washed three times with cold PBS, solubilized in 1.2 ml 1 N NaOH, and counted. The total cpm bound for each treatment are plotted versus length of incubation with radioligand. Each point represents the average of duplicate determinations, and the error bars represent the range.

**Figure 4.** Effect of anti-β, Fab fragments on 125I-fibronectin and 125I-70 kD binding to cell layers and on cell attachment to fibronectin-coated dishes. Confluent monolayers of human fibroblasts were incubated with 0.5 ml Ham's F-12 media containing 10 μg/ml cycloheximide, 0.2% BSA, and 125I-70 kD (160 ng, 300,000 cpm) or 125I-fibronectin (220 ng, 300,000 cpm) alone or in the presence of increasing concentrations of anti-β, Fab. Fab 6 h at 37°C. Binding medium was then removed, and cell layers were washed three times with cold PBS. Cell layers incubated with 125I-70 kD were solubilized in 1 N NaOH, and the radioactivity was counted. Cells incubated with 125I-fibronectin were extracted in 1% deoxycholate, and the amounts of fibronectin bound in the deoxycholate-soluble Pool I and deoxycholate-insoluble Pool II were determined as described in Materials and Methods. 125I-70 kD binding (open circles), and 125I-fibronectin binding in Pool I (open triangles) and Pool II (solid triangles) are expressed as the percent of control binding (see Fig. 1). In this experiment, the 125I-70 kD control cells bound 2,280 cpm (1.20 ng), and the 125I-fibronectin control cells bound 3,990 cpm (3.0 ng) in Pool I and 1,800 cpm (1.3 ng) in Pool II. Non-specific binding was measured in the presence of 500 μg/ml unlabeled fibronectin or 25 μg/ml unlabeled 70 kD. Non-specific binding was 22% of total binding for Pool I fibronectin binding, 13% for Pool II fibronectin binding, and 25% for 70 kD binding. Each point represents the average of duplicate determinations and the error bars indicate the range. Cell adhesion (open squares) was measured as described in Fig. 1. Each point represents the average of triplicate determinations and is expressed as a percent of control cell adhesion to fibronectin in the absence of antibody.
Figure 5. Effect of anti-βI Fab fragments on fibronectin binding in Pools I and II in the presence of increasing concentrations of fibronectin. Confluent monolayers of fibroblasts were incubated 7 h in 0.5 ml of F-12 media containing 0.2% BSA, 25 mM Hepes, pH 7.4, and 10 μg/ml cycloheximide (binding media) plus varying concentrations of 125I-fibronectin (80,000 cpm/μg) either alone or in combination with 5 μg/ml anti-βI Fabs. Binding medium was then removed, and cell layers were washed three times with cold PBS, and extracted with 1% deoxycholate as described in Materials and Methods. The graph shows specific ng of fibronectin bound in Pool I in the absence of Fabs (open circles) and in the presence of Fabs (open squares) and the ng of fibronectin accumulated in Pool II in the absence of Fabs (solid circles) and in the presence of Fabs (solid squares). Each point represents the average of duplicate determinations.

Fogerty et al. Inhibition of Fibronectin Binding by Antiintegrin

nectin and 70 kD binding than did anti-α5 suggested possible involvement of other α chains. Antibody blocking and affinity chromatography data suggest that integrin α5β1 is a low affinity receptor for fibronectin and also a receptor for other ligands (Wayner and Carter, 1987; Wayner et al., 1988). The site on fibronectin that α5β1 binds is not known though it appears not to bind to 120-kD fibronectin fragment or fibronectin affinity columns at physiological salt concentration (Wayner and Carter, 1987; Hynes et al., 1989; Plante-faber and Hynes, 1989). Two monoclonal antibodies specific for α5β1, J143 and PIB5, failed to block fibronectin and 70 kD binding to fibroblast cell layers. Since integrin α5, in association with a novel β subunit (β5), has been shown to promote cell attachment to fibronectin in addition to vitronectin (Cheresh et al., 1989), we examined whether antibodies specific for α5 or α5β1 complex inhibited binding of 70 kD. Two monoclonal antibodies, LMI42, which is directed to the α subunit of the vitronectin receptor (Cheresh and Harper, 1987), and LM609, which is directed to the α5β1 complex (Cheresh and Spiro, 1987), also failed to block 70 kD binding to fibroblast cell layers. Studies are ongoing to learn whether other available antiintegrin monoclonal antibodies are inhibitory.

Inhibition of fibronectin binding by anti-βI was demonstrable 20 min after the simultaneous addition of ligand and antibody. This result correlates well with the reported time Roman et al. (1989) found that it took another monoclonal antibody specific for α5β1 (PIF8) to reverse colocalization of the fibronectin receptor with a fibronectin-containing matrix at the immunofluorescence level. In their study fibronectin–α5β1 interactions were disrupted within 30 min after the addition of PIF8.

Figure 6. Autoradiograph of iodinated cell surface proteins immunoprecipitated by rabbit anti-α5β1 after chromatography on 120 kD- or 70 kD-Sepharose. Detergent extracts prepared from surface-labeled human foreskin fibroblasts were subjected to affinity chromatography on either immobilized 120-kD cell attachment fragment or immobilized fibronectin 70-kD amino-terminal fragment. Total cell lysate, unbound fraction and eluted fractions were immunoprecipitated with a polyclonal anti-α5β1 which recognizes primarily the β1 subunit and immunoprecipitates β1-containing integrins. The immunoprecipitated antigens were fractionated on SDS PAGE (80%) under nonreducing conditions. Lane 1, total lysate (starting material); lane 2, 120 kD–Sepharose unbound; lanes 3–7, fractions eluted with 10 mM EDTA from 120 kD–Sepharose; lane 8, 70 kD–Sepharose unbound; lanes 9–12, fractions eluted with 10 mM EDTA from 70 kD–Sepharose. The α5 and β1 subunits are indicated on the gel by the upper and lower arrowheads, respectively.
Increasing concentrations of exogenous fibronectin were unable to overcome inhibition by anti-β. Fab of fibronectin binding. One explanation of this result is that anti-β, Fabs have a much higher affinity for the receptor than does fibronectin. A second explanation is that fibronectin and anti-β, Fabs do not share the same binding site.

The discovery that a monoclonal antibody to the integrin β subunit could block 125I-70 kD binding to cell monolayers prompted us to examine whether any β-containing integrins bound to the immobilized 70-kD amino-terminal fragment. We were unable, however, to demonstrate binding of a β-containing integrin to 70 kD-Sepharose under conditions which resulted in the binding of α6β1 to immobilized cell adhesion fragment.

We should like to present three possible models to explain the inhibitory effect of the anti-β, antibody. (a) αβ, complex binds transiently to the cell adhesive domain of fibronectin causing it to unfold and expose its aminoterminal domain. This initial binding to the cell adhesion domain is rapid but of moderate affinity that has been reported for binding of soluble fibronectin to cells in suspension (Akiyama and Yamada, 1984). Such a moderate affinity interaction might explain why no direct binding of iodinated fibronectin cell adhesion fragments could be measured (McKeown-Longo and Mosher, 1985) and why large amounts of unlabeled cell adhesion fragments are required to compete fibronectin binding and matrix assembly (McDonald et al., 1987; our unpublished results1). The anti-β, would therefore block binding of soluble fibronectin to cell layers and its subsequent incorporation into the matrix by interfering with the initial transient binding event before the unfolding of the aminoterminal 70-kD domain.

After fibronectin unfolds, the amino-terminal domain can bind adjacent cell surface molecules, including adjacent fibronectin molecules. This binding increases the overall affinity of fibronectin binding and allows polymerization to begin. A possible role for fibronectin-fibronectin interactions in fibronectin binding to cell layers is supported by the finding that the amino-terminal region of horse fibronectin contains a self-association site that can interact with the carboxy-terminal regions of another fibronectin molecule in a head-to-tail manner (Ehrismann et al., 1982). The part of fibronectin mediating this interaction, however, is not in the most amino-terminal part of the protein but instead is probably located in a region spanning the ninth type I homology and the adjacent first type III homology unit (Chernousov, M. A., F. J. Fogerty, V. E. Koteliansky, and D. F. Mosher, manuscript in preparation). This model, furthermore, does not explain how the anti-β, can block binding of the isolated 70-kD fragment.

(b) β, complexed to an undetermined α subunit (α, ) or conformationally changed αβ, may bind the non-RGDS-containing amino-terminal region (70 kD) of fibronectin to mediate binding of exogenous fibronectin and its incorporation into the matrix. These are reasonable hypotheses to consider. Increasing numbers of integrins are reported to bind other sequences besides or in addition to RGDS. Examples of β-containing integrins that bind to their ligands in a RGDS independent manner include VLA3 (αβ3) binding to fibronectin, collagen, and laminin; VLA1 (αβ1) and VLA2 (αβ2) binding to type IV and type I collagen, respectively; and VLA4 (αβ4) binding to fibronectin (Wayner and Carter, 1987; Wayner et al., 1988, 1989; Kramer and Marks, 1989). The hypothesis that a conformationally changed αβ, is required to bind the amino-terminal region of fibronectin is also feasible. Platelet glycoprotein 11b/11a is one example of an integrin that must undergo a conformational change before it can bind its ligands (Plow and Ginsberg, 1981).

Cell attachment and spreading are probably mediated by the actin cytoskeleton that is linked to the integrins (Singer, 1979, 1989; Tamkun et al., 1986; Horwitz et al., 1986; Argraves et al., 1989). The binding and assembly site for fibronectin is not present on cells treated with cytochalasin (Barry and Mosher, 1988). Alterations in the actin cytoskeleton that occur during cell attachment and spreading could potentially alter the conformation of the integrin receptor resulting in exposure of a unique 70-kD binding site. The requirement for a conformationally changed receptor would explain why we were not able to detect binding of detergent solubilized β, containing integrin to a 70 kD-Sepharose affinity column.

In this second model anti-β, could inhibit binding because of steric hindrance if the binding sites for the antibody and ligand were overlapping or adjacent. Alternatively, occupancy of one binding site by anti-β, could produce a conformational change in the receptor complex that would then preclude occupancy of the second binding site by the fibronectin or 70-kD fragment (Santoro and Lawing, 1987).

(c) αβ, and/or αβ, may recruit a third component to form a binding site with specificity for the 70-kD region. The time that it takes the anti-β, to uncouple the complex or to interfere with recruitment would explain why the inhibitory effect of anti-β, lags behind the inhibitory effect of excess fibronectin. Higher concentrations of fibronectin would not be able to overcome the inhibitory effect of the antibody because the antibody and fibronectin are not competing for the same binding site. The identity of the third component and the mechanism of the uncoupling are unknown.

Cross-linking experiments indicate that the assembly of fibronectin is at the ends of growing fibrils (Barry and Mosher, 1989). One effect of anti-α and -β, could be dissociation of the fibril ends from assembly sites. Another effect could be to block some modification or rearrangement of preexisting matrix that allows assembly to proceed. The assembly sites seem catalytic, and there must be a mechanism to regenerate the site, such as the endocytic cycle for αβ, reported by Bretscher (1989). This mechanism may require cell locomotion. Interference with cycling could explain the peculiar differences in distribution of α2 and β1 after incubation of fibroblasts with the monoclonal antibodies.

In conclusion, our finding that a monoclonal antibody against the integrin β subunit completely blocks binding of the 70-kD amino-terminal fragment of fibronectin indicates that a β-containing integrin is either directly or peripherally involved in the binding of the aminoterminal "matrix assembly domain" of soluble fibronectin to cell layers. Further studies should elucidate more precisely the role β integrins play in fibronectin matrix assembly.

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