Stimulation of Integrin-mediated Cell Contractility by Fibronectin Polymerization*

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Ligation of integrins with extracellular matrix molecules induces the clustering of actin and actin-binding proteins to focal adhesions, which serves to mechanically couple the matrix with the cytoskeleton. During wound healing and development, matrix deposition and remodeling may impart additional tensile forces that modulate integrin-mediated cell functions, including cell migration and proliferation. We have utilized the ability of cells to contract floating collagen gels to determine the effect of fibronectin polymerization on mechanical tension generation by cells. Our data indicate that fibronectin polymerization promotes cell spreading in collagen gels and stimulates cell contractility by a Rho-dependent mechanism. Fibronectin-stimulated contractility was dependent on integrin ligation; however, integrin ligation by fibronectin fragments was not sufficient to induce either tension generation or cell spreading. Furthermore, treatment of cells with polyvalent RGD peptides or pre-polymerized fibronectin did not stimulate cell contractility. Fibronectin-induced contractility was blocked by agents that inhibit fibronectin polymerization, suggesting that the process of fibronectin polymerization is critical in triggering cytoskeletal tension generation. These data indicate that Rho-mediated cell contractility is regulated by the process of fibronectin polymerization and suggest a novel mechanism by which extracellular matrix fibronectin regulates cytoskeletal organization and cell function.

Fibronectin is a high molecular mass, multidomain glycoprotein that circulates in a soluble form in the plasma and is also found in an insoluble, multimeric form within the extracellular matrix (1). The polymerization of soluble fibronectin into insoluble fibrils within the extracellular matrix is a dynamic, cell-dependent process that is mediated by a series of events involving the actin cytoskeleton and integrin receptors (2). The adhesion of cells to fibronectin via integrin receptors has been shown to be important in the regulation and coordination of such complex processes as cell growth, differentiation, and migration (1). Ligation of integrins with extracellular matrix molecules, including fibronectin, induces the clustering of actin and actin-binding proteins to focal adhesions, which serves to mechanically couple the extracellular matrix with the actin cytoskeleton (3). Recent studies suggest that the interaction of cells with the extracellular matrix form of fibronectin triggers changes in cell cycle progression (4–7), cell migration (8), and actin filament organization (6, 9) that are distinct from those generated by the interaction of cells with nonpolymerized fibronectin. The mechanism by which the matrix form of fibronectin gives rise to cellular phenotypes distinct from protomer fibronectin is unknown.

Studies aimed at understanding the effect of extracellular matrix on cell function indicate that cells respond to fibronectin-coated beads with a local reinforcement of cytoskeletal links that are proportional to the strength of the force on the integrin (10). Similarly, cytoskeletal stiffness has been shown to increase in proportion to the stress applied to integrin receptors (11). Other studies suggest that cell spreading and migration (12) as well as cytoskeletal assembly (13) are regulated by the mechanical stiffness of the adhesive substrate. Taken together, these data suggest a model in which extracellular matrix fibronectin modulates cell function through local alterations in cytoskeletal organization that are mediated in part by changes in the rigidity of the insoluble fibronectin matrix. The cell-mediated polymerization of soluble fibronectin into an insoluble extracellular matrix is a tightly controlled process that can be rapidly up- or down-regulated (14–17). As such, the process of polymerizing an insoluble fibronectin matrix may serve as a distinct control mechanism by which the organization of the actin cytoskeleton remains tightly coupled to the organization of the extracellular environment.

The present study was undertaken to determine whether the polymerization of fibronectin into an insoluble extracellular matrix specifically influences the organization of the actin cytoskeleton by strengthening the linkage between the integrin and the cytoskeleton. Tension generation due to increases in cytoskeletal contractility may be determined directly by imbedding cells into three-dimensional, floating collagen gels and measuring the degree of gel contraction over time (18). In this well-characterized model of wound contraction, cells anchored in the collagen matrix organize and contract collagen fibrils (19–21) by an integrin-dependent mechanism (22–24). To determine the role of fibronectin deposition on actin organization and mechanical tension generation, fibronectin-null cells were imbedded into floating, native type I collagen matrices and the ability of fibronectin polymerization to stimulate cell spreading and integrin-mediated collagen gel contraction was assessed. Our data indicate that fibronectin polymerization stimulates cell spreading within collagen matrices and triggers a significant increase in cytoskeletal contractility. The increase in contractility induced by fibronectin was dependent on integrin ligation; however, integrin-binding fragments of fibronectin were not sufficient to induce either tension generation or cell spreading. Fibronectin-induced contractility was inhibited by...
agents that inhibit fibronectin polymerization, suggesting that the process of fibronectin polymerization is critical in triggering cytoskeletal tension generation. In addition, fibronectin-induced contractility was inhibited by pretreatment of cells with C3 exotransferrase, suggesting a role for RhoA in the contractile response of cells to fibronectin polymerization. Taken together, these data suggest that the polymerization of a fibronectin matrix triggers integrin-mediated cytoskeletal reorganization and enhanced cell contractility through a mechanism involving the activation of RhoA.

**EXPERIMENTAL PROCEDURES**

**Reagents—** Gel electrophoresis supplies were from Bio-Rad. Lipofectamine, GRGDSP, and GRGESP peptides were obtained from Life Technologies Inc. Laminin (purified from Engelbreth-Holm-Swam tumors) and type I rat tail collagen were purchased from Upstate Biotechnology (Lake Placid, NY). Vitronec was purified from fibronectin- and fibrinogen-depleted human plasma as described previously (25). Pertussis toxin was obtained from Calbiochem. C3 exoenzyme was obtained from List Biological Laboratories (Campbell, CA). Unless otherwise indicated, chemical reagents were obtained from Sigma.

**Cell Culture—** Mouse embryo cells, derived from fibronectin-null embryos and adapted to grow under serum-free conditions (4), were cultured under conditions where no exogenous source of fibronectin or other matrix protein is present.

**Purification of Fibronectin and Fibronectin Fragments—** Human plasma fibronectin was isolated from Cohn’s fraction I and II (a gift of Dr. Kenneth Ingham, American Red Cross, Rockville, MD) essentially as described previously (26). Figure 1 shows a schematic diagram of the various fibronectin fragments used in this study. The 70-kDa amino-terminal fragment of fibronectin was generated by limited digestion with cathepsin D, followed by gelatin affinity chromatography, as described previously (27). To cleave the 70-kDa fragment into the 27-kDa fragment, recombinant wild type and ΔRGD fibronectin were expressed in insect cells using a baculovirus expression system essentially as described (34). Insect cells do not produce detectable fibronectin and were maintained in defined media (SF-900-II, Life Technologies) lacking serum supplementation. Thus, recombinant fibronectins were produced under conditions where no nonrecombinant source of fibronectin was present. Purity of III-C and recombinant fibronectins was assessed by SDS-PAGE and aliquots were stored at −80 °C before use. Antibodies—9D2 hybridoma cells were a gift from Dr. Deane Mosher (University of Wisconsin, Madison, WI). The epitope recognized by 9D2 is found within the III-1 module of fibronectin (25). Activins were generated by TSD Bioservices (Newark, DE). Immunglobulins (Igs) and Fab’ fragments were purified as described previously (36). Nonimmune mouse Fab(α) and rabbit Fab’ fragments (Cappel, Durham, NC) were similarly reduced, alkylated, and dialyzed against PBS to produce Fab’ fragments. Purity was assessed by SDS-PAGE and aliquots were stored at −80 °C prior to use.

Anti-integrin αb1 (Ha2/5), αc1 (Ha31/8), αa2 (Hm10–27), and αb2 (H9.2B8) subunit antibodies were obtained from Pharmingen (San Diego, CA). Antibodies directed against the gelatin-binding domain (ISt-10) and III-5 module (ISt-5) of fibronectin were obtained from Chemicon International (Temecula, CA). The anti-fibronectin polyclonal antibody was purchased from Sigma. Purified rat IgG1, hamster IgG, and rat IgG2a were purchased from Pharmingen.

**Collagen Gel Contraction Assays—** Fibronectin-null cells were harvested from monolayer culture by treatment with 0.08% trypsin (Life Technologies) and 0.5 mM EDTA. Trypsin activity was neutralized with 2 mM NaOH, 2 mM CaCl2, 2 mM MgCl2, 50 mM HEPES, 10% dialyzed fetal bovine serum, and an equal volume of 10 mg/ml soybean trypsin inhibitor. Cells were washed once with 1 mg/ml soybean trypsin inhibitor in PBS and then resuspended in a 1:1 mixture of Cellgro/Aim V.

Native type I collagen gels were prepared by mixing collagen, 0.1 × NaOH, 2× concentrated Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) and 1× DMEM on ice such that the final mixture contained 0.8 mg/ml collagen and 1× DMEM (37). Fibronectin-null cells were added to the neutralized type I collagen solutions at 2 × 10^5 cells/ml. Control wells (no cells) received an equal volume of media alone. Fibronectin and fragments were added to the collagen solutions prior to the addition of cells. To form prepolymerized fibronectin, 20 nm soluble fibronectin was incubated for 30 min at 20 °C with 62.5 μg/m1 III-C (8) prior to its addition to the collagen solution. Control cells were incubated with either 20 nm fibronectin or 62.5 μg/m1 III-C incubated under identical conditions. In some experiments, aliquots of cells were incubated with anti-integrin antibodies for 30 min prior to their addition to the collagen solution. To form floating gels, aliquots (0.1 ml/well) of the collagen/cell mixtures were added to wells of 96-well tissue culture plates (Corning/Costar, Corning, NY) precoated with 2% BSA. The collagen/cell mixtures were allowed to polymerize for 1 h at 37 °C in 5% CO2. Following this incubation, 100 μl of Cellgro/Aim V was added to wells and the edges of the wells were scored to ensure that the gels were not attached to the wells. In experiments not shown, DMEM was substituted for Cellgro/Aim V and similar levels of contraction were observed.

To determine the extent of contraction, gels were incubated for various periods of time at 37 °C in 5% CO2 and then removed from the wells and weighed. Collagen gel contraction was measured as a decrease in gel weight, using a Mettler AE260 balance with a sensitivity of 0.1 mg.
Interaction of cells with fibronectin stimulates cell contraction. Measuring the diameter of contracted gels (data not shown). A previously been shown to parallel the extent of collagen gel in gel weight that occurs as a result of fluid extrusion has in an 83.9% decrease in gel weight. This corresponds to basal levels of contraction of between 25–50% (Figs. 2–4 and 6–11). Coincubation of fibronectin-null cells with plasma fibronectin triggered a further, dose-dependent decrease in gel weight (Fig. 2A); the addition of 80 nM of fibronectin (40 μg/ml) to collagen-imbedded cells resulted in an 83.9% ± 1.6% decrease in gel weight (Fig. 2A). The decrease in gel weight that occurs as a result of fluid extrusion has previously been shown to parallel the extent of collagen gel contraction (37, 38). Similar results were also obtained by measuring the diameter of contracted gels (data not shown). A similar, significant decrease in collagen gel weight was also observed following addition of fibronectin to human embryonic dermal fibroblasts (data not shown). These data indicate that the interaction of cells with fibronectin stimulates cell contraction.

RESULTS
Fibronectin Induces Contraction of Floating Type I Collagen Gels—To determine the effect of fibronectin on cytoskeletal tension generation, fibronectin-null cells were imbedded in floating collagen matrices in the absence and presence of increasing concentrations of intact fibronectin. Most adherent cells, including fibroblasts and endothelial cells, continually produce an extensive endogenous fibronectin matrix. Fibronectin-null cells do not produce any detectable fibronectin but are capable of assembling a fibronectin matrix when cultured in the presence of exogenously added fibronectin (4). Thus, in this system, the levels of extracellular matrix fibronectin can be precisely manipulated in order to determine the exact role of fibronectin polymerization on cytoskeletal organization. In the absence of fibronectin, fibronectin-null cells imbedded in collagen gels retained approximately 75–50% of the original gel weight. This corresponds to basal levels of contraction of between 25–50% (Figs. 2–4 and 6–11). Coincubation of fibronectin-null cells with plasma fibronectin triggered a further, dose-dependent decrease in gel weight (Fig. 2A); the addition of 80 nM of fibronectin (40 μg/ml) to collagen-imbedded cells resulted in an 83.9% ± 1.6% decrease in gel weight (Fig. 2A). The decrease in gel weight that occurs as a result of fluid extrusion has previously been shown to parallel the extent of collagen gel contraction (37, 38). Similar results were also obtained by measuring the diameter of contracted gels (data not shown). A similar, significant decrease in collagen gel weight was also observed following addition of fibronectin to human embryonic dermal fibroblasts (data not shown). These data indicate that the interaction of cells with fibronectin stimulates cell contraction.

To determine whether this stimulation of cell contractility is a property specific to fibronectin, the effect of other extracellular matrix molecules on cell-mediated collagen gel contraction was assessed. As demonstrated in Fig. 2B, coincubation of cells with equal molar concentrations of either vitronectin or laminin had no effect on collagen gel weight. These data suggest that stimulation of cell contractility is not a general property of extracellular matrix molecules.

Time Course of Fibronectin-induced Contraction—Previous studies indicate that treatment of fibroblasts with either lysophosphatidic acid (LPA) or platelet-derived growth factor results in a steady increase in the contraction of floating collagen
Fibronectin-null cells were imbedded into collagen gels as indicated in the legend to Fig. 2, in the absence (−Fn, □) or presence of 40 nM plasma-derived (+pFn, ○) or recombinant (+rFn, ⊗) fibronectin. Gels were allowed to polymerize at 37°C for 1 h after which time serum-free media was added to the wells. At various times, separate gels were removed and weighed. Data are presented as a percentage of the weight of gels not containing cells ± S.E.

**Fig. 3. Time course of fibronectin-induced collagen gel contraction.** Fibronectin-null cells were imbedded into collagen gels as indicated in the legend to Fig. 2. At the time of seeding, a 40-nM concentration of either intact fibronectin (+Fn) or various fibronectin fragments encompassing the entire fibronectin molecule were added to the collagen/cell mixture. Collagen gels were cast and processed as indicated in the legend to Fig. 2. Data are presented as a percentage of the weight of gels not containing cells ± S.E.

**Fig. 4. Effect of fibronectin fragments on collagen gel contraction.** Fibronectin-null cells were imbedded in collagen gels as indicated in the legend to Fig. 2. At the time of seeding, a 40-nM concentration of either intact fibronectin (+Fn) or various fibronectin fragments encompassing the entire fibronectin molecule were added to the collagen/cell mixture. Collagen gels were cast and processed as indicated in the legend to Fig. 2. Data are presented as a percentage of the weight of gels not containing cells ± S.E.
etrate the stacking gel. In the presence of a reducing agent, no high molecular mass aggregates were detected (Fig. 5). Furthermore, addition of the 9D2 mAb to fibronectin-treated cells inhibited the formation of these high molecular mass fibronectin aggregates. In contrast, addition of an equal concentration of nonimmune IgG to fibronectin-treated cells had no effect on fibronectin multimer formation (Fig. 5). These data indicate that fibronectin-null cells imbedded within a collagen lattice can polymerize fibronectin into high molecular mass multimers by a process that can be inhibited by the anti-fibronectin antibody, 9D2.

The fibronectin fragments assayed for their ability to increase cell contractility (Fig. 4) contain binding sites for cell surface receptors (32, 49) but do not become incorporated into the high molecular mass multimers typically observed in the extracellular matrix (27). To determine whether the ability of fibronectin to stimulate cell contractility depends on its incorporation into insoluble fibrils, the effect of inhibitors of fibronectin polymerization on fibronectin-induced collagen gel contraction was assessed. Fibronectin-null cells were imbedded in collagen gels in the presence of 40 nM fibronectin, and in the absence and presence of either 9D2 Fab′ fragments (Fig. 6A) or 70-kDa amino-terminal fibronectin fragments (27, 50) (Fig. 6B). Addition of either 9D2 Fab′ fragments (Fig. 6A) or 70-kDa fibronectin fragments (Fig. 6B) to collagen-imbedded fibronectin-null cells resulted in a dose-dependent inhibition of fibronectin-mediated collagen gel contraction. In contrast, addition of either nonimmune mouse Fab′ (Fig. 6A) or the nonblocking 40-kDa fibronectin fragment (Fig. 6B) had no effect on the fibronectin-induced decrease in collagen gel weight. Taken together, these data suggest that fibronectin polymerization is essential for the development of fibronectin-mediated contraction.

**Integrin Ligation Is Required but Not Sufficient for Fibronectin-mediated Collagen Gel Contraction.** Our data using integrin-binding fragments of fibronectin suggest that fibronectin-integrin ligation is not sufficient to trigger an increase in cell contractility. To determine whether Arg-Gly-Asp (RGD)-mediated integrin ligation is required for fibronectin-mediated tension generation, fibronectin-null cells were imbedded in collagen gels in the absence and presence of a recombinant fibronectin in which the RGD sequence had been deleted. As shown previously in Fig. 3, treatment of collagen-imbedded cells with either plasma-derived or recombinant, wild type fibronectin resulted in a significant decrease in collagen gel weight (Fig. 7). Deletion of the integrin-binding RGD repeat from fibronectin abolished the ability of fibronectin to induce collagen gel contraction (Fig. 7). Furthermore, addition of 850 μM RGD peptides to plasma fibronectin-treated cells completely inhibited the contractile response of cells to fibronectin (Fig. 7). Fibronectin-induced contraction was similarly inhib-
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![Graph](http://www.jbc.org/)

**Fig. 7. Role of fibronectin's integrin-binding RGD repeat in collagen gel contraction.** Fibronectin-null cells were imbedded in collagen gels as indicated in the legend to Fig. 2, in the absence (+0) or presence of 40 nM plasma fibronectin (pFn), recombinant wild type fibronectin (rFn), or a recombinant fibronectin lacking the RGD sequence (rFnΔRGD). In other wells, cells were incubated with 40 nM plasma fibronectin in the presence of 0.5 mg/ml (850 μM) GRGESP (RGD) or GRGDSP (RGE) peptides. Collagen gels were cast and processed as indicated in the legend to Fig. 2. Data are presented as a percentage of the weight of gels not containing cells ± S.E.

- The addition of soluble fibronectin or recombinant fibronectin (rFn) did not stimulate collagen gel contraction compared with cells incubated with untreated fibronectin. Moreover, addition of polymeric fibronectin to collagen-imbedded cells decreased the extent of collagen gel contraction compared with cells incubated with untreated fibronectin (5).

- Fibronectin-null cells were imbedded into collagen gels as indicated in the legend to Fig. 2. In A, either PBS, plasma fibronectin (+Fn; solid bar = 40 nM), monomeric RGD peptides (+RGD; solid bar = 80 μM, hatched bar = 80 μM), or polymeric RGD peptides (Poly-RGD; solid bar = 5.7 μM, hatched bar = 5.7 μM) were added to the cell/collagen mixture. Protein concentrations were adjusted to reflect equal molar ratios of RGD repeats versus 40 nM fibronectin (hatched bars). In B, 20 nM of untreated fibronectin (+Fn), fibronectin (20 nM) pretreated with 62.5 μM III-1C, III-1C (C5), or an equal volume of PBS (C5) were added to cell/collagen mixtures. Collagen gels were cast and processed as indicated in the legend to Fig. 2. Data are presented as a percentage of the weight of gels not containing cells ± S.E. *, different from the +Fn group (p < 0.01); #, different from the +Fn group (p < 0.05).

- Fibronectin-mediated collagen gel contraction, cells were incubated for various amounts of time with either soluble fibronectin or fibronectin that had been pretreated with III-1C (8). As shown in Fig. 8B, incubation of cells with prepolymerized fibronectin did not accelerate collagen gel contraction versus untreated fibronectin. Moreover, addition of prepolymerized fibronectin to collagen-imbedded cells decreased the extent of collagen gel contraction compared with cells incubated with untreated fibronectin (Fig. 8B). The ability of fibronectin polymerization inhibitors to block the contractile response of cells to fibronectin (Fig. 6), coupled with the inability of either monovalent fragments (Figs. 4 and 8A), polymeric RGD (Fig. 8A), or prepolymerized fibronectin (Fig. 8B) to stimulate cell contraction, suggests that the process of fibronectin polymerization...
In addition, recent studies suggest a role for integrins, can promote fibronectin polymerization (44, 54, 55).

Collagen Gel Contraction—Cell-mediated contraction of collagen gels has been shown to be dependent on $\alpha_1\beta_1$ (22) and/or $\alpha_2\beta_1$ integrin ligation (23, 24). Fibronectin polymerization by fibroblasts is mediated primarily by the $\alpha_5\beta_1$ integrin (44, 49). In the absence of $\alpha_5$, other integrins, including $\alpha_v$-containing integrins, can promote fibronectin polymerization (44, 54, 55).

In addition, recent studies suggest a role for $\alpha_v$$\beta_1$ integrin-fibronectin interactions in enhancing clot retraction (56). To determine the relative contributions of various integrin receptors to the fibronectin-mediated stimulation of cell contractility, adhesion-blocking anti-integrin antibodies were tested for their ability to inhibit fibronectin-induced collagen gel contraction. As expected, the addition of an anti-$\beta_1$ integrin subunit antibody completely inhibited collagen gel contraction of fibronectin-treated cells (Fig. 9). Addition of 10 $\mu$g/ml anti-$\alpha_5$ antibodies alone or in combination with anti-$\alpha_v$ antibodies resulted in a small but significant reduction in fibronectin-induced contraction (Fig. 9). No further inhibition of the fibronectin contraction response was observed when the anti-$\alpha_5$ and anti-$\alpha_v$ antibody concentrations were increased to 25 $\mu$g/ml (data not shown). In the presence of fibronectin, addition of a combination of anti-$\alpha_5$ and anti-$\alpha_v$ antibodies had only a slight inhibitory effect on collagen gel contraction. Addition of anti-$\alpha_5$ and anti-$\alpha_v$ antibodies to $\alpha_5/\alpha_v$ integrin-blocked cells further reduced collagen gel contraction to levels similar to those observed in the absence of fibronectin (Fig. 9). Incubation of cells with isotype-matched IgM or IgG antibodies did not alter fibronectin-induced gel contraction (Fig. 9). These data indicate that collagen- and fibronectin-integrin receptors act synergistically to mediate fibronectin-induced collagen fibril contraction. Furthermore, these data suggest that, in the presence of collagen-integrin receptor blockade, fibronectin-integrin receptors can mediate fibronectin-stimulated collagen gel contraction.

Role of Fibronectin’s Gelatin-binding Domain in Fibronectin-mediated Collagen Gel Contraction—Neither $\alpha_v$$\beta_1$ nor $\alpha_5$$\beta_1$ integrins bind directly to collagen (49). However, binding interactions between fibronectin and either individual collagen fibrils (types I, II, III, and IV) or denatured type I collagen have been demonstrated in vitro (57, 58). The binding site for gelatin has been localized to a 40-kDa domain in the amino-terminal region of fibronectin (Fig. 1) (59). To determine whether the gelatin-binding domain of fibronectin plays a role in fibronectin-mediated collagen gel contraction, fibronectin-null cells were imbedded in fibronectin-containing collagen gels in the absence and presence of various concentrations of either an anti-gelatin-binding domain monoclonal antibody or a control monoclonal antibody that recognizes an epitope within the III-5 module of fibronectin (IST-5) (60). As demonstrated in Fig. 10, addition of anti-gelatin-binding domain antibodies resulted in a dose-dependent inhibition of fibronectin-induced collagen gel contraction. In contrast, addition of the anti-III-5 monoclonal antibody had no effect on fibronectin-induced contraction (Fig. 10). These data indicate that the gelatin-binding domain of fibronectin plays a role in mediating fibronectin-stimulated contraction. The anti-gelatin-binding domain antibody also inhibits fibronectin binding to gelatin, as assessed by enzyme-linked immunoassay (D.C. Hocking, unpublished observation). Thus, it is possible that fibronectin-binding integrins mediate collagen gel contraction through the formation of a tri-molecular complex in which integrin-bound fibronectin interacts with collagen via the gelatin-binding domain of fibronectin.

**Effect of Anti-integrin Antibodies on Fibronectin-mediated Collagen Gel Contraction.—** Cell-mediated contraction of collagen gels has been shown to be dependent on $\alpha_1\beta_1$ (22) and/or $\alpha_2\beta_1$ integrin ligation (23, 24). Fibronectin polymerization by fibroblasts is mediated primarily by the $\alpha_5\beta_1$ integrin (44, 49). In the absence of $\alpha_5$, other integrins, including $\alpha_v$-containing integrins, can promote fibronectin polymerization (44, 54, 55). In addition, recent studies suggest a role for $\alpha_v$$\beta_1$ integrin-fibronectin interactions in enhancing clot retraction (56). To determine the relative contributions of various integrin receptors to the fibronectin-mediated stimulation of cell contractility, adhesion-blocking anti-integrin antibodies were tested for their ability to inhibit fibronectin-induced collagen gel contraction. As expected, the addition of an anti-$\beta_1$ integrin subunit antibody completely inhibited collagen gel contraction of fibronectin-treated cells (Fig. 9). Addition of 10 $\mu$g/ml anti-$\alpha_5$ antibodies alone or in combination with anti-$\alpha_v$ antibodies resulted in a small but significant reduction in fibronectin-induced contraction (Fig. 9). No further inhibition of the fibronectin contraction response was observed when the anti-$\alpha_5$ and anti-$\alpha_v$ antibody concentrations were increased to 25 $\mu$g/ml (data not shown). In the presence of fibronectin, addition of a combination of anti-$\alpha_5$ and anti-$\alpha_v$ antibodies had only a slight inhibitory effect on collagen gel contraction. Addition of anti-$\alpha_5$ and anti-$\alpha_v$ antibodies to $\alpha_5/\alpha_v$ integrin-blocked cells further reduced collagen gel contraction to levels similar to those observed in the absence of fibronectin (Fig. 9). Incubation of cells with isotype-matched IgM or IgG antibodies did not alter fibronectin-induced gel contraction (Fig. 9). These data indicate that collagen- and fibronectin-integrin receptors act synergistically to mediate fibronectin-induced collagen fibril contraction. Furthermore, these data suggest that, in the presence of collagen-integrin receptor blockade, fibronectin-integrin receptors can mediate fibronectin-stimulated collagen gel contraction.

**Effect of Pertussis Toxin and C3 Exotransferase Treatment on Collagen Gel Contraction.—** Fibronectin-null cells were imbedded in collagen gels as indicated in the legend to Fig. 2 in the absence (−) or presence (+) of 40 nM plasma fibronectin (Fn). At the time of imbedding, increasing concentrations of antibodies directed against either the gelatin binding domain (GBD) or the III-5 domain (IST-5; 50 $\mu$g/ml) of fibronectin were added to the gels. Collagen gels were cast and processed as indicated in the legend to Fig. 2. Data are presented as a percentage of the weight of gels not containing cells ± S.E. * different from the +Fn group ($p < 0.01$).
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**Fibronectin-mediated Gel Contraction**—To begin characterizing the intracellular signals involved in fibronectin-mediated gel contraction, fibronectin-null cells were pretreated with either the G_{i} protein inhibitor, pertussis toxin (61) or with the Rho inhibitor, C3 exotransferase (62). The involvement of these pathways in the contraction of floating collagen gels by human dermal fibroblasts has been recently demonstrated (40). Pre-treatment of fibronectin-null cells with pertussis toxin completely inhibited the LPA-induced contraction of floating collagen gels (Fig. 11A). In contrast, pertussis toxin pretreatment had no significant effect on fibronectin-mediated contraction (Fig. 11A), suggesting that the G_{i} class of heterotrimeric G proteins is not involved in the contractile response of cells to fibronectin. Inhibition of Rho activity by pretreatment of cells with C3 exotransferase completely blocked the fibronectin-mediated decrease in collagen gel weight (Fig. 11B), pretreatment of fibronectin-null cells with either DMEM or the Lipofectin vehicle alone had no effect on fibronectin-mediated contraction (Fig. 11B). These data suggest that activation of the small G protein, Rho, is required for the contraction of cells in response to fibronectin.

**Fibronectin Polymerization Induces Actin Cytoskeleton Rearrangement**—During collagen gel contraction, fibroblasts imbedded in three-dimensional, floating collagen gels progress from a round to a spread phenotype (63). As cells spread, tractional forces generated from cellular extensions are thought to be the primary mechanism by which contraction of collagen fibrils occurs (64). To correlate fibronectin-induced changes in mechanical tension generation with changes in actin organization, cell spreading, and extension formation, fibronectin-null cells were imbedded in collagen gels in the absence and presence of 40 nM intact fibronectin or the integrin-binding 120-kDa fibronectin fragment. To assess the role of fibronectin polymerization on cell morphology and actin organization, the anti-fibronectin III-1 antibody, 9D2, or the control, nonimmune IgG was added to fibronectin-treated cells. Following a 20-h incubation, cells imbedded in collagen gels were fixed and permeabilized, and actin filaments were visualized by staining with FITC-phalloidin. As demonstrated in Fig. 12, in the absence of fibronectin, collagen-imbedded fibronectin-null cells remained round (Fig. 12A). Similarly, treatment of cells with the 120-kDa integrin-binding fibronectin fragment did not promote cell spreading (Fig. 12B). In contrast, treatment of cells with fibronectin resulted in actin filament reorganization, cell spreading, and the formation of cell extensions (Fig. 12, C and D). These fibronectin-induced morphological changes were blocked by the addition of the 9D2 mAb, which inhibits fibronectin polymerization (35) (Fig. 12E). Addition of nonimmune mouse IgG had no effect on cell spreading or extension.
formation of fibronectin-treated cells (Fig. 12F). Similar results were observed following a 4-h incubation (not shown). Taken together, these data indicate that actin reorganization, cell spreading, and the formation of cellular extensions within a collagen lattice are stimulated by fibronectin polymerization.

**DISCUSSION**

In the present study, we have demonstrated that fibronectin polymerization stimulates the spreading of fibronectin-null cells imbedded in collagen gels and triggers a significant increase in cell contractility, as measured by collagen gel contraction. The increase in cell contractility induced by fibronectin was dependent on integrin ligation; however, ligation of integrin receptors by fibronectin fragments was not sufficient to induce either tension generation or cell spreading. Cell contractility could not be stimulated by treatment of cells with either polyvalent RGD peptides or prepolymerized fibronectin. Moreover, fibronectin-induced contractility was blocked by agents that inhibit fibronectin polymerization, suggesting that the process of fibronectin polymerization is critical in triggering cytoskeletal tension generation. Fibronectin-induced contractility was inhibited by pretreatment of cells with C3 exotransferase but not by pretreatment with pertussis toxin, suggesting a role for RhoA in the contractile response of cells to fibronectin polymerization. These data are the first to demonstrate that Rho-mediated cell contractility may be regulated by the process of fibronectin polymerization and suggest a novel mechanism by which extracellular matrix fibronectin regulates cytoskeletal organization and cell function.

Our data suggest that fibronectin-binding integrins may mediate native collagen fibril reorganization through an interaction of the gelatin-binding domain of integrin-bound fibronectin with collagen fibrils. Competition binding assays indicate that the affinity of fibronectin for native collagen fibrils is approximately 10- to 100-fold lower than that of gelatin (57), prompting the suggestion that fibronectin may not bind to native collagen under physiological conditions (65). However, previous studies have demonstrated colocalization of fibronectin and collagen fibers in cultured fibroblasts (66–68). In addition, microscopic analyses of cutaneous wounds indicate that fibronectin fibrils are associated with type I (69) and type III (68) collagen fibrils. Furthermore, inhibition of fibronectin polymerization by anti-fibronectin antibodies inhibits collagen deposition by lung fibroblasts (70). These data, together with the data presented here, suggest that the interaction of fibronectin with the cell surface may increase the affinity of fibronectin for native collagen. Previous studies have identified conformation-dependent binding sites within fibronectin (36, 39) that may be exposed by changes in cell contractility (71). Further studies will be required to explore the possibility that fibronectin polymerization or cell contracture induces a conformational change within the gelatin-binding domain of fibronectin to enhance its collagen-binding activity.

The interaction of cells with fibronectin via cell surface integrin receptors generates a series of complex signaling events that serve to regulate many aspects of cell behavior, including growth, differentiation, adhesion, and motility (44, 49). Data presented in this study extend these observations by suggesting that the polymerization of an insoluble fibronectin matrix provides a distinct control mechanism to tightly couple the organization of the actin cytoskeleton to the organization of the extracellular environment. Fibronectin deposition may be rapidly up- and down-regulated by such factors as protein kinase C, cyclic AMP, and LPA (14–17). Activators of protein kinase C, which affect both stress fiber and focal contact formation (72), enhance fibronectin matrix assembly (17). Conversely, increasing intracellular cAMP levels, which disrupt actin stress fibers and cause cell retraction (73), inhibits matrix assembly (14). In addition, studies have demonstrated decreased fibronectin deposition upon disruption of the actin cytoskeleton with cytochalasin D (74). In the present study, cell contractility was inhibited by agents previously shown to inhibit fibronectin polymerization. Moreover, cell contractility could not be stimulated by treatment of cells with either monovalent fibronectin fragments, polyvalent RGD peptides, or prepolymerized fibronectin, suggesting that the process of fibronectin polymerization is critical in triggering cytoskeletal tension generation. Taken together, these studies suggest that a dynamic, reciprocal relationship exists between fibronectin polymerization, cytoskeletal organization, and the contractile state of the cell that may serve to regulate cell behavior.

Previous studies have defined a role for Rho-mediated contractility in fibronectin polymerization (16, 71, 75). Increasing Rho-stimulated contraction through treatment of cells with either LPA (16, 71) or nocardazole (75) or by microinjection of recombinant, constitutively active Rho (71) results in enhanced fibronectin deposition. In the present study, cell contractility induced by fibronectin polymerization was inhibited by pretreatment of cells with the Rho inhibitor, C3 exotransferase. In addition, spreading and extension formation by cells imbedded in collagen gels were dependent on fibronectin polymerization, and could not be stimulated by integrin-binding fragments of fibronectin. These data suggest that fibronectin polymerization triggers integrin-mediated cytoskeletal reorganization and enhances cell contractility through a mechanism involving the activation of RhoA. Recent studies have demonstrated that disruption of preformed extracellular matrices by treatment with the III-1C fragment of fibronectin blocks the ability of LPA to stimulate stress fiber formation (6). In addition, other studies suggest that actin stress fiber formation follows fibronectin matrix assembly (6) and is dependent on the three-dimensional structure of extracellular fibronectin (9). Taken together, these data suggest that fibronectin polymerization and deposition into the extracellular matrix stimulates Rho activation and subsequent actin cytoskeleton organization. Moreover, these data suggest that Rho activity may be sustained or potentiated by the continued deposition or remodeling of a fibronectin matrix.

One of the histological hallmarks of chronic inflammatory diseases, including interstitial pulmonary fibrosis and asthma, is an excessive and/or inappropriate deposition of extracellular matrix molecules that is thought to arise as a consequence of unresolved wound repair (76, 77). In the present study, we have used a well-characterized model of wound contraction (19–21, 40, 41, 78, 79) to demonstrated that fibronectin stimulates the contraction of collagen matrices by a mechanism that requires both integrin ligation and fibronectin polymerization. These studies suggest that in normal wound healing, remodeling of the collagen matrix may be regulated in part, by the rate and extent of fibronectin polymerization into the extracellular matrix. In chronic inflammatory diseases, excessive fibronectin deposition into the extracellular matrix may contribute to the pathogenesis of the disease by triggering abnormal tissue remodeling through changes in the organization and contraction of collagen fibrils.

In summary, our data indicate that fibronectin enhances cell contractility and alters cytoskeletal organization by a mechanism that is dependent on fibronectin polymerization. Other studies have shown that cytoskeletal assembly and stiffness increase in proportion to the stress applied to integrin receptors (10, 11). In addition, cell spreading, migration (12), and cytoskeletal assembly (13) are regulated by the mechanical stiffness of the adhesive substrate. Therefore, it is possible that...
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fibronectin polymerization promotes cell contractility through local alterations in cytoskeletal organization that are mediated in part by changes in the rigidity of the insoluble fibronectin matrix. Additionally, cell-mediated fibronectin polymerization may trigger conformational changes within the fibronectin molecule (71, 80) resulting in the exposure of neoepitopes that have unique affects on actin cytoskeletal organization and, in addition, may suggest provide important insight into the extracellular factors that control cytoskeletal organization and, in addition, may suggest novel approaches for the development of therapeutic strategies aimed at preventing abnormal tissue remodeling following injury.

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Stimulation of Integrin-mediated Cell Contractility by Fibronectin Polymerization
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