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Matrix-driven translocation: Dependence on interaction of amino-terminal domain of fibronectin with heparin-like surface components of cells or particles

(extracellular matrix/mechanochemical coupling)

STUART A. NEWMAN*, DOROTHY A. FRENZ*, ETSUKO HASEGAWA†‡, AND STEVEN K. AKIYAMA†‡

*Department of Anatomy, New York Medical College, Valhalla, NY 10595; †Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and ‡Howard University Cancer Center, Howard University Medical School, Washington, DC 20060

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ABSTRACT **During the process of matrix-driven translocation, certain types of cells or polystyrene latex beads are transported between compositionally different regions of a collagen matrix. Under appropriate conditions this translocation depends on an interaction between the cell or particle surface and fibronectin. We now show that this interaction takes place at a site located within the first 31 kDa of the amino-terminal end of the fibronectin molecule. Using defined fibronectin fragments and monoclonal antibodies directed against specific fibronectin domains, this site is established as both necessary and sufficient for the promotion of matrix-driven translocation. Competition experiments using heparin, heparan sulfate, and other sulfated polysaccharides show that this fibronectin site interacts with heparin-like cell or particle surface components in promoting matrix-driven translocation. Treatment of cells with heparinase renders them unresponsive to the translocational effect. An antibody directed against the amino-terminal domain of fibronectin completely inhibits matrix-driven translocation without interfering with heparin binding, suggesting that a post-binding conformational change in fibronectin may be required for promotion of the effect.**

Matrix-driven translocation (MDT) is a biophysical process in which there is rapid unidirectional movement of certain types of cells or inert particles from one region to another of a compositionally nonuniform collagen–fibronectin matrix (1). Several characteristics of this phenomenon suggest that it may play a role in morphogenesis and related processes in living organisms: (*i*) MDT selectively translocates different types of cells. For example, chicken limb precartilage mesenchyme cells are translocated by assembling matrices containing chicken cellular fibronectin, but chicken embryo heart cells are not (1). (*ii*) MDT is selectively promoted by biologically distinct fibronectin molecules. For example, certain cells that are translocated by chicken cellular fibronectin are not translocated by chicken plasma fibronectin under similar conditions (1). (*iii*) MDT is dependent on physiological conditions. In particular, when the assay is done at nonphysiological pH or ionic strength, translocation of cells or particles does not occur (1).

Because MDT is evidently promoted by an interaction between fibronectin and cell or particle surfaces in an appropriate environment, we have now analyzed the specificity of this interaction in relation to current understanding of the domain organization of the fibronectin molecule. Using defined fragments of human plasma fibronectin (2–4) and monoclonal antibodies directed against the corresponding fibronectin domains, we have located a site within the first 31 kDa of the amino terminus of this molecule that is both necessary and sufficient to promote MDT under appropriate conditions. Molecular competition studies, studies using beads coated with defined macromolecules, and studies with heparinase-treated cells indicate that heparin-interacting properties of this amino-terminal domain play a central role in promoting MDT. Our results indicate that cell or particle translocation through an extracellular matrix can be promoted by a specific interaction between fibronectin and heparin-like components of the cell or particle surface.

MATERIALS AND METHODS

Translocation Assay. Collagen was extracted from rat tail tendons, dialyzed against 1:10 strength Ham's F-12 medium (GIBCO), and used to construct hydrated matrices as previously described (1, 5). Briefly, collagen gels containing chicken limb precartilage cells (6, 7) or 6-μm polystyrene latex beads (Polysciences, Warrington, PA), "primary gels," were made by adding to 0.7 ml of the collagen solution (4.4 mg/ml) on ice, 0.1 ml of 10-fold concentrated Ham's F-12 medium, and 0.2 ml of sodium bicarbonate solution (5.88 mg/ml); this solution was mixed rapidly with 1 ml of cells or beads (1–2 $\times$ 10^7 per ml) in Ham's F-12 medium. Collagen gels containing intact human plasma fibronectin, defined fragments of that molecule, or chicken plasma fibronectin—"secondary gels"—were prepared as above, but with 1 ml of Ham's F-12 medium containing fibronectin or fibronectin fragments at 25 μg/ml or 50 μg/ml instead of 1 ml of medium containing cells or beads. Gel preparations were photographed as described (1) after 10 min when translocation was completed. Translocation was considered to have occurred if movement of the front into the secondary gel was at least 3 mm.

In some experiments monoclonal antibodies directed against various domains of the human plasma fibronectin molecule were used to construct secondary gels containing fibronectin–antibody complexes. The antibodies were added at 30 μg/ml to 25 or 50 μg of fibronectin per ml in medium and incubated for 30 min at 37°C. These mixtures were substituted for the fibronectin-containing medium gel composition. In some experiments heparin (porcine intestine, 157 USP units/mg), beef lung heparan sulfate (gifts of S. Radoff and B. Lahiri, Department of Biochemistry, New York Medical College), or one of several dextran sulfates (Sigma no. D-0768, D-6393, and D-6001; from dextrans with average M_r of 5000, 8000, and 500,000, respectively) were added in various concentrations to secondary gels containing intact fibronectin. Each translocation experiment was done at least five times.

Preparation of Fibronectin and Fibronectin Fragments. Fibronectin was obtained from Bethesda Research Labora-

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Abbreviation: MDT, matrix-driven translocation.

tories or purified from citrated human plasma (Community Blood and Plasma Services, Baltimore, MD) by gelatin affinity chromatography using citric acid elution (8, 9). Fibronectins from both sources were equally active in the translocation assays.

The 31-kDa amino-terminal heparin-binding fragment, the 75-kDa cell-binding fragment, and the 146-kDa cell plus carboxyl-terminal heparin-binding fragment were purified from trypsin digests of fibronectin (4). The 43-kDa collagen-binding fragment was purified from thermolysin digests of fibronectin (2).

Preparation of Antifibronectin Monoclonal Antibodies. Hybridomas secreting the antifibronectin antibodies were produced by the fusion of Y3 rat myeloma cells with spleen cells from a Sprague–Dawley rat (10) that had been previously immunized with purified human fibronectin or with mouse 3T3 cell membranes (11, 12). Production and characterization of the clone designated 333 have been described elsewhere (13). Before being used in further studies, each hybridoma was subcloned four times by limiting dilution.

Monoclonal antibodies were purified from conditioned, serum-free medium, and their specificity was quantitated by an ELISA procedure as described (14, 15).

Preparation of Coated Latex Beads. Six-micrometer polystyrene latex beads were incubated in 0.5 mg of poly(L-lysine) per ml for 30 min at room temperature as previously described (1). This treatment renders the beads incapable of responding to MDT. Polylysine-coated beads were further coated with heparin, heparan sulfate, or one of several dextran sulfates by incubation for 30 min at room temperature in these substances (12.5 mg/ml).

Treatment of Cells with Heparinase and Chondroitinase ABC. Chicken limb mesenchyme cells (6, 7) were incubated at 1×10^7 cells per ml in 0.018 unit of *Flavobacterium* heparinase per ml (16; gift from J. Marcum, Massachusetts Institute of Technology) diluted in Earle's balanced salt solution for 30 min at 37°C. Cells were resuspended at 2×10^7 cells per ml in Ham's F-12 medium, mixed 1:1 with soluble type I collagen (3.4 mg per ml) in Ham's F-12 medium, and assayed for MDT as described (1). Similar experiments were done with cells incubated in 0.02 unit of chondroitinase ABC per ml (Sigma, C-2905) instead of heparinase.

Heparin-Binding Assay for Fibronectin 31-kDa Amino-Terminal Fragment. Fibronectin 31-kDa amino-terminal fragment was labeled using [^{14}C]formaldehyde (ICN) and sodium cyanoborohydride as described (17) to a specific radioactivity of 8.8×10^4 cpm/μg.

For the heparin-binding assay, tracer amounts of ^{14}C-labeled fragment were mixed with unlabeled fragment to yield a final protein concentration of 0.5 mg/ml in 10 mM sodium phosphate, pH 7.0. Forty microliters of fibronectin fragment was mixed either with 20 μl of monoclonal antibody (5 mg/ml) in 10 mM sodium phosphate, pH 7.0, or with 20 μl of sodium phosphate alone and incubated on ice for 1 hr. Packed heparin–Sepharose beads (0.1 ml) were added for another 1-hr incubation on ice. This mixture was stirred (Vortex) at 15-min intervals during the second incubation. The beads were washed with 0.5 ml of 10 mM sodium phosphate, pH 7.0, before liquid scintillation counting.

RESULTS

Sufficiency of the 31-kDa Amino-Terminal Fragment of Human Plasma Fibronectin for Promotion of MDT. Human plasma fibronectin is cleaved by proteases into several distinct fragments, each of which exhibits a characteristic array of binding activities (for review, see refs. 18–21; Fig. 1). Assays for MDT were done as previously described (1), but with fibronectin fragments used in the secondary gels rather than intact fibronectin. Chicken limb bud precartilage cells (6, 7) or polystyrene latex beads, each of which will translocate in response to intact human plasma fibronectin (1), were used in separate assays. Of the four fragments used, only the 31-kDa amino-terminal fragment was capable of promoting MDT (Fig. 1). Although the 43-kDa collagen-binding and 75-kDa cell-binding fragments and the fragment of 146 kDa that contains the strong heparin-binding site from the carboxyl-terminal half of fibronectin were used in these assays in a molar excess of 8- to 12-fold the threshold value for intact fibronectin (1), no translocation of either cells (data

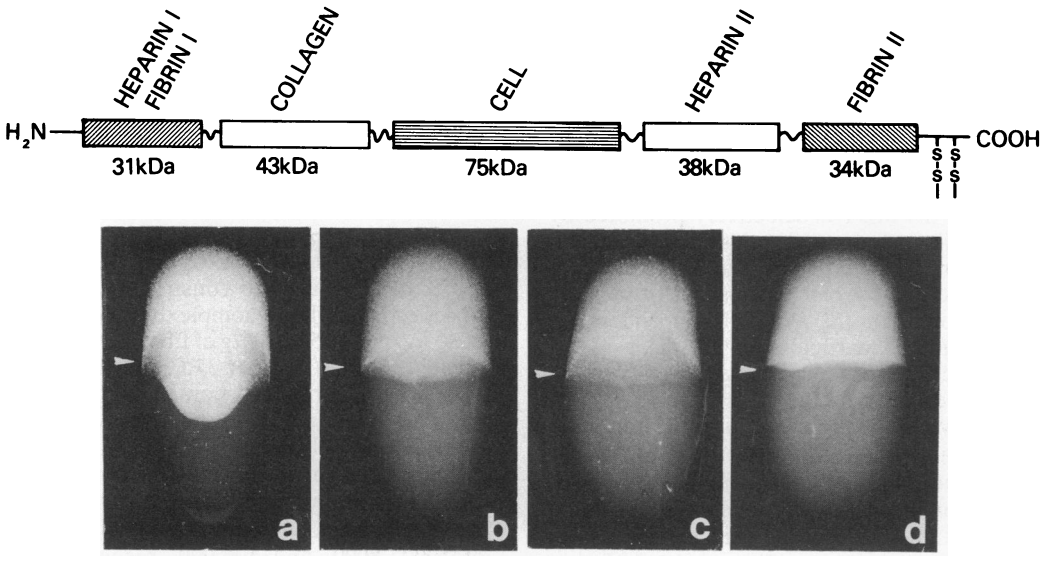

FIG. 1. (*Upper*) Map of functional binding domains of fibronectin. The major protease-resistant functional binding domains of fibronectin are shown along with their respective approximate sizes in kDa. Also indicated are the amino- and carboxyl-termini, denoted by H_2N- and -COOH, respectively, and the relative position of the interchain disulfide bond(s) (-S-S-). (*Lower*) MDT in response to defined fragments of human plasma fibronectin. All primary gels contained 6-μm polystyrene latex beads. Secondary gels contained defined fibronectin fragments at 25 μg/ml. The secondary gel contained the 31-kDa amino-terminal heparin/fibrin binding fragment (*a*), the 43-kDa collagen-binding fragment (*b*), the 75-kDa cell-binding fragment (*c*), and the 146-kDa fragment encompassing the cell-binding, strong heparin-binding, and carboxyl-terminal fibrin-binding domains (*d*). Translocation occurred over a period of 5–6 min, and preparations were photographed subsequently. Arrowheads, original positions of primary–secondary gel interfaces. Long dimension of all primary–secondary gel preparations was ≈12 mm.

not shown) or beads (Fig. 1) occurred. In 40 experiments using beads and the 31-kDa amino-terminal fragment, translocation occurred to an extent of 3–6 mm. In 12 or more experiments using beads and each of the other fragments, translocation was <0.5 mm. Similar results were obtained in 6 or more experiments using cells and each of the fragments.

Requirement of a Function in the Amino-Terminal Domain of Human Plasma Fibronectin for Promotion of MDT. Monoclonal antibodies were prepared that were reactive with the 31-kDa amino-terminal fragment, the 43-kDa collagen-binding fragment, and the 75-kDa cell-binding fragment of human plasma fibronectin. Characterization of the specificity of these antibodies by an enzyme-linked immunosorbent (ELISA) assay is shown in Fig. 2. Each of the fragment-binding antibodies is at least 100- to 1000-fold more reactive with its cognate fragment than with the other fragments (Fig. 2). By immunoblotting (data not shown), antibody 304 was not reactive with fibronectin fragments of 113 and 146 kDa that contain the heparin-binding site near the carboxyl terminus of the molecule. Antibody 191, which reacted with the collagen-binding fragment, was actually more reactive with intact fibronectin. Assays for MDT were done using intact human plasma fibronectin that had been mixed with varying amounts of each of the antibodies. Only antibody 304, which was directed against the 31-kDa amino-terminal fragment (and therefore the corresponding domain on the intact molecule), was capable of blocking MDT (Fig. 3). Translocation was 3–4 mm in the presence of antibodies 333 and 191 and <0.5 mm in the presence of antibody 304. Antibody 304 also blocked translocation promoted by the 31-kDa fragment alone. Each of these experiments was done 30 or more times using beads and 6 or more times using cells—with no qualitative variation in the outcome.

Dependence of MDT on Site of Heparin Interaction at the Amino-Terminal Domain of Fibronectin. By adding various substances to the secondary gel along with intact human plasma fibronectin in the translocation assays, we were able to explore which function or functions of the molecule are essential for the promotion of MDT. Introduction of fibrinogen, a ligand known to bind to the 31-kDa amino-terminal fragment of human fibronectin (22), into the secondary gel at concentrations of up to 50 μg/ml in the presence of fibronectin at 25 μg/ml had no effect on the rate or extent of MDT of cells or beads (data not shown). Heparin and heparan sulfate bind to fibronectin in its 31-kDa amino-terminal domain among other sites (for review see ref. 19). When heparin (average M_r 12,000, 2.5 μg/ml) was introduced into secondary gels in the presence of 25 μg of fibronectin per ml, MDT was completely blocked (Fig. 4*b*). The same result was obtained when heparan sulfate was added in this manner (data not shown). These substances also blocked MDT when an equimolar amount of the 31-kDa amino-terminal fibronectin fragment was substituted for intact fibronectin in the

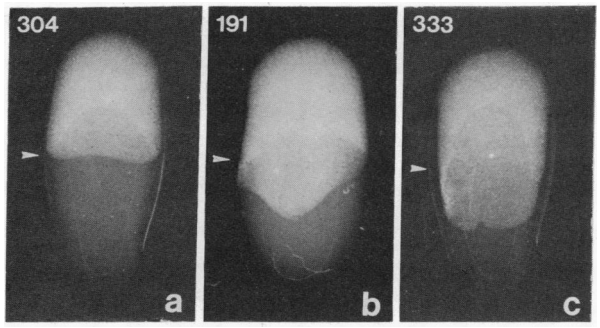

FIG. 3. MDT in response to fibronectin–monoclonal antibody complexes. All primary gels contained 6-μm polystyrene latex beads. All secondary gels contained human plasma fibronectin (25 μg per ml) in Ham's F-12 medium. The secondary gel contained antibody 304 (30 μg per ml) directed against the 31-kDa amino-terminal fragment (*a*), antibody 191 (30 μg per ml) directed against the 43-kDa collagen-binding fragment (*b*), and antibody 333 (30 μg per ml) directed against the 75-kDa cell-binding fragment (*c*).

secondary gels. Other sulfated polysaccharides, such as dextran sulfates, also bind to fibronectin in column-binding assays (23). However, dextran sulfates (M_r 5000 to 500,000; up to 4 mol of sulfate per mol of disaccharide) were completely inactive in inhibiting MDT when added at concentrations as high as 50 μg/ml to secondary gels together with 12.5 μg of fibronectin per ml (Fig. 4*a*). Furthermore, dextran sulfate at 50 μg/ml was unable to relieve inhibition of MDT by heparin at 2.5 μg/ml (data not shown). These experiments in conjunction with those described in the previous section indicate that a site of interaction with heparin or heparan sulfate located within the 31-kDa amino-terminal domain of human plasma fibronectin is required for MDT and also indicate that this site is different from those common for heparin and dextran sulfate (23).

Because the interactions of the cell surface and polystyrene latex with the amino-terminal heparin-binding site of fibronectin are respectively complex and poorly characterized, we constructed model particle surfaces by coating 6-μm polystyrene latex beads with either heparin, heparan sulfate, or dextran sulfate. Beads coated with heparin or heparan sulfate were capable of being translocated, whereas those coated with dextran sulfate were not (Fig. 4 *c–e*).

These experiments suggested the possibility that cells capable of undergoing MDT do so by virtue of heparan sulfate or other heparin-like molecules on their surfaces. To test this hypothesis directly, we incubated trypsinized chicken precartilage mesenchyme cells in heparinase (16) before assaying them for the capacity to undergo MDT. This treatment rendered them incapable of undergoing translocation in this assay (Fig. 4*g*), in contrast to treatment with trypsin alone (Fig. 4*f*), or with chondroitinase ABC (data not shown).

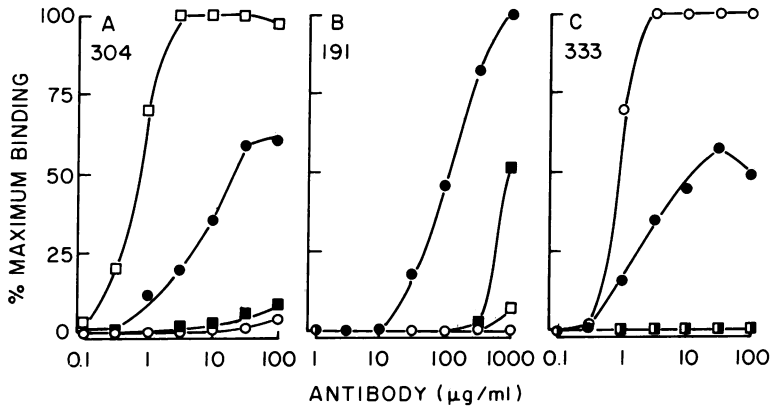

FIG. 2. ELISA assays of the monoclonal antibodies. The specificities of the monoclonal antibodies 304 (*A*), 191 (*B*), and 333 (*C*) are shown as determined by ELISA. Wells of a microtiter plate were coated with intact fibronectin (●) or purified amino-terminal heparin-binding (□), collagen-binding (■), or cell-binding (○) fragments. The concentration of antigen was 4 μg/ml in *A* and *C*, and 8 μg/ml in *B*. The indicated concentrations of the monoclonal antibody were added, and the extent of antibody binding was determined as described. Quantitation was done by measuring the amount of reaction product at 410 nm and normalizing to the maximum reading for the assay.

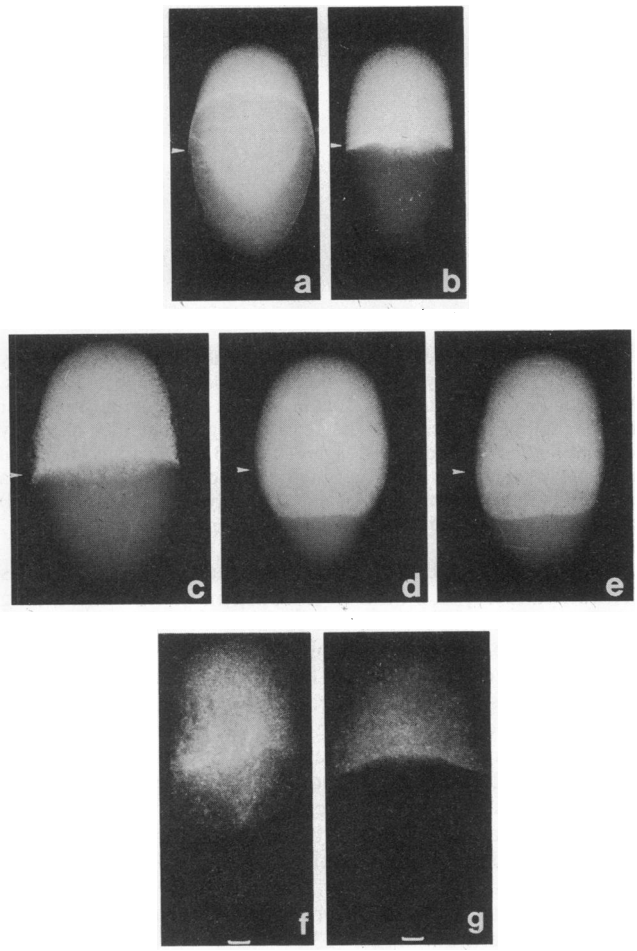

FIG. 4. (*Top*) Competition of heparin and dextran sulfate for fibronectin site required for MDT. Primary gels contained 6-μm polystyrene latex beads, and secondary gels contained human plasma fibronectin at a concentration of 25 μg/ml. The secondary gel contained dextran sulfate (50 μg per ml), derived from dextran of average M_r 500,000 (*a*), heparin (2.5 μg per ml), average M_r 12,000 (*b*). (*Middle*) MDT of 6-μm polystyrene latex beads coated with sulfated glycosaminoglycans. Six-μm polystyrene latex beads were coated with heparin, heparan sulfate, or one of several dextran sulfates (derived from dextrans of M_r 5000, 8000, and 500,000) as described. The primary gel contained 6-μm polystyrene latex beads coated with dextran sulfate (M_r 500,000) (*a*), 6-μm polystyrene latex beads coated with heparin or heparan sulfate, respectively (*d* and *e*). All secondary gels contained 25 μg of human plasma fibronectin per ml. (*Bottom*) Effect of pretreatment with heparinase on MDT of chicken precartilage mesenchyme cells. Primary gels contained 1×10^7 untreated mesenchyme cells per ml (*f*), or mesenchyme cells pretreated with heparinase (*g*). Secondary gels each contained 12.5 μg of human plasma fibronectin per ml.

MDT Depends on a Function of the Amino-Terminal Fibronectin Domain Distinct from Simple Binding of Heparin. The amino-terminal domain of fibronectin has been reported to undergo a conformational change upon binding heparin (24). It was therefore possible that antibody 304, which binds to the amino-terminal domain (Fig. 2) and inhibits MDT (Fig. 3), could do so by a mechanism other than interference with heparin binding. When labeled amino-terminal fragment was tested for its ability to bind to heparin–Sepharose after incubation with antibody 304, it was found to bind as well as unincubated fragment (Table 1). This shows that the MDT-promoting function of the amino-terminal domain of fibronectin that is blocked by antibody 304 is distinct from simple heparin binding.

Table 1. Effect of monoclonal antibodies on heparin-binding of 31-kDa amino-terminal fibronectin fragment

mAb	cpm bound	Mean cpm ± SEM	Rel. binding
None	2480		
	2140	2430 ± 160	1.00 ± 0.07
	2680		
304	2690		
	2050	2410 ± 190	0.99 ± 0.07
	2500		
333	2550		
	2380	2510 ± 60	1.03 ± 0.04
	2590		

Binding of the 31-kDa amino-terminal fibronectin fragment to heparin–Sepharose with and without antifibronectin monoclonal antibodies was assayed in triplicate as described. The preparation of mAb 304 that was used in the heparin-binding assay was then tested in the MDT assay and found to be fully inhibitory.

DISCUSSION

We have shown that the interaction of a site near the amino-terminal end of the fibronectin molecule with components on the surfaces of cells or polystyrene latex beads can promote translocation of cells or beads through an assembling type I collagen matrix. Because this interaction is competed with by exogenous heparin or heparan sulfate but not by fibrin, it seems likely that the interaction takes place at or near the site of heparin/heparan sulfate binding previously identified in this fibronectin domain (18–21). MDT promotes the movement of heparin- and heparan sulfate-coated, but not dextran sulfate-coated beads, and chicken limb mesenchyme cells, but not such cells when they have been incubated with heparinase. This indicates that the presence of heparin-like molecules on the bead or cell surfaces determines their capacity to undergo MDT. The translocation of uncoated polystyrene latex beads is not surprising in light of work demonstrating that commercial polystyrenes contain components that mimic heparin in a number of its protein-binding functions (25, 26).

Heparan sulfate proteoglycans (27, 28) are inserted into the plasma membranes of a variety of cell types (29, 30) and have been shown to mediate certain *in vitro* adhesive responses to fibronectin (31). Such responses are generally different from those mediated by the heparan sulfate-independent cell-surface receptor for fibronectin (31). Correspondingly, MDT is independent of the 140-kDa fibronectin cell-surface receptor system (32–34). The 75-kDa tryptic fragment of human plasma fibronectin, which contains the fibroblastic cell binding site (4), which can bind to cells in suspension (9), and which promotes cell spreading (4, 9), has no MDT-promoting activity. Moreover, intact fibronectin bound to monoclonal antibody 304, which leaves the cell binding site unblocked, does not promote MDT.

Our previous work has shown that MDT occurs with the same kinetics as collagen fibrillogenesis (1). While the presence of a critical concentration of collagen is, indeed, essential for fibronectin-dependent MDT (1), direct binding of fibronectin to collagen does not appear to play a role in this process because the collagen-binding domain of fibronectin is not required for the promotion of MDT. Heparin, at the concentrations used in these experiments, does not detectably affect the rate of collagen fibrillogenesis measured spectrophotometrically (N. S. Jaikaria and S.A.N., unpublished results). We therefore think it unlikely that the inhibitory effect of heparin on MDT is due to a direct effect on collagen.

Because heparin inhibits MDT even in the absence of the collagen-binding domain of fibronectin (i.e., when the 31-kDa fragment alone is used), it is unlikely that it acts through its

capacity to form a ternary complex with fibronectin and collagen (35–37). In any case, highly sulfated dextran sulfates of the types we have used are even more effective than heparin in promoting such complexes (36) but are, nonetheless, incapable of inhibiting MDT, even when the intact fibronectin molecule is present.

The interaction between fibronectin and cell or bead surfaces that promotes MDT appears to involve molecular interactions other than simple fibronectin–heparin binding because the amino-terminal fibronectin domain has only low affinity for heparin in physiological salt concentrations (3). In addition, under conditions permissive for the binding of the 31-kDa amino-terminal fragment to heparin, the MDT-inhibiting antibody 304 did not inhibit this binding in a simple Sepharose-column binding assay. Furthermore, the structurally different (38) heparin-binding site of fibronectin located near the carboxyl terminus of fibronectin is completely ineffective in promoting translocation, despite its much higher affinity for heparin under physiological ionic conditions than has the amino-terminal site (3). Finally, dextran sulfates would be expected to interfere with this interaction if simple binding were involved (22, 23, 39), but dextran sulfates are completely ineffective in blocking MDT. Whereas the heparin–fibronectin interaction that promotes MDT is thus more complex than simple binding, its mechanism remains obscure.

One possible consequence of the interaction of the amino-terminal domain of fibronectin with heparin-like components of the cell or bead surface could be a reduction in interfacial free energy between the particle-containing and fibronectin-containing regions of the two gel systems. If this were to occur, there would be a tendency for these two "quasi-phases" to increase (40) or even maximize (41) their area of mutual contact, and translocation would result. Such an effect would be directly analogous to the variation in capacities of tissues to spread upon one another, as described in the work of Steinberg (42). An alteration in interfacial free energy could arise from a conformational change in the amino-terminal fibronectin domain upon the binding of heparin-like molecules (24); an antibody, such as 304, could potentially block this change without directly interfering with heparin–fibronectin binding. Furthermore, if MDT is based on the thermodynamic properties of interfaces, cell surface–ligand interactions other than those described here could potentially promote analogous effects in other systems.

Promotion of extracellular-matrix assembly is another function that has been attributed to the amino-terminal fibronectin domain (43, 44). The relationship of this function to the promotion of MDT is unclear. While antibody 304 inhibits matrix assembly under certain assay conditions (13), matrix binding mediated by this site is unaffected by exogenous heparin under other conditions (44).

The idea of a force-generating cell microenvironment was suggested in the seminal studies of Bronner-Fraser (45) in which nonmotile cells and polystyrene latex beads were found to be conveyed through cell-free spaces in embryos. MDT represents a process that could plausibly promote such effects in living tissues. If so, the findings reported here imply that the modulation of cell-surface heparan sulfate content will prove to be an important determinant of cell translocatability during morphogenetic changes. Apart from this possibility, the molecular interaction delineated by these studies is likely to be a central feature in the understanding of energy coupling in this model system.

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