N-cadherin Cell-Cell Adhesion Complexes Are Regulated by Fibronectin Matrix Assembly

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Fibronectin is a principal component of the extracellular matrix. Soluble fibronectin molecules are assembled into the extracellular matrix as insoluble, fibrillar strands via a cell-dependent process. In turn, the interaction of cells with the extracellular matrix form of fibronectin stimulates cell functions critical for tissue repair. Cross-talk between cell-cell and cell-extracellular matrix adhesion complexes is essential for the organization of cells into complex, functional tissue during embryonic development and tissue remodeling. Here, we demonstrate that fibronectin matrix assembly affects the organization, composition, and function of N-cadherin-based adherens junctions. Using fibronectin-null mouse embryonic myofibroblasts, we identified a novel quaternary complex composed of N-cadherin, β-catenin, tensin, and actin that exists in the absence of a fibronectin matrix. In the absence of fibronectin, homophilic N-cadherin ligation recruited both tensin and α5β1 integrins into nascent cell-cell adhesions. Initiation of fibronectin matrix assembly disrupted the association of tensin and actin with N-cadherin, released α5β1 integrins and tensin from cell-cell contacts, stimulated N-cadherin reorganization into thin cellular protrusions, and decreased N-cadherin adhesion. Fibronectin matrix assembly has been shown to recruit α5β1 integrins and tensin into fibrillar adhesions. Taken together, these studies suggest that tensin serves as a common cytoskeletal link for integrin- and cadherin-based adhesions and that the translocation of α5β1 integrins from cell-cell contacts into fibrillar adhesions during fibronectin matrix assembly is a novel mechanism by which cell-cell and cell-matrix adhesions are coordinated.

Tissue development, remodeling, and homeostasis are governed by adhesion of cells to extracellular matrix (ECM) and to neighboring cells (1). Tight coordination between integrin-mediated cell-ECM adhesion and cadherin-mediated cell-cell adhesion is required during tissue morphogenesis to facilitate the formation of functional, multicellular structures (1, 2). Cell adhesion to ECM proteins has been shown to affect the expression, localization, composition, and function of cell-cell adhesions (2). However, neither the intracellular nor extracellular mechanisms that serve to coordinate the activities of these two adhesion systems are well understood. The adhesive functions of both integrins and cadherins require interactions with the actin cytoskeleton (3, 4). Further, the intracellular protein complexes that link integrins and cadherins to the actin cytoskeleton share several molecular components, including α-actinin and vinculin (3). As such, the exchange or sequestration of molecular constituents that are common to both adhesion systems provides an attractive mechanism by which cell-ECM adhesion can influence cell-cell adhesion and vice versa.

Cadherins are a family of transmembrane receptors that mediate calcium-dependent cell-cell adhesion by homophilic association of their ectodomains (3). The cadherin receptor associates intracellularly with several structural and signaling proteins, most notably the catenins, forming the adherens junction (A) (3). β-Catenin binds directly to the cadherin cytoplasmic tail and to α-catenin (3). In turn, the interaction of AJ with the actin cytoskeleton is mediated by α-catenin either directly or indirectly and is important for their adhesive function (3). N-cadherin is the predominant cadherin expressed by mesenchymal cells and is found in muscle and connective tissues, as well as in bone and cartilage (5). N-cadherin-dependent AJ play an important role in cell migration, differentiation, embryonic development, and metastasis (5). N-cadherin expression in vascular smooth muscle is up-regulated following arterial injury, whereas blocking N-cadherin function inhibits wound closure in vitro (6). N-cadherin is also required during early heart development (7).

Fibronectin is a modular ECM glycoprotein that plays a critical role in vascular development and angiogenesis (8–10). Soluble protomeric fibronectin circulates in the plasma at a high concentration and is subsequently deposited into the ECM in a fibrillar form by a tightly regulated, cell-dependent process (11). The ECM form of fibronectin stimulates changes in cell growth, migration, and cytoskeletal organization that are distinct from the effects of soluble fibronectin (12–18). Fibronectin has been implicated in regulating the localization, composition, and function of C-, E-, and VE-cadherin-containing AJ (19–21). Abnormal vascular morphogenesis in the absence of fibronectin is thought to result from impaired ves-
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N-cadherin-dependent adhesion also plays a critical role (22, 23). Similar effects on early heart formation are observed when either fibronectin- or N-cadherin-mediated adhesion is disrupted (7, 24, 25).

The current study was undertaken to determine the effect of ECM fibronectin on N-cadherin-based cell-cell contacts. Our data indicate that fibronectin matrix polymerization transiently disrupts the interaction of N-cadherin-containing AJs with the actin cytoskeleton, stimulates the reorganization of cell-cell contacts, and decreases N-cadherin-mediated adhesion. We describe α5β1 integrins and the actin-binding protein tensin as novel components of N-cadherin-based complexes formed in the absence of a fibronectin matrix and as possible targets for regulation of cell-cell adhesion by ECM fibronectin.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Human plasma fibronectin was isolated from Cohn’s fractions I and II (26). NH2-terminal 70- and 40-kDa fibronectin fragments were generated as described previously (15). Type I rat tail collagen was obtained from Upstate (Lake Placid, NY). Recombinant His-tagged functional upstream domain (FUD, also referred to as pUR-4) and the control peptide, Del29 (27) (provided by Dr. Deane Mosher, University of Wisconsin, Madison, WI) were expressed in bacteria and purified on nickel-Sepharose (GE Healthcare). Nonimmune mouse and 9D2 Fab’ fragments were produced as described (15). Antibodies and their sources are as follows: fibronectin monoclonal IgG (9D2) (28) was a gift from Dr. Deane Mosher, University of Wisconsin, Madison, WI; tensin polyclonal IgG was provided by Dr. Su Hao Lo (University of California-Davis); monoclonal N-cadherin (clone 32), monoclonal β-catenin (clone 14), monoclonal α-catenin (clone 5), monoclonal α5 integrin subunit (clone 5H10–27), and monoclonal paxillin (clone 349) IgGs were from BD Biosciences (San Jose, CA); monoclonal α-catenin, monoclonal β-actin (clone AC-15), monoclonal N-cadherin (clone GC-4), polyclonal pan-cadherin, polyclonal fibronectin, monoclonal vinculin (clone VIN 11.5), monoclonal talin (clone 8D4), and nonimmune mouse IgGs were from Sigma-Aldrich; polyclonal focal adhesion kinase, monoclonal tensin (clone 5B9), monoclonal cortactin (clone 4F11), and monoclonal phosphotyrosine (clone 4G10) IgG were from Upstate; monoclonal tensin (H-300) IgG was from Santa Cruz Biotechnology (Santa Cruz, CA); HRP-conjugated anti-mouse and anti-rabbit IgGs were from Bio-Rad; and Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa 488-conjugated phalloidin, and Alexa Fluor 594-conjugated goat anti-rat IgG were from Invitrogen. Tissue culture supplies were from Corning/Costar (Cambridge, MA). Latrunculin B and cytochalasin D were from Calbiochem. Unless otherwise indicated, the chemical reagents were from Sigma.

**Cell Culture**—Fibronectin-null mouse embryonic fibroblasts (FN-null MEFs) (18) were provided by Dr. Jane Sottile (University of Rochester, Rochester, NY). FN-null MEFs were cultured on collagen I-coated dishes under serum-free conditions using a 1:1 mixture of Cellgro® (Mediatech, Herndon, VA) and Aim V (Invitrogen). These media do not require serum supplementation. Thus, no exogenous source of fibronectin is present during routine culture.

Human aortic smooth muscle cells were obtained from Lonza (Walkersville, MD) and maintained in serum-containing smooth muscle cell growth media (Lonza). The experiments were performed using cells between passage 3 and 10. For experiments, the cells were serum-starved overnight in smooth muscle cell basal media and collected under trypsin-free conditions using PBS with 0.5 mM EDTA.

**Immunofluorescence Microscopy**—FN-null MEFs (2 × 10⁴ cell/cm²) were seeded onto 35-mm tissue culture plates precoated with collagen. In some experiments, the cells were prelabeled with CellTracker Green (Invitrogen) for 30 min in suspension, according to the manufacturer’s instructions. Labeled cells were then seeded 1:1 with unlabeled cells. The cells were grown to confluence and treated for various times with either fibronectin (20 μg/ml) or an equal volume of PBS. The cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100 (TX). In some experiments, the cells were extracted with 1% TX prior to fixation. The cells were incubated with primary antibodies followed by fluorophore-conjugated secondary antibodies. The samples were examined using an Olympus BX60 microscope equipped with epifluorescence and photographed with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). To quantify effects of fibronectin on N-cadherin-based contacts, N-cadherin-stained images of CellTracker Green-labeled cells were analyzed using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). For each cell, the length of the cell periphery to which N-cadherin localized was measured and expressed as a percentage of the total cell perimeter. Images of at least 15 cells were analyzed for each condition. The data presented represent the mean values of three independent experiments performed.

**Cell Fractionation, Immunoprecipitation, and Immunoblotting**—Collagen-adherent FN-null cell monolayers were washed with ice-cold PBS and extracted sequentially with 1% TX buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5 mM sodium orthovanadate, containing Complete protease inhibitor mixture (Roche Applied Science) for 10 min, followed by SDS-RIPA buffer (1% TX buffer containing 0.1% SDS and 0.5% deoxycholate) for 15 min. Protein concentrations were determined using a colorimetric biocinchoninic acid assay (Pierce). Unless otherwise indicated, 100 μg of protein were incubated with the specified antibodies bound to protein G-agarose (Upstate Biotechnology). Immunoprecipitates were washed three times with either 1% TX buffer or SDS-RIPA buffer and once with PBS. The proteins were solubilized with reducing sample buffer and analyzed by SDS-PAGE and immunoblotting, as described (15). Immunoblots were developed using enhanced chemiluminescence (Pierce). The blots were stripped, reblocked, and reprobed, as described previously (15).

**Cell Density Experiments**—FN-null MEFs were seeded onto collagen-coated tissue culture dishes (100 mm) at various initial densities to reach different stages of confluence after 48 h in culture. Cell density was monitored by phase contrast microscopy. Subconfluent cells (seeding density, 7.3 × 10⁵ cells/
jugated neutravidin (Pierce).

Sulfo-NHS-biotin (Pierce). The cells were washed three times bound after washing. The data presented are representative of the total number of microbeads added to cells remained with a hemacytometer. Under control conditions, the number of beads bound to each well was determined then treated with 0.25% trypsin to release the microbeads.

To determine whether cell-cell contacts of FN-null MEFs were similarly resistant to nonionic detergents, confluent monolayers treated with either fibronectin or the vehicle control, PBS, for an additional 2 h. Cell area was quantified as 100 cm²) and long confluent cells (seeding density, 1.8 × 10⁴ cells/cm²) were characterized by limited cell spreading and the absence of gaps.

Preparation of Ncad-Fc Substrates—Tissue culture dishes (35 mm) were coated with 50 μg/ml protein A in PBS followed by a recombinant chimeric protein, consisting of the extracellular domain of human N-cadherin fused to the Fc region of human IgG (Ncad-Fc; R & D Systems, Minneapolis, MN) at 10 μg/ml in PBS. FN-null MEFs were collected under trypsin-free conditions by washing and then incubating cells for 5 min with 10 mM EDTA in PBS with 2% BSA. Rounded cells were mechanically dislodged, centrifuged, and washed once with AIm V/Cellgro. The cells were seeded at 1.5 × 10⁴ cells/cm², allowed to adhere for 1 h, and then processed for immunofluorescence microscopy. In some experiments, adherent cells were treated with either fibronectin or the vehicle control, PBS, for an additional 2 h. Cell area was quantified as described previously (13). Briefly, phase contrast images of fixed cells from triplicate wells were obtained using a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). The areas of at least 20 randomly chosen cells/condition were determined using ImagePro-Plus software (Media Cybernetics, Silver Spring, MD) calibrated with a stage micrometer.

Ncad-Fc Bead-Cell Adhesion Assays—Recombinant Ncad-Fc was coated onto protein A-coupled microbeads (4-μm mean diameter; Spherotech, Lake Forest, IL) at 5 μg/ml. Ncad-Fc microbeads were washed and resuspended in DMEM at 2 × 10⁶ beads/ml. Collagen-adherent FN-null MEFs were grown to confluence on 96-well tissue culture plates and then treated with fibronectin (20 μg/ml) for 6 h in the presence of either anti-fibronectin 9D2 Fab’ or nonimmune mouse Fab’ (15 μg/ml). Control wells received an equal volume of PBS. To assess the specificity of Ncad-Fc microbead binding to cells, aliquots of microbeads were treated either with the anti-N-cadherin antibody, GC-4, or with nonimmune IgG (44 μg/ml) for 10 min prior to their addition to cells. 100 μl of the Ncad-Fc microbead suspension were added to each well, and microbeads were allowed to adhere for 20 min. Wells were washed three times with DMEM and then treated with 0.25% trypsin to release the microbeads. The number of beads bound to each well was determined with a hemacytometer. Under control conditions, ~20% of the total number of microbeads added to cells remained bound after washing. The data presented are representative of two independent experiments performed in quadruplicate.

Biotin Labeling of Cell Surface Proteins—Confluent FN-null cell monolayers were incubated for 30 min with 0.5 mg/ml Sulfo-NHS-biotin (Pierce). The cells were washed three times with PBS and extracted with SDS-RIPA buffer. The cell surface proteins were detected on immunoblots with HRP-conjugated neutravidin (Pierce).

Statistical Analysis—Comparisons between two groups were made using the unpaired Student’s t test, and among groups by one-way analysis of variance followed by Tukey’s post-test, with GraphPad Prism Version 4 software (La Jolla, CA). A p value of 0.05 was taken as the threshold for statistical significance. The images shown are representative of at least three independent experiments performed.

RESULTS

For this study, we utilized FN-null MEFs, which do not express fibronectin and have been adapted to grow under serum-free conditions (18). FN-null MEFs assemble exogenously added fibronectin into ECM fibrils via mechanisms utilized by fibronectin-expressing cells, including fibroblasts and smooth muscle cells (11, 18). Thus, FN-null MEFs are an ideal model to characterize cell behavior in the complete absence of fibronectin and to distinguish the effects of soluble fibronectin from ECM fibronectin. The FN-null MEFs used in this study express N-cadherin, but not E- or VE-cadherin (not shown).

Fibronectin Induces Cell Surface Reorganization of N-cadherin—Fibroblasts adhere to each other at the tips of thin cellular extensions, where AJs proteins concentrate (29). To determine the effect of fibronectin on the organizational pattern of N-cadherin-dependent AJs, N-cadherin and β-catenin were immunolocalized in confluent FN-null cell monolayers treated with either fibronectin or the vehicle control, PBS. In the absence of fibronectin, N-cadherin and β-catenin localized to cell-cell contacts at the cell periphery (Fig. 1A). In contrast, the addition of fibronectin to cell monolayers triggered a reorganization of both N-cadherin and β-catenin on cell surfaces (Fig. 1A).

To further characterize the reorganization of N-cadherin in response to fibronectin, a whole cell fluorescent label was used in conjunction with N-cadherin staining to delineate cell borders and thus clearly identify cell-cell contacts. As shown in Fig. 1B, N-cadherin was concentrated along areas of cell-cell contact in confluent monolayers devoid of fibronectin. The reorganization of N-cadherin away from cell borders in response to fibronectin was accompanied by the appearance of thin cellular extensions that contained N-cadherin (Fig. 1B, arrowheads). Quantitative analysis of this reorganization indicates that N-cadherin-based cell-cell contacts were found along ~45% of the cell periphery in the absence of fibronectin (Fig. 1C). The addition of fibronectin significantly decreased the peripheral localization of N-cadherin within cell-cell contacts to ~18% (Fig. 1C). These data indicate that N-cadherin localizes to cell-cell contacts at discrete cell borders in FN-null cell monolayers in the absence of fibronectin. The addition of fibronectin decreases the fraction of the cell periphery containing AJs and promotes the extension of N-cadherin-containing cellular processes.

Fibronectin Alters the Molecular Composition of N-cadherin Complexes and Transiently Disrupts the Association of N-cadherin with Actin—Protein complexes associated with the actin cytoskeleton, including AJs, are resistant to solubilization with nonionic detergents, such as Triton X-100 (30, 31). To determine whether cell-cell contacts of FN-null MEFs were similarly resistant to nonionic detergents, confluent monolayers were extracted with either 1% TX alone or 1% TX plus
N-cadherin staining was observed in cell-cell contacts in nonextracted and TX-extracted FN-null cell monolayers. In contrast, N-cadherin staining was lost from cells extracted with SDS (Fig. 2A). These data provide evidence that N-cadherin-based AJs of FN-null cell monolayers are associated with the actin cytoskeleton and are resistant to solubilization with nonionic detergents.

To analyze the molecular composition of N-cadherin-based AJ complexes, FN-null cell monolayers were sequentially extracted with 1% TX followed by SDS-RIPA buffer. N-cadherin was immunoprecipitated from both TX-soluble and TX-insoluble fractions. Proteins associated with N-cadherin were subsequently identified by immunoblotting. As expected (3), β-catenin co-precipitated with N-cadherin in both TX-soluble and TX-insoluble fractions (Fig. 2B). α-Catenin co-precipitated with N-cadherin primarily in the TX-soluble fraction; low levels of N-cadherin-association α-catenin were detected in the TX-insoluble fraction (Fig. 2B). The association of N-cadherin with α- or β-catenin was not affected by fibronectin treatment (Fig. 2B). In contrast, actin co-precipitated with N-cadherin within the cytoskeletal fraction only in the absence and not the presence of fibronectin (Fig. 2B). In the absence of fibronectin, N-cadherin immunoprecipitates of the TX-insoluble fraction also contained a protein with an approximate molecular mass of 200 kDa that was first observed as a heavy band on immunoblots stained with Ponceau S (not shown). We subsequently identified this protein as tensin (Fig. 2B), an actin-binding protein typically found in fibronectin-containing fibrillar adhesions (32, 33). The binding of tensin to the N-cadherin complex was specific, because other actin-binding proteins, including talin, vinculin, cortactin (Fig. 2B), and α-actinin (not shown) did not co-precipitate with N-cadherin. As with actin, tensin associated with N-cadherin only in the absence and not the presence of fibronectin (Fig. 2B). The loss of actin and tensin from N-cadherin immunoprecipitates in response to fibronectin was not due to a decrease in N-cadherin expression, because a small but reproducible increase (40.7 ± 3.7%) in the amount of N-cadherin immunoprecipitated from fibronectin-treated cell lysates was
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FIGURE 2. Effects of fibronectin on the molecular composition of N-cadherin complexes. A, untreated FN-null cell monolayers either were not extracted or were extracted with TX buffer (1% TX-extracted) or SDS-RIPA buffer (SDS-extracted) before fixation. The cells were labeled with N-cadherin mAb followed by fluorescein-conjugated anti-mouse IgG. Scale bar, 10 μm. B, collagen-adherent FN-null cell monolayers were treated with 40 nM (20 μg/ml) fibronectin (+) or an equal volume of PBS (−) for 24 h. The cells were sequentially extracted with 1% TX buffer (TX-soluble) followed by SDS-RIPA buffer (TX-insoluble). The proteins were immunoprecipitated (IP) from both fractions using an anti-N-cadherin mAb. Immunoblots (IB) were probed with antibodies to N-cadherin, β-actin, tensin, α-catenin, ε-catenin, vinculin, cortactin, and talin. C and D, FN-null cell monolayers were treated with fibronectin (40 nM; FN) or an equal volume of PBS for 0.5, 2, and 6 h (C), or 9, 27, 48, and 72 h (D). N-cadherin was immunoprecipitated from TX-insoluble fractions, and immunoprecipitates were probed for N-cadherin, β-actin, tensin, and cortactin.

routinely observed compared with untreated controls (Fig. 2B).

Time course studies show that partial dissociation of actin from N-cadherin complexes occurred within 30 min of fibronectin addition (Fig. 2C). Actin was completely dissociated from N-cadherin after 2 and 6 h of treatment with fibronectin, but not after 6 h of treatment with the vehicle control, PBS (Fig. 2C). The loss of actin from N-cadherin complexes in response to fibronectin was transient, because actin was again detected in N-cadherin immunoprecipitates 2 and 3 days after fibronectin addition (Fig. 2D). In contrast, tensin did not reassociate with N-cadherin. Instead, cortactin bound to N-cadherin complexes with prolonged fibronectin treatment (Fig. 2D). Thus, fibronectin causes a transient release of actin from N-cadherin complexes, alters the composition of actin-binding proteins associated with N-cadherin.

To further assess the interactions of tensin and actin with N-cadherin complexes, FN-null cell monolayers were treated with inhibitors of actin polymerization for 30 min prior to fractionation. Tensin and actin were clearly detected in N-cadherin immunoprecipitates following treatment with the vehicle control, Me2SO (supplemental Fig. S1A). In contrast, the addition of either cytochalasin D or latrunculin B disrupted the association of tensin and actin with N-cadherin (supplemental Fig. S1A). The interaction of tensin and actin with N-cadherin was also dependent on cell density, because N-cadherin immunoprecipitates from newly confluent cells contained greater levels of tensin and actin than immunoprecipitates from cells that had been confluent for more than 20 h (supplemental Fig. S1B). Together, these data indicate that tensin associates with N-cadherin complexes in an actin-dependent manner and that the association of tensin and actin with N-cadherin is dependent on the duration of cell-cell interactions.

Biological Characterization of Tensin-N-cadherin-β-Catenin Complexes—To further characterize the composition of N-cadherin complexes formed in the absence of fibronectin, immunoprecipitations of TX-insoluble fractions were performed using antibodies directed against tensin, β-catenin, or α-catenin. Tensin immunoprecipitates contained both N-cadherin and β-catenin, but not the focal contact protein, paxillin (Fig. 3A). β-Catenin immunoprecipitates contained both tensin and N-cadherin (Fig. 3B), confirming that tensin binds specifically to N-cadherin-β-catenin complexes. Similar to results obtained using N-cadherin antibodies (Fig. 2B), the associations of tensin and actin with β-catenin immunocomplexes were disrupted by 100 nM fibronectin (Fig. 3B). This disassociation of actin and tensin from β-catenin complexes in response to fibronectin was not associated with changes in the levels of β-catenin tyrosine phosphorylation (Fig. 3B).
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FIGURE 3. Tensin and actin immunoprecipitate with N-cadherin and β-catenin but not α-catenin. A, collagen-adherent FN-null cell monolayers were left untreated (A and C–E) or treated for 18 h with 10 or 100 nM fibronectin (FN) or an equal volume of PBS (B). The cells were fractionated as described in the legend to Fig. 2. The proteins were immunoprecipitated (IP) from TX-insoluble fractions using anti-tensin polyclonal antibodies (A), anti-β-catenin mAb (B), anti-α-catenin pAb (C), or anti-N-cadherin mAb (C, E). In D, TX-insoluble fractions (150 μg) were immunoprecipitated sequentially five times using α-catenin polyclonal antibodies. The α-catenin-depleted lysate was then immunoprecipitated with N-cadherin mAbs. In E, N-cadherin or nonimmune IgG immunoprecipitates were washed with SDS-RIPA buffer containing 0.1, 0.5, 1.5, or 2.5% SDS. Immunoblots (IB) were probed for tensin, β-actin, N-cadherin, phosphotyrosine, β-catenin, paxillin, α-catenin, and tensin.

together, these data suggest that biochemically distinct N-cadherin-based complexes may exist. One complex contains the traditional components, N-cadherin, β-catenin, and α-catenin, and is associated primarily with the TX-soluble (noncytoskeletal) fraction. A second, novel complex is associated with the cytoskeletal fraction and contains N-cadherin, β-catenin, tensin, and actin.

The stoichiometry and relative position of α- and β-catenin within AJs complexes have been analyzed by observing their association with cadherin precipitates under increasingly stringent conditions (34). To determine the hierarchy of protein-protein interactions within FN-null cell AJs, N-cadherin immunoprecipitates from untreated FN-null cell monolayers were washed with increasing concentrations of SDS and then subjected to immunoblot analysis. N-cadherin immunoprecipitates specifically contained β-catenin, as well as α-catenin and tensin, when washed with 0.1% SDS (Fig. 3E). Increasing the concentration of SDS to 0.5% resulted in a complex that contained N-cadherin and β-catenin but not tensin or α-catenin (Fig. 3E). β-Catenin was not observed in N-cadherin precipitates exposed to 1.5% SDS or above (Fig. 3E).

Tensin and α5β1 Integrins Co-localize with N-cadherin at Nascent Cell-Cell Adhesions—Although tensin typically co-localizes with fibronectin and α5β1 integrins in matrix adhesions (32, 33), others have reported tensin in cell-cell adhesions (35, 36). Our data indicate that tensin associates with N-cadherin in the absence of fibronectin. To visualize the fraction of tensin that associates specifically with N-cadherin at sites of cell-cell contact, FN-null MEFs were seeded onto substrates precoated with a recombinant chimeric protein consisting of the five extracellular N-cadherin modules fused to the Fc portion of human IgG (Ncad-Fc). FN-null MEFs were allowed to adhere and spread on Ncad-Fc-coated surfaces for 1 h. Previous studies have shown that cells adhere to Ncad-Fc surfaces and organize N-cadherin and other AJ proteins into radial “cadherin adhesions” reminiscent of focal adhesion staining of cell-ECM contacts (37). As observed previously (37), most cells on the Ncad-Fc substrate were egg-shaped and extended broad lamellipodia (Fig. 4). Adhesion of cell surface cadherins at the edge of lamellipodia to substrate-bound Ncad-Fc is thought to represent nascent cell-cell adhesions where actin nucleation occurs (37). In agreement, contact co-localized with α-catenin at the cell periphery (Fig. 4A, arrow) but not in radial cadherin adhesions (Fig. 4A, arrowhead). Tensin co-localized with N-cadherin primarily at the leading edge of spreading cells, with some staining observed in radial cadherin adhesions (Fig. 4B). Likewise, α5 integrin staining overlapped with N-cadherin staining at the cell periphery (Fig. 4B). In contrast, focal adhesion kinase did not co-localize with N-cadherin in either structure (Fig. 4B).

In the absence of fibronectin, F-actin was organized into both radial cadherin adhesions and circumferential bands at the periphery of N-cadherin-adherent cells (Fig. 5A, PBS). The addition of fibronectin to cells adherent to N-cadherin led to a decrease in actin organization at N-cadherin arrays and within the circumferential bands (Fig. 5A, FN). Quantitative analysis of cell area in the absence and presence of fibronectin indicates that the addition of fibronectin to N-cadherin-bound cells significantly decreased cell area (Fig. 5B), providing additional evidence that fibronectin disrupts N-cadherin adhesion by altering cytoskeletal organization.

Fibronectin Disrupts the Co-localization of α5β1 Integrins with N-cadherin—To examine further the cell surface distribution of α5β1 integrins with respect to AJs, in both the absence and presence of fibronectin, N-cadherin and α5 integrin subunit expression in collagen-adherent FN-cell monolayers was assessed. In the absence of fibronectin, α5β1 integrin co-localized with N-cadherin at regions of cell-cell contact (Fig. 6). We were unable to co-precipitate α5β1 integrins and N-
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Fibronectin Matrix Assembly Is Required for the Release of Actin and Tensin from N-cadherin Complexes—The accumulation of tensin in fibrillar adhesions is dependent on fibronectin matrix polymerization (38), suggesting the possibility that the active process of assembling a fibronectin matrix may initiate the release of tensin from AJs. To determine whether fibronectin matrix polymerization is required to induce the dissociation of tensin and actin from N-cadherin, we used two different reagents, 9D2 mAb and 70-kDa fibronectin fragments, to block fibronectin matrix polymerization. The 9D2 mAb blocks fibronectin matrix polymerization by binding to the first type III repeat of fibronectin and blocking multimer formation (28). The 70-kDa NH₂-terminal fragment of fibronectin inhibits fibronectin matrix assembly by blocking the initial binding of soluble fibronectin to cell surfaces (39, 40). Confluent FN-null cell monolayers were treated with fibronectin in the presence of either 9D2 or nonimmune IgG Fab’ fragments and in the presence of 70-kDa or control, 40-kDa amino-terminal fragments of fibronectin. N-cadherin was immunoprecipitated from TX-insoluble fractions, and precipitates were analyzed by immunoblotting. Similar to results shown in Fig. 2B, actin and tensin dissociated from N-cadherin in response to fibronectin when cells were treated with the control proteins, nonimmune IgG Fab’ fragments (Fig. 7A) and 40-kDa fibronectin fragments (Fig. 7B). In contrast, both actin and tensin remained associated with N-cadherin in the presence of fibronectin when fibronectin matrix polymerization was blocked with either 9D2 Fab’ fragments (Fig. 7A) or 70-kDa fragments (Fig. 7B). Neither 70- nor 40-kDa fragments had any affect on the association of tensin or actin with N-cadherin in the absence of fibronectin (Fig. 7B). Inhibiting fibronectin matrix polymerization with 9D2 Fab’ fragments also blocked the cell surface reorganization of N-cadherin in response to fibronectin (Fig. 7C). These data indicate that fibronectin matrix assembly is required for the dissociation of tensin and actin from N-cadherin and the rearrangement of N-cadherin molecules on cell surfaces.

To investigate the effects of fibronectin matrix assembly on the localization of tensin to N-cadherin-contacts in fibronectin-producing cells, human aortic smooth muscle cells were allowed to adhere to N-cadherin-coated surfaces in the absence and presence of the inhibitory peptide, FUD. FUD pep-
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FIGURE 6. Fibronectin disrupts \( \alpha_5\beta_1 \) integrin/N-cadherin co-localization. Collagen-adherent FN-null cells were grown to confluence and then treated with 40 nm fibronectin (FN) or an equal volume of PBS for 24 h. The cells were fixed, permeabilized, and probed with antibodies to N-cadherin and \( \alpha_5 \) integrin subunits, followed by Alexa 488- and Alexa 594-conjugated secondary antibodies. Scale bar, 10 \( \mu \)m.

FIGURE 7. Fibronectin matrix assembly is required for the release of actin and tensin from N-cadherin. FN-null cells were grown to confluence and treated for 4 h with either PBS or 20 \( \mu \)g/ml fibronectin (FN) in the presence of 10 \( \mu \)g/ml 9D2 mAb Fab\(^{\prime}\) or nonimmune mouse IgG Fab\(^{\prime}\) (A and C) or 50 \( \mu \)g/ml 70-kDa fibronectin fragments or an equimolar concentration of 40-kDa fibronectin fragments (B). In B, some wells received 70- or 40-kDa fragments alone. A and B, N-cadherin was immunoprecipitated from TX-insoluble fractions with an anti-N-cadherin mAb. Immunoprecipitates were probed with antibodies to N-cadherin, tensin, and \( \beta \)-actin. C, cells were processed for immunofluorescence and co-stained for N-cadherin and fibronectin. IP, immunoprecipitates; IB, immunoblot.

tides block polymerization of both endogenous and exogenous fibronectin by binding to the NH\(_2\) terminus of fibronectin (27). When aortic smooth muscle cells were treated with FUD peptides to block endogenous fibronectin matrix assembly, tensin was detected in long fibrillar N-cadherin-based adhesions (Fig. 8, arrows, + FUD). In contrast, only punctate tensin staining was detected on N-cadherin-adherent cells treated with 80 nm fibronectin (Fig. 8, + FN). Fibrillar tensin was again detected in N-cadherin contacts when FUD peptides were added to fibronectin-treated cells to block both endogenous and exogenous fibronectin matrix assembly (Fig. 8, arrows, + FN/FUD). Fibrillar tensin staining was absent from cells treated with fibronectin and the non-blocking peptide, Del29 (27), demonstrating specificity of the FUD peptide (Fig. 8, + FN/ Del29). These data confirm earlier reports (36) that tensin can localize to cell-cell adhesions of smooth muscle cells and further indicate that the loss of tensin from N-cadherin contacts in response to fibronectin requires fibronectin matrix polymerization.

Fibronectin Matrix Assembly Decreases N-cadherin-mediated Adhesion— Strengthening of cadherin-mediated cell-cell adhesion requires an intact actin cytoskeleton, suggesting that the association of cadherins with actin controls the strength of cell-cell adhesions (41, 42). Our data indicate that fibronectin matrix polymerization triggers the dissociation of actin from N-cadherin-\( \beta \)-catenin complexes and decreases the area of cells spread on an N-cadherin substrate, implying that fibronectin regulates cell-cell adhesion strength by controlling the interaction of N-cadherin complexes with the actin cytoskeleton. To determine whether fibronectin matrix polymerization affects N-cadherin-mediated cell-cell adhesion, adhesion of Ncad-Fc-coated microbeads to FN-null MEFs was measured. FN-null cell monolayers were treated with fibronectin in the absence and presence of 9D2 Fab\(^{\prime}\) fragments, and Ncad-Fc microbeads were allowed to bind to the cell surface. The addition of fibronectin caused a significant decrease in the adhesion of Ncad-Fc-coated beads to cell monolayers (Fig. 9A). Moreover, blocking fibronectin matrix polymerization with 9D2 Fab\(^{\prime}\) fragments abrogated the inhibitory effect of fibronectin on Ncad-Fc bead-cell adhesion (Fig. 9A). The addition of the N-cadherin function-blocking mAb, GC-4 (43), inhibited Ncad-Fc-bead binding to a similar extent as did
fibronectin (Fig. 9A). These data indicate that fibronectin matrix polymerization inhibits the adhesion of Ncad-Fc microbeads to FN-null MEFs in an N-cadherin-dependent manner. Fibronectin treatment did not affect the cell surface expression of N-cadherin (Fig. 9B). Thus, the fibronectin-induced decrease in N-cadherin adhesion was not due to decreased levels of N-cadherin on cell surfaces and hence was likely due to a decrease in the strength of N-cadherin adhesion.

DISCUSSION

We have identified α5β1 integrins and the actin-binding protein, tensin, as novel components of N-cadherin-adhesion complexes. Fibronectin induced the dissociation of both tensin and actin from N-cadherin–β-catenin complexes and stimulated the reorganization of N-cadherin from the cell periphery into thin cellular processes. Both α5β1 integrins, the major integrin receptor for fibronectin in fibroblasts (44), and tensin co-localized with N-cadherin in cell–cell contacts in the absence but not the presence of fibronectin. Further, fibronectin decreased the area of cells spread on an N-cadherin substrate and decreased the binding of N-cadherin-coated beads to cells without altering the cell surface expression of N-cadherin, suggesting that fibronectin decreases the strength of N-cadherin-mediated adhesion. The dissociation of actin and tensin from N-cadherin, the reorganization of N-cadherin on cell surfaces, and the decrease in N-cadherin-mediated adhesion in response to fibronectin were dependent on fibronectin matrix assembly. These data provide evidence that fibronectin matrix deposition affects the organization and function of N-cadherin-based cell–cell adhesions by regulating the interaction of N-cadherin protein complexes with the actin cytoskeleton.

Previous studies have localized tensin to cell–cell contacts in epithelial (35) and smooth muscle cell monolayers (36). Tensin has also been observed in the intercalated discs of cardiac muscle (35), where N-cadherin plays a critical role in myocyte cell–cell adhesion (45). In connective tissues, cell–cell adhesions formed by closely appositioned fibroblasts provide a conduit for cell–cell communication (46) and a structural network that mechanically couples cells subjected to mechanical loads (47). The pattern of N-cadherin-based cell–cell contacts formed in FN-null MEF monolayers lacking fibronectin is similar to that described by Yonemura et al. (29) in nonpolarized fibroblasts. Our data also demonstrate that fibronectin stimulates the extension of long, thin cellular processes that contain N-cadherin. Similar N-cadherin-containing extensions have been observed in Schwann cells co-cultured with dorsal root ganglia neurons (48). Upon contact with neurons, Schwann cells extended N-cadherin-containing cellular processes along axons that were perturbed by antibodies or peptides against N-cadherin (48). Cadherins can form transient bonds on the order of 1 s (49). Fibronectin-directed formation of transient, cadherin-mediated adhesions would allow for rapid extension and retraction of actin-enriched filopodia that would enable a cell to quickly sense its mechanical environment or detect ligands on nearby cells or matrices.

The precise role of tensin in N-cadherin-based adhesions remains to be elucidated. The dissociation of tensin from N-cadherin complexes in response to fibronectin occurred in parallel with the loss of actin and the decrease in N-cadherin-mediated adhesion. Furthermore, we provide preliminary evidence that tensin and α-catenin may reside in distinct N-cadherin complexes. Given the ability of tensin to bind to actin and the known sequence homology of tensin and α-catenin (50), we postulate that tensin stably links N-cadherin–β-catenin complexes to actin filaments and thus mediates strong intercellular adhesion. This concept is supported by an earlier study that found that vinculin, another actin-binding adaptor protein with homology to α-catenin, associates with E-cadherin–β-catenin complexes in cell lines deficient in α-catenin (51). Enhanced binding of vinculin to E-cadherin correlated with an increase in cell aggregation (51).

Recently, the view that cadherins are stably linked to the actin cytoskeleton through the catenins and other adaptor proteins has come into question. Despite biochemical and genetic data supporting this idea, an intact complex of proteins from cadherin to actin has yet to be isolated (52). Moreover, the turnover rate of actin at sites of cell–cell contact is significantly higher than that of the cadherin receptor or the catenins (53). However, the importance of the actin cytoskeleton in the formation and maintenance of AJs is well established (54). Here, we show that actin co-immunoprecipitates with N-cadherin, indicating that a stable protein complex containing both N-cadherin and actin exists. Although these
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FIGURE 10. Proposed model for the role of fibronectin in N-cadherin AJ dynamics. Distinct N-cadherin-based AJ complexes co-exist in the absence of active fibronectin matrix assembly. One complex contains N-cadherin, β-catenin, and α-catenin and likely associates indirectly with the actin cytoskeleton. A second N-cadherin-β-catenin AJ complex contains tensin and is stably linked to actin filaments. In the absence of fibronectin, unligated α5β1 integrins localize to cell-cell adhesions. Ligation of α5β1 integrins by fibronectin (A, (i)) and initiation of fibronectin matrix assembly (B, (iii)) disrupt the association of tensin with N-cadherin, promote the association of tensin with α5β1 integrins, and trigger the translocation of α5β1 integrins and tensin away from cell-cell contacts and into fibrillar ECM adhesions (B, (iii)). This sequestration of tensin by α5β1 integrins may release N-cadherin-β-catenin complexes from actin, transiently disrupting cell-cell adhesion and permitting the reorganization of N-cadherin into cell extensions.

As with the fibronectin-induced effects on AJ composition, the decrease in cell-cell adhesion in response to fibronectin was dependent upon its polymerization into the ECM. Strengthening of cadherin-mediated adhesion is dependent upon an intact actin cytoskeleton (41) and is regulated by proteins that control actin organization (57). Thus, these data suggest that the decrease in N-cadherin-mediated adhesion in response to fibronectin results from changes in the interaction of AJs with actin. However, it is unlikely that fibronectin eliminates the interaction of N-cadherin with the actin cytoskeleton. Indeed, N-cadherin still partitioned to the TX-insoluble fraction of proteins in the presence of fibronectin. The changes in the association of actin and actin-binding proteins with N-cadherin precipitates observed in our biochemical analyses may represent a switch to a less stable association between AJs and the cytoskeleton that may give rise to more transient or dynamic cell-cell adhesions. Several potential binding surfaces for actin have been identified within AJs, some of which likely involve lower binding affinities (57). Whether the fibronectin matrix assembly affects N-cadherin function directly through changes in cadherin signaling or indirectly through integrin-mediated changes in cytoskeletal signaling and/or organization remains to be determined.

The mechanisms that serve to coordinate cell-cell and cell-matrix adhesions are not well understood. Formation of α5β1 integrin-containing fibrillar adhesions requires fibronectin matrix assembly (38) and occurs via a tensin-dependent mechanism (58). The concomitant release of α5β1 integrins from cell-cell adhesions in response to fibronectin and the ability of tensin to bind directly to α5β1 integrins (59) suggest that physical associations among fibronectin fibrils, α5β1 integrins, and tensin may facilitate the movement of α5β1 integrins and tensin away from cell-cell adhesions and into fibrillar adhesions (Fig. 10). Fibronectin may bind directly to α5β1 integrins within cell-cell adhesions to trigger their translocation or alternatively, initiate the release of unligated integrins from cell-cell adhesions to cell-matrix adhesion indirectly via changes in intracellular signaling. Others have demonstrated inactive integrins, including α3β1 and αvβ3, at sites of cell-cell contact (60, 61). Hence, binding of soluble fibronectin to unligated α5β1 integrins within AJs may initiate the activation and directed movement of integrin receptors from cell-cell to cell-matrix adhesions. In support of this hypothesis, cadherin-dependent adhesion supports fibronectin fibril assembly during Xenopus embryogenesis (62). Further, pericellular fibronectin staining that co-localizes with C-cadherin is observed during gastrulation in Xenopus embryos; blocking fibronectin matrix polymerization disrupts gastrulation (63).

The mechanism by which fibronectin releases α5β1 integrins from cell-cell contacts is not known, nor is it known...
how α5β1 integrins are retained within cell-cell contacts in the absence of fibronectin. Possible signaling mechanisms downstream of fibronectin matrix assembly that may trigger integrin release include Src kinase activation and/or Rho family GTPase remodeling of the actin cytoskeleton (64). Activation of Src family kinases has been shown to play a role in the disruption of VE- and E-cadherin-mediated cell-cell junctions (21, 65). Local down-regulation of Rac activity by focal adhesion kinase is required for the maintenance of N-cadherin-mediated cell-cell contacts (66).

In summary, we have shown that fibronectin matrix assembly affects the organization and function of N-cadherin-based cell-cell adhesions. This study provides a mechanistic link between the formation of matrix adhesions and the disruption of cell-cell adhesion. Coordination between cell-ECM and cell-cell adhesion is critical for embryonic development and tissue remodeling (1, 63). The molecular composition and physical state of the ECM varies widely in the body. Likewise, the density of the fibronectin matrix varies in vivo. Branching morphogenesis of salivary glands is dependent on fibronectin and is associated with transient, local expression of fibronectin during cleft formation (20). As such, controlling the rate fibronectin matrix assembly or the physical state of the fibronectin matrix with novel therapeutics may provide a means to enhance tissue regeneration by stimulating key morphogenetic processes.

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