Cortactin associates with N-cadherin adhesions and mediates intercellular adhesion strengthening in fibroblasts

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Summary

The regulation of N-cadherin-mediated intercellular adhesion strength in fibroblasts is poorly characterized; this is due, in part, to a lack of available quantitative models. We used a recombinant N-cadherin chimeric protein and a Rat 2 fibroblast, donor-acceptor cell model, to study the importance of cortical actin filaments and cortactin in the strengthening of N-cadherin adhesions. In wash-off assays, cytochalasin D (1 µM) reduced intercellular adhesion by threefold, confirming the importance of cortical actin filaments in strengthening of N-cadherin-mediated adhesions. Cortactin, an actin filament binding protein, spatially colocalized to, and directly associated with, nascent N-cadherin adhesion complexes. Transfection of Rat-2 cells with cortactin-specific, RNAi oligonucleotides reduced cortactin protein by 85% and intercellular adhesion by twofold compared with controls (P<0.005) using the donor-acceptor model. Cells with reduced cortactin exhibited threefold less N-cadherin-mediated intercellular adhesion strength compared with controls in wash-off assays using N-cadherin-coated beads. Immunoprecipitation and immunoblotting showed that N-cadherin-associated cortactin was phosphorylated on tyrosine residue 421 after intercellular adhesion. While tyrosine phosphorylation of cortactin was not required for recruitment to N-cadherin adhesions it was necessary for cadherin-mediated intercellular adhesion strength. Thus cortactin, and phosphorylation of its tyrosine residues, are important for N-cadherin-mediated intercellular adhesion strength.

Key words: Cortactin, Cadherin, Actin, Adhesion strength

Introduction

Characterization of the proteins that regulate cadherin-dependent adhesion strength is central to the understanding of cell sorting events such as epithelial-mesenchymal transitions in embryogenesis, wound healing, tumorigenesis and metastasis, as well as to the maintenance of mature tissue architecture (Barth et al., 1997; Ben-Ze’ev and Geiger, 1998; Huber et al., 1996; Steinberg and McNutt, 1999; Takeichi, 1995; Yap et al., 1997a). Cadherins are a large, well-conserved family of calcium-dependent, single-pass transmembrane glycoproteins. N-cadherin (A-CAM) is the major cadherin of mesenchymal tissues and is thought to play a role in regulating cell attachment and migration during development (Akitaya and Bronner-Fraser, 1992; Bronner-Fraser et al., 1992; Duband et al., 1988), as well as mediating tissue organization and cell differentiation in muscle (Charrasse et al., 2002; Goichberg and Geiger, 1998; Redfield et al., 1997), cartilage (Haas and Tuan, 1999; Woodward and Tuan, 1999) and bone (Civitelli, 1995; Marie, 2002). Currently, the regulation of N-cadherin-dependent junctions in connective tissue cells is poorly defined.

N-cadherin is a member of the classical (or type I) cadherin family. These molecules are characterized by a highly conserved cytoplasmic tail to which are bound β, α and p120 catenins. α-Catenin is a linker protein that mediates the association of cadherins with the actin cytoskeleton. The insertion of cortical actin filament into cadherin adhesion complexes is thought to be crucial for cadherin-mediated adhesion strengthening and the subsequent formation of adherens junctions (Breen et al., 1993; Roe et al., 1998; Vasioukhin et al., 2000). Indeed, the anchorage of cadherins to the actin cytoskeleton is rate-limiting for epithelial cell adhesion (Vaezi et al., 2002). Sites of cadherin-mediated intercellular adhesion are also regions of rapid actin filament assembly and re-organization [our observations and (Kovacs et al., 2002b; Vasioukhin et al., 2000)], events that are mediated by actin binding proteins (dos Remedios et al., 2003). However, the recruitment of actin binding proteins during cadherin ligation and the functional importance of these proteins in the strengthening of cadherin-mediated adhesions are poorly characterized.

As the formation of adherens junctions is critically dependent on cortical actin filaments (Perez-Moreno et al., 2003), we investigated the role of the actin binding protein cortactin in the formation of early, N-cadherin-mediated...
intercellular adhesions. Cortactin regulates the assembly and organization of cortical actin filaments and has been implicated in the control of cell motility, growth factor signaling, osmotic responses and phagocytosis (Arora et al., 2003; Di Ciano et al., 2002; Huang et al., 1998; Kapus et al., 2000; Maa et al., 1992; Zhan et al., 1994). While cortactin has been implicated as a regulator of cell migration and invasiveness (Huang et al., 1998; Li et al., 2001), the potential role of cortactin in regulating N-cadherin mediated intercellular adhesions has not been determined.

To assess whether cortactin regulates the strength of N-cadherin adhesions in fibroblasts, we used plates or beads coated with a recombinant Ncad-Fc chimeric protein as well as a considerably refined intercellular adhesion model (Ko et al., 2000; Ko et al., 2001a; Ko et al., 2001b). While many in vitro models are available for the study of E-cadherin-mediated intercellular adhesions in epithelial cells, the dynamics of contact formation in these cells are considerably different from those of N-cadherin-expressing mesenchymal cells (Glowshankova et al., 1998). In vitro, spreading mesenchymal cells exhibit rapid rates of lamellipodial extension and transient intercellular adhesions in contrast to the broad surface areas of intercellular contact that are required for the formation of robust and stable adherens junctions necessary for the distinct apical-basal polarity of epithelial cells (Glowshankova et al., 1997; Glowshankova et al., 1998). While N-cadherin-expressing mesenchymal cells display an extensive network of intercellular contacts in vivo (Beertsen and Everts, 1980; Shore et al., 1981), in vitro these cells preferentially develop cell-matrix adhesions that facilitate their migratory phenotype. Thus cell-matrix adhesions compete with and restrict cadherin-dependent junction formation (Levenberg et al., 1998; Monier-Gavelle and Duband, 1995; Monier-Gavelle and Duband, 1997). Indeed, enforced N-cadherin expression in epithelial cells results in decreased E-cadherin expression and promotes motility and invasion, suggesting unique roles for each type of cadherin in regulating basic cellular processes (Kim et al., 2000; Nieman et al., 1999). These complicating factors limit the utility of many of the established models described above because of their inability to initiate stable, synchronized adhesions. These types of adhesions are required for the quantitative study of N-cadherin biology. In the donor-acceptor model (DAM) system, Rat-2 fibroblasts in suspension are added to the dorsal surfaces of substratum-bound, confluent monolayers of homotypic acceptor cells. The attachment of donor cells occurs via N-cadherin-mediated adherens junctions that are not confounded by integrin ligation of the underlying substratum. Quantification of spectrally discrete, fluorescently labeled cell populations permitted analyses of the kinetics and strength of large numbers of synchronized adherens junctions as they form and mature over time (Ko et al., 2000). With this model and the recombinant N-cadherin-Fc protein we found that cortactin directly associates with nascent N-cadherin-mediated intercellular adhesions following N-cadherin ligation and that tyrosine phosphorylation of cortactin regulates intercellular adhesion strength of fibroblasts.

Materials and Methods

Cell culture

Rat-2 cells were grown in T-75 flasks in DMEM supplemented with 5% (vol/vol) fetal bovine serum (FBS; ICN Biomedicals, Costa Mesa, CA) and antibiotics [0.017% penicillin G (Ayerst Lab, Montreal, PQ), 0.01% gentamycin sulfate (Life Technologies, Grand Island, NY, in DMEM). In some experiments, cells were cultured in calcium-deficient DMEM (Gibco, Grand Island, NY).

Quantification of intercellular adhesion

The DAM was used as described previously (Ko et al., 2000). Briefly, cultured completely confluent acceptor monolayers and donor cells were incubated overnight for ~12 hours in growth medium containing 1 mg/ml of dextran-conjugated fluorochromes. Donor cells were harvested in 0.01% trypsin (supplemented with 2 mM Ca2+) unless otherwise indicated, agitated vigorously and counted electronically to establish 1:1 acceptor:donor ratios. After designated incubation times samples were prepared for flow cytometry or fluorescence microscopy by jet washing three times to remove nonadherent cells. Samples were fixed for 10 minutes in 3.7% paraformaldehyde-5% sucrose for microscopic analysis and images were obtained with a cooled, charge-coupled device camera. Three randomly chosen, 40× fields were examined per sample for each experimental group. Digitized images were analyzed to enumerate the number of dextran-stained attached donor cells (Compix. Imaging Systems, Cranberry Township, PA). Intercellular adhesion data acquired by either flow cytometry or fluorescence microscopy is usually presented as a percentage of the starting number of donor cells that remained attached following jet washing.

Immunoprecipitation and immunoblotting

After treatments, samples were washed twice in PBS, extracted, pre-cleared and immunoprecipitated as reported elsewhere (Kapus et al., 2000). Briefly, following extraction, pre-cleared lysates containing equal amount of protein were incubated with pan-cadherin antibody (Sigma) for at least 1 hour at 4°C. Washed immunocomplexes were eluted from beads using 2% Laemmli sample buffer and boiled for 5 minutes. Samples were fractionated on 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked with 5% nonfat milk in 0.1% Tween 20 in Tris-buffered saline solution. Membranes were washed and immunoblotted for N-cadherin (GC-4: Sigma), β-catenin (E-5: Santa Cruz Biotechnology), cortactin (4F11: Upstate Biotechnology, Lake Placid, NY), phospho-cortactin (Tyr 421; AB3852, Chemicon, Temecula, CA), phosphotyrosine (PY-20, Chemicon) or myc-tag (9E10 Santa Cruz Biotechnology). Bound antibodies were detected with peroxidase-conjugated goat anti-mouse antibody (Jackson Laboratories, West Grove, PA) and chemiluminescence (Amersham, Oakville, ON).

For cadherin expression profiles and experiments assessing the detergent solubility of N-cadherin, whole cell lysates were prepared with 2% SDS Laemmli sample buffer. For examination of cytoskeletal pellets, cells were lysed with 1% Triton X-100 in PBS, 1 μM phalloidin (to stabilize actin filaments) supplemented with a protease inhibitor cocktail (Sigma). The detergent-insoluble pellet was obtained by centrifugation (20,000 g at 4°C for 30 minutes) and solubilized by boiling in 2% SDS Laemmli sample buffer. Protein concentrations of samples were standardized using the RC DC BioRad protein assay (BioRad Laboratories, Mississauga, ON) and equivalent amounts of proteins were loaded and fractionated, transferred onto nitrocellulose, blocked and developed as described. Membranes were probed for N-cadherin (GC-4: Sigma), P-cadherin ([26]: BD Transduction Laboratories, Lexington, KY) and E-cadherin (G-10: Santa Cruz Biotechnology, Santa Cruz, CA). β-Actin (AC-15: Sigma) antibody was used for co-blotting to verify equivalent gel loading.

Preparation of N-cadherin-Fc beads and dishes

The Ncad-Fc (chicken N-cadherin ectodomain fused to the Fc...
fragment of mouse IgG2b) protein was expressed in HEK-293 cells and collected as described (Lambert et al., 2000). Protein A-conjugated beads were used to concentrate the chimeric protein. The following types of protein A-conjugated beads were used: Sepharose beads (Zymed, San Francisco, CA) for trypsin digestion assays, magnetite smooth surface encapsulated beads for pull-off assays (3 μm mean diameter, Spherotech, Libertyville, Il), polystyrene beads for cortactin spatial localization and wash-off assays (5 μm mean diameter, Spherotech). To coat microbiological plastic plates, the N-cad Fc protein was purified from conditioned media using Immunopure® Plus immobilized protein G columns (Pierce Biotechnology) and exchanged into a sodium bicarbonate buffer (concentrations ~100 μg/ml). The protein was adsorbed onto plates by overnight incubation at 4°C and used immediately. Protein adsorption was quantified by dot blot and estimated at 1.25 μg/cm² based on densitometric comparisons with purified mouse IgG Fc fragment controls (Jackson Laboratories, West Grove, PA).

Immunofluorescence

N-cadherin and cortactin were localized in the DAM as well as in cells incubated with N-cad-Fc protein coated beads or plated on N-cad-Fc coated microbiological plastic culture dishes. For the DAM, Rat-2 fibroblasts were plated on nonconfluent monolayers of acceptor cells for 15 or 60 minutes in standard growth medium. Nonconfluent monolayers were used in these experiments to facilitate simultaneous analysis of donor to acceptor cell interactions and donor to substratum interactions to evaluate specificity of recruited proteins to nascent intercellular adhesions. Samples were fixed with 3.7% paraformaldehyde-5% sucrose solution for 1 hour, permeabilized with 0.2% Triton-X for 15 minutes and stained with cortactin antibody (Upstate Technologies) for 1 hour at 37°C. Washed samples were then incubated with Cy3 or FITC-conjugated goat anti-mouse Fab specific secondary antibody (Sigma) at a dilution of 1:100 at 37°C for 60 minutes. Donor-acceptor samples were then incubated with a TRITC-conjugated anti-β-catenin antibody (14; Transduction Laboratories, Mississauga, ON) for another hour at 37°C to permit colocalization of cortactin with the N-cadherin adhesion protein complex. Samples were visualized by wide-field fluorescence microscopy or, where indicated, analyzed with a Leica TCS SL confocal microscope with excitation set at 488 nm and emission collected at 520/10 nm for FITC. For Cy3 or TRITC excitation was set at 543 nm and emission was collected at 565/10 nm. Consecutive optical sections were obtained in the z-axis at a nominal thickness of 1 μm from the cell-substrate interface.

Cortactin videomicroscopy

Rat-2 donor cells were transfected with full-length EGFP-tagged cortactin constructs (1 μg of plasmid DNA per well; for 6-well plates) using Fugene reagent according to the manufacturer’s instructions (Roche, Basel, Switzerland) and allowed to attach to untransfected acceptor cells. Confocal images were acquired every 30 seconds for the initial 30 minutes of intercellular contact with settings as described for FITC fluorescence above.

Intercellular adhesion molecules

We used blocking antibodies for N-cadherin (GC-4; 1:10 dilution in medium) or β1 integrin (4B4; 1:10 dilution) in the donor cell suspension to determine which molecules contributed to intercellular adhesion. HAV mimetic or scrambled peptides were synthesized by Alberta Peptides (Edmonton, AB; sequences LFSHA VSSNG or LHSNSVGFS, respectively) (Ko et al., 2001a). Cells were incubated with peptides at 200 μg/ml for 60 minutes, jet-washed, fixed with 3.7% paraformaldehyde-5% sucrose for 10 minutes and counted with a fluorescence microscope.

RNA interference

A cortactin-specific (determined by BLAST query) siRNA oligonucleotide was synthesized using the following target sequence: AGGTTCTGAGAGAATGTCTTT (Dharmacon, Lafayette, CO). 3‘ Rhodamine sense strand tagged GFP siRNA for the target sequence: CGCGAAGCTGACCCCTGAAGTTCAT was purchased (Qiagen, Mississauga, ON) for a control reagent. Rat-2 cells seeded at 100,000 cells/35 mm dish were transfected with Oligofectamine reagent (Invitrogen Technologies, Burlington, ON) following the manufacturer’s instructions. Cells were assessed for cortactin protein content by western blot and immunofluorescence. Experiments with cortactin-silenced cells were performed 48 hours post-transfection.

Magnetic bead pull-off assay

Proteins enriched at sites of N-cadherin ligation through recombinant N-cadherin-coated bead-associated adhesion complexes were assessed by immunoblotting as described previously (Plopper and Ingber, 1993). Briefly, after designated incubation times, cells and attached N-cadherin-coated magnetic beads (Spherotech) were collected by scraping into ice-cold extraction buffer (CSKB). Beads were pelleted using a side-pull magnetic isolation apparatus (Dynal, Lake Placid, NY) and supernatants were collected. Isolated beads were resuspended, sonicated, homogenized and washed three times in CSKB before PAGE and western blot analysis.

Caderherin cleavage

Trypsin (0.01%) solutions were either supplemented with 2 mM CaCl₂ or 2 mM EGTA and were used to digest Ncad-Fc-coated protein A-conjugated sepharose beads for 10, 20 or 30 minutes. Western blots were performed on bead-bound proteins eluted by boiling. Blots were probed with GC-4 (Sigma) and peroxidase-conjugated goat anti-mouse antibody (Jackson Laboratories) as described above. For analysis of cell-surface cadherin expression, cell suspensions were prepared by trypsin solutions, washed and sedimented. Samples were stained with GC-4 antibody (Sigma; 1:20), counter-stained with FITC-conjugated goat anti-mouse antibody (Sigma; 1:40; both antibodies for 60 minutes at 37°C) and analyzed by flow cytometry.

Flow cytometry and cell viability

Analyses were conducted as previously described (Ko et al., 2000). Briefly, after designated incubation times and jet washing, DAM cultures were prepared as single-cell suspensions with 0.05% trypsin and 0.53 mM EDTA to minimize cell doublets. Cells that were labeled with FITC or RITC-dextran were analyzed by flow cytometry (FACSTAR Plus; Becton Dickinson FACS Systems, Mountain View, CA; excitation wavelength=488 nm). Thresholds for fluorescence were based on levels emitted by secondary antibody staining alone. Emitted fluorescence was collected at 530/15 nm and 630/15 nm. Propidium iodide (10 μM/L, Sigma) staining was used to determine if jet-washing adversely influenced cell viability in donor-acceptor cultures (Lee et al., 1996).

N-cadherin-mediated adhesion strength

Donor-acceptor cell preparations or N-cadherin-coated beads were subjected to shear forces by performing a logarithmic series of jet washes to estimate the strength of intercellular adhesion (Chou et al., 1996). To assess the importance of actin filaments, donor-acceptor cell samples were incubated for 15 minutes or 60 minutes in the presence or absence of cytochalasin D (1 μM). To assess the role of cortactin or phosphorylation of cortactin in adhesion strengthening, cells were transfected with either cortactin-specific or GFP control siRNA using oligofectamine (Invitrogen, Burlington, ON), or with a Myc epitope tagged Y421, 466, 482F plasmid encoding full-length cortactin.
intercellular N-cadherin staining at 15 minutes, which decreases following maturation of cell-substratum adhesions. Bars, 20

Ko et al., 2001a; Ko et al., 2001b). In contrast to substratum-established and characterized DAM was used (Ko et al., 2000; 1998). To circumvent the confounding effects of integrin ligation on our study of N-cadherin adhesions, the previously

consistent with previous work indicating that N-cadherin cadherins (Fig. 1). Confocal microscopy of samples of cells seeded on fibronectin in high density shows intense intercellular N-cadherin staining at 15 minutes, which decreases following maturation of cell-substratum adhesions. Bars, 20 µm.

Statistical analysis
For continuous variable data, means and standard errors of the mean were computed. Comparisons between two groups were made with the unpaired Student’s t-test and multiple comparisons by ANOVA. Statistical significance was set at P<0.05.

Results
Quantification of nascent intercellular adhesions with the donor-acceptor model
Rat-2 fibroblasts expressed N-cadherin but not E- or P-cadherins (Fig. 1i). Confocal microscopy of samples of cells seeded in high densities on a fibronectin matrix reveals decreasing intensity of intercellular N-cadherin staining as cell spreading and corresponding maturation of cell-substratum adhesions progressed (Fig. 1ii-iv). These observations are consistent with previous work indicating that N-cadherin adhesions are poorly developed in substratum-bound mesenchymal cells (Duband et al., 1988; Gloushankova et al., 1998). To circumvent the confounding effects of integrin ligation on our study of N-cadherin adhesions, the previously established and characterized DAM was used (Ko et al., 2000; Ko et al., 2001a; Ko et al., 2001b). In contrast to substratum-bound cells, there was sustained intense staining for N-cadherin at donor-acceptor cell interfaces (as identified by z-axis confocal optical sectioning); within 15 minutes of donor cell attachment to a subconfluent acceptor monolayer (Fig. 2Aii; i-open arrows). No N-cadherin staining was noted at sites of donor to substratum adhesion (Fig. 2Aii, i-closed arrow). In experiments using 60 or 180 minute attachments of donor cells to acceptor cells, the donor cells became more spread but exhibited a very similar staining pattern and staining intensity for N-cadherin to those at 15 minutes (data not shown). Immunohistochemically stained donor-acceptor samples which used completely confluent acceptor monolayers showed intense, continuous peripheral rings of N-cadherin staining in donor cells (not shown). These images, however, did not unequivocally show the specificity of protein recruitment to N-cadherin-mediated intercellular adhesion. (i) Whole-cell lysates of Rat-2 fibroblasts probed with monoclonal antibodies specific for N, E and P cadherins (top). A431 and LNCaP cell lysates used to verify specificity of antibodies used for cadherin expression profiling of Rat-2 fibroblasts. Equal loading of samples was optimized by measuring total amounts of protein (by BioRad assay) and co-blotted with β-actin. (ii-iv) Immunostaining of cells seeded on fibronectin in high density shows intense intercellular N-cadherin staining at 15 minutes, which decreases following maturation of cell-substratum adhesions. Bars, 20 µm.

Fig. 1. Fibroblasts express N-cadherin, which mediates intercellular adhesions compromised by matrix adhesions. (i) Whole-cell lysates of Rat-2 fibroblasts probed with monoclonal antibodies specific for N, E and P cadherins. A431 and LNCaP cell lysates used to verify specificity of antibodies used for cadherin expression profiling of Rat-2 fibroblasts. Equal loading of samples was optimized by measuring total amounts of protein (by BioRad assay) and co-blotted with β-actin. (ii-iv) Immunostaining of cells seeded on fibronectin in high density shows intense intercellular N-cadherin staining at 15 minutes, which decreases following maturation of cell-substratum adhesions. Bars, 20 µm.

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sites of nascent intercellular adhesions, as did samples in which subconfluent acceptor monolayers were used. Consequently in this report, subconfluent acceptor monolayers were used for spatial analysis to allow simultaneous study of intercellular donor-acceptor interactions and donor-substratum interactions. For all other experiments including those requiring quantification, donor cells were incubated onto a completely confluent acceptor monolayer.

To obtain reliable estimates of the proportion of donor and acceptor cells that formed stable intercellular adhesions, we used endocytosed, stable fluorescence markers to label separately the acceptor and donor cell populations. Staining
with dextran-conjugated fluorochromes permitted the maintenance of cell viability while simultaneously facilitating tracking, detection and quantification by fluorescence microscopy or flow cytometry. There was minimal signal crossover of the emission spectra of the fluorescent dyes when analyzed by flow cytometry or when examined with equivalent exposure times by microscopy (Fig. 2Bi-iv), allowing accurate quantification in mixed homotypic cultures. Sorting of cells by two-color flow cytometry in regions distinguished as exclusively RITC-labeled (R5-acceptor cells) or FITC-labeled (R6-donor cells) verified the accuracy and specificity of the analyses when sorted cells were sedimented and visualized by microscopy (Fig. 2Bv).

To ensure that donor-acceptor cell adhesion was largely mediated by cell-surface N-cadherins, we took advantage of the sensitivity of the extracellular cadherin domain to proteolytic digestion in the absence of calcium (Ozawa et al., 1990; Takeichi, 1977; Yap et al., 1997b). The intercellular adhesion kinetics of cells harvested in calcium-free trypsin and EGTA (2 mM) solutions were compared with those harvested in calcium-supplemented (2 mM calcium) trypsin solutions. Donor cells that were obtained using calcium-deficient trypsin solutions showed no increase of adhesion on completely confluent acceptor monolayers over time compared with controls (Fig. 2Ci). Immunofluorescence analysis by flow cytometry confirmed the surface cleavage of the extracellular domain of N-cadherin following the use of a calcium-free trypsin solution to prepare cell suspensions. Cells prepared with trypsin (0.01%) in the presence of 2 mM CaCl₂ exhibited more than fourfold higher cell-surface N-cadherin staining than cells prepared with trypsin and EGTA (Fig. 2Cii). The digestion efficiency of the trypsin solutions was also evaluated by SDS-PAGE analysis of recombinant N-cadherin extracellular domain, which showed cleavage only in solutions without calcium (data not shown).

Calcium not only provides resistance to cleavage by trypsin, but it is also required for cadherin function in mediating intercellular adhesion (Pertz et al., 1999; Pokutta et al., 1994). Indeed, calcium was required in the DAM because the use of a chemically defined, calcium-free culture medium supplemented with 2 mM EGTA reduced donor to acceptor adhesion by fivefold compared with controls using standard media (P<0.005; data not shown). We also analyzed donor-acceptor cell adhesion in the presence of blocking antibodies. Intercellular adhesion was dramatically reduced with an N-cadherin blocking antibody (GC-4; Fig. 2D; P<0.05). By contrast, cells treated with 4B4, a β1-integrin blocking antibody, caused no significant reductions of donor-acceptor cell adhesion (P>0.2). We examined further the role of cadherins in mediating donor-acceptor cell adhesion with blocking peptides. The HAV (Histidine79, Alanine80 and Valine81) motif is a highly conserved region in the first extracellular domain of all classical cadherins and is thought to be crucial for mediating homophilic cadherin-based intercellular adhesions (Shapiro et al., 1995). Accordingly, we measured the effect of an HAV-containing mimetic peptide (NH₂-LFHSHAVSSNG-amide; 200 µg/ml) and a corresponding ‘scrambled peptide’ on intercellular adhesion in the DAM (NH₂-LHSNSVGFSA-amide; 200 µg/ml) (Noe et al., 1999). The mimetic peptide decreased donor cell adhesion by more than tenfold compared with the scrambled peptide in which donor adhesion rates were similar to controls (Fig. 2D; P<0.01). Collectively these findings show that intercellular adhesion in the DAM is critically dependent on N-cadherin.

Role of actin in N-cadherin adhesion strength

Reorganization of the cortical actin cytoskeleton is thought to be necessary for maturation and strengthening of cadherin-mediated adherens junctions (Adams and Nelson, 1998; Perez-Moreno et al., 2003). To verify the functional importance of actin filament assembly in cadherin-mediated adhesion strengthening in the DAM, wash-off shear assays were conducted using cytochalasin D-treated and vehicle control samples (Chou et al., 1996). We found three- to fivefold reductions in the strength of adhesion after cytochalasin D treatment after 15 and 60 minutes of incubation (60 min – cytD: slope=−3.84, R²=0.701; 60 min+ cytD: slope=−0.776, R²=0.664; 15 min – cytD: slope=−1.42, R²=0.631; 15 min + cytD: slope=−0.388, R²=0.663; n=3 for all samples). We found distinct cortical rings of actin filaments within 15 minutes of incubation of donor cells on acceptor monolayers or onto N-cad-Fc plates, suggesting the reorganization and insertion of cortical actin filaments into the cytoplasmic domain of N-cadherin-mediated intercellular adhesions (Fig. 3A). Actin ring structures were not seen in samples treated with 1 µM cytochalasin D.

We confirmed that the increased strength of adhesion in wash-off assays of control samples was associated with enhanced recruitment of N-cadherin to the cytoskeletal fraction of cells from the DAM. Whole-cell lysates and Triton-insoluble cytoskeletal fractions of donor-acceptor culture model samples were immunoblotted for β-actin and N-cadherin, and the amount of protein in each lane was normalized (by BioRad assay) and quantified by densitometry. As expected, there was no change in β-actin and N-cadherin content over time in the whole-cell lysates. In the cytoskeletal fraction of donor-acceptor cells, compared with acceptor cell monolayer, there was a threefold increase of N-cadherin within 15 minutes after donor cell incubation, followed by stabilization at that level (Fig. 3B).

Cortactin colocalizes with N-cadherin adhesion complexes

In addition to the importance of actin filament integrity in N-cadherin adhesion noted above, recent evidence shows de novo actin formation at sites of early, N-cadherin-mediated adherens junctions (Adams and Nelson, 1998; Perez-Moreno et al., 2003). To verify the functional importance of actin filament assembly in cadherin-mediated adhesion strengthening in the DAM, wash-off shear assays were conducted using cytochalasin D-treated and vehicle control samples (Chou et al., 1996). We found three- to fivefold reductions in the strength of adhesion after cytochalasin D treatment after 15 and 60 minutes of incubation (60 min – cytD: slope=−3.84, R²=0.701; 60 min+ cytD: slope=−0.776, R²=0.664; 15 min – cytD: slope=−1.42, R²=0.631; 15 min + cytD: slope=−0.388, R²=0.663; n=3 for all samples). We found distinct cortical rings of actin filaments within 15 minutes of incubation of donor cells on acceptor monolayers or onto N-cad-Fc plates, suggesting the reorganization and insertion of cortical actin filaments into the cytoplasmic domain of N-cadherin-mediated intercellular adhesions (Fig. 3A). Actin ring structures were not seen in samples treated with 1 µM cytochalasin D.

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Cortactin colocalizes with N-cadherin adhesion complexes

In addition to the importance of actin filament integrity in N-cadherin adhesion noted above, recent evidence shows de novo actin formation at sites of early, N-cadherin-mediated adherens junctions (our observations) (Kovacs et al., 2002a; Lambert et al., 2002; Vasioukhin et al., 2000). Consequently, we determined if cortactin was colocalized to N-cadherin adhesion sites. Actin ring structures were not seen in samples treated with 1 µM cytochalasin D.
recombinant N-cadherin-Fc fusion protein, or poly-L-lysine, or IgG Fc fragments for 15 minutes. Following 15 minutes of attachment there was distinct immunostaining for cortactin at the cell periphery at areas of N-cadherin ligation (Fig. 3D). By contrast, there was punctate, perinuclear staining for cortactin in cells attached to poly-L-lysine. After 60 minutes of attachment, cells were more spread but displayed staining patterns similar to those found at 15 minutes (data not shown). No cellular attachment was detected on plates coated with Fc fragments. We also examined cortactin localization with N-cadherin-dependent adhesions following the incubation of cells with N-cadherin-Fc-coated beads for 15 minutes. There was distinct cortactin staining at the areas of N-cadherin ligation at the bead-cell interface (Fig. 3D), which also corresponded to intense F-actin staining.

To ensure that cortactin is also recruited to nascent junctions between substratum-bound cells, cell suspensions were plated at high densities onto a fibronectin matrix, a procedure that facilitates physical interactions between adjacent cells. Under these conditions, cortactin co-localized with N-cadherin (Fig. 3Ei-iii). However, as these cells spread and developed cell-matrix adhesions, the degree of co-localization at sites of intercellular contact decreased dramatically (Fig. 3Eiv-xi). These data support the established notion that N-cadherin intercellular adhesions are negatively regulated in migrating mesenchymal cells and that in these cells N-cadherin and cortactin are involved in early (nascent) intercellular contacts.

Cortactin association with N-cadherin adhesion complexes

We evaluated whether the localization of cortactin to intercellular adhesion complexes is mediated by the binding of cortactin to N-cadherin. Immunoprecipitations using
antibodies to the cytoplasmic domain of N-cadherin were conducted on samples of confluent acceptor monolayers without donor cells or following 15, 60, 180, 360 or 720 minute incubations with donor cells (Fig. 4A). Within 15 minutes of donor-acceptor cell adhesion, cortactin was co-immunoprecipitated with N-cadherin. By contrast, there was minimal cortactin association with N-cadherin in untreated, mature acceptor monolayers, further confirming that N-cadherin-cortactin association is a characteristic of early contacts. Accordingly, densitometry showed that the greatest amount of cortactin associated with N-cadherin was observed in the first hour of co-incubation, followed by a subsequent decrease over time.

To ensure that cell lysis conditions preserved the integrity of cadherin adhesion complexes, blots of immunoprecipitates were probed for β-catenin. The blots showed a constant amount of β-catenin co-precipitated with N-cadherin (data not shown). Reverse immunoprecipitations using cortactin antibodies showed that N-cadherin was detected in anti-cortactin immunoprecipitates (Fig. 4Aii), confirming the association of these proteins, and that cortactin was recruited to the nascent N-cadherin adhesion complex.

We examined cortactin recruitment to N-cadherin adhesion complexes after N-cadherin ligation. N-cad-Fc-conjugated protein A-coated magnetic beads were incubated with monolayers of Rat-2 cells. Saturation of available bead binding sites was confirmed by the minimal amounts of N-cad-Fc protein remaining in the supernatant after incubations (Fig. 4Aii). Proteins eluted from the beads and immunoblotted for β-catenin and cortactin showed that cortactin was recruited to areas of N-cadherin ligation within 15 minutes of incubation (Fig. 4Aiii). Further, real-time video microscopy of donor cells transfected with full-length cortactin-GFP constructs showed gradual increases of cortactin at sites of N-cadherin-mediated intercellular contact, from the time of initial attachment to the first 30 minutes of adhesion (Fig. 4B).

Cortactin expression is required for N-cadherin-mediated intercellular adhesion

Application of a post-transcriptional gene silencing technique using short, double-stranded RNA sequences specific for the cortactin gene in Rat-2 fibroblasts reduced cortactin content by ~85% compared with cells transfected with GFP dsRNA or in vehicle controls, as judged by densitometry of immunoblots and by immunofluorescence (Fig. 5A). To assess if cortactin was a necessary component of the N-cadherin adhesion complex, we determined whether downregulation of cortactin would have an impact on intercellular adhesion. When donor-acceptor cultures were established using cortactin gene-
silenced cells, the level of intercellular adhesion was reduced twofold compared with that of control samples treated with control GFP RNAi sequences (Fig. 5A; \( P < 0.005 \)). Conceivably, cortactin deficiency might affect surface expression of N-cadherin, which in turn may explain the reduced intercellular adhesion noted above. To assess if cortactin RNAi reduced surface-expressed N-cadherin, nonpermeabilized cortactin or control (GFP) RNAi-treated cells were surface labeled with anti N-cad antibody and analyzed by flow cytometry. Interestingly, cortactin-silenced samples showed 26% greater N-cadherin surface labeling compared with GFP-treated controls (Fig. 5B; \( P < 0.05 \)). Permeabilized samples (to estimate total cell cortactin) exhibited 83% less cortactin fluorescence in cortactin-silenced samples compared with controls, consistent with a reduction in cortactin expression.

Fig. 5. Cortactin is important for N-cadherin-mediated intercellular adhesion and surface expression. (A) Cortactin gene silenced cells show a twofold reduction of intercellular adhesion compared with GFP controls (\( P < 0.05 \)). Data are from \( n = 4 \) replicate samples, means±s.e.m. Immunofluorescence analysis shows lack of cortactin staining and alteration of actin filament network in cortactin knockdown cells. Bar, 20 \( \mu \)m. Immunoblotting shows the extent of cortactin knockdown by RNAi. Samples V, G and SR represent vehicle controls, GFP-RNAi transfected cells, and cortactin RNAi-transfected cells, respectively. Individual samples are corrected for total protein concentration and co-blotted for B-actin. (B) N-cadherin surface labeling of unpermeabilized cells reveals ~25% greater labeling in cortactin RNAi compared with GFP RNAi-treated samples (\( P < 0.05 \)). Permeabilized samples show ~85% reduction of cortactin expression in cortactin RNAi-treated vs. GFP RNAi-treated samples (\( P < 0.005 \)). (C) No effect noted of cortactin siRNA treatment on fibroblast spreading on fibronectin when compared with GFP siRNA-treated controls. (D) No effect noted of cortactin siRNA treatment on strength of cell-substratum adhesion when compared with GFP siRNA-treated controls.
with immunoblotting and immunofluorescence data (Fig. 5B; \( P<0.005 \)). These results imply that cortactin is an important component of the N-cadherin adhesion complex and regulates intercellular adhesion by a mechanism that does not reduce N-cadherin surface expression.

As cortactin is an important regulator of the dynamics of actin networks throughout the cell we investigated whether knockdown of cortactin expression also affects cell-matrix interactions. Fibroblast spreading on fibronectin surfaces from 15 to 180 minutes was not significantly different between samples treated with cortactin or control GFP RNAi sequences (Fig. 5C). We also found that the strength of cell matrix adhesions was not compromised in cortactin RNAi-treated samples as evaluated by a wash-off assay of substratum-bound cells when compared with GFP controls (Fig. 5D). Collectively, these results indicate that cortactin expression is not required for proper matrix attachment and spreading.

Role of cortactin in N-cadherin-dependent adhesion strengthening

As shown above, cortactin is physically associated with N-cadherin adhesion complexes. We next asked if cortactin is involved in actin-dependent, N-cadherin adhesion strengthening. N-cadherin-coated beads were seeded onto cells treated by RNAi for cortactin, and shear wash-off assays were conducted. RNAi treatment produced a heterogeneous population of cells that were divided into two subpopulations based on cortactin immunofluorescence staining intensity. In cells in which cortactin expression had been knocked down to background levels, there was much weaker binding to the N-cadherin-coated beads compared with cells in which cortactin expression was preserved (Fig. 6). Representative microscopic fields from the second wash and sixteenth wash-off samples showed the difference in bead-to-cell binding between the two cell populations (Fig. 6). In control experiments, no differences were noted in bead-binding affinities between GFP RNAi-transfected and untransfected cells (data not shown). These data indicate that cortactin contributes to strengthening of intercellular adhesions.

Tyrosine phosphorylation of cortactin

Tyrosine phosphorylation of cortactin has been detected in...
response to a varied range of stimuli (Weed and Parsons, 2001); however, its biological significance in these processes is unclear. We found that the cortactin associated with the N-cadherin adhesion complexes was tyrosine phosphorylated as shown when immunoprecipitates of donor-acceptor culture samples were probed with PY-20, a phosphotyrosine-specific monoclonal antibody (Fig. 7A). On the basis of molecular mass, cortactin appeared to be one of the major adhesion proteins phosphorylated on tyrosine residues during intercellular adhesion. Notably, bands corresponding to β-catenin and p120 catenin that are associated with the N-cadherin adhesion complex were not seen (Fig. 7A). Tyrosine phosphorylation of cadherin-associated cortactin in donor-acceptor cultures was verified in N-cadherin immunoprecipitates immunoblotted with an antibody specific for phospho-tyrosine residue 421 on cortactin (Fig. 7A).

We evaluated whether cortactin phosphorylation was required for association and recruitment to N-cadherin...
adhesions. DAMs were treated with a general tyrosine kinase inhibitor genistein (100 µM) or with an src-kinase specific inhibitor PP2 (25 µM). While these inhibitors abolished cortactin tyrosine phosphorylation, cortactin was still physically associated with the N-cadherin adhesion complex (Fig. 7A, genistein data not shown). We verified that tyrosine phosphorylation was independent of the recruitment of cortactin to the cadherin adhesion complex. Cells were transfected with a Myc-tagged cortactin construct in which tyrosine residues 421, 466 and 482 were mutated to phenylalanines (F-cort). N-cadherin immunoprecipitations of transfected donor-acceptor samples incubated for 60 minutes revealed that the cortactin construct indeed associated with the N-cadherin adhesion complex (Fig. 7C). Alternatively, immunoprecipitations using Myc antibodies also showed an association of the tagged construct with N-cadherin (Fig. 7C).

We next assessed the possible role of cortactin phosphorylation in adhesion strengthening by examining genistein or PP2-treated donor-acceptor cultures with a shear wash-off assay. Both genistein and PP2-treated samples exhibited dramatic reductions of adhesion strength compared with vehicle controls (Fig. 7B). To more precisely assess the role of cortactin phosphorylation in adhesion strengthening, cells were transfected with either the F-cort construct or a Myc-tagged full-length wild-type cortactin construct (WT-cort), and wash-off assays were conducted using beads coated with recombinant Ncad-Fc fusion protein. The numbers of beads bound to transfected cells detected by immunofluorescence staining were compared with untransfected controls in the same sample. F-cort transfected cells showed significantly reduced binding to the N-cadherin-coated beads compared with controls (Fig. 7D). WT-cort transfected cells showed a trend towards slightly enhanced N-cadherin bead binding but this was statistically insignificant compared with controls (Fig. 7D; P>0.2). The data from the WT-cort transfected samples supports the specificity of the results gathered from the F-cort transfected samples. Collectively, these results indicate that while tyrosine phosphorylation is not required for cortactin recruitment, it is extremely important for providing intercellular adhesive strength following N-cadherin ligation.

**Discussion**

We have used a recombinant N-cad-Fc chimeric protein and the DAM to study early N-cadherin-mediated adhesions in fibroblasts. The DAM is a simple, reliable and quantitative assay that facilitates the study of a large number of synchronized, cadherin-mediated intercellular adhesions independent of integrin ligation (Ko et al., 2000), while the recombinant N-cadherin chimeric protein enables studies of homogeneous N-cadherin contacts (Lambert et al., 2000). These complementary methods both generate N-cadherin-mediated intercellular adhesions and show that cortactin, an actin filament binding protein, spatially localizes to, and physically associates with, N-cadherin adhesion complexes. Our most important finding is that cortactin, a dynamic regulator of cortical actin assembly, is functionally important for N-cadherin-mediated intercellular adhesion strength in a phosphorylation-dependent mechanism. As cortactin is a substrate for numerous nonreceptor tyrosine kinase families and also functions in concert with the Arp2/3 actin nucleating complex, these data suggest that cadherin-mediated intercellular adhesions are centers for signaling and actin assembly.

Cortactin is a regulator of N-cadherin adhesions

Anchorage of surface-expressed cadherins to the actin cytoskeleton in response to intercellular adhesion is required for enhancing the strength of intercellular adhesions (Breen et al., 1993; Imamura et al., 1999; Roe et al., 1998). Indeed, we found that early N-cadherin ligation resulted in a marked reorganization of cortical actin filaments and a corresponding threefold increase in the abundance of the detergent-insoluble N-cadherin pool. This profile coincides with the recruitment and physical association of cortactin to N-cadherin adhesion complexes and suggests that cortactin plays a pivotal role in tethering N-cadherins to cortical actin filaments. Cortactin is an actin filament binding protein that enhances the actin nucleating activity of the Arp 2/3 complex (Kinley et al., 2003; Uruno et al., 2001). In this context, the Arp 2/3 complex physically associates with E-cadherin (Kovacs et al., 2002b) and is responsible for local actin nucleation at sites of cadherin ligation, a function in which cortactin has recently been shown to play a role. Indeed, while this manuscript was under review, a role for cortactin was found for E-cadherin-mediated intercellular adhesion that established a crucial role for cortactin in Arp2/3-dependent actin assembly at junctions between epithelial cells (Helwani et al., 2004). Our data show that in mesenchymal cells the cortactin association with the cadherin junctional complex is temporally limited to nascent contact formation and is central to the maturation and strengthening of contacts, but that this mechanism may not be important for the maintenance of stable and mature junctions.

Our data showed that knockdown of cortactin expression markedly reduces N-cadherin-dependent adhesion strength, a finding that supports a role for cortactin in mediating nascent intercellular contacts. By virtue of its role in regulating de novo actin polymerization, cortactin may regulate the attachment of cadherins to cortical actin filaments. Further, cortactin may help to stabilize local actin networks by inhibiting de-branching of newly formed filament networks, thereby contributing to actin-dependent strengthening of cadherin adhesions (Weaver et al., 2001). The recruitment of cortactin to intercellular adhesion complexes after N-cadherin ligation is consistent with the notion that intercellular contacts are active centers for actin assembly and organization (Perez-Moreno et al., 2003). Cortactin binds directly to proteins that play fundamental roles in the budding of vesicles in the process of receptor-mediated endocytosis (Schmid, 1997). In addition, cortactin is localized to post-endocytic vesicle actin tails, suggesting a role in stabilizing Arp2/3-nucleated actin networks (Kaksonen et al., 2000). Our results are consistent with these findings as downregulation of cortactin expression by RNA interference increased the surface expression of N-cadherin, suggesting reduced efficiency of endocytic recycling of cadherin molecules. In spite of the significantly higher surface expression of N-cadherin, we still found a dramatic reduction of N-cadherin-dependent adhesion strength. These data underscore the importance of cortactin as a regulator of actin filament dynamics at localized sites of cadherin-mediated intercellular adhesion.
Cortactin and N-cadherin adhesions

Cortactin tyrosine phosphorylation and cadherin-dependent signaling

Cortactin is a substrate for numerous tyrosine kinases including the Fes, Fyn and Src-family of nonreceptor tyrosine kinases (Calautti et al., 1995; Kapus et al., 2000; Zhan et al., 1993). While tyrosine phosphorylation of cortactin is a response to numerous stimuli (Weed and Parsons, 2001), the functional significance of these modifications, irrespective of the implicated kinase, is unclear. Recently, delta-catenin, a p120 catenin family member, which is expressed almost exclusively in neural tissues, has been shown to associate with cortactin in an src-kinase, tyrosine phosphorylation-dependent manner (Martinez et al., 2003). Our results are contrary to these findings and show that cortactin interacts with the N-cadherin adhesion complex in a phosphorylation-independent fashion. This discrepancy may be attributable to differences in cell type (neural cells vs. fibroblasts) (Braga et al., 1999), in homologous adhesion complex proteins (delta-catenin vs. p120 catenin) or in the cellular region that was studied. Notably, Martinez et al. report on the functional significance of nonjunctional delta catenin-cortactin association in the context of neural morphogenesis in contrast to the junctionally localized proteins reported here (Martinez et al., 2003).

We have shown through the use of chemical tyrosine kinase inhibitors and specifically using a transfected construct in which tyrosine residues have been mutated to phenylalanines that while tyrosine phosphorylation of cortactin was not required for association with the adhesion complex, it was required for N-cadherin-dependent adhesion strengthening. Phosphorylation of tyrosine residue 421 may be involved in cortactin function at local sites of N-cadherin ligation. This residue is adjacent to an src SH2 binding domain, which is thought to allow sequential phosphorylation of the other tyrosine residues further along the molecule and potentially for the stabilization of kinase-cortactin association (Huang et al., 1998; Weed and Parsons, 2001). The nonreceptor tyrosine kinase Fer is a probable candidate for mediating phosphorylation of N-cadherin-bound cortactin as it directly interacts with both cortactin (Kapus et al., 2000) and N-cadherin (Arregui et al., 2000), in addition to the phosphorylation of cadherin-bound catenins (Arregui et al., 2000; Piedra et al., 2003). Additionally, as protein phosphatases have been found to complex with and dephosphorylate N-cadherin junctional proteins (Balsamo et al., 2000; Piedra et al., 2003), the phosphorylation status of associated proteins is likely to serve an important signaling function, in addition to modulating the adhesion state (Lilen et al., 2002). Indeed, our results suggest that the regulation of tyrosine phosphorylation of cortactin, after association with the cadherin adhesion complex, may be an important mechanism for dynamically modulating intercellular adhesion strength. As loss of cadherin-mediated intercellular adhesion is implicated in wound healing, metastasis and tumor cell invasion, these findings may improve our understanding of the cellular outcomes preceding these events.

Collectively, these data indicate that N-cadherin ligation is required for cortactin translocation and association with the N-cadherin adhesion complex in a tyrosine phosphorylation-independent manner. At these adhesions, cortactin strengthens intercellular adhesion in a tyrosine phosphorylation-dependent mechanism. The remodeling of the cortical actin cytoskeleton, through the action of cadherin-associated actin binding, bundling and nucleating proteins after cadherin ligation, appears to be important in the process of cadherin-mediated intercellular adhesion.

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