Modulating the strength of cadherin adhesion: evidence for a novel adhesion complex

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Summary

Adherens junctions and desmosomes are critical for embryogenesis and the integrity of adult tissues. To form these junctions, classical cadherins interact via α- and β-catenin with the actin cytoskeleton, whereas desmosomal cadherins interact with the intermediate filament system. Here, we used a hormone-activated mutant N-cadherin expressed in fibroblasts to show the existence of a novel classical cadherin adhesion system. N-cadherin was fused at its C-terminus to a modified estrogen receptor ligand-binding domain (NcadER) that binds 4-hydroxytamoxifen (4OHT) and expressed in L cells, which lack an endogenous cadherin. Cells with the mutant cadherin (LNER cells) aggregated in the absence of 4OHT, but only in its presence formed tightly compacted aggregates like those formed by L cells expressing wild-type N-cadherin (LN cells). Compaction of LNER cells treated with 4OHT was accompanied by elevated levels of p120^{ctn} in NcadER immunoprecipitates, compared to immunoprecipitates of non-treated cells, but without changes in α- and β-catenin, or actin. Compaction induced by 4OHT was also accompanied by increased interaction of the NcadER with the cytoskeleton and increased vimentin organization. Vimentin co-immunoprecipitated with the NcadER/catenin complex, suggesting an interaction between cadherin and vimentin. The mechanism by which vimentin interacts with the cadherin appears to involve p120^{ctn} as it co-immunoprecipitates and colocalizes with vimentin in the parent L cells, which lack a cadherin and α- and β-catenins. Disrupting the actin cytoskeleton with cytochalasin B inhibited aggregation, whereas knocking down vimentin with specific siRNAs inhibited compaction. Based on our results we propose that a vimentin-based classical cadherin complex functions together with the actin-based complex to promote strong cell-cell adhesion in fibroblasts.

Key words: Cell-cell adhesion, Junctions, Cadherin, Catenins, Actin, Vimentin

Introduction

Cell-cell adhesion is critical for embryonic morphogenesis and for maintaining tissue integrity and function in adult organisms. Members of the cadherin family of transmembrane adhesion proteins form the hub for the multiprotein complexes comprising intercellular junctions detected at the ultrastructural level as adherens junctions, desmosomes, intercalated discs and endothelial junctions (Wheelock and Johnson, 2003). The exact protein composition and specific protein-protein interactions are largely understood for these junctions. In the case of the widely distributed adherens junctions, classical cadherins self-associate extracellularly, and intracellularly interact with β-catenin (or plakoglobin), which binds α-catenin and in turn interacts with the actin cytoskeleton (reviewed by Wheelock and Johnson, 2003; Nelson and Nusse, 2004). The p120 catenin (p120^{ctn}) also binds the cadherin intracellular domain at a juxtamembrane site, and is thought to regulate cadherin adhesive strength (Yap et al., 1998; Ohkubo and Ozawa, 1999; Thoreson et al., 2000). In addition to their roles in adhesion, p120^{ctn} and β-catenin, in particular, form signaling complexes that regulate transcription (reviewed by

Anastasiadis and Reynolds, 2000; Conacci-Sorrell et al., 2002; Nelson and Nusse, 2004). In the case of desmosomes, such as those found in the myocardium and epithelial tissues, desmocollins and desmogleins interact intracellularly with plakoglobin, which binds desmoplakin, and in turn links to the intermediate filament cytoskeleton (Green and Gaudry, 2000; Ishii and Green, 2001; Garrod et al., 2002; Wheelock and Johnson, 2003; Getsios et al., 2004). Cells and tissues frequently have more than one junction type; in addition, adhesion-related proteins can be found in more than one junction. For example, intercalated discs of the myocardium include adherens junctions containing the classical cadherin, N-cadherin, as well as desmosomes. In endothelial cells the classical cadherin VE-cadherin assembles an actin-based adherens junction, in addition to a specialized junction where VE-cadherin links to the vimentin intermediate filament cytoskeleton via plakoglobin and desmoplakin (Lampugnani and Dejana, 1997; Kowalczyk et al., 1998).

The strength of cadherin-mediated cell-cell adhesion is regulated in a dynamic manner to accommodate such processes as cell migration during embryogenesis, cell renewal in

epithelia and wound healing. In addition, abnormal changes in cadherin adhesion contribute to pathological processes, including cancer and its metastasis (Conacci-Sorrell et al., 2002; Hajra and Fearon, 2002; Christofori, 2003; Cavallaro and Christofori, 2004). Multiple mechanisms modulate the strength of adhesion, including the nature and level of the cadherin(s) expressed, cadherin clustering within the plasma membrane, presence of growth factors (e.g. EGF and scatter factor/HGF), phosphorylation state of catenins and anchorage to the cytoskeleton (Gumbiner, 2000). Additionally, the formation of multiple junction types in cells affects the strength of their interactions. In particular, desmosomes play an important role in stabilizing cell-cell interactions in tissues that are subject to mechanical stress, such as the skin and myocardium.

Studying the dynamic role of junctions within cells is complicated by the fact that under most physiological conditions, cells are in constant contact. To overcome this obstacle, several model systems have been established. One system involves exposing cadherin-expressing cells to a substrate comprised of the corresponding cadherin extracellular domain or to beads bearing immobilized anti-cadherin antibodies (Kovacs et al., 2002; Betson et al., 2002). This allows one to investigate initial cell-cell contacts in a system analogous to those used to study cell-extracellular matrix interactions. A second model involves a calcium switch. As cadherins require calcium for their activity, cell-cell contact can be initiated by elevating the concentration of extracellular calcium for cells grown under low-calcium conditions. This system has been used by a number of groups studying cadherin function (Nelson et al., 1990; Wheelock and Jensen, 1992). A third model involves plating cells in sparse cultures and observing them as they proliferate and finally make contact with one another (Ehrlich et al., 2002). Each model system has its limitations, and one goal of this study was to develop a new system in which the cadherin could be rapidly activated in order to further understand its function.

It has been established that fusion of the hormone-binding domain of the estrogen receptor to a heterologous protein can result in a protein whose function is hormone-regulated. Normally the estrogen receptor exists in cells as an inactive protein tightly associated with the HSP90 protein chaperone. When estrogen enters the cell and binds to its receptor, HSP90 is displaced, allowing the receptor to interact with DNA and initiate transcription of estrogen-responsive genes (Beato and Klug, 2000). One group has made use of this HSP90/estrogen receptor interaction to develop a chimeric system whereby heterologous proteins can be inactive or active, depending on the absence or presence of hormone (Picard et al., 1988). In the ideal case, the chimeric protein is expressed, but is inactive until hormone is added. Upon addition of hormone, HSP90 is displaced, and the hormone-binding domain undergoes a conformational change that relieves the inhibition of the heterologous protein and it becomes fully active. This system has mainly been used to create transcription factors and kinases that can be readily activated (Picard, 2000); however it has also been used to create a hormone-responsive form of two transmembrane proteins, Fas and the epidermal growth factor receptor (Picard, 2000), which suggested that we could use the system with a classical cadherin. Thus, we made a chimeric cDNA construct comprised of a modified estrogen receptor ligand binding domain (ER-LBD) fused to the 3′ end of the full-length N-cadherin cDNA (i.e. C-terminus of the protein). Our aim was to express the mutant N-cadherin (NcadER) in cells lacking an endogenous cadherin and to compare cell behavior in the absence and presence of 4-hydroxytamoxifen (4OHT) to activate the NcadER.

We expressed the mutant N-cadherin in mouse L fibroblasts, which lack an endogenous cell-cell adhesion system. Using this model system we discovered strong evidence for a novel vimentin-based classical cadherin adhesion system. We propose that the vimentin-based classical cadherin adhesion system acts in concert with the traditional actin-based system to strengthen cell-cell adhesion in fibroblasts, and perhaps other cell types.

## Materials and Methods

N-cadherin/ER-LBD construct

The cDNA encoding full-length human N-cadherin (a gift of Avri Ben Ze'ev, Weizman Institute of Science) (Salomon et al., 1992) was ligated at the 3′ end to a cDNA encoding a mutated estrogen receptor (ER) ligand-binding domain (LBD) (gift of Pierre Chambon, Institute de Genetique et de Biologie Moleculaire et Cellulaire) (Feil et al., 1997; Picard, 2000) (Fig. 1). The modified ER-LBD was mutated such that it binds 4-hydroxytamoxifen (4OHT) but not 17β-estradiol (Feil et al., 1997). To generate an expression vector for our studies with the L cells, the Moloney murine sarcoma virus (MSV) long terminal repeat (LTR) promoter was removed from the pEMSVscribeα2 expression vector (gift of Andres Lassar, Harvard University) (Davis et al., 1987) and moved to the backbone of the pLKneo expression vector (Hirt et al., 1992), replacing the MMTV promoter present in pLKneo, but retaining the SV40-derived poly A site, plus the neomycin resistance gene for selection. This expression vector also contained the N-cadherin/ER-LBD (NcadER). Details of the constructions are available upon request.

Cells

The mouse L fibroblast cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The L cells do not express an endogenous cadherin, nor do they have another cell-cell adhesion system. They are frequently used to evaluate proteins for their adhesive activity (Nagafuchi et al., 1987). The L cells were stably transfected with the NcadER construct using Lipofectamine (Invitrogen, Grand Island, NY), following the manufacturer's recommendations. Selection was accomplished with G418 at a concentration of 0.8-0.9 mg/ml. Drug-resistant cells were tested for expression of the NcadER fusion protein by immunofluorescent light microscopy using the 13A9 anti-N-cadherin monoclonal antibody (Knudsen et al., 1995). Multiple clones were chosen for our studies based on robust expression of the NcadER protein and several clones exhibiting similar characteristics were used. LNER cells were maintained in Ham's F12/DMEM (1:1) medium (Sigma, St Louis, MO) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), but lacking 4OHT, unless otherwise indicated. The LNER cells were studied in the absence and presence of 100 nM 4OHT, although 10 nM was similarly effective. Controls included the parent L cells (L cells) and L cells stably expressing the wild type, constitutively active mouse N-cadherin (LN cells) (gift of Masatoshi Takeichi, Kobe, Japan). For some immunoprecipitation experiments LNER cells were radiolabeled metabolically using [35S]methionine as described (Wahl et al., 2003).

Immunofluorescent light microscopy

Cells were grown on glass coverslips for 3-5 days. Fixation for vimentin immunofluorescence was done with ice-cold methanol for 5
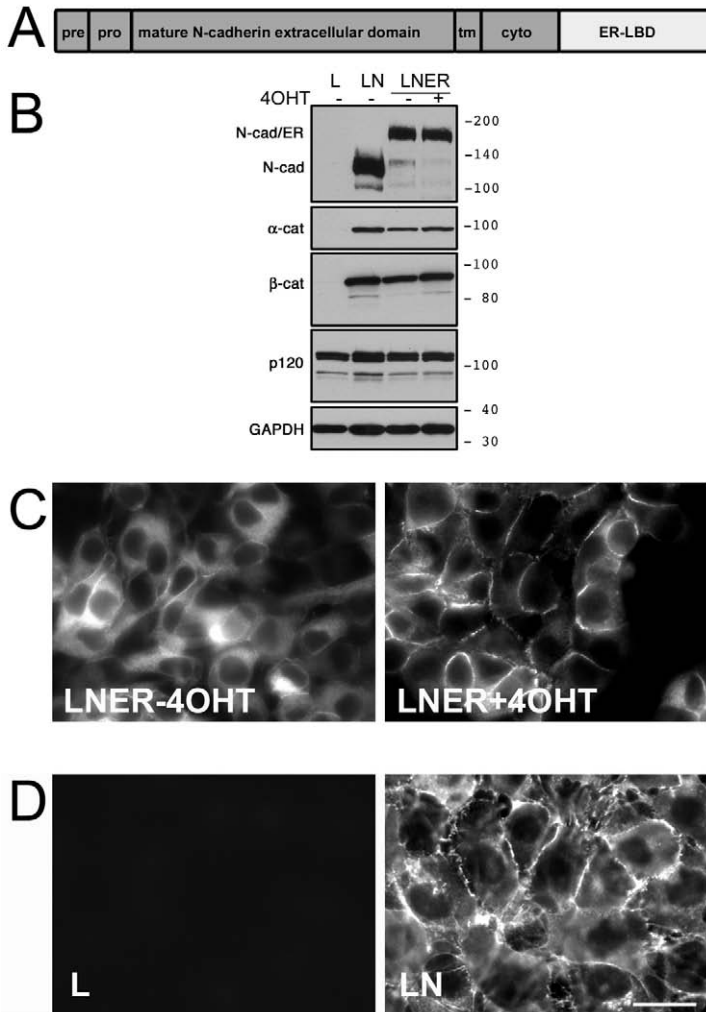
Fig. 1. N-cadherin/ER-LBD construct and its expression in L cells. (A) Full-length human N-cadherin (Ncad) cDNA fused at the 3′ end to a modified estrogen receptor ligand-binding domain (ER-LBD) that binds 4-hydroxytamoxifen (4OHT) but not 17β-estradiol. Pre and pro regions of the N-cadherin protein are cleaved off in cells to generate the mature N-cadherin. tm, transmembrane domain; cyto, cytoplasmic domain. (B) Western immunoblot analysis of N-cadherin, NcadER, α-catenin, β-catenin and p120^{ctn} with GAPDH as a control. L cells stably expressing the N-cadherin/ER-LBD construct (LNER) were treated with 100 nM 4OHT (+) or vehicle (−) for 5 hours. Non-transfected L cells (L) and L cells expressing wild-type N-cadherin (LN), in the absence of 4OHT (−), served as negative and positive controls, respectively. Cells were extracted with 0.5% NP40 and soluble proteins resolved by SDS-PAGE, transblotted electrophoretically to nitrocellulose and immunoblotted with antibodies to N-cadherin, the three catenins, and GAPDH. The parent L cells lacked endogenous N-cadherin, and α- and β-catenin were not detected in these cells as the proteins are degraded in the absence of a cadherin. Similar levels of NcadER (molecular weight ~150 kDa) were detected in LNER cells with or without 4OHT treatment. α- and β-catenin were detected in LN cells and at similar levels with or without 4OHT in LNER cells, suggesting formation of a cadherin/catenin complex under either condition. p120^{ctn}, which is stable in L cells lacking a cadherin, was present at similar levels in L, LN and LNER cells with or without 4OHT. Positions of molecular weight markers are indicated on the right. (C) Immunofluorescent light microscopic localization of NcadER in LNER cells with or without 4OHT. LNER monolayers plus or minus 100 nM 4OHT overnight were fixed and stained with anti-N-cadherin. The NcadER signal in cells with 4OHT appeared more organized and was particularly strong at sites of cell-cell contact, compared to cells without 4OHT. (D) Localization of N-cadherin in L cells and LN cells. As expected, L cells lacked N-cadherin, whereas LN cells exhibited strong N-cadherin staining, particularly at cell-cell borders. Bar, 50 μm.

minutes. Fixation for actin and tubulin immunofluorescence was done with 10% formaldehyde for 15 minutes followed by permeabilization with 0.2% Triton-X-100 for 15 minutes. Cells were fixed for N-cadherin and catenin immunofluorescence with ice-cold methanol for 10 minutes or Histochoice (Amresco, Solon, OH) for 15 minutes. Primary antibodies were diluted in 10% goat serum in phosphate-buffered saline (PBS) and applied to the cells at room temperature for 60 minutes. The cells were washed three times with PBS and exposed to Cy3- or FITC-conjugated, species-specific secondary antibodies for 60 minutes at room temperature. When indicated, live cells were exposed to 0.05% NP40 detergent for 1 minute at room temperature prior to being fixed and exposed to primary and secondary antibodies for antigen detection. Primary antibodies to N-cadherin included 13A9 (Knudsen et al., 1995) and 3B9 (Zymed Laboratories, South San Francisco, CA) developed by our laboratories. Other antibodies included: anti-β-catenin (15B8 or 5H10, BD PharMingen Transduction Laboratories, San Diego, CA), anti-plakoglobin (PG-11E4; Zymed Laboratories), anti-α-catenin (αCAT-7A4, Zymed Laboratories), monoclonal anti-p120 catenin (15D2, Zymed), polyclonal anti-p120 catenin (kind gift of Albert Reynolds, Vanderbilt University), anti-desmoplakin (multi-epitope cocktail to desmoplakin 1 and 2; Research Diagnostics, Flanders NJ), anti-vimentin (Vim 13.2; Sigma, St Louis, MO) and anti-actin (AC-15, Sigma). Rhodamine phalloidin used to detect filamentous actin (F-actin) was from Molecular Probes (Eugene, Oregon). Light microscopic images were acquired on a Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY) equipped with an ORCA-ER digital camera

(Hamamatsu, Houston, TX). Images were collected and processed using OpenLab software from Improvision (Boston, MA). Confocal images were acquired on an LSM 5 Pascal confocal microscope (Carl Zeiss, Thornwood, NY) by using a 63× 1.4 numerical aperture with appropriate filters.

Immunoblot analysis

Immunoblotting was performed essentially as described (Johnson et al., 1993). Briefly, cells were washed with phosphate-buffered saline, scraped from the culture dish, pelleted by centrifugation and extracted on ice in 10 mM Tris-acetate, pH 8.0, containing 0.5% Nonidet P-40 (NP40), 1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Cells were triturated vigorously and agitated for 20 minutes at 4°C. Non-solubilized material was removed by centrifugation at 14,000 *g* for 20 minutes at 4°C. Proteins in the supernatant fraction were separated by SDS-PAGE under reducing conditions, and for Western immunoblot analysis transblotted electrophoretically to nitrocellulose, which was then blocked with either 3% bovine serum albumin or 5% non-fat dry milk. Proteins were detected by the primary antibodies listed above, followed by alkaline phosphatase- or horseradish peroxidase-conjugated species-specific secondary antibodies (Southern Biotechnology Associates, Birmingham, AL) and substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT; Sigma) or Pierce supersignal substrate (Rockford, IL) as described (Radice et al., 2003; Maeda et al., 2005).

Immunoprecipitation and immunoblot analysis

Unlabeled or metabolically radiolabeled cells were extracted as described above for immunoblot analysis. Proteins were immunoprecipitated with specific antibodies as described (Wheelock et al., 1987; Knudsen et al., 1995; Wahl et al., 2003). Immunoprecipitates were resolved by SDS-PAGE. Radiolabeled bands were detected using Biomax MR1 film (Kodak). Non-radiolabeled immunoprecipitates were transferred to nitrocellulose and immunoblotted as described above for western immunoblot analysis. Primary and secondary antibodies were as listed above. When mouse monoclonal antibodies were used for immunoprecipitation and the immunoprecipitate subsequently immunoblotted using mouse antibodies, the heavy chain of the precipitating mouse antibody was routinely detected by the secondary anti-mouse IgG antibody. When immunoprecipitating vimentin, the cell extract was first centrifuged at 100,000 g for 60 minutes at 4°C then pre-cleared by incubating the cell extract with anti-mouse IgG beads (MP Biomedicals, Aurora, OH) for 60 minutes followed by a second incubation with anti-mouse IgG beads coated with anti-myc antibody (9E10; kindly provided by Kathleen Green, Northwestern University). The cleared cell extract was used for co-immunoprecipitation experiments. This was done to avoid the possibility that polymerized vimentin might contaminate the immunoprecipitates non-specifically during the immunoprecipitation procedure, which includes centrifugation steps.

Aggregation assays

Two types of aggregation assay were performed to evaluate cell-cell adhesion activity. In one case monolayer cultures were trypsinized, triturated into single cell suspensions, placed in suspension and mixed for up to 24 hours as described (Knudsen and Horwitz, 1978). In the second assay, trypsinized single cells were placed in hanging drop cultures for up to 24 hours as described (Redfield et al., 1997). At the end of the assay, cells and aggregates were collected by pipette, triturated to disperse loosely associated cells, visualized live by phase-contrast light microscopy and photographed.

siRNA targeting of mouse vimentin

Synthetic siRNAs were purchased from Dharmacon (Chicago, IL, M-061596-00-0005, mouse VIM, NM_011701). 7×10^4 LNER cells were transfected with 90 nM siRNA using siPort Amine (Ambion, Austin, TX) according to the manufacturer's instructions. Briefly, 90 nM mouse vimentin siRNA was mixed with siPort Amine/OPTI-MEM I (Gibco, Grand Island, NY) and incubated at room temperature for 10 minutes. The siRNA mixture was then added to a 12-well cell culture plate (90 nM of siRNA per well) followed by addition of 7×10^4 LNER cells. After overnight incubation at 37°C the transfection medium was replaced with HamsF12/DMEM (1:1) containing 10% fetal bovine serum. Target gene expression was assayed 96 hours after transfection with 90 nM RISC-Free siRNA (Dharmacon) as a control.

Results

### Characterization of L cells expressing the N-cadherin/ER-LBD (NcadER) fusion protein

The full-length human N-cadherin cDNA was fused at the 3′ end to the cDNA encoding a mutated estrogen receptor ligand-binding domain (ER-LBD) that binds 4-hydroxytamoxifen (4OHT) but not 17β-estradiol (Fig. 1A). The N-cadherin/ER-LBD construct was ligated into the pEMSVscribeα2 expression vector under the control of the Maloney murine sarcoma virus long terminal repeat and transfected into mouse

L fibroblast cells. Stable transfectants were selected, and the behavior of L cells expressing the N-cadherin protein fused at the C-terminus to the mutant ER-LBD (NcadER) was compared in the absence or presence of 100 nM 4OHT to activate the protein. The behavior of the NcadER-expressing L cells (LNER cells), plus or minus 4OHT treatment, was compared to that of parent L cells lacking a cadherin (L cells) and L cells expressing wild-type mouse N-cadherin (LN cells). The L cells were chosen for our studies because they lack an endogenous cadherin and have no other cell-cell adhesion mechanism (Nagafuchi et al., 1987).

Immunoblot analysis confirmed that the parent L cells lack N-cadherin (Fig. 1B). The L cells also lack α- and β-catenin, which are degraded in the absence of a cadherin (Nagafuchi et al., 1987). In contrast, the p120 catenin (p120^{ctn}) is stable in the absence of a cadherin (Thoreson et al., 2000) and was detected in the L cells (Fig. 1B). In the LN cells, which express wild-type N-cadherin, all three catenins, α-catenin, β-catenin and p120^{ctn}, were detected (Fig. 1B). The NcadER fusion protein was expressed by the LNER cells, and its level was not affected by 4OHT. In addition, all three catenins, α-catenin, β-catenin and p120^{ctn}, were detected in the LNER cells, and no significant difference was seen in the levels or molecular weights of these catenins when comparing the LNER cells treated or not with 4OHT. Plakoglobin was not detected (data not shown), consistent with its reported absence in L cells (Kowalczyk et al., 1996). Together, our data indicated that the cellular level of NcadER and catenins in LNER cells was not altered by 4OHT.

We examined localization of the NcadER in LNER cells with or without 4OHT treatment using immunofluorescent light microscopy (Fig. 1C). This revealed an interesting difference. In the absence of 4OHT, the NcadER signal was largely diffuse, whereas in its presence the NcadER staining was more organized and prominent at cell-cell borders. The pattern of NcadER in LNER cells treated with 4OHT closely resembled that in LN cells (Fig. 1D). As a control, the parent L cells lacked N-cadherin staining (Fig. 1D). The data suggested that 4OHT induces a change in the localization of the NcadER. We considered the possibility that in the absence of 4OHT the mutant cadherin might not be on the cell surface where it could promote cell-cell adhesion. To test this possibility we performed aggregation assays with LNER cells exposed or not to 4OHT, and compared their aggregate formation to that of parent L cells and LN cells expressing wild-type N-cadherin.

As expected, the parent L cells exhibited little or no propensity to adhere to one another (Fig. 2A). In contrast, the LN cells expressing wild-type N-cadherin formed tightly compacted cell aggregates (Fig. 2A). The LNER cells aggregated in the absence of 4OHT, indicating that even in the non-activated state the NcadER was present on the cell surface and was able to promote cell-cell adhesion (Fig. 2A). However, we noted that LNER cells without 4OHT treatment failed to form the tightly compacted aggregates formed by the LN cells. In contrast, the LNER cells treated with 4OHT, like the LN cells, formed tightly compacted aggregates (Fig. 2A). Compaction of cell aggregates was identified by two properties detected by phase-contrast light microscopy of live cells: (1) difficulty in discerning individual cells in the aggregate and (2) the presence of a phase-dense line at the periphery of the
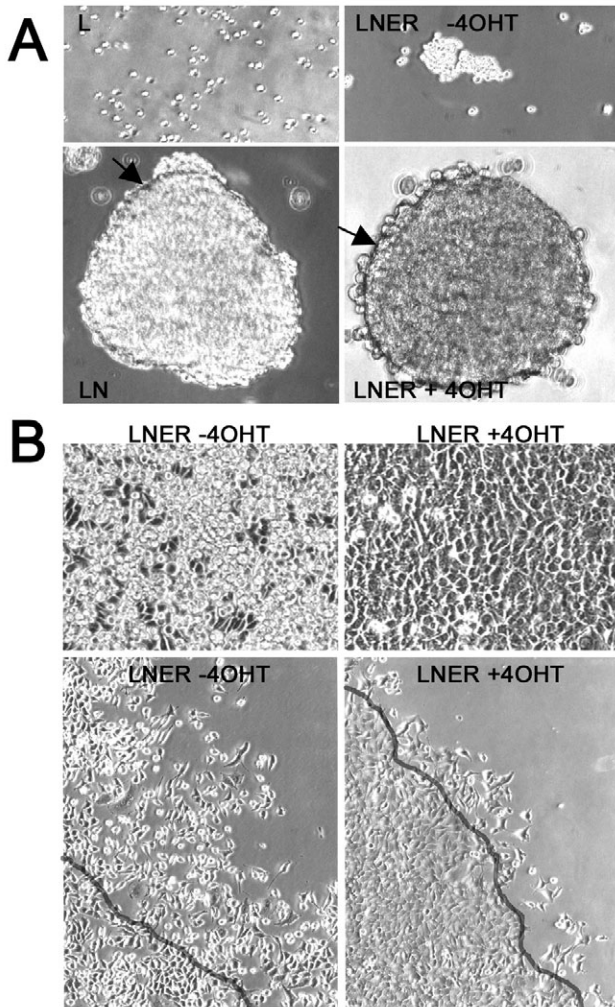
Fig. 2. Adhesive activity of NcadER activated or not by 4OHT. (A)
Aggregation of L cells, LN cells, and LNER cells with or without
4OHT treatment. Trypsin harvested cells were pipetted into a single
cell suspension and identical numbers placed in hanging drop
cultures, with LNER cells treated with or without 100 nM 4OHT.
After 24 hours the cells were collected, triturated and photographed
under phase-contrast microscopy. Non-transfected parent L cells (L)
failed to aggregate, whereas LN cells formed tightly compacted
aggregates. LNER cells without 4OHT (LNER – 4OHT) formed
large aggregates only partly dispersed by pipetting and where
individual cells were clearly discernable. LNER cells with 4OHT
(LNER + 4OHT) formed tightly compacted aggregates similar to
those formed by LN cells. Compacted aggregates were highly
resistant to dispersion by trituration, individual cells were difficult
to discern, and a phase-dense ring was observed around the
aggregate (arrows). (B) Behavior of LNER monolayers with or
without 4OHT. 50,000 cells were plated in 100 µl medium in wells
of six-well culture dishes. After the cells attached (~5 hours), the
dishes were gently flooded with 2 ml medium, with or without 100
nM 4OHT, and the cells were observed for 3 days. The cultures
were photographed within (top panels), and outside of (bottom
panels) the original plating area. The line defines the edge of the
plating area, which is left of the line. LNER cells without 4OHT
piled up on one another in the plating area, detached, and eventually
seeded new colonies outside the plating area. In the presence of
4OHT the LNER cells stayed predominantly as a monolayer within
the plating area, and seeded far fewer colonies outside this area,
compared to cells without 4OHT.

aggregate (arrows Fig. 2A). Compaction followed aggregation
in time, with aggregation of LNER cells detectable within ~30
minutes and compaction barely detectable by ~2 hours,
obvious by 6 hours and maximal by 12-24 hours. The
difference in the behavior of the LNER cells with or without
4OHT treatment suggested that cadherin-mediated adhesion
could be separated functionally into two processes, aggregation
followed by a strengthening of adhesion detected as
compaction.

A similar difference in the strength of LNER cell interaction
depending on 4OHT treatment was observed in monolayer
culture. Cells with or without 4OHT were plated at high
density (50,000 cells/100 µl medium) in a small circle in the
center of the well of a six-well culture plate. After the cells
attached to the plastic, the wells were gently flooded with 2 ml
medium, and the cells were observed for 3 days. In the absence
of 4OHT, the LNER cells piled up on one another, and although
most cells remained attached to one another in the original area
of plating, many were observed outside the area of plating. On
the other hand, the LNER cells treated with 4OHT remained
predominantly as a dense monolayer within the area of plating
(Fig. 2B). The cells did not pile up on one another and few
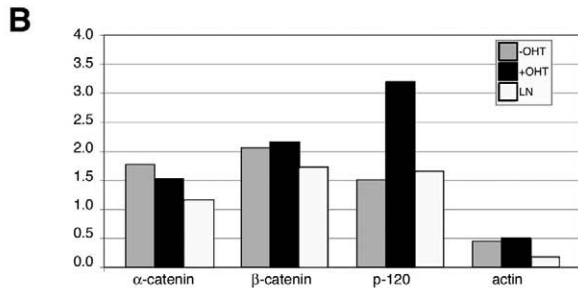cells were found outside the original plating area.

Analysis of proteins interacting with NcadER in LNER cells with or without 4OHT

Our data suggested that 4OHT induced a strengthening of
LNER cell-cell adhesion mediated by NcadER. To begin to
understand the mechanism involved we conducted a series of
experiments. We initially considered the possibility that the
ER-LBD, being located at the C-terminus of the N-cadherin,
altered β- and or α-catenin binding. Reduced α- and β-catenin
binding to NcadER would be predicted to affect its linkage to
actin, thereby altering the strength of adhesion. One argument
against this idea was that α- and β-catenin were stabilized at
similar levels in LNER cells, with or without 4OHT (Fig. 1B).
Nevertheless, to test the interaction of the mutant N-cadherin
with β- and α-catenin we immunoprecipitated the NcadER
from LNER cells treated with or without 4OHT, using anti-N-
cadherin antibodies. We then immunoblotted the
immunoprecipitate with antibodies to α-catenin, β-catenin and
actin, as well as p120[ctn], which is known to regulate cadherin
adhesive strength (Yap et al., 1998; Ohkubo and Awawa, 1999;
Thoreson et al., 2000). As a control, we immunoprecipitated
wild-type N-cadherin from the LN cells (Fig. 3A). Using wild-
type or mutant N-cadherin as a reference point, similar levels
of α- and β-catenin and actin, were detected in the
immunoprecipitates, comparing LN cells and LNER with or
without 4OHT treatment (Fig. 3A,B). Interestingly, the level of
p120[ctn] in immunoprecipitates of LNER cells treated with
4OHT was approximately twice that in immunoprecipitates of
LNER cells without 4OHT (Fig. 3A), although it was also
higher than for the LN cells. Thus, the level of p120[ctn] in the
immunoprecipitate relative to N-cadherin did not strictly
correlate with the compaction phenotype.

We also considered the possibility that compaction might
involve new protein synthesis or a new protein(s) docking to
the NcadER/catenin complex. Neither of these possibilities
was supported by our experiments. Treating the LNER cells
with cycloheximide to block protein synthesis had no effect on
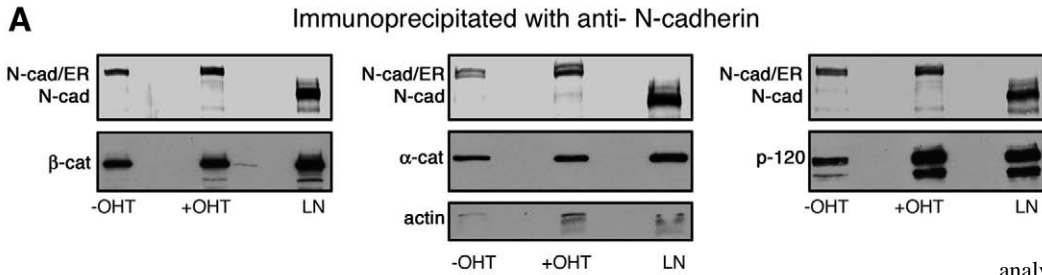
Fig. 3. Proteins co-immunoprecipitating with NcadER activated or not by 40HT. (A) Immunoblot analysis of N-cadherin immunoprecipitates from LN cells and LNER cells with or without 4OHT treatment. LNER cells without (–) or with (+) 100 nM 4OHT overnight were extracted with 0.5% NP40 and the NcadER immunoprecipitated with anti-N-cadherin using identical conditions. N-cadherin was immunoprecipitated from LN cells as a positive control. The immunoprecipitates were resolved by SDS-PAGE, transblotted to nitrocellulose and immunoblotted with antibodies to N-cadherin, β-catenin, α-catenin, actin and p120[ctn]. (B) Quantification of N-cadherin co-immunoprecipitating proteins normalized to N-cadherin. Means of two separate experiments are shown. (C) N-cadherin immunoprecipitation of ^{35}S-radiolabeled LNER cells, treated or not with 4OHT, analyzed by SDS-PAGE and autoradiography. The control lane was processed using hybridoma supernatant without
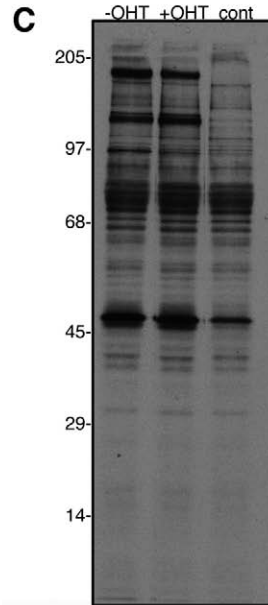
compaction, although with increasing time of exposure it decreased cell aggregation (data not shown). Metabolic radiolabeling of LNER cells with [^{35}S]methionine followed by immunoprecipitation of the NcadER and subsequent detection of radiolabeled bands in the immunoprecipitate by autoradiography revealed no striking differences comparing LNER cells with or without 4OHT treatment (Fig. 3C). These data suggested that if a change in the composition of cadherin/catenin complex occurs upon NcadER activation by 4OHT it is not reflected in immunoprecipitates of proteins solubilized by NP40 detergent. It remained possible that molecular changes of interest were to be found in the NP40-insoluble cadherin complex, i.e. that tightly bound to the cytoskeleton. It also remained possible that a slowly turning over protein was not radiolabeled under our conditions, and therefore was not detected.

Differences in association of NcadER with the cytoskeleton in LNER cells with or without 4OHT treatment

We considered that 4OHT might induce compaction by increasing the interaction of the NcadER/catenin complex with the cytoskeleton. It is well established that linkage to actin is involved in strengthening cadherin-mediated adhesion (Kemler, 1993). To probe for a 4OHT-induced increase in the

linkage of the NcadER/catenin complex to the cytoskeleton we briefly (1 minute) exposed live LNER cells, with or without prior 4OHT treatment, to a low concentration of NP40 (0.05%) and then fixed and stained the cells for NcadER. Little NcadER signal remained after NP40 treatment of LNER cells lacking 4OHT treatment (Fig. 4A). In contrast, the NcadER signal was strong and noticeably present at cell-cell borders in LNER cells treated with 4OHT (Fig. 4B). The results indicated that 4OHT activation of the NcadER rendered the mutant cadherin less soluble in NP40, which is generally thought to denote increased interaction of a cadherin with the cytoskeleton. Initially we thought that this must involve the actin cytoskeleton, although we saw no significant difference in actin present in NcadER immunoprecipitates from LNER cells, plus or minus 4OHT (Fig. 3B).
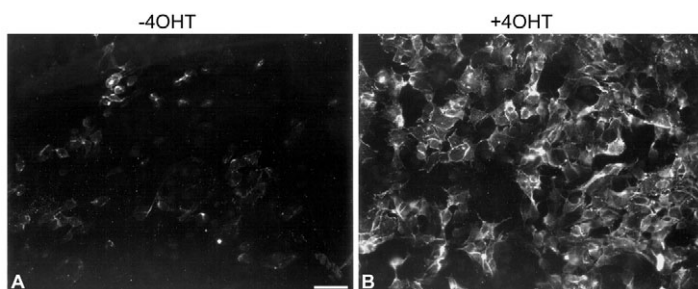
Fig. 4. Immunofluorescence light microscopic detection of NcadER following low concentration NP40 treatment of living LNER cells treated or not with 4OHT. Monolayer cultures of LNER cells previously treated or not with 100 nM 4OHT for 24 hours were exposed to 0.05% NP40 for 1 minute at room temperature prior to fixing and staining the cells with anti-N-cadherin. In the absence of 4OHT treatment much of the NcadER was removed by NP40 treatment of live cells (A). In contrast, the NcadER signal in LNER cells treated with 4OHT was significantly greater and was apparent at cell-cell borders (B). Bar, 50 μm.

Aggregation **Phalloidin**
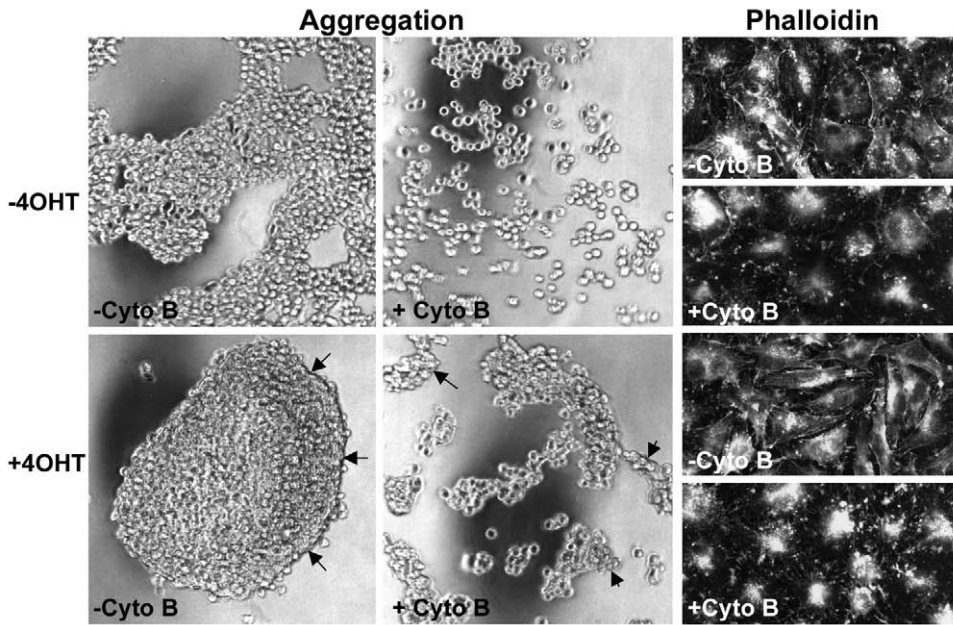
Fig. 5. Effect of cytochalasin B on aggregation and compaction of LNER cells treated or not treated with 4OHT. LNER cells were trypsinized and placed in suspension culture in the presence of 1 μM cytochalasin B, with (+) or without (−) 4OHT, for 24 hours. Cytochalasin B disrupted F-actin as evidenced by phalloidin staining (right panels). In the absence of 4OHT cytochalasin B had a negative impact on LNER aggregation, when compared to cells without either agent. In the presence of 4OHT the negative effect of cytochalasin B on LNER cell aggregation was attenuated. Moreover, the line of phase density (arrows) seen in compacted aggregates of 4OHT-treated LNER cells without cytochalasin B were observed in the smaller aggregates formed by LNER cells treated with both 4OHT and cytochalasin B.

As another approach to examining a possible role for actin in the 4OHT-induced strengthening of cell-cell adhesion, we treated LNER cells, plus or minus 4OHT, with cytochalasin B to disrupt actin filaments. Fluorescence microscopy confirmed that cytochalasin B treatment disrupted the actin cytoskeleton as detected by phalloidin staining (Fig. 5). In the absence of 4OHT treatment, the cytochalasin B had a striking negative impact on the aggregation of LNER cells, indicating that this aspect of cell-cell adhesion requires an intact actin cytoskeleton. However, the effect of cytochalasin B on LNER cells treated with 4OHT was tempered. Aggregation of these cells (i.e. +4OHT, +cytoB) was greater than that of cytochalasin B-treated LNER cells without 4OHT, although it was less than that of 4OHT-treated cells without cytochalasin B (Fig. 5). Moreover, the aggregates of 4OHT+cytochalasin B-treated LNER cells that did form showed evidence of compaction, as detected by the presence of the characteristic phase-dense lines at the periphery of aggregates (arrows).

As another way of testing the role of actin in LNER cells we examined the cellular localization of the cadherin/catenin complex in a monolayer of cells whose actin cytoskeleton was disrupted with cytochalasin B in the absence versus presence of 4OHT. We stained the cells for β-catenin as a marker of the NcadER/catenin complex. Without 4OHT treatment, the β-catenin staining in LNER cells treated with cytochalasin B appeared diffuse and weak, with little staining at cell-cell borders (Fig. 6A), perhaps suggesting that without an intact

actin cytoskeleton the non-activated NcadER/catenin complex was partially lost during fixation and staining of the cells. In contrast, in cytochalasin B-treated LNER cells exposed to 4OHT, the β-catenin staining was strong and well organized, with staining obvious at cell-cell borders (Fig. 6B). Together, the results presented in Figs 5 and 6 suggested that more than an interaction with actin is involved in linking NcadER to the cytoskeleton, strengthening cell-cell adhesion, and promoting the compaction observed in the 4OHT-treated LNER cells

We considered the possibility that, although actin was involved in aggregation, another cytoskeletal system was involved in compaction. To begin to explore this possibility we performed immunofluorescent light microscopy on monolayer cultures of LNER cells treated or not with 4OHT. We reasoned that we might detect a change in the organization of a particular cytoskeletal system if it interacted differently with the NcadER activated by 4OHT compared to the non-activated cadherin. We looked at F-actin, tubulin, and vimentin as markers of the actin, microtubule, and intermediate filament systems, respectively. No change was detected in the F-actin pattern of LNER cells upon 4OHT treatment as detected under lower magnification using conventional fluorescence microscopy (Fig. 7A) or higher magnification using confocal microscopy (Fig. 7B). This was predicted, as our data did not support a role for actin in 4OHT-induced compaction. Nor was there any noticeable difference in the tubulin pattern at low or high magnification, suggesting the microtubule system was not
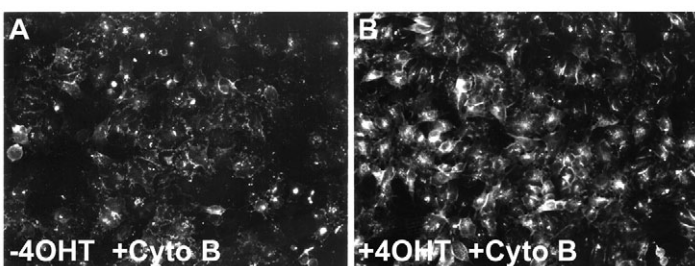
Fig. 6. Immunofluorescence light microscopic analysis of the effect of cytochalasin B on β-catenin in LNER cells with or without 4OHT. LNER cells with or without 4OHT treatment were cultured in the presence of 1 μM cytochalasin B (Cyto B) to disrupt the actin cytoskeleton (see Fig. 5). The cells were fixed and stained with anti-β-catenin to detect the NcadER/catenin complex. In LNER cells treated with Cyto B, but not 4OHT, the β-catenin staining was weak and not particularly distinct at cell-cell borders (A). However, in cells treated with 4OHT and Cyto B, the β-catenin staining was strong and distinct at cell-cell borders (B).

involved in compaction. On the other hand, the vimentin pattern was noticeably different comparing LNER cells with or without 4OHT treatment. In the absence of 4OHT much of the vimentin signal was perinuclear in the LNER cells, although vimentin extensions in the cell body were observed in some cells (Fig. 7A,B). In the presence of 4OHT the vimentin appeared more filamentous and more extended to the cell periphery (Fig. 7A,B). These data suggested to us that perhaps vimentin intermediate filaments were associating with the cadherin/catenin complex at the cell surface and were involved in strengthening the cadherin-mediated adhesion.

The increase in vimentin organization in LNER cells treated with 4OHT, compared to cells lacking 4OHT (Fig. 7 and Fig. 8A), did not result from a change in the level of vimentin. To determine this we extracted 4OHT- or vehicle-treated LNER cells with Laemmli sample buffer containing 2% SDS, as vimentin is only partially extracted with NP40. By immunoblot analysis no difference in the vimentin signal was noted between LNER cells with or without 4OHT (Fig. 8B). In addition, the better organized vimentin pattern seen in the 4OHT-treated LNER cells did not depend on the presence of the ER-LBD as the pattern was similarly organized in LN cells expressing wild-type N-cadherin (Fig. 8A). Conversely, the vimentin pattern in LNER cells lacking 4OHT treatment was more like the pattern in the parent L cells lacking a cadherin (Fig. 8A). Thus, the more organized vimentin pattern correlated with the compaction phenotype exhibited by both 4OHT-activated LNER cells and LN cells expressing wild-type N-cadherin.

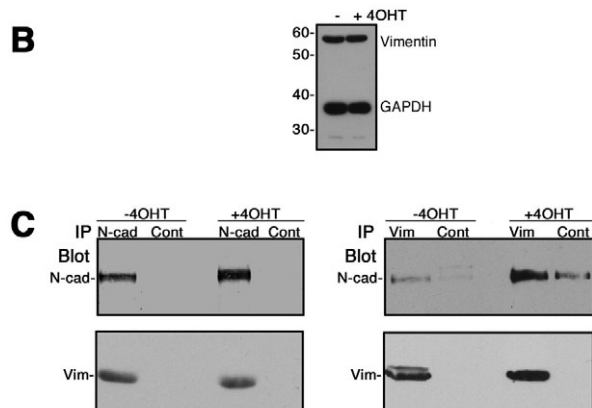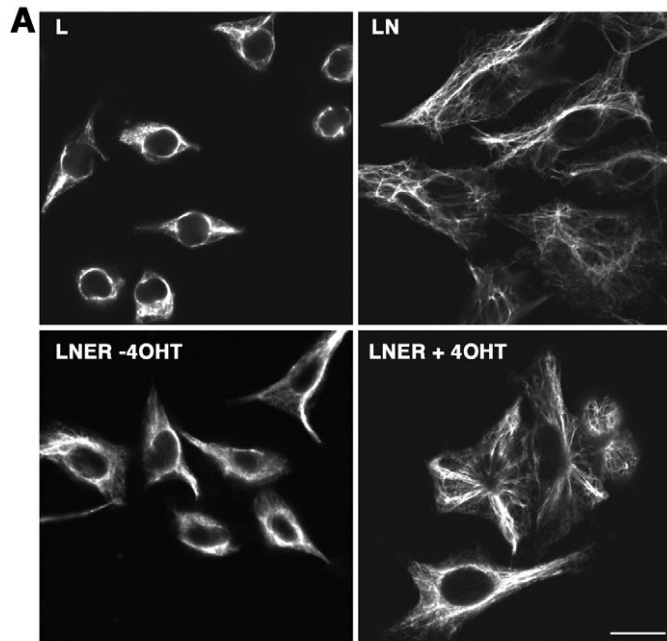### Evidence for a role for vimentin in strengthening cell-cell adhesion

Our data suggested that upon 4OHT activation NcadER might interact with vimentin intermediate filaments, thereby strengthening cell-cell adhesion. To test this idea we determined if vimentin co-immunoprecipitated with the mutant N-cadherin isolated from LNER cells treated or not with 4OHT. This was accomplished by immunoprecipitating NcadER or vimentin and subsequently immunoblotting for both proteins. When anti-N-cadherin was used to isolate the cadherin/catenin complex from LNER cells, vimentin was detected by immunoblotting (Fig. 8C). Conversely, NcadER was detected in vimentin immunoprecipitates, suggesting that vimentin interacts with the cadherin/catenin complex. The data were not entirely clear, however, because we expected to see more NcadER co-immunoprecipitating with vimentin in the case of the LNER cells treated with 4OHT, compared to cells
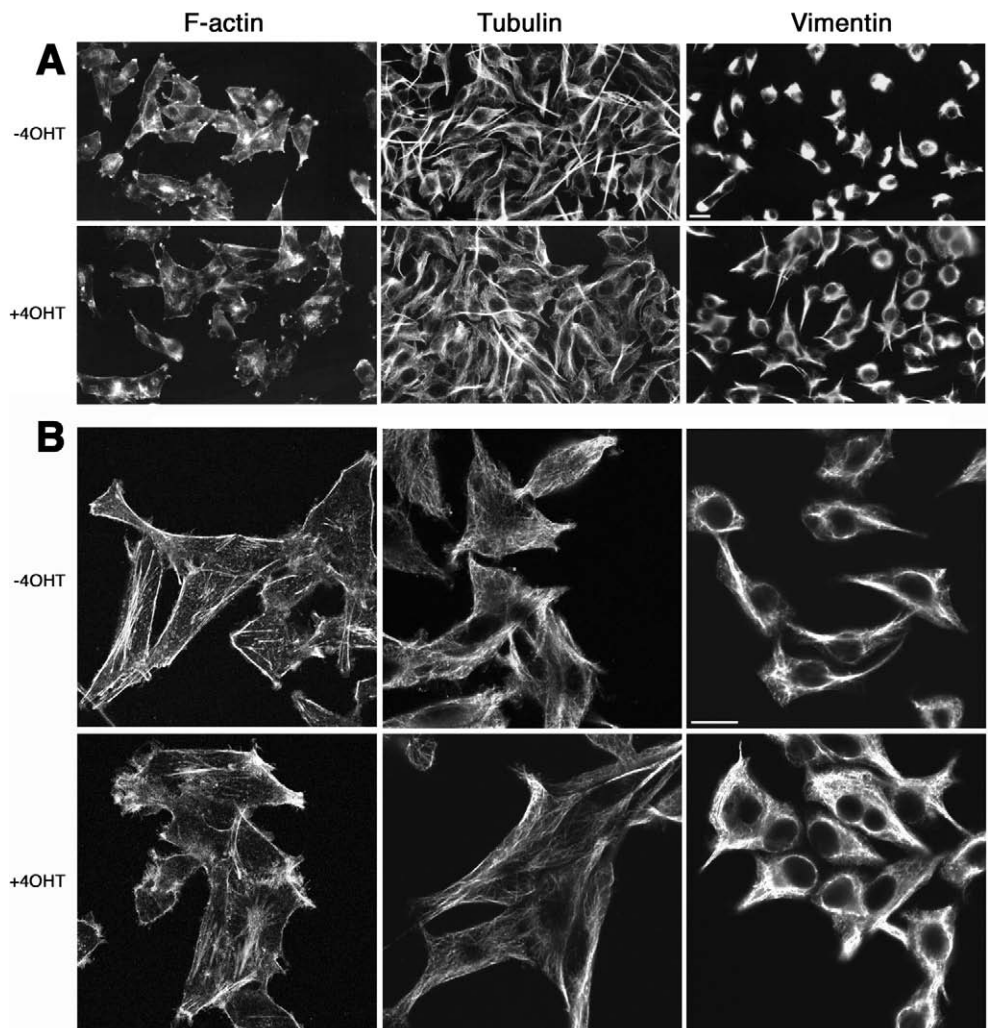
Fig. 7. Immunofluorescent light microscopic localization of F-actin, tubulin and vimentin in LNER cells treated or not with 4OHT. Monolayer cultures of LNER cells were treated with (+) or without (−) 100 nM 4OHT for 24 hours prior to being fixed and stained for F-actin, tubulin and vimentin as markers of the actin, microtubule, and intermediate filament systems, respectively. Rhodamine-phalloidin was used to detect F-actin, whereas antibodies were used to detect tubulin and vimentin. (A) Cells at low magnification. (B) Cells at higher magnification using confocal microscopy. Although no differences were apparent in F-actin or tubulin staining in LNER cells treated or not treated with 4OHT, the vimentin appeared better organized in the 4OHT-treated cells, compared to cells without 4OHT. Bar, 50 μm.
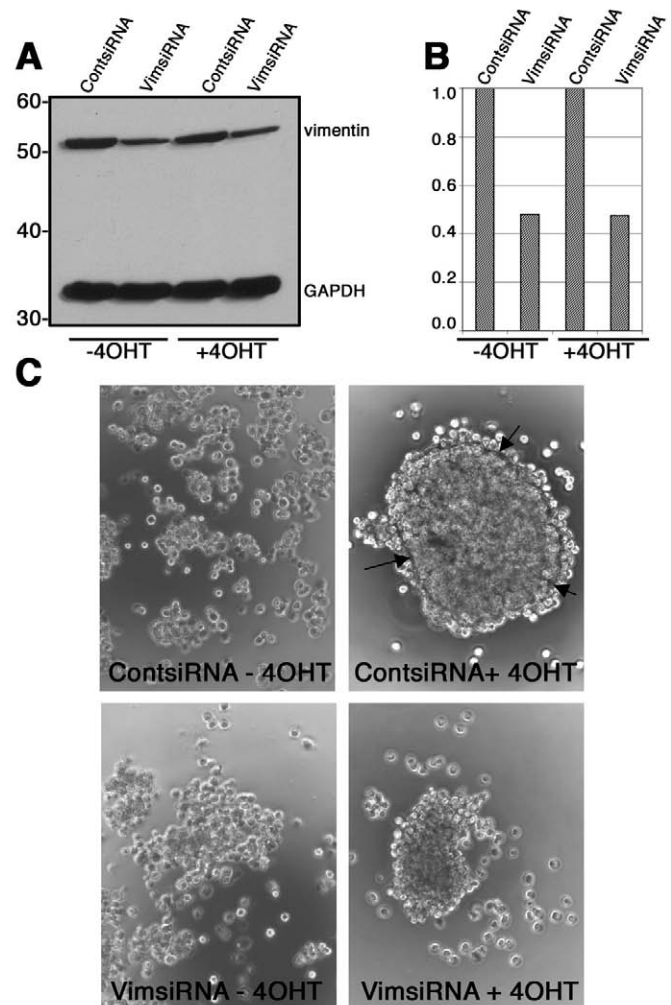
without 4OHT. These co-immunoprecipitations did not consistently bring down equivalent amounts of the co-immunoprecipitating protein and we think this is because we could only immunoprecipitate the vimentin that was in the NP40-soluble fraction, whereas the interactions we are most interested in might reside in the NP40-insoluble fraction.

Our data supported an interaction between NcadER and vimentin, and a role for vimentin intermediate filaments in strengthening cadherin-mediated cell-cell adhesion. To more directly test the role of vimentin intermediate filaments in aggregate compaction we knocked down vimentin using an siRNA strategy. LNER cells were transiently transfected with siRNAs directed against mouse vimentin, or with control siRNAs. The transfected cells were then evaluated for their levels of vimentin by immunoblot analysis and for their ability to aggregate and to form compacted aggregates (Fig. 9).

Fig. 8. Evidence for vimentin association with the cadherin/catenin complex. (A) Confocal microscopic localization of vimentin in L, LN and LNER cells treated or not treated with 4OHT. Monolayer cultures of parent L cells, LN cells expressing N-cadherin and LNER cells without (–) or with (+) 4OHT were fixed and stained with anti-vimentin. The vimentin in L cells, which lack a cadherin and do not aggregate, was largely perinuclear. In LN cells, which aggregate and compact, the vimentin was organized into filaments that extended throughout the cell body. In LNER cells lacking 4OHT treatment, which aggregate but do not compact, the vimentin pattern more closely resembled that in the L cells. In contrast, in LNER cells treated with 4OHT, which aggregate and compact, the vimentin pattern was closer to that seen in the LN cells. (B) Immunoblot analysis of vimentin in LNER cells with or without 4OHT. GAPDH was included as a sample loading control. (C) Immunoblot analysis of N-cadherin (N-cad) and vimentin (Vim) immunoprecipitates from LNER cells with or without 4OHT treatment. Control (Cont) was monoclonal anti-myc. Bar, 50 μm.


Fig. 9. siRNA knock down of vimentin and its effect on the aggregation and compaction of LNER cells treated or not with 4OHT. (A) Immunoblot analysis of vimentin expression in LNER cells treated or not with 4OHT and transfected with control or vimentin siRNAs. GAPDH served as a sample loading control. (B) Quantification of the vimentin signal (means of two experiments), showing a similar knockdown of vimentin (~50%) by vimentin siRNA, compared to control siRNA, in LNER cells treated or not with 4OHT. (C) Aggregation/compaction assay of LNER cells with or without 4OHT treatment and transfected with control or vimentin siRNAs. Control siRNA had no effect on the aggregation of LNER cells lacking 4OHT treatment, or on the aggregation or compaction (arrows) of LNER cells treated with 4OHT. Nor did vimentin siRNAs affect the aggregation of LNER cells lacking 4OHT treatment. In contrast, vimentin siRNAs inhibited the compaction of aggregates of 4OHT-induced LNER cells.

Similar vimentin levels were detected in LNER cells transfected with control siRNAs, whether or not the cells were exposed to 4OHT. In contrast, vimentin siRNAs knocked down the vimentin level by approximately 50% in LNER cells, regardless of 4OHT treatment (Fig. 9A,B). Neither control nor vimentin siRNAs affected the aggregation of LNER cells lacking 4OHT treatment (Fig. 9C). In contrast, vimentin siRNAs, but not control siRNAs, inhibited compaction of aggregates of LNER cells treated with 4OHT. This was determined by the absence of the characteristic phase-dense line at the periphery of compacted aggregates of 4OHT-treated, vimentin siRNA-transfected LNER cells, as was typically seen for 4OHT-activated LNER cells treated with control siRNAs (Fig. 9, arrows). Thus, our data show that with ~50% knockdown of vimentin protein, the 4OHT-treated LNER cells were unable to compact, providing strong evidence that vimentin plays an important role in the compaction process.

The question then arose as to the mechanism of interaction between NcadER and vimentin. As the NcadER immunoprecipitates from 4OHT-treated LNER cells had increased p120^{ctn} (Fig. 3), compared to non-4OHT-treated LNER cells, we considered the possibility that vimentin might interact with the cadherin through p120^{ctn}. We first examined the localization of p120^{ctn} in LNER cells in the presence or absence of 4OHT (Fig. 10A). Interestingly, p120^{ctn} was more diffuse in the absence of 4OHT and better organized at cell-cell borders in the presence of 4OHT. Although the difference in localization was not large, we thought it might be significant in light of the data shown in Fig. 3. Thus, we hypothesized that p120^{ctn} may serve as a link between N-cadherin and vimentin and predicted they would co-immunoprecipitate. To test this possibility we turned to the parent L cells because these cells express vimentin and p120^{ctn}, but do not have a cadherin, or α- and β-catenin, whose presence would confound the interpretation of co-immunoprecipitation data. Vimentin and p120^{ctn} co-immunoprecipitated from L cells, suggesting that there is a direct or indirect interaction between the two proteins (Fig. 10B). To confirm that p120^{ctn} and vimentin were in a similar location in L-cells, we carried out colocalization studies. As the L cells do not have a cadherin, we expected the p120^{ctn} to be diffuse, and it was. However, there was frequently a noticeable concentration of p120^{ctn} in the region of the cell where the vimentin was most highly stained (Fig. 10C, arrows). Moreover, in many cases the vimentin appeared to spread out from the area of p120^{ctn} density.
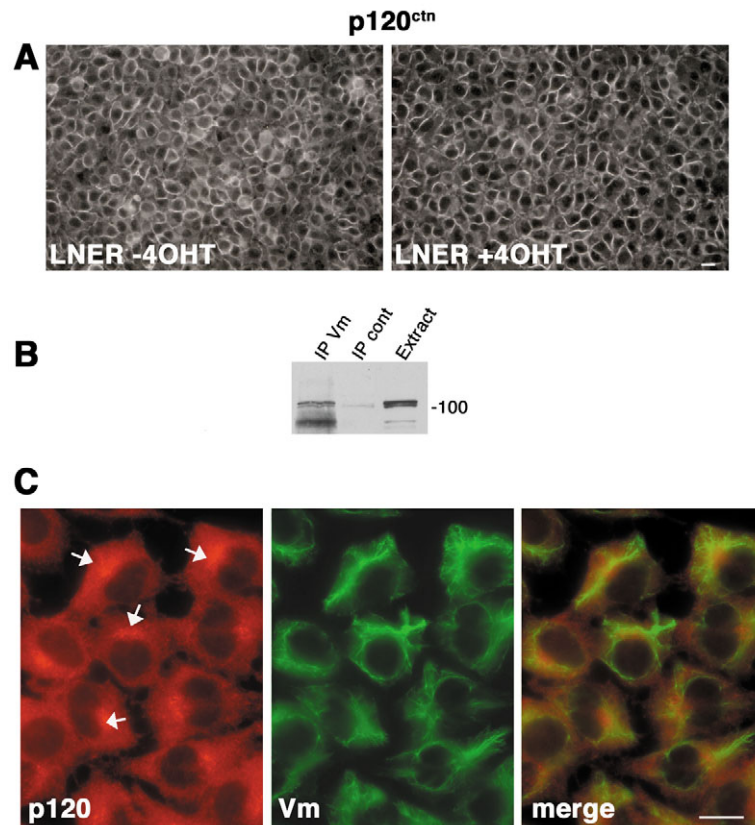
Fig. 10. Effect of 4OHT on p120^{ctn} localization in LNER cells and association of p120^{ctn} and vimentin in L cells. (A) Immunofluorescent light microscopic localization of p120^{ctn} in LNER cells treated (+) or not (−) with 4OHT. (B) Immunoblot analysis of p120^{ctn} in immunoprecipitates from non-transfected parental L cells immunoprecipitated with anti-vimentin (Vm) or control (Cont) anti-myc antibody. Immunoblot analysis of L cell extract (Extract) indicates the position of p120^{ctn}. (C) Non-transfected parental L cells were processed for immunofluorescence using rabbit anti-p120^{ctn} (p120) and mouse anti-vimentin (Vm) followed by Cy3-labeled anti-rabbit IgG and FITC-labeled anti-mouse IgG. Arrows indicate regions of the cell where both p120^{ctn} and vimentin are abundant and colocalize. Bar, 50 μm.

Discussion

The composition of multiprotein complexes comprising various cell-cell junctions is quite well understood (Wheelock and Johnson, 2003). In the case of adherens junctions, a transmembrane classical cadherin binds intracellularly to β-catenin (or plakoglobin), which in turn interacts with α-catenin and via α-catenin with actin (Knudsen et al., 1995; Rimm et al., 1995). In the case of desmosomes, desmocollin and desmoglein bind to plakoglobin, which in turn binds to desmoplakin and via desmoplakin to intermediate filaments. Cell-cell adhesion frequently involves more than one type of junction. For example, epithelia and the myocardium have both adherens junctions and desmosomes. Moreover, different junction types can share adhesion proteins. For example, plakoglobin is found in both adherens junctions and desmosomes in epithelial cells. Moreover, VE-cadherin is present in both actin-based adherens junctions and a specialized junction in endothelial cells where VE-cadherin interacts with the vimentin intermediate filament cytoskeleton via plakoglobin and desmoplakin (Kowalczyk et al., 1998). The strength of cell-cell adhesion is regulated by the presence of different types of junction, as well as their molecular composition. In particular, desmosomes are important for the integrity of tissues that are subject to mechanical stress, such as the heart and skin. From our current understanding, we can speculate that the composition of adhesion complexes and types of junctions that form will vary depending on the cell type and the particular repertoire of adhesion-related proteins expressed.

Here we present strong evidence that a novel classical cadherin adhesion system strengthens the

interaction of fibroblasts. Expression of a mutant, hormone-activated N-cadherin in mouse fibroblasts has revealed a vimentin-based cadherin adhesion complex that cooperates with the traditional actin-based complex to promote strong cell-cell adhesion. N-cadherin fused at the C-terminus to a mutant estrogen receptor ligand-binding domain (NcadER) that binds 4-hydroxytamoxifen (4OHT) was stably expressed in mouse L cells, which lack an endogenous cadherin. L cells expressing the NcadER (LNER cells) formed aggregates in suspension with or without 4OHT treatment. However, addition of 4OHT strengthened the adhesion of the LNER cells, causing the aggregates to compact, similar to L cells expressing wild-type N-cadherin (LN cells).

Compaction of 4OHT-treated LNER cells followed aggregation in time and did not correlate with a change in the cellular level of cadherin or catenins, including α-catenin, β-catenin and p120^{ctn}. Moreover, typical cadherin/catenin complexes formed in LNER cells, with or without 4OHT treatment, although when normalized to the NcadER, more p120^{ctn} was present in immunoprecipitates from 4OHT-treated cells, compared to non-treated cells. In addition, 4OHT-induced compaction of LNER cells correlated with enhanced interactions between the mutant N-cadherin and the cytoskeleton. Aggregation of LNER cells required an intact actin cytoskeleton, consistent with the known interaction of classical cadherins with the actin cytoskeleton, and its role in promoting cell-cell adhesion. In contrast, compaction clearly involved more than the actin cytoskeleton, which led us to consider other cytoskeletal systems. We noted that vimentin became more organized when the LNER cells were treated with 4OHT, compared to non-treated cells. Moreover, better-organized vimentin correlated with the compaction phenotype exhibited by both 4OHT-treated LNER cells and LN cells expressing wild-type N-cadherin. These data suggested that the vimentin intermediate filament system might interact with the cadherin to bring about compaction.

A physical interaction between vimentin and NcadER was supported by their co-immunoprecipitation from LNER cells. Moreover, a vital role for vimentin in compaction was shown by using siRNAs to knockdown vimentin. Decreasing vimentin by approximately 50% inhibited compaction, while having no effect on aggregation, which is actin-dependent. These data provide strong evidence that vimentin is involved in the compaction process. The mechanism by which vimentin interacts with cadherin appears to involve p120^{ctn}, as vimentin and p120^{ctn} co-immunoprecipitate even when a cadherin and α- and β-catenin are absent. Whether the interaction of vimentin and p120^{ctn} is direct or involves some linker protein is a topic for future study, as is regulation of the interaction by phosphorylation. We do know that plakoglobin is not involved in linking the cadherin to vimentin, as it is in endothelial cells (Kowalczyk et al., 1998), since the L cells do not express plakoglobin.

In summary, we propose that classical cadherins can form two types of adhesion complex in fibroblasts. One complex is comprised of the cadherin linked through β- and α-catenin to the actin cytoskeleton. This actin-based cadherin complex promotes the aggregation of cells. A second adhesion complex is comprised of the cadherin linked through p120^{ctn} to vimentin intermediate filaments. This adhesion system promotes compaction of fibroblast aggregates and may be functionally analogous to desmosomes in epithelial cells. Together, the actin- and vimentin-based classical cadherin adhesion systems initiate and strengthen cell-cell adhesion in fibroblasts, and perhaps other cell types.

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