Vinculin Is Associated with the E-cadherin Adhesion Complex*

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Cadherins mediate calcium-dependent cell-cell adhesion, and this activity is regulated by cytoplasmic interactions between cadherins, catenins, and the actin-based cytoskeleton. α-Catenin plays a critical role in the transmembrane anchorage of cadherins, and deletion of α-catenin has been shown to inactivate cadherin-mediated adhesion, resulting in a nonadhesive phenotype. Here we show that serum starvation increases E-cadherin expression and induces E-cadherin-dependent adhesion in the MDA-MB-468 breast cancer cell line. This adhesion occurred despite a lack of α-catenin expression, which was caused by mutations in the α-catenin gene. Coprecipitation analysis suggests that this adhesion may be mediated by cytoplasmic connections from cadherins to the cytoskeleton involving vinculin. A high level of vinculin associated with E-cadherin immunoprecipitates was observed in MDA-MB-468 cells. In contrast, vinculin was not detected in E-cadherin complexes in the A431 and MCF-7 epithelial carcinoma cell lines, which express α-catenin. However, in reciprocal immunoprecipitations using anti-vinculin antibodies, E-cadherin associated strongly with vinculin in MDA-MB-468 cells and, to a lesser extent, in A431 and MCF-7 cells. These results suggest that both α-catenin and vinculin may be present in the adhesion complex. To test the hypothesis that vinculin associates with E-cadherin complexes via β-catenin, excess recombinant β-catenin or α-catenin fusion protein was added to MDA-MB-468 cell lysates. Both specifically inhibited the coprecipitation of E-cadherin with vinculin, suggesting competition for the same binding site. These results suggest that vinculin plays a role in the establishment or regulation of the cadherin-based cell adhesion complex by direct interaction with β-catenin.

Cadherins are calcium-dependent cell-cell adhesion molecules that mediate homotypic interactions among cells and are essential for tissue morphogenesis (for review, see Refs. 1 and 2). Adhesion via cadherins involves the coordination of extracellular binding and intracellular anchorage to the actin-based cytoskeleton. The cytoplasmic domain of E-cadherin binds to either β-catenin or plakoglobin/γ-catenin (for review, see Refs. 3–5), and this complex is coupled to the actin cytoskeleton by α-catenin, which binds to both β-catenin and actin (6, 7). There is evidence that α-catenin also binds to α-actinin (8, 9) and spectrin (10), but the role of these interactions in adhesion is unknown. Disruption of α-catenin function by genetic deletion or mutation was shown to cause a loss of E-cadherin-dependent adhesion. PC-9 lung carcinoma cells, which lack detectable α-catenin expression, were shown to have aberrant cell-cell adhesion (11) that was restored by transfection of these cells with α-catenin cDNA (12). This resulted in the establishment of a polarized epithelium and inhibition of cell growth (13). This and other work suggest that the linkage of cadherin complexes to the cytoskeleton via α-catenin is essential for normal cell adhesion, morphogenesis, and cell growth.

α-Catenin shares sequence homology with vinculin (14, 15), a protein that is found in both focal contacts and adherens junctions (16, 17). Both α-catenin (6–9) and vinculin (18–24) are capable of binding to α-actinin and actin. In addition, vinculin has been shown to bind to talin (25) and to itself (26–28). Other than its well defined role in mediating the cytoplasmic anchorage of integrins, the role of vinculin in cell-cell adhesion is not clear. Except for its localization to adherens junctions, no evidence exists for its association with cadherins (17).

In this study, the use of cell lines deficient in α-catenin provided a good experimental system to study the role of vinculin in adherens junctions and to determine whether this protein plays a role in cadherin-based adhesion. We use the breast cancer cell line MDA-MB-468. We define two mutations in the mRNA for α-catenin in these cells that result in a lack of detectable α-catenin protein expression. Similarly to other α-catenin-deficient cell lines, MDA-MB-468 cells display a lack of cadherin-mediated adhesion under normal growth conditions. However, under serum starvation conditions, E-cadherin expression is up-regulated, and E-cadherin-dependent cell aggregation occurs. Examination of the cytoplasmic components of the adhesion complex reveals that both vinculin and α-catenin are associated with E-cadherin complexes in cell lines expressing both proteins. However, the association of vinculin with E-cadherin in MDA-MB-468 cells, lacking α-catenin expression, is dramatically increased. Moreover, we show that this interaction may be mediated by β-catenin and can be dissociated by the addition of α-catenin fusion protein. These results suggest that vinculin may function in place of α-catenin in cell-cell adhesion, therefore enabling the assembly of a functional adhesion complex.

MATERIALS AND METHODS

Cell Lines—The cancer cell lines MDA-MB-468, MCF-7, BT-549, MDA-MB-157, Caco2, PC-9, and A431 were all obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in Dulbecco’s modified Eagle’s/F-12 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in a humidified 5% CO2 atmosphere.

Antibodies—Monoclonal anti-human E-cadherin and β-catenin antibodies were acquired from Zymed Laboratories, Inc. (South San Francisco, CA). Monoclonal anti-α-catenin antibodies (αH4, α3C1, 5B11, α10E1, and TLx1) were made as described (29, 30), except for TLx1, which was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-α-catenin antibodies were raised against a syn-
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In the carboxy terminus of mouse α-catenin (31). Monoclonal antibodies to β-, γ-catenin and p120 were obtained from Transduction Laboratories. Rabbit polyclonal anti-cancer antibodies and anti-vinculin mouse ascites used for immunoblotting, anti-cancer antibodies and anti-vinculin mouse ascites, and monoclonal antibodies to N-cadherin (A-CAM) were purchased from Chemicon International (Temecula, CA). Anti-cancer antibodies and anti-vinculin mouse ascites used for immunoblotting were purchased from Uptown Biotechnology, Inc. (Lake Placid, NY). Rabbit anti-EGF receptor antisera was a gift from Dr. Joseph Schlessinger (New York University, New York).

Primer—Primers used for PCR are sequenced as described (30). RNA purification, RT-PCR, cloning, and sequencing of products—Total RNA was isolated from cells using the Trizol LS reagent (Life Technologies, Inc.). Subsequently, cDNA was synthesized from 3 μg of RNA using 2 μM oligo(dT) primer oligo(dT)12–18 (Pharmacia Biotech Inc.) and 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.) in reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 2 μM MgCl2 containing 0.01 μM diethiothreitol and 10 μM dNTPs. The reverse transcriptase reaction was incubated at 42 °C for 1 h. α-catenin cDNA was amplified using the designated primers. The PCR products resulting from amplification of the MDA-MB-468 α-catenin cDNA using primer pairs A/D and B/D (see Fig. 2) were ligated into the PCR II vector of the TA cloning kit (Invitrogen, La Jolla, CA). Then the PCR fragments containing the deleted regions were sequenced using primers A, B, and D (see Fig. 2) with Taq DNA polymerase and fluorescently labeled dideoxynucleotides in a thermal cycling protocol.

Northern Blotting—Total RNA was prepared from cell lines using the Trizol reagent according to the protocol on the package insert (32). 10 μg of total RNA was loaded in each lane as determined by absorbance at 260 nm. Total RNA was resolved on 1% formaldehyde-agarose gel and capillary-blotted to Duralon UV membrane (Stratagene) (33). After UV cross-linking, the membranes were hybridized in Rapid-Hyb™ buffer (Amersham Corp.) for 1.5–2.5 h with a fluorescently labeled N-terminus half of the α-catenin cDNA. Washes were done at high stringency (0.1 × SSC at 65 °C) for 20–30 min. The filters were then processed for the illuminator™ radioactivity detection system (Stratagene).

Immunoblotting—Cells were extracted in lysis buffer (1% Triton X-100, 10% glycerol, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM MgCl2, and 1 mM diethiothreitol) containing protease inhibitors (10 μg/ml each aprotinin and leupeptin, 5 μg/ml pepstatin, and 1 mM phenylmethlysulfonfluoride). 30 μg of protein from each indicated extract, as determined by the Bradford method (Bio-Rad), was boiled in SDS sample buffer for 10 min and loaded onto a 7.5% polyacrylamide minigel (Stratagene). Proteins were transferred onto Immobilon membranes (Millipore Corp.), which were subsequently blocked in 3% bovine serum albumin/phosphate-buffered saline and incubated for 2 h at 25 °C with the primary antibody (ranging from 10 to 80 μg/ml) were premixed with 2.5 mg of protein A-Sepharose. The beads were washed with lysis buffer, and the bound proteins were eluted by boiling the beads in sample buffer for 10 min. Transferred proteins were blotted with the designated antibodies at a 1:1000 dilution and processed as described above.

Cell-Cell Aggregation Assay—Confluent cell monolayers were incubated for 36 h in fresh medium containing 10% fetal bovine serum (serum-treated) or 0% fetal bovine serum (serum-starved). Cell monolayers were subsequently incubated in 0.02% crystalized trypsin (Worthington) and 10 mM CaCl2 in Hanks’ balanced saline solution (Life Technologies, Inc.) for 5 min at 37 °C and made into single cell suspensions by trituration with a Pasteur pipette. Cells were washed twice in Hanks’ balanced saline solution and incubated at 3 × 105 cells/well in 500 μl of Hanks’ balanced saline solution containing 1% bovine serum albumin and 100 μg/ml DNase (Worthington) with or without 100 μM CaCl2 in the presence or absence of 80 μg/ml antibodies to E-cadherin, P-cadherin, or N-cadherin. Aggregation assays were performed at 37 °C at 100 rpm for 20 min in triplicate wells on an

24-well non-tissue culture-treated plates (No. 1147; Becton Dickinson Labware, Franklin Lakes, NJ). Assays were stopped at 0 and 20 min by fixing the cells in 1% glacial acetic acid. The extent of cell-cell binding was monitored by measuring the disappearance of single cells using a Coulter counter.

Competitive Inhibition Studies—Glutathione S-transferase (GST)-α-catenin and GST-β-catenin fusion proteins as well as GST alone and GST-spectrin fusion proteins used as controls were prepared as described (53). The GST fusion proteins were isolated by affinity chromatography on glutathione-agarose (Sigma) and eluted with 5 mM glutathione in 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1 mM diethiothreitol, followed by dialysis in glutathione-free buffer. Protein concentration was determined (the Bradford method), and the identity of the eluted proteins was controlled by immunoblotting (data not shown). Increasing concentrations of α-catenin or β-catenin fusion protein (ranging from 10 to 80 μg/ml) were preincubated with 2.5 μg of serum-starved MDA-MB-468 cell lysate for 2 h at 4 °C. Control GST and GST-spectrin were added at 80 μg/ml. Lysates were subjected to immunoprecipitation with anti-vinculin antibodies as described above and were further analyzed by immunoblotting using anti-E-cadherin antibodies, followed by ECL detection.

RESULTS

MDA-MB-468 Cells Do Not Express α-Catenin—MDA-MB-468 cells lack detectable α-catenin protein expression as demonstrated by the absence of reactivity either with a mixture of five monoclonal antibodies that map to different sites within the α-catenin molecule (Fig. 1A, lane 2) or with a polyclonal antibody to a peptide near the carboxy terminus of α-catenin (lane 5). The lack of antibody reactivity for α-catenin with MDA-MB-468 cell lysates was compared with the antibody reactivity with lysates of the α-catenin-positive cell line MCF-7 (Fig. 1A, lanes 1 and 4) and the α-catenin-negative cell line PC-9 (lanes 3 and 6). The PC-9 cell line has been shown to have a homozygous deletion of the α-catenin gene (34) and is therefore used as a control for nonspecific reactivity of antibodies with cell lysates. These results were in agreement with previous studies that have demonstrated a lack of α-catenin expression in MDA-MB-468 cells using polyclonal antibodies against the carboxy terminus of the molecule (35, 36). An α-catenin transcript was present, however, in MDA-MB-468 cells at the appropriate molecular mass and at a level comparable to that

1 The abbreviations used are: EGF, epidermal growth factor; RT-PCR, reverse transcriptase-polymerase chain reaction; GST, glutathione S-transferase.

**Fig. 1. α-Catenin protein and mRNA expression in MDA-MB-468 breast cancer cells.** A, cell lysates from serum-starved monolayers of MCF-7, MDA-MB-468 (MDA-468), and PC-9 cells were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-α-catenin antibodies consisting of either a mixture of five monoclonal antibodies (M Ab) (29) or a polyclonal antibody (P Ab) (31). Blots were processed by the ECL method and exposed for 1 min. α-cat, α-catenin. B, Northern blotting shows a 3.7-kilobase band in MDA-MB-468 cells (lane 3). Caco2 cells were used as a positive control to show normal migration of α-catenin mRNA (lane 1), and MDA-MB-157 cells (MDA-157) were used as a negative control, showing a cell line with no α-catenin mRNA (lane 2) (36). Matching actin controls for gel loading are included.
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Fig. 2. Illustration of the characterization of the deletions in MDA-MB-468 cells. Primers used for RT-PCR of RNA from MDA-MB-468 cells are shown by direction and location along the α-catenin sequence (GenBank accession no. L23805) (A). RT-PCR products resolved by gel electrophoresis show pairs of lanes, where the left lane is a positive control template and the right lane is MDA-MB-468 RNA (B). The primer pair used for amplification is shown beneath each set of lanes. The PCR products shown in the MDA-MB-468 lanes for primer pairs A/D and B/D were subcloned and sequenced as described under “Materials and Methods” and showed two mutations, indicated as allele 1 (C) and allele 2 (D). Note that the allele 1 mutation does not result in a frameshift, but no protein product was detected. Allele 2 shows a frameshift mutation resulting in premature termination of the protein, as shown D. Finally the schematic (E) shows the relative location of each deletion mutation. nt, nucleotides.

found in Caco2 cells, which express normal α-catenin levels (Fig. 1B and data not shown).

Further evaluation of the mRNA for α-catenin in MDA-MB-468 cells by RT-PCR revealed only two mutations. Two prominent bands were observed in the PCR using the primer pair A/D, and one band was observed using the primer pair B/D (Fig. 2). The bands labeled 1 and 2 were subcloned and sequenced. The sequences of both of these PCR products showed deletion mutations of either 483 or 287 base pairs; both deletions were observed near the 5′-end of the α-catenin message (Fig. 2, C and D). An in-frame deletion was observed in allele 1, but unlike the truncated protein that has been shown to be expressed in the Clone A cell line as a result of a similar mutation in the 5′-end of the α-catenin message (30), no truncated protein was detected in MDA-MB-468 cell lysates by both monoclonal and polyclonal antibodies mapping to various regions of the α-catenin molecule (Fig. 1A, lanes 2 and 5, respectively). Since monoclonal antibody TLo1, which was present in the antibody mixture used for Western blotting, and the polyclonal antibody, both of which map to the carboxyl terminus of the α-catenin protein (amino acids 729–755 and 809–906, respectively), did not react with MDA-MB-468 cell lysates in both Western blotting (Fig. 1A) and immunofluorescence (data not shown), we conclude that allele 1 does not code for a viable α-catenin protein product. A frameshift mutation was observed in allele 2, resulting in a premature stop codon after 8 aberrant amino acids (Fig. 2D). A schematic showing the location of each mutation within the context of the full-length sequence is shown in Fig. 2E.

As has been previously observed in PC-9 cells (34), the MDA-MB-468 cell line appears to have no wild-type α-catenin. According to the American Type Culture Collection, the MDA-MB-468 cell line is predominantly hypodiploid, with a minor bi-modal component having 70 chromosomes. It may be possible that there are additional α-catenin alleles within the population of MDA-MB-468 cells. However, we found only two RT-PCR products related to α-catenin. The PCR gel shown in Fig. 2B is representative of multiple experiments, and in all cases, only two prominent bands are seen (numbered 1 and 2 on the gel). Subcloning of the PCR mixture did reveal other rare products that were apparently artifacts of the PCR as their sequence showed no resemblance to α-catenin.

Serum Withdrawal Induces E-cadherin-dependent Cell-Cell Aggregation in MDA-MB-468 Cells—Comparison of the ability of MDA-MB-468, A431, and MCF-7 cells to aggregate in the presence of calcium revealed that while A431 and MCF-7 cells were highly aggregated under these conditions, MDA-MB-468 cells displayed little or no aggregation (Fig. 3A). These results were consistent with previous reports on the inability of α-catenin-negative cells to aggregate (11, 37). We found, however, that removal of serum from the growth medium for 36 h caused a dramatic increase in the ability of MDA-MB-468 cells to aggregate, but had no effect on the aggregation of A431 or MCF-7 cells (Fig. 3A). To determine which cadherin mediates this aggregation, function-blocking antibodies to E-cadherin, P-cadherin, or N-cadherin (see “Materials and Methods”) were added to the cells during aggregation, and only the anti-E-cadherin antibodies inhibited the aggregation of serum-starved MDA-MB-468 cells even though these cells also express P-cadherin (38).

Serum withdrawal induced a 3-fold increase in E-cadherin expression levels in MDA-MB-468 cells, but had no effect on the expression of E-cadherin in MCF-7 and A431 cells (Fig. 3B), which was consistent with the effect of serum on the aggregation properties of these cells (Fig. 3A). Serum starvation affected the expression of only E-cadherin in MDA-MB-468 cells; the expression levels of other adhesion-related proteins such as β-catenin, plakoglobin/γ-catenin, and p120 were unaffected (Fig. 3C). Similarly, the levels of the EGF receptor and the cytoskeletal proteins vinculin, α-actinin, and actin remained unchanged by serum starvation (Fig. 3C). These results show a close relationship between serum withdrawal, levels of E-cadherin expression, and activation of E-cadherin-dependent adhesion.

Serum Starvation Increases the Association of Vinculin with E-cadherin Complexes—The preceding results demonstrate that E-cadherin-mediated cell aggregation can be stimulated in MDA-MB-468 cells, even in the absence of α-catenin expression. The similarities between α-catenin and vinculin in both amino acid sequence and subcellular localization to the adherens junction prompted us to examine whether vinculin is associated with cadherin complexes. In serum-starved MDA-MB-468 cells, a high level of vinculin was found to be associated
with immunoprecipitates of E-cadherin (Fig. 4A, lane 2). Vinculin was not initially detected in E-cadherin complexes in serum-treated MDA-MB-468 cells (Fig. 4A, lane 1), but the same levels of vinculin were found complexed with E-cadherin in serum-treated lysates when three times more lysate was used to compensate for the lower E-cadherin levels (data not shown). This suggested that E-cadherin levels restrict the interaction of vinculin with E-cadherin. By comparison, vinculin was not detected in E-cadherin immunoprecipitates of A431 and MCF-7 cells (Fig. 4A, lanes 4 and 6), although it was present at similar levels in total cell lysates from all three cell lines (lanes 3, 5, and 7).

To verify the mutual coprecipitation of E-cadherin with vinculin, immunoprecipitations with anti-vinculin antibodies were performed. We found the highest levels of E-cadherin complexed with vinculin in serum-starved MDA-MB-468 cells (Fig. 4B, lane 2), and to a lesser extent, this association was also present in serum-treated cells (lane 1) and in A431 and MCF-7 cells (lanes 3 and 4). The breast cancer line BT-549, which does not express E-cadherin (39), was used as a control to exclude any cross-reactivity of antibodies since vinculin (116 kDa) and E-cadherin (124 kDa) nearly comigrate on SDS-polyacrylamide gels (Fig. 4B, lane 5).

To determine whether vinculin is an integral component of the E-cadherin/β-catenin adhesion complex, immunoprecipitations from serum-starved MDA-MB-468 cell lysates were performed with anti-β-catenin antibodies. Both E-cadherin and vinculin were identified in these complexes (Fig. 4C, lanes 1 and 2). Reciprocally, immunoprecipitation with anti-vinculin antibodies verified the presence of β-catenin and E-cadherin in

FIG. 3. Serum starvation stimulates E-cadherin-dependent adhesion and increases E-cadherin expression. Confluent cell monolayers of MDA-MB-468 (MDA-MB-468), A431, or MCF-7 cells were incubated in medium with 10% or 0% serum for 36 h (A). Single cell suspensions obtained by low trypsin and 10 mM CaCl2 treatment were assayed for aggregation in the presence or absence of 80 μg/ml antibodies to E-cadherin (Anti-Ecad), P-cadherin (Anti-Pcad), or N-cadherin (Anti-Ncad). The accumulation of aggregates at 0 and 20 min was determined using a Coulter counter. The results are the means ± S.E. of eight separate experiments. Lysates (30 μg) from confluent MDA-MB-468, A431, or MCF-7 cell monolayers (B) or from MDA-MB-468 cell monolayers alone (C) that were either fetal bovine serum-treated (+FBS) or fetal bovine serum-starved (−FBS) for 36 h were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies to the designated molecules. Blots were processed by ECL and exposed for 1 min. The relative levels of E-cadherin in lane 2 and the same levels of vinculin were found complexed with E-cadherin in serum-treated lysates when three times more lysate was used to compensate for the lower E-cadherin levels (data not shown). This suggested that E-cadherin levels restrict the interaction of vinculin with E-cadherin. By comparison, vinculin was not detected in E-cadherin immunoprecipitates of A431 and MCF-7 cells (Fig. 4A, lanes 4 and 6), although it was present at similar levels in total cell lysates from all three cell lines (lanes 3, 5, and 7).

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A

| µg/ml | E-cad | GST-β-cat | GST-α-cat |
|-------|-------|-----------|-----------|
| 80    | 97    | 68        | 68        |
| 40    | 43    | 35        | 35        |
| 20    | 21    | 21        | 21        |
| 10    | 17    | 17        | 17        |
| 0     | 2    | 2         | 2         |

B

| µg/ml | E-cad | GST-α-cat | GST-spectrin |
|-------|-------|-----------|--------------|
| 80    | 97    | 97        | 97           |
| 40    | 43    | 43        | 43           |
| 20    | 21    | 21        | 21           |
| 10    | 17    | 17        | 17           |
| 0     | 2     | 2         | 2            |

**Fig. 5. Competitive inhibition of E-cadherin/vinculin coprecipitation with β-catenin and α-catenin fusion proteins.** Cell lysates from serum-starved MDA-MB-468 monolayers (2.5 mg) were premixed with 10, 20, 40, and 80 µg/ml concentrations of the following: A, β-catenin fusion protein (GST-β-cat; lanes 1–5); and B, α-catenin fusion protein (GST-α-cat; lanes 1–5), 80 µg/ml GST (lane 6), or GST-spectrin (lane 7). Lysates were incubated for 2 h at 4 °C prior to immunoprecipitation (IP) with anti-vinculin antibodies (a Vinculin), followed by blotting with anti-E-cadherin antibodies (a E-cadherin), and exposed for 1 min after ECL processing. E-cad, E-cadherin.

This complex (Fig. 4, C lane 2) and B lane 2, respectively.

These results suggest that both α-catenin and vinculin associate with the E-cadherin/β-catenin complex. The interaction of vinculin with E-cadherin in MDA-MB-468 cells is stimulated by increased E-cadherin expression and is likely to be facilitated by the absence of α-catenin, which may have generated additional binding sites for vinculin in the adhesion complex.

**β-Catenin Links Vinculin with E-cadherin in the Adhesion Complex—**To verify that the interaction of vinculin with E-cadherin is mediated by β-catenin in a manner analogous to α-catenin, excess recombinant β-catenin was added to serum-starved MDA-MB-468 cell lysates to competitively inhibit the coprecipitation of E-cadherin with vinculin. Increasing concentrations of recombinant β-catenin (ranging from 10 to 80 µg/ml) resulted in decreasing amounts of E-cadherin that coprecipitated with vinculin (Fig. 5A, lanes 1–5). In contrast, the addition of either GST alone or GST-spectrin fusion protein at 80 µg/ml did not affect the coprecipitation of E-cadherin with vinculin (Fig. 5B, lanes 6 and 7), indicating that this effect is specific for β-catenin.

To address the question of whether vinculin and α-catenin compete for binding to β-catenin, increasing amounts of α-catenin fusion protein were added to MDA-MB-468 cell lysates. α-Catenin inhibited the association of vinculin with E-cadherin in a dose-dependent manner (Fig. 5B, lanes 1–5). These results indicate that vinculin may be assembled in the adhesion complex by direct binding to β-catenin and by competing with α-catenin for the same binding site on β-catenin.

**DISCUSSION**

Vinculin and α-catenin share 25–30% sequence homology (14, 15), and both have been shown to mediate the association of integrins (18–24) and cadherins (6–10), respectively, with the actin-based cytoskeleton. Since vinculin has been found at both focal adhesions and adherens junctions (16, 17), it is tempting to speculate that it may perform a similar function in both structures. Vinculin has been well characterized as being present at adherens junctions (17) and is thought to bind to F-actin at these sites (21–24). In addition, colocalization studies using conventional immunofluorescence (40, 41) and resonance energy transfer imaging (42) indicate that vinculin, β-catenin, and E-cadherin are present at the same subcellular location. This study suggests that the localization of vinculin at the adherens junction may be a result of a direct interaction with β-catenin in the adhesion complex and represents the first biochemical evidence in support of a direct involvement of vinculin in cadherin-based cell-cell adhesion.

The absence of α-catenin in MDA-MB-468 cells is associated with increased vinculin in the adhesion complex, which suggests that α-catenin and vinculin may compete for the same binding site on β-catenin. In support of this hypothesis, we show that the coprecipitation of E-cadherin with anti-vinculin antibodies is prevented by exogenous recombinant β-catenin or α-catenin. These results are consistent with a model whereby vinculin, in a manner analogous to α-catenin, may also link actin filaments to E-cadherin via β-catenin. The sites within α-catenin and β-catenin (43, 44) that mediate their interactions have been mapped to specific peptides, and it will be revealing to determine whether they are identical for vinculin.

Vinculin was also found in E-cadherin complexes in epithelial cell lines that express α-catenin, suggesting that a fraction of the total cellular vinculin may be a component of cadherin complexes in cells with normal levels of α-catenin. It has been suggested that vinculin may interact with α-catenin at adhesion sites (14, 15, 4), and although there is no published evidence for this idea, it could account for its localization at adherens junctions. This study demonstrates, however, that vinculin binds to E-cadherin complexes in the absence of α-catenin, which eliminates binding to α-catenin as the sole mechanism for the targeting of vinculin to the adhesion complex. The possibility that vinculin may cross-link adhesion complexes is inferred from electron microscopy studies of rotary-shadowed vinculin that demonstrate the existence in vitro of vinculin complexes containing two to six individual molecules (45, 46). It remains to be determined whether this multimerization takes place in vivo.

This work suggests that while vinculin may participate in the anchorage of cadherins to the cytoskeleton, α-catenin is likely to mediate a more stable adhesion than vinculin. In support of this hypothesis, α-catenin induces a tight epithelial structure when reconstituted into deficient cells (12), and cadherin/α-catenin complexes with the actin cytoskeleton are detergent-insoluble (47). In contrast, while the morphologic appearance of adhesion-competent (serum-starved) MDA-MB-468 cells is epithelioid, these cells exhibit characteristic refractile cell-cell junctions (data not shown), which suggests that they may be arrested in a premature state of adhesion as compared with MCF-7 or A431 epithelial cells. A switch from a vinculin- to an α-catenin-based junction may therefore be a mechanism that regulates the transition from an initial to a stable adhesion. These two states of adhesion have been characterized by studies that have traced development of cell-cell contacts by video imaging and quantitative immunocytochemistry (48–50), correlating the maturation of cell-cell contacts with increased association of E-cadherin complexes with the cytoskeleton. We therefore propose that the strength of linkage of cadherin complexes to the actin cytoskeleton may determine
the strength of adhesion, and vinculin may play a modulatory role.

The mechanism by which serum starvation affects E-cadherin expression is not known, although evidence exists in support of a negative feedback loop exerted by the HER2/neu proto-oncogene, a close relative of the EGF receptor. Overexpression of HER2/neu has been shown to repress the promoter activity of E-cadherin, resulting in reduced E-cadherin expression (51). Since the EGF receptor is highly expressed in MDA-MB-468 cells (52), it is conceivable that a similar mechanism by serum withdrawal may cause an increase in E-cadherin expression and that this protein substitutes for HER2/neu. Increases in E-cadherin density may stimulate cooperation of vinculin to the adhesion complex and a surge in cell-cell adhesion. Finally, these findings implicate the strength of adhesion, and vinculin may play a modulatory role.

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