N-Cadherin Extracellular Repeat 4 Mediates Epithelial to Mesenchymal Transition and Increased Motility

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Abstract. E- and N-cadherin are members of the classical cadherin family of proteins. E-cadherin plays an important role in maintaining the normal phenotype of epithelial cells. Previous studies from our laboratory and other laboratories have shown that inappropriate expression of N-cadherin by tumor cells derived from epithelial tissue results in conversion of the cell to a more fibroblast-like cell, with increased motility and invasion. Our present study was designed to determine which domains of N-cadherin make it different from E-cadherin, with respect to altering cellular behavior, such as which domains are responsible for the epithelial to mesenchymal transition and increased cell motility and invasion. To address this question, we constructed chimeric cadherins comprised of selected domains of E- and N-cadherin. The chimeras were transfected into epithelial cells to determine their effect on cell morphology and cellular behavior. We found that a 69–amino acid portion of EC-4 of N-cadherin was necessary and sufficient to promote both an epithelial to mesenchymal transition in squamous epithelial cells and increased cell motility. Here, we show that different cadherin family members promote different cellular behaviors. In addition, we identify a novel activity that can be ascribed to the extracellular domain of N-cadherin.

Key words: N-cadherin • E-cadherin • cancer • motility • invasion

Introduction

Cadherins comprise a family of calcium-dependent cell–cell adhesion proteins that play important roles in embryonic development and maintenance of normal tissue architecture. As the transmembrane component of cellular junctions, the cadherins are composed of three segments: an extracellular domain comprised of five homologous repeats that mediate adhesion, a single pass transmembrane domain, and a conserved cytoplasmic domain that interacts with catenins to link cadherins to the actin cytoskeleton (for review see Wheelock et al., 1996). The catenins were first identified as proteins that coimmunoprecipitated with cadherins and were termed α-, β-, and γ-catenin, according to their mobility on SDS-PAGE. Either β- or γ-catenin binds directly to the cadherin and α-catenin, whereas α-catenin associates directly and indirectly with actin filaments (Stappert and Kemler, 1994; Knudsen et al., 1995; Rimm et al., 1995; Nieset et al., 1997). The ability of cadherins to simultaneously self-associate and link to the actin cytoskeleton mediates both the cell recognition required for cell sorting and the strong cell–cell adhesion needed to form tissues.

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In addition to their structural role in the adherens junction, catenins are thought to regulate the adhesive activity of cadherins. For example, phosphorylation of β-catenin in Src-transformed cells may contribute to the nonadhesive phenotype of these cells (Matsuyoshi et al., 1992; Hamaguchi et al., 1993). As a signaling molecule, β-catenin plays a critical role in patterning during development and in maintenance of the normal cellular phenotype during tumorigenesis (Cadigan and Nusse, 1997; Miller et al., 1999; Polakis et al., 1999). The signaling functions of β-catenin are due to its interactions with transcription factors of the lymphoid enhancer factor/T cell factor (LEF/TCF) family and with receptor tyrosine kinases. In addition, $p120^{ctn}$, originally identified as a Src substrate and subsequently shown to bind to the cytoplasmic domain of cadherins, has been suggested to play a role in regulating the adhesive activity of cadherins (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995). $p120^{ctn}$ binds to the juxtamembrane domain of cadherins, a domain that has been implicated in cadherin clustering and cell motility (Chen et al., 1997; Finnemann et al., 1997; Navarro et al., 1998; Yap et al., 1998). It is thought that $p120^{ctn}$ influences the strength of cadherin-mediated adhesion, perhaps by influencing the organization of the actin cytoskeleton (Aono et al., 1999; Ohkubo and Ozawa, 1999;

Thoreson et al., 2000). Thus, various studies have shown that the cytoplasmic domain of cadherins interacts with proteins that likely regulate adhesive function.

The extracellular domain of classic cadherins is involved in interactions that mediate adhesion. The earliest evidence for this came from studies demonstrating that antibodies produced against the extracellular domain of cadherins inhibit cell adhesion. The extracellular domain of cadherins can be divided into "extracellular cadherin structural domains" (EC)[1] each of which consists of ~110 amino acids and contains the conserved motifs LDRE, DXNDN, and DXD (Oda et al., 1994). EC-1 is the most NH_2-terminal domain and is responsible for adhesive activity (for review see Takeichi, 1990). The binding sites for most mAbs that block the adhesive function of E-, P-, and N-cadherin have been mapped to EC-1 (for review see Takeichi, 1990), a domain that contains an HAV tripeptide that has been implicated in adhesion. Synthetic peptides containing an HAV sequence inhibit cadherin-mediated adhesion, mimicking the activity of antibodies directed against EC-1 (Blaschuk et al., 1990). Structural studies have shown that the HAV tripeptide and its surrounding residues mediate self-association by interacting with a separate set of amino acids within EC-1 of the interacting cadherin on the adjacent cell (Shapiro et al., 1995). In addition, mutations in the NH_2 terminus of classical cadherins or deletion of EC-1 results in molecules that do not mediate cell adhesion (Nose et al., 1990; Ozawa et al., 1990; Ozawa and Kemler, 1990; Shan et al., 2000).

It was observed that cells expressing different members of the classical cadherin family segregate from one another when mixed together in culture (for review see Takeichi, 1990). It has been suggested that this preferential binding of cadherins plays an important role in the sorting activities of embryonic cells. Interestingly, the binding specificity of cadherin molecules also maps to EC-1. When the NH_2-terminal regions of E-cadherin were replaced with those of P- or N-cadherin, the chimeric molecules displayed P- or N-cadherin specificity, respectively (Nose et al., 1990; Shan et al., 2000). Thus, EC-1 of the classical cadherins is responsible not only for cadherin binding activity, but also for cadherin specificity.

Various studies have implicated E-cadherin in maintenance of the normal phenotype of epithelial cells (for reviews see El-Bahrawy and Pignatelli, 1998; Behrens, 1999). For example, invasive, fibroblast-like carcinoma cells could be converted to a noninvasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991), and forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Chen et al., 1997). Likewise, transfection of invasive E-cadherin–negative cell lines with E-cadherin resulted in cells that were less invasive in in vitro assays (Frixen et al., 1991; Luo et al., 1999). It has been suggested that, unlike E-cadherin, N-cadherin may promote motility and invasion in carcinoma cells. For example, Hazan et al. (1997) reported that expression of N-cadherin by breast carcinoma cells correlated with invasion and suggested that invasion was potentiated by N-cadherin–mediated interactions between the cancer and stromal cells. Studies from our laboratory suggest that N-cadherin plays a direct role in invasion. Expression of N-cadherin by squamous epithelial cells resulted in a scattered phenotype accompanied by an epithelial to mesenchyme transition. Here, forced expression of N-cadherin in cultured cells resulted in downregulation of the expression of E-cadherin (Islam et al., 1996). Thus, it was difficult to separate the characteristics due to decreased expression of E-cadherin from those due to increased expression of N-cadherin. In a second study, we showed that expression of N-cadherin by BT-20 human breast epithelial cells converted the cells to a motile and invasive phenotype. In this case, increased motility was not accompanied by decreased E-cadherin expression, suggesting that N-cadherin plays a direct role in epithelial cell motility (Nieman et al., 1999a). Hazan et al. (2000) confirmed our results using the MCF7 human breast carcinoma cell line. Importantly, these authors extended their studies to show that N-cadherin expression increased metastasis when the transfected cells were injected into nude mice. Thus, there is evidence that expression of an inappropriate cadherin may alter cellular behavior, suggesting that cadherins function as more than just cell–cell adhesion molecules.

Our study was designed to determine which domains of N-cadherin are responsible for both the epithelial to mesenchymal transition that we have seen in squamous epithelial cells and the increased motility seen in breast cancer cells. To address this question, we made use of chimeric cadherins constructed between N-cadherin and E-cadherin. The chimeras were transfected into the SCC1 oral squamous epithelial cell line, to determine their effect on cell morphology, and into the BT20 breast cancer cell line, to investigate influences on cell behavior. We found that a 69–amino acid portion of EC-4 of N-cadherin was both necessary and sufficient to promote motility. This study makes two important points: (a) it shows that cadherins promote differential cellular behavior and (b) it identifies a novel activity that maps to the extracellular domain of N-cadherin.

Materials and Methods

Antibodies and Reagents

Mouse mAbs against the cytoplasmic domain of human N-cadherin (13A9), α-catenin (1G5), and β-catenin (6E3) have been described previously (Johnson et al., 1993; Knudsen et al., 1995). Mouse mAbs against the extracellular amino acids 92–593 of human N-cadherin (8C11) and the cytoplasmic domain of human E-cadherin (4A2) were prepared as described previously (Johnson et al., 1993). Mouse mAb against the myc-epitope (9E10.2) was a gift from Dr. K. Green (Northwestern University, Chicago, IL). All reagents were from Sigma-Aldrich, unless otherwise indicated.

Cell Culture

The human squamous carcinoma cell line UM-SCC-1 (SCC-1) and the human breast cancer cell line BT20 were maintained in MEM 10% FBS (Hyclone Laboratories). A cadherin-negative derivative of A431 called A431D, which was described previously (Lewis et al., 1997), was maintained in DME 10% FBS.

Molecular Constructs

Human N-cadherin (sequence data available from GenBank/EMBL/DDBJ under accession no. S42303) (a gift of Dr. A. Ben Ze'ev, Weizmann Institute, Rehovot, Israel) and human E-cadherin (sequence data avail-

[1]*Abbreviations used in this paper:* EC, extracellular cadherin structural domains; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor.

Table I.

Chimera		Junction	Details
E/N		LLFL/KRRD	Joins E-cad 731 to N-cad 747
N/E and N/E myc		VVWM/RRRA	Joins N-cad 746 to E-cad 732
N/E5a myc		AGPF/TAEL	Joins N-cad 637 to E-cad 627
N/E5 myc		DNAP/IPEP	Joins N-cad 603 to E-cad 594
N/E4 myc		NIRY/RIWR	Joins N-cad 534 to E-cad 524
N/E3 myc		NAVY/TILN	Joins N-cad 420 to E-cad 414
N/E2 myc		MLRY/TILS	Joins N-cad 306 to E-cad 303
E/N/E myc	5′	KITY/TKLS	Joins E-cad 523 to N-cad 535
	3′	DNAP/IPEP	Joins N-cad 603 to E-cad 594
N/E/N myc	5′	NIRY/RIWR	Joins N-cad 534 to E-cad 524
	3′	DNAP/QVLP	Joins E-cad 593 to N-cad 604

In our N-cadherin cDNA, there is an additional leucine (CTG) after amino acid 11. Thus, the entire open reading frame is 906 codons. The numbers in the table reflect this change to S42303. The E-cadherin cDNA has an open reading frame of 882 codons.

able from GenBank/EMBL/DDBJ under accession no. Z13009) (Lewis et al., 1997) were used for construction of chimeric cadherins using recombinant PCR (Higuchi et al., 1988). In each case, the recombinant PCR product was subcloned and representatives were sequenced until one was identified that encoded the complete, correct amino acid sequence. Each full-length construct was assembled by joining restriction fragments from the correct recombinant PCR product and the cDNA clones. The full-length construct was moved into pLKneo (Hirt et al., 1992) or a derivative for transfection into cells. Amino acid sequences across the chimeric junctions are given in Table I. Brief descriptions of the constructions are given below; complete details are available upon request.

The E/N-chimera has the extracellular and transmembrane domains of E-cadherin and the cytoplasmic domain of N-cadherin, whereas the N/E-chimera has the extracellular and transmembrane domains of N-cadherin and the cytoplasmic domain of E-cadherin. To construct the E/N-chimera, recombinant PCR was used to generate a chimeric cDNA encoding a portion of E-cadherin's extracellular domain, including the unique Bsu36I site, plus its transmembrane domain and N-cadherin's entire cytoplasmic domain. To complete the full-length E/N-chimera, a 5′ E-cadherin cDNA fragment was ligated to the recombinant PCR product at the Bsu36I site. A similar strategy was employed to form the N/E-chimera, except the unique BglII site located in the N-cadherin sequence was used to join the 5′ N-cadherin cDNA fragment to the recombinant PCR product. The full-length chimeras were inserted into pLKneo for transfection.

To make the N/E-myc construct, the cytoplasmic domain of E-cadherin, including the unique SmaI site, was amplified such that the stop codon was replaced with a restriction site. The PCR product was inserted into a modified pSPUTK (Falcone and Andrews, 1991) to add a COOH-terminal 2X-myc tag (Nieman et al., 1999a). A 5′ restriction fragment from the N/E-chimera was ligated to the above construct at the SmaI site to make the full-length N/E-myc cDNA. To make N/E5a-myc, N/E5-myc, and N/E4-myc, recombinant PCR fragments were used to replace portions of the N/E-myc construct by using convenient restriction sites. To make N/E3-myc, a recombinant PCR fragment was used to replace a portion of the N/E4-myc construct. In a similar fashion, the N/E2-myc construct was made by replacing a portion of the N/E3-myc construct with a recombinant PCR fragment. Each of these full-length cadherins was then inserted into pLKpac (Islam et al., 1996) for transfection.

The E/N/E-myc chimera was generated by substituting nucleotides encoding N-cadherin amino acids 535–603 for the corresponding E-cadherin sequence. Recombinant PCR was performed to create the 5′ junction between E- and N-cadherin. The product of this reaction was used in a second recombinant PCR step to create the 3′ junction between N- and E-cadherin. The resulting PCR product was used to replace a portion of E-cadherin–2X-myc (Nieman et al., 1999b). The N/E/N-myc construct was prepared similarly. In this case, the final PCR product was used to replace a portion of the N-cadherin sequence in an N-cadherin–2X-myc construct. The chimeras were inserted into pLKpac for transfection.

Transfections

SCC1 and A431D cells were transfected, using calcium phosphate and BT20, by electroporation, as previously described (Nieman et al., 1999a). Stable clones were selected by growth in puromycin (1 μg/ml) or G418 (1 mg/ml). Clones were screened for transgene expression by immunoblot analysis. Clones that showed homogenous expression by immunofluorescence were selected. For morphological studies, at least three clones from each transfection were examined.

Microscopy

Cells were grown on glass coverslips, fixed with Histochoice (Amresco), blocked using PBS 10% goat serum, and stained with primary antibodies for 1 h, followed by treatment with a secondary antibody (Jackson ImmunoResearch Laboratories). Photos were taken with a ZEISS Axiophot microscope (ZEISS) equipped with a SPOT CCD camera (Spot Diagnostic).

Cell Fractionation and Protein Assays

Confluent monolayers were washed with PBS and extracted on ice with TNE buffer (10 mM Tris-acetate, pH 8.0, 0.5% NP-40, 1 mM EDTA, 2 mM PMSF). Extracts were mixed at 4°C for 30 min and centrifuged at 15,000 rpm for 15 min. Protein determinations were done using a Bio-Rad kit (Bio-Rad Laboratories).

Immunoprecipitations, Electrophoresis, and Immunoblot Analysis

A 300-μl aliquot of cell extract was incubated with 300 μl hybridoma supernatant for 30 min at 4°C. Protein A beads were added, and the incubation was continued for 30 min. Immune complexes were washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20) 5× at 4°C. Pellets were resolved by SDS-PAGE and immunoblotted as described previously (Johnson et al., 1993).

Aggregation Assays

Aggregation assays were done as described by Redfield et al. (1997), with minor modifications. In brief, cells were trypsinized and resuspended at 2.5×10^5 cells/ml in the appropriate medium containing 10% FBS. 20-μl drops of medium, containing 5,000 cells/drop, were pipetted onto the inner surface of the lid of a petri dish. The lid was then placed on the petri dish so that the drops were hanging from the lid with the cells suspended within them. To eliminate evaporation, 10 ml serum-free culture medium was placed in the bottom of the petri dish. After 24 h at 37°C, the lid of the petri dish was inverted and photographed using a ZEISS inverted tissue culture microscope at 100× magnification.

Motility Assays

For motility assays, 5×10^5 cells were plated in the top chamber of non-coated polyethylene teraphthalate membranes (six-well insert, pore size 8 mm) (Becton Dickinson). 3T3-conditioned medium was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h, and the cells that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transversing the membrane were stained with Diff-Quick (Dade). Cells in 10 random fields of view at 100× magnification were counted and expressed as the average number of cells/field of view. Three independent experiments were done in each case. The data was represented as the average of the three independent experiments with the standard deviation of the average indicated. When cells were induced with dexamethasone to express a transgene, the control cells were treated with the same level of dexamethasone.

Antibody Blocking Experiments

Ascites fluid generated from the 8C11 mAb or control ascites was diluted in culture medium. Cells were plated on membranes for motility assays, as described above, except that the cells were plated in medium-containing ascites fluid. After 24 h, the number of cells traversing the membrane was determined.

Results

Previous studies from our laboratory showed that expression of N-cadherin by squamous epithelial cells or breast cancer cells altered cellular behavior. In oral squamous epithelial cells, expression of N-cadherin produced a scattered phenotype with an epithelial to mesenchymal transi-

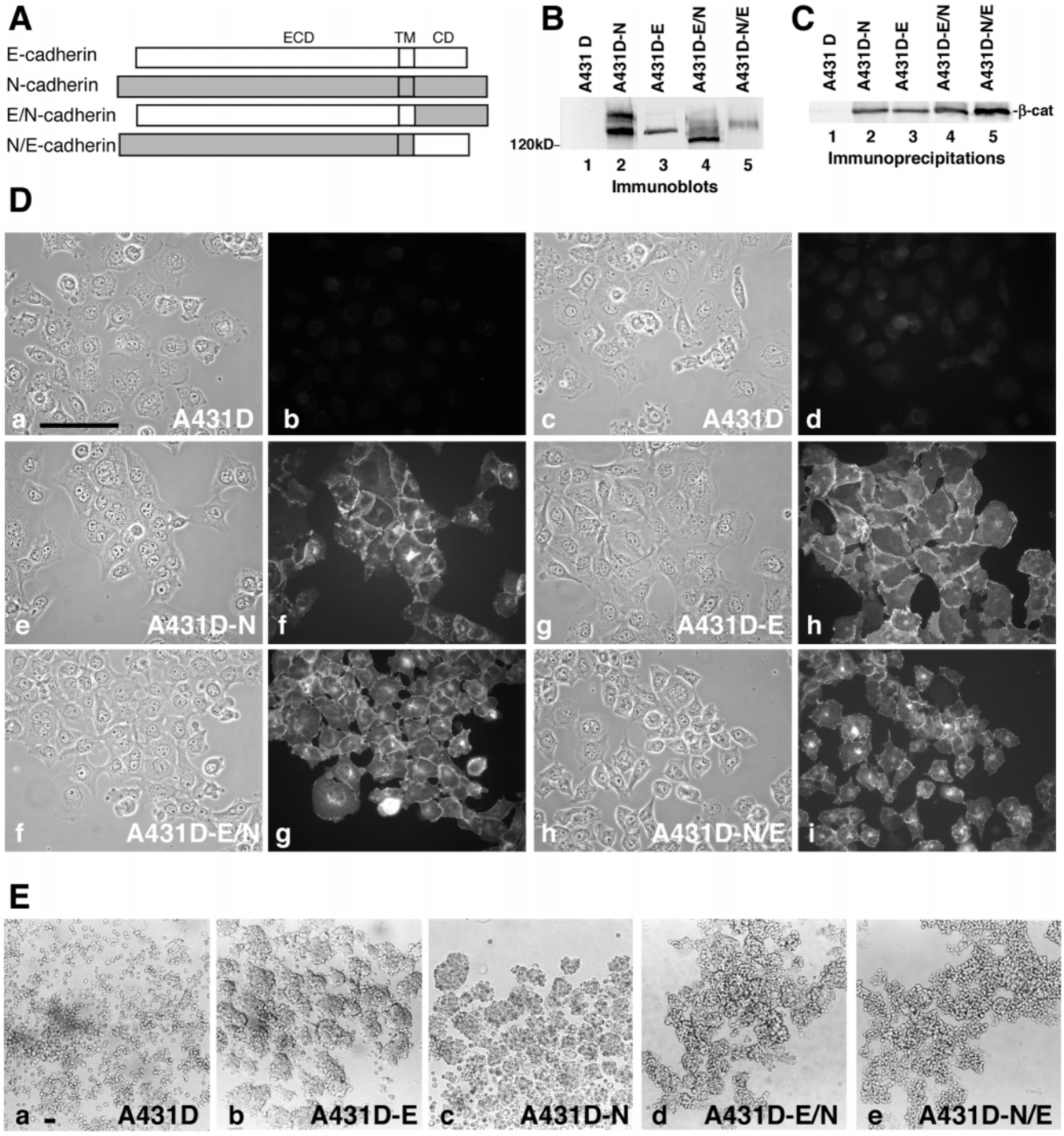

Figure 1. Expression of E/N- and N/E-cadherin in A431D cells. (A) Chimeric cadherins consisting of the extracellular and transmembrane domains of E-cadherin (white) and the cytoplasmic domain of N-cadherin (gray) or consisting of the extracellular and transmembrane domains of N-cadherin and the cytoplasmic domain of E-cadherin were cloned into pLKneo2. (B) A431D cells were transfected with N-cadherin, E-cadherin, E/N-cadherin, or N/E-cadherin and examined for transgene expression by immunoblotting with antibodies against the cytoplasmic domain of N-cadherin (lanes 1, 2, and 4), the extracellular domain of N-cadherin (lane 5), or the extracellular domain of E-cadherin (lane 3). Note, in some cases, we observed various processing variants when transfected cadherins were overexpressed in cells. (C) Extracts were immunoprecipitated, resolved by SDS-PAGE, and immunoblotted for β-catenin. (D) Untransfected A431D cells (a–d) or A431D cells expressing N-cadherin (e and f), E-cadherin (g and h), E/N-cadherin (f and g), or N/E-cadherin (h and i) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. (E) Untransfected A431 D cells (a) or A431D cells expressing E-cadherin (b), N-cadherin (c), E/N-cadherin (d), or N/E-cadherin (e) were tested for their ability to aggregate in a hanging drop aggregation assay. Bar, 10 μm.

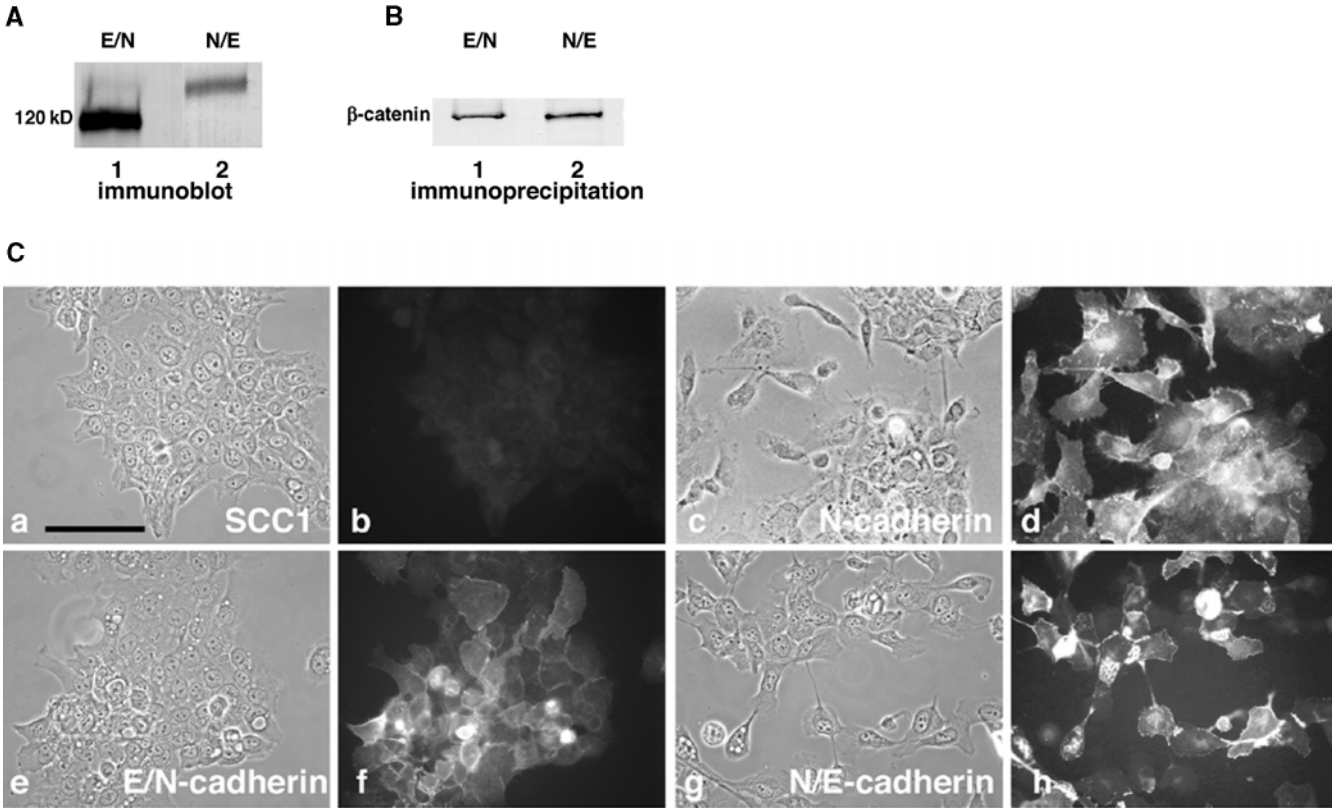

Figure 2. Expression of E/N- and N/E-cadherin in SCC1 cells. (A) SCC1 cells were transfected with E/N- or N/E-cadherin and examined for transgene expression by immunoblotting with antibodies against the cytoplasmic (lane 1) or extracellular (lane 2) domain of N-cadherin. (B) Extracts were immunoprecipitated, resolved by SDS-PAGE, and immunoblotted for β-catenin. (C) Untransfected SCC1 cells (a and b) or SCC1 cells expressing N-cadherin (c and d), E/N-cadherin (e and f), or N/E-cadherin (g and h) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. Bar, 15 μm.

tion (Islam et al., 1996). In breast cancer cells, expression of N-cadherin did not alter the morphology of the cells, but did induce cell motility and invasion (Nieman et al., 1999a). Here, we sought to determine how N-cadherin functioned to alter the phenotype of epithelial cells. We predicted that the cytoplasmic domain of N-cadherin was capable of initiating a signal transduction pathway that resulted in increased cellular motility. To determine if this was the case, we engineered two chimeric cadherins. The first, called E/N-cadherin, consisted of the extracellular and transmembrane domains of E-cadherin joined to the cytoplasmic domain of N-cadherin. The second chimera consisted of the extracellular and transmembrane domains of N-cadherin joined to the cytoplasmic domain of E-cadherin (N/E-cadherin). A schematic of these two chimeric cadherins is presented in Fig. 1 A.

The Extracellular Domain of N-Cadherin Influences Epithelial Cell Behavior

Our goal was to test E/N-cadherin and N/E-cadherin for effects on cellular morphology and behavior using two model systems we had already established. In the first model system, the oral squamous epithelial cell line SCC1 undergoes a significant and readily discernible morphological change from a typical epithelial cell to a fibroblastic cell, when transfected with N-cadherin (Islam et al., 1996). In the second model system, the human breast cancer cell line BT20 changes from a relatively nonmotile to a highly motile cell when transfected with N-cadherin (Nieman et al., 1999a). Interestingly, the BT20 cells do not undergo a morphological change when they are transfected with N-cadherin, suggesting that the effects of N-cadherin differ somewhat between these two different types of epithelial cells. Before testing the effect our chimeric cadherins had on the morphology and behavior of cells, it was important to show that each chimera was a functional adhesion molecule. To determine if the chimeras were functional, we transfected them into the cadherin-negative A431D cell line, which has been previously described by our laboratory (Lewis et al., 1997; Thoreson et al., 2000). Fig. 1 shows that the chimeric cadherins were expressed by the A431D cells at the expected size (Fig. 1 B), that they associated with catenins in an immunoprecipitation assay (Fig. 1 C), that they were located at the cell surface (Fig. 1 D), and that they mediated cell aggregation (Fig. 1 E). These data demonstrate that both E/N-cadherin and N/E-cadherin function as adhesion molecules in a manner similar to E-cadherin or N-cadherin. Surprisingly, the morphology of A431D cells transfected with E-cadherin did not differ significantly from that of A431D cells transfected with N-cadherin. In addition, the morphology of A431D cells transfected with the chimeras was similar to A431D cells transfected with either E-cadherin or N-cadherin.

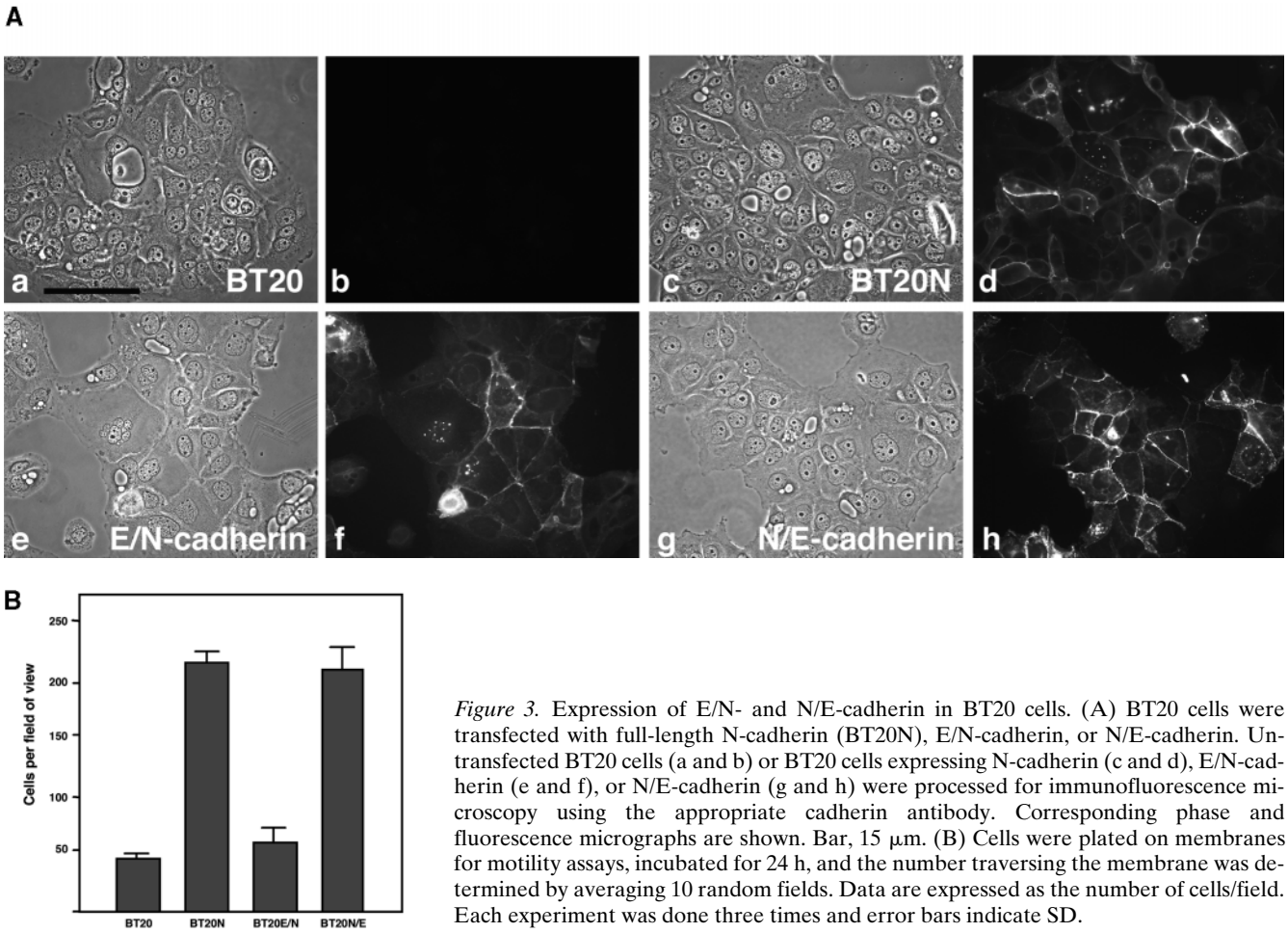

Figure 3. Expression of E/N- and N/E-cadherin in BT20 cells. (A) BT20 cells were transfected with full-length N-cadherin (BT20N), E/N-cadherin, or N/E-cadherin. Untransfected BT20 cells (a and b) or BT20 cells expressing N-cadherin (c and d), E/N-cadherin (e and f), or N/E-cadherin (g and h) were processed for immunofluorescence microscopy using the appropriate cadherin antibody. Corresponding phase and fluorescence micrographs are shown. Bar, 15 μm. (B) Cells were plated on membranes for motility assays, incubated for 24 h, and the number traversing the membrane was determined by averaging 10 random fields. Data are expressed as the number of cells/field. Each experiment was done three times and error bars indicate SD.

E/N-cadherin and N/E-cadherin were transfected into SCC1 cells and analyzed for their ability to induce an epithelial to mesenchymal transition. Each chimera was highly expressed (Fig. 2 A), coimmunoprecipitated with β-catenin (Fig. 2 B), and localized at the cell surface (Fig. 2 C, f and h). To our surprise, the N/E-cadherin (Fig. 2 C, g) produced a change in morphology similar to that seen with intact N-cadherin (Fig. 2 C, c), whereas the E/N-cadherin did not effect the morphology of these cells (Fig. 2 C, e). To determine if the extracellular domain of N-cadherin was also responsible for the change in motility of BT20 cells, we transfected N/E-cadherin and E/N-cadherin into these cells. Fig. 3 A shows that both chimeric cadherins were expressed at the cell surface and that neither chimera produced an effect on the morphology of these cells. This is consistent with our previous studies showing that N-cadherin did not effect the morphology of BT20 cells (Nieman et al., 1999a) (Fig. 3 A, c). Fig. 3 B shows that N/E-cadherin was as efficient as intact N-cadherin at inducing motility in BT20 cells, whereas E/N cadherin did not significantly alter the motile characteristics of BT20 cells. Thus, our hypothesis that the cytoplasmic domain of N-cadherin initiates a signaling pathway, resulting in increased cell motility, was not substantiated. Rather, it appeared that the extracellular domain of N-cadherin was responsible for the epithelial to mesenchymal transition in squamous epithelial cells and increased motility in breast cancer cells. The remainder of this study was aimed at determining which part of the extracellular domain of N-cadherin influenced cellular morphology and behavior.

Extracellular Domain 4 of N-Cadherin Confers a Motile Phenotype on Epithelial Cells

To further investigate the extracellular domain of N-cadherin, we constructed additional chimeric cadherins. We started with N/E-cadherin and moved the boundary between N- and E-cadherin progressively towards the NH_2 terminus (Fig. 4 A). We added a myc tag to the COOH terminus of the chimeras so that we could use the identical antibody to detect each chimera. We also constructed a chimeric N/E-cadherin with a myc tag (N/E-myc) to ensure addition of the tag did not alter the ability of N/E-cadherin to confer a motile phenotype on human epithelial cells. The chimeric cadherin that included approximately one third of EC5 of E-cadherin was designated N/E5a-myc; the chimeric cadherin that included EC5 of E-cadherin was designated N/E5-myc; the chimeric cadherin that included EC5 and most of EC4 of E-cadherin was designated N/E4-myc; the chimeric cadherin that included EC5, EC4, and most of EC3 of E-cadherin was designated N/E3-myc; and the chimeric cadherin that included EC5, EC4, EC3, and most of EC2 of E-cadherin was designated N/E2-myc (Fig. 4 A).

Each chimera was transfected into the cadherin-negative A431D cells to determine if it functioned properly as an adhesion molecule. The N/E-cadherin with a 2X-myc

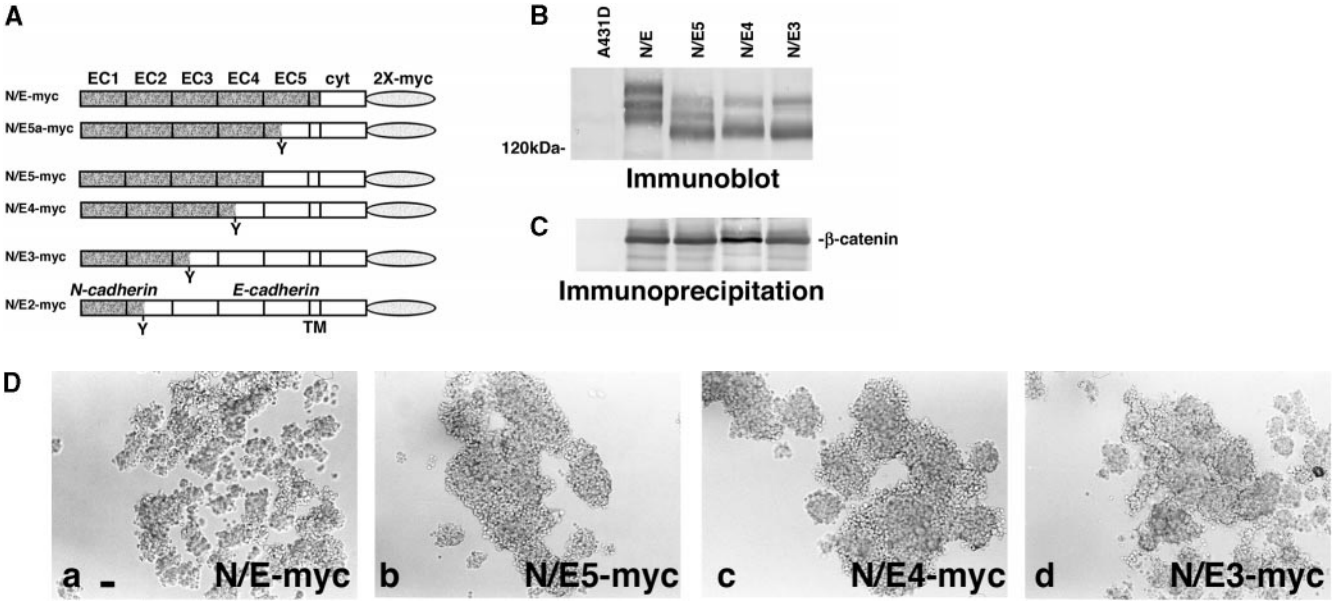

Figure 4. Generation of additional cadherin chimeras. (A) Chimeric cadherins, with a 2X-myc tag at the COOH terminus, consisting of E-cadherin (white) and N-cadherin (gray), were cloned into pLKpac. (B) A431D cells were transfected and examined for transgene expression by immunoblotting with anti-myc. Note, in some cases, we observed various processing variants when transfected cadherins were overexpressed in cells. (C) Extracts were immunoprecipitated using anti-myc, resolved by SDS-PAGE, and immunoblotted for β-catenin. (D) A431D cells expressing N/E-myc-cadherin (a), N/E5-myc-cadherin (b), N/E4-myc-cadherin (c), or N/E3-myc-cadherin (d) were tested for their ability to aggregate in a hanging drop aggregation assay. Bar, 15 μm.

tag (N/E-myc-cadherin) behaved exactly like N/E-cadherin, indicating that the myc tag did not influence the function of the chimeric cadherin. Chimeras N/E-myc, N/E5-myc, N/E4-myc, and N/E3-myc were each expressed at a high level, as indicated by immunoblot analysis using anti-myc antibodies (Fig. 4 B). The proteins were processed to the predicted size, though there was more unprocessed protein than was seen for endogenous cadherins, E/N-cadherin, or N/E-cadherin. Each chimera efficiently associated with β-catenin, as demonstrated by coimmunoprecipitation (Fig. 4 C). In addition, each chimera mediated cell aggregation (Fig. 4 D). Chimeras N/E5a-myc and N/E2-myc were not properly processed or did not mediate adhesion in A431D cells, so we did not use them in assays to map the domain of N-cadherin that functions to induce motility in epithelial cells.

When N/E-myc, N/E5-myc, N/E4-myc, and N/E3-myc chimeric cadherins were transfected into SCC1 cells, they were highly expressed (Fig. 5 A) and coimmunoprecipitated with β-catenin (Fig. 5 B). The N/E-myc and N/E5-myc chimeras produced the same morphological change in SCC1 cells that was seen with N/E-cadherin (Fig. 5 C, a and c). In contrast, the N/E4-myc and N/E3-myc chimeras had no effect on the morphology of SCC1 cells (Fig. 5 C, e and g).

We were equally interested in the ability of these additional chimeric cadherins to influence cellular motility. We typically use the BT20 cells for this assay, since we have established a clear difference between N-cadherin–expressing and –nonexpressing BT20 cells. In addition, we wanted to be sure we were looking at the same phenomenon we had previously published (Nieman et al., 1999a). However, the BT20 cells grow slowly in culture and are difficult to transfect. We have not been successful at establishing BT20 cell lines expressing the additional chimeras. Therefore, we established a motility assay that made use of the already transfected A431D cells. We first showed that A431D cells transfected with N-cadherin were more motile than untransfected A431D cells or A431D cells transfected with E-cadherin (Fig. 5 D). In addition, we showed that A431D cells transfected with E/N-cadherin behave similarly to A431 cells transfected with intact E-cadherin, and A431D cells transfected with N/E-cadherin behave like A431D cells transfected with intact N-cadherin. Thus, we believe we are testing the same N-cadherin–mediated effect on motility whether we use the BT20 system or the A431D system. A431D cells transfected with the N/E5 chimera were as motile as those transfected with full-length N-cadherin or with the N/E chimera, whereas the motility rates of cells transfected with the N/E4 and N/E3 chimeras were similar to the motility rates of cells transfected with E-cadherin or with the E/N chimera. Thus, we determined that the domain of N-cadherin, which is responsible for the epithelial to mesenchymal transition when expressed in squamous epithelial cells, is most likely the same domain that increases cell motility when N-cadherin is expressed in epithelial cells. This domain probably resides in EC4, most likely the region including amino acids 535–603.

Extracellular Domain 4 Is Sufficient to Confer a Motile Phenotype on Epithelial Cells

To confirm that extracellular domain 4 of N-cadherin alone was responsible for altering the behavior of epithelial cells, we constructed two additional chimeric cadherins. The first was E-cadherin, except that amino acids 535–603 of N-cadherin replaced the corresponding portion of E-cadherin and was called E/N/E-cadherin (Fig. 6 A). The second chimera was N-cadherin, except that amino acids 535–603 of N-cadherin were replaced by the corre-

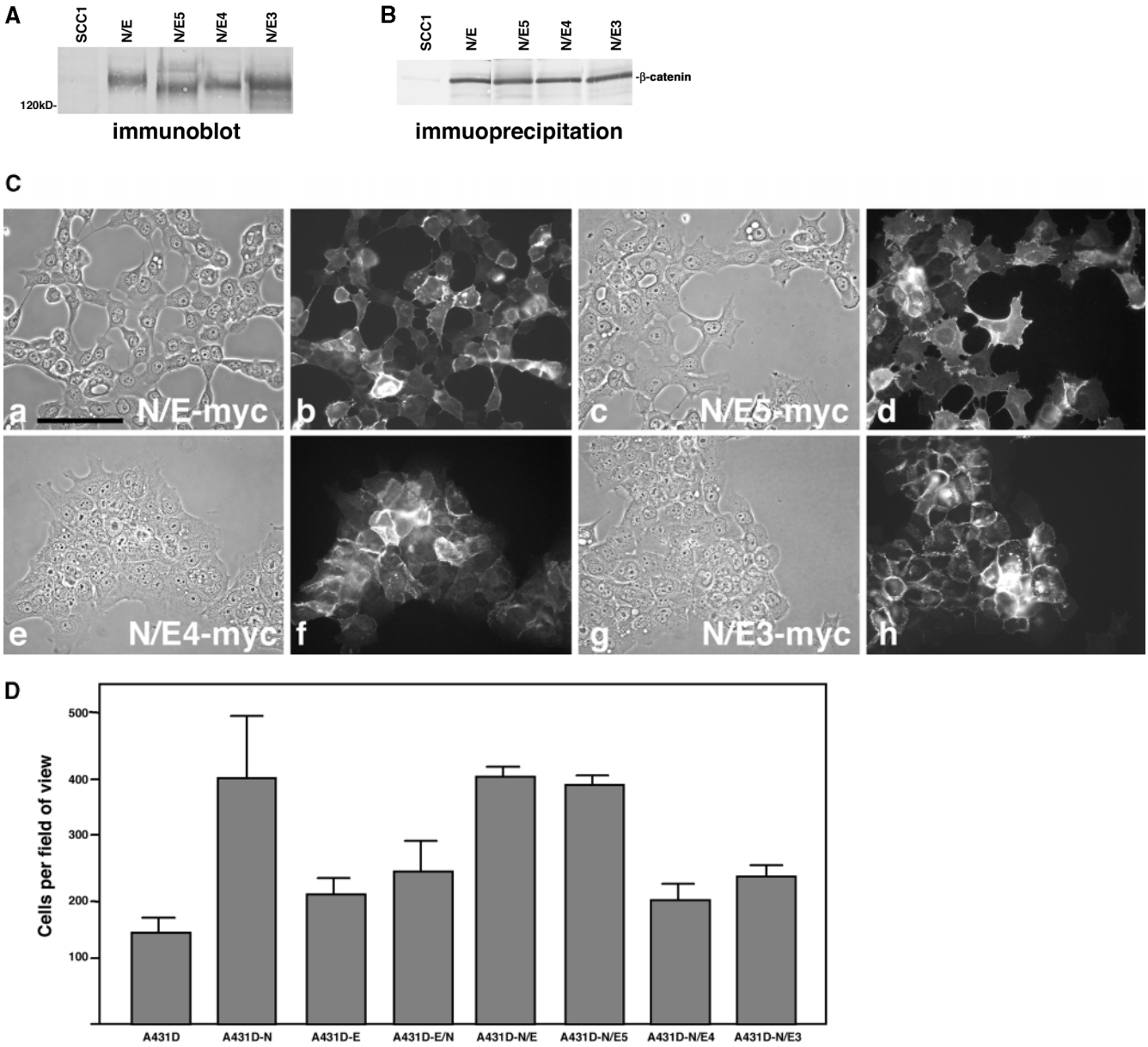

Figure 5. Expression of additional N/E-cadherin chimeras. (A) SCC1 cells were transfected with N/E-myc-cadherin, N/E5-myc-cadherin, N/E4-myc-cadherin, or N/E3-myc-cadherin and examined for transgene expression by immunoblotting with anti-myc. (B) Extracts were immunoprecipitated using anti-myc, resolved by SDS-PAGE, and immunoblotted for β-catenin. (C) SCC1 cells transfected with N/E-myc-cadherin (a and b) N/E5-myc-cadherin (c and d), N/E4-myc-cadherin (e and f), or N/E3-myc-cadherin (g and h) were processed for immunofluorescence microscopy using anti-myc. Corresponding phase and fluorescence micrographs are shown. Bar, 15 μm. (D) A431D cells either nontransfected or transfected with N-cadherin (A431D-N), E-cadherin (A431D-E), E/N-myc-cadherin (A431D-E/N), N/E-myc-cadherin (A431D-N/E), N/E5-myc-cadherin (A431D-N/E5), N/E4-myc-cadherin (A431D-N/E4), or N/E3-myc-cadherin (A431D-N/E3) were plated on membranes for motility assays, incubated for 24 h, and the number traversing the membrane was determined by averaging 10 random fields. Data are expressed as the number of cells/field. Each experiment was done three times and error bars indicate SD.

sponding amino acids of E-cadherin (N/E/N-cadherin). Both chimeras included a 2X-myc tag. When transfected into the cadherin-negative A431D cells, both the E/N/E-cadherin and the N/E/N-cadherin were highly expressed, coimmunoprecipitated with β-catenin (Fig. 6 B), and efficiently mediated cell aggregation (Fig. 6 C). In addition, each chimera was expressed at cell borders in SCC1 cells (Fig. 6 D). The E/N/E chimera produced the epithelial to mesenchymal transition seen with full-length N-cadherin (Fig. 6 D, a), whereas the N/E/N chimera did not (Fig. 6 D, c). When A431D cells were transfected with the E/N/E-cadherin, they showed motility rates similar to that seen when the cells were transfected with full-length N-cadherin. In contrast, the N/E/N transfected cells showed motility rates similar to E-cadherin–transfected cells (Fig. 6 E). Thus, this short 69–amino acid segment of N-cadherin was both necessary and sufficient to cause the morphological and behavioral changes seen in epithelial cells.

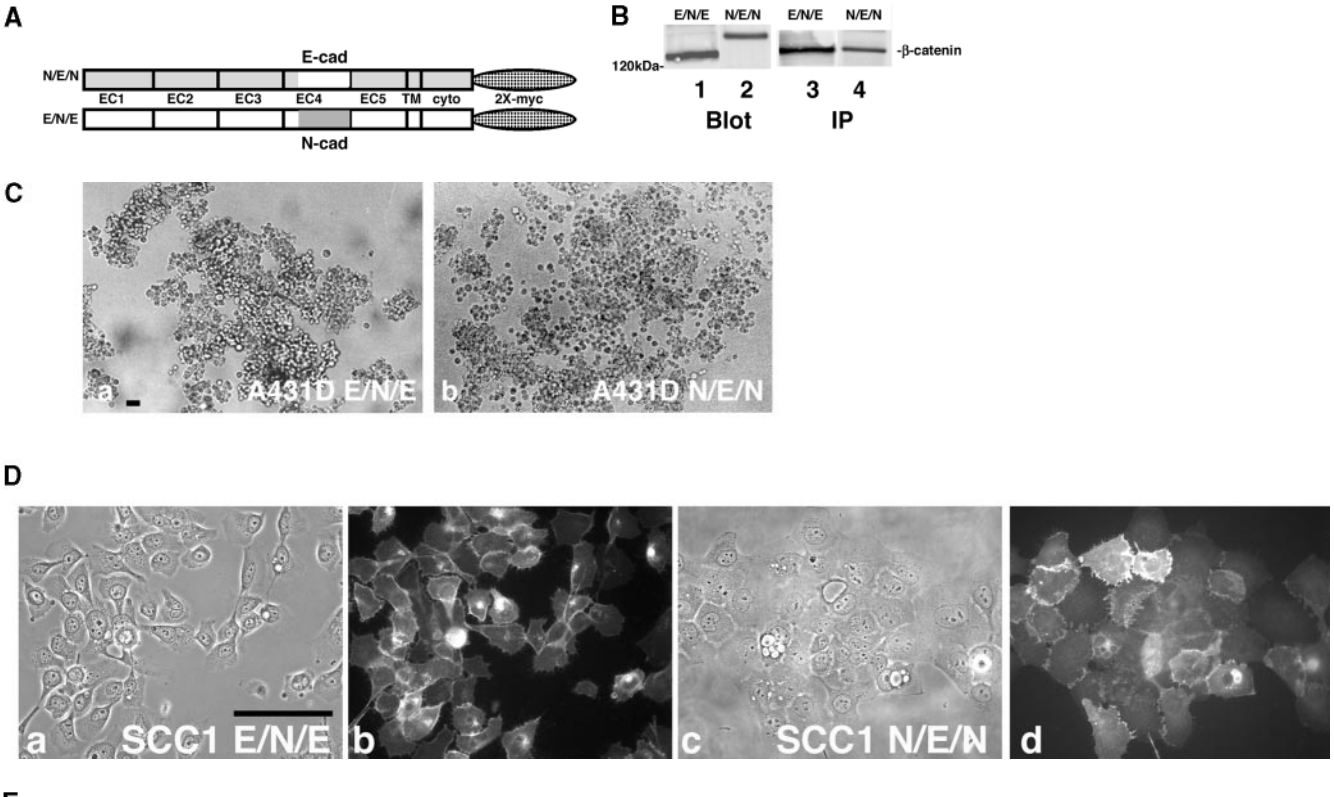

Figure 6. Generation and expression of E/N/E- and N/E/N-cadherins. (A) Chimeric cadherins consisting of E-cadherin (white) and N-cadherin (gray) were constructed and cloned into pLKpac with a 2X-myc tag at the COOH terminus. (B) A431D cells were transfected and examined for transgene expression by immunoblotting with anti-myc (lanes 1 and 2). Extracts were immunoprecipitated using anti-myc, resolved by SDS-PAGE, and immunoblotted for β-catenin (lanes 3 and 4). (C) A431D cells expressing E/N/E-myc-cadherin (a) or N/E/N-myc-cadherin (b) were tested for their ability to aggregate in a hanging drop aggregation assay. (D) SCC1 cells transfected with E/N/E-myc-cadherin (a and b) or N/E/N-myc-cadherin (c and d) were processed for immunofluorescence microscopy using anti-myc. Corresponding phase and fluorescence micrographs are shown. Bar, 15 μm. (E) A431D cells transfected with E-cadherin (A431D-E), N-cadherin (A431D-N), E/N/E-myc-cadherin (E/N/E), or N/E/N-myc-cadherin (N/E/N) were plated on membranes for motility assays, incubated for 24 h, and the number traversing the membrane was determined by averaging 10 random fields. Data are expressed as the number of cells/field. Each experiment was done three times and error bars indicate SD.

Antibodies Directed against the Extracellular Domain of N-Cadherin Inhibit Motility in Epithelial Cells

The domain of classical cadherins that is responsible for cell adhesion resides in EC1. Antibodies directed against EC1 inhibit cadherin-mediated cell–cell interactions. Thus, we sought to determine if the ability of N-cadherin to influence cellular behavior could be inhibited by antibodies that bind to EC4. We immunized mice with the entire extracellular domain of human N-cadherin and chose those antibodies that mapped near EC4 for these studies. Fig. 7 A shows that one antibody, 8C11, bound to chimeric cadherins N/E-myc, N/E5a-myc, N/E5-myc, and N/E4-myc, but not to N/E3-myc or N/E2-myc. The control anti-myc antibody recognized each chimeric cadherin. When 8C11 was added to BT20N cells in a motility assay, it inhibited motility in a dose-dependent manner, indicating that this antibody did bind near the domain of N-cadherin that was responsible for altering the behavior of these cells (Fig. 7 B). We used the antibody at a dilution of 1:10 to repeat the experiment and to determine if it had any effect on N-cadherin–negative cells. For this experiment, we used smaller filters and counted the number of cells traversing the entire filter. The 8C11 antibody had minimal effect on the motility of N-cadherin–negative cells (Fig. 7 C). In addition, an irrelevant ascites (4A2), used at a dilution of 1:10, had minimal effect on the motility of BT20N or on the motility of untransfected BT20 cells (Fig. 7 C). However, the mAb 8C11 significantly decreased cell motility in the N-cadherin–expressing BT20N cells. Importantly, even at a 1:10 dilution in the mAb 8C11 did not inhibit cell aggregation in N-cadherin–expressing cells (data not shown). In an initial experiment, the 8C11 antibody did not produce a significant change in morphology when applied to N-cadherin–expressing SCC1 cells (data not shown). These results are unexpected and are being further investigated in our laboratory.

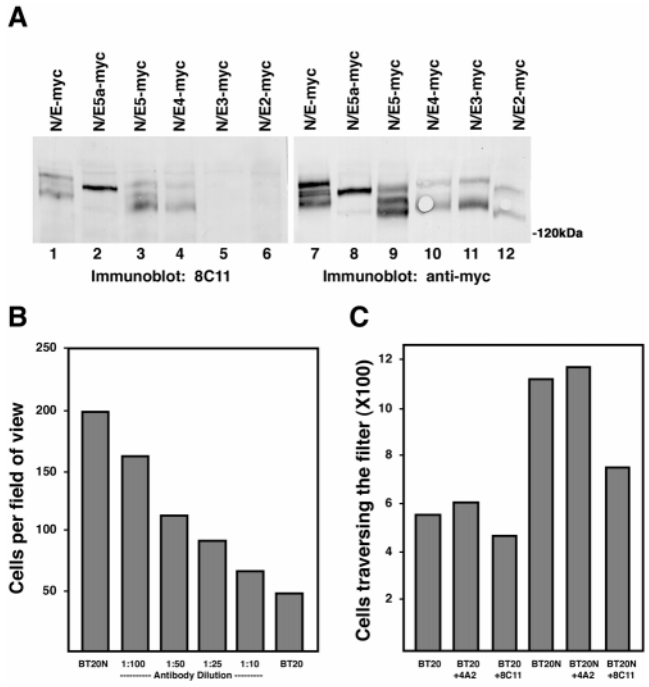

Figure 7. Anti–N-cadherin mAb 8C11 inhibits motility. (A) Extracts of A431D cells transfected with N/E-myc-cadherin (lanes 1 and 7), N/E5a-myc-cadherin (lanes 2 and 8), N/E5-myc-cadherin (lanes 3 and 9), N/E4-myc-cadherin (lanes 4 and 10), N/E3-myc-cadherin (lanes 5 and 11), or N/E2-myc-cadherin (lanes 6 and 12) were resolved by SDS-PAGE and immunoblotted with mAb 8C11 (lanes 1–6) or anti-myc (lanes 7–12). Note, in some cases, we observed various processing variants when transfected cadherins were overexpressed in cells. (B) BT20 cells, which were transfected with N-cadherin (BT20N), were plated on membranes for motility assays in the presence of no antibody or 8C11 ascites at a dilution of 1:10–1:100. Untransfected BT20 cells in the absence of antibody were included as a control. After 24 h, the number of cells traversing the membrane was determined by averaging 10 random fields at 100× magnification. Data are expressed as the number of cells/field. (C) Untransfected BT20 cells or BT20 cells transfected with N-cadherin (BT20N) were plated on membranes for motility assays in the presence of no antibody, irrelevant ascites 4A2 at a dilution of 1:10 or 8C11 ascites at a dilution of 1:10. After 24 h, the number of cells traversing the membrane was determined by counting the entire membrane. Data are expressed as the number of cells traversing the filter.

Discussion

We and others have shown that N-cadherin influences the morphology and behavior of epithelial cells (Islam et al., 1996; Hazan et al., 1997, 2000; Li et al., 1998). These studies implicate N-cadherin in an epithelial to mesenchymal transition in some cells, but not in others. In squamous epithelial cells, expression of N-cadherin results in downregulation of E-cadherin, which is most likely responsible for the change in cellular morphology. In other cells, such as breast cancer cells, expression of N-cadherin does not alter cell morphology, but does alter cellular behavior by inducing a motile phenotype. In breast cancer cells, expression of E-cadherin remains approximately the same when the cells are forced to express N-cadherin. This suggests that even in cells that express abundant E-cadherin, N-cadherin influences cell behavior. N-cadherin is often expressed by motile cells, such as fibroblasts, and a switch from E-cadherin expression to N-cadherin expression occurs when some cells become motile and/or invasive during normal developmental processes (Edelman et al., 1983; Hatta and Takeichi, 1986; Zhou et al., 1997; Huttenlocher et al., 1998). Thus, it is not unexpected that expression of N-cadherin by tumor cells alters cellular morphology and/or behavior.

The extracellular domain of a cadherin promotes cell–cell adhesion, whereas the cytoplasmic domain serves to link the cadherin to the cytoskeleton via interactions with catenins. These cytosolic interactions are critical to the adhesive function of the cadherin. Linkage to the cytoskeleton is necessary to promote strong cell–cell adhesion and to allow organization of the junction itself. In addition, the catenins have been implicated in signaling events that are thought to regulate the strength of the adhesive activity of the cadherin (for review see Gumbiner, 2000). This led us to propose that the cytoplasmic domain of N-cadherin was responsible for increasing the motility of epithelial cells. When we prepared two chimeric cadherins, one comprised of the extracellular domain of N-cadherin linked to the cytoplasmic domain of E-cadherin (N/E-cadherin) and the other comprised of the extracellular domain of E-cadherin linked to the cytoplasmic domain of N-cadherin (E/N-cadherin), we were surprised to find that it was the extracellular domain of N-cadherin that promoted cell motility. The extracellular domain of cadherins is comprised of five repeat regions with EC1 being the most NH_2-terminal. Most of the known activities of cadherins have been mapped to EC1. The best understood examples are those where cadherin molecules interact with other cadherin molecules. Structure determinations (Shapiro et al.,1995; Nagar et al., 1996; Tamura et al., 1998; Pertz et al., 1999) and biochemical characterization (Nose et al., 1990; Ozawa et al., 1990; Ozawa and Kemler, 1990; Koch et al., 1997; Shan et al., 2000) have demonstrated that EC1 is the site of the adhesion interface. Data from several laboratories have suggested that cadherins are displayed on the surface of cells as dimers (Shapiro et al., 1995; Brieher et al., 1996; Chitaev and Troyanovsky, 1998; Takeda et al., 1999; Shan et al., 2000). Although several differing pictures exist as to how these cis (also called lateral) dimers form and are maintained, the data point to EC1 and EC2 of the cadherins as playing major roles.

In some instances, it has been shown that cadherins can promote cell–cell adhesion via heterophilic interactions, for example N-cadherin can bind to R-cadherin (Inuzuka et al., 1991), B-cadherin can bind to L-CAM (Murphy-Erdosh et al., 1995), and cadherin-6B can bind to cadherin-7 (Nakagawa and Takeichi, 1995). Recently, Shimoyama et al. (2000) examined eight different type II cadherins and frequently observed interactions between L cells transfected with different cadherins. Another recent study showed that, in L cells expressing both N- and R-cadherins, the two cadherins formed cis heterodimers that functioned in cell adhesion (Shan et al., 2000). In this latter case, it was the NH_2 terminus of the cadherins that played a role in the formation of the cis heterodimers. It will be interesting to determine if other pairs of cadherins shown to mediate heterophilic cell–cell adhesion are able to form cis

heterodimers and what parts of the cadherins are involved. Here, we have shown that the ability of N-cadherin to promote cell motility resides in EC-4. Thus, this activity is distinct from the adhesive function of the cadherin.

In addition to the interaction of cadherins with themselves, various other interacting proteins have been described. The bacterium *Listeria monocytogenes* has been shown to use E-cadherin as a receptor. InlA, a surface protein on the bacterium, binds to E-cadherin. Lecuit et al. (1999) showed that changing a single amino acid in EC1 of E-cadherin (proline-16 of EC1) eliminated the binding of InlA and dramatically compromised internalization of *Listeria* by cells. In addition to being a target for *Listeria*, E-cadherin is the only cadherin that is known to be an integrin ligand. Integrin $\alpha_E\beta_7$ binds EC1 of E-cadherin, and glutamate-31 of EC1 plays a critical role in the interaction (Karecla et al., 1996). Since EC1 of cadherins has been shown to play a major role in their biological activities, all of the chimeras used here retained the intact EC1 of N-cadherin.

Although most activities have been mapped to the NH_2-terminal domains, there are several reports suggesting roles for EC3, EC4, and EC5 in cadherin adhesion. Zhong et al. (1999) have characterized a mAb (AA5) recognizing EC5 of C-cadherin that activates adhesion, perhaps by changing the cadherin's organization or altering its interaction with other cellular factors. Sivasankar et al. (1999) have studied the biophysical characteristics of adhesion mediated by layers of oriented recombinant C-cadherin ectodomains. They concluded that complete interdigitation of antiparallel ectodomains (i.e., where EC1 of one molecule interacted with EC5 of the antiparallel partner, EC2 interacted with EC4 of the partner, etc.) gave the strongest interactions. Their data also suggested that ratcheting the molecules one EC domain further apart (such that EC1 interacted with EC4 of its antiparallel partner, etc.) also resulted in an adhesive interaction. In addition, Troyanovsky et al. (1999) have reported that EC3 and EC4 of E-cadherin can mediate cis dimerization under some conditions.

A series of papers from Lilien's laboratory (for review see Lilien et al., 1999) have suggested that in neural retina cells, the ectodomain of N-cadherin is stably associated with and is a substrate for the cell surface enzyme *N*-acetylgalactosaminyphosphotransferase. The interaction of neurocan, a chondroitin sulfate proteoglycan, with *N*-acetylgalactosaminyphosphotransferase results in inhibition of N-cadherin–mediated cell adhesion. However, the site(s) on N-cadherin where this interaction takes place is unknown.

Investigators have suggested that N-cadherin can interact with and activate fibroblast growth factor receptors (FGFR) in neurons (Doherty and Walsh, 1996) and ovarian surface epithelial cells (Peluso, 2000). In the ovarian surface epithelial cell system, it has been reported that N-cadherin and FGFR coimmunoprecipitate. To date, this interaction has not been substantiated by other labs. Our laboratory recently showed that N-cadherin–mediated cell motility of breast cancer cells can be decreased by an inhibitor of the FGF-mediated signal transduction pathway, which has been characterized by the Walsh and Doherty labs (Nieman et al., 1999a). In addition, Hazan et al. (2000) showed that FGF caused a dramatic increase in motility in N-cadherin–expressing cells. The FGFRs contain an HAV sequence (Byers et al., 1992) that has been proposed to interact with EC4 of N-cadherin. It is interesting to note that the 69–amino acid segment of N-cadherin we have identified here includes the sequences proposed by Doherty and Walsh to interact with the FGFRs. The structure of a portion of FGFR1 bound to FGF2 has been determined (Plotnikov et al., 1999). The histidine and valine side chains of the HAV sequence in FGFR1 were involved in intradomain contacts and, thus, appear to be unavailable for interacting with partner molecules. Thus, the precise role the FGFR plays in N-cadherin–dependent cell motility is still unknown and it is not clear at this time whether N-cadherin and the FGFR directly interact with one another.

Many studies have shown that N-cadherin promotes cell motility that is dependent on the adhesive function of N-cadherin. The best studied example is that of N-cadherin–dependent neurite extension. In vitro experiments have demonstrated that N-cadherin promotes neurite outgrowth as a purified protein or when it is expressed by transfected cells. Importantly, antibodies that block the adhesive function of N-cadherin block this outgrowth, and it has been suggested that N-cadherin may guide axonal outgrowth in vivo (for review see Grunwald, 1996). In addition, Hazan et al. (1997) suggested that N-cadherin–mediated motility of tumor cells might be due to the interactions of N-cadherin–expressing epithelial cells with N-cadherin–expressing stromal cells. In contrast, the studies presented here, using the 8C11 mAb, provide evidence that N-cadherin may influence the motility of epithelial cells in a manner that is independent of cell–cell adhesion.

Since the 69–amino acid portion of N-cadherin can influence epithelial cell morphology and motility, we compared this portion of human N-cadherin to other cadherins. In this region, mouse and rat N-cadherin are identical to human N-cadherin, whereas 78% of the amino acids in human R-cadherin are identical. The corresponding region of human E-cadherin contains 70 amino acids and is 54% identical to N-cadherin. To further investigate the role this portion of N-cadherin plays in cell motility, we produced a mAb that binds near EC-4 of N-cadherin. When applied to cells in a motility assay, this antibody inhibited cell motility in N-cadherin–expressing cells, but not in N-cadherin–negative cells. In addition, this antibody inhibited motility without inhibiting cell–cell aggregation, providing further evidence that adhesion and motility are two separate properties of the extracellular domain of N-cadherin. It is important to remember that all the chimeras used here were full-length cadherins. Studies are in progress to determine if truncated cadherins can influence cell motility.

The authors thank Drs. K. Green and A. Ben Ze'ev for reagents. The authors thank Ms. Laura Sauppé and Ms. Jill Nieset for excellent technical help.

Support was provided by National Institutes of Health grants GM51188 and DE12308 to M.J. Wheelock and K.R. Johnson, respectively, and by Department of Defense grants DAMD-17-97-1-7298 and DAMD17-98-1-8252 to M.J. Wheelock.

Submitted: 24 July 2000
Revised: 5 October 2000
Accepted: 12 October 2000

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