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Disruption of Epithelial Cell-Cell Adhesion by Exogenous Expression of a Mutated Nonfunctional N-Cadherin

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Cadherins, a family of transmembrane cell-cell adhesion receptors, require interactions with the cytoskeleton for normal function. To assess the mechanisms of these interactions, we studied the effect of exogenous expression of a mutant N-cadherin, cN390Δ, on epithelial cell-cell adhesion. The intracellular domain of cN390Δ was intact but its extracellular domain was largely deleted so that this molecule was not functional for cell adhesion. cDNA of cN390Δ was attached to the metallothionein promoter, and introduced into the keratinocyte line PAM212 expressing endogenous E- and P-cadherin. When the expression of cN390Δ was induced by Zn^{2+}, cadherin-dependent adhesion of the transfected cells was inhibited, resulting in the dispersion of cell colonies, although their contacts were maintained under high cell density conditions. In these cultures, cN390Δ was expressed not only on the free surfaces of the cells but also at cell-cell junctions. The endogenous cadherins were concentrated at cell-cell junctions under normal conditions. As a result of cN390Δ expression, however, the endogenous cadherins localizing at the cell-cell junctions were largely diminished, suggesting that these molecules were replaced by the mutant molecules at these sites. As a control, we transfected the same cell line with cDNA of a truncated form of N-cadherin whose intracellular C terminus had been deleted leaving the extracellular domain intact. This molecule had no effect on cell-cell adhesion, nor did it localize to cell-cell contact sites. We also found that the association of the endogenous cadherins with α- and β-catenins and plakoglobin was not affected by the expression of cN390Δ, which also formed a complex with these molecules, suggesting that no competition occurred between the endogenous and exogenous cadherins for these cytoplasmic proteins. These and other additional results suggest that the nonfunctional cadherins whose intracellular domain is intact occupy the sites where the endogenous cadherins should localize, through interactions with the cytoskeleton, and inhibit the cadherin adhesion system.

INTRODUCTION

Cadherins are a family of transmembrane cell-cell adhesion receptors that are concentrated in a particular class of cell-cell junctions called adherens junction (AJ), intermediate junction, belt desmosome, or zonula adherens (Geiger and Ginsberg, 1991), and a subgroup of this molecular family localizes in desmosome (Buxton and Magee, 1992). In these cell-cell junctions, cadherins homophilically interact with each other via the extracellular domain in a Ca^{2+}-dependent manner (Takeichi, 1990, 1991). The cytoplasmic domain of cadherins is anchored to a complex of cytoskeletal proteins, and this anchoring is thought to be crucial for their cell adhesion function (Nagafuchi and Takeichi, 1988; Nagafuchi and Takeichi, 1989; Ozawa *et al.*, 1989; Ozawa and Kemler, 1992). Cadherin molecules whose cytoplasmic domain has been deleted cannot associate with the cytoskeletal protein complex, and coincidentally lose cell-cell binding ability (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990).

Proteins associated with the cadherin cytoplasmic domain include α-, β-, and γ-catenins, and plakoglobin

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(Ozawa *et al.*, 1989; McCrea and Gumbiner, 1990; Peifer *et al.*, 1992; Knudsen and Wheelock, 1992). α- and β-catenins have recently been cloned. α-Catenin is a vinculin-like protein that was identified as an E-cadherin-associated protein (Herrenknecht *et al.*, 1991; Nagafuchi *et al.*, 1991), and a neural subtype of this molecule was also identified (Hirano *et al.*, 1992). β-Catenin is a homologue of the Drosophila *armadillo* product, and resembles plakoglobin (McCrea *et al.*, 1991). These proteins bind to the carboxy half region of the cadherin cytoplasmic domain, and deletion of this region is sufficient to inhibit cell binding action of cadherins as well as their anchoring to the cytoskeleton (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990). Therefore, catenins are likely involved in the regulation of cadherin function, and a crucial role of α-catenin was recently demonstrated (Hirano *et al.*, 1992). To elucidate how cadherins interact with catenins and other cytoplasmic proteins is thus important in understanding the molecular mechanisms of cadherin-mediated cell-cell adhesion.

A recent work by Kintner (1992) demonstrated that exogenous expression of various mutant N-cadherins, whose extracellular domain was deleted, in *Xenopus* embryos caused dissociation of tissues. This interesting phenomenon was ascribed to a dominant negative effect of the introduced molecules on endogenous cadherin function, although evidence for this idea was not sufficient. A possible explanation for the dominant negative effect was such that the introduced cytoplasmic domain of N-cadherin competed with the endogenous cadherins for catenins or other cytoplasmic proteins, which were required for cadherin function. In accord with this idea, Kintner (1992) found that a competition occurred for α-catenin between the mutant and normal cadherins in *Xenopus* embryos.

The present study was designed to investigate in more detail how the expression of nonfunctional cadherins with the intact cytoplasmic domain interfered with cell-cell adhesion. To this aim, we constructed an expression vector of a mutant N-cadherin whose extracellular domain was largely deleted but leaving the cytoplasmic domain intact. This vector contained the metallothionein promoter, an inducible promoter (Stuart *et al.*, 1984), and it was introduced into an epithelial cell line with endogenous cadherins. The expression of these nonfunctional cadherins in the cells indeed perturbed cadherin mediated cell-cell adhesion. However, our results suggest that this perturbation is not due to a competition for the cadherin-associated proteins thus far identified between the endogenous and mutant cadherins, but more likely due to a substitution of the mutant nonfunctional cadherins for the endogenous functional cadherins at cell-cell junctions. The latter possibility was supported by the observation that the nonfunctional cadherins accumulated into cell-cell contact sites with a concomitant loss of endogenous cadherins from these sites.

MATERIALS AND METHODS

Construction of Expression Vectors

cDNA encoding a mutant N-cadherin whose extracellular domain was partially deleted was constructed as follows. Chicken N-cadherin cDNA cloned into Z10T6/BS (Hatta *et al.*, 1988) was digested with NspV, blunt ended with mungbean nuclease and partially digested using *Eco*RI, and then its 5040-bp fragment was purified using the DNA PREP (IATRON). This fragment was blunt ended with Klenow fragment and self-ligated, and the product was termed cN390Δ/BS. DNA sequence was checked at around the connection site by digestion with *EcoT22I*.

Inducible N-cadherin expression vectors, driven by the metallothionein-I promoter, which contain the neomycin resistant gene were constructed as below. The *Bam*HI-*Bgl* II fragment of the SV40 polyadenylation signal region of pSV2hph (Santerre *et al.*, 1984; Gorman, 1985) was cloned into the *Bam*HI site of pBluescript SK+. This vector was then digested with *Xba* I and *Spe* I, self-ligated to delete the *Xba* I recognition site, and termed pSV/BSx. The metallothionein-I promoter (MTID-113/-85) (Stuart *et al.*, 1984) was derived from pMTID13S (a gift from Kazuo Maruyama, University of Tokyo, Japan) by digestion with *Kpn* I and *Sal* I, and cloned into the *Kpn* I/*Sal* I site of pSV/BSx, and this vector was named pMTIDBS. Neomycin-resistant vector pSTneoB (Katoh *et al.*, 1987) in which the neomycin resistant gene is connected to the SV40/thymidine kinase promoter complex and SV40 polyadenylation signal was digested with *Xho* I, and the fragment obtained was cloned into the *Sal* I site of pUC19. Then this vector was digested with the use of *Xba* I, followed by blunt-ending with mungbean nuclease, and self-ligation was carried out to delete the *Xba* I recognition sequence. This vector was named as pSTB-19x. Expression vector pMT1neoB was made by ligating *Kpn* I-*Sac* I–digested pSTB-19x with the *Kpn* I-*Sac* I fragment of pMTIDBS.

To obtain an expression vector of normal or mutant N-cadherin cDNA, Z10T6BS or cN390Δ/BS was digested with *Sal* I and *Sma* I. The fragments were ligated to the *Sal I-Eco*RV digested pMT1neoB to give pMTcN and pMTcN390Δ, expression vectors for intact and deleted N-cadherins, respectively.

The β-actin promoter and RSV enhancer complex (Kato *et al.*, 1990) was used to make a constitutive expression vector of the above mutant N-cadherin, which was termed pMiwcN390Δ. To construct pMiwcN390Δ, cN390Δ/BS was digested with *Sma* I followed by partial digestion with *Hin*dIII, and with this fragment, the CAT gene of pMiwCAT (Kato *et al.*, 1990) was replaced by *Hin*dIII-*Hpa* I digestion and ligation.

Cell Culture

The mouse keratinocyte cell line PAM212 (Yuspa *et al.*, 1980) was used. For cell culture, a 1:1 mixture of Dulbecco's modified Eagle's minimal essential medium and Ham's F12 supplemented with 10% fetal calf serum (DH10) was used. DNA transfection by the calcium-phosphate precipitation method and selection of transfected cells were carried out as described previously (Nagafuchi *et al.*, 1987). To induce the expression of transfected N-cadherin molecules, cells were treated with 100 μM $ZnCl_2$ in DH10 at least for 24 h. This concentration of Zn^{2+} was not toxic for PAM212 cells.

Cell Aggregation Assay

PAM212 cells or their transfected lines were cultured with or without $ZnCl_2$ for 1 d and washed with Ca^{2+}- and Mg^{2+}-free *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered (pH 7.4) Hanks' saline (HBS) supplemented with 1 mM EDTA (HCMF-EDTA). Then the cultures were treated with 0.025% trypsin (Sigma T-8253,

St. Louis, MO) in HCMF-EDTA for 20 min at 37°C on a gyratory shaker and subsequently dissociated into single cells by pipetting. These cells were suspended in DH10 medium, and washed twice with the medium. Finally, 1×10^5 cells were suspended in 500 μl of DH10 with or without 100 μM $ZnCl_2$ and incubated on a gyratory shaker rotating at 80 rpm in a 5% CO_2 incubator. After 90 min, aggregates formed and were examined.

For detecting cadherin activity in L cells transfected with pMiwcN390Δ, termed cN390ΔL1, or L cells transfected with pMiwcN, termed cNLm-1 (Fujimori *et al.*, 1990), their cultures were treated with 0.01% trypsin in the presence of 1 mM Ca^{2+} to obtain single-cell suspension. This treatment generally leaves cadherins intact on cell surfaces (Takeichi, 1977). The cell suspension was incubated to allow cell aggregation on the gyratory shaker as above for 40 min.

Immunocytochemistry

For double immunofluorescence staining for N- and E-cadherins, cells were fixed with 3.5% paraformaldehyde in HBS containing 1 mM $CaCl_2$ at 4°C. After cells were permeabilized with 100% methanol at −20°C, and washed with tris(hydroxymethyl)aminomethane(Tris)-buffered saline (TBS) containing 1 mM $CaCl_2$ (TBS-Ca), they were incubated with 5% skim milk to prevent nonspecific binding of antibodies. Cells were then incubated with a mixture of anti-N- and E-cadherin antibodies each of which was appropriately diluted with TBS-Ca for 60 min, followed by washing with TBS-Ca. The rat monoclonal antibody NCD-2 (Hatta and Takeichi, 1986) and a rabbit anti-E-cadherin antiserum were used for staining for chicken N-cadherin and mouse E-cadherin, respectively. The samples were then incubated with biotinylated anti-rat IgG antibody (Amersham, Arlington Height, IL) and extensively washed. The staining signals were visualized by incubating the samples with a mixture of Texas-red conjugated streptavidin (Amersham) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (MBL) in TBS-Ca.

Detergent Extraction of Cadherins and Immunoprecipitation

Cells were washed with chilled HBS and extracted with TBS-Ca containing 1% NP40 and 1% Triton X-100 at 4°C with a gentle shaking for 30 min. After centrifuging at 14 000 rpm for 10 min, the supernatant was collected and subjected to immunoprecipitation of cadherins.

Immunoprecipitation of E- or N-cadherin was carried out as described (Nagafuchi and Takeichi 1989). To 1 ml of each detergent cell extract, 50 μl of 8% bovine serum albumin, 60 μl of 5 M NaCl, and 200 μl of Sepharose CL-4B (Pharmacia, Piscataway, NJ) were added and incubated for 30 min on ice to absorb materials, which bind nonspecifically to immunoaffiniy beads. The sample was then centrifuged at 10 000 rpm for 10 s, and the supernatant was incubated for 1 h on ice with NCD-2 or the rat monoclonal antibody ECCD-2 (Shirayoshi *et al.*, 1986) to immunoprecipitate chicken N-cadherin or mouse E-cadherin, respectively. To the solution, 50 μl of Sepharose 4B-conjugated anti-rat Ig (Zymed, San Francisco, CA) was added and incubated for 1 h on ice. After centrifugation, the beads were washed five times with TBS-Ca containing 1% NP40 and 1% Triton X-100. Then the immune complexes were boiled with a sodium dodecyl sulfate (SDS) sample buffer containing 5% 2-mercaptoethanol to release proteins, and the sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblot analysis.

Immunoblotting

Cell lysates or immunoprecipitates prepared with a SDS lysis buffer were boiled with 5% 2-mercaptoethanol. Proteins in the samples were separated by using 7.5% polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking with 5% skim milk in TBS-Ca, the membranes were incubated with antibodies. For detecting N-, E-, and P-cadherin, the rat monoclonal antibodies NCD-2, ECCD-2, and PCD-1 (Nose and Takeichi, 1986) were used, respectively. For staining α-catenin (CAP102) and plakoglobin, the rat monoclonal antibody 1809 (a gift from Akira Nagafuchi, National Institute of Physiological Science, Okazaki, Japan) and the mouse antibody 5A2 (a gift from Pamela Cowin, New York University, New York) was used, respectively. For detecting β-catenin, a rabbit anti-*armadillo* antiserum (a gift from Mark Peifer, University of North Carolina, Chapel Hill), which is known to cross-react with this catenin (Peifer *et al.*, 1992) was used. After washing, the membranes were incubated with peroxidase-conjugated anti-rat or anti-mouse IgG antibody (Amersham) or anti-rabbit Ig antibody (Cappel Cochranville, PA). For visualizing bands, the ECL Western blotting detection system (Amersham) was used.

RESULTS

Nonfunctional N-cadherin with a Deletion in the Extracellular Domain

We constructed cDNA encoding a mutant chicken N-cadherin in which a large portion of the extracellular domain was deleted but the other regions were intact (Figure 1A) and termed it cN390Δ. This mutant molecule retained the original *N*-terminal region consisting of 107 amino acids, which was recognized by the monoclonal antibody NCD-2 (Nose *et al.*, 1990). When this cDNA inserted into an expression vector was introduced into L cells without cadherins, the translation products did not confer any cadherin-mediated cell-cell adhesiveness on the cells (Figure 2, A–C), in spite of their expression on the cell surface (Figure 2D). This result showed that cN390Δ was not functional in cell-cell adhesion.

Effect of the Expression of cN390Δ on Epithelial Cell-Cell Adhesion

Next, we examined whether the nonfunctional N-cadherin cN390Δ had any effect on cell-cell adhesion when it was introduced into epithelial cells expressing functional endogenous cadherins. The inducible expression vector pMTcN390Δ was constructed (Figure 1B) in which cN390Δ expression was controlled by the metallothionein promoter that could be activated by Zn^{2+} (Stuart *et al.*, 1984). As a control, the vector pMTcN encoding intact N-cadherin, which contains the same metallothionein promoter, was used. With these vectors, PAM212, a keratinocyte cell line, was transfected, and stable transfectant lines were obtained. Among them, we chose two transfectant lines as representatives; PAMcNΔ2A transfected with cN390Δ, and PAMcN1-6 transfected with normal N-cadherin.

The transfected cells were treated with 100 μM $ZnCl_2$ to induce expression of the exogenous molecules. Immunoblot analysis showed that PAMcNΔ2A and PAMcN1-6 expressed 52 and 127 kDa proteins recognized by NCD-2, respectively, only when they were treated with Zn^{2+} (Figure 3A). The former band was assumed to be cN390Δ, and the latter was intact N-cadherin. The 52-kDa band was generally less intense

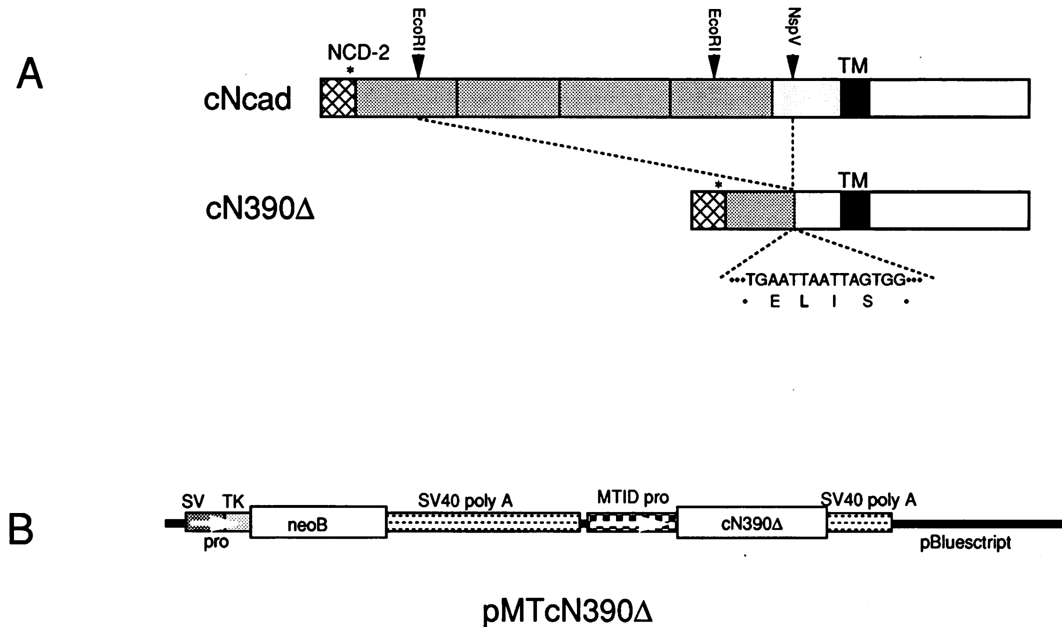

Figure 1. Construction of an inducible expression vector of the mutant N-cadherin cN390Δ. (A) Structure of cN390Δ in which the illustrated portion containing 390 amino acids of the extracellular domain of chicken N-cadherin was deleted by using *Eco*RI and *Nsp* V. This mutant molecule retained the site recognized by the anti-N-cadherin monoclonal antibody NCD-2, as shown by asterisks. Sequences of DNA and amino acids at the connection site are shown. Leucine (L) was newly introduced into the site during construction of the molecule. (B) Structure of the inducible expression vector pMTcN390Δ. cN390Δ cDNA was connected to the mouse metallothionein promoter (MTID-113/-85). The neomycin resistant gene, which is driven by the SV40 and thymidine kinase promoter, is located tandem on the same vector. For constructing pMTcN, cN390Δ cDNA was replaced with intact N-cadherin cDNA in the pMTcN390Δ. cNcad, intact chicken N-cadherin; TM, transmembrane domain; SV, SV40 promoter; TK, thymidine kinase promoter; neoB, neomycin resistant gene; MTID pro, metallothionein I promoter (MTID-113/-85).

than the 127-kDa band, although this did not necessarily indicate that a smaller amount of cN390Δ was expressed because this mutant protein might have a less immunoreactivity to NCD-2. Untransfected PAM212 cells expressed E- and P-cadherin endogenously, of which the former was more abundant (Figure 3B, lanes 1 and 3). Zn^{2+} treatment did not alter the expression of endogenous E- and P-cadherin (Figure 3B, lanes 2 and 4). PAMcNΔ2A also expressed similar amounts of E- and P-cadherin, and the expression of these molecules was not affected by Zn^{2+} treatment (Figure 3, lanes 5–8).

When expression of cN390Δ was induced in PAMcNΔ2A cells, they showed striking morphological changes; cells tended to disrupt their epithelial associations (Figure 4, A and B). This effect was most evident in small colonies growing at low-cell densities, and also in peripheral regions of large colonies. In centers of the large colonies where cells were confluent, the effect of cN390Δ expression was relatively small, and the cells maintained their mutual contacts, probably because of no physical spaces for cell separation. No morphological changes were induced either in untransfected PAM212 cells or in PAMcN1-6 cells with exogenous intact N-cadherin by Zn^{2+} treatment (Figure 4, C–F).

As another control, we transfected PAM212 cells with cDNA encoding a truncated form of N-cadherin, cNΔ, in which the C-terminal 36 amino acids had been removed (Fujimori *et al.*, 1990); this truncated N-cadherin was neither functional, nor did it associate with cytoskeletal proteins, as found with similar mutants of E-cadherin (Fujimori and Takeichi, unpublished data). The expression of this molecule had no effect on cell-cell adhesion. Thus cN390Δ specifically interfered with cell-cell adhesion.

Cell Aggregation Assay for Cadherin Activity

For direct measurement of the effect of cN390Δ expression on cell-cell adhesiveness, cell aggregation assay was performed. PAMcNΔ2A or PAM212 cells were cultured overnight with or without Zn^{2+}, trypsinized to make single-cell suspensions, and allowed to reaggregate in the presence or absence of Zn^{2+} in suspension cultures. These cells gradually aggregated, and the aggregation was cadherin-dependent at least for initial few hours of incubation, because it was completely blocked by a mixture of monoclonal antibodies to E- and P-cadherin (Figure 5, C and F). In these aggregation cultures, PAMcNΔ2A cells formed much smaller aggregates in

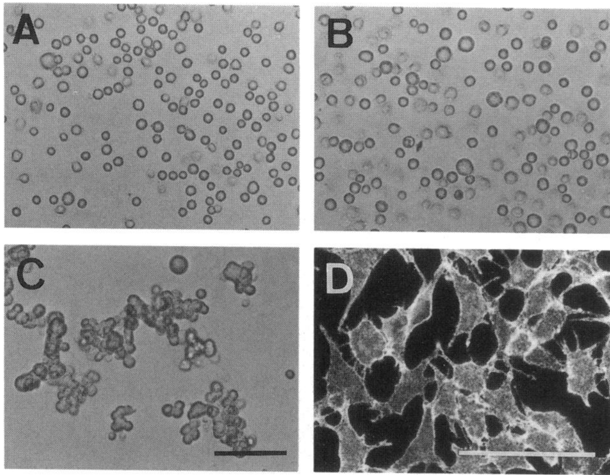

Figure 2. Aggregation of L cells transfected with cN390Δ or intact N-cadherin. (A) Parental L cells without cadherins. (B) L cells expressing cN390Δ (clone cN390ΔL1). (C) L cells expressing intact N-cadherin (clone cNLm-1). These cells were dissociated as described in MATERIALS AND METHODS and allowed to aggregate for 40 min. Only cNLm-1 cells aggregated. (D) Live cN390ΔL1 cells in a monolayer culture were immunofluorescently stained using NCD-2. cN390Δ is diffusely expressed on the surface. Bars, 100 μm.

the presence of Zn^{2+} (Figure 5B) than in the absence of this ion (Figure 5A). The aggregation of parental PAM212 cells was not affected by Zn^{2+} treatment (Figure 5, D and E), and this ion itself had no effect on these cell aggregations. These results indicated that the expression of cN390Δ suppressed endogenous cadherin activity, though not completely.

Immunofluorescence Localization of Endogenous and Mutant Cadherins

In parental PAM212 cells, E- and P-cadherin were concentrated at cell-cell boundaries, and this localization was not changed by addition of Zn^{2+}. PAMcNΔ2A cells that had not been treated with Zn^{2+} showed the same expression patterns of these cadherins (Figure 6B), and they did not express any anti-N-cadherin antibody positive signals (Figure 6A). Then we studied the localization of cN390Δ and the endogenous cadherins after Zn^{2+} treatment. We describe only the results on E-cadherin since essentially the same results were obtained for P-cadherin.

When expression of cN390Δ was induced by Zn^{2+} in PAMcNΔ2A cells, this molecule was detected on the cell surface, but its localization varied with cell densities. At low densities where cell-cell adhesion was visibly disrupted by cN390Δ expression, this mutant N-cadherin diffusely distributed on the cell surface (Figure 6C). In these cells, E-cadherin tended to disappear from their edges (Figure 6D), although it was still weakly concentrated at remaining cell-cell contact regions. At high cell densities where all cell-cell contacts were retained, the mutant N-cadherin localized at cell-cell contact sites as well as noncontact sites (Figure 6E). In these cells, E-cadherin signals were greatly decreased at the cell-cell boundaries (Figure 6F), as observed at low cell densities. This effect on E-cadherin expression was most clearly recognized when the culture was a mixture of cells expressing and not expressing cN390Δ, such an example is shown in Figure 6, E and F. In these cultures, E-cadherin signals decreased only in cell colonies expressing cN390Δ, resulting in a complementary expression pattern of the two molecules.

We also stained for intact N-cadherin and the C-terminus truncated N-cadherin, cNΔ, introduced into PAM212 cells. Intact N-cadherin localized at cell-cell boundaries as endogenous cadherins (Figure 7A). In contrast, the truncated N-cadherin did not localize to cell-cell boundaries, although it distributed on the cell surface (Figure 7B). The expression of this molecule had no effect on the distribution of endogenous cadherins.

Association of Mutant and Endogenous Cadherins with Cytoplasmic Proteins

Cadherins form a complex with a group of cytoplasmic proteins including α and β catenins (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990; Hirano *et al.*, 1992) and plakoglobin (Knudsen and Wheelock, 1992; Peifer *et al.*, 1992). We examined whether the expression of

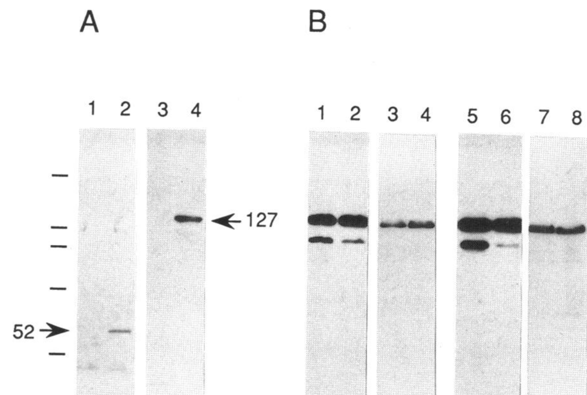

Figure 3. Immunoblot analyses of cadherins in the transfected PAM212 lines with or without Zn^{2+} treatment. (A) Expression of cN390Δ (52 kDa) and intact N-cadherin (127 kDa) in PAMcNΔ2A (lanes 1 and 2) and PAMcN1-6 (lanes 3 and 4), respectively. Cells in lanes 2 and 4 were treated with 100 μM Zn^{2+}, whereas cells in lanes 1 and 3 were not treated. (B) Expression of E- and P-cadherin in parental PAM212 (lanes 1–4) and PAMcNΔ2A (lanes 5–8) cells. Lanes 1, 2, 5, and 6, E-cadherin; lanes 3, 4, 7, and 8, P-cadherin. Cells in lanes 2, 4, 6, and 8 were treated with Zn^{2+}, and cells in lanes 1, 3, 5, and 7 were not. Zn^{2+} treatment had no effect on endogenous E- and P-cadherin expression. Lower bands are degradation products of E-cadherin. Molecular weight markers for 200, 116, 97, 66, and 45 × 10^3 are indicated on the left.

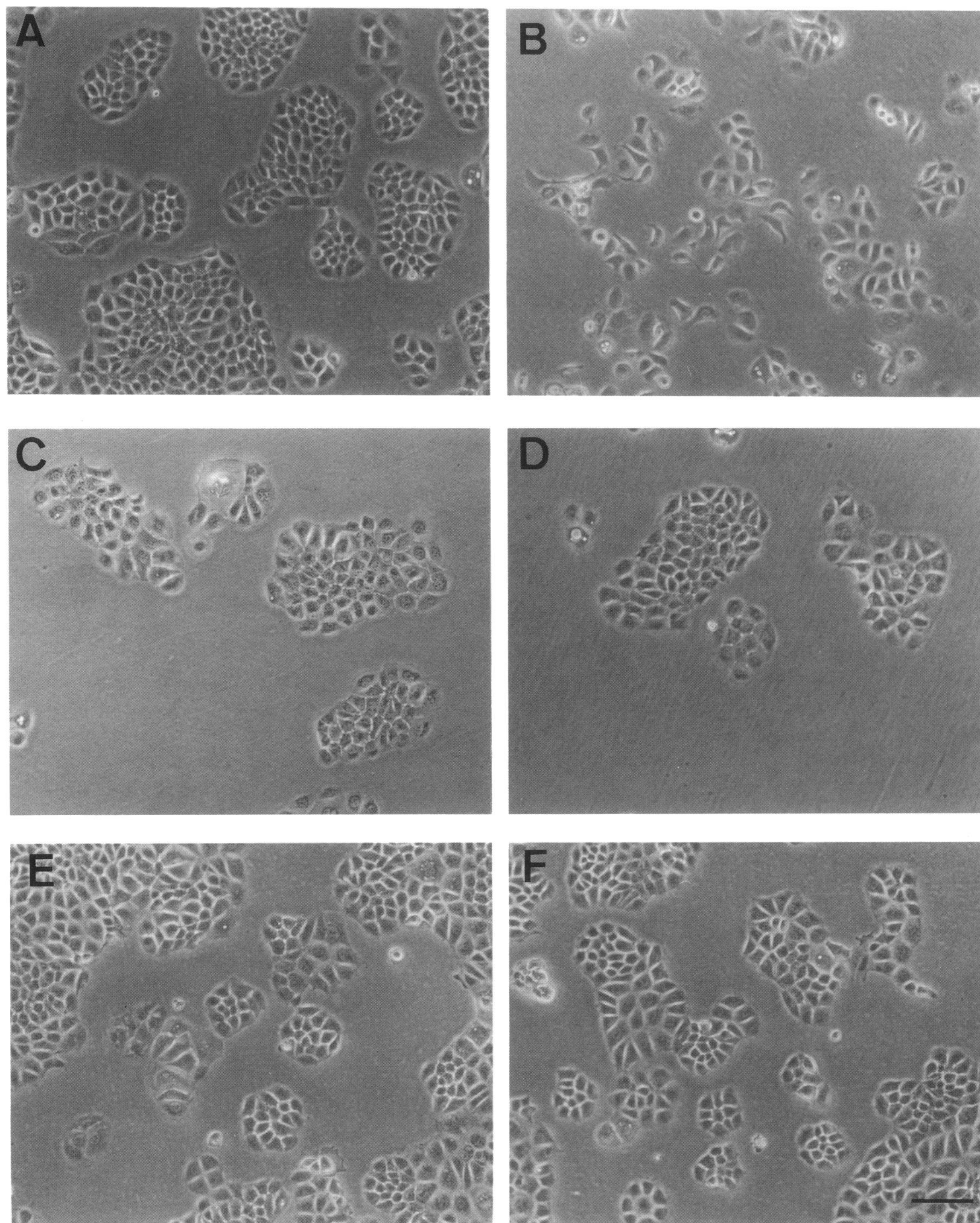

Figure 4. Effect of the induction of cN390Δ or intact N-cadherin expression by Zn^{2+} on cell-cell adhesion. (A and B) PAMcNΔ2A. (C and D) PAMcN1-6. (E and F) Control PAM212 cells. Left: cells without Zn^{2+} treatment; right: cells incubated with 100 μm Zn^{2+} for 24 h. Only PAMcNΔ2A cells, transfected with cN390Δ, responded to the Zn^{2+} treatment, and their cell-cell adhesion was disrupted. Bar, 100 μm.

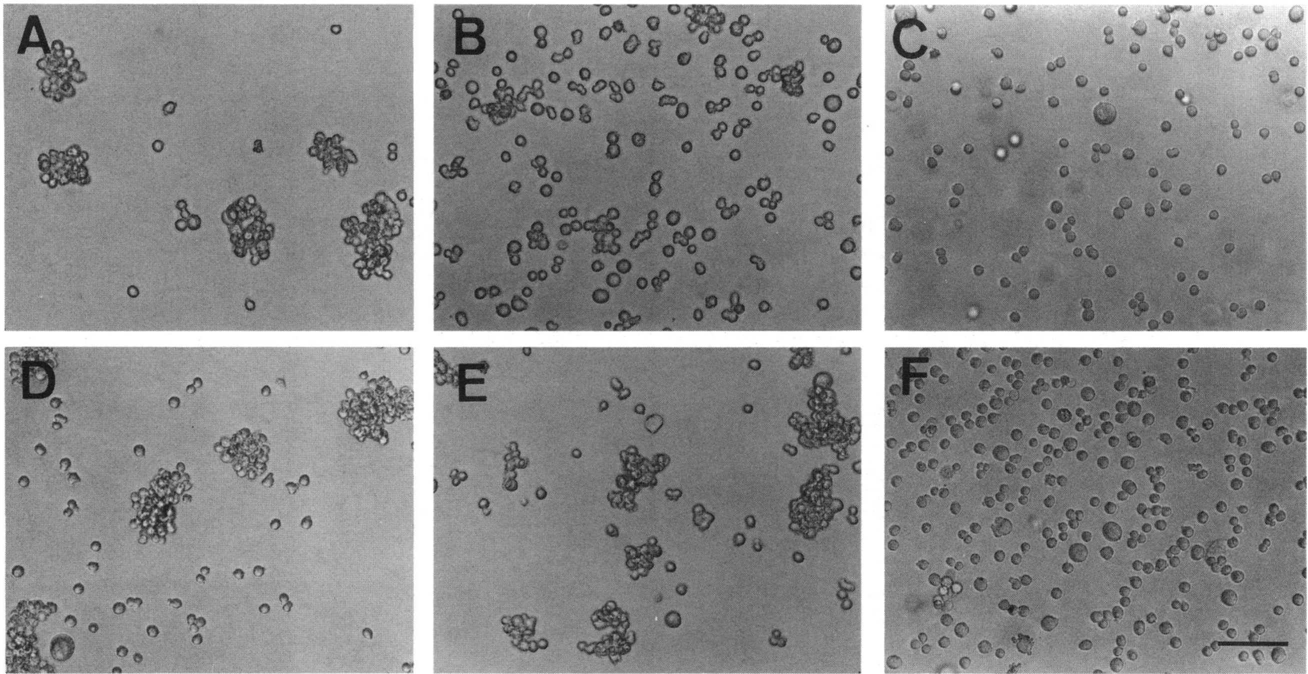

Figure 5. Effect of the expression of cN390Δ on cadherin dependent cell aggregation. (A–C) PAMcNΔ2A cells. (D–F) Control PAM212 cells. Cells in B and E were cultured overnight with 100 μM Zn^{2+}, whereas cells in other panels were not treated with Zn^{2+}. These cells were dissociated into single cells, and allowed to reaggregate for 90 min. The aggregation medium contained 100 μM Zn^{2+} in B and E. Note that Zn^{2+} treatment partly inhibited the aggregation of PAMcNΔ2A cells but not that of the parental cells. In C and F, cells were aggregated in the presence of both anti-E and P-cadherin monoclonal antibodies, ECCD-1 and PCD-1. Note the complete inhibition of cell aggregation by the antibodies. The concentration of each antibody was ~100 μg/ml. Bar, 100 μm.

cN390Δ had any effect on the organization of this system.

We first compared the solubility of endogenous E-cadherin and cN390Δ to a mixture of nonionic detergents before and after Zn^{2+} induction. Some fraction of cadherins, especially the ones localized at cell-cell junctions, are known to be resistant to extraction with nonionic detergents (Hirano *et al.*, 1987; Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989, 1990; Nelson *et al.*, 1990). We detected both detergent-soluble and insoluble fractions of E-cadherin in PAMcNΔ2A cells (Figure 8A, lanes 1 and 2). The ratio of the two fractions did not change after Zn^{2+} treatment (Figure 8A, lanes 3 and 4). cN390Δ was also divided into two fractions, soluble and insoluble, and their ratio was similar to that in the case of E-cadherin (Figure 8B). These results suggest that the nonfunctional cN390Δ molecules can be anchored to the cytoskeleton as equally as intact cadherins. We confirmed these observations by immunofluorescence staining for cN390Δ in the cells extracted with the nonionic detergent mixtures. This molecule was detected at cell-cell contact sites even after the detergent treatment.

In next experiments, binding of these cadherins with α or β catenin or plakoglobin was examined. E-cadherin or cN390Δ was extracted with nonionic detergents from PAMcNΔ2A cells, and were immunoprecipitated by using specific antibodies, and the coprecipitated materials were analyzed by immunoblotting. As shown in Figure 9, A and E, cadherin coprecipitated with α-catenin, β-catenin, and plakoglobin in samples without Zn^{2+} treatment. Interestingly, the amounts of these cytoplasmic proteins coprecipitating with E-cadherin were not changed after Zn^{2+} treatment (Figure 9B). Likewise, cN390Δ coprecipitated with all of these proteins (Figure 9C). Thus it seems that excess amounts of these cytoplasmic proteins were expressed in the cells even after the expression of cN390Δ.

DISCUSSION

A previous observation using *Xenopus* embryos (Kintner, 1992) suggested that the expression of nonfunctional N-cadherins with intact cytoplasmic domain perturbed the cadherin cell-cell adhesion system. In the present study, we provided evidence for this hypothesis, demonstrating that a similar form of N-cadherin indeed interfered with cadherin-mediated cell-cell adhesion. A suggested mechanism for this phenomenon was that the cytoplasmic domain of the introduced mutant N-cadherin competed with endogenous cadherins for cytoplasmic factors, which were essential for cadherin

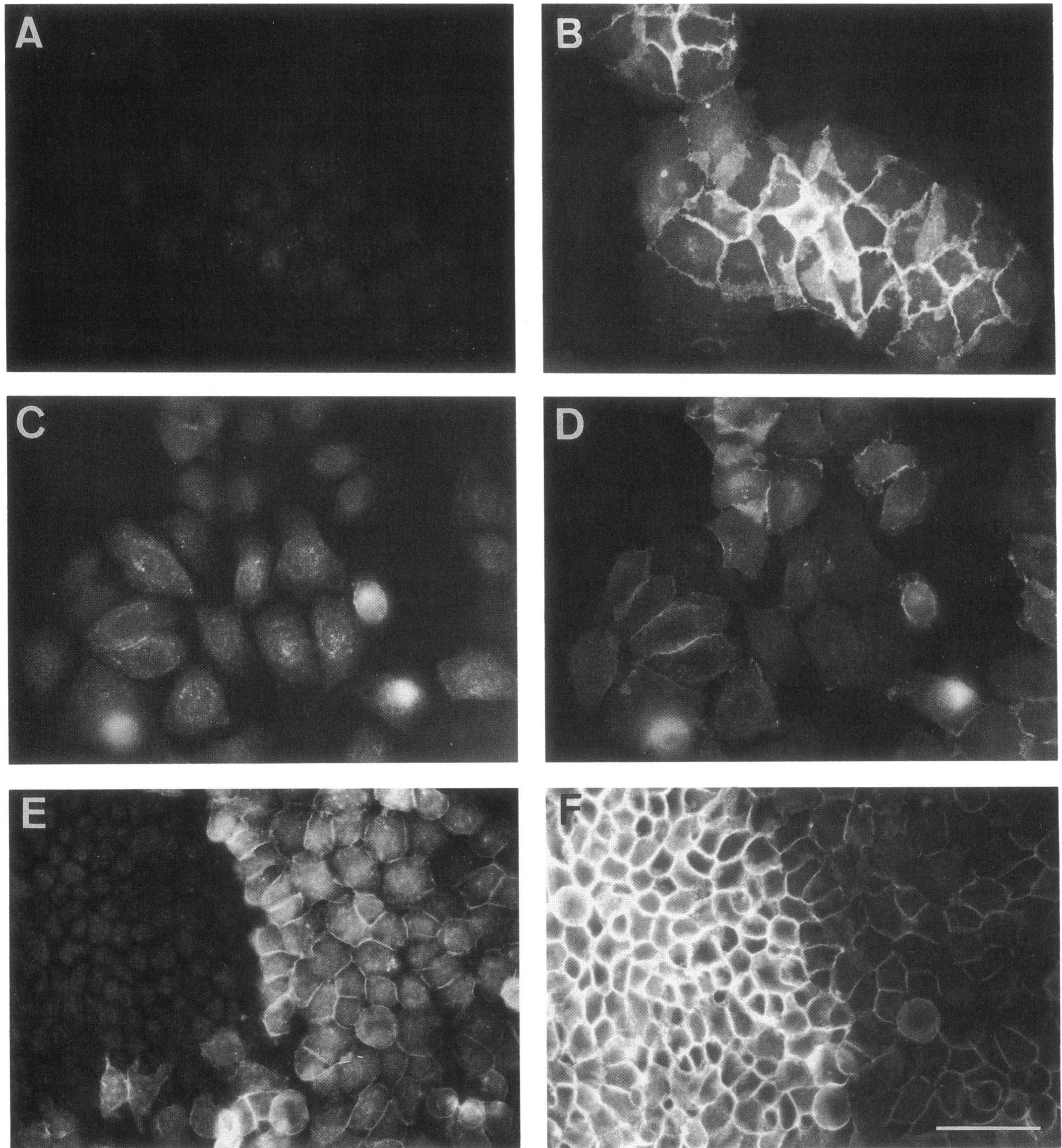

Figure 6. Immunofluorescence localization of cN390Δ and E-cadherin. Each specimen was double stained for cN390Δ (left) and E-cadherin (right). (A and B) PAMcNΔ2A cells not treated with Zn^{2+}. cN390Δ was not detected, and E-cadherin was localized at cell-cell boundaries. (C and D) PAMcNΔ2A cells at a low density treated with Zn^{2+}. (E and F) A mixed confluent culture of PAMcNΔ2A and untransfected PAM212 cells treated with Zn^{2+}. Expression of cN390Δ coincides with a disappearance or reduction of E-cadherin localizing at cell-cell boundaries. cN390Δ is detected at cell-cell boundaries as well as on free cell surfaces in the confluent cultures. Bar, 50 μm.

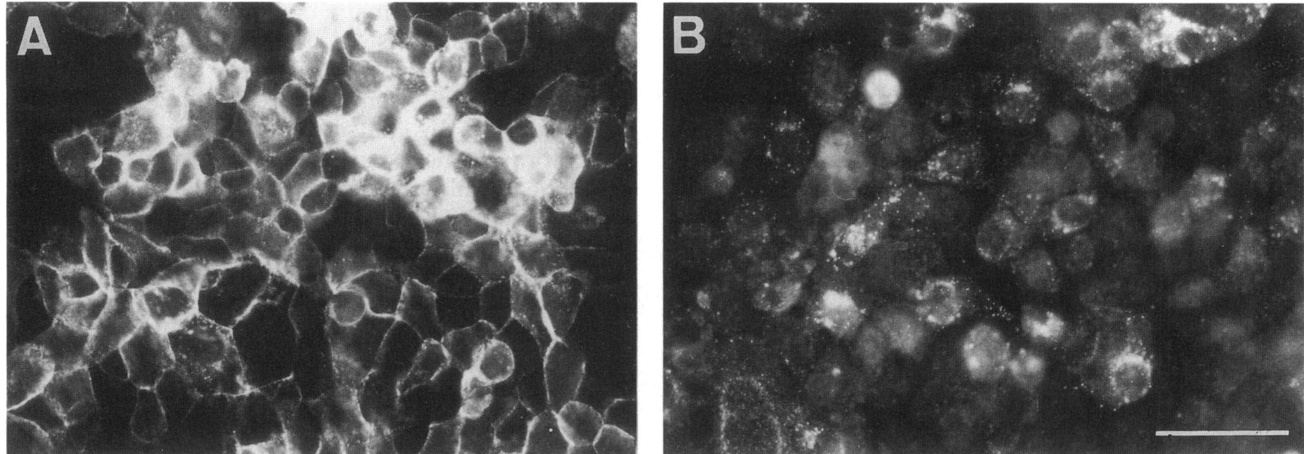

Figure 7. Immunofluorescence localization of intact N-cadherin and the C-terminus truncated cNΔ. (A) Intact N-cadherin expressed in PAMcN1-6 cells. (B) cNΔ expressed in a transfected PAM212 line. Note that this mutant molecule does not localize at cell-cell junctions. Bar, 50 μm.

function. However, we found that the amounts of three proteins, α- and β-catenin and plakoglobin, associated with endogenous E-cadherin were not altered by the expression of the mutant N-cadherin in PAM212 cells. It thus seems that sufficient amounts of these cadherin-associated proteins were supplied in the cells even after the expression of the mutant molecule. This finding is not consistent with the observation of Kintner (1992) that α-catenin was depleted in *Xenopus* embryos as a result of the overexpression of nonfunctional N-cadherins. In this study, however, α-catenin binding to *Xenopus* endogenous cadherins was not examined. Also, the *Xenopus* system could be exceptional; an excess α-catenin might not efficiently be supplied in early *Xenopus* embryos.

Thus we could not obtain any evidence for a possible competition for cytoplasmic factors between the exogenous and endogenous cadherins, as far as the three major cadherin-associated proteins were concerned.

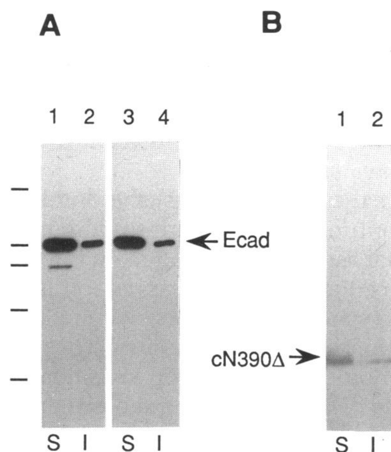

Figure 8. Detergent solubilities of E-cadherin and cN390Δ. PAMcNΔ2A cells were extracted with the nonionic detergent mixture, and the samples were centrifuged at 14 000 rpm for 10 min to separate the soluble (S) and insoluble (I) fractions. Each fraction was subjected to immunoblot analysis for detecting E-cadherin or cN390Δ. (A) E-cadherin. Cells in lanes 1 and 2 were not treated with Zn^{2+}, and cells in lanes 3 and 4 were treated with Zn^{2+}. (B) cN390Δ. E-cad, E-cadherin. Molecular weight markers are the same as shown in Figure 3.

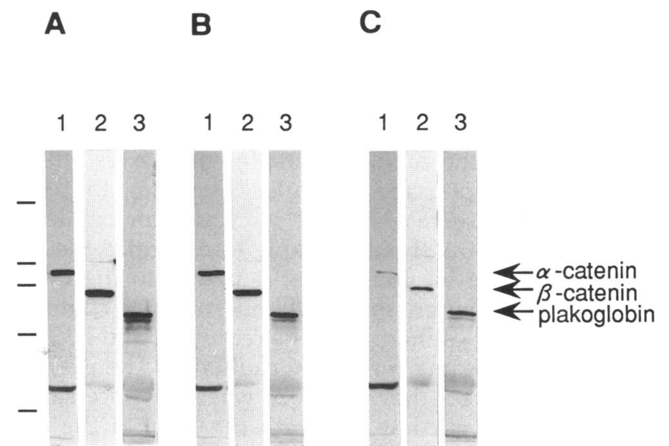

Figure 9. Immunoblot detection of cadherin-associated proteins. E-cadherin or cN390Δ was immunoprecipitated from the soluble fraction of detergent cell extracts, and the co-precipitated materials were subjected to immunoblot analyses for detection of α-catenin (lane 1), β-catenin (lane 2) and plakoglobin (lane 3). (A) E-cadherin co-precipitates from PAMcNΔ2A cells without Zn^{2+} treatment. (B) E-cadherin co-precipitates from PAMcNΔ2A cells after Zn^{2+} treatment. (C) cN390Δ co-precipitates from PAMcNΔ2A cells treated with Zn^{2+}. A lower major band seen in each lane is immunoglobulin brought into the samples through immunoprecipitation, and other bands were not identified. Molecular weight markers are the same as shown in Figure 3.

However, we still cannot rule out the possibility that the exogenous mutant N-cadherin deprived cells of some unknown cytoplasmic components. Kintner (1992) analyzed in detail the effect of various regions of the N-cadherin cytoplasmic domain on tissue dissociation. The carboxy half region that interacts with α and β catenins was effective, as suspected. In addition, however, some other regions of the cytoplasmic domain were also effective. This suggests that not only the proteins thus far identified but also other unknown molecules might be interacting with the cadherin cytoplasmic domain, and depletion of these molecules might have resulted in the perturbation of endogenous cadherin function. This possibility, however, requires experimental supports.

An important finding in the present study was that cN390Δ was expressed at cell-cell contacts sites, and coincidentally, the expression of E-cadherin at these sites was suppressed. This observation suggests that some of the endogenous E-cadherins were replaced by the nonfunctional mutant N-cadherin at cell-cell boundaries, raising another possible mechanism of disrupting cell-cell adhesion. How can this replacement occur? We demonstrated that a C-terminus truncated form of N-cadherin had no effect on cell-cell adhesion nor was it transported into cell-cell contact sites, although this molecule had the intact extracellular domain. This truncated N-cadherin is not only unable to associate with catenins but also unable to bind to the cytoskeleton, as found with similar truncated forms of E-cadherin (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1990). These observations imply that the adhesion inhibitory action of cN390Δ is closely associated with its ability to interact with the cytoskeletal elements.

We here define the putative cytoskeletal structure interacting with the cadherin-catenin complex as an intracellular receptor for the latter, assuming that the binding of these two systems is crucial for cadherin function. cN390Δ should be able to recognize the receptor, because we found that it bound to the cytoskeleton. This mutant molecule, thus, ought to compete with functional cadherins for the receptors, and occupy the positions for the functional cadherins. The present results suggested that cN390Δ was not necessarily overexpressed as compared with the level of the endogenous cadherins. Therefore, we have to postulate that even a relatively small amount of cN390Δ could compete with the endogenous molecules for the receptors. This could be achieved if the mutant molecule acquired a higher affinity to the receptor than the endogenous ones, as a result of its conformational changes induced by deletion in the extracellular domain.

Another question is how the substitution of the mutant cadherins for intact cadherins can occur at the cell-cell contact sites. Without this process, the substitution cannot be effective in inhibiting cell-cell adhesion. Possibly, the cadherin-catenin complexes have a property to form molecular clusters via lateral interactions in the plasma membrane, as inferred from the fact that cadherins are generally concentrated in adherens junctions (see discussion by Matsuyoshi *et al.*, 1992). cN390Δ could form chimeric clusters with intact cadherins by using the cytoplasmic tail or the associated cytoplasmic components. We then can assume that once some of the intact cadherins have accumulated into cell-cell contact sites as the result of their intercellular homophilic interactions, the mutant cadherins should be attracted to these "cores," interfering with another accumulation of intact molecules into these sites. This process could even actively remove some of the preexisting endogenous cadherins from the cell contact sites if the mutant cadherins have a higher affinity to the intracellular receptor as hypothesized above. Endogenous cadherins, which have been excluded from these sites probably remain in the cells, as we observed no significant difference in the total amount of E-cadherin between cells with and without exogenous cN390Δ. A similar molecular behavior was demonstrated for a β chain of the integrin. Integrins form focal contacts where these molecules are concentrated. LaFlamme *et al.*, (1992) found that the integrin $\beta 1$ chain without the extracellular domain can accumulate into focal contacts, suggesting that its cytoplasmic domain plays a major role in the molecular assembly of integrins to form focal contacts.

The exogenous expression of intact N-cadherin in PAM212 cells might have the same effect on the distribution of endogenous cadherins. In this case, however, the exogenous molecule should not cause the inhibition of cell adhesion, as in fact observed, because the introduced molecule itself is a functional adhesion molecule. The above hypothetical mechanism is partly reminiscent of the dominant negative effect of mutated FGF receptors, that is, an intact FGF receptor associates with a mutated counterpart to form a nonfunctional dimer (Amaya *et al.*, 1991).

Whatever the mechanism is, the dominant negative action of nonfunctional cadherins is also intriguing from applicational aspects. By introducing into animals nonfunctional cadherins whose expression is regulated by some tissue-specific promoters, we could inhibit cadherin function in particular tissues. These kinds of approaches should be useful for investigating developmental roles of this interesting molecular family.

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