

Modulation of Cell–Cell Adherens Junctions by Surface Clustering of the N-Cadherin Cytoplasmic Tail

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Cadherins mediate the formation of cell–cell adherens junctions (AJ) by homophilic interactions through their extracellular domains as well as by interacting with the actin cytoskeleton via their cytoplasmic portions. Cadherin clustering initiates cytoplasmic signaling that results in the assembly of structural components into cell–cell AJ. To elucidate the function of the cytoplasmic tail of cadherins in initiating the assembly signal, we generated and characterized a chimeric cadherin tail fused to an inert transmembrane anchor. The chimera enabled us to cluster the cadherin cytoplasmic tail in the absence of extracellular portions of the molecule. The transfected cadherin tail chimera localized to cell–cell AJ of epithelial cells, indicating that the submembrane junctional plaque has the capacity to recruit additional cadherins, with no involvement of their extracellular domains. Expression of the chimera in cells of mesenchymal origin resulted in dominant negative effects on the formation of cell–cell AJ. Surface clustering of cadherin cytoplasmic tails induced the recruitment of components and structural assembly of cell–cell AJ, thereby reversing the initial dominant–negative effects. We conclude that the cadherin cytoplasmic tail contains information required to direct the molecule to cell–cell AJ. Its function as modulator of cell–cell AJ depends on cell type and on whether the tail is clustered. © 1998

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INTRODUCTION

Cadherins are transmembrane adhesion molecules which mediate cell-to-cell homophilic interactions [1–4]. The capacity of a cadherin to form stable adhesions depends not only on its specific homophilic bind-

ing site in the extracellular portion of the molecule, but also on interactions with the actin cytoskeleton via its cytoplasmic domain [3, 5, 6]. Recent studies indicate that cell adhesion plays a central role not only in the formation of cell–cell adherens junctions (AJ) but also in the generation of specific transmembrane signals which affect cell behavior and fate [7, 8]. To study possible signaling events triggered by cadherins, we have recently investigated the effects of cadherin clustering induced by reacting cells expressing N-cadherin with synthetic beads covalently linked to the extracellular domain of N-cadherin [9]. The results indicated that cadherin clustering by these beads induced specific global enhancement of cadherin junction assembly [9]. This enhancement apparently depended on tyrosine phosphorylation associated with the AJ in the induced cells [9]. While clustering of an intact cadherin may result in a long-range assembly signal, a recent study demonstrated that experimental clustering of the C-cadherin ectodomain results in an increase of cell–cell binding strength, independent of the cytoplasmic associations of the cadherin molecule [10].

Our main objective in the present study was to determine whether local clustering of the cadherin cytoplasmic domain, per se, can result in transduction of cell–cell AJ assembly signals. To test this hypothesis directly, cells expressing low levels of N-cadherin were transfected with a chimeric molecule that contains the extracellular and transmembrane domains of the non-signaling α subunit of the IL-2 receptor (IL-2R), to serve as an inert transmembrane anchor, and the cytoplasmic domain of N-cadherin. We show that while the IL-2R/N-cadherin tail chimera segregated into cell–cell AJ and perturbed them (especially when overexpressed in mesenchymal cells), clustering of the chimeric molecules, using beads coated with a monoclonal anti IL-2R antibody, dramatically enhanced the assembly of cell–cell AJ. This study indicates that the cytoplasmic portion of cadherin can transduce a cytoplasmic signal upon clustering, in analogy with signals delivered by aggregation of integrin cytoplasmic tails.

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MATERIALS AND METHODS

Cells. NIH 3T3 cells were kindly provided by J. Silvio Gutkind (NIDR, NIH, Bethesda, MD). CaCo-2 cells were obtained from the American Type Culture Collection (Rockville, MD). NBT-II rat bladder carcinoma cells were kindly provided by Pierre Savagner (NIDR, NIH, Bethesda, MD). CHO cells stably expressing chicken N-cadherin (CHO-Ncad cells line FL4) were previously described [9]. These cells were transfected with pIL-2R/N-cadherin to generate stable cell lines expressing the chimera. All the cells used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, at 37°C under 5% CO₂ and 95% air.

Generation of the IL-2R/N-cadherin cytoplasmic domain chimera. The cDNA coding for the cytoplasmic domain of chicken N-cadherin was amplified using full-length chicken N-cadherin cDNA as a template and the following oligonucleotides: the sense primer 5'-AAG-CTTAAGCGCCGTGATAAGGAG-3' containing the *Hind*III restriction site followed by a sequence coding for the first six amino acids of chicken N-cadherin cytoplasmic domain (underlined) and the antisense primer 5'-TCTAGATCAGTCATCACCTCCACC-3' containing the *Xba*I restriction site followed by a complementary sequence coding for a termination codon and the last six amino acids of chicken N-cadherin (underlined). The PCR product was restricted with *Hind*III/*Xba*I and ligated into a *Hind*III/*Xba*I restricted pCMV/IL-2R expression vector. This vector is coding for the nonsignaling subunit of the IL-2R and was used in previous studies [11].

Antibodies. Monoclonal mouse anti IL-2R α and mouse anti-phosphotyrosine (4G10) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal mouse anti β -catenin antibody was a gift from Dr. M. Wheelock (Department of Biology, University of Toledo). Monoclonal mouse anti N-cadherin cytoplasmic tail antibody (CH-19) was purchased from Sigma (St. Louis, MO).

Preparation and application of IL-2R reactive and ConA coated beads. Polybead amino microspheres (mean diameter 6 μ m; Polysciences Inc., PA), were coated with either mouse anti IL-2R monoclonal antibody or ConA (Worthington Biochemical, USA) by washing 10⁸ beads with PBS at pH 7.4, incubating with 8% glutaraldehyde for 16 h with gentle mixing, washing with PBS, followed by incubation with 500 μ g/ml antibody or ConA for 5 h. The beads were then incubated with 0.5 Methanolamine in PBS for 30 min, followed by incubation with 10 μ g/ml BSA for 30 min and then resuspended in a storage buffer containing 10 mg/ml BSA, 0.1% NaN₃, and 5% glycerol in PBS, pH 7.4.

Indirect immunofluorescence. Cultured cells were stained by indirect immunofluorescence. Cells were fixed with 4% formaldehyde with 5% sucrose in PBS for 20 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. The cells were then incubated for 1 h with the indicated antibodies in PBS at 10 μ g/ml and then stained with a 1:100 dilution of either fluorescein- or rhodamine-conjugated goat F(ab')₂ anti-mouse or anti-rabbit IgG (Biosource International, Camarillo, CA). Coverslips were mounted in Gel-mount (Biomedica Corp., Foster City, CA) containing *p*-phenylenediamine (Fluka, Ronkonkoma, NY) at 1 mg/ml to inhibit photobleaching, and viewed at 630 \times magnification using a Zeiss Axiophot photomicroscope equipped with epifluorescence. In some cases the cells were examined by confocal microscopy (Leica, model TCS 4D; Deerfield, IL) at 1000 \times magnification. Immunofluorescence was photographed using Kodak TMAX 3200 film.

Digital immunofluorescence microscopy. The computerized microscopic system used here was based on the design of Agard *et al.* [12], consisting of a Zeiss AxioScope and Micro VAX III worksta-

tion which controls image acquisition, light shutters, filter wheels, and focus. Images were recorded with a cooled, scientific grade, charge-coupled device (CCD) camera (Photometrics, Tucson, AZ). Images were recorded into an array processor (Mercury Computer Systems, Chelmsford, MA) which calculated on-the-fly pixel-per-pixel correction for illumination and CCD sensitivity and scaled and deconvoluted images, essentially in real time [12].

Calculation of fluorescence intensities. Cell-cell AJ (identified by labeling for associated molecules, i.e., cadherin and β -catenin) were manually marked by polygons enclosing the entire relevant area, and the net intensity of labeling (minus background) in them was determined. The average intensity values of the enclosed pixels displaying signal above the threshold level was then calculated. The effects mediated by the anti IL-2R antibody- and ConA-coated beads on cell-cell AJ was examined in five independent experiments. Variations between experiments were typically lower than 20%. In each experiment, data were collected from 15–20 microscopic images containing a total number of about 50 marked cell-cell adhesions, all in selected beads-associated cells. To compare the results obtained following treatment with IL-2R-reactive beads to those found in control cells (associated with ConA beads), the Student *t* test was performed and significance values were calculated for each pair.

RESULTS

Expression and Junctional Localization of IL-2R/N-Cadherin Tail Chimera in Epithelial Cells

The intracellular tail of the cadherin molecule provides the linkage between the extracellular portion that is mediating the cell-cell adhesion response and the physiological machinery in the cytoplasm. The binding of the cadherin cytoplasmic tail to catenins is essential for the association of the cadherin with the cytoskeleton and is also important for the extracellular cadherin/cadherin association [3, 5, 13, 14].

In the present study, we analyzed signals that are initiated by clustering of the cytoplasmic tail of N-cadherin. The experimental approach was based on generation of transmembrane cadherin tail chimera, similar to the one used to study the functions of integrin cytoplasmic tails [11]. Figure 1 describes the

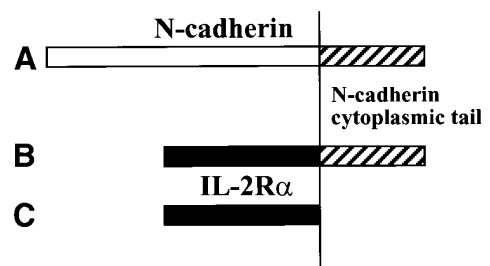


FIG. 1. N-cadherin transmembrane chimera. Plasmids coding for the following proteins were used in this study: (A) full-length chicken N-cadherin; (B) the extracellular and transmembrane portions of IL-2R α were fused to the cytoplasmic tail of chicken N-cadherin; and (C) tailless IL-2R α was used as a negative control for the chimera.

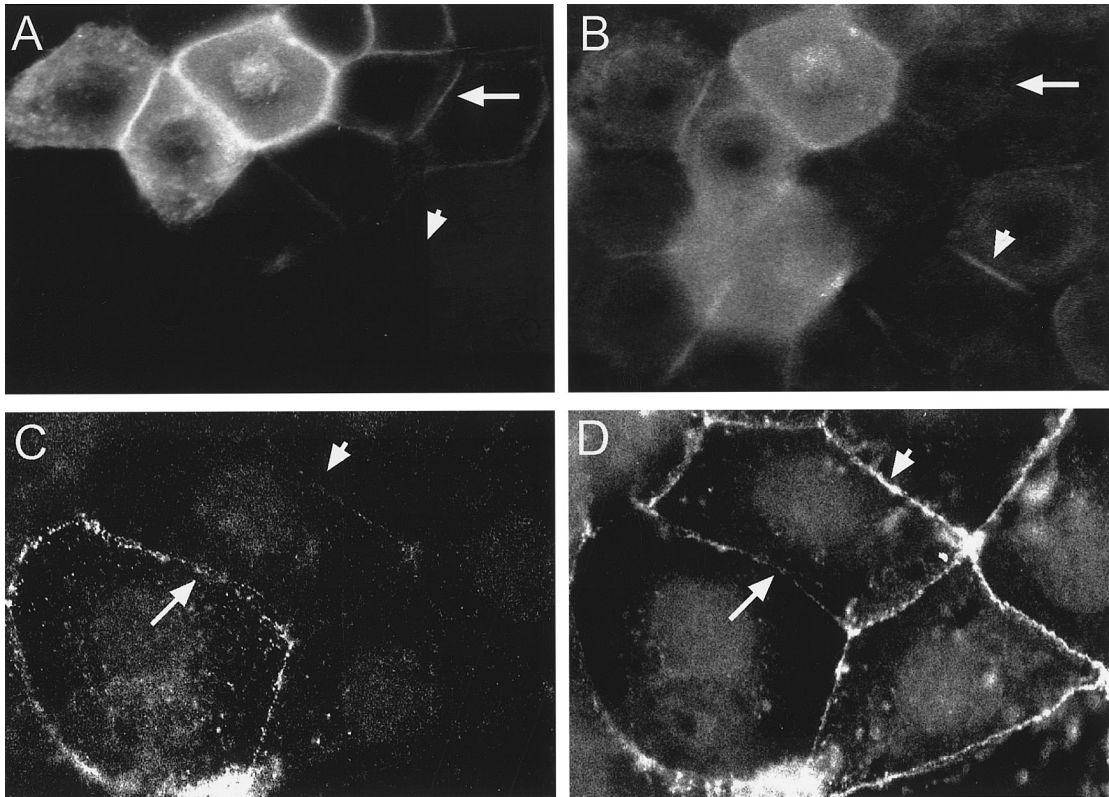


FIG. 2. Localization of the cadherin tail chimera in cell-cell AJ of epithelial cells. IL-2R/N-cadherin tail chimera was transiently transfected into NBT-II rat bladder carcinoma cells (A and B) and human colon carcinoma CaCo-2 cells (C and D). Forty-eight hours later immunofluorescence staining with monoclonal anti IL-2R antibody (A and C) or with polyclonal anti pan-cadherin antibody (B and D) was performed. Note the localization of the chimera to cell-cell AJ (A and C, arrows) and the reduction of cadherin staining in the transfected cells (B versus D, arrows) compared to nontransfected cells (B and D, arrowheads).

different versions of the cadherin constructs that were used in this study. Full-length N-cadherin (Fig. 1A) was stably expressed in CHO cells as previously described [9]. The cytoplasmic tail of N-cadherin was fused to the extracellular and transmembrane domains of the nonsignaling α subunit of the IL-2R (Fig. 1B). The same portions of the IL-2R, without any cytoplasmic domain (Fig. 1C), served as a negative control in the following experiments.

First, the chimera was expressed in several epithelial and mesenchymal cell lines, and its subcellular localization was examined. Previous studies showed that cadherin molecules that lack most of the extracellular portion may, in some cases, have a dominant negative effect on the cell-cell AJ [15, 16]. Localization of cadherin tails to cell-cell AJ was also observed [15]. We found different localization patterns of the IL-2R/N-cadherin tail chimera, depending on the cell type in which the molecule was expressed and the level of expression. The chimera colocalized with endogenous cadherins in cell-cell AJ in NBT-II rat bladder carcinoma cells (Figs. 2A and 2B) and

CaCo-2 human colon carcinoma cells (Figs. 2C and 2D). Similar localization of the chimera at cell-cell AJ was also observed in MCF-7 human breast carcinoma cells and human salivary gland (HSG) cells (not shown). Next, we evaluated the cadherin chimera distribution in NBT-II rat bladder carcinoma cells using confocal analysis. Figure 3 shows an optical section at the ventral portion of cells expressing the IL-2R/N-cadherin tail chimera. The distribution of the chimera was restricted to cell-cell contacts (Fig. 3, empty arrowheads), while regions of the plasma membrane that were not interacting with adjacent cells contained substantially lower amounts of the chimera (Fig. 3, full arrowheads). No dominant negative effects on the structure of cell-cell AJ were observed in the epithelial cells expressing the chimera. However, a reduction of total cadherin localization in cell-cell AJ in the chimera-transfected epithelial cells was apparent, compared to nontransfected adjacent cells (Fig. 2). In all of these cells, the control tailless IL-2R had a nonlocalized, diffuse distribution (not shown).

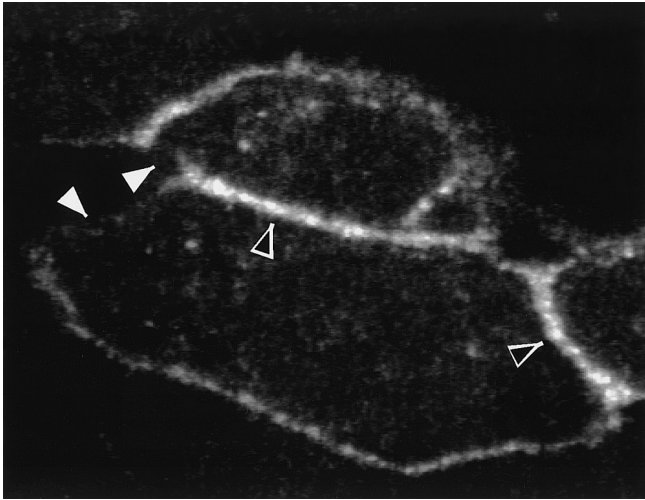


FIG. 3. Confocal analysis of the IL-2R/N-cadherin tail chimera distribution in NBT-II rat bladder carcinoma cells. IL-2R/N-cadherin tail chimera was transiently transfected into NBT-II rat bladder carcinoma cells. Forty-eight hours later immunofluorescence staining with monoclonal anti IL-2R antibody was performed. An optical section at the bottom of the cells is shown. Note that the chimera is localized at cell-cell contacts (empty arrowheads), and absent from regions of the cell membrane that do not associate with adjacent cells (full arrowheads).

Possible dominant negative effects of the chimera on cell-cell AJ may depend on the levels of endogenous cadherin expression. These levels are high in epithelial cells; therefore, the chimera may be incorporated into these sites with only minor structural effects. We examined the localization of the chimera in cells of mesenchymal origin that express relatively low levels of cadherins [17]. As shown in Fig. 4B, 3T3 cells form cell-cell AJ upon short-term (8 min) induction with 1 mM pervanadate, as previously reported [17]. Expression of the cadherin tail chimera in pervanadate-induced 3T3 cells resulted in detachment of the transfected cells from the monolayer (Fig. 4B), and no localization of the chimera to cell-cell AJ was observed. The control tailless IL-2R did not localize to cell-cell AJ and did not affect cell morphology (Figs. 4C and 4D).

Clustering of IL-2R/N-Cadherin Tail Results in the Enhancement of Cadherin and Catenin Labeling within Cell-Cell AJ of the Same Cells

In a previous study we demonstrated that surface clustering of intact N-cadherin in CHO cells (CHO-Ncad cells stably expressing N-cadherin) resulted in a major increase in the levels of AJ-associated cadherin and β -catenin [9]. To examine whether clustering of the cadherin cytoplasmic tail can also result in intracellular long-range effects on cell-cell AJ, we

first expressed the chimera in CHO-Ncad cells. As shown in Fig. 5, a cell line stably expressing the IL-2R/N-cadherin tail chimera was generated. The chimera localized to cell-cell AJ in these cells (Fig. 5B); however, a notable dominant negative effect was observed with respect to the number, size, and cadherin staining of these sites (Fig. 5D), compared to nontransfected cells (Fig. 5C). As shown in Fig. 5 (inserts), expression of the chimera did not result in any reduction of endogenous cadherin levels in transfected compared to control cells.

While transfection of the IL-2R/N-cadherin tail chimera into CHO-Ncad cells resulted in the inhibition of AJ formation, incubation of the transfected cultures with monoclonal anti IL-2R antibody-coated beads dramatically enhanced the formation of cadherin and β -catenin containing junctions. As shown in Fig. 6, clustering of the cadherin tail chimera resulted in the accumulation of cadherins in cell-cell AJ. Also, the sizes of these sites in the induced cells increased (Fig. 6F) compared to cells incubated with ConA-coated beads (Fig. 6B). The accumulation of cadherins in cell-cell AJ upon clustering of the cadherin tail was accompanied by increases in quantities of β -catenin at these sites (Fig. 7F). No increase in β -catenin accumulation was observed in cells incubated with ConA-coated beads (Fig. 7B) or in control cells expressing the tailless IL-2R (Figs. 7A, 7C and 7E).

To obtain quantitative information about the changes in junction labeling following the clustering of the IL-2R/N-cadherin chimera, we have determined the intensity of junctional fluorescence labeling for cadherin and β -catenin, using digital microscopy as previously performed [9]. The results indicate a significant ($P < 0.001$), fourfold increase in cadherin (Fig. 8A) and β -catenin (Fig. 8B) immunofluorescence staining intensity in AJ of cells induced by the clustering of the IL-2R/N-cadherin tail chimera, using IL-2R beads compared to ConA beads (Figs. 8A and 8B). No such increase was observed in control cells expressing the tailless IL-2R (Figs. 8A and 8B).

DISCUSSION

Clustering of cytokine and growth factor receptors is a critical event in generating a variety of signal transduction responses [18, 19]. Similarly, clustering of β -integrin cytoplasmic tails, even in the absence of any extracellular integrin domain, promotes tyrosine phosphorylation of focal adhesion kinase [20]. Clustering also appears to be important for the initiation of cadherin-mediated signaling, which results in tyrosine phosphorylation and assembly of cell-cell junctions [9, 21]. To investigate the impor-

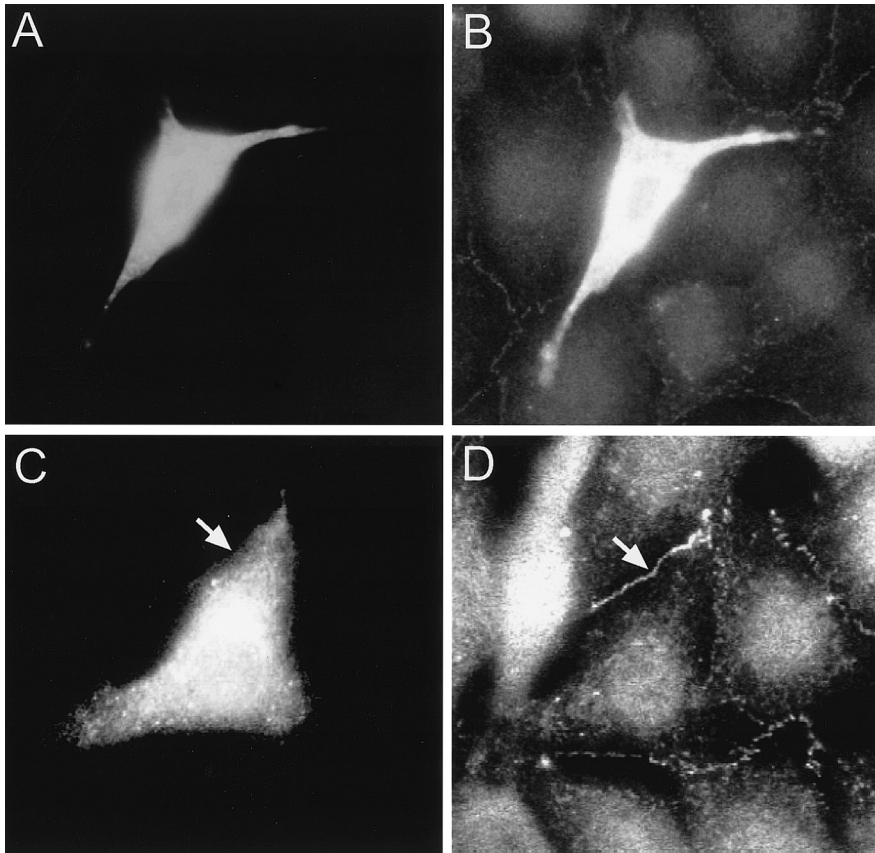


FIG. 4. Dominant negative effect of the IL-2R/N-cadherin tail chimera on cell-cell AJ in 3T3 cells. 3T3 cells were transfected with the chimera (A and B) or control tailless IL-2R (C and D). Forty-eight hours later the cells were treated with pervanadate (1 mM orthovanadate and 1 mM H_2O_2) for 8 min and then fixed, permeabilized, and stained for immunofluorescence with monoclonal anti IL-2R antibody (A and C) or with polyclonal anti pan-cadherin antibody (B and D).

tance of clustering of the cytoplasmic portion of the cadherin molecule, we generated a chimera of the cytoplasmic tail of N-cadherin fused to the extracellular and transmembrane domains of the nonsignaling α subunit of the IL-2 receptor. The IL-2R portion serves as a transmembrane anchor, and the extracellular domain functions as a tag for tracking the localization of the chimera, as well as being a target for antibody-induced clustering. This construct differed from previous cadherin tail truncations that deleted most, but not all, of the cadherin extracellular portion (most of the EC1 and EC5 domains were retained in those studies) [15, 16]. The cadherin tail chimera was expressed in several cell types to evaluate its subcellular distribution and its possible interactions with cell-cell AJ.

The transfected IL-2R/N-cadherin tail chimera specifically localized to cell-cell AJ in several cell lines, as confirmed by confocal analysis and double immunofluorescence staining with anti-cadherin and anti β -catenin antibodies. The localization studies

suggest that cell-cell AJ have the capacity to recruit cadherin molecules via their cytoplasmic tails irrespective of their extracellular composition or interactions. Immunolocalization of total cellular cadherins in transiently transfected cultures of NBT-II and CaCo-2 cells using a polyclonal pan-cadherin antibody showed that the chimera can replace substantial amounts of intact endogenous cadherins without causing dominant negative effects on the structure of cell-cell AJ, at least in epithelial cells. The localization of a cadherin chimera to cell-cell contacts of epithelial cells without dominant negative effects was observed previously [15] and may be explained by the fact that such cells express high levels of cadherins and also form desmosomes. Indeed, NBTII have well-organized desmosomes [22] that may continue to maintain cell-cell contacts in the chimera-transfected cells.

In contrast, transfection of the same IL-2R/N-cadherin tail chimera into N-cadherin-transfected CHO cells resulted in a marked inhibition of cell-cell AJ

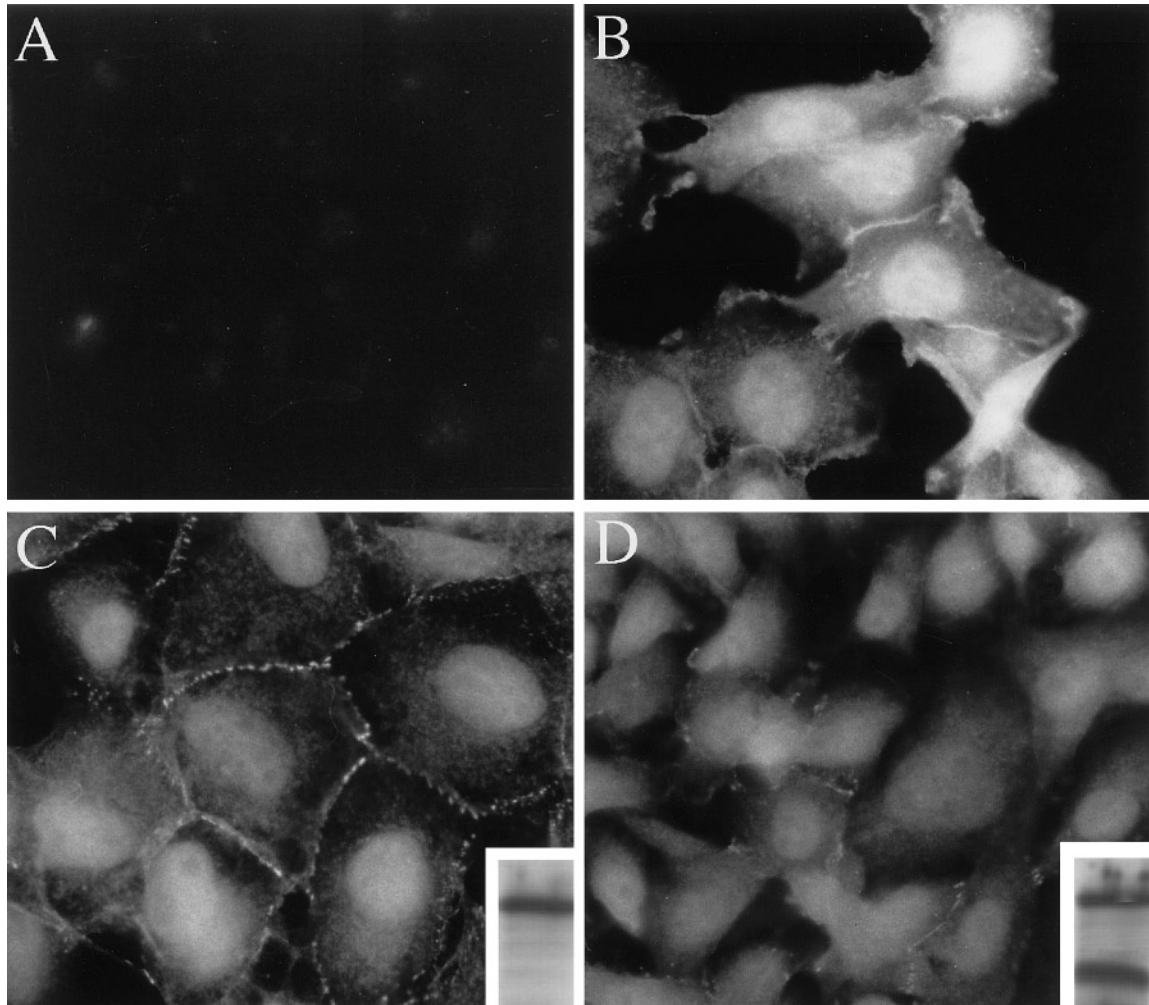


FIG. 5. Expression of the IL-2R/N-cadherin tail chimera in CHO cells expressing N-cadherin. Nontransfected cells (A) and cells from a clone expressing the IL-2R/N-cadherin tail chimera (B) cultured on coverslips were fixed, permeabilized, and immunostained with a monoclonal anti IL-2R antibody followed by rhodamine-labeled goat anti-mouse IgG. Note that the chimera is located on the cell membrane at a cell-cell contact. Nontransfected cells (C) and cells from a clone expressing the IL-2R/N-cadherin tail chimera (D) cultured on coverslips were fixed, permeabilized, and immunostained with a monoclonal anti N-cadherin antibody that reacts with the extracellular domain of N-cadherin, followed by rhodamine-labeled anti-mouse IgG. Insets: Expression of the IL-2R/N-cadherin tail chimera in N-cadherin expressing CHO cells. Equal amounts of total cell protein from nontransfected cells or from cells of a positive clone were analyzed by SDS-PAGE and Western blotting with a monoclonal mouse anti N-cadherin antibody that reacts with the intracellular domain of N-cadherin. Note the dominant negative effect of the IL-2R/N-cadherin tail chimera on N-cadherin staining in cell-cell junctions.

formation. The localization pattern and dominant negative effects of the IL-2R/N-cadherin tail chimera were similar to those previously described using truncated cadherin molecules [15, 16]. Therefore, cadherin replacement may be the mechanism for the chimera-induced dominant negative effect, in a manner similar to the actions of previously described truncated cadherins [16]. The effects of the IL-2R/N-cadherin tail chimera on cell-cell AJ differ markedly from the dominant negative effects on cell-substrate adhesion of our IL-2R/integrin tail chimeras described previously [11].

Subsequent effects of membrane clustering of the chimera were studied by incubating transfected cells with beads coated with anti IL-2R monoclonal antibody. This experimental approach is similar to that used previously to induce surface clustering of intact cadherins, which resulted in the induction of long-range recruitment of components to cell-cell AJ [9]. We observed a similar induction of accumulation of cadherins and β -catenin in cell-cell AJ of cells after bead-induced clustering of chimeras containing only the cadherin cytoplasmic tail. Control ConA-coated beads had no effects on AJ structure. These data

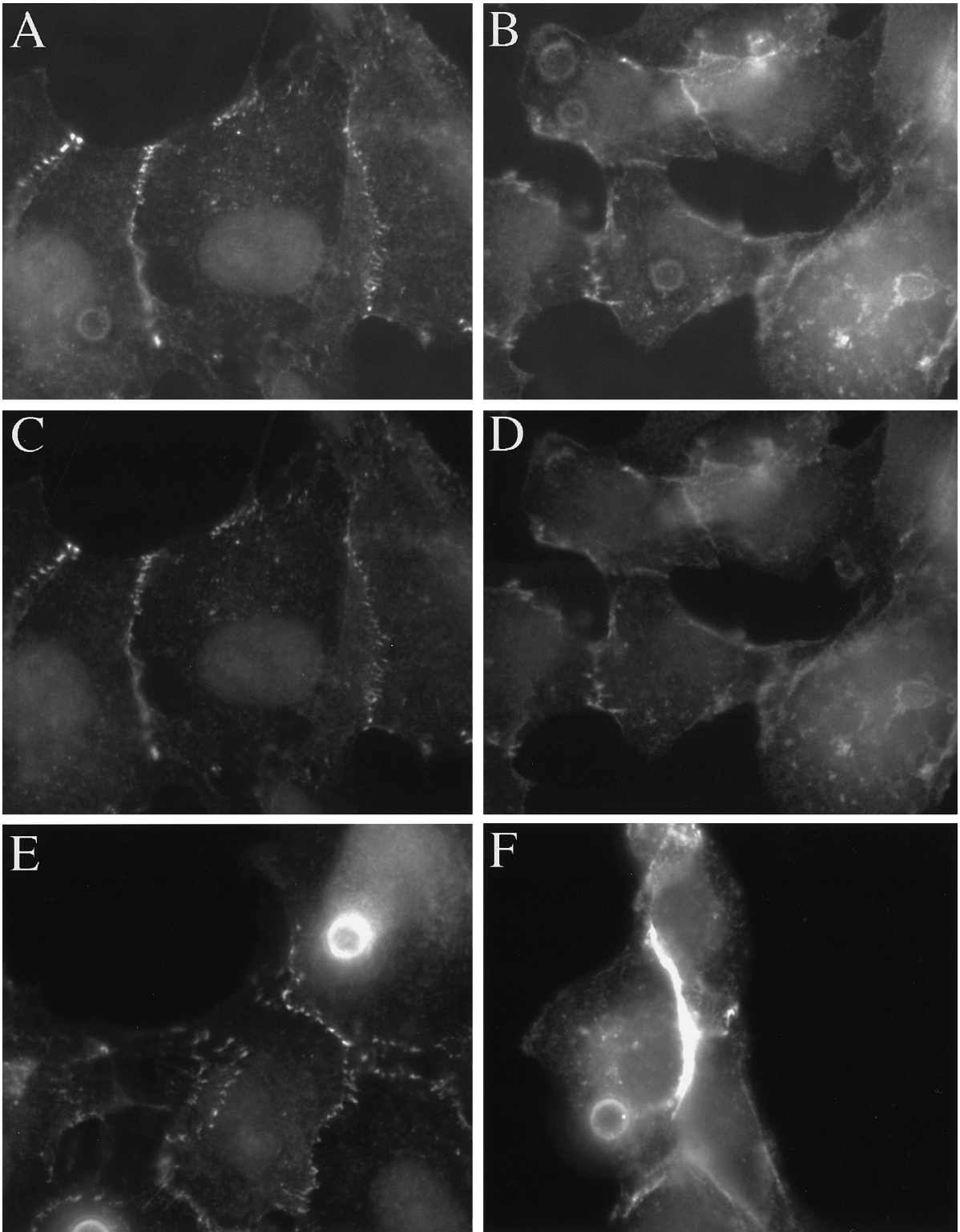


FIG. 6. Experimental clustering of the IL-2R/N-cadherin cytoplasmic tail induces the accumulation of cadherins in cell-cell AJ. CHO-Ncad cells expressing IL-2R/N-cadherin tail chimera (B, D, F) or tailless IL-2R as a control (A, C, E) were plated on glass coverslips, and 24 h later beads coated with monoclonal anti IL-2R antibody (E, F) or ConA (A-D) were added to the cells. Following 20 min of incubation at 37°C, the cells were fixed and stained with anti N-cadherin antibody. A and B pictures were taken using concurrent phase contrast under low illumination in order to see the ConA beads.

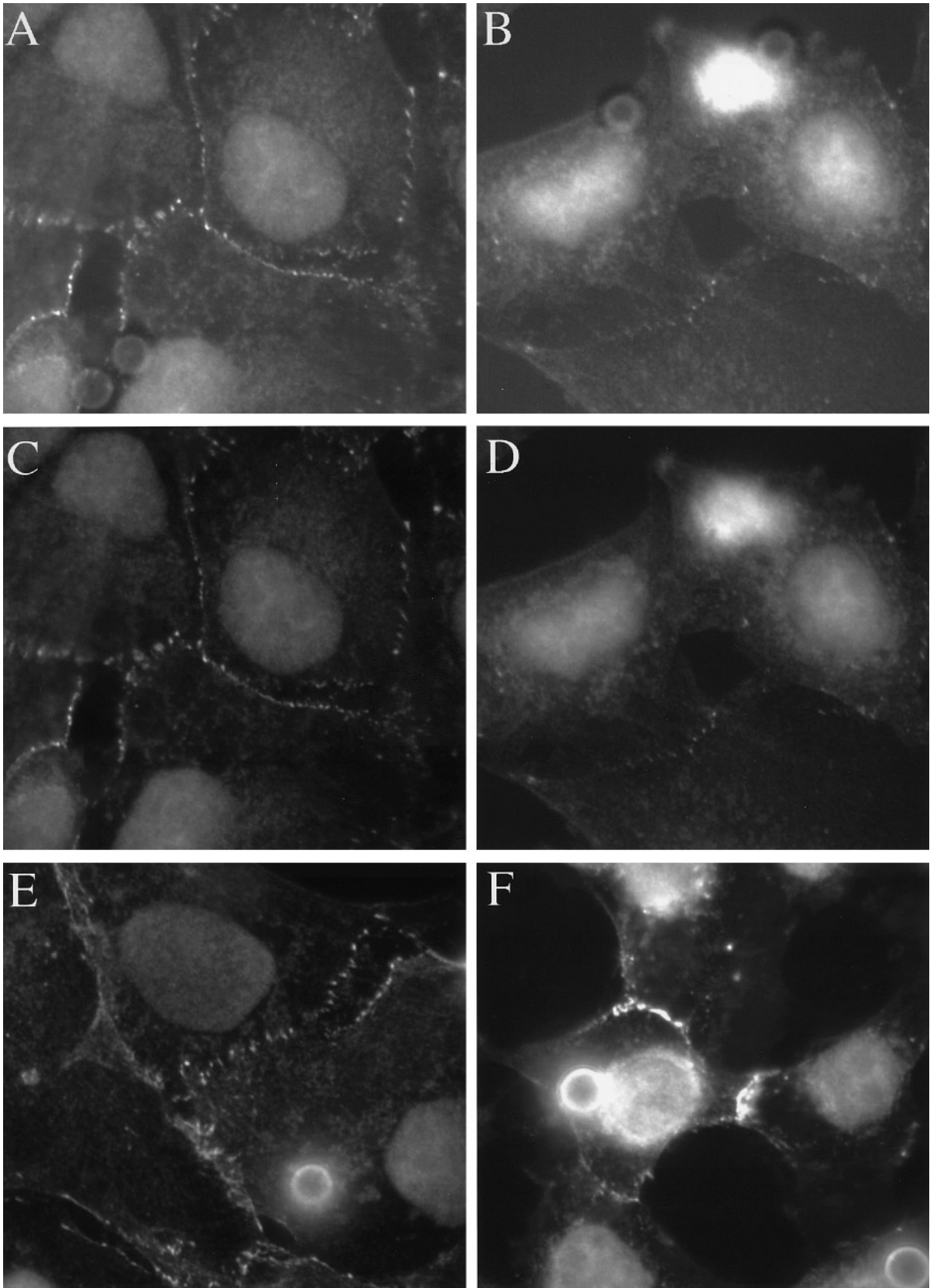


FIG. 7. Clustering of the IL-2R/N-cadherin cytoplasmic tail chimera induces the accumulation of β -catenin in cell-cell AJ. CHO-Ncad cells expressing IL-2R/N-cadherin tail chimera (B, D, F) or tailless IL-2R as a control (A, C, E) were plated on glass coverslips, and 24 h later beads coated with monoclonal mouse anti IL-2R antibody (E, F) or ConA (A–D) were added to the cells. Following 20 min of incubation at 37°C, the cells were fixed and stained with monoclonal anti β -catenin antibody. A and B pictures were taken using concurrent phase contrast under low illumination in order to see the ConA beads.

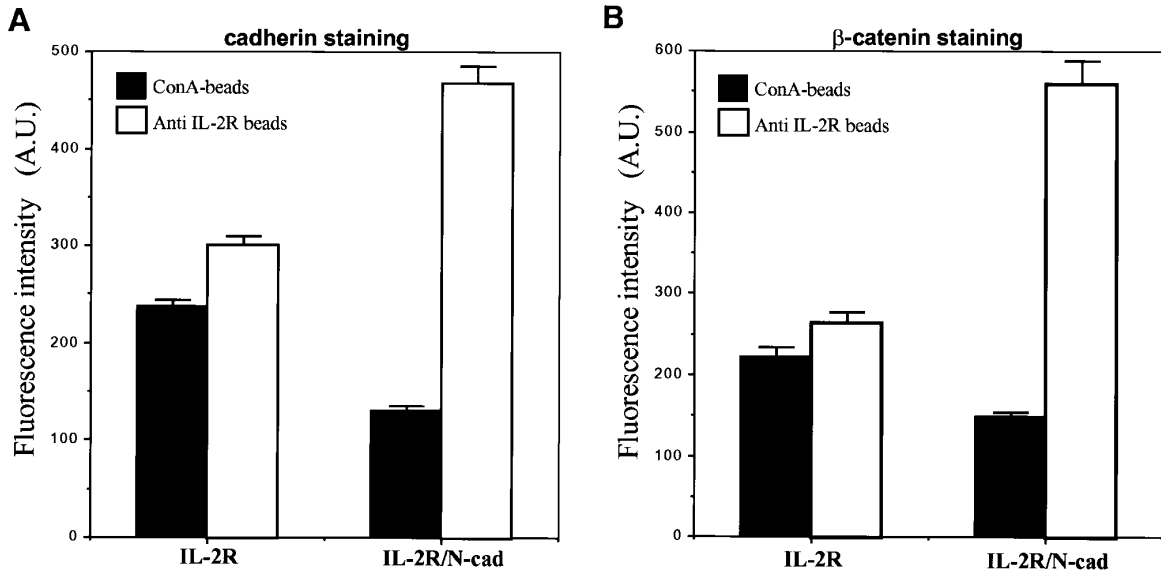


FIG. 8. Quantitative analysis of cadherin and β -catenin accumulation in cell-cell AJ induced by N-cadherin tail chimera clustering. The bars represent the mean immunofluorescence intensity and SD (expressed in arbitrary units) of: (A) cadherin staining; (B) β -catenin staining, in cells treated with monoclonal anti IL-2R antibody-coupled beads (open bars) or ConA-coupled beads (solid bars).

demonstrate that the signal for long-range cell-cell AJ assembly can be triggered by experimental aggregation of just the cadherin cytoplasmic tail, without any contribution from the extracellular portions of cadherins. The signals triggered by cadherin tail clustering not only resulted in the recruitment of components into preexisting cell-cell AJ, but could also enhance the extent of structural assembly of these sites and even reverse the dominant negative effect of overexpression of cadherin tail chimeras on cell-cell AJ.

This study indicates that clustering of the cytoplasmic domain of a cell-cell adhesion receptor, in a similar manner to cell-matrix adhesion receptors, may serve as a biochemical trigger for adhesion-mediated signaling responses. Future studies may reveal other signal transduction pathways initiated by cadherin clustering.

In certain situations, both pathological and physiological, cadherin molecules that lack parts or all of the extracellular domains are generated [23]. Expression of a truncated form of cadherin may result in dominant negative effects on cell-cell AJ structures, although the mechanism is not yet clear [15, 16]. In cells derived from the central nervous system, proteolytic cleavage of the N-cadherin molecule results in a cell-free N-cadherin extracellular domain and a residual membrane-associated N-cadherin tail. Both fragments may have physiological roles [24]. The present study demonstrates the context-dependent ability of cadherin tails to mediate either

positive or negative effects on AJ formation, composition, and function.

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