

# Two Cell Adhesion Molecules, Nectin and Cadherin, Interact through Their Cytoplasmic Domain-associated Proteins

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**Abstract.** We have found a new cell–cell adhesion system at cadherin-based cell–cell adherens junctions (AJs) consisting of at least nectin and l-afadin. Nectin is a Ca<sup>2+</sup>-independent homophilic immunoglobulin-like adhesion molecule, and l-afadin is an actin filament-binding protein that connects the cytoplasmic region of nectin to the actin cytoskeleton. Both the trans-interaction of nectin and the interaction of nectin with l-afadin are necessary for their colocalization with E-cadherin and catenins at AJs. Here, we examined the mechanism of interaction between these two cell–cell adhesion systems at AJs by the use of  $\alpha$ -catenin-deficient F9 cell lines and cadherin-deficient L cell lines stably expressing their various components. We showed here that nec-

tin and E-cadherin were colocalized through l-afadin and the COOH-terminal half of  $\alpha$ -catenin at AJs. Nectin trans-interacted independently of E-cadherin, and the complex of E-cadherin and  $\alpha$ - and  $\beta$ -catenins was recruited to nectin-based cell–cell adhesion sites through l-afadin without the trans-interaction of E-cadherin. Our results indicate that nectin and cadherin interact through their cytoplasmic domain-associated proteins and suggest that these two cell–cell adhesion systems cooperatively organize cell–cell AJs.

**Key words:** immunoglobulin superfamily • afadin • ponsin • catenin • cell–cell adherens junctions

## Introduction

We have recently identified a novel cell–cell adhesion system at cadherin-based cell–cell adherens junctions (AJs)<sup>1</sup> consisting of at least nectin and l-afadin (Mandai et al., 1997; Takahashi et al., 1999). Nectin is a Ca<sup>2+</sup>-independent homophilic cell adhesion molecule that belongs to the immunoglobulin (Ig) superfamily (Takahashi et al., 1999). l-Afadin is an actin filament (F-actin)-binding protein with one PDZ domain, and connects the cytoplasmic region of nectin to the actin cytoskeleton (Mandai et al., 1997; Takahashi et al., 1999). Nectin consists of three extracellular Ig-like loops, a single transmembrane segment, and a cytoplasmic region. Nectin is identical to the poliovirus receptor-related protein (Eberlé et al., 1995; Lopez et al., 1995), and recently has been shown to serve as the

$\alpha$ -herpes virus entry and cell–cell spread mediator (Geraghty et al., 1998; Warner et al., 1998; Cocchi et al., 2000). Nectin comprises a family consisting of at least three members: nectin-1, -2, and -3. Each member has two or three splicing variants: nectin-1 has two splicing variants, nectin-1 $\alpha$  and -1 $\beta$ /HIgR (Lopez et al., 1995; Cocchi et al., 1998); nectin-2 also has two splicing variants, nectin-2 $\alpha$  and -2 $\delta$  (Morrison and Racaniello, 1992; Aoki et al., 1994; Eberlé et al., 1995); and nectin-3 has three splicing variants, nectin-3 $\alpha$ , -3 $\beta$ , and -3 $\gamma$  (Satoh-Horikawa et al., 2000). The extracellular regions of splicing variants of each member are identical, but their transmembrane segments and cytoplasmic regions are different. The cytoplasmic regions of nectin-1 $\alpha$ , -2 $\alpha$ , -2 $\delta$ , -3 $\alpha$ , and -3 $\beta$ , but not nectin-1 $\beta$ /HIgR and -3 $\gamma$ , have a COOH-terminal conserved motif of four amino acid (aa) residues (E/A-X-Y-V) that interacts with the PDZ domain of l-afadin (Takahashi et al., 1999). l-Afadin has a splicing variant, s-afadin (Mandai et al., 1997). Human s-afadin is identical to the AF-6 protein, the gene of which is originally found to be fused to the *ALL-1* gene in acute leukemia (Prasad et al., 1993). It has been shown that the PDZ domain of the AF-6 protein/s-afadin also interacts with the Eph receptor tyrosine kinases (Hock et al., 1998; Buchert et al., 1999).

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<sup>1</sup>Abbreviations used in this paper: aa, amino acid(s); Ab, antibody; AJ, adherens junction; F-actin, actin filament; gD, glycoprotein D; GFP, green fluorescent protein; GST, glutathione-S-transferase; HA, hemagglutinin; His6, hexa-histidine; HSV1, herpes simplex virus type 1; Ig, immunoglobulin; TJ, tight junction.

We have isolated another l-afadin-binding protein, named ponsin, which is colocalized with nectin and l-afadin at cadherin-based AJs (Mandai et al., 1999). Furthermore, ponsin binds to vinculin, which is known to interact with both F-actin and  $\alpha$ -catenin (BurrIDGE and Feramisco, 1982; Menkel et al., 1994; Watabe-Uchida et al., 1998; Weiss et al., 1998; Imamura et al., 1999), but ponsin forms a binary complex with either l-afadin or vinculin and does not form a ternary complex (Mandai et al., 1999). Ponsin is a member of the family having three SH3 domains, including ArgBP2 (Wang et al., 1997), nArgBP2 (Kawabe et al., 1999), and vinexin (Kioka et al., 1999).

The cadherin/catenin system plays essential roles not only in the formation and maintenance of cell-cell AJs, but also in the organization of tight junctions (TJs; Takeichi, 1991, 1995; Gumbiner, 1996, 2000). Cadherin consists of five extracellular tandemly repeated domains, EC1-EC5, a single transmembrane segment, and a cytoplasmic region (Takeichi, 1991, 1995; Gumbiner, 1996, 2000). Cadherin shows through EC1 homophilic cis-dimerization and trans-interaction, which causes  $\text{Ca}^{2+}$ -dependent cell-cell adhesion (Shapiro et al., 1995; Nagar et al., 1996; Tomschy et al., 1996; Tamura et al., 1998; Pertz et al., 1999). The cis-dimerization is essential for trans-interaction (Briehner et al., 1996; Tomschy et al., 1996; Tamura et al., 1998; Pertz et al., 1999). The cytoplasmic region of cadherin regulates the cis-dimerization and/or trans-interaction by interacting with many peripheral membrane proteins (Takeichi, 1991, 1995; Gumbiner, 1996, 2000). The distal portion of the cytoplasmic region interacts with  $\beta$ -catenin, which in turn interacts with  $\alpha$ -catenin (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989).  $\alpha$ -Catenin interacts with F-actin (Rimm et al., 1995). Furthermore,  $\alpha$ -catenin interacts with other F-actin-binding proteins,  $\alpha$ -actinin and vinculin, through the  $\text{NH}_2$ -terminal half (Knudsen et al., 1995; Nieset et al., 1997; Watabe-Uchida et al., 1998; Imamura et al., 1999) and ZO-1 through the COOH-terminal half (Itoh et al., 1997; Imamura et al., 1999). The juxtamembrane portion of cadherin interacts with p120<sup>cas</sup> (Reynolds et al., 1994).

By analogy with cadherin, we have shown that nectin exhibits cis-dimerization, which may be a key regulatory step for its trans-interaction (Miyahara et al., 2000). The first and third Ig-like domains may be responsible for its trans-interaction and cis-dimerization, respectively (Miyahara et al., 2000). The interaction of nectin with l-afadin is not essential for its cis-dimerization or trans-interaction (Miyahara et al., 2000), but is essential for the colocalization of nectin and l-afadin with E-cadherin at cell-cell AJs (Takahashi et al., 1999). The interaction of nectin with l-afadin is furthermore necessary for their compact clustering at cell-cell adhesion sites (Miyahara et al., 2000). The colocalization of these two cell-cell adhesion systems at AJs, moreover, requires the trans-interaction of nectin (Miyahara et al., 2000). Studies on epithelial cells in afadin ( $-/-$ ) mice and ( $-/-$ ) embryoid bodies have shown that E-cadherin-based AJs are impaired in these mutant cells, suggesting that afadin plays a key role in the proper organization of E-cadherin-based cell-cell AJs (Ikeda et al., 1999). Thus, evidence is accumulating that the nectin/afadin system plays a key role at cadherin-based AJs. Furthermore, we have shown that the interaction of nectin-1 $\alpha$

with l-afadin and their colocalization with the cadherin/catenin system are necessary for efficient cell-cell spread of herpes simplex virus type 1 (HSV1; Sakisaka, T., T. Taniguchi, H. Nakanishi, K. Takahashi, M. Miyahara, W. Ikeda, S. Yokoyama, Y.-F. Peng, K. Yamanishi, and Y. Takai, manuscript submitted for publication). However, it has not been understood which component(s) of the cadherin/catenin system is required for the colocalization with the nectin/afadin system. It has not been determined how these two cell adhesion systems are functionally correlated to each other. In this study, we have examined the mechanism of interaction between the nectin/afadin and cadherin/catenin systems by the use of  $\alpha$ -catenin-deficient F9 cell lines and cadherin-deficient L cell lines stably expressing their various components.

## Materials and Methods

### Construction, Expression, and Purification

Mammalian expression vectors were constructed with pPGKIH (Miyahara et al., 2000), pEGFP-N1 (CLONTECH Laboratories, Inc.), pPGKIZ-HA, pEF-MC1neo (Visvader et al., 1992), pFLAG-CMV2 (Eastman Kodak Co), and pPGKIH-FLAG using standard molecular biology methods (Sambrook et al., 1989). pEGFP-N1 was designed to express a COOH-terminal green fluorescent protein (GFP)-tagged protein. pPGKIZ-HA was constructed as follows: pPGKIZ was first constructed by replacing the CAG promoter of pPCAGIZ (Niwa et al., 1998) with the murine phosphoglycerate kinase promoter (Adra et al., 1987). A cDNA fragment encoding the hemagglutinin (HA) epitope and multicloning site (5'-GAATTGTTAATACGACTCACTATAGGCTAGCGGTACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TTG GGT GGT GAA TTC CTC GAG ACG CGT GGT ACC TCT AGA GTC GAC CCG GGC GGC CGC TTC CCT TTA GTG AGG GTT AAT GCA ATT C-3') was inserted into pPGKIZ to express an  $\text{NH}_2$ -terminal HA-tagged protein. pFLAG-CMV2 was designed to express an  $\text{NH}_2$ -terminal FLAG-tagged protein. pPGKIH-FLAG was constructed by inserting a cDNA fragment encoding the pre-protrypsin signal peptide and FLAG epitope of pFLAG-CMV1 (Eastman Kodak Co.) into pPGKIH as previously described (Takahashi et al., 1999; Miyahara et al., 2000; Satoh-Horikawa et al., 2000).

Expression vectors for human nectin-1 $\alpha$  and mouse nectin-2 $\alpha$  (shown in Fig. 1) contained the following aa: pPGKIH-nectin-1 $\alpha$ , aa 1-518 (full-length); pPGKIH-nectin-1 $\alpha$ - $\Delta$ C, aa 1-514 (deletion of the COOH-terminal four aa residues); pPGKIH-FLAG-nectin-1 $\alpha$ , aa 27-518; pPGKIH-FLAG-nectin-1 $\alpha$ - $\Delta$ C, aa 27-514; pPGKIH-nectin-2 $\alpha$ , aa 1-467 (full-length); and pPGKIH-nectin-2 $\alpha$ - $\Delta$ C, aa 1-463 (deletion of the COOH-terminal four aa residues). An expression vector for mouse ponsin-2 (pEGFP-ponsin) contained aa 1-724 (full-length; see Fig. 1). An expression vector for l-afadin (pFLAG-CMV2-l-afadin) contained aa 1-1,829 (full-length; see Fig. 1). Expression vectors for mouse E-cadherin and  $\alpha$ -catenin contained the following aa: pEF-tEC, aa 562-728 (cytoplasmic region and partial transmembrane segment of E-cadherin); pPGKIZ-HA- $\alpha$ -catenin, aa 1-906 (full-length); and pPGKIZ-HA- $\alpha$ -catenin-C, aa 509-906 (COOH-terminal half; see Fig. 1). pEF-tEC was designed to express the  $\text{NH}_2$ -terminal T7 peptide (M-A-S-M-T-G-G-Q-Q-M-G)-tagged protein.

Baculovirus transfer vectors were constructed with pFastBac1-Myc-His6 and -Msp-Fc. pFastBac1-Myc-His6 was constructed to express a COOH-terminal Myc- and His6-tagged protein by inserting a cDNA fragment encoding the Myc epitope and His6 (hexahistidine) tags of pcDNA3.1(-)/Myc-His (Invitrogen) into pFastBac1 (GIBCO BRL). pFastBac1-Msp-Fc was constructed to express a chimeric protein fused with the  $\text{NH}_2$ -terminal honeybee melittin signal peptide and the COOH-terminal human IgG Fc by inserting cDNA fragments encoding the signal peptide (5'-ATG AAA TTC TTA GTC AAC GTT GCC CTT GTT TTT ATG GTC GTG TAC ATT TCT TAC ATC TAT GCG-3'; Tessier et al., 1991) and the IgG Fc into pFastBac1. Baculovirus transfer vectors for l-afadin and glycoprotein D (gD), an envelope component of HSV1, contained the following aa: pFastBac1-Myc-His6-l-afadin, aa 1-1,829 (full-length; see Fig. 1); and pFastBac1-Msp-Fc-gD, aa 1-285. A baculovirus bearing the l-afadin or gD cDNA was prepared with pFastBac1-Myc-

His6-l-afadin or -Msp-Fc-gD, respectively, according to the manufacturer's protocol (GIBCO BRL). The Myc- and His6-tagged protein of l-afadin (Myc-His6-l-afadin) was expressed in High Five insect cells (Invitrogen) infected with the baculovirus bearing the l-afadin cDNA and purified by the use of TALON metal affinity beads (CLONTECH Laboratories, Inc.). The chimeric protein of gD fused with IgG Fc was expressed in High Five insect cells (Invitrogen) infected with the baculovirus bearing the gD cDNA and purified by the use of protein A-Sepharose beads (Amersham Pharmacia Biotech).

Glutathione S-transferase (GST) fusion vectors for  $\alpha$ -catenin contained the following aa: GST- $\alpha$ -catenin, aa 1-906 (full-length; Itoh et al., 1997); and GST- $\alpha$ -catenin-C, aa 509-906 (COOH-terminal half; see Fig. 1). A GST fusion vector for ZO-1 (GST-ZO-1-N) contained aa 1-862 (NH<sub>2</sub>-terminal half; Itoh et al., 1997; Sakisaka et al., 1999). The GST fusion proteins were purified by the use of glutathione-Sepharose beads (Amersham Pharmacia Biotech).

### Cell Culture and DNA Transfection

F9, L, EL, and COS7 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. F9 cells were cultured in gelatin-coated (0.1%) culture dishes.  $\alpha$ -Catenin-deficient F9 cells (F9D $\alpha$ (-/-) cells) and F9D $\alpha$ (-/-) cells reexpressing  $\alpha$ -catenin ( $\alpha$ F9D $\alpha$ (-/-) cells) were obtained as previously described (Maeno et al., 1999). EL cells were cloned by the introduction of E-cadherin cDNA into L cells (Nagafuchi et al., 1987; see Fig. 1). The following L cell lines stably expressed the fusion molecules (see Fig. 1): nE $\alpha$ -L cells, the fusion molecule consisting of nonfunctional E-cadherin lacking its catenin-binding domain (aa 1-657) and full-length  $\alpha$ -catenin (aa 1-906; Nagafuchi et al., 1994; Imamura et al., 1999); nE $\alpha$ N-L cells, the fusion molecule consisting of nonfunctional E-cadherin lacking its catenin-binding domain and the NH<sub>2</sub>-terminal half of  $\alpha$ -catenin (aa 1-508; Nagafuchi et al., 1994; Imamura et al., 1999); and nE $\alpha$ C-L cells, the fusion molecule consisting of nonfunctional E-cadherin lacking its catenin-binding domain and the COOH-terminal half of  $\alpha$ -catenin (aa 509-906; Nagafuchi et al., 1994; Imamura et al., 1999). nE $\alpha$ -L, nE $\alpha$ N-L, and nE $\alpha$ C-L cells were obtained as previously described (Nagafuchi et al., 1994; Imamura et al., 1999).

The following L and EL cell lines were cloned by the introduction of the pPGKIH and pPGKIH-FLAG vectors, respectively, as described previously (Takahashi et al., 1999; Miyahara et al., 2000; Satoh-Horikawa et al., 2000): nectin-1 $\alpha$ -L cells, pPGKIH-nectin-1 $\alpha$  (aa 1-518); nectin-1 $\alpha$ - $\Delta$ C-L cells, pPGKIH-nectin-1 $\alpha$ - $\Delta$ C (aa 1-514); nectin-2 $\alpha$ -L cells, pPGKIH-nectin-2 $\alpha$  (aa 1-467); nectin-2 $\alpha$ - $\Delta$ C-L cells, pPGKIH-nectin-2 $\alpha$ - $\Delta$ C (aa 1-463); nectin-1 $\alpha$ -EL cells, pPGKIH-FLAG-nectin-1 $\alpha$  (aa 27-518); and nectin-1 $\alpha$ - $\Delta$ C-EL cells, pPGKIH-FLAG-nectin-1 $\alpha$ - $\Delta$ C (aa 27-514). In brief, L and EL cells were transfected with the vectors described above using Lipofectamine reagent (GIBCO BRL) according to the manufacturer's protocol. The cells were cultured for 1 d, replated, and selected by culturing in the presence of 500  $\mu$ g/ml of hygromycin (GIBCO BRL). To prepare nectin-1 $\alpha$ -L and -2 $\alpha$ -L cells, both of which transiently expressed  $\alpha$ - and/or  $\beta$ -catenins, the cells were transfected with pPGKIZ-HA- $\alpha$ -catenin or pEF-tEC using Lipofectamine reagent. The cells were cultured for 1 d, replated, and cultured for 3 d.

### Antibodies

One rabbit polyclonal anti-nectin-1 $\alpha$  antibody (Ab) was raised against GST-nectin-1 $\alpha$ -CPN (aa 379-438) as previously described (Takahashi et al., 1999; Satoh-Horikawa et al., 2000) and used as the polyclonal anti-nectin-1 $\alpha$  Ab1. Another rabbit polyclonal anti-nectin-1 $\alpha$  Ab was raised against the 19-mer synthetic peptide (corresponding to aa 450-468 of nectin-1 $\alpha$ ) as previously described (Satoh-Horikawa et al., 2000) and used as the polyclonal anti-nectin-1 $\alpha$  Ab2. A rabbit polyclonal anti-nectin-2 $\alpha$  Ab was prepared as described previously (Takahashi et al., 1999). A rat anti-nectin-2 mAb, which recognizes both nectin-2 $\alpha$  and -2 $\delta$ , was prepared as described previously (Aoki et al., 1997; Takahashi et al., 1999). A mouse anti-l-afadin mAb (Sakisaka et al., 1999) and a rabbit polyclonal antiponsin Ab (Mandai et al., 1999) were prepared as previously described. A rat anti-E-cadherin mAb (ECCD-2) was supplied from Dr. M. Takeichi (Kyoto University, Kyoto, Japan). A mouse antivinculin mAb was purchased from Sigma Chemical Co. A mouse anti-HA mAb was prepared as previously described (Yamochi et al., 1994). Mouse anti- $\beta$ -catenin and anti-FLAG mAbs were purchased from Zymed and Eastman Kodak Co., respectively.

### Yeast Two-Hybrid Interaction and $\beta$ -Galactosidase Assay

The bait vectors, pBTM116-HA- $\alpha$ -catenin (full-length, aa 1-906), -HA- $\alpha$ -catenin-N (NH<sub>2</sub>-terminal half, aa 1-508), -HA- $\alpha$ -catenin-C (COOH-terminal half, aa 509-906), and -HA-nectin-2 $\alpha$ -CP (cytoplasmic region, aa 387-467) were constructed by inserting cDNA fragments encoding the respective aa residues of  $\alpha$ -catenin and nectin-2 $\alpha$  into pBTM116-HA (see Fig. 1; Imamura et al., 1997). The prey vector, pGAD424-HA-l-afadin (aa 1-1,829, full-length), was constructed with pGAD424-HA (see Fig. 1). pGAD424-HA was constructed to express an HA-tagged protein fused with GAL4 activation domain by inserting a cDNA fragment encoding the HA epitope into pGAD424 (CLONTECH Laboratories, Inc.). The yeast two-hybrid  $\beta$ -galactosidase activity was measured according to the ONPG assay method (Guarente, 1983).

### Affinity Chromatography and Immunoprecipitation

Myc-His6-l-afadin (20  $\mu$ g of protein) was immobilized on TALON metal affinity beads (CLONTECH Laboratories, Inc.) (100  $\mu$ l of wet volume). GST- $\alpha$ -catenin and - $\alpha$ -catenin-C (100  $\mu$ g of protein each) were separately applied to the Myc-His6-l-afadin-immobilized beads equilibrated with buffer A (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10  $\mu$ g/ml leupeptin, 1 mM PMSF, and 1  $\mu$ g/ml pepstatin A). After the beads were extensively washed with buffer A, elution was performed with buffer A containing 100 mM imidazole chloride, pH 7.5. Each fraction was subjected to SDS-PAGE (10% polyacrylamide gel), followed by protein staining with Coomassie brilliant blue. Where indicated, GST-ZO-1-N and - $\alpha$ -catenin-C (100  $\mu$ g of protein each) were mixed and subjected to affinity chromatography.

Immunoprecipitation was performed as described previously (Takahashi et al., 1999). In brief, COS7 cells transiently expressing FLAG-l-afadin and HA- $\alpha$ -catenin or - $\alpha$ -catenin-C were prepared with transfection of pFLAG-CMV2-l-afadin and pPGKIZ-HA- $\alpha$ -catenin or -HA- $\alpha$ -catenin-C, respectively, using Lipofectamine reagent. The cells were sonicated in buffer B (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10  $\mu$ g/ml leupeptin, 1 mM PMSF, and 1  $\mu$ g/ml pepstatin A), followed by centrifugation at 100,000 *g* for 30 min. The supernatant was incubated with the anti-FLAG or anti-HA mAb at 4°C for 2 h. Protein G-Sepharose beads (Amersham Pharmacia Biotech) were added to this sample, and incubation was further performed at 4°C for 1 h. After the beads were extensively washed with buffer B, the bound proteins were eluted by boiling the beads in an SDS sample buffer (60 mM Tris-Cl, pH 6.7, 3% SDS, 2% [vol/vol] 2-mercaptoethanol, and 5% glycerol), and subjected to SDS-PAGE, followed by Western blot analysis.

### Coculture of L and EL Cells Stably Expressing Nectin

Nectin-1 $\alpha$ -L, -1 $\alpha$ -EL, and -1 $\alpha$ - $\Delta$ C-EL cells were washed with PBS, incubated with 0.2% trypsin and 1 mM EDTA at 37°C for 5 min, and dispersed by gentle pipetting to obtain single cell suspensions. The cell number of each cell line was counted, and nectin-1 $\alpha$ -EL or -1 $\alpha$ - $\Delta$ C-EL cells were mixed with a 10-fold number of nectin-1 $\alpha$ -L cells. These mixed cells were plated at  $2 \times 10^5$  cells on a 35-mm culture dish and cultured for 2 d. Where indicated, gD was added to the mixed cell suspension to give a final concentration of 0.3  $\mu$ M. The cells were further cultured with the same concentration of gD for 2 d.

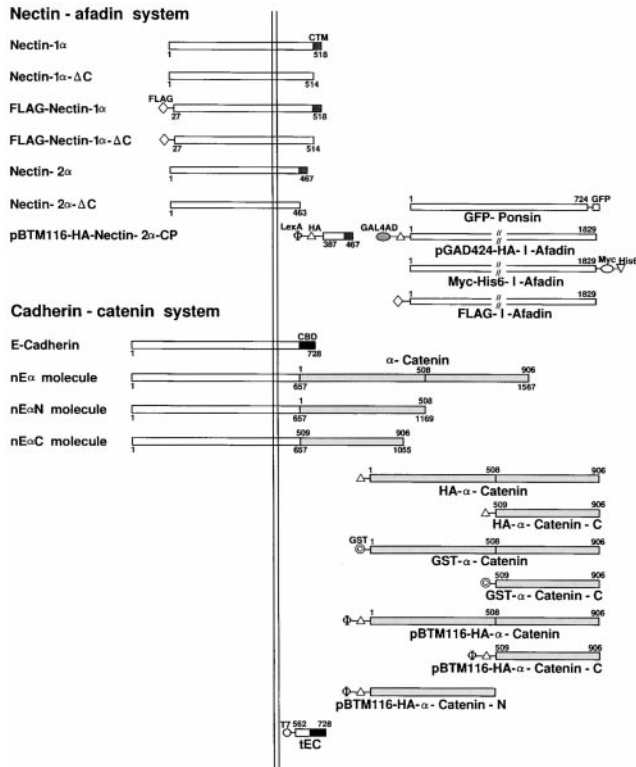
### Other Procedures

Immunofluorescence microscopy of cultured cells was done as described previously (Mandai et al., 1997, 1999; Takahashi et al., 1999). Protein concentrations were determined with BSA as a reference protein (Bradford, 1976). SDS-PAGE was done as previously described (Laemmli, 1970).

### Results

#### $\alpha$ -Catenin-dependent Colocalization of Nectin and l-Afadin with E-Cadherin

We first examined whether  $\alpha$ -catenin is involved in the



**Figure 1.** Structures of various constructs of nectin, l-afadin, ponsin, E-cadherin, and  $\alpha$ -catenin. CTM, COOH-terminal motif of four aa residues; GAL4AD, GAL4 activation domain; and CBD, catenin-binding domain.

colocalization of nectin and l-afadin with E-cadherin. For this purpose, we used an  $\alpha$ -catenin-deficient F9 cell line, F9D $\alpha$ ( $-/-$ ) cells (Maeno et al., 1999). F9 cells are mouse teratocarcinoma-derived embryonal carcinoma cells. F9D $\alpha$ ( $-/-$ ) cells were generated by a disruption of both  $\alpha$ -catenin alleles with a targeting vector. As a control, we used  $\alpha$ F9D $\alpha$ ( $-/-$ ) cells, which were generated by the introduction of an expression vector encoding full-length  $\alpha$ -catenin into F9D $\alpha$ ( $-/-$ ) cells (Maeno et al., 1999). It has been shown that  $\alpha$ F9D $\alpha$ ( $-/-$ ) cells reexpress  $\alpha$ -catenin in an amount similar to that of wild-type F9 cells (Maeno et al., 1999). In  $\alpha$ F9D $\alpha$ ( $-/-$ ) cells, E-cadherin was localized at beltlike cell-cell adhesion sites, where nectin-2 and l-afadin were colocalized (Fig. 2 A). In F9D $\alpha$ ( $-/-$ ) cells, E-cadherin showed the similar staining pattern, but nectin-2 and l-afadin were hardly concentrated at cell-cell adhesion sites between two cells, where E-cadherin was concentrated (Fig. 2 B). Nectin-2 and l-afadin were colocalized with E-cadherin only at spotlike cell-cell adhesion sites where  $>2$  cells adhered to each other. Western blot analysis indicated that the protein levels of nectin-2 $\alpha$  and l-afadin were similar between  $\alpha$ F9D $\alpha$ ( $-/-$ ) and F9D $\alpha$ ( $-/-$ ) cells (data not shown). It has been shown that the protein levels of E-cadherin and  $\beta$ -catenin in  $\alpha$ F9D $\alpha$ ( $-/-$ ) cells are comparable to those in F9D $\alpha$ ( $-/-$ ) cells (Maeno et al., 1999). Taken together, these results indicate that the colocalization of nectin and l-afadin with E-cadherin is dependent on  $\alpha$ -catenin, but not on the cytoplasmic region of E-cadherin or  $\beta$ -catenin, in F9 cells.

### *$\beta$ -Catenin-independent Colocalization of Nectin and l-Afadin with E-Cadherin*

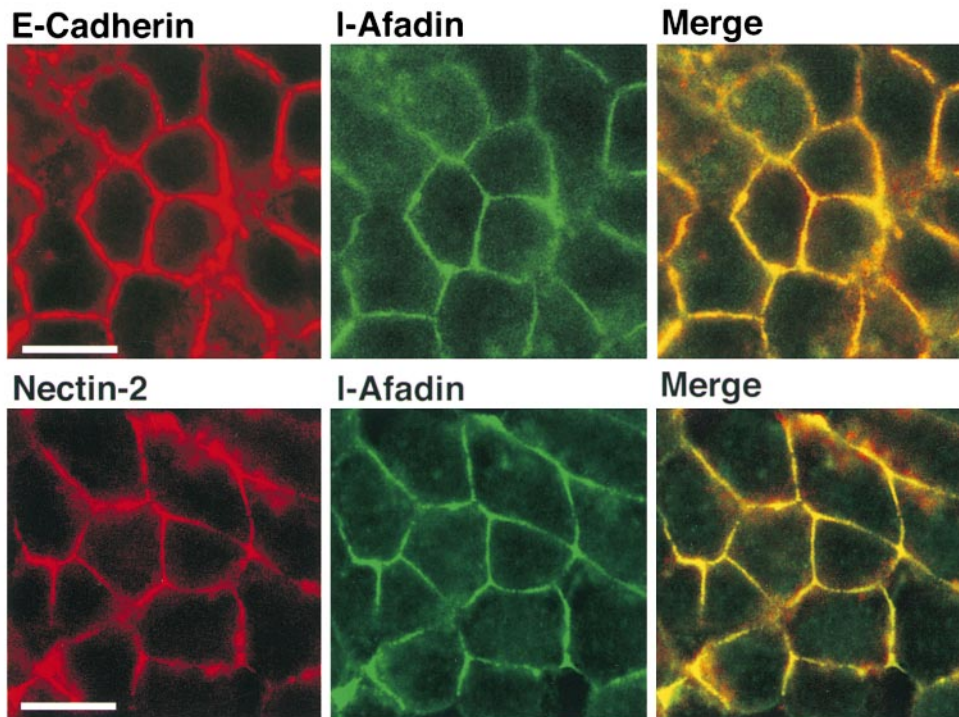
We have previously shown that nectin-2 and l-afadin are colocalized with E-cadherin at cell-cell AJs in EL cells (Takahashi et al., 1999), which have been established as L cells stably expressing E-cadherin (Nagafuchi et al., 1987). In EL cells, ponsin and vinculin were colocalized and concentrated with E-cadherin at cell-cell AJs and focal contacts (data not shown). To further confirm the results obtained with the two F9 cell lines described above, we used L cells stably expressing a chimeric protein (nE $\alpha$ ) of catenin-binding domain-deleted E-cadherin fused with full-length  $\alpha$ -catenin (nE $\alpha$ -L cells). In the junctional level of nE $\alpha$ -L cells, nectin-2 and l-afadin were colocalized at cell-cell adhesion sites where the nE $\alpha$  molecule was localized (Fig. 3 A). Ponsin and vinculin were also colocalized with nE $\alpha$  there. In the basal level, nE $\alpha$ , nectin-2, or l-afadin was not concentrated at focal contacts (data not shown), whereas ponsin and vinculin were colocalized there (Fig. 3 B). These results are consistent with those obtained with the two F9 cell lines described above, and indicate that  $\beta$ -catenin is not essential for the colocalization of nectin, l-afadin, ponsin, and vinculin with E-cadherin at cell-cell adhesion sites.

### *Ponsin- and Vinculin-independent Colocalization of Nectin and l-Afadin with E-Cadherin*

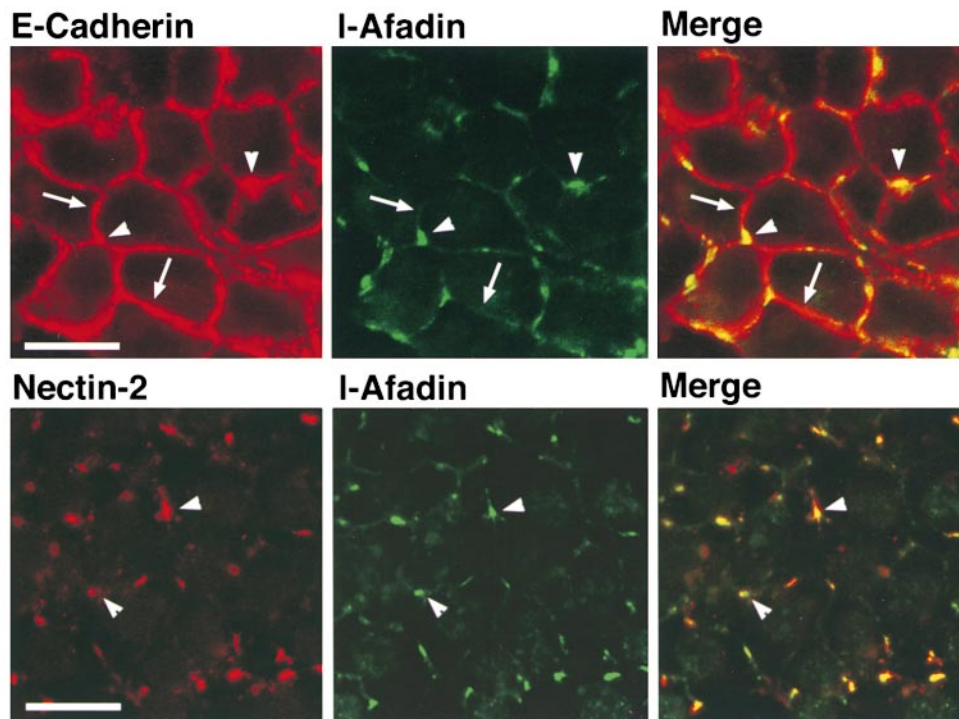
We next examined whether ponsin and vinculin are necessary for the colocalization of nectin and l-afadin with E-cadherin. For this purpose, we took advantage of two L cell lines. One cell line (nE $\alpha$ N-L) is an L cell clone stably expressing a chimeric protein (nE $\alpha$ N) of catenin-binding domain-deleted E-cadherin fused with the NH<sub>2</sub>-terminal half of  $\alpha$ -catenin, which is capable of binding vinculin and  $\alpha$ -actinin but not ZO-1 (Nagafuchi et al., 1994; Imamura et al., 1999). The other cell clone (nE $\alpha$ C-L) is an L cell clone stably expressing a chimeric protein (nE $\alpha$ C) of catenin-binding domain-deleted E-cadherin fused with the COOH-terminal half of  $\alpha$ -catenin, which is capable of binding ZO-1, but not vinculin or  $\alpha$ -actinin (Nagafuchi et al., 1994; Imamura et al., 1999). In the junctional level of nE $\alpha$ N-L cells, the nE $\alpha$ N molecule was localized at cell-cell adhesion sites where neither nectin-2 nor l-afadin was concentrated (Fig. 4 A). However, vinculin was colocalized with nE $\alpha$ N there. Ponsin appeared to be also colocalized with nE $\alpha$ N there, but the staining was not clear (data not shown). When a GFP-tagged protein of full-length ponsin (GFP-ponsin) was transiently expressed, it was clearly colocalized with nE $\alpha$ N at cell-cell adhesion sites (Fig. 4 A). In the basal level of nE $\alpha$ N-L cells, ponsin and vinculin were colocalized at focal contacts (Fig. 4 B).

In the junctional level of nE $\alpha$ C-L cells, the nE $\alpha$ C molecule was localized at cell-cell adhesion sites, where nectin-2 and l-afadin were colocalized (Fig. 5 A). However, neither ponsin nor vinculin was concentrated with nE $\alpha$ C there (Fig. 5 A), although they were colocalized at focal contacts (Fig. 5 B). We have previously shown that the protein levels of nectin-1 $\alpha$  and l-afadin are similar among nE $\alpha$ -L, nE $\alpha$ N-L, and nE $\alpha$ C-L cells (Sakisaka, T., T. Taniguchi, H. Nakanishi, K. Takahashi, M. Miyahara, W. Ikeda, S. Yokoyama, Y.-F. Peng, K. Ya-

### A $\alpha$ F9D $\alpha$ (-/-) cells



### B F9D $\alpha$ (-/-) cells

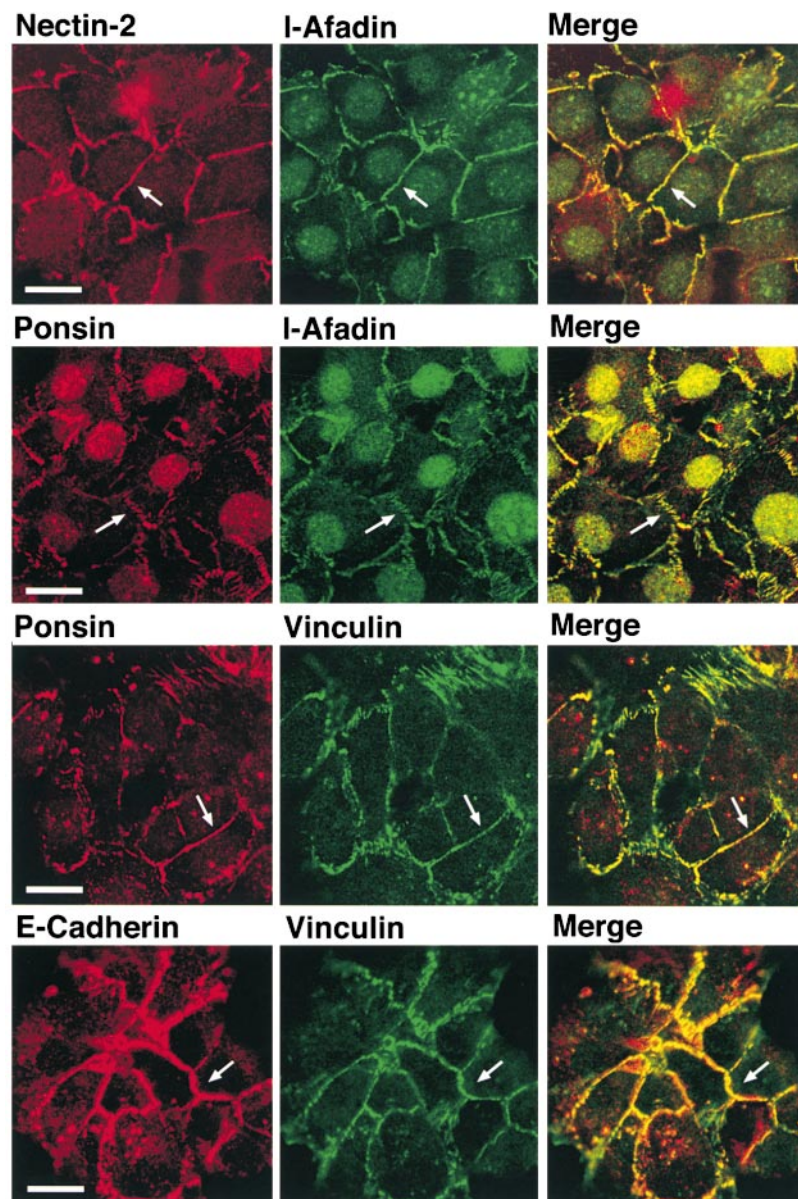


*Figure 2.*  $\alpha$ -Catenin-dependent colocalization of nectin and I-afadin with E-cadherin.  $\alpha$ F9D $\alpha$ (-/-) and F9D $\alpha$ (-/-) cells were double stained with various combinations of the anti-E-cadherin, anti-I-afadin, and anti-nectin-2 Abs. (arrows) Cell-cell adhesion sites between two cells; and (arrowheads) cell-cell adhesion sites where >2 cells adhere to each other. The results shown are representative of three independent experiments. Bars, 10  $\mu$ m.

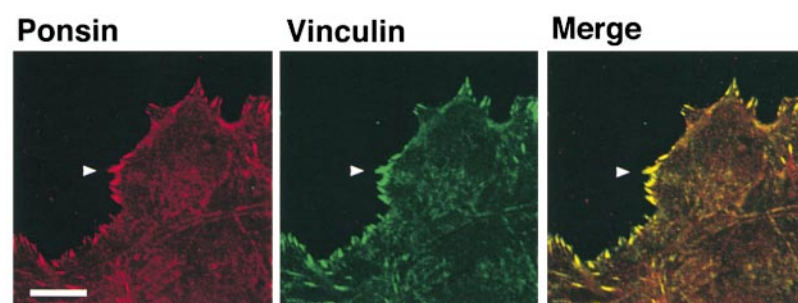
manishi, and Y. Takai, manuscript submitted for publication). In addition, Western blot analysis indicated that the protein levels of nectin-2 $\alpha$ , ponsin, and vinculin were also similar among these three L cell lines (data not shown). These results indicate that neither ponsin nor vinculin is

necessary for the colocalization of nectin and I-afadin with E-cadherin at cell-cell adhesion sites, and that their colocalization is dependent on the COOH-terminal half of  $\alpha$ -catenin, but not on the cytoplasmic region of E-cadherin or the NH<sub>2</sub>-terminal half of  $\alpha$ -catenin.

## A Junctional level



## B Basal level



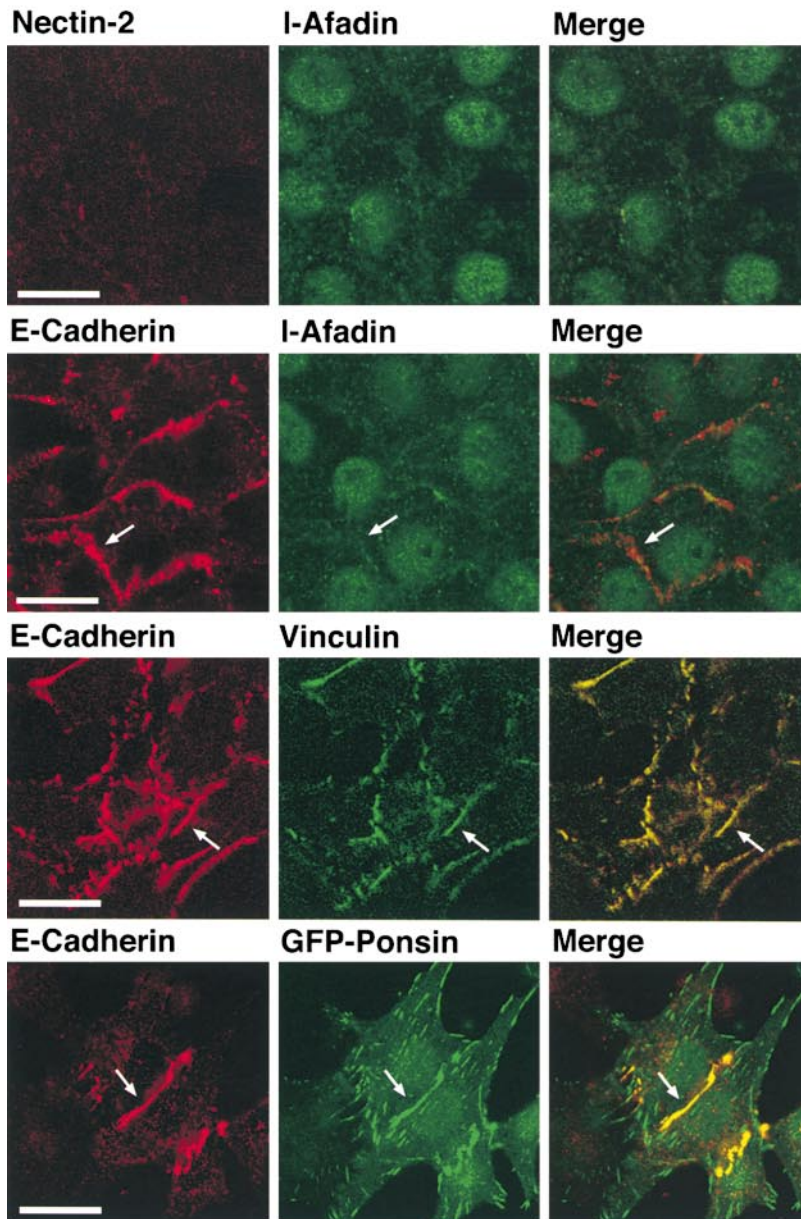
**Figure 3.**  $\beta$ -Catenin-independent colocalization of nectin, l-afadin, ponsin, and vinculin with E-cadherin. nE $\alpha$ -L cells were double stained with various combinations of the anti-nectin-2, anti-l-afadin, antiponsin, antivinculin, and anti-E-cadherin Abs. There was nuclear staining with the anti-l-afadin or antiponsin Ab, but the nuclear staining was not an artifact of second antibodies, although its significance is not known. (arrows) Cell-cell adhesion sites; and (arrowhead) focal contacts. The results shown are representative of three independent experiments. Bars, 10  $\mu$ m.

### *Molecular Linkage between l-Afadin and $\alpha$ -Catenin*

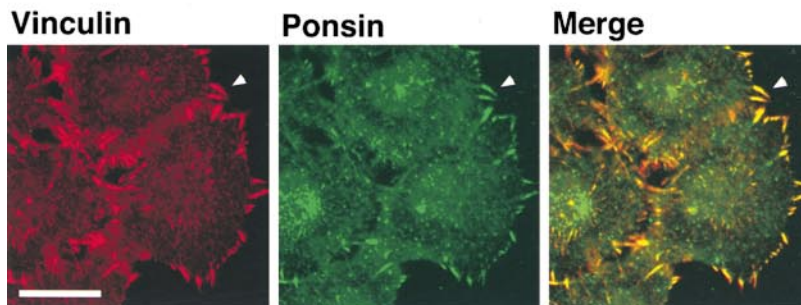
We have previously shown that l-afadin does not directly interact with  $\alpha$ -catenin by affinity chromatography using the purified samples (Sakisaka et al., 1999). In these exper-

iments, we used full-length proteins. It has been shown that  $\alpha$ -catenin is homologous to vinculin (Nagafuchi et al., 1991; Herrenknecht et al., 1991; Pokutta and Weis, 2000), and that vinculin shows the intramolecular association of its

## A Junctional level

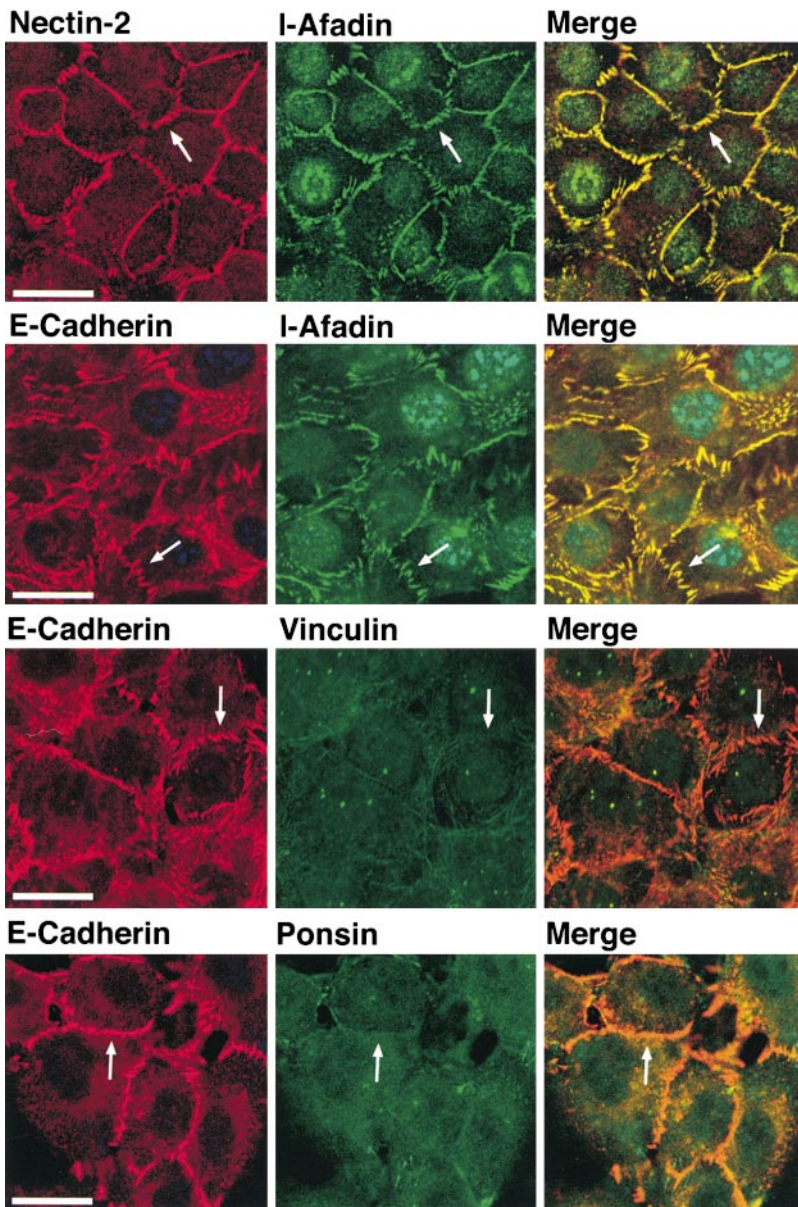


## B Basal level

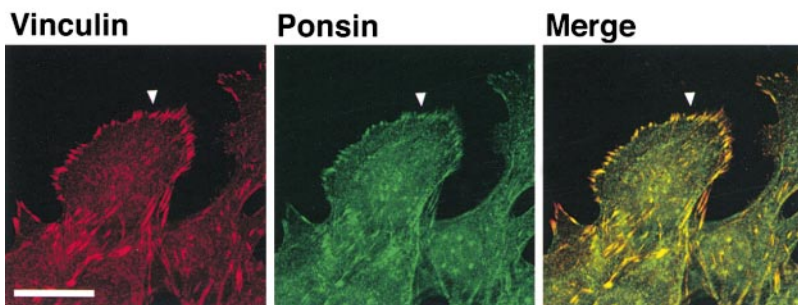


*Figure 4.* Nectin- and I-afadin-independent colocalization of ponsin and vinculin with E-cadherin. nEαN-L cells were double stained with various combinations of the anti-nectin-2, anti-I-afadin, antiponsin, antivinculin, and anti-E-cadherin Abs. Alternatively, nEαN-L cells were transfected with pEGFP-ponsin and stained with the anti-E-cadherin Ab. (arrows) Cell-cell adhesion sites; and (arrowhead) focal contacts. The results shown are representative of three independent experiments. Bars, 10 μm.

## A Junctional level



## B Basal level

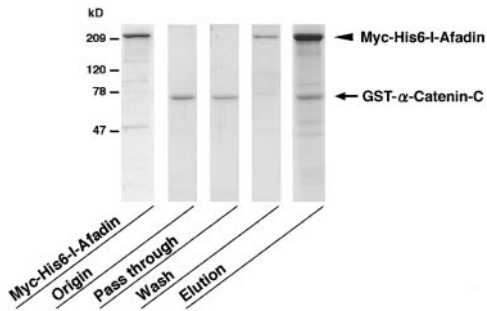


**Figure 5.** Ponsin- and vinculin-independent colocalization of nectin and I-afadin with E-cadherin. nE $\alpha$ C-L cells were double stained with various combinations of the anti-nectin-2, anti-I-afadin, anti-ponsin, anti-vinculin, and anti-E-cadherin Abs. (arrows) Cell-cell adhesion sites; and (arrowhead) focal contacts. The results shown are representative of three independent experiments. Bars, 10  $\mu$ m.

NH<sub>2</sub>-terminal and COOH-terminal halves (Johnson and Craig, 1995). Therefore, we examined whether I-afadin directly interacts with the COOH-terminal half of  $\alpha$ -catenin. The yeast two-hybrid analysis revealed that I-afadin

(pGAD424-HA-I-afadin) interacted with the COOH-terminal half (pBTM-116-HA- $\alpha$ -catenin-C), but not with the full-length (pBTM-116-HA- $\alpha$ -catenin) or the NH<sub>2</sub>-terminal half (pBTM-116-HA- $\alpha$ -catenin-N; Table I). This yeast





**Figure 6.** Interaction of l-afadin with  $\alpha$ -catenin on affinity chromatography. GST- $\alpha$ -catenin-C was applied to metal affinity beads on which Myc-His6-l-afadin was immobilized. After the beads were extensively washed, elution was performed with 100 mM imidazole chloride, pH 7.5. Each fraction was subjected to SDS-PAGE (10% polyacrylamide gel), followed by protein staining with Coomassie brilliant blue. The results shown are representative of three independent experiments.

two-hybrid interaction of l-afadin with the COOH-terminal half of  $\alpha$ -catenin was comparable to that of l-afadin with the cytoplasmic region of nectin-2 $\alpha$  (pBTM-116-HA-nectin-2 $\alpha$ -CP). We further confirmed the *in vitro* direct interaction of l-afadin with  $\alpha$ -catenin by affinity chromatography. A GST fusion protein of the COOH-terminal half of  $\alpha$ -catenin (GST- $\alpha$ -catenin-C) bound to a Myc- and His6-tagged protein of full-length l-afadin (Myc-His6-l-afadin) immobilized on metal affinity beads (Fig. 6), whereas a GST fusion protein of the full-length (GST- $\alpha$ -catenin) hardly bound to the beads (data not shown). However, the stoichiometry of the interaction of GST- $\alpha$ -catenin-C with Myc-His6-l-afadin was  $\sim 0.1:1$ . It has been shown that the NH<sub>2</sub>-terminal half of ZO-1 directly interacts with the AF-6 protein/s-afadin (Yamamoto et al., 1997) and the COOH-terminal half of  $\alpha$ -catenin (Itoh et al., 1997). To examine whether the NH<sub>2</sub>-terminal half of ZO-1 enhances the stoichiometry of the interaction of the COOH-terminal half of  $\alpha$ -catenin with l-afadin, a GST fusion protein of the NH<sub>2</sub>-terminal half of ZO-1 (GST-ZO-1-N) was mixed with GST- $\alpha$ -catenin-C, and the mixture was subjected to the Myc-His6-l-afadin-immobilized affinity chromatography. However, GST-ZO-1-N did not enhance the stoichiometry of the interaction of GST- $\alpha$ -catenin-C with Myc-His6-l-afadin (data not shown). To further confirm the interaction of l-afadin with the COOH-terminal half of  $\alpha$ -catenin in intact cells, a FLAG-tagged protein of full-length l-afadin (FLAG-l-afadin) and an HA-tagged protein of the COOH-terminal half of  $\alpha$ -catenin (HA- $\alpha$ -catenin-C) were coexpressed in COS7

cells and immunoprecipitated by either the anti-FLAG or anti-HA Ab, but they were not coimmunoprecipitated to a significant extent (data not shown). However, negative results obtained from immunoprecipitation experiments do not necessarily reflect *in vivo* negative protein-protein interactions. Therefore, these results suggest that l-afadin directly interacts with  $\alpha$ -catenin under appropriate conditions.

### ***l-Afadin-dependent Recruitment of $\alpha$ -Catenin to Nectin-based Cell-Cell Adhesion Sites in the Absence of E-Cadherin***

In the next series of experiments, we examined whether  $\alpha$ -catenin and  $\beta$ -catenin are recruited through l-afadin to nectin-based cell-cell adhesion sites in the absence of E-cadherin. For this purpose, we used L cells stably expressing full-length nectin-2 $\alpha$  (nectin-2 $\alpha$ -L cells) or the COOH-terminal four aa-deleted nectin-2 $\alpha$  (nectin-2 $\alpha$ - $\Delta$ C-L cells). We have previously shown that nectin-2 $\alpha$ -L and -2 $\alpha$ - $\Delta$ C-L cells show similar cell adhesion activity, as estimated by a cell aggregation assay (Miyahara et al., 2000). The protein levels of nectin-2 $\alpha$  and l-afadin in nectin-2 $\alpha$ -L cells are similar to those in nectin-2 $\alpha$ - $\Delta$ C-L cells, respectively. However, in nectin-2 $\alpha$ -L cells, l-afadin is concentrated at nectin-2 $\alpha$ -based cell adhesion sites, whereas in nectin-2 $\alpha$ - $\Delta$ C-L cells, l-afadin is not recruited to nectin-2 $\alpha$ - $\Delta$ C-based cell adhesion sites (Miyahara et al., 2000). We first confirmed this result (Fig. 7). When an HA-tagged protein of full-length  $\alpha$ -catenin (HA- $\alpha$ -catenin) was transiently expressed in nectin-2 $\alpha$ -L cells, HA- $\alpha$ -catenin was concentrated at nectin-2 $\alpha$ -based cell-cell adhesion sites (Fig. 8, top). However, when HA- $\alpha$ -catenin was transiently expressed in nectin-2 $\alpha$ - $\Delta$ C-L cells, HA- $\alpha$ -catenin was not concentrated at nectin-2 $\alpha$ - $\Delta$ C-based cell-cell adhesion sites (Fig. 8, bottom). Similar results were obtained with L cells stably expressing full-length nectin-1 $\alpha$  (nectin-1 $\alpha$ -L cells) or the COOH-terminal four aa-deleted nectin-1 $\alpha$  (nectin-1 $\alpha$ - $\Delta$ C-L cells; data not shown). These results indicate that  $\alpha$ -catenin is recruited through l-afadin to nectin-based cell-cell adhesion sites in the absence of E-cadherin.

### ***l-Afadin-dependent Recruitment of $\beta$ -Catenin to Nectin-based Cell-Cell Adhesion Sites without the trans-Interaction of E-Cadherin***

In L cells, endogenous  $\beta$ -catenin was reduced to a practically undetectable level, but the amount of  $\beta$ -catenin as well as  $\alpha$ -catenin was increased upon expression of a T7 peptide-tagged protein of the cytoplasmic region and partial transmembrane segment of E-cadherin (tEC; data not

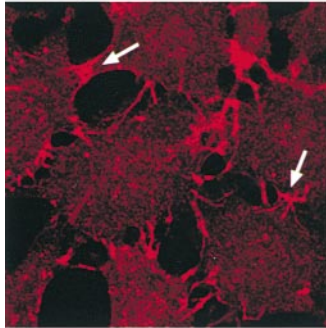
**Table I.** Yeast Two-Hybrid Interaction of l-Afadin with  $\alpha$ -Catenin

	$\alpha$ -Catenin				Nectin-2 $\alpha$
	Control (pBTM116-HA)	Full-length (pBTM116-HA- $\alpha$ -catenin)	COOH-terminal (pBTM116-HA- $\alpha$ -catenin-C)	NH <sub>2</sub> -terminal (pBTM116-HA- $\alpha$ -catenin-N)	Cytoplasmic region (pBTM116-HA-nectin-2 $\alpha$ -CP)
Control (pGAD424-HA)	<1	<1	<1	<1	<1
l-Afadin (pGAD424-HA-l-afadin)	1 $\pm$ 0.5	<1	30 $\pm$ 2	<1	41 $\pm$ 2

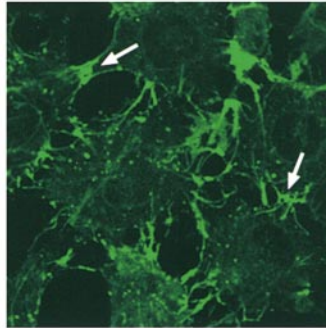
The data list units of  $\beta$ -galactosidase activity of yeast strains harboring the respective bait and prey plasmids. The values are means  $\pm$  SEM of three independent experiments.

## Nectin-2 $\alpha$ -L cells

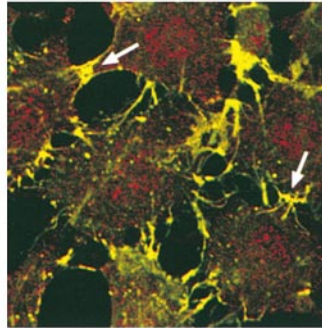
I-Afadin



Nectin-2

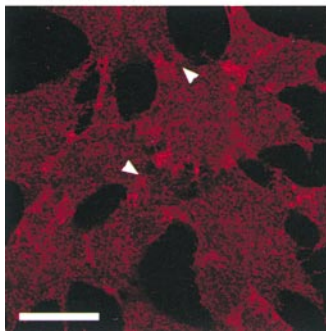


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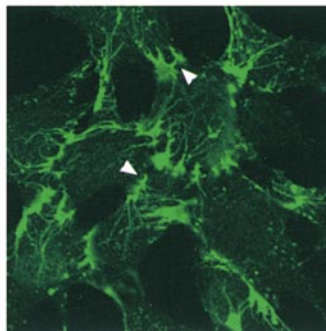


## Nectin-2 $\alpha$ - $\Delta$ C-L cells

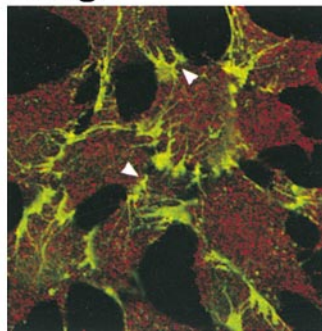
I-Afadin



Nectin-2



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**Figure 7.** Localization of nectin and I-afadin in nectin-2 $\alpha$ -L and -2 $\alpha$ - $\Delta$ C-L cells. Nectin-2 $\alpha$ -L and -2 $\alpha$ - $\Delta$ C-L cells were double stained with the anti-nectin-2 and anti-I-afadin Abs. (arrows) Nectin-2 $\alpha$ -based cell-cell adhesion sites; and (arrow-heads) nectin-2 $\alpha$ - $\Delta$ C-based cell-cell adhesion sites. The results shown are representative of three independent experiments. Bar, 10  $\mu$ m.

shown). In nectin-2 $\alpha$ -L cells transiently expressing tEC,  $\beta$ -catenin was recruited to nectin-2 $\alpha$ -based cell-cell adhesion sites (Fig. 9, top). In contrast, in nectin-2 $\alpha$ - $\Delta$ C-L cells transiently expressing tEC,  $\beta$ -catenin was not concentrated at nectin-2 $\alpha$ - $\Delta$ C-based cell-cell adhesion sites (Fig. 9, bottom). The amounts of increased endogenous  $\beta$ -catenin were similar between these two types of cells (data not shown). These results indicate that  $\beta$ -catenin is recruited through I-afadin to nectin-based cell-cell adhesion sites without the trans-interaction of E-cadherin.

### ***I-Afadin-dependent Recruitment of E-Cadherin to Nectin-based Cell-Cell Adhesion Sites without the trans-Interaction of E-Cadherin***

In the last set of experiments, we examined whether E-cadherin is recruited to nectin-based cell-cell adhesion sites without the trans-interaction of E-cadherin. For this purpose, we cocultured nectin-1 $\alpha$ -L cells with EL cells stably expressing a FLAG-tagged protein of full-length nectin-1 $\alpha$  (nectin-1 $\alpha$ -EL cells) or a FLAG-tagged protein of the COOH-terminal four aa-deleted nectin-1 $\alpha$  (nectin-1 $\alpha$ - $\Delta$ C-EL cells). When nectin-1 $\alpha$ -L cells were cocultured with nectin-1 $\alpha$ -EL cells, nectin-1 $\alpha$  was concentrated at adhesion sites between the same type of cells and between two different types of cells (Fig. 10, top). E-Cadherin was concentrated at adhesion sites between two nectin-1 $\alpha$ -EL cells. In addition, E-cadherin was concentrated at adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ -EL cells. When

nectin-1 $\alpha$ -L cells were cocultured with nectin-1 $\alpha$ - $\Delta$ C-EL cells, nectin-1 $\alpha$  and -1 $\alpha$ - $\Delta$ C were also concentrated at adhesion sites between the same type of cells and between the two different types of cells (Fig. 10, middle). Consistent with our previous report that the interaction of nectin with I-afadin is necessary for its clustering (Miyahara et al., 2000), the concentration of nectin-1 $\alpha$  and -1 $\alpha$ - $\Delta$ C at adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ - $\Delta$ C-EL cells was weaker than that of nectin-1 $\alpha$  at adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ -EL cells. Moreover, the recruitment of E-cadherin to adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ - $\Delta$ C-EL cells was significantly reduced when compared with that between nectin-1 $\alpha$ -L and -1 $\alpha$ -EL cells. Western blot analysis indicated that the protein levels of nectin-1 $\alpha$  or -1 $\alpha$ - $\Delta$ C among these three cell lines were similar (data not shown). The polyclonal anti-nectin-1 $\alpha$  Ab2 recognized two or three protein bands. Their relationship is not clear, but this may be due to different levels of the post-translational modifications such as glycosylation.

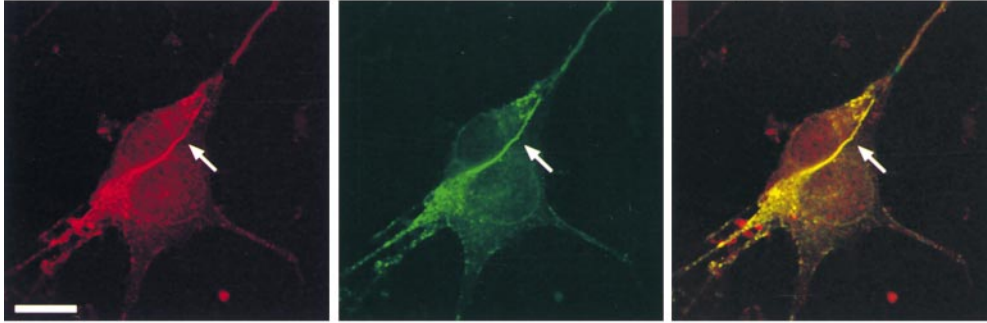
We have previously shown that gD, an envelope component of HSV1, specifically inhibits nectin-1 $\alpha$ -mediated cell adhesion activity as estimated by a cell aggregation assay (Sakisaka, T., T. Taniguchi, H. Nakanishi, K. Takahashi, M. Miyahara, W. Ikeda, S. Yokoyama, Y.-F. Peng, K. Yamanishi, and Y. Takai, manuscript submitted for publication). When gD was added to the coculture system of nectin-1 $\alpha$ -L and -1 $\alpha$ -EL cells, nectin-1 $\alpha$  was hardly concentrated at adhesion sites between the same type of cells or between two different types of cells (Fig. 10, bottom).

## Nectin-2 $\alpha$ -L cells

HA- $\alpha$ -Catenin

Nectin-2

Merge

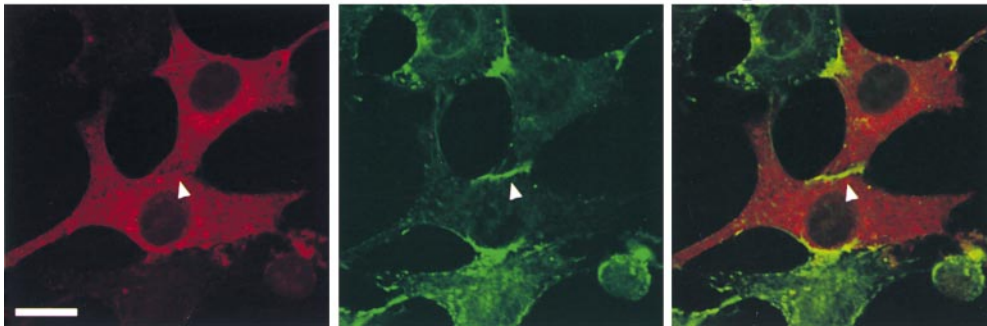


## Nectin-2 $\alpha$ - $\Delta$ C-L cells

HA- $\alpha$ -Catenin

Nectin-2

Merge



**Figure 8.** l-Afadin-dependent recruitment of  $\alpha$ -catenin to nectin-based cell-cell adhesion sites in the absence of E-cadherin. Nectin-2 $\alpha$ -L and -2 $\alpha$ - $\Delta$ C-L cells were transfected with pPGKIZ-HA- $\alpha$ -catenin and double stained with the anti-nectin-2 and anti-HA Abs. (arrow) Nectin-2 $\alpha$ -based cell-cell adhesion sites; and (arrowhead) nectin-2 $\alpha$ - $\Delta$ C-based cell-cell adhesion sites. The results shown are representative of three independent experiments. Bars, 10  $\mu$ m.

The recruitment of E-cadherin to adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ -EL cells was remarkably reduced when compared with that in the absence of gD. However, the concentration of E-cadherin at adhesion sites between two nectin-1 $\alpha$ -EL cells was not affected. These results indicate that E-cadherin is recruited through l-afadin to nectin-based cell-cell adhesion sites without the trans-interaction of E-cadherin, and that nectin, of which trans-interaction is inhibited, is not recruited to cadherin-based cell-cell adhesion sites.

## Discussion

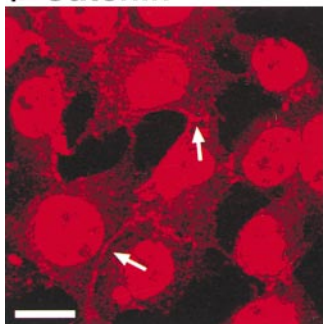
Our previous series of studies have shown that l-afadin is essential for the colocalization and compact clustering of nectin with E-cadherin at cell-cell AJs and proper organization of E-cadherin-based cell-cell AJs (Ikeda et al., 1999; Takahashi et al., 1999; Miyahara et al., 2000). Extending these earlier observations, we have shown here that the COOH-terminal half of  $\alpha$ -catenin is, furthermore, essential for this colocalization. This colocalization is not mediated through the cytoplasmic region of E-cadherin or  $\beta$ -catenin.  $\alpha$ -Catenin directly interacts with vinculin and  $\alpha$ -actinin through the NH<sub>2</sub>-terminal half (Knudsen et al., 1995; Nieset et al., 1997; Watabe-Uchida et al., 1998; Imamura et al., 1999) and with ZO-1 through the COOH-terminal half (Itoh et al., 1997; Imamura et al., 1999). All of these  $\alpha$ -catenin-binding proteins directly interact with F-actin (Burrige and Feramisco, 1982; Menkel et al., 1994; Johnson and Craig, 1995; Itoh et al., 1997; Fanning et

al., 1998). The present results show that the NH<sub>2</sub>-terminal half of  $\alpha$ -catenin is not essential for the colocalization of nectin and l-afadin with E-cadherin, indicating that neither vinculin,  $\alpha$ -actinin, nor ponsin is necessary for this colocalization. This result is consistent with our previous observation that ponsin forms a binary complex with either l-afadin or vinculin, and does not form a ternary complex (Mandai et al., 1999).

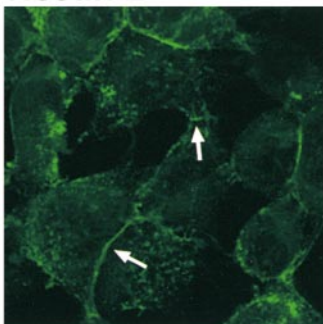
It is of crucial importance to clarify the molecular linkage between l-afadin and the COOH-terminal half of  $\alpha$ -catenin. We have previously shown that l-afadin does not directly interact with  $\alpha$ -catenin as estimated by affinity chromatography using the purified samples (Sakisaka et al., 1999). Therefore, we assumed that there might be a protein that interacts with both l-afadin and  $\alpha$ -catenin and connects them. On the basis of this assumption, we have been attempting to isolate an l-afadin-binding protein in the presence of  $\alpha$ -catenin or an  $\alpha$ -catenin-binding protein in the presence of l-afadin by using various methods currently available, including yeast two-hybrid, affinity chromatography, immunoprecipitation, and blot overlay. We have not yet obtained any candidate proteins, but we have found, by the yeast two-hybrid method, that full-length l-afadin directly interacts with the COOH-terminal half of  $\alpha$ -catenin. We have confirmed this interaction by affinity chromatography, although the stoichiometry of this interaction is small. We have attempted to increase this small stoichiometry by the use of the NH<sub>2</sub>-terminal or COOH-terminal half of l-afadin. The COOH-terminal half directly interacted with the COOH-terminal half of  $\alpha$ -catenin, but

## Nectin-2 $\alpha$ -L cells

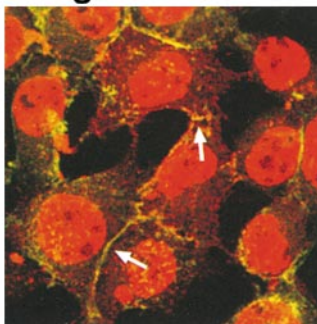
$\beta$ -Catenin



Nectin-2

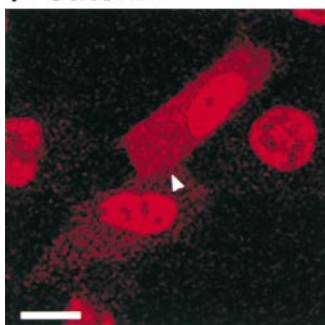


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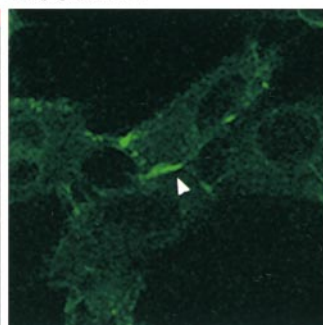


## Nectin-2 $\alpha$ - $\Delta$ C-L cells

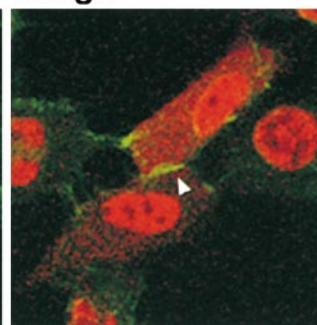
$\beta$ -Catenin



Nectin-2



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*Figure 9.* I-Afadin-dependent recruitment of  $\beta$ -catenin to nectin-based cell-cell adhesion sites without the trans-interaction of E-cadherin. Nectin-2 $\alpha$ -L and -2 $\alpha$ - $\Delta$ C-L cells were transfected with pEF-tEC and double stained with the anti-nectin-2 and anti- $\beta$ -catenin Abs. (arrows) Nectin-2 $\alpha$ -based cell-cell adhesion sites; and (arrowhead) nectin-2 $\alpha$ - $\Delta$ C-based cell-cell adhesion sites. The results shown are representative of three independent experiments. Bars, 10  $\mu$ m.

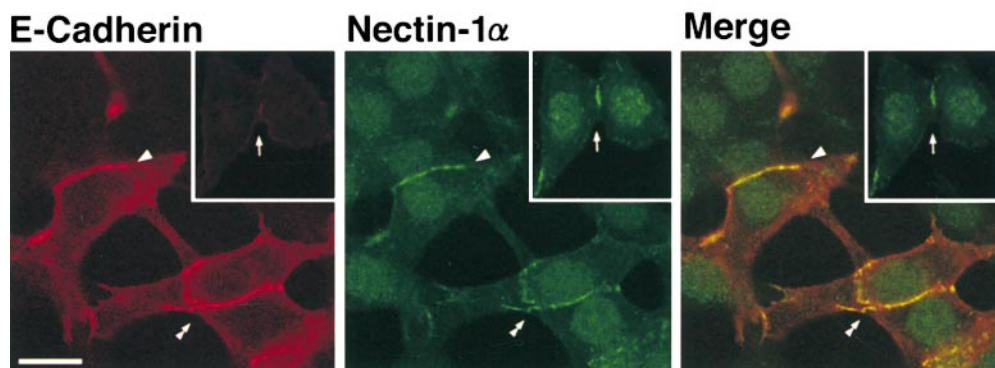
the stoichiometry did not increase (data not shown). We have added the NH<sub>2</sub>-terminal half of ZO-1 because it has been shown to directly interact with the AF-6 protein/s-afadin (Yamamoto et al., 1997) and with the COOH-terminal half of  $\alpha$ -catenin (Itoh et al., 1997), but the NH<sub>2</sub>-terminal half of ZO-1 does not increase the stoichiometry. We have not yet obtained the conditions where the stoichiometry is increased. Moreover, I-afadin and the COOH-terminal half of  $\alpha$ -catenin overexpressed in COS7 cells are not coimmunoprecipitated to a significant extent. Thus, our results concerning the interaction of I-afadin with the COOH-terminal half of  $\alpha$ -catenin are apparently inconsistent depending on the methods used: one positive, one semi-positive, and one negative. However, the negative results obtained from the immunoprecipitation experiments do not necessarily reflect *in vivo* negative protein-protein interactions, because coimmunoprecipitation of two proteins is sometimes affected by an extraction buffer used in experiments, and sometimes is not observed when they form a very complicated multicomplex. Taken together, I-afadin may directly interact with  $\alpha$ -catenin under appropriate conditions, but the molecular linkage between these two proteins may not be so simple and another factor and/or posttranslational modifications of I-afadin and  $\alpha$ -catenin may be necessary for this linkage.

Several other possible mechanisms of the linkage between the nectin/afadin and cadherin/catenin systems are conceivable. One possibility is that I-afadin indirectly interacts with  $\alpha$ -catenin through ZO-1, because it has been shown that ZO-1 directly interacts *in vitro* with the AF-6

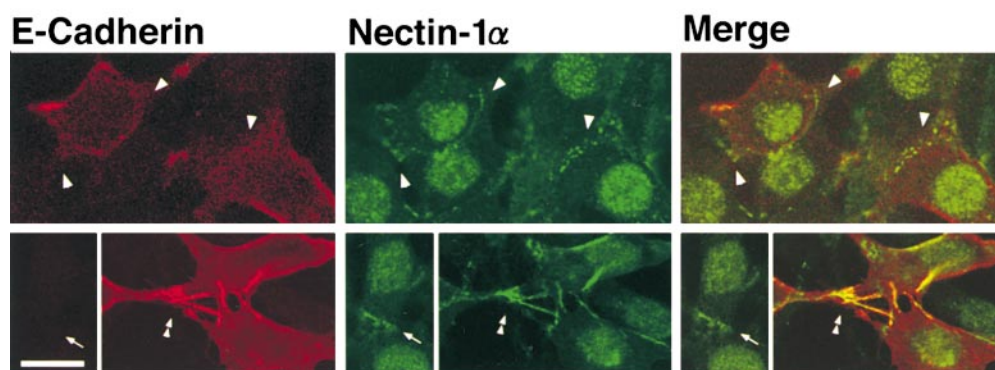
protein/s-afadin (Yamamoto et al., 1997) and with the COOH-terminal half of  $\alpha$ -catenin (Itoh et al., 1997). However, we have previously shown that the stoichiometry of the interaction of I-afadin with ZO-1 is negligible as estimated by affinity chromatography (Sakisaka et al., 1999). We have shown here that ZO-1 does not affect the interaction of I-afadin with the COOH-terminal half of  $\alpha$ -catenin. Therefore, this possibility is unlikely. The second possibility is that I-afadin indirectly interacts with  $\alpha$ -catenin through vinculin, because it has been shown that the COOH-terminal half of  $\alpha$ -catenin directly interacts with vinculin *in vitro* (Weiss et al., 1998) and that vinculin interacts with ponsin (Mandai et al., 1999). However, consistent with previous reports (Watabe-Uchida et al., 1998; Imamura et al., 1999), we have shown by the use of nE $\alpha$ C-L and nE $\alpha$ N-L cells that vinculin is colocalized with the NH<sub>2</sub>-terminal half of  $\alpha$ -catenin (nE $\alpha$ N), but not with the COOH-terminal half (nE $\alpha$ C) or I-afadin. Therefore, this possibility is unlikely either. The third possibility is that F-actin is involved in the linkage between I-afadin and  $\alpha$ -catenin, because I-afadin (Mandai et al., 1997) and the COOH-terminal half of  $\alpha$ -catenin (Rimm et al., 1995) directly interact with F-actin. This possibility cannot be excluded, but the *in vivo* interaction of  $\alpha$ -catenin with F-actin remains unknown.

It may be noted that nectin has a potency to recruit  $\alpha$ - and  $\beta$ -catenins and E-cadherin through I-afadin at nectin-based cell-cell adhesion sites in the absence of E-cadherin or without its trans-interaction. It is likely that  $\alpha$ -catenin is recruited there by the direct or indirect interaction with

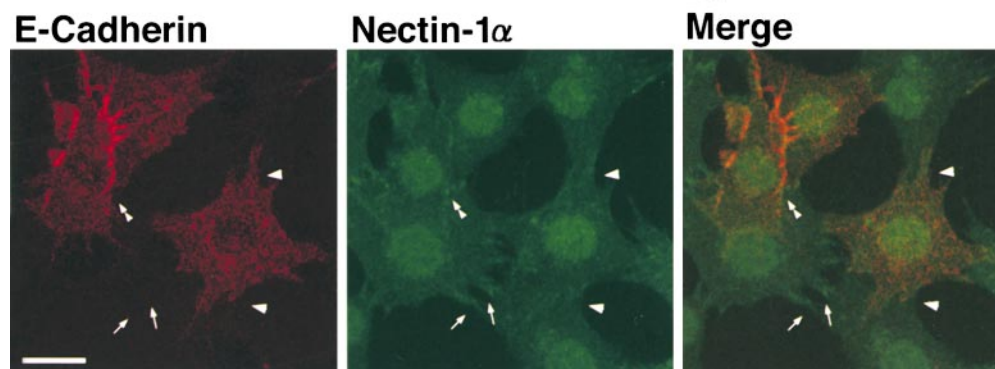
### Nectin-1 $\alpha$ -EL cells + Nectin-1 $\alpha$ -L cells



### Nectin-1 $\alpha$ - $\Delta$ C-EL cells + Nectin-1 $\alpha$ -L cells



### Nectin-1 $\alpha$ -EL cells + Nectin-1 $\alpha$ -L cells + gD



**Figure 10.** 1-Afadin-dependent recruitment of E-cadherin to nectin-based cell-cell adhesion sites without the trans-interaction of E-cadherin. Nectin-1 $\alpha$ -L cells were cocultured with nectin-1 $\alpha$ -EL or -1 $\alpha$ - $\Delta$ C-EL cells in the presence or absence of gD and double stained with the anti-nectin-1 $\alpha$  Ab1 and the anti-E-cadherin Ab. In the absence of gD, E-cadherin was concentrated at  $\sim$ 40% of the adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ -EL cells. The recruitment of E-cadherin was observed in  $\sim$ 20% of the adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ - $\Delta$ C-EL cells. In the presence of gD, the recruitment of E-cadherin was observed in  $\sim$ 8% of the adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ -EL cells. (arrows) Cell-cell adhesion sites between two nectin-1 $\alpha$ -L cells or between two nectin-1 $\alpha$ - $\Delta$ C-L cells; (arrowheads) cell-cell adhesion sites between nectin-1 $\alpha$ -L and -1 $\alpha$ -EL or -1 $\alpha$ - $\Delta$ C-EL cells; and (double arrowheads) cell-cell adhesion sites between two nectin-1 $\alpha$ -EL cells or between two nectin-1 $\alpha$ - $\Delta$ C-EL cells. The results shown are representative of three independent experiments.

1-afadin, and that  $\beta$ -catenin is recruited there by the direct interaction with  $\alpha$ -catenin. E-cadherin may be recruited there by the direct interaction with the  $\alpha$ - and  $\beta$ -catenin complex through 1-afadin. However, it remains unknown whether E-cadherin, which is concentrated at adhesion sites between L and EL cells stably expressing nectin, forms a cis-dimer or not.

In contrast to the recruitment of the cadherin-catenin complex to nectin-based cell-cell adhesion sites without the trans-interaction of E-cadherin, we have shown that nectin-1 $\alpha$ , of which trans-interaction is inhibited by gD, is not recruited to E-cadherin-based cell-cell adhesion sites between two nectin-1 $\alpha$ -EL cells. Thus, nectin trans-inter-

acts independently of the trans-interaction of E-cadherin, and nectin, of which trans-interaction is inhibited or which lacks the ability to interact with 1-afadin, does not appear to be recruited to E-cadherin-based cell-cell adhesion sites. However, it remains unresolved whether the trans-interaction of E-cadherin is dependent on the trans-interaction of nectin, because nectin has three isoforms, and cell adhesion activities of all the isoforms cannot be inhibited simultaneously at this time (Sato-Horikawa et al., 2000).

On the basis of these present and previous observations, we propose here at least two models for the formation of cell-cell AJs. One model is that nectin and E-cadherin in-

dependently form the respective trans-interactions and the nectin/afadin and cadherin/catenin systems recruit each other to form compact cell-cell AJs. The other model is that nectin first forms a trans-interaction that recruits, through l-afadin, the cadherin-catenin system in which E-cadherin does not trans-interact, followed by the trans-interaction of E-cadherin at nectin-based cell-cell adhesion sites, finally leading to the formation of compact cell-cell AJs. It is currently unknown which is the case, but it is likely that the nectin/afadin system plays a key role in the organization of cell-cell AJs in cooperation with the cadherin-catenin system.

We have previously shown by the use of epithelial cells in afadin (-/-) mice and (-/-) embryoid bodies that not only cadherin-based AJs, but also claudin/occludin-based TJs are impaired in these mutant cells (Ikeda et al., 1999). Claudin and occludin are Ca<sup>2+</sup>-independent homophilic cell adhesion molecules at TJs, of which cytoplasmic domains interact with ZO-1, -2, and -3 (Tsukita and Furuse, 1999; Tsukita et al., 1999). ZO-1 and -2 are F-actin-binding proteins that connect the cytoplasmic regions of claudin and occludin to the actin cytoskeleton (Tsukita et al., 1999). Moreover, we have shown that behavior of nectin and l-afadin is different from that of E-cadherin and similar to that of ZO-1 during the formation of TJs in cultured MDCK cells (Sakisaka et al., 1999; Asakura et al., 1999). These results suggest that the nectin/afadin system plays a key role in proper organization of not only cadherin-based cell-cell AJs, but also claudin/occludin-based TJs. It remains unknown how the nectin/afadin system organizes TJs properly, but l-afadin may also indirectly connect nectin to the component of TJs through an unidentified factor. It is of crucial importance to clarify the molecular linkages among these three different cell-cell adhesion systems.

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