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α-Catenin-independent Recruitment of ZO-1 to Nectin-based Cell-Cell Adhesion Sites through Afadin

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ZO-1 is an actin filament (F-actin)–binding protein that localizes to tight junctions and connects claudin to the actin cytoskeleton in epithelial cells. In nonepithelial cells that have no tight junctions, ZO-1 localizes to adherens junctions (AJs) and may connect cadherin to the actin cytoskeleton indirectly through β- and α-catenins as one of many F-actin–binding proteins. Nectin is an immunoglobulin-like adhesion molecule that localizes to AJs and is associated with the actin cytoskeleton through afadin, an F-actin–binding protein. Ponsin is an afadin- and vinculin-binding protein that also localizes to AJs. The nectin-afadin complex has a potency to recruit the E-cadherin–β-catenin complex through α-catenin in a manner independent of ponsin. By the use of cadherin-deficient L cell lines stably expressing various components of the cadherin-catenin and nectin-afadin systems, and α-catenin–deficient F9 cell lines, we examined here whether nectin recruits ZO-1 to nectin-based cell-cell adhesion sites. Nectin showed a potency to recruit not only α-catenin but also ZO-1 to nectin-based cell-cell adhesion sites. This recruitment of ZO-1 was dependent on afadin but independent of α-catenin and ponsin. These results indicate that ZO-1 localizes to cadherin-based AJs through interactions not only with α-catenin but also with the nectin-afadin system.

INTRODUCTION

ZO-1 is an actin filament (F-actin)–binding protein, containing three PDZ domains, one SH3 domain, and one guanylate kinase (GK) domain in this order from the N terminus (Itoh *et al.*, 1993; Willott *et al.*, 1993). In epithelial cells, ZO-1 exclusively localizes to claudin-based tight junctions (TJs) and directly binds to claudin at the first PDZ domain (Itoh *et al.*, 1999) and F-actin at the C-terminal half (Itoh *et al.*, 1997; Fanning *et al.*, 1998) and connects claudin to the actin cytoskeleton (Tsukita *et al.*, 1999). Occludin localizes to TJs, and ZO-1 directly binds to it at the GK domain (Fanning *et al.*, 1998), although the function of occludin at TJs is currently unknown. ZO-1 is excluded from cadherin-based adherens junctions (AJs) that are aligned at the basal side of TJs along the lateral membranes. In nonepithelial cells that have no TJs, however, ZO-1 localizes to cadherin-based AJs and directly binds to α-catenin (Itoh *et al.*, 1991, 1993, 1997), which is associated with cadherin through β-catenin (Nagafuchi and Takeichi, 1989; Ozawa *et al.*, 1989; Takeichi, 1991). α-Catenin is an F-actin–binding protein (Rimm *et al.*, 1995) and furthermore binds two other F-actin–binding proteins, vinculin and α-actinin (Nieset *et al.*, 1997; Weiss *et al.*, 1998). As one of the many F-actin–binding proteins, ZO-1 may indirectly connect cadherin to the actin cytoskeleton (Itoh *et al.*, 1997). Thus, the localization of ZO-1 is different between epithelial and nonepithelial cells, but the mechanism of this different localization is unknown.

In the initial stage during the formation of cell-cell junctions of epithelial cells, primordial spot-like cell-cell junctions are first formed at the tips of the cellular protrusions radiating from adjacent cells (Yonemura *et al.*, 1995; Ando-Akatsuka *et al.*, 1996; Adams *et al.*, 1998; Vasioukhin *et al.*, 2000). Cadherin and ZO-1 colocalize to the primordial junctions where occludin is not concentrated (Ando-Akatsuka *et al.*, 1996). As cellular polarization proceeds, occludin gradually accumulates at the spot-like junctions to form TJs, and

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Abbreviations used: aa, amino acid(s); AJs, adherens junctions; F-actin, actin filament(s); GFP, green fluorescent protein; GK, guanylate kinase; GST, glutathione *S*-transferase; mAb, monoclonal antibody; MBP, maltose-binding protein; pAb, polyclonal antibody; TJs, tight junctions.

cadherin is sorted from occludin and ZO-1 to form AJs. To elucidate the mechanism of the formation of TJs and AJs, it is of importance to understand the mechanism of the different localization of ZO-1 between epithelial and nonepithelial cells.

We have recently found a novel cell-cell adhesion system at AJs that may regulate the formation of cadherin-based AJs and claudin-based TJs (Mandai *et al.*, 1997; Asakura *et al.*, 1999; Ikeda *et al.*, 1999; Mandai *et al.*, 1999; Sakisaka *et al.*, 1999; Takahashi *et al.* 1999; Miyahara *et al.*, 2000; Satoh-Horikawa *et al.*, 2000; Tachibana *et al.*, 2000). This system consists of at least three components, nectin, afadin, and ponsin. Nectin is a Ca^{2+}-independent cell-cell adhesion molecule that belongs to the immunoglobulin superfamily (Takahashi *et al.*, 1999). This adhesion molecule is identical to the poliovirus receptor-related protein and has recently been shown to serve as the α-herpesvirus 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 of which has two or three splicing variants (Morrison and Racaniello, 1992; Aoki *et al.*, 1994; Eberlé *et al.*, 1995; Lopez *et al.*, 1995; Cocchi *et al.*, 1998; Satoh-Horikawa *et al.*, 2000). Most members have a C-terminal conserved motif of four amino acid (aa) residues (E/A-X-Y-V) that interacts with the PDZ domain of afadin (Takahashi *et al.*, 1999; Satoh-Horikawa *et al.*, 2000). Afadin has at least two splicing variants, l- and s-afadins. l-Afadin, a larger splicing variant, is an F-actin–binding protein with one PDZ domain and three proline-rich domains and connects nectin to the actin cytoskeleton (Mandai *et al.*, 1997; Takahashi *et al.*, 1999). s-Afadin, a smaller splicing variant, has one PDZ domain but lacks the F-actin–binding domain and the third proline-rich domain (Mandai *et al.*, 1997). Human s-afadin is identical to the AF6 protein, the gene of which is originally found to be fused to the ALL-1 gene in acute leukemia (Prasad *et al.*, 1993). In this study, we use "afadin" as l-afadin. Ponsin is an afadin-binding protein that colocalizes with nectin and afadin to cadherin-based AJs (Mandai *et al.*, 1999; Tachibana *et al.*, 2000). Furthermore, ponsin binds to vinculin, which is known to interact with both F-actin and α-catenin (Burridge and Feramisco, 1982; Menkel *et al.*, 1994; Weiss *et al.*, 1998) and colocalizes with vinculin to not only cadherin-based AJs but also focal contacts (Mandai *et al.*, 1999). However, ponsin forms a binary complex with either afadin or vinculin and does not form a ternary complex (Mandai *et al.*, 1999).

The nectin-afadin complex has a potency to recruit the E-cadherin-β-catenin complex through α-catenin in a ponsin- and vinculin-independent manner and is involved in the formation of AJs in cooperation with the cadherin-catenin complex (Tachibana *et al.*, 2000). In epithelial cells of afadin (−/−) mice and (−/−) embryoid bodies, the proper organization of AJs and TJs is impaired (Ikeda *et al.*, 1999). In spermatozoa of nectin-2 (−/−) mice, the nuclear and cytoskeletal morphology and mitochondrial localization are impaired (Bouchard *et al.*, 2000). Nectin-1 has recently been determined by positional cloning to be responsible for cleft lip/palate-ectodermal dysplasia (Suzuki *et al.*, 2000). Thus, evidence is accumulating that the nectin-afadin system plays an important role in the organization of AJs and TJs, although the function of ponsin is currently unknown.

Nectin-2, afadin, and ponsin colocalize with E-cadherin and ZO-1 to the primordial junctions during the formation of cell-cell junctions of MTD-1A epithelial cells (Yonemura *et al.*, 1995; Ando-Akatsuka *et al.*, 1996; Asakura *et al.*, 1999). In MDCK epithelial cells, reduction of medium Ca^{2+} concentrations to a micromolar range causes disruption of AJs and TJs (Kartenbeck *et al.*, 1991). Nectin-1α and afadin as well as ZO-1 are observed on the plasma membrane, whereas E-cadherin is not (Asakura *et al.*, 1999; Sakisaka *et al.*, 1999). Addition of a protein kinase C-activating phorbol ester induces the formation of a TJ-like structure (Balda *et al.*, 1993), where nectin-1α and afadin as well as ZO-1, but not E-cadherin, are recruited (Balda *et al.*, 1993; Asakura *et al.*, 1999; Sakisaka *et al.*, 1999). These observations have increased the possibility that the nectin-afadin system is involved in the localization of ZO-1.

On the basis of this assumption, we have examined here whether nectin has a potency to recruit ZO-1 to nectin-based cell-cell adhesion sites and have found that nectin has the potency to recruit not only α-catenin but also ZO-1 there. We discuss a possible role of the nectin-afadin system during the formation of AJs and TJs.

MATERIALS AND METHODS

Construction and Purification

Mammalian expression vectors were constructed with pPGKIH (Miyahara *et al.*, 2000), pPGKIH-FLAG (Tachibana *et al.*, 2000), pPGKIZ-Myc, and pEGFP-N1 (CLONTECH Laboratories, Palo Alto, CA) by the use of standard molecular biology methods (Sambrook *et al.*, 1989). pPGKIH-FLAG was designed to express an N-terminal FLAG-tagged protein in which the preprotrypsin signal peptide precedes the FLAG tag. pPGKIZ-Myc was constructed to express an N-terminal Myc-tagged protein by replacing the hemagglutinin tag of pPGKIZ-HA (Tachibana *et al.*, 2000) with the Myc tag. pEGFP-N1 was designed to express a C-terminal green fluorescent protein (GFP)-fusion protein. Mammalian expression vectors of mouse nectin-2α, α-catenin, and ponsin-2 contained the following aa: pPGKIH-nectin-2α, aa 1–467 (full length); pPGKIH-nectin-2α-ΔC, aa 1–463 (deletion of the C-terminal four aa residues); pPGKIH-FLAG-nectin-2α, aa 28–467; pPGKIZ-Myc-α-catenin, aa 1–906 (full length), and pEGFP-ponsin, 1–724 (full length).

Baculovirus transfer vectors were constructed with pFastBacHT (GIBCO-BRL, Grand Island, NY) and pFastBac1-Myc-His6 (Tachibana *et al.*, 2000). pFastBacHT was designed to express an N-terminal His6-tagged protein. pFastBac1-Myc-His6 was designed to express a C-terminal Myc- and His6-tagged protein. Baculovirus transfer vectors of ZO-1 and afadin contained the following aa: pFastBacHT-ZO-1, aa 1–1745 (full length); and pFastBac1-Myc-His6-afadin, aa 1–1829 (full length). The His6-tagged protein of ZO-1 (His6-ZO-1) and the Myc- and His6-tagged protein of afadin (Myc-His6-afadin) were expressed in High Five insect cells (Invitrogen, San Diego, CA) and purified by the use of TALON metal affinity beads (CLONTECH Laboratories) as described (Tachibana *et al.*, 2000).

Prokaryote expression vectors were constructed with pGEX-KG (Guan and Dixon, 1991) and pMal-C2 (New England Biolabs, Beverly, MA). The glutathione *S*-transferase (GST)-fusion or maltose-binding protein (MBP)-fusion vector of nectin-2α, afadin, and ZO-1 contained the following aa: GST-nectin-2α-CP, aa 387–467 (cytoplasmic region); MBP-afadin-PDZ, aa 1007–1125 (PDZ domain); MBP-ZO-1-PDZ1–2, aa 1–292 (first and second PDZ domains); and MBP-ZO-1-PDZ2–3, aa 181–503 (second and third PDZ domains). The GST- and MBP-fusion proteins were purified by the use of glutathione-Sepharose beads (Amersham-Pharmacia Biotech, Pisca-

taway, NJ) and amylose resin beads (New England Biolabs), respectively.

Cell Culture and DNA Transfection

L, EL, and F9 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. EL cells were cloned by introduction of the E-cadherin cDNA into L cells (Nagafuchi *et al.*, 1987). EL cell lines stably expressing a FLAG-tagged protein of full-length nectin-2α (nectin-2α-EL cells) were prepared by the use of pPGKIH-FLAG-nectin-2α as described previously (Takahashi *et al.*, 1999; Tachibana *et al.*, 2000). L cell lines stably expressing full-length nectin-2α (nectin-2α-L cells) or C-terminal four-aa–deleted nectin-2α (nectin-2α-ΔC-L cells) were prepared as described before (Takahashi *et al.*, 1999; Miyahara *et al.*, 2000; Tachibana *et al.*, 2000). The following L cell lines stably expressed the fusion molecules: nEα-L cells, the fusion molecule consisting of E-cadherin lacking its β-catenin–binding domain (aa 1–657) and full-length α-catenin (aa 1–906) (Nagafuchi *et al.*, 1994; Imamura *et al.*, 1999); nEαN-L cells, the fusion molecule consisting of E-cadherin lacking its β-catenin–binding domain and the N-terminal half of α-catenin (aa 1–508) (Nagafuchi *et al.*, 1994; Imamura *et al.*, 1999); and nEαC-L cells, the fusion molecule consisting of E-cadherin lacking its β-catenin–binding domain and the C-terminal half of α-catenin (aa 509–906) (Nagafuchi *et al.*, 1994; Imamura *et al.*, 1999). nEα-L, nEαN-L, and nEαC-L cells were obtained as described previously (Nagafuchi *et al.*, 1994; Imamura *et al.*, 1999). Nectin-2α-L and -2α-ΔC-L cells, both of which transiently expressed a Myc-tagged protein of full-length α-catenin (Myc-α-catenin), were prepared by the use of pPGKIZ-Myc-α-catenin as described by Tachibana *et al.* (2000). Nectin-2α-L cells transiently expressing a GFP-fusion protein of full-length ponsin (GFP-ponsin) were prepared by the use of pEGFP-ponsin as described by Tachibana *et al.* (2000). Where indicated, nectin-2α-L cells were cultured with 50 nM latrunculin A for 45 min or with 2 μM cytochalasin D for 15 min. F9 cells were cultured in gelatin-coated (0.1%) culture dishes. α-Catenin–deficient F9 cells [F9Dα(−/−) cells] and F9Dα(−/−) cells re-expressing α-catenin [αF9Dα(−/−) cells] were obtained as described by Maeno *et al.* (1999).

Antibodies

A rabbit anti-nectin-2α polyclonal antibody (pAb) was prepared as described by Takahashi *et al.* (1999). A rat anti-nectin-2 monoclonal antibody (mAb), which recognizes both nectin-2α and -2δ, was prepared as described by Takahashi *et al.* (1999). A mouse anti–ZO-1 mAb (Itoh *et al.*, 1991) was kindly supplied by Drs. S. Tsukita and M. Itoh (Kyoto University, Kyoto, Japan). A rabbit anti–ZO-1 pAb was purchased from Zymed (San Francisco, CA). A mouse anti-afadin mAb was prepared as described by Sakisaka *et al.* (1999). A rat anti–E-cadherin mAb (ECCD-2) was kindly supplied by Dr. M. Takeichi (Kyoto University, Kyoto, Japan). A mouse anti-Myc mAb was from American Type Culture Collection (Manassas, VA). Mouse anti-vinculin and anti-GFP mAbs were purchased from Sigma Chemicals (St. Louis, MO) and CLONTECH, respectively. The specificity of each antibody was examined by Western blotting of various cell lines described above (Figure 1).

Subcellular Fractionation and Immunoprecipitation

Confluent cells cultured on a 10-cm dish were washed with phosphate-buffered saline and scraped. The cells were again washed with phosphate-buffered saline and sonicated in a buffer (10 mM HEPES/NaOH at pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, and 25 mM $NaHCO_3$) on ice for 15 s five times at 3-min intervals. The homogenate was subjected to centrifugation at 100,000 × *g* for 30 min. A comparable amount of each fraction (each 20 μg of protein) was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting.

Immunoprecipitation was performed as described previously (Takahashi *et al.*, 1999; Miyahara *et al.*, 2000). Briefly, nectin-2α-L or -2α-ΔC-L cells were sonicated in buffer A (10 mM HEPES/NaOH at pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, and 25 mM $NaHCO_3$, 1% Triton X-100, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A), followed by centrifugation at 100,000 × *g* for 30 min. The supernatant (2 mg of protein) was incubated with the anti–nectin-2 mAb at 4°C for 2 h. Anti-rat immunoglobulin beads (American Qualex International, San Clemente, CA; 20 μl of wet volume) were added to this sample, and incubation was further performed at 4°C overnight. After the beads were extensively washed with buffer A, the bound proteins were eluted by boiling the beads in an SDS sample buffer (60 mM Tris/Cl at pH 6.7, 3% SDS, 2% [vol/vol] 2-mercaptoethanol, and 5% glycerol), and subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting.

Affinity Chromatographies

To examine the interaction of full-length ZO-1 with full-length afadin, Myc-His6-afadin (20 μg of protein) was immobilized on anti-Myc mAb-coupled beads (20 μl of wet volume) prepared as described by Takahashi *et al.* (1999). His6-ZO-1 (100 μg of protein) was applied to the Myc-His6-afadin–immobilized beads equilibrated with buffer B (20 mM Tris/Cl at pH 7.5, 150 mM NaCl, and 0.1% Triton X-100). After the beads were extensively washed with buffer B, the bound proteins were eluted by boiling the beads in the SDS sample buffer. The sample was then subjected to SDS-PAGE (8% polyacrylamide gel), followed by staining with Coomassie brilliant blue.

To examine the interaction of ZO-1 or afadin with nectin-2α, MBP-ZO-1-PDZ1–2, MBP-ZO-1-PDZ2–3, or MBP-afadin-PDZ (each 20 μg of protein) was immobilized on amylose resin beads (20 μl of wet volume). His6-ZO-1 (20 μg of protein) was also immobilized on TALON metal affinity beads (20 μl of wet volume). GST-nectin-2α-CP (100 μg of protein) was applied to the MBP-fusion protein-immobilized beads equilibrated with buffer B. After the beads were extensively washed with buffer B, elution was performed with buffer B containing 10 mM maltose. GST-nectin-2α-CP (100 μg of protein) was also applied to the His6-ZO-1–immobilized beads equilibrated with buffer B. After the beads were extensively washed with buffer B, elution was performed with buffer B containing 100 mM imidazole/Cl at pH 7.5. Each eluate was subjected to SDS-PAGE (10 or 13% polyacrylamide gel), followed by protein staining with Coomassie brilliant blue.

Other Procedures

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

RESULTS

Colocalization of ZO-1 with Nectin-2 and Afadin at E-Cadherin–based AJs

It has been shown that ZO-1 localizes to E-cadherin–based AJs in EL cells (Itoh *et al.*, 1993). L cells are cadherin-deficient mouse fibroblasts, and EL cells are an L cell line stably expressing E-cadherin (Nagafuchi *et al.*, 1987). We have previously shown that endogenous nectin-2 and afadin localize to E-cadherin–based cell-cell AJs in EL cells (Mandai *et al.*, 1997; Takahashi *et al.*, 1999). We first examined the localization of ZO-1 in

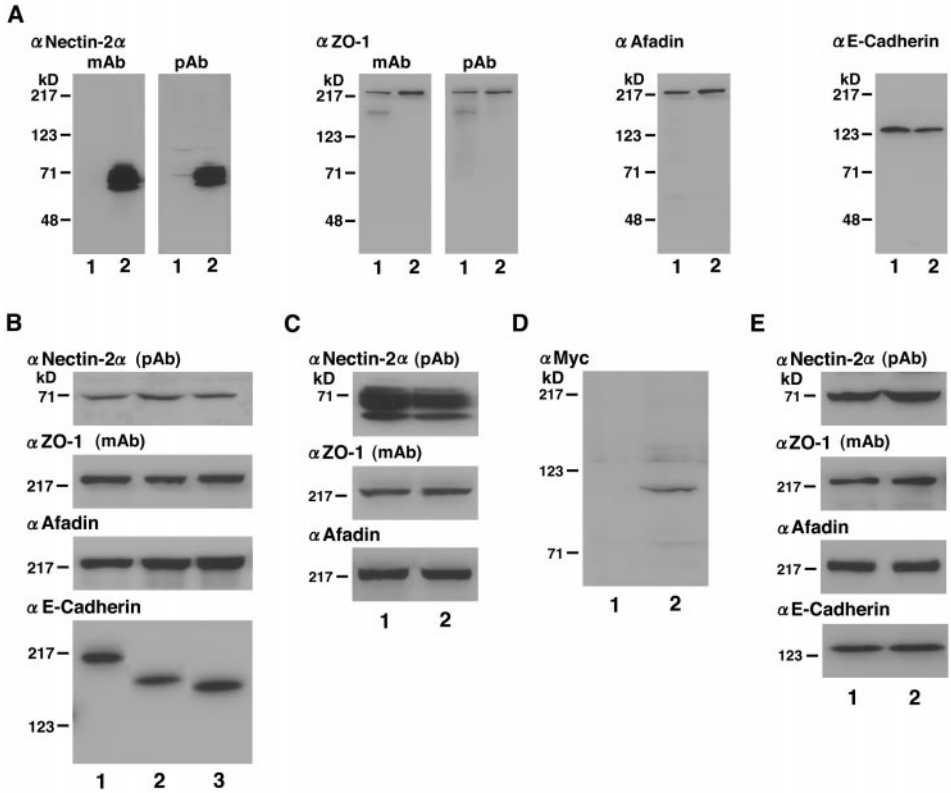

Figure 1. Specificity of antibodies and expression levels of various constructs. The cell lysates of various L cell lines and F9 cell lines (each 20 μg of protein) were subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti–nectin-2 mAb, the anti–nectin-2α pAb, the anti–ZO-1 mAb, the anti–ZO-1 pAb, the anti-afadin mAb, the anti–E-cadherin mAb, or the anti-Myc mAb. (A) EL and nectin-2α-EL cells. Lane 1, EL cells; lane 2, nectin-2α-EL cells. (B) nEα-L, nEαN-L, and nEαC-L cells. Lane 1, nEα-L cells; lane 2, nEαN-L cells; lane 3, nEαC-L cells. (C) Nectin-2α-L and -2α-ΔC-L cells. Lane 1, nectin-2α-L cells; lane 2, nectin-2α-ΔC-L cells. (D) Nectin-2α-L cells transfected with pPGKIZ-Myc-α-catenin or the empty vector. Lane 1, empty vector; lane 2, pPGKIZ-Myc-α-catenin. (E) αF9Dα(−/−) and F9Dα (−/−) cells. Lane 1, αF9Dα(−/−) cells; lane 2, F9Dα(−/−) cells. The results shown are representative of three independent experiments.

an EL cell line stably expressing a FLAG-tagged protein of full-length nectin-2α (nectin-2α-EL cells). The expression level of the nectin-2α protein in nectin-2α-EL cells was much higher than that in EL cells, but the expression level of the ZO-1, afadin, or E-cadherin protein in nectin-2α-EL cells was similar between these two types of EL cell lines (Figure 1A). ZO-1 colocalized with nectin-2α and afadin to E-cadherin–based cell-cell AJs in nectin-2α-EL cells (Figure 2).

It has been shown by the use of three types of L cell lines, nEα-L, nEαC-L, and nEαN-L cells, that the localization of ZO-1 to E-cadherin–based AJs is mediated through the C-terminal half of α-catenin (Nagafuchi *et al.*, 1994; Imamura *et al.*, 1999). nEα-L cells express a chimeric protein (nEα) of β-catenin–binding domain-deleted E-cadherin fused with full-length α-catenin; nEαN-L cells express a chimeric protein (nEαN) of β-catenin–binding domain-deleted E-cadherin fused with the N-terminal half of α-catenin; and nEαC-L cells express a chimeric protein (nEαC) of β-catenin–binding domain-deleted E-cadherin fused with the C-terminal half of α-catenin. The expression level of the nectin-2α, ZO-1, afadin, or chimeric protein was similar among these three types of L cell lines (Figure 1B). We have previously shown that nectin-2 and afadin localize to nEα- and nEαC-based cell-cell adhesion sites but not to nEαN-based adhesion sites (Tachibana *et al.*, 2000). Ponsin and vinculin localize to nEα-based and nEαN-based cell-cell adhesion sites but not to nEαC-based adhesion sites. These results indicate that the localization of nectin-2 and afadin at E-cadherin–based AJs is mediated through the C-terminal half of α-catenin (Tachibana *et al.*, 2000). They furthermore indicate that the localization of nectin-2 and afadin at E-cadherin–based AJs is not mediated through ponsin or vinculin. Consistent with these earlier observations, ZO-1 colocalized with nectin-2 to nEα-based and nEαC-based cell-cell adhesion sites, whereas neither ZO-1 nor nectin-2 was concentrated at nEαN-based adhesion sites (Figure 3). Taken together, these results indicate that ZO-1 colocalizes with nectin-2 and afadin to E-cadherin–based AJs and that the colocalization of these proteins there is mediated through the C-terminal half of α-catenin. The localization of ZO-1 to E-cadherin–based AJs is, furthermore, independent of vinculin and ponsin.

Afadin-dependent Recruitment of ZO-1 to Nectin-2α–based Cell-Cell Adhesion Sites

We have previously shown that L cells expressing full-length nectin-2α (nectin-2α-L cells) form nectin-2α–based cell-cell adhesion sites, where afadin is recruited through its direct interaction with full-length nectin-2α, whereas L cells expressing C-terminal four-aa–deleted nectin-2α (nectin-2α-ΔC; nectin-2α-ΔC-L cells) also form nectin-2α-ΔC–based cell-cell adhesion sites, but afadin does not interact with nectin-2α-ΔC or is not recruited to the adhesion sites (Miyahara *et al.*, 2000; Tachibana *et al.*, 2000). The expression level of the nectin-2α protein in nectin-2α-L cells was similar to that of the nectin-2α-ΔC protein in nectin-2α-ΔC-L cells (Figure 1C). The expression level of the ZO-1 or afadin protein was also similar between these L cell lines. In these cell lines, cadherin was not expressed (Yokoyama, Tachibana, Nakanishi, Yamamoto, Irie, Mandai, Nagafuchi, Monden, and Takai, unpublished results). Endogenous α-catenin was expressed

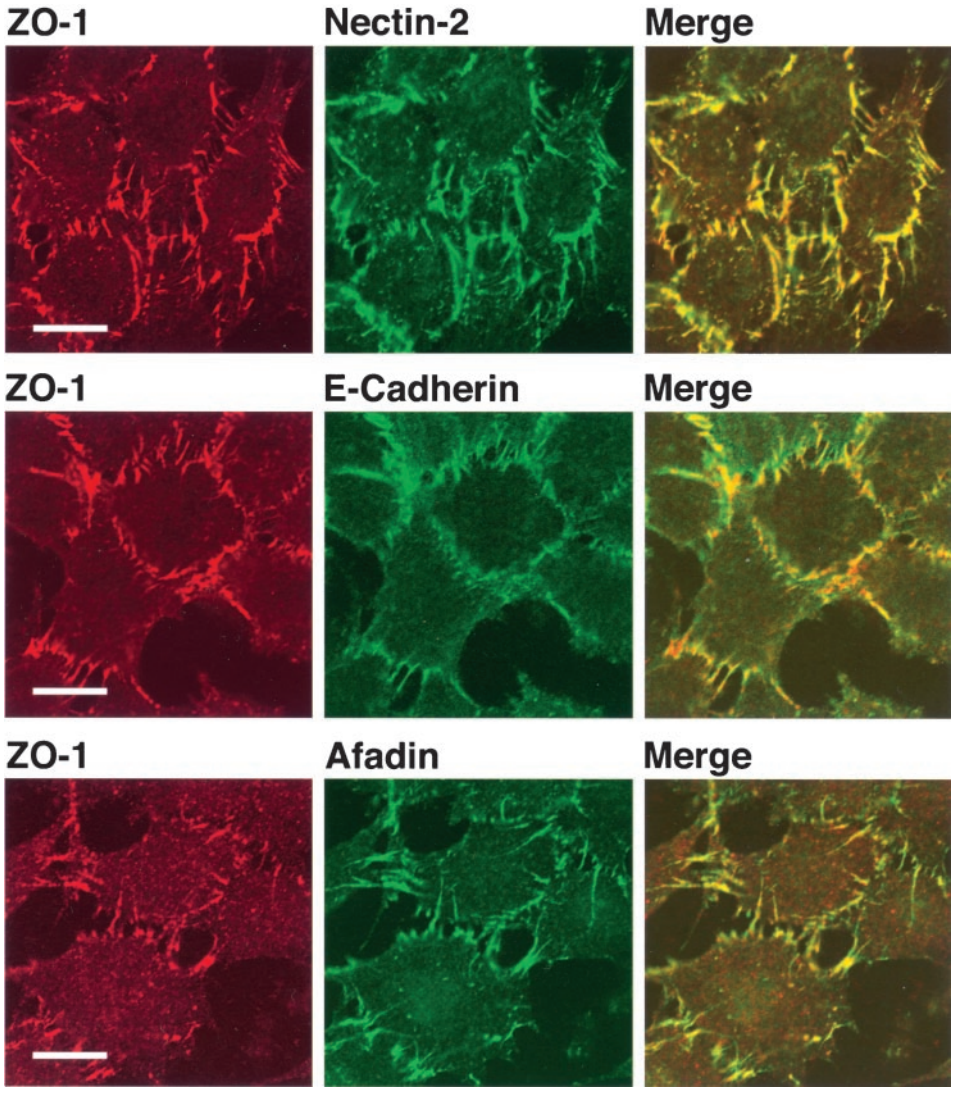

Figure 2. Colocalization of ZO-1 with nectin-2α and afadin at E-cadherin–based AJs. Nectin-2α-EL cells were doubly stained with various combinations of the anti–ZO-1 pAb, the anti–nectin-2 mAb, the anti–E-cadherin mAb, and the anti-afadin mAb. Bars, 10 μm. The results shown are representative of three independent experiments.

but the amount was remarkably less than that in EL cells and negligible (Yokoyama, Tachibana, Nakanishi, Yamamoto, Irie, Mandai, Nagafuchi, Monden, and Takai, unpublished results). We have shown that transiently expressed α-catenin is recruited to nectin-2α–based, but not nectin-2α-ΔC–based, cell-cell adhesion sites (Tachibana *et al.*, 2000). We examined whether ZO-1 is recruited to nectin-2α–based cell-cell adhesion sites even in the absence of the cadherin-catenin system. In nectin-2α-L cells, ZO-1 and afadin colocalized to nectin-2α–based cell-cell adhesion sites (Figure 4A), whereas in nectin-2α-ΔC-L cells, neither ZO-1 nor afadin localized to nectin-2α-ΔC–based cell-cell adhesion sites (Figure 4B). These results indicate that ZO-1 is recruited to nectin-2α–based cell-cell adhesion sites in an afadin-dependent manner even in the absence of the cadherin-catenin system.

Afadin-dependent Association of ZO-1 with Nectin-2α

We next examined whether ZO-1 is associated with nectin-2α through afadin. For this purpose, we performed subcellular fractionation and immunoprecipitation analyses of nectin-2α-L and -2α-ΔC-L cells. When these cells were sonicated, followed by ultracentrifugation, similar amounts of nectin-2α and -2α-ΔC were recovered only in the membrane fraction (Figure 5A). However, ZO-1 and afadin were recovered in both the membrane and cytosol fractions, but the amounts of ZO-1 and afadin recovered in the membrane fraction in nectin-2α-L cells were more than those in nectin-2α-ΔC-L cells. When the cell extracts of nectin-2α-L and -2α-ΔC-L cells were subjected to immunoprecipitation by the use of the nectin-2 mAb, similar amounts of nectin-2α and -2α-ΔC were precipitated (Figure 5B). ZO-1 and afadin were coimmunoprecipitated with nectin-2α, whereas they were not coimmunoprecipitated with nectin-2α-ΔC. The reason why some amounts of ZO-1 and afadin were recovered in the membrane fraction in nectin-2α-ΔC-L cells is not known.

We have previously shown by affinity chromatography with the use of the N-terminal and C-terminal fragments of ZO-1 and the N-terminal, middle, and C-terminal fragments

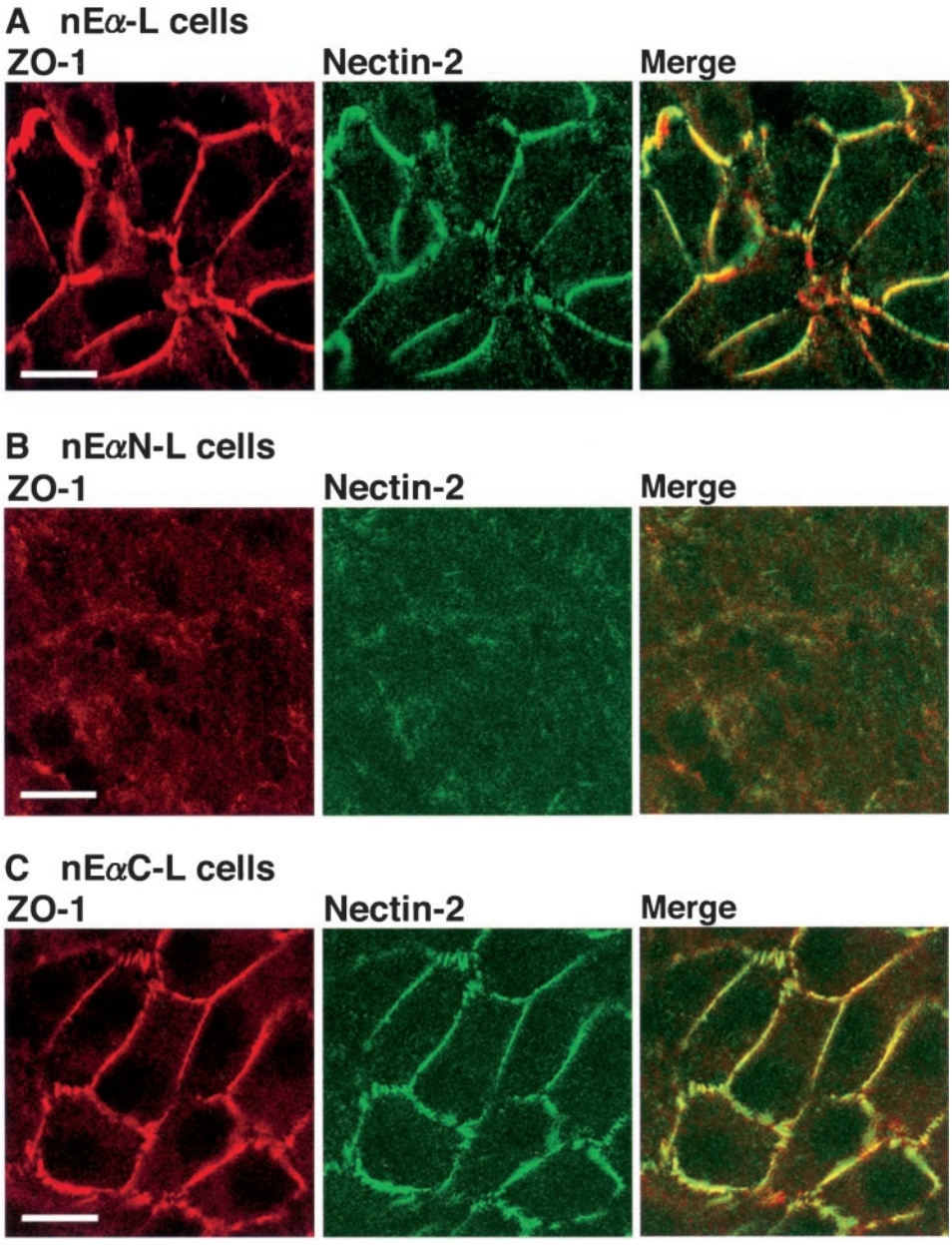

Figure 3. Localization of ZO-1 and nectin-2 at E-cadherin–based AJs through the C-terminal half of α-catenin. nEα-L, nEαN-L, and nEαC-L cells were doubly stained with the anti–ZO-1 mAb and the anti–nectin-2 mAb. (A) nEα-L cells. (B) nEαN-L cells. (C) nEαC-L cells. Bars, 10 μm. The results shown are representative of three independent experiments.

of afadin that ZO-1 does not directly interact with afadin (Sakisaka *et al.*, 1999). We confirmed here this conclusion by affinity chromatography with the use of full-length ZO-1 and full-length afadin. A His6-tagged protein of full-length ZO-1 (His6-ZO-1) did not bind to a Myc- and His6-tagged protein of full-length afadin (Myc-His6-afadin) immobilized on affinity beads (Yokoyama, Tachibana, Nakanishi, Yamamoto, Irie, Mandai, Nagafuchi, Monden, and Takai, unpublished results). We next examined whether ZO-1 directly interacts with nectin-2α. A GST-fusion protein of the cytoplasmic region of nectin-2α (GST-nectin-2α-CP) bound to an MBP-fusion protein of the first and second PDZ domains of ZO-1 (MBP-ZO-1-PDZ1–2) immobilized on amylose resin beads. However, the stoichiometry of the interaction of GST-nectin-2α-CP with MBP-ZO-1-PDZ1–2 was ~0.1:1, whereas that of GST-nectin-2α-CP with a MBP-fusion protein of the PDZ domain of afadin (MBP-afadin-PDZ) was ~1:1 (Figure 6). GST-nectin-2α-CP did not bind to a MBP-fusion protein of the second and third PDZ domains of ZO-1 (MBP-ZO-1-PDZ2–3). A similar result was obtained with full-length ZO-1 (His6-ZO-1; Yokoyama, Tachibana, Nakanishi, Yamamoto, Irie, Mandai, Nagafuchi, Monden, and Takai, unpublished results). These results indicate that ZO-1 does not directly interact with nectin-2α or afadin, although ZO-1 is recruited to nectin-2α–based cell-cell adhesion sites in an afadin-dependent manner.

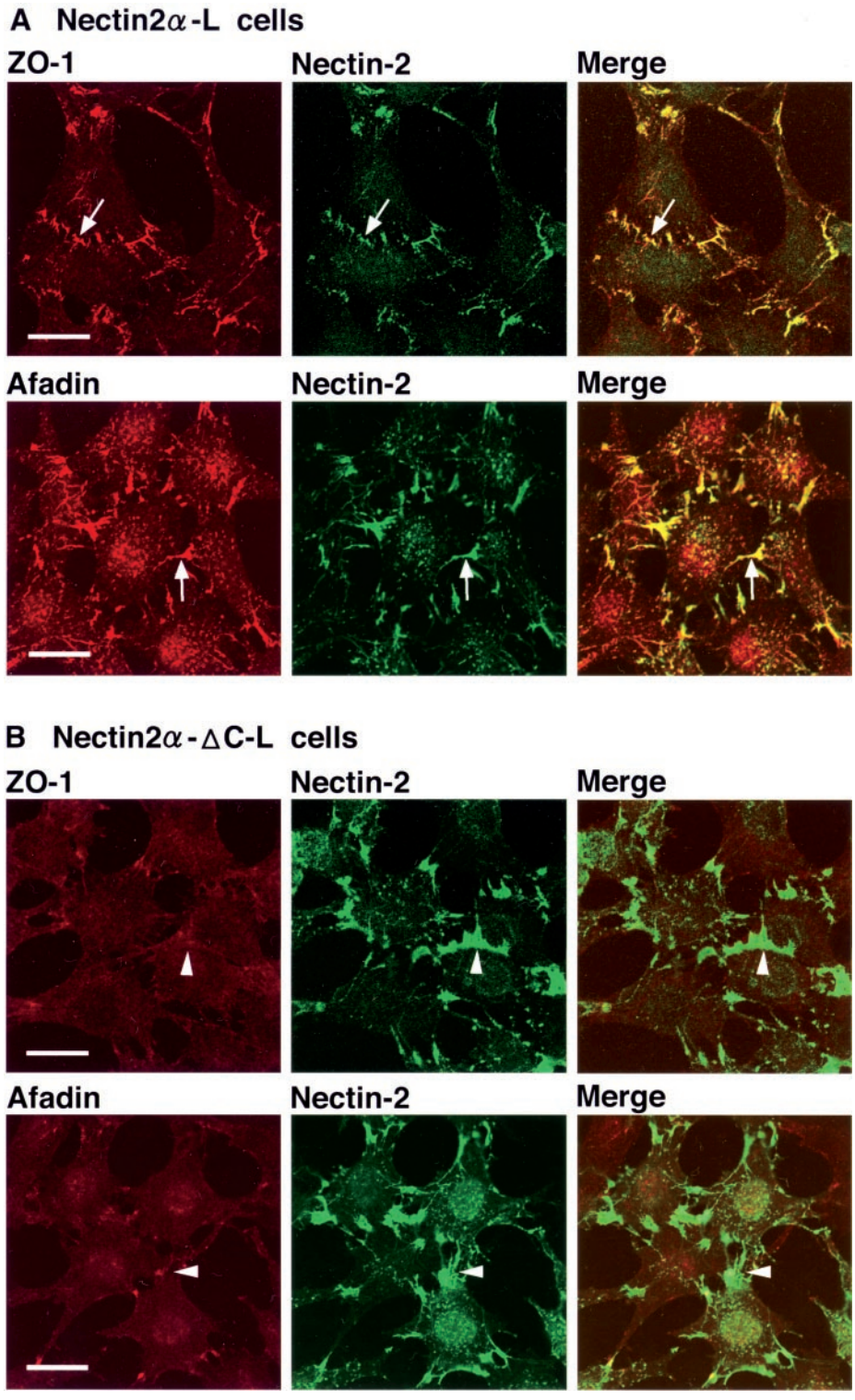

Figure 4. Afadin-dependent recruitment of ZO-1 to nectin-2α–based cell-cell adhesion sites. Nectin-2α-L and -2α-ΔC-L cells were doubly stained with various combinations of the anti–ZO-1 mAb, the anti–nectin-2 mAb, and the anti-afadin mAb. (A) Nectin-2α-L cells. (B) Nectin-2α-ΔC-L cells. Arrows, nectin-2α–based cell-cell adhesion sites; arrowheads, nectin-2α-ΔC–based cell-cell adhesion sites. Bars, 10 μm. The results shown are representative of three independent experiments.

Ponsin- and Vinculin-independent Recruitment of ZO-1 to Nectin-2α–based Cell-Cell Adhesion Sites

Ponsin and vinculin colocalize to nEαN-based cell-cell adhesion sites but not to nEαC-based adhesion sites (Tachibana *et al.*, 2000). Conversely, ZO-1 colocalized with nectin-2 and afadin to nEαC-based cell-cell adhesion sites but not to nEαN-based adhesion sites (Figure 3, B and C). As described above, these results indicate that ZO-1 is recruited to cell-cell

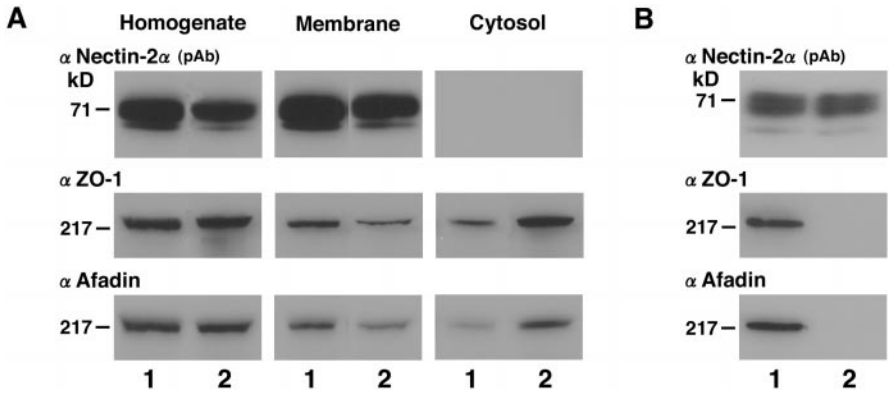

Figure 5. Afadin-dependent association of ZO-1 with nectin-2α. (A) Subcellular fractionation analysis. Nectin-2α-L and -2α-ΔC-L cells were sonicated, followed by ultracentrifugation. A comparable amount of each fraction (each 20 μg of protein) was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti–nectin-2α pAb, the anti–ZO-1 mAb, and the anti-afadin mAb. (B) Immunoprecipitation analysis. The cell extracts of nectin-2α-L and -2α-ΔC-L cells (each 2 mg of protein) were separately subjected to immunoprecipitation with the anti–nectin-2 mAb. The immunoprecipitate was then subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti–nectin-2α pAb, the anti–ZO-1 mAb, and the anti-afadin mAb. Lane 1, nectin-2α-L cells; lane 2, nectin-2α-ΔC-L cells. The results shown are representative of three independent experiments.

adhesion sites, where nectin-2 and afadin colocalize, in a manner independent of ponsin and vinculin. To further confirm these results, we prepared nectin-2α-L cells where a GFP-fusion protein of full-length ponsin (GFP-ponsin) was transiently expressed. In these cells, neither GFP-ponsin nor vinculin localized to nectin-2α–based cell-cell adhesion sites, although both proteins colocalized to focal contacts (Yokoyama, Tachibana, Nakanishi, Yamamoto, Irie, Mandai, Nagafuchi, Monden, and Takai, unpublished results). These results provide additional evidence that neither ponsin nor vinculin is necessary for the recruitment of ZO-1 to nectin-2α–based cell-cell adhesion sites.

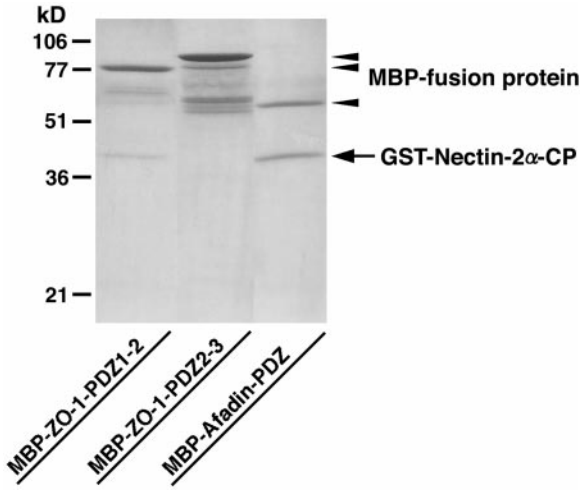

Figure 6. Inability of ZO-1 to directly interact with nectin-2α. MBP-ZO-1-PDZ1–2, MBP-ZO-1-PDZ2–3, or MBP-afadin-PDZ (each 20 μg of protein) was immobilized on amylose resin beads. GST-nectin-2α-CP (100 μg of protein) was applied to the affinity beads. After the beads were extensively washed, elution was performed with 10 mM maltose. The eluate was subjected to SDS-PAGE (13% polyacrylamide gel), followed by protein staining with Coomassie brilliant blue. The results shown are representative of three independent experiments.

Afadin-dependent Corecruitment of ZO-1 and α-Catenin to Nectin-2α–based Cell-Cell Adhesion Sites

We have previously shown that α-catenin is recruited to nectin-2α–based cell-cell adhesion sites (Tachibana *et al.*, 2000). We next examined whether ZO-1 and α-catenin are corecruited. For this purpose, a Myc-tagged protein of full-length α-catenin (Myc-α-catenin) was transiently expressed in nectin-2α-L cells (Figure 1D). In these cells, both ZO-1 and Myc-α-catenin localized to nectin-2α–based cell-cell adhesion sites (Figure 7A). When Myc-α-catenin was transiently expressed in nectin-2α-ΔC-L cells, neither ZO-1 nor Myc-α-catenin localized to nectin-2α-ΔC–based cell-cell adhesion sites (Figure 7B). The expression level of the Myc-α-catenin protein was similar between these two types of L cell lines (Yokoyama, Tachibana, Nakanishi, Yamamoto, Irie, Mandai, Nagafuchi, Monden, and Takai, unpublished results). These results indicate that ZO-1 and α-catenin are corecruited to nectin-2α–based cell-cell adhesion sites in an afadin-dependent manner.

α-Catenin–independent Colocalization of ZO-1 with Nectin-2 and Afadin

We have previously shown that nectin-2 and E-cadherin colocalize to cell-cell AJs in an α-catenin-dependent manner and that nectin-2 and E-cadherin exhibit different localization in an α-catenin–deficient F9 cell line, F9Dα(−/−) cells (Tachibana *et al.*, 2000). The F9 cell line is derived from mouse teratocarcinoma-derived embryonal carcinoma cells.

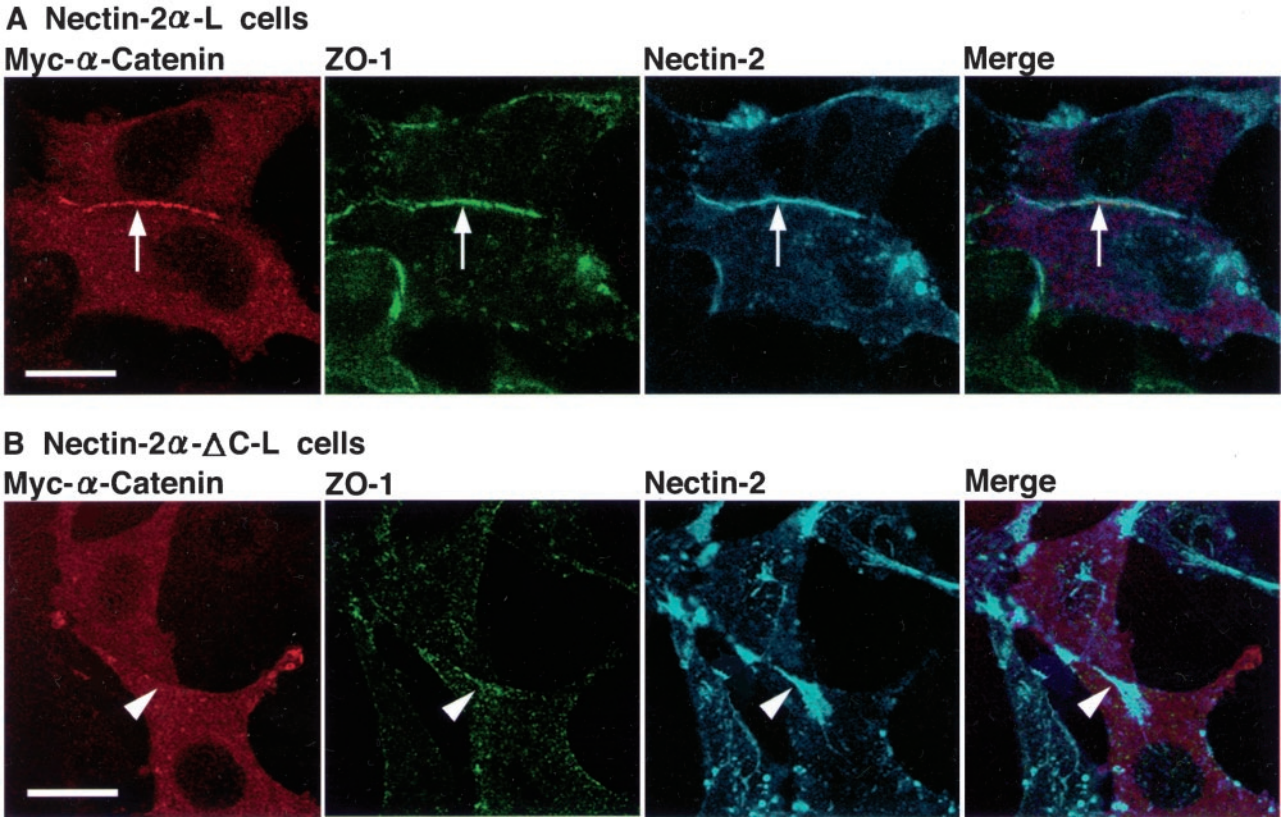

Figure 7. Afadin-dependent corecruitment of ZO-1 and α-catenin to nectin-2α–based cell-cell adhesion sites. Nectin-2α-L and -2α-ΔC-L cells were transfected with pPGKIZ-Myc-α-catenin and triply stained with the anti-Myc mAb, the anti–ZO-1 pAb, and the anti–nectin-2 mAb. (A) Nectin-2α-L cells. (B) Nectin-2α-ΔC-L cells. Arrows, nectin-2α–based cell-cell adhesion sites; arrowheads, nectin-2α-ΔC–based cell-cell adhesion sites. Bars, 10 μm. The results shown are representative of three independent experiments.

F9Dα(−/−) cells have been generated by disruption of both of the α-catenin alleles with a targeting vector (Maeno *et al.*, 1999). We then examined whether ZO-1 is recruited to nectin-2–based or E-cadherin–based cell-cell adhesion sites in F9Dα(−/−) cells. As a control, we used αF9Dα(−/−) cells, which have been generated by introduction of an expression vector encoding full-length α-catenin into F9Dα(−/−) cells (Maeno *et al.*, 1999). It has been shown that αF9Dα(−/−) cells re-express α-catenin in an amount similar to that of wild-type F9 cells (Maeno *et al.*, 1999). The expression level of the ZO-1, afadin, E-cadherin, and nectin-2α protein was similar between these two types of F9 cell lines (Figure 1E). In αF9Dα (−/−) cells, E-cadherin localized to belt-like cell-cell adhesion sites where ZO-1 colocalized with nectin-2 and afadin (Figure 8A). In F9Dα(−/−) cells, E-cadherin showed a similar staining pattern, but ZO-1 was hardly concentrated at cell-cell adhesion sites between two cells where E-cadherin was concentrated (Figure 8B). ZO-1 was concentrated with nectin-2 and afadin at spot-like cell-cell adhesion sites where more than two cells adhered to each other. These results indicate that ZO-1 is recruited to E-cadherin–based cell-cell AJs in an α-catenin–dependent manner, whereas it is recruited to nectin-2–based cell-cell adhesion sites in an α-catenin–independent manner.

Recruitment of ZO-1 to Nectin-2α–based Cell-Cell Adhesion Sites in a Manner Insensitive to F-Actin–disrupting Agents

Both afadin and ZO-1 are F-actin–binding proteins (Itoh *et al.*, 1997; Mandai *et al.*, 1997; Fanning *et al.*, 1998). In the last set of experiments, therefore, we examined by the use of F-actin–disrupting agents, latrunculin A and cytochalasin D, whether actin cytoskeletal structures are involved in the recruitment of ZO-1 to nectin-2α–based cell adhesion sites. In nectin-2α-L cells, F-actin was associated with nectin-2α–based cell-cell adhesion sites where ZO-1 and afadin colocalized (Figure 9). When these cells are incubated with latrunculin A for 45 min, the cells became round and most of actin cytoskeletal structures were disrupted (Figure 9A). Under these conditions, nectin-2α formed cell adhesion sites where ZO-1 and afadin colocalized (Figure 9, B and C). When the cells were cultured with the agent for a longer time, the cells detached from dishes (Yokoyama, Tachibana, Nakanishi, Yamamoto, Irie, Mandai, Nagafuchi, Monden, and Takai, unpublished results). Essentially similar results were obtained with cytochalasin D (Yokoyama, Tachibana, Nakanishi, Yamamoto, Irie, Mandai, Nagafuchi, Monden, and Takai, unpublished results). These results suggest that latrunculin A- or cytochalasin D-sensitive actin cytoskeletal

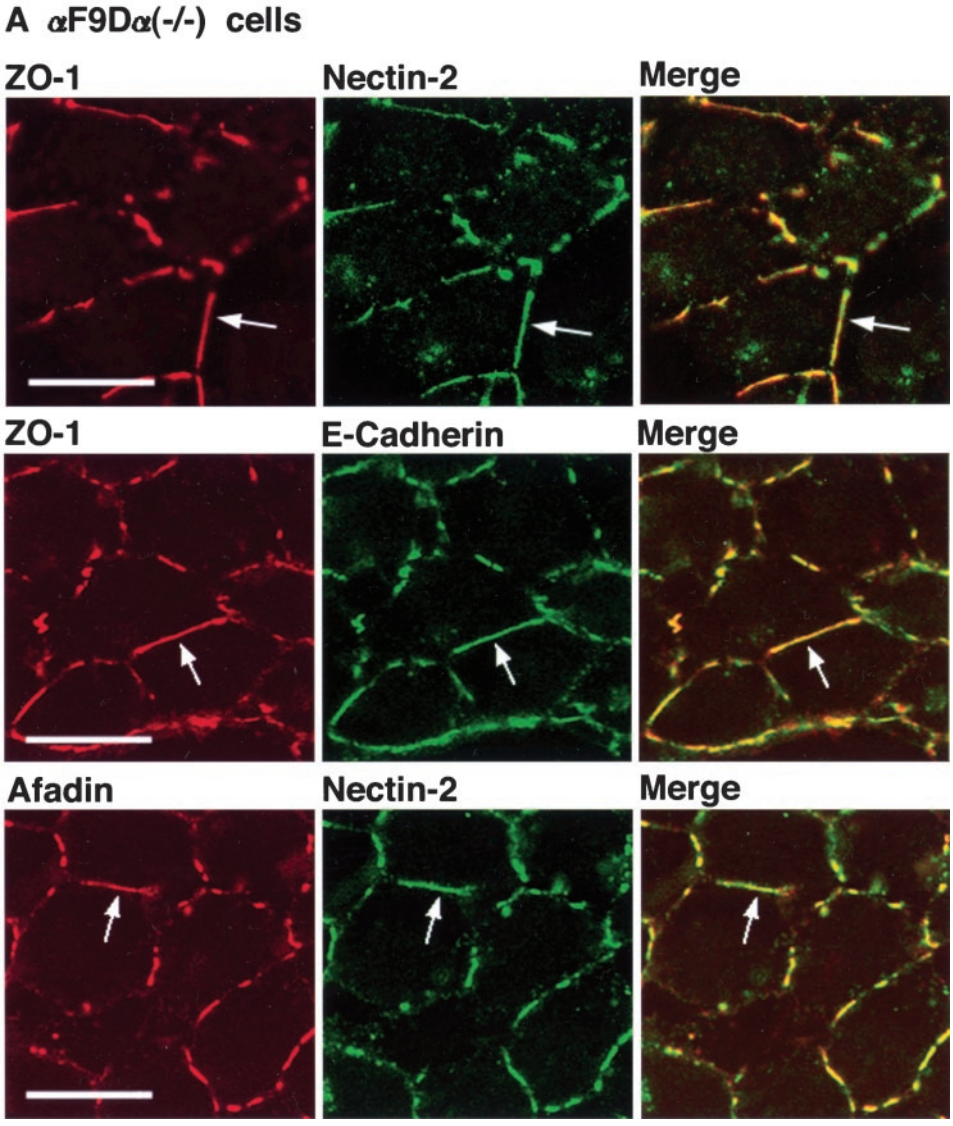

Figure 8. α-Catenin-independent colocalization of ZO-1 with nectin-2 and afadin. αF9Dα(−/−) and F9Dα(−/−) cells were doubly stained with various combinations of the anti–ZO-1 mAb, the anti–nectin-2 mAb, the anti–E-cadherin mAb, and the anti-afadin mAb. (A) αF9Dα(−/−) cells. (B) F9Dα(−/−) cells. Arrows, cell-cell adhesion sites between two cells; arrowheads, cell-cell adhesion sites where more than two cells adhere to each other. Bars, 10 μm. The results shown are representative of three independent experiments.

structures are not essential for the recruitment of ZO-1 to nectin-2α–based cell-cell adhesion sites, although it could not be concluded from these results that any actin cytoskeletal structure is not involved in this recruitment.

DISCUSSION

We have previously shown that nectin has a potency to recruit α-catenin to nectin-based cell-cell adhesion sites in an afadin-dependent manner in L cells (Tachibana *et al.*, 2000). We have shown here that nectin has a potency to recruit not only α-catenin but also ZO-1 to nectin-based cell-cell adhesion sites in L cells. This recruitment of ZO-1 is dependent on the interaction of nectin with afadin, because full-length nectin capable of interacting with afadin recruits ZO-1 but nectin-ΔC incapable of interacting with afadin does not. It has been shown that ZO-1 directly interacts with α-catenin (Itoh *et al.*, 1997; Imamura *et al.*, 1999). It is therefore possible that the recruitment of ZO-1 to nectin-based cell-cell adhesion sites is mediated through α-catenin, but this possibility is unlikely, because in F9Dα(−/−) cells that are deficient of α-catenin, ZO-1 still colocalizes with nectin.

It is of crucial importance to clarify the molecular mechanism of the linkage between afadin and ZO-1. It has previously been reported that the AF6 protein, a smaller splicing variant of afadin, directly binds ZO-1 (Yamamoto *et al.*, 1997). However, our present results, together with our previous reports (Sakisaka *et al.*, 1999; Tachibana *et al.*, 2000), indicate that afadin does not directly binds ZO-1 to a significant extent. We cannot currently exclude the possibility that the posttranslationally modified forms of these proteins directly interact with each other or that these proteins interact with each other in the presence of an unidentified cofactor.

The second possible molecular mechanism of the linkage between afadin and ZO-1 is that ponsin and/or vinculin are

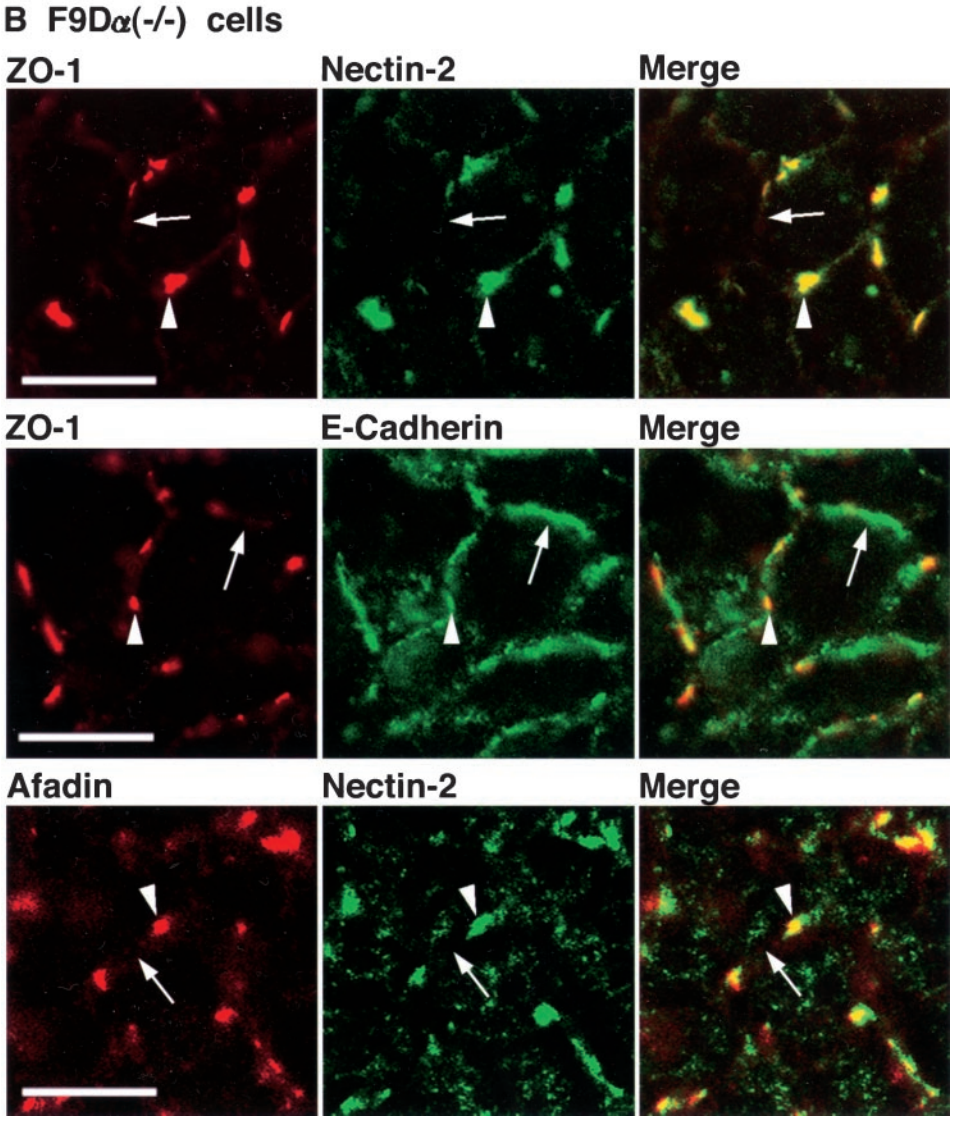

Figure 8 (cont)

involved in it. We have previously shown that ponsin and vinculin colocalize with nectin-2 and afadin to nEα-based cell-cell adhesion sites (Tachibana *et al.*, 2000). We have shown here that ZO-1 colocalizes there with nectin-2 and afadin. However, ponsin or vinculin does not localize to nEαC-based cell-cell adhesion sites where nectin-2 and afadin colocalize (Tachibana *et al.*, 2000), although ZO-1 colocalizes there with nectin-2 and afadin. Furthermore, neither ponsin nor vinculin is recruited to nectin-2α–based cell-cell adhesion sites where afadin and ZO-1 colocalize. Thus, the second possibility is negated.

The third possible molecular mechanism of the linkage is that actin cytoskeletal structures are involved in it, because afadin (Mandai *et al.*, 1997) and ZO-1 (Itoh *et al.*, 1997; Fanning *et al.*, 1998) are F-actin–binding proteins. However, we have shown here that afadin and ZO-1 colocalize to nectin-based cell-cell adhesion sites where actin cytoskeletal structures are mostly disrupted by an inhibitor of actin polymerization, latrunculin A or cytochalasin D, indicating that at least these agent-sensitive actin cytoskeletal structures are not involved in the linkage between afadin and ZO-1. Nectin forms cell-cell adhesion sites even under the conditions where actin cytoskeletal structures are disorganized by latrunculin A or cytochalasin D. This result is consistent with our earlier observation that full-length nectin and nectin-ΔC exhibit similar cell adhesion activity (Miyahara *et al.*, 2000). It is likely that the connection of nectin to the actin cytoskeleton through afadin is not essential for cell adhesion activity. It has recently been shown that disorganization of actin cytoskeletal structures shifts cadherin-based cell adhesion activity from the strong to the weak state (Imamura *et al.*, 1999). By analogy with cadherin, the connection of nectin to the actin cytoskeleton may be required for stronger cell adhesion activity. Thus, further

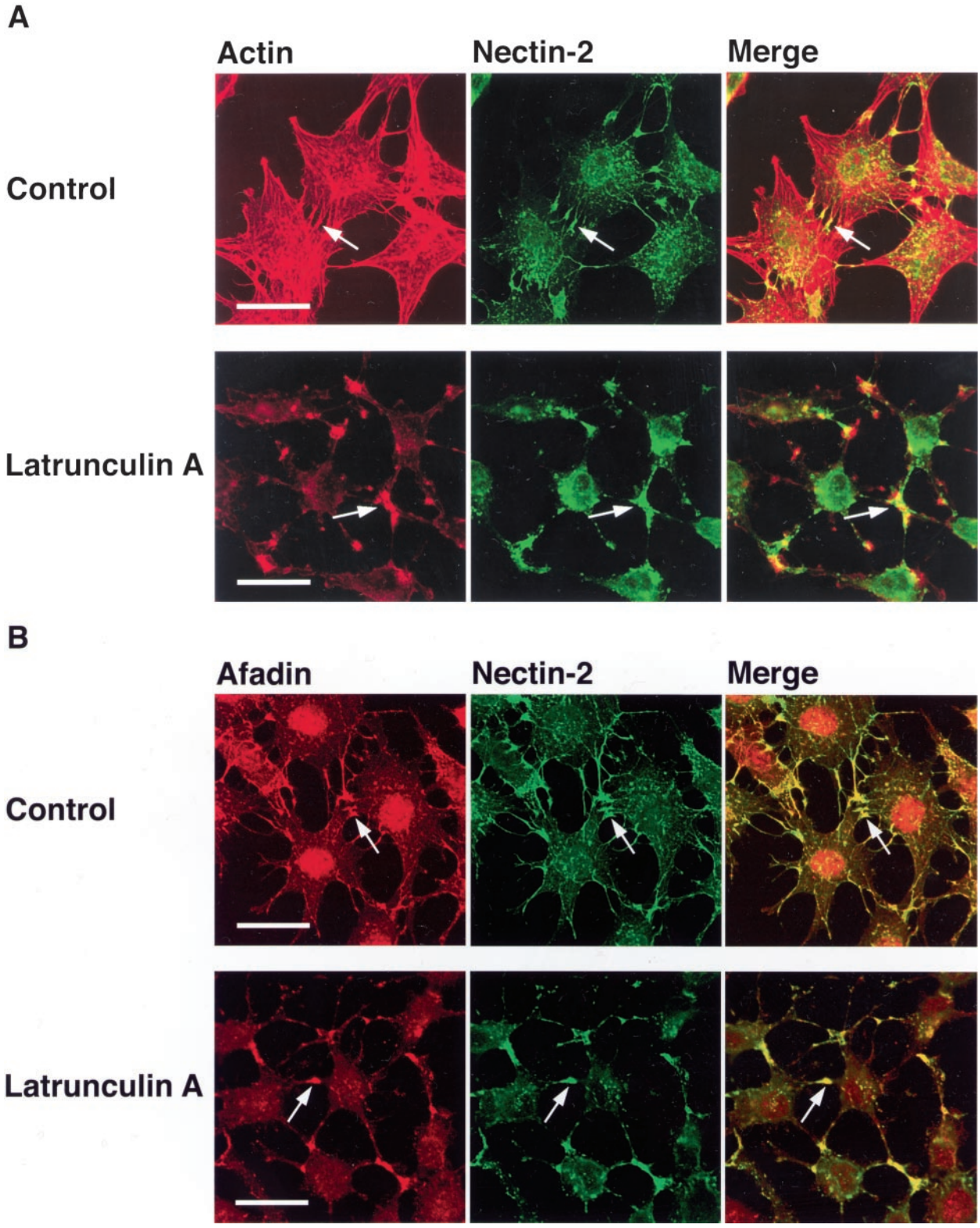

Figure 9. Recruitment of ZO-1 to nectin-2α–based cell-cell adhesion sites in a manner insensitive to latrunculin A. Nectin-2α-L cells were incubated in the presence or absence of 50 nM latrunculin A for 45 min, followed by double staining with various combinations of rhodamine-phalloidin, the anti–nectin-2 mAb, the anti-afadin mAb, and the anti–ZO-1 mAb. (A) F-actin and nectin-2. (B) Afadin and nectin-2. (C) ZO-1 and nectin-2. Arrows, nectin-2α–based cell-cell adhesion sites. Bars, 10 μm. The results shown are representative of three independent experiments.

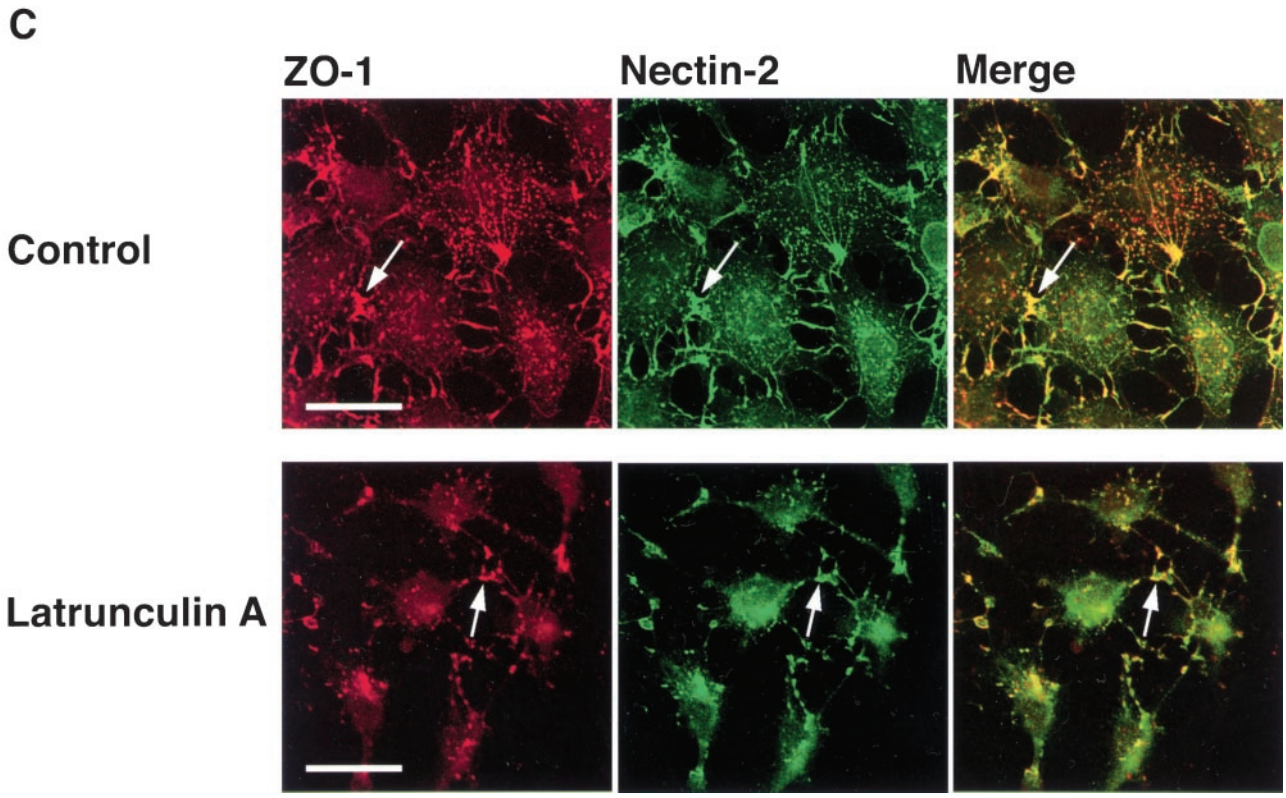

Figure 9 (cont)

studies are necessary for our understanding of the molecular mechanism of the linkage between afadin and ZO-1.

In EL cells, endogenous nectin-2, afadin, and ZO-1 all colocalize with E-cadherin and α- and β-catenins at the same cell-cell adhesion sites (Itoh *et al.*, 1993; Mandai *et al.*, 1997; Imamura *et al.*, 1999; Takahashi *et al.*, 1999). This colocalization of the two cell-cell adhesion systems is mediated through at least afadin and α-catenin (Tachibana *et al.*, 2000). ZO-1 has been shown to be associated with E-cadherin through direct interaction with α-catenin (Itoh *et al.*, 1997; Imamura *et al.*, 1999). This result, together with our present results, suggests that, in cell-cell adhesion sites where both nectin and cadherin colocalize, ZO-1 may localize there through interactions with both afadin and α-catenin. We cannot currently exclude the possibility that the recruitment of α-catenin to nectin-based cell-cell adhesion sites is mediated through ZO-1, but one possible function of ZO-1 is a connector between the nectin-afadin and cadherin-catenin systems. Another possible function of ZO-1 is that it connects these two cell-cell adhesion systems to the actin cytoskeleton, because ZO-1 as well as afadin and α-catenin is an F-actin–binding protein (Rimm *et al.*, 1995; Itoh *et al.*, 1997; Mandai *et al.*, 1997).

In well-polarized epithelial cells, ZO-1 is not associated with the cadherin-catenin system or the nectin-afadin system but is associated with the claudin-occludin system at TJs (Stevenson *et al.*, 1986; Itoh *et al.*, 1993; Mandai *et al.*, 1997, 1999; Asakura *et al.*, 1999; Sakisaka *et al.*, 1999; Takahashi *et al.*, 1999; Tsukita *et al.*, 1999). During the formation of cell-cell junctions including AJs and TJs, E-cadherin, β- and α-catenins, nectin-2, afadin, and ZO-1 first accumulate at the spot-like junctions, followed by the recruitment of occludin and probably claudin (Yonemura *et al.*, 1995; Ando-Akatsuka *et al.*, 1996; Asakura *et al.*, 1999). Once TJs are separated from AJs, ZO-1 is translocated to TJs. Our present results have increased the possibility that nectin has a potency to recruit claudin and occludin to nectin-based cell-cell adhesion sites through afadin and ZO-1. However, our preliminary analysis has revealed that nectin-2α does not show this activity in L cells expressing both nectin-2α and claudin-1, but this may be simply because many other components of TJs, such as ZO-2 (Jesaitis and Goodenough, 1994), ZO-3 (Haskins *et al.*, 1998), JAM (Marîn-Padura *et al.*, 1998), cingulin (Citi *et al.*, 1988), MAGI (Dobrosotskaya *et al.*, 1997; Ide *et al.*, 1999), symplekin (Keon *et al.*, 1996), and 7H6 antigen (Zhong *et al.*, 1993), may be absent in nonepithelial cells, such as L cells. It is of crucial importance to identify such components for our understanding of the mechanism of the formation of the junctional complex in epithelial cells.

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