Involvement of LMO7 in the Association of Two Cell-Cell Adhesion Molecules, Nectin and E-cadherin, through Afadin and α-Actinin in Epithelial Cells

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Nectins are Ca2+-independent immunoglobulin-like cell-cell adhesion molecules that are involved in formation of cadherin-based adherens junctions (AJs). The nectin-based cell-cell adhesion induces activation of Cdc42 and Rac small G proteins, which eventually enhances the formation of AJs through reorganization of the actin cytoskeleton. Although evidence has accumulated that nectins recruit cadherins to the nectin-based cell adhesion sites through their cytoplasm-associated proteins, afadin and catenins, it is not fully understood how nectins are physically associated with cadherins. Here we identified a rat counterpart of the human LIM domain only 7 (LMO7) as an afadin- and α-actinin-binding protein. Rat LMO7 has two splice variants, LMO7a and LMO7b, consisting of 1,729 and 1,395 amino acids, respectively. LMO7 has calponin homology, PDZ, and LIM domains. Western blotting revealed that LMO7 was expressed ubiquitously in various rat tissues. Immunofluorescence and immunoelectron microscopy revealed that LMO7 localized at cell-cell AJs, where afadin localized, in epithelial cells of rat gallbladder. In addition, LMO7 localized at the cytoplasmic faces of apical membranes in the same epithelial cells. We furthermore revealed that LMO7 bound α-actinin, an actin filament-bundling protein, which bound to α-catenin. Immunoprecipitation analysis revealed that LMO7 was associated with both the nectin-afadin and E-cadherin-catenin systems. LMO7 was assembled at the cell-cell adhesion sites after both the nectin-afadin and E-cadherin-catenin systems had been assembled. These results indicate that LMO7 is an afadin- and α-actinin-binding protein that connects the nectin-afadin and E-cadherin-catenin systems through α-actinin.

Cells in multicellular organisms recognize their neighboring cells, adhere to them, and form intercellular junctions. Such junctions have essential roles in various cellular functions, including morphogenesis, differentiation, proliferation, and migration (for reviews, see Refs. 1–3). In polarized epithelial cells, cell-cell adhesion is mediated through a junctional complex comprised of tight junctions (TJs), adherens junctions (AJs), and desmosomes (3). These junctional structures are typically aligned from the apical to basal sides, although desmosomes are distributed independently in other areas (3). AJs were originally defined using ultrastructural analysis as closely apposed plasma membrane domains reinforced by a dense cytoplasmic plaque where actin filament (F-actin) bundles are undercoated (3). Molecular analysis shows that AJs are cell-cell adhesion sites where classic cadherins function as cell-cell adhesion molecules and where the actin-based cytoskeleton and several cytoplasmic components are assembled (for reviews, see Refs. 4 and 5). E-cadherin, like other classical cadherins, is a single-pass transmembrane protein whose extracellular domain mediates homophilic recognition and adhesive binding in a Ca2+-dependent manner (1). E-cadherin associates with the actin cytoskeleton through peripheral membrane proteins, including α-, β-, and γ-catenins, α-actinin, and vinculin (4, 5). β-Catenin interacts directly with the cytoplasmic tail of E-cadherin and connects E-cadherin to α-catenin, which binds directly to F-actin. α-Actinin and vinculin are also F-actin-binding proteins that bind directly to α-catenin. The association of E-cadherin with the actin cytoskeleton through these peripheral membrane proteins strengthens the cell-cell adhesion activity of E-cadherin (4, 5).

We have found that Ca2+-independent immunoglobulin-like cell-cell adhesion molecules, nectins, and their associated F-actin-binding protein, afadin, localize at AJs (for reviews, see Refs. 6 and 7). Nectins comprise a family of four members, nectin-1, -2, -3, and -4. All nectins form homo-cis-dimers and then homo-trans-dimers (trans-interactions), causing cell-cell adhesion. Nectin-3 furthermore forms hetero-trans-dimers with nectin-1 and -2. Nectin-4 also forms hetero-trans-dimers with nectin-1. Afadin has at least two splice variants, l- and s-afadin (6, 7). Unless otherwise specified, afadin refers to l-afadin in this paper. Nectins recruit cadherins to the nectin-based cell-cell adhesion through afadin and catenins, resulting in formation of AJs in epithelial cells and fibroblasts and in formation of synapses in neurons (for reviews, see Refs. 6–8). Nectins also recruit first junctional adhesion molecules and
then cadulins and occludin to the apical side of AJs in cooperation with cadherins, resulting in formation of TJs (6, 7). Kinetically, nectins increase the velocities of the formation of AJs and TJs. Claudulins are key Ca\(^{2+}\)-independent cell-cell adhesion molecules at TJs, but the function of occludin has not yet been established (for a review, see Ref. 9). Junctional adhesion molecules are Ca\(^{2+}\)-independent Ig-like cell-cell adhesion molecules that recruit the cell polarity protein complex, consisting of Par-3, atypical protein kinase C, and Par-6, by directly binding Par-3 (for a review, see Ref. 10). We have demonstrated recently that nectin-1 and -3, but not nectin-2 furthermore play a role in cell polarization by binding Par-3 directly (11). On the other hand, nectins induce activation of Cdc42 and Rac small G proteins, which regulates cell-cell adhesion through reorganization of the actin cytoskeleton (for reviews, see Refs. 7 and 12). Rac is also activated by E-cadherin (for a review, see Ref. 13). Cdc42 activated by nectins increases the number of filopodia and cell-cell contact sites such as “Fork initiation.” Rac activated by either nectins and/or E-cadherin induces formation of lamellipodia and efficiently zips the cell-cell adhesion between the filopodia such as “Zipper.” The Cdc42 activated by nectins is furthermore involved in the formation of the claudin-based TJs in Madin-Darby canine kidney cells (14). Non-trans-interacting nectins inhibit the E-cadherin-induced activation of Rac and the subsequent formation of AJs, until the non-trans-interacting nectins trans-interact with other nectins and induce the activation of Cdc42 (15).

Although evidence has accumulated that nectins recruit cadherins to the nectin-based cell-cell adhesion sites, resulting in the formation of AJs (6, 7), it is not fully understood how nectins are physically associated with cadherins. Afadin binds \(\alpha\)-catenin directly in vitro, but this binding is not strong (6, 7). The direct binding of these proteins may occur in vivo, but it is more likely that a post-translational modification(s) of either or both proteins and/or an unidentified molecule(s) is required for the binding of \(\alpha\)-catenin and afadin. Consistently, we have identified two connector units for nectins and cadherins (16, 17). One is a ponsin-vinculin unit. Ponsin is an afadin- and vinculin-binding protein, and vinculin is a F-actin- and \(\alpha\)-catenin-binding protein. The other is an afadin dilute domain-interacting protein (ADIP)-\(\alpha\)-actinin unit. ADIP is an afadin- and \(\alpha\)-actinin-binding protein, and \(\alpha\)-actinin is an \(\alpha\)-catenin-binding protein. However, we have not obtained direct evidence that the nectin-afadin system is associated with the cadherin-cadherin systems in vivo through these two connector units. Therefore, there may be another connector unit for nectins and cadherins. It also remains unknown which protein(s) is the downstream target(s) of Cdc42 and Rac activated by nectins during the formation of AJs and TJs.

To obtain the molecule(s) that acts as a connector for nectins and cadherins, or which functions in the regulation of cell-cell adhesion by Cdc42 and Rac, we attempted here to identify an afadin-binding protein using yeast two-hybrid screening. We identified a rat counterpart of human LIM domain only 7 (LMO7) as an afadin- and \(\alpha\)-actinin-binding protein. Human LMO7 was first reported to be a human cDNA containing a tissue-specific alternative spliced LIM domain (18). Human and mouse LMO7 are candidate genes for the breast cancer development in human chromosomal region 13q21-q22 and for the embryonic lethality in the mouse Ednrbs-1Acrg deletion, respectively (19–21). However, no functional analysis of LMO7 protein has been performed. Here we have shown that LMO7 is an afadin- and \(\alpha\)-actinin-binding protein that connects the necdin-afadin and E-cadherin-catenin systems through \(\alpha\)-actinin.

EXPERIMENTAL PROCEDURES

**Yeast Two-hybrid Screening**—One bait vector, pGBD-nectin-2-cytosolic domain (CP) (amino acids 387–467), was constructed by subcloning the insert encoding the amino acid residue of nectin-2 into pGBD-C1 (22). The yeast expression plasmids expressing 1-afadin (amino acids 1–1829) and s-afadin (amino acids 1–1863) were constructed with the pADHU-Myc vector, which was designed to express an N-terminal Myc-tagged protein. Another bait vector, pGBD-LMO7-FDZ (amino acids 1030–1052), was constructed by subcloning the insert encoding the amino acids residue of LMO7a into pGBD-C1. Yeast two-hybrid libraries constructed from rat brain and rat lung cDNAs were purchased from Clontech (Palo Alto, CA). The yeast strain P369-4A (MATa trpl1–901 leu2–3, 112 ura3–52 his3–200 gal4a1 gal80a GAL2-ADE2 LYS2: GAL1-HIS3 met2: GAL7-lacZ) harboring pGBD-nectin-2-CP and pADHU-Myc-l-afadin was transformed with the rat brain and rat lung cDNAs libraries. 16 and 18 positive clones were obtained from 3 × 10⁶ clones of a rat lung library and 5 × 10⁵ clones of a rat brain library, respectively. Two-hybrid analysis revealed that the proteins encoded by these 32 clones bound to afadin, but not to nectin-2-CP (see Fig. 1A and data not shown). Standard procedures for yeast manipulations were as described previously (23).

**Construction of Expression Vectors**—Full-length cDNAs of LMO7a and LMO7b were obtained from a rat heart Marathon-Ready cDNA (Clontech) by PCR. The obtained full-length cDNAs encoded two splice variants of rat LMO7, LMO7a (GenBank™ accession number AY533473) and LMO7b (GenBank™ accession number AY533474), consisting of 1,729 and 1,395 amino acids, respectively. The mammalian expression vectors pCMV-FLAG, pCMV-HA, and pCMV were used to express N-terminal (pCMV-FLAG, hemagglutinin) and N-terminal Myc-tagged proteins, respectively (24). The mammalian expression vector pEGFP-C1 (Clontech) was used to express an N-terminal enhanced green fluorescent protein (EGFP)-tagged protein. The mammalian expression vector expressing afadin (amino acids 1–1829) was constructed with pCMV-Myc. The mammalian expression vectors expressing LMO7a and LMO7b, pCMV-FLAG-LMO7a (amino acids 1–1729), pCMV-FLAG-LMO7b (amino acids 1–1829), pCMV-FLAG-LMO7-C (1318–1729), pEGFP-LMO7a (1–1729), and pEGFP-LMO7b (1–1729) were constructed with pCMV-FLAG and pEGFP-C1. The mammalian expression vector expressing \(\alpha\)-actinin-1 (GenBank™ accession number B0C015766), pCMV-HA-\(\alpha\)-actinin-1-C (amino acids 406–892) were constructed with pCMV-HA. The glutathione S-transferase (GST) fusion or maltose-binding protein (MBP) fusion vectors of LMO7, MBP-LMO7-C1 (amino acids 1320–1412), MBP-LMO7-C2 (1573–1723), GST-LMO7-C1 (1320–1412), GST-LMO7-C2 (1573–1723), and GST-LMO7-C3 (1540–1729) were constructed with pGEX4T-1 (Amersham Biosciences) and PMal-C2 (New England Biolabs, Beverly, MA). The GST-MBP fusion protein was purified using glutathione beads (Amersham Biosciences) and amylose resin beads (New England Biolabs), respectively. pFastBac1-Myc-His\(_{6}\)-afadin was designed to express a C-terminal Myc- and Hist\(_{6}\)-tagged afadin (Myc-His\(_{6}\)-afadin; amino acids 1–1829). The Myc-His\(_{6}\)-afadin was expressed in High Five insect cells (Invitrogen) and purified by the use of TALON metal affinity beads (Clontech) as described previously (25).

**Antibodies**—GST-LMO7-C1 (amino acids 1320–1412) and GST-LMO7-C2 (1573–1723) were used as both antigens to raise polyclonal antibodies (pAbs) in rabbits. Two pAbs against LMO7-C1 (amino acids 1320–1412) and LMO7-C2 (1573–1723), N02 and C05, were used after affinity purification with MBP-LMO7-C1 (amino acids 1320–1412) and MBP-LMO7-C2 (1723–1729), respectively. A mouse anti-afadin monoclonal Ab (mAb) was prepared as described previously (26). A rat anti-ZO-1 mAb was purchased from Chemicon (Temecula, CA). A mouse anti-E-cadherin mAb and a rabbit anti-\(\beta\)-catenin mAb were purchased from BD Biosciences (San Jose, CA). A mouse anti-FLAG-M2 mAb and a rabbit anti-\(\alpha\)-catenin pAb were purchased from Sigma. A rabbit anti-HA Ab was purchased from BabCO (Richmond, CA). A rabbit anti-\(\alpha\)-actinin pAb and a mouse anti-Myc mAb were purchased from Santa Cruz (Santa Cruz, CA).

**Cell Culture and Transfection**—MTD-1A cells were kindly supplied by Dr. S. Tsuchika (Kyoto University). MTD-1A and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Afadin- and \(\alpha\)-catenin E cells were generated as described previously (27). Wild-type and afadin\(^{-/-}\) E cells were cultured without feeder cells on gelatin-coated 10-cm dishes for 2 days in high glucose Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 0.1 mM 2-mercaptoethanol, 1,000 units/ml leukemia inhibitory factor, 0.1 mM nonessential amino acids, 3 mM adenosine, 3 mM cysteine, 3 mM guanosine, 3 mM

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uridine, and 1 mM thymidine (27). HEK293 cells were transfected using the CalPhos mammalian transfection kit (Clontech). MTD-1A cells were transfected using the LipofectAMINE 2000 reagent (Invitrogen). Assay for Coimmunoprecipitation of LMO7 with Afadin and α-Ac- 

tinin—Coimmunoprecipitation experiments using HEK293 cells were done as follows. HEK293 cells were transfected with the expression plasmids in various combinations. The cells were suspended in 1 ml of buffer A (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10 μM α-phenylmethylsulfonyl fluoride hydrochloride, and 10 μg/ml aprotinin). The cell extract (1.2 mg of protein) was obtained by centrifugation at 100,000 × g for 15 min. The cell extract was incubated with 4 μl of the anti-FLAG M2 mAb-coated protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) at 4°C for 18 h. After the beads were washed with buffer A, the bound proteins were eluted by boiling the beads in the SDS sample buffer for 10 min. The samples were then subjected to SDS-PAGE followed by Western blotting with the anti-FLAG, anti-HA, anti-Myc, anti-afadin, and anti-α-actinin Abs.

Coimmunoprecipitation experiments using MTD-1A cells and wild-type and afadin−/− ES cells were done as follows. MTD-1A cells, wild-type ES cells, and afadin−/− ES cells on two 10-cm dishes were suspended in 1 ml of buffer A, followed by ultracentrifugation at 100,000 × g for 15 min. The cell extract was incubated with 20 μl of the anti-LMO7 pAb (N02)-coated protein A-Sepharose CL-4B beads (Amersham Biosciences) at 4°C for 18 h. After the beads were washed with buffer A, the bound proteins were eluted by boiling the beads in the SDS sample buffer for 10 min. The samples were then subjected to SDS-PAGE, followed by Western blotting with the anti-LMO7, anti-afadin, anti-α-actinin, anti-E-cadherin, anti-α-catenin, and anti-β-catenin Abs.

Assay for the Binding of LMO7 to Afadin—To examine the interaction of LMO7 with Myc-Hisα-afadin (12.5 pmol of protein fixed on 5.7 μl of beads) was immobilized on the anti-Myc mAb-coupled beads prepared by cross-linking of the anti-Myc mAb with protein A-Sepharose beads (Amersham Biosciences) via dimethylpimelimidate (28). GST-LMO7-C3 (amino acids 1540–1729, 252 pmol of protein) was applied to the Myc-Hisα-afadin-immobilized beads equilibrated with buffer B (20 mM Tris-HCl at pH 8.0, 0.2 mM EDTA, 150 mM NaCl, 0.1% CHAPS, 12 mM MgCl2, 1 mM dithiothreitol, 5 μM GTP[S]). After the beads were extensively washed with buffer B, the bound proteins were eluted by boiling the beads in the SDS sample buffer. The samples were then subjected to SDS-PAGE (10% polyacrylamide gel), followed by staining with Coomassie Brilliant Blue.

Other Methods—Subcellular fractionation of rat liver was performed as described (29). Immunofluorescence microscopy of cultured cells and frozen sections of mouse tissues were done as described (30, 31). Immunelectron microscopy of epithelial cells of gallbladder using the ultrathin cryosection technique was done as described (32). GST-LMO7C was cloned as a fusion protein to GST (C05) against the C-terminal part of LMO7a (amino acids 1412–1729) and against the C-terminal part of LMO7b (amino acids 1723–1956) via thrombin cleavage site, which was then purified by a GST affinity column. The concentration was determined by a bicinchoninic acid assay.

RESULTS

Identification of an Afadin-binding Protein—To identify an afadin-binding protein, we performed the yeast two-hybrid screening using full-length afadin bound to the cytoplasmic domain of nectin-2 as a bait (Fig. 1A). We screened 4 × 10^5 clones of a rat lung library and 5 × 10^6 clones of a rat brain library and obtained 16 and 16 positive clones, respectively. Seven clones encoded proteins highly similar to the C-terminal portion of human LMO7 (GenBankTM accession number AF33045). Rat full-length clones of LMO7 were obtained by reverse transcription-PCR. Two splice variants were isolated, and they encoded proteins composed of 1,729 amino acids with a calculated molecular weight of 195,568 and 1,395 amino acids with a calculated molecular weight of 156,646, respectively (Fig. 1B). We named these rat counterparts of human LMO7, LMO7a and LMO7b. Both LMO7a and LMO7b have calponin homology (amino acids 17–129 in LMO7a and LMO7b), PDZ (amino acids 1076–1152 in LMO7a; 742–818 in LMO7b), and LIM (amino acids 1659–1723 in LMO7a; 1325–1389 in LMO7b) domains (Fig. 1B). LMO7a also has an F-box (amino acids 549–590), which is shown to be critical for degradation of cellular regulatory proteins (35) (Fig. 1B). The amino acid sequences of human LMO7 and rat LMO7b were 75% identical to each other.

To confirm whether the isolated cDNAs encode full-length LMO7a and LMO7b, HEK293 cells were transfected with pCMV-FLAG-LMO7a and pCMV-FLAG-LMO7b, which expressed the FLAG-tagged full-length proteins of LMO7a and LMO7b, respectively. The cell extract was subjected to SDS-PAGE followed by Western blotting with the anti-LMO7 pAbs. Two anti-LMO7 pAbs, N02 against the central portion of LMO7a (amino acids 1320–1412) and C05 against the C-terminal portion of LMO7a (amino acids 1659–1723), were generated. Proteins with a molecular mass of about 220 and 200 kDa were detected in the extracts of HEK293 cells expressing pCMV-FLAG-LMO7a and pCMV-FLAG-LMO7b, respectively, by these two pAbs (Fig. 1C and data not shown). The band of endogenously expressed FLAG-LMO7b was slightly higher than that of endogenous LMO7 in rat liver. This difference is probably because of the molecular mass of FLAG tag. Thus, the isolated cDNAs encode full-length LMO7a and LMO7b.

In Vitro and in Vivo Binding of LMO7 to Afadin—Two-hybrid analysis revealed that LMO7 specifically bound to afadin, but not to s-afadin (Fig. 1A). The region containing the LIM domain (amino acids 1659–1723) of LMO7a was required for the binding to afadin (Fig. 1B). To confirm the binding of LMO7 to afadin in intact cells, we performed immunoprecipitation analysis. HEK293 cells were cotransfected with the Myc-tagged full-length of afadin (Myc-afadin) and the FLAG-tagged C-terminal portion of LMO7 containing the LIM domain (FLAG-LMO7-C; amino acids 1318–1729), which was isolated in the two-hybrid screening. When FLAG-LMO7-C was immunoprecipitated from the cell extract with the anti-FLAG mAb, Myc-afadin was coimmunoprecipitated as detected by Western blotting with the anti-Myc mAb (Fig. 2A). When FLAG-LMO7-C was immunoprecipitated with the anti-FLAG mAb from the extract of HEK293 cells transiently expressing FLAG-LMO7-C alone, endogenous afadin was coimmunoprecipitated (Fig. 2B). We next examined whether endogenous afadin was coimmunoprecipitated with endogenous LMO7 from the extract of MTD-1A cells. MTD-1A cells expressed two isoforms of LMO7, which were detected by two anti-LMO7 pAbs (Fig. 1C and data not shown). When the two isoforms of endogenous LMO7 were immunoprecipitated from the extract of MTD-1A cells with anti-LMO7 pAb, endogenous afadin was coimmunoprecipitated (Fig. 2C). Afadin was not coimmunoprecipitated with control IgG. Finally, we examined whether LMO7 interacted directly with afadin. A GST fusion protein of the region containing the LIM domain (GST-LMO7-C3; amino acids 1540–1729) stoichiometrically bound to the Myc- and Hisα-tagged protein of afadin (Myc-Hisα-afadin) immobilized on protein A-Sepharose beads through the anti-Myc mAb (Fig. 2D). These results together with the two-hybrid analysis indicate that LMO7 binds directly to afadin both in vitro and in vivo.

Tissue and Subcellular Distribution of LMO7—Western blotting with the anti-LMO7 pAb detected ~180-kDa protein in rat heart, lung, kidney, and small intestine (Fig. 3A lanes 2, 3, 5, and 7). The larger and smaller bands (~210 and ~115 kDa in brain, ~200 kDa in kidney) were detected, suggesting that the larger and smaller splice variants of LMO7 may be expressed (Fig. 3A, lanes 1 and 5). After long exposure, the immunoreactive band of LMO7 was detected in other tissues including liver, spleen, testis, and skeletal muscle (data not shown). Subcellular fractionation analysis of LMO7 in rat liver indicated that it was enriched in the fraction rich in AJs and TJs, where afadin was also enriched (Fig. 3B, lanes 4 and 5). The reason why the Western blotting of tissue distribution with short exposure did not detect ~180-kDa protein in the liver...
Fig. 3 A, yeast two-hybrid assay showing the specific binding of LMO7 to afadin. Yeast transformants with the indicated plasmids were streaked on the synthetic complete medium lacking adenine to score the ADE2 reporter activity and incubated at 30 °C for 3 days. B, schematic structure of LMO7. CH, calponin homology domain; F-box, F-box domain; PDZ, PDZ domain; LIM, LIM domain. Yeast two-hybrid analysis of the afadin- or α-actinin-binding regions of LMO7 is shown. LMO7, afadin, and α-actinin were constructed into the pGBD, pADHU, or pGAD vector and cotransformed into the reporter yeast strains. Binding of LMO7 to afadin or α-actinin, as shown by expression of the HIS3 and ADE2 reporter genes, was monitored by scoring growth on synthetic complete medium lacking histidine and adenine, respectively. +, interacted; −, not interacted; NT, not tested. C, comparison of molecular masses of native and recombinant LMO7 proteins. pCMV-FLAG-LMO7a and pCMV-FLAG-LMO7b were transfected to HEK293 cells, and the cell extract was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-LMO7 pAb (N02). The extracts of control HEK293, MTD-1A cells, and the fraction rich in AJs and TJs of the isolated rat liver (10 μg of protein each) were similarly subjected to SDS-PAGE followed by Western blotting with the anti-LMO7 pAb (N02). Results are representative of three independent experiments.

(Fig. 3A, lane 4) was probably because of its low expression level. These results indicate that LMO7 is expressed widely, although its expression levels vary depending on tissues.

Localization of LMO7 at AJs and at the Cytoplasmic Faces of Apical Membranes in Epithelial Cells—Because afadin has been shown to localize strictly at AJs undercoated with F-actin bundles (30), we examined by immunofluorescence microscopy whether LMO7 colocalized with afadin at AJs in MTD-1A cells. LMO7 localized at the sites of cell-cell contacts (Fig. 4A). The cross-sectional analysis using a confocal microscopy revealed that LMO7 localized at cell-cell AJs where afadin localized (data not shown). In addition, the signal for LMO7 was observed along the apical membranes. The essentially same results were obtained with two anti-LMO7 pAbs, N02 and C05 (data not shown). To confirm the localization of LMO7 at the sites of cell-cell contacts, the EGFP-tagged LMO7a and LMO7b were transiently expressed in MTD-1A cells, and the localization of the expressed proteins was examined. Exogenously expressed EGFP-LMO7a and EGFP-LMO7b were concentrated at the sites of cell-cell contacts (Fig. 4B).

To examine the precise localization of LMO7 at the junctional complex region, the frozen sections of gallbladder were triple stained with the anti-LMO7 pAb, the anti-afadin mAb, and the anti-ZO-1 mAb because TJs and AJs are well separated in this cell type. ZO-1 is known to be a marker for TJs (9). Immunofluorescence microscopy revealed that LMO7 localized at cell-cell AJs, where afadin localized, in epithelial cells of gallbladder (Fig. 5A, arrows). The signals for LMO7 at AJs were distributed at the slightly more basal side than those of ZO-1. In addition, the signal for LMO7 was observed along the apical membrane (Fig. 5A, arrowheads). Immunoelectron microscopy revealed that LMO7 localized at AJs in epithelial cells of gallbladder (Fig. 5B, arrowheads). The signal for LMO7 at AJs was distributed thicker from the membrane to the cytoplasm, whereas that of afadin was distributed along the membrane of AJs (30). In addition, the signal for LMO7 was also observed at the cytoplasmic face of the apical membrane, and the faint signal was observed furthermore at that of the basolateral membrane (Fig. 5B, arrowheads and double arrowheads). The signal for LMO7 was not observed at TJs or desmosomes. These results indicate that LMO7 localizes at AJs where afadin localizes and at the cytoplasmic faces of apical membranes in epithelial cells.

Binding of LMO7 to α-Actinin—To gain the insight into the function of LMO7, we attempted to identify a LMO7-binding protein(s). As described above, LMO7 has calponin homology,
PDZ, and LIM domains, and the region containing the LIM domain (amino acids 1660–1724) of LMO7 was required for the binding to afadin (Fig. 1B). We performed the yeast two-hybrid screening using the central region of LMO7 containing the PDZ domains (amino acids 796–1540) as a bait (Fig. 1B). We screened 4/H11003104 clones of a rat lung library and obtained 12 positive clones. Three clones encoded the C-terminal portion of LMO7, an Afadin- and α-Actinin-binding Protein

**Fig. 2. In vitro and in vivo binding of LMO7 to afadin.** A, coimmunoprecipitation of the Myc-tagged afadin with the FLAG-tagged LMO7-C. Expression vectors were transfected into HEK293 cells as indicated. The extracts of the HEK293 cells were immunoprecipitated (IP) with the anti-FLAG mAb and analyzed by Western blotting (WB) with the anti-Myc and anti-FLAG mAbs. **B**, coimmunoprecipitation of endogenous afadin with the FLAG-tagged LMO7. Expression vectors were transfected into HEK293 cells as indicated. The extracts of the HEK293 cells were immunoprecipitated with the anti-FLAG mAb and analyzed by Western blotting with the anti-afadin and anti-FLAG mAbs.

**Fig. 3. Tissue and subcellular distribution of LMO7.** A, Western blotting. The homogenates of various rat tissue (30 μg of protein each) were subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-LMO7 pAb (N02). Lane 1, brain; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, kidney; lane 6, spleen; lane 7, small intestine; lane 8, testis; lane 9, skeletal muscle; and lane 10, MTD-1A cells. B, subcellular distribution of LMO7 in rat liver. Subcellular fractionation of rat liver was performed, and each fraction (10 μg of protein each) was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-LMO7 pAb (N02) or the anti-afadin mAb. Lane 1, the homogenate fraction; lane 2, the pellet fraction; lane 3, the soluble fraction; lane 4, the fraction rich in bile canaliculi; and lane 5, the fraction rich in AJs and TJs. Results are representative of three independent experiments.

**Fig. 4. Localization of LMO7 at the cell-cell contact sites in MTD-1A cells.** A, colocalization of LMO7 and afadin at the cell-cell contact sites in MTD-1A cells. MTD-1A cells were double stained with the anti-LMO7 (N02) and anti-afadin Abs. **B**, localization of EGFP-LMO7a and EGFP-LMO7b at the cell-cell contact sites in MTD-1A cells. MTD-1A cells expressing exogenous EGFP-LMO7a and EGFP-LMO7b were stained with the anti-afadin mAb. Bars, 20 μm. Results are representative of three independent experiments.
α-actinin-1 (amino acids 320–892; GenBank™ accession number NM031005). α-Actinin is a well characterized protein that shows F-actin cross-linking activity (36). Four isoforms of human α-actinin have been identified: non-muscle α-actinin-1 and α-actinin-4, and muscle α-actinin-2 and α-actinin-3 (37–40). LMO7 bound α-actinin-4 in addition to α-actinin-1 as estimated by yeast two-hybrid analysis (data not shown), indicating that the binding of LMO7 to α-actinin is not specific for α-actinin-1. Two-hybrid analysis revealed that the region adjacent to the PDZ domain (amino acids 796–1076) of LMO7 bound α-actinin-1 (Fig. 1B).

To confirm the binding of LMO7 to α-actinin-1 in intact cells, we performed the immunoprecipitation analysis. HEK293 cells were cotransfected with the HA-tagged C-terminal portion of α-actinin-1 (HA-α-actinin-1-C; amino acids 406–892) and FLAG-tagged LMO7b. When FLAG-tagged LMO7b was immunoprecipitated from the cell extract with the anti-FLAG mAb, HA-α-actinin-1-C was coimmunoprecipitated as detected by Western blotting with the anti-HA mAb (Fig. 6A). When FLAG-LMO7b was immunoprecipitated with the anti-FLAG Ab from the extract of HEK293 cells transiently expressing the FLAG-tagged LMO7b alone, endogenous α-actinin was coimmunoprecipitated (Fig. 6B). Finally, when endogenous LMO7 was immunoprecipitated from the extract of MTD-1A cells with the anti-LMO7 pAb, endogenous α-actinin was coimmunoprecipitated (Fig. 6C). α-Actinin was not coimmunoprecipitated with control IgG. These results indicate that LMO7 binds α-actinin in vivo and in vitro.

Association of LMO7 with the E-cadherin-Catenin System—Because α-actinin is known to be indirectly associated with E-cadherin through its direct binding to α-catenin (4), we next examined whether LMO7 was associated with E-cadherin, α-catenin, or β-catenin through α-actinin. When endogenous LMO7 was immunoprecipitated from the extract of MTD-1A cells with the anti-LMO7 pAb, endogenous E-cadherin, α-catenin, and β-catenin were coimmunoprecipitated (Fig. 7A). E-cadherin, α-catenin, or β-catenin was not coimmunoprecipitated with control IgG. These results, together with the observation that LMO7 directly binds to α-fadin, suggest that LMO7 links α-fadin to the cadherin-catenin system through α-actinin.

To exclude the possibility that LMO7 was associated with E-cadherin, α-catenin, or β-catenin through α-fadin, we next examined whether LMO7 was associated with E-cadherin, α-catenin, or β-catenin in the afadin−/− ES cells. When endogenous LMO7 was immunoprecipitated from the extract of the wild-type ES cells with the anti-LMO7 pAb, endogenous E-cadherin, α-catenin, and β-catenin as well as endogenous α-fadin were coimmunoprecipitated (Fig. 7B). Afadin, E-cadherin, α-catenin, or β-catenin was not coimmunoprecipitated with control IgG. When endogenous LMO7 was immunoprecipitated from the extract of the afadin−/− ES cells with the anti-LMO7
LMO7, an Afadin- and α-Actinin-binding Protein

Assembly of LMO7 at Cell-Cell Junctions at the Later Stage of Their Formation—Wound healing assays using MTD-1A cells have revealed that nectin, afadin, ZO-1, E-cadherin, and α- and β-catenins are first assembled at the primordial, spot-like adhesion sites including AJs (6, 7, 41). A putative connector for nectins and cadherins, ponsin is also first assembled at the primordial, spot-like adhesion sites (41). These primordial adhesion sites fuse with each other to form short line-like adhesion sites, which develop into more matured AJs. During and/or after the formation of AJs, junctional adhesion molecule is first assembled at these adhesion sites. Finally, occludin and claudin are recruited at the apical side of AJs, resulting in the formation of TJs. We examined which stage LMO7 is recruited to the adhesion sites. LMO7 was not concentrated at the spot-like adhesion sites where afadin was concentrated, although it was distributed weakly along F-actin bundles in thin cellular protrusions (Fig. 8A). At the next stage of this wound healing process, the spot-like junctions began to fuse to form short line-like junctions. Claudins and occludin accumulated at this type of junction (32). LMO7 was not concentrated at the line-like junctions, although it was distributed weakly along F-actin bundles in thin cellular protrusions (Fig. 8B). At the later stage of this process, the line-like junctions grow up to complete cell-cell junctions. LMO7 was finally concentrated at these junctions (Fig. 8C). These results suggest that LMO7 is assembled at the cell-cell adhesion sites at the very late stage, and its behavior is different from that of afadin or ponsin.

DISCUSSION

We have isolated here a rat counterpart of human LMO7 as an afadin-binding protein. Although previous papers had reported the partial sequence of LMO7 and tissue distribution of the LMO7 mRNA (18–21), no functional analysis of LMO7 protein has been performed. In this paper, we have provided several lines of evidence suggesting that LMO7 binds directly to afadin: (i) LMO7 binds to afadin as estimated by the yeast two-hybrid and coimmunoprecipitation from the extracts of cells exogenously expressing the fragments of LMO7; (ii) endogenous LMO7 and afadin are coimmunoprecipitated from the extracts of MTD-1A cells; (iii) recombinant LMO7 binds directly to recombinant afadin in a cell-free system; and (iv) LMO7 localizes at AJs where afadin localizes in epithelial cells of rat gallbladder. We have furthermore shown that LMO7 binds α-actinin on the basis of the following evidence: (i) LMO7 binds α-actinin as estimated by the yeast two-hybrid and coimmunoprecipitation from the extracts of HEK293 cells exogenously expressing the full-length of LMO7; (ii) endogenous LMO7 and α-actinin are coimmunoprecipitated from the extracts of MTD-1A cells; (iii) recombinant LMO7 binds directly to recombinant α-actinin in a cell-free system; and (iv) LMO7 localizes to AJs where α-actinin localizes in endothelial cells of mouse aorta. We have previously shown that nectins have a potency to recruit the E-cadherin-β-catenin complex to the nectin-based cell-cell adhesion sites through afadin and α-catenin in fibroblasts and epithelial cells and that there are two connector units for nectins and cadherins, the ponsin-vinculin unit and the ADIP-α-actinin unit (6, 7, 16, 17). Thus, the LMO7-α-actinin unit is a third connector unit for nectins and cadherins. Although we have not obtained direct evidence that ponsin or ADIP is associated with both nectin-afadin and E-cadherin-catenin systems in vivo, our immunoprecipitation analysis in the present study revealed that LMO7 is associated with both nectin-afadin and E-cadherin-catenin systems. We have not detected the association of ponsin or ADIP with the cadherin-catenin system by the immunoprecipitation analysis (data not shown). One possibility is that LMO7 is associated more tightly with the nectin-afadin and E-cadherin-catenin systems than...
ponsin or ADIP. These three connector units function in the different stage of the formation of AJs (Fig. 9). During the formation of cell-cell junctions, nectin, afadin, ZO-1, E-cadherin, $\alpha$- and $\beta$-catenins, and ponsin are first assembled at the primordial, spot-like adhesion sites (Fig. 9B). In contrast, LMO7 is assembled at the cell-cell adhesion sites at the very late stage (Fig. 9C). It has not been determined which stage the ADIP-$\alpha$-actinin unit is assembled. Thus, the LMO7-$\alpha$-actinin
unit does not seem to function in the recruitment of the E-cadherin-catenin complex to the nectin-based cell-cell adhesion sites during the formation of cell-cell junctions. The ponsin-vinculin unit may act as a connector unit at the early stage during the formation of cell-cell junctions, whereas the LMO7-α-actinin unit may act as a stabilizer of the nectin-afadin and cadherin-catenin systems after they are recruited. Because the association between afadin and LMO7 was detected in the dissociated cells by the immunoprecipitation analysis (data not shown), LMO7 might be able to bind to the afadin that does not localize at cell-cell junctions. The precise mechanism of the assembly of these connector units remains to be elucidated.

LMO7 binds two F-actin-binding proteins, afadin and α-actinin. Afadin binds along the sides of F-actin but does not have a marked F-actin cross-linking activity (30). α-Actinin has an F-actin cross-linking activity (36). Thus, LMO7 links afadin to α-actinin possibly together with another afadin- and α-actinin-binding protein, ADIP, and may function in the formation of the specialized actin structure at the nectin- and E-cadherin-based AJs (Fig. 9C). This specialized actin structure at AJs is critical for stabilizing the junctions (3). Afadin, α-actinin, LMO7, and ADIP may also function in the regulation of actin dynamics at AJs. These proteins may act downstream of the Cdc42 activated by nectins and the Rac activated by nectins and/or E-cadherin, which regulate cell-cell adhesion through reorganization of the actin cytoskeleton.

Although we have identified LMO7 as an afadin-binding protein, the localization of LMO7 is not the same as that of afadin. LMO7 is distributed thicker from the membranes to the cytoplasm than afadin at AJs. In addition, LMO7 localizes at the cytoplasmic faces of apical membranes. The physiological significance of this localization of LMO7 is currently unknown. It may be noted that ponsin localizes at AJs and cell-matrix junctions and that ADIP localizes at AJs and the Golgi apparatus (16, 17). Thus, these three connectors may have additional roles, which differ from the role in the formation of AJs.

The cross-talk between the cell-cell and cell-matrix adhesions has been known to play important roles for regulation of cell migration, adhesion, and polarization. Protein sorting in the trans-Golgi network also has an important role for regulation of cell polarization in epithelial cells (42). Components of apical membranes such as Crb have been suggested to have an important role for regulation of cell polarization (43). However, their molecular mechanisms are currently unknown. Further studies of these connectors may give us deeper insights into regulation of cell migration, adhesion, and polarization.

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Involvement of LMO7 in the Association of Two Cell-Cell Adhesion Molecules, Nectin and E-cadherin, through Afadin and α-Actinin in Epithelial Cells
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