Implications of Nectin-like Molecule-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1 in Cell-Cell Adhesion and Transmembrane Protein Localization in Epithelial Cells*

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Nectins are Ca2+-independent immunoglobulin-like cell-cell adhesion molecules that play roles in organization of a variety of cell-cell junctions in cooperation with or independently of cadherins. Four nectins have been identified. Five nectin-like molecules, which have domain structures similar to those of nectins, have been identified and we characterized here nectin-like molecule-2 (Necl-2/IGSF4/RA175/SgIGSF/TSLC1/SynCAM1. Necl-2 showed Ca2+-independent homophilic cell-cell adhesion activity. It furthermore showed Ca2+-independent heterophilic cell-cell adhesion activity with Necl-1/TSSL1/SynCAM3 and nectin-3. Necl-2 was widely expressed in rat tissues examined. Necl-2 localized at the basolateral plasma membrane in epithelial cells of the mouse gall bladder, but not at specialized cell-cell junctions, such as tight junctions, adherens junctions, and desmosomes. Nectins bind afadin, whereas Necl-2 did not bind afadin but bound Pals2, a membrane-associated guanylate kinase family member known to bind Lin-7, implicated in the proper localization of the Let-23 protein in Caenorhabditis elegans, the homologue of mammalian epidermal growth factor receptor. These results indicate the unique localization of Necl-2 and its possible involvement in localization of a transmembrane protein(s) through Pals2.

Cell-cell adhesion is critical for tissue patterning and morphogenesis as well as for maintenance of normal tissues. In polarized epithelial cells, intercellular adhesion is mediated through a junctional complex comprised of tight junctions (TJs), adherens junctions (AJs), and desmosomes (DSs) (1). These junctional structures are typically aligned from the apical to basal sides, although DSs are independently distributed in other areas. The formation and maintenance of TJs and DSs depend upon the formation and maintenance of AJs. At TJs, claudins are key cell-cell adhesion molecules that form TJ strands (1). At AJs, E-cadherin is a key Ca2+-dependent cell-cell adhesion molecule (2, 3). TJs and AJs are undercoated with actin filament (F-actin) bundles. At DSs, desmosomal cadherins, desmocollin and desmoglein, are key Ca2+-dependent cell-cell adhesion molecules (4). DSs are linkers of the intermediate filament cytoskeleton.

Nectins are emerging cell-cell adhesion molecules that play roles in the organization of a variety of cell-cell junctions, such as TJs and AJs in epithelial cells, synaptic junctions in neurons, and heterotypic junctions formed between the Sertoli cells and spermatids in the testis, in cooperation with or independently of cadherins (5). Although cadherins are Ca2+-dependent cell-cell adhesion molecules, nectins are Ca2+-independent cell-cell adhesion molecules that comprise a family of four members, nectin-1, -2, -3, and -4 (5). All nectins have one extracellular region with three Ig-like loops, one transmembrane region, and one cytoplasmic region (5). Each nectin forms homois-dimers followed by formation of homo-trans-dimers, causing cell-cell adhesion (5). Nectin-3 furthermore forms hetero-trans-dimers with either nectin-1 or -2, and the adhesion activity of each hetero-trans-dimers is stronger than that of each homo-trans-dimers (5). Nectin-4 also forms hetero-trans-dimers with nectin-1 (5). Nectins except nectin-4 have a C-terminal conserved motif of four amino acid (aa) residues that interacts with the PDZ domain of afadin (5). Nectin-4 does not have this consensus motif but binds afadin. Afadin is an F-actin-binding protein with one PDZ domain and three other domains and connects nectins to the actin cytoskeleton (5).

Five molecules with one extracellular region containing three Ig-like loops, one transmembrane region, and one cytoplasmic region have thus far been identified. We have proposed, based on their domain structures which are similar to those of nectins, that these molecules are called nectin-like proteins.
molecules (Necls) (6). These include Necl-1/TSLL1/SynCAM3 (7, 8), Necl-2/IGSF4/RA175/SigIGSF/TSLC1/SynCAM1 (8–12), Necl-3/similar to Necl-3/SynCAM2 (8), Necl-4/TSLL2/SynCAM4 (7, 8), and Necl-5/Tage-1/PVR/CD155 (6, 13–16). In this section, we focus on Necl-2/IGSF4/RA175/SigIGSF/TSLC1/SynCAM1. Necl-2 was directly submitted to GenBank TM (GenBank TM/EMBL/DBJ accession number AF601260 (1998); GenBank TM/EMBL/DBJ accession number AF132811 (1999)); IGSF4 was identified as a candidate for a tumor suppressor gene associated with loss of heterozygosity of chromosome 11q23.2 (9); RA175 was identified to be a gene highly expressed during neuronal differentiation of embryonic carcinoma cells (10); SigIGSF was identified to be a gene expressed in spermatogenic cells during earlier stages of spermatogenesis (11); and TSLC1 was identified to be a tumor suppressor in human non-small cell lung cancer (12). TSLC1 shows Ca2 ++-binding tumor suppressor DAL-1, one of the band 4.1 family members, which connects TSLC1 to the actin cytoskeleton (18); SynCAM1 was identified to be a brain-specific synaptic adhesion molecule (8). SynCAM1 shows Ca2 ++-independent homophilic cell-cell adhesion activity (8). SynCAM1 forms synapses between HEK293 cells expressing exogenous SynCAM1 and primary cultured hippocampal neurons in vitro (8). SynCAM1 has been shown to be specifically expressed in mouse brain as estimated by Western blotting (8), but TSLC1 and SigIGSF have been shown to be expressed ubiquitously as estimated by Northern blotting (11, 19). This molecule with six different nomenclatures is referred to Necl-2.

We have studied the properties of Necl-2, including its cell-cell adhesion activity, its localization, and its binding partners. We have found that Necl-2 localizes at the extra-junctional region of the basolateral plasma membrane of epithelial cells and directly binds Pals2. Pals2 was originally isolated as a Lin-7-binding protein (20). Lin-7 is a PDZ domain-containing protein that forms a heterotrimeric complex with Lin-2 and -10. This Lin-2/Lin-7/Lin-10 protein complex is involved in organization of epithelial and neuronal junctions in Caenorhabditis elegans and mammals (21). Pals2 belongs to the membrane-associated guanylate kinase family and consists of two Lin-2/7 homology domains, one PDZ domain, one Src-homology 3 domain, one homology domain, one Src-homology 2 domain, and one guanylate kinase domain. Pals2 as well as Pals1 and Lin-2 constitutes a subfamily that binds to Lin-7 (20). Here we describe these novel properties of Necl-2.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning of Mouse Necl-1 and -2 cDNAs**—We performed reverse transcription-PCR from mouse brain total RNA using the Iso-GEN RNA extraction kit (Nippon Gene), Ready-To-Go You-Prime First-Strand Beads and pd(N)6 (Amersham Biosciences), Pfu turbo DNA polymerase (Stratagene), and the specific primers of mouse Necl-1 or -2. The primers were designed based on Necl-1 (GenBank TM/EMBL/DBJ accession number AF195662) and Necl-2 (GenBank TM/EMBL/DBJ accession number AB052293). The forward and reverse primers used were: Necl-1 5′- GCCAACGACCTGCCGGAGCAAGTC-3′ and 5′-GGGTCACTAGTATAGAATTTCTGTC-3′; Necl-2 5′- GCAGAACGACCTGCCGGAGCAAGTC-3′ and 5′-GGGTCACTAGTATAGAATTTCTGTC-3′; respectively. The reverse transcription-PCR products were cloned using ZeroBlunt TOPO PCR cloning kit (Invitrogen). DNA sequencing was performed by the dideoxy nucleotide termination method using a sequencing ABI Prism 3100 Genetic Analyzer. Pals2 was cloned using Necl-2 DNA clone that we isolated was identical to AF195662 (data not shown). The Necl-2 cDNA clone that we isolated was identical to AB052293 except for the deletion of 336–363 as at the extracellular region; data not shown, GenBank TM/EMBL/DBJ accession number AT351388). This deletion has been reported as one of the splicing variants (8).

**Construction of Plasmids**—Expression vectors were constructed in pFLAG-CMV1 (Sigma), pCAGIPuro (22), pCAGIgzeo (23), pGBD-C1 (24), pGEX4T-1 (Amersham Biosciences), pMAL-C2 (New England Biolabs Inc.), pGAD424-HA (25), pFastBac1-Msp-Fc (26), and pCMV-HA (27). Various constructs of Necl-1 and -2 contained the following aa: pFLAG-CMV1-Necl-1, aa 20–396 (deleting the signal peptide); pCAGIPuro-FLAG-Necl-1, aa 20–396 (including the preprotrypsin signal peptide); pCAGIgzeo-Necl-2, aa 2–417 (full-length); pFLAG-CMV1-Necl-2, aa 43–417 (deleting the signal peptide); pCAGIgIcuro-FLAG-Necl-2, aa 43–417 (including the preprotrypsin signal peptide); pCAGIguro-Necl-2 ΔC, aa 1–413 (deleting the C-terminal 4 aa); pFastBac1-Msp-Fc-Necl-2 EC, aa 43–438 (the extracellular region deleting the signal peptide); pGBD-C1-Necl-2 ΔEC, aa 335–417 (deleting the extracellular region); pGEX4T-1-Necl-2 CP, aa 372–417 (the cytoplasmatic region); pGEX4T-1-Necl-2 CP, aa 372–417 (the cytoplasmatic region deleting the C-terminal aa); the cDNA of mouse Pals2 was kindly provided by Dr. B. Margolis (University of Michigan Medical Center, Ann Arbor, MI). Constructs of mouse Pals2 contained the following aa: pCMV-HA-Pals2, aa 1–539 (full-length); pMAL-C2-Pals2, aa 1–539 (full-length); pMAL-C2-Pals2-PDZ, aa 361–639 (PDZ domain). Various constructs of necl-2 and I-afadin contained the following aa: pGEX4T-1-necl-2 CP, aa 387–467 (the cytoplasmatic region); pGBD-C1-necl-2 CP, aa 387–467 (the cytoplasmatic region); pGBD-C2-afadin-PDZ, aa 1007–1125 (PDZ domain); and pGAD424-HA-1-afadin, aa 1–1829 (full-length) (25, 28). The IgG Fc fusion proteins were prepared as a secreted protein from the baculovirus expression system (Invitrogen) and purified by use of protein A-Sepharose beads (Amersham Biosciences) as described (29). The glutathione S-transferase (GST) and maltose-binding protein (MBP) fusion proteins were purified by use of glutathione-Sepharose beads (Amersham Biosciences) and amylose resin beads (New England Biolabs, Inc.).

**Cell Culture and Establishment of Transfectants**—L and MTD-1A cells were kindly supplied by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). L and MTD-1A cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. L cells lines stably expressing human nectin-1o, mouse nectin-2o, mouse nectin-3a, or mouse Nect-5 (nectin-1L, -2L, -3L, and -5L cells, respectively) were prepared as described (6, 22, 28, 29). An L cell line stably expressing FLAG-Necl-1 (Necl-1-L cells), full-length Necl-2 (non-tagged Necl-2-L cells), and FLAG-Necl-2 C-terminal (Necl-2C-L cells) was obtained by transfection with pCAGIguro-FLAG-Necl-1, pCAGIgzeo-Necl-2, pCAGIguro-FLAG-Necl-2, or pCAGIguro-Necl-2 ΔC, respectively, using LipofectAMINE PLUS reagent (Invitrogen). We mostly used Necl-2-L cells (FLAG-tagged Necl-2) in the present study, but the essentially similar results were obtained with non-tagged Necl-2-L cells.

**Antibodies**—A rat anti-Necl-2 monoclonal Ab (mAb) (1C4-2) was raised against the fusion protein of the extracellular region of Necl-2 (aa 43–348) with IgG Fc. The anti-Necl-2 mAb was used for both Western blotting and immunostaining. An anti-I-afadin polyclonal antibody (pAb) was prepared as described (30). An anti-GF Fab mAb (M1) and pAb were purchased from Sigma. Anti-ZO-1 mAb was purchased from Azam Laboratories Inc., respectively. An anti-HA mAb was purchased from Berkeley Antibody Co.

**Yeast Two-hybrid Screening**—The yeast two-hybrid library constructed from mouse testis cDNA was purchased from Clontech, and a Necl-2-binding protein(s) was screened using pGBD-C1-Necl-2 ΔEC as bait as described (24). Two-hybrid screening using the yeast strain JY69-4A (MATα trp1-901 leu2-3, 112 ura3-52 his3-b200 gal4a gal80 GAL2-ADE2 LYS2: GAL1-HIS3 met2: GAL7-lacZ) was done as described (24).

**Procedures**—Immunofluorescence microscopy of cultured cells and coinoculation assay were done as described (28). The cell aggregation assay, chemical cross-linking, and affinity chromatography were done as described (29). Immunoelectron microscopy of mouse tissues was done using the silver enhancement technique as described (31). SDS-PAGE was done as described (32). Protein concentrations were determined with bovine serum albumin as a reference protein as described (33).

**RESULTS**

**Ca2 ++-independent Homophilic Cell-Cell Adhesion Activity of Necl-2—**TSLC1 and SynCAM1, which are identical with Necl-2, have been shown to have Ca2 ++-independent homophilic cell-cell adhesion activity by aggregation assay using Madin-Darby canine kidney (MDCK) cells expressing green fluorescence protein (GFP)-tagged TSLC1 (17) and Drosophila S2 cells expressing SynCAM1 (8). We first confirmed these earlier re-
sults by measuring the aggregation activity of Necl-2-L cells (cadherin-deficient L cells stably expressing Necl-2). Wild-type L cells endogenously expressed nectin-1 and -2 (22, 26), but expression of Necl-2 was undetectable by Western blotting (see Fig. 5A). Wild-type L cells did not form visible cell aggregates (Fig. 1, Aa), but Necl-2-L cells formed aggregates (Fig. 1, Ab).

The sizes of the aggregates were not significantly affected by the presence of Ca²⁺/H₁₁₀₀₁ or EDTA (data not shown). These results are consistent with the earlier observation (8, 17) and indicate that Necl-2 has Ca²⁺/H₁₁₀₀₁-independent homophilic cell-cell adhesion activity. This cell-cell adhesion activity of Necl-2 was furthermore confirmed by immunofluorescence microscopy. When Necl-2-L cells were cultured, the immunofluorescence signal for Necl-2 was concentrated at cell-cell contact sites (Fig. 1B).

We have previously shown that each nectin forms cis-dimers followed by formation of trans-dimers, eventually causing cell-cell adhesion (22). Similarly, Necl-2 formed cis-dimers (Fig. 1C). These results are consistent with the earlier observation using HEK293 cells expressing TSLC1 (17). It is likely by analogy with the mode of action of nectins that Necl-2 forms first cis-dimers followed by formation of trans-dimers, eventually causing cell-cell adhesion.

**Ca²⁺-independent Heterophilic Cell-Cell Adhesion Activity of Necl-2**—Nectins show both homophilic and heterophilic cell-cell adhesion activities (5). We next examined by the cell aggregation assay whether Necl-2 shows heterophilic cell-cell adhesion activity with other nectins and Necls. Necl-2-L cells formed heterophilic cell aggregates with L cells stably expressing Necl-1 or nectin-3 (Necl-1-L and nectin-3-L cells, respectively) but not with L cells stably expressing Necl-5, nectin-1, or nectin-2 (Necl-5-L, nectin-1-L, or -2-L cells, respectively) (Fig. 2, A–E). Necl-1-L cells formed homophilic cell aggregates (Fig. 2F). The sizes of the aggregates were not significantly affected by the presence of Ca²⁺ or EDTA (data not shown). The detailed properties of Necl-1 will be described elsewhere. Necl-5-L cells did not form homophilic cell aggregates as described (Fig. 2G) (6). Nectin-1-L, -2-L, and -3-L cells formed homophilic cell aggregates as described (Fig. 2, H–J) (28, 29). The size of the cell aggregates formed between nectin-1-L and nectin-3-L cells was the biggest among various combinations (5). The sizes of the cell aggregates formed between Necl-2-L and Necl-2-L cells, between Necl-2-L and Necl-1-L cells, and between Necl-
2-L and nectin-3-L cells were about 5, 10, and 10% that of the aggregates formed between nectin-1-L and nectin-3-L cells, respectively. These results indicate that Necl-2 has both Ca\textsuperscript{2+}-independent homophilic and heterophilic cell-cell adhesion activities.

**Tissue Distribution and Subcellular Localization of Necl-2—** TSLC1 has been shown by Northern blotting to be expressed ubiquitously, except the skeletal muscle, in which the expression of TSLC1 was not detected (11, 19). We confirmed these earlier results by Western blotting using the anti-Necl-2 mAb. Western blotting showed that an immunoreactive band at a molecular mass of 92 kDa was detected in various tissues thus far examined, including the brain, the lung, and the kidney (Fig. 3). In the testis, a larger band at 105 kDa was detected and might be one of the alternative splicing variants as described (8). After long exposure, the immunoreactive band of Necl-2 was detected in other tissues including the heart, the spleen, and the liver but was not detected in the skeletal muscle (data not shown). Immunofluorescence microscopy revealed that the signal for Necl-2 was highly concentrated at the basolateral plasma membrane of the epithelial cells of the mouse gall bladder, liver, and pancreas (Fig. 4A, a1, b1, and c1). It may be noted that the signal for Necl-2 was not overlapped with the signal for afadin, which is known to be confined to AJs undercoated with the F-actin bundles (Fig. 4A, a1–a3), nor the signal for ZO-1, which is known to be confined to TJs (Fig. 4A, b1–b3 and c1–c3). Consistently, immuno electron microscopy showed that the immunogold particles for Necl-2 were indeed concentrated at the basolateral plasma membrane of the epithelial cells of gall bladder, but was undetectable at the areas of TJs, AJs, and DSs (Fig. 4B, a–c). These findings about Necl-2 showed sharp contrast to the localization of nectins and afadin that are strictly confined to AJs, which are undercoated with F-actin bundles (5).

**Expression of Necl-2 in an Epithelial Cell Line, but Not in Fibroblast Cell Lines**—We next examined whether Necl-2 is differentially expressed in cells in culture. Western blotting revealed that Necl-2 was detected in mouse MTD-1A epithelial cells but not in mouse fibroblastic L, NIH3T3, or Swiss3T3 cells (Fig. 5A). The larger protein of 105 kDa detected in MTD-1A cells appeared to be an alternative splicing variant of Necl-2 as detected in the testis. In MTD-1A cells the immunofluorescence signal for Necl-2 was concentrated at the basolateral plasma membrane of cell-cell contact sites (Fig. 5B, a1–a3 and b1–b3). In contrast, the signal for Necl-2 was not observed in L, NIH3T3, or Swiss3T3 cells (data not shown). These results suggest that Necl-2 is dominantly expressed in epithelial cells but not in fibroblasts.
FIG. 5. Expression of Necl-2 in an epithelial cell line, but not in fibroblast cell lines. A, expression levels of the Necl-2 protein in cell lines. Cell lysates of various cell lines (each 10 μg of protein) were subjected to SDS-PAGE (10% polyacrylamide gel) followed by Western blotting with the anti-Necl-2 mAb. B, localization of Necl-2 in MTD-1A cells. The samples were doubly stained with the anti-Necl-2 mAb and rhodamine-phalloidin. Confocal images of cells. The samples were doubly stained with the anti-Necl-2 mAb and rhodamine-phalloidin. A, spot-like adhesion sites; B, C, and D, line-like adhesion sites. Aa and Ba, actin; Ac, ZO-1; Da, 1-afadin; Ab, Bb, Cb, and Db, Necl-2; Ac, Bc, Cc, and Dc, merge. Arrows, adhesion sites. Bars, 10 μm. The results shown are representative of three independent experiments.

FIG. 6. Assembly of Necl-2 to cell-cell junctions at the initial stage of their formation. Confluent cell layers were manually scratched with a needle and cultured for 6 h (spot-like adhesion sites) and for 8 h (line-like adhesion sites) followed by double-staining with various combinations of the anti-Necl-2, anti-1-afadin, and anti-ZO-1 Abs and rhodamine-phalloidin. A, spot-like adhesion sites; B, C, and D, line-like adhesion sites. Aa and Ba, actin; Ac, ZO-1; Da, 1-afadin; Ab, Bb, Cb, and Db, Necl-2; Ac, Bc, Cc, and Dc, merge. Arrows, adhesion sites. Bars, 10 μm. The results shown are representative of three independent experiments.

and afadin (Fig. 6, Ca-Cc and Da-De). These results suggest that Necl-2 is assembled to the cell-cell adhesion sites at the very early stage together with the components of the nectin-afadin and E-cadherin-catenin units, and thereafter, is translocated to the other sites, presumably the extra-junctional region of the basolateral plasma membrane.

Direct Binding of Pals2 to Necl-2—Nectins except nectin-4 have a C-terminal conserved motif of four aa residues that interacts with the PDZ domain of afadin (5). Although Necl-2 has this motif, Necl-2 did not bind afadin, as estimated by the yeast two-hybrid assay and the affinity chromatography assay (Fig. 7, A and D). Therefore, in the last set of experiments we attempted to isolate a Necl-2-binding protein(s). By use of the transmembrane and cytoplasmic region of Necl-2 (Necl-2-SEC) as bait, we searched a Necl-2-binding protein(s) by the yeast two-hybrid screening and isolated one positive clone from a mouse testis library (Fig. 7A). It encoded Pals2 (lacking the N-terminal 1–31 aa). We then examined whether Necl-2 directly binds Pals2 in vitro and in vivo using the constructs of full-length Pals2. FLAG-tagged Necl-2 and HA-tagged Pals2 were coexpressed in HEK293 cells, and FLAG-tagged Necl-2 was immunoprecipitated with the anti-FLAG mAb. HA-tagged Pals2 was co-immunoprecipitated with FLAG-tagged Necl-2 (Fig. 7B). When HA-tagged Pals2 was overexpressed in Necl-2-L cells, the signal for Pals2 was co-localized with that for Necl-2 at the cell-cell contact sites (Fig. 7C, a1–a3). However, when HA-tagged Pals2 was overexpressed in Necl-2-ΔC-L cells (L cells stably expressing the C-terminal four aa-deleted Necl-2), the signal for Necl-2-ΔC was concentrated at the cell-cell contact sites, but the signal for Pals2 was not concentrated there (Fig. 7C, b1–b3). The pure recombinant protein of the cytoplasmic region of Necl-2 (GST-Necl-2-CP) bound the pure recombinant protein of full-length Pals2 (MBP-Pals2) (Fig. 7D) and the PDZ domain of Pals2 (MBP-Pals2-PDZ) (data not shown). However, the pure recombinant protein of the cytoplasmic region of Necl-2, of which the C-terminal four aa were deleted (GST-Necl-2-CPΔC), did not bind MBP-Pals2 (Fig. 7D) or MBP-Pals2-PDZ (data not shown). These results indicate that Necl-2 directly binds Pals2 and that this binding is mediated through the C-terminal four aa of Necl-2 and the PDZ domain of Pals2.

DISCUSSION

Studies on Necl-2-mediated cell aggregation using MDCK-cells expressing GFP-tagged TSLC1 and Drosophila S2 cells expressing SynCAM1 have shown that Necl-2 has Ca\textsuperscript{2+}-independent homophilic cell-cell adhesion activity (8, 17). We have confirmed here these earlier observations by the aggregation assay using L cells expressing each cell-cell adhesion molecule (17). Then we have shown that Necl-2 has furthermore Ca\textsuperscript{2+}-independent heterophilic cell-cell adhesion activity with Necl-1/TSLLI/SynCAM3 and nectin-3 but not with Necl-5/Tag/ CD155/PVR, nectin-1, or nectin-2, suggesting that Necl-2 is capable of organizing cell-cell adhesion by interactions with these molecules.
Necl-2 and Pals2

TSCL1 and SgIGSF have been shown to be expressed ubiquitously as estimated by Northern blotting (11, 19), but SynCAM1 has been shown to be specifically expressed in the mouse brain, as analyzed by Western blotting (8). Our present result indicates that Necl-2 is expressed in a wide variety of mouse tissues thus far examined and is consistent with the results of TSCL1 and SgIGSF but not with that of SynCAM1 (8). The exact reason for this inconsistency between the result of SynCAM1 and those of TSCL1, SgIGSF, and Necl-2 is not known, but it could be attributed to the specificity of the Ab used for SynCAM1.

Immunofluorescence microscopic analysis indicates that Necl-2 localizes at the basolateral plasma membrane of many epithelial cell types, and this result is consistent with the earlier observation that TSCL1 localizes at the basolateral plasma membrane of MDCK cells expressing GFP-tagged TSCL1 (17). Detailed analysis by immunoelectron microscopy indicates that Necl-2 localizes at the basolateral plasma membrane except for specialized cell-cell junctions, such as AJs, TJs, and DSs. This unique localization pattern of Necl-2 is quite different from those of any other known cell-cell adhesion molecules: claudins, occludin, and JAM at TJs, nectins at AJs, E-cadherin at AJs and the lateral plasma membrane, and desmocollin and desmoglein at DSs (1, 4, 5, 39, 40).

Nectin-3 has been shown to be involved in the formation of AJs in epithelial cells (5). Therefore, the ability of Necl-2 to interact with nectin-3 suggests that Necl-2 may be recruited to the nectin-3-based cell-cell adhesion in the process of forming AJs. Consistently, wound healing assay analysis using MTD-1A cells indicates that Necl-2 is assembled to the cell-cell adhesion sites at the very early stage together with the components of the nectin-afadin and E-cadherin-catenin units. After Necl-2 is assembled to the primordial cell-cell adhesion sites, it may be translocated from there to the extra-junctional region of the basolateral plasma membrane. Nectins are confined to AJs undercoated with F-actin bundles and are absent from the areas where Necl-2 localizes (5). Therefore, Necl-2 is likely to form homodimers at these areas lacking nectins. The mechanism of segregation of Necl-2 from nectin-3 at the plasma membrane is currently unknown, but it is of crucial importance for our understanding of how the membrane domains of epithelial cells are organized.

It has been shown that TSCL1 binds DAL-1 through a band 4.1-binding motif at the juxtamembrane region (18). We have shown here that Necl-2 does not bind afadin but directly binds Pals2. This binding is mediated through the C-terminal consensus motif of four aa of Necl-2 and the PDZ domain of Pals2. Thus, Necl-2 appears to bind both DAL-1 and Pals2. DAL-1 belongs to the band 4.1 family, connects TSCL1/Necl-2 to the actin cytoskeleton (18), and is known as a tumor suppressor (41). In contrast, Pals2 is a membrane-associated guanylate kinase family member and binds to Lin-7, of which the C. elegans homologue is implicated in the proper localization of the Let-23 protein, the homologue of mammalian epidermal growth factor receptor (42). Taken together, Necl-2 directly binds to Necl-1 and nectin-3 extracellularly, mediating cell adhesions, and also binds Pals2 intracellularly, mediating localization of transmembrane proteins. Further studies would be necessary for our understanding of the physiological role of Necl-2, which regulates cell-cell adhesion and localization of transmembrane proteins in mammals.

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