Cell polarity is fundamental not only for cell functions but also for development and tissue maintenance (1, 2). Studies in mammalian epithelial cells have revealed that establishment of cell polarity depends upon cell adhesion, intracellular signaling, cytoskeletal organization, and protein sorting. Mechanisms of establishment of cell polarity have been studied most extensively in Caenorhabditis elegans and Drosophila (3). PAR proteins, PAR-1 to -6, were first identified in C. elegans as indispensable proteins that are involved in establishment of the anterior-posterior cell polarity of the one-cell embryo (4). Thereafter, homologous proteins have been identified in Drosophila and mammals (5). In mammals, PAR-3, a mammalian homologue of the par-3 gene product, was first identified as an atypical protein kinase C (aPKC)β-binding protein (6).

PAR-3 localizes at tight junctions (TJs) and forms a ternary complex with PAR-6 and aPKC in mammalian epithelial cells (5). These three proteins directly interact with each other and play a critical role in the apico-basal polarization of mammalian epithelial cells (5). This PAR-3-aPKC-PAR-6 complex is an evolutionarily conserved cell polarization machinery that works ubiquitously in a variety of biological context from warm embryos to differentiated mammalian cells (5). PAR-3 has recently been shown to be directly bound to junctional adhesion molecule-1 (JAM-1), a Ca2+-independent immunoglobulin (Ig)-like cell-cell adhesion molecule that localizes at TJs in epithelial cells (7, 8). PAR-3 has three PDZ domains and one aPKC-binding domain (6), and this binding to JAM-1 is mediated through the first PDZ domain of PAR-3 and the C-terminal four aa (amino acids) of JAM-1 (7). The PAR-3-aPKC-PAR-6 complex is tethered to TJs through its binding to JAM-1. At TJs, Claudin is a key cell-cell adhesion molecule which forms TJ strands (9). Claudin is associated with the actin cytoskeleton through ZO-1. ZO-1 furthermore binds both to the C-terminal four aa of JAM-1 through the third PDZ domain of ZO-1 (8) and to the cytoplasmic tail of Claudin through the first PDZ domain of ZO-1 (10). Through these interactions, JAM-1 might be recruited and tethered to TJ strands.

In epithelial cells, TJs constitute a junctional complex with adherens junctions (AJs) and desmosomes (9). These junctional structures are typically aligned from the apical to the basal side, although desmosomes are independently distributed in other areas. The formation and maintenance of TJs are dependent on the cell-cell adhesion activity of E-cadherin. E-Cadherin is a key Ca2+-dependent cell-cell adhesion molecule at AJs (11, 12). E-Cadherin is associated with the actin cytoskeleton through peripheral membrane proteins, including α-, β-, and γ-catenins, α-actinin, and vinculin (11, 12). Nectin and afadin constitute another cell-cell adhesion unit at cell-cell AJs (13). Nectin is a Ca2+-independent Ig-like cell-cell adhesion molecule which comprises a family of four members, nectin-1, -2, -3, and -4. Nectins are associated with the actin cytoskeleton at TJs in neuroepithelial cells of the embryonic telencephalon. Nectin comprises a family of four members, nectin-1, -2, -3, and -4. Nectins are associated with the actin cytoskeleton through afadin, of which the PDZ domain binds to nectins through their C-terminal four amino acids. We show here that PAR-3 binds to nectin-1 and -3 in neuroepithelial cells of the embryonic telencephalon, which are equipped with AJs, but not with typical TJs. Nectin-1, -2, -3, and afadin, but not JAM-1, were concentrated at AJs in neuroepithelial cells of the embryonic telencephalon at E13.5 and PAR-3 co-localized with nectins. PAR-3 was co-immunoprecipitated with nectin-1 and -3, but not with nectin-2 or JAM-1, from the mouse whole brain at E13.5. Recombinant PAR-3 stoichiometrically bound to recombinant nectin-1 and -3. The first one of the three PDZ domains of PAR-3 bound to the C-terminal four amino acids of nectin-1 and -3. The affinities of PAR-3 and afadin for nectin-1 and -3 were similar. Cadherin-deficient L cells expressing nectin-1 and -3 formed nectin-1- and -3-based cell-cell junctions, respectively, where PAR-3 as well as afadin was recruited. These results indicate that nectin-1 and -3 are involved in the localization of PAR-3 at AJs in the neuroepithelial cells of the embryonic telencephalon.

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The abbreviations used are: aPKC, atypical protein kinase C; TJs, tight junctions; JAM-1, junctional adhesion molecule-1; Ig, immunoglobulin; aa, amino acid(s); AJs, adherens junctions; Ab, antibody; mAb, monoclonal antibody; pAb, polyclonal antibody; GST, glutathione S-transferase; MBP, maltose-binding protein; PBS, phosphate-buffered saline.
domains and connects nectin to the actin cytoskeleton. The nectin-afadin unit has roles in the organization of E-cadherin-based AJs and claudin-based TJs in epithelial cells (14–17), in the organization of AJ-s in fibroblasts (14, 15, 17), in the formation of synapses in neurons (18), and in the formation of Sertoli cell-spermatids junctions in the testis (19), in cooperation with or independently of cadherin.

To determine the localization of the PAR-3-aPKC-PAR-6 complex at TJs in epithelial cells, this complex localizes at AJs in fibroblasts (6) and in the neuroepithelial cells of the embryonic telencephalon during E10.5 to E14.5 (20). The localization of nectins, afadin, ZO-1, occludin, and JAM-1 at AJs, which are highly concentrated at the lumenal surface of neuroepithelial cells (21, 22), and the mechanism of the localization of the PAR-3-aPKC-PAR-6 complex remains unknown in this cell type. We show here that nectin-1 and -3, but not JAM-1, are involved in the localization of PAR-3 at AJs in the neuroepithelial cells of the embryonic telencephalon.

EXPERIMENTAL PROCEDURES

Antibodies—Rat anti-nectin-1, -2, and -3 monoclonal antibodies (mAbs) were prepared as described (18, 23, 24). Rabbit anti-nectin-1a and -3a polyclonal antibodies (pAbs) were prepared as described (23, 24). A rabbit anti-afadin pAb and a mouse anti-afadin mAb were prepared as described (25, 26). A rabbit anti-PAR-3 pAb was kindly provided by Dr. S. Ohno (Yokohama City University, Yokohama, Japan) (6). A rabbit anti-occludin pAb was purchased from Zymed Laboratory Inc. A mouse anti-ZO-1 mAb was purchased from Sanko-junyaku. A rabbit anti-PAR-3 pAb was kindly provided by Dr. M. Aurrand-Lions (27).

Construction and Purification—pCAGI PurO-nectin-3aCP, containing aa 1–545 (deletion of the C-terminal four aa residues), was constructed according to the standard molecular biology methods (28). Prokaryote expression vectors were constructed in pGEX (Amersham Biosciences) and pMAL-C2 (New England Biolabs Inc.). Various constructs of mouse nectin-1a, -2a, and -3a and rat afadin containing the following aa: glutathione S-transferase (GST-nectin-1a-CP, aa 431–515; GST-nectin-1a-CPAC, aa 431–511; GST-nectin-2a-CP, aa 387–467; GST-nectin-3a-CP, aa 433–549; GST-nectin-3a-CPAC, aa 433–545; and maltose-binding protein (MBP)-afadin-PDZ, aa 1067–1125 (PDZ domain) (23, 24). The cDNA of rat PAR-3 was kindly provided by Dr. S. Ohno (Yokohama City University, Yokohama, Japan). Various constructs of mouse PAR-3 containing the following aa: MBP-PAR-3-CPDI-3, aa 257–717; MBP-PAR-3-CPDI2-3, aa 257–460; MBP-PAR-3-CPDZ2, aa 360–590; and MBP-PAR-3-CPDZ3, aa 534–717. The GST and MBP fusion proteins were expressed in RosettaBlue (Novagen) and purified by the use of glutathione-Sepharose beads (Amersham Biosciences) and amylose resin beads (New England Biolabs Inc.), respectively.

Cell Culture and Establishment of Transformants—L cells were kindly supplied by Dr. T. Tsukita (Kyoto University, Kyoto, Japan). L cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Various L cell lines, nectin-1a-L, -1aΔC-L, -2a-L, and -3a-L cells were prepared as described (14, 23, 24). An L cell line stably expressing C-terminal four-aa-deleted nectin-3a (nectin-3aΔC-L cells) was also similarly prepared with pCAGI PurO-nectin-3aΔC.

Immunoprecipitation—The whole brain was dissected from mouse embryo at E13.5, and the sample was incubated in 0.75 m M dithiothreitol (DTT) for 5 min and subjected to SDS-PAGE, followed by Western blotting.

Affinity Chromatography—To determine the binding domains of PAR-3 and nectin-1a, -2a, or -3a, various MBP-fusion fragments of PAR-3 (200 pmol each) were immobilized on amylose resin beads. GST-nectin-1a-CP, -3a-CPAC, -1a-CP, -3a-CP, or -3a-CP (2 nmol each) was applied to the MBP-fusion protein-immobilized beads equilibrated with PBS containing 0.1% Triton X-100. After the beads were extensively washed with the same buffer, elution was performed with the same buffer containing 10 mM maltose. The eluate was then subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue.

To compare the affinities of PAR-3 and afadin for nectin-1a or -3a, GST-nectin-1a-CP or -3a-CP (200 pmol) was immobilized on glutathione-Sepharose beads. Various amounts (200 pmol or 2 nmol) of MBP-PAR-3-CPDI-3 and MBP-afadin-PDZ were mixed and applied to the GST fusion protein-immobilized beads equilibrated with PBS containing 0.1% Triton X-100. After the beads were extensively washed with the same buffer, elution was performed with the same buffer containing 20 mM glutathione. The eluate was then subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue.

RESULTS

In neuroepithelial cells of the embryonic telencephalon at E13.5, the immunofluorescence signals for nectin-1, -2, and -3 and afadin were highly concentrated at the luminal surface of neuroepithelial cells (Fig. 1, A–C). All of these signals apparently co-localized. The signal for PAR-3 colocalized with the signal for nectin-3 (Fig. 1D). The signals for ZO-1 and β-catenin colocalized with the signal for afadin as described (20) (data not shown). However, the signal for occludin or JAM-1 was not detected (Fig. 1, E and F).

We then examined by the immunoprecipitation assay whether PAR-3 binds to nectins in the neuroepithelial cells of the embryonic telencephalon at E13.5. When PAR-3 was immunoprecipitated by its Ab from the embryonic mouse whole brain at E13.5, including telencephalon, nectin-1 and -3, but not nectin-2 or JAM-1, were co-immunoprecipitated with PAR-3 (Fig. 2A). These results suggest that PAR-3 binds to nectin-1 and -3 in the neuroepithelial cells of the embryonic telencephalon.

It has been shown that AJs, but not typical TJs, are observed in neuroepithelial cells (21, 22). Consistently, AJs, which are undercoated with actin filament bundles, but not typical TJs,
were observed (Fig. 3). Immunogolds of nectin-3, PAR-3, and afadin localized at AJs. Immunogolds of nectin-1 also localized at AJs (data not shown). These results, together with the result obtained by the co-immunoprecipitation experiment, suggest that PAR-3 binds to nectin-1 and -3 and co-localize with them at AJs of neuroepithelial cells of the embryonic telencephalon.

We next examined whether PAR-3 directly binds to nectin-1 and -3, using the recombinant PAR-3 fragment containing all three PDZ domains (PAR-3-PDZ1–3) and the cytoplasmic tails of recombinant nectin-1, -2, and -3 (nectin-1-CP, -2-CP, and -3-CP, respectively). PAR-3-PDZ1–3 stoichiometrically bound to nectin-3-CP (Fig. 2B). PAR-3-PDZ1–3 also stoichiometrically bound to nectin-1-CP, but not to nectin-2-CP (data not shown). These results indicate that the binding of PAR-3 to nectin-1 and -3 is direct. We then examined the binding sites of PAR-3 and nectin-1 and -3. We prepared pure samples of each PDZ domain of recombinant PAR-3 and the C-terminal four aa-deleted cytoplasmic tails of nectin-1 and -3 (nectin-1CPΔC and -3CPΔC, respectively). PAR-3-PDZ1–3 did not bind to nectin-3-CP, while PAR-3-PDZ1–3 bound to nectin-1-CP and nectin-3-CP (Fig. 2C). Thus, both afadin and PAR-3 bind to the C-terminal four aas of nectin-1 and -3.

Thus, both afadin and PAR-3 bind to the C-terminal four aa of nectin-1 and -3. We therefore compared the affinities of afadin and PAR-3 for nectin-1 and -3. Various amounts of either afadin or PAR-3 alone or both were incubated with nectin-3-CP. When the same amounts of afadin and PAR-3 were mixed with nectin-3-CP, apparently the same amounts of afadin and PAR-3 bound to it, and 1 mol of each maximally bound to 2 mol of nectin-3-CP (Fig. 2D). The essentially same results were obtained for nectin-1 (data not shown). These results indicate that the first PDZ domain of PAR-3 directly binds to the C-terminal four aa of nectin-1 and -3.

In the last set of experiments, we confirmed whether PAR-3 binds to nectin-1 and -3 in an intact cell assay system, using cadherin-deficient L cells stably expressing full-length nectin-1 or -3 (nectin-1-L or -3-L cells, respectively) or the C-terminal four aa-deleted nectin-1 or -3 (nectin-1ΔC-L or -3ΔC-L cells, respectively). Nectin-3-L cells and nectin-3-L cells formed cell-cell junctions, and PAR-3 was concentrated there (Fig. 4). Afadin was also concentrated there (data not shown). Nectin-3ΔC-L cells and nectin-3ΔC-L cells formed cell-cell junctions, but PAR-3 was not concentrated there. Afadin was not concent-

Fig. 2. Direct binding of PAR-3 to nectin-3. A, co-immunoprecipitation of nectin-3 with PAR-3. A tissue extract from the whole brain of mouse embryo at E13.5, including telencephalon, was subjected to immunoprecipitation with the anti-PAR-3 pAb or the control IgG. The immunoprecipitates were then subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti-PAR-3 pAb, the anti-nectin-1 pAb, the anti-nectin-2 pAb, or the anti-JAM-1 pAb. Lane 1, the anti-PAR-3 pAb; lane 2, the control IgG.

B, direct binding of PAR-3 to nectin-3 through the C-terminal four aa of nectin-3. GST-nectin-3-CP, GST-nectin-3ΔC-CP, or GST-nectin-3ΔC-CP was applied to the MBP-PAR-3-PDZ1–3-immobilized beads. After the beads were extensively washed, the bound proteins were subjected to SDS-PAGE (10% polyacrylamide gel), followed by protein staining with Coomassie Brilliant Blue. Lane 1, GST-nectin-3a-CP; lane 2, GST-nectin-3a-CP, arrowheads, recombinant proteins of MBP-PAR-3.

C, affinities of PAR-3 and afadin for nectin-3a. Various combinations of the mixtures of MBP-PAR-3-PDZ1–3 and MBP-afadin-PDZ were applied to the GST-nectin-3a-CP-immobilized beads. After the beads were extensively washed, the bound proteins were subjected to SDS-PAGE (10% polyacrylamide gel), followed by protein staining with Coomassie Brilliant Blue. Lane 1, GST-nectin-3a-CP; lane 2, GST-nectin-3a-CP, arrowheads, recombinant proteins of MBP-PAR-3.

D, affinities of PAR-3 and afadin for nectin-3a. Various combinations of the mixtures of MBP-PAR-3-PDZ1–3 and MBP-afadin-PDZ were applied to the GST-nectin-3a-CP-immobilized beads. After the beads were extensively washed, the bound proteins were subjected to SDS-PAGE (10% polyacrylamide gel), followed by protein staining with Coomassie Brilliant Blue. Lane 1, GST-nectin-3a-CP; lane 2, GST-nectin-3a-CP, arrowheads, recombinant proteins of MBP-PAR-3.

Fig. 3. Recruitment of PAR-3 to the nectin-3a-based cell-cell junctions in L cells. Nectin-3-L cells and nectin-3ΔC-L cells were double stained with the anti-nectin-3 mAb and the anti-PAR-3 pAb. Bars, 10 µm. The results shown are representative of three independent experiments.

Fig. 4. Recruitment of PAR-3 to the nectin-3a-based cell-cell junctions in L cells. Nectin-3a-L and nectin-3aΔC-L cells were double stained with the anti-nectin-3 mAb and the anti-PAR-3 pAb. Bars, 10 µm. The results shown are representative of three independent experiments.

The localization of nectin-3, PAR-3, and afadin at the neuroepithelial cells of the embryonic telencephalon at E13.5 was analyzed by immunoelectron microscopy. A, nectin-3; B, PAR-3; C, afadin; A1, B1, and C1, low magnification; A2, B2, and C2, high magnification. Bars, 300 nm. The results shown are representative of three independent experiments.

3-CPAC under the conditions where PAR-3-PDZ1–3 bound to nectin-3-CP (Fig. 2B). The first PDZ domain of PAR-3 bound to nectin-3-CP (Fig. 2C). PAR-3-PDZ1–3 did not bind to nectin-1-CPΔC under the conditions where PAR-3-PDZ1–3 bound to nectin-3-CP (Fig. 2B). The first PDZ domain of PAR-3 bound to nectin-3-CP (Fig. 2C). PAR-3-PDZ1–3 did not bind to nectin-1-CPΔC under the conditions where PAR-3-PDZ1–3 bound to nectin-1-CP, and the first PDZ domain bound to nectin-1-CP (data not shown). These results indicate that the first PDZ domain of PAR-3 directly binds to the C-terminal four aa of nectin-1 and -3.

Thus, both afadin and PAR-3 bind to the C-terminal four aa of nectin-1 and -3. We therefore compared the affinities of afadin and PAR-3 for nectin-1 and -3. Various amounts of either afadin or PAR-3 alone or both were incubated with nectin-3-CP. When the same amounts of afadin and PAR-3 were mixed with nectin-3-CP, apparently the same amounts of afadin and PAR-3 bound to it, and 1 mol of each maximally bound to 2 mol of nectin-3-CP (Fig. 2D). The essentially same results were obtained for nectin-1 (data not shown). These results indicate that the affinities of PAR-3 and afadin for nectin-1 and -3 were apparently similar.

In the last set of experiments, we confirmed whether PAR-3 binds to nectin-1 and -3 in an intact cell assay system, using cadherin-deficient L cells stably expressing full-length nectin-1 or -3 (nectin-1-L or -3-L cells, respectively) or the C-terminal four aa-deleted nectin-1 or -3 (nectin-1ΔC-L or -3ΔC-L cells, respectively). Nectin-3-L cells and nectin-3-L cells formed cell-cell junctions, and PAR-3 was concentrated there (Fig. 4). Afadin was also concentrated there (data not shown). Nectin-3ΔC-L cells and nectin-3ΔC-L cells formed cell-cell junctions, but PAR-3 was not concentrated there. Afadin was not concent-
trated there, either (data not shown). Nectin-1-L cells and nectin-1-L cells formed cell-cell junctions, and PAR-3 as well as afadin was concentrated there (data not shown). Nectin-1L cells and nectin-1ΔC-L cells formed cell-cell junctions, but PAR-3 or afadin was not concentrated there (data not shown).

Nectin-3 forms not only homo-trans-dimers with nectin-3 but also hetero-trans-dimers with nectin-1 and -2 (19). PAR-3 as well as afadin was concentrated at the junctions formed between nectin-3-L cells and L cells stably expressing nectin-1 or -2 (data not shown). These results are consistent with the above biochemical results and indicate that PAR-3 binds to nectin-1 and -3 in intact cells.

**DISCUSSION**

We have shown here that PAR-3 directly binds to nectin-1 and -3 and localizes with it at TJs in epithelial cells (7, 8). In the neuroepithelial cells of the embryonic telencephalon, TJs are not well developed, and we could not significantly observe the immunofluorescence signal for occcludin or JAM-1 at the cell-cell junction area. Furthermore, JAM-1 was not co-immunoprecipitated with PAR-3 under the conditions where nectin-1 and -3 were co-immunoprecipitated with PAR-3 from the embryonic mouse whole brain at E13.5 including telencephalon. It has been shown that PAR-3 forms a ternary complex with aPKC and PAR-6 (5) and that this complex localizes at AJs in the neuroepithelial cells of the embryonic telencephalon (20). Taken together, it is likely that the PAR-3-aPKC-PAR-6 complex localizes at AJs in the neuroepithelial cells by directly binding of PAR-3 to nectin-1 and -3. The physiological function of this direct binding remains unknown, but it may recruit aPKC and PAR-6 and play a role in the formation and maintenance of polarity of the neuroepithelial cells.

We have shown here that all nectin-1, -2, and -3 localize at AJs of the neuroepithelial cells of the embryonic telencephalon, but that the first PDZ domain of PAR-3 directly binds to the C-terminal four aa of nectin-1 and -3, but not to that of nectin-2. All nectin-1, -2, and -3 have a C-terminal consensus motif with four aa for the binding to PDZ domains and bind afadin. The aa sequences of the motif of nectin-1 and -3 are the same, whereas that of nectin-2 is different from them (24). This difference may determine the specific binding of PAR-3 to nectin-1 and -3.

All nectins directly bind afadin, and this binding is mediated through the PDZ domain of afadin and the C-terminal four aa of nectins (13). This paper demonstrates for the first time that nectin-1 and -3 directly bind not only afadin but also PAR-3. We have shown here that the affinities of PAR-3 and afadin for nectin-1 and -3 are roughly similar and that 1 mol of each afadin and PAR-3 binds to 2 mol of nectin-1 and -3. All nectins first form cis-dimers, followed by the formation of trans-dimers, eventually inducing cell-cell adhesion (13). It remains unknown whether both afadin and PAR-3 bind to the same cis-dimer or either afadin or PAR-3 alone binds to the different cis-dimer.

Nectin-1 and -3 are expressed in many cell types in addition to the neuroepithelial cells (13). They may be involved in the localization of the PAR-3-aPKC-PAR-6 complex in cells where JAM-1 is not expressed. We have previously shown that the nectin-afadin unit plays a role in the localization of JAM-1 at TJs (16), where the PAR-3-aPKC-PAR-6 complex is associated with JAM-1. Another possible function of nectin-1 and -3 is that PAR-3 first binds to nectin-1 or -3 and then transferred to JAM-1 during the organization of the junctional complex in the epithelial cells equipped with TJs. Further studies are necessary for our understanding of the physiological role of the direct binding of PAR-3 to nectin-1 and -3 in the neuroepithelial cells and other cell types.

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Direct Binding of Cell Polarity Protein PAR-3 to Cell-Cell Adhesion Molecule Nectin at Neuroepithelial Cells of Developing Mouse
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