MAGI1 Recruits Dll1 to Cadherin-based Adherens Junctions and Stabilizes It on the Cell Surface*

Received for publication, January 11, 2005, and in revised form, May 16, 2005
Published, JBC Papers in Press, May 20, 2005, DOI 10.1074/jbc.M500375200

Eri Mizuhara†, Tomoya Nakatani‡, Yasuko Minaki‡, Yoshimasa Sakamoto‡, Yuichi Ono¶, and Yoshimi Takaï†

From the ¶KAN Research Institute Inc., 93 Chudoji-Awata-cho, Shimogyo-ku, Kyoto 600-8815, Japan and \textdegree Departement of Molecular Biology and Biochemistry, Faculty of Medicine, Osaka University Graduate School of Medicine, Suita 565-0871, Japan

Delta-Notch signaling plays an essential role in cell fate determination in many tissue types, including the central nervous system. Although the signaling mechanism of Notch has been extensively studied, the behaviors of its ligands are not well understood. In the present study, we found that, in the developing neural tube, Dll1 (Delta-like 1) was mainly localized on the processes extending from nascent neurons toward both the pia and the ventricle and accumulated at apical termini, where adherens junctions (AJs) were formed. To understand the mechanism of Dll1 localization, we searched for binding proteins for Dll1 and identified a scaffolding molecule, MAGI1. In the developing spinal cord, MAGI1 mRNA was highly expressed in the ventricular zone, where Dll1 mRNA was expressed. MAGI1 protein accumulated at the AJs formed around the termini of apically extending processes and was partially colocalized with Dll1. MAGI1 bound not only to Dll1 but also to N-cadherin-\(\beta\)-catenin complexes. In cultured AJ-forming fibroblasts, MAGI1 was localized at AJs, and Dll1 was recruited to these AJs through binding to MAGI1. In addition, Dll1 was stabilized on the cell surface by MAGI1. Taken together, these results suggest that Dll1 is presented on the surface of AJs formed at the apical termini of processes through interaction with MAGI1 to activate Notch on neighboring cells in the developing central nervous system.

ML and differentiates into mature neurons (4). These migrating precursor cells in the VZ transiently express Notch ligand molecules, such as Dll1 (Delta-like 1) and Jagged1, to activate Notch receptors expressed on the neighboring progenitors (5–9). Activation of Notch receptors mediated by the downstream target genes, such as the Hes family basic helix-loop-helix transcriptional repressors, prevents the cells from undergoing neuronal differentiation by repressing proneural basic helix-loop-helix factors, such as Mash1 (1, 3). In addition to these “lateral inhibition” functions, constructive roles of Notch signaling in gliogenesis have also been reported (10, 11).

The mechanism of signal transduction by the Notch pathway has been extensively studied. Ligand binding on the cell surface leads to proteolytic cleavage of the Notch receptor at an extracellular region by tumor necrosis factor \(\alpha\)-converting enzyme metalloprotease (12). This triggers a second cleavage at a transmembrane domain by a presenilin-dependent protease to release the Notch intracellular domain from the plasma membrane. The Notch intracellular domain is then translocated into the nucleus, where it acts as a transcriptional coactivator for a DNA-binding factor, RBP-J/CBF1, to induce expression of downstream target genes (12).

The Delta gene is well conserved from flies to mammals and encodes a transmembrane ligand for the Notch receptor (5–7). Thus, Delta-Notch signaling mediates local cell-cell communication. Regulation of the ligand as well as the receptor seems to play important roles in signal transduction by the Delta-Notch pathway. The soluble extracellular domain of Notch ligands can bind to the Notch receptor; however, it cannot activate Notch \emph{in vitro} (13) but rather acts as a dominant-negative mutant that inhibits the native Delta-Notch pathway \emph{in vivo} (14, 15). Furthermore, deletion mutants of Notch ligands lacking the intracellular domain also act as dominant-negative mutants (6, 8, 16). These findings suggest the possible importance of the intracellular domain of Delta in Notch activation. In support of this, one of the factors involved in the Delta-Notch signaling cascade in \textit{Drosophila}, Neuralyzed (Neur), has been shown to bind the cytoplasmic domain of Delta and act as an E3 ubiquitin ligase for Delta protein (17–19). Neur-dependent internalization, possibly triggered by its ubiquitination, facilitates Notch activation (18, 19). Consistent with this, dynamin-dependent endocytosis in Delta-expressing signaling cells is required for Notch activation (20). Furthermore, \textit{trans}-endocytosis of the Notch extracellular domain by its ligand has been observed (21, 22). In vertebrates, similar E3 ligases, such as Neur and Mind bomb, have been identified as regulators of Delta protein and Notch signaling (23, 24). Thus, endocytosis of the ligand regulated by its intracellular domain seems to be an important mechanism of Notch activation, and this mechanism is conserved from flies to vertebrates.
The expression pattern and function of the Delta gene have been extensively elucidated. However, the high internalization efficiency that is required for its function and the resulting instability of the Delta protein on the cell surface make it difficult to understand the molecular behavior of Delta protein. In particular, where and how Delta protein is presented to Notch-expressing signal-receiving cells remains largely unknown. Recently, Delta-induced filopodia formation was reported to mediate long-range lateral inhibition of neural differentiation of sensory organ precursors in Drosophila (25), but it is currently unknown whether a similar cellular structure is involved in the presentation of Delta protein to neighboring cells in vertebrate systems.

The adherens junction (AJ) is a specialized structure for the cell-cell adhesion machinery in epithelial cells and consists of cadherin and nectin family cell adhesion molecules, which are linked to the actin cytoskeleton through their binding proteins catenins and afadin, respectively (26, 27). Neuroepithelial cells in the developing neural tube extend processes toward both the ventricle and the pia to form the bipolar morphology and then form AJs at the termini of their apically extending processes with the termini of neighboring progenitors (28). AJs are thought to be involved in maintenance of the undifferentiated progenitor pool and neural production by asymmetric cell division at the neurogenesis stage (29–31). Recently, we observed that migrating nascent neural precursors expressingDll1 mRNA in the developing spinal cord extended processes toward the ventricle and formed AJs at the apical termini of the extending processes (32). AJs are known to both regulate cell-cell adhesion and mediate the cell-cell signaling involved in cell differentiation of sensory organ precursors in Drosophila (26). Thus, it is possible that Dll1-expressing nascent progenitors in the VZ communicate with neighboring progenitors through the AJs to regulate the differentiation of these cells. In addition, Dll1 mRNA was selectively transported to the apically extending processes (32), raising the possibility that Dll1 functions on these processes to activate Notch on neighboring progenitors.

To address these issues, we first examined the localization of Dll1 in the developing mouse spinal cord and observed that it was mainly localized at the processes. To understand the mechanism of Dll1 localization and regulation of its activity in the Notch signaling pathway, we screened for proteins that interacted with the intracellular domain of Dll1 and identified the multiple PDZ domain-containing scaffolding molecule MAGI1 as a binding partner for the C terminus of Dll1. During the course of this study, Wright et al. (33) reported that zebrafish MAGI proteins bind to DeltaC and DeltaD proteins and that the MAGI1-binding domain of DeltaD is required for proper development of Rohon-Beard neurons. However, the mode of action of MAGI1 in Delta-Notch signaling has not yet been clarified. Thus, in the current study, we tried to address this issue by focusing on the role of MAGI1 in regulating Dll1 localization and regulation of its activity in the Notch signaling pathway.

### Experimental Procedures

**Plasmid Construction**—pDNA-SS-FLAG was constructed by ligating the annealed oligonucleotides 5’-GAT-CGG-ATNT-ACA-AGG-ATG-AGC-AGA-TGA-AGG-TAC-CCG-GAG-GTC-GTA G-CCC-AG-3’ and 5’-GAG-GCA-GTC-GAC-ATG-GCT-ACT-CA T-ACA-CAG-AAA-GTG-ATC-CAG-AAG-AAG-AAC-CAC-3’ into the BamHI/XhoI site of pcDNA3.1+ (Invitrogen). pMX-S-S-HA and pDNA-SS-HA were constructed by ligating the annealed oligonucleotides 5’-GAT-CGG-CGA-CCA-ATA-TGT-CTG-CAG-TTC-TGA-GAC T-GAT-TCC-CTG-TGT-GTC-GGA-TGC-GTC-GAT-GCA-GTC-ACC-TGA-TGG-GTA-GTC-GAC-TGG-GAC-GTC-GTA TG-GTA-GTA-GCA-AGC-AAC-TGG-GAC-GTC-TGA-CAT-CCA-GCA-AAG-CAG-3’, respectively. cDNAs of Dll1, MAGI1a, Jagged1, b-catenin, and N-cadherin were amplified by PCR using the following primer sets: Dll1 WT, 5’-GAG-CGC-GGC-GGC-GTA-TTT-GTC-GAT-CAG-CAC-3’ and 5’-GAG-GAA-TTA-TAC-CTG-TAT-ACC-ACT-ACA-CAT-C-3’; Dll1 ΔC, 5’-GAG-CGC-GGC-GGC-GTA-TTT-GTC-GAT-CAG-CAC-3’ and 5’-GAG-GAA-TTA-TAC-CTG-TAT-ACC-ACT-ACA-CAT-C-3’; Dll1 C-terminal fragment was digested with XhoI/EcoRI and cloned into the pMX vector. pcDNA-HA-NII, pcDNA-HA-NII, and pMX-Myc-NII (34). The cDNAs of Dll1 were amplified by PCR using the following primer sets: Dll1 P1, aa 1–459; Dll1 P2, aa 1–630; Dll1 P3, aa 1–828; Dll1 P4, aa 906–1138; and Dll1 P5, aa 1–1235; and 5’-GAG-GCA-GTC-GAC-ATG-GCT-ACT-CA T-ACA-CAG-AAG-CAC-3’ and 5’-GAG-GCA-GTC-GAC-ATG-GCT-ACT-CA T-ACA-CAG-AAG-CAC-3’ and 5’-GAG-GCA-GTC-GAC-ATG-GCT-ACT-CA T-ACA-CAG-AAG-CAC-3’ and 5’-GAG-GCA-GTC-GAC-ATG-GCT-ACT-CA T-ACA-CAG-AAG-CAC-3’. The PDZ, guanylate kinase (GK), WW, and transmembrane (TM) domains and the signal sequence (SS) are indicated.
pMX-derived constructs were used for the subcellular localization experiments, and pcDNA-derived constructs were used for the coimmunoprecipitation assays.

For in situ hybridization probes, Dll1 and MAGI1 cDNAs were amplified by PCR using the following primers: Dll1, 5′-GGG-AGA-AGG-AGG-TTT-CTG-TTA-GCA-TC-3′ and 5′-ATA-TAG-CTA-CAT-AGA-CCC-GAG-GTG-CC-3′; and MAGI1, 5′-TCA-TGC-ACA-GCT-GCA-AGG-AGG-CCG-TC-3′ and 5′-TTT-CTG-TCC-TGA-GAG-TCC-CTG-TGA-TC-3′. The amplified PCR fragments were cloned into pcRII (Invitrogen) and used as templates for the transcription of digoxigenin-labeled probes.

In Situ Hybridization and Immunohistochemistry—In situ hybridization and immunohistochemistry were performed as described previously (34). Polyonal rabbit anti-MAGI1 and anti-Dll1 antibodies were raised against GST-MAGI1 (aa 379–454) and GST-Dll1 (aa 646–722), respectively, and affinity-purified. The primary antibodies used for double staining included anti-ZO1 (Zymed Laboratories Inc.), anti-N-cadherin (BD Biosciences), anti-β-catenin (Santa Cruz), and anti-Neph3 (32).

Immunofluorescence analysis of the transfected cells was performed as described previously (35). Briefly, cells were transfected with the indicated combinations of plasmids using Lipofectamine (Invitrogen). After culture for 24 h, the transfected cells were replated onto glass coverslips, cultured for an additional 16 h, and subjected to immunofluorescence analyses using anti-Myc (Roche Applied Science), anti-E-cadherin (Takara), anti-Dll1, and anti-MAGI1 antibodies.

Immunoprecipitation—Immunoprecipitation experiments using 293E cells and detection by Western blotting were performed as described previously (34). Briefly, transfected 293E cells were lysed with a lysis buffer containing 10 mM HEPES (pH 7.6), 250 mM NaCl, 5 mM EDTA, and 1% TX-100 for 1 h at 4 °C, and immunoprecipitation was performed with anti-FLAG M2 beads (Sigma). Western blotting was carried out with anti-FLAG M2 (Sigma), anti-MAGI1, anti-β-catenin (Sigma), and anti-N-cadherin (BD Biosciences) antibodies.

Embryonic day (E) 12.5 mouse neural tubes were dissected and sonicated in a lysis buffer containing 10 mM HEPES (pH 7.6), 250 mM NaCl, 5 mM EDTA, and 1% TX-100. The cell extract was incubated with either an anti-MAGI1 antibody or control IgG at 4 °C for 16 h. The antibody-protein complexes were collected with protein G-Sepharose beads and subjected to Western blotting using anti-Dll1 and anti-MAGI1 antibodies.

Reverse Transcription-PCR—Reverse transcription-PCR was performed as described previously (34). ExTaq polymerase (Takara) was used for amplification, which was carried out by denaturation at 94 °C for 30 s (2 min in the first cycle), annealing at 65 °C for 30 s, and extension at 72 °C for 1 min (3 min in the last cycle). There were 26 cycles for MAGI1 and 30 cycles for Dll1. The primer sequences were as follows: MAGI1, 5′-CTG-TCA-ACA-CTG-TGA-GCT-CTG-GCA-TC-3′ and 5′-GTC-AGT-TTG-TGG-TGG-TGG-TGG-TGG-TGG-CC-3′; and Dll1, 5′-GGC-AGG-AGG-TCT-TTG-GCA-TC-3′ and 5′-CTT-CTC-TCC-TCC-TGG-AAG-GCT-TGG-TGG-3′.

TX-100 Extraction—TX-100 extraction experiments were carried out as described previously (36). Briefly, transfected EL cells on coverslips were extracted with CSK buffer (50 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM MgCl2, and 0.5% TX-100) for 5 min at 4 °C, fixed with 1% formaldehyde, and subjected to immunofluorescence analyses using anti-E-cadherin (Takara), anti-Dll1, and anti-MAGI1 antibodies.

Surface Protein Labeling—Transfected EL cells were surface-labeled with EZ-link Sulfo-NHS-SS-Biotin (Pierce) and sonicated in a lysis buffer containing 1% SDS, 10 mM Tris- HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA. Biotinylated proteins were collected with streptavidin beads, eluted by boiling in SDS sample buffer, and subjected to Western blotting with anti-HA (Roche Applied Science) and anti-E-cadherin (Takara) antibodies.

RESULTS

Dll1 Protein Is Localized on the Processes Extending from Nascent Neuronally Focused Precursors in the Developing Spinal Cord VZ—We previously observed that Dll1 mRNA was selectively transported into the processes apically extending from nascent neural precursors in the developing spinal cord VZ (32). This suggests that Dll1 functions on the processes. To examine this possibility, we first examined the localization of Dll1 protein in the developing mouse spinal cord. In the E11.5 spinal cord, Dll1 mRNA was expressed only in a subset of VZ cells (see Fig. 4B). Consistent with this, a high level of anti-Dll1 immunoreactivity was detected in the VZ region (Fig. 2A). Furthermore, the immu

nereactivity was not detected in the ventral striped region where Dll1 mRNA was absent (compare Figs. 2A and 4B). Together with the observation that this anti-Dll1 antibody did not cross-react with the most homologous protein (Dll4) and the Jagged family proteins in Western blotting (data not shown), these results confirm the specificity of the antibody. Importantly, Dll1 was detected not only at the cell body but also in the processes. In contrast to its mRNA, which was detected only in the apical processes extending toward the ventricle (32), Dll1 protein was distributed in both the apical processes and the basal processes extending toward the pia. These results suggest that the localization of Dll1 is not simply regulated by local protein synthesis from localized mRNA.

Our previous observation that Dll1 mRNA accumulated at the termini of the apically extending processes (32) suggests a possible involvement of the process termini, where AJ is formed, in Dll1 function. In support of this, Dll1 was detected in the processes contacting the ventricle, and its accumulation near the ventricle was observed (Fig. 2B). These signals were specific because apical signals were not detected at Dll1 mRNA-negative regions (Fig. 2C). Furthermore, Dll1 was partially colocalized with an AJ marker, ZO1 (zonula occludens 1), at the termini (Fig. 2D). These results suggest that Dll1 is presented on the processes to activate Notch expressed on neighboring cells.

MAGI1 Interacts with the C Terminus of Dll1—The selective localization of Dll1 in the processes suggests possible cell-intrinsic regulation through interacting proteins. To understand the mechanism of Dll1 localization, we searched for interacting partners for the ICD of Dll1 by yeast two-hybrid screening of an E12.5 central nervous system cell line. Sixteen of the 17 clones obtained encoded the multiple PDZ domain-containing scaffolding molecule, MAGI1 (38). Most of the clones were derived from the cDNA portion encoding the fifth and sixth PDZ domains (PDZ4 and PDZ5) (data not shown). Although MAGI1 proteins have been reported to bind to the cytoplasmic domain of Delta proteins in vitro (33, 37), whether the full-length Delta interacts with MAGI1 in vivo has not yet been investigated. To examine this, we performed coimmunoprecipitation experiments using transfected 293E cells. It has been reported that many alternative splicing variants of MAGI1 are expressed in the brain (38). We used two alternative isoforms, MAGI1a and MAGI1c (GenBank™ acce-
In the interaction. To directly test this possibility, we strongly suggesting that the PDZ domain of MAGI1 is involved

1) were indispensable for its association with MAGI1 (Fig. 3A). This prompted us to examine whether MAGI1 binds to the C terminus of Dll1. As revealed by coimmunoprecipitation experiments, the C-terminal four amino acids of Dll1 (see Fig. 3C) were specifically recognized by PDZ4 of MAGI1. These results strongly suggest that Dll1 associates with MAGI1 in vivo.

The amino acid sequence of the C terminus of Dll1 is well matched with the consensus PDZ domain recognition sequence (37, 39). This prompted us to examine whether MAGI1 binds to the C terminus of Dll1. As revealed by coimmunoprecipitation experiments, the C-terminal four amino acids of Dll1 (see Fig. 1) were indispensable for its association with MAGI1 (Fig. 3A), strongly suggesting that the PDZ domain of MAGI1 is involved in the interaction. To directly test this possibility, we constructed six MAGI1 mutants, each lacking a different PDZ domain (Fig. 1). As shown in Fig. 3C, deletion of the fifth PDZ domain (PDZ4) completely abolished coimmunoprecipitation with Dll1, whereas the mutants lacking the other PDZ domains retained the ability to interact with Dll1. Furthermore, PDZ4 was sufficient for the interaction with Dll1 (Fig. 3D). These results clearly demonstrate that the C terminus of Dll1 is specifically recognized by PDZ4 of MAGI1.

As previously reported, Dll1 is expressed in the VZ region of the developing neural tube with regional selectivity (5, 40) (see Figs. 3A and 4B), whereas another Notch ligand, Jagged1 (Jag1) is complementarily expressed in Dll1-negative regions and is thought to play similar roles in the lateral inhibition of neuronal differentiation (9). Therefore, we next examined whether Jag1 could also interact with MAGI1. As shown in Fig. 3E, Jag1 ICD did not coimmunoprecipitate MAGI1 under the conditions in which Dll1 ICD efficiently associated with MAGI1. Thus, MAGI1 specifically binds to Dll1, even though Dll1 and Jag1 have similar expression profiles and functions in neuronal development.

MAGI1 Is Coexpressed with Dll1 in the Developing Spinal Cord—We next examined the expression pattern of MAGI1 to further confirm the functional interaction between Dll1 and MAGI1. MAGI1 mRNA was detected in both embryonic and adult central nervous system regions (Fig. 4A). Because many alternatively spliced variants of MAGI1 mRNA, including iso-

**Fig. 3. Interaction between the C terminus of Dll1 and PDZ4 of MAGI1.** A, MAGI1 binds to the C terminus of Dll1. 293E cells were transiently transfected with an expression vector for each tagged protein as indicated. The cell lysates were immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates and the cell lysates were analyzed by Coomassie Brilliant Blue (CBB) staining and Western blotting with the anti-FLAG or anti-HA antibody as indicated. B, association of Dll1 with MAGI1 in vivo. The cell lysates from E12.5 mouse neural tubes were immunoprecipitated with an anti-MAGI1 antibody. The immunoprecipitates and the cell lysates were analyzed by Western blotting with the anti-Dll1 or anti-MAGI1 antibody as indicated. C and D, domains of MAGI1 required for the interaction with Dll1. Coimmunoprecipitation experiments were carried out as described in A. PDZ4 is necessary and sufficient for the association with Dll1. E, MAGI1 specifically interacts with Dll1. Coimmunoprecipitation experiments were carried out as described in A using tagged Dll1 intracellular domain and Jagged1. Jagged1 cannot bind to MAGI1.

**Fig. 4. Expression of MAGI1 mRNA in the central nervous system.** A, expression patterns of MAGI1 alternative variant transcripts in the embryonic or adult brain. Total RNA from E12.5 or adult brain was subjected to reverse transcription-PCR analysis for MAGI1 and Dll1 mRNA. The amplified products were electrophoresed in 2% agarose gels and stained with ethidium bromide. B, in situ hybridization analysis with a MAGI1 (a, c, and e) or Dll1 (b, d, and f) antisense riboprobe. Images of transverse sections of E11.5 mouse spinal cord are shown. c–f show magnified images of the boxed regions in a and b. The bracket indicates the Dll1 mRNA-negative VZ region. Bars: a and b, 100 μm; c–f, 40 μm.
forms without PDZ4 that may not recognize the C terminus of Dll1, have been reported (38), we designed primers for reverse transcription-PCR to detect alternative splicing around the exon encoding PDZ4. Interestingly, exclusion of this exon was stage-dependent. At E12.5, when Dll1 mRNA was progressively expressed, almost all the MAGI1 mRNA contained this exon, whereas at the adult stage, when Dll1 mRNA expression was remarkably down-regulated, MAGI1 mRNA without the PDZ4 exon were detected (Fig. 4A). These results further support the idea that MAGI1 functions together with Dll1 through PDZ4.

To analyze the expression pattern of MAGI1 mRNA in more detail, we performed in situ hybridization. At E11.5, MAGI1 mRNA expression was observed throughout the neural tube (data not shown). In the spinal cord, MAGI1 mRNA was detected throughout the neural tube including both the VZ and ML, but the expression level was not uniform (Fig. 4B). Bc in situ detail, we performed with that of Bc, ML, but the expression level was not uniform (Fig. 4tected throughout the neural tube including both the VZ and (data not shown). In the spinal cord, mRNA expression was observed throughout the neural tube suggestive that control of mRNA-negative regions, including the floor plate, roof plate, and ventral Dll1 mRNA-negative VZ regions (Fig. 4B, a, b, e, and f). These results demonstrate that MAGI1 mRNA is coexpressed with Dll1 mRNA in the developing spinal cord VZ and suggest that control of MAGI1 mRNA expression is coupled with that of Dll1 mRNA.

MAGI1 Is Localized at AJs in the Developing Spinal Cord—It has been reported that MAGI1 is localized at tight junctions in epithelial cells (41, 42). However, neuroepithelial cells do not form tight junctions (28). Therefore, we examined the localization of MAGI1 in the developing spinal cord. As shown in Fig. 5A, the anti-MAGI1 immunoreactivity was detected at the margin of the ventricle. This pattern was highly similar to that of ZO1, and colocalization of these proteins was observed (Fig.

FIG. 5. Localization of MAGI1 at AJs in the developing spinal cord. Images of transverse (A–D) or sagittal (E and F) sections of E11.5 spinal cord double-stained for MAGI1 (green) and ZO1 (A, B, and E, red). N-cadherin (C, red), β-catenin (D, red), or Neph3 (F, red) are shown. Bars: A, 100 μm; B–D, 20 μm; E and F, 10 μm. Arrowheads indicate the processes extending from Neph3-positive cells.

FIG. 6. Association of Dll1 with the N-cadherin-β-catenin complex through MAGI1. 293E cells were transiently transfected with an expression vector for each tagged protein as indicated. The cell lysates were immunoprecipitated with the anti-FLAG antibody. The immunoprecipitates and the cell lysates were analyzed by Western blotting with the anti-FLAG, anti-MAGI1, anti-N-cadherin, or anti-β-catenin antibody as indicated.

5, A and B). Furthermore, in sagittal sections of the E11.5 spinal cord providing a horizontal view of the apical termini of processes, accumulation of MAGI1 was detected around the processes, and it was again colocalized with ZO1 (Fig. 5E), clearly demonstrating that MAGI1 is localized at AJs formed by VZ cells at the termini of apically extending processes. This was further confirmed by double staining of MAGI1 and N-cadherin or β-catenin (Fig. 5, C and D).

Recently, we observed that Dll1 mRNA-expressing cells in the VZ formed AJs at the apical termini of the processes (32). The observation that MAGI1, similarly to ZO1, accumulated at virtually all the cell-cell contact sites at the apical termini of processes (Fig. 5E) suggests that MAGI1 is expressed in proliferating progenitors and Dll1 mRNA-expressing nascent neural precursors in the VZ, both of which extend processes toward the ventricle and form AJs there (28, 29), and is accumulated at the apical AJs formed by these cells. To examine whether this is the case, we performed double staining for MAGI1 and a nascent neural precursor marker, Neph3, which is coexpressed with Dll1 mRNA (32). As shown in Fig. 5E, MAGI1 accumulated around the processes extending from cells positive for Neph3. Together, these results suggest that MAGI1 is expressed by nascent neurally fated precursors in the VZ, which transiently express Dll1 mRNA, and accumulates at AJs formed by these cells. It should be noted that MAGI1 may be partially colocalized with Dll1 at AJs (compare Figs. 2D and 5B).

Dll1 Forms a Complex with β-Catenin and N-Cadherin through MAGI1—It has been reported that MAGI1 can interact with β-catenin through its sixth PDZ domain (PDZ5) (43). The observation that Dll1 binds to PDZ4 of MAGI1 raises the possibility that Dll1 interacts with the cadherin-catenin complex through MAGI1. To test this possibility, these proteins were transiently expressed in 293E cells, and immunoprecipitation assays were performed. As shown in Fig. 6, N-cadherin and β-catenin were efficiently immunoprecipitated with Dll1 ICD only in the presence of MAGI1. As expected, PDZ4 was re-
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Fig. 7. Localization of MAGI1 at AJs in EL cells. Myc-tagged wild-type MAGI1 (A, C, and D) or a MAGI1 mutant lacking PDZ5 (ΔP5) (B) was transiently expressed in EL (A and B), L (C), or nEoL (D) cells and then immunostained with the anti-Myc (green) and anti-E-cadherin (red) antibodies. Wild-type MAGI1 is colocalized with E-cadherin in EL cells. In contrast, the ΔP5 mutant is localized at cell-cell contact sites as dots, but it is not colocalized with E-cadherin. In L or nEoL cells, wild-type MAGI1 is localized at cell-cell contact sites as dots, similar to the ΔP5 mutant in EL cells. Bars, 10 μm.

required for the coimmunoprecipitation of MAGI1, N-cadherin, and β-catenin. In addition, PDZ5 was also required for this bridging activity of MAGI1, even though the MAGI1 mutant lacking PDZ5 was efficiently associated with DIll1, confirming that the N-cadherin-β-catenin complex interacts with MAGI1 through PDZ5. Taken together, these results suggest that DIll1 and β-catenin interact with PDZ4 and PDZ5 of MAGI1, respectively, and that DIll1 and the components of the cadherin-based AJs form a complex through interaction with MAGI1.

DIll1 Is Recruited to AJs by MAGI1 in Cultured Fibroblasts—
The above observation that DIll1 interacts with the AJ components through MAGI1 led us to examine whether MAGI1 affects the subcellular localization of DIll1. For this purpose, we used EL cells (E-cadherin-expressing fibroblasts) as a model for AJ-forming cells (44) because MAGI1 and DIll1 were not endogenously expressed in EL cells (data not shown). We first confirmed that the AJ localization of MAGI1 was reproduced in EL cells. When MAGI1 was expressed in EL cells, accumulation of MAGI1 at cell-cell contact sites and colocalization of MAGI1 and E-cadherin were observed (Fig. 7A), indicating that MAGI1 is localized at AJs in EL cells. This is consistent with the previous observation that MAGI1 is localized at AJs in cultured cells that form AJs but not tight junctions (45). To further confirm the selective localization of MAGI1 at AJs, we next examined the localization of MAGI1 in cadherin-deficient L cells. MAGI1 was localized at cell-cell contact sites only when expressed at a very low level (Fig. 7C; data not shown). It should be noted that the pattern of MAGI1 accumulation in L cells was dotty and quite different from that in EL cells. This suggests that MAGI1 is not simply localized at cell-cell contact sites but rather is localized at AJs in EL cells. However, these results indicate that MAGI1 can be localized at cell-cell contact sites without AJs. Therefore, we next examined whether the interaction between MAGI1 and β-catenin is required for the localization of MAGI1 at AJs in EL cells. In most cases, the MAGI1 mutant lacking PDZ5 was diffusely distributed throughout cells, and localization at cell-cell contact sites was not observed (data not shown). In some cases, a small amount of mutant MAGI1 was accumulated at cell-cell contacts as dots, similar to the case in L cells, but did not completely colocalize with E-cadherin (Fig. 7B), indicating that PDZ5 is required for the localization of MAGI1 at AJs. To examine whether β-catenin is involved in the localization of MAGI1, we used nEoL cells, which express E-cadherin that lacks the intracellular domain and is fused with α-catenin instead (46). These cells can form AJ-like structures, but β-catenin is not recruited to the cell-cell contact sites (46). In nEoL cells, MAGI1 was not efficiently localized at the cell-cell contact sites, although some cases showed accumulation of MAGI1 as dots, again similar to the case in L cells (Fig. 7D). Taken together, these results suggest that MAGI1 is recruited to AJs through the interaction between PDZ5 of MAGI1 and β-catenin.

We next examined the effect of MAGI1 on DIll1 localization. Transiently expressed N-terminally HA-tagged DIll1 (Fig. 1) was mainly localized at the perinuclear cytoplasm in EL cells (Fig. 8A). In contrast, when HA-DIll1 and Myc-MAGI1 were coexpressed, accumulation of HA-DIll1 at cell-cell contact sites was observed (Fig. 8B). This accumulated HA-DIll1 was apparently colocalized with Myc-MAGI1 and E-cadherin, indicating that HA-DIll1 is only recruited to AJs in the presence of MAGI1. It should be noted that MAGI1 was mainly localized at AJs, but a significant portion of HA-DIll1 was still localized at the cytoplasm, similar to the pattern in the absence of MAGI1. This pattern of HA-DIll1 localization was consistent with DIll1 localization in vivo (see Fig. 2). To examine the involvement of the interaction between DIll1 and MAGI1 in DIll1 localization, we performed similar experiments using mutant versions of DIll1 and MAGI1. When HA-DIll1 lacking the C-terminal four amino acids, which could not bind to MAGI1, and wild-type Myc-MAGI1 were coexpressed in EL cells, HA-DIll1C was not recruited to AJs, even though Myc-MAGI1 was localized at the AJs (Figs. 8C). Similarly, mutant MAGI1 lacking PDZ4, which could not bind to DIll1 but was still localized at AJs, did not recruit wild-type HA-DIll1 (Fig. 8D). Collectively, these results strongly suggest that DIll1 is recruited to AJs through its interaction with MAGI1.

It has been reported that molecules tightly associated with the cytoskeleton, such as the AJ component molecules, are resistant to extraction with TX-100 (36, 47). As shown in Fig. 8E, staining of MAGI1 at AJs persisted after TX-100 extraction, similar to E-cadherin. Furthermore, HA-DIll1 was retained at AJs, whereas cytoplasmic DIll1 staining was clearly reduced. These observations suggest that DIll1 is tightly linked to the AJ components and the cytoskeleton through MAGI1.

DIll1 Is Stabilized on the Cell Surface by MAGI1—Given that DIll1 functions as a ligand for the Notch receptor, cell surface expression of DIll1 may be important for its activity. However, DIll1 is not stable on the cell surface due to its high efficiency of internalization, which is possibly regulated by ubiquitination (24). The above observation that DIll1 is accumulated at AJs by MAGI1 led us to examine whether the surface expression of DIll1 is also regulated by MAGI1. EL cells were transiently transfected with expression vectors for HA-DIll1 and Myc-MAGI1, and cell surface proteins were labeled with biotin. Next, the biotinylated proteins were collected by streptavidin beads and detected by Western blotting. As shown in Fig. 9, the surface HA-DIll1 level was apparently increased by coexpression of MAGI1, and the C terminus of HA-DIll1 was required for...
in the developing mouse neural tube. It has been reported that MAGI2/Avrinp1 could bind to Dll1 in vitro (37). However, in the developing central nervous system, MAGI2 mRNA is only expressed in the ML, where Dll1 mRNA is not expressed. Thus, at least in the developing central nervous system, it is likely that MAGI1 is a binding partner for Dll1. Furthermore, by using the in vitro culture system involving AJ-forming fibroblasts, we have revealed that MAGI1 functions to recruit Dll1 to AJs. These observations indicate a possible role of MAGI1 and the cadherin-based AJs in Delta-Notch signaling.

Dll1 encodes a transmembrane ligand for the Notch receptor. Thus, Delta-Notch signaling is thought to be involved in local cell-cell signaling. In the developing neural tube, Delta-Notch signaling is thought to play a major role in regulating neural differentiation and maintaining the undifferentiated progenitor pool (2, 3). Notch is expressed throughout the VZ (8), whereas Dll1 is transiently expressed by neuronally fated precursors until they exit the VZ (5–9). Thus, it is thought that Dll1-expressing precursors activate Notch receptors on neighboring progenitors in the VZ to repress their differentiation. One of the most important issues for understanding the mechanism of the Delta-Notch signaling in this process is how and where Dll1 is presented on the cell surface and binds to Notch.
Drosophila nilin 1, is associated with the cadherin molecule and selectively (Fig. 10). This idea is supported by previous observations that suggests the possible involvement of AJs in Delta-Notch signaling and recruited to AJs through its interaction with MAGI1 supported by experiments further suggesting the possible involvement of AJs in the developing neural tube.

In the developing neural tube (24) makes it difficult to detect its localization on the cell surface. Whether Dll1 localized at processes is presented on the cell surface and can activate the Notch receptors there may be an important issue. Further detailed analysis of Dll1 localization in vivo is required to understand the mechanism and location of Dll1 presentation in the developing neural tube.

Although direct evidence supporting the idea that Dll1 is presented on the cell surface of processes has not yet been obtained, our observation that Dll1 can be linked to cadherin and recruited to AJs through its interaction with MAGI1 suggests the possible involvement of AJs in Delta-Notch signaling (Fig. 10). This idea is supported by previous observations that one of the key components in the Delta-Notch pathway, presenilin 1, is associated with the cadherin molecule and selectively localized at AJs in epithelial cells (36). In addition, although Delta protein is not detected on the cell surface in wild-type Drosophila embryos, mutants without Neur activity, which is required for Delta ubiquitination and its resulting internalization, accumulate Delta and Notch proteins at apical cell-cell junctions (19). These observations support the idea that Delta-Notch signaling occurs at apical AJs. However, Drosophila Delta does not have a PDZ domain recognition sequence at its C terminus. Thus, the localization of Delta protein may be regulated by different mechanisms in vertebrates and invertebrates. Recent studies by Zechner et al. (30) and Chae et al. (31) have revealed that correct localization of the AJ components at apical junctions in the developing cortex and β-catenin activity in the developing neural tube are required for maintaining the neural progenitors in an undifferentiated state. These observations further suggest the possible involvement of AJs in Delta-Notch signaling for lateral inhibition.

The regulation of cell-cell Delta-Notch signaling using a specialized cell adhesion apparatus appears to be a reasonable strategy. In addition, this idea may explain why migrating neuronally fated precursors in the VZ extend processes in the opposite direction to the migration and form strong cell-cell adhesions at AJs until they exit the VZ (32). If Dll1 was only presented on the cell body, Notch receptors on surrounding cells would only be transiently activated due to the rapid movement of Dll1-expressing cells toward the ML. In contrast, if Dll1 is presented on the AJ surface, continuous contact during the migration of Dll1-expressing cells through the termini of the apical processes may increase the time and efficiency of Notch activation. However, the question remains as to whether MAGI1 facilitates Delta-Notch signaling. Wright et al. (33) recently reported that injection of a morpholino that blocks mRNA splicing required for the synthesis of the intact C terminus of Delta protein into zebrafish embryos caused a slight increase in neurogenesis, suggesting a possible involvement of the Delta-MAGI1 interaction in lateral inhibition by Delta-Notch signaling in the developing central nervous system. We tried to address this issue via the most established reporter system using C2C12 myoblasts (48). This cell line has been reported to form N-cadherin-based AJs (49). However, overexpression of MAGI1 in the Dll1-expressing signaling cells did not affect the efficiency of Notch activation in the receiving cells. One possible explanation is that MAGI1 regulates Dll1 activity in a cell type-specific manner. This is consistent with the observation by Wright et al. (33) that treatment of zebrafish embryos with the above-described DeltaD morpholino only affected central nervous system development. This includes the possibility that an as yet unidentified component(s), which functionally associates with MAGI1, may be involved in the regulation of Dll1 activity and required for the effect in the C2C12 assay system. Alternatively, functionally redundant factor(s) may be highly active in this system. In support of this, another member of the membrane-associated guanylate kinase family, Dlg1, has recently been reported to associate with the Dll1 C terminus (50). The fact that not all Notch ligands have the PDZ domain recognition sequence at their C terminus (39) may support this hypothesis. Additional expression and subcellular localization studies of MAGI1 and Notch ligands in other tissues are necessary to clarify this point.

Undifferentiated neural progenitors are thought to be maintained by Delta-Notch signaling; thus, how do they escape to generate neurons? Asymmetric cell division is thought to be involved in this step (51). It has been reported that the basally positioned daughter cells that are fated to become neurons lose their apical membrane contacting the ventricle and their AJs forms with neighboring cells (29, 52). Thus, asymmetrically inherited molecule(s) associated with these AJs may regulate neuronal differentiation. If AJs are involved in Delta-Notch signaling through MAGI1, loss of AJs in the basally positioned daughter cells may release them from the lateral inhibition and trigger the differentiation program of these cells.

Our results obtained from in vitro cell culture assays have revealed that MAGI1 not only recruits Dll1 to AJs but also stabilizes it on the cell surface. These observations suggest that MAGI1 inhibits Dll1 internalization by anchoring Dll1 at AJs. This phenomenon may increase the efficiency of binding to Notch on neighboring cells at AJs, in addition to the effect on the subcellular localization where signal transduction occurs. However, endocytosis of Dll1 is necessary for its activity. Thus, release of Dll1 from AJs is possibly regulated by binding to Notch. Alternatively, MAGI1 may be involved in the clustering of Dll1, which seems to be required for its activity (13, 53), by accumulating Dll1 at AJs. On the other hand, MAGI1 may play a role in the signal transduction from Delta protein (54–56). In any case, additional experiments, including loss of function experiments using MAGI1 knock-out mice, are required to understand the physiological role of the Dll1-MAGI1 interaction.

Acknowledgments—We thank Dr. T. Imai (KAN Research Institute Inc.) for helpful comments and encouragement. We also thank Dr. S. Tsukita (Kyoto University) for providing us with the L, EL, and nEaL cells.

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