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The Notch Ligand Delta1 Recruits Dlg1 at Cell-Cell Contacts and Regulates Cell Migration*[S]

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Emmanuelle M. Six‡, Delphine Ndiaye‡, Guido Sauer§, Yacine Laâbi¶‖, Rafika Athman, Ana Cumano¶, Christel Brou‡, Alain Israël‡ ‡‡, and Frédérique Logeat‡**

*From the ‡Unité de Biologie Moléculaire de l'Expression Génique, URA 2582 CNRS, ¶Unité du Développement des Lymphocytes, URA 1961 CNRS, and **Groupe Immunité Innée et Signalisation, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex 15, France and §Max-Planck-Institut für Biochemie, Abteilung Zellbiologie, Am Klopferspitz 18, D-82152 Martinsried, Germany*

Delta1 acts as a membrane-bound ligand that interacts with the Notch receptor and plays a critical role in cell fate specification. By using peptide affinity chromatography followed by mass spectrometry, we have identified Dlg1 as a partner of the Delta1 C-terminal region. Dlg1 is a human homolog of the *Drosophila* Discs large tumor suppressor, a member of the membrane-associated guanylate kinase family of molecular scaffolds. We confirmed this interaction by co-immunoprecipitation experiments between endogenous Dlg1 and transduced Delta1 in a 3T3 cell line stably expressing Delta1. Moreover, we showed that deletion of a canonical C-terminal PDZ-binding motif (ATEV) in Delta1 abrogated this interaction. Delta4 also interacted with Dlg1, whereas Jagged1, another Notch ligand, did not. In HeLa cells, transfected Delta1 triggered the accumulation of endogenous Dlg1 at sites of cell-cell contact. Expression of Delta1 also reduced the motility of 3T3 cells. Finally, deletion of the ATEV motif totally abolished these effects but did not interfere with the ability of Delta1 to induce Notch signaling and T cell differentiation in coculture experiments. These results point to a new, probably cell-autonomous function of Delta1, which is independent of its activity as a Notch ligand.

Notch receptors and their ligands are essential in many binary cell fate decisions in various tissues from worm to vertebrates. Mechanisms through which Notch signaling regulates cell fate include lateral inhibition and inductive signaling (1). The Notch gene that encodes a type I transmembrane protein was first identified in *Drosophila*, and in mammals four Notch paralogs have been isolated (2). In fly the two structurally related Notch ligands, Delta and Serrate, have both redundant and nonredundant functions (1, 3). Notch ligands are conserved in vertebrates and include the Serrate orthologs Jagged-1 and -2 and the Delta orthologs Delta-1, -3, and -4 (4, 5). The plurality of Notch ligands and receptors may account for the functional diversity of Notch signaling. All Notch ligands are single pass transmembrane polypeptides, and their intracellular domains vary in length and do not display any significant sequence similarity except at their very C-terminal end. In *Xenopus* and mammals several lines of evidence suggest that the intracellular domain of Delta is required for its normal function (6–8). The extracellular domain of the Notch ligands interacts with the Notch receptors expressed on adjacent cells and consists of multiple highly conserved epidermal growth factor-like motifs and a conserved DSL domain. No specificity of a ligand for a given Notch receptor has been demonstrated so far, and the transcriptional program elicited by a given Notch/ligand interaction has not been investigated either (9). Upon ligand binding, the Notch receptor undergoes two proteolytic cleavages (10, 11), which release the intracellular region of the receptor that translocates into the nucleus, where it participates in transcriptional activation of target genes together with CSL and Mastermind (12–15). Notch ligands have been shown to constitutively undergo the same two proteolytic events that affect the receptor during activation. They are first cleaved by a metalloprotease of the ADAM family, resulting in the shedding of the extracellular region (16). We and others (17–20) have shown that Notch ligands are then processed by a presenilin-dependent γ-secretase-like activity, which releases their intracellular region.

The function of Notch ligands in the emitting cell remains to be determined, both for the full-length molecule and the cleavage products. Comparative analysis of their intracellular amino acid sequences (Fig. 1) reveals a highly conserved ATEV motif at the C-terminal end of the Delta molecules, which corresponds to a canonical PDZ[1] (PSD-95/DLG/ZO-1)-binding motif (21). This motif differs from Jagged PDZ-binding motif (PBM) and could be a binding site for specific partners that might regulate the *cis* or *trans* activity of the ligands (or both). Ascano *et al.* (22) have recently shown that Jagged1 interacts with the PDZ domain of AF6, the mammalian ortholog of *Drosophila* Canoe, and they proposed a bi-directional signaling model according to which Notch ligands would signal both in *trans* through Notch receptors located on adjacent cells and in

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[S] The on-line version of this article (available at http://www.jbc.org) contains Table I and Fig. 1.

‖ Supported by grants from the Fondation pour la Recherche Médicale. Present address: Laboratoire de Biologie des Cellules Souches Humaines, CNRS-UPR 9045, Institut André Lwoff, 7 Rue Guy Môquet, 94801 Villejuif Cedex, France.

‡‡ To whom correspondence should be addressed: Unité de Biologie Moléculaire de l'Expression Génique, URA 2582, CNRS, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex 15, France. Tel.: 33-1-45-68-85-53; Fax: 33-1-40-61-30-40; E-mail: aisrael@pasteur.fr.

[1] The abbreviations used are: PDZ, PSD-95/DLG/ZO-1; Dlg1, Discs large homolog 1; MAGUK, membrane-associated guanylate kinase; MIG, MSCV-IRES-GFP; PBM, PDZ-binding motif; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HA, hemagglutinin; IP, immunoprecipitation; IB, immunoblot; IF, immunofluorescence; VSV, vesicular stomatitis virus; GFP, green fluorescent protein; MS, mass spectrometry; FCS, fetal calf serum; APC, allophycocyanin; HSC, hematopoietic stem cells.

cis in the emitting cell itself. We undertook a proteomic approach to identify proteins interacting with the cytoplasmic region of Delta1 based on peptide affinity chromatography. Here we report the identification of Dlg1 as an interacting partner for the PDZ-binding motif of Delta1. Dlg1 is a human homolog of the product of the *Drosophila discs large* tumor suppressor gene and is a member of the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins. Dlg1 is essential for the assembly of multiprotein complexes at cell-cell junctions and is involved in maintenance of cell adhesion, cell polarity, growth control, and cell invasion (23). We demonstrated that Delta1 recruits Dlg1 at cell-cell contacts, thereby resulting in the association of Delta1 with a signaling complex, eventually leading to specific cellular effects in the emitting or in the receiving cell. Deletion of the four C-terminal residues encompassing the Delta1 PDZ-binding motif abolished the Delta1/Dlg1 interaction but did not affect Notch activation in a T cell differentiation assay. However, this mutation abolished the inhibitory activity that Delta1 exerts in *cis* on the motility of 3T3 cells, suggesting that Delta ligands somehow control cell motility through interaction with cytoskeletal proteins such as Dlg1.

	X	S/T	X	V/I	ClassI PDZ BM
Q S V Y V L S A E K D E C V I	A	T	E	V	mDelta1
Q S V Y V L S A E K D E C V I					mDelta1$^{\Delta ATEV}$
Q S V Y V I S E E K D E C V I	A	T	E	V	hDelta1
H P I Y I L P E - P E Q C I F	A	T	E	V	xDelta2
Q S V C L I S E E R N E C V I	A	T	E	V	mDelta4
H P V Y I I P E H I E Q R V F	A	T	E	V	zDeltaC
G D S R S I Y V I P A P S I Y	*A*	*R*	*E*	A	mDelta3
	X	Φ	X	Φ	ClassII PDZ BM
Q D N R D L E S A Q S L N R M	E	Y	I	V	mJagged1
K V D N R A V R S T K D V R R	*A*	*G*	*R*	*E*	mJagged2

FIG. 1. **Alignment of the PBM of Notch ligands.** C-terminal amino acid sequence of murine (*m*) Delta1 and its orthologs (*h*, human; *x*, *Xenopus,* and *z,* Zebrafish) which harbor a class I PBM except for Delta3. The C-terminal amino acid sequence of Jagged1, but not Jagged2, presents a class II PBM. The consensus for both PBM is indicated (Φ represents hydrophobic residues).

EXPERIMENTAL PROCEDURES

Affinity Purification Procedure—A crude membrane fraction (in 50 mM Tris, pH 7.9, 10 mM NaCl, 1% Nonidet P-40), prepared from 10^{10} HeLa cells (as described in Brou *et al.* (10)), was diluted two times with 20 mM Tris, pH 7.4, 0.3 M NaCl, 10 mM $MgCl_2$, 10% glycerol and applied to a column containing 5 ml of Affi-Gel-15 resin (Bio-Rad). The flow-through was then loaded, overnight at 4 °C, onto a Delta peptide resin (5 mg of peptide LSAEKDECVIATEV per ml of Affi-Gel-15 coupled according to the manufacturer's protocol) and onto a control peptide resin with a similar pI (5 mg of peptide TEVKEDSAYGSQSVE per ml of Affi-Gel-15). The resins were washed extensively with 20 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM $MgCl_2$, 0.2% Nonidet P-40, 5% glycerol. Bound proteins were eluted with a buffer containing 2 M NaCl. Column eluates were precipitated with trichloroacetic acid, analyzed by SDS-PAGE (Criterion XT gel 4–12%, Bio-Rad), and stained with colloidal Coomassie.

Mass Spectrometry—Protein bands were in-gel digested by trypsin as reported by Shevchenko *et al.* (24) and subsequently desalted and concentrated by using C18 extraction tips (25). The peptide mixture was eluted from a pulled fused silica capillary with an internal diameter of 75 μm and a tip opening of 8 μm (New Objective, Woburn, MA) and filled with ReproSil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany) 3-mm reverse-phase material, directly into a quadrupole time-of flight mass spectrometer (Ultima, Micromass, UK). Peptides were separated by a 60-min linear gradient of 0–100% buffer B (80% acetonitrile, 0.5% acetic acid) from a CAPLC HPLC system (Waters) at a flow rate of ~200 nl/min. Combined peak lists were searched in the MSDB data base using Mascot (www.matrixscience.com) allowing ±0.15-Da tolerance for both peptide and MS/MS fragment ion mass values.

In Vitro Interaction with Biotinylated Peptides—HeLa and 3T3 cell extracts prepared in 20 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM $MgCl_2$, 1% CHAPS were diluted to 0.25% CHAPS final concentration and incubated with various concentrations of NH_2-biotinylated peptides (Eurogentec). Two peptides were used; one corresponds to the last 14 amino acids of Delta1 (see above), the second is a scrambled one (LVSEATEAKIDVEC). Proteins bound to the peptides were immobilized on streptavidin-agarose. The beads were washed with a buffer containing 0.25 M NaCl and 0.3% CHAPS. Bound proteins were eluted with sample buffer and analyzed by immunoblotting.

Plasmids and Delta1 Constructs—Delta1 and VSV-Delta1 constructs in the pcDNA6 or MSCV-IRES-GFP (MIG) vector were described previously (17). Delta1$^{\Delta ATEV}$ mutant was generated by PCR amplification using the following primer, 5′-cgcggatccaccatgggccgtcggagcg-3′ and 5′-ccgctcgagcttatataacacactcatccttttctg-3′ (with the Delta1 or the VSV-Delta1 matrix). The Delta1$^{\Delta ATEV}$ mutant was cloned into the BamHI/XhoI sites of pcDNA6 and into the BglII/XhoI sites of MIG. Rat Jagged1 cDNA was cloned into the BamHI/XhoI sites of pcDNA6. Murine Delta4 cDNA was the kind gift from D. Henrique and was cloned into the EcoRI site of pcDNA6. Rat HA-Dlg1 construct was the kind gift of L. Banks (HA-SAP97/pGW1-CMV).

Antibodies—For Dll1 and Dll1CT (C terminal) antisera, the peptides encoding Delta1 amino acids 676–696 and 709–722, respectively, were coupled to keyhole limpet hemocyanin and injected into rabbits. Dll1 antiserum recognizes both Delta1 and Delta1$^{\Delta ATEV}$, and Dll1CT antiserum recognizes both Delta1 and Delta4 but not Delta1$^{\Delta ATEV}$. These serum were diluted 1:2000 for immunofluorescence and 1:8000 for immunoblots. The following antibodies were used in immunoblot (IB), immunoprecipitation (IP), and/or immunofluorescence (IF) with the indicated dilutions: anti-Jagged1 (IB, 1:1000; R & D Systems), anti-VSV (IB, 1:5000; P5D4), anti-HA (IP, 1:200; 12CA5), anti-Dlg1 (IB, 1:1000, and IP, 1:500; Transduction Laboratories), anti-ZO-1 (IB, 1:500, IF, 1:300; Transduction Laboratories), and anti-GFP (IB, 1:2000; Oncogene Research Products).

Cell Extracts, Immunoprecipitation, and Immunoblots—Whole cell extracts, immunoprecipitation, and immunoblots were carried out as described previously (26).

Cell Culture and Transfection—293T, HeLa, Plat-E, and 3T3 cells were cultured in DMEM supplemented with 10% FCS and blasticidin and puromycin for the Plat-E cell line. OP9 cells were cultured in Opti-MEM supplemented with 10% FCS and 50 μM β-mercaptoethanol. 293T cells were transfected with the calcium phosphate co-precipitation procedure and harvested 24 h later. HeLa and Plat-E cells were transfected with FuGENE 6 (Roche Applied Science).

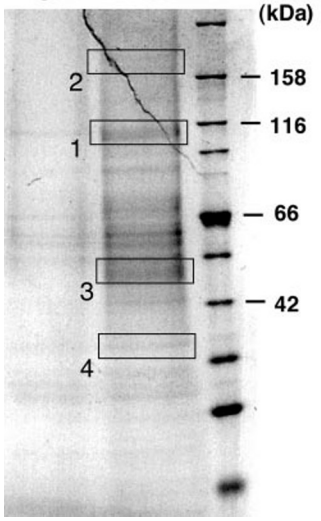
Retroviral Transduction of 3T3 and OP9 Cells and Cell Sorting—High titers of empty (MIG) or recombinant (MIG-Delta1 or MIG-Delta1$^{\Delta ATEV}$) viruses were obtained after transfection of the Plat-E ecotropic packaging cell line. Retroviruses containing supernatants were collected 48 h after transfection and added to 5×10^5 3T3 or OP9 cells. Retrovirally transduced cells were collected 48 h later and analyzed for GFP expression by flow cytometry. GFP-positive cells were enriched by sorting using a MoFlo cytometer (BD Biosciences), giving rise to a ≥98% pure population as determined by post-sort analysis.

Hematopoietic Stem Cell and OP9 Cell Co-cultures—Day 14.5 fetal livers were isolated from C57BL/6 mice. After red cell depletion using Ter119-biotin antibody and streptavidin-coated magnetic Dynabeads (Dynal), HSC were sorted for expression of c-*kit* and Sca-1 using c-*kit*-APC, Sca-1-FITC antibodies, and a MoFlo cytometer (BD Biosciences). c-*kit*$^+$ Sca-1$^+$ HSC were seeded at 10^3 cells/well into 24-well tissue culture plates containing a confluent monolayer of OP9 cells. These co-cultures were performed in Opti-MEM medium (Invitrogen) with 10% FCS, 50 μM β-mercaptoethanol and in the presence of interleukin 7 and Flt3 ligand (27).

Flow Cytometry—Cell surface staining was performed using the appropriate combinations of phycoerythrin and allophycocyanin (APC)-conjugated antibodies (Pharmingen). The following antibodies were used: anti-CD4 (RM4–5), anti-CD8α (53–6.7), anti-TCRγδ chain (H57–597), anti-CD3ε chain (145–2C11), anti-CD19 (1D3), anti-Mac-1. Acquisitions and data analysis were performed with a FACSCalibur interfaced to CellQuest software. Analysis was gated on viable cells based on propidium iodide staining and GFP$^+$ cells (OP9) were excluded from the analysis.

Immunofluorescence—Briefly, HeLa cells were fixed with 4% paraformaldehyde/phosphate-buffered saline, quenched in 50 mM NH_4Cl, permeabilized in 0.1% Triton X-100, 0.1% Tween 20, 1% gelatin, incubated with the first antibody and then with the appropriate dye-labeled secondary antibodies as follows: Cy3-conjugated anti-mouse (1:1600; Amersham Biosciences) and AlexaFluor488-conjugated anti-rabbit (1:800; Molecular Probes). Cell preparations were mounted in Mowiol, and images were acquired by using a Zeiss axioplan 2 micro-

A



B

Observed m/z	Experimental mass	Calculated mass	Δmass	Modification	Position	Peptide
558.31	1114.61	1114.63	-0.03		512-521	IISVNSVDLR
565.29	**1128.56**	**1128.59**	**-0.03**		**258-269**	**IITGGAAAQDGR**
584.29	1166.57	1166.60	-0.03		522-533	AASHEQAAAALK
631.81	1261.60	1261.63	-0.03		597-608	DSGLPSQGLNFK
654.28	1306.54	1306.57	-0.03	MOx	666-677	DKGEIPDDMGSK
703.33	1404.64	1404.68	-0.04		727-738	INDDLISEFPDK
568.23	1701.68	1701.73	-0.05	MOx	562-577	EQMMNSSISSGSGSLR
638.31	1911.89	1911.95	-0.06		628-645	QVTPDGESDEVGVIPSKR

C

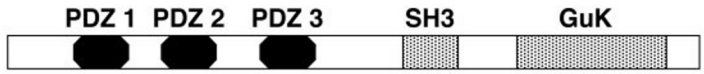


FIG. 2. **Peptide affinity chromatography using Delta1 C terminus and identification of human Dlg1 by mass spectrometry.** *A,* HeLa cells membrane preparation was applied on a Delta1 peptide column (*Dll1*) or a control column (*Cl*). The retained proteins were eluted with 2 M NaCl and after trichloroacetic acid precipitation were loaded on an SDS-PAGE that was stained with colloidal Coomassie. Bands of interest (*boxed*) were excised for subsequent mass spectrometry analysis. Molecular mass markers are indicated on the *right. B,* human Dlg1 peptides identified from quadrupole time-of flight peptide mass spectrum obtained after tryptic digestion of band 1 in *A* (GenBank™ accession number Q12959; molecular mass, 100.6 kDa; total score, 323). The amino acid position in hDlg1 is indicated for each peptide. *m/z* is the mass over charge ratio. *MOx*, oxidized methionine. *C,* schematic structure of the Dlg1 protein, which contains the characteristic domains of MAGUK family members: PDZ (PSD-95/DLG/ZO-1) domains, an Src homology 3 (*SH3*) domain, and a guanylate kinase-like (*GuK*) domain.

scope with the Zeiss ApoTome system for optical sectioning.

Wound-filling Motility Assay—3T3 cells stably transfected with Delta1 and Delta1$^{\Delta ATEV}$ were cultured until they reached confluence. A cell-free area was introduced by scraping the monolayer with a plastic pipette tip. The medium was then changed. The wound area was photographed after 0 and 20 h under standard culture conditions.

Time-lapse Videomicroscopy—3T3 cells were grown on coverslips and placed in a videomicroscopy chamber (Attofluor, Molecular Probes) containing 1 ml of culture medium at 37 °C and 8% CO_2. One acquisition was taken every minute for 2 h. Cells were tracked using the MetaMorph software.

RESULTS

Isolation and Identification of Dlg1—In order to identify Delta1 partners, we concentrated on the highly conserved C-terminal domain that is a canonical class I PDZ-binding motif (S/T)*X*(V/I) (Fig. 1). Delta1-binding proteins were isolated from HeLa crude membranes preparation by retention on a column containing a peptide encompassing the 14 last C-terminal amino acids of mouse Delta1 (see under "Experimental Procedures"). The elution profile of the Delta1 peptide column (Fig. 2*A, Dll1 lane*) was compared with that of a column carrying an irrelevant peptide (Fig. 2*A*, *Cl lane*). After loading on an SDS-polyacrylamide gel and staining with Coomassie Blue, four major bands present only in the *Dll1 lane* of Fig. 2*A* were excised and identified by mass spectrometry (supplemental Table I). Among the proteins unambiguously identified, we focused on the only PDZ-containing protein, Dlg1, isolated from band 1 (Fig. 2*A*). Eight of the measured peptides masses matched the theoretical tryptic peptides masses calculated for the PDZ domain-containing protein Dlg1 (Fig. 2*B*). The fragmentation spectrum, which enabled the sequence identification, is shown for one of them (supplemental Fig. 1). Dlg1 is a scaffolding protein of the MAGUK family that contains three PDZ domains, as shown on Fig. 2*C*. To confirm the specificity of the interaction between Dlg1 and the C-terminal domain of Delta1, HeLa whole cell extracts were incubated with increasing concentrations of an *N*-biotinylated Delta1 C-terminal peptide or a biotinylated scrambled peptide as a control. Proteins retained on streptavidin-agarose were analyzed by Western blot with an anti-Dlg1 antibody. As shown in Fig. 3, endogenous Dlg1 of HeLa cells (Fig. 3, *lane 7*) interacted specifically and in a saturable manner with the C-terminal cytoplasmic tail of Delta1 (Fig. 3, *lanes 1, 3,* and *5*). To verify the specificity of this interaction, we performed the same experiment by using another MAGUK protein, ZO1. By using an anti-ZO1 antibody, we did not detect any specific interaction (data not shown).

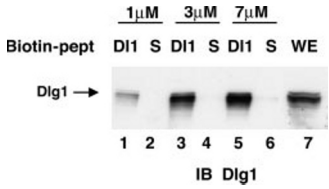


FIG. 3. ***In vitro* interaction of Dlg1 with the Delta1 C-terminal domain.** HeLa whole cell extracts were incubated with increasing concentrations of *N*-biotinylated peptides (Biotin-pep, 1, 3, and 7 μM): Delta1 C-terminal peptide (*Dl1*, *lanes 1, 3,* and *5*) and a scrambled peptide (*S*, *lanes 2, 4,* and *6*). After immobilization on streptavidin-agarose, proteins were analyzed by immunoblotting (*IB*) using an anti-Dlg1 antibody. A whole cell extract (*WE*) was loaded on *lane 7*.

The Delta1 PDZ-binding Motif, ATEV, Is Responsible for the Interaction with Dlg1 in Cells—To confirm a physical association between Delta1 and Dlg1 in cells, we co-transfected 293T cells with VSV-Delta1 and HA-Dlg1 and performed co-immunoprecipitation experiments. Dlg1 immunoprecipitation with an anti-HA antibody followed by immunoblotting with an anti-VSV antibody revealed an interaction between the two molecules (Fig. 4*A, lane 4*).

In order to determine whether the canonical C-terminal PDZ-binding motif of Delta1 (ATEV) was implicated in this association, we generated a mutant deleted of these four amino acids, Delta1$^{\Delta ATEV}$. The correct membrane localization of this protein was confirmed by immunofluorescence analysis (data

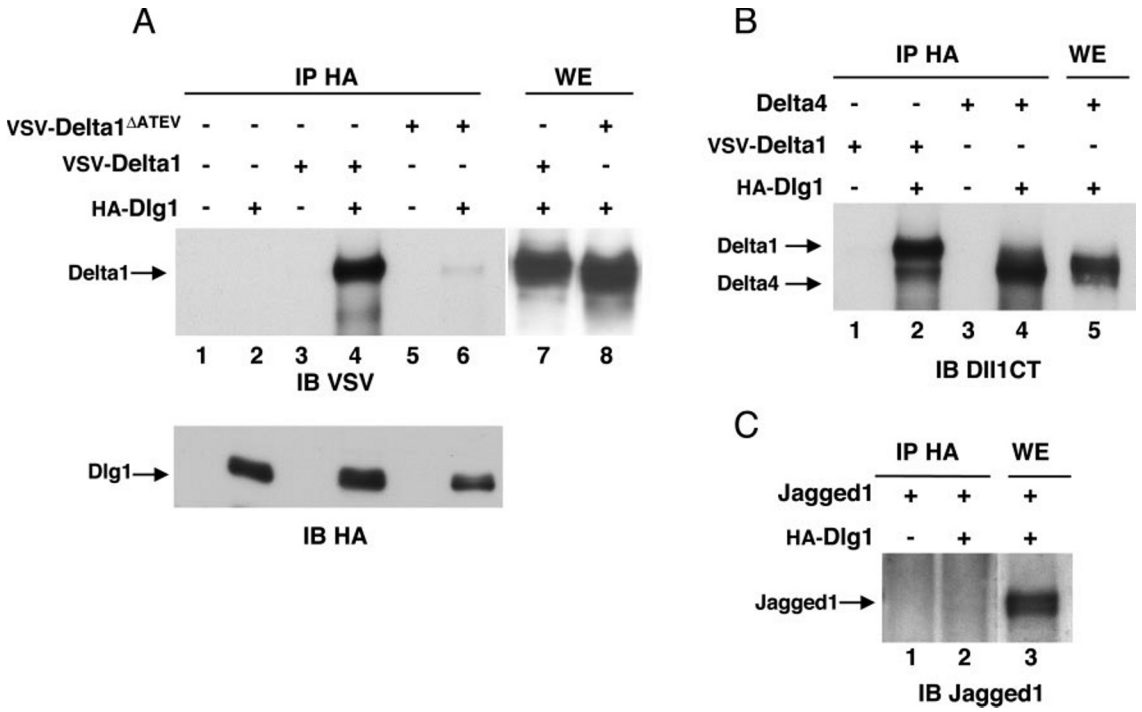


FIG. 4. **Dlg1 interacts specifically with the PBM of Delta1 and Delta4 but not with Jagged1.** *A,* 293T cells were co-transfected with HA-Dlg1 and VSV-Delta1 or VSV-Delta1$^{\Delta ATEV}$. Whole cell extracts (*WE*, *lanes 7* and *8*) and anti-HA-Dlg1 immunoprecipitates (*IP HA*, *lanes 1–6*) were analyzed on SDS-PAGE and immunoblotted (*IB*) with anti-VSV antibody. Dlg1 expression was controlled by immunoblotting with an anti-HA antibody (*lower panel*). *B,* 293T cells were co-transfected with HA-Dlg1 and VSV-Delta1 or Delta4. Whole cell extracts of Delta4 transfected cells (*WE*, *lane 5*) and anti-HA-Dlg1 immunoprecipitates (*IP HA*, *lanes 1–4*) were analyzed by SDS-PAGE and immunoblotted (*IB*) with an anti-Dll1CT antibody. *C,* 293T cells were co-transfected with HA-Dlg1 and Jagged1. Whole cell extracts (*WE*, *lane3*) and anti-HA-Dlg1 immunoprecipitates (*IP HA*, *lanes 1* and *2*) were analyzed on SDS-PAGE and immunoblotted (*IB*) with an anti-Jagged1 antibody.

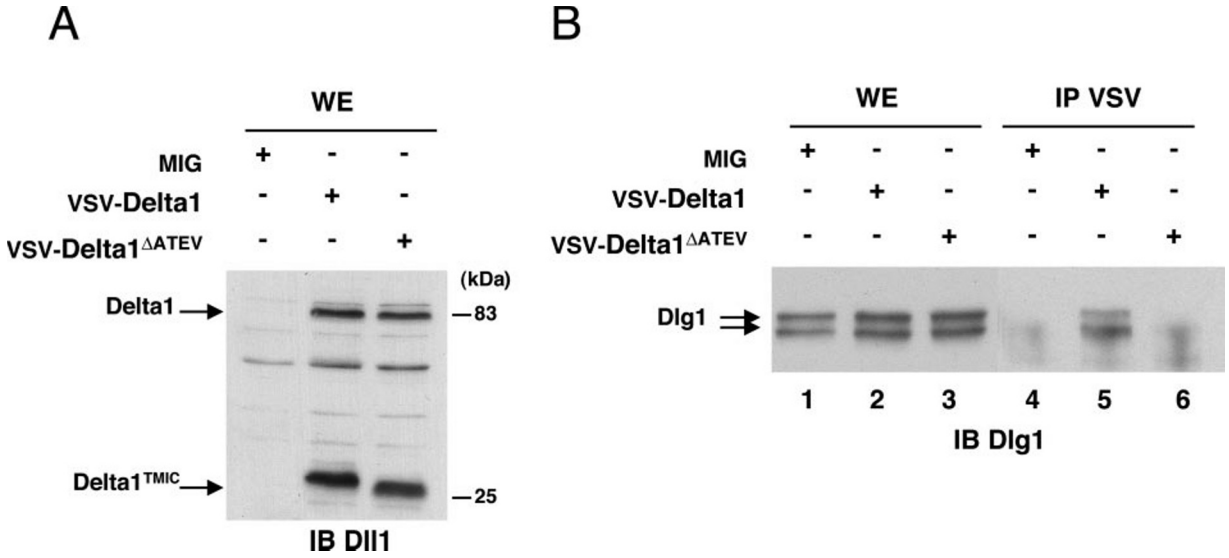


FIG. 5. **Endogenous Dlg1 interacts with Delta1 but not with Delta1$^{\Delta ATEV}$ in 3T3 stable cell lines.** *A,* Delta1 expression in the 3T3 cell lines (MIG, Delta1, and Delta1$^{\Delta ATEV}$) revealed by immunoblotting with an anti-Dll1 antibody (Delta1^{TMIC} is a processing product of Delta1). *B,* whole cell extracts of the 3T3 cell lines (*WE*, *lanes 1–3*) and anti-VSV-Delta1 immunoprecipitates (*IP VSV*, *lanes 4–6*) were analyzed on SDS-PAGE and immunoblotted (*IB*) with anti-Dlg1 antibody.

not shown). Co-immunoprecipitation experiments clearly showed that the Delta1/Dlg1 interaction was completely abolished by deletion of the ATEV motif (Fig. 4*A, lane 6*).

We then generated by retroviral transduction stable 3T3 cell lines expressing VSV-Delta1, VSV-Delta1$^{\Delta ATEV}$, or the control retroviral vector MIG (Fig. 5*A*). Endogenous Dlg1 was expressed in equivalent amounts in the three cell lines (Fig. 5*B, lanes 1–3*). VSV-Delta1 immunoprecipitation with an anti-VSV antibody followed by immunoblotting with an anti-Dlg1 antibody revealed a specific interaction between endogenous Dlg1 and Delta1 (Fig. 5*B, lane 5*) but not with the Delta1$^{\Delta ATEV}$ mutant (Fig. 5*B, lane 6*). This result further confirmed the specificity of the PDZ-mediated interaction in the context of a stable cell line.

To complete this analysis, we studied the interaction of HA-tagged Dlg1 with two other Notch ligands, Delta4 and Jagged1. Extracts from cells co-transfected with Dlg1 and Delta4 were first immunoprecipitated with an anti-HA antibody followed by immunoblotting with a Dll1CT antiserum that recognizes the C terminus of Delta1 and Delta4. Delta4 was found to interact

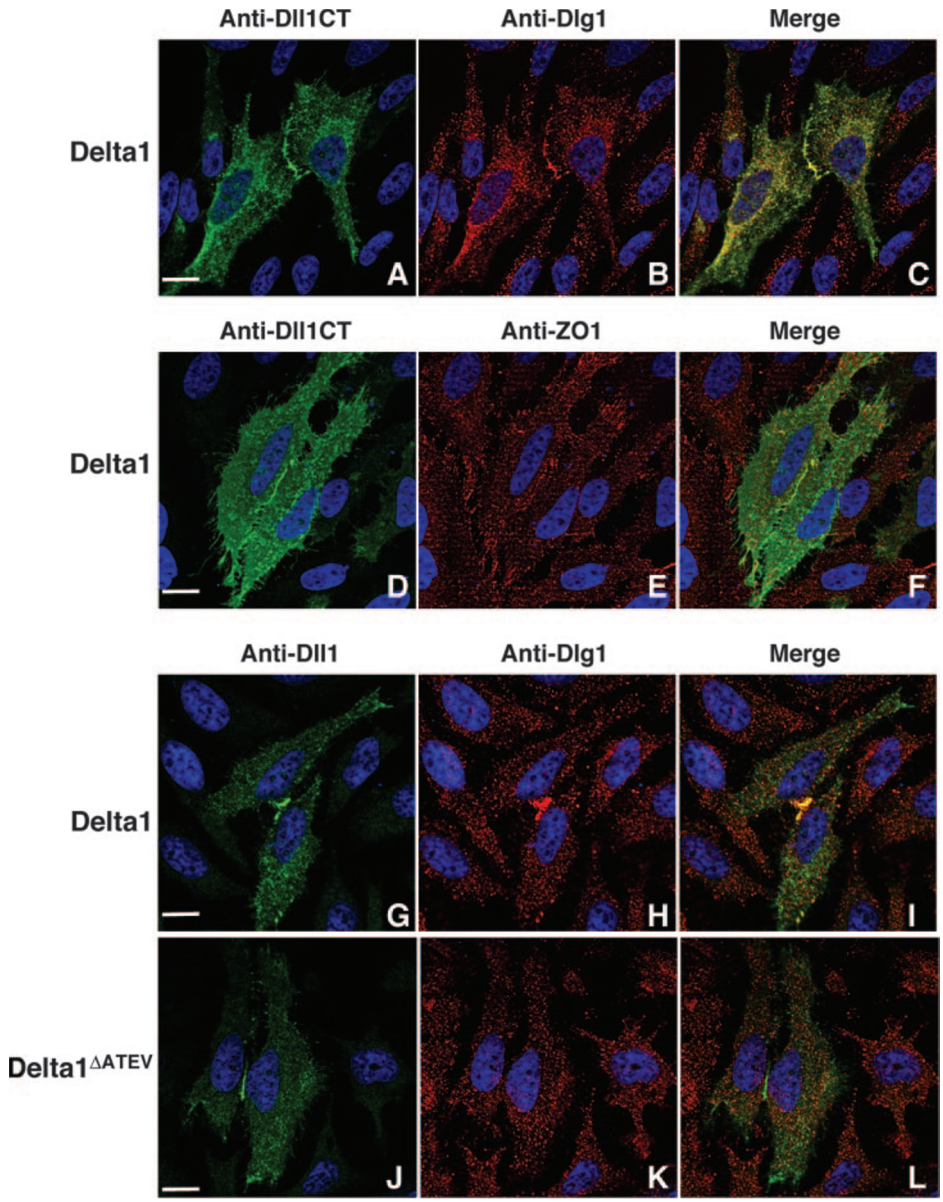


FIG. 6. **Delta1 recruits Dlg1 but not ZO1 to cell-cell contacts in HeLa cells.** *A–F,* HeLa cells were transfected with Delta1. Cells were immunostained with an anti-Dll1CT antibody revealed with Alexa Fluor488-coupled secondary antibody (*A* and *D*), an anti-Dlg1 antibody (*B*), or an anti-ZO1 antibody (*E*) both revealed with Cy3-coupled secondary antibody. The yellow staining (*C*) indicates a co-localization of Delta1 and Dlg1. *G–L,* HeLa cells transfected with Delta1 (*G–I*) or Delta1$^{\Delta ATEV}$ (*J–L*) were immunostained with an anti-Dll1 antibody (*G* and *J*) and an anti-Dlg1 antibody (*H* and *K*). The *yellow staining* (*I*) indicates a co-localization between Delta1 and Dlg1. *Scale bar,* 10 μm.

with Dlg1 (Fig. 4*B, lane 4*). On the contrary, no interaction could be detected between Dlg1 and Jagged1 (Fig. 4*C, lane 2*). Thus Delta1 and Delta4, which have the same C-terminal ATEV motif and Jagged1 which has a distinct motif EYIV (Fig. 1), do not exhibit the same binding specificity. All together these results suggest that Delta1 PBM is specifically responsible for the association with Dlg1.

Co-localization of Delta1 and Dlg1—To test if Delta1 and Dlg1 co-localize in cells, we performed immunolocalization experiments. HeLa cells transfected with Delta1 revealed a striking concentration of Delta1 at cell boundaries (Fig. 6, *A, D* and *G*). This strand-like structure was variable in length but seemed to be associated with the presence of tight contacts between two Delta1-expressing cells. Moreover, this structure did not require the presence of a Notch receptor, which was not detected in HeLa cells (28). Following this observation, HeLa cells transfected with Delta1 were double-labeled with anti-Dll1CT and anti-Dlg1 antibodies. Endogenous Dlg1 was mainly detected in the cytoplasm of HeLa cells (in *red*, Fig. 6, *B, H,* and *K*). However, in the presence of wild type Delta1 (Fig. 6*A*), Dlg1 concentrated at cell-cell junctions where it co-localized with Delta1 (Fig. 6*C*). This co-localization was strictly limited to the region of cell-cell contacts between Delta1-expressing cells and not to other membranous regions. This observation demonstrated that Delta1 is able to trigger Dlg1 localization to sites of cell-cell contacts. Moreover, this recruitment is specific for Dlg1, because Delta1 was not able to recruit another PDZ-containing protein ZO1 (Fig. 6, *D–F*). ZO1 displayed the same cytoplasmic distribution, irrespective of Delta1 expression, and did not co-localize with Delta1 at cell-cell contacts. We then compared the immunolocalization of Delta1 with the Delta1$^{\Delta ATEV}$ mutant by using another antibody which recognizes both proteins (anti-Dll1). We confirmed that Dlg1 is recruited by wild type Delta1 (Fig. 6, *G–I*), and we demonstrated that endogenous Dlg1 remained totally cytoplasmic in cells expressing the Delta1$^{\Delta ATEV}$ mutant (Fig. 6, *J–L*). All together these results showed that Dlg1 is specifically recruited by Delta1 PBM and co-localizes with Delta1 at cell-cell contacts formed between two Delta1-expressing cells.

Induction of T Cell Differentiation Is Not Affected by the Absence of the ATEV Motif—In co-culture experiments Shimizu *et al.* (29) have shown recently that deletion of the whole intracellular region of Delta1 completely abolished the activation of the Notch2 receptor. As PDZ-containing proteins are known to be involved in the organization of signaling complexes (30), the Delta1-triggered recruitment of Dlg1 to cell-cell

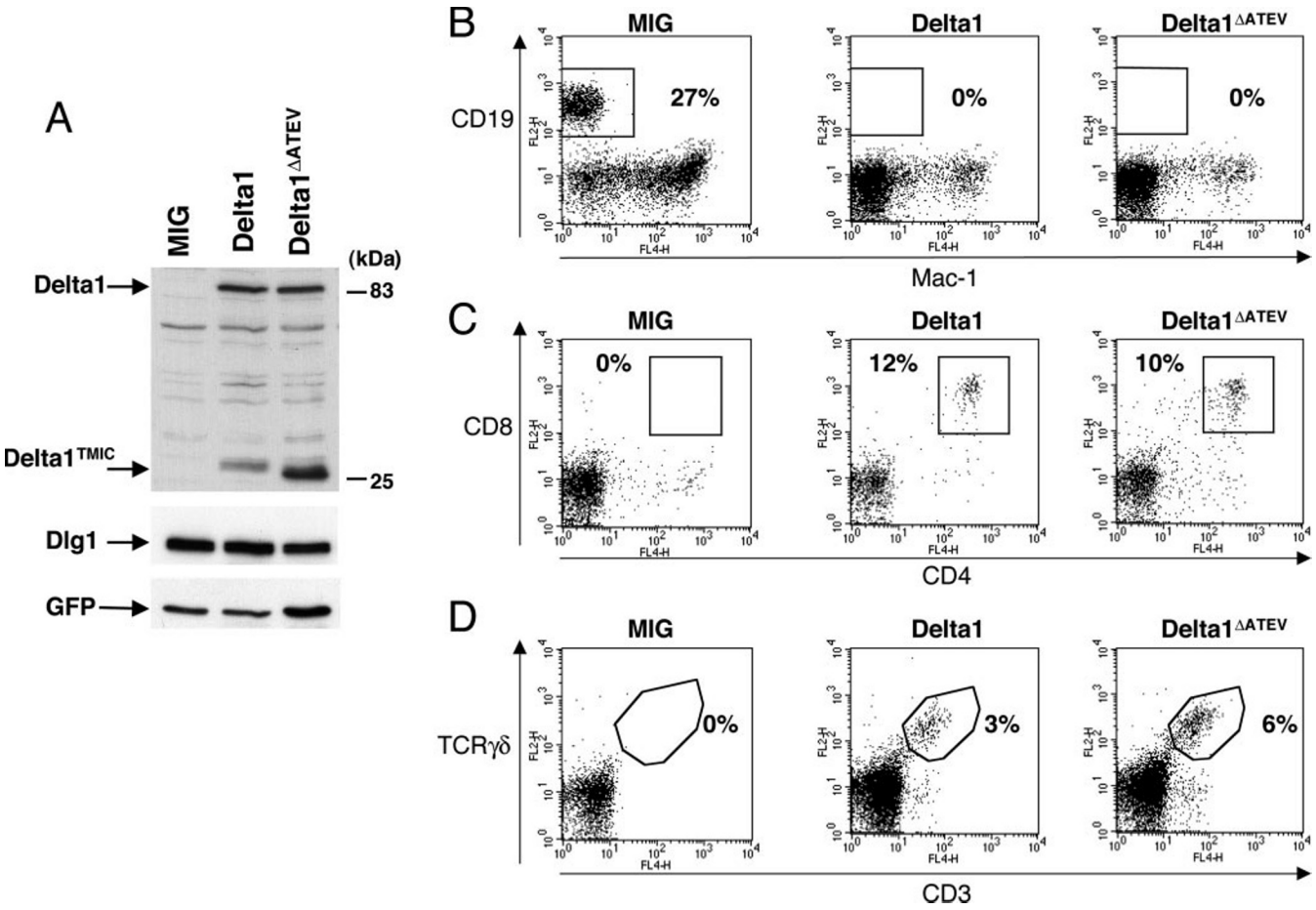


FIG. 7. **Delta1 and Delta1$^{\Delta ATEV}$ inhibit B cell development and induce T cell differentiation.** *A,* Delta1 and Dlg1 expression were checked in the OP9 stromal cell lines (MIG, Delta1, and Delta1$^{\Delta ATEV}$) by immunoblotting with an anti-Dll1 antibody and an anti-Dlg1 antibody. GFP expression is shown as internal control (Delta1^{TMIC} is a processing product of Delta1 (17)). *B–D,* hematopoietic stem cells were co-cultured with OP9-MIG (MIG), OP9-Delta1 (Delta1), or OP9-Delta1$^{\Delta ATEV}$ (Delta1$^{\Delta ATEV}$) stromal cell lines. After 15 days, cells were analyzed by flow cytometry using the B cell marker (anti-CD19) and monocyte/macrophage marker (anti-Mac-1) (*B*). *C,* $\alpha\beta$ T cell markers (anti-CD4 and anti-CD8 antibodies). *D,* $\gamma\delta$ T cell marker (anti-TCR$\gamma\delta$) and anti-CD3 antibody. The percentage of the B cells and T cells populations are indicated and were confirmed in two independent co-culture experiments.

contacts raises the question of its potential indirect role in the Notch signaling pathway.

In order to address this question, we took advantage of the OP9-Delta1 co-culture system that enables *in vitro* T cell differentiation of HSC through activation of the Notch receptor (27, 31). OP9 cells stably expressing either Delta1 or Delta1$^{\Delta ATEV}$ were generated by retroviral transduction together with a control cell line (MIG, transduced with the empty retroviral vector). Delta1 as well as endogenous Dlg1 expression were checked by immunoblotting (Fig. 7*A*). Sorted fetal liver Scal$^+$ c-Kit$^+$ HSC were then co-cultured with the OP9 cell lines. 15 days later, flow cytometry analysis showed that OP9-Delta1 cells totally inhibited CD19$^+$ B cell differentiation in contrast to the control OP9-MIG cells (Fig. 7*B*). Moreover, OP9-Delta1 induced differentiation of double positive CD4$^+$ CD8$^+$ T cells (Fig. 7*C*) as well as $\gamma\delta$ T cells (Fig. 7*D*). HSC differentiation elicited by the OP9-Delta1$^{\Delta ATEV}$ cells showed a similar block in B cell differentiation (Fig. 7*B*) and a concomitant induction of T cell development (Fig. 7, *C* and *D*). These results led us to the conclusion that the deleted mutant Delta1$^{\Delta ATEV}$ is able to activate the Notch signaling pathway in an identical manner as the wild type ligand, suggesting that Delta1/Dlg1 interaction is not essential for Notch activation, at least for B and T cell development *in vitro*.

The PDZ-binding Motif Is Required for Delta1-induced Reduction of 3T3 Cell Motility—To investigate the cell-autonomous role of the Delta1 PBM, we took advantage of the stable 3T3 cell lines expressing wild type Delta1 or the C-terminally deleted molecule (Delta1$^{\Delta ATEV}$). Because both Delta1 and Dlg1 have been implicated in cell migration (8, 32, 33), we monitored the migratory behavior of 3T3 cells expressing Delta1 or Delta1$^{\Delta ATEV}$ and endogenously expressing Dlg1 (Fig. 5*B*). We tested the motility of these cells by using both a wound-filling assay (Fig. 8*A*) and time-lapse videomicroscopy tracking the movement of individual cells at 1-min intervals over a 2-h time course (Fig. 8*B*). As shown in Fig. 8*A*, cells expressing Delta1 or Delta1$^{\Delta ATEV}$ migrated in the wound at different rates. In 3T3-Delta1$^{\Delta ATEV}$ cells, the wound closure was nearly complete after 20 h (Fig. 8*A, d*), whereas the wound was not filled by 3T3-Delta1 cells (Fig. 8*A, b*). Cell tracking analysis showed that 3T3-Delta1 moved at a lower velocity (100 nm/min) than 3T3-Delta1$^{\Delta ATEV}$ (145 nm/min) which is consistent with the wound-filling assay. 3T3-Delta1 cells also showed a significantly ($p < 0.01$) reduced motility (30%) when compared with the control 3T3-MIG cells. The slight difference in motility observed between control cells (130 nm/min) and cells expressing Delta1$^{\Delta ATEV}$ (145 nm/min) was not significant (Student's *t* test). Thus deletion of the ATEV motif abolished the effect of Delta1 on motility and restored a normal rate of migration. These results indicate that Delta1 expression leads to a reduced cell motility and that this effect is mediated by its PBM. We propose that Delta1/Dlg1 interaction is responsible for this behavior.

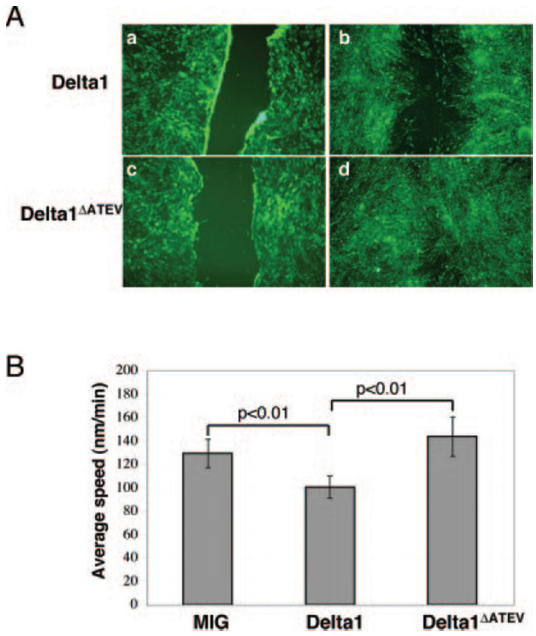


FIG. 8. **PBM is required for Delta1 reduction of 3T3 cells motility.** *A,* wound-filling capacity of stably transfected 3T3 cells expressing Delta1 (*a* and *b*) or Delta1$^{\Delta ATEV}$ (*c* and *d*). Confluent monolayers of 3T3 cells were scraped to create a clear area (*a* and *c*). After 20 h, the wound closure was monitored (*b* and *d*). Two distinct pools of stably transduced cells were used in at least four independent experiments each. *B,* 3T3 cell migration was followed by time-lapse videomicroscopy for 2 h. Ten cells were tracked over this period to determine the average speed of the population (nm/min). This experiment was conducted twice with two distinct pools of 3T3 cells. *Error bars* indicate S.D. Data were analyzed for significance by the Student's *t* test (3T3-MIG and 3T3-Delta1$^{\Delta ATEV}$ do not show a significant difference).

DISCUSSION

The Notch ligands are well known as activators of the Notch cascade by an intercellular mechanism, but some results suggest that they might also exhibit an intrinsic function in the signaling cell (8, 22, 34). To elucidate the possible cell-autonomous role of Delta1, one of the mammalian Notch ligands, we have used affinity chromatography to isolate intracellular proteins interacting with its C-terminal extremity. This approach led us to the identification of Dlg1, which belongs to the family of MAGUK scaffolding proteins.

Dlg1 is a mammalian homolog of the product of the *Drosophila* tumor suppressor gene *discs large* (*dlg*). Mutation of this gene leads to recessive lethal phenotype associated with neoplastic growth of epithelial imaginal discs cells. In *dlg* mutants, epithelial cells also demonstrated abnormalities in septate junction formation, cell polarity, and cell adhesion. At least four mammalian homologs of *Drosophila* Discs large have been described (23). Rodent Dlg1 or synapse-associated protein 97 (SAP97) can functionally substitute for *Drosophila* Discs large in establishing cell polarity (35), suggesting that the function of this protein is conserved between *Drosophila* and mammals. Indeed, in epithelial cells human Dlg1 can be recruited to sites of cell-cell contact by E-cadherin where it regulates adherens junction integrity (36, 37). Mammalian Dlg1 is also involved in the negative regulation of cell growth through its association with the APC tumor suppressor (38).

We demonstrated that Delta1 specifically interacts with endogenous Dlg1 and that both proteins co-localize at cell-cell contacts in HeLa cells. The specificity of this interaction was further confirmed by the fact that neither *in vitro* interaction nor co-localization could be observed with ZO1, another member of the MAGUK family. The conserved C-terminal valine, present in all Notch ligands studied so far (except for Delta3 and Jagged2) (Fig. 1), is part of a canonical PBM (21). By using deleted constructs, we demonstrated that, in transiently transfected 293T cells and in stably transduced 3T3 cell lines, Delta1 ATEV motif was required for Delta1/Dlg1 interaction. We showed that Delta4, which shares the same motif, also interacts with Dlg1. Delta1 has been shown by a yeast two-hybrid screen to interact with another scaffolding protein of the MAGUK family, Acvrinp1 (39). The existence of several PDZ partners could correspond to different cell types studied or to different functions elicited by a single ligand. Moreover, we demonstrated that Jagged1, which presents a distinct PBM, does not interact with Dlg1 (Fig. 4*C*). The PDZ-containing protein AF6 was identified previously by two-hybrid experiments as a partner of Jagged1 (22, 40). The observation that Delta and Jagged probably interact with distinct PDZ proteins may constitute another step toward understanding the functional differences between Notch ligands.

To assess further the importance of the interaction between Delta1 and Dlg1, we took advantage of the C-terminally deleted construct Delta1$^{\Delta ATEV}$, and we compared its activity with that of the wild type molecule in a series of cellular assays. The first assay was aimed at testing the activity of the two Delta1 constructs in activating the Notch pathway in an hematopoietic co-culture system (27, 31). We demonstrated that both Delta1 and Delta1$^{\Delta ATEV}$ are able to induce T cell and inhibit B cell differentiation, therefore indicating that at least in this assay the ATEV motif (and therefore the interaction with Dlg1) is not essential for Delta1 to induce Notch signaling. A similar conclusion was reached, using a different assay, by deleting the C-terminal PBM of Jagged1 (22).

Deletion of the intracellular domain of Notch ligands has a dominant negative effect on Notch activation in *Drosophila* (41, 42) as well as in *Xenopus* (6). However, contradictory results have been obtained in other systems depending on the ligand, the cell type, or the organism studied (43). Therefore, we cannot totally exclude the possibility that deletion of the ATEV motif might interfere with Notch signaling in some other type of assay.

The second series of assays was designed to test the hypothesis that Delta1, through its interaction with Dlg1, might be involved in some cell-autonomous, possibly Notch-independent, activity. Immunofluorescence analysis demonstrated that ectopic expression of Delta1 in HeLa cells induces a major change in the localization of a fraction of endogenous Dlg1, which accumulated together with Delta1 in areas of cell-cell contacts. Deletion of the C-terminal PBM did not affect the localization of Delta1 at cell-cell junctions but totally abolished Dlg1 recruitment, suggesting that Delta1 may be implicated in cellular adhesion through its ability to recruit Dlg1.

In polarized epithelial cells, like many other PDZ and MAGUK proteins, Dlg1 localizes mainly at the basolateral membrane, where it forms protein complexes involved in the establishment of cell-cell adhesion (44, 45). Loss of Dlg1 has indeed been shown to provoke unstable cell-cell adhesion and cell spreading (37). On the other hand in fibroblasts as well as in weakly differentiated epithelial cells such as HeLa cells, which do not form tight intercellular contacts in culture, it was reported that Dlg1 remains cytoplasmic even in cells that adhere to each other (36, 46).

Recruitment of Dlg1 to the plasma membrane has been shown to be triggered by multiple partners, resulting in the formation of molecular scaffolds that control cytoarchitecture and maintain cell-cell junctions (47). For example, it has been demonstrated recently that Dlg1 can be recruited to the plasma membrane by the tumor endothelial marker 5 (48) as well as to cell-cell contacts by E-cadherin (36).

Our observations and the suggested role of Delta1 in the establishment of cell-cell junctions led us to investigate the effect of this molecule on cell motility. We tested this effect in stably transfected 3T3 cells expressing either wild type Delta1 or its mutant derivative Delta1$^{\Delta ATEV}$, using either videomicroscopy or a wound-filling assay. We observed that cells expressing wild type Delta1 exhibit a reduced mobility compared with mock-transfected cells, whereas deletion of the ATEV motif abolished this inhibitory effect. The difference observed was less dramatic for the videomicroscopy assay, probably as a consequence of the multiple signaling pathways that are specifically turned on in the wound-filling assay.

Our findings support the notion that the ligand Delta1 behaves as an adhesion molecule. Indeed the recruitment of Dlg1 at cell-cell junctions has been shown to correlate with the establishment of tight contacts between cells (44–46). In addition, the deletion of Delta1 extracellular region abolishes the localization of this truncated molecule at cell-cell junctions (data not shown), suggesting that intercellular Delta1/Delta1 homotypic interactions might be involved in the formation of the strand-like structure observed. Indeed homotypic Delta/Delta interactions have been visualized by aggregation of ligand-expressing S2 cells (49, 50), but although this type of interaction has been suggested to negatively regulate Notch signaling (51), no firm demonstration of this activity has been reported so far.

A role for a ligand of the Delta family in regulating cell adhesion has been suggested before by two studies (7, 8), which provided evidence that Delta1 intracellular region enhances the cohesiveness of stem cells and maintains tissue integrity. Most interestingly, a similar situation has been observed with E-cadherin, which mediates cell adhesion through homotypic binding of molecules present on adjacent cells and has been shown to recruit Dlg1 into the cortical cytoskeleton at the sites of cell-cell junctions (36).

Taken together these results show that the ligand Delta1 can mediate adhesion and modify cell motility, and we propose that this effect is mediated in part through its association with Dlg1. Indeed, it has been reported in fly that Discs large inhibits epithelial cell cluster motility and thereby is required to block cell invasion (33, 52).

One intriguing question relates to the role of the cleavages undergone by Delta1 in modulating this Delta1/Dlg1 interaction. We and others (17–20) showed previously that Delta1 constitutively undergoes two successive cleavage events; the first one takes place in the extracellular region, is caused by a membrane metalloprotease of the ADAM family; and results in shedding of the extracellular region. It would therefore interrupt the homotypic Delta1/Delta1 interaction mentioned above. Following this event, Delta1 undergoes a second cleavage caused by a γ-secretase-like activity, which liberates the intracellular region of the molecule. Again the fate as well as the role of this region remains unclear, but in any case this cleavage would abolish Delta1-mediated recruitment of Dlg1 to cell-cell contacts. Therefore, these cleavage events, beside terminating Notch signaling, would abolish any role of Delta1 in cell adhesion and motility.

Most interestingly, it has been shown that presenilin, an important component of the γ-secretase complex, associates with the cadherin/catenin cell-cell adhesion system and accumulates at intercellular contacts in epithelial cells (53). More recently, E-cadherin has been demonstrated to be a substrate for γ-secretase, thus regulating disassembly of adherens junctions (54). These data raise the possibility that a single complex (or distinct complexes) containing E-cadherin, Delta1, and γ-secretase might be involved in the formation of intercellular junctions in epithelial cells, and that the proteolytic activity of γ-secretase would participate in the disappearance of these junctions under normal or pathological conditions.

Another intriguing point relates to the possible modulation of this Delta1/Dlg1 interaction by the Notch receptor. The idea of bi-directional signaling by a Notch/Jagged1 interaction has been suggested recently by Ascano *et al.* (22). If this is correct, the cell-autonomous role of Delta1 as a regulator of the adhesion and motility properties of the emitting cell could then be regulated by the Notch receptor.

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