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Delta1 family members are involved in filopodial actin formation and neuronal cell migration independent of Notch signaling

Kazuya Sugiyama [b], Kenji Nishide [b], Hiromi Matsuo [a], Sayumi Okigawa [b], Makoto Okano [b], Tohru Ishitani [b], Kunihiro Matsumoto [b], Motoyuki Itoh [a,b,*]

[a] Institute for Advanced Research, Nagoya University, Nagoya, Aichi 464-8602, Japan
[b] Graduate School of Science, Nagoya University, Nagoya, Aichi 464-8602, Japan

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ABSTRACT

Delta family proteins are transmembrane molecules that bind Notch receptors and activate downstream signaling events in neighboring cells. In addition to serving as Notch ligands, Notch-independent roles for Delta have been suggested but are not fully understood. Here, we demonstrate a previously unrecognized role for Delta in filopodial actin formation. Delta1 and Delta4, but not Delta3, exhibit filopodial protrusive activity, and this activity is independent of Notch signaling. The filopodial activity of Delta1 does not depend on the PDZ-binding domain at the C-terminus; however, the intracellular membrane-proximal region that is anchored to the plasma membrane plays an important role in filopodial activity. We further identified a Notch-independent role of DeltaD in neuronal cell migration in zebrafish. These findings suggest a possible functional link between Notch-independent filopodial activity of Delta and the control of cell motility.

1. Introduction

Evolutionarily conserved Notch signaling plays critical roles in cell growth and differentiation in many cell types during development [1,2]. Notch is a transmembrane receptor that is activated by several ligands. Two groups of Notch ligands that are also transmembrane proteins, Delta and Jagged/Serrate, have been identified in numerous species. In the canonical Notch signaling pathway, ligand-stimulated Notch receptors are cleaved, and their intracellular fragments enter the nucleus to activate downstream effector genes.

In addition to acting as Notch ligands, accumulating evidence suggests that Delta and Jagged function independently of Notch in different processes such as cell motility, oncogenic transformation and neurogenesis [3–8]. For example, the potential involvement of the PDZ-binding domain (PDZ-BD) of Delta1 in cell adhesion and motility has been well-studied [3,5,6,9]. These studies suggest that PDZ proteins such as MAGI and Dlg stabilize surface expression of Delta1 and increase the efficiency of binding of Delta or Notch on neighboring cells, which promotes the cohesiveness between cells. However, Delta3 and Jagged2 do not have PDZ-BDs, which are required for binding to PDZ proteins [10], and the function of intracellular regions other than the PDZ-BD of Delta proteins is not fully understood.

Individual cell motility requires rearrangement of the cytoskeleton. The actin cytoskeleton is a dynamic structure that is involved in cell motility in response to various signals. Cells extend two types of protrusions containing actin, sheet-like protrusions called lamellipodia and spine-like protrusions called filopodia [11–14]. A previous study showed that Delta1 co-localized with actin filaments in keratinocytes [3]. In addition, *Drosophila* Delta, which does not have a PDZ-BD, promotes filopodia formation [15]. However, the function of Delta in relation to actin-driven filopodia formation is not known.

In this study, we uncovered a new role for Delta family proteins in filopodial actin extension. Delta1 and Delta4, but not Delta3, promote filopodial protrusions in neuro2a cells, and their functions are independent of Notch signaling activity. This filopodial activity does not require the PDZ-BD of Delta1; however, it does require the membrane-anchored intracellular domain of Delta1. We further identified Notch-independent activity of DeltaD, a Delta1 family protein in zebrafish, in neuronal cell migration *in vivo*. Collectively, these data suggest an unidentified mechanism mediates the actin regulatory function of Delta1 through a Notch-independent pathway that contributes to neuronal cell migration *in vivo*.

Abbreviations: EGF, epidermal growth factor; DSL, Delta/Serrate/Lag-2; PDZ-BD, PSD-95/Discs-large/ZO-1-binding domain; MAGI, MAGUKS with inverted domain structure; Dlg, Discs large; mib, mind bomb; hpf, hours post-fertilization.

* Corresponding author. Address: Institute for Advanced Research, Nagoya University, IAR Hall, Rm. 208, Furou-cho, Chikusa-ku, Nagoya, Aichi 464-8602, Japan. Fax: +81 52 788 6179.
E-mail address: mito@iar.nagoya-u.ac.jp (M. Itoh).

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2. Materials and methods

2.1. Plasmids and expression constructs

In full-length mDelta1-Myc (M1 to V722), the stop codon of mouse Delta1 was replaced by histidine to allow fusion with a 6× Myc tag, and the fusion was inserted into pcDNA3 (Invitrogen). Delta1 Myc ΔICD is a deletion form lacking a cytoplasmic region (R570 to D714). Delta1 Myc TMICD is a deletion form lacking the extracellular region (V12 to H535). Delta1 Myc TMICDΔMPR-A is a deletion construct of TMICD lacking the membrane-proximal region A (R570 to C583). Delta1 Myc TMICDΔMPR-B is a deletion form of TMICD lacking the membrane-proximal region B (G584 to A594). Delta1 Myc ECD is a deletion construct lacking the transmembrane domain and intracellular region (M536 to V722). Delta1 Myc ICD is a form containing the intracellular region (V567 to V722). Delta1 HA wt is a full-length form in which an HA tag is inserted between A594 and N595 in pcDNA3.1. Delta1 HA del1 is an HA-tagged deletion form lacking an intracellular region (N595 to V722). mDelta3-Myc was made in a manner similar to mDelta1-Myc. The hDelta4-Flag plasmid was obtained from S. Sakano (Asahi Kasei, Japan). Zebrafish DeltaA-HA and DeltaD-HA expression plasmids were created by replacing the stop codons with HA tags, and the entire coding fragments were inserted into the pCS2 + vector. HA-DeltaD ICD is an HA-tagged form containing the intracellular region (I570 to V717). DeltaD TMICD is a deletion form lacking the extracellular region (G21 to C514). Xsu(H)DN and Notch1ICD (pEFBOSneo-RAMIC) have been previously described [16,17].

2.2. Cell culture, transfection, immunofluorescence, phalloidin staining and chemical treatment

Neuro2a cells were transfected using Fugene6 (Roche, Switzerland). Cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Myc-tagged or HA-tagged Delta- or Xsu(H)DN-expressing cells were visualized with primary antibodies, anti-Myc (9E10, Santa Cruz, CA, USA) or anti-HA (3F10, Roche, Switzerland) and then with secondary antibodies, containing Alexa-488 mouse or rat IgG, respectively (Invitrogen, CA, USA). Actin filaments were visualized with rhodamine–phalloidin (Invitrogen, CA, USA) or DY590-phalloidin (Dyomics GmbH, Germany). Twenty-four hours after transfection, cells were treated with 2 μM cytochalasin D (Sigma, MO, USA) or 5 μM nocodazole (Sigma, MO, USA) for 24 h and were then fixed for staining.

2.3. Reporter gene assays

Neuro2a cells (4.5×10^4) were transfected with Xsu(H)DN, Notch1ICD (pEFBOS-neo RAMIC), a wild-type or mutant 8× su(H) Notch reporter (0.1 μg of JH26 or JH28 plasmid, respectively), and pRL-EF (5 ng). Forty-eight hours after transfection, firefly and *Renilla* luciferase activities were determined with the Promega Dual luciferase assay system (Madison, WI, USA). The Notch activity of each transfection was calculated as the ratio of values determined from the wild-type reporter compared to the values determined by the mutant reporter.

2.4. Fish maintenance and mutants

Zebrafish were raised and maintained under standard conditions. The *mib*^{ta52b} mutant line has been previously described [18].

2.5. mRNA injection

mRNAs were synthesized by transcription with SP6 RNA polymerase using the SP6 mESSAGE mACHINE kit (Applied Biosystems, CA, USA), and 600 pg of mRNA were injected into one-cell-stage embryos.

2.6. Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed as previously described, except a different blocking buffer was used (5% sheep serum, 1% BMB, 0.1% Tween 20, 1% DMSO, 150 mM NaCl and 100 mM maleic acid, pH 7.5) [19]. *elavl3* utilized in the experiment were previously published [20].

3. Results

3.1. Delta1 induces filopodial protrusions in neuro2a cells

An earlier study demonstrated that overexpression of dominant negative or full-length forms of Delta1 in neuro2a neuroblastoma cells induces neurite extension in reduced serum at low plating density [21]. We performed a similar experiment in regular media containing 10% fetal calf serum and found that overexpression of full-length mouse Delta1 increased the number of cells with short cellular protrusions, as shown by staining with fluorescently labeled phalloidin (85%, $n = 708$) (Fig. 1A). To further define the cellular protrusions induced by Delta1, we treated Delta1 overexpressing cells with cytochalasin D, a potent inhibitor of actin polymerization, and nocodazole, which interferes with the polymerization of microtubules. Treatment with cytochalasin D but not nocodazole inhibited Delta1-induced protrusions (Fig. 1B). These data suggest that the filopodial protrusions induced by Delta1 are composed of actin filaments.

3.2. Delta1 and Delta4 family proteins, but not Delta3, induce cellular protrusions

More than three Delta family members (e.g., Delta-like 1, 3 and 4 in mammals; DeltaA, B, C, D and 4 in zebrafish) have been identified in vertebrates. These proteins show considerable structural homology within their extracellular regions and share a common domain architecture, including multiple epidermal growth factor (EGF)-like repeats and a DSL domain [10]. Therefore, we hypothesized that other Delta family proteins may possess similar protrusive activities. Transfection of Delta1, Delta3 and Delta4 into neuro2a cells revealed differing activities among the Delta protein family, namely Delta1 and Delta4 have protrusive activity but Delta3 does not (Fig. 2A). We next examined if zebrafish homologs of Deltal possess protrusive activity. Although zebrafish DeltaA and DeltaD do not have strong protrusion activity like Delta1, they showed a substantial effect on the formation of filopodial protrusions (Fig. 2B). These data suggest that Delta1 family proteins have conserved functions in inducing actin-based cell protrusions in a cell-autonomous manner.

3.3. A membrane-proximal domain anchored to the plasma membrane is required for the protrusive activity of Delta1

A previous study suggested the potential involvement of Delta in actin cytoskeletal organization [3]. The mechanism of Delta1-mediated regulation of actin dynamics remains unclear; therefore, we sought to identify regions important for the cellular protrusive activity of Delta1. The PDZ-binding domain at the Delta1 C-terminus is important for the cohesiveness of keratinocytes [3]; however, deletion of 128 C-terminal amino acids of Delta1, including the PDZ-binding domain, did not affect its protrusive activity (Delta1 HA del1 in Fig. 2C). In contrast, deletion of almost the entire cytoplasmic region, except the PDZ-binding domain, resulted in a