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Conversion of plasma membrane topology during epithelial tube connection requires Arf-like 3 small GTPase in *Drosophila*

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ABSTRACT

The development of tubular organs often involves the hollowing of cells into a torus (doughnut shape), as observed in blood vessel formation in vertebrates and tracheal development in insects. During the fusion of *Drosophila* tracheal branches, fusion cells located at the tip of migrating branches contact each other and form intracellular luminal cavities on opposite sides of the cells that open to connect the tubule lumens. This process involves the intracellular fusion of plasma membranes associated with microtubule tracks. Here, we studied the function of an evolutionarily conserved small GTPase, Arf-like 3, in branch fusion. Arf-like 3 is N-terminally acetylated, and associates with both intracellular vesicles and microtubules. In Arf-like 3 mutants, the cell adhesion of fusion cells, specification of apical membrane domains, and secretion of luminal extracellular matrix proceeded normally, but the luminal cavities did not open due to the failure of intracellular fusion of the plasma membranes. We present evidence that the Arf-like 3 mutation impairs the localized assembly of the exocyst complex, suggesting that the targeting of exocytosis machinery to specific apical domains is the key step in converting the plasma membrane topology in fusion cells.

1. Introduction

The variety of cell morphology is sometimes understood as the changes in cell surface topology, which is defined as the properties of the plasma membrane that are independent of size or shape and are changed only by fission or fusion. The topology of plasma membrane is highly stable, but in special situations, the plasma membrane changes its topology. For example, cellularization of the insect blastoderm proceeds by ingression of the plasma membrane of the syncytium around each nucleus, and its fusion to form individual cells (Foe et al., 1993). The fusion of the plasma membrane via its inner layer takes place in every round of the cell cycle, in the process called abscission, at the finishing stage of cytokinesis (Gromley et al., 2005). On the other hand, the fusion of myoblasts via the outer layer of the plasma membranes forms multinucleated myotubes, and the mechanism underlying this process is currently being elucidated (Kim et al., 2007; Massarwa et al., 2007). During the development of tubular organs, such as inter-segmental vessel formation in the zebrafish (Kamei et al.,

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2006), intracellular vesicles align along the path of the future lumen; the subsequent fusion of each cell's vesicles with the plasma membrane converts each spherical cell into a hollow unicellular tubule. Despite its importance in vasculogenesis and cell topology studies, research progress toward an understanding of intracellular plasma membrane fusion has been slow.

Fusion of the *Drosophila* tracheal tubules offers a unique opportunity to study internal plasma membrane fusion in a genetically tractable model system (Samakovlis et al., 1996b; Tanaka-Matakatsu et al., 1996). The *Drosophila* tracheal system is derived from invagination of the tracheal primordium in ten metameres on each side of the embryo (Samakovlis et al., 1996a). Each primordium extends tubular branches in stereotypical patterns. Branches for the dorsal trunk (DT) and lateral trunk (LT) meet at specific locations at segment boundaries, and the dorsal branch (DB) and ventral branch (VB) meet at the dorsal and ventral midline, respectively. Fusion is initiated by the contact of specialized fusion cells located at the tip of each branch. Detailed studies on fusion of the DT have revealed that the fusion cells in contact establish a new adherens junction between them. Inside the fusion cells, tracks of microtubules associated with F-actin and vesicles align in a pattern prefiguring the future luminal axis (Lee and Kolodziej, 2002). After this alignment, plasma membrane of the fusion cells invaginates along the future luminal axis, from the lumen toward the newly formed adherens junction. Finally lumen passes through the fusion cell by localized plasma membrane fusion, and the topology of cell changes from a sphere to a torus (a doughnut-shape). Although less is known, a similar intracellular event is believed to mediate the fusion of other branches. In each case, a change in cell surface topology of fusion cells is the key event for the connection of two closed branches into one continuous tube. But the molecular machinery of the cell topology conversion process remained completely unknown.

One proposed mechanism for the cell-shape conversion of tracheal fusion cells is that microtubule tracks guide vesicle assembly and facilitate the internal fusion of plasma membranes bridged by the tracks (Samakovlis et al., 1996b). This model predicts that these vesicles fuse with the plasma membrane by exocytosis. The exocyst complex is involved in polarized membrane fusion and exocytosis in various systems, such as the nervous system and epithelial tissues (Hsu et al., 2004; Langevin et al., 2005; Lipschutz and Mostov, 2002; Murthy et al., 2003). The exocyst complex also localizes to the mid-body of the cell, which is the site of abscission, during cytokinesis (Gromley et al., 2005), and is required for abscission, suggesting that it is a good candidate regulator for internal plasma membrane fusion. Here we report that Arf-like 3 (Arl3) plays a major role in the internal plasma membrane fusion of tracheal fusion cells, probably by recruiting the exocyst complex.

2. Results

2.1. *Identification of Arf-like 3 as a fusion cell vesicle component*

To examine the membrane dynamics in fusion cells, we observed the intracellular membrane dynamics by time-lapse imaging of DT fusion cells labeled with membrane-targeted GFP (GFP-CAAX, Ikeya and Hayashi, 1999). After the initial contact between fusion cells (Fig. 1A time 0′00″, contact site is shown by arrowhead), an intracellular array of vesicles (Fig. 1A, time 15′00″, arrows) appeared between the contact site of the fusion cells and the tips of the lumens (Fig. 1A, time 15′00″, Fig. 1D, arrows). Finally, the lumen penetrated through the fusion cells where GFP-CAAX accumulated (Fig. 1A, time 69′00″, arrowhead). These observations led us to investigate the role of intracellular vesicles during tracheal tube connection.

To identify genes involved in tracheal branch fusion, we searched the BDGP *in situ* database (Tomancak et al., 2002) for genes expressed in a subset of *Drosophila* tracheal cells, and identified Arf-like 3 (*Arl3*, CG6560, Crosby et al., 2007). An antibody against Arl3 detected its specific expression in all the tracheal fusion cells (Fig. 1B, data not shown), and this pattern was consistent with the RNA expression pattern (Tomancak et al., 2002; data not shown). The accumulation of Arl3 became prominent when a pair of fusion cells approached within ~10 μm of each other, and persisted after the completion of fusion (Fig. 1B, data not shown). A high-magnification view revealed Arl3 to be in a vesicular pattern within the fusion cells, and co-localized with the intracellular vesicles marked by GFP-CAAX (Fig. 1D). In addition Arl3 was detected in the sensory organs from embryonic stage 15 (data not shown).

2.2. *Arl3 is an N-terminally acetylated microtubule-associated protein*

Arl3 is a member of the ADP-ribosylation factor (Arf) subfamily of Ras-like small GTPases, and its structure is evolutionarily conserved from yeast to humans (Kahn et al., 2005, Fig. 2A). Arf family members play various roles in intracellular vesicle traffic (Burd et al., 2004; Kahn et al., 2005; Kawasaki et al., 2005; Nie et al., 2003). Arf-like family members, such as human Arl8 and *S. cerevisiae* Arl3p (also known as Arfrp, Arf-related protein, in higher eukaryotes), are reported to be post-translationally modified at their N-terminus (Behnia et al., 2004; Hofmann and Munro, 2006; Setty et al., 2004). To detect post-translational modification, Arl3 expressed in *Drosophila* S2 cells was immunopurified and subjected to mass spectrometric analysis. A number of peptide fragments corresponding to the mass of the acetyl group (Ac-) linked to the second glycine (G2) residue of the N-terminus of Arl3 were detected (Ac-GLLSLLR, precursor ion: 813.64 *m/z* (1+), Fig. 2B, arrows). In addition, unmodified peptides missing the N-terminal methionine were also detected (GLLSLLR, precursor ion: 771.98 *m/z* (1+), data not shown). These data suggest that *Drosophila* Arl3 undergoes the removal of methionine and subsequent acetylation at the N-terminus. No peptide peaks corresponding to N-terminal myristoylation were detected. N-terminal acetylation is reported to be crucial for yeast Arl3p to localize to the Golgi apparatus, and N-terminal aceylation and N-terminal hydrophobic residues are required for human Arl8 to localize at lysosomes (Behnia et al., 2004; Hofmann and Munro, 2006; Setty et al., 2004). To address the functional significance of the N-terminal acetylation, GFP was fused to the N-terminus of Arl3. GFP-Arl3 migrated in SDS–PAGE with