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Nak regulates Dlg basal localization in *Drosophila* salivary gland cells

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ABSTRACT

Protein trafficking is highly regulated in polarized cells. During development, how the trafficking of cell junctional proteins is regulated for cell specialization is largely unknown. In the maturation of *Drosophila* larval salivary glands (SGs), the Dlg protein is essential for septate junction formation. We show that Dlg was enriched in the apical membrane domain of proximal cells and localized basolaterally in distal mature cells. The transition of Dlg distribution was disrupted in *nak* mutants. Nak associated with the AP-2 subunit α-Ada and the AP-1 subunit AP-1γ. In SG cells disrupting AP-1 and AP-2 activities, Dlg was enriched in the apical membrane. Therefore, Nak regulates the transition of Dlg distribution likely through endocytosis of Dlg from the apical membrane domain and transcytosis of Dlg to the basolateral membrane domain during the maturation of SGs development.

Introduction

Most cells exhibit subdivisions in plasma membranes and underlying subcortical regions with specialized compositions and structures. Typical examples include the apical and basolateral domains of polarized epithelial cells and the axonal and somatodendritic domains of neurons that bear distinct and vital functions to the cells. During the establishment of the epithelial cell polarity, different cell junctions that function in cell–cell contact and tissue integrity are organized. Many junctional components also play essential roles in the establishment and the maintenance processes [1–3]. Depositions of junctional components and organization of specialized cellular domains rely on endocytosis, protein sorting, endosomal recycling, and polarized transportation processes [4,5]. Little is known for the reorganization of junctional proteins and the underlying mechanism regulating this process.

In clathrin-mediated trafficking processes, AP complexes recruit cargos, clathrin, and other accessory proteins to form clathrin-coated vesicles. The AP-2 complex is localized at the subcortical region of the plasma membrane and required for endocytosis. In mammalian cells, the AP-1A complex is ubiquitously expressed in all cell types and involved in trafficking between TGN and endosomes. The AP-1B complex is only present in polarized cells and required for sorting from the recycling endosomes [6–9]. In *Drosophila*, the only AP-1 complex might play dual roles performed by AP-1A and AP-1B in mammalian cells. In addition, the *Drosophila* AP-1 and AP-2 complexes share the β subunit, but differ in other subunits: the AP-1 complex includes γ, μ1, and σ1 subunits; the AP-2 complex includes α, μ2, and σ2 subunits [10].

Activities of the AP complexes are regulated by the Ark/Prk family of protein kinases which share sequence homology in the serine/threonine kinase domains [11]. In endocytosis, yeast Ark1p and Prk1p negatively regulate the formation of protein complexes that include the Eps-15 homolog Pan1p, Sla1p, and End3p [12,13]. Mammalian AAK1 and GAK/auxilin2 have multiple and partially redundant roles in clathrin-mediated trafficking processes, such as endocytosis, endosomal recycling, or lysosomal sorting [14–18]. AAK1, GAK, or both regulate these processes through phosphorylations of the μ subunits of AP-1 and AP-2 complexes by which facilitates the cargo binding efficiency [15,19–22]. Other mechanisms involve recruitment of adaptors through protein–protein interactions, phosphorylation of accessory proteins, and uncoating the clathrin-coated vesicles [16,18,23].

We show that the *Drosophila* Ark/Prk homolog Nak is involved in the polarization of SG cells. In *nak* mutants, basolateral membrane distribution of Dlg and apical-to-basal transition in SG cells were disrupted, and the growth of SG cells was severely retarded in *nak* mutants. We identified the interaction between Nak and the α subunit of the AP-2 complex and the γ subunit of AP-1 complex. Consistently, mutants for components of AP-1 or AP-2 complexes photocopied *nak* mutants, displayed similar Dlg distribution and cellular growth defects. Therefore, Nak regulates SGs

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development and the transition of Dlg likely through endocytosis and transcytosis.

Materials and methods

Drosophila strains. Wild type flies used in this study were w^{1118}. *Sgs3-GAL4*, $AP47^{EP1112}$, and $AP47^{SHE11}$ were from Bloomington *Drosophila* Stock Center. α-ada^{1} and α-ada^{3} were described previously [24]. The RNAi transgenic lines for knockdown of *AP-1*γ (#3275) and *AP-2*σ (#34148) were from Vienna *Drosophila* RNAi Center. Flies were maintained at standard food vials at 25 °C except for α-$ada^{1/3}$ that was raised at 22 °C.

Immunostaining. SGs were isolated from third instar larvae, and fixed and immunostained following standard procedures. Antibodies used were mouse α-Dlg (1:50, DSHB), α-aPKC (1:50, Santa Cruz), and α-Syx 1A (1:50, DSHB). TRITC-conjugated Phalloidin and Hoechst 33258 were used to mark the filamentous actin and nuclei, respectively. Fluorescence intensities of Dlg along the apical and basal membrane were measured by the Metamorph software and the *a*pical and *b*asal intensity per pixel were used to calculate for the A/B ratios.

S2 cell transfection, immunoprecipitation, and Western blots. Plasmids of p*WA-GAL4* and p*UAST-Myc-nak* or p*UAST-Myc-nakΔC* were mixed with Cellfectin (Invitrogen) and transfected into S2 cells. Two days after, the transfected cells were lysed for immunoprecipitation or Western blots. Antibodies used for immunoprecipitation were α-c-Myc antibody (A14, Santa Cruz). Primary antibodies used for immunoblots were α-Chc (1:250, Sigma), α-Eps15 (1:250, Covance Research), α-α-Ada (1:20,000, [24]), α-14-3-3ε [25], α-gamma Adaptin (1:1000, Abcam), and α-c-Myc (1:1000, A14). Secondary antibodies used were α-mouse-HRP or α-rabbit-HRP (1:5000, Jackson ImmunoResearch Laboratories).

Results

Dlg is mislocalized in the distal SG cells in nak mutants

Dlg is a founding member of the membrane-associated guanylate kinase (MAGUK) family proteins and is involved in the establishment of cell polarity, stabilization of septate junction, and inhibition of cell proliferation [26,27]. In distal part of SGs in *Drosophila* third instar larvae, Dlg localized at the subcortical region of the basal membrane (Fig. 1A). When viewing sections through distal SG cells, Dlg was found tightly associated with the basal membrane (Fig. 1A1). In the lateral membrane, Dlg expression was presented in an apicobasal gradient with diminishing concentration toward the apical end (arrowheads). In addition, cytoplasmic Dlg also formed a similar gradient (arrow) but with lower protein levels than the membrane-associated ones.

The polarized distribution of Dlg was altered in *nak* mutants, in which the SG cell growth was also hindered (Supplementary Fig. 1 and Table 1). Dlg localization in the basal membrane region was still observed; the protein levels, however, were reduced (Fig. 1B and B1). Dlg was enriched at the apical membrane region (arrow in Fig. 1B1) and the Dlg gradient along the lateral membrane domain was reversed with higher concentration toward the apical end (arrowhead). The gradient in the cytoplasm was no longer present. In addition, Dlg punctas were detected near the apical membrane (empty arrowhead). The fluorescence intensities of Dlg immunoreactivity in apical and basal membrane domains were measured, and the apical-to-basal ratio of Dlg immunoreactivity is expressed as the A/B ratio. In the WT and heterozygous $nak^{MR6/+}$ and $nak^{Del3/+}$ control cells, the A/B ratios are 55–65% (Fig. 1C). In $nak^{MR6/MR6}$ and $nak^{MR6/Del3}$ mutant cells (see Supplementary Fig. 2A for mutant description), however, the A/B ratios of Dlg immunoreactivity reached 3 and 2.4, respectively. These results

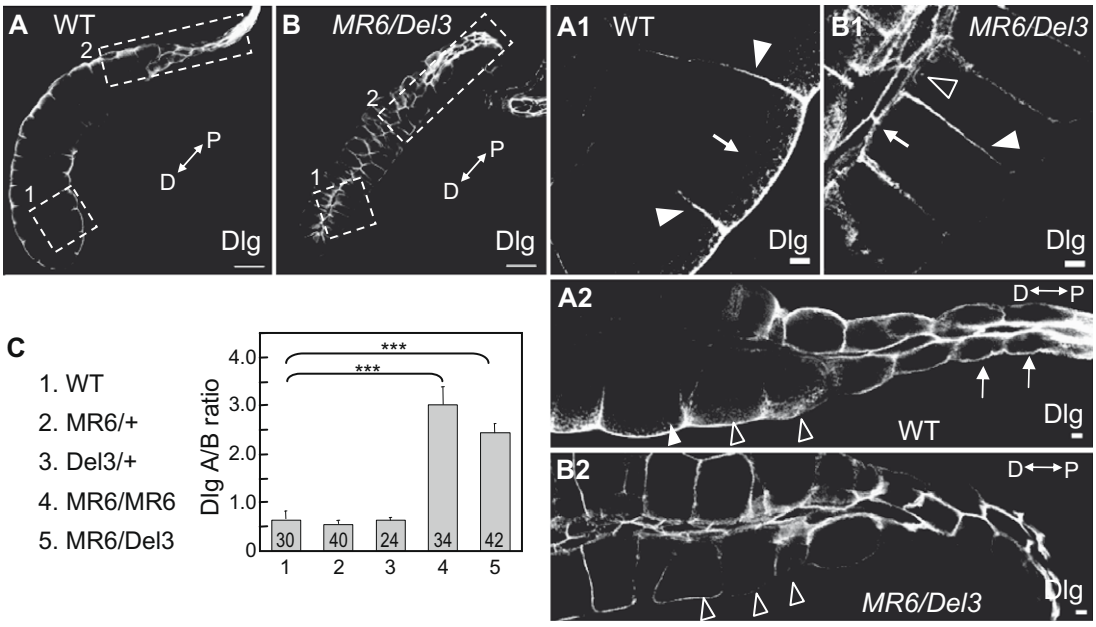

Fig. 1. Dlg mislocalization in *nak* mutant SG cells. Images for third instar larval SGs of WT (A) and $nak^{MR6/Del3}$ (B) stained for Dlg. In this and all following figures, SG images are oriented with proximal (P) to right and distal (D) to left (bi-headed arrows). (A1,B1) and (A2,B2) are enlarged images of regions (dashed outlined boxes, labeled 1 or 2) in (A,B), respectively. Apical direction is toward the left-top facing the lumen of SGs and basal direction is to the right-bottom. (A1) Dlg gradients in lateral membrane and basal cytoplasm are indicated by arrowheads and arrow, respectively. (B1) A arrow, apical Dlg; arrowhead, lateral Dlg; empty arrowhead, Dlg punctas. (A2) A arrows, proximal cells; empty arrowheads, transition zone cells; arrowhead, slightly distal cell. (B2) Empty arrowheads, transition zone cells. In these and all following figures, thin scale bars are 100 μm such in (A,B) and thick scale bars are 10 μm such as in (A1–B2). (C) A/B ratios of Dlg immunoreactivity in WT, $nak^{MR6/+}$, $nak^{Del3/+}$, $nak^{MR6/MR6}$, and $nak^{MR6/Del3}$ distal SG cells. In this and all following statistics, averages are mean ± SEMs; numbers of SG cells scored are indicated in the bars; and Student's *t* test is used for comparisons (significance: $^{***}p < 0.0001$; $^{**}p < 0.001$; and $^{*}p < 0.01$).