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Activation of *Aplysia* ARF6 induces neurite outgrowth and is sequestered by the overexpression of the PH domain of *Aplysia* Sec7 proteins

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ABSTRACT

ADP-ribosylation factors (ARFs) are small guanosine triphosphatases of the Ras superfamily involved in membrane trafficking and regulation of the actin cytoskeleton. *Aplysia* Sec7 protein (ApSec7), a guanine nucleotide exchange factor for ARF1 and ARF6, induces neurite outgrowth and plays a key role in 5-hydroxyltryptamine-induced neurite growth and synaptic facilitation in *Aplysia* sensory-motor synapses. However, the specific role of ARF6 signaling on neurite outgrowth in *Aplysia* neurons has not been examined. In the present study, we cloned *Aplysia* ARF6 (ApARF6) and revealed that an overexpression of enhanced green fluorescent protein (EGFP)-fused constitutively active ApARF6 (ApARF6-Q67L-EGFP) could induce neurite outgrowth in *Aplysia* sensory neurons. Further, we observed that ApARF6-induced neurite outgrowth was inhibited by the co-expression of a Sec7 activity-deficient mutant of ApSec7 (ApSec7-E159K). The pleckstrin homology domain of ApSec7 may bind to active ApARF6 at the plasma membrane and prevent active ApARF6-induced functions, including intracellular vacuole formation in HEK293T cells. The results of the present study suggest that activation of ARF6 signaling could induce neurite outgrowth in *Aplysia* neurons and may be involved in downstream signaling of ApSec7-induced neurite outgrowth in *Aplysia* neurons.

1. Introduction

Memory-related long-term synaptic plasticity involves a variety of structural changes to neurites, including the formation of synaptic varicosities, in the neurons of mammals and some invertebrates, such as *Aplysia* (Kandel, 2001, 2012; Lee, 2014). Application of 5-hydroxytryptamine (5-HT) induces rapid and permanent changes in the structure of the plasma membrane, leading to synaptic growth and long-term synaptic facilitation (LTF) at sensory-motor synapses involved in *Aplysia* gill and siphon withdrawal reflexes (Bailey, Kandel, & Si, 2004; Mayford, Barzilai, Keller, Schacher, & Kandel, 1992; Udo et al., 2005). The *Aplysia* Sec7 protein (ApSec7) belongs to the *Aplysia* homolog of the mammalian cytohesin family and, as such, has a guanine nucleotide exchange factor (GEF) for ADP-ribosylation factor 1 (ARF1) and 6 (ARF6). This Sec7 protein is crucially involved in 5-HT-induced synaptic growth and LTF in *Aplysia* sensory-motor synapses (Lee et al., 2012).

ARFs comprise a small guanosine triphosphatase (GTPase) family responsible for the regulation of the synaptic membrane and vesicle trafficking (D'Souza-Schorey & Chavrier, 2006). ARF6, the only member of ARF class III, plays a vital role in exchanges between the plasma membrane and endocytic compartments (Jaworski, 2007). ARFs are activated by specific families of GEFs (Casanova, 2007), with the cytohesin family typically being responsible for activation of ARF1 and ARF6 (Cohen et al., 2007;

Abbreviations: 5-HT, 5-hydroxyltryptamine; PH, pleckstrin homology; ARF, ADP-ribosylation factor; GTPase, guanosine triphosphatase; GEF, guanine nucleotide exchange factor; PI(3,4,5)P_3, phosphatidylinositol (3,4,5)-trisphosphate; KD, kinase-dead; ApARF6, *Aplysia* ARF6; ApSec7, *Aplysia* Sec7; PIP5KI, type I phospha tidylinositol-4-phosphate 5-kinase; apCAM, *Aplysia* cell adhesion molecules; EGFP, enhanced green fluorescent protein; DIC, differential interference contrast; mRFP, monomeric red fluorescent protein; LTF, long-term synaptic facilitation; Co-IP, co-immunoprecipitation.

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Frank, Upender, Hansen, & Casanova, 1998). The cytohesin family and downstream ARF6 play many roles in the regulation of dendrite and axon branching as well as in dendritic spine formation in mammalian neurons (Choi et al., 2006; Hernandez-Deviez, Casanova, & Wilson, 2002; Hernandez-Deviez, Roth, Casanova, & Wilson, 2004). Overexpression of catalytically inactive ARNO/cytohesin-2 or dominant-negative ARF6 can induce dendrite and axonal branching in cultured hippocampal neurons (Hernandez-Deviez et al., 2002, 2004). As previously reported, Rac1—another member of the Rho family of GTPases—is a downstream effector of dendrite branching: Hernandez-Deviez et al. reported that Rac1-N17, a dominant-negative mutant of Rac1, increased dendrite branching, whereas the type I phosphatidylinositol-4-phosphate 5-kinase (PIP5KI) was observed to be involved in axon growth and branching, based on the observation that co-expression of PIP5KIα could block axonal growth and branching induced by catalytically inactive ARNO (ARNO-E156K) or dominant-negative ARF6 (ARF6-T27N) (Hernandez-Deviez et al., 2004). Further, overexpression of either EFA6A or fast cycling ARF6 (ARF6-T157A) promotes the formation of dendritic spines, with some involvement from the downstream activation of Rac1 (Choi et al., 2006). Thus, ARF6 signaling regulates different aspects of neuronal morphology via a variety of distinct downstream signaling pathways.

Meanwhile, in contrast with patterns observed for mammalian neurons, overexpression of a wild-type mSec7-1, mouse cytohesin-1, or ARNO/cytohesin-2 induces neurite outgrowth in *Aplysia* neurons (Huh et al., 2003). Similarly, overexpression of ApSec7 has been shown to induce neurite outgrowth and contribute to 5-HT-induced synaptic growth and facilitation in *Aplysia* sensory neurons, whereas the catalytically inactive mutant ApSec7-E159K has not been observed to induce neurite outgrowth, but rather to block 5-HT-induced synaptic growth and LTF (Jun et al., 2015; Lee et al., 2012). PIP5KIα is a major downstream signaling component of ApSec7-induced neurite outgrowth and 5-HT-induced synaptic facilitation, since overexpression of the kinase-dead (KD) form of PIP5KIα (PIP5KIα-KD) leads to the inhibition of ApSec7-induced neurite outgrowth and 5-HT-induced LTF (Lee et al., 2012). Since overexpression of ApSec7 has been observed to induce neurite outgrowth, enhance synaptic growth, and occlude LTF in *Aplysia* neurons, and since overexpression of the Sec7 activity-deficient mutant ApSec7-E159K has further been observed to inhibit 5-HT-induced internalization of *Aplysia* cell adhesion molecules (apCAMs) and synaptic growth (Lee et al., 2012), researchers have suggested that ApSec7-E159K acts as a dominant-negative mutant. However, the mechanism behind this dominant-negative action is poorly understood. In addition, unlike ApSec7, another isoform, ApSec7(VPKIS), in which the VPKIS amino acid sequence is inserted into the pleckstrin homology (PH) domain at a site near the β1/β2 loop of ApSec7, has been observed to impair phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$) binding, and does not induce neurite outgrowth in *Aplysia* neurons (Jun et al., 2015). Thus, plasma membrane localization and Sec7 activity of ApSec7 play important roles for neurite outgrowth in *Aplysia* neurons. Considering that the GEFs for ARF1 and ARF6 are of the cytohesin family, it is possible that ARF6 may be one target of downstream signaling for neurite outgrowth induced by ApSec7 in *Aplysia* neurons.

To examine the roles of ARF6 on neurite outgrowth, we cloned ARF6 (ApARF6) in *Aplysia* and examined the neurite outgrowth effects of ApARF6 overexpression in sensory neurons. As a result, we found that overexpression of enhanced green fluorescent protein (EGFP)-fused constitutively active ApARF6 (ApARF6-Q67L-EGFP) could induce neurite outgrowth in *Aplysia* sensory neurons. Such neurite outgrowth, however, could be blocked by co-expression of a Sec7-deficient mutant of ApSec7 (ApSec7-E159K). The PH domain of ApSec7 could further interact with active ApARF6 at the plasma membrane. Co-expression of monomeric red fluorescent protein (mRFP) fused to ApSec7-E159K (mRFP-ApSec7-E159K) or the PH domain of ApSec7 (mRFP-ApSec7(PH)) with ApARF6-EGFP could block ApARF6-induced intracellular vacuole formation in HEK293T cells. The overall results of the present study suggest that the activation of ARF6 signaling could induce neurite outgrowth in *Aplysia* sensory neurons and may be involved in downstream signaling of ApSec7-induced neurite outgrowth in *Aplysia* neurons.

2. Materials and methods

2.1. DNA constructions

We used previously described methods for the preparation of EGFP-ApSec7, EGFP-ApSec7(VPKIS), pNEXδ-EGFP and EGFP-ApSec7-E159K (Jun et al., 2015; Lee et al., 2012).

To generate the pmRFP-C1 backbone, the mRFP PCR product was obtained by PCR with mRFP-NheI-S (5′-AGCTAGCTAGCGCCAC CATGGCCTCCTCCGAGGAC-3′). The mRFP-HindIII-A (5′-CGCCCAAG CTTGGGCGCCGGTGGAGTGGCG-3′) was then inserted between the NheI-HindIII-digested pEGFP-C1 vectors. The region encoding ApSec7, ApSec7-E159K, ApSec7(VPKIS), and ApSec7(PH) was inserted between the SalI-Xba1 sites of the pmRFP-C1 vector. The mutant fragments of ApSec7-E159K(VPKIS) were generated by recombinant PCR using specific sense or antisense primers (sense primer containing E159K, 5′-CCCGGTAAAGCACAGAAGA-3′; antisense primer containing E159K, 5′-TCTTCTGTGCTTTACCGGG-3′). The region encoding AKT1(PH) (Addgene, #21218) was inserted between the BamHI-Xba1 sites of the pmRFP-C1 vector.

To identify the ARF6 protein in *Aplysia*, the DNA sequence of ApARF6 (GenBank #: KU724187) was cloned into pNEXδ-EGFP at the HindIII and Xba1 sites. Based on the nucleotide sequences of identified clones, recombinant PCR was used to generate the active and inactive forms of ApARF6. The mutant fragments—the active form ApARF6-Q67L and inactive form ApARF6-T27N—were generated by recombinant PCR using specific sense or antisense primers (sense primer containing ApARF6-Q67L, 5′-GTTGGTGGTCTGGACAA GATCCGT-3′; antisense primer containing ApARF6-Q67L, 5′-GATC TTGTCCAGACCACCAACATC-3′; sense primer containing ApARF6-T27N, 5′-GCTGGAAAAAATACTTTATTATAC-3′; antisense primer containing ApARF6-T27N, 5′-TAATAAAGTATTTTTTCCAGCAGC-3′). The PCR products containing mutant forms (Q67L and T27N) in ApARF6 amino acid residues were also subcloned into the HindIII- and Xba1-digested pNEXδ-EGFP vectors.

2.2. Cell culture and confocal microscopy

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. Cells were seeded in a sticky-Slide 8-well system (Catalog #: 80828, Ibidi, Martinsried, Germany), such that they could reach 40–60% confluence on the day of imaging. In the 24–26-h period prior to imaging, the cells were transfected with DNA constructs using calcium phosphate or Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). The relative amount of each construct was empirically determined on the basis of the relative expression of each construct combination.

Cells were then analyzed with an inverted Zeiss LSM-700 confocal scanning laser microscope operated by ZEN software (Carl Zeiss). Respective laser lines for excitation and spectral detection windows for the fluorochromes were as follows: 488 nm and 508–543 nm for GFP; 561 nm and 578–649 nm for mRFP. Appropriate GFP (500–550 nm) and mRFP (575–625 nm) emission filters