PI4KII activity-dependent Golgi complex targeting of Aplysia phosphodiesterase 4 long-form mutant

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ABSTRACT

The compartmentalization of cAMP by specifically targeted phosphodiesterases (PDEs) contributes to signal regulation in defined regions of cells. We previously demonstrated that the 20 N-terminal amino acids of Aplysia PDE4 (ApPDE4) long-form (L(N20)) and the two mutants of L(N20) were localized to the Golgi complex. However, the molecular mechanisms underlying the Golgi complex targeting of ApPDE4 long-form and its mutated forms are not clear. In the present study, we show that the Golgi complex targeting of L(N20/C14,15S)-enhanced green fluorescent protein (EGFP) was antimycin A- and adenine-sensitive, but insensitive to high concentrations of wortmannin. On the other hand, the Golgi complex targeting of L(N20)-EGFP and L(N20/C3,14S)-EGFP was antimycin A- and PAO-insensitive. These results suggest that the Golgi-localized lipid kinase protein, phosphatidylinositol 4-kinase type II alpha (PI4KII), the activity of which is inhibited by PAO and adenine, but not by high concentrations of wortmannin, is likely involved in the Golgi complex targeting of L(N20/C14,15S)-EGFP. In addition, subcellular localization of L(N20/C14,15S)-EGFP, but not L(N20)-EGFP or L(N20/C3,14S)-EGFP, was changed from the Golgi complex only to both the endoplasmic reticulum (ER) and the Golgi complex following treatment with a palmitoylation inhibitor, 2-bromo palmitate. Taken together, our results suggest that L(N20/C14,15S)-EGFP, but not L(N20)-EGFP or L(N20/C3,14S)-EGFP, is targeted to the Golgi complex in a PI4KII activity- and palmitoylation-dependent manner. Therefore, phosphatidylinositol 4-phosphate (PI4P) generated by PI4KII at the Golgi complex might play a key role in the Golgi complex targeting of L(N20/C14,15S)-EGFP.

Introduction

Phosphodiesterases (PDEs) are critical regulators of cAMP signaling, which modulate numerous physiological functions such as learning and memory in mammals and invertebrates, including Drosophila and Aplysia (Lee et al. 2008; Kandel 2012; Lee 2014; Lee et al. 2016; Naganos et al. 2016). Among the 11 families of PDEs, PDE4, which specifically degrades cAMP, is important for many physiological functions, including memory and cognition (Richter et al. 2013). The overexpression or disruption of ApPDE4s in sensory neurons impairs 5-HT-induced synaptic facilitation in sensory-to-motor synapses (Park et al. 2005; Jang et al. 2010; Jang et al. 2011). The unique N-terminal region (NTR) of a PDE4 protein determines its subcellular localization through specific protein interactions or lipid binding (Houslay 2010). For example, the NTR of PDE4D4 interacts with the SH3 domains of tyrosine kinases, such as Lyn, Fyn, and Src, which results in the localization of PDE4D4 to the plasma membrane (Beard et al. 1999). In contrast, the NTR of PDE4A1 is targeted to the trans-Golgi network (TGN) through direct membrane-binding involving phosphatidic acid (PA) (Baillie et al. 2002; Houslay and Adams 2003; Huston et al. 2006). In addition, we recently demonstrated that the NTRs of the long- and short-forms of ApPDE4 are involved in their intracellular membrane targeting through distinct membrane associations (Jang et al. 2010; Kim et al. 2014). The NTR of the ApPDE4 short-form is targeted solely to the plasma membrane primarily by nonspecific electrostatic interactions, whereas the ApPDE4 long-form is localized to the plasma membrane through hydrophobic interactions in HEK293T cells (Kim et al. 2014). However, given that the N-terminus of the ApPDE4 long-form binds to numerous lipids, including sulfatide,
phosphatidylinositol 4-phosphate (PI4P), and cardiolipin in vitro (Jang et al. 2010), specific lipid binding may be required for the specific intracellular membrane targeting of the ApPDE4 long-form.

Phosphoinositides, which are derivatives of phosphatidylinositol (PI), are minor lipid components of eukaryotic membranes that have important roles in many cellular functions, including as specific markers of intracellular membrane organelles (Di Paolo and De Camilli 2006; Lemmon 2008). Phosphoinositides are not randomly distributed within intracellular membranes; each molecular species has a specific cellular localization (Di Paolo and De Camilli 2006; Jang et al. 2009). For example, phosphatidylinositol 3-phosphate (PI3P) is localized to the cytoplasmic surface of early endosomes, while phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3) are primarily restricted to the cytoplasmic surface of the plasma membrane. PI4P is enriched in TGN and the plasma membranes of cells (D’Angelo et al. 2008). A PI4P-specific probe, the enhanced green fluorescent protein (EGFP)-fused PI4P-binding SidM (P4M) domain of SidM (EGFP-P4M-SidM), was recently described (Hammond et al. 2014). Experiments with this probe showed that PI4P is enriched in the TGN and the plasma membranes of cells (D’Angelo et al. 2008). A PI4P-specific probe, the enhanced green fluorescent protein (EGFP)-fused PI4P-binding SidM (P4M) domain of SidM (EGFP-P4M-SidM), was recently described (Hammond et al. 2014). Experiments with this probe showed that PI4P is enriched in the TGN and the plasma membranes of cells as well as in late endosomes/lysosomes, which are associated with subcellular compartments known to contain PI4-kinases (PI4Ks), which generate PI4P from PI (Hammond et al. 2014). Thus, PI4P may have additional roles in the endosomes/lysosomes.

In this study, we examined the molecular mechanism of the Golgi complex targeting of the ApPDE4 long-form and its mutants. We found that, although ApPDE4 long-form and the two mutants showed targeting to the Golgi complex, the molecular mechanism of the Golgi complex targeting is classified into either PI4KII activity/palmitoylation-dependent or phosphoinositide/palmitoylation-independent.

Materials and methods

Cloning of DNA constructs

We used the following previously described DNA constructs: enhanced EGFP- or mRFP-fused ApPDE4 long-form (L(N20)-EGFP), ApPDE4 long-form mutants (L(N20/C14,15S)-EGFP and L(N20/C3,14S)-EGFP), GalT-mRFP, and human PDE4A1 (Kim et al. 2014). To generate pcDNA3.1-GAD65(N50)-EGFP, the GAD65(N50) PCR product obtained by PCR with hGAD65-HindIII-S (5′-CG AAGCTTGGCCACCATGGCATCCTCCGGGCTCTGGCTTTGTC TTTCCGGTCCGAA-3′) and hGAD65-XbaI-A (5′-GCTCTA GATCCG TAGACGAGGGCGACAGTTTGTTCGCCGGATGCC GCGT-3′) was inserted into the HindIII-XbaI-digested pcDNA3.1-EGFP vector. Arf1-EGFP (#39554) (Chun et al. 2008), GFP/mCherry-P4M-SidM (#51469 and #51471) (Hammond et al. 2014), and mCherry-Sec61β (#49155) (Zurek et al. 2011) were obtained from Addgene (Cambridge, MA, USA).

Cell culture

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) CO2 at 37°C. For transient transfection, HEK293T cells were plated at a density of 2.5 × 10⁴ cells per well in μ-Slide 8-Well (Ibidi) chambers and cultured for 24 h. The cells were then transfected with DNA constructs using calcium phosphate (Clontech), and incubated for an additional 24 h. Images of the cells were captured under a laser scanning confocal microscope (Carl Zeiss, LSM700).

Drug treatment

To disrupt palmitoylation, HEK293T cells were treated with 100 μM 2-bromopalmitate (2-BrP) in culture media for 4 h. The lipid derivatives generated by various lipid kinases, including phosphoinositides, were depleted by treating the cells with 200 nM antimycin A in PBS for 1 h. The cells were treated with 10 μM phenylarsine oxide (PAO) for 30 min to inhibit the activities of PI4KIIs and PI4KIIIs. To inhibit the activity of PI4KIIIs further, the cells were incubated with 10 μM wortmannin in PBS for 30 min. The Golgi complex targeting of ARF1-EGFP was disrupted by treating the cells with 10 μg/mL brefeldin A (BFA) for 5 min. Adenosine, which is a PI4KII-specific inhibitor, was administered through a combined treatment of 0.01% digitonin and 500 μM adenosine in culture media for 30 min.

Results and discussion

Antimycin A-sensitive or -insensitive Golgi complex targeting of ApPDE4 long-form and the two mutants

We previously reported that ApPDE4 wild-type and its mutants were localized to the Golgi complex in mammalian cells, including HEK293T cells (Jang et al. 2010; Kim et al. 2014). To investigate the molecular mechanisms of the Golgi complex targeting of ApPDE4 long-form and several mutant versions of the protein, we first examined the Golgi complex targeting of ApPDE4...
long-form in HEK293T cells. To do this, galactose-1-phosphate uridylyltransferase-monomeric red fluorescent protein (GalT-mRFP), a TGN marker, was co-expressed with the two mutants of ApPDE4 long-form. In agreement with the previous reports (Jang et al. 2010; Kim et al. 2014), EGFP fused to the 20 N-terminal amino acids of ApPDE4 long-form (L(N20)-EGFP) localized to both the plasma membrane and the Golgi complex, whereas the two mutated forms, L(N20/C14,15S)-EGFP and L(N20/C3,14S)-EGFP, were localized only to the Golgi complex in HEK293T cells (Figure 1(A, B)).

Next, to decipher the molecular mechanism of the localization to the Golgi complex, we examined the effects of pharmacological inhibitors on the cellular localization of various constructs. First, antimycin A, which is an ATP synthesis inhibitor, was used to deplete the lipid derivatives generated by various lipid kinases, including various phosphoinositides such as PI3P, PI4P, and PI(4,5)P2 within cells (Kim et al. 2014). EGFP-P4M-SidM, which shows PI4P-dependent targeting to the plasma membrane and the Golgi complex, was used as a control. As shown in Figure 1(C), the Golgi complex targeting of L(N20/C14,15S)-EGFP and EGFP-P4M-SidM, but not of L(N20)-EGFP or L(N20/C3,14S)-EGFP, was disrupted by antimycin A treatment (Figure 1(C)). In addition, the Golgi complex targeting of EGFP fused to the 25 N-terminal amino acids of PDE4A1 (PDE4A1(N25)-EGFP) and the 50 N-terminal amino acids of glutamic acid decarboxylase 65 (GAD65(N50)-EGFP) was antimycin A-insensitive (Figure 1(C)). These results suggest that phosphoinositides or other lipids that are synthesized or generated by lipid kinases in an ATP-dependent manner may be involved in the Golgi complex targeting of L(N20/C14,15S)-EGFP, but not of L(N20)-EGFP, L(N20/C3,14S)-EGFP, PDE4A1(N25)-EGFP, or GAD65(N50)-EGFP.

Figure 1. Antimycin A-sensitive or -insensitive Golgi complex targeting of ApPDE4 long-form constructs. (A) Schematic diagram of the amino acid sequences of ApPDE4 long-form L(N20)-EGFP and derived constructs, (L(N20/C14,15S)- and L(N20/C3,14S)-EGFP). (B) ApPDE4 long-form and constructs co-localized with GalT-mRFP, a trans-Golgi network (TGN) marker, in HEK293T cells. (C) Antimycin A-sensitive- or -insensitive Golgi complex targeting of various Golgi complex targeting proteins. The Golgi complex targeting of L(N20/C14,15S)-EGFP and EGFP-P4M-SidM was disrupted by antimycin A treatment, whereas L(N20)-EGFP, L(N20/C3,14S)-EGFP, PDE4A1(N25)-EGFP, and GAD65(N50)-EGFP targeting to the Golgi complex were antimycin A-insensitive. Scale bar, 20 μm.
**PI4KII activity-dependent Golgi complex targeting of L(N20/C14,15S)-EGFP**

We next investigated whether lipid kinases, PI4Ks, contributed to the Golgi complex targeting of L(N20/C14,15S)-EGFP, since PI4P is known to be highly concentrated in Golgi complexes, especially in the TGN. To test this possibility, we applied PAO, which inhibits PI4KIIIs and PI4KIIIs, to cells expressing L(N20/C14,15S)-EGFP, L(N20/C3,14S)-EGFP, or PDE4A1(N25)-EGFP. We observed that L(N20/C14,15S)-EGFP, but not L(N20/C3,14S)-EGFP or PDE4A1(N25)-EGFP, showed changes in localization from the Golgi complex to the cytosol and nucleus upon PAO treatment, which was similar to the effects of PAO on EGFP-P4M-SidM localization (Figure 2(A)).

To further discern the molecular mechanism of the Golgi complex targeting of L(N20/C14,15S)-EGFP, we treated cells with adenosine, which inhibits PI4KII selectively (Dickson et al. 2014). However, adenosine itself is not able to diffuse through the plasma membrane. To overcome this limitation, we co-treated adenosine with a low concentration of digitonin to disrupt the plasma membrane partially. As shown in Figure 2(B), in the absence or presence of digitonin, L(N20/C3,14S)-EGFP, and L(N20/C3,14S)-EGFP were co-localized with P4M-SidM-mRFP, indicating that digitonin treatment has no effects on the Golgi complex targeting of the constructs. However, the addition of adenosine with digitonin impaired the Golgi complex targeting of L(N20/C14,15S)-EGFP and P4M-SidM-RFP, but not of L(N20/C3,14S)-EGFP. These results indicate that the Golgi complex targeting of L(N20/C14,15S)-EGFP and P4M-SidM-RFP, but not of L(N20/C3,14S)-EGFP, are PI4KII activity-dependent. We next used a high concentration of wortmannin to inhibit PI4KIIIs selectively, while not affecting PI4KIIIs (Dickson et al. 2014). However, wortmannin treatment (10 μM) did not disrupt the Golgi complex targeting of L(N20/C14,15S)-EGFP in HEK293T cells (Figure 2(C)).

PI4P can be generated by PI4Ks from PI on the cytoplasmic side of the cell membrane (D’Angelo et al. 2008). Mammals have four different PI4K genes, which can be classified as type II and type III based on their sensitivity to adenosine and wortmannin, respectively. Both PI4K type II and III are sensitive complex to PAO (Balla

![Figure 2](https://example.com/figure2.png)

**Figure 2.** PI4KII activity-dependent Golgi complex targeting of L(N20/C14,15S)-EGFP. (A) Effects of PAO treatment on the Golgi complex targeting of L(N20/C14,15S)-EGFP, L(N20/C3,14S)-EGFP, and EGFP-P4M-SidM. (B) Effects of adenosine treatment on the Golgi complex targeting of L(N20/C14,15S)-EGFP, which was co-expressed with mCherry-P4M-SidM in the presence of digitonin. (C) Effect of 10 μM wortmannin (Wm) on the cellular localization of L(N20/C14,15S)-EGFP. (D) Effects of BFA on the cellular localization of L(N20/C14,15S)-EGFP and human ARF1-EGFP. Scale bar, 20 μm.
et al. 2007). PI4KIIα and PI4KIIIβ are both found in Golgi complexes (D’Angelo et al. 2008). The Golgi targeting by L(N20/C14,15S) was sensitive to antimycin A, PAO, and adenosine, but insensitive to a high concentration of wortmannin (10 μM) (Figure 2), indicating that PI4P generated by PI4KIIα in the Golgi complex might be involved in the Golgi complex targeting of L(N20/C14,15S) in HEK293T cells. However, PI4P is also enriched in the plasma membrane, where EGFP-P4M-SidM is weakly localized, but L(N20/C14,15S)-EGFP was only localized to the Golgi complex. These results suggest the possibility that additional factors are involved in the Golgi complex targeting of L(N20/C14,15S)-EGFP.

ADP-riboylolation factor 1 (ARF1), which is a well-known Golgi complex targeting protein, is a good candidate protein for the additional factor in the Golgi complex targeting of L(N20/C14,15S)-EGFP. Therefore, to examine whether ARF1 is involved in the Golgi complex targeting of L(N20/C14,15S)-EGFP, we applied BFA, which is an inhibitor of ARF-GTP exchange factor (ARF-GEF) activity. Figure 2(D) shows that the Golgi complex localization of ARF1-EGFP was reduced in the presence of BFA, whereas L(N20/C14,15S)-EGFP was still targeted to the Golgi complex, indicating that the Golgi complex localization of L(N20/C14,15S)-EGFP in HEK293T cells is ARF1-independent. Overall, our results suggest that L(N20/C14,15S)-EGFP localizes to the Golgi complex in a PI4KII activity-dependent manner, whereas L(N20)-EGFP and L(N20/C3,14S)-EGFP localize to the Golgi complex in phosphoinositide-independent manner.

Disruption of Golgi complex targeting of L(N20/ C14,15S)-EGFP in the presence of 2-BrP

Next, we investigated the roles of palmitoylation on the Golgi complex targeting of the ApPDE4 long-form constructs, since these proteins contain cysteine residues that can be palmitoylated. Palmitoylation of proteins increases their hydrophobicity and leads to their incorporation into membranes (el-Husseini Ael and Bredt 2002). To examine this, we used 2-BrP, a reversible palmitoylation inhibitor. In the presence of 100 μM 2-BrP, the localization of L(N20/C14,15S)-EGFP shifted from the Golgi complex to the cytoplasm, but not to the nucleus, whereas the Golgi complex targeting of L(N20/C3,14S)-EGFP was not changed by 2-BrP treatment (Figure 3(A)).

To further confirm the cellular localization of L(N20/C14,15S)-EGFP, this construct was co-expressed with mCherry-Sec61β, an ER marker. As shown in Figure 3

![Figure 3](image-url)

**Figure 3.** Palmitoylation-dependent Golgi complex targeting of L(N20/C14,15S)-EGFP. (A) Cellular localization of L(N20/C14,15S)-EGFP and L(N20/C3,14S)-EGFP in the presence or absence of 2-BrP in HEK293T cells. (B) Co-localization of L(N20/C14,15S)-EGFP with GalT-mRFP and with mCherry-Sec61β in the absence or presence of 2-BrP. (C) Co-localization of L(N20/C14,15S)-mRFP with GAD65(N50)-EGFP in the absence or presence of 2-BrP. Scale bar, 20 μm.
(B), in the absence of 2-BrP, L(N20/C14,15S)-EGFP was co-localized only with GalT-mRFP. However, in the presence of 2-BrP, L(N20/C14,15S)-EGFP was co-localized with mCherry-Sec61β as well as with GalT-mRFP. These results indicate that cellular localization of L(N20/C14,15S)-EGFP shifted from the Golgi complex to the ER/Golgi complex in a palmitoylation-dependent manner.

These results are similar to the results obtained in a study of GAD65 (Kanaani et al. 2008), which is a palmitoylation-dependent, Golgi-targeted protein. To confirm these results, we investigated the localization of GAD65 (N50)-EGFP in HEK293T cells. As shown in Figure 3(C), GAD65(N50)-EGFP co-localized with L(N20/C14,15S)-mRFP, indicating its Golgi complex targeting in the HEK293T cells. In the presence of 100 μM 2-BrP, the localization of GAD65(N50)-EGFP shifted from the Golgi complex to the ER/Golgi complex, similar to L(N20/C14,15S)-mRFP (Figure 3(C)). However, although cellular localization of GAD65(N50)-EGFP was similar to that of L(N20/C14,15S)-mRFP, it seems that the molecular mechanism of the Golgi complex targeting of GAD65(N50)-EGFP is different from that of L(N20/C14,15S)-EGFP. As shown in Figure 1(A), unlike L(N20/C14,15S)-mRFP, the Golgi complex targeting of GAD65(N50)-EGFP was not changed following antimycin A treatment. Thus, although 2-BrP could induce a similar change in localization from the Golgi complex to the ER/Golgi complex, the molecular mechanism of the Golgi complex targeting of GAD65(N50)-EGFP or L(N20/C14,15S)-EGFP appears to be different.

We previously showed that L(N20)-EGFP, L(N20/C14,15S)-EGFP, and L(N20/C3,14S)-EGFP were localized to the Golgi complex, whereas L(N20/C3,14,15S)-EGFP was localized to cytosol (Jang et al. 2010; Kim et al. 2014). In addition, in the current study, we showed that the Golgi complex localization of L(N20/C14,15S)-EGFP shifted to the ER/Golgi complex in the presence of 2-BrP. From these results, we could postulate that at least the third cysteine residue (C3) in the L(N20) protein might be palmitoylated. Considering the results that L(N20/C3,14,15S)-EGFP was localized to the cytosol (Jang et al. 2010), while L(N20/C14,15S)-EGFP was localized to the ER/Golgi complex in the presence of 2-BrP, the 20 N-terminal amino acids in ApPDE4 long-form might be a binding site for the ER membrane itself or the site of another unknown modification important for ER localization within the third cysteine residue. It has been reported that a synthesized GAD65 undergoes an irreversible hydrophobic modification in the cytosol, and that hydrophobic GAD65 then reversibly associates with the ER and Golgi membranes (Kanaani et al. 2008). Newly synthesized ApPDE4 long-form may also undergo hydrophobic modification and become anchored to ER/Golgi membranes prior to palmitoylation.

Although GAD65(N50)-EGFP, L(N20)-EGFP, and L(N20/C3,14S)-EGFP are localized to the Golgi complex in mammalian cells, the finding that antimycin A and PAO treatments did not disrupt the TGN targeting in HEK293T cells (Figure 1) indicates that the Golgi complex targeting of these constructs is PI4P-independent. Palmitoylation is a crucial component of the membrane targeting of proteins, including GAD65 (el-Husseini Ael and Bredt 2002; Jang et al. 2009), and it has been suggested that the palmitoylation of proteins enhances the incorporation of the targeted protein into lipid rafts within the membrane. Therefore, future studies will need to examine whether lipid rafts are involved in the Golgi complex targeting of L(N20)-EGFP and L(N20/C3,14S)-EGFP.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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