Intracellular Membrane Association of the Aplysia cAMP Phosphodiesterase Long and Short Forms via Different Targeting Mechanisms*

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**Background:** Phosphodiesterases play a role in cAMP regulation through specific targeting.

**Results:** Membrane targeting of the Aplysia phosphodiesterase long and short forms is mediated hydrophobically and electrostatically, respectively.

**Conclusion:** The Aplysia phosphodiesterase long and short forms are targeted to the intracellular membranes by different mechanisms.

**Significance:** This is the first report demonstrating that phosphodiesterase is targeted to the membranes by hydrophobic or electrostatic interactions.

Phosphodiesterases (PDEs) play key roles in cAMP compartmentalization, which is required for intracellular signaling processes, through specific subcellular targeting. Previously, we showed that the long and short forms of Aplysia PDE4 (ApPDE4), which are localized to the membranes of distinct subcellular organelles, play key roles in 5-hydroxytryptamine-induced synaptic facilitation in Aplysia sensory and motor synapses. However, the molecular mechanism of the isoform-specific distinct membrane targeting was not clear. In this study, we further investigated the molecular mechanism of the membrane targeting of the ApPDE4 long and short forms. We found that the membrane targeting of the long form was mediated by hydrophobic interactions, mainly via 16 amino acids at the N-terminal region, whereas the short form was targeted solely to the plasma membrane, mainly by nonspecific electrostatic interactions between their N termini and the negatively charged lipids such as the phosphatidylinositol polyphosphates PI4P and PI(4,5)P2, which are embedded in the inner leaflet of the plasma membrane. Moreover, oligomerization of the long or short form by interaction of their respective upstream conserved region domains, UCR1 and UCR2, enhanced their plasma membrane targeting. These results suggest that the long and short forms of ApPDE4 are distinctly targeted to intracellular membranes through their direct association with the membranes via hydrophobic and electrostatic interactions, respectively.

Phosphodiesterases (PDEs) are important regulators of signal transduction mediated by cAMP, which is a key signaling molecule modulating physiological functions, including learning and memory (1–3). Among the 11 families of PDEs, PDE4 degrades cAMP specifically, is a four-gene family (A, B, C, and D) in mammalian cells, and is important for various functions, including cognition (3). The role of PDE4 in synaptic plasticity has been investigated in various organisms, including Drosophila, Aplysia, and mammals. A mutation of dunce encoding a Drosophila PDE4 isoform impaired synaptic facilitation and olfactory learning (4–6). In Aplysia, knockdown of the Aplysia PDE4s (ApPDE4s) impaired 5-HT-induced synaptic facilitation (7, 8), and overexpression of the ApPDE4 supershort form impaired 5-HT-induced synaptic facilitation in a non-de...

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**The abbreviations used are: PDE, phosphodiesterase; ApPDE4, Aplysia PDE4; NTR, N-terminal region; UCR, upstream conserved region; PI, phosphoinositide; dansyl-PE, N-(5-(dimethylamino)naphthalene-1-sulfonyl)-1,2-di-hexadecanoyl-sn-glycero-3-phosphoethanolamine, PS, 1-α-phosphatidylserine; EGFP, enhanced GFP; mRFP, monomeric red fluorescent protein; PH, pleckstrin homology; TGN, trans-Golgi network; ANOVA, analysis of variance; PJ, pseudojacin; PLC, phospholipase C; mTOR, mammalian target of rapamycin; FRB, rapamycin binding domain of mTOR; 5-HT, 5-hydroxytryptamine.
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pressed, but not in a depressed, synapse (9). In mammals, treatment with a PDE4-specific inhibitor, rolipram, enhanced performances in hippocampus-dependent memory tasks such as context-dependent fear conditioning and the object recognition task (10, 11). On the other hand, rolipram impaired prefrontal cortical function in aged rats and monkeys (12). Therefore, PDE4s play crucial roles in learning and memory but can regulate PKA differently in different species.

PDE4 isoforms can usually be classified into three major categories: the long, short, and supershort forms (13, 14). All isoforms contain the PDE catalytic domain. The long form contains a unique N-terminal region (NTR) and upstream conserved regions (UCRs) 1 and 2, the short form contains an NTR and UCR2, and the supershort form contains an NTR and part of UCR2. It has been reported that the NTRs of PDE4s determine their subcellular localization through binding with specific proteins or lipids (15). For example, the NTR of PDE4A4/5 and PDE4D4 interacts with the SH3 domain of the Src family of tyrosine kinases such as Lyn, Fyn, and Src (16, 17). The NTR of PDE4D5 interacts with β-arrestin (18) or with the receptor for activated protein kinase C (RACK1) (19), and the NTR of PDE4D4/5 interacts with Aryl hydrocarbon receptor-interacting protein (AIP) (20). The NTR of PDE4A1, a brain-specific isoform, is known to be the only PDE4 isoform that interacts with the membrane directly and is targeted to the Golgi complex and its associated vesicles through the two helices (helices 1 and 2) within the NTR (21, 22). Recently, we showed that the NTRs of the ApPDE4 long and short forms might be involved in intracellular membrane targeting, probably through lipid binding (8).

Many proteins are targeted to the plasma membrane via various targeting mechanisms, including specific lipid binding, nonspecific electrostatic interactions, or hydrophobic interactions. Proteins can be targeted to the plasma membrane via direct lipid binding. Phosphoinositides (PIs) play a key role in protein targeting in cells (23). PI(4,5)P2 and PI(3,4,5)P3 are abundant on the cytoplasmic surface of the plasma membrane and play an important role in the plasma membrane targeting of many proteins, including phospholipase C δ1 (PLCδ1) and AKT, via direct interactions with those proteins (23). Proteins can also be targeted to the plasma membrane via nonspecific electrostatic interactions (24). The cytosolic surface of the plasma membrane is the most acidic membrane in the cell because negatively charged lipids such as PI(4,5)P2 and PI(3,4,5)P3 are enriched in the inner leaflet (23). Therefore, many proteins containing a polybasic motif and a hydrophobic domain are localized to the cytosolic side of the plasma membrane through nonspecific electrostatic interactions (25). For example, K-Ras, which has a C-terminal hydrophobic prenylation site and a polybasic motif near this site, can be localized to the cytosolic plasma membrane via nonspecific electrostatic interactions with PIs, including PI(4,5)P2 (25). Recently, it has been reported that PI4P is involved in these electrostatic interactions. Depletion of PI4P and PI(4,5)P2 simultaneously, but not depletion of either PI4P or PI(4,5)P2 alone, could change the localization of many plasma membrane-targeted proteins, such as myristoylated alanine-rich C-kinase substrate (MARCKS), Ritz, and K-Ras (26). Proteins can also be targeted to the plasma membrane via hydrophobic interactions. For example, the plasma membrane targeting of H-Ras is mainly mediated by hydrophobicity caused by the combined prenylation and palmitoylation of the protein (27, 28).

We previously cloned all three isoforms of ApPDE4, which are mammalian PDE4D homologues (7, 8). Unlike the mammalian PDE4 short form, the ApPDE4 short form contains an NTR, UCR2, and truncated UCR1 (the C-terminal half. We also described that the ApPDE4 short and long forms were localized to the plasma membrane and to both the plasma membrane and presynaptic terminals, respectively (8). However, the molecular mechanisms of the distinct membrane targeting of the ApPDE4 long and short forms was not clear. In this study, we further examined the molecular mechanisms of membrane targeting of the ApPDE4 short and long forms. We found that the ApPDE4 long form was localized to the intracellular membranes by a 16-amino acid hydrophobic motif within the N-terminal region, whereas the ApPDE4 short form was targeted to the plasma membrane via nonspecific electrostatic interactions. Moreover, oligomerization of the short or the long form through an interaction between UCR1 and UCR2 further increased plasma membrane targeting. This body of evidence shows, for the first time, that PDE4 can be targeted to specific cell membranes by direct membrane association through either hydrophobic or electrostatic interactions.

EXPERIMENTAL PROCEDURES

DNA Constructs—We used the EGFP-fused ApPDE4 isoforms described previously in the pNEXδ vector (8), the FLAG-fused ApPDE4 short form in the pNEXδ vector (7), pcDNA3.1-FRB (29), and EGFP-PLCδ1(PH) (7, 30). pNEXδ-ApPDE4 L(N-UCR1–2)-EGFP, pNEXδ-ApPDE4 S(N-UCR1–2)-EGFP, and pNEXδ-ApPDE4 S(N-UCR1–2)-FLAG were digested with HindIII/Kpn1 and subcloned into HindIII-Kpn1-digested pcDNA3.1(+/-) to create pcDNA3.1-ApPDE4 L(N-UCR1–2)-EGFP, pcDNA3.1-ApPDE4 S(N-UCR1–2)-EGFP, and pcDNA3.1-ApPDE4 S(N-UCR1–2)-FLAG, respectively. pNEXδ-ApPDE4 S(N-UCR1–2)-FLAG was digested with HindIII/Kpn1 and subcloned into HindIII-Kpn1-digested pcDNA3.1(+/-) to create pcDNA3.1-ApPDE4 L(N-UCR1–2)-FLAG and pcDNA3.1-ApPDE4 S(N-UCR1–2)-FLAG. The PCR product of mRFp, which was obtained by performing PCR using mRFp-Xbαl-S (5’-GCTTCAATGGCCCTCCTCCGAGGAC-3’)/mRFp-Apal-A (5’-CGTAGGGCCCTTACGCGCCGTT-GGAATGGT-3’), was subcloned into XbaI-Apal-digested pcDNA3.1(+/-) to create pcDNA3.1-mRFp. pNEXδ-ApPDE4 L(N-UCR1–2)-EGFP and pNEXδ-ApPDE4 S(N-UCR1–2)-EGFP were digested with HindIII/Xbαl and subcloned into HindIII-Xbαl-digested pcDNA3.1(+/-)-mRFp to create pcDNA3.1-ApPDE4 L(N-UCR1–2)-mRFp and pcDNA3.1-ApPDE4 S(N-UCR1–2)-mRFp. The mutants of the long and short forms of ApPDE4 were obtained by performing PCR using specific primer sets. The PCR products were subcloned separately into HindIII-Xbαl-digested pcDNA3.1-mRFp to create pcDNA3.1-ApPDE4 mutantr-mRFp. We also generated 25 N-terminal amino acids of human PDE4A1 using primer extensions and subcloned them into pEGFP-N3 using EcoRI/Kpn1 sites.
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ApPDE4 L(N20)-EGFP was used as the template for PCR using the specific primer sets L-D3-S (5'-GCAGCAAGGTCATGTGG-ACTTGTACAGCTCGTCCAT-3')/EGFP-Xhol-A (5'-CCCTCTAGACTTGGTACAGCTGGTCCTC-3') and subcloned into pcDNA3.1-FRB, generating pcDNA3.1-L(N20)-EGFP-FRB. EGFP-P-EEA1 (plasmid 42307) (31), EGFP-GaIt (plasmid 11929) (32), EGFP-Rab7 (plasmid 12605) (33), PI-Dead (plasmid 37999) (26), GFP-Lact-C2 (plasmid 22852) (34), EGFP-AKT (plasmid 21218) (35), and Lyn11-FRB (plasmid 20147) (36) were obtained from Addgene.

Cell Culture and Immunocytochemistry—HEK293T cells were grown in DMEM supplemented with 10% v/v FBS and penicillin/streptomycin in a humidified atmosphere of 5% v/v CO₂ at 37 °C.

Anti-GM130 (BD Biosciences) antibody was used as a cis-Golgi marker in HEK293T cells. Fluorescence images were obtained with a confocal laser-scanning microscope (Radiance 2000, Zeiss) and analyzed with ImageJ software (National Institutes of Health).

Immunoprecipitation—For transient transfection, HEK293T cells were plated at a density of 5–7 × 10⁵ cells/well in 6-well plates and cultured for 24 h. The cells were then transfected with DNA constructs using calcium phosphate (Clontech) and incubated for 24 h. For FLAG immunoprecipitation, the transfected HEK293T cells were washed twice with 1× PBS and lysed with a buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, and a protease inhibitor mixture (Roche). The cell lysate was incubated with 50 µl of mouse anti-FLAG M2 antibody-conjugated beads (Sigma) at 4 °C overnight. Subsequently, the beads were washed three times with lysis buffer. Finally, the immunoprecipitate was eluted by adding 2 µg/ml of 3× FLAG peptides and analyzed by Coomassie Blue staining.

Lipid-coated Bead Assay—For the lipid-coated bead assay, briefly, the proteins of transfected HEK293T cells, 20 µl of lipid-coated beads, and 400 µl of binding buffer (0.1 M Hepes (pH 7.4), 150 mM NaCl, and 0.0025% Nonidet P-40) were incubated for 1 h at 4 °C and centrifuged at 5400 rpm for 2 min. The bead pellet was resuspended in 1 ml of binding buffer and then centrifuged. This step was repeated three times. The bound and flow-through samples were resolved by 1× SDS sample buffer, and the transfected proteins were detected by Western blot analysis using anti-GFP antibody.

Preparation of Liposomes and Assay for Peptide-Liposome Binding—All lipids were purchased from Avanti Polar Lipids, except for N-((5-(dimethylamino) naphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (dansyl-PE), which was purchased from Invitrogen. Liposomes consisted of l-GAGCTTGTACAGCTCGTCCAT-3'/EGFP-Xhol-A (5'-CCCTCTAGACTTGGTACAGCTGGTCCTC-3'), and subcloned into pcDNA3.1-FRB, generating pcDNA3.1-L(N20)-EGFP-FRB. EGFP-P-EEA1 (plasmid 42307) (31), EGFP-GaIt (plasmid 11929) (32), EGFP-Rab7 (plasmid 12605) (33), PI-Dead (plasmid 37999) (26), GFP-Lact-C2 (plasmid 22852) (34), EGFP-AKT (plasmid 21218) (35), and Lyn11-FRB (plasmid 20147) (36) were obtained from Addgene.

Results

Intracellular Localization of the ApPDE4 Long and Short Forms—As shown in Fig. 1A, there are two differences between the molecular structures of the ApPDE4 short and long forms: the NTR and UCR1. There is no sequence similarity between the NTRs of these two forms. The short form contains the C-terminal half of UCR1, whereas the long form contains the full-length UCR1.

To investigate the molecular mechanism of intracellular membrane targeting of the ApPDE4 long and short forms, we examined the detailed intracellular localization in HEK293T cells. Because the NTR-UCR1–2 of the ApPDE4 long form was sufficient for its subcellular targeting (7, 8), enhanced GFP (EGFP) was fused to the C-terminal region of NTR-UCR1–2 to create L(N-UCR1–2)-EGFP and expressed in HEK293T cells. As shown in Fig. 1B, L(N-UCR1–2)-EGFP showed plasma membrane and intracellular organelle localization. To clarify the intracellular localization of the long form in HEK293T cells, NTR-UCR1–2 of the ApPDE4 long form, which was fused with the monomeric red fluorescent protein (mRFP) (L(N-UCR1–2)-mRFP), was coexpressed with various subcellular markers, including the pleckstrin homology (PH) domain of PLCβ1 fused to EGFP (EGFP-PLCβ1(PH)), a plasma membrane marker; EGFP-GaIt, a trans-Golgi network (TGN) marker; EGFP-EEA1, an early endosome marker; and EGFP-Rab7, a late endosome marker; or in cells stained with anti-GM130, a cis-Golgi marker. As shown in Fig. 1C, L(N-UCR1–2)-mRFP was colocalized with EGFP-PLCβ1(PH), and partially with EGFP-GaIt and GM130, but not with Rab7-EGFP, indicating that the ApPDE4 long form was localized to the plasma membrane and Golgi complex in HEK293T cells.

Next, we examined the subcellular localization of the ApPDE4 short form in HEK293T cells. We fused EGFP to the C terminus of NTR-UCR1–2 of the ApPDE4 short form (S(N-UCR1–2)-EGFP). As shown in Fig. 1B, S(N-UCR1–2)-EGFP was detected in the plasma membrane. To further clarify its intracellular localization, S(N-UCR1–2)-mRFP was coexpressed with EGFP-PLCβ1(PH). As shown in Fig. 1D, S(N-UCR1–2)-mRFP was colocalized with EGFP-PLCβ1(PH), indicating the plasma membrane targeting of the ApPDE4 short form. Taken together, these results suggest that the long form is...
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Mapping the Minimal Domains for Membrane Targeting of the ApPDE4 Long Form—Next, we examined the minimal domain of the long form for membrane targeting. We first generated and expressed serial deletion mutants of the long form, as shown in Fig. 2A. L(N-UCR1–2)-mRFP, L(N116)-mRFP, L(N20)-mRFP, and L(N16)-mRFP were localized to the plasma membrane and intracellular organelles. L(N13)-mRFP was mainly localized to the cytosol, intracellular organelles, and, weakly, to the plasma membrane. L(N10)-mRFP showed exclusively cytoplasmic localization. N-terminal deletion of three amino acids from L(N20), yielding L(N4/20)-mRFP, resulted in cytoplasmic localization. Taken together, our results suggest that 13 N-terminal amino acids are minimally required for membrane association. However, at least 16 N-terminal amino acids are required for sufficient plasma membrane targeting of the long form.

To identify which amino acids were important for membrane targeting of the long form, we generated various point mutants using L(N20)-mRFP, as shown in Fig. 2B. L(N20/W11A)-mRFP, L(N20/C14S)-mRFP, L(N20/C15S)-mRFP, and L(N20/AA)-mRFP were localized to the plasma membrane and intracellular organelles, similar to L(N20)-mRFP. L(N20/C14S)-mRFP was localized weakly to the plasma membrane and intracellular organelles, which is different from the finding for L(N20)-mRFP. Interestingly, L(N20/C3,14S)-mRFP, L(N20/C3,14,15S)-mRFP, and L(N20/AAA) were impaired for plasma membrane targeting but could be targeted to intracellular organelles. On the other hand, L(N20/C3,15S)-mRFP, L(N20/C3,14,15S)-mRFP, and L(N20/SAAA)-mRFP were completely localized to the plasma membrane and intracellular organelles, including the Golgi complex, whereas the short form is localized only to the plasma membrane in HEK293T cells.

FIGURE 1. Intracellular localization of the ApPDE4 long and short forms. A, schematic of the ApPDE4 long and short forms (top panel) and multiple sequence alignment of a unique N terminus (NTR) and UCR1 domain of the ApPDE4 long and short forms (bottom panel). B, L(N-UCR1–2)-EGFP or S(N-UCR1–2)-EGFP was expressed in HEK293T cells. L(N-UCR1–2)-EGFP is localized to the plasma membrane and intracellular organelles, whereas S(N-UCR1–2)-EGFP is localized to the plasma membrane. Scale bar = 20 μm. C, cellular localization of the ApPDE4 long form. C1, L(N-UCR1–2)-EGFP was coexpressed with various intracellular markers. L(N-UCR1–2)-mRFP is localized to the plasma membrane and, partially, to the Golgi complex in HEK293T cells. EGFP-PLC1(PH), plasma membrane marker; EGFP-EEA1, early endosome marker; EGFP-Rab7, late endosome marker; EGFP-GaT1, TGN marker. C2, L(N-UCR1–2)-EGFP-expressed cells were stained with GM130, a cis-Golgi marker. L(N-UCR1–2)-EGFP is localized to the plasma membrane and, partially, to the cis-Golgi complex in HEK293T cells. Scale bar = 20 μm. D, cellular localization of the ApPDE4 short form. S(N-UCR2)-mRFP was coexpressed with EGFP-PLC1(PH), S(N-UCR2)-mRFP is localized to the plasma membrane. Scale bar = 20 μm.
impaired for membrane targeting. These results suggest that, among the 20 amino acids of the N terminus, Arg9/His10/Trp11 and Cys3/Cys14/Cys15 are important for membrane targeting, therefore indicating that a combination of amino acids is essential for membrane targeting of the long form.

To further examine the intracellular localization of L(N20/C3,14S)-mRFP and L(N20/C14,15S)-mRFP, we coexpressed them with EGFP-GalT. As shown in Fig. 2, L(N20/C3,14S)-mRFP and L(N20/C14,15S)-mRFP were colocalized with EGFP-GalT, indicating Golgi targeting. It has been reported that 25 N-terminal amino acids of mammalian PDE4A1 were sufficient for Golgi targeting (37). Therefore, to examine whether PDE4A1 colocalized with L(N20/C3,14S)-mRFP or L(N20/C14,15S)-mRFP, a segment containing the 25 N-terminal amino acids of human PDE4A1 that was fused to EGFP (hPDE4A1(N25)-EGFP) was coexpressed with L(N20/C3,14S)-mRFP or L(N20/C14,15S)-mRFP, respectively. Interestingly, hPDE4A1(N25)-EGFP was colocalized with L(N20/C3,14S)-mRFP or L(N20/C14,15S)-mRFP (Fig. 2D). Although there is a lack of sequence similarity, these results suggest the possibility that similar molecular mechanisms might be involved in the membrane targeting of the N terminus region of human PDE4A1 and ApPDE4 long form mutants.

Mapping the Minimal Domains for Membrane Targeting of the ApPDE4 Short Form—We generated serial deletion mutants of the short form. As shown in Fig. 3A, S(N-UCR1–2)-mRFP and S(N52)-mRFP were still targeted to the plasma membrane but less efficiently than S(N-UCR1–2)-mRFP. On the other hand, S(N30)-mRFP was mainly localized to the cytoplasm (Fig. 3A). These results suggest that the N-terminal hydrophobic region might be critically involved in membrane association of the ApPDE4 short form.

![FIGURE 2. Characterizations of the membrane targeting domains of the ApPDE4 long form. A, schematics (left panel) and cellular localization (right panel) of deletion mutants of the ApPDE4 long form. L(N-UCR1–2)-mRFP, L(N5)-mRFP, L(N20)-mRFP, and L(N16)-mRFP are localized to the plasma membrane and intracellular organelles in HEK293T cells. On the other hand, L(N13)-mRFP is mainly localized to the cytosol and intracellular organelles and, weakly, to the plasma membrane. L(10)-mRFP and L(4/20)-mRFP are localized to the cytosol. Scale bar = 20 μm. B, schematics (left panel) and cellular localization (right panel) of various point mutants generated by L(N20)-mRFP in HEK293T cells. L(N20/W11A)-mRFP, L(N20/C3S)-mRFP, L(N20/C15S)-mRFP, and L(N20/AA)-mRFP are localized to the plasma membrane and intracellular organelles. L(N20/C14S)-mRFP is localized weakly to the plasma membrane and intracellular organelles, which is different from L(N20)-mRFP. L(N20/C3,14S)-mRFP, L(N20/C14,15S)-mRFP, and L(N20/AAA) are only localized to intracellular organelles. L(N20/C3,14S)-mRFP, L(N20/C3,14,15S)-mRFP, and L(N20/AAA)-mRFP are localized to the cytoplasm. Scale bar = 20 μm. C, the Golgi localization of L(N20/C3,14S)-mRFP and L(N20/C3,14S)-mRFP with human PDE4A1(N25)-EGFP (top panel) and their sequence alignments (bottom panel). Scale bars = 20 μm.]
Because S(ΔN6/N-UCR1–2)-mRFP showed cytosolic localization, we investigated further to identify the amino acids important for plasma membrane targeting. For this, we generated point mutants, including Lys3 to alanine (S(N-UCR1–2/K3A), Asn5 to alanine (S(N-UCR1–2/N5A), and Phe6 to alanine (S(N-UCR1–2/F6A), using S(N-UCR1–2)-mRFP. As shown in Fig. 3B, only S(N-UCR1–2/F6A)-mRFP showed increased cytoplasmic localization, not S(N-UCR1–2)-mRFP, indicating that Phe6 is involved in plasma membrane association. We also replaced Lys3 with aspartic acid in S(N-UCR1–2), yielding S(N-UCR1–2/K3D)-mRFP. Interestingly, this mutant was localized to the cytosol (Fig. 3B). Taken together, these results suggest that the NTR of the ApPDE4 short form is necessary for plasma membrane targeting because it includes a hydrophobic region and basic motifs. In addition, Phe6 plays a role for the plasma membrane targeting of ApPDE4 short form. However, the full-length NTR-UCR1–2 domain of the short form is required for the full plasma membrane targeting.

**Oligomerization of the ApPDE4 Short and Long Forms by the Interaction between UCR1 and UCR2**—It has been reported that UCR1 can interact with UCR2, causing oligomerization (38). Unlike the mammalian PDE4 short form, which only contains UCR2, the ApPDE4 short form contains the C-terminal half region of UCR1 and the entire UCR2 (Figs. 1A and 4A), which are highly homologous to human PDE4 (Fig. 4A). The amino acid sequence of the C-terminal UCR1 domain of the ApPDE4 short form is 76.7% identical with human PDE4D and 77.4% identical with human PDE4A (Fig. 4A). The UCR2 domain of ApPDE4 short form is 59.5% identical with human PDE4D and 65.8% identical with human PDE4A (Fig. 4A).

Therefore, it is plausible that oligomerization of the ApPDE4 short form through the interaction of the C terminus of UCR1 with the N terminus of UCR2 enhances membrane targeting. Therefore, it is highly plausible that both the ApPDE4 short and long forms can produce oligomerization, thereby enhancing their membrane localization.

To test this, we generated S(N-UCR1–2)-3×FLAG and L(N-UCR1–2)-3×FLAG. First, S(N-UCR1–2)-3×FLAG was coexpressed with S(N-UCR1–2)-EGFP or S(N52)-EGFP in HEK293T cells and subjected to FLAG coimmunoprecipitation. As shown in Fig. 4B, S(N-UCR1–2)-EGFP, not S(N52)-EGFP, could be associated with S(N-UCR1–2)-3×FLAG. Next, L(N-UCR1–2)-3×FLAG was coexpressed with L(N-UCR1–2)-EGFP or L(N20)-EGFP. As shown in Fig. 4C, L(N-UCR1–2)-EGFP, not L(N20)-EGFP, could be associated with L(N-UCR1–2)-3×FLAG. These results indicate that the short or the long form could associate with itself, probably through the interaction between UCR1 and UCR2, respectively.

It has been shown previously that the interaction between UCR1 and UCR2 was blocked by the mutation of two positively charged amino acids (RSVR sequence into ASVA) within UCR1 or three negatively charged amino acids (EELDW into AALAW) in UCR2 (39). As shown in Fig. 4A, these residues are conserved in the ApPDE4 long and short forms. Therefore, to examine the roles of the oligomerizations of the ApPDE4 long and short forms in plasma membrane localization, we mutated the RSVR sequence within UCR1 of the long form to ASVA, and EELDW within UCR2 of the short form to AALAW, thereby generating the oligomerization-deficient mutants L(N-UCR1*-2*)-EGFP and S(N-UCR1*-2*)-EGFP. We first exam-
ined the interaction between the wild-type and the oligomerization-deficient mutant using FLAG coimmunoprecipitation. As expected, neither L(N-UCR1–2)-3/FLAG nor S(N-UCR1–2)-3/FLAG could interact with L(N-UCR1*–2*)-EGFP or S(N-UCR1*–2*)-EGFP, respectively (Fig. 4, B and C). These results indicate that L(N-UCR1*–2*)-EGFP and S(N-UCR1*–2*)-EGFP could not form oligomers.

Next, we examined the cellular localization of the oligomerization-deficient mutants. To quantify the plasma membrane localization, we measured the ratio of the fluorescent intensity on the plasma membrane and in the cytoplasm of the constructs using ImageJ. As shown in Fig. 4D, the membrane-cytoplasmic ratio of S(N-UCR1*–2*)-EGFP was significantly different from that of S(N-UCR1–2)-EGFP ($p < 0.001$, one-way ANOVA, $F = 23.39$, Tukey’s post hoc test) but not from that of S(N52)-EGFP ($p > 0.05$, one-way ANOVA, $F = 8.6$, Tukey’s post hoc test). We also examined the cellular localization of the long form. As shown in Fig. 4E, the membrane-cytoplasmic ratio of L(N-UCR1*–2*)-EGFP was significantly different from that of L(N-UCR1–2)-EGFP ($p < 0.01$, one-way ANOVA, $F = 8.6$, Tukey’s post hoc test) but not from that of L(N20)-EGFP ($p > 0.05$, one-way ANOVA, $F = 8.6$, Tukey’s post hoc test).

FIGURE 4. Oligomerization of the ApPDE4 short and long forms. A, multiple sequence alignment of UCR1 (A1) and UCR2 (A2) of various PDE4s, including human PDE4A-D, the ApPDE4 long form, and the ApPDE4 short form. Arrows indicate the residues required for the interaction between UCR1 and UCR2. We generated oligomerization-deficient mutants of the short form (S(N-UCR1*–2*)) and the long form (L(N-UCR1*–2*)), with replacement of the residues indicated by arrows in A1 and A2 into alanine. B, oligomerization of the short form. S(N-UCR1–2)-3/FLAG was coexpressed with S(N-UCR1–2)-EGFP, S(N52)-EGFP, S(N-UCR1*–2*)-EGFP, or EGFP in HEK293T cells, and FLAG coimmunoprecipitation (IP) was performed. S(N-UCR1–2)-EGFP, but not S(N52)-EGFP or S(N-UCR1*–2*)-EGFP, associated with S(N-UCR1–2)-3/FLAG. In a control experiment, EGFP did not associate with S(N-UCR1–2)-3/FLAG. C, oligomerization of the long form. L(N-UCR1–2)-3/FLAG was coexpressed with L(N-UCR1–2)-EGFP, L(N20)-EGFP, L(N-UCR1*–2*)-EGFP, or EGFP in HEK293T cells, and FLAG coimmunoprecipitation was performed. L(N-UCR1–2)-EGFP, but not L(N20)-EGFP or L(N-UCR1*–2*)-EGFP, associated with L(N-UCR1–2)-3/FLAG. In a control experiment, EGFP did not associate with L(N-UCR1–2)-3/FLAG. D, cellular localization (top panel) and quantification of the ratio between the fluorescent intensity at the plasma membrane and at the cytosol of the short form mutants. ***, $p < 0.001$, one-way ANOVA; $F = 23.39$, Tukey’s post hoc test. Data are mean ± S.E. Scale bar = 20 μm. E, cellular localization (top panel) and quantification of the ratio between the fluorescent intensity at the plasma membrane and at the cytosol of the long form mutants. *, $p < 0.05$; **, $p < 0.01$; one-way ANOVA; $F = 8.6$, Tukey’s post-hoc test. Data are mean ± S.E. Scale bar = 20 μm.
These results suggest that oligomerization of the long and short forms plays a role in plasma membrane targeting. However, oligomerization-deficient mutations were still mainly localized to the membrane, indicating that the NTR sequences of the ApPDE4 long and short forms play key roles in membrane targeting and that the oligomerization of the long and short forms further enhances the membrane targeting.

Membrane Targeting of the Short Form, but Not the Long Form, Was Impaired by the Depletion of PIs—

PIs, including PI(4,5)P₂ and PI(3,4,5)P₃, are located mainly in the plasma membrane, where they play important roles in membrane targeting of many proteins via direct interaction (24). First, to examine whether PIs are involved in the localization of the ApPDE4 short or long forms, we performed an in vitro lipid-binding assay using lipid-coated beads. EGFP-PLC₅₁(PH) or EGFP-AKT(PH), which can bind directly to PI(4,5)P₂ and PI(3,4,5)P₃, respectively (40–42), were used as controls. As shown in Fig. 5B, the ApPDE4 short and long forms bind directly to PI(4,5)P₂ and PI(3,4,5)P₃, respectively (40–42), whereas the ApPDE4 short form, but not the long form, was changed by treatment with sphingosine, one of the basic lipids. The images were acquired before and after treatment with 75 μM sphingosine in PBS. Scale bar = 20 μm. D, plasma membrane localization of the ApPDE4 short form, but not the long form, is changed by treatment with a Ca²⁺ ionophore. The images were acquired before and after treatment with 10 μM Ca²⁺ ionophore. Scale bar = 20 μm.
did not interact directly with PI(4,5)P_2 and PI(3,4,5)P_3, whereas EGFP-PLC\_δ1(\text{PH}) and EGFP-AKT(\text{PH}) did. These results suggest that the plasma membrane targeting of the ApPDE4 long and short forms are not mediated by direct PI(4,5)P_2 or PI(3,4,5)P_3 binding.

The cytoplasmic layer of the plasma membrane is the most negatively charged membrane in the cells because PS, PI4P, PI(4,5)P_2, and PI(3,4,5)P_3 are enriched in the inner leaflet of the plasma membrane (25, 26, 34). Therefore, we investigated whether the plasma membrane targeting of the ApPDE4 short and long forms was mediated by electrostatic interactions. To examine this possibility, we neutralized negative charges on the plasma membrane by the addition of sphingosine, a membrane-permeating basic lipid. In the presence of sphingosine, targeting of S(N-UCR1–2)-mRFP, but not L(N-UCR1–2)-mRFP and L(N20)-mRFP, was switched from the plasma membrane to the cytosol (Fig. 5C), indicating that the short form, but not the long form, was targeted to the plasma membrane via electrostatic interactions.

We also neutralized negative charges on the plasma membrane by increasing the cytosolic Ca^{2+} concentration using a Ca^{2+} ionophore, A23187. As shown in Fig. 5D, in the presence of the Ca^{2+} ionophore, the localization of S(N-UCR1–2)-mRFP was changed from the plasma membrane to cytosol, whereas the localization of L(N20)-mRFP was partially changed from the plasma membrane to the cytosol by this treatment (Fig. 5D). Taken together, these results suggest that the ApPDE4 short form might be localized to the plasma membrane via nonspecific electrostatic interactions, whereas the ApPDE4 long form uses other targeting mechanisms.

**Liposome Binding of Peptides Derived from the N Termini of the ApPDE4 Long or Short Forms**—To verify clearly the lipid-binding properties of ApPDE4 long and short forms, we performed peptide-liposome binding assays using peptides derived from the N termini of the ApPDE4 long or short form because this domain plays a key role in membrane association, as shown in Figs. 2 and 3. We have shown previously that the membrane localization of L(N20)-EGFP was not changed by 2-bromo-palmitate, a reversible palmitoylation inhibitor (8), and that L(N20/C3,14,15S)-EGFP was localized to the cytoplasm (Fig. 2B). Normally, cysteine residues might be modified by palmitoylation, which is important for membrane association. However, although having no direct evidence that cysteine residues within the N-terminal are palmitoylated, we concluded that palmitoylation itself has no effect on the intracellular localization of the long form. Therefore, for the ApPDE4 long form study, we generated an L(N16) peptide (MSCLLPAIRHWSCCM) without any lipid modification and an L(N16/C3,14,15S) peptide (MSCLLPAIRHWSSSSM) as a control. We found that the L(N16) peptide, but not the L(N16/C3,14,15S) peptide, was bound to all liposomes irrespective of lipid makeup (Fig. 6A). These results indicate that the 16 N-terminal amino acids of the ApPDE4 long form could be associated with liposomes mainly via hydrophobic interactions. Considering that the L(N16/C3,14,15S) peptide failed to associate with liposomes, cysteine residues within the wild-type peptide might be involved in this hydrophobic interaction, but directly, not as an indirect result of providing prenylation or palmitoylation sites. Taken together, these results suggest that the N terminus of the long form can be associated with the membrane via hydrophobic interactions.

To examine the lipid-binding properties of the short form, we generated the S(N15) and S(N15/K3D) peptides, the latter a control, and performed liposome-binding assays. To perform the liposome assay, we added tryptophan to the C termini of both peptides artificially because tryptophan was required for liposome-binding assays. We found that, with liposomes containing neutral lipids, neither S(N15) nor S(N15/K3D) bound to the liposomes (Fig. 6B). On the other hand, with liposomes containing acidic lipids, i.e., 15% PS and 1% PI(4,5)P_2, binding of the S(N15) peptide increased compared with binding of the S(N15/K3D) peptide (Fig. 6B). These results indicate that the S(N15) peptide can associate with acidic but not neutral lipids, suggesting that the 15 N-terminal amino acids of the short form are involved in specific association with acidic membranes, including plasma membranes within cells.
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Effect of an Acute Depletion of PIs on Cellular Localization of the ApPDE4 Long and Short Forms—To selectively manipulate PIs in the plasma membrane, we used pseudojanin (PJ) system, which is useful for transient membrane lipid depletion in living cells (43) (Fig. 7A). The ApPDE4 short or long form was cotransfected with Lyn11-FRB (rapamycin-binding domain of mTOR) and mRFP-FKBP (FK506 binding protein 12)-PJ, which can be recruited to the plasma membrane in an inducible manner in response to rapamycin (Fig. 7A). PJ, which contains active yeast Sac1, which dephosphorylates PI4P, and active phosphatase E (INPP5E), which converts PI(4,5)P2 to PI4P, can deplete PI4P and PI(4,5)P2 (top panel). In the absence of rapamycin (Rapa), Lyn11-FRB and mRFP-FKBP-PJ are localized to the plasma membrane and cytosol, respectively (bottom panel). In the presence of rapamycin, Rapa-FRB can be associated through FKBP, leading to the plasma membrane targeting of mRFP-FKBP-PJ, depleting PIs. B, cellular localization of the ApPDE4 long or short form in the absence or presence of rapamycin in HEK293T cells. The colored lines in the confocal fluorescence images indicate the paths along which the fluorescence intensities of the corresponding images were plotted on the right. S(N-UCR1–2)-mRFP was cotransfected with PJ, PJ-Sac, INPP5E, and PJ-Dead. Membrane localization of the short form is switched to the cytoplasm by PJ and PJ-Sac recruitment but not by INPP5E (right panel). As a control, PLCδ1(PH)-EGFP was used. Therefore, an electrostatic interaction contributes to short form recruitment to the plasma membrane, and PI4P and PI(4,5)P2 are also involved in charging the plasma membrane. Scale bar = 20 μm. C, activation of the PLC pathway by treatment with carbachol, an M1 receptor agonist, disrupts the plasma membrane localization of the ApPDE4 short form. The colored lines in the confocal fluorescence images indicate the paths along which the fluorescence intensities of the corresponding images were plotted on the right. Scale bar = 20 μm.

FIGURE 7. Localization of the short form, but not the long form, is changed by the depletion of PIs on the plasma membrane by the PJ system. A, schematic of experimental models of the Lyn11-FRB/PJ system (modified from Ref. 26). Yeast Sac1 dephosphorylates PI4P, and INPP5E converts PI(4,5)P2 to PI4P (top panel). In the absence of rapamycin (Rapa), Lyn11-FRB and mRFP-FKBP-PJ are localized to the plasma membrane and cytosol, respectively (bottom panel). In the presence of rapamycin, Rapa-FRB can be associated through FKBP, leading to the plasma membrane targeting of mRFP-FKBP-PJ, depleting PIs. B, cellular localization of the ApPDE4 long or short form in the absence or presence of rapamycin in HEK293T cells. The colored lines in the confocal fluorescence images indicate the paths along which the fluorescence intensities of the corresponding images were plotted on the right. S(N-UCR1–2)-mRFP was cotransfected with PJ, PJ-Sac, INPP5E, and PJ-Dead. Membrane localization of the short form is switched to the cytoplasm by PJ and PJ-Sac recruitment but not by INPP5E (right panel). As a control, PLCδ1(PH)-EGFP was used. Therefore, an electrostatic interaction contributes to short form recruitment to the plasma membrane, and PI4P and PI(4,5)P2 are also involved in charging the plasma membrane. Scale bar = 20 μm. C, activation of the PLC pathway by treatment with carbachol, an M1 receptor agonist, disrupts the plasma membrane localization of the ApPDE4 short form. The colored lines in the confocal fluorescence images indicate the paths along which the fluorescence intensities of the corresponding images were plotted on the right. Scale bar = 20 μm.
recruitment of YFP-FKBP-PJ to Lyn11-FRB in the plasma membrane depleted PI4P and PI(4,5)P2 from the membrane. We conjectured that the 20 N-terminal amino acids of the ApPDE4 long form could play the role of Lyn11. To test this, we generated L(N20)-mRFP-FRB and coexpressed it with YFP-FKBP-PJ in HEK293T cells (Fig. 8B). As shown in Fig. 8B, left panel, in the absence of rapamycin, YFP-FKBP-PJ was expressed in the cytosol, and L(N20)-mRFP-FRB was localized to the plasma membrane and intracellular organelles, as in the case of L(N20)-mRFP. In the presence of rapamycin, the localization of L(N20)-mRFP-FRB was shifted from the cytosol to the plasma membrane and intracellular organelles, where L(N20)-mRFP-FRB was localized. These results show clearly that L(N20)-mRFP-FRB was localized to the plasma membrane and intracellular organelles. Therefore, rapamycin treatment could relocate YFP-FKBP-PJ to the plasma membrane to the cytosol (Fig. 8C). Therefore, L(N20) can be used to target FRB to the plasma membrane and intracellular organelles. We also found that L(N16)-mRFP and L(N20)-mRFP were colocalized with EGFP-Lact-C2, a PS-binding probe, in HEK293T cells is shown. Scale bar = 20 μm.

**DISCUSSION**

In this study, we investigated the molecular mechanisms of membrane targeting of the ApPDE4 long and short forms. We first showed that, in HEK293T cells, the ApPDE4 long form was localized to the plasma membrane and Golgi apparatus, whereas the short form was targeted to the plasma membrane only. Second, we showed that the 16 N-terminal amino acids of the long form were sufficient for membrane targeting, whereas the full length of the NTR of the short form was required for plasma membrane targeting. Third, the long form was targeted to the plasma membrane via N-terminal hydrophobic interactions, whereas the short form was localized to the plasma membrane via electrostatic interactions. Fourth, oligomerization of the short and the long forms, probably through UCR1-UCR2.
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interaction, enhances plasma membrane localization. Therefore, the ApPDE4 long and short forms were localized to the intracellular membranes via different targeting mechanisms. Taken together, this is the first report to demonstrate that PDE4 can be targeted to the plasma membrane by direct membrane association through hydrophobic or electrostatic interactions.

**Plasma Membrane Targeting of the ApPDE4 Short Form via Nonspecific Electrostatic Interactions**—In our previous paper, we speculated that the plasma membrane localization of the short form was mediated by interactions with PIs, including P(4,5)P₂ (8). This was motivated by the fact that, in the in vitro lipid strip assay, the ApPDE4 short form could bind to various PIs, including PIP₄, P(4,5)P₂, and P(3,4,5)P₃, and that the N terminus of the ApPDE4 short form contains many positively charged amino acids (14 Lys/Arg of 52 amino acids within the NTR) (8). In this study, we show clearly that the short form was localized to the plasma membrane via nonspecific electrostatic interactions between polybasic amino acids within the NTR and acidic lipids, including PIP₄ and P(4,5)P₂. Similarly, many proteins having basic amino acids are targeted to the plasma membrane by electrostatic interactions. For example, cAMP-dependent protein kinase isomerizes positively charged motifs are targeted to the plasma membrane via electrostatic interactions (44). However, in many cases, this targeting requires a hydrophobic domain, either a lipid-modified motif such as a myristoylation site or a non-lipid-modified hydrophobic domain, as well as polybasic amino acids. Otherwise, the proteins could be localized to the nucleus. For example, MARCKS, which has an N-terminal myristoylation site that provides hydrophobic interaction plus basic motifs within the middle part, is targeted to the plasma membrane by electrostatic interactions (45). K-Ras, which has a polybasic motif and a farnesylation site within the C terminus, can be localized to the plasma membrane (25). In the case of Rit, a non-lipid hydrophobic domain and a polybasic amino acid composition in the C terminus are necessary for localization to the plasma membrane (25). Similarly, the ApPDE4 short form has a hydrophobic domain and basic amino acids within the N terminus. Therefore, the deletion of these domains independently abolished the membrane association of the short form (Fig. 3). Thus, the flexible N-terminal hydrophobic domain can be associated hydrophobically with membranes in general, whereas the basic amino acids in the NTR can be associated electrostatically with acidic lipids in the plasma membrane, leading to stable plasma membrane association of the short form. This is the first report showing that a PDE4 is localized to the plasma membrane through nonspecific electrostatic interactions.

We also found that oligomerization of the short form further enhanced the plasma membrane targeting (Fig. 4, B and D). An oligomerization-deficient mutant, S(N-UCR1⁎-2⁎)-EGFP, which could not bind to S(N-UCR1₋2₋3₋4)-FLAG, was more localized to the cytosol compared with S(N-UCR1₋2₋3₋4)-EGFP (Fig. 4, B and D). It has been reported previously that the N-terminal region of UCR2 can associate with the C-terminal region of UCR1 in human PDE4D, eventually inducing oligomerization (38). Therefore, PDE4D3, which belongs to the long form containing UCR1 and UCR2, is oligomeric, whereas PDE4D2, which belongs to the short form containing UCR2 only, is monomeric (38). Interestingly, the ApPDE4 short form contains a truncated C-terminal region of UCR1 and a full-length UCR2 (Figs. 1A and 3A). Therefore, unlike the mammalian long form, the ApPDE4 short form could form an oligomer, leading to further enhancement the plasma membrane localization (Fig. 4D).

**Membrane Targeting of the ApPDE4 Long Form via Hydrophobic Interactions**—PDEs can be localized to specific intracellular organelles through direct membrane associations. For example, N-terminal hydrophobic regions of PDEs are involved in its association to the endoplasmic reticulum membrane (46). PDE2A3, which is abundantly expressed in the brain, is localized to the intracellular membrane, including the plasma membrane in HEK293T and PC12 cells, and to the synaptic membranes of neurons through N-terminal dual acylation (47). HEKPD4E-A1, which belongs to the supershort form category, can be associated directly with the Golgi membrane. The N-terminal 25 amino acids of PDE4A1 bind preferentially to phosphatidylinerically-rich regions of membranes and is redistributed dynamically by perturbing both the phosphatidic acid and calcium signaling systems (22, 37). Similarly, we found that the N-terminal 16 amino acids of the ApPDE4 long form were sufficient to be localized to the intracellular membranes via direct membrane association through hydrophobic interactions (Fig. 2A). Furthermore, oligomerization of the long form further enhanced membrane targeting (Fig. 4, C and E).

Interestingly, although there is no sequence similarity between the N terminus of hPDE4A1 and the ApPDE4 long form, ApPDE4 L(N20/C14,15S)-mRFP was colocalized with hPDE4A1(N25)-EGFP in HEK293T cells (Fig. 2D). These results suggest that the molecular mechanism(s) of the membrane targeting of PDE4A1 and ApPDE4 long form mutants might be similar and through direct membrane association. The N terminus of PDE4A1 is involved in membrane association via two different motifs, namely, helix 1 and helix 2, that are separated by a hinge region (22, 48). Our results showed that at least 16 N-terminal amino acids are required for sufficient membrane targeting of the long form. We also showed that N-terminal deletion of three amino acids from L(N20), ApPDE4 L(N4/20)-mRFP resulted in an exclusive localization to cytoplasm (Fig. 2A). Therefore, considering that proline is known as potent breaker of both α-helix and β-sheet structure, the N terminus of the ApPDE4 long form might be divided into two separate motifs: motif 1 (MSCLL) and motif 2 (PAIRHWISCCM). In PDE4A1, helix 1 is involved in facilitating membrane association and its targeting to the TGN. Helix 2 contains TAPAS-1, which allows membrane association via its binding with calcium ions and phosphatidic acid (37). Helix 2 contains a membrane insertion unit (Leu₁₆, Val₁₁₇–Trp₁₁₉, Trp₂₀₀) that is stabilized by Pro⁴-Trp¹⁵, indicating that the sequence P-W-L-V-G-W-W in helix 2 is the key unit for membrane association. Considering that the localization pattern of ApPDE4 L(N20/AA)-mRFP is similar to that of the wild type (Fig. 2B), the sequence PALXXWI (where X is hydrophobic or basic) is responsible for the membrane association. Therefore, the conserved motif can be proposed as follows: Pro-hydrophobic-hydrophobic-X-X-Trp-hydrophobic (where X is hydrophobic or...
basic). Considering the Golgi localization of ApPDE4 L(N20/C14,15S)-mRFP, the downstream sequence SCCM of ApPDE4 might contribute to specific cellular localization, whereas the downstream sequence Lys<sup>24</sup>,Arg<sup>25</sup> of PDE4A1 is required for the preferential association with phosphatidic acid. These results indicate that the ApPDE4 long form and PDE4A1 might share similar core membrane insertion motifs but have distinct modes of dynamic targeting, such as the lipid binding preference and intracellular organelle targeting. Furthermore, we showed previously that L(N20)-EGFP and L(N20/C14,15S)-EGFP were localized to the plasma membrane and presynaptic terminals in Aplysia sensory neurons (8). Therefore, it is possible that hydrophobic interactions or the association of the 20 N-terminal amino acids of the ApPDE4 long form with the phosphatidic acid-rich region might be responsible for synaptic membrane targeting in Aplysia neurons. It will be of interest to examine this in future studies.

Our results suggest that the 16 N-terminal amino acids of the long form are sufficient for membrane targeting. Considering the result that the L(N16) peptide can be associated with liposomes irrespective of lipid composition (Fig. 6A) and that the depletion of PI<sub>3</sub>ks using antiamycin and the neutralization of the plasma membrane by sphingosine had no effect on long form localization (Fig. 5B), we can conclude that the N terminus of the ApPDE4 long form is associated with the intracellular membranes via hydrophobic interactions. Similarly, the C terminus of H-Ras that is fused to EGFP (EGFP-H-RassCAAX) is targeted to the plasma membrane mainly through hydrophobic interactions. Therefore, depletion of plasma membrane PI<sub>4</sub> by the PI<sub>1</sub> system, has no effect on cellular localization of H-Ras.

However, we could not exclude the possibility that electrostatic interactions might be involved in the intracellular targeting of the ApPDE4 long form. Although the L(N16) peptide of the long form did not bind to PS-containing liposomes more strongly than to neutral liposomes in vitro (Fig. 6A), L(N16)-mRFP or L(N20)-mRFP was colocalized with PS-enriched membrane in HEK293T cells (Fig. 8D). It has been reported that surface charge biosensors with a progressively lower positive charge showed different cellular localizations (34). Strongly positively charged probes (8+) are localized mainly to the plasma membrane. On the other hand, intermediate biosensor charges (6+ or 3+) are localized to the PS-enriched membrane, similar to the ApPDE4 long form. Similarly, c-Src, which contains five positive charges within the myristoylated N terminus, is localized to the PS-enriched membrane. Within the N terminus of the ApPDE4 long form, a strongly basic amino acid (Arg<sup>9</sup>) next to a mildly positively charged amino acid (His<sup>10</sup>) is located within the run of hydrophobic amino acids. Therefore, it might be possible that L(N20)-mRFP and L(N16)-mRFP were localized to the PS-enriched membrane via the N-terminal region through combined hydrophobic and intermediate-strength electrostatic interactions. Consistent with this, L(N20)-mRFP-FRB could recruit YFP-FKBP-PJ to deplete PI<sub>4</sub>P and PI(4,5)P<sub>2</sub> from the plasma membrane (Fig. 8C). These results suggest that L(N20)-mRFP-FRB was localized near PI<sub>4</sub>P- and PI(4,5)P<sub>2</sub>-enriched membranes. PI(4,5)P<sub>2</sub> is a minor lipid within cells, constituting about 1% of the plasma membrane. It is believed that PI<sub>2</sub> is sequestered and clustered within the plasma membrane. Therefore, we can conclude that L(N20)-mRFP-FRB is targeted to acidic, membrane-enriched regions.

PI<sub>4</sub>P and PI(4,5)P<sub>2</sub> as the Key Lipids for Plasma Membrane Targeting of the ApPDE4 Short Form by Nonspecific Electrostatic Interactions—In the plasma membrane targeting of many proteins by nonspecific electrostatic interactions, acidic lipids, including PS, PI<sub>4</sub>P, PI(4,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub>, play key roles (24–26, 34). Especially the inner leaflet of the plasma membrane is the only reservoir of polyanionic inositol lipids. It is well known that PI(4,5)P<sub>2</sub> is a main contributor to plasma membrane targeting by electrostatic interaction (24). For example, the plasma membrane targeting of MARCKS or GAP43 is mediated by nonspecific PI(4,5)P<sub>2</sub> binding. However, recent papers have shown that PI<sub>4</sub>P as well as PI(4,5)P<sub>2</sub> contribute independently to electrostatic plasma membrane targeting (26). Our results also showed that the plasma membrane targeting of the short form is mainly mediated by PI<sub>4</sub>P and PI(4,5)P<sub>2</sub> because the localization of the short form was shifted significantly from the plasma membrane to the cytosol by PI<sub>1</sub> but not by either INPP5E or PI-Sac recruitment to the plasma membrane by rapamycin in HEK293T cells (Fig. 7B). Therefore, we can conclude that PI<sub>4</sub>P and PI(4,5)P<sub>2</sub> are major contributors to the plasma membrane targeting of the short form via nonspecific electrostatic interactions.

Application of the N Termini of the ApPDE4 Long and Short Forms as New Peptides for Selective Cellular Targeting—As shown in Fig. 8, we showed that L(N20)-mRFP-FRB can functionally replace Lyn11-FRB-mRFP. These results suggest that the N-terminal 20 amino acids of the ApPDE4 long form can be used as a peptide tool for selective targeting to the cytoplasmic surface of intracellular organelles. We showed that the C14S/C15S mutant was localized to the Golgi apparatus, including the TGN, but not to the plasma membrane, in HEK293T cells (Fig. 2). Using this mutant peptide, we can deliver cytoplasmic proteins specifically to the Golgi complex (Fig. 8B, right panel). Therefore, it will be interesting to develop a specific cellular delivery system using selective intracellular targeting peptides derived from mutation of the N-terminal 20 amino acids of the long form.

Normally, phospholipid-binding domains that have low lipid-binding affinity cannot associate stably with the membrane. Therefore, to be stably localized to the membrane, additional binding, such as self-oligomerization or cooperation with other lipid-binding domains, is required (49). As shown in Fig. 3A, the N-terminal 30 amino acid segment of the ApPDE4 long form by itself could not be localized to the intracellular membrane. However, if this domain is combined with another low-affinity lipid-binding domain, specific membrane targeting might be enhanced. Therefore, the N termini of the ApPDE4 long and short forms might be useful for engineering specific cellular membrane targeting or enhancing the membrane affinity of lipid-binding proteins.

REFERENCES
1. Lee, Y. S., Bailey, C. H., Kandel, E. R., and Kaang, B. K. (2008) Transcriptional regulation of long-term memory in the marine snail Aplysia. Mol.
Intracellular Targeting of ApPDE4s by Membrane Association

Brain 1, 3

2. Kandel, E. R. (2012) The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. Mol. Brain 5, 14

3. Richter, W., Menniti, F. S., Zhang, H. T., and Conti, M. (2013) PDE4 as a target for cognition enhancement. Expert. Opin. Ther. Targets 17, 1011–1027

4. Dudai, Y., Jan, Y. N., Byers, D., Quinn, W. G., and Benzer, S. (1976) dunce, a mutant of Drosophila deficient in learning. Proc. Natl. Acad. Sci. U.S.A. 73, 1684–1688

5. Zhong, Y., and Wu, C. F. (1991) Altered synaptic plasticity in a mutant of Drosophila melanogaster. Nature 289, 79–81

6. Byers, D., Davis, R. L., and Kiger, J. A., Jr. (1981) Defect in cyclic AMP phosphodiesterase due to the dune mutation of learning in Drosophila melanogaster. Nature 289, 79–81

7. Park, H., Lee, J. A., Lee, C., Kim, M. J., Chang, D. J., Kim, H., Lee, S. H., Lee, Y. S., and Kaang, B. K. (2005) An Aplysia type 4 phosphodiesterase homolog localizes at the presynaptic terminals of Aplysia neuron and regulates synaptic facilitation. J. Neurosci. 25, 9037–9045

8. Jang, D. J., Park, S. W., Lee, J. A., Lee, C., Chae, Y. S., Park, H., Kim, M. I., Choi, S. L., Lee, N., Kim, H., and Kaang, B. K. (2010) N termini of apPDE4 isoforms are responsible for targeting the isoforms to different cellular membranes. Learn. Mem. 17, 469–479

9. Jang, D. J., Lee, J. A., Chae, Y. S., and Kaang, B. K. (2011) State-dependent disruption of short-term facilitation due to overexpression of the apPDE4 supershort form in Aplysia. Mol. Cells 31, 175–180

10. Barad, M., Bourouchdade, R., Winder, D. G., Golan, H., and Kandel, E. (1998) Rolipram, a type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory. Proc. Natl. Acad. Sci. U.S.A. 95, 15020–15025

11. Rutten, K., Prickaerts, J., and Blokland, A. (2006) Rolipram reverses scopolamine-induced and time-dependent memory deficits in object recognition by different mechanisms of action. Neurobiol. Learn. Mem. 85, 132–138

12. Ramos, B. P., Birnbaum, S. G., Lindenmayer, I., Newton, S. S., Duman, R. S., and Arnsten, A. F. (2003) Disregulation of protein kinase a signaling in the aged prefrontal cortex: new strategy for treating age-related cognitive decline. Neuron 40, 835–845

13. Houslay, M. D., and Adams, D. R. (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. Biochem. J. 370, 1–18

14. Bolger, G., Michaela, T., Martins, T., St John, T., Steiner, B., Rodgers, L., Riggs, M., Wigler, M., and Ferguson, K. (1993) A family of human phosphodiesterase RPDE-6 region of the PDE4A cAMP-specific phosphodiesterase confers preferential interaction with specific SH3 domains. Proc. Natl. Acad. Sci. U.S.A. 90, 651–657

15. Houslay, M. D. (2010) Underpinning compartmentalised cAMP signalling through targeted C(4)orF34 as a novel endoplasmic reticulum resident protein. BMB Rep., in press

16. Lawe, D. C., Patki, V., Heller-Harrison, R., and Corvera, S. (2000) The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding: critical role of this dual interaction for endosomal localization. J. Biol. Chem. 275, 3699–3705

17. Cole, N. B., Smith, C. L., Sciaky, N., Terasaki, M., Edidin, M., and Lippincott-Schwartz, J. (1996) Diffusional mobility of Golgi proteins in membranes of living cells. Science 273, 797–801

18. Lawe, D. C., Patki, V., Heller-Harrison, R., and Corvera, S. (2000) The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding: critical role of this dual interaction for endosomal localization. J. Biol. Chem. 275, 3699–3705

19. Yeung, T., Gilbert, G. E., Shi, J., Silvius, J., Kapus, A., and Grinstein, S. (2008) Membrane phosphatidyserine regulates surface charge and protein localization. Science 319, 210–213

20. Baillie, G. S., Huston, E., Scotland, G., Hodgkin, M., Gall, I., Peden, A. H., Mackenzie, C., Houslay, E. S., Currie, R., Pettit, T. R., Walmsley, A. R., Wakelam, M. J., Warwicker, J., and Houslay, M. D. (2002) TAPAS-1, a novel microdomain within the unique N-terminal region of the PDE4A1 cAMP-specific phosphodiesterase that allows rapid, Ca2+-triggered membrane association with selectivity for interaction with phosphatidic acid. J. Biol. Chem. 277, 28298–28309
specific phosphodiesterases is mediated by the upstream conserved regions (UCRs). J. Biol. Chem. 277, 40212–40221
39. Beard, M. B., Olsen, A. E., Jones, R. E., Erdogan, S., Houslay, M. D., and Bolger, G. B. (2000) UCR1 and UCR2 domains unique to the cAMP-specific phosphodiesterase family form a discrete module via electrostatic interactions. J. Biol. Chem. 275, 10349–10358
40. Garcia, P., Gupta, R., Shah, S., Morris, A. J., Rudge, S. A., Scarlata, S., Petrova, V., McLaughlin, S., and Rebecchi, M. J. (1995) The pleckstrin homology domain of phospholipase C-\(\gamma\) binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. Biochemistry 34, 16228–16234
41. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science 275, 665–668
42. Lemmon, M. A., Ferguson, K. M., O’Brien, R., Sigler, P. B., and Schlessinger, J. (1995) Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. Proc. Natl. Acad. Sci. U.S.A. 92, 10472–10476
43. Suh, B. C., Inoue, T., Meyer, T., and Hille, B. (2006) Rapid chemically induced changes of PtdIns(4,5)P2 gate KCNQ ion channels. Science 314, 1454–1457
44. Fairn, G. D., Ogata, K., Botelho, R. J., Stahl, P. D., Anderson, R. A., De Camilli, P., Meyer, T., Wodak, S., and Grinstein, S. (2009) An electrostatic switch displaces phosphatidylinositol phosphate kinases from the membrane during phagocytosis. J. Cell Biol. 187, 701–714
45. McLaughlin, S., and Aderem, A. (1995) The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. Trends Biochem. Sci. 20, 272–276
46. Shakur, Y., Takeda, K., Kenan, Y., Yu, Z. X., Rena, G., Brandt, D., Houslay, M. D., Degerman, E., Ferrans, V. J., and Manganiello, V. C. (2000) Membrane localization of cyclic nucleotide phosphodiesterase 3 (PDE3): two N-terminal domains are required for the efficient targeting to, and association of, PDE3 with endoplasmic reticulum. J. Biol. Chem. 275, 38749–38761
47. Russwurm, C., Zoidl, G., Koesling, D., and Russwurm, M. (2009) Dual acylation of PDE2A splice variant 3: targeting to synaptic membranes. J. Biol. Chem. 284, 25782–25790
48. Smith, K. J., Scotland, G., Beattie, J., Trayer, I. P., and Houslay, M. D. (1996) Determination of the structure of the N-terminal splice region of the cyclic AMP-specific phosphodiesterase RD1 (RNPD4A1) by 1H NMR and identification of the membrane association domain using chimeric constructs. J. Biol. Chem. 271, 16703–16711
49. Lemmon, M. A. (2008) Membrane recognition by phospholipid-binding domains. Nat. Rev. Mol. Cell Biol. 9, 99–111