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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)P_2, 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.

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Phosphodiesterases (PDEs)[6] 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-

[6] 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-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; PS, l-α-phosphatidylserine; EGFP, enhanced GFP; mRFP, monomeric red fluorescent protein; PH, pleckstrin homology; TGN, trans-Golgi network; ANOVA, analysis of variance; PJ, pseudojanin; PLC, phospholipase C; mTOR, mammalian target of rapamycin; FRB, rapamycin binding domain of mTOR; 5-HT, 5-hydroxytryptamine.

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 commonly 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. Phosphoinosites (PIs) play a key role in protein targeting in cells (23). $PI(4,5)P_2$ and $PI(3,4,5)P_3$ 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 cytoplasmic surface of the plasma membrane is the most acidic membrane in the cell because negatively charged lipids such as $PI(4,5)P_2$ and $PI(3,4,5)P_3$ 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 $PI(4,5)P_2$ (25). Recently, it has been reported that PI4P is involved in these electrostatic interactions. Depletion of PI4P and $PI(4,5)P_2$ simultaneously, but not depletion of either PI4P or $PI(4,5)P_2$ alone, could change the localization of many plasma membrane-targeted proteins, such as myristoylated alanine-rich C-kinase substrate (MARCKS), Rit, 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/KpnI and subcloned into HindIII-KpnI-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/KpnI and subcloned into HindIII-KpnI-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-XbaI-S (5′-GCTCTAGAATGGCCTCCTCCGAGGAC-3′)/mRFP-ApaI-A (5′-CGTAGGGCCCTTAGGCGCCGGT-GGAGTG-3′), was subcloned into XbaI-ApaI-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/XbaI and subcloned into HindIII-XbaI-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-XbaI-digested pcDNA3.1-mRFP to create pcDNA3.1-ApPDE4 mutant-mRFP. We also generated 25 N-terminal amino acids of human PDE4A1 using primer extensions and subcloned them into pEGFP-N3 using EcoRI/KpnI sites.

ApPDE4 L(N20)-EGFP was used as the template for PCR using the specific primer sets L-D3-S (5′-CGCCCAAGCTTGCCAC-CATGTCTTGCTTGCTTCCC-3′)/EGFP-XhoI-A (5′-CCCTC-GAGCTTGTACAGCTCGTCCAT-3′) and subcloned into pcDNA3.1-FRB, generating pcDNA3.1-L(N20)-EGFP-FRB. EGF-P-EEA1 (plasmid 42307) (31), EGFP-GalT (plasmid 11929) (32), EGFP-Rab7 (plasmid 12605) (33), PJ-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 CO_2 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 × 10^5 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 (bead volume) 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-α-phosphatidylcholine, l-α-phosphatidylethanolamine, PS (l-α-phosphatidylserine), cholesterol, 1% PI(4,5)P_2, and dansyl-PE in the ratio of 44:10:15:25:1:5 mol %, respectively. In cases where no PS/PI(4,5)P_2 was used, l-α-phosphatidylcholine contents were adjusted accordingly.

Liposome binding of peptide was monitored using FRET measurements, in which the dansyl-PE incorporated into the liposome quenches the tryptophan residues of the peptide. All measurements were carried out in a Fluoromax (Horiba Jobin Yvon) in 1 ml of buffer containing 100 mM KCl and 20 mM HEPES-KOH (pH 7.4). The excitation wavelength used for tryptophan was 280 nm (slit width, 5 nm), and the emission spectra were recorded from 320–420 nm (slit width, 5 nm), with the peak at 355 nm. FRET was normalized as F_0/F, where F_0 and F represent the fluorescence intensity at 355 nm before and after liposome addition, respectively. Peptide-liposome interaction increases FRET (F_0/F) because of tryptophan quenching.

Drug Treatments—ATP depletion was performed by incubating cells with 200 nM anti-mycin A (Sigma) in a calcium- and glucose-free medium (PBS) for at least 40 min. A sphingosine (Sigma) was added to cells incubated in DMEM to a final concentration of 75 μM. Unless indicated otherwise, all treatments and assays were performed at 37 °C. Cells were treated with wortmannin, a PI3K inhibitor, in glucose-free medium for at least 40 min at a concentration of 200 nM.

RESULTS

Intracellular Localization of the ApPDE4 Long and Short Forms—As shown in Fig. 1*A*, 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. 1*B*, 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 PLCδ1 fused to EGFP (EGFP-PLCδ1(PH)), a plasma membrane marker; EGFP-GalT, 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. 1*C*, L(N-UCR1–2)-mRFP was colocalized with EGFP-PLCδ1(PH), and partially with EGFP-GalT 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. 1*B*, 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-PLCδ1(PH). As shown in Fig. 1*D*, S(N-UCR1–2)-mRFP was colocalized with EGFP-PLCδ1(PH), indicating the plasma membrane targeting of the ApPDE4 short form. Taken together, these results suggest that the long form is

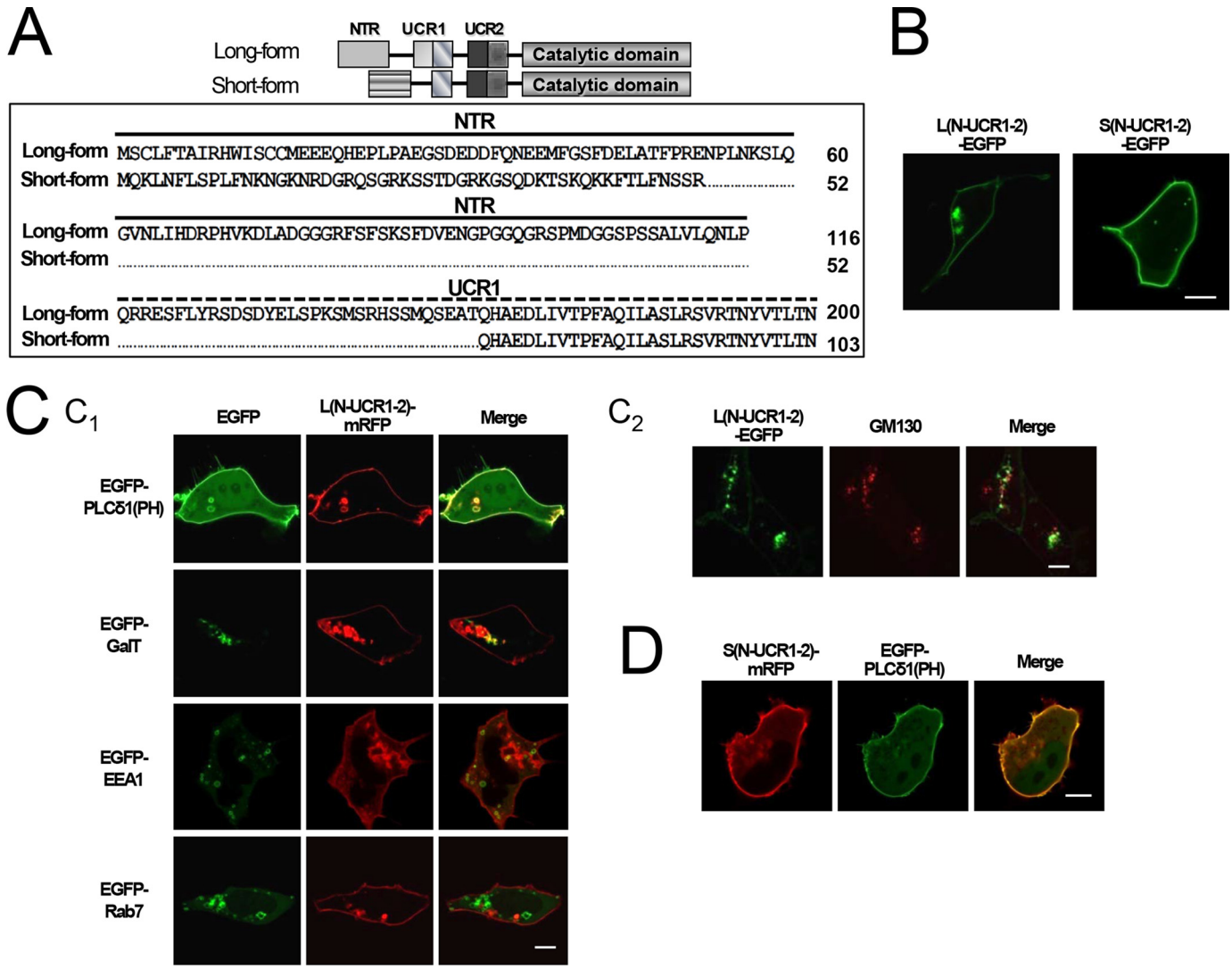

FIGURE 1. **Intracellular localization of the ApPDE4 long and short forms.** *A*, schematic of the ApPDE4 long and short forms (*top panel*) and multiple sequence alignment of a unique N terminus (NTR) and UCR1 domain of the ApPDE4 long and short forms (*bottom panel*). *B*, L(N-UCR1–2)-EGFP or S(N-UCR1–2)-EGFP 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. C_1, 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-PLCδ1(PH)*, plasma membrane marker; *EGFP-EEA1*, early endosome marker; *EGFP-Rab7*, late endosome marker; *EGFP-GalT*, TGN marker. C_2, 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-PLCδ1(PH). S(N-UCR2)-mRFP is localized to the plasma membrane. *Scale bar* = 20 μm.

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.

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. 2*A*. 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. 2*B*. L(N20/W11A)-mRFP, L(N20/C3S)-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/C14,15S)-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

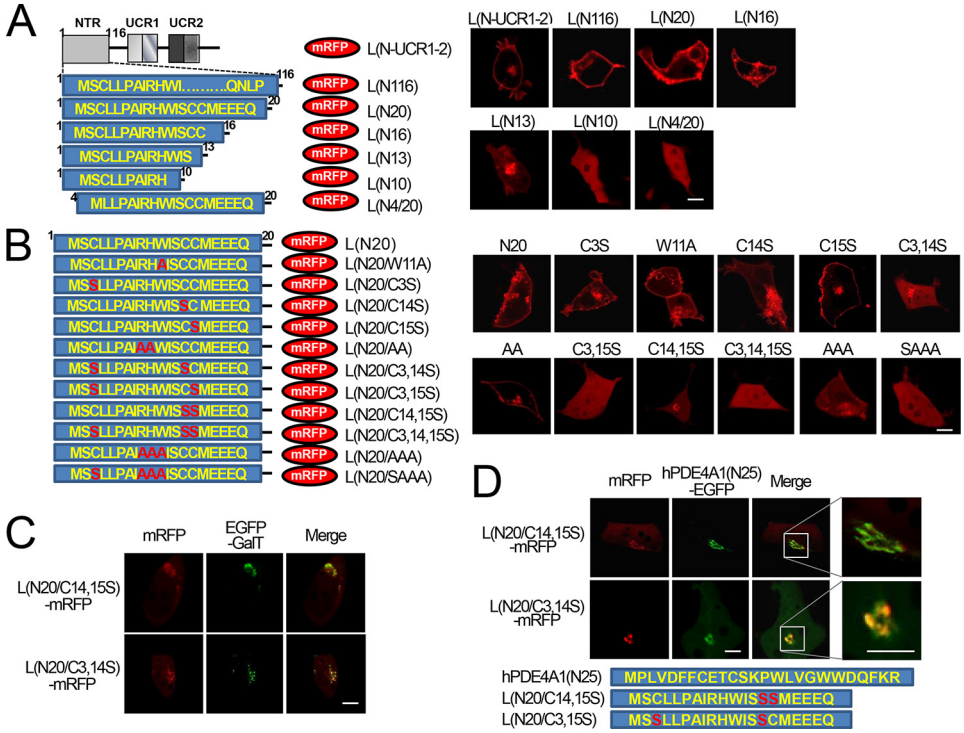

FIGURE 2. **Characterizations of the membrane targeting domains of the ApPDE4 long form.** *A*, schematics (*left panel*) and cellular localization (*right panel*) of deletion mutants of the ApPDE4 long form. L(N-UCR1–2)-mRFP, L(N)-mRFP, L(N20)-mRFP, and L(N16)-mRFP are localized to the plasma membrane 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/C3,15S)-mRFP, L(N20/C3,14,15S)-mRFP, and L(N20/SAAA)-mRFP are localized to the cytoplasm. *Scale bar* = 20 μm. *C*, the Golgi localization of L(N20/C14,15S)-mRFP and L(N20/C3,14S)-mRFP. GalT-EGFP was used as the Golgi marker. L(N20/C14,15S)-mRFP and L(N20/C3,14S)-mRFP are colocalized with GalT-EGFP. *D*, colocalization of L(N20/C14,15S)-mRFP or L(N20/C3,14S)-mRFP with human PDE4A1(N25)-EGFP (*top panel*) and their sequence alignments (*bottom panel*). *Scale bars* = 20 μm.

impaired for membrane targeting. These results suggest that, among the 20 amino acids of the N terminus, Arg^9/His^{10}/Trp^{11} and Cys^3/Cys^{14}/Cys^{15} 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*C*, 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. 2*D*). 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. 3*A*, S(N-UCR1)-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. 3*A*). These results indicate that UCR1, UCR2, and the domain between the 31st and 52nd amino acids within the NTR of the short form might be involved in plasma membrane targeting. Interestingly, the domain between the 31st and 52nd amino acids is highly positively charged: seven of 22 amino acids are basic (Lys/Arg).

Sequence analysis of the short form showed that, within the N-terminal-20-amino acids, eight hydrophobic residues are positioned between the lysine residues at positions 3 and 13 (MQ$\underline{K}^3$LNFLSPLFN$\underline{K}^{13}$NG). It is known that targeting to acidic membranes, including the plasma membrane, requires a hydrophobic domain and basic amino acids (25). Therefore, we generated N-terminal serial deletion mutants using S(N-UCR-1–2). An N-terminal four-amino acid deletion mutant of S(N-UCR1–2), S(ΔN4/N-UCR1–2)-mRFP, showed plasma membrane localization similar to S(N-UCR1–2)-mRFP (Fig. 3*A*). However, an N-terminal five-amino acid deletion mutant, S(ΔN5/N-UCR1–2)-mRFP, showed cytosolic and weaker plasma membrane localization. An N-terminal six-amino acid deletion mutant, S(ΔN6/N-UCR1–2)-mRFP, showed strictly cytosolic localization (Fig. 3*A*). These results suggest that the N-terminal hydrophobic region might be critically involved in membrane association of the ApPDE4 short form.

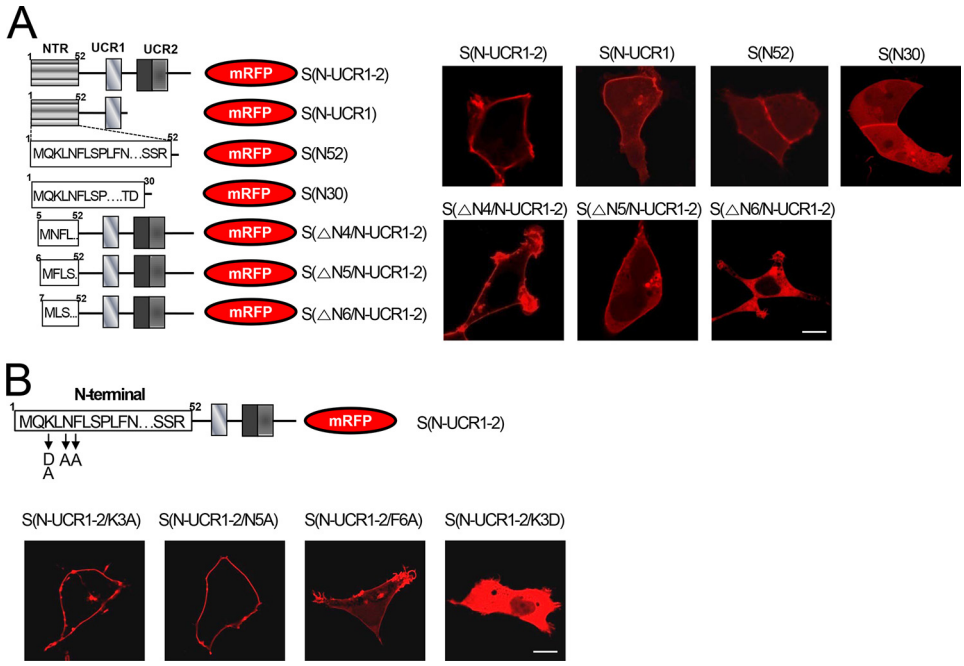

FIGURE 3. **Characterizations of the plasma membrane targeting domains of the ApPDE4 short form.** *A*, schematics (*left panel*) and cellular localization (*right panel*) of deletion mutants of the ApPDE4 short form. S(N-UCR1–2)-mRFP and S(Δ4/N-UCR-1–2)-mRFP are mainly localized to the plasma membrane. S(N-UCR1)-mRFP, S(N52)-mRFP, and S(Δ5/N-UCR1–2)-mRFP are localized to the cytosol and plasma membrane. On the other hand, S(N30)-mRFP and S(Δ6/N-UCR1–2)-mRFP are mainly localized to the cytosol. *Scale bar* = 20 μm. *B*, schematics (*left panel*) and cellular localization (*right panel*) of various point mutants of S(N-UCR1–2)-mRFP. S(N-UCR1–2/K3A)-mRFP and S(N-UCR1–2/N5A)-mRFP are localized to the plasma membrane much the same as S(N-UCR1–2)-mRFP, whereas S(N-UCR1–2/F6A)-mRFP shows more diffusible cytoplasmic localization, and S(N-UCR1–2/K3D) shows impairment of plasma membrane targeting. *Scale bar* = 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 Lys^3 to alanine (S(N-UCR1–2/K3A), Asn^5 to alanine (S(N-UCR1–2/N5A), and Phe^6 to alanine (S(N-UCR1–2/F6A), using S(N-UCR1–2)-mRFP. As shown in Fig. 3*B*, only S(N-UCR1–2/F6A)-mRFP showed increased cytoplasmic localization, not S(N-UCR1–2)-mRFP, indicating that Phe^6 is involved in plasma membrane association. We also replaced Lys^3 with aspartic acid in S(N-UCR1–2), yielding S(N-UCR1–2/K3D)-mRFP. Interestingly, this mutant was localized to the cytosol (Fig. 3*B*). 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, Phe^6 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. 1*A* and 4*A*), which are highly homologous to human PDE4 (Fig. 4*A*). 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. $4A_1$). The UCR2 domain of ApPDE4 short form is 59.5% identical with human PDE4D and 65.8% identical with human PDE4A (Fig. $4A_2$). 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 form and the long form 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. 4*B*, 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. 4*C*, 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. 4*A*, 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-

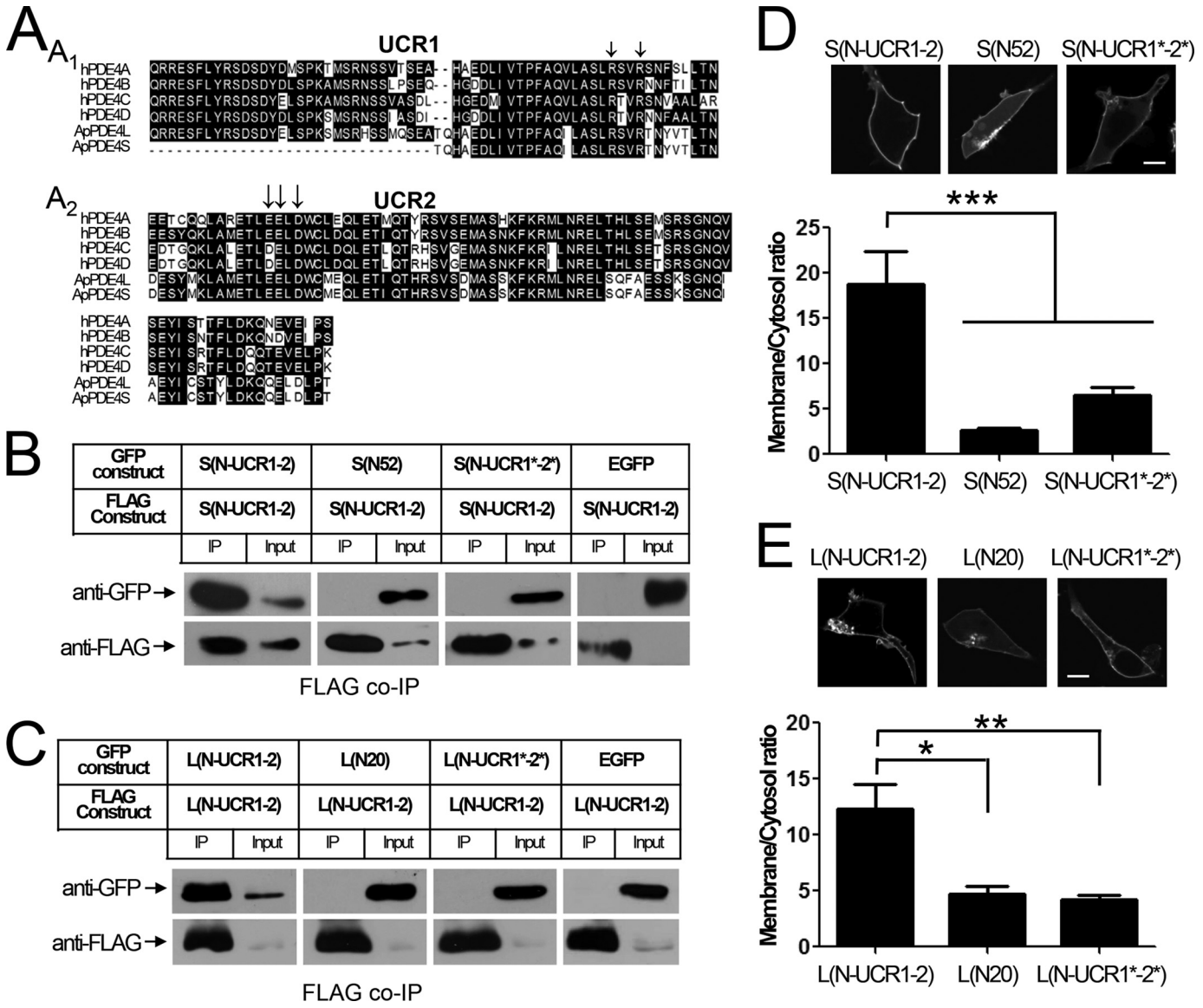

FIGURE 4. **Oligomerization of the ApPDE4 short and long forms.** *A*, multiple sequence alignment of UCR1 (A_1) and UCR2 (A_2) of various PDE4s, including human PDE4A-D, the ApPDE4 long form, and the ApPDE4 short form. *Arrows* indicate the residues required for the interaction between UCR1 and UCR2. 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 A_1 and A_2 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.

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. 4*D*, 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 = 23.39, Tukey's post hoc test). We also examined the cellular localization of the long form. As shown in Fig. 4*E*, 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).

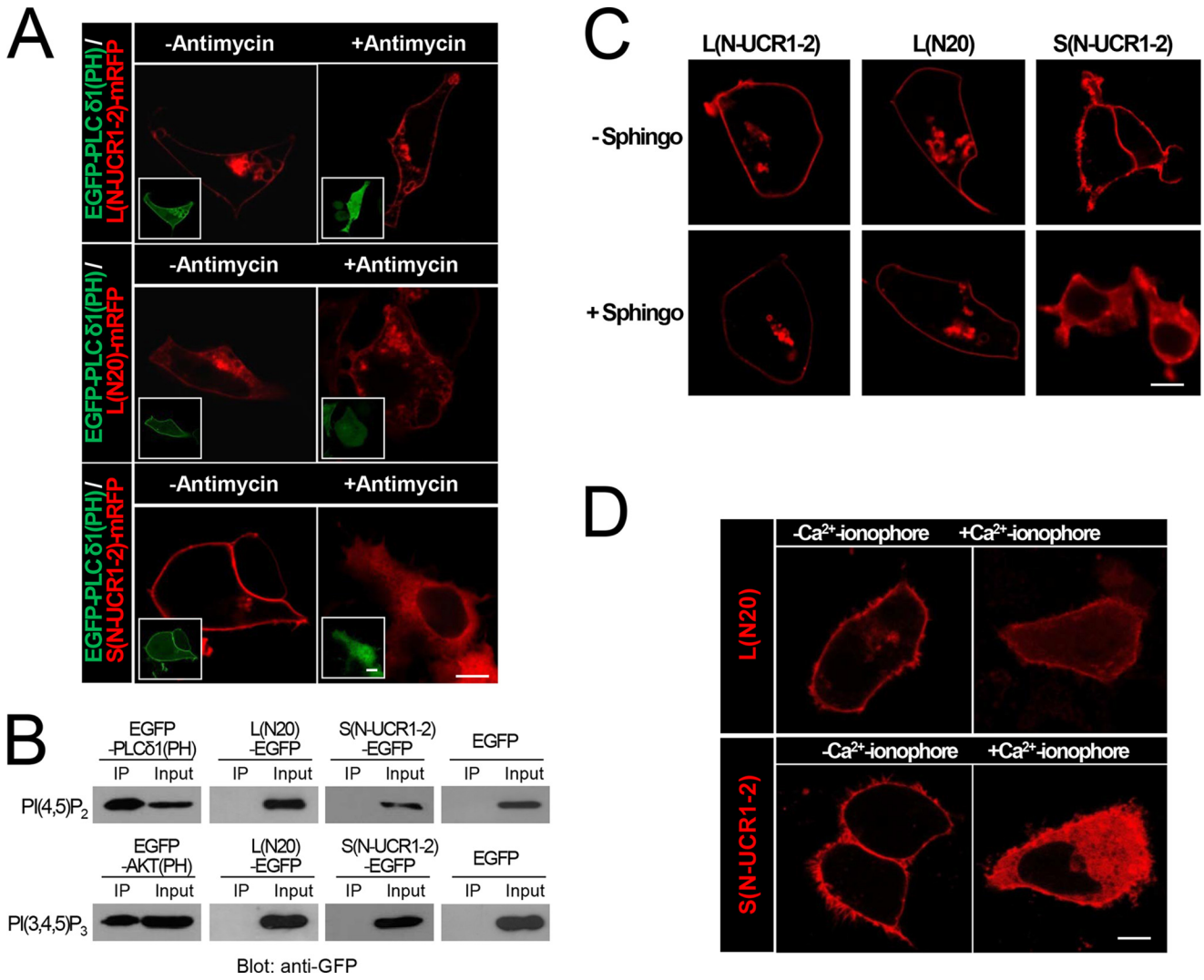

FIGURE 5. **Plasma membrane localization of the short form, but not the long form, is changed by decreasing the surface charges of the membrane.** *A*, effects of PI depletion by antimycin on plasma membrane localization of the long and short forms. L(N20)-mRFP, L(N-UCR1–2)-mRFP, or S(N-UCR1–2)-mRFP was coexpressed with PLCδ1(PH)-EGFP in HEK293T cells. Images were acquired before and after 10 μM antimycin treatment for 40 min. Membrane localization of S(N-UCR1–2)-mRFP, but not L(N20)-mRFP or L(N-UCR1–2)-mRFP, is changed from the membrane to the cytosol by antimycin A treatment. *Scale bars* = 20 μm. *B*, *in vitro* lipid-binding assay of the ApPDE4 long and short forms using lipid-coated beads. Total lysates of the HEK293T cells expressing L(N20)-EGFP or S(N-UCR1–2)-EGFP were incubated with $PI(4,5)P_2$- or $PI(3,4,5)P_3$-coated beads. As controls, EGFP-PLCδ1(PH) or EGFP-AKT(PH) were used. *IP*, immunoprecipitation. *C*, plasma membrane localization of the ApPDE4 short form, but not the long form, is 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 Ca^{2+} ionophore. The images were acquired before and after treatment with 10 μM Ca^{2+} ionophore. *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 $PI(4,5)P_2$ and $PI(3,4,5)P_3$, are located mainly in the plasma membrane, where they play important roles in the plasma membrane targeting of many proteins via direct interaction (24). First, to examine whether PIs are involved in the localization of ApPDE4s, we depleted cellular PI derivatives such as PI4P, $PI(4,5)P_2$, and $PI(3,4,5)P_3$ by incubation with antimycin, an ATP synthesis inhibitor. As a control, EGFP-PLCδ1(PH), which is known to bind $PI(4,5)P_2$ directly (40), was cotransfected. Similar to EGFP-PLCδ1(PH), the plasma membrane localization of S(N-UCR1–2)-mRFP was changed to cytoplasmic localization by antimycin treatment, whereas the plasma membrane targeting of L(N-UCR1–2)-mRFP and L(N20)-mRFP was not changed by antimycin treatment (Fig. 5*A*). These results suggest that PIs in the plasma membrane might be involved in the plasma membrane targeting of the ApPDE4 short form but not the long form.

To examine whether the ApPDE4 short or long form could bind directly to $PI(4,5)P_2$ and $PI(3,4,5)P_3$, we performed an *in vitro* lipid-binding assay using lipid-coated beads. EGFP-PLCδ1(PH) and EGFP-AKT(PH), which can bind directly to $PI(4,5)P_2$ and $PI(3,4,5)P_3$, respectively (40–42), were used as controls. As shown in Fig. 5*B*, the ApPDE4 short and long forms

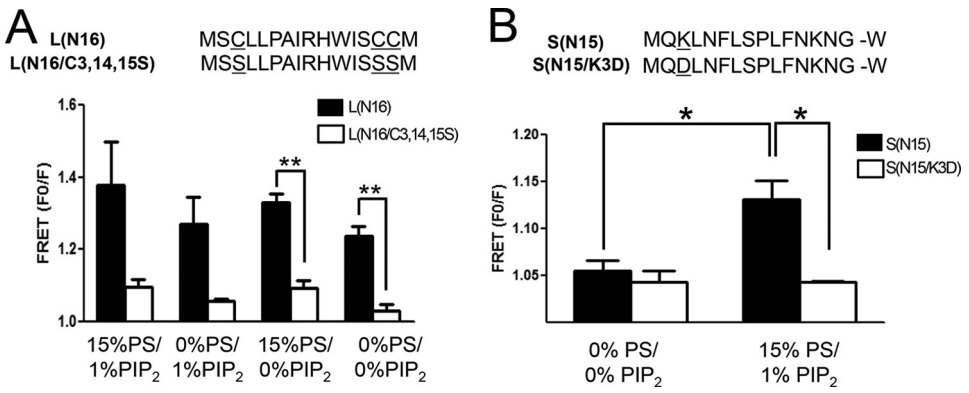

FIGURE 6. **Liposome binding properties of the ApPDE4 long and short forms.** *A*, liposome binding of ApPDE4 L(N16) and ApPDE4 L(N16/C3,14,15S) peptides. The L(N16) peptide, but not the L(N16/C3,14,15S) peptide, associates with various liposomes irrespective of lipid components. **, $p < 0.01$; two-tailed unpaired Student's *t* test. *B*, liposome binding of the S(N15) and S(N15(K3D)) peptides. The S(N15) peptide, but not the S(N15(K3D) peptide, associates with liposomes containing only 1% PIP_2 and 15% PS (*, $p < 0.05$; two-tailed unpaired Student's *t* test). Binding was tested using FRET between peptides that contain tryptophan and liposomes labeled with dansyl-PE as the donor and acceptor, respectively. Peptide binding to liposomes of different lipid compositions is revealed by quenching of tryptophan fluorescence by dansyl-PE. A control spectrum of tryptophan was determined in the absence of liposomes (F_0), and the subsequent test spectrum was recorded after liposome addition (*F*). FRET is expressed as F_0/F at 355 nm. Data are mean ± S.D. from three independent experiments.

did not interact directly with $PI(4,5)P_2$ and $PI(3,4,5)P_3$, whereas EGFP-PLCδ1(PH) and EGFP-AKT(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 cytoplasm (Fig. 5*C*), 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. 5*D*, 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. 5*D*). 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-bromopalmitate, a reversible palmitoylation inhibitor (8), and that L(N20/C3,14,15S)-EGFP was localized to the cytoplasm (Fig. 2*B*). 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 (MS*S*LLPAIRHWS*SS*M) 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. 6*A*). 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. 6*B*). 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. 6*B*). 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.

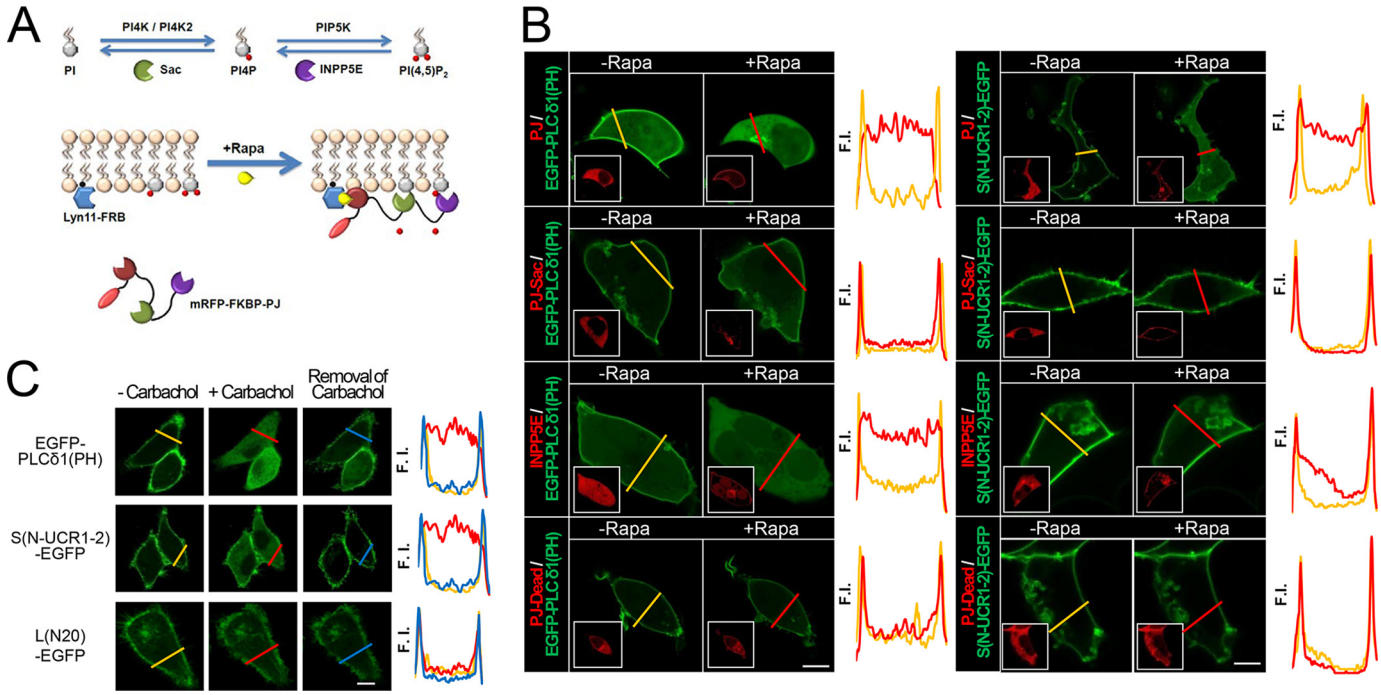

FIGURE 7. **Localization of the short form, but not the long form, is changed by the depletion of PIs on the plasma membrane by the PJ system.** *A*, schematic of experimental models of the Lyn11-FRB/PJ system (modified from Ref. 26). Yeast Sac1 dephosphorylates PI4P, and INPP5E converts $PI(4,5)P_2$ 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 (*F.I.*) 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 $PI(4,5)P_2$ 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.

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. 7*A*). 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. 7*A*). PJ, which contains active yeast Sac1, which dephosphorylates PI4P, and active polyphosphate 5-phosphatase E (INPP5E), which converts $PI(4,5)P_2$ to PI4P, can deplete PI4P and $PI(4,5)P_2$ (Fig. 7*A*). Controls were PJ-Sac, which contains active Sac1 and inactive INPP5E; INPP5E, containing inactive Sac1 and active INPP5E; and PJ-Dead, containing inactive Sac1 and inactive INPP5E (26). The localization of EGFP-PLCδ1(PH) was shifted from the plasma membrane to the cytosol by rapamycin treatment in cells expressing PJ or INPP5E but not PJ-Sac or PJ-Dead (Fig. 7*B*, *left panel*), indicating that EGFP-PLCδ1(PH) was localized to the plasma membrane via direct $PI(4,5)P_2$ binding. As shown in Fig. 7*B* (*right panel*), rapamycin switched the localization of S(N-UCR1–2)-EGFP from the plasma membrane to the cytosol in cells expressing PJ but not INPP5E, PJ-Sac, or PJ-Dead, indicating that PI4P as well as $PI(4,5)P_2$ were involved independently in the plasma membrane targeting of the ApPDE4 short form. Overall, these results indicate that the targeting mechanism of the ApPDE4 short form to the plasma membrane might be determined by nonspecific electrostatic interactions through PI4P and $PI(4,5)P_2$ but not by specific lipid binding.

Next, we investigated whether the activation of the PLC pathway could regulate the localization of the ApPDE4 short form. To do this, we stimulated the M1 muscarinic receptor with carbachol to transiently activate the PLC pathway, by which $PI(4,5)P_2$ on the plasma membrane is degraded to inositol-1,4,5-trisphosphate and diacylglycerol. L(N20)-EGFP, S(N-UCR1–2)-EGFP or EGFP-PLCδ1(PH) was transfected into HeLa cells. Localization of S(N-UCR1–2)-EGFP, but not L(N20)-EGFP, was partially shifted from the membrane to the cytoplasm after carbachol treatment, whereas the localization of EGFP-PLCδ1(PH) was fully shifted from the plasma membrane to the cytosol by this treatment (Fig. 7*C*). Subsequent removal of carbachol relocated S(N-UCR1–2)-EGFP and EGFP-PLCδ1(PH) from the cytosol back to the plasma membrane. These results show clearly that the cellular localization of S(N-UCR1–2)-EGFP, which degrades cAMP, can be changed from the plasma membrane to the cytosol by activation of the PLC pathway.

Applications of the N Terminus of ApPDE4 Long Form as New Targeting Peptides—In the PJ system, the 11 N-terminal amino acids of Lyn kinase (MGCIKSKGKDS, Lyn11), which has myristoylation and palmitoylation sites and basic amino acids, was used to target FRB to the plasma membrane. A subsequent

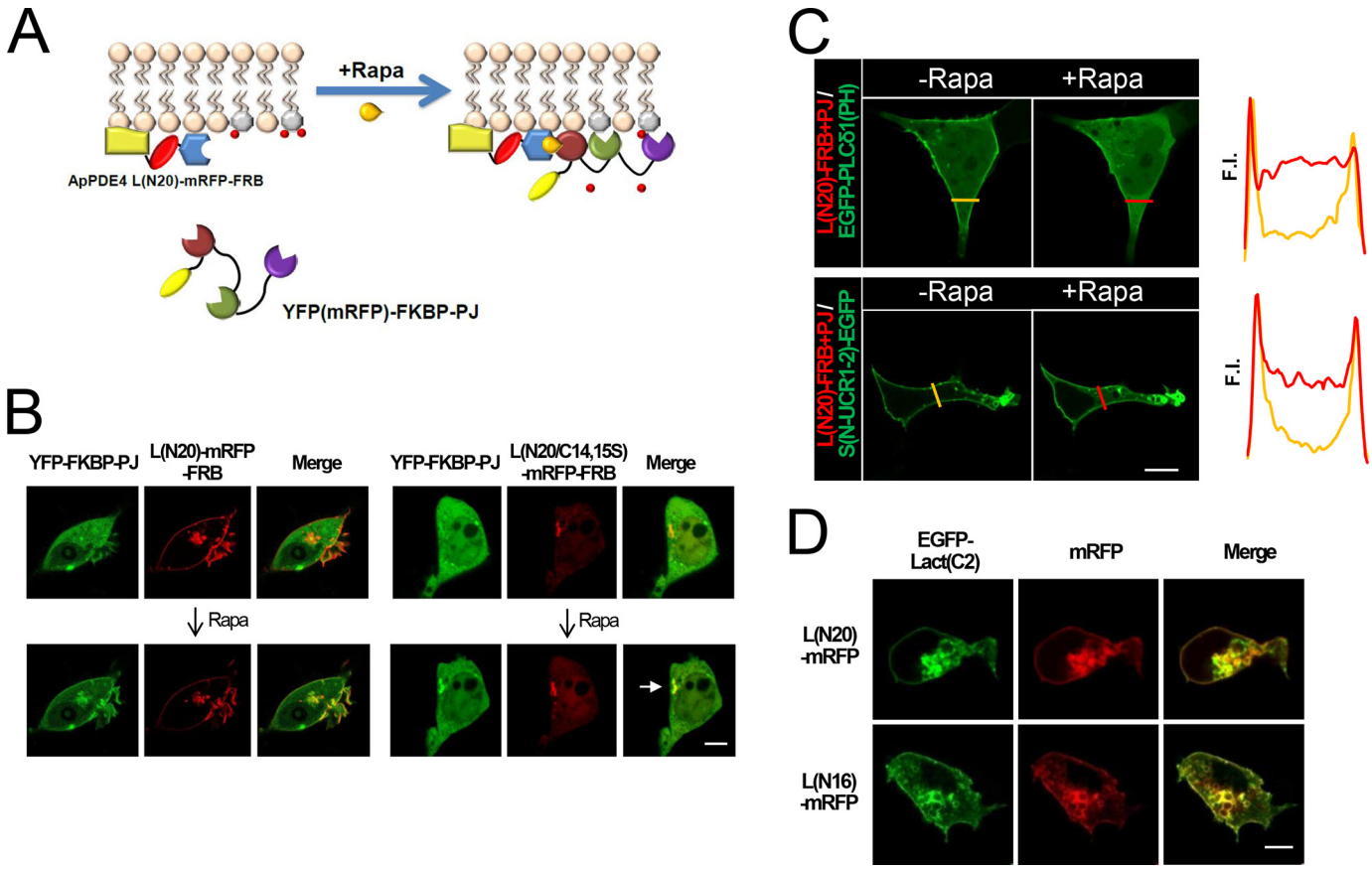

FIGURE 8. **Application of the N-terminal 20 amino acids of the ApPDE4 long form as a new target mediator.** *A*, schematic of the L(N20)-mRFP-FRB/YFP(mRFP)-FKBP-PJ system. *Rapa*, rapamycin. *B*, the localization shift of YFP-FKBP-PJ before (*top*) and after (*bottom*) rapamycin treatment. L(N20)-mRFP-FRB (*top*) or the L(N20/C14,15S)-mRFP-FRB was coexpressed with YFP-FKBP-PJ in HEK293T cells. The *arrow* indicates colocalization between YFP-FKBP-PJ and L(N20)-mRFP-FRB. *Scale bar* = 20 μm. *C*, effects of rapamycin treatment on the localization of EGFP-PLCδ1(PH) or S(N-UCR1–2)-EGFP in cells expressing L(N20)-mRFP-FRB/mRFP-FKBP-PJ. The *colored lines* in the confocal fluorescence images indicate the paths along which the fluorescence intensities (*F.I.*) of the corresponding images were plotted on the *right*. *Scale bar* = 20 μm. *D*, localization of L(N20)-mRFP or L(N16)-mRFP in PS-enriched membranes. Colocalization of L(N20)-mRFP or L(N16)-mRFP with EGFP-Lact-C2, a PS-binding probe, in HEK293T cells is shown. *Scale bar* = 20 μm.

recruitment of YFP-FKBP-PJ to Lyn11-FRB in the plasma membrane depleted PI4P and PI(4,5)P_2 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. 8*B*). As shown in Fig. 8*B*, *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 YFP-FKBP-PJ 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 inner leaflet of the plasma membrane and the cytoplasmic surfaces of intracellular membrane organelles. Therefore, rapamycin treatment could relocate YFP-FKBP-PJ to the plasma membranes, where L(N20)-mRFP-FRB was localized.

Therefore, we asked whether YFP-FKBP-PJ recruited by L(N20)-mRFP-FRB could deplete PIs in the plasma membrane. To do this, L(N20)-mRFP-FRB was coexpressed with mRFP-FKBP-PJ + EGFP-PLCδ1(PH) or with mRFP-FKBP-PJ + S(N-UCR1–2)-EGFP in HEK293T cells. As with Lyn11-FRB, rapamycin treatment could shift the localization of either PLCδ1(PH)-EGFP or S(N-UCR1–2)-EGFP from the plasma membrane to the cytosol (Fig. 8*C*). 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 (Fig. 8*D*), which was used as a fluorescent biosensor of PS. These results also suggest that L(N20)-mRFP might be located close to the clusters of acidic lipids, such as PS, PI4P, and PI(4,5)P_2, in the plasma membrane.

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

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 $PI(4,5)P_2$ (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 PI4P, $PI(4,5)P_2$, and $PI(3,4,5)P_3$, 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 PI4P and $PI(4,5)P_2$. Similarly, many proteins having basic amino acids are targeted to the plasma membrane by electrostatic interactions. For example, PI4P 5-kinase isoforms that have conserved 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×FLAG, was more localized to the cytosol compared with S(N-UCR1–2)-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. 1*A* and 3*A*). Therefore, unlike the mammalian short form, the ApPDE4 short form could form an oligomer, leading to further enhancement the plasma membrane localization (Fig. 4*D*).

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 PDE3 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). HEKPDE4A1, 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 phosphatidic acid-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. 2*A*). 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. 2*D*). 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. 2*A*). 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 (Leu^{16}-Val^{17}-Trp^{19}-Trp^{20}) that is stabilized by Pro^{14}-Trp^{15}, indicating that the sequence PWLVGWW in helix 2 is the key unit for membrane association. Considering that the localization pattern of ApPDE4 L(N20/AA)-mRFP was similar to that of the wild type (Fig. 2*B*), the sequence PA*LXX*WI (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^{24}-Arg^{25} 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. 6*A*) and that the depletion of PIs using antimycin and the neutralization of the plasma membrane by sphingosine had no effect on long form localization (Fig. 5*B*), 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-Ras-C*AAX*) is targeted to the plasma membrane mainly through hydrophobic interactions. Therefore, depletion of plasma membrane PI, by the PJ 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. 6*A*), L(N16)-mRFP or L(N20)-mRFP was colocalized with PS-enriched membrane in HEK293T cells (Fig. 8*D*). 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^9) next to a mildly positively charged amino acid (His^{10}) 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 PI4P and $PI(4,5)P_2$ from the plasma membrane (Fig. 8*C*). These results suggest that L(N20)-mRFP-FRB was localized near PI4P- and $PI(4,5)P_2$-enriched membranes. $PI(4,5)P_2$ is a minor lipid within cells, constituting about 1% of the plasma membrane. It is believed that PIP_2 is sequestered and clustered within the plasma membrane. Therefore, we can conclude that L(N20)-mRFP-FRB is targeted to acidic, membrane-enriched regions.

PI4P and PI(4,5)P_2 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, PI4P, $PI(4,5)P_2$, and $PI(3,4,5)P_3$, 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 $PI(4,5)P_2$ 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 $PI(4,5)P_2$ binding. However, recent papers have shown that PI4P as well as $PI(4,5)P_2$ 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 PI4P and $PI(4,5)P_2$ because the localization of the short form was shifted significantly from the plasma membrane to the cytosol by PJ but not by either INPP5E or PJ-Sac recruitment to the plasma membrane by rapamycin in HEK293T cells (Fig. 7*B*). Therefore, we can conclude that PI4P and $PI(4,5)P_2$ 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. 8*B*, *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. 3*A*, the N-terminal 30 amino acid segment of the ApPDE4 short 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.

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