TAPAS-1, a Novel Microdomain within the Unique N-terminal Region of the PDE4A1 cAMP-specific Phosphodiesterase That Allows Rapid, Ca\(^{2+}\)-triggered Membrane Association with Selectivity for Interaction with Phosphatidic Acid*

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Here we identify an 11-residue helical module in the unique N-terminal region of the cyclic AMP-specific phosphodiesterase PDE4A1 that determines association with phospholipid bilayers and shows a profound selectivity for interaction with phosphatidic acid (PA). This module contains a core bilayer insertion unit that is formed by two tryptophan residues, Trp19 and Trp20, whose orientation is optimized for bilayer insertion by the Leu16:Val17 pairing. Ca\(^{2+}\), at submicromolar levels, interacts with Asp21 in this module and serves to gate bilayer insertion, which is completed within 10 ms. Selectivity for interaction with PA is suggested to be achieved primarily through the formation of a charge network of the form (Asp21:-Ca\(^{2+}\):PA2:-Lys24*) with overall neutrality at the bilayer surface. This novel phospholipid-binding domain, which we call TAPAS-1 (tryptophan anchoring phosphatidic acid selective-binding domain 1), is here identified as being responsible for membrane association of the PDE4A1 cAMP-specific phosphodiesterase. TAPAS-1 may not only serve as a paradigm for other PA-binding domains but also aid in detecting related phospholipid-binding domains and in generating simple chimeras for conferring membrane association and intracellular targeting on defined proteins.

It is becoming increasingly recognized that the compartmentalization of proteins within specific regions of the cell is pivotal to the appropriate functioning of many, if not all, signaling pathways. For example, the localization of the RII subunits of PKA\(^1\) to distinct intracellular sites, achieved by binding to A-Kinase-Anchor Proteins (AKAPs), allows for the localized activation of this protein kinase and functionally compartmentalized signaling (1). Activation of signaling processes themselves can lead to changes in the organization of intracellular proteins. This can take the form of protein-protein interactions, of which the SH2 and SH3 modules provide mechanistic and structural examples (2, 3). However, more recently, lipid-directed re-organization of protein components within cells has been recognized, such as that seen in the binding of phosphoinositides to pleckstrin homology (PH) and FYVE domain modules, as well as the interaction of C2 domains with phospholipids (4, 5). To appreciate the ways in which various signaling processes are connected in the cell, it is important to appreciate the range of protein modules that allow for interaction with intracellular anchors.

Stimulation of many cells with a plethora of growth factors, cytokines, and hormones activates phospholipase D (PLD). This enzyme catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) which is presumed to mediate downstream signaling effects such as secretion, vesicle trafficking, cytoskeletal reorganization, apoptosis, and mitogenesis (6–8). PA has been reported to bind to and activate Raf-1, and a neutrophil PA-regulated protein kinase has been described (9, 10). In addition, PA has been shown to activate “long form” cAMP phosphodiesterases (11–14). However, how PA specifically interacts with signaling proteins is not yet clear. In particular, no module akin to either the pleckstrin homology or the FYVE domains involved in selectively binding 3-phosphorylated phosphoinositides or the C2 domains that are able to bind

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1 The abbreviations used are: PKA, protein kinase A; SH, Src homology domain; PLD, phospholipase D; PA, phosphatidic acid; PC, phosphatidylcholine; TRITC, tetramethylrhodamine isothiocyanate; bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxyethyl)propane-1,3-diol; MES, 4-morpholinethanesulfonic acid; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; GFP, green fluorescent protein; NT, N-terminal; DOTAP, N1,N1-trimethylammonium methyl sulfate.

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certain phospholipid molecules (5, 15) has been recognized. cAMP phosphodiesterases provide the sole means of degrading cAMP in cells and are thus poised to regulate the cAMP signaling system (16–19). There is currently great interest in PDE4 cAMP phosphodiesterases (17, 20), as selective inhibitors for these enzymes appear to have a potential therapeutic benefit in a number of major disease areas, such as asthma and chronic obstructive pulmonary disease (20–23). In addition to this, disruption of the gene for the cognate family in Drosophila melanogaster causes memory and learning defects (24). Four genes, each of which encode multiple isoforms, provide a complex family of PDE4 enzymes (17, 20). Each isoform is characterized by a unique N-terminal region that is believed to be involved with intracellular targeting and complex formation. Thus the N-terminal regions of the PDE4A4/5 and PDE4D4 isoforms confer interaction with the SH3 domains of the Src family tyrosyl kinases (25, 26), that PDE4D5 interacts with the signaling scaffold protein RACK1 (27), and that PDE4D3 interacts with the PKA anchor proteins, AKAP-450 (28) and m-AKAP (29) as well as the Golgi/centrosomal protein, myo-megalin (30). However, the PDE4A4D1 isoform is unique in that its specific N-terminal region, which is encoded by a single exon (31), makes it exclusively membrane-associated (32). Here we identify a novel helical microdomain, called TAPAS-1 that is located within the N-terminal membrane-anchoring region of PDE4A1 (32–35). TAPAS1 consists of a bilayer insertion module that shows selectivity for interaction with PA and whose interaction with lipid bilayers is gated by Ca2+ binding to a single aspartate residue.

**EXPERIMENTAL PROCEDURES**

### Total Lipid Analysis

**Lipid Extraction**—500 ng of 12/0:12/0 phosphatidic acid (sodium salt) was added to each sample as internal standard, followed by 1.5 ml of methanol. This was transferred to a glass tube-capped tube, then 3 ml of chloroform was added, mixed, and left to stand for 10 min. 1.5 ml of 0.88% KCl in 0.1 M HCl was added, mixed, and left to split into two phases. The upper aqueous phase was discarded, whereas the lower phase was dried under a stream of nitrogen, resuspended in 15 μl methanol, and used for lipid analysis.

**LC-MS Analysis**—Total lipid extracts in chloroform/methanol (2:1, v/v) were separated and characterized by LC-MS (QP8000alpa, Shimadzu) using 1-μl injection volumes onto a Luna silica column (3 μm, 1.0 × 150 mm; Phenomenex) with a solvent gradient of 100% chloroform/methanol/water/ammonia solution (90:9.5:0.5:0.32, by volume) changing to 100% chloroform/methanol/water/ammonia solution (50:48:2.0:32, by volume) over 40 min at 0.1 ml/min. Detection (nitrogen flow, 41/min; curved desolvation line temperature, 300 °C; probe voltage, ±45 kV) in negative electrospray ionization (ESI) mode allowed characterization of phosphatidylbutanol, phosphatidic acid, phosphatidylcholine, and sphingomyelin. This has been described in detail previously (36).

### Preparation of Lipid Vesicles

Phospholipids (Avanti) were dissolved in chloroform/methanol (2:1) and dried in a glass tube under oxygen-free nitrogen gas. Lipids were then resuspended by repeat pipetting in 1 ml of vesicle buffer (12.5 mM HEPES, pH 7.4, 20 mM KCl, 200 mM sucrose, 2 mM MgCl2, 2 mM CaCl2) and snap frozen in liquid nitrogen. Lipid suspensions were thawed in a 40 °C water bath, resuspended once again, and snap frozen. This procedure was repeated 10 times to produce multilamellar liposomes that were reduced in size by extrusion through a Lipofast extruder (Avestin, USA) containing a polycarbonate filter with defined pores of 100 nm (37). Liposomes were stored at 4 °C and used within 48 h of manufacture. Total lipid analysis of the extruded liposomes showed that they had the same composition as the mixtures used to generate them (±3% difference).
chloroquine. 10 μg of DNA was diluted to 250 μl with TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.6) and 200 μl of 10 mg/ml DEAE dextran was then added. The mixture was incubated at room temperature for 15 min before addition to the culture medium. Cells were incubated at 37 °C with gentle shaking before the medium was aspirated and the cells were shocked for 2 min with 10% dimethylsulfoxide in a phosphate-buffered saline solution. The culture was then rinsed twice in phosphate-buffered saline solution before Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum was added, and the cells were incubated at 37 °C in a 5% CO2 atmosphere for 72 h. Disruption of cells and the isolation of particulate and high speed supernatant fractions is described above based upon our previous studies (25, 42). In other instances cells were used for confocal analyses (25, 39, 42) as described below.

**Constructs**

Site-directed mutagenesis was performed using a QuickChange DNA mutagenesis kit (Stratagene) according to the manufacturer’s instructions. All mutagenesis and deletion constructs were confirmed by DNA sequencing.

**Full-length PDE4A1—Full-length PDE4A1 (GenBank accession number M26715/43) was used. This was cloned into the pcDNA3 vector for COS1 cell expression studies, either with or without a HA epitope tag at its extreme C terminus.**

**NT-4A1-CAT Constructs**—We have described previously in some detail (33) a modified version of the pBlCAT2 plasmid containing the CAT gene of the T7 virus plasmid, which encodes the protein comprising the first 25 amino acids of PDE4A1. This allows for the generation of a chimeric form of CAT that has the first 25 amino acids of PDE4A1 at its N terminus. The Sp6 promoter in this plasmid was used to drive the coupled transcription translation reaction.

**NT-4A1-GFP—**The N-terminal first 25 amino acids of PDE4A1 (RD1) were cloned as an in-frame fusion with the GFP gene in the vector pEGFP-N1, via the BamHI/EcoRI sites. The methionine at the start of EGFP was then mutated to alanine. This was done to prevent “false” initiation, at this point because of a preceding weak Kozak sequence, as occurred with the NT-4A1-CAT constructs (33, 34, 44). This modified construct was then used as the template for all GFP mutations and deletions in NT-4A1-GFP.

**NT-4A1-GST—**The N-terminal first 25 amino acids of PDE4A1 (RD1), which had been generated by PCR, were cloned as an in-frame fusion with GST in pGEX-3X-1, via the BamHI/EcoRI sites. This construct was then used as the template for generating all the NT-4A1-GST mutations. ARF1 as an HA epitope-tagged form was a kind gift from Dr. R. Lefkowitz (Duke University, Durham, NC).

**Purification of GST Fusion Proteins**

Purification of GST or MBP fusion proteins was carried out as described in Ref. 45. Briefly, frozen aliquots of bacteria expressing GST fusion proteins were thawed at room temperature and then held on ice and sonicated for 100 s in 20-s pulses, separated by 20-s intervals. Bacterial debris was then pelleted by centrifugation for 30 min at 9,000 × g in a refrigerated centrifuge and the supernatant was transferred to a fresh tube. Glutathione-Sepharose beads were equilibrated by washing twice with 20 bed volumes of ice-cold phosphate-buffered saline and once with 10 bed volumes of complete phosphate-buffered saline (containing 1 mM dithiothreitol and protease inhibitor mixture). The equilibrated beads were added to the sonicated bacterial supernatant (200 μl bed volume of Sepharose beads per 4 ml of supernatant) and incubated end over end at 4 °C for 2 h. Following incubation, the beads were collected by centrifugation for 5 min at 2900 rpm in a Jouan C2 “swing-out” centrifuge and the supernatant was discarded. The beads were washed 4 times with 10 bed volumes of ice-cold complete phosphate-buffered saline per wash. Each wash step was carried out end over end for 30 min at 4 °C. Incubating the beads with 1 bed volume of elution buffer 3 times at 4 °C for 15 min allowed for fusion protein elution. This procedure routinely generated a homogeneous preparation (>96% purity on SDS-PAGE). However, in some instances the above extract was subjected to a further purification by ion-exchange chromatography using a Mono Q column on a Bio-Rad Biologic system using a gradient of NaCl to elute.

**Membrane Binding Assay using GST Fusion Proteins**

In a total volume of 100 μl a P2 pellet (1 mg/ml) fraction in complete KHEM buffer was mixed with the appropriate GST fusion protein or GST itself (50 μM/ml). The free Ca2+ was in the range 26–30 μM. After incubation at 4 °C for 30 min they were then centrifuged at 100,000 × g for 30 min. The pellets were washed three times with 200 μl of complete, ice-cold KHEM buffer before analysis. This was done by SDS-PAGE followed by transfer onto nitrocellulose membranes and visualization using an anti-GST polyclonal antibody.

**Thermal Denaturation Experiments**

This was done essentially as described before (46), but here with samples (50 μl) of COS1 cell membranes (P2 fraction; 25 μg/ml) in KHEM buffer (with protease inhibitor mixture) and incubated at the indicated temperature for the indicated times before being removed to ice for rapid cooling prior to use in binding assay. For protease sensitivity experiments, P2 membranes (20 μl; 27 μg/ml) in buffer without protease inhibitors, were incubated for 30 min at 30 °C with 10 μl of protease (0.5 mg/ml; chymotrypsin, V8, or trypsin). Membranes were then harvested by centrifugation, as above, before being resuspended in 200 μl of KHEM buffer containing complete inhibitor mixture together with added phenylmethylsulfonyl fluoride (10 mM), 3,4-dichlorocumarin (10 mM), Pefabloc SC (10 mM), and soybean trypsin inhibitor (1 mg/ml). They were then subjected to a further round of harvesting before final resuspension in 200 μl of KHEM buffer, containing the above listed protease inhibitors, and used in the binding assay described above.

**Fluorescence Measurements**

Time-resolved fluorescence measurements were carried out in an Applied Photophysics (London, UK) SX.18MV stopped-flow instrument, operated at 20 °C, essentially as we have described for other proteins (47). For measurements of the change in tryptophan fluorescence, the samples were excited at 285 nm, selected with a cut-off filter. Invariably, equal volumes of the reactants were mixed together in the stopped-flow instrument, using two syringes of equal volume. The final concentration of the 25-mer peptide reflecting the N-terminal region of PDE4A1 was 5 μM and that of the total vesicle lipid was 0.12 μM, all in vesicle buffer. Stopped-flow traces were analyzed by non-linear regression fitting to a single exponential.

**Confocal Microscopy**

This was done as before (25). Briefly, cells were seeded at about 40% confluence, onto 22-mm diameter coverslips, 24 h prior to transfection. For single transfections, 2 μg of DNA was added to each coverslip and transfection was achieved using a DOTAP liposomal transfection reagent for 16 h (Roche Molecular Biochemicals). Where two constructs were to be co-transfected then 4 μg of total DNA was used and the DOTAP mixture altered accordingly. Expression was allowed to progress for a further 32 h and then cells were examined using a Zeiss laser scanning microscope and analysis was carried out using the Improvisation Open Lab system as described before (40). Where Mitotracker (Molecular Probes) was used, this was added to cells at a concentration of 10 nM 5 min before the medium was added. When fixed cells were used, they were permeabilized with 3 changes of 0.2% Triton in TBS for 15 min and, following four 5-min blocking incubations with 20% goat serum and 4% bovine serum albumin, were labeled for 2 h with polyclonal antibodies raised against specific peptide sequences of the C-terminal region of PDE4A1 as described before by us (32, 40). Labeling was detected using an Alexa 488 or an Alexa 594 (Molecular Probes, Eugene, OR) conjugated goat anti-rabbit IgG for 1 h. Co-staining of cells was achieved using a monoclonal mouse anti-HA antibody (Transduction Laboratories, Lexington, KY) at a dilution of 1:100. Localization of proteins was visualized using the complementary fluorescein isothiocyanate- or TRITC-conjugated goat anti-mouse IgG to the polyclonal staining. All incubations were carried out at room temperature. Cells were visualized using a laser-scanning confocal microscope using 0.1× objective. 10 μm tissue sections with a X56/1.4NA plan apochromat lens, as described before by us (40, 42).

**Modeling**

Possible interactions between helix-2 of PDE4A1 and a phospholipid bilayer were modeled with molecular graphics using the program QUANTA (Molecular Simulations Inc.). Modeling incorporated both the irregular helix of the published NMR structure (Protein Data Bank accession code 1JUX) and the putative polyethylene glycol side chains in the solution structure in the absence of membrane insertion may lead to overhanging of the helix. Polyethylene backbone frameworks other than helical have not been considered in the modeling. Application of helical geometry, supported by NMR (34), and placement of the helix axis along a model membrane surface are the key overall constraints. A cluster of non-polar residues follows from this basis, and
when the helical template is oriented to allow for membrane insertion of these residues, a putative charge cluster involving Asp$^{21}$, calcium ion, and membrane phosphate can be constructed, following established geometry for such a system (48). It is reasonable to model the helix axis along the membrane surface because it maximizes potential interactions with the helix-2 segment, whereas allowing neighboring protein regions to be located away from the membrane, consistent with their lack of influence on membrane association. At a general level, our model depends on helical polypeptide geometry, which is reasonable from the available data. Membrane influence that causes a gross deviation from the conformation of helix-2 is not included in the current modeling. At the detailed level of atomic coordinates, the modeling is not precise. Rather it seeks to establish general principles that can be tested, such as non-polar side chain clustering and charge proximity and neutralization in the polar cluster. Phospholipid coordinates were derived from the crystal structure and crystal packing of 1,2-dilauroyl-DL-phosphatidylethanolamine (49) with reduction of the head group to mimic that of PA.

RESULTS

NMR studies (34) have shown that the unique 25-residue N-terminal region of PDE4A1 consists of two distinct helical domains that are separated by a mobile hinge (Fig. 1). Of these, it is helix-2 that confers association of PDE4A1 with membranes (34), although the molecular basis for this interaction is unknown. We originally postulated (34) that PDE4A1 might associate with membranes by interaction with membrane proteins rather than by inserting directly into lipid bilayers. This was because for a peptide to span a bilayer then about 6 turns of an α-helix containing predominantly hydrophobic amino acids are required. Clearly, helix-2, the membrane-association region of PDE4A1 is far too small to achieve this. Furthermore, it was considered difficult to imagine how helix-2 could insert into bilayers when it contained the charged Asp$^{21}$, Lys$^{24}$, and Arg$^{25}$ residues. However, here we identify the apparent thermostability of the binding of a chimera formed from the N-terminal first 25 amino acids of PDE4A1 with CAT (NT-4A1-CAT; Fig. 3a) (data not shown). In marked contrast to this, expressing the N-terminal portion of PDE4A5, which has a role in membrane targeting (40, 50, 51), as a fusion protein with GST (NT-4A5-GST; Fig. 2b), generated a species that bound to membranes in a highly thermolabile fashion (Fig. 2e). We consider it unlikely that PDE4A1 binds to a membrane protein that is highly thermostable as we were unable to ablate binding of either NT-4A1-GST or NT-4A1-CAT by pretreating membranes with either trypsin or chymotrypsin, under conditions where severe membrane protein degradation had occurred as assessed by SDS-PAGE and staining with Coomassie Blue (data not shown). These various data led us to explore the notion that PDE4A1 might associate with membranes by interacting with phospholipid bilayers.

The N-terminal Region of PDE4A1 Binds to Phospholipid Vesicles and Shows Selectivity for Phosphatidic Acid—TNT transcription-translation reactions were used to generate [35S]methionine-labeled chimera formed from the 25-amino acid N-terminal region of PDE4A1 (NT-4A1), fused to the normally soluble, bacterial protein, CAT (33, 34). Lack of fidelity in this in vitro reaction system causes CAT to be generated as well as the NT-4A1-CAT chimera because of the presence of a suboptimal Kozak sequence (33, 44). As before (33, 44), we exploited this as an internal control, where membranes are provided with the opportunity of interacting with both CAT and the NT-4A1-CAT chimera present together in the binding assay. As before (33, 34), when COS1 cell membranes were incubated with the CAT and NT-4A1-CAT mixture, then only NT-4A1-CAT associated with membranes (Fig. 3c). However, we see here (Fig. 3b) that NT-4A1-CAT, but not CAT, was able to interact with phosphatidylcholine (PC) vesicles. Fascinatingly, a profound increase in the binding of NT-4A1-CAT to the lipid vesicles was seen (Fig. 3, b and c) upon the incorporation of low levels of phosphatidic acid (PA) into the PC vesicles, whereas keeping the total lipid concentration constant. Indeed, PA dose dependently increased binding of NT-4A1-CAT to phospholipid vesicles (Fig. 3d), with half-maximal binding at 0.9 ± 0.1 mol % PA (mean ± S.D.; n = 3). This compares favorably with the half-maximal binding of 5–8 mol % reported for PA binding to Raf (9).
Fig. 2. Membrane binding of a fusion protein formed from GST and the unique N-terminal region of PDE4A1. Panel a shows a Coomassie Blue (protein)-stained SDS-PAGE of purified GST and a purified fusion protein formed between GST (26.5 kDa) and the first 25-amino acid N-terminal region of PDE4A1 (NT-4A1-GST) (29.6 kDa). Panel b shows a Coomassie Blue (protein)-stained SDS-PAGE of purified GST and a purified fusion protein formed between GST (26.5 kDa) and the N-terminal first 256 amino acids of PDE4A5 (NT-4A5-GST) (56 kDa). Panel c shows the binding of purified NT-4A1-GST, but not GST, to a P2 membrane fraction from COS7/1 cells. After incubation of a mixture (M) of the fusion protein and membranes, the membrane pellet fraction (P) and soluble, supernatant (S) fraction were analyzed by SDS-PAGE with immunoblotting using a polyclonal antiserum specific for GST. In panel d membranes were incubated for the indicated times, at either 50 or 70 °C, prior to being cooled on ice. Membranes were then assayed for their ability to bind NT-4A1-GST and this was expressed as log % of binding to the non-heat-treated control. In panel e the binding of NT-4A5-GST was assessed after pretreating membranes at 50 °C. These various binding experiments were done in complete KHEM, with free Ca\(^{2+}\) in the 27–30 μM range. Experiments shown were either representative of ones done at least three times (a, b, and c) or means with errors (S.D.) of three different experiments (d and e).

Vesicle binding of NT-4A1-CAT equates to a half-maximal value of 900 ± 100 nm PA, which is similar to a value of 390 ± 85 nm PA seen for binding to COS1 cell membranes (mean ± S.D.; n = 3 separate preparations). This latter value is based upon COS1 cell P2 membranes containing 2.6 ± 0.3 nmol of PA/mg of membrane protein (mean ± S.D.; n = 3 separate preparations). Note then that our standard membrane binding assay contains membrane PA at a concentration of around 780 nM.

There was no increase in NT-4A1-CAT binding upon incorporating other acidic phospholipids, such as phosphatidylserine, phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate into the PC vesicles (Fig. 3d). Neither was there any increase in binding seen with added phosphatidylethanolamine (Fig. 3d).

In an independent strategy the NT-4A1-GST chimera (Fig. 2a) was used to probe for binding to immobilized phospholipid vesicles in surface plasmon resonance analyses (Fig. 3e). Again, there was a marked selectivity for interaction with PA (Fig. 3e).

The Trp\(^{19}\):Trp\(^{20}\) Pair Are Required for Binding of the N-terminal Region of PDE4A1 to Membranes—Scanning alanine mutagenesis was used to identify residues in helix-2 that are critically involved in the binding of NT-4A1-CAT to COS1 cell membranes (Figs. 3a and 4, a and b). The most profound effect related to the adjacent Trp\(^{19}\):Trp\(^{20}\) pairing, whose individual mutation led to an approximately 70% reduction in binding (Fig. 4, a and b). Indeed, binding was virtually ablated in the W19A/W20A double mutant (about 85–90% reduction; Fig. 4, b and c), and lost in the triple W19A/L16A/V17A mutant (Fig. 4, b and c). In marked contrast, mutation of Trp\(^{19}\) had no effect on binding (Fig. 4a). A second mutation identified was the Leu\(^{16}\):Val\(^{17}\) pairing, whose individual mutation reduced binding by about 40% (Fig. 4, a and b). However, the L16A/V17A double mutant caused no further reduction in binding (Fig. 4c). Similarly, the W19A/W20A and L16A/V17A double mutant forms of either NT-4A1-CAT in sedimentation assays (Fig. 5a) or NT-4A1-GST in surface plasmon resonance analyses (Fig. 5b) showed comparable reductions in their binding to PA:PC vesicles.

The Trp\(^{10}\):Trp\(^{20}\) Pair Are Required for Binding of the N-terminal Region of PDE4A1 to Membranes in Living COS1 Cells—Both sedimentation and surface plasmon resonance data (Figs. 4, a-c, and 5, a and b) identified the Trp\(^{10}\):Trp\(^{20}\) pairing as being critical for membrane/bilayer insertion. To evaluate whether mutation of this pairing affected membrane association in living cells we generated a chimera (NT-4A1-GFP) formed between the 25-amino acid N-terminal region of PDE4A1 and green fluorescent protein (GFP). Whereas wild-type GFP expressed in COS1 cells showed a generalized fluorescence throughout the cell (Fig. 6a), the NT-4A1-GFP chimera exhibited a highly localized distribution abutting the nucleus (Fig. 6b). Indeed the NT-4A1-GFP chimera co-localized (Fig. 6b) with the Golgi-specific protein, GRASP-55 (52).

In complete contrast to this the W19A/W20A double mutant form of NT-4A1-GFP was distributed throughout the cell (Fig. 6a) in a similar fashion to GFP itself (Fig. 6c). This was confirmed in subcellular fractionation studies (Fig. 6d), where full-length wild-type PDE4A1 was localized exclusively to the...
membrane pellet, whereas W19A/W20A-PDE4A1 was exclusively found in the soluble supernatant.

In both sedimentation and surface plasmon resonance studies (Figs. 4, a–c, and 5, a and b), the alanine mutation of Leu\(^{16}\) and Val\(^{17}\), either individually or together, failed to ablate completely membrane association. Analysis of the distribution of the L16A/V17A double mutant form of NT-4A1-CAT (Fig. 6c) in living cells showed that a Golgi-associated fraction remained evident when analyzing localization with respect to GRASP-55. However, unlike wild-type NT-4A1-CAT (Fig. 6a), fluorescence was also seen throughout the cell interior, including the nucleus (Fig. 6c). Similarly, the L16A/V17A double mutant form of full-length PDE4A1 was clearly found distributed between both membrane and soluble fractions in COS1 cells (Fig. 6c).

These data are consistent with the lipid and membrane binding studies where mutation of Leu\(^{16}\) and Val\(^{17}\) either alone or together, failed to ablate interaction completely but, rather, reduced it. The Leu\(^{16}\)/Val\(^{17}\) pairing orients the ring structures of the Trp\(^{19}\)/Trp\(^{20}\) pairing in helix-2 (34) and we suggest that Leu\(^{16}\)/Val\(^{17}\) may act to optimize membrane insertion driven by Trp\(^{19}\)/Trp\(^{20}\).

A Model for the Association of PDE4A1 with Phospholipid Bilayers—Fig. 1 shows a general model that may account for the binding of helix-2 of PDE4A1 to membranes based upon the conformation determined by NMR (Fig. 1b) and that modeled as a regular \(\alpha\)-helix (Fig. 1c). The tightly wound helix of the NMR structure is likely to be influenced by clustering of non-polar side chains, leading to the hypothesis that a membrane-bound peptidase, in which these side chains are solvated by the membrane interior rather than by water, may adopt a more regular helical structure. Overall, the helical nature of the NMR structure suggests that modeling this peptidase on a helical backbone is a reasonable approach to analysis of potential peptide-membrane interactions. It is notable that helical geometry, for helix-2, places those residues implicated in this study as being involved in non-polar bilayer insertion (Leu\(^{16}\), Val\(^{17}\), Trp\(^{19}\), and Trp\(^{20}\)) on one helical face (Fig. 1, b and c). The importance of Trp\(^{19}\)/Trp\(^{20}\) in our model for association is of particular interest given the apparently preferred location of tryptophan side chains at interfaces in membrane/aqueous systems (53).

Although both modeled structures indicate that Phe\(^{23}\) penetrates into the bilayer (Fig. 1, b and c), mutation of this residue to alanine had no discernible effect upon membrane association (Fig. 4a). This indicates that, unlike Trp\(^{19}\)/Trp\(^{20}\), Phe\(^{23}\) does not contribute to the basic insertion module. Thus, mutation to aliphatic, hydrophobic alanine will be tolerated as it can be accommodated within the hydrophobic bilayer core. However, as our model predicts entry of Phe\(^{23}\) into the bilayer core, one might expect that mutation of Phe\(^{23}\) to Asp, with its negatively charged side chain at physiological pH, would pose a serious problem in being accommodated within the bilayer core. Indeed, membrane association of the F23A-NT-4A1-CAT mutant (Fig. 4d, F23) was severely attenuated. Contrast this with the
The ability of various mutations in the PDE4A1 N-terminal region to associate with P2 membranes from COS1 cells was assessed in sedimentation assays using $[^{35}S]$methionine-labeled NT-4A1-CAT chimera. $[^{35}S]$Methionine-labeled CAT was used as an internal, non-membrane associating control (see "Experimental Procedures" and Ref. 33). Panel a shows screening of a panel of NT-4A1-CAT constructs with the indicated amino acid in helix-2 of PDE4A1 mutated to alanine. The amount of these species that associated with the P2 pellet fraction is given as a percentage of that seen for wild-type NT-4A1-CAT. Panel b shows examples of the distribution, between the pellet (p) and soluble (s) fractions, of wild-type and the indicated alanine mutated forms of NT-4A1-CAT after being subjected to SDS-PAGE with identification by PhosphorImager analysis. The upper arrow indicates the NT-4A1-CAT chimera (30 kDa) and the lower arrow indicates CAT itself (26 kDa). The amount of CAT varied between 70 and 90% with that of the chimera. When CAT was generated by itself and added at a 10-fold excess over that used here it did not associate with membranes or vesicles. Panel c shows similar analyses done with the indicated multiple alanine mutations of NT-4A1-CAT. Panel d shows a sedimentation assay done using P2 membranes from COS1 cells. In this instance the indicated residues were mutated to the negatively charged aspartate forms of NT-4A1-CAT (F23D, Q22D, G18D, and W15D). Binding experiments were done in complete KHEM, with free Ca$^{2+}$ in the 27–30 μM range. The data are mean ± S.D. of at least three separate experiments done using different membrane preparations.

**Fig. 4. Scanning mutational analysis of helix-2.**
lack of effect seen (Fig. 4d, Q22) with the Q22D mutant, where Gln22 is proposed to locate in the aqueous phase (Fig. 1, b and c). These data support a model (Fig. 1, b and c) where Phe23 inserts into the bilayer and adds to the stabilization of the system but, unlike the Leu16:Val17 and Trp19:Trp20 pairings, is not involved in the insertion process per se. Our model also suggests (Fig. 1, b and c) that neither the side chain of Trp15 nor that of Gly18, both of which are hydrophobic, insert into the bilayer. To evaluate this we also mutated these residues to aspartate. Doing this, both the W15A (Fig. 4d, W15) and G18A (Fig. 4d, G18) mutants showed similar or only slightly reduced binding to membranes compared with “wild-type” NT-4A1-CAT (Fig. 4d). This suggests that a negatively charged side chain can readily be accommodated at these positions in helix-2, consistent with their being located (Fig. 1, b and c) at the face of this helix that interacts with aqueous milieu rather than inserted into the hydrophobic membrane bilayer.

The N-terminal Region of PDE4A1 Associates with Membranes and Lipid Vesicles through a Rapid Ca2+/H11001-dependent Process—Penetration of tryptophan residues into an apolar environment, such as a phospholipid bilayer, can be expected to generate an increase in their fluorescence with a concomitant increase in their apparent size (Fig. 5, Mutations of the Leu16:Val17 and the Trp19:Trp20 pairings on the binding of the N-terminal region of PDE4A1 to phospholipid vesicles. Panel a shows sedimentation assays evaluating the binding of the indicated alanine mutant forms of NT-4A1-CAT to PC vesicles with 10 mol % PA (0.1 mM lipid) in the presence of added Ca2+ (total 2 mM, free 1.99 mM; although similar data were obtained using 30 µM free Ca2+) in vesicle buffer. The data are the mean ± S.D. of three separate experiments with binding expressed as % of wild-type NT-4A1-CAT. Panel b shows a surface plasmon resonance analysis of binding of GST and the indicated wild-type and alanine mutant NT-4A1-GST forms to PC vesicles containing 10 mol % of PA. Vesicle buffer contained Ca2+ (total 1 mM, free 0.99 mM; although similar data were obtained using 30 µM free Ca2+). The data are representative of triplicate experiments.

FIG. 5. Mutations of the Leu16:Val17 and the Trp19:Trp20 pairings on the binding of the N-terminal region of PDE4A1 to phospholipid vesicles. Panel a shows sedimentation assays evaluating the binding of the indicated alanine mutant forms of NT-4A1-CAT to PC vesicles with 10 mol % PA (0.1 mM lipid) in the presence of added Ca2+ (total 2 mM, free 1.99 mM; although similar data were obtained using 30 µM free Ca2+) in vesicle buffer. The data are the mean ± S.D. of three separate experiments with binding expressed as % of wild-type NT-4A1-CAT. Panel b shows a surface plasmon resonance analysis of binding of GST and the indicated wild-type and alanine mutant NT-4A1-GST forms to PC vesicles containing 10 mol % of PA. Vesicle buffer contained Ca2+ (total 1 mM, free 0.99 mM; although similar data were obtained using 30 µM free Ca2+). The data are representative of triplicate experiments.

FIG. 6. Intracellular localization of PDE4A1-GFP chimera in living cells. Panels a–c show confocal microscopy analyses of single optical sections through the center of living COS1 cells. Panel a shows cells transfected to express GFP, which is distributed throughout the cell. In panel b, cells were co-transfected to express both ds-red-GRASP55 (red) and NT-4A1-GFP (green) chimeric constructs, shown separately for two cells in each panel. The relative distribution of both wild-type NT-4A1-GFP and ds-red-GRASP55 is shown across these two cells (upper and lower) for the overlay of both channels. Panel c shows, as indicated, the distribution of both the W19A/W20A and the L16A/V17A-NT-4A1-GFP mutants (green) together with their distribution (green) relative to that of the co-transfected Golgi marker, ds-red-GRASP55 (red). These data are representative of experiments done at least six times. Panel d shows separate experiments where, as indicated, COS1 cells were transfected to express the full-length, wild-type PDE4A1 enzyme (Wt-4A1) or the indicated double alanine mutant forms of this full-length enzyme. These were either (W19A/W20A)-PDE4A1 or (L16A/V17A-4A1)-PDE4A1. After disruption, the P2 and S2 fractions were isolated, subjected to SDS-PAGE, and then immunothed using a PDE4A-specific antiserum to detect the single 68-kDa PDE4A1 species (32). These data are typical of experiments done at least three times.
blue shift in their emission spectrum (54). The unique N-terminal region of PDE4A1 contains three tryptophan residues, all of which are located in helix-2 (Fig. 1a) and are thus potentially poised to act as highly sensitive indicators of the interaction of helix-2 with lipid bilayers. Indeed, we see here that the negatively charged Asp21 juxtaposes the insertion module formed by the Trp19:Trp20 pairing (Fig. 1a), signifying an extremely rapid and efficient process. In addition to this, the magnitude of the fluorescence change was enhanced in vesicles to which PA was added (Fig. 7a), providing a third independent assessment of the selectivity of the PDE4A1 N-terminal region for interaction with PA. Intriguingly, with either PC or PA:PC vesicles, we failed to observe any change in fluorescence over a 30-ms period when Ca\textsuperscript{2+} was omitted from the reaction mixture (Fig. 7a). This suggests that Ca\textsuperscript{2+} was required for the interaction of the PDE4A1 N-terminal region with membranes. Indeed, the presence of Ca\textsuperscript{2+} was found to be essential for NT-4A1-CAT to bind to phospholipid vesicles in sedimentation assays (Fig. 7c) and for NT-4A1-GST to bind to phospholipid vesicles in surface plasmon resonance analyses (Fig. 7d). Additionally, Ca\textsuperscript{2+} increased the binding of the NT-4A1-CAT chimera to COS1 cell membranes in a dose-dependent fashion (Fig. 7e), with an EC\textsubscript{50} value of 0.36 ± 0.08 µM free Ca\textsuperscript{2+} (mean ± S.D.; n = 3 separate determinations). This did not reflect a general requirement for a divalent cation as Mg\textsuperscript{2+} failed to substitute for free Ca\textsuperscript{2+} in the various experiments reported here (data not shown). Thus low, physiologically relevant levels of free Ca\textsuperscript{2+} gate the insertion of the unique N-terminal region of PDE4A1 into lipid bilayers and biological membranes. In this regard, we note that the negatively charged Asp\textsuperscript{21} juxtaposes the insertion module formed by the Trp\textsuperscript{19}:Trp\textsuperscript{20} pairing (Fig. 1a). To test whether the carboxylate group of Asp\textsuperscript{21} mediates the role of Ca\textsuperscript{2+} we analyzed the D21A mutant form of NT-4A1-CAT (Fig. 8a, D21). In profound contrast to wild-type 4A1-CAT (Fig. 7), this mutant species was able to bind to membranes in a Ca\textsuperscript{2+}-independent fashion (Fig. 8a). Similarly, the D21A-NT-4A1-CAT mutants was able to bind
TAPAS-1, a Novel Phosphatidic Acid-binding Domain

Fig. 8. TAPAS1, a Ca^{2+} gated, phosphatidic acid selective-binding domain. Panel a shows sedimentation assays in both P2 membranes from COS1 cells and in phospholipid vesicles (0.12 mM; PC with 10 mol % PA) with the D21A (D21), L6A/V16A (L16/V17) and K24A/R25A (K24/R25) mutant forms of NT-4A1-CAT. Binding of these mutant forms is expressed as a ratio of that observed in the presence of added Ca^{2+} (1 mM total) to that seen in its absence. Thus a value of unity indicates Ca^{2+}-independent binding. P2 membranes were generated and washed in Ca^{2+}-free KHEM prior to use, as described under “Experimental Procedures.” Vesicles were prepared in Ca^{2+}-free vesicle buffer prior to use, as described under “Experimental Procedures.” Data are the mean ± S.D. of at least 3 separate experiments. Panel b is a model of the proposed charge neutralization network in TAPAS-1, with D21(-1), Ca^{2+}(+2), PA(-2), and K24(+1) shown left to right. The peptide backbone is shown in purple with a purple sphere for Ca^{2+} that is coordinated by Asp^{21}(-1; green) and a phosphate group (+2; dark blue) with Lys^{24}(+1; green). Panel c uses a sedimentation assay to compare the binding of both wild-type and mutant forms of NT-4A1-CAT to vesicles (0.1 mM phospholipid; 1 mM total (0.99 mM free) Ca^{2+}; vesicle buffer) made of either PC + 10 mol % PA or PC alone. The relative binding is expressed as a ratio of that observed using PA:PC vesicles to that observed with PC vesicles alone. The NT-4A1-CAT forms were: K24A/R25A (K24A/R25A (KR), D21A (D), L16A/V17A (LV) mutants, and wild-type (wt)). Panel d shows confocal microscopy-derived sections of fixed COS1 cells expressing wild-type, full-length PDE4A1 (red; rhodamine) together with hemagglutinin epitope-tagged versions of either PLD1b (green; fluorescein) (upper section) or ARF1 (green; fluorescein) (lower section). The arrow indicates co-localization (yellow) within the Golgi. Panel e shows a confocal microscopy study of COS1 cells expressing the D21A-NT-4A1-GFP mutant (green) together with either ds-red-GRASP55 (red) (upper section) or Mitotracker (red) (lower section) with overlay analyses. Co-localization is shown as yellow. Confocal data are typical of the various transfected cells identified in separate experiments done at least 3 times.

Figures show the binding of various mutants of NT-4A1-CAT to phospholipid vesicles in the presence and absence of calcium. The binding is expressed as a ratio to wild-type binding, and the data are shown as mean ± S.D. of at least 3 separate experiments. Panel b is a model of the proposed charge neutralization network in TAPAS-1, with D21(-1), Ca^{2+}(+2), PA(-2), and K24(+1) shown left to right. The peptide backbone is shown in purple with a purple sphere for Ca^{2+} that is coordinated by Asp^{21}(-1; green) and a phosphate group (+2; dark blue) with Lys^{24}(+1; green). Panel c uses a sedimentation assay to compare the binding of both wild-type and mutant forms of NT-4A1-CAT to vesicles (0.1 mM phospholipid; 1 mM total (0.99 mM free) Ca^{2+}; vesicle buffer) made of either PC + 10 mol % PA or PC alone. The relative binding is expressed as a ratio of that observed using PA:PC vesicles to that observed with PC vesicles alone. The NT-4A1-CAT forms were: K24A/R25A (K24A/R25A (KR), D21A (D), L16A/V17A (LV) mutants, and wild-type (wt)). Panel d shows confocal microscopy-derived sections of fixed COS1 cells expressing wild-type, full-length PDE4A1 (red; rhodamine) together with hemagglutinin epitope-tagged versions of either PLD1b (green; fluorescein) (upper section) or ARF1 (green; fluorescein) (lower section). The arrow indicates co-localization (yellow) within the Golgi. Panel e shows a confocal microscopy study of COS1 cells expressing the D21A-NT-4A1-GFP mutant (green) together with either ds-red-GRASP55 (red) (upper section) or Mitotracker (red) (lower section) with overlay analyses. Co-localization is shown as yellow. Confocal data are typical of the various transfected cells identified in separate experiments done at least 3 times.

to PA:PC vesicles in a Ca^{2+} independent fashion (Fig. 8a). We propose that a charge interaction domain forms at the membrane surface, where Asp^{21} interacts with Ca^{2+}, as shown in Fig. 8b. In this model we envisage that the interaction of Ca^{2+} with Asp^{21} allows the appropriate orientation of helix-2 for bilayer insertion through the Trp 19:Trp 20 pairing. In this way Ca^{2+} serves to gate bilayer insertion of helix-2 in wild-type PDE4A1. That substitution of the negatively charged Asp 21 with alanine allows insertion to occur independent of added Ca^{2+} (Fig. 8a), consistent with a key role for Asp^{21}.

A Model for the Phosphatidic Acid Selectivity of the N-terminal Region of PDE4A1—An additional feature of this lipid-binding domain in PDE4A1 is its apparent selectivity for PA. In the case of FYVE domains, a complex protein structure scans the inositol ring to define specificity (15). In marked contrast, helix-2 is merely a 12-residue region (Fig. 1a) and PA a very simple phospholipid. However, a notable feature of PA at neutral pH is a potential for an overall charge of −2 compared with, for example, PS with a charge of −1, PI with a charge of −1, phosphatidylinositol 4-phosphate with a charge of −3, and phosphatidylinositol 4,5-bisphosphate with a charge of −4. We suggest that this factor, along with location of the double negative charge on a single phosphate group, may be crucially exploited in helix-2 to confer selectivity for PA over other acidic phospholipids. Although it is possible that steric hindrance, because of the nature of the head groups of various other acidic phospholipids, may also play a role in deselecting them from interaction. In an extension (Fig. 8b) of our proposed charge interaction domain (Fig. 1, b and c), we consider that the second phosphate pK_a in free PA is around neutral pH, so that stabilization in a charge network leads to a double negative charge through reduction of this pK_a. Summing the charges within the (Asp^{21}−Ca^{2+}−PA^2−) network, then a further positive charge is required for neutralization. This could be supplied by either Lys^{24} or Arg^{25} (Fig. 8b), giving the charge network (Asp^{21}−Ca^{2+}−PA^2−B^−), where B represents a basic residue. To evaluate this we analyzed (Fig. 8c) the effect of mutations within this proposed network (Figs. 1c and 8b) on the selectivity for binding to PA:PC vesicles compared with PC vesicles. PA selectivity was greatly reduced using the D21A mutant form of NT-4A1-CAT (Fig. 8c), which inserts into membranes in a Ca^{2+} independent fashion (Fig. 8a), consistent with a key role for Asp^{21}.
in establishing the charge network that determines head group specificity. In addition, selectivity for interaction with PA vesicles was abolished in the K24A/R25A mutant form of NT-4A1-CAT (Fig. 8c), where the possibility for interaction with a positively charged side chain was ablated (Fig. 8b). However, unlike the D21A mutant (Fig. 8a), membrane binding of the K24A/R25A double mutant remained clearly Ca$^{2+}$-dependent (Fig. 8a). These results are in agreement with a model in which either Lys$^{24}$ or Arg$^{25}$ can complete the charge coordination of PA (Fig. 8b), but are not required for the binding of non-PA head groups. The loss of specificity for PA in the K21A/R25A mutant and in the D21A mutant (Fig. 8c) may thus be viewed in terms of this model of balanced charge solvation (Figs. 1c and 8b).

Selectivity for PA was maintained at wild-type levels in the L16A/V17A double mutant form of NT-4A1-CAT (Fig. 8c), despite the fact that the efficiency of this mutant for associating with membranes was compromised (Fig. 4, a and b). This would be consistent with the notion that the insertion module, formed from the Leu$^{16}$:Val$^{17}$ and Trp$^{19}$:Trp$^{20}$ pairings (Fig. 1b, c), does not underpin PA selectivity.

Expressed in COS1 cells, PDE4A1 is primarily associated with Golgi (Fig. 6b). Intriguingly, in COS1 cells the Golgi (Fig. 8d) is also the main focus of localization of both PLD1 and ARF1, a mini G-protein (55) implicated in mediating PLD1 activation (56–59). Intriguingly, the D21A-NT-4A1-GFP mutant, which lacks selectivity for PA, had a dramatically different intracellular distribution (Fig. 8e) from wild-type NT-4A1-GFP (Fig. 6b). Rather than being found exclusively in the Golgi, it was also distributed among various other membranous structures, including mitochondria as specifically identified here using Mitotracker (Fig. 8e).

**DISCUSSION**

Here we present a molecular description of a novel PA selective binding domain that we have called TAPAS-1, for tryptophan anchoring phosphatidic acid selective-binding domain 1. The geometry of the small helix (Fig. 1, b and c) that provides this domain places those residues implicated in non-polar insertion (Leu$^{16}$, Val$^{17}$, Trp$^{19}$, Trp$^{20}$, and Phe$^{25}$) on an opposite face to those (Asp$^{21}$, Lys$^{24}$, and Arg$^{25}$) implicated in head group and Ca$^{2+}$-dependent interactions, leading to separation of these interaction domains. We suggest that the primary insertion phase is driven by the Trp$^{19}$:Trp$^{20}$ pairing, which is held in an optimal configuration by the Leu$^{16}$:Val$^{17}$ pairing. The insertion of Phe$^{25}$ then supplies additional hydrophobic interactions. A striking feature of this domain is the requirement for sub-micromolar free Ca$^{2+}$ levels to trigger bilayer insertion. The “gating” of this insertion process, by Ca$^{2+}$, may involve a conformational change in helix-2. However, we suggest that a critical feature of the process is the establishment, at the bilayer interface region, of a charge network having overall neutrality (Figs. 1e and 8b). We believe that this requirement for a neutral charge network, rather than the nature of the insertion module, underpins the selectivity of TAPAS-1 for interaction with PA. However, steric hindrance may also play a role in deselecting various other acidic phospholipids from interaction. Thus the TAPAS-1 domain, which allows for Ca$^{2+}$-dependent, PA selective binding in a single helix consisting of a mere 12 amino acids, provides an example of a molecular machine of exquisite simplicity.

TAPAS-1 bears functional, if not structural, similarities to C2 domains whose association with phospholipid bilayers is invariably Ca$^{2+}$-dependent (15, 60). C2 domains are large complex structures formed from an anti-parallel $\beta$-sandwich where it is believed that the interaction of Ca$^{2+}$ with various asparagine residues causes a conformational change that allows apolar residues, located within the $\beta$-hairpin loops or “jaws” of the $\beta$-sheets, to interact with membranes (60). Thus mutation of the aspartate residues in C2 domains to uncharged species leads to functional inactivation of the C2 domain rather than to Ca$^{2+}$-independent membrane association as we see with TAPAS-1 (Fig. 6, c and d). Nevertheless, in the C2 domain of PKC-$\alpha$ (61) it has been suggested that Ca$^{2+}$ binding (2–3 $\mu$M affinity) to a pair of aspartate residues (Asp$^{246}$:Asp$^{248}$) located within the loops connecting the $\beta$-sheets may re-orientate these residues to allow the insertion of the interposed Trp$^{245}$:Trp$^{247}$ residues into the lipid bilayer. Such bilayer association was suggested (61) to be further facilitated by electrostatic interactions provided by a nearby charged cluster (Arg$^{249}$:Arg$^{252}$).

Insertion of tryptophan residues into bilayers has also been shown for melittin (62) and annexin V (63), where Ca$^{2+}$-binding causes the bilayers to Trp$^{185}$ to be exposed at the protein surface where it can then associate with membranes. In the case of PDE4A1, Ca$^{2+}$ does not control a reversible membrane association-disassociation process. Rather it gates the essentially irreversible insertion of hydrophobic residues within helix-2 of PDE4A1 into the lipid bilayer. Thus chelation of Ca$^{2+}$ does not lead to the release of PDE4A1 chimera from either bilayers or membranes (data not shown), neither does it allow solubilization of PDE4A1 from membranes (32, 35). In this way, membrane-associated PDE4A1 provides a long-term “memory” of an event in which a cell was activated by Ca$^{2+}$.

The key motif of TAPAS-1 may be represented by the motif LVX$_{aa}$-WWDX$_{aa}$X$_{aa}$/X$_{aa}$(K/R), where the LVX$_{aa}$-WW unit provides the core insertion module and the DX$_{aa}$/X$_{aa}$X$_{aa}$ scheme join the Ca$^{2+}$-gating/specificity region. Nevertheless, we were unable to identify any similar region in either Raf-1 (9) or the UCR1 region of the long PDE4 cAMP phosphodiesterases (64), which are both considered capable of binding to PA. However, unlike PDE4A1, upon cellular disruption these proteins are found in both particulate and soluble fractions of cells. Thus, as suggested (9, 64), basic patches on Raf-1 and PDE4 long isoforms are likely to be responsible for allowing a primarily electrostatic interaction with PA. Indeed, whereas the PDE4D3 isoform has been shown to be complexed with PA in cells (12), it has also been shown to interact similarly with the acidic phosphatidylserine in vitro (13), identifying another fundamental difference in its lipid interaction site compared with that of TAPAS-1. PA activates PDE4 isoforms by binding to the UCR1 module that characterizes long, but not short isoforms, to elicit a conformational change (13) akin to that achieved by stimulatory PKA phosphorylation of UCR1 (65, 66). Here, the super-short PDE4A1 isoform, which lacks UCR1, has a unique N-terminal region that binds phospholipids, with a preference for PA, as a means of anchoring PDE4A1 to lipid bilayers.

We analyzed PDE4A1 distribution in COS1 cells merely as a device to show that mutations in helix-2 that prevented bilayer insertion in vitro also prevented membrane association in living cells (Fig. 6). In doing this we noticed that PDE4A1 appears to locate primarily to the Golgi, as it does when expressed in both FTC133 and FTC236 cells (67). The precise mechanism that underpins such localization remains to be elucidated. However, we show here that fidelity of localization to the Golgi is diminished in the D21A-NT-4A1-GFP mutant, which shows lack of selectivity for PA (Fig. 8). This implies that PA selectivity may, at least in part, contribute to targeting. In this regard, both PLD and ARF1, a regulatory, GTP-binding protein able to activate PLD, are localized to the Golgi in COS1 cells (Fig. 8). As recruitment to the Golgi is synonymous with ARF1 being in its activated GTP-bound state (68), then it appears likely that Golgi-associated PLD provides an active focus of PA generation within these cells. This may explain why we did not
find any re-localization of either PDE4A1 or PLD upon treat-
ment of COS1 cells with phorbol 12-myristate 13-acetate (data
not shown). Furthermore, the possibility that the N-ter-
mino region of PDE4A1, after insertion into the phospho-
lipid bilayer, interacts with proteins that further influence its
intracellular localization. Such a targeted PDE4A1 isomor
may serve to shape local intracellular gradients of cAMP that
are then sampled by PKA isomers bound to anchor proteins
(AKAPs) (1, 71).

The Ca2+-dependent, PA selective-binding domain TAPAS-1
defines a novel domain that shows selectivity for binding the
signaling lipid, PA. As such it may serve(233,440),(757,992)