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Membrane Targeting by APPL1 and APPL2: Dynamic Scaffolds that Oligomerize and Bind Phosphoinositides

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Abstract

Human adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1 (APPL1) and adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2 (APPL2) are homologous effectors of the small guanosine triphosphatase RAB5 that interact with a diverse set of receptors and signaling proteins and are proposed to function in endosome-mediated signaling. Herein, we investigated the membrane-targeting properties of the APPL1 and APPL2 Bin/Amphiphysin/Rvs (BAR), pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains. Coimmunoprecipitation and yeast two-hybrid studies demonstrated that full-length APPL proteins formed homooligomers and heterooligomers and that the APPL minimal BAR domains were necessary and sufficient for mediating APPL-APPL interactions. When fused to a fluorescent protein and overexpressed, all three domains (minimal BAR, PH and PTB) were targeted to cell membranes. Furthermore, full-length APPL proteins bound to phosphoinositides, and the APPL isolated PH or PTB domains were sufficient for *in vitro* phosphoinositide binding. Live cell imaging showed that full-length APPL-yellow fluorescent protein (YFP) fusion proteins associated with cytosolic membrane structures that underwent movement, fusion and fission events. Overexpression of full-length APPL-YFP fusion proteins was sufficient to recruit endogenous RAB5 to enlarged APPL-associated membrane structures, although APPL1 was not necessary for RAB5 membrane targeting. Taken together, our findings suggest a role for APPL proteins as dynamic scaffolds that modulate RAB5-associated signaling endosomal membranes by their ability to undergo domain-mediated oligomerization, membrane targeting and phosphoinositide binding.

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Keywords

APPL1; APPL2; BAR domain; endocytosis; PH domain; phosphoinositide binding; PTB domain; RAB5; signaling endosome

Human APPL1 (adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1)/APPL (adaptor protein containing PH domain, PTB domain and Leucine zipper motif)/DIP13α (DCC-interacting protein 13α) and APPL2/DIP13β (referred to herein as APPL1 and APPL2, respectively) are two highly homologous proteins that contain three domains: an N-terminal Bin/Amphiphysin/Rvs (BAR) domain, a central pleckstrin homology (PH) domain and a C-terminal phosphotyrosine binding (PTB) domain. APPL1 interacts with a diverse set of receptors, including the netrin-1 receptor DCC (deleted in colorectal carcinoma) (1), the nerve growth factor (NGF) receptor TrkA (2,3), the follicle-stimulating hormone (FSH) receptor (FSHR) (4,5) and the AdipoR1 and AdipoR2 adiponectin receptors (6,7). APPL1 also associates with signaling proteins, including AKT (v-akt murine thymoma viral oncogene homolog) proteins (5,8,9), phosphatidylinositol 3-kinase (PI3K) subunits (8) and the OCRL (oculocerebrorenal syndrome of Lowe) and INPP5B (inositol polyphosphate-5-phosphatase, 75 kDa) phosphatidylinositol 5-phosphatases (10). Many of these interactions are mediated by the APPL1 PTB domain, suggesting that APPL1 may function as an adaptor linked to distinct signaling pathways. APPL2 was originally identified based on its high level of homology to APPL1 (1). APPL1 and APPL2 each contain a C-terminal PDZ binding motif, which mediates interaction between APPL1 and GIPC1 (GIPC PDZ domain containing family, member 1) (2,3).

APPL proteins were also identified as RAB5 effectors: both APPL1 and APPL2 interact directly with GTP-bound RAB5 (11), which is a small guanosine triphosphatase (GTPase) that regulates early endocytosis. Activated RAB5 regulates the localization of oligomeric complexes of over 20 different effector proteins (12) to early endosomal membrane subdomains enriched for phosphatidylinositol (PtdIns) 3-phosphate [PtdIns (3)P] (13). The level of RAB5 on individual early endosomes is in constant flux and is accompanied by frequent fusion and fission events. As a result, RAB5-containing endosomes are generated continuously at the cell periphery and increase in size as they migrate toward the middle of the cell (14). Early endosomes are dynamic organelles that undergo short-range movement along actin filaments (15) and long-range movement along microtubules (16-18).

APPL1 and APPL2 colocalize with each other and with activated RAB5 on punctate cytosolic structures often concentrated at the inner surface of the plasma membrane, which appear distinct from canonical early endosome antigen 1 (EEA1)-positive early endosomes, caveolae or clathrin-coated vesicles (11). Dynamic changes in APPL1 localization suggest a role in endosome-mediated signaling: in response to epidermal growth factor (EGF) stimulation, APPL1 translocates from punctate cytosolic structures to a subset of EGF-containing endosomes and then to the nucleus. In the nucleus, APPL proteins interact with the NuRD/MeCP1 nucleosome remodeling and histone deacetylase multiprotein complex (11). Release of APPL1 from endosomal membranes to the nucleus requires GTP hydrolysis by RAB5 (11). The subpopulation of EGF-containing endosomes that APPL1 associates

with is predicted to act as an intermediate signaling compartment that relays information from the cell surface to the nucleus (11).

Interaction between APPL1 and GTP-bound RAB5 requires the APPL1 N-terminal BAR domain and the adjacent PH domain; it has been suggested that APPL1 membrane targeting requires interaction with GTP-RAB5 (11). BAR domains are known for their ability to form crescentshaped dimers (19-23). Several proteins with BAR domains also contain neighboring lipid-binding domains, such as PH or phox homology (PX) domains, allowing for coordinated regulation of their activities (24,25). PH and PTB domains exhibit a strikingly similar overall structure, each consisting of a seven-stranded β-sandwich that is capped at one end by a C-terminal α-helix (reviewed in 26,27). Both domains have been shown to bind phosphoinositides and proteins, and some PH and PTB domains can simultaneously interact with their protein and phosphoinositide targets using separate binding surfaces (28-30).

Here, we investigated the membrane-targeting functions of the APPL1 and APPL2 BAR, PH and PTB domains. We found that full-length APPL proteins formed homooligomers and heterooligomers and that the APPL minimal BAR domains were necessary and sufficient for mediating these interactions. APPL1 and APPL2 minimal BAR, isolated PH and isolated PTB domains were each capable of membrane targeting. Furthermore, our data demonstrated that full-length APPL proteins bound to phosphoinositides and that APPL isolated PH or PTB domains were sufficient for *in vitro* phosphoinositide binding. Full-length APPL-yellow fluorescent protein (YFP) fusion proteins associated with cytosolic membrane structures that recruited endogenous RAB5 and underwent striking changes in shape as well as movement, fusion and fission events. Collectively, our findings suggest that APPL1 and APPL2 may function as protein scaffolds that contribute to the dynamic organization of RAB5-associated membrane subdomains on signaling endosomes.

Results

BAR domains mediate homotypic and heterotypic APPL-APPL interactions

While a previous study showed colocalization of APPL1 with APPL2 (11), no direct interaction between these proteins has been demonstrated. We found that full-length APPL proteins interacted with each other in a homotypic and heterotypic manner in the yeast two-hybrid system (Figure 1). Based on the ability of BAR domains to form dimers, we hypothesized that APPL-APPL interactions may be mediated by the BAR domains. To map these interactions, we used full-length APPL proteins (1-FL and 2-FL) and their respective minimal BAR domains (1-BAR and 2-BAR) as baits and 12 deletion constructs for APPL1 (1-1 to 1-12) and APPL2 (2-1 to 2-12) as prey (Figure 1A). Recent crystal structures of the APPL1 BAR-PH domain show a fourth α-helix that extends the BAR domain (residues 17-268) (31,32). The isolated BAR domains used in our studies lack the fourth α-helix, and we refer to them as `minimal' BAR domains (residues 18-226 and 18-225 for APPL1 and APPL2, respectively).

Diploid yeast strains coexpressing all combinations of bait and prey proteins were plated to control (control) or experimental (Ade^-His^-) plates to test for two-hybrid interactions (Figure

1B). We detected weak auto-activation activity for the full-length APPL2 bait protein (2-FL); however, the interactions shown here with APPL1 deletion clones (1-1 to 1-5) and APPL2 deletion clones (2-1 to 2-5) occurred much earlier than background signal, as shown by comparison to the prey vector-alone controls (V). Results of these experiments demonstrated that the APPL minimal BAR domains were necessary and sufficient for mediating APPL1-APPL1, APPL2-APPL2 and APPL1-APPL2 interactions in the yeast two-hybrid system (Figure 1C).

Identification of APPL1-APPL1, APPL2-APPL2 and APPL1-APPL2 interactions in vivo

We carried out coimmunoprecipitation experiments to determine whether APPL-APPL homotypic or heterotypic interactions occur *in vivo*. We used the human DLD-1 colorectal cancer cell line in these experiments because it expresses APPL1 and APPL2 (1). A previous study of APPL1-AdipoR1 interactions demonstrated that overexpressed APPL1 lacking the PTB domain interacted with endogenous APPL1 in C2C12 myoblasts (6). To determine whether full-length APPL1 associates with full-length APPL1 *in vivo*, we tested whether endogenous APPL1 coimmunoprecipitates with APPL1 expressed as a C-terminal YFP fusion protein (APPL1-YFP). We transfected the DLD-1 cell line with a vector to overexpress APPL1-YFP or with a vector to overexpress YFP as a negative control. Endogenous APPL1 specifically coimmunoprecipitated with APPL1-YFP (Figure 2A). We consistently observed some slower migrating forms of endogenous APPL1 in these immunoprecipitates, suggesting possible posttranslational modification. The anti-APPL1 and anti-APPL2 antibodies were highly specific and did not cross-react with APPL2 or APPL1, respectively (Figure S1).

In order to determine whether APPL2 associates with APPL2 *in vivo*, we tested whether APPL2 fusion proteins coimmunoprecipitate. We used two different APPL2 fusion proteins in these experiments as endogenous APPL2 is difficult to detect using our rabbit polyclonal anti-APPL2 antibodies. We cotransfected DLD-1 cells with vectors to overexpress a C-terminal fusion of APPL2 with YFP (APPL2-YFP) and a C-terminal fusion of APPL2 with the V5 and His6 epitopes (APPL2-V5-His). As a negative control, we cotransfected DLD-1 cells with vectors to overexpress YFP and APPL2-V5-His to ensure that interactions are not mediated by the tags. APPL2-V5-His specifically coimmunoprecipitated with APPL2-YFP and vice versa (Figure 2B).

To determine whether APPL1 associates with APPL2 *in vivo*, we tested whether APPL1 and APPL2 fusion proteins coimmunoprecipitate. We cotransfected DLD-1 cells with vectors to overexpress APPL1-YFP and APPL2-V5-His. As a negative control, we cotransfected DLD-1 cells with vectors to overexpress YFP and APPL2-V5-His. APPL2-V5-His specifically coimmunoprecipitated with APPL1-YFP and vice versa (Figure 2C). We detected a strong band corresponding to endogenous APPL1 protein in the immunoprecipitate with APPL1-YFP, as shown earlier (Figure 2A). We also detected a faint band corresponding to endogenous APPL1 protein in both immunoprecipitates with APPL2-V5-His. These results are supported by a recent study showing that APPL1 and APPL2 proteins coimmunoprecipitate when co-overexpressed in a human embryonic kidney-293 (HEK-293) cell line stably overexpressing the FSHR; this interaction did not require the

APPL1 PTB domain (5). As a complementary approach, we examined the subcellular localization of full-length APPL proteins when they were coexpressed as YFP and V5-His fusion proteins. These experiments confirmed that moderate overexpression did not disrupt APPL localization to cytosolic membranes and the nucleus and that coexpressed APPL proteins colocalized (Figure S2).

APPL1 and APPL2 mRNAs are widely expressed in human and mouse tissues

APPL1 is expressed in several human and mouse tissues (8). To address the potential relevance of APPL-APPL homotypic and heterotypic interactions, we examined APPL1 and APPL2 messenger RNA (mRNA) levels in a panel of human and mouse tissues using a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method. APPL1 and APPL2 mRNAs were widely expressed in human and mouse tissues (Figure S3A and B, respectively), although the relative amounts of APPL1 and APPL2 mRNAs varied from tissue to tissue. In some cases, only one APPL transcript was present. These data suggest that APPL homooligomers and heterooligomers may be present in numerous tissues, whereas a subset of tissues may be enriched for APPL1 homooligomers (human: kidney and lung; mouse: lung, uterus, prostate and certain breast tissues) or APPL2 homooligomers (human: bone marrow and fetal brain; mouse: none).

APPL minimal BAR domains associate with cell membranes and affect cellular morphology

To determine whether APPL BAR domains associate with cell membranes, we overexpressed APPL1 or APPL2 minimal BAR domains (lacking the fourth α-helix) as C-terminal YFP fusion proteins in DLD-1 cells (APPL1 BAR-YFP or APPL2 BAR-YFP, respectively). APPL1 and APPL2 minimal BAR domain YFP fusion proteins exhibited distinct membrane-associated patterns of subcellular localization when compared with control cells transfected with YFP alone or with full-length APPL-YFP fusion proteins (Figure 3A-C). The majority of APPL1 minimal BAR domains localized to large clusters of round vesicles and ring-like structures that appeared similar to concave discs (56% localized to large clusters containing more than five vesicular structures and 13% localized to smaller clusters containing five or fewer vesicular structures). In the remaining cells, we observed APPL1 minimal BAR domains associated with individual medium-sized round vesicles (16%), with individual small-sized round vesicles (4%) or with both a cluster of vesicles and with individual round vesicles within the same cell (11%) (Figure 3D; $n = 109$ cells). APPL2 minimal BAR domains associated primarily with curved networks of elongated membrane structures (59%), individual medium-sized round vesicles (8%), individual small-sized vesicles (9%), a mix of medium- and small-sized vesicles (8%) or to a combination of curved networks of elongated membrane structures and individual round vesicles within the same cell (16%) (Figure 3E; $n = 111$ cells). Differential interference contrast (DIC) images showed alterations in cellular morphology that coincided with BAR domain localization, and DAPI (4′,6-diamidino-2-phenylindole) staining showed that the enlarged membrane structures were often perinuclear.

We also examined the subcellular localization of APPL1 and APPL2 minimal BAR domains relative to a set of cell compartment markers including α-tubulin, GM130, TGN38,

caveolin-1, BiP/Grp78, RAB5 and actin (merged images are shown in Figure S4C and D, respectively). The APPL minimal BAR domains often localized to perinuclear regions that also contained Golgi markers (GM130 and TGN38), although their localization did not overlap significantly with most of the cell compartment markers employed. The APPL minimal BAR domains showed some overlapping signal with RAB5. However, the RAB5 signal was relatively diffuse and quite different from the strong overlapping signal observed for full-length APPL-YFP fusion proteins. In agreement with previous studies (11), our yeast two-hybrid studies showed that APPL deletion clones containing both the BAR and the PH domains interacted with activated RAB5, whereas the APPL minimal BAR domains did not interact with RAB5 (Figure S5).

APPL proteins exhibit PH and PTB domain-mediated phosphoinositide binding

We used affinity-purified APPL1 and APPL2 proteins (full-length, isolated PH domains or isolated PTB domains) in protein overlay experiments to test for binding to membrane-immobilized phosphoinositides and other phospholipids using PIP Strips™ (Echelon Biosciences, Inc.). APPL1 and APPL2 full-length proteins, isolated PH domains and isolated PTB domains all bound to membrane-immobilized PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P_2 and PtdIns(3,5)P_2 (Figure 4A). APPL isolated PTB domains also bound to membrane-immobilized phosphatidylserine (PS, spot 15). It is possible that APPL-APPL interactions and/or the overall conformation of the full-length proteins may inhibit PTB domain-mediated PS binding.

APPL isolated PH and PTB domains associate with cell membranes in vivo

Based on deletion analyses, it has been suggested that interaction with GTP-RAB5 is required for APPL1 membrane targeting: only deletion clones that retained both the BAR and the PH domains associated with cell membranes (11). However, the APPL1 deletion clones used in the previous study included residues adjacent to the APPL1 BAR, PH and PTB domains, and APPL2 deletion clones were not examined. We overexpressed APPL1 and APPL2 isolated PH and PTB domains as N-terminal cyan fluorescent protein (CFP) fusion proteins in DLD-1 cells to determine whether they associate with cell membranes. Although membrane targeting can be mediated by interactions with targets other than phosphoinositides, membrane association is suggestive of phosphoinositide binding *in vivo*.

We found that APPL1 and APPL2 isolated PH domains localized to the plasma membrane, cytosolic vesicles and distinct nuclear and perinuclear structures (Figure 4C and D, respectively), whereas the APPL1 and APPL2 isolated PTB domains localized to cytosolic membrane structures (Figure 4E and F, respectively), and the APPL1 PTB domain also localized to the nucleus. In contrast, CFP alone displayed diffuse whole-cell localization (Figure 4B). Our yeast two-hybrid studies demonstrated that the APPL isolated PH and PTB domains did not interact with activated RAB5 (Figure S5). Therefore, APPL PH and PTB domain-mediated membrane targeting is not likely to involve direct interaction with RAB5.

We also examined the subcellular localization of the overexpressed APPL1 and APPL2 isolated PH and PTB domains relative to a series of cell compartment markers, including α-tubulin, GM130, TGN38, caveolin-1, BiP/Grp78, RAB5 and actin (merged images are

shown in Figure S4E-H, respectively). The isolated PH domains localized to the plasma membrane where caveolin-1 signal was also detected, but we did not observe significant overlap between the APPL isolated PH and PTB domains and the other cell compartment markers employed.

APPL-YFP fusion proteins associate dynamically with cytosolic membrane structures that undergo changes in shape, movement, fusion and fission events

We examined the subcellular localization of overexpressed full-length APPL1-YFP and APPL2-YFP in living cells. Both APPL1-YFP and APPL2-YFP showed dynamic associations with cytosolic membrane structures that underwent movement, fusion and fission events (Videos S1-S3 and S4-S6, respectively). Additionally, both APPL1-YFP and APPL2-YFP associated with membrane structures that exhibited striking changes in their shape, regardless of their size. We examined the subcellular localization of APPL1-YFP and APPL2-YFP fusion proteins relative to a set of cell compartment markers including α-tubulin, GM130 (*cis* Golgi), TGN38 (*trans* Golgi), caveolin-1 (caveosomes), BiP/Grp78 (endoplasmic reticulum), RAB5 and phalloidin (actin). With the exception of RAB5 (discussed below), we did not observe significant overlap between full-length APPL-YFP proteins and the cell compartment markers employed (merged images are shown in Figure S4A,B).

Overexpression of APPL-YFP proteins leads to the recruitment of endogenous RAB5 to APPL-associated membrane structures

In order to determine whether the enlarged APPL-YFP associated membrane structures exhibit features of early endosomes, we examined endogenous RAB5 localization. Overexpression of APPL1-YFP or APPL2-YFP fusion proteins led to alterations in endogenous RAB5 localization that were distinct from neighboring untransfected cells (Figure 5A and B, respectively). In untransfected cells, endogenous RAB5 localization was cytosolic and was associated with small cytosolic puncta. However, overexpression of APPL-YFP fusion proteins led to a redistribution of endogenous RAB5 to enlarged cytosolic membrane structures that contained APPL-YFP; this effect was most pronounced in cells expressing APPL1-YFP. In APPL1-YFP expressing cells, 99% of the APPL1-YFP-positive structures were RAB5 positive and 94% of the RAB5-positive structures were APPL1-YFP positive (n = 15 cells, 849 vesicles counted; Figure 5A). APPL2-YFP colocalized strongly with RAB5 to smaller cytosolic vesicular structures: 100% of the APPL2-YFP-positive vesicles were RAB5 positive, and 98% of the RAB5-positive vesicles were APPL2-YFP positive (n = 13 cells, 448 vesicles counted; Figure 5B). However, both APPL2-YFP and endogenous RAB5 exhibited additional nonoverlapping subcellular localization distinct from that associated with the cytosolic vesicles. Results of these studies suggest that overexpression of APPL-YFP fusion proteins is sufficient to direct the localization of endogenous RAB5 to enlarged APPL-associated cytosolic membrane structures.

APPL1 is not required for RAB5 membrane targeting

Based on the ability of overexpressed APPL-YFP fusion proteins to facilitate the recruitment of endogenous RAB5 to APPL-associated membranes, we carried out small

interfering RNA (siRNA)-mediated silencing experiments to determine whether APPL1 is required for RAB5 membrane targeting. DLD-1 cells were transfected with a non-specific siRNA (NS siRNA) reagent, an APPL1 siRNA reagent or were mock transfected. At 2 days post-transfection, the cells were trypsinized and replated: (i) for immunoblot (IB) analysis, (ii) to coverslips to examine the subcellular localization of endogenous RAB5 in starving cells following 15 min of serum stimulation or (iii) to coverslips for transfection with CFP-RAB5 as a more rigorous test to determine whether loss of APPL1 expression affects CFP-RAB5 membrane targeting. All three sets of cells were analyzed at 4.5 days after the initial siRNA transfection. We found that the APPL1 siRNA-transfected cells exhibited no overt deficits in cell growth or viability (data not shown), the APPL1 siRNA reagent very effectively inhibited APPL1 protein expression [undetectable levels of APPL1 protein by IB analysis with 20 μg total protein loaded (Figure 6A), and 95% of APPL1 siRNA-transfected cells grown on coverslips did not express significant levels of APPL1 as determined by indirect immunofluorescence microscopy] and the loss of APPL1 expression did not impact endogenous RAB5 protein levels (Figure 6A). In control (NS siRNA) cells, endogenous APPL1 and endogenous RAB5 proteins colocalized extensively on cytosolic puncta following 15 min of serum stimulation (Figure 6B, upper panel). In the absence of APPL1, endogenous RAB5 maintained its association with cytosolic puncta (Figure 6B, lower panel). Similar to the ability of overexpressed APPL-YFP proteins to recruit endogenous RAB5 to cytosolic membranes, we found that overexpressed CFP-RAB5 recruited endogenous APPL1 to enlarged CFP-RAB5-associated membrane structures in control (NS siRNA) cells (Figure 6C, upper panel). In the absence of APPL1, CFP-RAB5 continued to exhibit membrane targeting that was indistinguishable from APPL1-expressing cells (Figure 6C, lower panel). Taken together, the results of these experiments suggest that APPL1 is not required for RAB5 membrane targeting.

Discussion

We report the identification and functional characterization of domain-mediated membrane targeting, oligomerization and phosphoinositide binding by human APPL1 and APPL2 proteins. The crystal structures of APPL1 BAR-PH, BAR, PH and PTB domains have been solved recently (31,32), and our studies are the first to systematically examine the membrane-targeting properties of the APPL1 and APPL2 minimal BAR, isolated PH and isolated PTB domains *in vivo*. APPL1 BAR and BAR-PH domains form homodimeric crystal structures (31,32), and our studies demonstrate that APPL1 and APPL2 minimal BAR domains are necessary and sufficient for mediating all APPL-APPL interactions. Furthermore, we show that the APPL1 and APPL2 minimal BAR, isolated PH and isolated PTB domains are each capable of membrane targeting. We show that APPL full-length proteins bind phosphoinositides and that APPL isolated PH or PTB domains are sufficient for *in vitro* phosphoinositide binding. Our live cell imaging studies demonstrate that APPL-YFP fusion proteins associate with cytosolic membrane structures that undergo dynamic changes in shape as well as movement, fusion and fission events. Furthermore, we found that overexpression of APPL-YFP fusion proteins leads to the recruitment of endogenous RAB5 to APPL-associated cytosolic membrane structures, although APPL1 is not necessary for RAB5 membrane targeting. Our findings provide new insights about the *in vivo*

membrane-targeting properties of APPL1 and APPL2 minimal BAR, PH and PTB domains and uncover new mechanisms that may regulate the dynamic association between APPL proteins and signaling endosomal membranes.

BAR domain-mediated APPL-APPL interactions and membrane targeting

The minimal activities associated with functional BAR domains include the ability to (i) form dimers, (ii) bind cell membranes and (iii) sense membrane curvature (19-21). Taken together, our yeast two-hybrid, coimmunoprecipitation and live cell imaging studies provide evidence that full-length APPL proteins interact directly in a homotypic and heterotypic manner and associate with curved cell membranes that recruit RAB5 and undergo movement, fusion and fission events. The crystal structure of the APPL1 BAR-PH domain shows the presence of a fourth α-helix that extends the BAR domain and leads to a BAR domain dimer that contains two bundles of four α-helices (31,32). The fourth α-helix of the APPL1 BAR domain is not expected to contribute to the structure of the concave inner surface of the BAR domain dimer (31,32), and our studies demonstrate that it is not required for membrane targeting or dimerization.

APPL domain-mediated membrane targeting without RAB5 binding

A common feature among BAR domain-containing proteins is their ability to bind small GTPases. Whether membrane targeting and GTPase binding are mutually exclusive events or are co-ordinately regulated *in vivo* is not known (20,33). Interaction between APPL1 and RAB5 requires the N-terminal BAR domain and the adjacent PH domain (11). Based on the crystal structure and *in vitro* binding studies, the APPL1 BAR-PH dimer is believed to generate two RAB5-binding surfaces: each end of the dimer is predicted to form a distinct heterotypic RAB5 binding platform that consists of PH domain residues from one monomer and BAR domain residues from the second monomer (32). Additionally, mutational analyses predict that APPL1 may compete with the RAB5 effector rabaptin-5 for binding to RAB5 (32).

Based on previous deletion analyses, it has been suggested that interaction with GTP-bound RAB5 is required for APPL1 membrane targeting (11). However, our data show that the APPL minimal BAR, isolated PH and isolated PTB domains are each capable of membrane targeting, even though they do not interact directly with activated RAB5 [(11) and Figure S5]. The ability of the isolated domains to associate with cell membranes suggests that APPL proteins may be capable of membrane targeting in a RAB5-independent manner *in vivo* and provides evidence that APPL proteins may interact with cell membranes prior to the recruitment of their protein-binding partners. Indeed, a role for APPL proteins in modulation of RAB5 membrane targeting is supported by our discovery that overexpression of APPL-YFP fusion proteins leads to the recruitment of endogenous RAB5 to APPL-associated cytosolic membrane structures. Although APPL proteins may facilitate the recruitment of RAB5 to endosomal membranes, our siRNA experiments demonstrate that APPL1 is not required for RAB5 membrane targeting. This finding is not surprising because RAB5 is known to interact with a number of additional effectors on cell membranes, and RAB5 is also independently capable of membrane targeting through lipid modification at its C terminus. APPL1 was shown recently to bind GTP-bound RAB21 *in vitro* (32), suggesting

that APPL proteins may interact with additional RAB5-related small GTPases on cell membranes.

APPL phosphoinositide binding

Our *in vitro* phosphoinositide-binding studies suggest that the APPL1 and APPL2 full-length proteins, isolated PH domains and isolated PTB domains all bind to PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P_2 and PtdIns(3,5)P_2. Taken together, these five phosphoinositides are associated with the plasma membrane, early endosomes, late endosomes/multivesicular bodies, endoplasmic reticulum, Golgi and the nucleus (reviewed in 34,35). In addition, APPL-binding partners, including AKT proteins, PI3K subunits, phosphatidylinositol 5-phosphatases and RAB5, localize to membrane domains enriched for some of these phosphoinositides. Although they interact with the same set of phosphoinositides *in vitro*, overexpressed APPL isolated PH domains, isolated PTB domains and full-length proteins exhibit distinct patterns of membrane-associated subcellular localization *in vivo*; it is likely that membrane curvature and interactions with protein-binding partners contribute to these differences.

A recent study tested for binding of a subset of APPL domains to a small number of phospholipids [PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, PtdIns(3,5)P_2, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol]. Results of these studies showed that the APPL1 BAR, BAR-PH and PTB domains could bind PtdIns(3,4,5)P_3, whereas the APPL2 PH domain could bind PtdIns(3,4,5)P_3 and PtdIns(3,5)P_2 (31). Unlike the previous study, we did not detect significant binding to PtdIns(3,4,5)P_3; this difference may be because of the fact that our experiments included all the phosphoinositides on a single membrane and would require competitive binding.

Although canonical early endosomes are enriched for PtdIns(3)P, it is possible that APPL proteins may associate with distinct membrane subdomains on signaling endosomal membranes. Two separate pathways lead to PtdIns(3)P enrichment on early endosomal membranes because of associations between RAB5 and two classes of PI3K: Vps34 and PI3Kβ (36). Vps34 directly converts PtdIns to PtdIns(3)P, whereas PI3Kβ converts plasma membrane-associated PtdIns(4)P and PtdIns(4,5)P_2 to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, respectively. A second pair of RAB5 effectors, phosphatidylinositol 4-phosphatase and phosphatidylinositol 5-phosphatase (INPP5B), are believed to convert PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 to PtdIns(3)P in a stepwise manner (37). A recent study identified direct interactions between APPL1 and INPP5B as well as between APPL1 and a second related phosphatidylinositol 5-phosphatase called OCRL (10); this interaction is mediated through a motif between the APPL1 PH and PTB domains that is not conserved in APPL2. Although additional experiments will be required to characterize the binding of APPL proteins to cell membrane-associated phosphoinositides, results from our studies suggest that the APPL phosphoinositide-binding profiles may impart them with regulatory properties distinct from those of the canonical RAB5 effector EEA1 that only binds PtdIns(3)P.

APPL-mediated organization of RAB5 subdomains on signaling endosomal membranes

Dynamic changes in APPL1 localization suggest a role in endosome-mediated signaling: following EGF stimulation, APPL1 translocates from punctate cytosolic structures to a subset of EGF-containing endosomes and then to the nucleus, where APPL proteins interact with the NuRD/MeCP1 nucleosome remodeling and histone deacetylase multiprotein complex (11). The subpopulation of EGF-containing endosomes that APPL1 associates with is predicted to act as an intermediate signaling compartment that relays information from the cell surface to the nucleus. Although APPL proteins are predicted to localize to a specialized endosomal compartment (11), they may also associate with canonical early endosomes. APPL1 shows some colocalization with EEA1 in EGF-stimulated HeLa cells (11), and APPL1 colocalizes more extensively with EEA1 in NGF-stimulated PC12 cells (3). Furthermore, endogenous APPL1 colocalizes with overexpressed wild-type and constitutively active RAB5 on enlarged endosomal membranes [Figure 6C and (11), respectively].

Early evidence for compartmentalized signaling came from studies of the epidermal growth factor receptor (EGFR): the majority of the EGFR and its downstream signaling proteins were found to be associated with endosomes shortly after ligand stimulation (38), and inhibition of clathrin-mediated endocytosis led to decreased mitogen-activated protein kinase (MAPK) and PI3K signaling downstream of EGFR activation (39). The `signaling endosome hypothesis' was proposed originally based on observations that purified endosomes from NGF-stimulated PC12 cells contained activated NGF receptors (TrkA) bound to both NGF and phospholipase C-γ1 (40) and that activated TrkA undergoes retrograde axonal transport (41), suggesting that these endosomal compartments may transduce signals over long distances. Recent studies demonstrate that APPL1 interacts directly with TrkA and the TrkA-associated protein GIPC1 (2,3); APPL2 was also detected in GIPC1 immunoprecipitates (3). Furthermore, these studies demonstrate a functional role for APPL1 in NGF-mediated signal transduction, TrkA trafficking, recruitment of GIPC1 to TrkA-containing membranes and neurite outgrowth (2,3). However, whether APPL proteins associate with NGF-containing signaling endosomes that undergo retrograde axonal transport is not known, and nuclear translocation of APPL proteins following NGF stimulation has not been described.

In summary, we show here that APPL1 and APPL2 undergo domain-mediated oligomerization, phosphoinositide binding and membrane targeting, all of which contribute to the organization of functional RAB5-containing endosomal membrane subdomains (42,43). Our studies suggest that the APPL BAR, PH and PTB domains act in concert to impart distinct properties to full-length APPL proteins. The activities of the APPL BAR, PH and PTB domains are likely to be co-ordinately regulated in response to membrane curvature, localized populations of phosphoinositides and protein-binding partners. Furthermore, our findings inform us of previously unknown mechanisms by which APPL proteins may regulate endosome-mediated signaling and provide evidence for RAB5-independent membrane targeting by APPL proteins. Our results support a role for APPL1 and APPL2 as protein scaffolds that contribute to the dynamic organization of RAB5-associated membrane subdomains on signaling endosomes.

Materials and Methods

Cloning

Full-length APPL1 (accession number AF424738), APPL2 (accession number AY113704) and RAB5A (accession number NM004162) clones were generated by RT-PCR using total RNA from a normal human mammary epithelial cell line as a template with primers specific for each gene. We used PCR amplification to introduce attB1 and attB2 recombination sites at the 5′ and 3′ ends of each gene, respectively, to facilitate cloning into Gateway Cloning System vectors (Invitrogen Corporation). Each APPL1 and APPL2 clone was generated with or without a stop codon and cloned into the pDONR207 or pDONR201 vectors (Invitrogen Corporation) to allow expression as N- or C-terminal fusion proteins, respectively. The resulting full-length complementary DNA (cDNA) clones were then used as templates for PCR amplification to generate 11 deletion clones of APPL1 and APPL2 (Figure 1A) that were also cloned with or without stop codons into the pDONR207 or pDONR201 vectors, respectively. APPL deletion clones were designed to include the amino acid residues that constitute the predicted APPL minimal BAR (APPL1 BAR: residues 18-226; APPL2 BAR: residues 18-225), isolated PH (APPL1 PH: residues 280-375; APPL2 PH: residues 278-376) and isolated PTB (APPL1 PTB: residues 502-634; APPL2 PTB: residues 493-624) domains as well as the residues flanking these domains. RAB5A constitutively active or dominant-negative clones, Q79L or S34N, respectively (44,45), were generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, Inc.). All APPL1 and APPL2 full-length, APPL1 and APPL2 deletion clones, RAB5A wild-type and RAB5A mutant clones (Q79L and S34N) were fully sequenced on both DNA strands. We also generated pDONR207 and pDORN201 control vectors containing only the attB1 and attB2 flanking sequences with or without a stop codon, respectively, which we used to generate the vector-only controls. The resulting vectors were then used as templates for recombination-based cloning into vectors compatible with the Gateway System (Invitrogen Corporation), including the pdEYFP and pdECFP vectors (46) to express C-terminal YFP and N-terminal CFP fusions, respectively, the pcDNA-DEST40 vector (Invitrogen Corporation) for expression of C-terminal V5-His6 fusion proteins and the pDEST15 vector (Invitrogen Corporation) for bacterial expression of N-terminal glutathione S-transferase (GST) fusion proteins. We used the Gateway Vector Conversion System (Invitrogen Corporation) to generate Gateway-compatible versions of the Yeast Matchmaker GAL4 Two-Hybrid System 3 (BD Biosciences) bait and prey vectors, pGBKT7 and pGADT7, respectively, that were then used to generate the bait and prey vectors containing full-length and deletion clones of APPL1 and APPL2 (Figure 1A).

Yeast two-hybrid experiments

We used the Yeast Matchmaker GAL4 Two-Hybrid System 3 (BD Biosciences) to analyze APPL-APPL and APPL-RAB5A interactions. As described above, we generated 12 different prey constructs for both APPL1 (1-1 to 1-12) and APPL2 (2-1 to 2-12) using the Gateway-converted pGADT7 prey vector. We generated APPL1 and APPL2 full-length and minimal BAR domain-containing bait vectors using the Gateway-converted pGBKT7 vector. We used the boundaries of the three main domains (BAR, PH and PTB) and the regions between these domains as a guide for generating the deletion constructs (Figure 1A).

The AH109 and Y187 yeast strains were transformed with bait and prey vectors, respectively. Whole-cell lysates from yeast strains expressing each bait and prey vector were analyzed by immunoblotting with anti-c-Myc or anti-hemagglutinin antibodies, respectively, to ensure that each bait and prey protein was expressed and of the correct molecular weight. All possible combinations of bait and prey matings were tested, and diploid yeast strains were selected on Trp$^-$Leu$^-$plates. Diploids were then grown in Trp$^-$Leu$^-$ liquid media and stamped onto either Trp$^-$Leu$^-$ plates or Trp$^-$Leu$^-$ Ade$^-$His$^-$ plates to test for interaction between the bait and the prey proteins. We used the pGBKT7-Lam control vector expressing lamin C (Lamin) as a negative control bait to ensure that prey proteins do not exhibit non-specific activation, and we used our Gateway-converted pGADT7 control prey vector (V) to ensure that the bait proteins do not show auto-activation activity.

Coimmunoprecipitation experiments

Coimmunoprecipitation experiments were carried out using Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc.) according to the manufacturer. Briefly, whole-cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF and 0.5% IGEPAL CA630) containing 1 mM Na_3VO_4,1 mM DTT, 1 mM phenylmethylsulphonyl fluoride and 1× complete protease inhibitor cocktail (Roche Diagnostics Corporation) at 24 h post-transfection. Whole-cell lysates (0.5 mg total protein) were precleared by preincubation with control mouse IgG and Protein A/G PLUS-Agarose beads for 30 min at 4°C. Precleared lysates were then incubated with 2 μg primary antibody for 1 h at 4°C, followed by the addition of 20 μL beads and incubation at 4°C for 1 h. The beads were washed four times with PBS, and 2× Laemmli sample buffer was added prior to boiling and loading for SDS-PAGE. Antibodies used for the immunoprecipitation experiments include control mouse IgG (Santa Cruz Biotechnologies, Inc.), mouse anti-green fluorescent protein (GFP) monoclonal antibody (Sigma-Aldrich) and mouse anti-V5 monoclonal antibody (Invitrogen Corporation). Antibodies used only for IB analysis include an affinity-purified rabbit anti-APPL1 polyclonal antibody generated by our lab against a C-terminal APPL1 peptide sequence (SSSQSEESDLGEGGKKR), an affinity-purified rabbit anti-APPL2 polyclonal antibody generated by our lab against a C-terminal APPL2 peptide sequence (TNDGKYVLLNDQPDD) and a rabbit anti-GFP polyclonal antibody (AbCam). We have tested the specificity of our anti-APPL1 and anti-APPL2 rabbit polyclonal antibodies using affinity-purified full-length GST-APPL1 and GST-APPL2 proteins, and we do not detect any cross-reactivity (Figure S1B).

Reverse transcriptase-polymerase chain reaction

Commercially available cDNA from human or mouse tissues (Origene) was used in semiquantitative PCR reactions with APPL1 (human: 5′-ATGGCTGTGGACTGTGAA-3′ and 5′-TGCCCTACGATCCAGTTC-3′; mouse: 5′-GGGGGGTGATGATGAAGTTAT-3′ and 5′-TTTCCACCCTGCGTGAGTAA-3′, APPL2 (human: 5′-GCGGAAGCAGCACCTCTC-3′ and 5′-GCCCGAGCAGCCAATACT-3′; mouse: 5′-TGGCACCCTCACAGATTACAC-3′ and 5′-CTGCCTGGAGATGTTGTTTAC-3′) or actin primer sets using Platinum Taq enzyme (Invitrogen Corporation) for 35 cycles.

Fluorescence microscopy

Standard fluorescence microscopy studies using living and fixed cells were carried out using a Leica DMI6000 B inverted microscope equipped with an EL6000 external light source and a metal halide bulb (Leica Microsystems), a CTR6000 electronics box (Leica Microsystems), an HCX PL APO 100×/ 1.46 OIL CORR 23-37°C objective (Leica Microsystems) and a Rolera-MGi Fast Mono EMCCD camera (QImaging). DIC images were collected using a DIC analyzer (Leica 11513900), and fluorescence images were collected using the following filters: DAPI/ultraviolet (Leica 11513874 A4), fluorescein isothiocyanate (FITC)/GFP (Leica 11513880 L5), HC RED (Chroma 41043; Chroma Technology), Cyan GFP V2 (Chroma 31044 V2) and Yellow GFP BP 10C/Topaz (Chroma 41028). Images were collected and analyzed using QED INVITRO 3 Imaging software (MediaCybernetics) and IMAGE-PRO ANALYZER 6.1 (MediaCybernetics), respectively.

For experiments to examine the subcellular localization of YFP and CFP fusion proteins in fixed cells, DLD-1 cells were grown on coverslips and transfected using Lipofectamine 2000 (Invitrogen Corporation) according to the manufacturer. After 24 h, the cells were rinsed with PBS and fixed with 2% formaldehyde for 15 min, followed by PBS washes. Coverslips were mounted on slides using Prolong Gold antifade reagent (Invitrogen Corporation). Indirect immunofluorescence microscopy experiments were carried out using primary antibodies against the V5 epitope (Invitrogen Corporation), α-tubulin (DM1A; Santa Cruz Biotechnology, Inc.), GM130 (BD Biosciences Pharmingen), TGN38 (BD Biosciences Pharmingen), caveolin-1 (Santa Cruz Biotechnology, Inc.), BiP/Grp78 (Calbiochem) and RAB5 (Santa Cruz Biotechnology, Inc.). Texas Redconjugated donkey anti-mouse or donkey anti-rabbit antibodies and FITC-conjugated donkey anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. Actin was visualized using Alexa-Fluor 568-Phalloidin according to the manufacturer (Invitrogen Corporation). Live cell imaging experiments were carried out using DLD-1 cells grown on coverslips transfected as described above; coverslips were transferred cell-side down to a dish with a glass coverslip bottom-containing media. Images were collected at a rate of 0.3-1 frame/second. The lowest light intensity setting and exposure times were used initially; however, light intensity was increased and the focus was adjusted as the experiment progressed in order to compensate for photobleaching and changes in the focal plane.

siRNA-mediated APPL1 silencing experiments

Reagents for siRNA-mediated silencing, including a non-specific SMARTpool for human cell lines and an APPL1 custom SMARTpool were purchased from Dharmacon. DLD-1 cells were transfected using Lipofectamine 2000 reagent (Invitrogen Corporation) according to the manufacturer using 100 nM siRNA reagent. At 2 days post-siRNA transfection, cells were replated: (i) for IB analysis, (ii) to coverslips to examine endogenous RAB5 localization or (iii) to coverslips to examine CFP-RAB5 localization as a more rigorous test of RAB5 membrane targeting. Whole-cell lysates were collected at 4.5 days post-siRNA transfection, followed by IB analysis using antibodies against APPL1, RAB5 and actin. To examine the subcellular localization of endogenous RAB5, cells were grown overnight in media lacking serum, followed by 15 min of serum stimulation and fixation at 4.5 days post-siRNA transfection; indirect immunofluorescence microscopy was carried out using rabbit

anti-APPL1 and mouse-anti-RAB5 antibodies, followed by incubation with anti-rabbit-FITC- and anti-mouse-Texas Red-conjugated secondary antibodies and DAPI staining. At 3.5 days post-siRNA transfection, the second set of cells plated to coverslips was transfected with a plasmid to express CFP-RAB5 (pdECFP-RAB5A) using Lipofectamine 2000 reagent. Cells expressing CFP-RAB5 were fixed at 4.5 days post-siRNA transfection, followed by indirect immunofluorescence microscopy using a rabbit anti-APPL1 antibody, anti-rabbit-Texas Red-conjugated secondary antibody and DAPI staining. The efficiency of APPL1 siRNA-mediated silencing was determined by first counting the cell number within a field using DAPI staining, followed by examination of APPL1 signal within each cell [95% of the cells showed APPL1 silencing at 4.5 days post-transfection with the APPL1 siRNA SMARTpool reagent (n = 556 cells counted on coverslips to examine endogenous RAB5 localization; n = 502 cells counted on coverslips to examine CFP-RAB5 localization)].

In vitro *phosphoinositide-binding assays*

APPL1 and APPL2 full-length proteins, isolated PH domains and isolated PTB domains were affinity-purified as N-terminal GST fusion proteins following isopropyl-β-D-1-thiogalactopyranoside (IPTG)-induced expression in bacteria grown at 30°C or 15°C using Glutathione Sepharose 4B Micro-Spin or Bulk Purification Modules (Amersham Biosciences). Protein yield and purification were monitored by SDS-PAGE, followed by IB analysis and Coomassie Blue staining. All incubations and rinses with PIP Strips (Echelon Biosciences, Inc.) were carried out using TBST buffer (20 mM Tris, 0.8% NaCl, 0.1% Tween-20, pH 7.6) containing 3% fatty acid-free BSA (TBST/3%). PIP Strips were blocked for 3 h in TBST/3%, followed by incubation in TBST/3% containing the purified GST fusion protein of interest (0.01-1 μg/mL) overnight at 4°C. Membranes were rinsed (3 × 10 min), followed by incubation with goat-anti-GST antibody (Amersham Biosciences) for 2 h at room temperature, rinsing (3 × 10 min), incubation with anti-goat-horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature and rinsing (3 × 10 min). Bound GST fusion proteins were detected using enhanced chemiluminescence (ECL-Plus; Amersham Biosciences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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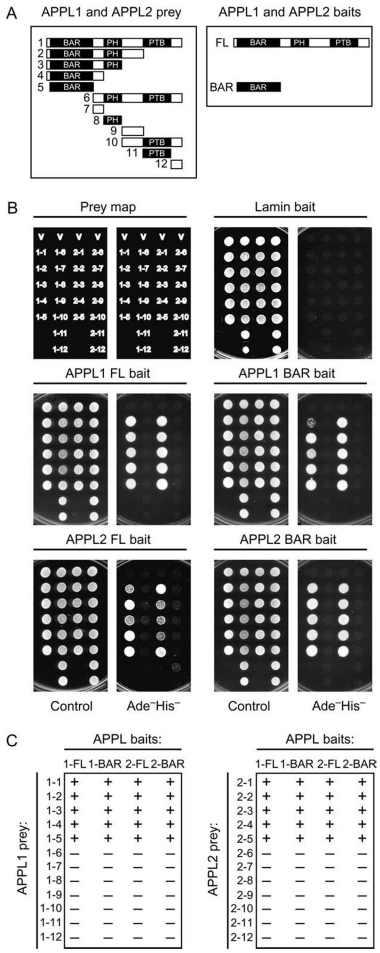

Figure 1. Minimal BAR domains are necessary and sufficient for mediating homotypic and heterotypic APPL-APPL interactions

A) Twelve prey constructs were generated for APPL1 (1-1 to 1-12) and APPL2 (2-1 to 2-12). Bait constructs include full-length APPL1 (1-FL) and APPL2 (2-FL) and their respective minimal BAR domains (1-BAR and 2-BAR). B) Diploid yeast strains coexpressing all possible combinations of bait and prey proteins were stamped onto control plates (Control) or onto plates that require interaction between the bait and the prey proteins ($Ade^{-}His^{-}$). Diploids coexpressing the empty prey vector with each bait protein (V, top row of each plate) and diploids coexpressing the lamin bait with each prey protein (Lamin bait) serve as negative controls. C) Summary of yeast two-hybrid mapping data.

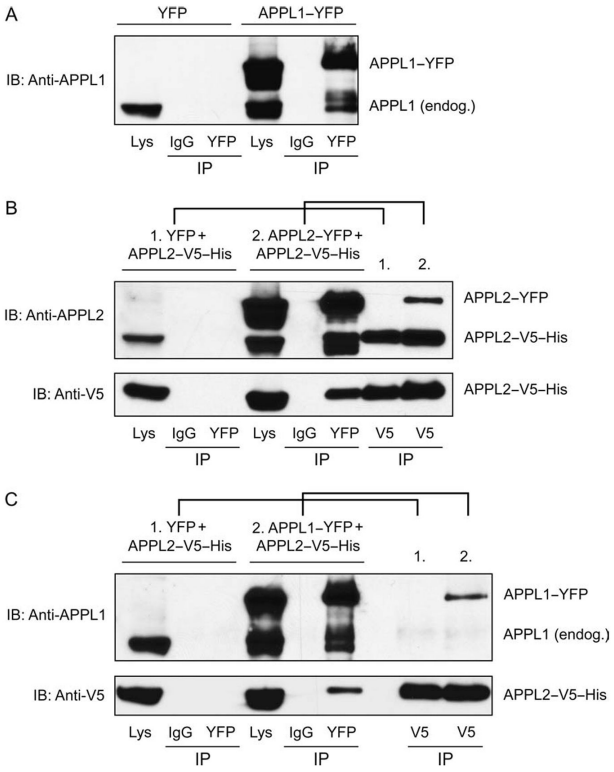

Figure 2. APPL1 and APPL2 exhibit homotypic and heterotypic protein-protein interactions *in vivo*

Whole-cell lysates from DLD-1 cells were collected at 24 h post-transfection, and immunoprecipitation experiments were carried out using the indicated antibodies [mouse anti-YFP monoclonal antibody (YFP), mouse anti-V5 monoclonal antibody (V5) or mouse IgG control (IgG)]. Shown are IBs of whole-cell lysates (Lys) and immunoprecipitates (IP) probed with anti-APPL1 (A and C), anti-APPL2 (B) or anti-V5 (B and C) antibodies. A) APPL1 coimmunoprecipitates with APPL1-YFP. Cells were transfected with a vector to overexpress YFP as a negative control or with a vector to overexpress APPL1-YFP. B) APPL2-YFP coimmunoprecipitates with APPL2-V5-His and vice versa. Cells were cotransfected with vectors to overexpress YFP and APPL2-V5-His as a negative control (1) or with vectors to overexpress APPL2-YFP and APPL2-V5-His (2). C) APPL1-YFP coimmunoprecipitates with APPL2-V5-His and vice versa. Cells were cotransfected with vectors to overexpress YFP alone and APPL2-V5-His as a negative control (1) or with vectors to overexpress APPL1-YFP and APPL2-V5-His (2).

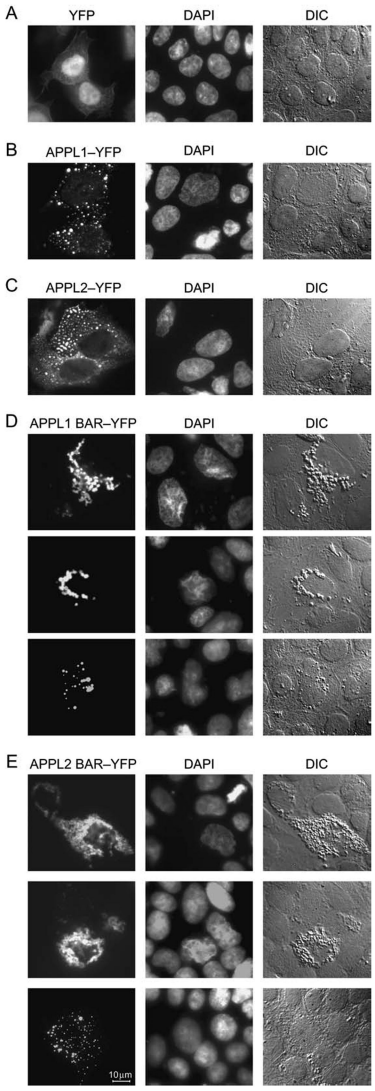

Figure 3. APPL1 and APPL2 minimal BAR domains localize to cell membrane structures and affect cellular morphology

DLD-1 cells were transfected with vectors to overexpress (A) YFP alone (B) full-length APPL1-YFP (APPL1-YFP) (C) full-length APPL2-YFP (APPL2-YFP) (D) the minimal BAR domain of APPL1 fused to YFP (APPL1 BAR-YFP) or (E) the minimal BAR domain of APPL2 fused to YFP (APPL2 BAR-YFP). Cells grown on coverslips were fixed at 24 h post-transfection, and the localization of the YFP fusion proteins was determined. Shown are YFP autofluorescence, DAPI staining and DIC images for each cell. Bar, 10 μm.

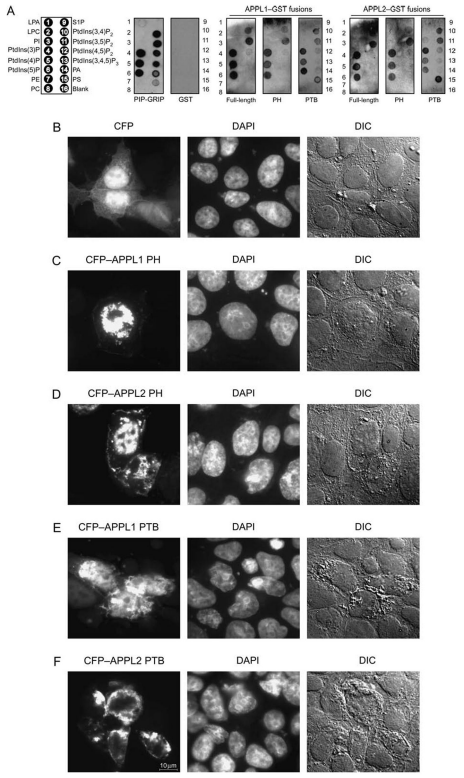

Figure 4. APPL1 and APPL2 proteins exhibit PH and PTB domain-mediated phosphoinositide binding *in vitro* and membrane targeting *in vivo*

A) Protein overlay experiments were carried out by incubating PIP Strips (Echelon Biosciences, Inc.) containing 100-pmol spots of the indicated lipids with purified bacterially expressed GST fusion proteins, followed by probing with anti-GST antibody, incubation with HRP-conjugated secondary antibodies and detection using enhanced chemiluminescence. A cocktail of the Multi-PIP GRIP™ and PIP2 GRIP™ (Echelon Biosciences, Inc.), which together bind to all phosphorylated PIPs, served as the positive control, whereas GST alone served as the negative control (GST) to insure that binding was not mediated by the GST tag. Purified GST fusion proteins corresponding to APPL1 or APPL2 full-length proteins and their respective isolated PH domains or isolated PTB domains were tested for PIP binding. B-F) Shown are standard fluorescence microscopy images of DLD-1 cells grown on coverslips and fixed at 24 h post-transfection to examine the subcellular localization of overexpressed (B) CFP alone or N-terminal CFP fusions with the (C) APPL1 isolated PH domain (CFP-APPL1 PH), (D) APPL2 isolated PH domain (CFP-APPL2 PH), (E) APPL1 isolated PTB domain (CFP-APPL1 PTB) and (F) APPL2 isolated PTB domain (CFP-APPL2 PTB). Shown are CFP autofluorescence, DAPI staining and DIC images for each cell. Bar, 10 μm. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PI, PtdIns; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine.

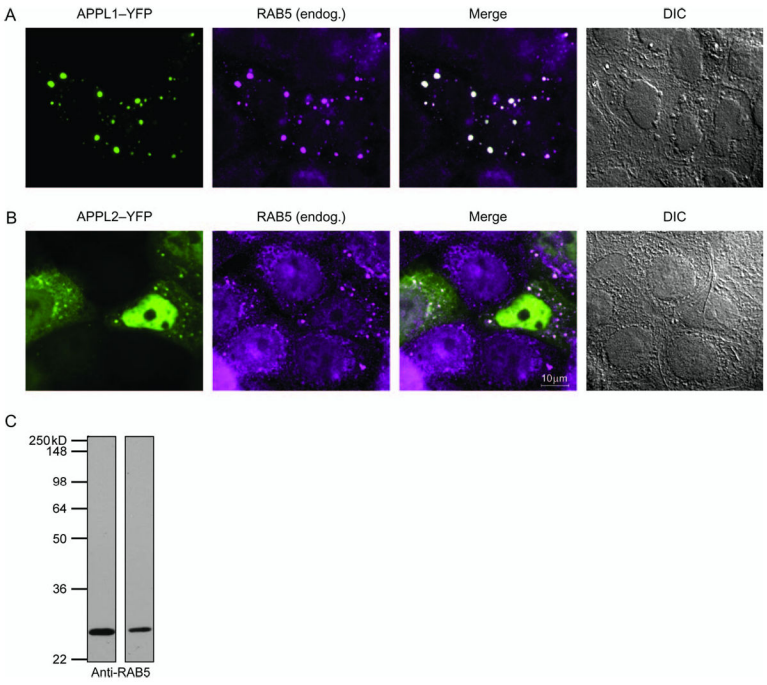

Figure 5. Overexpression of APPL-YFP fusion proteins leads to the recruitment of endogenous RAB5 to cytosolic membrane structures

DLD-1 cells were transfected with vectors to overexpress APPL1-YFP or APPL2-YFP, and the cells were fixed at 24 h post-transfection. Indirect immunofluorescence microscopy using an anti-RAB5 antibody and a Texas Red-conjugated secondary antibody shows that endogenous RAB5 colocalizes with (A) APPL1-YFP and (B) APPL2-YFP on enlarged cytosolic membrane structures (white indicates overlap). C) IB of DLD-1 whole-cell lysate probed with the mouse monoclonal anti-RAB5 antibody used in these experiments showing long and short exposures of the same blot (left and right, respectively). Bar, 10 μm.

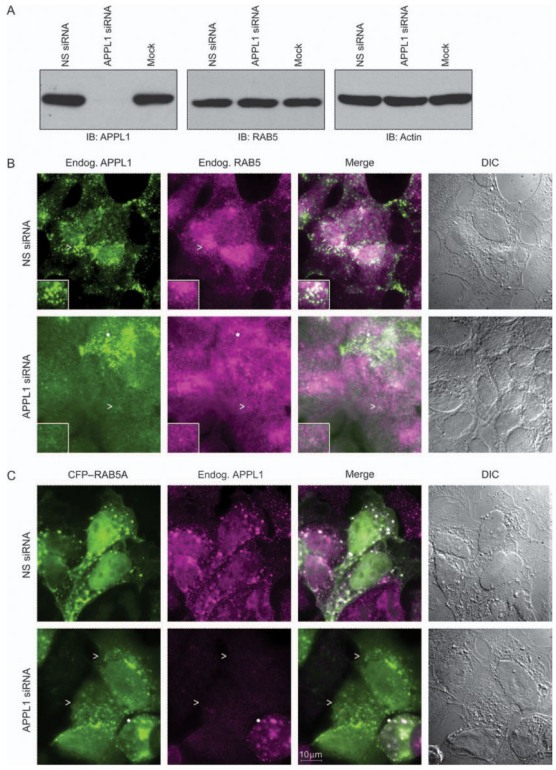

Figure 6. APPL1 is not required for RAB5 membrane targeting

Cells were transfected with a NS siRNA reagent, an APPL1 siRNA reagent or were mock transfected. At 2.5 days post-siRNA transfection, cells were replated (A) for IB analysis to examine APPL1, RAB5 and actin levels (20 μg total protein loaded per well), (B) to coverslips to examine endogenous RAB5 and APPL1 localization in starving cells following 15 min of serum stimulation (lower left insets show enlarged views of cell region indicated by `>', and `*' indicates non-siRNA-inhibited cells in the lower APPL siRNA panel) or (C) to coverslips for transfection to examine CFP-RAB5 and endogenous APPL1 localization (in the lower APPL1 siRNA panel, `>' indicates siRNA-inhibited cells and `*' indicates non-siRNA-inhibited cells). Lysates were collected, and cells were fixed at 4.5 days post-siRNA transfection. Bar, 10 μm.