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ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis

Yoshikatsu Aikawa and Thomas F.J. Martin

Department of Biochemistry, University of Wisconsin, Madison, WI 53706

ADP-ribosylation factor (ARF) 6 regulates endosomal plasma membrane trafficking in many cell types, but is also suggested to play a role in Ca^{2+}-dependent dense-core vesicle (DCV) exocytosis in neuroendocrine cells. In the present work, expression of the constitutively active GTPase-defective $ARF6^{Q67L}$ mutant in PC12 cells was found to inhibit Ca^{2+}-dependent DCV exocytosis. The inhibition of exocytosis was accompanied by accumulation of ARF^{Q67L}, phosphatidylinositol 4,5-bisphosphate (PIP_2), and the phosphatidylinositol 4-phosphate 5-kinase type I (PIP5KI) on endosomal membranes with their corresponding depletion from the plasma membrane. That the depletion of PIP_2 and PIP5K from the plasma membrane caused the inhibition of DCV exocytosis was demonstrated directly in permeable cell reconstitution studies in which overexpression or addition of PIP5KIγ restored Ca^{2+}-dependent exocytosis. The restoration of exocytosis in $ARF6^{Q67L}$-expressing permeable cells unexpectedly exhibited a Ca^{2+} dependence, which was attributed to the dephosphorylation and activation of PIP5K. Increased Ca^{2+} and dephosphorylation stimulated the association of PIP5KIγ with ARF6. The results reveal a mechanism by which Ca^{2+} influx promotes increased ARF6-dependent synthesis of PIP_2. We conclude that ARF6 plays a role in Ca^{2+}-dependent DCV exocytosis by regulating the activity of PIP5K for the synthesis of an essential plasma membrane pool of PIP_2.

Introduction

The release of neurotransmitters from neural and neuroendocrine cells is mediated by the exocytotic fusion of synaptic vesicles and dense-core vesicles (DCVs) with the plasma membrane in a process activated by cytoplasmic Ca^{2+} elevations (Rettig and Neher, 2002). Secretory vesicles dock at the plasma membrane and undergo priming, which confers competence for Ca^{2+}-triggered fusion (Robinson and Martin, 1998). Identification of phosphatidylinositol transfer protein and the phosphatidylinositol 4-phosphate 5-kinase type I (PIP5KI) as factors required for ATP-dependent priming indicated that phosphatidylinositol 4,5-bisphosphate (PIP_2) is essential for the Ca^{2+}-dependent exocytosis of docked DCVs (Eberhard et al., 1990; Hay and Martin, 1993; Hay et al., 1995). A type II PI 4-kinase on DCVs (Wiedemann et al., 1996; Barylko et al., 2001) and a cytosolic or plasma membrane type I PIP5K (Hay et al., 1995; Wenk et al., 2001) have been implicated in the synthesis of plasma membrane PIP_2 (Holz et al., 2000) required for DCV exocytosis.

Plasma membrane PIP_2 is also essential for mediating the sequential recruitment of adaptor and accessory proteins to sites of clathrin-dependent endocytosis (Cremona and DeCamilli, 2001). The requirement for PIP_2 as a cofactor for both endocytosis and exocytosis suggests that its synthesis at localized sites of fission and fusion is tightly regulated, but little is known about the control of PIP_2 synthesis. Recent analyses identified protein kinases and phosphatases that regulate the type I PIP5K (Park et al., 2001). An important advance was the finding that ADP-ribosylation factor (ARF) GTPases directly activate type I PIP5K enzymes (Honda et al., 1999; Jones et al., 2000).

The ARF proteins are GTPases that function as regulators of membrane traffic (Randazzo et al., 2000). ARF6, the sole member of the class III ARF family, resides on endosomes and the plasma membrane and regulates membrane traffic between these compartments (D'Souza-Schorey et al., 1995; Peters et al., 1995). ARF6 functions as a switch that cycles through GTP for GDP exchange and GTP hydrolysis. Correspondingly, studies of its function have used expression of dominant-

Address correspondence to Thomas F.J. Martin, Dept. of Biochemistry, University of Wisconsin, 433 Babcock Drive, Madison, WI 53706. Tel.: (608) 263-2427. Fax: (608) 262-3453. email: tfmartin@facstaff.wisc.edu

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Abbreviations used in this paper: ARF, ADP-ribosylation factor; BFA, brefeldin A; DCV, dense-core vesicle; hGH, human growth hormone; PH, pleckstrin homology; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP5KI, phosphatidylinositol 4-phosphate 5-kinase type I.

negative GTP-binding deficient mutants (e.g., ARF6^{T27N}) or of constitutively active GTPase-deficient mutants (e.g., ARF6^{Q67L}) to perturb membrane trafficking. Activation of ARF6 promotes peripheral actin assembly (Schafer et al., 2000), plasma membrane protrusions and invaginations (Peters et al., 1995; D'Souza-Schorey et al., 1998), pinocytosis (Brown et al., 2001), and the internalization of plasma membrane proteins via clathrin-dependent (D'Souza-Schorey et al., 1995; Altschuler et al., 1999; Claing et al., 2001; Palacios et al., 2002) or clathrin-independent (Radhakrishna et al., 1996; Radhakrishna and Donaldson, 1997; Brown et al., 2001; Delaney et al., 2002) endocytosis.

Unlike the results in other cell types, ARF6 was reported to localize to DCVs in adrenal chromaffin cells and to undergo translocation from DCVs to the plasma membrane in response to the stimulation of exocytosis (Galas et al., 1997; Caumont et al., 1998). Whether ARF6 plays a direct role in regulated DCV exocytosis or exerts indirect effects through cytoskeletal rearrangements or plasma membrane-endosomal trafficking remains to be determined. We found that ARF6 localizes to the plasma membrane and recycling endosomes, but not to DCVs in neuroendocrine PC12 cells. Cells expressing the GTPase-deficient ARF6^{Q67L} mutant exhibited reduced rates of Ca^{2+}-triggered DCV exocytosis resulting from the diversion of plasma membrane PIP_2 and PIP5K to endosomal membranes. The restoration of DCV exocytosis by addition of PIP5K to permeable cells required Ca^{2+}, which activated PIP5K and increased its association with ARF6. These results indicate that ARF6 plays a role in DCV exocytosis by controlling the activity of PIP5K and the synthesis of a required plasma membrane pool of PIP_2.

Results

Expression of ARF6^{Q67L} inhibits Ca^{2+}-activated DCV exocytosis in PC12 cells

To determine if ARF6-dependent cellular activities play a role in Ca^{2+}-activated DCV exocytosis, wild-type and mu-

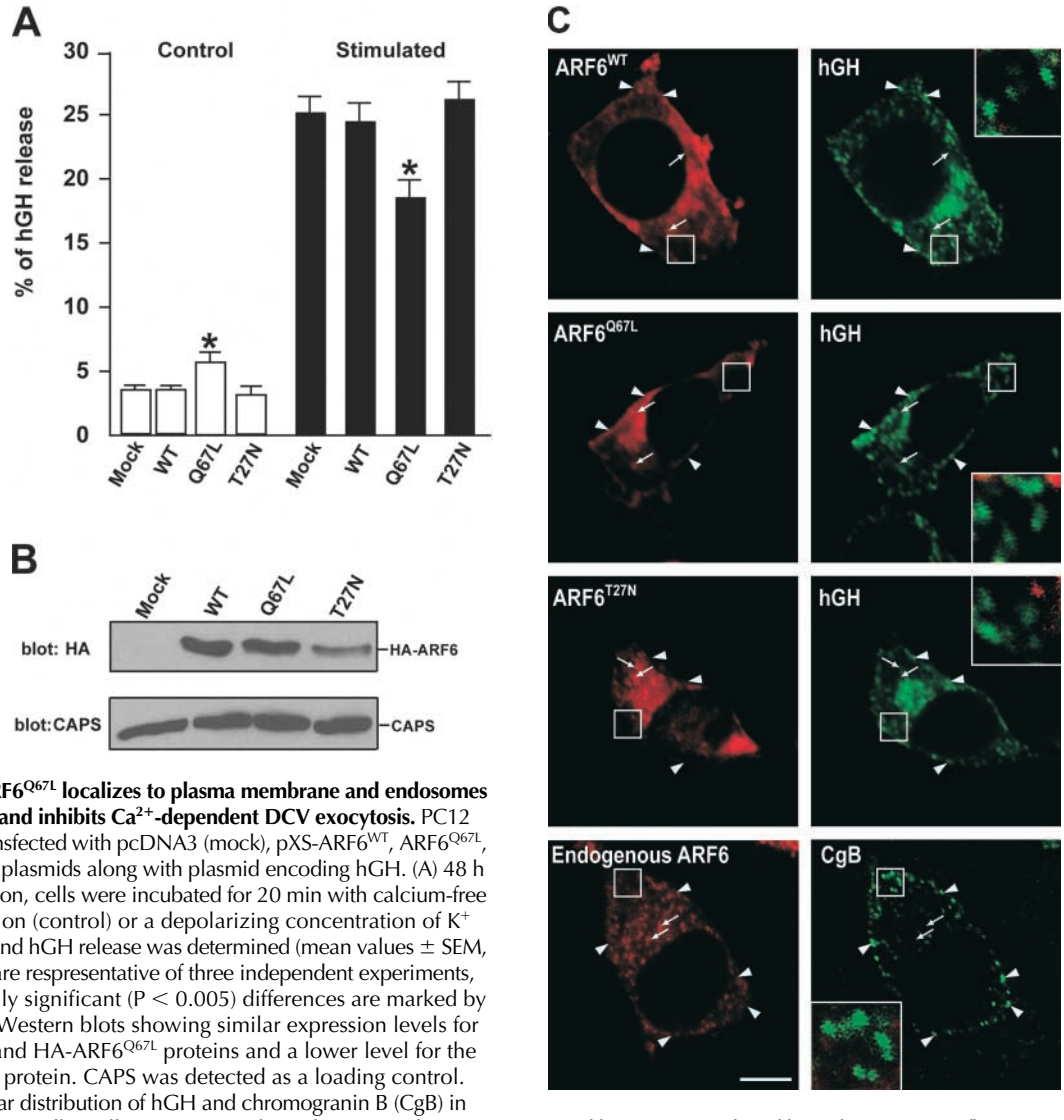


Figure 1. **ARF6^{Q67L} localizes to plasma membrane and endosomes in PC12 cells and inhibits Ca^{2+}-dependent DCV exocytosis.** PC12 cells were transfected with pcDNA3 (mock), pXS-ARF6^{WT}, ARF6^{Q67L}, and ARF6^{T27N} plasmids along with plasmid encoding hGH. (A) 48 h after transfection, cells were incubated for 20 min with calcium-free Locke's solution (control) or a depolarizing concentration of K^+ (stimulated), and hGH release was determined (mean values ± SEM, $n = 9$). Data are respresentative of three independent experiments, and statistically significant ($P < 0.005$) differences are marked by asterisks. (B) Western blots showing similar expression levels for HA-ARF6^{WT} and HA-ARF6^{Q67L} proteins and a lower level for the HA-ARF6^{T27N} protein. CAPS was detected as a loading control. (C) Intracellular distribution of hGH and chromogranin B (CgB) in transfected PC12 cells. Cells expressing indicated HA-tagged ARF6 proteins and hGH were analyzed by indirect immunofluorescence with HA (for ARF6, red) and hGH (green) antibodies or with ARF6 (red) and CgB (green) antibodies. Arrowheads indicate docked hGH- or CgB-containing DCVs that lack ARF6, and arrows indicate ARF6-positive cytoplasmic vesicles that lack hGH and CgB. Insets show magnified boxed regions. Bar, 5 μm.

tant ARF6 proteins (GTPase-deficient ARF^{Q67L} or GTP binding–deficient $ARF6^{T27N}$) were expressed in PC12 cells (Fig. 1 B). Human growth hormone (hGH), which is stored in DCVs, was coexpressed to enable the detection of regulated secretion in the transfected cells (Zhang et al., 2002). Ca^{2+}-dependent hGH secretion was elicited in brief incubations in high K^+-containing buffer to promote Ca^{2+} influx, and was compared with basal secretion in Na^+-containing buffer. Expression of wild-type and GTP binding–deficient $ARF6^{T27N}$ did not affect the basal release of hGH, but expression of the GTPase-deficient $ARF6^{Q67L}$ increased basal release approximately twofold (Fig. 1 A, open bars), which may correspond to the Ca^{2+}-independent, GTP-dependent stimulation of DCV exocytosis characterized in permeable PC12 cells (Banerjee et al., 1996). Ca^{2+} influx–dependent hGH secretion stimulated by K^+ depolarization was unaffected by expression of either wild-type or $ARF6^{T27N}$, but was consistently inhibited in $ARF6^{Q67L}$-expressing cells (Fig. 1 A, closed bars). The Ca^{2+}-dependent release of hGH, calculated by subtracting basal release from high K^+-induced release measured in the presence of high K^+, was inhibited by 43% (24.4 ± 1.1% and 13.8 ± 2.8% hGH release for wild-type and $ARF6^{Q67L}$, respectively). Because $ARF6^{Q67L}$, reported to alter peripheral membrane trafficking in other cell types, affected DCV exocytosis in PC12 cells, subsequent analyses were undertaken to determine the basis for the inhibition.

Expression of wild-type or either of the ARF6 mutants did not affect the cellular distribution of hGH (Fig. 1 C, right panels) in the Golgi or in DCVs, indicating that the synthesis and transport of hGH through the secretory pathway were unaffected by ARF6 expression. The wild-type and ARF^{Q67L} expressed proteins localized to the plasma membrane and to cytoplasmic vesicular membranes, whereas the $ARF6^{T27N}$ protein was predominantly localized to cytoplasmic vesicular membranes (Fig. 1 C, left panels), as reported for other cell types (D'Souza-Schorey et al., 1995; Peters et al., 1995). ARF6-containing cytoplasmic vesicles (Fig. 1 C, arrows) were readily distinguished from hGH-immunoreactive DCVs. Neither docked DCVs containing hGH (Fig. 1 C, arrowheads) nor cytoplasmic DCVs (Fig. 1 C, insets) colocalized with ARF6-containing cytoplasmic vesicles. Endogenous ARF6 was weakly detected in cytoplasmic vesicular membranes and on the plasma membrane (Fig. 1 C), and this localization was clearly distinct from that of chromogranin B, an endogenous DCV constituent (Fig. 1 C). Overall, these results indicated that ARF6 proteins are not present on DCVs, and that the inhibition of Ca^{2+}-dependent DCV exocytosis by $ARF6^{Q67L}$ was unlikely the result of either an altered DCV localization or of a direct effect of ARF6 on DCVs.

ARF6 colocalizes with transferrin- and Rab11-positive endosomes in PC12 cells

In other cell types, ARF6 is present in endosomal compartments and on the plasma membrane (D'Souza-Schorey et al., 1995; Peters et al., 1995; Radhakrishna and Donaldson, 1997). Although ARF6 was reported to be present on DCVs in chromaffin cells (Galas et al., 1997; Caumont et al., 1998), this was not the case for neuroendocrine PC12 cells (Fig. 1 C). To identify the cytoplasmic vesicles containing ARF6, colocalization studies were conducted for proteins characteristic of secretory and endocytic compartments. Cytoplasmic ARF6 (Fig. 2 A, arrows) did not colocalize with either TGN38 or with mannosidase II. ARF6-containing vesicles were more diffusely localized around the perinuclear region after treatment with brefeldin A (BFA; Fig. 2 A), which causes tubulation of endosomes, the TGN, and lysosomes (Lippincott-Schwartz et al., 1991). BFA

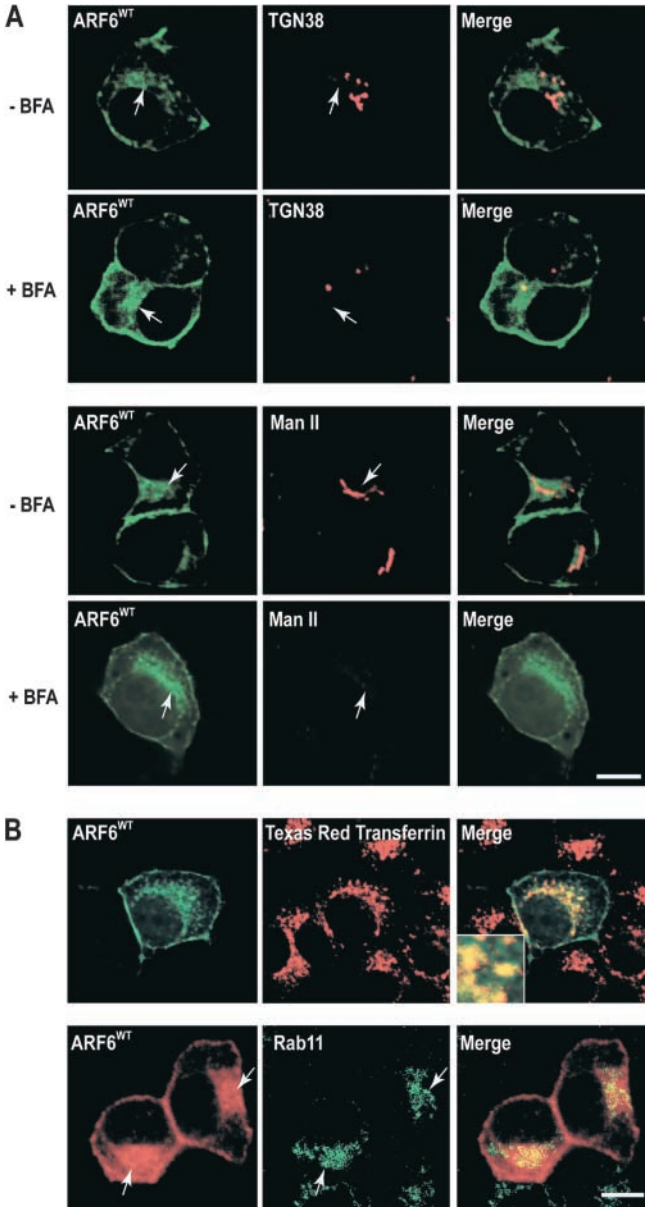


Figure 2. **ARF6 localizes to the endocytic pathway in PC12 cells.** (A) PC12 cells were transfected for 48 h with wild-type EGFP-ARF6 (green) and treated with 5 μg ml^{-1} BFA where indicated (BFA). Immunocytochemistry was conducted to localize TGN38 (red) and mannosidase II (red). Arrows indicate ARF6-containing compartments that lack TGN38 and mannosidase II. (B) Top row, internalized Texas red transferrin (red) exhibits extensive colocalization with EGFP-ARF6 (green). Inset shows magnified perinuclear region. Bottom row, HA-ARF6 (red) exhibits extensive colocalization with Rab11 (green). Arrows indicate ARF6-containing compartments that colocalize with transferrin and Rab11. Bars, 10 μm.

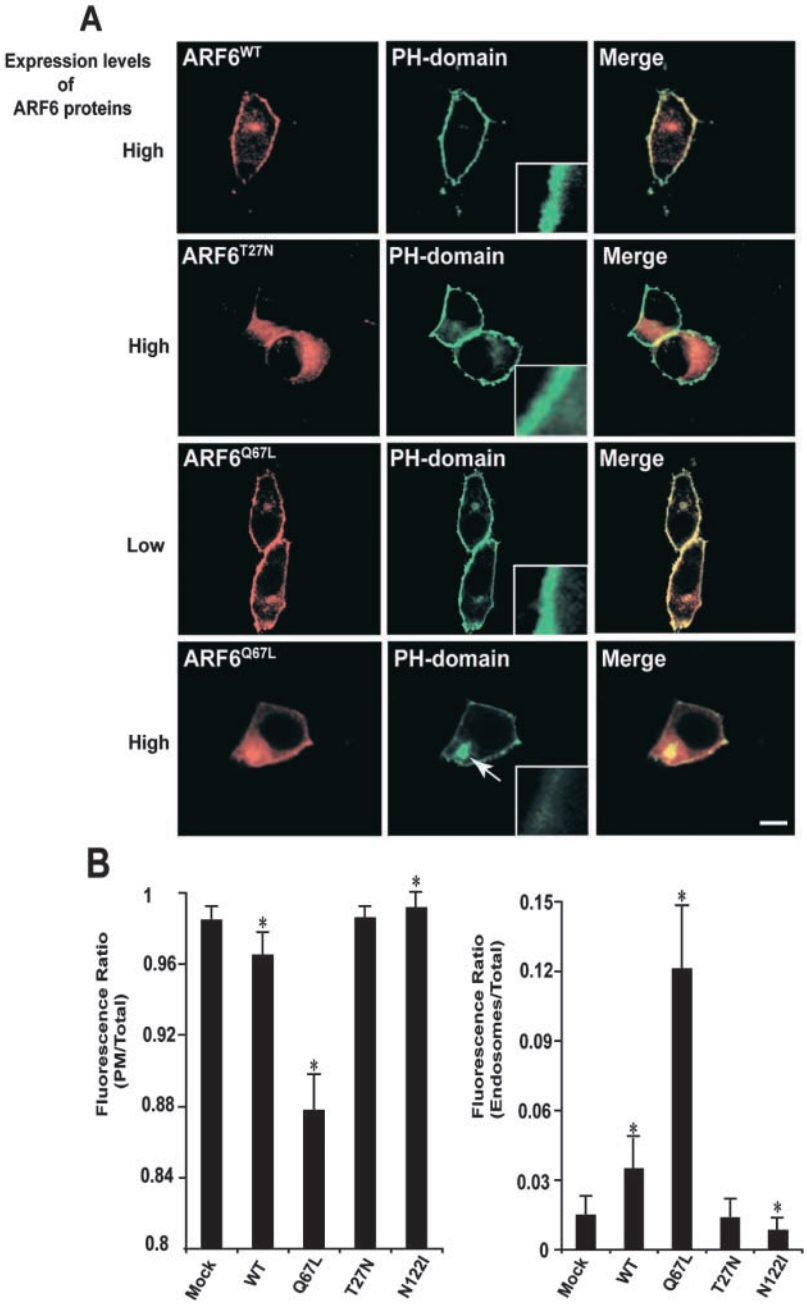
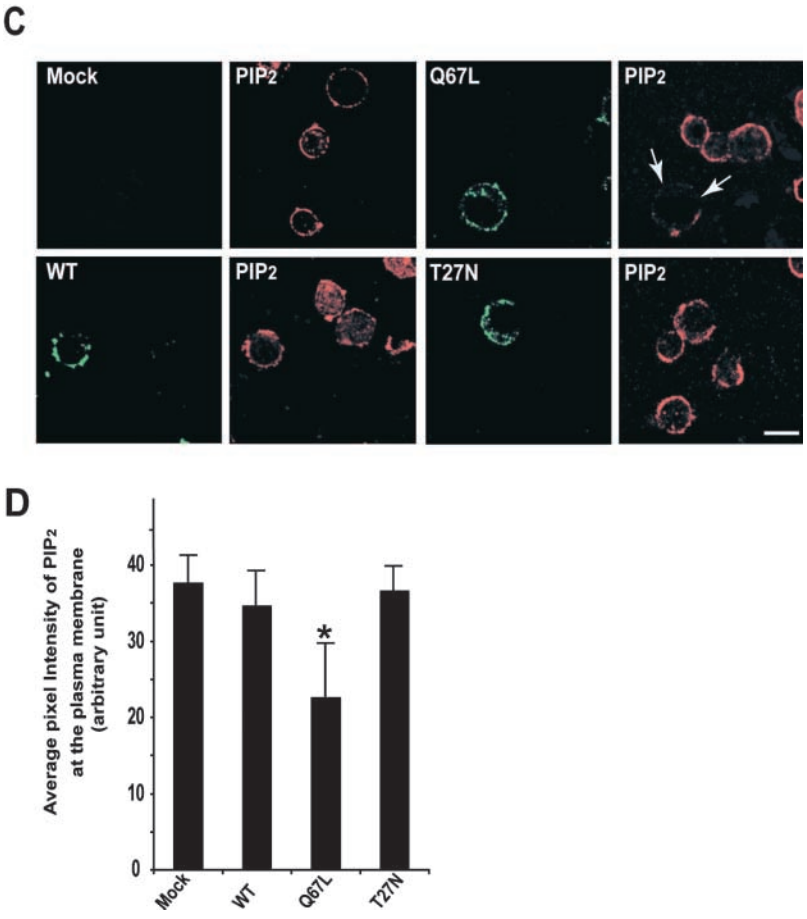


Figure 3. **PIP_2 redistributes from plasma membrane to endosomes in $ARF6^{Q67L}$-expressing PC12 cells.** (A) PC12 cells were cotransfected for 48 h with plasmids encoding PH-GFP (green) and HA-tagged $ARF6^{WT}$, $ARF6^{Q67L}$, or $ARF6^{T27N}$ (detected with HA antibody, red). Insets show enlarged regions of the plasma membrane. Cells expressing relatively high levels of $ARF6^{Q67L}$ exhibited endosomal accumulation of PH-GFP (arrow) and decreased plasma membrane fluorescence (inset). (B) The fluorescence intensity of PH-GFP was quantified in 50 randomly selected cells, and the ratio of plasma membrane (left) or endosomal (right) to total fluorescence was plotted as mean values ± SEM (asterisk indicates $P < 0.005$ for comparison with mock-transfected by *t* test). (C) Immunoreactive PIP_2 (red) was detected in permeable PC12 cells expressing $ARF6^{WT}$, ARF^{Q67L}, or $ARF6^{T27N}$ (green). Immunoreactive PIP_2 was decreased in $ARF6^{Q67L}$-expressing cells (arrows). (D) The fluorescence intensity of PIP_2 antibody staining was quantified and normalized to membrane area in 50 randomly selected cells. Values are means ± SEM with asterisk corresponding to $P < 0.005$ for comparison with mock-transfected by *t* test). Bar, 20 μm.

treatment also affected mannosidase II–containing and TGN38-containing Golgi elements (Fig. 2 A), as well as transferrin-positive endosomal compartments (unpublished data). The distributions of wild-type and $ARF6^{Q67L}$ (unpublished data) and endocytosed transferrin, a marker for recycling endosomes, overlapped extensively, but not precisely (Fig. 2 B, inset), indicating that cytoplasmic ARF6 proteins were localized to a subdomain of recycling endosomes. Consistent with this, ARF6-containing membranes colocalized extensively with Rab11-positive late endosomes (Fig. 2 B, arrows). The localization of ARF^{Q67L} at the plasma membrane and in recycling endosomes suggested that the $ARF6^{Q67L}$ inhibition of DCV exocytosis was indirect and caused by alterations in endosomal plasma membrane trafficking.

Recruitment of PIP5K by $ARF6^{Q67L}$ alters the cellular distribution of PIP_2

In other cell types, the expression of the GTPase-deficient $ARF6^{Q67L}$ induces an accumulation of endosomal membranes leading to the intracellular trapping of plasma membrane constituents that normally recycle (Brown et al., 2001; Naslavsky et al., 2003). We considered the possibility that a plasma membrane constituent essential for regulated DCV exocytosis was depleted from the plasma membrane in cells expressing $ARF6^{Q67L}$. Indeed, SNAP-25, a plasma membrane SNARE required for DCV exocytosis (Banerjee et al., 1996), was found to substantially relocalize to an endosomal compartment in $ARF6^{Q67L}$-expressing cells (unpublished data); however, we were unable to rescue regulated hGH release by overexpressing SNAP-25. Another



plasma membrane constituent required for DCV exocytosis is PIP_2 (Hay et al., 1995), and we found that plasma membrane pools of PIP_2 were dramatically altered by $ARF6^{Q67L}$ expression. A PLC δ1 pleckstrin homology (PH) domain–GFP fusion protein was expressed to detect membrane-associated PIP_2 as described previously (Varnai and Balla, 1998). PC12 cells expressing high levels of wild-type ARF6 exhibited a uniform distribution of the PH-GFP at the plasma membrane (Fig. 3 A), which colocalized with plasma membrane ARF6. In contrast, cells expressing $ARF6^{Q67L}$ exhibited a very different distribution of the PH-GFP protein. At low expression levels, the PH-GFP localized to the plasma membrane as well as to intracellular endosomal membranes that contained $ARF6^{Q67L}$ (Fig. 3 A). Cells expressing higher levels of $ARF6^{Q67L}$, representing ~25% of the cell population, accumulated intracellular inclusions enriched in the PH-GFP protein as well as $ARF6^{Q67L}$ (Fig. 3 A, arrow). Plasma membrane–associated PH-GFP was markedly decreased in these cells (Fig. 3 A, insets), and the cells resembled $ARF6^{Q67L}$-expressing fibroblasts in which PIP_2-positive vacuoles accumulated (Brown et al., 2001). This feature was unique for $ARF6^{Q67L}$ expression and was not observed for $ARF6^{WT}$ or $ARF6^{T27N}$ (Fig. 3 A). Quantitation of digital images documented that the PH-domain of PLCδ1 was depleted from the plasma membrane (Fig. 3 B, left) and preferentially accumulated on endosomal membranes (Fig. 3 B, right) in cells expressing $ARF6^{Q67L}$ and, to a much lesser extent, wild-type ARF6, but not $ARF6^{T27N}$ or $ARF6^{N122I}$, another GTP-binding–defective mutant (Fig. 3 B, $P < 0.005$).

A shift of the PH-GFP protein from plasma membrane to endosomes in $ARF6^{Q67L}$-expressing cells implied that the distribution of PIP_2 was markedly altered. To confirm this directly, we determined the plasma membrane content of PIP_2 in permeable cells using a PIP_2 antibody that revealed a punctate staining pattern (Fig. 3 C, red channel). The PIP_2 content of the plasma membrane was not altered in cells expressing $ARF6^{WT}$ or $ARF6^{T27N}$ (Fig. 3 C), but was strongly decreased in cells expressing $ARF6^{Q67L}$ (Fig. 3 C, arrows). Quantitation of digital images documented a highly significant decrease for plasma membrane PIP_2 in cells expressing $ARF6^{Q67L}$ (Fig. 3 D). Overall, the results indicated that $ARF6^{Q67L}$ expression promotes a redistribution of PIP_2 in PC12 cells from the plasma membrane to an intracellular endosomal compartment.

The preferential accumulation of PIP_2 in an endosomal compartment might result from either the translocation of plasma membrane containing PIP_2 or the redirected synthesis of PIP_2 on endosomes. ARF proteins directly regulate PIP5K activity (Honda et al., 1999; Jones et al., 2000), and the constitutively active $ARF6^{Q67L}$ localized to endosomes may persistently activate PIP5K on endosomal membranes. To examine this possibility directly, we coexpressed HA-tagged PIP5KIγ with ARF6-GFP and determined their distribution (Fig. 4). PIP5KIγ preferentially localized to the plasma membrane in cells expressing wild-type ARF6 or low

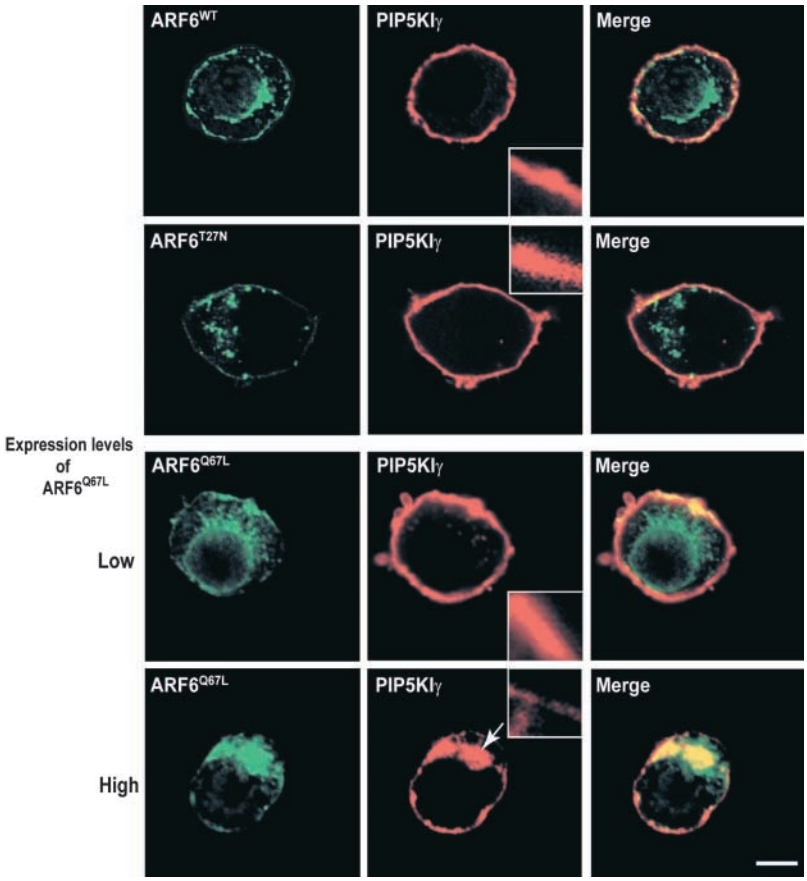


Figure 4. **PIP5KIγ is recruited to an endosomal compartment in ARF6^{Q67L}-expressing PC12 cells.** PC12 cells were cotransfected for 48 h with plasmids encoding EGFP-ARF6 proteins (green) and HA-PIP5KIγ (detected by HA antibody, red). Insets show magnified regions of the plasma membrane. Cells expressing relatively high levels of ARF6^{Q67L} exhibited an endosomal accumulation of PIP5KIγ (arrow) and its depletion from the plasma membrane (inset). Bar, 10 μm.

levels of ARF6^{Q67L} where there was extensive colocalization of PIP5KIγ with the ARF6 proteins (Fig. 4). Cells expressing higher levels of ARF6^{Q67L} exhibited a striking redistribution of PIP5KIγ to intracellular membranes where the enzyme colocalized with the ARF6 protein (Fig. 4, arrow). Plasma membrane PIP5KIγ was substantially decreased in these cells (Fig. 4, insets), indicating that ARF6^{Q67L} diverts PIP5K to endosomal membranes. This redistribution of PIP5K would account for the accumulation of PIP_2 in an endosomal compartment (Fig. 3 A, arrow). The results indicated that the GTPase-deficient ARF6^{Q67L} induces a shift in the distribution of PIP5K and PIP_2 from the plasma membrane to intracellular membranes.

Restoration of Ca^{2+}-dependent exocytosis by PIP5K by a Ca^{2+}-dependent mechanism

To further probe the mechanism by which ARF6^{Q67L} inhibited Ca^{2+}-dependent DCV exocytosis, we determined whether inhibition was preserved in permeable PC12 cells where DCV exocytosis can be directly triggered by Ca^{2+} addition (Hay and Martin, 1992). Expression of ARF6^{Q67L} (but not wild-type ARF6 or ARF6^{T27N}) resulted in the inhibition of Ca^{2+}-triggered hGH release by ~48% in permeable cells (Fig. 5 A, middle bars), which corresponded closely to the intact cell assay results (Fig. 1 A). However, when MgATP and rat brain cytosol were included in the incubations to fully reconstitute Ca^{2+}-dependent exocytosis, the inhibitory effect of ARF6^{Q67L} was not observed (Fig. 5 A, right bars). This reversal of inhibition in the permeable cells was dependent on MgATP concentration (Fig. 5 C) and the presence of cytosol (compare Fig. 5 D with Fig. 5 A).

To determine whether MgATP and cytosol were sufficient for reversing the inhibitory effects of ARF6^{Q67L}, a two-stage assay was used. MgATP- and cytosol-dependent priming reactions were conducted before Ca^{2+}-triggering incubations, which contained Ca^{2+} and cytosol, but not MgATP (Hay and Martin, 1992). We found that the inhibition by ARF6^{Q67L} was preserved in the two-stage assay over the entire time course of Ca^{2+}-evoked hGH release (Fig. 5 B). Together with the other findings, this result indicates that all three constituents, MgATP, cytosol, and Ca^{2+}, but no two of these alone, were essential for restoring regulated DCV exocytosis in ARF6^{Q67L}-expressing cells.

Because the reversal of ARF6^{Q67L} inhibition was dependent on MgATP and cytosol in permeable cells in which plasma membrane PIP_2 and PIP5K had been depleted, it was very likely that the soluble PIP5K enzymes present in cytosol were responsible for the restoration of DCV exocytosis. To test the role of PIP5K directly, we attempted to rescue the inhibitory effect of ARF6^{Q67L} by expressing the type Iγ PIP5K. Expression of PIP5KIγ successfully counteracted the inhibitory effect of ARF6^{Q67L} on Ca^{2+}-activated hGH release in permeable cells (Fig. 6 A). As anticipated, rescue by PIP5KIγ was only evident in incubations that included MgATP and failed to occur with expression of a kinase-dead truncation mutant of PIP5KIγ Δ345 (Fig. 6 A), which did not localize to the plasma membrane (unpublished data). In addition, the reversal of ARF6^{Q67L} inhibition by expression of the wild-type PIP5KIγ enzyme did not require addition

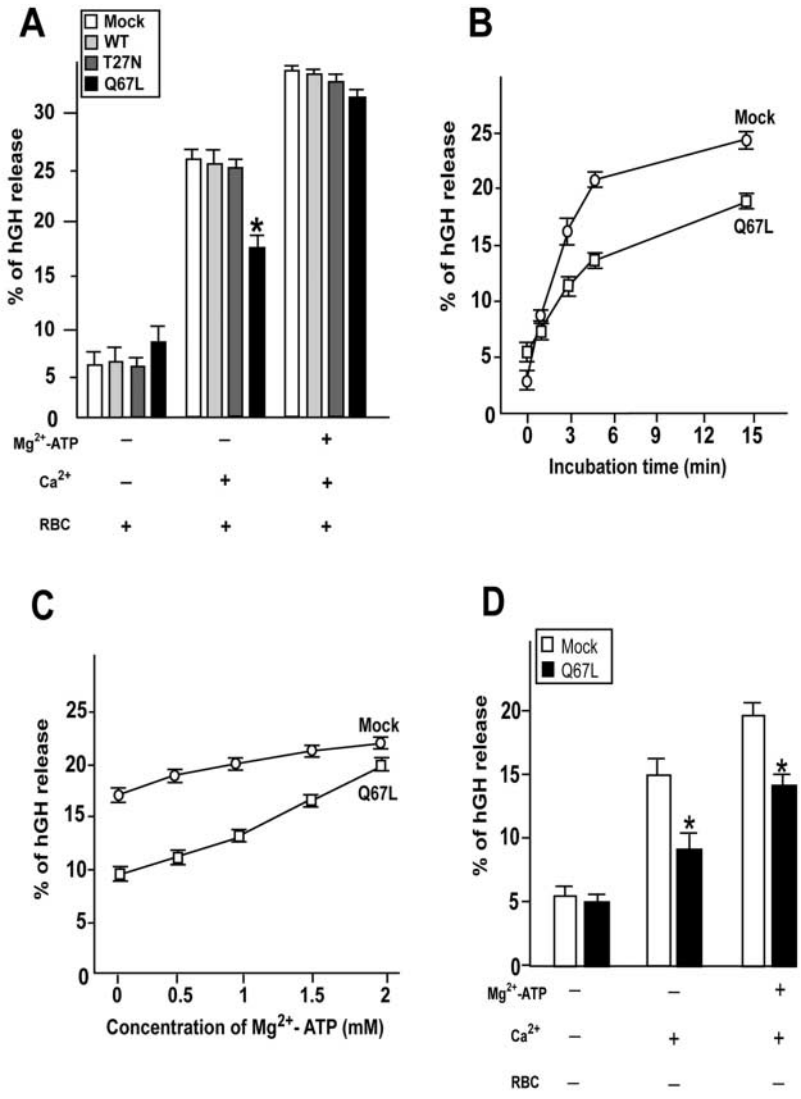


Figure 5. **The inhibition of DCV exocytosis by $ARF6^{Q67L}$ is preserved in permeable cells and reversed in incubations containing Ca^{2+}, MgATP, and cytosol.** PC12 cells were cotransfected for 48 h with plasmids encoding hGH and $ARF6^{WT}$, $ARF6^{T27N}$, or $ARF6^{Q67L}$ as indicated, and permeable cell hGH secretion assays were conducted (shown as mean ± SEM, $n = 4$). (A) Inhibition of DCV exocytosis in $ARF6^{Q67L}$-expressing cells was preserved on permeabilization, but only in incubations lacking MgATP. Incubations contained Ca^{2+}, MgATP, and rat brain cytosol (RBC) as indicated. (B) Reversal of inhibition in $ARF6^{Q67L}$-expressing cells required all three components. Two-stage secretion assays were conducted with MgATP and cytosol in priming incubations and Ca^{2+} and cytosol in triggering incubations (for times indicated). (C) Reversal of inhibition in $ARF6^{Q67L}$-expressing cells required MgATP. Incubations similar to those in A were conducted with Ca^{2+}, cytosol, and indicated concentrations of MgATP. (D) Reversal of inhibition in $ARF6^{Q67L}$-expressing cells was not observed in the absence of cytosol. Incubations similar to those in A were conducted with Ca^{2+} or MgATP in the absence of cytosol (RBC). Asterisks indicate significant ($P < 0.01$) differences with mock-transfected cells.

of cytosol (Fig. 6 A). These results indicate that reversal of $ARF6^{Q67L}$ inhibition can be achieved by PIP5KIγ overexpression, and they imply that the factor in cytosol required for the reversal is one of the abundant cytosolic PIP5K enzymes (Hay et al., 1995; Wenk et al., 2001). Indeed, direct addition of a pure recombinant PIP5KIγ protein to the permeable cell assay was effective in counteracting the inhibitory effects of $ARF6^{Q67L}$ (Fig. 6 B).

Multiple isoforms of PIP5K have been characterized consisting of types Iα, Iβ, and Iγ (Ishihara et al., 1998; Anderson et al., 1999). We found that expression of the type Iα (human) PIP5K also reversed the $ARF6^{Q67L}$ inhibition, whereas the kinase-dead (D309N/R427Q) PIP5KIα failed to provide rescue (Fig. 6 C). Both active and inactive enzymes were equally well expressed and associated with the plasma membrane (Fig. 6 D). Expression of the type Iβ (human) PIP5K provided only weak rescue, but was poorly expressed (unpublished data). Overall, the results strongly support the conclusion that the depletion of PIP_2 from the plasma membrane induced by long-term expression of $ARF6^{Q67L}$ was responsible for the inhibition of regulated DCV exocytosis.

PIP5KIγ is regulated by phosphorylation- and ARF6-dependent mechanisms

These results on PIP5K provided an explanation for why MgATP and cytosol counteracted the inhibition of exocytosis mediated by $ARF6^{Q67L}$ expression, but they did not account for the additional requirement for Ca^{2+} in the rescue. Thus, we determined whether a requirement for Ca^{2+} in the restoration was related to the regulation of PIP5K activity. The phosphorylation of Ser214 in mouse PIP5KIα was reported to decrease lipid kinase activity, whereas PKC was reported to stimulate lipid kinase activity by promoting protein phosphatase–mediated dephosphorylation of the enzyme (Park et al., 2001). Thus, the requirement of Ca^{2+}, in addition to MgATP and cytosol, for counteracting $ARF6^{Q67L}$ inhibition might be mediated by the Ca^{2+}-dependent activation of PKC, which may enhance PIP5K activity. Consistent with this, we found that the specific pseudosubstrate inhibitor of PKC fully blocked the reversal of $ARF6^{Q67L}$ inhibition observed with Ca^{2+}, MgATP, and cytosol in permeable cell incubations (Fig. 7 A).

To determine the role of dephosphorylation in the regulation of PIP5K activity, we constructed the cognate nonphos-

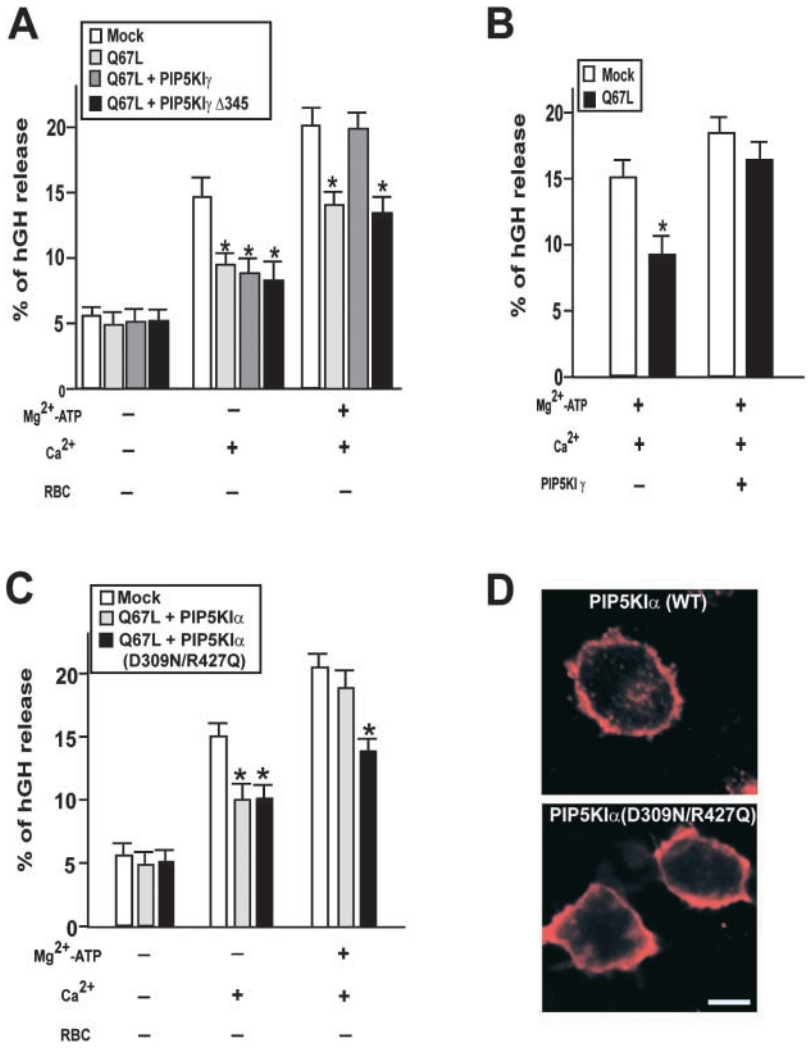


Figure 6. **Expression or addition of PIPKIγ to permeable cells replaces cytosol in restoring DCV exocytosis in ARF6^{Q67L}-expressing cells.** PC12 cells were transfected for 48 h with plasmids encoding hGH and either ARF6^{Q67L}or PIP5KIγ as indicated, and permeable cell hGH secretion assays were conducted. (A) Expression of PIP5KIγ restored Ca^{2+}-dependent hGH secretion in ARF6^{Q67L}-expressing cells in the presence (but not in the absence) of MgATP. Expression of the Δ345 PIP5KIγ mutant failed to restore Ca^{2+}-dependent secretion. (B) Purified PIP5KIγ replaced cytosol and restored Ca^{2+}-dependent hGH secretion in ARF6^{Q67L}-expressing cells. Incubations similar to those of A were conducted with Ca^{2+}, MgATP, and 0.6 μg/ml (~7 nM) PIP5KIγ where indicated. (C) Expression of PIP5KIα, but not a kinase-dead (D309N/R427Q) mutant, replaced cytosol in restoring Ca^{2+}-dependent hGH secretion in ARF6^{Q67L}-expressing cells in the presence (but not in the absence) of MgATP. Results are representative of at least two experiments with mean hGH values ± SEM ($n = 4$). (D) Wild-type and kinase-dead FLAG-tagged PIP5KIα proteins were expressed at similar levels and were plasma membrane localized. Immunocytochemistry with FLAG antibodies was conducted. Bar, 10 μm. Asterisks indicate significant ($P < 0.01$) differences with mock-transfected cells.

phorylatable S264A mutant of PIP5KIγ, which was anticipated to be active relative to the wild-type enzyme. Both wild-type and S264A PIPKIγ were expressed at similar levels in transfected PC12 cells, and each localized to the plasma membrane (Fig. 7 D, left panels). Expression of the S264A PIP5KIγ mutant enhanced Ca^{2+}-dependent hGH secretion by ~30% (Fig. 7 B), whereas expression of wild-type PIP5KIγ had little effect, consistent with the possible constitutive activity of the S264A PIPKIγ enzyme. Indeed, cells expressing the S264A mutant PIPKIγ exhibited substantially greater increases in plasma membrane PIP_2 than those expressing the wild-type enzyme (Fig. 7 C and Fig. 7 Da, right). Quantitation of plasma membrane immunoreactive PIP_2 (Fig. 7 Db) indicated that >32% of S264A PIP5KIγ-expressing cells exceeded threshold values of immunoreactive PIP_2, compared with <10% of wild-type PIPKIγ-expressing cells. Overall, the results indicate that phosphorylation at Ser264 may control the activity of PIP5KIγ and that plasma membrane PIP_2 levels regulate Ca^{2+}-dependent exocytosis.

ARF proteins were reported to directly activate purified type Iα (mouse) PIP5K (Honda et al., 1999; Jones et al., 2000) and ARF6 was shown to be recruited with PIP5K Iα to plasma membrane sites in fibroblasts (Honda et al., 1999), but a direct interaction of ARF6 with PIP5K has not been demonstrated. To determine whether ARF6 associates with PIP5K, coimmunoprecipitation experiments were conducted (Fig. 8 A). ARF6 was found to co-immunoisolate with PIP5KIγ in resting cells, and this was enhanced by Ca^{2+} influx stimulated by K^+ depolarization (Fig. 8 A, a and b). ARF6^{Q67L} and ARF6^{N122I} proteins exhibited similar associations with PIP5KIγ that were enhanced by Ca^{2+} influx (Fig. 8 Ba). The results indicate that the association of ARF6 with PIP5K is regulated by a Ca^{2+}-dependent mechanism.

To determine if this Ca^{2+}-dependent mechanism involves PIP5K dephosphorylation, immunoprecipitations were conducted with cells expressing the nonphosphorylatable S264A PIP5KIγ mutant. ARF6 exhibited substantial association with the S264A enzyme in resting cells, and this was not further enhanced by K^+ depolarization and Ca^{2+} influx (Fig. 8 A, a and b). ARF6^{Q67L} and ARF6^{N122I} proteins exhibited a similar association with the S264A PIP5KIγ that was not enhanced by Ca^{2+} influx (Fig. 8 Bb). The results indicate that, in addition to enhancing PIP5K activity, Ca^{2+} increases the association of PIP5K with ARF6. Interactions between ARF6 and PIP5K appear to be governed principally by the phosphorylation state of PIP5K rather than the guanine nucleotide–bound state of ARF6. The association of PIP5K with membrane-bound ARF6 may be important for targeting the

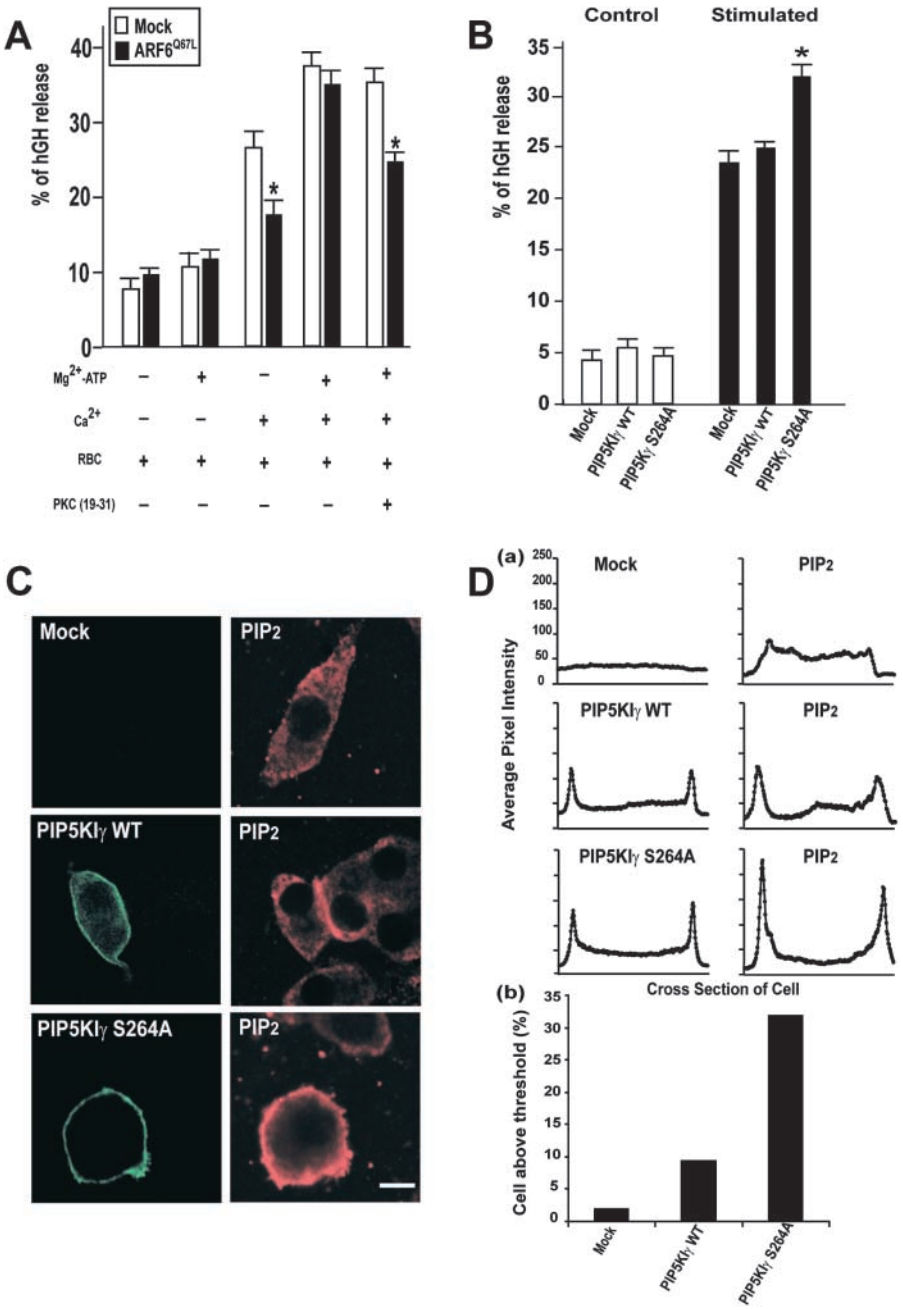


Figure 7. **PIP5KIγ is regulated by Ca^{2+} and dephosphorylation.** (A) The restoration of Ca^{2+}-dependent hGH secretion in permeable $ARF6^{Q67L}$-expressing cells by Ca^{2+}, MgATP, and cytosol is prevented by inhibition of PKC. Permeable cell incubations were conducted 48 h after transfection with hGH- and $ARF6^{Q67L}$-encoding plasmids. Restoration of Ca^{2+}-dependent hGH release was observed with MgATP and cytosol, but not in the presence of 5 μg/ml PKC(19–31) pseudosubstrate inhibitor. (B) Expression of S264A PIP5KIγ (but not wild-type PIP5KIγ) enhanced Ca^{2+}-dependent hGH secretion. PC12 cells were cotransfected with plasmids encoding hGH and wild-type or S264A PIP5KIγ as indicated. hGH release from intact cells in Na^+ (control) or K^+ (stimulated) buffers was determined. Asterisks indicate significant ($P < 0.05$) differences with mock-transfected cells. (C) PC12 cells were probed with HA antibody to detect PIP5KIγ (green) and PIP_2 antibody to detect PIP_2 (red). Plasma membrane immunoreactive PIP_2 increased in cells expressing S264A PIP5KIγ. Bar, 10 μm. (D, a) Quantitation of PIP_2 in cells expressing PIP5KIγ or S264A PIP5KIγ. A representative density profile plot was generated using SCION Image for green channel (PIP5K, left) and red channel (PIP_2, right). Pixel intensity was determined by a "row average plot" of a section selected from a confocal slice through the center of the cell. (D, b) The percentage of cells exhibiting average pixel intensities above 150 for immunoreactive PIP_2 is shown for mock transfectants and cells expressing wild-type or S264A PIP5KIγ. 50 randomly selected cells of each type were quantitated.

enzyme to its membrane PI(4)P substrate. Overall, the results suggest a pathway by which Ca^{2+} enhances PIP_2 synthesis for DCV exocytosis via an ARF6-dependent PIP5K (Fig. 9).

Discussion

Basis of the inhibition of DCV exocytosis by $ARF6^{Q67L}$ expression

In this work, we sought to characterize the role of ARF6 in the mechanisms that regulate Ca^{2+}-dependent DCV exocytosis in neuroendocrine PC12 cells. Expression of the constitutively active GTPase-deficient $ARF6^{Q67L}$ mutant exerted a strong inhibitory effect on regulated DCV exocytosis. Subsequent analyses clarified the nature of the inhibitory effect as consisting of a diversion of plasma membrane constituents that are required for DCV exocytosis, PIP5K, and PIP_2, to endosomal membranes with their corresponding depletion from the plasma membrane.

As reported for other cell types (D'Souza-Schorey et al., 1995; Peters et al., 1995), the distribution of ARF6 proteins in PC12 cells was restricted to the plasma membrane and endosomal membranes. The restricted localization of ARF6 to these compartments in PC12 cells was unanticipated in view of the reported biochemical localization of ARF6 to the DCVs of chromaffin cells (Galas et al., 1997; Caumont et al., 1998). However, the degree of purity of DCVs was not assessed in those experiments, and immunocytochemical localization analyses were not reported. Vitale et al. (2002) reported immunocytochemical experiments in PC12 cells indicating that ARF6 proteins colocalize with DCV markers.

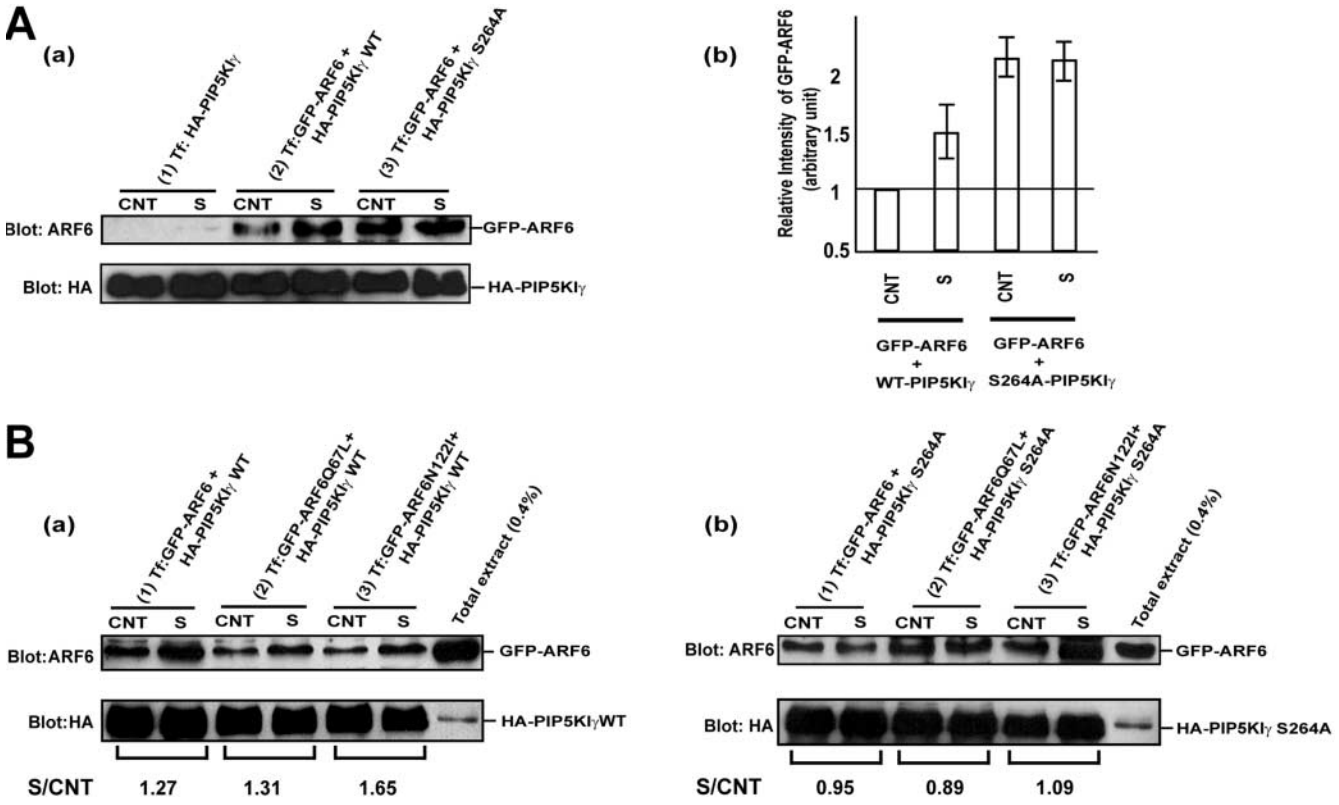


Figure 8. **ARF6 associates with PIP5KIγ.** PC12 cells were cotransfected with plasmids encoding EGFP-ARF6^{WT}, EGFP-ARF6^{Q67L}, or EGFP-ARF6^{N122I} and HA-tagged wild-type or S264A PIP5KIγ as indicated. (A, a) Ca^{2+} influx and dephosphorylation promote the association of ARF6 with PIP5KIγ. Cells cotransfected for 48 h with indicated plasmids were incubated in Na^{+}-containing (control, CNT) or K^{+}-containing (stimulated, S) buffers for 5 min. HA antibody immunoprecipitates were prepared from detergent lysates and analyzed by Western blotting for EGFP-ARF6 and HA-PIP5KIγ. Co-immunoprecipitation of EGFP-ARF6 with HA antibody was enhanced by K^{+} depolarization in PIP5KIγ-expressing cells. In S264A PIP5KIγ-expressing cells, coimmunoprecipitation was increased in resting cells and not enhanced by K^{+} depolarization. (A, b) Quantitation of ARF6 coimmunoprecipitation with PIP5KIγ from 3 experiments (± SEM) similar to that in panel a. (B) Co-immunoprecipitation studies similar to those in panel A were conducted for cells cotransfected with plasmids encoding ARF6^{WT}, ARF6^{Q67L}, and ARF6^{N122I} with (a) wild-type HA-PIP5KIγ or (b) HA-S264A PIP5KIγ. Values under each lane pair indicate fold stimulation by K^{+} depolarization.

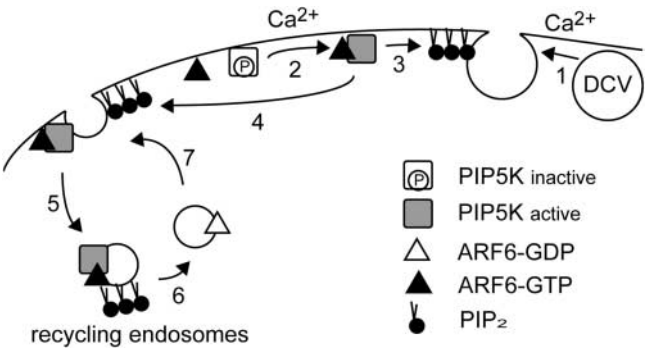


Figure 9. **ARF6 regulation of Ca^{2+}-dependent DCV exocytosis in PC12 cells.** (1) Ca^{2+} influx stimulates exocytosis of docked DCVs, but only if plasma membrane PIP_2 is available. (2) Ca^{2+} influx promotes the dephosphorylation of PIP5KI. (3) Dephospho-PIP5KI associates with ARF6 and is activated to synthesize PIP_2 for DCV exocytosis. (4) Increased PIP_2 synthesis drives endocytosis and retrieval of the DCV membrane after exocytosis. (5) Constitutive endocytosis may be enhanced by ARF6^{Q67L} stimulation of PIP5K. (6 and 7) Lack of GTP hydrolysis on ARF6^{Q67L} and constitutive PIP_2 production on endosomes prevents recycling to the plasma membrane. Entrapment of plasma membrane constituents and diversion of PIP5K and PIP_2 to endosomes in ARF6^{Q67L}-expressing cells results in the inhibition of DCV exocytosis.

However, there was a broad cytoplasmic distribution of both ARF6 and DCV markers by fluorescence microscopy that would preclude resolution of the clearly distinct distributions that we observed (Fig. 1 C). Moreover, an immunogold electron microscopic analysis (Vitale et al., 2002) was consistent with the localization of ARF6 proteins to cytoplasmic membranes other than DCVs.

In other cell types, ARF6 traffics between endosomal and plasma membrane compartments and regulates endosome-plasma membrane trafficking. Perturbations in endosome-plasma membrane trafficking that result from expression of the constitutively active ARF6^{Q67L} mutant vary with cell type, internalized protein, time of expression, and distribution of the actin cytoskeleton (Radhakrishna et al., 1996; Radhakrishna and Donaldson, 1997; Altschuler et al., 1999; Brown et al., 2001; Delaney et al., 2002; Palacios et al., 2002). Long-term expression of the constitutively active ARF6^{Q67L} protein in PC12 cells resulted in the accumulation of a subset of plasma membrane constituents in an endosomal compartment. This likely resulted from both accelerated internalization as well as inhibited recycling of constituents back to the plasma membrane. Activated ARF6 likely enhances both clathrin-dependent and -independent pathways of inter-

nalization in PC12 cells. Plasma membrane SNAP-25 is co-internalized with PIP_2-containing, $ARF6^{Q67L}$-enriched membranes by a dynamin-independent pathway (unpublished data), but $ARF6^{Q67L}$-expressing cells also exhibit increased initial rates of transferrin uptake (unpublished data) presumed to be via clathrin- and dynamin-mediated endocytosis. $ARF6^{Q67L}$ expression also accelerates the formation of synaptic vesicle-like endosomes in PC12 cells (Powelka and Buckley, 2001) that are formed by a clathrin-dependent pathway (Strasser et al., 1999). These results indicate that multiple endocytic pathways are enhanced by expression of the constitutively active $ARF6^{Q67L}$ in PC12 cells.

ARF proteins directly activate PIP5K enzymes and regulate the synthesis of PIP_2 (Honda et al., 1999; Jones et al., 2000). Plasma membrane PIP_2 is an essential cofactor for clathrin-dependent endocytosis (Barbieri et al., 2001; Cremona and DeCamilli, 2001; Galiano et al., 2002), phagocytosis (Botelho et al., 2000), pinocytosis (Schafer et al., 2000; Brown et al., 2001; Galiano et al., 2002), and endosome motility (Rozelle et al., 2000; Schafer et al., 2000). Thus, constitutive activation of PIP5K by $ARF6^{Q67L}$ could enhance the internalization of plasma membrane constituents through multiple pathways (Radhakrishna and Donaldson, 1997; Brown et al., 2001; Claing et al., 2001; Palacios et al., 2002; Naslavsky et al., 2003). However, ARF6-GTP cycling is essential for the trafficking of recycling endosomes back to the cell surface (Franco et al., 1999; Naslavsky et al., 2003). Because this fails to occur in the GTPase-deficient $ARF6^{Q67L}$ mutant, plasma membrane constituents are entrapped in an endosomal pool (Brown et al., 2001; Delaney et al., 2002; Palacios et al., 2002; Naslavsky et al., 2003). In PC12 cells expressing $ARF6^{Q67L}$, an endosomal compartment containing $ARF6^{Q67L}$, PIP_2, and PIP5K formed, which resulted in the depletion of these constituents from the plasma membrane.

Plasma membrane PIP_2 is essential for Ca^{2+}-dependent DCV exocytosis at the plasma membrane (Hay et al., 1995; Holz et al., 2000). That the depletion of PIP_2 from the plasma membrane in $ARF6^{Q67L}$-expressing PC12 cells was responsible for the inhibition of DCV exocytosis was established by demonstrating that expression or addition of PIP5K was capable of restoring regulated exocytosis in permeable cells incubated with MgATP. These results accounted for the absence of inhibition in permeable cell reconstitutions in which cytosol and MgATP were included, and strongly support the conclusion that plasma membrane PIP_2 is rate-limiting for DCV exocytosis in $ARF6^{Q67L}$-expressing cells. These results reinforce the conclusion that PIP_2 is essential for DCV exocytosis and indicate that ARF6 regulation of PIP5K plays a role in maintaining plasma membrane pools of PIP_2 required for exocytosis.

Regulation of PIP5K by ARF6 for Ca^{2+}-dependent DCV exocytosis

The restoration of Ca^{2+}-dependent DCV exocytosis in $ARF6^{Q67L}$-expressing permeable cells by PIP5K expression or addition explained the roles of cytosol and MgATP in the restoration, but not that of Ca^{2+}. The role of Ca^{2+} in the restoration may be mediated by PKC based on the effectiveness of a specific PKC pseudosubstrate inhibitor in blocking it. Although the involvement of PKC in Ca^{2+}-dependent DCV exocytosis is well established (Nishizaki et al., 1992; Chen et al., 1999), its precise role and the nature of PKC substrates involved in DCV exocytosis remain unknown. Park et al. (2001) reported that PKC contributes to the maintenance of cellular PIP_2 levels by regulating protein phosphatase-1, which catalyzes the dephosphorylation and activation of PIP5KI. The dephospho-PIP5KIα enzyme exhibits increased lipid kinase activity in vitro (Park et al., 2001), and we found that the cognate nonphosphorylatable S264A PIP5KIγ enzyme was activated to synthesize plasma membrane PIP_2 and enhance Ca^{2+}-dependent DCV exocytosis in PC12 cells. This result indicates that a nonphosphorylatable, activated PIP5KIγ enzyme is a positive effector for regulated exocytosis, which is particularly significant because Wenk et al. (2001) found that PIP5KIγ undergoes dephosphorylation in response to Ca^{2+} influx into synaptosomes. Eberhard and Holz (1991) also reported that Ca^{2+} influx increases PIP_2 synthesis in chromaffin cells. Together, these results indicate that PIP5K enzymes are Ca^{2+}-dependent effectors that are activated to promote PIP_2 synthesis at the plasma membrane.

ARF6 at the plasma membrane likely mediates the membrane targeting and localization of the activated PIP5K enzymes (Honda et al., 1999), and we found that wild-type PIP5K exhibited an increased association with ARF6 in response to Ca^{2+} influx into PC12 cells. The Ca^{2+}-dependent increase in ARF6 association may result from PIP5K dephosphorylation because the S264A mutant exhibited a strongly enhanced association with ARF6 that was not further increased by Ca^{2+} influx. These results are consistent with a model (Fig. 9) in which Ca^{2+} influx triggers the dephosphorylation of PIP5K, its increased association with ARF6, and its activation for plasma membrane PIP_2 synthesis.

Although the ATP-dependent priming of DCVs for Ca^{2+}-dependent exocytosis is known to require PIP_2 synthesis (Hay et al., 1995), our results provide the first indication that PIP_2 synthesis is regulated through an ARF6-dependent Ca^{2+}-triggered mechanism. The reported Ca^{2+} regulation of DCV priming (Bittner and Holz, 1992; Rettig and Neher, 2002) may in part be mediated by this ARF6-dependent pathway. The Ca^{2+}-triggered stimulation of PIP5K activity, particularly of the type Iγ isoform that is enriched at synapses (Wenk et al., 2001), would play an important role in enhancing Ca^{2+}-triggered exocytosis and endocytosis. In nerve terminals, plasma membrane PIP_2 levels decrease immediately upon Ca^{2+} influx (Micheva et al., 2001), and the Ca^{2+}-dependent recruitment and stimulation of PIP5K may be essential for the synthesis of additional PIP_2 needed to maintain rates of vesicle exocytosis and the endocytic retrieval of the vesicle membrane.

In conclusion, we present evidence that ARF6, through its regulation of PIP5K activity and PIP_2 synthesis, controls at least two types of peripheral membrane trafficking events in PC12 cells (Fig. 9). Ca^{2+}-dependent DCV exocytosis requires a plasma membrane pool of PIP_2 whose synthesis may be acutely regulated by Ca^{2+}-dependent activation of ARF6-dependent PIP5K enzymes. ARF6-dependent PIP5K enzymes also regulate endosome-plasma membrane trafficking. An imbalance in the ARF6-regulated endosome

recycling pathway can result in the depletion of plasma membrane constituents such as PIP_2 that are essential for DCV exocytosis.

Materials and methods

Constructs

The plasmid pXS-SRα containing COOH-terminal HA-tagged human ARF6 wild type, Q67L, and T27N were provided by J. Donaldson of the National Institutes of Health (NIH), Bethesda, MD. Constructs encoding COOH-terminal fusions of wild-type, Q67L, and T27N ARF6 proteins with EGFP were generated by PCR using the primers 5′-CGATAAGCTTATATC-CGACC-3′ and 5′-GGATACTGCAGGGATTTG-3′. The PCR product was cleaved with HindIII and PstI and inserted into HindIII-PstI–digested pEGFP-N1 (CLONTECH Laboratories, Inc.). All constructs were confirmed by sequencing. Coding sequences from a pBS plasmid containing HA-tagged murine full-length PIP5KIγ provided by H. Ishihara (University of Tokyo, Tokyo, Japan) were excised with HindIII and VspI and subcloned into pcDNA 3.1 (Invitrogen). HA-tagged murine Δ 344–635 PIP5KIγ was excised with HindIII and Alw441 and subcloned into pcDNA 3.1. The QuikChange® Site-Directed Mutagenesis method (Stratagene) was used to generate the PIP5KIγ (S264A) mutant using the sense primer 5′-CACGTA-CAAGCGCAGGGCCGCCAAGAAGGAGAAG-3′ (mutation site underlined). The pCMVGH plasmid encoding hGH was provided by H. Herweijer (Mirus Corp., Madison, WI) and an expression vector encoding PLC δ1 PH-GFP fusion protein provided by T. Balla (NIH).

Cell culture, transfection, and immunofluorescence

PC12 cells were cultured as described previously (Hay and Martin, 1992). Cells were transfected with 50 μg DNA in cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM KH_2PO_4, 2 mM EGTA, 5 mM $MgCl_2$, 2 mM MgATP, 5 mM glutathione, and 25 mM Hepes, pH 7.6) using an electroporator apparatus (Electroporator II; Invitrogen) set at 1,000 μF and 330 V. 48 h after transfection, cells were washed with PBS, fixed with 4% formaldehyde (wt/vol), permeabilized with PBS containing 0.3% Triton X-100 or 0.2% saponin, and blocked in 10% FBS in PBS before antibody incubations. For PIP_2 localization in intact cells using anti-PIP_2 antibody, we used methods described previously (Laux et al., 2000). For PIP_2 localization in permeable PC12 cells, the permeable cells were tethered to glutaraldehyde-activated, poly-L-lysine–coated glass coverslips (Wiedman et al., 1993). Antibodies used were polyclonal ARF6 (J. Donaldson and V. Klenchin; University of Wisconsin, Madison, WI); monoclonal and polyclonal HA (BAbCO); monoclonal FLAG M2 (Sigma-Aldrich); monoclonal SNAP-25 (Sternberger); monoclonal TGN38 (K. Howell, University of Colorado, Denver, Co); polyclonal mannosidase II (K. Moremen, University of Georgia, Athens, GA); monoclonal Rab11 (Zymed Laboratories); monoclonal PIP_2 (K. Fukami, University of Tokyo); and monoclonal chromogranin B (W. Huttner, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). For transferrin uptake, transfected cells were incubated in medium containing 50 μg/ml Texas red–conjugated transferrin (Molecular Probes, Inc.) for 30 min at 37°C, washed in PBS, and fixed. Images were obtained on an laser-scanning confocal microscope (MRC-600; Bio-Rad Laboratories) with a 63× oil immersion objective. Quantitation of fluorescence was conducted with MetaMorph® software (Universal Imaging Corp.) or SCION Image software (SCION Corp.).

Preparation of permeable PC12 cells and hGH secretion assays

hGH release experiments were performed 48 h after transfection. Adherent PC12 cells were incubated in medium containing 5 μg/ml BFA (Epicentre) for 30 min at 37°C to inhibit constitutive hGH secretion without affecting Ca^{2+}-dependent exocytosis. Cells were washed with Locke's solution (15 mM Hepes, pH 7.4, 145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 5.6 mM glucose, 0.5 mM ascorbic acid, and 0.1% BSA) and incubated for 20 min with calcium-free Locke's solution or with elevated K^+ solution (Locke's containing 59 mM KCl and 85 mM NaCl). Cell permeabilization and secretion assays were performed as described previously (Hay and Martin, 1992; Zhang et al., 2002) in KGlu buffer (50 mM Hepes, pH 7.2, 105 mM potassium glutamate, 20 mM potassium acetate, and 2 mM EGTA) with 0.1% BSA at 30°C. Single-stage assays contained 2 mM MgATP, 0.7 mg/ml rat brain cytosol, and 10 μM free Ca^{2+}. Two-stage assays used sequential incubations with MgATP plus cytosol and with Ca^{2+} plus cytosol with intervening washes. hGH secreted into medium or low speed supernatants and in 0.1% SDS-lysed cells was measured by radioimmunoassay (Nichols Institute Diagnostics).

Immunoprecipitation assays

After transfection, cells were washed and incubated for 5 min with Locke's or elevated K^+ buffer before harvesting in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 or NP-40, 50 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and Boehringer complete protease inhibitor cocktail). Lysates were sedimented and supernatants were adsorbed with protein G–Sepharose beads before conducting 3–16-h incubations with HA tag mAbs immobilized on protein G–Sepharose beads. Beads were washed five times with lysis buffer and eluted in sample buffer containing 0.2 M DTT for analysis by immunoblotting with ARF6 or HA antibodies.

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References

Altschuler, Y., S. Liu, L. Katz, K. Tang, S. Hardy, F. Brodsky, G. Apodaca, and K. Mostov. 1999. ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* 147:7–12.

Anderson, R.A., I.V. Boronenkov, S.D. Doughman, J. Lunz, and J.C. Loijens. 1999. Phosphatidylinositol phosphate kinases, a multifaceted family of signaling enzymes. *J. Biol. Chem.* 274:9907–9910.

Banerjee, A., J.A. Kowalchyk, B.R. DasGupta, and T.F. Martin. 1996. SNAP-25 is required for a late postdocking step in Ca^{2+}-dependent exocytosis. *J. Biol. Chem.* 271:20227–20230.

Barbieri, M.A., C.M. Heath, E.M. Peters, A. Wells, J.N. Davis, and P.D. Stahl. 2001. Phosphatidylinositol-4-phosphate 5-kinase-1beta is essential for epidermal growth factor receptor-mediated endocytosis. *J. Biol. Chem.* 276: 47212–47216.

Barylko, B., S.H. Gerber, D.D. Binns, N. Grichine, M. Khvotchev, T.C. Sudhof, and J.P. Albanesi. 2001. A novel family of phosphatidylinositol 4-kinases conserved from yeast to humans. *J. Biol. Chem.* 276:7705–7708.

Bittner, M.A., and R.W. Holz. 1992. Kinetic analysis of secretion from permeabilized adrenal chromaffin cells reveals distinct components. *J. Biol. Chem.* 267:16219–16225.

Botelho, R.J., M. Teruel, R. Dierckman, R. Anderson, A. Wells, J.D. York, T. Meyer, and S. Grinstein. 2000. Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. *J. Cell Biol.* 151:1353–1367.

Brown, F.D., A.L. Rozelle, H.L. Yin, T. Balla, and J.G. Donaldson. 2001. Phosphatidylinositol 4,5-bisphosphate and ARF6-regulated membrane traffic. *J. Cell Biol.* 154:1007–1017.

Caumont, A.S., M.C. Galas, N. Vitale, D. Aunis, and M.F. Bader. 1998. Regulated exocytosis in chromaffin cells. Translocation of ARF6 stimulates a plasma membrane-associated phospholipase D. *J. Biol. Chem.* 273:1373–1379.

Chen, Y.A., V. Duvvuri, H. Schulman, and R.H. Scheller. 1999. Calmodulin and protein kinase C increase Ca^{2+}-stimulated secretion by modulating membrane-attached exocytotic machinery. *J. Biol. Chem.*, 274:26469–26476.

Claing, A., W. Chen, W.E. Miller, N. Vitale, J. Moss, R.T. Premont, and R.J. Lefkowitz. 2001. β-Arrestin-mediated ADP-ribosylation factor 6 activation and β2-adrenergic receptor endocytosis. *J. Biol. Chem.* 276:42509–42513.

Cremona, O., and P. De Camilli. 2001. Phosphoinositides in membrane traffic at the synapse. *J. Cell Sci.* 114:1041–1052.

Delaney, K.A., M.M. Murph, L.M. Brown, and H. Radhakrishna. 2002. Transfer of M2 muscarinic acetylcholine receptors to clathrin-derived early endosomes following clathrin-independent endocytosis. *J. Biol. Chem.* 277: 33439–33446.

D'Souza-Schorey, C., G. Li, M.I. Colombo, and P.D. Stahl. 1995. A regulatory role for ARF6 in receptor-mediated endocytosis. *Science.* 267:1175–1178.

D'Souza-Schorey, C., E. van Donselaar, V.W. Hsu, C.D. Yang, P.D. Stahl, and P.J. Peters. 1998. ARF6 targets recycling vesicles to the plasma membrane: insight from an ultrastructural investigation. *J. Cell. Biol.* 140:603–616.

Eberhard, D.A., C.L. Cooper, M.G. Low, and R.W. Holz. 1990. Regulation of the

formation of inositol phosphates by calcium, guanine nucleotides and ATP in digitonin-permeabilized bovine adrenal chromaffin cells. *Biochem. J.* 268: 15–25.

Eberhard, D.A., and R.W. Holz. 1991. Calcium promotes the accumulation of polyphosphoinositides in intact and permeabilized bovine adrenal chromaffin cells. *Cell. Mol. Neurobiol.* 11:357–370.

Franco, M., P.J. Peters, J. Boretto, E. van Donselaar, A. Neri, C. D'Souza-Schorey, and P. Chavrier. 1999. EFA6, a Sec7 domain-containing exchange factor for ARF6, coordinates membrane recycling and actin cytoskeleton organization. *EMBO J.* 18:1480–1491.

Galas, M.C., J.B. Helms, N. Vitale, D. Thierse, D. Aunis, and M.F. Bader. 1997. Regulated exocytosis in chromaffin cells. A potential role for secretory granule-associated ARF6 protein. *J. Biol. Chem.* 272:2788–2793.

Galiano, F.J., E.T. Ulug, and J.N. Davis. 2002. Overexpression of murine phosphatidylinositol 4-phosphate 5-kinase type 1 disrupts a phosphatidylinositol 4,5 bisphosphate regulated endosomal pathway. *J. Cell. Biochem.* 85:131–145.

Hay, J.C., and T.F.J. Martin. 1992. Resolution of regulated secretion into sequential MgATP-dependent and calcium-dependent stages mediated by distinct cytosolic proteins. *J. Cell Biol.* 119:139–151.

Hay, J.C., and T.F.J. Martin. 1993. Phosphatidylinositol transfer protein required for Ca^{2+}-activated secretion. *Nature.* 366:572–575.

Hay, J.C., P.L. Fisette, G.H. Jenkins, K. Fukami, T. Takenawa, R.A. Anderson, and T.F.J. Martin. 1995. ATP-dependent inositide phosphorylation required for Ca^{2+}-activated secretion. *Nature.* 374:173–177.

Holz, R.W., M.D. Hlubek, S.D. Sorensen, S.K. Fisher, T. Balla, S. Ozaki, G.D. Prestwich, E.L. Stuenkel, and M.A. Bittner. 2000. A pleckstrin homology domain specific for phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5 P_2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P_2 as being important in exocytosis. *J. Biol. Chem.* 275:17878–17885.

Honda, A., M. Nagomi, T. Yokozeki, M. Yamazaki, H. Nakamura, H. Watanabe, K. Kawamoto, K. Nakayama, A.J. Morris, M.A. Frohman, and Y. Kanaho. 1999. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell.* 99: 521–532.

Ishihara, H., Y. Shibasaki, N. Kizuki, T. Wada, Y. Yazaki, T. Asano, and Y. Oka. 1998. Type I phosphatidylinositol-4-phosphate 5-kinases. *J. Biol. Chem.* 273:8741–8748.

Jones, D.H., J.B. Morris, C.P. Morgan, H. Kondo, R.F. Irvine, and S. Cockcroft. 2000. Type I phosphatidylinositol 4-phosphate 5-kinase directly interact with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the Golgi compartment. *J. Biol. Chem.* 275: 13962–13966.

Laux, T., K. Fukami, M. Thelen, T. Golub, D. Frey, and P. Caroni. 2000. GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J. Cell Biol.* 149:1455–1472.

Lippincott-Schwartz J., L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R.D. Klausner. 1991. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell.* 67:601–616.

Micheva, K.D., R.W. Holz, and S.J. Smith. 2001. Regulation of presynaptic phosphatidylinositol 4,5-bisphosphate by neuronal activity. *J. Cell Biol.* 154: 355–368.

Naslavsky, N., R. Weigert, and J.G. Donaldson. 2003. Convergence of non-clathrin- and clathrin-derived endosomes involves Arf6 inactivation and changes in phosphoinositides. *Mol. Biol. Cell.* 14:417–431.

Nishizaki, T., J.H. Walent, J.A. Kowalchyk, and T.F.J. Martin. 1992. A key roe for a 145 kD cytosolic protein in the stimulation of Ca^{2+}-dependent secretion by protein kinase C. *J. Biol. Chem.* 267:23972–23981.

Palacios, F., J.K. Schweitzer, R.L. Boshans, and C. D'Souza-Schorey. 2002. ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. *Nat. Cell Biol.* 4:929–936.

Park, S.J., T. Itoh, and T. Takenawa. 2001. Phosphatidylinositol 4-phosphate 5-kinase type I is regulated through phosphorylation response by extracellular stimuli. *J. Biol. Chem.* 276:4781–4787.

Peters, P.J., V.W. Hsu, C.E. Ooi, D. Finazzi, S.B. Teal, V. Oorschot, J.G. Donaldson, and R.D. Klausner. 1995. Overexpression of wild-type and mutant ARF1 and ARF6: distinct perturbations of nonoverlapping membrane compartments. *J. Cell Biol.* 128:1003–1017.

Powelka, A.M., and K.M. Buckley. 2001. Expression of ARF6 mutants in neuroendocrine cells suggests a role for ARF6 in synaptic vesicle biogenesis. *FEBS Lett.* 501:47–50.

Radhakrishna, H., and J.G. Donaldson. 1997. ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. *J. Cell Biol.* 139:49–61.

Radhakrishna, H., R.D. Klausner, and J.G. Donaldson. 1996. Aluminium fluoride stimulates surface protrusions in cells overexpressing the ARF6 GTPase. *J. Cell Biol.* 134:935–947.

Randazzo, P.A., Z. Nie, K. Miura, and V.W. Hsu. 2000. Molecular aspects of the cellular activities of ADP-ribosylation factors. *Sci. STKE.* 2000:RE1.

Rettig, J., and E. Neher. 2002. Emerging roles of presynaptic proteins in Ca^{2+}-triggered exocytosis. *Science.* 298:781–785.

Robinson, L.J., and T.F.J. Martin. 1998. Docking and fusion in neurosecretion. *Curr. Opin. Cell Biol.* 10:483–492.

Rozelle, A.L., L.M. Machesky, M. Yamamoto, M.H.E. Driessens, R.H. Insall, M.G. Roth, K. Luby-Phelps, G. Marriot, A. Hall, and H.L. Yin. 2000. Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Curr. Biol.* 10:311–320.

Schafer, D.A., C. D'Souza-Schorey, and J.A. Cooper. 2000. Actin assembly at membranes controlled by ARF6. *Traffic.* 1:892–903.

Strasser, J.E., M. Arribas, A.D. Blagoveshchenskaya, and D.F. Cutler. 1999. Secretagogue-triggered transfer of membrane proteins from neuroendocrine secretory granules to synaptic-like microvesicles. *Mol. Biol. Cell.* 10:2619–2630.

Varnai, P., and T. Balla. 1998. Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamics changes and relationship to myo-[3H] inositol-labeled phosphoinositide pools. *J. Cell Biol.* 143:501–510.

Vitale, N., S. Chasserot-Golaz, Y. Bailly, N. Morinaga, M.A. Frohman, and M.F. Bader. 2002. Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane. *J. Cell Biol.* 159:79–89.

Wenk, M.R., L. Pellegrini, V.A. Klenchin, G. DiPaolo, S. Chang, L. Daniell, M. Arioka, T.F.J. Martin, and P. DeCamilli. 2001. PIP kinase Iγ is the major PI(4,5)P_2 synthesizing enzyme at the synapse. *Neuron.* 32:79–88.

Wiedemann, C., T. Schafer, and M.M. Burger. 1996. Chromaffin granule-associated phosphatidylinositol 4-kinase activity is required for stimulated secretion. *EMBO J.* 15:2094–2101.

Wiedman, P., R. Roth, and J. Heuser. 1993. Golgi membrane dynamics imaged by freeze-etch electron microscopy: views of different membrane coatings involved in tubulation versus vesiculation. *Cell.* 75:123–133.

Zhang, X., M.J. Kim-Miller, M. Fukuda, J.A. Kowalchyk, and T.F.J. Martin. 2002. Ca^{2+}-dependent synaptotagmin binding to SNAP-25 is essential for Ca^{2+}-triggered exocytosis. *Neuron.* 34:599–611.