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

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The ADP ribosylation factor (ARF) GTP binding proteins are believed to mediate cytoskeletal remodeling and vesicular trafficking along the secretory pathway. Here we show that ARF6 is specifically associated with dense-core secretory granules in neuroendocrine PC12 cells. Stimulation with a secretagogue triggers the recruitment of secretory granules to the cell periphery and the concomitant activation of ARF6 by the plasma membrane-associated guanine nucleotide exchange factor, ARF nucleotide binding site opener (ARNO). Expression of the constitutively inactive ARF6(T27N) mutant inhibits secretagogue-dependent exocytosis from PC12 cells. Using a mutant of ARF6 specifically impaired for PLD1 stimulation, we find that ARF6 is functionally linked to phospholipase D (PLD)1 in the exocytotic machinery. Finally, we show that ARNO, ARF6, and PLD1 colocalize at sites of exocytosis, and we demonstrate direct interaction between ARF6 and PLD1 in stimulated cells. Together, these results provide the first direct evidence that ARF6 plays a role in calcium-regulated exocytosis in neuroendocrine cells, and suggest that ARF6-stimulated PLD1 activation at the plasma membrane and consequent changes in membrane phospholipid composition are critical for formation of the exocytotic fusion pore.

Introduction
Calculated release of hormones and peptides from neuroendocrine cells is mediated by the fusion of large, dense-core secretory granules with the plasma membrane, a spatially and temporally controlled process based on a complex network of interactions between regulatory molecules and mechanical components. Cytoskeletal rearrangements are a prerequisite for exocytosis. Actin filaments form a cortical network that separates secretory granules into a small release-ready granule pool and a larger reserve pool, subjecting the transit of granules to the plasma membrane to a carefully orchestrated regulation (Trifaro et al., 2000). Classically viewed as a barrier that hinders the movement of reserve granules to the plasma membrane (Aunis and Bader, 1988), it is now believed that the actin cortex provides also tracks along which the granules are pulled to the sites of exocytosis (Lang et al., 2000). Once docked at the plasma membrane, granules undergo membrane fusion through a mechanism governed by SNAREs (Jahn and Südhof, 1999; Chen and Scheller, 2001). The role of the membrane lipid composition at the site of fusion is less well understood, but several reports suggest that lipids are essential partners for proteins in the basic fusion machinery (Schmidt et al., 1999; Weigert et al., 1999; Vitale et al., 2001). A specific feature of the exocytotic release in secretory cells is its tight regulation, as the speed, length, and number of exocytotic events must be controlled. Thus, additional proteins able to link and coordinate the successive recruitment, docking, and fusion steps into the integrated secretion machinery are necessarily required but most of them remain to be identified.
ADP ribosylation factor (ARF)* GTPases play important roles in membrane trafficking events (Moss and Vaughan, 1998; Donaldson and Jackson, 2000). ARF6, the most structurally divergent member of the family (Chavrier and Goud, 1999), is found exclusively at the plasma and/or endosomal membranes in various cell types (Peters et al., 1995; Cavenagh et al., 1996). The protein has been proposed to play a role in cell motility (Song et al., 1998; Santy and Casanova, 2001), vesicle recycling at the plasma membrane (Radhakrishna and Donaldson, 1997; D’Souza-Schorey et al., 1998), Fc-mediated phagocytosis (Zhang et al., 1999a) and insulin-regulated secretion (Yang and Muekler, 1999), and Glut-4 translocation (Millar et al., 1999). Taken together, these findings suggest that ARF6 is an ubiquitous regulator of vesicle transport at the plasma membrane.

ARF6 is unique among the six mammalian ARFs in its ability to stimulate cortical actin rearrangements (Radhakrishna et al., 1999; Boshans et al., 2000). Because ARF6 also activates phospholipase D (PLD; Massenburg et al., 1994), a lipid-modifying enzyme recently described as a key factor for exocytosis in neurons and neuroendocrine cells (Humeau et al., 2001; Vitale et al., 2001), it would be attractive to hypothesize that ARF6 functions by coordinating reorganization of the actin cytoskeleton with the lipid modifications required for neuronal exocytosis. We demonstrate here that ARF6 is the sole member of the ARF family able to exert direct control on the exocytotic machinery. We show that ARF6 is in its inactive GDP-bound state when associated with secretory granules but then switches to its active GTP-bound state at the plasma membrane upon cell stimulation. Our results indicate that the guanine nucleotide exchange factor, ARF nucleotide binding site opener (ARNO), is a regulator of ARF6 functions in this setting. Finally, we show that ARF6 participates in the regulation of secretion, most likely through the direct activation of PLD1 at the site of exocytosis.

Results
Secretagogue-evoked stimulation activates ARF6 at the plasma membrane
To probe the role of ARF proteins in dense-core granule exocytosis, we first investigated their intracellular distribution. The relatively low level of endogenous ARFs and the difficulty of generating antibodies able to discriminate between the six members of the ARF family (which share ~70% identity), prompted us instead to visualize them using overexpression of epitope-tagged isoforms. PC12 cells were cotransfected with an HA-tagged representative of each ARF class and with growth hormone (GH) to visualize dense-core secretory granules (Caumont et al., 2000; Vitale et al., 2001). ARF1, ARF3, and ARF5 are concentrated in the perinuclear region (Fig. 1), which contains the Golgi as identified with the pECFP-Golgi marker (unpublished data). Neither ARF1, ARF3, nor ARF5 colocalized with the GH-labeled secretory granules distributed in the cytoplasm (Fig. 1). In contrast, ARF6 colocalized both with GH (Fig. 1) and GFP–chromogranin A (unpublished data) throughout the cell, indicating that it represents most likely the sole ARF protein associated with mature secretory granules in PC12 cells.

We then compared the distribution of overexpressed WT and mutant ARF6 in resting and stimulated PC12 cells using in addition anti-SNAP25 antibodies to visualize the plasma membrane. In resting cells, WT ARF6, the inactive ARF6(T27N) mutant unable to bind GTP, and the constitutively active ARF6(Q67L) mutant unable to hydrolyze GTP (Peters et al., 1995) associated with GH-containing secretory granules (Fig. 2). Membrane depolarization by high potassium concentrations initiates calcium-dependent exocytosis in PC12 cells. Accordingly, a 5-min stimulation with 59 mM K+/H11601 triggered a partial movement of secretory granules to the cell periphery and the docking of a portion of the granules at the plasma membrane, as indicated by the increased colocalization of GH with SNAP-25 (Fig. 2, mask GH/SNAP-25). WT and mutant ARF6 displayed a staining pattern very similar to GH in K+/H11001-treated cells, indicating that ARF6 accompanied the secretory granules to the periphery (Fig. 2, mask ARF6/SNAP-25). Immunoelectron microscopy confirmed the association of ARF6 with the plasma membrane.
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membrane after stimulation with elevated K\(^+\) (Fig. 3). ARF6 was detected not only on secretory granules within the cytoplasm and on granules docked at the plasma membrane, but also on portions of the plasma membrane seemingly free of granules. However, coated pits and vesicles were not decorated by gold particles, suggesting that ARF6 does not undergo clathrin-dependent endocytosis in this cell type.

To correlate the intracellular distribution of ARF6 with its nucleotide status, we used a biochemical approach that in the first phase offers an alternative method to probe ARF6 subcellular distribution: PC12 cells expressing WT or mutated ARF6 were incubated with \(^{32}\)Porthophosphate to label the ARF6-bound nucleotide, subjected to subcellular fractionation, and assayed for ARF6 distribution using immunoprecipitation and quantitation of the coprecipitated labeled nucleotide. As illustrated in Fig. 4 B, the labeled nucleotide was not recovered from the cytosol, signifying, as expected, that ARF6 is membrane associated. Instead, under resting conditions, most of the labeled guanine nucleotides were immunoprecipitated from the secretory granule fractions (Fig. 4 B), in agreement with the granule association of ARF6 observed by confocal and electron microscopy. However, stimulation with elevated K\(^+\) increased the amount of labeled guanine nucleotides detected in the plasma membrane fraction at the expense of the granule fraction (Fig. 4 B), consistent with the immunofluorescent results shown in Fig. 2 and the Western blot results in Fig. 4 A. This redistribution was exaggerated by ARF6(Q67L) and blunted by ARF6(T27N). Note that endogenous ARF6 similarly translocated from secretory granules to the plasma membrane upon cell stimulation (Fig. 4 A). The observation of ARF6 at the plasma membrane in stimulated cells could result from the fusion of granules into the plasma membrane at the sites of exocytosis. In support of this hypothesis, dopamine-\(\beta\)-hydroxylase, a granule membrane protein, could be detected in the plasma membrane-containing fraction when the membrane fractions were prepared from stimulated cells (Fig. 4 A). However, note the low level of GH in this fraction (Fig. 4 A), suggesting that most of the granule membrane recovered with the plasma membrane originated from granules that had already released their content. ARF6 could also directly interact with a plasma membrane–bound protein after granule docking, and thereby be retained at the plasma membrane upon cell fractionation.

In a second phase, this method permits the nucleotide status of ARF6 in resting and stimulated cells to be assessed by analyzing on chromatography plates the guanine nucleotides bound to the immunoprecipitated WT and mutant ARFs. As shown in Fig. 5, in resting cells, only GDP was found to be associated with the ARF6 on secretory granules, and this was true even for ARF6(Q67L) which exhibits a dramatic preference for GTP. Some ARF6(Q67L) was also detected on the plasma membrane in resting cells (Fig. 4 B), but it was found to be loaded both with GDP and GTP (Fig. 5). Stimulation with K\(^+\) triggered a partial translocation of the ARF6s to the plasma membrane, but only WT ARF6 and ARF6(Q67L) switched to their active GTP-bound state. 97% of the ARF6(T27N) remained in the GDP-bound state despite its association with the plasma membrane (Fig. 5). Taken together, these results indicate that the secretory granule–associated ARF6 protein is in its inactive GDP-bound form. Stimulation with a secretagogue triggers the activation of ARF6 but the GDP to GTP exchange occurs only at or after arrival to the plasma membrane.

**ARNO activates ARF6 at the plasma membrane in stimulated PC12 cells**

In chromaffin cells, ARNO, a nucleotide exchange factor for ARF proteins (Chardin et al., 1996; Frank et al., 1998b), is localized at the plasma membrane (Caumont et al., 2000).
To establish whether ARNO is the activator of ARF6 at the cell surface in stimulated PC12 cells, we compared the intracellular distribution of ARNO and ARF6. In resting PC12 cells, ARF6 associates with secretory granules as described earlier, whereas ARNO is restricted to the plasmalemma (Fig. 6 A). In contrast, in K⁺-stimulated cells, ARF6 colocalized with ARNO at the plasma membrane (Fig. 6 A). To investigate directly whether ARNO triggers nucleotide exchange on the plasma membrane–associated ARF6, we determined the nature of the guanine nucleotide bound to immunoprecipitated ARF6 in cells cotransfected with ARNO or the catalytically inactive ARNO(E156K) mutant that lacks nucleotide exchange activity (Frank et al., 1998a). Overexpression of ARNO or ARNO(E156K) modified neither the level of expression of ARF6 (unpublished data) nor its subcellular distribution in resting and stimulated PC12 cells (Fig. 6 B, top); as was shown earlier, ARF6 was detected exclusively in the fraction containing secretory granules and it was in its GDP-bound state (Fig. 6 B, bottom). Stimulation with potassium triggered ARF6 translocation to the plasma membrane to a similar extent in all of the cells (Fig. 6 B). However, in cells overexpressing ARNO, all of the plasma membrane–associated ARF6 was in its GTP-bound state, whereas in cells expressing ARNO(E156K) the plasma membrane–bound ARF6 remained in its GDP-bound form (Fig. 6 B). Thus, ARNO stimulates GDP/GTP exchange on ARF6 in PC12 cells. Critically, overexpression of ARNO did not induce GTP loading of the secretory granule–associated ARF6 in resting or stimulated cells, indicating that ARNO can activate ARF6 only at or near the plasma membrane.
ARF6 is a functional element of the exocytotic machinery in PC12 cells

To establish whether ARF6 plays a role in exocytosis, we examined the effect of overexpressing the various ARF isoforms using GH as a secretory reporter in PC12 cells (Vitale et al., 2001). As illustrated in Fig. 1, overexpression of ARF1, ARF3, ARF5, or ARF6 did not apparently modify the distribution of GH. However, expression of the constitutively active ARF1(Q71L) and ARF3(Q71L) proteins and the dominant-negative ARF1(T31N) and ARF3(T31N) slightly but significantly reduced the total cellular GH measured by radioimmunoassay (Fig. 7 B), in agreement with the participation of ARF1 and ARF3 in the biosynthetic pathway of secretory granules (Peters et al., 1995). Of the ARFs tested, only ARF6 mutants modified the GH secretion in response to elevated K+/H11001 (Fig. 7 C). Expression of the inactive ARF6(T27N) strongly inhibited GH secretion, whereas ARF6(Q67L) significantly stimulated it (Fig. 7 C). The degree of inhibition observed is consistent with other studies using this assay to characterize molecular components of regulated exocytosis (Holz et al., 1994; Sugita et al., 1999).

It is also noteworthy that expression of the catalytically inactive ARNO(E156K) mutant resulted in a comparable inhibition of K+-evoked GH secretion in PC12 cells (Caumont et al., 2000), most likely because ARNO(E156K) forms an abortive ternary complex with endogenous ARF6 in which GDP remains bound to the nucleotide binding site (Béraud-Dufour et al., 1998).

To confirm that ARF6 plays a role in the exocytic machinery, we examined the effect of the ARF6 mutants on exocytic activity as visualized by the binding of fluorescent annexin 5 to patches of phosphatidylserine exposed on the cell surface by secretory granule fusion (Demo et al., 1998; Caumont et al., 2000). Resting PC12 cells showed no fluorescent annexin 5 patches (Fig. 7 D), indicating that the annexin 5 binding assay does not detect baseline activity in the absence of secretagogue. Stimulation with elevated K+ triggered the appearance of a patchy pattern of surface annexin 5 binding in cells expressing ARF6 or ARF6(Q67L), but not in cells expressing the inactive ARF6(T27N) mutant (Fig. 7 D). In line with the finding that ARF6(T27N) inhibits GH secretion from PC12 cells, this observation suggests that activation of ARF6 is critically important for calcium-evoked exocytosis of dense-core secretory granules.
PLD1 is a molecular partner of ARF6 in the exocytotic machinery

ARF proteins including ARF6 are established activators of PLD. In neurons and neuroendocrine cells, we previously described that a plasma membrane–associated PLD1 plays an important function in a late postdocking step of exocytosis (Humeau et al., 2001; Vitale et al., 2001). In order to test whether PLD1 might be an effector of ARF6 in the exocytotic process, we generated a mutant of ARF6 unable to stimulate PLD activity, namely ARF6(N48I), based on analogy with a similar mutant generated for ARF1 by Jones et al. (1999). As illustrated in Fig. 8 A, GTPγS binding to recombinant myrARF6(N48I) was indistinguishable from that of WT myrARF6. Dissociation of GDP from myrARF6(N48I) and myrARF6 was also very similar (unpublished data). ARNO stimulated GTPγS binding to myrARF6(N48I) and to myrARF6 to a similar extent (Fig. 8 A). Additionally, GIT1, a GAP protein for ARF6 (Vitale et al., 2000a), stimulated GTP hydrolysis on myrARF6(N48I) (Fig. 8 B). Together, these experiments indicate that the N48I mutation does not affect the activation/inactivation cycle of ARF6 and its regulation by endogenous accessory proteins. We next tested the ability of myrARF6(N48I) to stimulate known ARF effectors. In presence of GTP, cholera toxin ADP-ribosyltransferase activity was stimulated similarly by myrARF6(N48I) and myrARF6 (Fig. 8 C). However, as intended, the mutation N48I abolished almost completely the ability of ARF6 to stimulate PLD1 (Fig. 8 D), making ARF6(N48I) an ideal tool to test the involvement of the ARF6-PLD pathway in a cellular function.

We next examined whether ARF6 and PLD1 colocalize, and could thereby physically interact in stimulated PC12 cells. In agreement with the presence of endogenous PLD1 at the plasma membrane in chromaffin cells (Vitale et al., 2001), GFP-PLD1 was found at the plasma membrane in both resting and stimulated PC12 cells (Fig. 9 A). Little ARF6 colocalized with PLD1 in resting cells. In contrast, stimulation with K⁺ triggered the colocalization of a fraction of ARF6 with GFP-PLD1 at the plasma membrane (Fig. 9 B).
A), thereby making feasible a potential interaction of ARF6 with PLD1 near the site of exocytosis. Direct interaction of ARF6 and PLD1 in secretagogue-activated cells was assessed by immunoprecipitation. As illustrated in Fig. 9 B, immunoprecipitation of WT ARF6 or the active ARF6(Q67L), but not the inactive ARF6(T27N), coprecipitated PLD1 from lysates of K+/H11001-stimulated PC12 cell but not from lysates of resting cells (Fig. 9 B), confirming the binding of activated ARF6 to PLD1 upon cell activation.

Next we examined the effects of ARF6(N48I) and ARF6(N48I/Q67L) on K+-evoked GH secretion. ARF6(N48I) expressed in PC12 cells associated with secretory granules (Fig. 10 A) and, similar to WT ARF6, became activated at the plasma membrane upon cell stimulation (Fig. 10 B). However, as shown in Fig. 10 C, expression of ARF6(N48I) in PC12 cells clearly decreased GH secretion by ~50%. Furthermore, superimposing the N48I mutation onto the constitutively active ARF6(Q67L) caused it to lose its ability to stimulate exocytosis; instead, ARF6(N48I/Q67L) behaved as a dominant negative, inhibiting secretion to an extent similar to the PLD1 nonstimulating ARF6(N48I) mutant (Fig. 10 C) and the inactive GDP-bound ARF6(T27N) mutant (Fig. 7 C). Immunoprecipitation experiments indicate that ARF6(N48I) still binds to PLD1 upon cell stimulation (unpublished data), suggesting that the inhibition of secretion after ARF6(N48I) expression is likely to be due to the partial sequestration and inactivation of endogenous PLD1. These results argue for a major contribution of PLD1 in the pathway by which ARF6 regulates exocytosis in neuroendocrine cells.

Discussion

ARF proteins have been proposed as ubiquitous regulators of vesicle transport within cells. Here, we investigated their possible function in calcium-regulated exocytosis of dense-core secretory granules, using PC12 cells which have proved to be a good model for the study of neuroendocrine secretion. We find that ARF6 is the sole member of the ARF family specifically associated with secretory granules and that it is activated upon secretagogue-evoked stimulation, most likely after targeting and docking of granules onto the plasma membrane. Transfection experiments with ARF6 mutants confirm the importance of ARF6 in the exocytotic reaction in PC12 cells. Our results suggest an intimate connection between ARF6 and the plasma membrane-bound PLD1 that we recently described as a key factor in the late fusion reaction in neurons (Humeau et al., 2001) and neuroendocrine cells (Vitale et al., 2001).
The granule-associated ARF6 is activated by ARNO at the plasma membrane upon PC12 cell stimulation

We previously compared the distribution of ARF6 in subcellular fractions obtained from resting and stimulated chromaffin cells and found that ARF6 apparently dissociates from secretory granules and binds to the plasma membrane upon cell stimulation (Caumont et al., 1998). Similarly, we found here that overexpressed ARF6 proteins translocate from secretory granule–to plasma membrane–containing fractions in stimulated PC12 cells. Immunofluorescence and ultrastructural analysis confirm the association of ARF6 with secretory granules and its relocation at the plasma membrane upon cell stimulation. A reasonable explanation is that the plasma membrane location of ARF6 results from the docking of secretory granules to sites of exocytosis. The observation that ARF6 remains attached to the plasma membrane upon subcellular fractionation of stimulated cells may reflect the direct interaction of ARF6 with a plasma membrane–bound protein. The nucleotide exchange factor ARNO is an attractive candidate to be the plasma membrane-bound partner of ARF6 (Frank et al., 1998b). Indeed, ARNO colocalizes with ARF6 in stimulated PC12 cells. Furthermore, we show here that ARF6 is in its inactive GDP-bound state when associated with secretory granules, but becomes activated when recruited to the plasma membrane. Conversely, overexpression of ARNO increases GTP binding to ARF6 at the plasma membrane whereas a catalytically inactive ARNO mutant dramatically inhibits it. It is interesting to note that the constitutively GDP-bound ARF6(T27N) could also be found associated to the plasma membrane in stimulated cells, suggesting that GDP/GTP exchange is not required for the ARF6 association with plasma membrane. This observation is in line with the idea that granules bring ARF6 to the plasma membrane, making the protein transiently available for ARNO upon docking at the exocytotic sites.

GDP-bound ARFs interact weakly with membranes through hydrophobic interactions of the myristate and electrostatic interactions of cationic residues with anionic lipids (Antonny et al., 1997). Binding of GTP releases the NH$_2$-terminal helix from the protein core allowing a stronger interaction with membranes through the NH$_2$-terminal hydrophobic residues (Antonny et al., 1997). Using purified chromaffin granules, we previously described that ARF6 coimmunoprecipitates with trimeric G protein $\beta$$\gamma$ subunits (Galas et al., 1997), suggesting that $\beta$$\gamma$ subunits could serve as anchors for GDP-bound ARF6 in the secretory granule membrane. An appealing speculation is that the ARF6/ARNO interaction is in some way under the control of the granule-associated G protein $\beta$$\gamma$ subunits. Thus, activation of ARF6 would require the docking of granules to appropriate ARNO-containing domains on the plasma membrane and concomitant changes in the interactions of ARF6 with $\beta$$\gamma$ subunits. In favor of this idea, several recent reports describe $\beta$$\gamma$ subunit regulation of the exocytotic fusion machinery, downstream of calcium entry, in secretory cells (Pirxteren et al., 1998; Gensse et al., 2000) and as well as in neuronal presynaptic terminals (Blackmer et al., 2001).

**ARNO-induced ARF6 activation is critical for exocytosis in PC12 cells**

We previously reported that a synthetic peptide, corresponding in sequence to the myristoylated NH$_2$-terminal domain of ARF6 inhibited, in a dose-dependent manner, calcium-evoked catecholamine secretion from permeabilized chromaffin cells (Galas et al., 1997; Caumont et al., 1998). More recently, we described the presence of ARNO at the plasma
membrane in chromaffin cells and demonstrated that over-
expression of WT ARNO enhanced secretagogue-evoked se-
cretion from PC12 cells whereas the catalytically inactive
ARNO(E156K) mutant inhibited it (Caumont et al., 2000).
These results suggested, but did not prove, that an ARNO-
regulated ARF6 plays an important role in exocytosis. In-
deed, ARNO is exchange factor for both ARF1 and ARF6,
and both ARFs can be detected at the plasma membrane in
chromaffin cells (Galas et al., 1997). Moreover, although
ARF peptides have been described to block ARF activities in
various cellular processes (Barr and Huttner, 1996; Le
Stunff et al., 2000; Mukherjee et al., 2000), their specificity
has also been questioned (Fensome et al., 1994). Using di-
rect means, we demonstrate here for the first time that ARF6
is the sole member of the ARF family involved in the molec-
ular pathway underlying calcium-regulated exocytosis. Al-
though the involvement of ARF6 in peripheral vesicle traf-
ficking has been reported previously in various cell types,
this specific requirement for ARF6 in secretion is surprising
and may be linked to its granular location and its specific ac-
tivation at granule targeting sites.

**PLD1 is a major downstream effector of ARF6 in the exocytotic process**

One of the proposed functions of ARF6 is to mediate actin
rearrangements (Chavrier and Goud, 1999). Activation of
ARF6 can also lead to significant changes in the lipid
composition of specific membrane domains, as the pro-
tein is known to directly activate two lipid-modifying en-
zymes, PLD and phosphatidylinositol-4-phosphate 5-kinase
(Honda et al., 1999). Thus, activation of ARF6 at the exocy-
totic sites may provide a mechanism whereby secretory cells
can engineer a localized remodeling of the actin cytoskeleton
with phospholipid modifications, so that the fusion mach-
inery can negotiate the exocytotic event. Using rhodamine-
conjugated phalloidin to visualize actin filaments, we have
investigated whether ARF6 mutants affect the depolymeriza-
tion of cortical actin observed in stimulated cells. However,
we could not correlate the strong inhibition of secretion in-
duced by ARF6(T27N) to a stabilization of the cortical ac-
tin barrier (unpublished data). Accordingly, we found that
the secretagogue-dependent movement of secretory gran-
ules to the cell periphery was similar in control and in
ARF6(T27N)-expressing cells. Thus, ARF6 is probably not
involved in the partial depolymerization of the cortical actin
cytoskeleton that enables recruitment of the reserve pool of
secretory granules to the plasma membrane. This finding is
in line with the idea that ARF6 is activated after docking of
secretory granules to the sites of exocytosis. Whether ARF6
mediates some other subtle modifications of the actin cyto-
skeleton functionally required in late stages of the exocytotic
machinery cannot be excluded and will be an interesting fu-
niture issue.

We recently demonstrated that PLD1 constitutes a critical
factor for regulated exocytosis in neuroendocrine cells (Vi-
tale et al., 2001) and neurons (Humeau et al., 2001), operat-
ing subsequent to the cytoskeletal-mediated recruitment of
secretory granules to exocytotic sites (Vitale et al., 2001). To
directly probe the idea that ARF6 is the upstream activator
of the plasma membrane-bound PLD1 in the exocytotic
pathway, we generated a novel ARF6(N48I) mutant that
specifically lost its ability to stimulate PLD1, based on anal-
ogy with a similar ARF1 mutant defective in PLD activation
but still able to activate phosphatidylinositol-4-phosphate
5-kinase (Jones et al., 1999; Skippen et al., 2002). Expres-
sion of ARF6(N48I) inhibited secretagogue-evoked GH se-
cretion to an extent similar to the constitutively inactive
ARF6(T27N), suggesting that the stimulation of PLD1 is
the major function ARF6 undertakes in regulated exocytosis.
Accordingly, ARF6 interacts directly with PLD1 at the
plasma membrane in stimulated cells. Our previous obser-
vation that the introduction of specific anti-ARNO antibodies
into permeabilized chromaffin cells inhibited in a similar
manner catecholamine secretion and PLD activation (Ca-
umont et al., 2000) further corroborates the ARNO–ARF6–
PLD1 cascade in the exocytotic process.

How does PLD1 relate to our current understanding of
the protein machinery responsible for regulated exocytosis?
PLD1 catalyzes the hydrolysis of phosphatidylcholine to
produce membrane-localized phosphatidic acid (PA). An ex-
citing speculation relates to the changes in the lipid bilayer
properties created by the activation of PLD. PA is a cone-
shaped lipid which promotes membrane curvature and the
formation of hemifusion intermediates required for the fu-
sion of two membranes (Monck and Fernandez, 1994).
Thus, the predicted effect of a local elevation of PA due to
the activation of PLD1 at exocytotic sites would be to pro-
mote membrane bending, particularly in the presence of cal-
cium, thereby facilitating membrane breakdown and subse-
quent formation of the exocytotic fusion pore. Accordingly,
data obtained by amperometry on isolated chromaffin cells
suggest that PLD1 affects the kinetics of the initial fusion
event and/or the rate of the fusion pore opening (Vitale et
al., 2001). Similarly, electrophysiological analysis suggest
that the role played by PLD1 in neurotransmission is related
to the fusogenic status of the presynaptic release sites (Hu-
meau et al., 2001). Hence, the ARF6–PLD1 complex may
provide to the protein scaffold pulling membrane together
the lipid counterparts required to open and/or expand the
fusion pore.

**Materials and methods**

**Antibodies, DNA constructs, and proteins**

The following antibodies were used: rabbit polyclonal anti-ARF6 antibod-
ies, provided by J. Donaldson (National Institutes of Health, Bethesda,
MD); (Radhakrishna and Donaldson, 1997); rabbit polyclonal anti-human
GH antibodies, provided by Dr. A.F. Parlow (Los Angeles County Harbor–
UCLA Medical Center, Torrance, CA) and the National Institute of Diabe-
tes and Digestive and Kidney Diseases, National Hormone and Pituitary
Program; polyclonal anti-Rab5 antibodies, provided by M. Zerial (Max
Planck Institute for Molecular Cell Biology and Genetics, Dresden, Ger-
many); monoclonal anti–human GH antibodies (Nichols Institute); mono-
clonal anti-SNAP-25 antibodies (Stemberger Monoclonals, Inc.); mono-
clonal anti-HA antibodies (BabCO); monoclonal anti-myc antibodies
(Novacastra Laboratories). Cy2-anti–mouse, Cy3-anti–rabbit, and Cy5-
anti–mouse were obtained from Amersham Biosciences. pECFP-Golgi was
purchased from Stratagene.

The ARF and ARNO constructs, provided by J.E. Casanova (Univer-
sity of Virginia Health System, Charlottesville, VA), were described pre-
vously (Frank et al., 1998a; Morinaga et al., 2001). pXS-ARF6(N48I)
and pXS-ARF6(N48I-Q67L) were generated by site-directed mutagen-
esis with a Quickchange mutagenesis kit from Stratagene (Vitale et al.,
2000a).

Recombinant myrARF6 and myrARF6(N48I) were expressed and puri-
fied as described previously (Vitale et al., 2000b). Recombinant ARNO was expressed as described (Pacheco-Rodriguez et al., 1998).

**Culture, transfection, and assay of GH release from PC12 cells**

PC12 cells were grown in DME supplemented with glucose (4,500 mg/L) and containing 30 mM NaHCO3, 3% fetal bovine serum, 10% horse serum and 100 U/ml penicillin/streptomycin. Mammalian expression vectors were introduced into PC12 cells together with the GH plasmid pGHS-6-well assay kit (Nichols Institute). The amount of GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

**Distribution and nucleotide status of ARF6 in PC12 cell subcellular fractions**

PC12 cells grown in 100-mm plates were transfected with various plasmids (for each 10 μg per plate) using GenePorter. 36 h after transfection, cells were incubated in 2 ml plate phosphate- and serum-free DME medium, supplemented with 25 mM Hepes, pH 7.2, 2 mM pyruvate and 175 μl of 10 mM PIP2 phosphophosphate for 24 h. PC12 cells were washed twice with Locke’s solution and then incubated for 15 min with calcium-free Locke’s solution (basal release) or stimulated with an elevated K+ solution (Locke’s containing 59 mM KC1 and 85 mM NaCl). The supernatant was collected and the cells harvested by scraping in 10 mM phosphate buffered saline. The amounts of GH secreted into the medium and retained in the cells were measured using a radioimmunossay kit (Nichols Institute). The amount of GH secretion is expressed as a percentage of total GH present in the cells before stimulation.

**Molecular characterization of myrARF6(N48I)**

Time-course GTPγS binding assays were performed with 0.5 μg of myrARF6 or myrARF6(N48I) essentially as described (Vitale et al., 1997). In the presence or absence of Gβ1, provided by R.T. Premont (Duke University Medical Center, Durham, NC), GTPase activity of 0.5 μg of myrARF6 or myrARF6(N48I) was measured for 10 min at 30°C as described previously (Vitale et al., 2000b). ARF stimulation of cholera toxin-catalyzed ADP-ribosylagmatine formation was performed with 0.5 μg of myrARF6 or myrARF6(N48I) as described (Vitale et al., 1997). PLD activity assays were carried out using the in vitro head-group release assay for a 30-min time period as previously described (Zhang et al., 1996). Recombinant WT and mutant ARF6s were loaded using 50 μM GTPγS as previously described (Frohman et al., 2000) and were used at 0.5 μM (1 μg/100 μl reaction). Assay blanks were subtracted. The source of PLD1 protein was a tet-regulated stable CHO cell line that had been induced for 24 h in a 33-mm dish before harvesting and sonication. The sonicated lysate was centrifuged for 30 min at 4°C and 20,000 g to pellet the PLD1, and the supernatant containing the Rho and ARF small GTP proteins removed. 100 μl of PBS containing protease inhibitors was added to the pellet which was then sonicated briefly to evenly resuspend it. 1 μl of the resulting PLD1 source was used for each assay sample.

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