Distribution of ARF6 between Membrane and Cytosol Is Regulated by Its GTPase Cycle*

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The ADP-ribosylation factor (ARF) subfamily of small GTPases regulates intracellular transport. Although much is known about how ARF1 regulates transport in the secretory pathways, regulation of the endocytic pathways by ARF6 remains less understood. In particular, whereas cycling of ARF1 between membrane and cytosol represents a major mechanism of regulating its function, this regulation has been questioned for ARF6. In this study, we found that ARF6 is distributed both on membranes and in the cytosol. Cytosolic ARF6 is recruited to membranes in a GTP-dependent manner that is fundamentally similar to ARF1. However, unlike ARF1, release of membrane-bound ARF6 to the cytosol requires hydrolysis of GTP that is sensitive to the level of magnesium. These findings suggest that the GTPase cycle of ARF6 also regulates its distribution between membrane and cytosol and that this form of regulation will also likely be important for the function of ARF6. Moreover, as ARF6 has little intrinsic ability to hydrolyze GTP, magnesium concentration most likely affects the release of membrane-bound ARF6 by altering the activity of its GTPase-activating protein.

Intracellular transport by vesicular carriers is initiated from a membrane compartment by the recruitment of cytosolic coat proteins to their target membranes. These local membrane sites become transformed into coated buds that then mature into coated transport vesicles. Subsequently, the membrane-bound coat proteins must be released to the cytosol before a vesicle can fuse with its target compartment. Thus, regulating movement of coat proteins between membranes and cytosol represents a major mechanism of regulating intracellular transport pathways (1, 2).

The recruitment of cytosolic coat proteins to membranes is regulated by the ADP-ribosylation factor (ARF)3 subfamily of small GTPases. Among members of this family, ARF1 serves as the prototype and has been shown to mediate coat proteins regulated by ARF1 are also recruited from the cytosol to target membranes (3, 4, 9, 10). Subsequently, hydrolysis of its bound GTP deactivates ARF1 and releases ARF1 and its coat proteins from their target membranes to the cytosol (11–13). Thus, as with coat proteins, translocation of ARF1 between membranes and cytosol also represents a major mechanism of regulating its function. Moreover, like all members of the Ras-like small GTPase family, interconversion of ARF1 between its active and inactive states requires catalysis whereby a guanine nucleotide exchange factor (GEF) catalyzes the exchange of GDP for GTP (14–18), and a GTPase-activating protein (GAP) catalyzes the hydrolysis of GTP to GDP (19–22).

ARF6 is the most distant relative of ARF1 and is the first member of the ARF subfamily characterized to regulate both membrane transport (23, 24) and cytoskeletal organization (25). ARF6 induces actin rearrangement through POR-1 (26), a protein that is predicted to poorly hydrolyze GTP blocks internalization of the transferrin receptor from the cell surface to the early endosome (23). Overexpression of a mutant ARF6 (T27N) that is predicted to prevent exchange of GDP for GTP blocks the internalized transferrin receptor from recycling to the cell surface (23). A recent ultrastructural study suggests that membrane-bound ARF6 localizes primarily to the recycling early endosome, suggesting that activation of ARF6 occurs at this compartment (28). Thus, inhibition of internalization by the activating mutant of ARF6 (Q67L) may be an indirect result of the redistribution of the early endosome to the plasma membrane such that material from the cell surface can no longer be internalized (24).

Moreover, an initial fractionation study on ARF6 suggested that it is all membrane-bound, whereas all other ARFs have a significant cytosolic pool that can be recruited to membranes in a GTP-dependent manner (29), suggesting that ARF6 does not move between membrane and cytosol like all other ARF members. A subsequent study revealed a cytosolic form of ARF6, and its abundance varied by tissue type and developmental stage (30). It remains unclear how this finding can be reconciled with the earlier finding that ARF6 is all membrane-bound and whether the GTPase cycle of ARF6 regulates its distribution between membrane and cytosol.

In this study, we found that the distribution of ARF6 between membrane and cytosol is regulated by its GTPase cycle. Recruitment of cytosolic ARF6 to membranes requires its binding of GTP. However, unlike ARF1, release of membrane-bound ARF6 to the cytosol that requires hydrolysis of GTP is sensitive to changes in magnesium concentration, and this effect is attributable to alterations in the GAP activity on ARF6. Thus, as

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3 The abbreviations used are: ARF, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; GAP, GTP-activating protein; CHO, Chinese hamster ovary; GTP-S, guanosine 5’-O-(3-thiotriphosphate); GDP-S, guanosine 5’-O-(2-thiodiphosphate); AP, adaptor protein.
the GTPase cycle also regulates the distribution of ARF6 between membrane and cytosol, our finding also suggests that this form of regulation is a general characteristic of all members of the ARF subfamily.

**MATERIALS AND METHODS**

**Cells, Reagents, and Transfection—**All cell lines were grown in media that were supplemented with 10% fetal calf serum, 100 μM glutamine, and 20 μg/ml gentamycin. Dulbecco’s modified Eagle’s medium was used for HeLa, RBL, and RD4 cells. a-Minimal essential medium was used for HEK 293 cells whereas RPMI 1640 medium was used for Jurkat cells. All 100 μl of either cytosol prepared in the magnesium-containing buffer was centrifuged at 200,000 g for 10 min to obtain a post-nuclear supernatant. This supernatant was further centrifuged at 200,000 × g for 1 h (TFT 80.4 rotor, Sorvall, Newtown, CT) to obtain the cytosol (supernatant) and membranes (pellet). Equal fractions of both were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using an anti-ARF6 antibody and an anti-transferrin receptor antibody that assessed for quantitative recovery of transferrin receptor in the cytosol and membranes. The EDTA-containing buffer consisted of 10 mM triethanolamine (pH 7.4), 1 mM EDTA, and 250 mM sucrose, and was referred to as TEAS buffer previously (29). Cells that were washed in either of these two buffers were then resuspended in 4 volumes of either buffer and subjected to shearing using a ball-bearing homogenizer (EMBL, Heidelberg, Germany) with four passes at 36-μm clearance. The resulting homogenate was centrifuged at 1000 × g for 10 min to obtain the post-nuclear supernatant. This supernatant was further centrifuged at 200,000 × g for 1 h (TFT 80.4 rotor, Sorvall, Newtown, CT) to obtain the cytosol (supernatant) and membranes (pellet). Equal fractions of both were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using an anti-ARF6 antibody and an anti-transferrin receptor antibody that assessed for quantitative recovery of total membranes. Blots were then visualized using enhanced chemiluminescence (ECL, NEN Life Science Products).

**Preparation of Cytosol and Total Membranes for Recruitment Assays—**CHO cells (5 × 10⁶) were washed as described above and then resuspended in 4 volumes of either the magnesium- or EDTA-containing buffer and homogenized, followed by centrifugation as described above. The resulting post-nuclear supernatant was layered onto a sucrose step gradient in an SW 41 tube (Beckman Instruments). Sucrose solutions were prepared in either the magnesium- or EDTA-containing buffer and layered as follows: 1 ml of 60% sucrose at the bottom, followed by 1.5 ml of 50% sucrose, 1.5 ml of 20% sucrose, and then 7 ml of homogenate. The step gradient was centrifuged (SW 41 rotor, Beckman Instruments) at 200,000 × g for 2.5 h. The interface between 30% and 60% sucrose of the step gradient was collected as the membrane fraction, whereas those above the 20% sucrose interface was collected as cytosol. Both fractions were stored at −80 °C. After thawing, the cytosol fraction was centrifuged at 200,000 × g for 40 min, and the supernatant was used for assays.

**Assay for Recruitment of Cytosolic ARF6 to Membranes—**100 μl of cytosol (total protein concentration of 6–7 mg/ml) and 6 μl of total membranes (total protein concentration of 2–3 mg/ml), prepared as described above, were thawed and incubated together with various concentrations of nucleotides (as indicated in figure legends) for 45 min at 37 °C. The incubation mixture was then centrifuged at 200,000 × g for 30 min at 4 °C. The supernatant was collected, and the remaining membrane pellet was washed once. Equal fractions of both membranes and supernatant were then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted using an anti-ARF6 antibody. Blots were visualized using ECL and then scanned for densitometry using ScionImage.

**Assay for Release of Membrane-bound ARF6—**Total membranes (6 μl) prepared in the EDTA-containing buffer, as described above, were incubated with either cytosol prepared in the magnesium-containing buffer or simply the magnesium-containing buffer alone at 4 °C for 1 h. After incubation, the incubation mixture was centrifuged at 200,000 × g for 30 min at 4 °C and analyzed as membrane and cytosol fractions as described above. In experiments in which cytosol was used, calculation of membrane-bound ARF6 released into the cytosol was performed by quantifying the amount of ARF6 already in the cytosol and then subtracting this value from the total amount of soluble ARF6 obtained after the incubation.

**RESULTS**

**Magnesium Concentration Affects the Distribution of ARF6 between Membrane and Cytosol—**A subcellular fractionation study that had revealed ARF6 as all membrane-bound was performed in the presence of EDTA, where cells were homogenized in a 4-fold volume of buffer that contained 1 mM EDTA (29). As physiologic magnesium is in the range of 1 mM (31), its dilution into the EDTA-containing buffer would likely result in a free magnesium concentration substantially <1 mM (probably in the submicromolar range). However, studies on how the GTPase cycle of ARF1 regulates its distribution between membrane and cytosol were performed using magnesium concentrations in the millimolar range (3, 4, 9, 10, 31). Thus, we first examined whether this difference in the concentration of magnesium affected the distribution of ARF6 between membrane and cytosol.

Subcellular fractionation that separated total membranes from the cytosol was performed initially on a CHO cell line. ARF6 was detected by Western blotting using an anti-ARF6 antibody that was generated as described previously (23). Consistent with previous observation using CHO cells (29), when fractionation was performed in the EDTA-containing buffer, ARF6 remained virtually all membrane-bound. However, using a buffer that contained physiologic levels of magnesium (3, 4), we found a significant pool of cytosolic ARF6 (Fig. 1). In contrast, the distribution of ARF1 remained unaffected (Fig. 1), suggesting that the effect of increased magnesium was selective for ARF6. This ability of increased magnesium to alter the ARF6 distribution between membrane and cytosol was not unique to CHO cells. Subcellular fractionation using the magnesium-containing buffer revealed a significant level of cytosolic ARF6 in human embryonal kidney cells (293), human cervical epithelial cells (HeLa), human fibrosarcoma cells (RD4), human T cells (Jurkat), and rat basophilic granulocytic cells (RBL) (Fig. 2).

**Binding to GTP Recruits ARF6 from the Cytosol to Membranes—**As the GTPase cycle regulates the distribution of ARF1 between membrane and cytosol (3, 4, 9, 10, 31), we examined whether the cytosolic form of ARF6 induced by increased magnesium could be recruited to membranes in a GTP-dependent manner. In a recruitment assay, we found that more cytosolic ARF6 was recruited to membranes with increasing concentrations of GTPyS (Fig. 3). Significantly, this recruitment assay was performed using the magnesium-containing buffer. Thus, the finding that the effect of GTPyS in promoting more membrane-bound ARF6 dominated over the effect of magnesium that induced more cytosolic ARF6 suggested that the ARF6 distribution was regulated more directly by its GTPase cycle than by the effect of magnesium.

![Fig. 1. Subcellular fractionation using a magnesium-containing buffer reveals a cytosolic form of ARF6. CHO cells were homogenized in either the EDTA- or magnesium-containing buffer and fractionated into the cytosol (C) and total membranes (M), followed by immunoblotting for either ARF6 or ARF1.](http://www.jbc.org/Downloaded from http://www.jbc.org/)
The cell lines, as shown were homogenized with the magnesium-containing buffer and then fractionated into total membranes (M) and the cytosol (C), followed by immunoblotting with an anti-ARF6 antibody (lower panel) and an anti-transferrin receptor (TfR) antibody to assess quantitative recovery of total membranes (upper panel). The relative fractions of ARF6 on membranes and in the cytosol were then quantified by densitometry.

In support of this finding, we sought to inhibit hydrolysis of GTP bound to ARF6 more selectively by examining a GTPase mutant of ARF6 (Q67L) that is predicted to poorly hydrolyze GTP (24). Transfecting ARF6 Q67L into cells followed by subcellular fractionation using the magnesium-containing buffer, we found that the Q67L mutant localized on membranes more efficiently than wild-type ARF6 (Fig. 4). This difference was similar to that observed between an activating mutant of ARF1 (Q71I) that poorly hydrolyzed GTP (12) and wild-type ARF1 (Fig. 4). For both ARF1 and ARF6, the activating mutation caused a relative, but not complete, shift in the distribution of the mutants to membranes. This partial shift can be attributed to the previous finding that the activating point mutation on the distribution of ARF1 and ARF6 further suggested that the GTPase cycle of ARF6, like that of ARF1, directly regulated its movement between membrane and cytosol.

In contrast to GTPγS, GDPβS did not stabilize ARF6 on membranes (Fig. 5). Thus, this result corroborated with the above findings suggesting that the GTP-bound form stabilized ARF6 on membranes. Significantly, GTP was also unable to induce the membrane-bound form of ARF6 (Fig. 5). This result was reminiscent of the recruitment of cytosolic ARF1 to membranes. GTPγS, but not GTP, stabilized ARF1 on membranes because its GAP was too active to allow accumulation of membrane-bound ARF1 in the presence of GTP (3, 4). These findings suggested why subcellular fractionation revealed mainly cytosolic ARF1, as the ARF1 GAP activity appeared highly active even at 4 °C during subcellular fractionation (32).

**Magnesium-sensitive GAP Activity Releases Membrane-bound ARF6 to the Cytosol**—To begin investigating whether increasing magnesium used a similar mechanism to redistribute ARF6 from membranes to the cytosol, we first isolated the membrane-bound fraction of ARF6 in the EDTA-containing buffer that allowed significant accumulation of ARF6 on membranes. This membrane was then incubated at 4 °C with cytosol that was prepared in the magnesium-containing buffer.

When incubated together, a significant fraction of membrane-bound ARF6 became soluble (Fig. 6). To ensure that this redistribution of ARF6 is not simply due to a re-mixing of membranes with cytosol, total membranes and cytosol were both prepared in the EDTA-containing buffer and then incubated together. This incubation revealed no significant redistribution of ARF6 to the cytosol (Fig. 6), suggesting that increasing magnesium also induced an activity at 4 °C to redistribute ARF6 from membranes to the cytosol.

As cytosolic ARF1 could be induced by either inhibiting its GEF activity that prevents its recruitment from the cytosol to membranes or enhancing its GAP activity that facilitates its release from membranes to the cytosol, we also examined which regulatory activity on ARF6 was affected by magnesium to induce cytosolic ARF6. Thus, to examine whether increasing magnesium induced cytosolic ARF6 by inhibiting its GEF activity, we performed a recruitment assay in the presence of GTPγS that blocked the possibility of magnesium acting through a GAP activity to induce more cytosolic ARF6. Under this condition, more membrane-bound ARF6 was observed using the magnesium-containing buffer compared with the EDTA-containing buffer (Fig. 7). Thus, cytosolic ARF6 induced by increasing magnesium could not be attributed to inhibition of the GEF activity on ARF6, suggesting that magnesium most likely induced its effect by enhancing the GAP activity on ARF6.
To test more directly whether a GAP activity on ARF6 would enhance its release from membranes, we prepared total membranes in the EDTA-containing buffer to accumulate a significant fraction of membrane-bound ARF6. This membrane fraction was then incubated with the magnesium-containing buffer that lacked cytosol. In this type of simplified incubation, a significant fraction of membrane-bound ARF6 was released (Fig. 8). Thus, as this incubation started with ARF6 only in the membrane-bound fraction, its result suggested that a membrane-localized activity was activated by magnesium to release ARF6 from membranes. Significantly, the degree of this release was less than that seen when incubation was performed using cytosol prepared from the magnesium-containing buffer (compare Figs. 6 and 8), suggesting that the cytosol had additional factor(s) that potentiated a membrane-localized activity to release membrane-bound ARF6.

To show that the magnesium-induced release of membrane-bound ARF6 was attributable to hydrolysis of GTP, we next examined whether ARF6 that had been loaded onto membranes with GTP$_\gamma$S could be released when subsequently incubated in the magnesium-containing buffer. For this purpose, membranes and cytosol that were prepared in the magnesium-containing buffer were incubated with GTP$_\gamma$S, and membrane-bound ARF6 was isolated by centrifugation. When isolated in this manner, membrane-bound ARF6 became resistant to release by the magnesium-containing buffer (Fig. 8), suggesting

![Image](http://www.jbc.org/Downloaded/fig5.jpg)

**FIG. 5.** Recruitment of cytosolic ARF6 to membranes depends specifically on GTP$_\gamma$S. Cytosol and total membranes prepared from the magnesium-containing homogenization buffer were incubated with 200 μM GTP$_\gamma$S, GTP, ATP, or GDP$_\beta$S or with no added nucleotides and then fractionated into the cytosol (C) and total membranes (M), followed by immunoblotting for ARF6. ARF6 in the membrane fractions from three separate experiments was then calculated for the mean ± S.E. and for -fold increase over the base line when no nucleotides were added.

![Image](http://www.jbc.org/Downloaded/fig6.jpg)

**FIG. 6.** Cytosol prepared in the magnesium-containing buffer redistributes ARF6 from membranes to the cytosol. Cytosol prepared in either the magnesium- or EDTA-containing buffer was incubated with total membranes prepared in the EDTA-containing buffer, followed by fractionation into the cytosol (C) and total membranes (M) and then immunoblotting for ARF6. Protein levels were quantified by densitometry for three separate experiments. To calculate release of membrane-bound ARF into magnesium-containing cytosol, the amount of ARF6 already in the cytosol was quantified and subtracted from the total amount of soluble ARF6 detected after the incubation.

![Image](http://www.jbc.org/Downloaded/fig7.jpg)

**FIG. 7.** Increasing magnesium does not inhibit the recruitment of cytosolic ARF6 to membranes. Total membranes (prepared in the magnesium-containing buffer to deplete the level of membrane-bound ARF6) were incubated with cytosol that was prepared in either the magnesium-containing (●) or EDTA-containing (∆) buffer in the presence of increasing concentrations of GTP$_\gamma$S (as indicated). The incubation was then fractionated into the cytosol (C) and total membranes (M) and immunoblotted for ARF6. ARF6 in the membrane fraction from three separate experiments was calculated for the mean ± S.E. and for -fold increase over the base line when no GTP$_\gamma$S was added.

![Image](http://www.jbc.org/Downloaded/fig8.jpg)

**FIG. 8.** The magnesium-containing buffer induces release of ARF6 from membranes, and this release is blocked when membrane-bound ARF6 is generated using GTP$_\gamma$S. Left, total membranes prepared in the EDTA-containing buffer were incubated with the magnesium-containing buffer and centrifuged to obtain membrane and soluble fractions, followed by immunoblotting for ARF6; right, total membranes and cytosol that were prepared in the magnesium-containing buffer were incubated with GTP$_\gamma$S. The membrane fraction was isolated from this incubation and re-incubated with the magnesium-containing buffer, followed by fractionation into membrane and soluble fractions and immunoblotting for ARF6. For both sets of experiments, protein levels were quantified and calculated for mean ± S.E. based on three separate experiments.
that increasing magnesium enhanced the release of membrane-bound ARF6 by facilitating the hydrolysis of GTP.

**DISCUSSION**

In this study, we found that a difference in the level of magnesium alters the distribution of ARF6 between membrane and cytosol. Elucidating the underlying mechanism, we found that ARF6 is recruited from the cytosol to membranes in a GTP-dependent manner and is released back to the cytosol by increasing magnesium, which facilitates hydrolysis of GTP. Thus, these findings suggest that translocation of ARF6 between membrane and cytosol is regulated by its GTPase cycle. As ARF6 has little intrinsic ability to hydrolyze GTP, our findings also suggest that increasing magnesium induces cytosolic ARF6 by enhancing its GAP activity.

**GTPase Cycle of ARF6 Regulates Its Distribution between Membrane and Cytosol**—Our study used indirect approaches to show that the ARF6 distribution is regulated by its GTPase cycle because approaches that might be more direct are also technically unfeasible. For example, one can envisage immunoprecipitating ARF6 from membrane and cytosol fractions and then examining the guanine nucleotide bound to ARF6 derived from these two fractions. However, this direct approach requires an immunoprecipitating antibody that is capable of stabilizing binding of a small GTPase to its guanine nucleotide (34), and such an antibody has not been identified for small GTPases other than Ras (35). Thus, as done for ARF1 (3, 4, 9, 10), we also used indirect approaches to show that binding of GTP localizes ARF6 to membranes. We found that GTPyS, but not GDPyS, supports recruitment of cytosolic ARF6 to membranes. Moreover, a point mutant of ARF6 (Q67L) that is predicted to poorly hydrolyze its bound GTP associates with membranes better than its wild-type counterpart. Thus, these results suggest that the GTPase cycle of ARF6 regulates its distribution between membrane and cytosol in a manner that is fundamentally similar to that seen for ARF1. Significantly, these results also suggest that potential mechanisms independent of the GTPase cycle as an explanation for how magnesium redistributes ARF6 would be unlikely. For example, one can envisage a mechanism of proteolysis whereby a magnesium-sensitive protease is activated to directly cleave a proteinaceous docking site used by ARF6 to bind membranes. However, such a mechanism cannot readily explain our finding that the magnesium-induced redistribution of membrane-bound ARF6 to the cytosol is blocked when GTP hydrolysis is inhibited.

As magnesium appears to affect the ARF6 distribution through its GTPase cycle, this finding has mechanistic parallels to how brefeldin A affects the ARF1 distribution. Brefeldin A induces the cytosolic form of ARF1 by inhibiting its GEF activity, which then allows the GDP-bound form of ARF1 to predominate (36, 37). In this regard, increasing magnesium can potentially induce cytosolic ARF6 by either inhibiting a GEF activity to prevent the recruitment of cytosolic ARF6 to membranes or enhancing a GAP activity to facilitate the release of membrane-bound ARF6 to the cytosol. To address this issue, we assessed whether increasing magnesium inhibits the recruitment of cytosolic ARF6 to membranes by examining its recruitment in the presence of GTPyS, which blocks the contribution of the ARF6 GAP activity. As this recruitment is enhanced upon increasing magnesium (see Fig. 7), this result suggests that the redistribution of ARF6 from membrane to cytosol cannot be explained by inhibition of the ARF6 GEF activity by magnesium. Thus, this result suggests that magnesium affects the ARF6 distribution mainly by enhancing its GAP activity.

A seeming contradiction to our findings is the previous observation that both the activating (Q67L) and deactivating (T27N) mutants of ARF6 are localized on membranes (23, 24). This observation has the appearance that both the GTP- and GDP-bound forms of ARF6 are localized on membranes and thus reinforces the previous notion that the GTPase cycle of ARF6 does not regulate its distribution between membrane and cytosol (29). An explanation for this apparent contradiction is suggested, however, by the elicitation of how the equivalent point mutations in Ras affect its GTPase cycle (38). Significantly, whereas the equivalent mutation of ARF6 Q67L in Ras represents a GTP-bound form of Ras, the equivalent mutation of ARF6 T27N in Ras does not appear to represent a GDP-bound form of Ras. The exchange of GDP for GTP on Ras represents a two-step mechanism whereby a GEF activity displaces GDP bound to Ras and results in a transition state of Ras that has an empty GTP-binding pocket. Subsequently, GTP fills the pocket to generate the GDP-bound form of Ras. Thus, ARF6 T27N is likely to represent a transition state of ARF6 that is devoid of a guanine nucleotide, rather than its GDP-bound form.

An important implication for the transition state of Ras is that it interacts more tightly with its GEF than the wild-type counterpart. Thus, overexpression of the transition state mutant results in a dominant-negative phenotype because the GEF can no longer act on endogenous wild-type Ras. In this regard, the phenotypic manifestations of ARF6 T27N could be attributed to its ability to block the function of endogenous wild-type ARF6 by sequestering GEFs that act on ARF6 (23, 24). Thus, because this GEF activity is likely to be membrane-bound, as seen for ARF1 GEF (36, 37, and 39), ARF6 T27N would be predicted to associate with membranes rather than to reside in the cytosol. Indeed, we have found recently, by both subcellular fractionation and immunogold electron microscopy, that ARF6 T27N and its equivalent mutant in ARF1 (T31N) are associated with membranes to similar degrees. Thus, the fundamental parallels that we have seen for how the GTPase cycle regulates the distribution of both ARF1 and ARF6 are further supported by the behavior of their point mutants that affect the GTPase cycle.

**Potential Roles for the GTPase Cycle in Regulating ARF6 Function**—As cytosolic magnesium is thought to exist in the millimolar range (31) rather than the submicromolar range, which was used initially to study the distribution of ARF6 (29), our study suggests that ARF6 is likely to have a significant cytosol fraction in vivo. In considering how our findings might relate to regulation of endocytic transport by ARF6, we are led by the recent suggestion that ARF6 is likely to be activated on endosomal membranes to regulate the recycling pathway of endocytosis (28). Thus, we propose that a GEF activates ARF6 for its recruitment from the cytosol to endosomal membranes to initiate the formation of transport carriers from this compartment. However, given what is currently known, the role of a GAP that acts on ARF6 in this transport pathway is more difficult to pinpoint. Although studies on ARF1 suggest that GAP should act to release ARF6 from its transport vesicles prior to their fusion with the plasma membrane (11), the activating mutant of ARF6 (Q67L) has been shown to reside on the plasma membrane (23, 24). This observation suggests that GAP acts to release ARF6 only after its transport vesicles have fused with the plasma membrane. Because coated vesicles are thought to fuse only with their target compartment after uncoating (1, 2), the effect of ARF6 Q67L suggests the possibility that deactivation of ARF6 does not regulate the uncoating of its transport vesicles. As current evidence does not support this possibility, an alternative explanation that seems more likely

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is that constitutive activation of ARF6 through the use of the Q67L mutant may lead to secondary effects that confound the true nature of how GAP acts. Significantly, ARF6 Q67L also induces the redistribution of the early endosome to the plasma membrane (24). Thus, although this activating mutant can localize on the resulting mixed membrane compartment, it might not be capable of recruiting the ARF6-regulated coat to this compartment because this coat recruitment might occur only in an endosome-specific context. This explanation suggests that the GT-Pase cycle of ARF6 can be uncoupled with respect to its ability to recruit coat, when a mixed compartment of plasma membrane and early endosome is induced. Insight into the actual explanation will likely be forthcoming once the putative coat regulated by ARF6 is identified and characterized.

On the other hand, another aspect of our study may provide insight into potential regulatory mechanisms that govern the GAP activity on ARF6. Although more cytosolic ARF6 induced by increasing magnesium could be attributed to enhancing the ARF6 GAP activity, the ARF1 distribution is not affected similarly, suggesting that its GAP activity does not vary over the range of magnesium concentrations examined in our study. This disparity between the GAP activities of ARF1 and ARF6 suggests that the GAPs for ARF1 and ARF6 may be regulated in significantly different ways. Relevant to this possibility, ARF6 has been shown recently to regulate not only endocytic transport, but also actin rearrangement (25, 26), and these two events are also regulated by the Rho family of small GTPases during the formation of surface membrane protrusions (40, 41). Thus, an intriguing possibility is that upstream regulation of ARF6 through its GAP might be subject to intracellular signaling mechanisms that regulate membrane protrusions (42, 43). Consistent with this possibility, ARF6 has been shown recently to regulate cell spreading (44), a process that occurs through the formation of membrane protrusions. Moreover, this possibility would explain why different cell types exhibit different degrees of membrane-bound ARF6 (see Fig. 2), as different cell types are likely to exhibit different capacities to form membrane protrusions. Finally, as this process is regulated by calcium signaling (42, 43), it will be interesting to examine in the future whether calcium also affects the distribution of ARF6 between membrane and cytosol.

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REFERENCES
1. Rothman, J. E., and Wieland, F. T. (1996) Science 272, 227–234
2. Schekman, R., and Orci, L. (1996) Science 271, 1526–1532
3. Donaldson, J. G., Cassel, D., Kahn, R. A., and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6408–6412
4. Palmer, D. J., Helms, J. B., Beckers, C. J., Orci, L., and Rothman, J. E. (1993) J. Biol. Chem. 268, 12083–12089
5. Stamnes, M. A., and Rothman, J. E. (1993) Cell 73, 999–1005
6. Traub, I. M., Ostrow, J. A., and Kernfeld, S. (1993) J. Cell Biol. 123, 561–573
7. West, M. A., Bright, N. A., and Robinson, M. S. (1997) J. Cell Biol. 138, 1239–1254
8. Ooi, C. E., Dell’Angelica, E. C., and Bonifacino, J. S. (1998) J. Cell Biol. 142, 391–402
9. Randazzo, P. A., Yang, Y. C., Rulka, C., and Kahn, R. A. (1993) J. Biol. Chem. 268, 9555–9563
10. Tsai, S.-C., Adamik, R., Haun, R. S., Moss, J., and Vaughan, M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9272–9276
11. Tanigawa, G., Orci, L., Amherdt, M., Ravazzola, M., Helms, J. B., and Rothman, J. E. (1995) J. Cell Biol. 123, 1365–1371
12. Teal, S. B., Hsu, V. W., Peters, P. J., Klausner, R. D., and Donaldson, J. G. (1994) J. Biol. Chem. 269, 3135–3138
13. Randazzo, P. A., and Kahn, R. A. (1994) J. Biol. Chem. 269, 10758–10763
14. Peyroche, A., Paris, S., and Jackson, C. L. (1996) Nature 384, 479–481
15. Chardin, P., Paris, S., Antony, R., Robineau, S., Beraud-Dufour, S., Jackson, C. L., and Chabre, M. (1996) Nature 384, 481–484
16. Klarlund, J. K., Guilintera, A., Holik, J. J., Virbasius, J. V., Chawla, A., and Czech, M. P. (1997) Science 275, 1927–1930
17. Mecari, E., Tsai, S.-C., Adamik, R., Moss, J., and Vaughan, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1745–1748
18. Morinaga, N., Moss, J., and Vaughan, M. (1997) Proc Natl. Acad. Sci U. S. A. 94, 12926–12931
19. Cukierman, E., Huber, I., Rotman, M., and Cassel, D. (1995) Science 267, 1490–1499
20. Ding, M., Vitale, N., Tsai, S.-C., Adamik, R., Moss, J., and Vaughan, M. (1996) J. Biol. Chem. 271, 24005–24009
21. Randazzo, P. A. (1997) Biochem. J. 324, 413–419
22. Premont, R. T., Clagin, A., Vitale, N., Freeman, J. L. R., Pitcher, J. A., Patton, W. A., Moss, J., Vaughan, M., and Letkovitz, R. W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14082–14087
23. D’Souza-Schorey, C., Li, G., Colombo, M. I., and Stahl, P. D. (1995) Science 267, 1175–1178
24. Peters, P. J., Hsu, V. W., Ooi, C. E., Finazzi, D., Teal, S. B., Oorschot, V., Donaldson, J. G., and Klausner, R. D. (1995) J. Cell Biol. 128, 1003–1017
25. Radhakrishna, H., Klausner, R. D., and Donaldson, J. G. (1996) J. Cell Biol. 134, 935–947
26. D’Souza-Schorey, C., Boshans, R. L., McDonough, M., Stahl, P. D., and Van Aelst, L. (1997) EMBO J. 16, 5445–5454
27. Van Aelst, L., Joneson, T., and Bar-Sagi, D. (1996) EMBO J. 15, 3778–3786
28. D’Souza-Schorey, C., van Denselaar, E., Hsu, V. W., Yang, C., Stahl, P. D., and Peters, P. J. (1998) J. Cell Biol. 140, 603–616
29. Cavenagh, C. M., Whitney, J. A., Carroll, K., Zang, C., Boman, A. L., Rosenwald, A. G., Mellman, I., and Kahn, R. A. (1996) J. Biol. Chem. 271, 21767–21774
30. Yang, C. Z., Heimberg, H., D’Souza-Schorey, C., Muckler, M. M., and Stahl, P. D. (1998) J. Biol. Chem. 273, 4006–4011
31. Franco, M., Chardin, P., Chabre, M., and Paris, S. (1995) J. Biol. Chem. 270, 1337–1341
32. Ostermann, J., Orci, L., Tani, K., Amherdt, M., Ravazzola, M., Elazar, Z., and Rothman, J. E. (1993) Cell 75, 1015–1025
33. Welch, C. F., Moss, J., and Vaughan, M. (1994) J. Biol. Chem. 269, 15583–15587
34. Downward, J., Graves, J. D., Warne, P. H., Rayter, S., and Cantrell, D. A. (1990) Nature 346, 719–723
35. Ren, X. D., Kiessow, B. W., and Schwartz, M. A. (1999) EMBO J. 18, 578–585
36. Donaldson, J. G., Finazzi, D., and Klausner, R. D. (1992) Nature 350, 350–352
37. Helms, J. B., and Rothman, J. E. (1992) Nature 350, 352–354
38. Boguski, M. S., and McCormick, F. (1993) Nature 366, 643–654
39. Dascher, C., and Balch, W. E. (1994) J. Biol. Chem. 269, 1437–1448
40. Nobs, C. D., and Hall, A. (1995) Cell 81, 53–62
41. Lamaze, C., Chuan, T.-H., Terlecky, L. J., Bokoch, G. M., and Schmid, S. L. (1999) Nature 392, 177–179
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