

# Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic

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**A**DP-ribosylation factor (Arf) 6 regulates the movement of membrane between the plasma membrane (PM) and a nonclathrin-derived endosomal compartment and activates phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase), an enzyme that generates phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). Here, we show that PIP<sub>2</sub> visualized by expressing a fusion protein of the pleckstrin homology domain from PLC $\delta$  and green fluorescent protein (PH-GFP), colocalized with Arf6 at the PM and on tubular endosomal structures. Activation of Arf6 by expression of its exchange factor EFA6 stimulated protrusion formation, the uptake of PM into macropinosomes enriched in PIP<sub>2</sub>, and recycling of this membrane back to the PM. By contrast, expression

of Arf6 Q67L, a GTP hydrolysis-resistant mutant, induced the formation of PIP<sub>2</sub>-positive actin-coated vacuoles that were unable to recycle membrane back to the PM. PM proteins, such as  $\beta$ 1-integrin, plakoglobin, and major histocompatibility complex class I, that normally traffic through the Arf6 endosomal compartment became trapped in this vacuolar compartment. Overexpression of human PIP 5-kinase  $\alpha$  mimicked the effects seen with Arf6 Q67L. These results demonstrate that PIP 5-kinase activity and PIP<sub>2</sub> turnover controlled by activation and inactivation of Arf6 is critical for trafficking through the Arf6 PM-endosomal recycling pathway.

## Introduction

Cells interact with their environment at the plasma membrane (PM)\* and respond to stimuli by rearranging their surfaces through modification of cell junctions, cell polarity, and the organization of the cortical actin cytoskeleton. Although signaling pathways and actin dynamics have well-documented roles in these responses, the contribution of membrane traffic to these processes is less clear. Nonetheless, evidence is accumulating that the insertion and uptake of membrane at the PM is important in regulating PM morphology (Mellman, 2000). Requirements for membrane traffic in neutrophil migration (Lawson and Maxfield, 1995) and phagocytosis (Bajno et al., 2000) have been demonstrated. Similarly, GTPases involved in the regulation of membrane

traffic, such as Rab5 (Spaargaren and Bos, 1999), Rab8 (Peranen et al., 1996), and ADP-ribosylation factor (Arf) 6 (Radhakrishna et al., 1996) have been shown to influence cortical actin structure at the PM, lending support to the concept that membrane trafficking can influence the composition and structure of the plasma membrane.

We have been studying the Arf6 GTPase in cells to elucidate the relationship between Arf6-regulated membrane traffic and cell morphology at the PM. In HeLa and many other cells, Arf6 regulates the movement of membrane between the PM and a nonclathrin-derived endosomal compartment (Radhakrishna and Donaldson, 1997). PM proteins and lipids that are internalized by this endocytic pathway can be recycled back to the PM. This membrane recycling requires activation of Arf6 and the presence of actin filaments (Radhakrishna and Donaldson, 1997). Activation of Arf6 has also been implicated in regulated exocytosis (Galas et al., 1997), suggesting that recycling of plasma membrane and regulated exocytosis may be related or share some properties.

Several investigations have shown that Arf6-dependent regulation of membrane recycling influences the actin cytoskeleton along the PM. Expression of a dominant negative mutant of Arf6, T27N, that inhibits membrane recycling inhibits cell spreading (Song et al., 1998), Rac-mediated

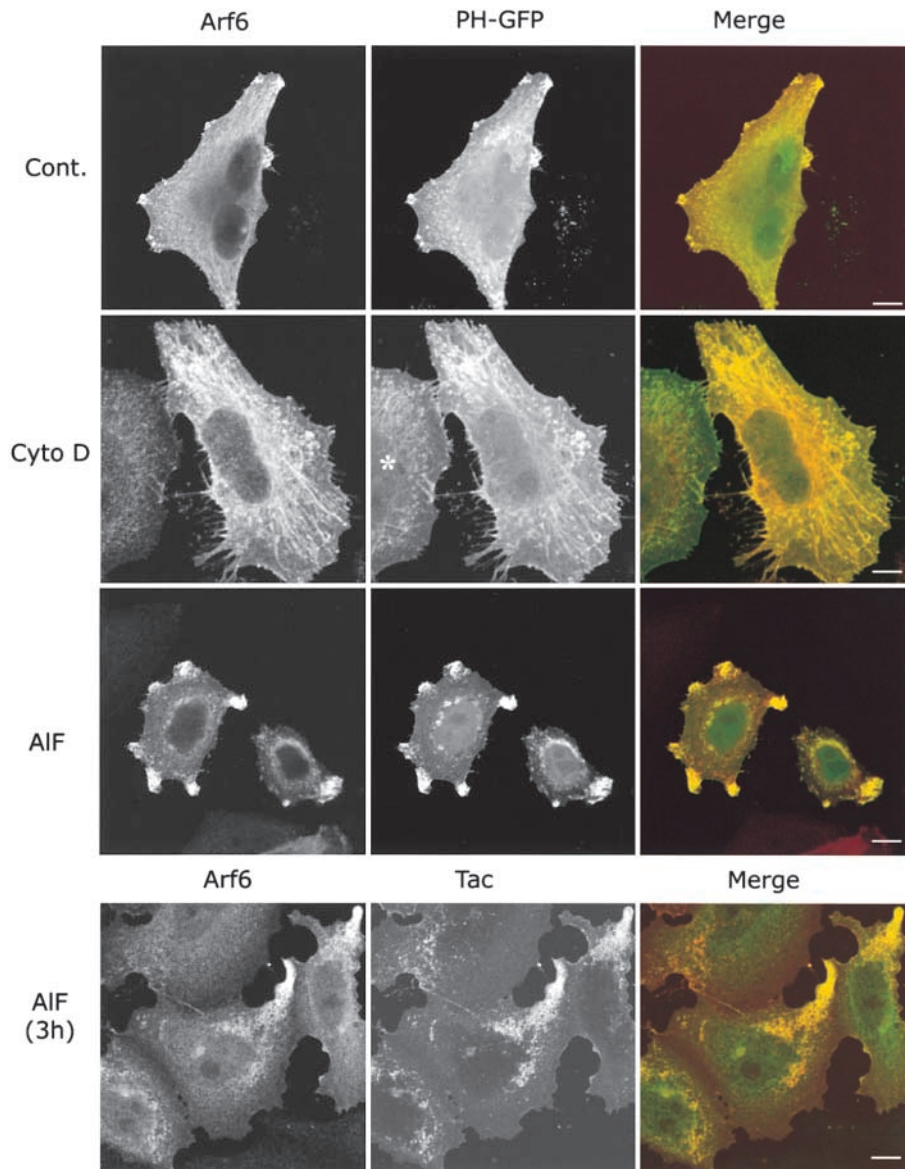
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\*Abbreviations used in this paper: AlF, aluminum fluoride; Arf, ADP-ribosylation factor; CD, cytochalasin D; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; MHC I, major histocompatibility complex class I; PH, pleckstrin homology; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PIP 5-kinase, phosphatidylinositol 4-phosphate 5-kinase; PM, plasma membrane.

Key words: Arf6; membrane traffic; phosphatidylinositol 4,5-bisphosphate; PIP<sub>2</sub>; PIP 5-kinase

**Figure 1. Arf6 and PH-GFP colocalize at the PM and on distal portions of the tubular endosome.**

HeLa cells were cotransfected with Arf6 and PH-GFP and then incubated for 30 min with either no addition (Cont., top), 0.2  $\mu$ M CD (Cyto D, middle), or 30 mM NaF and 50  $\mu$ M AlCl<sub>3</sub> (AIF, bottom) and then fixed and processed for immunofluorescence labeling of Arf6. Membrane internalization into the Arf6 compartment during extended AIF treatment was assessed by examining uptake of Tac antibody in cells cotransfected with Arf6 and Tac (AIF 3 h, bottom). Asterisk denotes cell transfected with PH-GFP alone, displaying enhanced tubular localization after CD treatment. Bars, 10  $\mu$ m.



ruffling (Radhakrishna et al., 1999), and Fc-mediated phagocytosis (Zhang et al., 1998). Acute activation of Arf6 by either treatment of cells with aluminum fluoride (AIF) (Radhakrishna et al., 1996) or coexpression of guanine nucleotide exchange factors (GEFs) for Arf6, ARNO (Frank et al., 1998), or EFA6 (Franco et al., 1999) results in ruffling protrusive structures along the PM. Although expression of the constitutively active Arf6 mutant, Q67L, also generates protrusive structures and alterations in cortical actin (Radhakrishna et al., 1996), a variety of changes in cell morphology is observed, and the phenotype has not been explored thoroughly. Some studies investigating the morphology of cells expressing Arf6 Q67L report extensive invaginations of the PM, often tortuous and complex (Peters et al., 1995; D'Souza-Schorey et al., 1998). In another recent report, constitutively active Arf6 was shown to decrease stress fiber formation, suggesting antagonism of Rho function (Boshans et al., 2000). The variable morphology of cells expressing Arf6 Q67L can be attributed to the consequence of persistent activation of Arf6 effectors over ex-

tended periods during transient transfection. Understanding how activated Arf6 causes these morphological changes would clarify the normal functions of Arf6 in cells.

An important advance in understanding Arf function was the recent demonstration that members of the Arf family can activate type I phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase), an enzyme that catalyses the formation of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) from phosphatidylinositol 4-phosphate (Honda et al., 1999; Godi et al., 1999; Jones et al., 2000). Synthesis and turnover of PIP<sub>2</sub> have been implicated in a variety of cellular events including membrane trafficking (Martin, 1997), control of actin polymerization (Janmey, 1994; Yamamoto et al., 2001), regulation of ion channel transporters (Kobrinisky et al., 2000), and signal transduction where it serves as a substrate for enzymes such as PLC and phosphatidylinositol 3-kinase (Czech, 2000). This pleiotropic role of PIP<sub>2</sub> illustrates the importance of understanding where in the cell PIP<sub>2</sub> is synthesized and how this process is regulated spatially. Although all Arfs have been shown to stimulate PIP 5-kinase activity in vitro,

in cells it is Arf6 that colocalizes with PIP 5-kinase at the PM (Honda et al., 1999). Protrusions and ruffles generated by activation of Arf6 result in the recruitment of PIP 5-kinase and PLD2 into the PM structures (Honda et al., 1999), suggesting that Arf6 might function in cells through activation of PIP 5-kinase. However, no data has been published showing the distribution of these proteins and their lipid product, PIP<sub>2</sub>, in this membrane trafficking pathway.

Here, we have investigated the effects of Arf6 activation and PIP 5-kinase on the distribution and local formation of PIP<sub>2</sub> in order to better understand the role of this lipid in the regulation of membrane traffic through the Arf6 pathway. We find that the Arf6 PM-endosomal recycling pathway can serve as a mechanism to regulate the availability and activity of PIP 5-kinase and therefore PIP<sub>2</sub> levels at the PM. We show that activation of Arf6 by expression of its exchange factor, EFA6, allows for uptake and recycling of membrane through this pathway, whereas expression of constitutively active Arf6Q67L or PIP 5-kinase alters the trafficking of membrane such that PM proteins enter the cell and become trapped in PIP<sub>2</sub>-positive actin-coated vacuolar structures. These results establish that Arf6-dependent regulation of PIP 5-kinase and PIP<sub>2</sub> levels and their localization is crucial for proper regulation of trafficking through the Arf6 PM-endosomal recycling pathway.

## Results

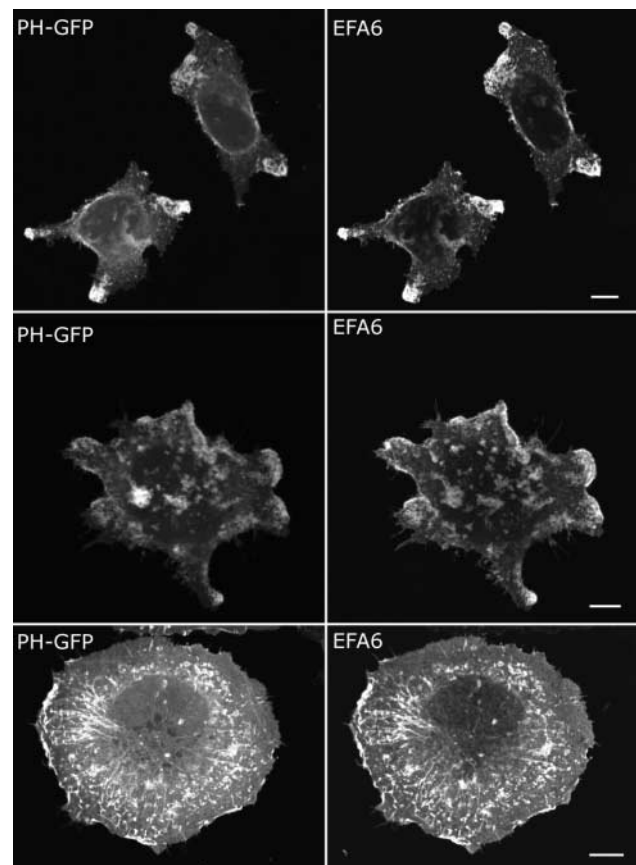
To understand the relationship between Arf6 function and PIP 5-kinase, we first compared the distribution of Arf6 and PIP<sub>2</sub> in cells under conditions where we could manipulate trafficking through the Arf6-regulated recycling pathway with pharmacologic reagents. To localize PIP<sub>2</sub>, we expressed a chimeric protein of the pleckstrin homology domain of PLC $\delta$  coupled to the green fluorescent protein (PH-GFP), previously characterized as a marker for membrane-associated PIP<sub>2</sub> (Varnai and Balla, 1998). We examined the cellular distribution of PIP<sub>2</sub> by monitoring PH-GFP localization in cells expressing wild-type Arf6 after treatment with cytochalasin D (CD) or AIF to impose blocks in the recycling pathway.

Cells cotransfected with plasmids encoding PH-GFP and wild-type Arf6 exhibited a remarkable colocalization of PH-GFP with Arf6 along the plasma membrane, and some associated with endocytic structures (Fig. 1). The Arf6 endosomal compartment was more readily observed upon treatment of cells with CD. Under these conditions, Arf6 and PIP<sub>2</sub> were observed on the tubular endosomes emanating from the juxtannuclear region. These tubular endosomes have been shown previously to represent the accumulation of endocytosed membrane within the Arf6 recycling compartment, since CD blocks return of this membrane back to the PM (Radhakrishna and Donaldson, 1997). The PH-GFP showed striking colocalization with Arf6-labeled tubules, especially on their distal portions. Labeling of the tubular endosomes with PH-GFP in response to CD treatment did not require overexpression of Arf6, since it was observed in cells expressing endogenous levels of Arf6 (Fig. 1, Cyto D, asterisk).

Recruitment of PH-GFP to membrane was dependent on fusion to an intact PH domain specific for PIP<sub>2</sub>. Much reduced PM labeling and no endosomal tubule localization

was observed with the phosphatidylinositol 3,4,5-trisphosphate-specific Btk PH domain-GFP chimera nor was any membrane localization detected with GFP fused to the R40L mutant of the PLC $\delta$  PH domain, a mutation that abolishes PIP<sub>2</sub> binding (Varnai and Balla, 1998; unpublished data). Thus, PH-GFP serves as a marker for most of the membrane trafficking pathway regulated by Arf6.

To investigate the role of PIP<sub>2</sub> and PIP 5-kinase in Arf6 function, we activated Arf6 by incubation of cells with AIF or by coexpression of an Arf6-specific GEF, EFA6. AIF treatment of cells resulted in the formation of protrusive structures along the cell's edge and caused Arf6 and PH-GFP to colocalize in these structures consistent with previous reports (Honda et al., 1999). During extended AIF treatment up to 3 h, we observed protrusive structures and increased amounts of endocytic vesicles associated with these protrusions. In cells coexpressing Tac, the  $\alpha$ -subunit of the interleukin 2 receptor and a marker for the Arf6 endosome (Radhakrishna and Donaldson, 1997), these endosomes accumulated Tac antibody by internalization during the 3-h AIF treatment (Fig. 1, bottom). We had previously observed these macropinosomes during protrusion formation induced by AIF treatment (Radhakrishna et al., 1996). During short AIF treatment, these structures recycle membrane and fluid back to the PM (Radhakrishna et al., 1996). However, on



**Figure 2. EFA6 induces formation of PIP<sub>2</sub>-enriched protrusions and the appearance of PIP<sub>2</sub>-labeled endosomal structures.** HeLa cells were transfected with plasmids encoding EFA6 and PH-GFP and then fixed (top and middle) or treated with CD (100 nM for 30 min) before fixing (bottom). Bars, 10  $\mu$ m.

Table I. Percentage of cells exhibiting morphology

	Protrusions only	Vacuoles only	Protrusions and vacuoles
20 h			
PH-GFP alone	3.3 (2.2)	1.1 (1.3)	0
plus EFA6	53.8 (8.7)	2.5 (0.8)	3.5 (1.9)
plus Arf6 Q67L	4.2 (1.8)	56.2 (9.5)	5.8 (1.9)
plus PIP 5-kinase	0.1 (0.1)	36.1 (2.6)	1.1 (0.7)
44 h			
PH-GFP alone	2.8 (2.3)	1.4 (0.4)	0
plus EFA6	68.2 (5.6)	3.6 (0.1)	8.3 (2.1)
plus Arf6 Q67L	2.1 (1.4)	71.9 (3.9)	8.1 (2.8)
plus PIP 5-kinase	0	51.7 (3.3)	1.1 (1.4)

Numbers are displayed as average with SEM shown in parentheses. 200 cells clearly expressing the indicated protein(s) were counted per experiment. Vacuoles were scored by observation of intracellular PIP<sub>2</sub>-positive ring-like structures. Data are representative of at least three independent experiments.

longer AIF activation as shown here these macropinosomes tend to be more evident (Fig. 1, bottom).

As an alternative to AIF stimulation, we expressed EFA6 shown to be a specific GEF for Arf6 in *in vitro* assays and in cells (Franco et al., 1999). The majority of cells expressing EFA6 exhibited protrusions around the cell periphery where EFA6 and PIP<sub>2</sub> colocalized (Fig. 2). This phenotype could be observed in 57% of cells at 20 h and increased to 76% at 44 h after transfection (Table I). In about 5% of the cells, vacuolar endocytic structures that were positive for EFA6 and PH-GFP were observed in the central portion of the cells and also associated with the protrusions (Fig. 2 and Table I). However, this is an underestimate, since imaging of live cells expressing EFA6 revealed that all cells forming protrusions were undergoing active endocytosis and recycling (see Fig. 7). These endosomal membranes also contained endogenous molecules that traffic through the Arf6 endosome such as major histocompatibility complex class I (MHC I; see Fig. 6). EFA6-induced protrusion formation was inhibited in cells coexpressing the dominant negative mutant of Arf6, T27N (unpublished data), indicating that endogenous Arf6 mediates the effects of EFA6 as described previously (Franco et al., 1999). CD treatment also inhibited protrusions and resulted in localization of EFA6 on the tubular endosomal membranes (Fig. 2, bottom) as observed for Arf6 in Fig. 1.

#### Accumulation of PIP<sub>2</sub>-positive vacuoles in cells expressing Arf6 Q67L and PIP 5-kinase

We next examined the distribution of PH-GFP in cells expressing the constitutively active mutant of Arf6, Q67L. Previous attempts to understand the phenotype of cells expressing Arf6 Q67L have been hampered by the lack of a clear characterization of the onset and nature of the membrane traffic block imposed. These cells are somewhat protrusive, although not particularly F-actin rich (Radhakrishna et al., 1996), and often take on distorted cell morphology. Furthermore, immunofluorescent localization of Arf6 is poorly resolved in these cells (Peters et al., 1995; Radhakrishna et al., 1996). We assessed the distribution of PIP<sub>2</sub> in cells expressing Arf6 Q67L after a 44-h transfection and found that PH-GFP clearly labeled distinct membrane structures in these cells (Fig. 3). The vacuoles, particularly those in large clusters, were coated with actin and were inside the cells as labeling of the cell surface with fluorescent lectin in fixed cells (shown in Fig. 3) or with the lipid dye,

DiI, in live cells (unpublished data) did not label these structures. These actin-coated, PH-GFP-labeled structures were also observed in other cell types including BHK (unpublished data) and Cos cells (see Fig. 8). However, induction of PIP<sub>2</sub>-positive vacuoles was specific to Arf6 and was not observed with Arf1. Expression of the constitutively active Arf1 mutant, Q71L, which stably associates with the Golgi complex and causes alterations in Golgi morphology (Zhang

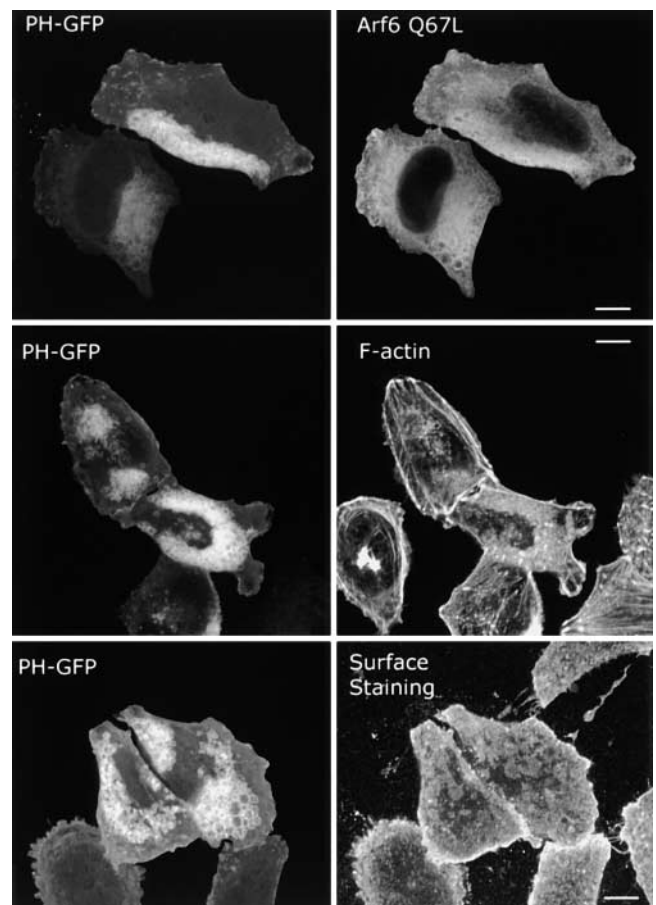
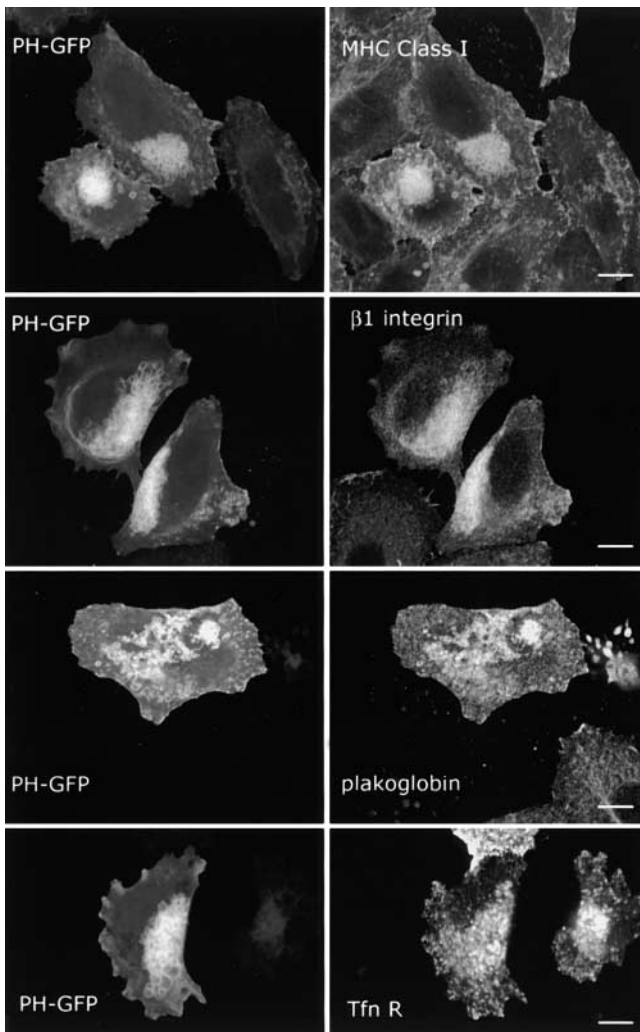


Figure 3. **Constitutively active Arf6 induces the accumulation of PIP<sub>2</sub>-positive actin-coated vacuoles.** HeLa cells were transfected with Arf6 Q67L and PH-GFP for 44 h and then fixed and probed with antibody to Arf6 (top), with phalloidin (middle), or the surface was stained using Alexa 594-conjugated concanavalin A (bottom). Bars, 10  $\mu$ m.

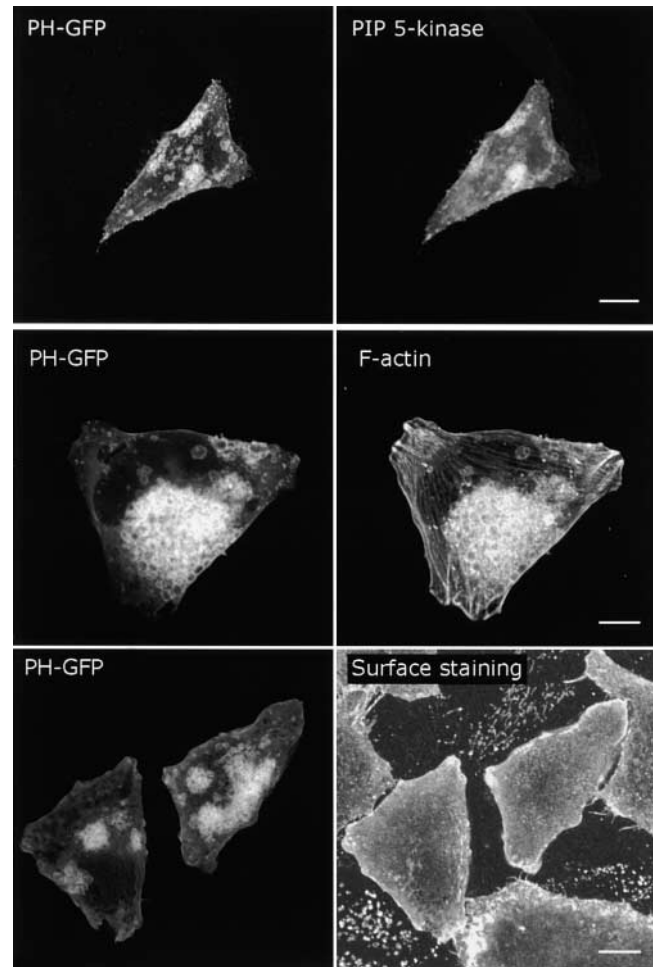


**Figure 4. Overexpression of Arf6 Q67L traps proteins that traffic through the Arf6 compartment but not the transferrin receptor into PIP<sub>2</sub>-positive vacuoles.** HeLa cells were transfected with Arf6 Q67L and PH-GFP and probed with antibodies against MHC I, β1-integrin, plakoglobin, and the transferrin receptor as shown. Bars, 10 μm.

et al., 1994), did not induce the formation of PIP<sub>2</sub>-positive vacuoles nor did the PH-GFP construct label the Golgi complex (unpublished data).

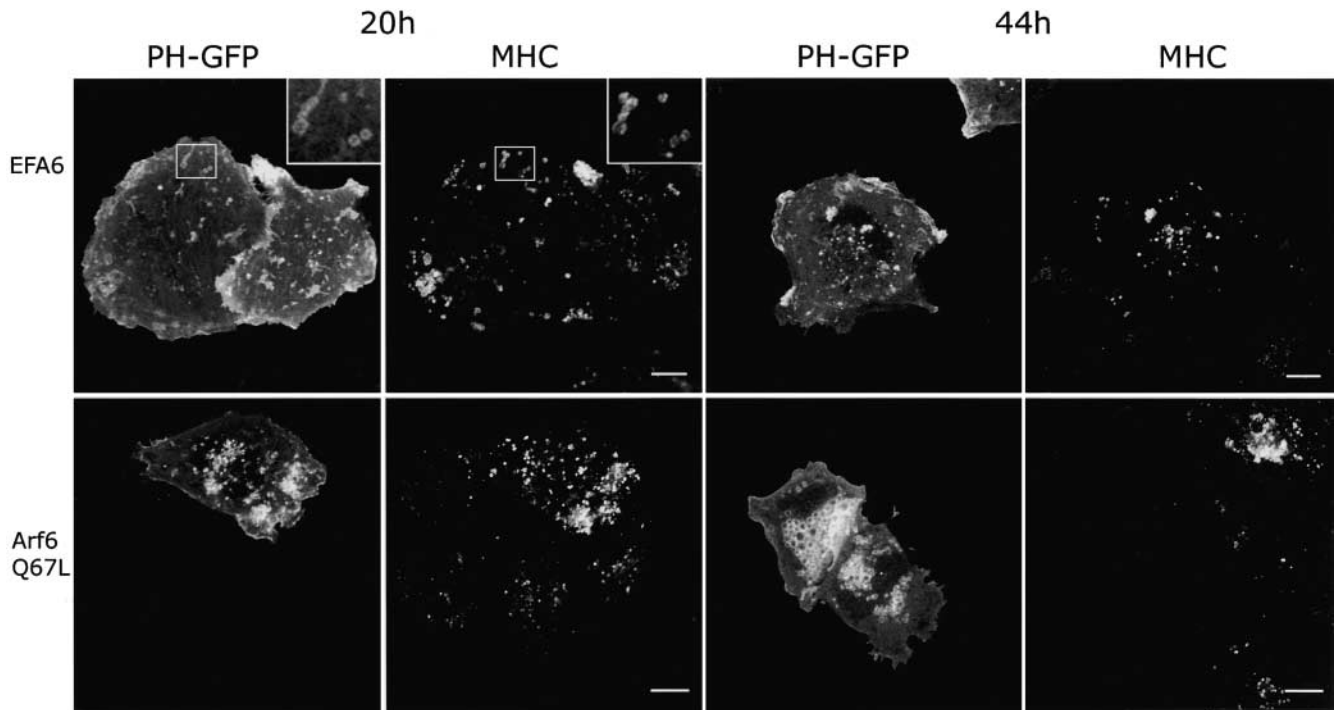
The vacuolar membranes accumulated integral and peripheral membrane proteins that traffic through the Arf6 endosome in untransfected cells. As can be seen in Fig. 4, MHC I, β1-integrin, and plakoglobin (γ-catenin) were all enriched in the PH-GFP-labeled structures induced by expression of Arf6 Q67L. The normal distribution of these proteins in cells not expressing Q67L can also be observed (Fig. 4). In contrast, transferrin receptor distribution in cells expressing Q67L was similar to that in untransfected cells, and transferrin receptor was absent from these vacuolar structures, consistent with the distinction between the clathrin-coated and Arf6-regulated endocytic recycling systems in HeLa cells as observed previously (Radhakrishna and Donaldson, 1997).

The PH-GFP labeling of the vacuoles that accumulate in cells expressing Arf6 Q67L suggests the involvement of PIP<sub>2</sub>



**Figure 5. Overexpression of PIP 5-kinase induces the accumulation of PIP<sub>2</sub>-positive actin-coated vacuoles.** HeLa cells were cotransfected with PIP 5-kinase and PH-GFP for 44 h, fixed, and probed with myc antibody to detect PIP 5-kinase (top), with phalloidin (middle), and the surface was stained using Alexa 594-conjugated concanavalin A (bottom). Bars, 10 μm.

and therefore a type I PIP 5-kinase in Arf6 function. Overexpression of human PIP 5-kinase α (homologue of the mouse PIP 5-kinase β) alone resulted in cells containing vacuolar structures that were indistinguishable from those formed in cells expressing Arf6 Q67L. The vacuoles labeled with PH-GFP were actin coated and within the cell (Fig. 5). These vacuoles were also formed in Cos cells expressing PIP 5-kinase (see Fig. 7). Kinase activity was necessary for the formation of vacuoles, since expression of a PIP 5-kinase mutant, D270A, which lacks kinase activity (Mejillano et al., 2001), did not induce their formation (unpublished data). Vacuoles induced in cells expressing human PIP 5-kinase labeled with antibodies to MHC I, integrins, and plakoglobin but not with antibodies to the transferrin receptor (unpublished data) similar to that observed for the expression of Arf6 Q67L (Fig. 4). Induction of vacuoles by PIP 5-kinase appeared to occur downstream of Arf6 activation, since expression of dominant negative Arf6 did not block vacuole formation (unpublished data). Furthermore, at low expression levels barely detected by antibody PIP 5-kinase had no effect on cell morphology but could syner-



**Figure 6. Surface MHC I is internalized and gains access to PIP<sub>2</sub> vacuoles in cells expressing EFA6 at all times but is only loaded into cells expressing Arf6 Q67L early in transfection.** HeLa cells were transfected with PH-GFP and EFA6 or Arf6 Q67L. At the indicated time (20 or 44 h), they were allowed to internalize MHC I antibody for 30 min and then were fixed and processed for visualizing internalized antibody. (Inset) Enlargement of box showing MHC antibody internalized into PIP<sub>2</sub>-positive macropinosomes. Bars, 10  $\mu$ m.

gize with wild-type Arf6 to form protrusive structures (unpublished data). In contrast to the results with human PIP 5-kinase  $\alpha$ , expression of the mouse  $\alpha$  isoform used by Honda et al. (1999), a homologue of human PIP 5-kinase  $\beta$ , was less effective at inducing the vacuolar structures, although it synergized with Arf6 to form protrusions (unpublished data).

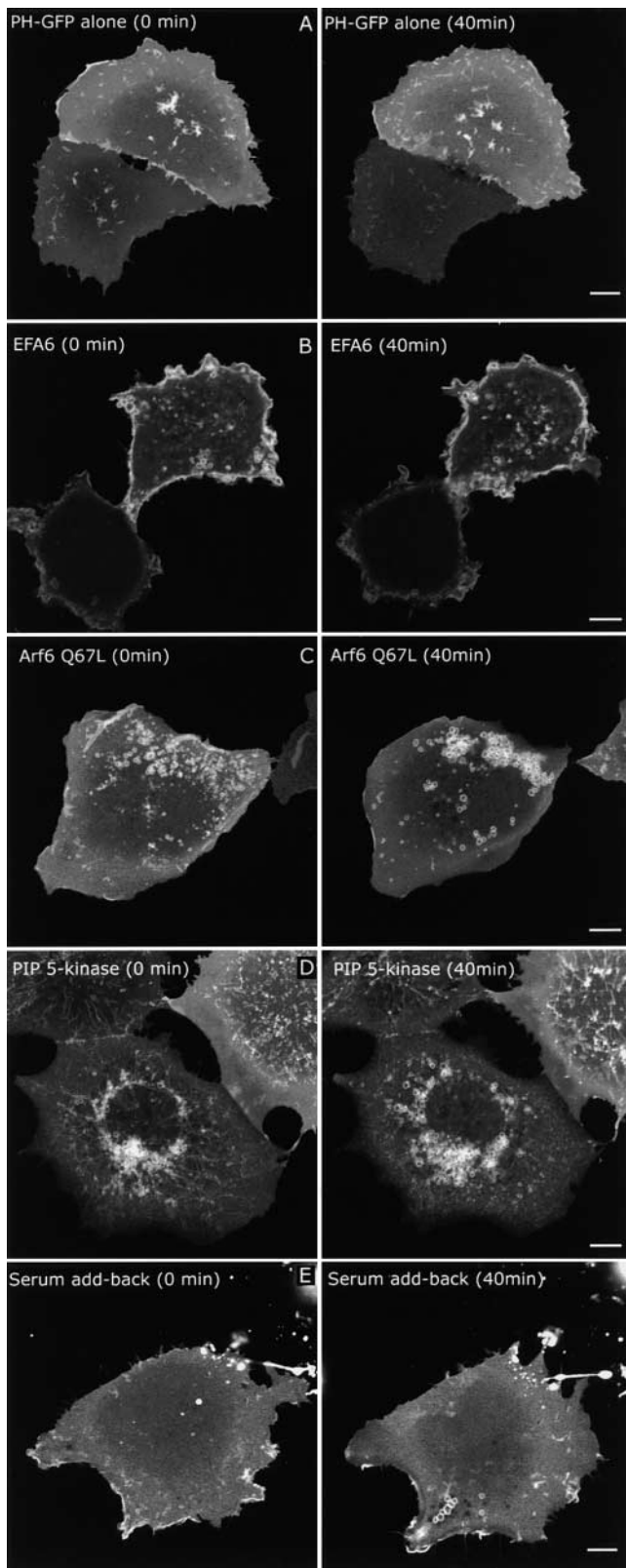
#### PIP<sub>2</sub> vacuoles are endosomally derived

To further characterize the nature of the vacuoles observed in cells expressing EFA6 and Arf6 Q67L, we examined whether these structures could be loaded with membrane from the cell surface. Since MHC I is a marker for the Arf6 endosomal pathway (Radhakrishna and Donaldson, 1997), we used antibody internalization over 30 min to test whether surface MHC I can enter the cell and be delivered to the PIP<sub>2</sub> vacuoles. In cells expressing EFA6, antibody to MHC I was observed in many of the PIP<sub>2</sub>-positive vacuoles and macropinosomes observed at both 20 and 44 h of expression (Fig. 6). In cells expressing Arf6 Q67L, MHC I antibody was found loaded into small PIP<sub>2</sub>-positive endosomal structures in cells after a 20-h transfection (Fig. 6). However, at 44 h no uptake of MHC I was observed in cells expressing Arf6 Q67L where large vacuolar membranes were apparent. It should be noted that at 20 h some cells that contain numerous large vacuolar structures did not take up antibody, suggesting that as internal membranes accumulate the cells stop internalizing membrane. Although endocytosis through the Arf6 pathway is apparently blocked at this late stage, endogenous (steady-state) MHC I was observed in these vacuolar structures (Fig. 4), presumably having been internalized earlier in transfection. These observations dem-

onstrate that EFA6-induced vacuoles readily labeled with MHC I from the PM, whereas Arf6 Q67L-induced vacuoles could be labeled with surface antibody early but not at later times in transfection.

#### Arf6 activation and membrane dynamics

To understand the dynamics of membrane trafficking and the effects of expressing EFA6, Arf6 Q67L, or PIP 5-kinase on membrane movement, live cells expressing PH-GFP were imaged. As the bulk of PH-GFP-labeled endosomes appear to be identical to the Arf6 recycling membrane compartment (Fig. 1), the PH-GFP can be used as a probe to monitor membrane traffic throughout the Arf6 pathway. Here, we show images taken of transfected Cos cells 20 h after transfection, since their more flattened appearance facilitated image acquisition. Similar observations were made in HeLa cells. In Cos cells expressing PH-GFP alone, the edge of the cells was quiet with little ruffling activity (Fig. 7 A). Internal structures labeled with PH-GFP consisted of some vesicular structures and membranous tubules. The tubules could be seen moving throughout the cytoplasm along curvilinear tracts, sometimes appearing to fuse with the PM (Fig. 7 A; video 1 available at <http://www.jcb.org/content/vol154/issue5/1007>). Cells coexpressing EFA6 and PH-GFP exhibited extensive ruffling and protrusion activity at the peripheral edge of the cells (Fig. 7 B; video 2). Along with the ruffling, these cells internalized membrane via macropinocytosis, and these PH-GFP-labeled internal structures were observed forming and disappearing again. The addition of CD to these cells immediately halted ruffling activity, and internal PIP<sub>2</sub>-labeled endosomes extended tu-



**Figure 7. Live cell dynamics of PIP<sub>2</sub>-labeled membranes after Arf6 activation.** Cos cells were transfected with PH-GFP alone (A), EFA6 (B), or Arf6 Q67L (C) or PIP 5-kinase (D) and then imaged 18 h after transfection for ~40 min. For serum add-back (E), PH-GFP-expressing cells were serum starved overnight. Serum (20%) was added immediately before imaging. Images of the subject cells taken at 0 and 40 min are shown. See also QuickTime videos 1–5 of each condition available at <http://www.jcb.org/content/vol154/issue5/1007>. Each

bules (unpublished data), resembling the appearance of EFA6-expressing HeLa cells treated with CD (Fig. 2, bottom row). In Fig. 7 B (video 2), some of the membrane appeared to be discharged through tubules extending from crescent-shaped wave-like structures along the ventral surface of the cells. These waves are strongly induced by EFA6 expression (Fig. 2) and after serum stimulation (see Fig. 7 E; video 5) and are found to a lesser extent in cells expressing PH-GFP alone. However, over the course of the 40-min imaging period it was notable that cells expressing EFA6 continued to protrude and take in PM, indicating that membrane recycling back to the PM was occurring. In contrast, there was an increase in the number and size of vacuoles that label with PH-GFP in cells expressing Arf6 Q67L over the 40-min time period of imaging (Fig. 7 C; video 3). At the start of imaging, these Arf6 Q67L cells exhibited modest ruffling and contained small vesicles and tubulo-vesicular structures. However, over time vacuoles accumulated within the cell, arising through an increase in both the numbers and size of the vesicles. Indeed, the larger vacuoles appeared to be forming through fusion of small vesicles. By the end of imaging, the cell was filled with vacuoles that often aggregated to one side, and little ruffling was observed along the PM. Cells expressing PIP 5-kinase underwent a similar transition, resulting in the accumulation of vacuoles that increased in number and size (Fig. 7 D; video 4). However, at early time points it was notable that numerous tubules that labeled with PH-GFP were present throughout the cell. The number and extent of these tubules diminished over time, since the amount of membrane contained in the PH-GFP-labeled vacuoles accumulated.

It is clear from the videos (available at <http://www.jcb.org/content/vol154/issue5/1007>) that stimulation of Arf6 activation influences the trafficking through and appearance of the Arf6 endosome and recycling pathway. Expression of EFA6 activates Arf6 but also allows for inactivation; therefore, the cells ruffle and take in membrane via macropinocytosis that is recycled, allowing continuous protrusion activity. To demonstrate that this ruffling activity could be observed in cells not overexpressing EFA6, we imaged serum-starved Cos cells expressing PH-GFP after readdition of serum (Fig. 7 E). In the absence of serum, the cells were not ruffling nor undergoing macropinocytosis, but within minutes after serum addition active ruffling and accumulation of macropinosomes in the cells were evident (Fig. 7 E; video 5). Again, as in Fig. 7 B and video 2, macropinosomes coming into the cell were turned over during the course of the video, and dissipation of membrane was observed via tubules and wave-like structures along the ventral surface.

## Discussion

In this study, we provide evidence that Arf6 regulation of membrane traffic is coupled to PIP<sub>2</sub> generation and removal. Under normal growth conditions, Arf6 constitutively under-

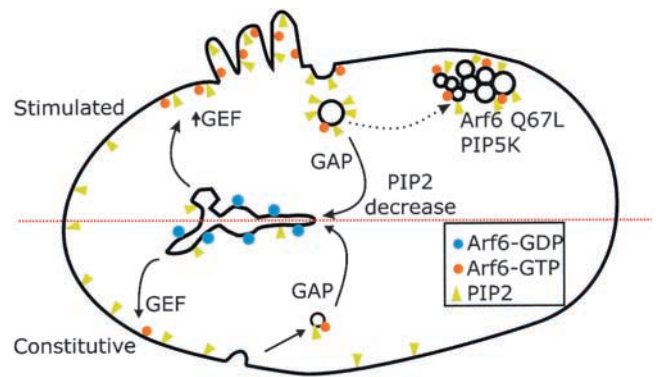
video shows the time points between the still images, are played at equivalent frame rates, and were recorded over the same approximate length of time. Bars, 10  $\mu$ m.

goes activation and inactivation, allowing membrane to recycle into and out of the cell (Fig. 8). Although activation of Arf6 was stimulated by overexpression of EFA6, membrane was internalized into PIP<sub>2</sub>-labeled macropinosomes induced by protrusive activities in these cells, but importantly, since Arf6 could be inactivated by an Arf GTP hydrolysis-activating protein, this membrane was efficiently recycled back to the PM to allow continual rounds of protrusions and membrane traffic. By contrast, overexpression of Arf6 Q67L, a GTP-hydrolysis-resistant mutant of Arf6, resulted in the internalization and trapping of membrane in PIP<sub>2</sub>-positive and actin-coated vacuolar structures. The vacuoles became enlarged from fusion of smaller PIP<sub>2</sub>-labeled structures (Fig. 7 C; video 3). Overexpression of PIP 5-kinase induced an essentially identical morphology, suggesting that it is the Arf6-GTP-stimulated PIP 5-kinase activity and therefore elevated PIP<sub>2</sub> which induces the block in membrane trafficking. Together, these data show that inactivation of Arf6 and subsequent loss of PIP<sub>2</sub> is an obligate step during normal trafficking from this compartment (Fig. 8).

The formation of these vacuolar structures represents a new aspect of constitutively active Arf6 phenotype not appreciated previously. EM studies have suggested that the surface of cells expressing Arf6 Q67L consists of numerous membrane folds that label in HEK 293 cells with extracellular tracers (D'Souza-Schorey et al., 1998). The vacuoles we observed in HeLa and Cos cells were often clustered together and positioned directly below the PM and from our imaging and surface labeling studies appear to be separated from the PM. Whether Arf6 Q67L acts at the PM or immediately after endocytosis may vary with cell type. Previously, we reported that internalization into the Arf6 recycling compartment was blocked in HeLa cells expressing Arf6 Q67L 36–40 h after transfection (Radhakrishna and Donaldson, 1997). In the present study, we find that by 44 h 80% of the cells already contain large accumulations of vacuoles (Fig. 3), and in these cells antibody internalization from the cell surface is greatly inhibited (Fig. 6). However, at earlier times in transfection there are many cells with small PIP<sub>2</sub>-labeled endosomes that can internalize antibody into the forming vacuoles (Fig. 6 and Fig. 7 C; video 3 available at <http://www.jcb.org/content/vol154/issue5/1007>).

The clear labeling of the vacuoles with PH-GFP and the ability to image cells expressing Arf6 Q67L during the onset of vacuole formation has assisted us in elucidating the alteration in membrane trafficking induced by this active mutant. We do not know why the onset of the Arf6 Q67L phenotype occurs for different cells at different times such that at 20 h after transfection there are a few cells already loaded with vacuoles. What is clear is that once vacuoles accumulate within the cell, endocytosis via the nonclathrin pathway is greatly reduced, presumably as a mechanism to maintain the cell surface area.

Our studies implicate PIP<sub>2</sub> and the ability to generate and remove PIP<sub>2</sub> as being critical for maintaining trafficking through the Arf6 endosomal recycling pathway. However, we have not been able to demonstrate directly this biochemically in intact cells. A plausible explanation for this is that PIP<sub>2</sub> increases may occur in small microdomains on membranes that would be difficult to measure but that nevertheless could influence membrane activities such as the fusion and generation of PIP<sub>2</sub>-labeled vacuoles observed here.



**Figure 8. Model for the roles of PIP 5-kinase and PIP<sub>2</sub> on Arf6-regulated membrane traffic.** Under normal growth conditions (Constitutive, bottom half), membrane is transported from the tubular endosome and fuses with the plasma membrane in an Arf6-GTP-dependent step that activates PIP 5-kinase. Upon internalization from the plasma membrane, GTP on Arf6 is hydrolyzed, generating Arf6-GDP, allowing PIP<sub>2</sub> levels to be lowered. The membrane fuses with the recycling endosome and then returns to the PM completing the cycle. When GEF activity is elevated, as in overexpression of EFA6 (Stimulated, top half) or transiently upon addition of serum to serum-starved cells, protrusions are formed. This leads to the uptake of membrane into dynamic PIP<sub>2</sub>-positive macropinosomes that can rapidly turn over, since Arf6-GTP can be converted to Arf6-GDP, allowing PIP<sub>2</sub> levels to decrease and sorting through the recycling endosome to proceed. However, expression of Arf6Q67L or PIP 5-kinase results in an accumulation of PIP<sub>2</sub> endosomes that tether to and fuse with one another, forming large vacuolar membrane structures (dotted arrow), preventing membrane from recycling back to the PM.

The trafficking block imposed by overexpression of PIP 5-kinase and the accumulation of PIP<sub>2</sub>-positive actin-coated endosomal structures observed here may be related to phenotypes observed in cells from mice in which PIP<sub>2</sub> 5-phosphatases, which reduce PIP<sub>2</sub> levels, have been eliminated. For example, clathrin-coated vesicles accumulate in nerve terminals of mice lacking synaptojanin (Cremona et al., 1999). Moreover, a recent study describing enlarged actin-coated vacuolar membranes in sertoli cells from mice lacking another PIP<sub>2</sub> 5-phosphatase, InPP5b, may be more directly related to the phenotype observed here (E. Hellsten and R. Nussbaum, personal communications).

### Arf6 functions through PIP 5-kinase

Our results are consistent with an earlier report that showed that PIP 5-kinase is a significant downstream effector of Arf6. Studies by Honda et al. (1999) identified Arf proteins as activators of PIP 5-kinase in vitro, and their in vivo studies also suggested that PIP 5-kinase is a mediator of Arf6 functions in cells (Honda et al., 1999). The observation made here that overexpression of PIP 5-kinase can induce the same morphological changes as activated Arf6 and that these effects are not blocked by expression of the dominant negative mutant of Arf6 indicates that PIP 5-kinase indeed acts downstream of Arf6-GTP. The high level expression of PIP 5-kinase may result in increased PIP<sub>2</sub> such that there is no effect of dominant negative Arf6. Furthermore, in our studies low level expression of PIP 5-kinase with wild-type Arf6, conditions under which neither one alone has any effect, synergizes to induce



protrusions similar to AIF-induced membrane accumulations. The morphological data presented here together with the biochemical evidence of Honda et al. (1999) suggests that Arf6-GTP can bind to and activate PIP 5-kinase in cells, leading to locally elevated PIP<sub>2</sub>.

The overexpressed PIP 5-kinase localizes to the PM, and at low levels of expression it is observed on the Arf6 recycling compartment, especially when recycling is blocked with CD. Furthermore, the localization of PIP 5-kinase to these membranes is independent of the nucleotide status of Arf6 (unpublished data). Our data suggests that PIP 5-kinase activity is responsible for mediating trafficking through the Arf6 pathway and that the level of PIP 5-kinase activity is regulated by the nucleotide status of Arf6.

Previous reports on overexpression of PIP 5-kinase have focused on changes to the actin cytoskeleton such as formation of stunted actin filaments (Shibasaki et al., 1997), microvilli (Matsui et al., 1999), stress fibers (Yamamoto et al., 2001), and exocytic and endocytic vesicles propelled by actin rockets (Rozelle et al., 2000). We have focused on the influence of PIP 5-kinase expression on membranes and suggest that the effects of PIP 5-kinase in the earlier studies and in our report may be related. We occasionally observed rocketing PIP<sub>2</sub>-labeled vesicles that extended out from the PM as microvillar-like projections in cells expressing PIP 5-kinase (Fig. 7 D; video 4 available at <http://www.jcb.org/content/vol154/issue5/1007>) like those of Rozelle et al. (2000). Interestingly, Schafer et al. (2000) have now reported that microinjection of Arf6 Q67L into cells induces the formation of actin comet tails on endocytic vesicles. These rocketing endosomes may represent an early step that precedes vacuole formation and the block in membrane traffic. Indeed, they may fuse with each other to generate the vacuoles that we see developing at later times in HeLa and Cos cells.

### Cellular functions of Arf6-generated PIP<sub>2</sub>

The ability of Arf6 to modulate PIP<sub>2</sub> levels and regulate trafficking of critical PM proteins could serve several cellular processes that involve changes in PM architecture. The trapping of proteins involved in cell adhesion into Arf6 vacuoles observed in cells expressing Arf6 Q67L or PIP 5-kinase (Fig. 4) had an effect on adhesion and morphology of these cells after 24–40 h, resulting in a reduced footprint on the substratum (unpublished data). Zhang et al. (1998) have reported that Fc-mediated phagocytosis in macrophages requires Arf6 function. Both constitutively active and dominant negative Arf6 mutants blocked phagocytosis, showing that Arf6 must complete GTP-GDP cycling for particle internalization. Consistent with this and our data, generation and removal of PIP<sub>2</sub> has been shown recently to be crucial for phagocytosis. PIP<sub>2</sub> levels are transiently enhanced beneath the phagocytic cup before internalization and then return to normal PM levels most likely through stimulation of PLC as the particle is engulfed (Seastone et al., 1999; Botelho et al., 2000). Together with our studies, this suggests that Arf6 and PIP 5-kinase must be activated to increase PIP<sub>2</sub> and then inactivated to facilitate PIP<sub>2</sub> removal in order for the engulfment phase of internalization to occur. Additionally, several studies have implicated turnover of various phosphoinositides including PIP<sub>2</sub> during macropinocytosis

(Amyere et al., 2000; Malecz et al., 2000), raising the possibility of a role for Arf6 in this process. Finally, there is an intriguing parallel between the formation and fusion of PIP<sub>2</sub> vesicles in our system and the role of PIP<sub>2</sub> during yeast vacuole fusion where PIP<sub>2</sub> is required during tethering and fusion of vacuoles (Mayer et al., 2000). In both cases, larger vacuoles formed as a result of the association and fusion of small vesicles.

At the molecular level, formation and turnover of PIP<sub>2</sub> could facilitate the recruitment and activities of numerous proteins. Indeed PIP<sub>2</sub> and PIP<sub>3</sub> have been shown both to recruit and regulate the activity of proteins that regulate membrane traffic such as Arf GEFs and GT hydrolysis-activating protein (Toker, 1998). Thus the generation of PIP<sub>2</sub> at specific sites followed by its rapid removal may provide a mechanism for spatial and temporal regulation by controlling the recruitment and activity of proteins involved in these processes (Martin, 1998).

### Parallel roles for Arfs at the plasma membrane and the Golgi complex

The activities associated with Arf6 at the plasma membrane are related to those observed for Arf1 at the Golgi complex. Arf1-regulated membrane traffic and the assembly of cytoskeletal elements onto the Golgi complex is facilitated by Arf1-dependent binding of coat protein complexes to Golgi membranes and through Arf1-stimulated changes in lipid composition at Golgi membranes (Donaldson and Jackson, 2000). In addition to PIP 5-kinases, PI 4-kinases can also contribute to the formation of PIP<sub>2</sub>, and Arf1 has been shown to recruit a PI 4-kinase to Golgi membranes, which results in an increase in PIP<sub>2</sub> levels (Stamnes et al., 1998; Godi et al., 1999; Jones et al., 2000). Arf1 can recruit actin and spectrin to Golgi membranes, possibly through PIP<sub>2</sub>-enriched domains on Golgi membranes (Godi et al., 1998; Fucini et al., 2000). Importantly, for both Arf1 and Arf6 the ability of these GTPases to cycle between active and inactive forms is crucial for their ability to regulate membrane traffic and structure in cells.

An emerging theme in the function of Arf family members is regulation of membrane lipid composition. Here, we have demonstrated that Arf6 controls traffic through a clathrin-independent PM/endosomal pathway by regulating PIP<sub>2</sub>. Localized transient activation of Arf6 and therefore elevation of PIP<sub>2</sub> levels can regulate membrane traffic and PM morphology during cellular processes such as phagocytosis and cell migration. Understanding how members of the Arf family regulate these pathways and how this contributes to membrane traffic presents a major challenge in cell biology.

## Materials and methods

### Cells, reagents, and antibodies

Cos, BHK, and HeLa cells were grown in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO<sub>2</sub>. Rabbit polyclonal antibodies were used as described previously (Radhakrishna and Donaldson, 1997). Mouse monoclonal antibody (16B12) against the influenza HA epitope was purchased from BabCo. Mouse monoclonal antibody (9E10) against the myc epitope was purchased from Zymed Laboratories. Human Tac antigen (IL-2 receptor α subunit) was detected with mouse monoclonal anti-Tac antibodies (7G7). Tac uptake was performed as described previously (Radhakrishna and Donald-

son, 1997). Mouse hybridoma cells generating antibodies against human MHC I W6/32 were provided by Dr. Paul Roche (National Institutes of Health). Alexa 594 concanavalin A and secondary antibodies (Alexa 594, Alexa 488, anti-mouse, and anti-rabbit) were from Molecular Probes, Inc. All other reagents including M5 anti-FLAG antibody and TRITC-phalloidin were purchased from Sigma-Aldrich.

### DNA manipulations and transient transfections

EFA6 was cloned from Marathon human fetal brain cDNA library (CLONTECH Laboratories, Inc.) by PCR and then shuttled into pFLAG-cmv6 (Sigma-Aldrich), placing the FLAG epitope on the NH<sub>2</sub> terminus of the protein. EFA6 sequence was confirmed correct by sequencing. Wild-type and mutant Arf6 genes were shuttled from pXS into pCDNA3.1. Identical results were achieved using Arf6 genes in either vector. *myc*-tagged human type I PIP 5-kinase  $\alpha$  was as described previously (Rozelle et al., 2000). The PH domain from PLC $\delta$ 1 cloned into pEGFP-N1 for use as a high affinity marker for PIP<sub>2</sub> was described previously (Varnai and Balla, 1998). This fusion protein can associate with soluble inositol 1,4,5-trisphosphate (Hirose et al., 1999); however, when membrane bound it specifically recognizes PIP<sub>2</sub> among phosphoinositides. At high expression levels, PH-GFP can interfere with activities requiring PIP<sub>2</sub> through sequestration (Raucher et al. 2000), and we observed increased blebbing in cells expressing high levels of PH-GFP. However, low level expression of the chimera as used here is a useful indicator of PIP<sub>2</sub> distribution (Botelho et al., 2000; Tall et al., 2000).

HeLa cells were grown on glass coverslips and transfected using the calcium phosphate method as described previously (Bonifacino et al., 1989) or Fugene 6 (Roche) according to the manufacturers instructions. Typically, the cells were then examined the following day (~20 h) or the second day (~44 h) after DNA addition.

### MHC I antibody internalization

Serum-containing medium with MHC monoclonal antibody (clone W6/32) was preequilibrated to 37°C for 30 min before addition of cells. Cells were allowed to internalize antibody for 30 min and then fixed. To mask antibody bound to the cell surface, cells were probed with unconjugated goat anti-mouse antibody in the absence of saponin for 30 min and then re-fixed briefly. Internalized MHC antibody was then visualized by probing with Alexa 594-conjugated GAM in the presence of saponin.

### Microscopy

Either 20 or 44 h after transfection, cells were treated as indicated, fixed with 2% formaldehyde in PBS for 10 min at room temperature, and rinsed with 10% FBS and 0.02% azide in PBS (PBS/serum). Cells were incubated with primary antibodies diluted in PBS/serum containing 0.2% saponin for 1 h at room temperature and then washed (three times for 5 min each) with PBS/serum. The cells were then incubated with fluorescently labeled secondary antibodies diluted in PBS/serum plus 0.2% saponin for 1 h, washed again, and mounted on glass slides. Note that the onset of the phenotype occurs after overnight (20-h) transfection. However, cells were frequently fixed at 44 h when more cells exhibited the phenotype. Images were taken on a ZEISS 510 laser scanning microscope using a 63 $\times$  1.3 NA PlanApo objective. Figures were constructed using Adobe Photoshop<sup>®</sup> 5.5. For live cell imaging, Cos cells were plated in Lab-tek coverglass chambers with glass of no. 1 thickness (Nunc). Cells were cultured overnight, transfected the following day, and then imaged after a further 20 h. 25 mM Hepes, pH 7.4, was added to maintain pH while the chamber was on the microscope.

### Online supplemental material

Live cell images shown in Fig. 7 were acquired at 37°C every 10 s. In each case, images were acquired over a period of ~40 min at 20 h after transfection. Videos were generated with Adobe Premiere<sup>®</sup>, using every third frame due to file size constraints. Videos are played at rates such that timing is comparable between each condition. Videos 1–5 are available at <http://www.jcb.org/content/vol154/issue5/1007>.

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