

A role for POR1, a Rac1-interacting protein, in ARF6-mediated cytoskeletal rearrangements

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The ARF6 GTPase, the least conserved member of the ADP ribosylation factor (ARF) family, associates with the plasma membrane and intracellular endosome vesicles. Mutants of ARF6 defective in GTP binding and hydrolysis have a marked effect on endocytic trafficking and the gross morphology of the peripheral membrane system. Here we report that expression of the GTPase-defective mutant of ARF6, ARF6(Q67L), remodels the actin cytoskeleton by inducing actin polymerization at the cell periphery. This cytoskeletal rearrangement was inhibited by co-expression of ARF6(Q67L) with deletion mutants of POR1, a Rac1-interacting protein involved in membrane ruffling, but not with the dominant-negative mutant of Rac1, Rac1(S17N). A synergistic effect between POR1 and ARF6 for the induction of actin polymerization was detected. Furthermore, we observed that ARF6 interacts directly with POR1 and that this interaction was GTP dependent. These findings indicate that ARF6 and Rac1 function on distinct signaling pathways to mediate cytoskeletal reorganization, and suggest a role for POR1 as an important regulatory element in orchestrating cytoskeletal rearrangements at the cell periphery induced by ARF6 and Rac1.

Keywords: ARF6/cytoskeletal rearrangements/POR1/Rac

Introduction

The Ras superfamily of GTP-binding proteins can be classified into at least five subfamilies, Ras, Rho, ARF, Rab and Ran. Diligent work from a number of laboratories over the past decade has unraveled the numerous aspects of cellular functions which are controlled by the Ras superfamily of low molecular weight (20–30 kDa) GTPases (Zerial and Huber, 1995). These functions include cellular proliferation and differentiation (Ras), intracellular trafficking (Rab and ARF), cytoskeletal remodeling (Rho) and nuclear transport (Ran). A unique feature of these low molecular weight GTPases is that they function as molecular switches, cycling between their inactive GDP-bound and active GTP-bound forms.

The ADP ribosylation factor (ARF) subfamily currently includes six proteins (ARFs 1–6) that are highly conserved

in amino acid sequence (Tsuchiya *et al.*, 1991). Originally identified as a co-factor required for the cholera toxin-catalyzed ADP ribosylation of Gs (Kahn and Gilman, 1986), the ARFs have been shown to play critical roles in vesicular transport (Donaldson and Klausner, 1994; Bowman and Kahn, 1995). The best characterized ARF protein is ARF1, which is localized to the Golgi complex and is critical for transport along the secretory pathway (Donaldson and Klausner, 1994). Elucidation of the functional roles of the other ARF proteins remains a challenge. Previous studies have shown that ARF6, the least well conserved of the human ARF proteins, is localized at the cell periphery and cycles between the plasma membrane and intracellular endosomal vesicles, depending on its nucleotide status (D'Souza-Schorey *et al.*, 1995; Peters *et al.*, 1995). ARF6 mutants defective in GTP binding or hydrolysis have a marked effect on receptor-mediated endocytic trafficking (D'Souza-Schorey *et al.*, 1995) and on the gross morphology of the peripheral membrane system (Peters *et al.*, 1995). Expression of the wild-type and the GTPase-defective mutant, ARF6(Q67L), results in the elaboration of the plasma membrane characterized by the formation of extensive membrane vaginations, while expression of the ARF6(T27N) mutant defective in GTP binding results in the massive accumulation of coated endosomes around the pericentriolar region of the cell. Here we report that the expression of the GTPase-defective mutant of ARF6, ARF6(Q67L) in Chinese hamster ovary (CHO) cells induces a rearrangement of the actin cytoskeleton with a redistribution of cortical actin to the cell periphery. The actin cytoskeleton has been implicated in many cellular functions, including endocytosis, cell division, cell proliferation and cell motility. Therefore, an understanding of how actin filament organization is orchestrated is a central question in cell biology.

A number of observations have implicated the Rho family of GTPases and growth factors in signal transduction pathways that regulate the actin cytoskeletal network (Hall, 1994). In Swiss 3T3 cells, the activation of Rho results in the formation of stress fibers (Ridley and Hall, 1992), whereas activation of Rac leads to polymerization of actin at the plasma membrane, producing lamellipodia and membrane ruffles (Ridley *et al.*, 1992). Activation of Cdc42Hs results in the extension of microspikes and filopodia (Kozma *et al.*, 1995; Nobes and Hall, 1995). As judged by the respective cytoskeletal readouts in Swiss 3T3 fibroblasts, Cdc42, Rac and Rho can be placed in a cascade wherein Cdc42 activates Rac, which in turn activates Rho. GTPase cascades have tremendous potential for choreographing cellular responses. Until recently, very little was known about the mechanism by which these GTPases induce changes in cellular morphology. Several potential targets of Rho, Rac and Cdc42, implicated in actin polymerization, have now been isolated. Rho-kinase

(ROK), myosin light chain (MLC) phosphatase and PIP5 kinase have been shown to interact with Rho and to play a role in mediating Rho-induced stress fiber formation (Chong *et al.*, 1994; Gilmore and Burridge, 1996; Ishizaki *et al.*, 1996; Kimura *et al.*, 1996; Leung *et al.*, 1996; Matsui *et al.*, 1996; Ren *et al.*, 1996). It has been suggested that WASP, the Wiskott–Aldrich syndrome protein, may link Cdc42 to the actin cytoskeleton. WASP binds in a GTP-dependent manner to Cdc42 and induces actin cluster formation (Aspenstrom *et al.*, 1996; Symons *et al.*, 1996). However, a role for WASP in Cdc42-induced filopodia formation remains to be defined. Recently, a novel Rac-interacting protein, POR1, has been isolated and shown to play a role in membrane ruffling (Van Aelst *et al.*, 1996). Deletion mutants of POR1 inhibit the induction of membrane ruffling by an activated mutant of Rac1, Rac1(G12V). Our observation that an activated mutant of ARF6 induces a redistribution of cortical actin to the cell periphery led us to investigate the relationship between the ARF6 and Rac1 GTPases. We have made use of the dominant-negative mutant of Rac1, Rac1(S17N), and deletion mutants of POR1 to examine their effects on ARF6-induced actin polymerization. We demonstrate that Rac1(S17N) fails to block ARF6-mediated actin redistribution, whereas the POR1 deletion mutants interfere with ARF6-induced cytoskeletal rearrangements. Furthermore, we show that ARF6 interacts directly with POR1 in a GTP-dependent manner. Our results indicate that ARF6 regulates actin cytoskeletal organization by a mechanism independent of Rac1 and suggest a role for POR1 in ARF6-mediated signal transduction pathways.

Results

Activation of ARF6 induces actin rearrangements in CHO cells

The expression of ARF6(Q67L), the GTPase-defective mutant of ARF6, and to a lesser extent, expression of wild-type ARF6, causes a dramatic alteration at the cell periphery, by inducing the formation of numerous plasma membrane folds and a depletion of endosomal compartments (Peters *et al.*, 1995). These observations prompted us to analyze the cytoskeletal architecture in cells expressing ARF6 and its mutants defective in GTP binding and hydrolysis. Using the Sindbis virus as an expression vector, we used immunofluorescence microscopy to examine the structure of the actin cytoskeleton in CHO cells transfected with (i) wild-type ARF6, (ii) the GTPase-defective mutant, ARF6(Q67L), (iii) the GTP-binding-defective and dominant-negative mutant, ARF6(T27N), and (iv) ARF6(G2A), the non-myristoylated, cytosolic and inactive form of the protein. Four to five hours post-viral infection, the cells were fixed, labeled with rhodamine–phalloidin and viewed with a confocal immunofluorescence microscope. Figure 1A depicts actin rearrangements in cells infected with the vector virus alone. This pattern of actin filament distribution was identical to that seen in uninfected CHO cells (data not shown). Thus, under the described experimental conditions, any effect of the vector virus on the actin cytoskeleton can be excluded. Expression of the GTPase-defective mutant, ARF6(Q67L), resulted in a redistribution of cortical actin to the cell periphery. As shown in Figure 1B, cells expressing ARF6(Q67L) exhibited prominent

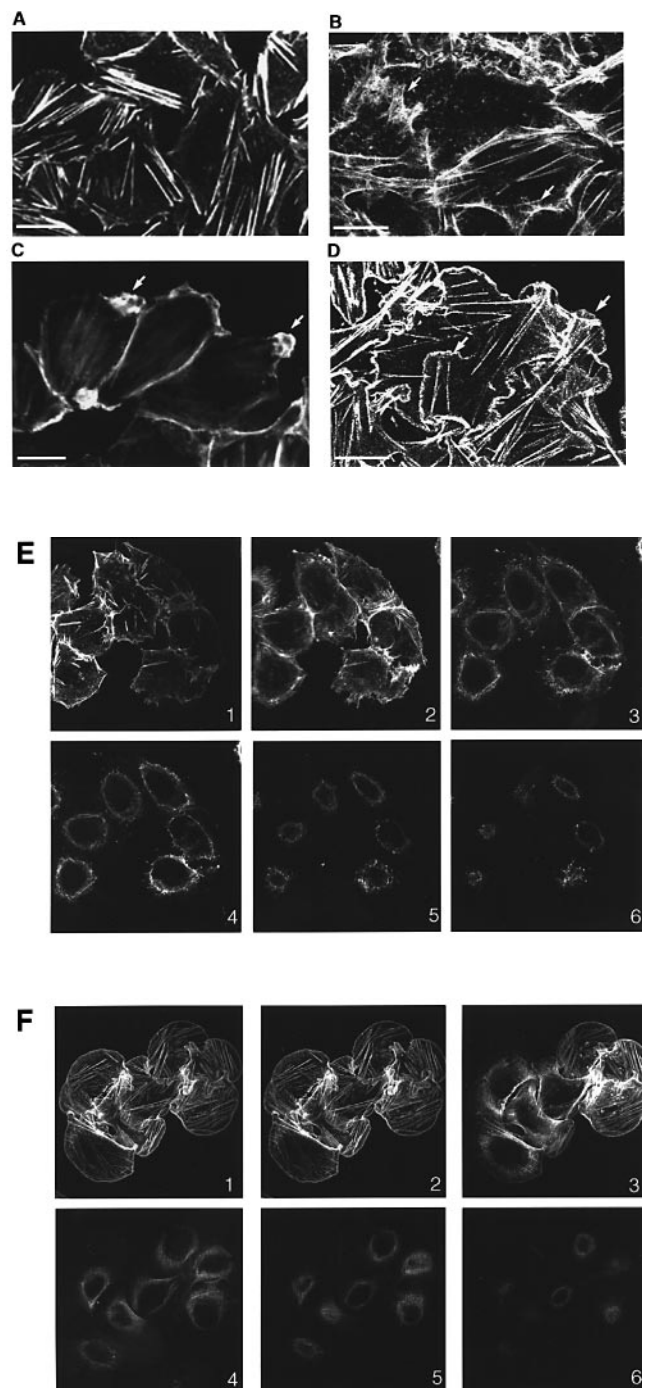


Fig. 1. ARF6 and Rac1 induce distinct actin rearrangements in CHO cells. Cells on coverslips were infected with Sindbis virus alone (A) and with recombinant Sindbis virus expressing ARF6(Q67L) (B and C) and Rac1(G12V) (D). At 4.5 h post-viral infection, cells were incubated with (C) or without aluminum fluoride (AIF) for 20 min, fixed in 2% paraformaldehyde and labeled with rhodamine–phalloidin to visualize actin filament rearrangements. Cells expressing ARF6(Q67L) exhibit increased staining for actin at the cell periphery (arrows). This pattern of actin filament organization is distinct from actin clusters (arrows) seen with AIF treatment and from Rac1(G12V)-induced lamellipodia formation (arrows). Using confocal laser scanning microscopy, fixed cells expressing ARF6(Q67L) (E) and Rac1(G12V) (F) were scanned from the bottom (1) to the top (6), through the z-axis. While spread out lamellipodia project out from the dorsal surface in Rac1(G12V)-expressing cells, actin-rich microspikes emanate from the dorsal surface of ARF6(Q67L)-expressing cells. Bar = 10 μ m

phalloidin staining at the peripheral edges of the cell, indicative of increased actin polymerization at the cell surface. The formation of microspike-like extensions at the sites of actin assembly was observed. The number of stress fibers in cells expressing ARF6(Q67L) appeared to be reduced compared with control cells infected with the vector virus alone. The latter observation may be a consequence of titrating out cortical F-actin required for actin polymerization at the cell periphery, although we cannot exclude the possibility that this may indeed be a specific effect of the ARF6 mutant. Similar actin rearrangements were seen at relatively higher levels of expression of wild-type ARF6 (data not shown). Actin redistribution was not elicited by overexpression of ARF6(T27N), or with ARF6(G2A). Furthermore, we did not observe any peripheral actin rearrangements with expression of wild-type ARF1 or its GTPase-defective mutant, ARF1(Q71L), suggesting that this event is specific for ARF6 (data not shown). The ARF6-induced cytoskeletal rearrangements were not restricted to CHO cells, as other cell types such as BHKs and Rat-1 fibroblasts exhibited the same phenotype.

The actin filament architecture described above for cells expressing ARF6(Q67L) was distinct from the actin-rich surface protrusions formed when wild-type ARF6-transfected HeLa cells were treated with aluminum fluoride (AIF) (Radhakrishna *et al.*, 1996). To compare the actin rearrangements in CHO cells expressing ARF6(Q67L) with those induced by AIF, we treated uninfected CHO cells and CHO cells expressing wild-type ARF6 or ARF6(Q67L) with AIF. Whereas AIF did not have any effect on uninfected cells, we observed that AIF treatment of cells expressing wild-type ARF6 as well as ARF6(Q67L) (Figure 1C) resulted in the clustering of actin filaments in bundles at discrete sites on the cell surface. These structures were similar to the actin pseudopodia described in HeLa cells, but clearly distinct from those observed in untreated ARF6(Q67L)-expressing cells (Figure 1B and C), suggesting that AIF triggers the activation of other signaling molecules in addition to the ARF6 GTPase. Consistent with the latter, AIF has been shown to evoke several effects on cellular metabolic pathways, among which include activation of the heterotrimeric G proteins (Higashijima *et al.*, 1991) as well as the Ras GTPase (Mittal *et al.*, 1996).

Members of the Rho family of GTPases have been shown to regulate the organization of the actin cytoskeleton (Hall, 1994). Activation of each of these GTPases results in the induction of unique cellular morphological changes (Nobes and Hall, 1995). As seen in Figure 1, ARF6(Q67L) elicited unique morphological changes that were distinct from those induced by members of the Rho family. The ARF6(Q67L)-expressing cells exhibited a reduction in the number of stress fibers rather than an induction of stress fiber formation characteristic of cells expressing the activated mutants of RhoA. Although both Rac1(G12V) and ARF6(Q67L) triggered actin polymerization at the cell periphery, the resulting actin structures were not identical. Rac1(G12V) induced the formation of spread out, fan-shaped lamellipodia that appeared to fold back on themselves to form membrane ruffles. Lamellipodia were not seen in ARF6(Q67L)-expressing cells. The differences in the actin rearrangements induced by ARF6(Q67L) and

Rac1(G12V) are clearly discernible from serial confocal optical sections through the z-axis of cells expressing each of these GTPases (Figure 1E and F).

Effect of POR1 on ARF6-induced actin rearrangements

It has been proposed that the Rho GTPases regulate cytoskeletal architecture by functioning in a cascade. In such cascades, one GTPase controls the function of the next. Besides Ras and the Rho GTPases, ARF6 is the only other GTPase shown to regulate the formation of polymerized actin structures. This prompted us to determine whether ARF6 was linked to the Rho GTPase cascade. We were particularly interested in examining the relationship between the GTPases, ARF6 and Rac1. In addition to eliciting a centrifugal flux of cortical actin, the ARF6 and Rac GTPases share several other phenotypes. The activated forms of these GTPases have been shown to reduce the efficiency of receptor-mediated endocytosis (D'Souza-Schorey *et al.*, 1995; Lamaze *et al.*, 1996) and have been implicated in the outward flow of membrane traffic (Price *et al.*, 1995; Norman *et al.*, 1996; Galas *et al.*, 1997). To investigate whether the function of one GTPase is dependent on the other, either the dominant-negative mutant of either GTPase or the interfering mutant forms of their target proteins have been used previously. To examine whether the ARF6(Q67L)-elicited effect on the cytoskeleton is dependent on Rac1 function, we investigated the effects of the N- and C-terminal deletion mutants of POR1 on ARF6(Q67L)-induced cytoskeletal rearrangements. POR1 recently was identified as a novel Rac1-interacting protein that binds Rac1 in a GTP-dependent manner (Van Aelst *et al.*, 1996). The protein has been shown to localize both in the cytoplasm and along the plasma membrane. In Rac1(G12V)-transfected cells, POR1 expression was detected in membrane ruffles. Furthermore, POR1 has been demonstrated to play a role in Rac1-induced membrane ruffling. Expression of N- and C-terminal deletion mutants of POR1, POR1 Δ N1 and POR1 Δ C1 respectively, blocked the induction of lamellipodia formation and membrane ruffling by the activated mutant of Rac1, Rac1(G12V) (Van Aelst *et al.*, 1996).

To test the effects of POR1 deletion mutants on ARF6-elicited actin rearrangements, CHO cells on coverslips were co-infected with recombinant Sindbis virus capable of expressing ARF6(Q67L) and viruses encoding either the POR1 Δ N1 or POR1 Δ C1 mutant. Cells were labeled with affinity-purified anti-ARF6 antisera and stained with rhodamine-phalloidin to visualize actin filament rearrangements. Approximately 60–70% of the cells on the coverslip labeled positively for ARF6. When ARF6(Q67L) was co-expressed with the vector virus alone, all the ARF6-positive cells showed actin rearrangements. However, when ARF6(Q67L) was co-expressed with recombinant viruses encoding either POR1 Δ N1 or POR1 Δ C1, about half of the ARF6-positive cells did not show actin rearrangements. The other half of ARF6-labeled cells exhibited a rearrangement of actin, but to a much lesser extent than in cells transfected with ARF6(Q67L) alone (not shown). To confirm these observations, ARF6(Q67L) and the POR1 mutants were cloned into mammalian expression vectors and microinjected into the nuclei of CHO cells. Cytoskeletal rearrangements were monitored

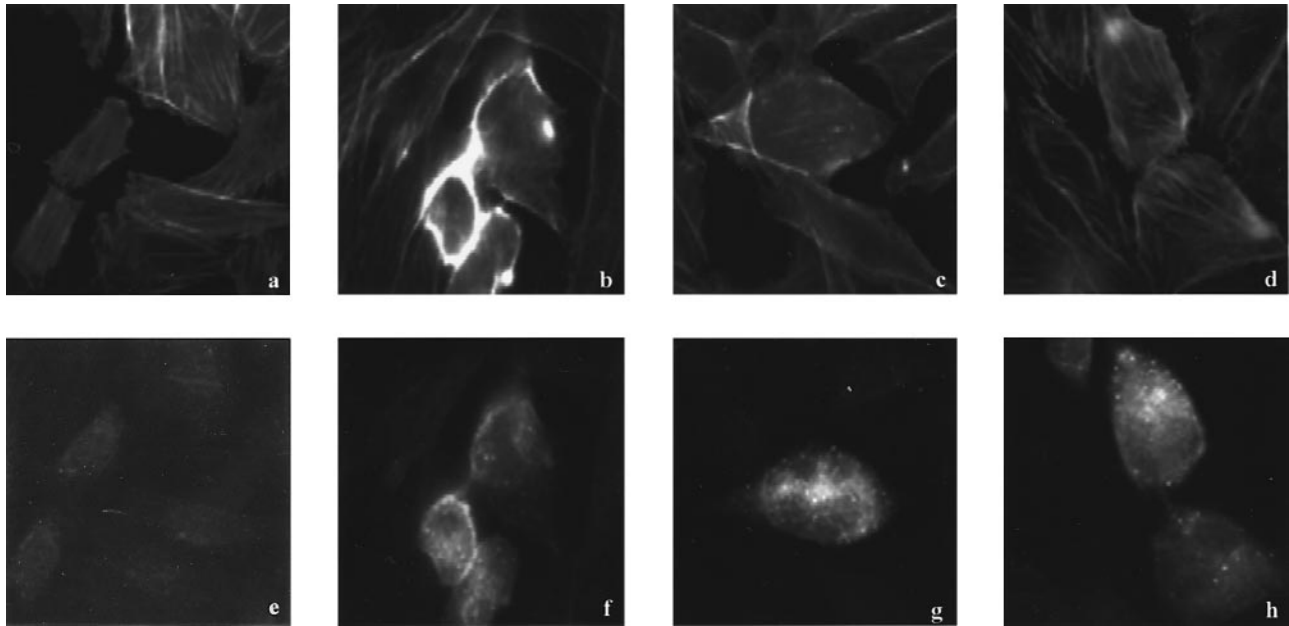


Fig. 2. Effects of POR1 truncation mutants, POR1 Δ N1 and POR1 Δ C1, on ARF6(Q67L)-induced cytoskeletal rearrangements. CHO cells were microinjected with (a and e) empty vector alone, (b and f) pcDNA3-ARF6(Q67L) and empty vector, (c and g) pcDNA3-ARF6(Q67L) and pcDNA3-POR1 Δ N1, (d and h) pcDNA3-ARF6(Q67L) and pcDNA3-POR1 Δ C1. At 4–5 h after injection, the cells were fixed and labeled with anti-ARF6 polyclonal antisera followed by goat anti-rabbit IgG conjugated to FITC to monitor ARF6 expression, and stained with rhodamine-labeled phalloidin to visualize actin rearrangements. Microinjection of expression plasmids encoding ARF6(Q67L) induced actin polymerization at the cell periphery, a response that was inhibited by co-injection of the POR1 deletion mutants.

4–5 h after injection. As shown in Figure 2, microinjection of an expression plasmid encoding ARF6(Q67L) resulted in actin polymerization at the cell edges. Consistent with the results described above, co-injection of expression plasmids encoding ARF6(Q67L) and POR1 Δ N1 resulted in an inhibition of ARF6-induced actin redistribution (Figure 2c). Also, expression of POR1 Δ C1 blocked ARF6-induced cytoskeletal redistribution (Figure 2d). The inhibitory effects of the POR1 truncated mutants were dose dependent. In all cases, expression of ARF6(Q67L) was confirmed by immunostaining of fixed cells with anti-ARF6 antisera (Figure 2).

Our previous studies have shown that expression of POR1 alone in REF-52 fibroblasts caused a very small induction of membrane ruffles. However, co-expression of POR1 with an activated mutant of Ras, Ras(G12V), resulted in extensive membrane ruffling. We analyzed the effect of POR1 expression on cytoskeletal rearrangements induced by wild-type ARF6. Consistent with what was seen previously in other cell types, expression of POR1 alone did not cause any cytoskeletal rearrangements in CHO cells (data not shown). Also, at 4 h post-viral expression, a very modest effect, if any, of wild-type ARF6 expression on the actin cytoskeleton was observed (Figure 3A and B). However, when POR1 and wild-type ARF6 were co-expressed under the same experimental conditions, a synergistic effect on actin polymerization was detected at the cell periphery that resulted in a ruffle-like appearance at the cell edges (Figure 3D). ARF6 localized to these peripheral structures (Figure 3C). Furthermore, similarly to what was observed previously for the localization of POR1 in Rac1(G12V)-transfected REF-52 cells (Van Aelst *et al.*, 1996), POR1 localized to the peripheral surface rearrangements in cells expressing ARF6 and POR1 (data not shown). These actin rearrange-

ments were not identical to those seen in cells expressing ARF6(Q67L). The reason for this is unclear. One possible explanation is that POR1 participates in other signaling pathways that may contribute to the observed phenotype. Indeed, as mentioned above, POR1 plays a role in the Rac1 signaling pathway leading to actin polymerization (Van Aelst *et al.*, 1996). On co-expression of POR1 with ARF6(Q67L), some synergy in the induction of cytoskeletal alterations was also seen, although this was less readily detectable by our assay conditions since ARF6(Q67L) alone induced actin rearrangements. The findings described above are consistent with the involvement of POR1 in regulating actin organization at the cell periphery induced by the ARF6 GTPase.

ARF6 and Rac function on separate signaling pathways

The observations that POR1 has been shown to interact with Rac1-GTP and that truncated mutants of POR1 interfere with cytoskeletal rearrangements induced by both the ARF6 and Rac1 GTPases suggest that ARF6 may act in coordination with Rac1, perhaps via a linear pathway, to orchestrate actin filament organization, with POR1 as a downstream target of Rac1. We therefore tested the ability of the dominant-negative mutant of Rac1, Rac1(S17N), to block the phenotype induced by the activated form of ARF6, ARF6(Q67L). For these studies, CHO cells on coverslips were co-infected with equal titers of recombinant virus expressing Rac1(S17N) and ARF6(Q67L), and the effects on the cytoskeleton were determined by staining with phalloidin. As shown in Figure 4A and B, all transfected cells exhibited the ARF6(Q67L) phenotype, indicating that Rac1(S17N) had no effect on ARF6(Q67L)-induced cytoskeletal rearrangements. Under the same experimental conditions, however, Rac1(S17N) did inter-

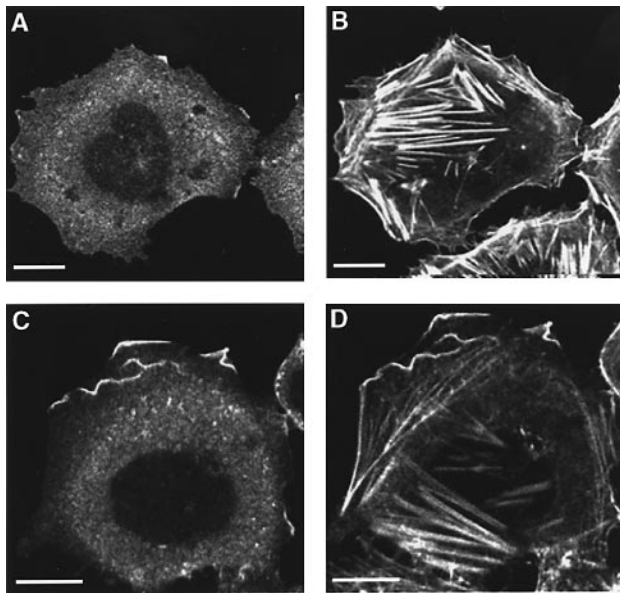


Fig. 3. Synergy between ARF6 and POR1 for the induction of membrane ruffles. CHO cells on coverslips were infected with recombinant virus expressing ARF6 (A and B) and equal titers of viruses encoding ARF6 and POR1 (C and D). At 4.5 h post-infection, cells were fixed with 2% paraformaldehyde and labeled with either affinity-purified rabbit polyclonal anti-ARF6 antibody (A and C) followed by goat anti-rabbit IgG conjugated to FITC, or with rhodamine-phalloidin to visualize actin filament organization (B and D). Under these experimental conditions, cytoskeletal alterations are barely detectable with expression of wild-type ARF6 alone. On co-expression of ARF6 and POR1, actin rearrangements at the cell edge are seen. ARF6 localizes to these peripheral structures. Bar = 10 μ m

ferre with the Ras(G12V)-induced membrane ruffling (data not shown). Expression of Rac1(S17N) was examined by immunofluorescence staining using an anti-Rac peptide antibody. Although Rac1 expression could be confirmed, unfortunately with the Rac1 antibody used we were not able to define clearly the localization of the Rac1 mutant. Rac1-transfected cells fluoresced bright green, with diffuse staining throughout the cell (Figure 4A). Similarly, we tested the effect of ARF6(T27N), the GDP-bound form of ARF6, on cytoskeletal changes induced by Rac1(G12V), the GTPase-defective mutant of Rac1. As shown in Figure 4C and D, expression of ARF6(T27N) did not cause any significant changes to Rac1-induced lamellipodia formation. Lamellipodia were clearly discernible in cells co-infected with ARF6(T27N) and Rac1(G12V) (Figure 4D). The expression of ARF6(T27N) was ensured by antibody staining using an affinity-purified antibody directed against ARF6. As previously shown, ARF6(T27N) localized to the perinuclear region of the cell. Taken together, our observations indicate that ARF6 and Rac1 function on parallel pathways to regulate cytoskeletal architecture.

ARF6 interacts with POR1

The results described above suggest that POR1 may regulate ARF6-induced cytoskeletal modeling via a Rac1-independent pathway. This raised the question of whether POR1 interacts directly with ARF6. To assess whether POR1 associates with ARF6, we first used an *in vitro* binding assay. ARF6 protein, purified as a recombinant

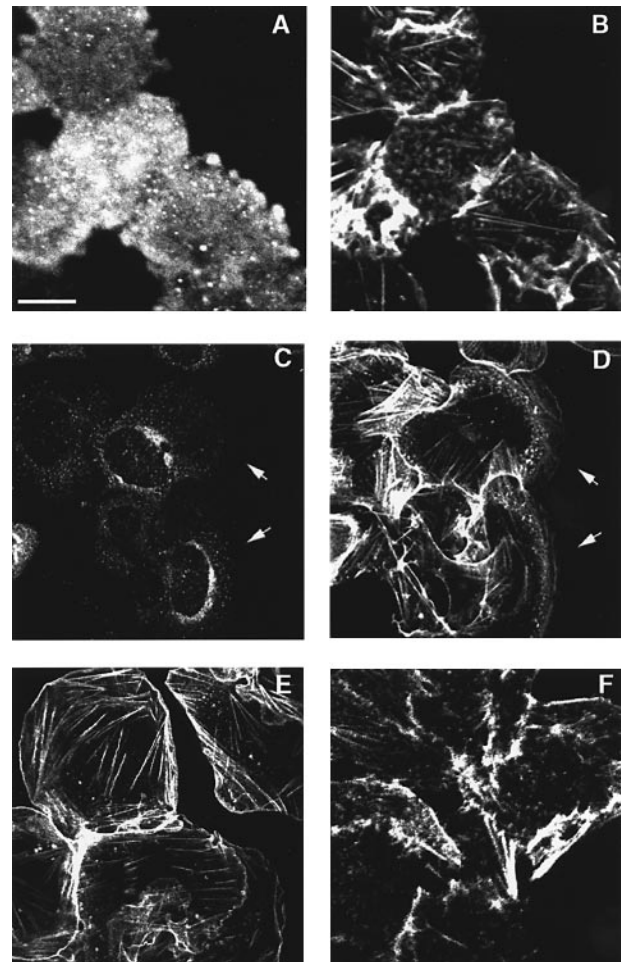


Fig. 4. ARF6 and Rac1 function on separate signaling pathways. Cells were co-infected with equal titers of recombinant virus expressing either ARF6(T27N) and Rac1(G12V) (A and B) or ARF6(Q67L) and Rac1(S17N) (C and D), or with recombinant virus expressing either Rac1(G12V) (E) or ARF6(Q67L) (F). At 4.5 h post-viral infection, cells were fixed and labeled with affinity-purified rabbit anti-ARF6 antibody (A) or a rabbit anti-Rac peptide antibody (C), followed by goat anti-rabbit IgG conjugated to FITC, and with rhodamine-phalloidin to visualize actin filament organization (B, D, E and F). Arrows indicate double transfectants. Rac1(G12V)-induced lamellipodia are clearly seen in cells expressing ARF6(T27N) and are similar to those seen in cells expressing Rac1(G12V) alone, while ARF6-induced cytoskeletal alterations are seen in cells expressing Rac1(S17N) and are similar to those seen in cells expressing ARF6(Q67L) alone. Bar = 10 μ m

GST fusion protein from *Escherichia coli*, was loaded with either GTP γ S or GDP β S (non-hydrolyzable analogs of GTP and GDP respectively), and then incubated with POR1 coupled to maltose-binding protein (MBP-POR1) immobilized on amylose resin. After several washes to remove non-specifically bound material, proteins bound to the resin were resolved by SDS gel electrophoresis, and the presence of ARF6 was detected by immunoblotting using anti-ARF6 polyclonal antiserum. As shown in Figure 5, POR1 binds directly to ARF6. Furthermore, as previously observed for the interaction between Rac1 and POR1 (Van Aelst *et al.*, 1996), POR1 bound preferentially to the GTP-bound form of ARF6 (Figure 5).

The interaction between ARF6 and POR1 was also observed using the yeast two-hybrid system (Chien *et al.*, 1991). POR1 was expressed as a *GAL4* activation domain

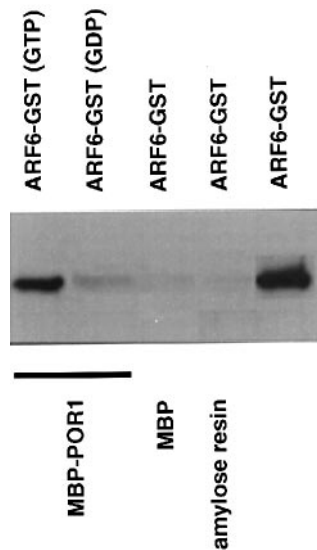


Fig. 5. *In vitro* binding of ARF6 to POR1. POR1 protein purified as an MBP fusion protein was immobilized on amylose resin and incubated with 3 μ g of ARF6-GST loaded with either GTP γ s (lane 1) or GDP β s (lane 2). As a control, ARF6-GST was incubated with MBP-amylose resin and with resin alone. After binding for 90 min at 4°C, the resin was washed and bound ARF6 was detected by SDS-PAGE followed by immunoblotting with anti-ARF6 antibody. Lane 5 contains 3 μ g of purified ARF6-GST. Approximately 70% of the ARF6-GST bound MBP-POR1 in the presence of GTP γ s.

fusion protein (GAD) whereas ARF(Q67L) and ARF6(T27N) were fused to the LexA DNA-binding domain (LBD) in the yeast strain L40 (Vojtek *et al.*, 1993). L40 permits the detection of protein-protein interaction by transcriptional activation of both *HIS3* and *LacZ* reporter genes. As depicted in Figure 6, the activated form of ARF6 was able to interact with POR1. As previously shown, Rac1(G12V) associated with POR1, whereas no interaction was detected between POR1 and Ras(G12V) (Figure 6). No association of POR1 was observed with the ARF6(T27N) mutant (data not shown).

To determine which region of POR1 binds to ARF6, we tested the ability of ARF6(Q67L) to interact with a POR1 mutant that lacked the first 207 bp (POR1 Δ N1), and a POR1 clone that lacked 257 bp of the C-terminal end (POR1 Δ C1). As shown in Figure 6, ARF6(Q67L) bound to the N-terminal truncation mutant (POR1 Δ N1), but failed to bind to the C-terminal truncation mutant (POR1 Δ C1), indicating that the 207 bp are not required for ARF6 binding. This binding pattern was similar to that observed for Rac1 (Van Aelst *et al.*, 1996). To compare the strength of the ARF6-POR1 interaction with the Rac1-POR1 interaction, we performed a liquid β -galactosidase assay using Rac1(G12V) and ARF6(Q67L) fused to the LBD and POR1 Δ N1 fused to the GAD. Similar values were obtained for the ARF6(Q67L)-POR1 Δ N1 (71 ± 1.5 Miller units) and for Rac1(G12V)-POR1 Δ N1 (75 ± 1.1 Miller units) interactions. Interestingly, we observed an inhibition of ARF6-induced cytoskeletal rearrangements with expression of POR1 Δ N1 as well as with POR1 Δ C1 deletion mutants (Figure 2). The inhibitory effect of POR1 Δ C1, the fragment that does not interact with ARF6, could arise from the non-productive interaction of POR1 Δ C1 with other regulatory elements that are required to manifest ARF6-mediated cytoskeletal

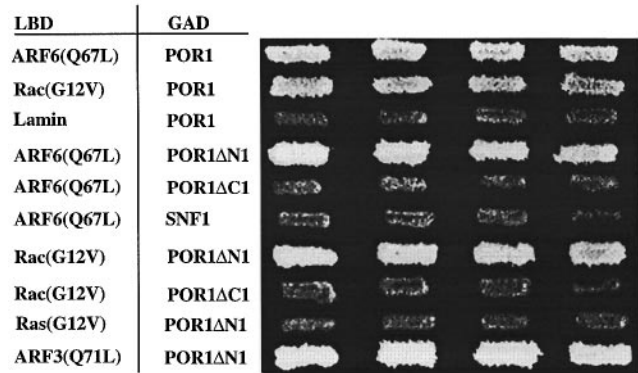


Fig. 6. Two-hybrid interactions between ARF6(Q67L), Rac1(G12V), Ras(G12V) and POR1. ARF6(Q67L), ARF3(Q71L), Ras(G12V) and Rac1(G12V) cDNAs fused to the LexA DNA-binding domain (LBD) were transformed into the yeast reporter strain L40 with the *GAL4* activation domain (GAD) fusions containing POR1 and POR1 deletion mutants, POR1 Δ N1 and POR1 Δ C1. POR1 Δ N1 and POR1 Δ C1 contain base pairs 208–912 and 1–654 respectively. SNF1-GAD and lamin-LBD fusions were used as negative controls for LBD-ARF6(Q67L) and GAD-POR1 respectively. For each transformation, four independent clones were picked and tested for growth on medium lacking histidine.

remodeling. Thus POR1 may function as part of a multi-molecular complex responsible for mediating signals induced by the GTPases ARF6 and Rac1.

To determine whether POR1 interacts with other members of the ARF family, we tested the interaction of ARFs 1 and 3 with POR1 using the *in vitro* binding assay described above and the yeast two-hybrid system respectively. ARF1-GST bound MBP-POR1 and the interaction was GTP dependent (data not shown). Also, ARF3(Q71L) was able to interact with the C-terminal fragment of POR1 (Figure 6). This observation was not surprising since the ARF proteins are highly homologous in amino acid sequence. Further experiments on the effects of POR1 on ARF1 (or other ARF proteins) function *in vivo* will be required to address the physiological relevance, if any, of the ARF1-POR1 interaction.

Discussion

The ARF6 GTPase belongs to the ARF family of proteins which are believed to function as regulators of organelle assembly and traffic. The best characterized ARF protein, ARF1, has been shown to be required for the recruitment of coat proteins (COPI) onto Golgi membranes (Donaldson and Klausner, 1994). Although the function of the other ARFs remain to be elucidated, it appears that ARFs 3, 4 and 5 are localized along the secretory pathway (Peters, personal communication). ARF6, the least distinct member of the ARF family, is localized at the cell periphery (D'Souza-Schorey *et al.*, 1995; Peters *et al.*, 1995) and regulates trafficking and the organization of the plasma membrane, most likely by eliciting the targeted delivery of intracellular membrane to the cell surface. In this study, we examined the effects of ARF6 on the cytoskeleton and its relationship with the Rho GTPases. We show that ARF6-induced elaboration of the plasma membrane is accompanied by a redistribution of actin to the cell periphery. Expression of ARF6(Q67L) induces actin polymerization at the cell surface. Consistent with previous

observations in HeLa cells (Radhakrishna *et al.*, 1996), no actin rearrangements at the cell periphery were observed when ARF1 was expressed, indicating that this phenomenon was specific for ARF6. These results are suggestive; however, it remains to be proven that membrane and actin redistribution are co-dependent processes.

The Rho GTPases function as important regulators of cytoskeletal reorganization in response to growth factors. In Swiss 3T3 cells, Rho proteins mediate the lysophosphatidic acid- and bombesin-induced formation of stress fibers (Ridley and Hall, 1992; Nobes *et al.*, 1995). Rac1 is required for platelet-derived growth factor-, insulin- and bombesin-stimulated actin polymerization at the plasma membrane that results in membrane ruffling (Ridley *et al.*, 1992; Nobes *et al.*, 1995), whilst Cdc42 mediates bradykinin-induced formation of filopodia (Kozma *et al.*, 1995). Furthermore, microinjection studies have defined a hierarchical relationship between the three GTPases such that Cdc42 activates Rac and Rac activates Rho, resulting in coordinated changes on the actin cytoskeleton (Nobes and Hall, 1995). The ARF6-induced reorganization of actin filaments prompted us to examine the possibility of ARF6 functioning in coordination with members of the Rho GTPase family, in particular Rac1. We have shown here that ARF6 and Rac1 act via parallel pathways to regulate the organization of the actin cytoskeleton. The dominant-negative mutant of Rac1, Rac1(S17N), fails to block actin rearrangements induced by ARF6 although, under the same conditions, Rac1(S17N) inhibits Ras(G12V)-induced membrane ruffling. Also, the dominant-negative mutant of ARF6 fails to block Rac1(G12V)-induced lamellipodia formation.

The mechanism by which ARF6 controls actin assembly remains to be defined. A step in this direction is our observation that ARF6 interacts directly with POR1 in a GTP-dependent manner. POR1 was isolated previously as a Rac1-interacting protein and was shown to play a role in membrane ruffling (Van Aelst *et al.*, 1996). Our findings that (i) expression of truncated versions of POR1, POR1 Δ N1, a fragment that retains the ability to interact with ARF6, as well as POR1 Δ C1, a mutant that fails to bind ARF6, interfere with ARF6(Q67L)-induced cytoskeletal rearrangements; (ii) POR1 synergizes with ARF6 to induce actin polymerization; and (iii) ARF6 and POR1 localize to the peripheral rearrangements, suggest that POR1 interacts with ARF6 to mediate downstream signaling. The fact that POR1 associates with both ARF6 and Rac1 is somewhat surprising. It is possible that depending on the nature of the extracellular stimuli, POR1 could interact with either ARF6 or Rac1 or both to establish highly specified patterns of cytoskeletal rearrangements and plasma membrane architecture. POR1 was also capable of interacting with ARFs 1 and 3 in the *in vitro* binding assay and the yeast two-hybrid system. It should be noted, however, that the ARFs are structurally a highly homologous family of proteins (Tsuchiya *et al.*, 1991). Therefore, *in vitro*, any one ARF may be able to replace/complement the function of another ARF protein. To address the physiological relevance of the interaction between two proteins, it is essential to perform functional assays *in vivo*. Our results clearly point to a physiological role for the ARF6–POR1 interaction. We have not tested the effect of POR1 on ARF1 function *in vivo* but, given that the ARF1 localizes

to the Golgi and that expression of ARF1 or its activated mutant does not induce peripheral cytoskeletal remodeling, a physiological interaction for ARF6 and POR1 seems rather unlikely, although we cannot preclude the possibility that, under some circumstances, ARF1/ARF3 may be able to interact with POR1 *in vivo*. In light of this, several instances where an effector molecule of a particular GTPase also interacts with other members of the same subfamily have been reported. For example, the kinase Raf1 not only interacts with H-Ras but also with R-Ras and Rap1A (Van Aelst *et al.*, 1995). Pak and S6 kinases have been shown to interact both with Rac1 and Cdc42 (Manser *et al.*, 1994; Bagrodia *et al.*, 1995; Knaus *et al.*, 1995; Martin *et al.*, 1995; Chou and Blenis, 1996), while citron and Rho-kinase (or its isoform p160ROCK) have been shown to interact with Rac1 and Rho (Madaule *et al.*, 1995; Joneson *et al.*, 1996; Lamarche *et al.*, 1996). The physiological relevance of some of these interactions such as that between Rac1 and Rho-kinase is unclear.

Our studies described here demonstrate that ARF6, a GTPase previously shown to play a role in endocytic trafficking, is also involved in cytoskeletal remodeling. More recently, several reports suggest the possible interdependence or cross-talk between signaling pathways that regulate cytoskeletal architecture and membrane trafficking. A new member of the Rho family, RhoD, has been suggested to provide a molecular link between transport and the cytoskeleton. Activated RhoD regulates early endosome dynamics and distribution and causes rearrangements of the cytoskeleton (Murphy *et al.*, 1996). Rac1 and Rho have also been shown to regulate the secretion of granules in mast cells, in addition to eliciting cytoskeletal organization (Norman *et al.*, 1996). Furthermore, it has been reported that expression of the activated form of Rac1 in HeLa cells dramatically decreases the efficiency of receptor-mediated endocytosis (Lamaze *et al.*, 1996), a phenotype exhibited by the activated mutant of ARF6 in CHO cells (D'Souza-Schorey *et al.*, 1995). As mentioned above, the alterations in membrane trafficking observed in cells expressing ARF6 and ARF6(Q67L) are accompanied by marked perturbations of membrane structure at the cell surface. Interestingly, at low levels of wild-type ARF6 expression, ARF6 localizes predominantly to intracellular compartments with little or no effect on plasma membrane morphology (C.D'Souza-Schorey, E.Van Donselaar, C.Yang, P.D.Stahl and P.J.Peters, in preparation). However, at higher levels of expression, ARF6 induces plasma membrane vaginations characteristic of what is observed in cells expressing the Q67L, GTPase defective mutant. Thus, the appearance of ARF6-elicited actin remodeling and membrane rearrangements appears to follow a similar pattern in terms of levels of protein expression required to elicit these changes. Whether the induction of actin and membrane rearrangements are two distinct downstream effects of ARF6 or both processes are dependent on a common downstream target such as POR1 requires further investigation. Our studies provide new directions to investigate further the complex regulation of cytoskeletal modeling and its relationship to membrane trafficking.

Materials and methods

Plasmid constructions

For two-hybrid screening constructs, fusions to the activating domain of GAL4 (GAD) were constructed using pGADGH (Van Aelst *et al.*, 1993);

fusions to the DNA-binding domain of LexA (LBD) were made in pVJLII, a derivative of pBTM116 (Vojtek *et al.*, 1993). ARF6(Q67L) and ARF6(T27N) were amplified by PCR from cDNA clones (D'Souza-Schorey *et al.*, 1995) and subcloned as *EcoRI-SalI* fragments in pVJLII. LBD Rac1(G12V), LBD Rac(G12V), LBD Rac(Lamin), GADPOR1, GADPOR1 Δ N1 and GADPOR1 Δ C1 constructs were generated as previously described (Van Aelst *et al.*, 1996). ARF3(Q71L) was amplified by PCR from pAB3-2 (a generous gift from Rick Kahn) and subcloned as a *BamHI-SalI* fragment in pVJLII. To generate ARF6-GST, the ARF6 cDNA was amplified by PCR with sequences encoding *EcoRI* restriction sites on the N- and C-terminal ends respectively, and cloned into the bacterial expression vector pGEX-3X (Pharmacia-LKB Biotechnology Inc.). The ARF1-GST construct was generated by PCR amplification of the bovine ARF1 cDNA (kindly provided by Richard Klausner) with sequences encoding *BamHI* and *EcoRI* restriction sites on the N- and C-terminal ends respectively, and then cloned into the bacterial expression vector pGEX-2T (Pharmacia-LKB Biotechnology Inc.). To generate Rac1-GST and MBP-POR1, wild-type Rac1 was cloned into the vector pRP259, a derivative of pGEX-2T, and POR1 was cloned into pMAL-c2 as previously described (Van Aelst *et al.*, 1996). The mammalian expression constructs pcDNA3-POR1 Δ C1 and pcDNA3-POR1 Δ N1 were previously described (Van Aelst *et al.*, 1996). pcDNA3-ARF6(Q67L) was constructed by inserting a PCR-generated ARF6(Q67L) cDNA as an *EcoRI-SalI* fragment into the *EcoRI-XhoI* sites of the pcDNA3 vector (Van Aelst *et al.*, 1996).

Recombinant Sindbis virus production

The wild-type and mutant cDNAs of Rac1, ARF6 and POR1 were subcloned into the *XbaI* restriction site of the Sindbis vector pto1000:3'2J as previously described (D'Souza-Schorey *et al.*, 1995). Briefly, plasmids were linearized by *XhoI* digestion and used as a template for *in vitro* transcription using SP6 RNA polymerase. The resulting RNA transcripts were used for transfection of confluent BHK-21 cell monolayers using a lipofectin-mediated procedure (Life Technologies Inc.). The cells were maintained in α -minimal essential medium (α -MEM) containing 3% fetal bovine serum (FBS) at 37°C. At 40 h post-transfection, the medium containing the released viruses was harvested and titered on fresh BHK-21 cell monolayers. The virus titers were generally between 10⁷ and 10⁹ plaque-forming units (p.f.u.) per ml. Virus stocks were aliquoted and frozen at -70°C and thawed just before use.

Examination of protein expression

CHO cell monolayers in 35 mm dishes (~5 × 10⁵ cells/dish) were infected with recombinant Sindbis virus capable of expressing ARF6, Rac1 or POR1 proteins and their mutant derivatives or with vector virus as a negative control. Virus adsorption was carried out as described below. Cells were incubated at 37°C for 3 h in growth medium containing 3% serum. The medium was then replaced with 1 ml of serum-free RPMI medium containing 50 μ Ci/ml [³⁵S]methionine (ICN ³⁵S-translabel) and maintained at 37°C for another 2 h. The cells were lysed in 1% SDS and the cell lysates were analyzed by SDS-PAGE followed by autoradiography. Alternatively, proteins resolved on SDS gels were transferred to nitrocellulose membranes and blotted with polyclonal antisera directed against ARF6, Rac or POR1 proteins. ARF6 and Rac1 wild-type and mutant proteins migrated as single ~20 kDa bands and POR1 migrated as a 33 kDa band on SDS gels (Figure 7). Protein synthesis was detected 3–3.5 h post-viral infection and peaked at ~6–7 h post-viral infection. Our studies were carried out 4.5–5 h post-viral infection; at this time point we were able to observe all the phenotypic changes induced by the ARF6 and Rac1 GTPases.

Virus infection procedures

TRVb-1 cells (a CHO cell line that overexpresses the human transferrin receptor, and kindly provided by Tim McGraw, Columbia University) were grown to 60–70% confluence on glass coverslips, in Ham's F-12 medium containing 100 μ g/ml G418 and 5% FBS. The medium was aspirated, cells were rinsed briefly with phosphate-buffered saline (PBS) and recombinant virus (50 p.f.u./ml) was added to the cells, in 250 ml of PBS containing 1% serum. The cells were maintained at 4°C for 45 min, followed by incubation at room temperature for an additional 15 min. The viral infection mixture was replaced with 3 ml of Ham's F12 containing 1% FBS and the cells were maintained at 37°C in a cell culture incubator. At 4.5–5 h post viral infection, cells were washed and fixed for immunofluorescence procedures.

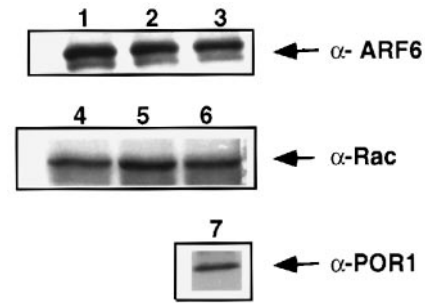


Fig. 7. Expression of ARF6, Rac1 and POR1 proteins. Lysates of cells expressing ARF6, Rac1 and POR1 proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes and immunoblotting was carried out with the antibodies indicated. Lanes 1–7 show expression of ARF6, ARF6(Q67L), ARF6(T27N), Rac1, Rac1(G12V), Rac1(S17N) and POR1 respectively.

Fluorescence microscopy procedures

CHO cells grown on coverslips were either mock infected or infected with vector virus alone or with recombinant virus expressing the appropriate GTPase as described above. At 4.5 h post-infection, the cells were fixed with 2% paraformaldehyde for 30 min, permeabilized and quenched with PBS containing 0.05% Triton X-100, 0.1 M NH₄Cl and 0.2% gelatin, followed by staining with rhodamine-phalloidin to visualize actin filament organization. In double labeling experiments after permeabilization, cells were first incubated with either an anti-Rac peptide rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.), anti-ARF6 peptide rabbit polyclonal antibody (D'Souza-Schorey *et al.*, 1995) or an anti-POR1 rabbit polyclonal antibody (Van Aelst *et al.*, 1996) for 2 h at room temperature, followed by incubation with rhodamine-phalloidin (Molecular Probes) and goat anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC; Cappel). In all experiments (unless indicated otherwise), cells were mounted in 70% glycerol (in PBS) and visualized using a Zeiss axiovert microscope and a Biorad confocal scanning imaging system.

In vitro binding assay

Rac1 and ARF6 proteins were affinity purified as GST fusion proteins from *E. coli*. The GST fusion proteins were eluted from glutathione-Sepharose resin with 50 mM Tris-HCl pH 7.0, containing 10 mM reduced glutathione. The eluate was dialyzed against 20 mM Tris-HCl buffer pH 7.4, containing 1 mM dithiothreitol (DTT) and 5 mM EDTA, and the dialyzed protein was incubated with 200 μ M GDP β s or GTP γ s at room temperature for 30 min. The reaction was stopped by addition of MgCl₂ to a final concentration of 10 mM. For binding experiments, GST, GST-Rac or GST-ARF6 were incubated with MBP or MBP-POR1 immobilized on amylose resin using ~3 μ g of each protein. The binding reactions contained 20 mM Tris pH 7.4, 1 mM DTT, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 and 10% glycerol, and was performed by mixing the resin with the GST proteins at 4°C for 90 min. The resin was then sedimented and washed 4–5 times with reaction buffer. The resin was boiled for 3 min in SDS gel sample buffer and then resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and bound proteins were detected by immunoblotting with anti-ARF6 polyclonal antisera.

Detection of protein complex formation using the two-hybrid system

LBD and GAD fusion constructs were co-transformed in the yeast reporter strain L40 (MATA *trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) (Vojtek *et al.*, 1993). Transformants were plated on synthetic medium lacking leucine and tryptophan (DO-Leu-Trp). Individual colonies were patched on DO-Leu-Trp plates and the following day replica plated onto DO-Leu-Trp-His plates. After 48 h, growth was evaluated (Van Aelst *et al.*, 1993). For the liquid β -galactosidase assay, transformants were grown in selective medium, and β -galactosidase activity was assayed with *o*-nitrophenyl β -D-galactoside; values (mean \pm SD of triplicate determinations) are given in Miller units (Miller, 1972).

Microinjection assay

CHO cells were plated onto glass coverslips and cultured in Ham's F12 medium containing 5% FBS. The cells were serum starved overnight in Ham's F12 containing 1% FBS. The pcDNA3-ARF6(Q67L), pcDNA3-

POR1ΔN1 and pcDNA3-POR1ΔC1 plasmids were microinjected into the nuclei at concentrations of 20, 100 and 120 μg/ml respectively. The cells were fixed 4–5 h after injection in 3.7% formaldehyde in PBS for 30 min at room temperature. Subsequently, the cells on coverslips were incubated with anti-ARF6 antibody for 2 h, followed by incubation for 45 min with 0.1 mg/ml rhodamine-phalloidin. The coverslips were mounted and the cells were visualized using a Zeiss Axiophot fluorescent microscope and photographed with a photometric cooled CCD camera.

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