Arfs, phosphoinositides and membrane traffic

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Abstract

Arf (ADP-ribosylation factor) GTP-binding proteins function in cells to regulate membrane traffic and structure. Arfs accomplish this task through modification of membrane lipids and the recruitment of proteins, including coat proteins and actin, to membrane surfaces. Arf1 and Arf6 are the most divergent and most studied human Arf proteins that localize predominantly to the Golgi complex and plasma membrane respectively. We have been studying the targeting of Arf1 and Arf6 to these specific compartments and the common and divergent activities that they exert on these membranes. We have found that Arf6 acts through activation of type I phosphatidylinositol 4-phosphate 5-kinases to generate phosphatidylinositol 4,5-bisphosphate and that this activity is instrumental in facilitating the actin cytoskeletal rearrangements and alterations in endosomal membrane trafficking observed with increased Arf6 activation. Arf1 can also stimulate the activity of phosphatidylinositol kinases and recruit coat proteins and actin cytoskeletal elements to the Golgi complex.

Introduction

The Arf (ADP-ribosylation factor) family of GTP-binding proteins are conserved, low molecular mass Ras-related GTPases involved in regulation of membrane traffic and organelle structure. There are six mammalian Arf proteins and many more Arf-related proteins called Arls. All Arfs are myristoylated at the N-terminus, a lipid modification required for membrane association and biological function. Arfs have been divided into three classes based on amino acid identity with class I containing Arfs 1, 2 and 3, class II containing Arfs 4 and 5 and class III containing only Arf6. Yeast have both class I and III Arf members, but all metazoans have at least one Arf from each class. Arf function at membrane surfaces to modify the lipid composition and recruit cytosolic proteins onto membranes to facilitate membrane traffic and organelle structure.

Like all GTPases, Arfs are activated by GEFs (guanine nucleotide-exchange factors) that catalyse the exchange of GTP for GDP bound to the protein. Arfs are then inactivated by GTP hydrolysis catalysed by GAPs (GTPase-activating proteins), returning Arf to the inactive, GDP-bound state. Many Arf GEFs have been identified that contain the conserved Sec7 domain that has the catalytic activity. There are several families of Arf GEFs including: Gea/GBF1 [Golgi-associated BFA (Brefeldin A)-resistant GEF], BIG1 (BFA-inhibited GEF 1)/BIG2/Sec7, ARNO (Arf nucleotide-binding site opener)/cytohesin and EFA6 (exchange factor for Arf6). The GBF1 and BIG families are all Golgi localized and inhibited by BFA [1]. The ARNO and EFA6 families are localized to the cytosol and PM (plasma membrane), not inhibited by BFA and do not seem to be present in yeast or plant cells. There is an even larger number of Arf GAPs, all containing the conserved zinc-finger catalytic domain found in Arf GAPI [2]. Many of the Arf GAP proteins contain numerous domains that could form scaffolds for recruitment of many regulatory and effector proteins [2]. A major challenge for the scientists working on Arf function is to determine which GEFs and GAPs are acting on which Arfs and where these cycles of activation and inactivation are occurring in cells.

Arf-GTP acts at membranes to recruit cytosolic coat proteins, activate lipid-modifying enzymes and modify actin structure. The recruitment of coat proteins, including the COPI (coat protein complex I), GGA and AP1 (adaptor protein 1), AP3 and AP4 to Golgi membranes has been most clearly demonstrated for Arf1. Although Arf6 can in cell-free assays work to recruit COPI and AP1 to Golgi membranes [3], it does so poorly and in cells Arf6 is not localized to the Golgi complex, but rather to the PM. Since class I Arfs localize to the Golgi, in both mammalian cells and in yeast, they are most likely the Arfs carrying out functions on Golgi membranes.

The first hint that Arf GTPases could affect membrane lipid composition came from studies that identified Arfs as activators of PLD (phospholipase D) [4,5], an enzyme that hydrolyses phosphatidylcholine to form PA (phosphatidic acid). This activity has been implicated in mast cell signalling [6], regulated exocytosis [7], GLUT4 exocytosis [8] and cell migration [9]. In most cases, there is some evidence that it is Arf6 that is stimulating PLD to generate PA. Honda et al. [10] identified Arfs as the proteins in cytosol responsible for activation of the type 1 PIP 5-kinase (phosphatidylinositol 4-phosphate 5-kinase). Although both Arf1 and Arf6 could

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Arf6 and PIP 5-kinase association with clathrin-independent endocytosis

Endocytic cargo proteins such as MHCI and CD59 enter cells by clathrin-independent endocytosis. After endocytosis, Arf6-GTP gets hydrolysed and PIP2 is lost prior to fusion with the common early endosome. From there, MHCI and CD59 can either be routed to late endosomes for degradation or recycled back to the PM in tubular membranes that arise from the juxtanuclear endocytic recycling compartment (ERC). PIP2 presence is indicated at the PM and on endosomes by solid lines; compartments marked in dashed lines are devoid of PIP2. Failure to inactivate Arf6 or modify PIP2 leads to the accumulation of PIP2 and actin-coated vacuoles that contain clathrin-independent cargo, movement out of these vacuoles is blocked.

Arf6 activation of PIP 5-kinase regulates endosomal membrane traffic

Arf6 is the most divergent from Arf1 in sequence and in localization. Arf6 is found associated with the PM, and in some cells endosomal membranes that contain PM proteins that enter cells independently of AP2 and clathrin [12–14]. We have studied this clathrin-independent endosomal system for over 10 years and have shown that integral membrane proteins such as the MHCI, integrins [15,16] and GPI (glycosylphosphatidylinositol)-anchored proteins such as CD59 [14] enter cells through this pathway (Figure 1). We have mostly used HeLa cells for these studies as the clathrin-independent endosomal pathway is prominent and distinctive in this cell type. Membrane carriers containing these proteins fuse 5–10 min after internalization with the Rab5-associated classical early endosome that contains cargo-entering cells via clathrin-dependent endocytic mechanisms [13]. After convergence with the classical 'early endosome', these cargo molecules can either move into late endosomes and lysosomes for degradation or be routed to the juxtanuclear ERC (endocytic recycling compartment) for recycling back to the PM (Figure 1). In HeLa cells, once at the ERC, membrane containing MHCI and CD59 emerges and is directed towards the PM in tubular structures, whose formation is dependent upon Rab22 and microtubules [17]. Fusion of membrane back to the PM requires activation of both Rab11 and Arf6 [12,16,17].

In contrast with Arf1, Arf6 appears to be retained on membranes following GTP hydrolysis and we observe that Arf6 is localized both to the PM and on these endosomal structures. In order to understand whether Arf6 activities can modulate the flow of membrane through this pathway, we examined whether the Arf6 activation of PIP 5-kinase is a major determinant of Arf6 function. We monitored the effects of increasing activation of Arf6 on PIP2 levels and movement of membrane through the pathway. To do this, we expressed the GFP (green fluorescent protein) fusion protein containing the phospholipase Cδ PH (pleckstrin homology) domain (PH–GFP), which specifically recognizes PIP2. In HeLa cells expressing wild-type Arf6 or the GTP hydrolysis-defective, constitutively active Arf6 mutant, Arf6Q67L. We also looked at expression of an Arf6-specific GEF that would allow increased activation but still inactivation of Arf6. In this study, we found that PH–GFP is present at the PM and on the distal portion of the tubular, recycling endosomal membranes [15], indicating that PIP2 is present on these membranes (Figure 1). Expression of EFA6 induces the formation of PM ruffling and protrusion formation and a significant shift from pinocytosis to macropinocytosis. Incoming macropinosomes label with PH–GFP initially but within minutes PH–GFP dissociates from these membranes allowing rapid recycling of this membrane back to the PM to allow continued ruffling [15]. In contrast, expression of the constitutively active mutant, Arf6Q67L, caused ruffling initially and increased macropinocytosis, but these internalized vesicles fused with other vesicles and PH–GFP remained associated, suggesting that failure to inactivate Arf6 results in continued production of PIP2 (Figure 1). Remarkably, overexpression of human PIP 5-kinase Iα leads to the same end stage phenotype of accumulation of actin-coated vacuoles that contain PIP2. Interestingly, the PIP 5-kinase expression did not lead to early membrane ruffling. Instead, rocketing membrane vesicles are observed early during the transfection [15,18]. This suggests that activated Arf6 has additional functions that mediate the change in PM actin dynamics.

The observation that expression of Arf6Q67L or PIP 5-kinase, a downstream target for Arf6-GTP, leads to the same accumulation of PIP2-associated endocytic vesicles demonstrates that it is the unregulated production of PIP2 that is responsible for this phenotype. The transient appearance and disappearance of PIP2-positive macropinocytic structures in cells expressing EFA6 demonstrates that Arf6
GTP hydrolysis, which still occurs in cells expressing EFA6, is necessary for membrane recycling. Presumably, by turning off Arf6, PIP5-kinase is no longer active.

The block in trafficking imposed by Arf6Q67L leads to the accumulation of non-clathrin cargo proteins in these vesicles [13] (Figure 1). Both GPI-anchored proteins such as CD59 and non-raft partitioning proteins such as MHCI accumulate in the Arf6Q67L vacuoles and their subsequent trafficking to Rab5 endosomes, late endosomes or to recycling is also blocked [14]. Since PM PIP5-kinase and PIP2 accumulate on these structures, the PM may be depleted of PIP2 [19]. Hence investigators should be aware of indirect consequences of expression of Arf6Q67L, for example on clathrin endocytosis that requires PIP3. Indeed, after longer periods of expression, internalization of clathrin-independent pathways is inhibited [12,15].

Future studies will focus on the function of PIP2 in this endocytic pathway, which proteins are recruited to the endosomal membranes by PIP2 and by what mechanism PIP2 is lost from the endosome. Another question to address is whether Arf6 activation of PLD is required for any aspect of this clathrin-independent pathway.

References