

Advantages and limitations of cell-based assays for GTPase activation and regulation

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Abbreviations: Arf, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; GGA, Golgi-associated gamma adaptin ear containing protein; FRET, fluorescence recovery after photobleaching

Small GTPases of the Ras superfamily are important regulators of many cellular functions, including signal transduction, cytoskeleton assembly, metabolic regulation, organelle biogenesis and intracellular transport. Most GTPases act as binary switches, being “on” in the active, GTP-bound state and “off” in the inactive, GDP-bound state, and cycle between the two states with the aid of accessory proteins, referred to as guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). This review will focus on the ADP-ribosylation factors (Arfs), a family of G-proteins that are essential regulators of carrier vesicle formation during vesicular transport. As for most other GTPases, the Arfs themselves are vastly outnumbered by the proteins that regulate them, and a major focus in the field has been to define the functional relationships between individual GEFs and GAPs and their substrates at the cellular level. Over the years, a variety of methods have been developed to measure GTPase activation *in vitro* and *in vivo*. *In vitro* analysis will be discussed in the accompanying article by Randazzo and colleagues. Here we will focus on cell- and tissue-based assays and their advantages/disadvantages relative to cell-free systems.

Introduction

ADP-ribosylation factors (Arfs) are a family of closely related GTP-binding proteins that are best known for their roles in the regulation of vesicular transport. In the active, GTP-bound state, Arfs nucleate the assembly of a variety of coat protein complexes at sites of carrier vesicle formation. These include the COPI coat-omer, AP-1, AP-3 and AP-4 adaptor complexes, and the monomeric GGA adaptors. They accomplish this in part by direct physical interactions with components of the coat, and also by modulation of the local lipid microenvironment through activation of both phospholipase D and phosphoinositide kinases (for review, see ref. 1).

Like other GTPases, regulatory control of Arf activation requires the activities of Arf-specific guanine nucleotide exchange factors (GEFs), which catalyze GTP loading, and GTPase activating proteins (GAPs), which promote GTP hydrolysis.

While the human genome encodes five Arfs (Arf1, 3, 4, 5 and 6, which are expressed in all cells), it contains 15 recognizable Arf GEFs² and an even larger number of GAPs (31 proteins with identifiable Arf-GAP domains, and two structurally unrelated proteins with Arf-GAP activity, ELMOD2³ and ELMOD1⁴ (R. Kahn, personal communication)). At least some of this diversity can be accounted for by tissue-specific expression, but all cells express more GEFs and GAPs than they do Arfs. A major challenge in this field has been to determine the substrate specificity of each of these regulatory molecules, where they act in the cell and how their activities are influenced by other regulatory inputs.

In the accompanying article, Paul Randazzo describes the utility of *in vitro* assays to determine substrate specificity and regulatory mechanisms. Such assays have the distinct advantage that they are highly quantitative, and by definition reductionist; the use of highly purified

components and controlled conditions allows the measurement of binding constants, rate constants and the influence of cofactors such as phospholipids on enzyme function without interference from other cellular factors. However, they also have several clear limitations. First, many Arf GEFs and GAPs are large, multi-domain proteins that are difficult to produce in recombinant or highly purified form. For this reason many *in vitro* studies have used truncated forms, which may lack important regulatory domains. Second, post-translational modifications, which can have a significant impact on enzymatic activity, are not present in recombinant proteins produced in bacteria. Third, subcellular localization can have a significant impact on substrate specificity. For example, Arf6 is more abundant than Arf1 at the plasma membrane, and an enzyme that can act on both *in vitro* may have a quantitatively larger effect on Arf6 if it colocalizes with Arf6 in an intact cell.

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It is therefore important to complement *in vitro* assays with whole-cell (or tissue) based assays when possible. This allows the characterization of substrate specificity in a more physiological context, as well as the dissection of biological function. Cell-based assays fall into two general categories: (1) biochemical (e.g., pulldowns) and (2) *in situ* (morphological). Here we will describe the practical aspects of each assay, and the advantages and disadvantages that they offer in dissecting the mechanisms of Arf activation.

Pulldown Assays

Perhaps the earliest effort to quantify the levels of an activated GTPase in a cell lysate were those that made use of a monoclonal antibody (Y13–259) that bound over the guanine nucleotide binding site of Ras to prevent nucleotide dissociation.^{5,6} Unfortunately, such antibodies have not been found for any other GTPases. For many years, investigators measured the levels of radiolabeled GTP/GDP after immunoprecipitation of their favorite GTPase from metabolically labeled cells. This required extraction of the bound nucleotide, and chromatography on thin-layer TLC plates followed by autoradiography. More recently, this cumbersome procedure has been replaced by pulldown assays, which make use of the increased affinity of active GTPases for their effectors to specifically precipitate the activated proteins from cell lysates. The first such assay used a GST fusion containing the CRIB (Cdc42/Rac Interaction and Binding) domain of the serine/threonine kinase PAK to measure the activity of both Cdc42 and Rac in cell lysates.⁷ We subsequently developed a similar assay for Arfs, which takes advantage of the ability of the adaptor protein GGA3 to bind all Arf isoforms.⁸ In these assays, cells (or tissues) are lysed in buffer containing detergent (typically Triton X100 or NP-40) and a high concentration of magnesium (~10 mM) to inhibit spontaneous nucleotide exchange. Lysates are then incubated with the immobilized GST fusion of choice (in this case containing residues 1–313 of GGA3), and precipitates are immunoblotted to detect bound Arf-GTP. A sample pulldown for Arf6 is shown in **Figure 1**. As mentioned above, an advantage of using

GGA3 for this purpose is its ability to bind all Arf isoforms, which can then be distinguished by immunoblotting with isoform-specific antibodies. It should be noted however that related reagents have been developed using fragments of the Cdc42-GAP ARHGAP21,⁹ which binds Arf1 and Arf6, and metallothioneine-2 (MT-2)¹⁰ or JIP3,¹¹ which bind selectively to Arf6. Such assays have been used extensively to measure Arf activation in response to extracellular cues such as receptor agonists/antagonists, cell-cell or cell-matrix adhesion, and infection with bacterial or viral pathogens.¹²⁻²³

Variations on the theme. In the simplest form of pulldown assay all components (Arfs, GEFs and GAPs) are endogenous, which has the advantage that they are expressed at normal levels and are localized to the appropriate subcellular compartment(s). Cells/tissues can be exposed to stimuli, and the activation of endogenous Arfs measured using an appropriate combination of GST-effector and antibody. One limitation of this approach is the availability of high quality isoform-specific antibodies. At this writing, good antibodies are available that selectively recognize either Arf1 or Arf6. Although there are numerous commercial

antibodies that are described as Arf3-, Arf4- or Arf5- specific, their cross-reactivity with other Arfs is not known, and has not been tested by the companies that sell them. Buyer beware!

An alternative approach is to express individual Arfs in epitope-tagged form. Although there are caveats associated with epitope tagging (see ref. 24) this approach allows the activation state of individual Arfs to be compared directly to each other, assuming they all have the same tag. Care must be taken to keep expression levels low, to assure targeting of exogenous Arfs to the appropriate subcellular compartment(s). This can be achieved by using vectors with weak promoters, by varying the time of analysis after transfection or by selecting stable transfectants that exhibit near-endogenous levels of expression.

Analysis of GEFs and GAPs. Most Arf GEFs and GAPs are expressed at low levels relative to their substrates and, as for the Arfs, results of assays using overexpressed proteins must be interpreted with caution. In some cases, such as the GEF EFA6 and the GAPs ACAP1 and ACAP2, clear-cut specificities for Arf6 are apparent even when both the GEF/GAP and the Arfs are overexpressed.²⁵ However in other cases, such as the GEF BRAG2, at least some activation of all Arfs is observed upon overexpression.²⁶ For this reason, we prefer to analyze GEF and/or GAP activity after knockdown of the endogenous protein by RNAi. When this approach was applied to BRAG2, we observed a 50% decrease in Arf6-GTP, with no change in the levels of Arf1-GTP.²⁶ This result highlights one of the important advantages of whole cell assays; although BRAG2 has the capacity to activate Arf1 when overexpressed it does not appear to do so at endogenous levels of expression, presumably because it is more restricted in its localization. It also tells us that roughly half of the pool of active Arf6 is generated through the activity of BRAG2.

One important limitation of such assays is that small or highly localized changes in Arf activation may be difficult to resolve. For example, there is a large pool of active Arf1 associated with the Golgi complex, but Arf1 is also found in smaller amounts on endosomal membranes and the plasma membrane. If a particular GEF or GAP acts only on the endosomal pool of Arf1,

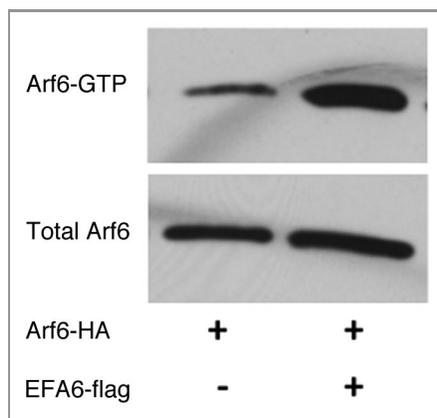


Figure 1. Activation of Arf6 by the Arf6-specific GEF EFA6. HeLa cells were transfected with plasmids encoding Arf6-HA and either empty vector or a vector encoding EFA6. After 24 h, cells were lysed and incubated with 30 μ g of GST-GGA3 fusion protein coupled to glutathione-sepharose beads. Washed precipitates (top panel) and aliquots of total cell lysates (bottom panel) were immunoblotted with anti-HA antibody to detect active, GTP-bound Arf6, and total Arf6 respectively.

knockdown may have a significant biological effect, but only a minor effect on the total cellular pool of Arf1-GTP.

Reporter Assays

FRET. Fluorescence resonance energy transfer (FRET) has been used by many investigators to visualize GTPase activation at specific locations within the cell. Such assays typically utilize a donor fluorophore fused to the GTPase, and an acceptor fluorophore fused to the GTPase-binding domain of a known effector (often referred to as a biosensor). When the GTPase is activated, binding to its effector brings the two fluorophores close enough together that light emitted by the donor activates the acceptor. Many variations on this theme have been developed to track the activation of Ras, Rho, Rab and Arf family GTPases (for review, see ref. 27).

In general, FRET biosensors can be classified as either bi-molecular, in which donor and acceptor are encoded by separate constructs, or unimolecular, in which donor and acceptor are encoded as a single multidomain construct, where the GTPase-donor cassette is connected through a flexible linker to an effector-acceptor cassette.²⁷ Unimolecular biosensors have the advantage that donor and acceptor are by definition expressed at equivalent levels and are physically linked to each other in space. However, to date only bimolecular biosensors have been applied to the study of Arfs. Joel Swanson and colleagues made elegant use of a single biosensor based on GGA3 to measure and localize the activation of both Arf1 and Arf6 during Fc γ -mediated phagocytosis, showing that Arf6 was activated in the leading edge of the phagocytic cup, while Arf1 was activated on the rim of the nascent phagosome.²⁸ An Arf6-specific biosensor, MT-2, has been used by Vitale and colleagues to demonstrate Arf6 activation during regulated secretion.²⁹

Because Arfs are myristoylated at their N-terminus, fluorophores must be introduced elsewhere in the molecule. While some Arf isoforms (e.g., Arf1) tolerate C-terminal fluorophores (CFP, GFP and mCherry) reasonably well, Arf6 apparently does not. Endogenous Arf6 is primarily membrane-bound even in its GDP-bound state, and

this is not significantly affected by small C-terminal epitope tags such as HA.²⁴ However, C-terminally-tagged Arf6-GFP is largely cytosolic, presumably due to steric interference from the fluorophore.³⁰ Insertion of GFP or the FRET-optimized donor fluorophore CyPet into a loop between the α 4 helix and β 6 strand in Arf6 (residues 140–148) yields a construct that associates with membranes efficiently and is appropriately regulated by GEFs and GAPs.³⁰ This construct has been used to monitor Arf6 activation in fibroblasts treated with PDGF³⁰ and neuronal growth cones.³¹ A representative set of images demonstrating Arf6 FRET is shown in **Figure 2**.

The activation of endogenous Arfs can also be monitored in situ, using fluorophore-tagged fragments of effector proteins similar to the FRET biosensors described above. Vitale and colleagues have used an MT-2-GFP probe to examine the activation of endogenous Arf6 in PC12 cells undergoing regulated exocytosis.²⁹ Similarly, Montagnac et al. made use of

the Arf6-binding domain of the scaffolding protein JIP3 to demonstrate the presence of active Arf6 in clathrin-coated vesicles.³² Both reagents are useful for this purpose because they bind selectively to Arf6. In contrast, GGA3 binds to all Arfs, and therefore cannot be used to distinguish among Arf isoforms in in situ assays. The discovery of new isoform-specific effectors in the future will help expand the arsenal of tools available for this type of analysis.

Summary

As noted above, a major focus in this field has been to identify which Arfs are acted upon by which GEFs and GAPs, where these activities are focused in the cell, and how they are regulated by upstream signals. Although careful in vitro analysis of the catalytic properties of these enzymes is important to understanding their function, the biological roles of these important regulatory proteins can best be defined in the context of the cell.

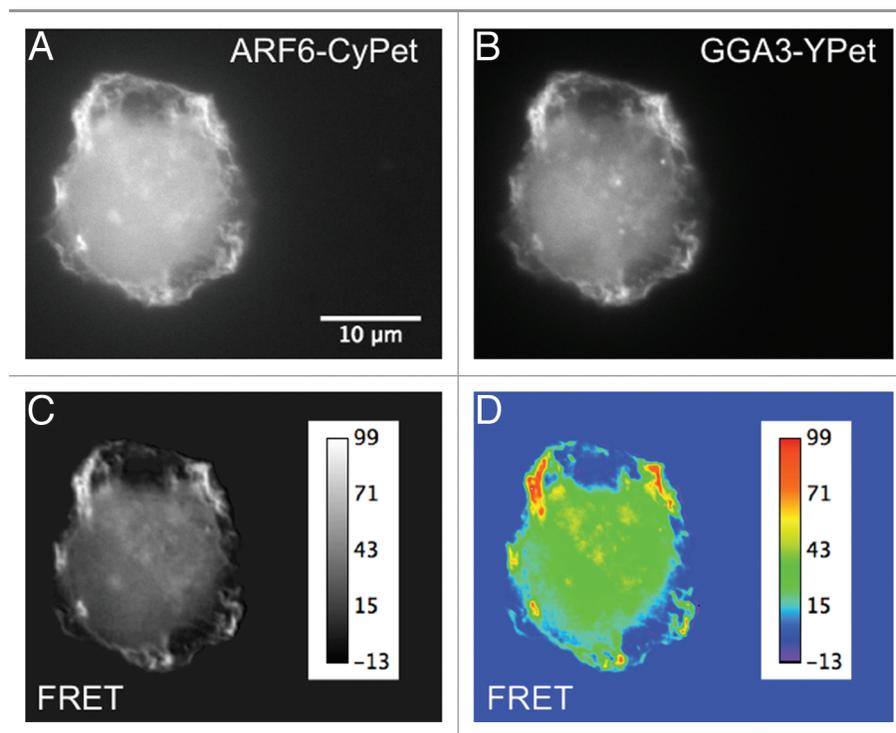


Figure 2. A set of images from a FRET experiment showing activation of Arf6 by the Arf GEF ARNO. HeLa cells were co-transfected with donor plasmid encoding Arf6-CyPet (inserted into the α 6/ β 4 loop as described in reference 30) and an acceptor plasmid encoding GGA3-YPet. Separate images for CyPet (A) and YPet (B) are shown, as well as the corresponding grayscale FRET image (C) and a pseudocolored version of the same image (D). Images courtesy of Dr Lorraine Santy, Pennsylvania State University.

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