

Kinetic and cell-based analyses of GTPase regulators

Nava Segev^{1,2} and Richard A. Kahn^{3,*}

¹Landes Bioscience; Chicago, IL USA; ²Department of Biological Sciences; University of Illinois at Chicago; Chicago, IL USA;

³Department of Biochemistry; School of Medicine; Emory University; Atlanta, GA USA

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Regulatory GTPases are often portrayed as binary molecular switches that control a wide range of cellular processes, including, but not limited to, the generation of second messengers (e.g., cAMP and inositol phosphates), intracellular traffic, cytoskeleton organization and cell proliferation. GEF stimulators and GAP inhibitors regulate the nucleotide-bound state of these proteins. Because of the relevance of GTPases and their regulators to human diseases, they comprise a major therapeutic target. Currently, most of the information about GTPase regulators comes from structure analyses. Such structural information is limited to certain conditions and does not necessarily reflect specificity or physiological activity. To address questions about specificity and mechanisms of action, kinetic and cell-based analyses of GTPase regulators is necessary. Here, we compare these two approaches in the context of regulators of Arf and heterotrimeric G-proteins.

Introduction

Regulatory GTPases in the heterotrimeric G-protein and ADP-ribosylation factor (Arf) families provide some interesting parallels and contrasts in their origins but also in the issues that drive current research and the construction of molecular models of their regulation. All GTPases cycle between GTP- and GDP-bound states, with consequent changes in protein conformation and binding partners. Because the GTP/GDP ratio in cytosol is thought to be about 10, regulatory GTPases would be overwhelmingly in the active conformation except the rate of spontaneous nucleotide exchange is quite low, especially relative to the biological process they control. Today it is generally assumed that activation by a guanine nucleotide exchange factor (GEF) is required for the biological function of each GTPase. Indeed, nature has devised families of GEFs, often quite large, that are specific to families or sub-families of GTPases. There are typically more GEFs than their GTPase substrates and because the GEFs themselves are regulated and often contain additional domains and

activities, they provide specificity as well as spatial and temporal regulation of GTPase activation.

G protein coupled receptors (GPCRs) act as G-protein GEFs and the Arf GEFs to date all contain a conserved Sec7 domain. As described in Northup et al.,¹ GPCRs are tremendously important for clinical medicine and the pharmaceutical industry as the number one class of target for drugs today. Though far less developed in chemotherapeutics, the Arf GEFs have been mimicked by human pathogens (including *Vibrio cholera* and *Legionella pneumophila*), targeted for inhibition by fungi (Brefeldin A), or in small molecule screens (Golgicide A). As a result, spectacular advances have been achieved in the detailed structural analyses of each. These have allowed testable predictions of the role of specific amino acid side chains in binding and hydrolysis.

Structural analyses of GTPases and their regulators were instrumental in our current understanding of their mechanisms of action. However, sole structure approaches have their limitations. Both crystallography and NMR usually employ truncated or modified proteins in higher

than physiological concentrations. To reveal the basis of interactions between GTPases and their regulators, two additional approaches are currently being employed: kinetic analyses of in vitro reactions using purified proteins, and monitoring interactions in the context of cells or cell lysates. While each approach has its advantages, it also has limitations that need to be considered. We think that the article from Northup et al.¹ argues convincingly for both continued structural work but also a clear need to couple it to kinetic studies of enzymatic properties. In parallel, the article from Casanova² summarizes nicely both the importance of cell-based assays and a couple of the approaches that are increasingly important to research into GTPase regulation and biology, as well as cell biology in general. As always, the strongest arguments and soundest models are those that encompass as many different approaches as possible.

Kinetic Analyses

In this issue, Northup et al.¹ make a case for studying the kinetics of GEF-stimulated nucleotide exchange on Arfs

*Correspondence to: Richard A. Kahn; Email: rkahn@emory.edu

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and heterotrimeric G-proteins. Such studies can be used to test mechanistic hypotheses that emerge from structural studies or obtain structure-function information not available from structural studies. One nice example is using such assays to address the question whether the GTPase-nucleotide-free form is a free intermediate or exists mostly when bound to the GEF. Determining reaction rates and affinities is important not only for elucidating mechanisms of action, but also for pharmacological design and testing of potential therapeutics that target these hubs of cell signaling.

While kinetic studies can be used also for determining GEF-GTPase specificity, these analyses should be taken with caution due to their reductionist nature. Kinetic studies employ purified proteins, usually expressed in bacteria, and depend on the availability of soluble and stable proteins. One important and underappreciated aspect of such kinetic studies is the ability (indeed the need) to characterize each preparation and confirm that it recapitulates the native protein. For practical reasons, these assays frequently use truncated proteins and therefore miss effects of other domains of the regulator (GEF) or the substrate (GTPase), or miss effects of post-translational modifications, or of other molecules (proteins, lipids and cofactors) important for the interaction.

Cell-Based Analyses

Casanova² discusses the importance of cell-based assays for regulation of Arfs by GEFs. These assays can be used for determining the physiological specificity of regulator-GTPase interactions. This article also makes the point that the issues are largely the same for the GEFs, which activate the GTPase and downstream pathways, and the GAPs that may terminate or mediate the GTPase signal. Two basic

types of cell-based assays are discussed, with variations on each type.

The first type discussed is arguably not a cell-based assay but a biochemical one that is intended to capture changes in the activation status of a GTPase occurring in the intact cells via specific pulldown from cell lysates. Such pulldowns require the generation of reagents that are capable of specific binding to the activated (GTP-bound) GTPase with quantitative recovery. An antibody with a conformational epitope specific to the activated species would be a wonderful reagent but, to our knowledge, exists only for Ras. Instead, the most common reagents used in these assays consist of fusion proteins of the domain from an effector that binds the active GTPase, fused to a tag that can be used to quantitatively purify the complex from solution. GTPase-binding domains from effectors typically have the specificity and affinity required of such reagents and we can expect to see many more generated for different GTPases. The utility of such assays is not questionable. Neither are their limitations, as pointed out by Casanova. Caution should be used when interpreting experiments that employ overexpressed proteins as there are both theoretical and practical reasons to believe that endogenously and exogenously expressed GTPases or their modulators behave, localize and are regulated distinctly.

The second type is a microscopic approach that determines regulator-GTPase interaction inside cells and requires fluorescent reporters. Such assays follow bi-molecular interactions of a GTPase with a GEF or an effector using fluorescence microscopy. The requirements of these assays for fluorescent tagging and (typically) protein overexpression are key limitations. While these are always issues of concern to researchers using fluorescent tags, it is particularly acute when the tag (GFP) is larger than the protein

being studied (Arf family members). When done without overexpression and in combination with compartmental markers, these assays are the gold standard for determining the cellular location where a certain GTPase is stimulated by a certain GEF. Both types of cell-based analyses address regulator-GTPase specificity in a physiological context. Obviously, the imaging assays can also address the location of the interaction and, therefore, even highly localized interactions can be detected.

Perspectives

While both kinetic and cell-based approaches have their advantages, they each have limitations. Therefore, getting information from both approaches is essential to the generation of hypotheses focused on mechanisms at atomic resolution and roles in biology/live cells as well as the ability to test them. Cell-based approaches can better resolve issues concerning physiological regulator-GTPases specificity. Pulldown assays from cell lysates circumvent the need of other proteins or protein domains, whereas microscopy-based assays also determine the site of interaction and are superior in cases of localized stimulation of a globally expressed GTPase. On the other hand, kinetic analyses of purified regulators or their domains with GTPases are instrumental for dissecting molecular mechanisms suggested by structural studies, which are essential for designing drug therapies. We hope that the two accompanying reasoned debate papers in this issue would encourage researchers to consider including these approaches in future studies aimed at unraveling GTPase regulation as well as drive further research into improved methods capable of addressing these important issues that are highly relevant to a large fraction of cell signaling and regulation studies.

References

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