Cellular Logistics 2:3, 140–146; July/August/September 2012; © 2012 Landes Bioscience

Nucleotide exchange factors
Kinetic analyses and the rationale for studying kinetics of GEFs

John K. Northup,[1,†] Xiaoying Jian[2] and Paul A. Randazzo[2,*]

[1]Laboratory of Cellular Biology; National Institute of Deafness and Other Communication Disorders; Rockville, MD USA;
[2]Laboratory of Cellular and Molecular Biology; National Cancer Institute; Bethesda, MD USA

[†]Current affiliation: Laboratory of Membrane Biochemistry and Biophysics; National Institute on Alcohol Abuse and Alcoholism; Rockville, MD USA

Keywords: G-protein couple receptor, guanine nucleotide binding protein, ADP-ribosylation factor, exchange factor, kinetics

Exchange factors are enzymes that catalyze the exchange of GTP for GDP on guanine nucleotide binding proteins. Progress in understanding the molecular basis of action and the cellular functions of these enzymes has largely come from structural determinations (e.g., crystal structures) and studying effects on cells when expression levels of the exchange factors are perturbed or mutated exchange factors are expressed. Proportionally little effort has been expended on studying the kinetics of exchange; however, reaction rates are central to understanding enzymes. Here, we discuss the importance of kinetic analysis of exchange factors for guanine nucleotide binding proteins, with a focus on ADP-ribosylation factor (Arf) and heterotrimeric G proteins, for providing unique insights into molecular mechanisms and regulation as well as how kinetic analyses are used to complement other approaches.

Introduction

Guanine nucleotide binding proteins, such as heterotrimeric G and Ras superfamily proteins control cellular responses and behaviors. In this reasoned debate, we focus of ADP-ribosylation factor (Arf) family guanine nucleotide proteins[1-4] and the heterotrimeric G proteins.[5,6] Six mammalian genes encode Arfs. They are divided into three classes based on primary structure. They regulate membrane traffic and actin cytoskeleton remodeling and work in two capacities. One function is to restrict the localization of vesicle coat proteins that are considered the machinery of membrane traffic. The second is to activate enzymes in signaling pathways, such as PIP kinase and phospholipase D. Arf6 has been reported to be necessary for the invasion of mammary carcinomas.[7-9] The heterotrimeric G proteins are comprised of α, β and γ subunits, each arising from one of a multigene family. Humans express 20 α subunits, 5 β subunits and 12 γ subunits. It is currently not known how many combinations of αβγ form in differentiated cells. These complexes mediate signaling necessary for diverse processes ranging from cognition to chemotaxis. Aberrant G-protein signaling has been implicated in diseases ranging from psychiatric disorders to cancer, with specific mutations associated with malignancies such as thyroid cancer and melanoma.

Common to guanine nucleotide binding proteins is that their function depends on controlled binding and hydrolysis of GTP, which converts the proteins between two states: protein bound to GTP (abbreviated here as G•GTP) and protein bound to GDP (G•GDP). G•GTP is often referred to as the active form. It binds to other proteins to affect their function or cellular distribution, e.g., adenylate cyclase is activated when GTP is bound to the α subunit of the heterotrimeric G protein Gs,[5] and proteins necessary for membrane traffic are recruited to membranes by the GTP-bound Arfs.[10] For the typical guanine nucleotide binding protein, nucleotide affinities are high and nucleotide dissociation rates and, consequently, spontaneous nucleotide exchange, are slow compared with the biological processes being controlled. Therefore, the functions of guanine nucleotide binding proteins critically depend on additional proteins called guanine nucleotide exchange factors (GEFs) for Ras superfamily proteins and G-protein-coupled receptors (GPCRs) for heterotrimeric G proteins, which catalyze the exchange of GTP for GDP to generate G•GTP. We will focus our discussion on GEFs that function with Arf guanine nucleotide binding proteins (Arf GEFs) and GPCRs.

Exchange factors are important to human health and for understanding human disease. The 15 human Arf GEFs outnumber the Arfs that they regulate.[11,12] The encoded proteins all contain a sec7 domain comprised of 10 α helices with a prominent hydrophobic groove, but are otherwise structurally diverse.[12-18] Brag2 is an Arf GEF that has been reported to drive invasion of some human cancers.[7] Mutations in the Arf GEF Big2 have been

*Correspondence to: Paul A. Randazzo; Email: randazzp@mail.nih.gov
Submitted: 07/02/12; Revised: 07/21/12; 07/24/12; Accepted: 07/25/12
http://dx.doi.org/10.4161/cl.21627

found to underlie an autosomal microcephaly and periventricular heterotopia found in two families.[19] The GPCRs far outnumber the G proteins that they control. Humans have over 800 genes for GPCRs. All GPCRs contain a canonical transmembrane helix bundle formed by 7 α-helical segments that is necessary for G-protein activation. The diverse GPCR structures are variously classified into six groups based upon the extracellular N-terminal domains, sequence diversity in the transmembrane helices and the intracellular C-terminal domains. About 90% of GPCR genes fall into a family that bears the name of its prototypical GPCR rhodopsin, and this thoroughly investigated GPCR has provided much of the structural and molecular insight into the mechanism of G-protein signaling. Underscoring the importance of these proteins, they are the targets for some 50% of the currently used therapeutics and are a major focus in the pharmaceutical industry's efforts to develop future therapeutic agents. Mutations in GPCRs are responsible for inherited diseases including disorders of calcium homeostasis associated with mutations of the calcium-sensing receptor[20] and diabetes insipidus arising from mutation of the V1 vasopressin receptor.[21] Mutant GPCRs are also identified as oncogenes and are also found in transforming retroviruses.[22,23]

Our understanding of the exchange factors has mostly derived from two approaches. First, crystal structures have been used to define the molecular basis of catalysis and have also provided information about regulation for GEFs.[14,16] Second, the effects of perturbing expression levels of the GEFs and GPCRs or expressing mutant GEFs or GCPRs in cells on enzymatic activity and cellular functions have been examined (e.g., see refs. 7 and 24–29). Still, the GEFs and GPCRs, other than several studied in solution,[30-35] are relatively poorly understood in terms of the molecular bases for substrate specificity and regulatory mechanisms. Although some in vitro biochemistry has been reported, kinetic approaches have been more limited to date. They have been used to complement crystallography and the study of the proteins in cells. Although often seen as a means of confirming conclusions from other approaches, examination of kinetics provides insights that are not accessible by other approaches.

Structure/Function Analysis

Determination of crystal structures is a powerful approach to learn about molecular mechanisms of catalysis and has provided researchers with considerable details of the chemistry involved. Based upon contacts between the enzyme and the substrate, predictions can be made about binding, catalytic and regulatory interactions. However, crystal structures are frozen. Even if a series of crystal structures can be identified, these represent single states among a continuum of structures from which mechanisms are not determined, only inferred. In addition, although estimates of the energetics of the different states can be made, the prediction of energetic barriers and, therefore, reaction rates, is not reliable at this time. Finally, the structures are determined from proteins under extreme conditions and usually with modified proteins that favor crystallization. Consequently, the binding interfaces observed may not represent the physiologically relevant structures. For these reasons, kinetics analyses are necessary to test conclusions based on crystallographic structures.

The literature contains numerous examples of testing inferences based on structural studies. Typically, recombinant proteins in which residues involved in protein-protein binding or catalysis are either mutated or deleted. The effect of these changes on reaction rates are then determined. Work on the ARF GEF Grp1 is one example in which the kinetic analysis confirmed conclusions based on the crystal structure.[36] From the crystal structure of Grp1, a polybasic motif and the linker between the catalytic sec7 domain and the PH domain were predicted to be autoinhibitory, blocking access of switch 1 and switch 2 of Arf to the sec7 domain. Determination of the relative k_{cat}/K_m ratio for a number of mutants provided a robust test of the hypothesis, supporting the conclusion.[36] In another example, based on a crystal structure of Big2, the loop after helix J of the sec7 domain was predicted to interact with Arf and consequently to facilitate activity. The prediction was tested and confirmed by determining the effect of mutations in the loop using an in vitro assay to determine reaction rates.[27]

The importance of kinetic analysis is emphasized by those instances in which predictions based on a crystal structure were not substantiated. The requirements of crystallization often necessitate mutant and/or truncated protein constructs which may not reflect the regulated properties of the wild-type gene product. As an example, the mechanism for Gq activation of phospholipase Cβ (PLCβ) was proposed to be the recruitment of the enzyme to the inner leaflet of the plasma membrane based upon the structure determined for a truncated PLCβ.[37,38] However, the kinetic analysis of Gq activation and the intrinsic kinetic properties of the full-length PLCβ compared with a truncated construct used for crystallization revealed that that an additional sequence element not in the crystallized PLCβ acts as an auto-inhibitory constraint and that Gq binding of that sequence released PLCβ from inhibition as the mechanism of activation.[39]

In many cases kinetic analysis can provide more structure/function information than available by structural approaches alone. As described above, one shortcoming of crystallography is the proteins are often truncated and modified to enable crystallization. NMR determination of structure is currently limited to proteins under 50 kDa. Kinetic analysis requires only that proteins be soluble and stable, but usually at much lower concentrations than those required for crystallography or NMR, and thus, can be valuable for examining structures, e.g., outside of the Sec7 domain of Brag2, that may contribute to catalysis but have not been visualized either by crystallography or NMR. Recent kinetic analysis of Brag2 identified the linker between neighboring sec7 and PH domains as a positive contributor to the exchange reaction[40] The model that is being tested by kinetic strategies is that the linker binds directly to two motifs within Arf.

The use of kinetics to examine structural requirements for activity is not

dependent on having a structure determined by X-ray crystallography or NMR. Based on analysis of primary sequences of a class of proteins, a structural feature may be hypothesized as important. This can be easily tested by mutagenesis and kinetic analysis. Because of the myriad challenges to their crystallization, only a limited number of crystal structures for GPCRs are currently solved, many of them involving the replacement of native structure with foreign sequences to enhance crystallization. However, numerous studies employing site-directed mutagenesis have identified common critical residues essential for G-protein activation.

The use of kinetics to examine the contribution of protein motifs outside that which can be visualized by crystallography or NMR extends to the substrate Arf. Most crystal structures have used a truncated form of Arf, [Δ17]Arf1, lacking the critical N-terminal extension, and, consequently, much of the kinetics that have been done have used [Δ17]Arf1 and equivalent Arf5 and Arf6 recombinant proteins as substrates. Important information about catalytic mechanism and regulation may be missed, however, by using the truncated Arfs. The deleted motif can be considered an additional switch motif as its conformation and position change dramatically on switching between GDP and GTP bound forms of Arf.[41-43] Any mechanism of GDP to GTP switching has to account for these changes in the N-terminal motif. Direct comparison with full length N-myristoylated Arf1 (myrArf1) revealed that truncated Arf is two orders of magnitude less efficient a substrate than is myrArf1 for Brag2[40] and some early evidence supported the idea that the N-terminus of Arf interacts with the exchange factor ARNO.[44] These results motivated studies that revealed the N-terminus of Arf may contact an extra catalytic motif within Brag2.[40] Thus, understanding the complete catalytic mechanism and regulation will be facilitated by using full length myristoylated Arf1 in kinetic studies.

A similar situation applies to the heterotrimeric G proteins. The N-termini of Gα proteins are post-translationally modified with two distinct lipids—myristate as an amide-linkage to an N-terminal glycine and/or palmitate thio-esterified to cysteines near the N-terminus. In addition the C-termini of Gγ of the Gβγ dimer are modified with thio-ether linked isoprenoids followed by cleavage of the terminating three amino acids and methylation of the resulting C-terminal, prenylated cysteine. To obtain crystals, the N-terminus of native Gαt and C-terminus of native Gγ1 were proteolytically cleaved,[45] or recombinant $G\alpha_{i1}$ and Gβ1γ2 were expressed without the lipid modifications[46] so that the native structures of these terminal sequences remain unknown in relation to GPCR contact. To date, a single crystal structure has resolved the isoprenoid (farnesyl) modification in contact the retinal Gβγ effector phosducin.[47] It remains undetermined if the isoprenoid of Gβγ adopts a similar contact with GPCRs. Further, interaction of Gαi family proteins with Gβγ[48,49] and GPCRs[50] (Gutierrez and Northup, unpublished) and the Gβγ interaction with GPCRs[51] are critically dependent upon the lipid modifications. Since bacteria do not carry out these post-translational modifications natively and the G proteins expressed under high-level expression promoters in mammalian cells may be incompletely or incorrectly modified, in vitro kinetic analysis using G proteins with confirmed, homogeneous lipid modification may be necessary to clearly determine molecular mechanisms in G protein regulation.

Examination of Mechanism with Kinetics

Even if several crystal structures presumably defining states of a reaction path can be determined, the energy barriers and, therefore, the rate of reaction and particular paths are not defined by the structures. The kinetic scheme labeled "Model 1" in **Figure 1**, which is generally accepted, is an allosteric competitive mechanism and has been tested for some Ras superfamily members.[31,32,52] Exchange factor binds to G•GDP, reducing the affinity of the guanine nucleotide binding protein for GDP (or GTP as the case might be) by accelerating dissociation. A nucleotide free guanine nucleotide binding protein•exchange factor complex is formed (E•G in the schematic). GTP binds to the guanine nucleotide pocket of the proteins in complex, forming E•G•GTP. GTP binding has been reported to be accelerated by the exchange factor.[35] The complex E•G•GTP dissociates to form free E and G•GTP.

An alternative mechanism, the GTP/GDP displacement model, was proposed in which a quaternary intermediate is required for exchange (see Model 2 in **Fig. 1**). In this model, the guanine nucleotide binding proteins with GDP (G•GDP) binds to the exchange factor (E),

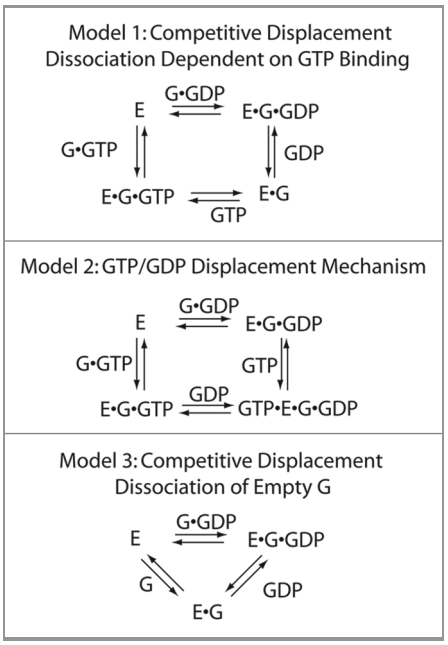

Figure 1. Schemes for three possible kinetic mechanisms. E, GEF; G, empty guanine nucleotide binding protein; G•GTP, guanine nucleotide binding protein with bound GTP; G•GDP, guanine nucleotide binding protein with bound GDP. Model 1: competitive displacement with dissociation dependent on GTP binding. In this reaction scheme, G•GDP binds to exchange factor, resulting in the release of GDP. Empty G has a higher affinity for the exchange factor than either G•GDP or G•GTP, and remains associated until GTP binds. G•GTP, with a lower affinity than G for the exchange factor, dissociates. Model 2: GTP/GDP displacement. In this scheme, GTP binds to the E•G•GDP complex and subsequently displaces GDP. The E•G•GTP complex then dissociates into free E and G•GTP. Model 3: competitive displacement with dissociation of empty G. In this variation of reaction scheme 1, empty G has a low affinity for the exchange factor and can be considered a product of the reaction. GTP binds to G while free in solution to form G•GTP.

forming the ternary complex of E•G•GDP. GTP then binds forming the quaternary complex GTP•E•G•GDP. GTP then displaces GDP, with the latter leaving the complex, to form E•G•GTP, which subsequently dissociates into E and G•GTP. A similar model has often implicitly been implied for GPCR-Gα activation based upon the enhancement observed upon addition of GDP for GPCR-catalyzed GTP binding to membrane fractions. The GTP/GDP displacement mechanism was conclusively excluded for the Ras exchange factor Cdc25 by evaluating the kinetics of GDP dissociation: the rate constant for GDP release was found to be insensitive to the concentration of GTP under single turnover conditions.[53]

Although the competitive allosterism mechanism as drawn for Model 1 is generally accepted, this represents a subset of an equilibrium system that includes free empty guanine nucleotide binding protein (G) (i.e., not in complex with the exchange factor or nucleotide). Another subset of equilibria within the competive allosteric binding model could be considered a variation of the mechanism, which is schematically represented in Model 3 of **Figure 1**. In this variation, nucleotide free guanine nucleotide binding proteins can dissociate and bind to GTP, similar to the mechanism for ATP exchange seen for motor proteins.[54] Thus, Models 1 and 3 represent two extreme cases of a single equilibrium system, one in which the affinity of empty G for E is much higher than the affinity of G•GXP for E (Model 1) and the other in which the affinity of G and G•GTP for E is similar. GPCRs would be of particular interest to test since nucleotide exchange on heterotrimeric G proteins is linked to subunit dissociation, which influences the equilibria and hence kinetic mechanism. As far as we are aware, tests to discriminate between the mechanisms for GPCRs have yet to be performed.

More Ras superfamily proteins should also be tested, especially from the practical consideration of experimental design. Indeed, the mutation introduced in the P-loop to make the presumed dominant negative (T or S to N) is thought to work by sequestration of Arf GEFs. This dominant negative effect requires the exchange factor function as described in the scheme in Model 1, i.e., that the affinity of G for E is much greater than the affinity of G•GTP for E. Thus, G with the P-loop mutation does not efficiently bind nucleotide, and dissociates slowly from E, preventing E to catalyze exchange for endogenous G. If the difference between the affinities is not great, the extreme case being the exchange factor functioning by the kinetic mechanism described in Model 3, this mutant guanine nucleotide binding protein would not function as a dominant negative. Other factors independent of the kinetic mechanism are also important for the function of the P-loop mutant as a dominant negative, such as relative expression levels and the stability of the protein. In the case of Rap1, the mutation did not result in a dominant negative using C3G as an exchange factor.[55] Distinguishing among the models requires the determination of the dependence of reaction rates on protein and nucleotide concentrations, thus the importance of kinetics for understanding molecular mechanism of exchange factors. The kinetic studies can also help assess the value of a P-loop mutant as a dominant negative.

Biological Function: Relating Enzymatic Activity to Biological Effects

Establishing kinetic constants is central to understanding the biological function of the GEFs. The structural determinations for the molecular mechanisms involved in the reaction and the energetics to understand binding and chemical reactions are important, but the biologically relevant activity is the control of the rate of the reaction. The K_m should be reasonable given the estimated concentrations of proteins in the cell. Similarly, the rate of reaction should be at least as fast as the biological process putatively controlled by the enzyme. Differences among heterotrimeric G proteins interactions with GPCRs illustrate the significance of the kinetics to biological processes. While the heterotrimeric G-proteins share considerable homology of both structure and mechanism, their biological roles have dictated the evolution of significant quantitative differences in the kinetics of their activation/de-activation. The Gαi1 and Gαt gene products, while both members of the αi gene family, display about two orders of magnitude difference in rates of spontaneous GDP-dissociation (i.e., not catalyzed by GPCR) with αi1 $>>$ αt, while the GDP-dissociation rate from αt catalyzed by rhodopsin greatly exceeds that measured for GPCR-αi1. In this case, the visual response of a vertebrate rod cell which can detect single photons under conditions of full dark adaptation dictates a near zero "basal" activity of the signaling cascade in the absence of rhodopsin activation and a robust exchange rate on photon activation of rhodopsin.

If the kinetics of guanine nucleotide exchange in vitro are slower than the rate of the biological processes that they control then several possibilities exist: (1) an enzyme is inactive due to misfolding of the recombinant protein, (2) conditions are not optimal for activity, e.g., need for an activator or (3) the GEF does not control the process in question. Misfolding can be excluded by comparing substrate saturation kinetics to single turnover kinetics. If the two types of experiments yield similar parameter estimates, then the GEF is a single active population and possibilities 2 and 3 should be considered. GEFs may be activated either by a covalent modification or by interaction with a protein or small molecule. Possibilities 2 and 3 have important ramifications for design of experiments in cells, e.g., expression of the GEF from a plasmid. If the activation is through binding to a protein, it is plausible that the activator will be titrated and most of the GEF expressed will be relatively inactive. Although some activity may be observed, there are a number of artifacts associated with partially inactive proteins; one artifact is related to determining substrate specificity.

Kinetic experiments are ideal for determining substrate specificity and, importantly, for determining if appropriate experiments, given available reagents and knowledge, can be designed to determine substrate specificity in vitro and in vivo. Assuming completely myristoylated Arf proteins are available and the GEF is active, the Arfs can be directly compared with determination of kinetic parameters; however, if the GEF has low activity, the results should be interpreted cautiously.

Work on GTPase-activating protein for Arfs (ArfGAPs) illustrates the potential pitfalls of interpreting the results of experiments with enzymes of low activity.[56,57] In the case of the ArfGAPs, ASAP1 and AGAP1, the full length proteins are relatively inactive. An autoinhibitory motif in ASAP1 has been identified. Neither ASAP1 nor AGAP1, in their inactive forms, distinguish between Arf1 and Arf6 as substrates. In contrast, in active form, both GAPs use Arf1 30- to 200-fold more efficiently than Arf6. Perhaps analogously, examination of the exchange factor Brag2 revealed both smaller than expected catalytic power and lack of Arf specificity.[40] Without knowing the means to activate the GEF, simply expressing the protein in mammalian cells may not resolve the issue of specificity. Brag2 overexpressed in HeLa cells had the same lack of specificity (Jian and Randazzo, unpublished), as was observed with purified protein. Although the promiscuity of Brag2 cannot be excluded at this time, further work is needed to assess substrate specificity.

Realizing the Power of Kinetic Analysis: Clean Reagents, Titrations and Time

The power of in vitro analysis is realized by including (1) proper quantification and validation of reagents, (2) titrations and (3) time courses, which is difficult or impossible to do with cell-based assays. Arf concentrations can be determined by titrating active sites. Comparisons of substrate titrations and single turnover results can provide an assessment of the quality of the GEF. The rigor prevents pursuit of artifacts in in vitro analysis and can raise concerns about artifacts that may be observed using cell-based assays. There are several examples in the GPCR literature. One approach often utilized to investigate GPCR-G-protein selectivity is the co-transfection of GPCR and Gα constructs designed to re-direct the signaling to cellular calcium mobilization, which can readily be detected by fluorescent dyes. While this strategy has succeeded powerfully in identifying activating ligands for "orphan" GPCRs it has often misreported the G-protein selectivity. Initial studies for GPCRs linking to Gαi signaling concluded that the receptor-selective sequence of a G-protein was limited to the C-terminal four amino acid residues.[58] However, studies with T2R bitter taste receptors seem to identify a significantly larger region of the carboxyl terminus of Gustducin α involved in taste signaling.[59] In vitro examination of this[60] and a recent crystal structure of the β2AR-Gs complex identify a significantly greater surface of interaction including contributions of residues from the C-terminal 50 amino acids and the N-terminal α helix of $G\alpha_s$[61] (Gutierrez and Northup, unpublished).

Lack of quantification and/or characterization of reagents can provide misleading results. For example, because of convenience, use of epitope tags and kits is widespread. Unfortunately the tags often affect the proteins to which they are fused. Glutathione S-transferase (GST) has a large effect on the ability of Arf to bind to GDP and GTP and both GST and green fluorescent protein have large effects on interaction with the exchange factor ARNO.[62] Truncation of Arf also has a large effect on interaction with ARNO and Brag2 as described above. These reagents would not be appropriate for assessing Arf specificity for the reasons described in the discussion above about determining Arf specificity with an inactive GEF. The quality of the GEF is equally important. We recognize that sometimes the use of truncated or modified proteins is difficult to avoid and, despite the shortcomings of these reagents, often does provide valuable insights into the function of the exchange factors. The opportunity for insights is maximized by carrying out substrate titrations to determine K_m and V_{max} and, where possible, single turnover studies to determine k_{cat}. The effect of modifiers, comparison to other exchange factors and limitations of the experiments are most robustly assessed by expressing activity as the ratio of k_{cat}/K_m.

Conclusions

Many GPCRs and some ArfGEFs (e.g., Brag2) are GEFs that represent important target for the pharmaceutical industry. These proteins are enzymes and as enzymes, their function is related to controlling reaction rates. Consequently, studies aimed at understanding of the molecular basis of the activity and biological function must include kinetic analyses.

Acknowledgments

We thank Dr Victor Rebois for critical review of the manuscript. The work was supported by the Intramural Program of the National Institutes of Health.

References

1. Kahn RA, Cherfils J, Elias M, Lovering RC, Munro S, Schurmann A. Nomenclature for the human Arf family of GTP-binding proteins: ARF, ARL, and SAR proteins. J Cell Biol 2006; 172:645-50; PMID: 16505163; http://dx.doi.org/10.1083/jcb.200512057
2. Donaldson JG, Jackson CL. ARF family G proteins and their regulators: roles in membrane transport, development and disease. Nat Rev Mol Cell Biol 2011; 12:362-75; PMID:21587297; http://dx.doi.org/10.1038/nrm3117
3. Gillingham AK, Munro S. The small G proteins of the arf family and their regulators. Ann Rev Cell Dev Biol, 2007:579-611.
4. D'Souza-Schorey C, Chavrier P. ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol 2006; 7:347-58; PMID:16633337; http://dx.doi.org/10.1038/nrm1910
5. Gilman AG. G proteins: transducers of receptor-generated signals. Annu Rev Biochem 1987; 56:615-49; PMID: 3113327; http://dx.doi.org/10.1146/annurev.bi.56.070187.003151
6. Sprang SR. G protein mechanisms: insights from structural analysis. Annu Rev Biochem 1997; 66:639-78; PMID: 9242920; http://dx.doi.org/10.1146/annurev.biochem.66.1.639
7. Morishige M, Hashimoto S, Ogawa E, Toda Y, Kotani H, Hirose M, et al. GEP100 links epidermal growth factor receptor signalling to Arf6 activation to induce breast cancer invasion. Nat Cell Biol 2008; 10:85-92; PMID:18084281; http://dx.doi.org/10.1038/ncb1672
8. Hu B, Shi B, Jarzynka MJ, Yiin J-J, D'Souza-Schorey C, Cheng SY. ADP-ribosylation factor 6 regulates glioma cell invasion through the IQ-domain GTPase-activating protein 1-Rac1-mediated pathway. Cancer Res 2009; 69:794-801; PMID:19155310; http://dx.doi.org/10.1158/0008-5472.CAN-08-2110
9. Tague SE, Muralidharan V, D'Souza-Schorey C. ADP-ribosylation factor 6 regulates tumor cell invasion through the activation of the MEK/ERK signaling pathway. Proc Natl Acad Sci U S A 2004; 101:9671-6; PMID:15210957; http://dx.doi.org/10.1073/pnas.0403531101

10. Nie ZZ, Randazzo PA. Arf GAPs and membrane traffic. J Cell Sci 2006; 119:1203-11; PMID: 16554436; http://dx.doi.org/10.1242/jcs.02924
11. Casanova JE. Regulation of Arf activation: the Sec7 family of guanine nucleotide exchange factors. Traffic 2007; 8:1476-85; PMID:17850229; http://dx.doi.org/10.1111/j.1600-0854.2007.00634.x
12. Cherfils J, Chardin P. GEFs: structural basis for their activation of small GTP-binding proteins. Trends Biochem Sci 1999; 24:306-11; PMID:10431174; http://dx.doi.org/10.1016/S0968-0004(99)01429-2
13. Renault L, Christova P, Guibert B, Pasqualato S, Cherfils J. Mechanism of domain closure of Sec7 domains and role in BFA sensitivity. Biochemistry 2002; 41:3605-12; PMID:11888276; http://dx.doi.org/10.1021/bi012123h
14. Renault L, Guibert B, Cherfils J. Structural snapshots of the mechanism and inhibition of a guanine nucleotide exchange factor. Nature 2003; 426:525-30; PMID: 14654833; http://dx.doi.org/10.1038/nature02197
15. Goldberg J. Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. Cell 1998; 95:237-48; PMID:9790530; http://dx.doi.org/10.1016/S0092-8674(00)81754-7
16. Mossessova E, Corpina RA, Goldberg J. Crystal structure of ARF1*Sec7 complexed with Brefeldin A and its implications for the guanine nucleotide exchange mechanism. Mol Cell 2003; 12:1403-11; PMID:14690595; http://dx.doi.org/10.1016/S1097-2765(03)00475-1
17. Mossessova E, Gulbis JM, Goldberg J. Structure of the guanine nucleotide exchange factor Sec7 domain of human arno and analysis of the interaction with ARF GTPase. Cell 1998; 92:415-23; PMID:9476900; http://dx.doi.org/10.1016/S0092-8674(00)80933-2
18. Cherfils J, Ménétrey J, Mathieu M, Le Bras G, Robineau S, Béraud-Dufour S, et al. Structure of the Sec7 domain of the Arf exchange factor ARNO. Nature 1998; 392:101-5; PMID:9510256; http://dx.doi.org/10.1038/32210
19. Sheen VL, Ganesh VS, Topcu M, Sebire G, Bodell A, Hill RS, et al. Mutations in ARFGEF2 implicate vesicle trafficking in neural progenitor proliferation and migration in the human cerebral cortex. Nat Genet 2004; 36:69-76; PMID:14647276; http://dx.doi.org/10.1038/ng1276
20. Hannan FM, Nesbit MA, Zhang C, Cranston T, Curley AJ, Harding B, et al. Identification of 70 calcium-sensing receptor mutations in hyper- and hypo-calcaemic patients: evidence for clustering of extracellular domain mutations at calcium-binding sites. Hum Mol Genet 2012; 21:2768-78; PMID: 22422767; http://dx.doi.org/10.1093/hmg/dds105
21. Babey M, Kopp P, Robertson GL. Familial forms of diabetes insipidus: clinical and molecular characteristics. Nat Rev Endocrinol 2011; 7:701-14; PMID:21727914; http://dx.doi.org/10.1038/nrendo.2011.100
22. van Rhee AM, Jacobson KA. Molecular architecture of G protein-coupled receptors. Drug Dev Res 1996; 37:1-38; PMID:21921973; http://dx.doi.org/10.1002/(SICI)1098-2299(199601)37:1<1::AID-DDR1>3.0.CO;2-S
23. Parnot C, Miserey-Lenkei S, Bardin S, Corvol P, Clauser E. Lessons from constitutively active mutants of G protein-coupled receptors. Trends Endocrinol Metab 2002; 13:336-43; PMID:12217490; http://dx.doi.org/10.1016/S1043-2760(02)00628-8
24. Dunphy JL, Moravec R, Ly K, Lasell TK, Melancon P, Casanova JE. The Arf6 GEF GEP100/BRAG2 regulates cell adhesion by controlling endocytosis of beta1 integrins. Curr Biol 2006; 16:315-20; PMID:16461286; http://dx.doi.org/10.1016/j.cub.2005.12.032
25. Manolea F, Claude A, Chun J, Rosas J, Melançon P. Distinct functions for Arf guanine nucleotide exchange factors at the Golgi complex: GBF1 and BIGs are required for assembly and maintenance of the Golgi stack and trans-Golgi network, respectively. Mol Biol Cell 2008; 19:523-35; PMID:18003980; http://dx.doi.org/10.1091/mbc.E07-04-0394
26. Zhao X, Claude A, Chun J, Shields DJ, Presley JF, Melançon P. GBF1, a cis-Golgi and VTCs-localized ARF-GEF, is implicated in ER-to-Golgi protein traffic. J Cell Sci 2006; 119:3743-53; PMID:16926190; http://dx.doi.org/10.1242/jcs.03173
27. Lowery J, Szul T, Seetharaman J, Jian X, Su M, Forouhar F, et al. Novel C-terminal motif within Sec7 domain of guanine nucleotide exchange factors regulates ADP-ribosylation factor (ARF) binding and activation. J Biol Chem 2011; 286:36898-906; PMID:21828055; http://dx.doi.org/10.1074/jbc.M111.230631
28. Szul T, Grabski R, Lyons S, Morohashi Y, Shestopal S, Lowe M, et al. Dissecting the role of the ARF guanine nucleotide exchange factor GBF1 in Golgi biogenesis and protein trafficking. J Cell Sci 2007; 120:3929-40; PMID: 17956946; http://dx.doi.org/10.1242/jcs.010769
29. Li HS, Shome K, Rojas R, Rizzo MA, Vasudevan C, Fluharty E, et al. The guanine nucleotide exchange factor ARNO mediates the activation of ARF and phospholipase D by insulin. BMC Cell Biol 2003; 4: 13; PMID:12969509; http://dx.doi.org/10.1186/1471-2121-4-13
30. Itzen A, Rak A, Goody RS. Sec2 is a highly efficient exchange factor for the Rab protein Sec4. J Mol Biol 2007; 365:1359-67; PMID:17134721; http://dx.doi.org/10.1016/j.jmb.2006.10.096
31. Klebe C, Prinz H, Wittinghofer A, Goody RS. The kinetic mechanism of Ran–nucleotide exchange catalyzed by RCC1. Biochemistry 1995; 34:12543-52; PMID: 7548002; http://dx.doi.org/10.1021/bi00039a008
32. Lenzen C, Cool RH, Prinz H, Kuhlmann J, Wittinghofer A. Kinetic analysis by fluorescence of the interaction between Ras and the catalytic domain of the guanine nucleotide exchange factor Cdc25Mm. Biochemistry 1998; 37:7420-30; PMID:9585556; http://dx.doi.org/10.1021/bi972621j
33. Gromadski KB, Wieden HJ, Rodnina MV. Kinetic mechanism of elongation factor Ts-catalyzed nucleotide exchange in elongation factor Tu. Biochemistry 2002; 41:162-9; PMID:11772013; http://dx.doi.org/10.1021/bi015712w
34. Hutchinson JP, Eccleston JF. Mechanism of nucleotide release from Rho by the GDP dissociation stimulator protein. Biochemistry 2000; 39:11348-59; PMID: 10985780; http://dx.doi.org/10.1021/bi0007573
35. Chin HF, Cai YY, Menon S, Ferro-Novick S, Reinisch KM, De La Cruz EM. Kinetic analysis of the guanine nucleotide exchange activity of TRAPP, a multimeric Ypt1p exchange factor. J Mol Biol 2009; 389:275-88; PMID:19361519; http://dx.doi.org/10.1016/j.jmb.2009.03.068
36. DiNitto JP, Delprato A, Gabe Lee MT, Cronin TC, Huang S, Guilherme A, et al. Structural basis and mechanism of autoregulation in 3-phosphoinositide-dependent Grp1 family Arf GTPase exchange factors. Mol Cell 2007; 28:569-83; PMID:18042453; http://dx.doi.org/10.1016/j.molcel.2007.09.017
37. Jezyk MR, Snyder JT, Gershberg S, Worthylake DK, Harden TK, Sondek J. Crystal structure of Rac1 bound to its effector phospholipase C-beta2. Nat Struct Mol Biol 2006; 13:1135-40; PMID:17115053; http://dx.doi.org/10.1038/nsmb1175
38. Waldo GL, Ricks TK, Hicks SN, Cheever ML, Kawano T, Tsuboi K, et al. Kinetic scaffolding mediated by a phospholipase C-beta and Gq signaling complex. Science 2010; 330:974-80; PMID: 20966218; http://dx.doi.org/10.1126/science.1193438
39. Lyon AM, Tesmer VM, Dhamsania VD, Thal DM, Gutierrez J, Chowdhury S, et al. An autoinhibitory helix in the C-terminal region of phospholipase C-β mediates Gαq activation. Nat Struct Mol Biol 2011; 18:999-1005; PMID:21822282; http://dx.doi.org/10.1038/nsmb.2095
40. Jian X, Gruschus JM, Sztul E, Randazzo PA. The pleckstrin homology (PH) domain of the Arf exchange factor Brag2 is an allosteric binding site. J Biol Chem 2012; 287:24273-83; PMID:22613714; http://dx.doi.org/10.1074/jbc.M112.368084
41. Randazzo PA, Terui T, Sturch S, Fales HM, Ferrige AG, Kahn RA. The myristoylated amino terminus of ADP-ribosylation factor 1 is a phospholipid- and GTP-sensitive switch. J Biol Chem 1995; 270:14809-15; PMID:7782347; http://dx.doi.org/10.1074/jbc.270.24.14809
42. Liu Y, Kahn RA, Prestegard JH. Structure and membrane interaction of myristoylated ARF1. Structure 2009; 17:79-87; PMID:19141284; http://dx.doi.org/10.1016/j.str.2008.10.020
43. Liu Y, Kahn RA, Prestegard JH. Dynamic structure of membrane-anchored Arf*GTP. Nat Struct Mol Biol 2010; 17:876-81; PMID:20601958; http://dx.doi.org/10.1038/nsmb.1853
44. Pacheco-Rodriguez G, Patton WA, Adamik R, Yoo HS, Lee FJS, Zhang GF, et al. Structural elements of ADP-ribosylation factor 1 required for functional interaction with cytohesin-1. J Biol Chem 1999; 274: 12438-44; PMID:10212218; http://dx.doi.org/10.1074/jbc.274.18.12438
45. Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE, Sigler PB. The 2.0 A crystal structure of a heterotrimeric G protein. Nature 1996; 379:311-9; PMID:8552184; http://dx.doi.org/10.1038/379311a0
46. Wall MA, Coleman DE, Lee E, Iñiguez-Lluhi JA, Posner BA, Gilman AG, et al. The structure of the G protein heterotrimer Gi alpha 1 beta 1 gamma 2. Cell 1995; 83:1047-58; PMID:8521505; http://dx.doi.org/10.1016/0092-8674(95)90220-1
47. Loew A, Ho YK, Blundell T, Bax B. Phosducin induces a structural change in transducin beta gamma. Structure 1998; 6:1007-19; PMID:9739091; http://dx.doi.org/10.1016/S0969-2126(98)00102-6
48. Linder ME, Pang IH, Duronio RJ, Gordon JI, Sternweis PC, Gilman AG. Lipid modifications of G protein subunits. Myristoylation of Go alpha increases its affinity for beta gamma. J Biol Chem 1991; 266: 4654-9; PMID:1900297
49. Muntz KH, Sternweis PC, Gilman AG, Mumby SM. Influence of gamma subunit prenylation on association of guanine nucleotide-binding regulatory proteins with membranes. Mol Biol Cell 1992; 3:49-61; PMID: 1550955
50. Saidak Z, Blake-Palmer K, Hay DL, Northup JK, Glass M. Differential activation of G-proteins by mu-opioid receptor agonists. Br J Pharmacol 2006; 147:671-80; PMID:16415903; http://dx.doi.org/10.1038/sj.bjp.0706661
51. Jian XY, Sainz E, Clark WA, Jensen RT, Battey JF, Northup JK. The bombesin receptor subtypes have distinct G protein specificities. J Biol Chem 1999; 274: 11573-81; PMID:10206964; http://dx.doi.org/10.1074/jbc.274.17.11573
52. Goody RS, Hofmann-Goody W. Exchange factors, effectors, GAPs and motor proteins: common thermodynamic and kinetic principles for different functions. Eur Biophys J 2002; 31:268-74; PMID:12122473; http://dx.doi.org/10.1007/s00249-002-0225-3
53. Guo Z, Ahmadian MR, Goody RS. Guanine nucleotide exchange factors operate by a simple allosteric competitive mechanism. Biochemistry 2005; 44: 15423-9; PMID:16300389; http://dx.doi.org/10.1021/bi0518601
54. Sweeney HL, Houdusse A. Structural and functional insights into the Myosin motor mechanism. Annu Rev Biophys 2010; 39:539-57; PMID:20192767; http://dx.doi.org/10.1146/annurev.biophys.050708.133751

55. van den Berghe N, Cool RH, Horn G, Wittinghofer A. Biochemical characterization of C3G: an exchange factor that discriminates between Rap1 and Rap2 and is not inhibited by Rap1A(S17N). Oncogene 1997; 15:845-50; PMID:9266971; http://dx.doi.org/10.1038/sj.onc.1201407
56. Jian X, Brown P, Schuck P, Gruschus JM, Balbo A, Hinshaw JE, et al. Autoinhibition of Arf GTPase-activating protein activity by the BAR domain in ASAP1. J Biol Chem 2009; 284:1652-63; PMID:19017632; http://dx.doi.org/10.1074/jbc.M804218200
57. Luo R, Akpan IO, Hayashi R, Sramko M, Barr V, Shiba Y, et al. GTP-binding protein-like domain of AGAP1 is protein binding site that allosterically regulates ArfGAP protein catalytic activity. J Biol Chem 2012; 287:17176-85; PMID:22453919; http://dx.doi.org/10.1074/jbc.M111.334458
58. Conklin BR, Farfel Z, Lustig KD, Julius D, Bourne HR. Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha. Nature 1993; 363:274-6; PMID:8387644; http://dx.doi.org/10.1038/363274a0
59. Ueda T, Ugawa S, Yamamura H, Imaizumi Y, Shimada S. Functional interaction between T2R taste receptors and G-protein alpha subunits expressed in taste receptor cells. J Neurosci 2003; 23:7376-80; PMID:12917372
60. Okada M, Goldman D, Linnoila M, Iwata N, Ozaki N, Northup JK. Comparison of G-protein selectivity of human 5-HT2C and 5-HT1A receptors. In: Ali SFNTYT, ed. Current Status of Drug Dependence / Abuse Studies: Cellular and Molecular Mechanisms of Drugs of Abuse and Neurotoxicity, 2004:570-7.
61. Rasmussen SGF, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the β2 adrenergic receptor-Gs protein complex. Nature 2011; 477:549-55; PMID:21772288; http://dx.doi.org/10.1038/nature10361
62. Jian X, Cavenagh M, Gruschus JM, Randazzo PA, Kahn RA. Modifications to the C-terminus of Arf1 alter cell functions and protein interactions. Traffic 2010; 11:732-42; PMID:20214751; http://dx.doi.org/10.1111/j.1600-0854.2010.01054.x