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Enzymology and Regulation of ArfGAPs and ArfGEFs

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1. Introduction

Arf family GTP-binding proteins, a subfamily of the Ras superfamily, are critical regulators of membrane traffic and actin remodeling (Kahn, 2009; Kahn et al., 2006; Gillingham and Munro, 2007; Donaldson and Jackson, 2011). The Arf family contains six Arf proteins in most mammals (five in humans) that are divided into three classes based on primary sequence and phylogenetic considerations (Kahn et al., 2006). The function of the Arf proteins requires switching between GDP and GTP bound forms. The accessory proteins that mediate the transitions between Arf•GDP and Arf•GTP function as enzymes and can be studied using the formalisms of enzymology.

Like other GTP binding proteins, switching between Arf•GDP and Arf•GTP is achieved by a controlled cycle of GTP binding and hydrolysis. The two steps are catalyzed by distinct enzymes. The conversion of Arf•GDP to Arf•GTP is accomplished through nucleotide exchange, with the apo form of Arf as an intermediate. Nucleotide, however, binds tightly to Arf, resulting in very slow intrinsic exchange rates, and the apo form of Arf is unstable. Guanine nucleotide exchange factors (GEFs) are enzymes that accelerate the reaction, by decreasing affinity for nucleotide and stabilizing the apo form of Arf (Casanova, 2007; Gillingham and Munro, 2007; Renault et al., 2003). Arf proteins are unusual among Ras superfamily proteins in having no detectable GTPase activity. Conversion of Arf•GTP to Arf•GDP is catalyzed by GTPase-activating proteins (GAPs;Gillingham and Munro, 2007; Donaldson and Jackson, 2011; Ha et al., 2008b; Kahn et al., 2008; Randazzo and Hirsch, 2004; Spang et al., 2010). The GEFs and GAPs are both large families of proteins with diverse structural features. The control of binding and hydrolysis of GTP by Arf is thought to be achieved by regulation of the ArfGAPs and ArfGEFs. The study of the ArfGAPs and ArfGEFs as allosterically controlled enzymes is providing valuable information about their regulation and insights into the roles in cell physisology.

2. ArfGAP family of proteins

Thirty-one genes encode proteins with Arf GAP domains in humans (Kahn et al., 2008). The proteins are divided into 10 groups (Figure. 1) based on domain structure and phylogenetic analysis (Kahn et al., 2008). Six groups have the ArfGAP catalytic domain at the extreme N-terminus of the protein. In the other four groups, which comprise 20 proteins, the ArfGAP

domain is sandwiched between a PH and Ankyrin repeat domains. Other than having the common ArfGAP catalytic domain, the groups are structurally diverse. Some of the structural differences are thought to contribute to differential regulation of the catalytic activity of the GAPs through lipid or protein binding, which has been tested for several ArfGAPs. The molecular mechanism for catalysis by ArfGAPs remains unclear. The first reported structure of ArfGAP/Arf complex is for the ArfGAP domain of ArfGAP1 in complex with Arf1 bound to GDP, the product of the reaction (Goldberg). This structure argued against the general "Arginine-finger" mechanism for the GAPs that has been described for GAPs for other Ras superfamily members (Scheffzek et al., 1998). Studies of the enzymology of ArfGAP1 and ASAP1 first revealed that the available crystal structure may have to be reinterpreted.
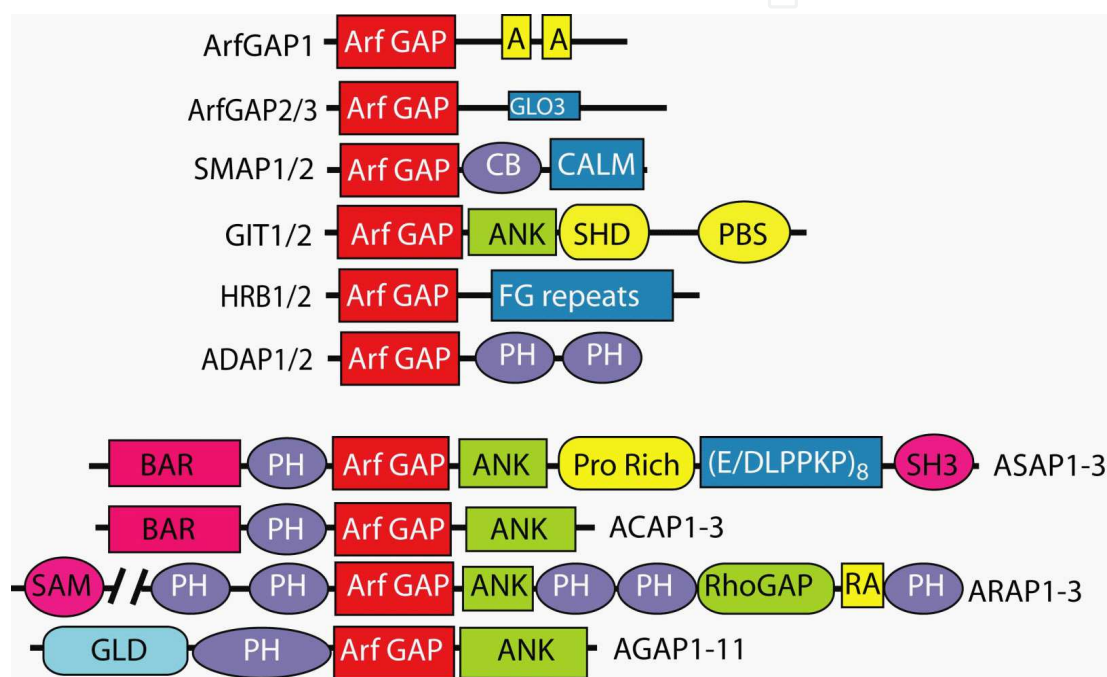

Fig. 1. Schematic of human ArfGAPs. Abbreviations-A, Ankyrin repeat; BAR, Bin/Amphiphysin/Rvs; PBS, paxillin-binding site; PH, pleckstrin homology; SAM, sterile alpha motif; SH3, Src-homology 3; SHD, Spa2 homology; CB, clathrin box; CALM, CALM-binding domain; GLD, GTP-binding protein-like domain; RA, Ras association domain; and GLO3, GLO3 motif.
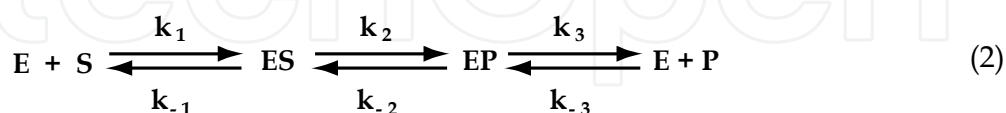
The ArfGAPs contained all necessary elements for efficiently inducing GTP hydrolysis (Luo et al., 2007; Jian et al., 2009). In addition, arginine 497 of ASAP1 and arginine 50 of ArfGAP1 likely serve in the catalytic capacity described for the arginine finger in Ras GAPs. Indeed, recent structural studies of another ArfGAP/Arf complex reveals an arginine that is catalytic (Ismail et al., 2010). The enzymology indicates that our current understanding of the catalytic mechanism is still incomplete. The proteins used for the crystal (Ismail et al., 2010) have $1/10^5$ the optimal activity of the ASAP3. Thus, in addition to providing insights into the cellular functions of ArfGAPs, which will be described in more detail below, the enzymology has provided important information about the molecular basis of catalysis. Here, we discuss general considerations about the enzymology of these proteins and then discuss two specific examples.

## 2.1 General considerations in the kinetic analysis of ArfGAPs

ArfGAPs catalyze what can be considered a single substrate reaction (the second substrate is water), schematized as

$$\text{Arf} \bullet \text{GTP} \xrightarrow{\text{GAP}} \text{Arf} \bullet \text{GDP} + \text{P}_i \tag{1}$$
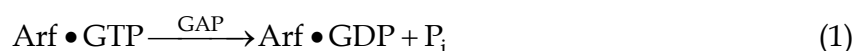
For more complex schemes, we simplify the notation. If we let E =GAP, S=Arf•GTP and P=Arf•GDP, then the scheme is

$$\text{E} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{EP} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \text{E} + \text{P} \tag{2}$$

If we consider that ES and EP rapidly isomerize, then

$$\text{E} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{E} + \text{P} \tag{3}$$
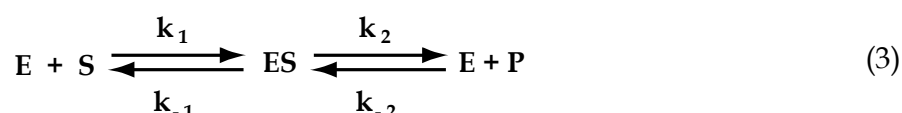
The kinetics can be complex for several reasons. Excluding other factors such as dimerization of the ArfGAP and allosteric modifiers, the first possible additional complexity of the simple E+S$\rightarrow$ ES scheme is that the substrate is restricted to a surface and the reaction occurs on the same membrane surface. The restriction to the surface is important for two reasons related to analyzing GAP activity. First, if the enzyme is also restricted to the surface, the collision rate of enzyme and substrate will be determined by the surface concentration, i.e. mass/area, rather than mass/volume. Second, the quality of the surface is important. In the few cases examined, the quality of the surface is a more important consideration than the total surface area (Jian et al., 2009). With Arf GAPs that reversibly associate with surfaces, surface dilution does not seem to affect reaction rate so long as surface area is about 5 fold greater than the surface occupied by the maximum amount of Arf present. The quality of surface, however, is critical with different parameters determined when using mixed micelles of Triton X-100, LUVs containing all saturated acyl groups in the phospholipids or LUVs containing unsaturated lipids. Catalysis and regulation of the ArfGAPs can be analyzed without invoking surface dilution kinetics but comparisons between proteins are only valid when the same quantity and composition of lipid or detergent are used as the hydrophobic surface to support the reaction. Keeping the surface constant, the initial velocity equation is

$$v_i = \frac{V_{max} \cdot \text{Arf} \bullet \text{GTP}}{K_m + \text{Arf} \bullet \text{GTP}}, \tag{4}$$

the Michaelis-Menten equation for initial reaction velocity. Using the symbol S for the substrate Arf•GTP gives the familiar notation used in the equation.

$$v_i = \frac{V_{max} \cdot S}{K_m + S} \tag{5}$$

The effect of an allosteric modifier is schematized

$$(6)$$
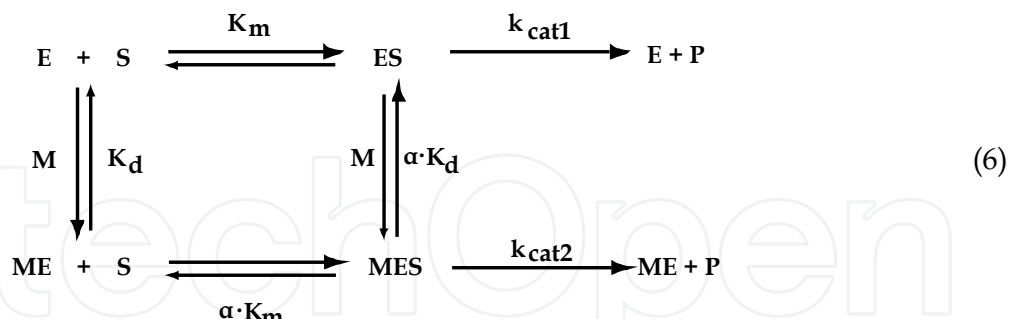
The initial velocity equation is:

$$v_i = \frac{\dfrac{E_t \cdot k_{cat1} \cdot S}{K_m} + \dfrac{E_t \cdot k_{cat2} \cdot M \cdot S}{\alpha \cdot K_m \cdot K_d}}{1 + \dfrac{S}{K_m} + \dfrac{M}{K_d} + \dfrac{S \cdot M}{\alpha \cdot K_m \cdot K_d}} \qquad (7)$$

Where E is the GAP (enzyme), S is Arf•GTP and M is the allosteric modifier. K_m is the concentration of substrate at which the enzyme proceeds with half maximal velocity, k_{cat} is the turnover number, α is the effect of the modifier on substrate binding, E_t is the total GAP in the reaction, and $E_t \cdot k_{cat} = V_{max}$.

As briefly discussed for ASAP1 in this chapter, a second consideration for ArfGAPs is the potential role of dimerization in regulating the reaction. We are not aware of any description, to date, of an ArfGAP that requires the consideration of dimerization to explain the kinetics, but only a few ArfGAPs have been analyzed.

2.2 ASAP1: Examination of putative lipid binding domain leads to model of activation by two signals

ASAP1 is of interest to cell biologists because it has been implicated as one critical factor for cancer cell invasion and metastasis (Ha et al., 2008b; Sabe et al., 2006) with the most compelling evidence coming from studies of uveal melanoma (Ehlers et al., 2005). Consistent with a potential role in cancer, ASAP1 affects cellular adhesions and cell migration (Randazzo et al., 2000; Onodera et al., 2005; Bharti et al., 2007; Ha et al., 2008b; Ha et al., 2008a). Recently, additional interest in ASAP1 comes from the study of ciliogenesis (Ward et al., 2011; Mazelova et al., 2009). ASAP1 is necessary for the delivery of proteins to primary cilia. The molecular bases for the contribution of ASAP1 to the pathologic behavior of cells or the physiological cellular function are not known. The mechanisms by which catalytic activity is regulated are being defined with the hope of furthering the understanding of the role of ASAP1 in cell physiology.

The regulation of ArfGAPs by phosphoinositides was an early finding that led to the purification of ASAP family Arf GAPs (Randazzo and Kahn, 1994). ASAPs contain, from the N-terminus, a BAR, PH, ArfGAP, Ank repeat, Proline rich, E/DLPPKP repeat and SH3 domains, while ASAP1 also has a 30 amino acid extension on the N-terminus of the BAR

domain (Jian et al., 2009). Although many Arf GAPs have PH domains, which can bind to phosphoinositides, the hypothesis that phosphoinositide binding to the PH domain regulates catalysis by the GAP domain has been most extensively examined for ASAP1.

The PH domain is one of two potential lipid binding domains in ASAP1, the other being the BAR domain. The PH domain of ASAP1 has some consensus with PIP_2 binding PH domains and was found to bind to phosphatidylinositol 4,5-bisphosphate (PI4,5P$_2$). Initial analysis revealed that the PH domain was important for catalytic activity. Recombinant protein with the PH domain had 3 – 4 orders of magnitude greater activity than recombinant protein lacking the PH domain (Kam et al., 2000; Che et al., 2005; Luo et al., 2008). Extrapolating from other PH domain proteins, the function of the PH domain was thought to be recruitment of the protein to PIP_2-containing membranes, which also contained the substrate, Arf•GTP; however, in the case of ASAP1, recruitment could be uncoupled from activation (Che et al., 2005). PIP_2 binding was found to cause conformational changes in the PH domain in ASAP1 and in more than 100-fold stimulation of catalytic activity (Che et al., 2005). Changing residues in the PH domain that reduced PIP_2 binding resulted in a change in both K_m and k_{cat} for the reaction consistent with the concept that PIP_2 binding induces conformational changes in the protein leading to increased activity. A simple recruitment mechanism would lead to an isolated change in K_m for the enzymatic reaction. In addition, although ASAP1 is recruited to membrane ruffles, the recruitment is independent of the PH domain. PIP_2 binding to the PH domain of ASAP1 may be necessary for enzymatic activity, but it may not be sufficient to regulate the protein. There must be a signal to at least recruit ASAP1 to the site of action. Studies examining the BAR domain reveal regulation may be more complex.

BAR domains are bundles of 3 α helices that homodimerize to form banana shaped structures (Zhu et al., 2007). The function of some BAR domains is related to binding membranes where they are thought to either induce or sense curvature (Habermann, 2004; McMahon and Gallop, 2005; McMahon and Gallop, 2005) (Figure 2). This hypothesis was tested for ASAP1. The BAR domain of ASAP1 dimerizes with a dissociation constant of less than 10 nM (Nie et al., 2006). The isolated BAR was not found to be stable, but the isolated BAR-PH tandem was stable and could induce membrane curvature (Nie et al., 2006). Any
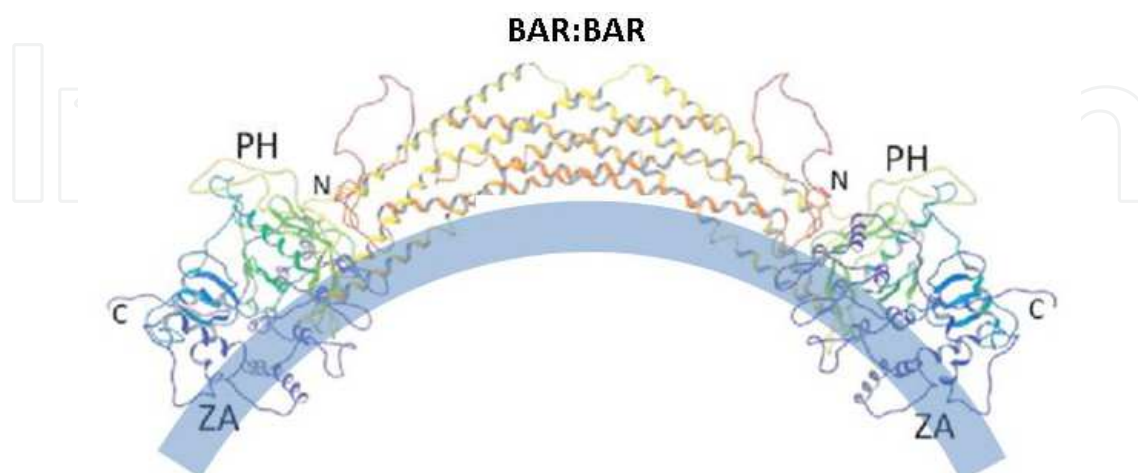

Fig. 2. BAR domain sense or induce the curvature of the membranes. A homology model of the BAR-PH-ArfGAP-Ankyrin repeat domains of ASAP1. The protein forms homodimer through the BAR domains. Light blue curve represents the membrane.

additional domains, e.g. a construct comprised of the BAR, PH, ArfGAP and Ank repeat domains did not induce membrane curvature nor sense membrane curvature. Comparisons of recombinant proteins derived from ASAP1 containing or lacking the BAR domain revealed that the extension from the BAR domain inhibited GAP activity, presumably acting in trans within the homodimer of ASAP1 (Jian et al., 2009) (Figure. 3). Neither lipid composition nor curvature of vesicles affected the autoinhibition. This leads to the still untested hypothesis that proteins that bind to the BAR domain of ASAP1 may stimulate activity by relieving the autoinhibition. The current model for regulation of ASAP1 is that simultaneous binding of protein to the BAR domain and to the PH domain leads to activation.

The contribution of the domains C-terminal of the ankyrin repeats in the regulation of GAP activity has not been extensively examined. Plausible models include the SH domain interacting with PXXP motifs in the N-terminus of the molecule or molecules that bind to the PXXP motifs c-terminal of the ank repeat domains interacting with one of the N-terminal domain. There is support for the idea that in mouse ASAP1, src binding to the PXXP motif can phosphorylate residues near the PH domain resulting is reduced GAP activity (Kruljac-Letunic et al., 2003).

The ACAP subfamily is similar to the ASAPs having a structure comprised of BAR, PH, ArfGAP and Ank repeat domains. Similar to ASAPs, the ACAPs are regulated by phosphoinositides, which was expected for the PH domain. Different than ASAPs (Figure. 3), the ACAPs do not contain the N-terminal extension of the BAR domain that has an autoinhibitory function in ASAPs. The ACAPs, therefore, are likely to be regulated by distinct mechanisms from those used by the ASAPs. Inhibition by proteins that associate with the BAR-PH domain is one possibility.
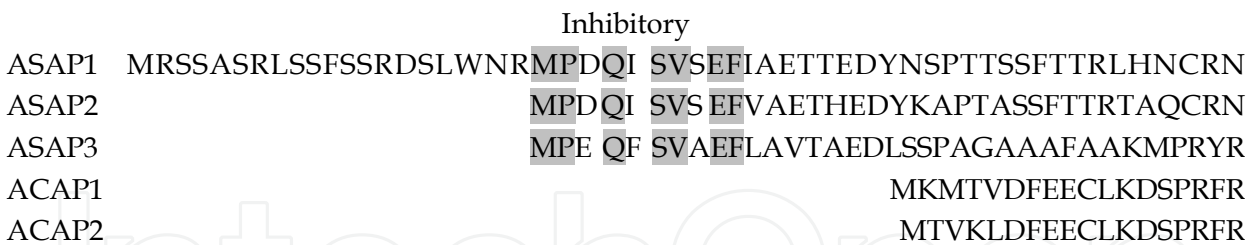
```
                                          Inhibitory
ASAP1    MRSSASRLSSFSSRDSLWNRMPDQI SVSEFIAETTEDYNSPTTSSFTTRLHNCRN
ASAP2                       MPDQI SVS EFVAETHEDYKAPTASSFTTRTAQCRN
ASAP3                       MPE QF SVAEFLAVTAEDLSSPAGAAAFAAKMPRYR
ACAP1                                       MKMTVDFEECLKDSPRFR
ACAP2                                       MTVKLDFEECLKDSPRFR
```

Fig. 3. Alignment of the N-termini of ASAPs and ACAPs. The autoinhibitory fragment identified in ASAP1 is indicated as "Inhibitory." Identities among ASAPs are indicated by gray shading. Loci numbers: ASAP1, NP_060952; ASAP2, NP_003878; ASAP3, NP_060177; ACAP1, NP_055531; ACAP2, NP_036419.

Other examples of ArfGAPs regulated by phosphoinositides include ARAPs, ACAPs and AGAPs. ARAPs contain, from the N-terminus, SAM, two PH, ArfGAP, two PH, RhoGAP RA, and PH domains (total of 5 PH domains). PH domains 1 and 3 (see schematic in Figure 1) have consensus for PIP3 binding PH domains and PIP3-binding to PH domain 1 stimulates GAP activity (Campa et al., 2009). Like the PH domain for ASAP1, the PH domain does not mediate recruitment to the membrane surface containing Arf•GTP. AGAPs are also stimulated by phosphoinositides but specificity among the phosphoinositides is not apparent.

2.3 AGAP1: Allosteric regulation through a GTP-binding protein like domain

The AGAP proteins are comprised of a GTP-binding protein-like domain (GLD), split PH, ArfGAP and ankyrin repeat domains (Nie et al., 2002). Two AGAPs bind clathrin adaptor proteins and have effects on endocytic membrane traffic (Nie et al., 2003; Nie et al., 2005). One of these, AGAP2, has also been implicated in the progression of glioblastoma (Ye and Snyder, 2004). Defining the regulation of GAP activity is of significance both to understanding membrane traffic and cancer.

Initial examination of regulation of the AGAPs focused on phosphoinositides and later, with the discovery that clathrin adaptor proteins bind to the PH domain, on the adaptors (Nie et al., 2005; Nie et al., 2002). GLD was at first discounted as a regulator of the ArfGAP catalytic activity because deletion of the domain did not affect activity. Recombinant AGAPs, with or without the GLD have less than 1 % of the activity of ASAP1 and no apparent substrate specificity, indicating that there was likely a means of increasing AGAP catalytic activity, as explained below. Two-hybrid screening revealed that the GLD is a protein binding site. When a complex is formed, the GAP activity is increased for Arf1 and decreased for Arf6. The mechanistic basis still needs to be determined (Luo et al., submitted).

The additional value of the work on AGAP1 is that it illustrated two concepts important to studying enzymes that regulate proteins. First, turnover number, or at least catalytic power, is relevant. Low activity could indicate a poorly folded protein, in which case the data obtained may not be physiologically relevant. On the other hand, if the protein is properly folded, as was the case for AGAP1, low activity may indicate that positive regulatory mechanisms remain to be discovered. Second, studying an inactive enzyme could be misleading. Although enough activity may be present to make measurements, the enzyme may not optimally recognize the physiological substrate. Other proteins with similarity to the physiological substrate may be fortuitously used. These properties of the GAPs need to be considered when expressing the proteins in cells, since activators may be titrated away, and the bulk of the GAP may be relatively inactive.

2.4 ArfGAP1, 2 and 3: Control by two interacting proteins

ArfGAP1 was the first identified ArfGAP and the first GAP found to regulate membrane traffic, although its precise role remains unknown (Hsu, 2011; Hsu et al., 2009;Kahn, 2009; East and Kahn, 2011; Kahn, 2011;Beck et al., 2011; Weimer et al., 2008; Beck et al., 2009b; Shiba et al., 2011; Spang et al., 2010). The protein is approximately 50 kDa with the Arf GAP domain at the N-terminus and a unique C-terminus that contains two ALPS motifs that are described below. ArfGAP2 and 3 have a similar overall structure but in place of the ALPS motifs contain a Glo3 homology domain (Figure 1). ArfGAP1, ArfGAP2 and ArfGAP3 localize to the Golgi where they regulate Golgi-to-ER membrane traffic. Early work reported that PIP_2 could activate ArfGAP1, despite lack of a PH domain, but that result was later found to result from the use of nonmyristoylated Arf as a substrate, which is recruited to membrane surfaces by PIP_2 (Randazzo, 1997). Later, diacyglycerol was found to activate ArfGAP1. The effect was attributed to effects on lipid packing in the vesicles used in the experiments (Antonny et al., 1997). Subsequently, increasing vesicle curvature, which also results in loosened packing of the lipid head groups, was found to increase activity (Bigay et al., 2003). The effect depends on two ArfGAP Lipid Packing Sensor (ALPS) motifs in

ArfGAP1. The model based on this result was embraced as it could explain the timing of GTP hydrolysis on Arf during coated vesicle formation (for review see Nie and Randazzo, 2006). However, the idea of curvature sensitivity of ArfGAP1 has been difficult to reconcile with current models of coatomer and ArfGAP1 function, and regulation by interaction with proteins may be a plausible alternative model for the regulation of this protein. Furthermore, the curvature sensing model has not been tested in vivo and there is little kinetic support of the model. It is not known, for instance, how the change in curvature affects enzymatic parameters K_m and k_{cat}.

A second model for the regulation of ArfGAP1 has been proposed, which also seems to apply to ArfGAP2 and ArfGAP3. ArfGAP1 binds to the vesicle coat protein coatomer and to cargo proteins (Hsu et al., 2009; Lee et al., 2005). Coatomer is a protein that drives formation of transport intermediates that carry material between the Golgi apparatus and the endoplasmic reticulum (ER) and cargo proteins are the material carried in the transport intermediates. ArfGAP2 and ArfGAP3 were subsequently discovered and found to also bind coatomer and cargo (Frigerio et al., 2007; Kliouchnikov et al., 2009; Weimer et al., 2008). As early as 1999, coatomer was found to stimulate GAP activity of ArfGAP1 (Goldberg, 1999; Goldberg, 2000). The following model was formulated to help analyze the kinetics (note that we abbreviate coatomer as C in the schematic, instead of M which is used for allosteric modifier in other sections of this chapter).

$$
\begin{array}{cccc}
 & \xrightarrow{\quad S \quad} & ES & \longrightarrow \quad E \;+\; P \\
E & \rightleftharpoons & & \\
\end{array}
\tag{8}
$$
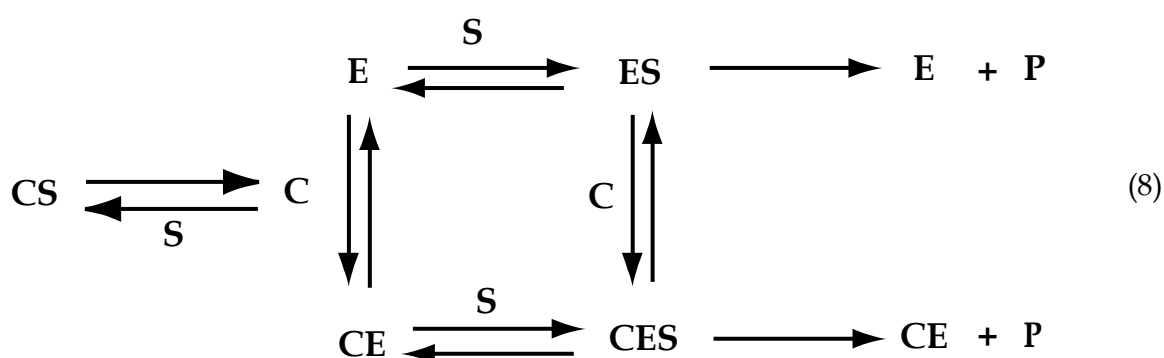
Coatomer is unusual as an allosteric modifier because it could bind the substrate Arf•GTP independently of ArfGAP1. Therefore, the possibility of substrate sequestration by the allosteric modifier had to be taken into account when analyzing kinetics. If coatomer activated ArfGAP1 by direct interaction but was also able to sequester Arf•GTP, titration of coatomer would result in a biphasic curve as was observed, described by the following initial rate equation:

$$
v_i = \frac{K_{cs} \cdot (V_{max1} \cdot K_{m2} \cdot K_c + V_{max2} \cdot K_{m1} \cdot C) \cdot S}{K_{m1} \cdot K_{m2} \cdot (K_{cs} + C) \cdot (K_c + C) + K_{cs} \cdot (K_{m2} \cdot K_c + K_{m1} \cdot C) \cdot S}
\tag{9}
$$

Where K_{m1} is the Michaelis constant for the Arf with GAP in the absence of coatomer, K_{m2} is the Michaelis constant for Arf with GAP in complex with coatomer, V_{max1} is is the limiting rate of the reaction in the absence of coatomer, V_{max2} is the limiting rate for the GAP in complex with coatomer, K_c is the affinity for coatomer in the absence of Arf, K_{cs} is the affinity for coatomer in the presence of Arf, C is coatomer, E is GAP and S is Arf•GTP. Consistent with the prediction of the equation, Luo and colleagues (Luo and Randazzo, 2008; Luo et al., 2009)

found a biphasic coatomer dependence under conditions of limiting substrate. At low substrate concentration, the sequestration effect is dominant at high coatmer concentration, while at the high substrate concentration, the activation effect is dominant.

ArfGAP2 and ArfGAP3 were found to be similar in that GAP activity depended on binding to coatomer. Titration revealed coatomer affected the K_m for both ArfGAP1 and ArfGAP2. ArfGAP2 and ArfGAP3 bind coatomer more tightly than does ArfGAP1. Consequently, the coatomer•ArfGAP2/3 complex could be formed at concentrations of coatomer low enough for substrate sequestration to be ignored. For this reason, ArfGAP2 was used for subsequent studies examining the effect of cargo on GAP activity. In the experiments a peptide from cargo was used as a model of cargo because of the challenges of expressing recombinant transmembrane proteins in bacteria. Cargo was found to act as an allosteric modifier, increasing the k_{cat} of the reaction. The effect depended on the presence of coatomer. ArfGAP1 was also stimulated by cargo in the presence of coatomer. Therefore, rather than curvature, ArfGAPs that function with coatomer are stimulated by the coat-cargo complex. These results have implications important to our understanding of membrane traffic. Previously Arf was thought to function as a bridge between coat proteins and membranes. Arf•GTP, in this model, is required to hold coat on the membrane through the process of trapping cargo and forming a vesicle. GTP hydrolysis would trigger the dissociation of coat necessary after a vesicle is formed. The curvature sensing model fit this paradigm, since the GAP would be most active on the highly curved vesicle and would have little activity on the flat surface on which the vesicle is formed. However, coat-cargo complex is formed prior to making a vesicle, so that activation of the GAP would also occur prior to vesicle formation. The competing models of the role of Arf and ArfGAPs for the formation of coated vesicles are discussed in more detail in a series of papers published from 2009 to 2011 (Shiba et al., 2011; Hsu, 2011; Hsu et al., 2009; Beck et al., 2009b; Beck et al., 2009a). Importantly, the enzymology has been found valuable to gain insights into biological processes.

3. The ArfGEF family of proteins

There are at least 16 proteins with Arf GEF, also called sec7, domains in humans (Casanova, 2007; Donaldson and Jackson, 2011). They are divided into 5 groups: BIG1/2 and GBF; Brag;
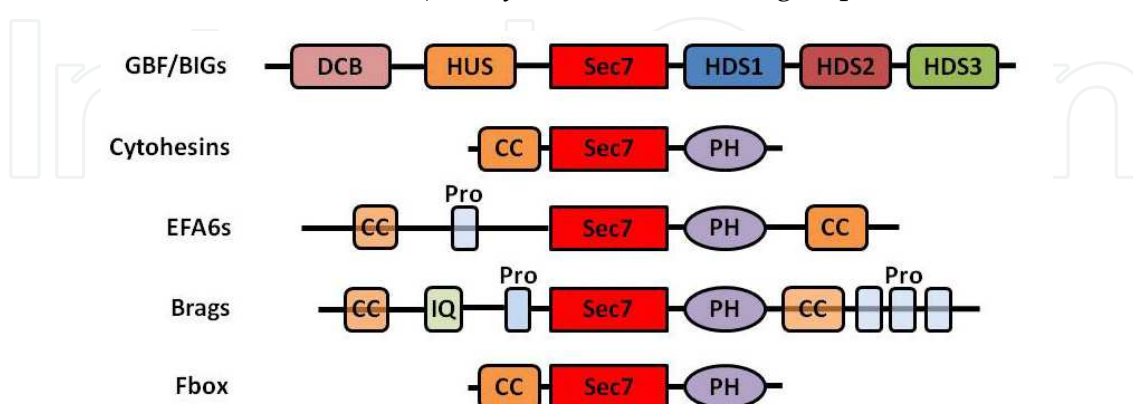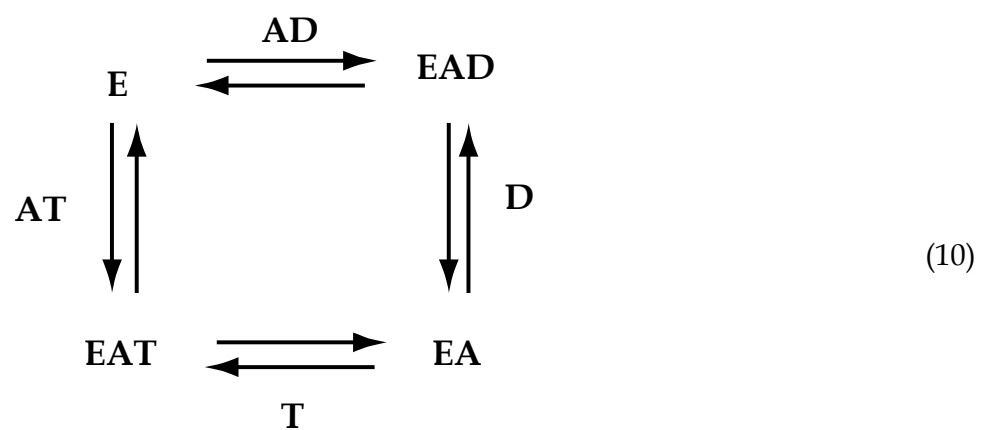

Fig. 4. Schematic of ArfGEFs. Abbreviations-CC, coiled-coil ; DCB, dimerization and cyclophilin-binding domain; HDS, Homology Downstream of Sec7 domain; HUS, Homology Upstream of Sec7 domain; IQ, IQ motif; PH, pleckstrin homology; Pro, Proline-rich. The semi-transparent means not universally present in all subfamily members.

Cytohesins/ARNO, EFA6 and Fbox. Like the ArfGAPs, the GEFs are a family of structurally diverse and complex proteins (Figure 4).
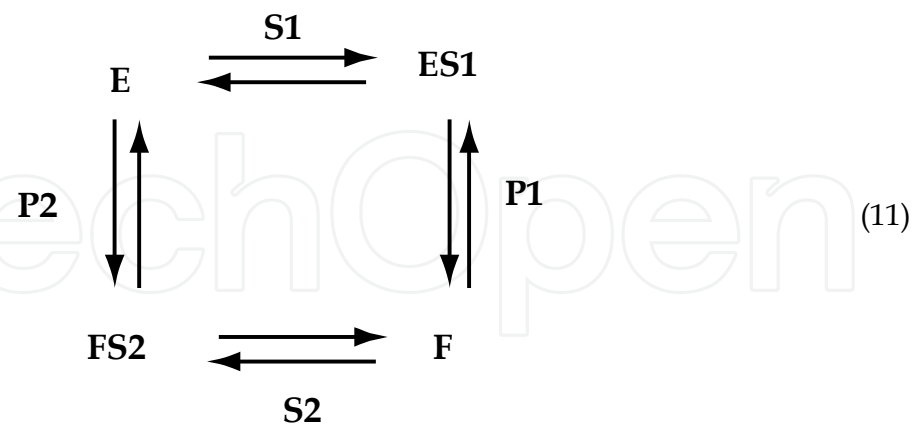
3.1 General considerations in the kinetic analysis of ArfGEFs

The exchange factors catalyze the exchange reaction by a bi bi ping pong mechanism, referring to two substrates (Arf•GDP and GTP), two products (GDP and Arf•GTP) and a reaction that proceeds with binding of the first substrate (Arf•GDP), followed by release of the first product (GDP) and formation of a distinct enzyme intermediate (EA as shown in scheme 10, or F in scheme 11 as it is often presented) prior to binding of the second substrate (GTP) and release of the second product (Arf•GTP). The essential elements of the reaction are schematized as

$$
\begin{array}{ccc}
 & \xrightarrow{\text{AD}} & \\
\text{E} & \rightleftarrows & \text{EAD} \\
\text{AT} \Big\uparrow\Big\downarrow & & \Big\uparrow\Big\downarrow \text{D} \\
\text{EAT} & \underset{\text{T}}{\overset{}{\rightleftarrows}} & \text{EA}
\end{array}
\qquad (10)
$$

E = ArfGEF; AD=Arf1•GDP; AT = Arf1•GTP; T = GTP; D = GDP

The general scheme often shown for bi bi ping pong is

$$
\begin{array}{ccc}
 & \xrightarrow{\text{S1}} & \\
\text{E} & \rightleftarrows & \text{ES1} \\
\text{P2} \Big\uparrow\Big\downarrow & & \Big\uparrow\Big\downarrow \text{P1} \\
\text{FS2} & \underset{\text{S2}}{\overset{}{\rightleftarrows}} & \text{F}
\end{array}
\qquad (11)
$$

In the forward direction, both substrates are soluble but the ES1 and EP2 complexes are membrane restricted as is the second product (Arf•GTP). When studying initial rates, the reaction can be treated as a soluble system, without invoking surface dilution kinetics. Nevertheless, the quality of the surface may affect stability of the enzyme complexes starting with ES1 through FS2 and the relaxation of the transition state of the enzyme to the ground state. A similar situation was found for ASAP1, an ArfGAP (Jian et al.,2009; Luo et al., 2007).

The initial velocity of the forward reaction based on the scheme above, excluding the presence of products, is:

$$v_i = \frac{V_1 \cdot S1 \cdot S2}{K_{S2} \cdot S1 + K_{S1} \cdot S2 + S1 \cdot S2} \tag{12}$$

where S1 is Arf•GDP and S2 is GTP. Holding S1 or S2 constant at saturating concentrations gives the following two equations:

$$v_i = \frac{V_{max,app1} \cdot S2}{K_{m,app1} + S2} \tag{13}$$

$$v_i = \frac{V_{max,app2} \cdot S1}{K_{m,app2} + S1} \tag{14}$$

Note that these equations do not account for allosterism. The kinetic consequences of dimerization have not been examined. However, other allosteric modifiers have been considered and have been treated as described for GAPs, represented in schematic 6 and equation 7.

As for the GAPs, the allosteric modifier could potentially modify the K_m or the k_{cat} for one or both substrates. The full equation for allosteric modification, examining a single substrate (e.g. Arf•GDP), holding the second substrate constant and saturating, is:

$$v_i = \frac{\dfrac{\alpha \cdot K_d}{\alpha \cdot K_d + M} \cdot V_{max1} \cdot Arf1 \bullet GDP + \dfrac{M}{\alpha \cdot K_d + M} \cdot V_{max2} \cdot Arf1 \bullet GDP}{\dfrac{\alpha \cdot K_m \cdot (K_d + M)}{\alpha \cdot K_d + M} + Arf1 \bullet GDP} \tag{15}$$

where M is the modifier.

The potential effect on k_{cat} is given by the equation:

$$k_{cat,obs} = \frac{\alpha \cdot K_d \cdot k_{cat1} + M \cdot k_{cat2}}{\alpha \cdot K_d + M} \tag{16}$$

And the potential effect on the K_m is given by:

$$K_{m,app} = \frac{K_m \cdot \alpha \cdot (K_d + M)}{\alpha \cdot K_d + M} \tag{17}$$

Note, in this latter case, the K_d for the modifier should be affected by the substrate described in following equation,

$$K_{d,app} = \frac{K_d \cdot \alpha \cdot (K_m + S1)}{\alpha \cdot K_m + S1} \tag{18}$$

which provides an additional test of the model for allostery. We described this below for Brag2.

The enzymology of two ArfGEFs has been examined using some of these principles.

3.2 ARNO/cytohesin/Grp1: Example of ArfGEF regulated by relief of autoinhibition

ARNO proteins are comprised of coiled coil, sec7 (catalytic), PH and polybasic (PB) domains. The ARNO group of proteins has roles in diverse cellular processes: regulation of cell adhesion and migration (Goldfinger et al., 2003;Santy and Casanova, 2001;Nagel et al., 1998; Geiger et al., 2000;Hernandez-Deviez et al., 2004); insulin signaling (Fuss et al., 2006;Hafner et al., 2006); and; vesicle transport (Hurtado-Lorenzo et al., 2006; Merkulova et al., 2010; Merkulova et al., 2011;Caumon et al., 2000). The regulation described for ARNO is complex. The effect of protein and lipid binding to the PH domain is discussed here. Protein binding to the coiled-coil domain (Esteban et al., 2006;Goldfinger et al., 2003) and PKC mediated phosphorylation (DiNitto et al., 2007) also contribute to regulating ARNO.

The molecular basis for the effect of protein and lipid binding to the PH domain has been examined in some detail (DiNitto et al., 2007; Cohen et al., 2007). ARNO is autoinhibited by the linker region between the sec7 and PH domains and a C-terminal amphipathic helix, which physically block the Arf binding site. Binding of Arl4•GTP, Arf6•GTP and phosphoinositides to the PH domain has two functions. One is to recruit ARNO to the membrane surface on which it is active and the second to induce a conformational change in the PH domain that relieves autoinhibition. Phosphorylation of ARNO by protein kinase C (PKC) also alleviates autoinhibition (DiNitto et al., 2007; Frank et al., 1998). The characterization was done primarily with a truncated form of Arf, lack an N-terminal extension that is unique to the Arf family of GTP binding proteins. A possible function of N-terminus – Arno interaction in regulation will be interesting to examine.

3.3 Brag2: PIP_2 acts as allosteric modifier binding to the PH domain

The Brag subgroup of GEF proteins has three members characterized by the presence of IQ, sec7, PH and coiled-coil domains (Casanova, 2007). Brag1 and 3 are found primarily in brain. Brag2, although enriched in brain, is ubiquitously expressed. Brag2 affects endocytosis of cell adhesion molecules, including cadherins and integrins, and has been implicated in antiangiogenic signaling in endothelial cells and invasion of breast cancer cells.

Recent work examining Brag2 supports the idea that PIP_2 allosterically modifies activity by binding to the PH domain (Jian and Randazzo, manuscript in preparation). The work was an extension of work examining signaling by semaphorin. Sema3E is an antiangiogenic factor that binds to Plexin D1 resulting in recruitment of PIP kinase and increased Arf6 exchange factor activity mediated by Brag2 (Sakurai et al., 2010; Sakurai et al., 2011). Brag2 was found to bind to PIP_2, which stimulated exchange factor activity in vitro. Subsequent work identified residues within the PH domain that bound to PIP_2. PH domains are thought to be recruitment domains, but the two substrates for Brag2, Arf•GDP and GTP, are soluble, so recruitment to a membrane by itself would not result in increased activity. PIP_2 was found to increase both the K_m and k_{cat} for the exchange reaction, and, consistent with behavior as an allosteric modifier with an effect on K_m, the substrate Arf•GDP affected the

K_d for the ligand PIP_2. Based on these results, PIP_2 must induce some rearrangement of the catalytic pocket. One possibility is that PIP_2 stabilizes the transition state, which is restricted to the membrane. Given that PIP_2 has also been found to stabilize the apo form of Arf (Terui et al., 1994), it is possible that PIP_2 binds to Arf, in addition to the PH domain of Brag2, within the transition complex.

4. Conclusions

The knowledge of kinetic parameters is limited to a few GAPs and GEFs. The information available has provided a number of insights into the biological function of the proteins and potential regulation. For instance, the effect of cotaomer and cargo on ArfGAP1 led to the idea that it may act prior to transport vesicle formation rather than after vesicle formation as has been generally accepted. Activation of ARNO by Arf6 and Arl4 has led to the idea of sequential signaling functions of Arf proteins.

Other aspects of the known enzymology of GAPs and GEFs, such as the discrepant turnover numbers among the GAPs, are intriguing. The slow turnover number could result from a lack of understanding of optimal conditions for a particular ArfGAP, including potential allosteric modifiers that may stimulate activity. Also possible, the different turnover numbers may be related to the biological process being controlled. In addition to examination of additional GAPs and GEFs and further characterization of individual proteins to find optimal conditions for enzymatic activity, identification of GEF/GAP pairs will be important for understanding the function of the Arf proteins in biological processes.

5. Acknowledgment

6. References

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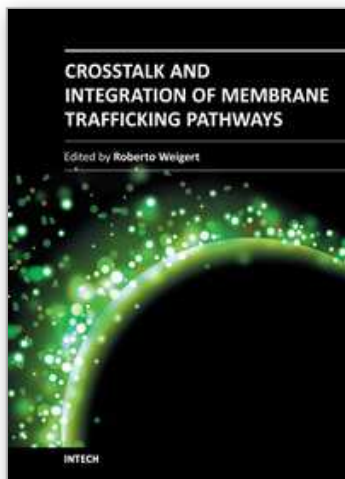
Crosstalk and Integration of Membrane Trafficking Pathways
Edited by Dr. Roberto Weigert

Membrane traffic is a broad field that studies the complex exchange of membranes that occurs inside the cell. Protein, lipids and other molecules traffic among intracellular organelles, and are delivered to, or transported from the cell surface by virtue of membranous carriers generally referred as "transport intermediates". These carriers have different shapes and sizes, and their biogenesis, modality of transport, and delivery to the final destination are regulated by a multitude of very complex molecular machineries. A concept that has clearly emerged in the last decade is that each membrane pathway does not represent a close system, but is fully integrated with all the other trafficking pathways. The aim of this book is to provide a general overview of the extent of this crosstalk.

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