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Snapshots of ARF1: Implications for Mechanisms of Activation and Inactivation

Minireview

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Usually by the time enough pure protein is available to grow crystals and solve a structure, biochemical experiments have produced sufficient data to allow hypotheses to be formulated and models of protein function to be proposed. The atomic structure then becomes the best test of aspects of an existing model. Often, the structure confirms in an elegant way what we thought we already knew. Occasionally, the structure of a protein, or complex of proteins, is incompatible with preconceived ideas and forces an existing model to be modified. In rare cases, the structure is so unexpected that it requires a complete reevaluation of preexisting data. In two recent papers in *Cell*, Jonathan Goldberg has provided us with the structures of the small GTPase, ADP-ribosylation factor 1 (ARF1), in complexes with important regulators: the exchange domain that activates ARF1, and the GTPase-activating protein (GAP) domain that inactivates it (Goldberg, 1998, 1999). The first structure supports previous biochemical studies that raised questions about the ability of the ARF1 exchange protein to control where ARF1 binds to membranes (Franco et al., 1996; Antonny et al., 1997; Paris et al., 1997). The second structure indicates that the ARF1 GTPase activating protein (ARF GAP) functions differently from previously studied GAPs (Scheffzek et al., 1998). Taken together, these new data suggest that the mechanisms by which ARF controls vesicle coat formation are more complicated than previously was imagined.

ARF Proteins

ARFs belong to the superfamily of small GTPases related to Ras (Moss and Vaughan, 1998). There are six ARF proteins identified in mammalian cells, and two in *S. cerevisiae*. The mammalian ARFs fall into three classes by sequence comparison. Class I ARFs (ARF1–ARF3) are the best studied and appear to be functionally redundant. These proteins control the formation of at least three different vesicle coats that act at distinct steps in intracellular membrane transport. Vesicle coats are thought to collect cargo proteins and to impart the curvature to lipid bilayers necessary for pinching off membrane vesicles (see Springer et al., this issue of *Cell*, for a review of vesicle formation). The function of the class II ARFs, ARF4 and ARF5, is not known. ARF6 (class III) is found at the plasma membrane and regulates in some way either membrane traffic or cytoskeleton organization, or perhaps both. The two yeast ARFs are functionally redundant and are required for maintenance of the organization of secretory and endocytic pathways. ARF activity is necessary for vegetative growth of *S. cerevisiae* and each of the six mammalian ARFs can complement the deletion of the two yeast genes.

ARF1 was discovered to be a component of the coat found on vesicles produced from isolated Golgi membranes and was subsequently found to be necessary to form those vesicles, called COPI vesicles, in vitro (see Rothman and Wieland, 1996 and references therein). ARF1 was also later found to be required to form clathrin-coated vesicles containing AP1 or AP3 adaptor complexes at the *trans*-Golgi network (TGN) and on endosomes (Stamnes and Rothman, 1993; Ooi et al., 1998). The role for ARF1 for regulating vesicle traffic in vivo was confirmed by expressing in intact cells mutants of ARF1 that were defective in either GTP binding or GTP hydrolysis. The mutant defective in binding GTP prevented coat proteins from binding to the Golgi, resulting in the migration of Golgi membranes back to the endoplasmic reticulum (ER), a phenotype similar to that observed in cells treated with brefeldin A (see review by Chardin and McCormick, this issue of *Cell*). The mutant defective in GTP hydrolysis caused COPI proteins to bind irreversibly to membranes and to prevent subsequent delivery of vesicles into the Golgi (reviewed by Rothman and Wieland, 1996; Schekman and Orci, 1996). Although there is some evidence for a role for COPI vesicles in anterograde transport between the ER and Golgi, it is clear that COPI vesicles are required for retrograde transport between the Golgi and the ER (reviewed by Springer et al., 1999). For the control of COPI vesicle formation, ARF1 operates as a binary switch, active when bound to GTP and inactive when bound to GDP.

The Prevailing Model of Activation of ARF

When bound to GDP, ARF1 is found in the cytosol. ARF1 binds GDP with high affinity and in order to become active must interact with guanine nucleotide exchange factors (GEFs) that stimulate the exchange of the GDP on ARF for GTP in the cytosol. ARFs are myristoylated at the amino terminus and tight membrane binding by ARF requires that the myristate insert into the lipid bilayer. Since ARF1 is an abundant protein found throughout the cytosol, and the coat proteins regulated by ARF1 must form only at certain membrane locations, it was proposed that the site of ARF1 activity is determined by the location of the GEF responsible for the release of GDP from ARF. Originally it was imagined that the ARF GEF would be a transmembrane protein located on Golgi membranes, similar to the transmembrane protein, Sec12p, that acts at the ER as the GEF for the small GTPase, Sar1p. Sar1p regulates formation of COPII vesicles that are responsible for anterograde transport between the ER and the Golgi (reviewed by Springer et al., 1999). It was imagined that the ARF GEF would recruit ARF-GDP from the cytosol and stimulate the release of the GDP. GTP would bind in its place, inducing a conformational change that would cause the myristoylated amino terminus of ARF to insert into the nearby membrane. In this way, activated ARF would be delivered to the correct membrane.

However, subsequent experimental data have raised several questions about this simple, straightforward

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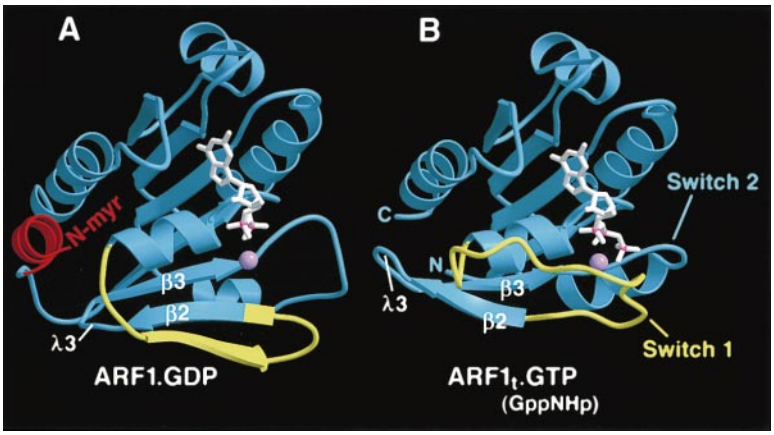

Figure 1. Structure of ARF1 in the Triphosphate Conformation

Please see text for details. The figure was kindly provided by Jonathan Goldberg.

model. Seven proteins capable of stimulating guanine nucleotide exchange on ARF have been identified and all are soluble proteins found in the cytosol. These proteins are divided by sensitivity to the drug brefeldin A into a sensitive class, which contains the yeast proteins Sec7, Gea1, Gea2, and mammalian p200, and an insensitive class, which contains the much smaller proteins ARNO1, GRP-1, and cytohesin-1 (reviewed by Chardin and McCormick, 1999; Moss and Vaughan, 1998). The latter proteins each contain a pleckstrin homology domain and may associate with specific membrane sites in response to the production of phosphoinositides. It is not known if the large GEFs can bind to specific lipids and there is no information as to how they associate with membranes.

The ARF GEFs have in common a small catalytic domain, the Sec7 domain, that is sufficient for their guanine nucleotide exchange activity. When the small ARF GEF, ARNO1, or its isolated Sec7 domain, is reacted with myristoylated ARF-GDP in vitro, it fails to catalyze nucleotide exchange (Paris et al., 1997). Addition of lipid micelles to the two proteins allows the exchange reaction to proceed. These observations suggest that the myristoylated amino terminus of ARF inhibits the exchange reaction on ARF, unless there are lipids nearby.

Structural Insights into the Nucleotide Exchange Reaction on ARF

Two recent structures of ARF provided by Jonathan Goldberg give insight into the mechanism of nucleotide exchange on ARF and suggest that this reaction probably occurs after ARF has bound to membranes. The first structure, that of ARF bound to the nonhydrolyzable analog GppNHp, resembles the structures of other proteins related to Ras, particularly in the arrangement of the switch 1 and switch 2 regions that interact with the effectors of these proteins (Figure 1B). In contrast, the switch 1 and switch 2 regions in the structure of ARF bound to GDP, which had been solved previously by two laboratories (Amor et al., 1994; Greasley et al., 1995), do not resemble other proteins of the Ras superfamily (Figure 1A). In the GDP conformation, switch 1 of ARF rotates down to form strand β1 of a seven-strand β sheet. Loop λ3 between strands β2 and β3 forms the base of a pocket that holds the myristoylated amino terminus (Figure 1A). When bound to GTP, switch 1 rotates up toward the active site and strands β2 and β3 translate 7 Å parallel to the sheet, pushing loop λ3 into the space that was the binding pocket for the amino terminus (Figure 1B). This conformational change could drive the amino terminus from its binding pocket, allowing it to interact with membranes, or might occur only after the amino terminus has left the binding pocket and made space for loop λ3. Clues to distinguishing between these possibilities are provided by the structure of nucleotide-free ARF bound to a Sec7 domain.

For this structure, Goldberg crystallized a complex of the 24 kDa Sec7 domain from the Gea2 protein and an ARF lacking the first 17 amino acids (Goldberg, 1998). The structure presents us with a snapshot of the intermediate between inactive and active ARF. Most importantly, it is a structure relevant to the ARF activation mechanism before ARF has bound GTP. Among the many interesting details of this structure, two stand out in their implications for the mechanism of ARF activation. The first is that the nucleotide-free ARF in the complex is in the ARF-GTP conformation. If this is not an artifact of having deleted the amino terminus and its myristic acid from ARF, then this structure suggests that the conformational change that occurs on ARF that is usually ascribed to the binding of GTP occurs before GTP binds to ARF. In this model, GTP may lock ARF in its active conformation but would not be necessary to stimulate formation of the active conformation. The second observation is, to quote Goldberg, that "the structure of ARF-GDP appears to be incompatible with the contours of the recognition site" of the Sec7 domain. This implies that conformational changes on ARF must precede entry of ARF into the Sec7-binding domain. In the case of nucleotide-free, soluble ARF lacking its first 17 amino acids, these conformational changes may occur spontaneously or through initial contacts with Sec7. However, biochemical data indicate that native, myristoylated ARF will not interact with Sec7 without high concentrations of lipids being present. Either Sec7 has a weak ability to dislodge the myristoylated amino terminus of ARF if there are lipids nearby to capture it, or the lipids themselves begin the process by binding to the amino terminus and facilitating its exit from the binding pocket (see Roth, 1999 for more details). In either case, the difference between these two mechanisms is major when one considers the question of how ARF is activated on the correct membrane in vivo, and needs to be addressed experimentally.

GTPase Activation on ARF

In the prevailing model of ARF activation, once it binds GTP and is recruited to the membrane, ARF becomes a binding site for coat proteins, such as Golgi COPI coat protomer (coatomer) or the AP1 clathrin adaptor. In the most highly purified system in vitro, COPI vesicles will form from pure lipids to which are added coatomer and ARF-GTP-γS (Spang et al., 1998). In the purified system, coatomer will bind to membranes that contain some acidic lipids but will not form vesicles in absence of activated ARF. ARF must therefore be able to bind coatomer and arrange it in some way that curves membranes. Once bound to GTP, the intrinsic rate of GTP hydrolysis on ARF is exceedingly slow. Since either mutants of ARF that fail to hydrolyze GTP or nonhydrolyzable analogs of GTP inhibit secretion when added to cells, it was logical to conclude that a GTPase-activating reaction must occur in vivo to allow coat proteins to disassemble and ARF to recycle back to the ground state. In vitro experiments for producing vesicles indicated that a GAP protein was not necessary before vesicle budding, suggesting that the GAP reaction would be a late event and occur after the vesicle had budded (Ostermann et al., 1993; Spang et al., 1998). However, several simple predictions of this model have not been observed. Although COPI vesicles are commonly isolated after they are made with GTP-γS, published gradient profiles of purified COPI vesicles do not contain a major polypeptide migrating with the apparent molecular weight of ARF GAP (Serafini et al., 1991a, 1991b). This suggests that the interaction between ARF GAP and activated ARF is not stable. In addition, when the first ARF GAP was identified, it was found to bind to Golgi membranes in a manner acutely sensitive to brefeldin A, similar to ARF and COPI. This suggested that the GAP might enter the vesicle on the Golgi before the budding step. Finally, for both COPI coats and clathrin/AP1 coats, vesicles can be made in vitro with ARF-GTP under conditions where the vesicles isolated do not contain detectable ARF (Ktistakis et al., 1996; Zhu et al., 1998; but see Stamnes et al., 1998 for an alternative explanation). One interpretation of these results is that the GAP reaction on ARF might occur early, before vesicles had budded (see also Springer et al., 1999).

Structure of the Complex of ARF-GDP and a GAP Domain

The minimum domain of rat p47 ARF GAP containing GAP activity for ARF is approximately 130 N-terminal amino acids that contain the zinc finger motif that is a signature for ARF GAP proteins (Cukierman et al., 1995). In a recent article in *Cell*, Goldberg (1999) has provided us with the crystal structure of ARF-GDP bound to the 130–amino acid GAP catalytic domain. This structure represents the product complex of the GTPase reaction and so only inferences can be drawn about the active site alignment in the transition state where hydrolysis would occur. The structure is remarkable and unlike the complexes between other GAP proteins and G proteins large or small. For other Ras-related proteins, the GAP-binding domain overlaps the effector-binding site, precluding simultaneous interactions between the GTP-binding protein, effector, and GAP. In those proteins, the hydrolysis reaction is accelerated by residues from the GAP protein, usually including an arginine, that extend into the nucleotide-binding pocket of the G protein (Tesmer et al., 1997; Scheffzek et al., 1998). In contrast, the GAP-binding site on ARF does not overlap with its effector-binding site, nor does ARF GAP supply any residues to the active site that could be involved in hydrolysis (Figure 2). The major direct contribution of ARF GAP to the hydrolysis reaction appears to be through stabilizing the switch 2 region of ARF to align glutamine 71 to participate in the hydrolysis reaction. With all of these differences from the standard mechanism by which GAP proteins act on small G proteins, how then does ARF GAP accelerate GTP hydrolysis?

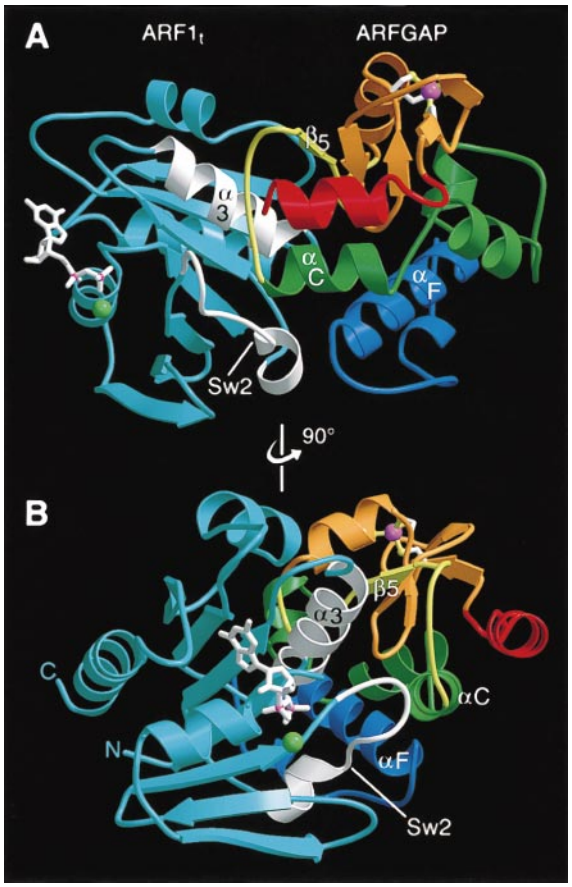

Figure 2. Structure of the ARF1–ARF GAP Complex

(A) Ribbon diagram of human ARF1 bound to the catalytic domain of rat ARF-GAP.

(B) The complex is rotated 90° about the vertical axis.

(Reprinted from Goldberg, 1999.)

When coatomer is added to ARF-GTP and the GAP, the GTPase reaction in vitro is accelerated 1000-fold (Goldberg, 1999). One simple mechanism for this rate enhancement is for coatomer to provide the residues that enter the active site and accelerate hydrolysis. It is possible that it is the complex of coatomer and ARF that is recognized by the GAP, leading to an orderly sequence of events compatible with prevailing ideas of the role of ARF GAP. However, if the interaction of GAP with ARF leads to a slow rate of hydrolysis, there is no reason a priori to propose that the GAP interaction with

ARF must follow the coatomer interaction. Another possibility is that the GAP interacts with ARF earlier than coatomer, and that the complex of ARF and GAP provides the binding site for the coat proteins (see also Zhu et al., 1998 and Springer et al., 1999). Once the coat has formed and become stable (through interlocking subunits perhaps) ARF might hydrolyze GTP and both ARF and GAP might leave the coat, possibly to recycle for more rounds of coatomer addition.

Rather than merely confirming what we thought that we knew, the snapshots of ARF provided to us by Goldberg challenge our ideas of how ARF works. Clearly structures of additional complexes in the pathway of ARF action will provide major insight into its mechanism. One of these will certainly be the trimeric complex of ARF, ARF GAP, and coatomer subunits. Others will require that the major technical hurdle of the insolubility of myristoylated full-length ARF is solved, so that, among other things, we might learn how nucleotide hydrolysis on ARF can be coupled to extraction of the amino terminus of ARF from the membrane. The uniqueness of the ARF exchange and GAP reactions in relation to those that have been studied with other G proteins reflects the fact that the membrane is also a player in the cycle of ARF activation and inactivation (Goldberg, 1998). Why this is the case, and why ARF is myristoylated whereas Sar1p, a small G protein with a similar function, is not, remain major questions for the future.

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