Characterization of a Fast Cycling ADP-ribosylation Factor 6 Mutant*

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Lorraine C. Santy‡
From the Department of Cell Biology,
Health Sciences Center, University of Virginia,
Charlottesville, Virginia 22908

Studies of GTPase function often employ expression of dominant negative or constitutively active mutants. Dominant negative mutants cannot bind GTP and thus cannot be activated. Constitutively active mutants cannot hydrolyze GTP and therefore accumulate a large pool of GTP-bound GTPase. These mutations block the normal cycle of GTP binding, hydrolysis, and release. Therefore, although the GTPase-deficient mutants are in the active conformation, they do not fully imitate all the actions of the GTPase. This is particularly true for the ADP-ribosylation factors (ARFs), GTPases that regulate vesicular trafficking events. In Ras and Rho GTPases replacement of phenylalanine 28 with a leucine residue produces a “fast cycling” mutant that can undergo spontaneous GTP-GDP exchange and retains the ability to hydrolyze GTP. Unfortunately this phenylalanine residue is not conserved in the ARF family of GTPases. Here we report the design and characterization of a novel activated mutant of ARF6, ARF6 T157A. In vitro studies show that ARF6 T157A can spontaneously bind and release GTP more quickly than the wild-type protein suggesting that it is a fast cycling mutant. This mutant has enhanced activity in vivo and induces cortical actin rearrangements in HeLa cells and enhanced motility in Madin-Darby canine kidney cells.

Members of the Ras superfamily of small GTPases function as switches to regulate a wide variety of processes within cells. When bound to GTP these proteins bind to and activate a variety of downstream effector proteins. Hydrolysis of the bound GTP to GDP returns the GTPase to an inactive state. The interconversion of these two states depends upon the action of several accessory proteins. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of bound GDP for GTP, thereby activating the G-protein, whereas GTPase-activating proteins (GAPs) stimulate the hydrolysis of the bound GTP.

The ADP-ribosylation factors (ARFs), a family of small GTPases, have been well characterized as regulators of vesicular trafficking. This family consists of six isoforms that can be divided into three classes. Class 1 ARFs (ARFs 1–3) regulate trafficking in the secretory pathway and in endosomes, whereas little is known about the functions of the class II ARFs (ARFs 4 and 5). ARF6, the sole class III ARF, is located at the plasma membrane and regulates some aspects of the endosomal and recycling pathways (1). Additionally, the ARFs, particularly ARF6, have been shown to modulate cortical actin assembly (2–6).

GTPase functions are most often studied using expression of dominant negative or constitutively active mutants. Dominant negative mutants cannot bind GTP and therefore cannot be activated. Constitutively active mutants, on the other hand, cannot hydrolyze the bound GTP leading the cell to accumulate a large pool of activated GTPase. Both of these mutations block the normal cycle of GTP binding followed by hydrolysis. Therefore, constitutively active mutants cannot necessarily recapitulate all of the actions of the normal GTPase.

This is particularly true of ARF-regulated trafficking events, such as budding of COPI-coated vesicles from the Golgi. Assembly of the COPI coat and vesicle budding requires activation of ARF1 (7, 8), whereas uncoating requires GTP hydrolysis (9). Therefore, expression of constitutively active ARF1 Q71L induces the accumulation of COPI-coated vesicles that cannot fuse with target membranes (10, 11). Similarly, ARF6 Q67L induces the accumulation of endosomally derived vacuolar clusters and blocks the endocytosis and recycling of certain proteins (12–14). Additionally, ARF6 Q67L alters cell morphology and has toxic effects with extended expression (13, 15, 16).

For the Rho family of GTPase these types of problems can be avoided with the use of fast cycling mutants (17, 18). These mutants have reduced affinity for nucleotides and spontaneously release GDP and bind GTP, thereby increasing levels of active GTPase within the cell (17, 19). Importantly this pool of active GTPase can still hydrolyze GTP and go through the entire normal GTPase cycle (17, 20).

Although most members of the Ras superfamily of small GTPases share a similar structure and nucleotide-binding pocket, ARFs have some divergent characteristics. For example, most Ras family members are C-terminally lipid-modified, whereas the ARFs are myristoylated at their N terminus. ARFs also contain alterations in the nucleotide-binding pocket with respect to other Ras family proteins (21, 22). For example, the canonical activating mutation in Ras is an alteration of glycine 12 to valine; however, this glycine residue is not conserved in the ARFs. Mutation of the ARF residue located in the equivalent position (Asp-28) produces an inactive protein rather than an activated mutant (23).

The known Ras and Rho fast cycling mutants are mutations of a phenylalanine in the nucleotide-binding site to leucine (Phe-28 in Ras, Rac1, Cdc42 and Phe-30 in RhoA) (18, 20). Phenylalanine 28 is widely conserved among members of the Ras superfamily of small GTPases, it is not conserved in the ARF family. X-ray crystal structure analysis of Ras determined that phenylalanine 28 sits at the base of the nucleotide-binding pocket, forming van der Waals contacts...
with the surface of the guanine ring (24). The crystal structure of ARF1 demonstrated that this portion of the ARF nucleotide-binding pocket was not formed by a phenylalanine near the N terminus of the protein but rather consisted of a threonine residue located at the ARF C terminus (21, 22). This threonine (Thr-161 in ARF1, Thr-157 in ARF6) sits in a position analogous to Ras Phe-28 (21, 22). Therefore ARF6 with a threonine 157 to alanine substitution was investigated as a possible fast cycling mutant.

The experiments presented here demonstrate that ARF6 T157A does indeed have the properties of a fast cycling GTPase. It is more active than the wild-type protein in vitro while still able to undergo normal GAP-mediated hydrolysis and has an enhanced rate of GTP binding and release. Expression of this mutant induces phenotypes that have previously been attributed to ARF6 activation without the toxic effects of the constitutively active Q67L mutant. Therefore, we conclude that ARF6 T157A is a fast cycling mutant and should prove useful in future studies to elucidate specific ARF6 function.

MATERIALS AND METHODS

Mutagenesis and Protein Expression—The ARF6 T157A mutation was generated by site-directed mutagenesis of HA-tagged ARF6 as described in the QuikChange kit (Stratagene, La Jolla, CA). Recombinant adenovirus encoding this mutant under the control of a tetracycline-repressible promoter was produced as previously described (25). HeLa or MDCK cells expressing this tetracycline-regulated transactivator were used for protein expression. Expression of proteins was carried out either by infection with recombinant adenovirus as previously described (26) or by transient transfection with the Effectene reagent (Qiagen, Valencia, CA) for 18 h.

ARF Activation Assay—Activation of ARF6 was assayed using a previously described pulldown assay (26). ARF-GTP was isolated by binding to glutathione S-transferase-GGA3 and quantitated by Western blotting with a monoclonal anti-HA antibody (antibody 16B12, Covance, Berkeley, CA).

GTP Loading and Release Assays—Myristoylated ARF6 (WT and T157A) was produced by co-expression with yeast N-myristoyltransferase in Escherichia coli and purified as previously described (27). For in vitro loading, recombinant ARF6 (2.5 μg, 0.125 mg/ml) was incubated in 25 mM Hepes pH 7.4, 100 mM NaCl, 0.5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 100 μM [35S]GTPγS, 0.7 mg/ml liposomes (5:2 phosphatidylcholine:phosphatidic acid) at 30 °C (28). At various times of incubation the GTPase and bound nucleotide were isolated by filtration through nitrocellulose filters and quantitated by liquid scintillation counting.

For measurement of the rate of GTP release, ARF6 was first loaded with [35S]GTPγS for 1 h as described above. Then it was diluted 100-fold into 25 mM Hepes pH 7.4, 100 mM NaCl, 0.5 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol, 1 mM GTP, and 0.2 mg/ml liposomes (5:2 phosphatidylcholine:phosphatidic acid). During the incubation bound nucleotide was measured by filter binding.

Transwell Migration Assay—Motility of T23 cells was tested using a transwell migration assay as previously described (26). Briefly, cells were infected with adenoviruses encoding the various ARF6 mutants for 6 h and then plated on transwell filters that had been coated on the lower surface with 5 μg/cm² fibronectin. After an 18-h incubation, cells on the upper surface of the filter were removed and those remaining on the lower side were quantitated by staining with crystal violet.

RESULTS

The fast cycling Ras mutation, F28L, reduces the size of the side chain at this position, which removes contacts between this residue and the guanine ring and weakens the affinity of the protein for guanine nucleotides (19). This reduced affinity allows the GTPase to spontaneously release GDP and bind GTP, even in the absence of a GEF. The functionally equivalent residue in ARF6 is Thr-157. In an attempt to generate a fast cycling ARF6 mutant, Thr-157 was mutated to alanine, which should eliminate the van der Waals interactions between this side chain and the guanine ring.

If ARF6 T157A truly is a fast cycling mutant, it should have enhanced rates of GTP binding and release when compared with the wild-type protein. The loading of ARF6 T157A with GTP was investigated in vitro to determine whether this mutation enhances the spontaneous rate of GTP binding. Recombinant, myristoylated wild-type and T157A ARF6 were purified from E. coli as previously described (27). These proteins were incubated with [35S]GTPγS, and at various times after the initiation of this loading reaction, samples were removed, and the protein was isolated on nitrocellulose filters. The bound nucleotide was then quantitated by liquid scintillation counting. As is shown in Fig. 1A, ARF6 T157A binds GTPγS at a significantly enhanced rate compared with wild-type ARF6 (2.4–6.9-fold, n = 3 experiments). The release of nucleotide by these proteins was assayed by continuing this loading reaction for an extended period and then diluting the loaded GTPase into a solution containing an excess of unlabeled GTP. After various times of incubation samples were removed, and the amount of [35S]GTPγS remaining bound to the protein was determined as described above. The ARF6 T157A mutant also increased the rate of GTP release compared with the wild-type protein (Fig. 1B) (rate increase, 1.9–3.0-fold; n = 4 experiments). These results suggest that ARF6 T157A could act as a fast cycling mutant.

To determine whether ARF6 T167A acts as a fast cycling mutant in vivo the activation state of this mutant was compared with that of the wild-type ARF6, dominant negative ARF6 T27N, and constitutively active ARF6 Q67L using a previously described ARF pulldown assay (26). GTP-bound ARF was isolated by specific binding to the ARF effector GGA3. Fig. 2A shows that ARF6 T157A is significantly more active than WT ARF6 (1.6–18-fold more active, n = 8, p < 0.05 (paired t test)). As is expected for a fast cycling mutant ARF6 T157A is less active than ARF6 Q67L, which cannot hydrolyze
FIG. 2. ARF6 T157A is more active than wild-type ARF6 and is still subject to normal regulation. A, MDCK cells were infected with adenovirus encoding HA-tagged WT, dominant negative (T27N), constitutively active (Q67L), or T157A ARF6 for 4 h. Cells were lysed and GTP-ARF6 isolated by incubation with glutathione S-transferase-GGA3 as previously described (26). ARF6 levels were quantitated by Western blotting with monoclonal anti-HA antibody. B, MDCK cells were transfected with plasmids encoding ARF6 T157A either alone or in combination with the GEF ARNO or the GAP ACAP1. ARF6-GTP was isolated and quantitated as described above. Gels are representative of at least 4 separate experiments.

GTP. Moreover, this assay demonstrates that ARF6 T157A retains the capacity to bind a known ARF effector, suggesting that the overall structure of this protein is not grossly altered by this mutation.

A true fast cycling mutant remains subject to the normal mechanisms of regulation; therefore, it can be activated by GEFs and inactivated by GAPs. The ARF6 T157A mutant was co-expressed either with the ARF GEF ARNO (29) or the ARF GAP ACAP1 (30), and the ARF pulldown assay was then used to measure GTP-ARF6 T157A levels. Fig. 2B shows that co-expression with ARNO leads to enhanced activation of ARF6 T157A (2.1–6.0-fold increase, n = 6, p < 0.05 (paired t test)), whereas co-expression with ACAP1 reduces levels of GTP-bound ARF6 T157A (1.8–4.9-fold decrease, n = 4, p < 0.01 (paired t test)). Importantly this experiment demonstrates that ARF6 T157A can still interact with both GAPs and GEFs and clearly cycles. These properties are characteristic of a fast cycling mutant.

A fast cycling GTPase mutant should reproduce the phenotype of activating the wild-type protein. In HeLa cells activation of ARF6 either by expression of a GEF or by the addition of aluminum fluoride induces the formation of actin-rich surface protrusions or ruffles (3, 31, 32). HeLa cells expressing either wild-type ARF6, ARF6 T157A, or ARF6 Q67L were stained for the exogenous ARF6 protein and for polymerized actin (Fig. 3). Cells expressing wild-type ARF6 have smooth edges and few surface ruffles. In contrast cells expressing ARF6 T157A exhibit numerous actin-rich ruffles, suggesting that this protein is indeed behaving similarly to activation of the wild-type protein. Cells expressing the constitutively active ARF6 Q67L, on the other hand, are largely rounded up. At later times these cells actually detach from the substrate. This phenotype, which is commonly seen in cells expressing ARF6 Q67L, does not resemble that produced by activation of the wild-type protein. Therefore, the full GTPase cycle including both GTP loading and hydrolysis is necessary to reproduce the functions of ARF6. Furthermore this rounded phenotype demonstrates one of the persistent problems of using this constitutively active mutant to study ARF6 function; it often has toxic effects when expressed for extended periods of time. The inability of this protein to hydrolyze GTP likely prevents the completion of the membrane trafficking and down-regulation of signal transduction pathways that are regulated by ARF6. For this reason ARF6 T157A should prove to be a more accurate and useful tool in the study of ARF6 functions.

We have previously shown that activation of ARF6 by the exchange factor ARNO enhances the migratory potential of MDCK cells (26). Expression of ARF6 T157A also increases the migration of MDCK cells by 3-fold in a transwell migration assay, whereas expression of wild-type ARF6 does not alter cell motility (Fig. 4). Expression of ARF6 Q67L similarly increases the percentage of cells migrating through the filter in the transwell assay; however, the number of cells surviving to the end of the experiment is substantially reduced by expression of this mutant (data not shown).

DISCUSSION

The experiments presented here demonstrate that ARF6 T157A is a fast cycling ARF6 mutant. ARF6 T157A is spontaneously more active than the wild-type protein; it binds and releases GTP more quickly and induces phenotypes characteristic of ARF6 activation. Previously the only known activating ARF6 mutation was the constitutively active Q67L, and T157A offers significant advantages over this mutant for studies of ARF6 function.

The constitutively active mutants ARF6 Q67L and ARF1 Q71L both block the vesicular transport processes regulated by these GTPases (10–14). These observations demonstrate that inactivation of a GTPase, as well as activation, can be a critical part of GTPase function. T157A can undergo the complete cycle of GTP binding, hydrolysis, and release and, therefore, maintains all of the GTPase actions.

Interestingly, although Ras and ARF have significant differences in the structure of their nucleotide-binding pockets, the known Ras P28L fast cycling mutant was able to guide the design of a fast cycling ARF6 mutant. Both Ras Phe-28 and...
**Fig. 4. Expression of ARF6 T157A enhances MDCK cell migration.** MDCK cells were infected with adenoviruses encoding wild type or T157A ARF6 under the control of the tetracycline-regulated trans-activator for 6 h. Control cells were infected with wild-type ARF6 virus in the presence of doxycycline to prevent transgene expression. Cells were then replated on transwell filters coated on the underside with fibronectin and incubated for 18 h. Cells migrating through the filter were then quantitated by crystal violet staining. Data shown are mean ± S.D. of quadruplicate samples and are representative of 5 experiments.

ARF6 Thr-157 form the base of the nucleotide-binding pocket and form van der Waals contacts with the face of the guanine ring. A mutation reducing the side chain size of either of these residues results in a fast cycling mutant by weakening the binding of nucleotide by the protein.

ARF6 T157A seems to more faithfully recapitulate the effects of ARF6 activation than does ARF6 Q67L. These observations and the frequent toxicity of Q67L suggest that GTP hydrolysis is an important component of ARF function. However, this may not be the case for all GTPases. The Ras constitutively active mutations were first isolated as oncogenes and have been successfully used to study Ras function and to reproduce Ras-mediated phenotypes (33). Therefore, hydrolysis may not be a necessary component of any known Ras functions. The constitutively active Rho family (RhoA, Rac1, Cdc42) mutants induce the formation of characteristic F-actin-rich structures and have been successfully used to study many Rho family functions. However, many Rho GEFs are proto-oncogenes whereas the constitutively active Rho family mutants are only weakly transforming and can be toxic in certain circumstances (18). Rho family fast cycling mutants, on the other hand, are oncogenic and more similar to GEF overexpression (18). These data suggest that cycling is important for at least some of the oncogenic activities of the Rho family proteins.

Why is cycling seemingly more important for the ARF and Rho GTPases than for Ras? It is interesting to speculate that the critical difference may be that Ras primarily regulates signal transduction cascades, whereas in addition to signaling cascades the ARF and Rho proteins regulate the assembly of large structural protein complexes, namely vesicle coats and F-actin networks. For ARF1 it has been shown that GTP hydrolysis is necessary for the disassembly of the induced protein coat. Continuous assembly without simultaneous disassembly of previously formed complexes may deplete the cells of the protein components that compose these structures. This depletion could send the cells into a rigor-like state. Therefore, GTP hydrolysis by ARF and Rho proteins may be critically important for maintaining some ARF and Rho functions, whereas Ras-activated signals can be maintained in the absence of GTP hydrolysis.

The ARF6 T157A mutant characterized here is an activated mutant that retains the ability to hydrolyze GTP. This will allow future studies of ARF function to avoid the complications arising from interruption of the normal ARF GTPase cycle. This should prove advantageous in elucidating ARF function and the specific roles of the various ARF proteins.

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