Communication

An Activating Mutation in ARF1 Stabilizes Coatomer Binding to Golgi Membranes*

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The Ras-related protein ADP-ribosylation factor 1 (ARF1) is a low molecular weight GTP binding protein, which in its GTP state supports the binding of coatomer, a cytosolic coat protein complex, to Golgi membranes. To create an "active" ARF, we constructed a point mutation in ARF1, Q71I, which was predicted to slow the rate of GTP hydrolysis. We demonstrate that Q71I, in contrast to wild type ARF1, exhibits a 2-3-fold increase in the half-life of ARF-GTP and is able to promote stable coatomer binding to Golgi membranes in the presence of GTP in vitro. Additionally, Q71I is able to support the binding of a significant amount of coatomer to membranes in the absence of added nucleotides, effectively bypassing the brefeldin A (BFA)-sensitive exchange activity. Furthermore, transfection of cells with Q71I, but not ARF1, renders the Golgi association of coatomer resistant to the effects of BFA in vivo. These observations provide compelling evidence that ARF1 is a necessary GTP binding protein that regulates the reversible binding of coat proteins to Golgi membranes and that the effects of BFA on this process in living cells must be a consequence of BFA's inhibition of guanine nucleotide exchange onto ARF1.

The reversible binding of cytosolic coat proteins (coatomer) to Golgi membranes is believed to function in the maintenance of structure and regulation of membrane traffic in the Golgi complex (1). The assembly of coatomer onto Golgi membrane requires GTP and a small GTP binding protein, ADP-ribosylation factor 1 (ARF1) (2, 3). The ability of ARF1 to mediate the assembly of coatomer onto Golgi membranes is believed to require the activation of ARF1 by a membrane nucleotide exchange protein (4-6) which results in the association of ARF1-GTP with the membrane followed by coatomer binding (2, 3).

Hydrolysis of the ARF-bound GTP is proposed to be coupled to the release of both ARF-GDP and coatomer from the membrane (7).

Pharmacologic reagents that perturb the activation/inactivation cycle of GTPases have proved to be valuable tools for dissecting their functions. Addition of the nonhydrolyzable GTP analogue GTPγS, which persistently activates GTPases, results in enhanced and irreversible binding of ARF and coatomer to Golgi membranes (8-10). In contrast, addition of brefeldin A (BFA) inhibits the activation of ARF catalyzed by the Golgi-associated nucleotide exchange protein and prevents the binding of ARF and, therefore, coatomer to membrane (8, 9, 11). While it is tempting to conclude that ARF is the sole GTP binding protein required for coatomer binding, previous experiments do not exclude a requirement for other GTP binding proteins known to be associated with the Golgi membrane (12-14).

To determine whether ARF1 is both the sole, GTP-requiring component for coatomer binding and the sole target of BFA in this process, we constructed a point mutation in human ARF1 that was predicted to inhibit the rate of GTP hydrolysis, creating a persistently active ARF1. Characterization of this mutant ARF is presented below and confirms that ARF1 is the key BFA-sensitive component required for coat protein binding to Golgi membranes.

EXPERIMENTAL PROCEDURES

Purification of ARF, Golgi Membranes, and Cytosol—Wild type ARF1 and the mutant ARF1, Q71I, were co-expressed in Escherichia coli with N-myristoyltransferase and isolated as previously described (15, 16). Golgi-enriched membrane fractions from rat liver (17) or Chinese hamster ovary cells (18) were obtained as described. Cytosol was isolated from bovine brains (19).

Nucleotide Exchange Assays—Nucleotide exchange was measured by measuring nucleotide binding to ARF and/or rat liver Golgi membranes using a filter binding assay as described previously (4). Incubations were carried out in a 0.1-mL reaction volume containing 25 mM Hepes-KOH, pH 7.0, 25 mM KCl, 2.5 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM ATP (Sigma A2583), 2-5 μg of recombinant ARF protein, and 4-6 μg of Golgi membranes and [α-32P]GTP or [γ-32P]GTP (1 μM, 0.7-1.4 Ci mmol⁻¹) at 30 °C for the indicated times. The reaction was stopped by the addition of cold buffer, the mixture was filtered on BAB5 nitrocellulose filters and washed with five 2-ml volumes of wash buffer, and the amount of radioactivity trapped to the filter was measured as described (4). The amount of membrane-catalyzed nucleotide bound to ARF was calculated by subtracting the nucleotide bound after incubations of ARF alone and Golgi membranes alone from the amount bound after incubations of ARF plus Golgi membranes (4).

Coatomer Binding Assay—Membranes from Chinese hamster ovary cells (8 μg of protein) and ARF1 or Q71I (10 μg of protein each) were incubated under conditions described above for nucleotide exchange assays with and without activators prior to the addition of cytosol (600 μg of protein) and further incubation to allow for coatomer binding. The membranes and bound material were pelleted at 14,000 g, and the amount of β-COP associated with the pellet was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot, and quantitated by PhosphorImager (Molecular Dynamics) (2). The background amount of β-COP binding to membranes that occurred in the absence of added ARF was subtracted from values obtained with ARF1 and Q71I.

Transfection of Cells and Immunofluorescence Assays—ARF1 and Q71I containing the hemagglutinin epitope at the C terminus were subcloned into the expression vector PCDL52R (20). COS-1 cells grown on coverslips were transiently transfected by the calcium phosphate precipitation method and were analyzed 40 h later to assess sensitivity to BFA. For immunofluorescence, cells on coverslips were fixed in 2% formaldehyde and double labeled with a monoclonal antibody to the HA epitope (12CA5) to detect transfected HA-tagged ARF protein and a polyclonal anti-β-COP peptide (EAGE) antisem as described (9).
RESULTS AND DISCUSSION

To create a persistently active ARF1, we selected a point mutation based upon the homology of ARF1 protein sequence domains with Ras (21). In Ras the conserved glutamine at position 61 is required for efficient GTP hydrolysis; mutations at this position stabilize the Ras-GTP state, resulting in a persistently activated protein (22, 23). The analogous position in ARF1 is glutamine 71, and this was changed to an isoleucine. This mutant, called Q711, was co-expressed in E. coli with N-myristoyltransferase in order to produce the biologically active N-myristoylated form of ARF (15). The recombinant protein was isolated and its activity monitored in nucleotide exchange, GTP hydrolysis, and coatomer binding assays in vitro, as well as in transfected cells.

Isolated Golgi membranes catalyzed nucleotide exchange onto both ARF1 and Q711 with similar kinetics using α-labeled [γ-32P]GTP (Fig. 1, A and B, open squares). This exchange activity was inhibited for both ARF1 and Q711 in a dose-dependent manner by BFA (Fig. 1C). During the membrane-catalyzed nucleotide exchange assay, GTP is bound to ARF and is then hydrolyzed to GDP (4, 7). Thus, using α-labeled [γ-32P]GTP, total exchange events onto ARF are recorded even if the ARF-bound GTP has been hydrolyzed to GDP. When γ-labeled [32P]GTP was used in the exchange assay to assess the amount of GTP exchanged onto ARF that was not hydrolyzed (Fig. 1, A and B, solid squares), a larger fraction of nucleotide bound to Q711 remained as GTP as compared with ARF1. To further assess this distinction, we measured the rate of hydrolysis of γ-[32P]GTP that had been preloaded onto ARF1 and Q711 by monitoring the loss of ARF-bound radioactivity after the addition of unlabeled GTP. GTP that was bound to ARF was hydrolyzed with a t1/2 of less than 2 min for ARF1 and approximately 5 min for Q711 (Fig. 1D). The addition of BFA, in place of unlabeled GTP, yielded similar results. Thus, while there was no observed change in the BFA-sensitive nucleotide exchange onto Q711, there was a 2–3-fold decrease in the rate of membrane-dependent GTP hydrolysis associated with the Q711 mutation in vitro.

![Figure 1: Golgi membrane-catalyzed GTP exchange and hydrolysis onto ARF1 and Q711](image)

![Figure 2: Nucleotide requirements for preactivation of Golgi membranes by ARF1 and Q711](image)
Incubation of Golgi membranes with ARFl in the presence of GTPyS is sufficient to preactivate the membranes, making them competent for binding of subsequently added coatomer in the absence of free nucleotide (2). While BFA inhibits the initial activation step, it has no effect on subsequent coatomer binding to such “preactivated” membranes. Importantly, this preactivation is not observed with hydrolyzable GTP. The relative stability of GTP bound to Q71I suggested that the mutant might, in contrast, preactivate the membranes for coatomer binding with GTP alone. Indeed, incubation of Q71I with Golgi membranes in the presence of GTP allowed maximal levels of binding of the coatomer protein β-COP to occur during a second incubation in the presence of coatomer (Fig. 2B). The level of β-COP binding achieved was nearly that achieved with GTPyS. In contrast, as observed previously (2), preactivation of Golgi membranes by wild type ARFl was only observed if GTPyS was included in the first incubation (Fig. 2A).

Another difference between Q71I and wild type ARFl was the significant level of BFA-resistant activation of membranes by Q71I that was observed in the absence of any added nucleotide (Fig. 2B, open bars). This activity of Q71I increased with longer incubation but was never observed with ARFl. Possible explanations for this observation are that the preparation of Q71I contains “activated” ARF that effectively bypasses any BFA-sensitive step in the membrane. Thus, Q71I appears to have the characteristics of both a longer lived, persistent ARF-GTP as well as a constitutively active ARFl.

Preincubation of permeabilized cells with GTPyS blocks the effects of subsequent addition of BFA on coatomer dissociation from the Golgi apparatus (8). Since Q71I has the characteristic of acting in vitro like ARF-GTPyS in the absence of added GTPyS, we could now test whether the expression of this “active” ARFl in cells would confer resistance to BFA’s effects on coatomer association with the Golgi apparatus. COS-1 cells were transfected with either ARFl or Q71I containing an HA epitope at the carboxyl terminus so that the transfected protein could be immunologically detected. In cells transfected with either ARFl or Q71I, the HA tagged ARFs were localized to a perinuclear region, which co-localized, by immunofluorescence, with the coatomer component β-COP (Fig. 3) and Golgi resident markers (not shown). β-COP localization in untransfected cells was indistinguishable from that in the transfected cells. When BFA was added to cells transfected with wild type ARFl, both ARFl and β-COP rapidly became diffusely distributed to the cytosol, effects indistinguishable from those seen in untransfected cells. The effects of BFA on cells transfected with Q71I were quite different. The distribution of neither the introduced Q71I nor β-COP changed in response to the addition of the drug. Even after extended incubations of 1 h in the presence of 10 μg/ml BFA, β-COP and Q71I remained co-localized to perinuclear structures. Other markers of the Golgi complex also remained in these β-COP-labeled structures (not shown). The presence of the HA epitope had no effect on the phenotype since transfection of untagged ARFs resulted in similar effects (not shown). Myristoylation of the proteins was required for their biological function, since cells transfected with ARFl sequences that contained an additional mutation at position 2 from a glycine to an alanine abolished myristoylation and Golgi localization for both ARFl and Q71I and eliminated BFA protection in cells transfected with Q71I. The BFA-resistant phenotype of Q71I cannot be explained by overexpression of ARF proteins per se since it was observed in all cells expressing variable amounts of Q71I and never observed in cells overexpressing wild type ARFl.

Our expectation in creating Q71I was that this mutation in ARF, analogous to Q61L in Ras, would inhibit hydrolysis of GTP bound to the protein, creating a persistently active GTP-ARF. If the inhibition of hydrolysis was sufficiently strong, resistance to the effects of BFA on coatomer binding would be expected. We measured, however, only a 2–3-fold increase in the half-life of GTP bound to Q71I in our in vitro assays, which would predict a delay in, but not the complete inhibition of, the effects of BFA on coatomer binding that was observed in cells transfected with Q71I. Although it is possible that the half-life of Q71I-GTP in vivo may be longer, studies with the analogous mutation in Rab3A have shown that the rates of GTP hydrolysis as measured in vivo are actually similar for the wild type and mutant proteins, and these authors caution that the analogy made to the observation with Ras mutations may not always be valid (24). This raises the possibility that this mutation in ARFl, while it does affect to some extent GTP hydrolysis, may also result in a conformational change in the protein that effectively makes it “active” regardless of the nucleotide bound. In this regard, it might be significant that the site of this mutation is adjacent to a critical glycine (at position 70 in ARFl, 60 in Ras), which is believed to make contact with the γ-phosphate and be involved in the GDP-GTP-induced conformational change in the protein (21).
Regardless of the mechanism, the effect of expressing this activated ARF is comparable with selectively activating ARF with GTPyS, rendering the cell resistant to the effects of BFA on both coat assembly and redistribution of Golgi membrane into the endoplasmic reticulum. Furthermore, these results demonstrate that the only process inhibited by BFA in the ARF-coatomer binding reaction is the nucleotide exchange activity. ARF thus becomes one of the few small GTPases for which an effector function is identified; its cycle of activation and inactivation regulates the binding of coatomer to Golgi membranes.

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