Effectors Increase the Affinity of ADP-ribosylation Factor for GTP to Increase Binding*

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The stoichiometry of the binding of GTP to ADP-ribosylation factor (ARF) proteins, normally quite low at ~0.05 mol/mol protein, was found to increase to a maximum of 1 mol/mol in the presence of effectors. The mechanism of this action was found to result from the ability of these effectors to increase the affinity of ARF for activating guanine nucleotide triphosphates. The existence of a conformation of ARF with low affinity (>100 μM) for GTP is proposed. The actions of effectors to increase the equilibrium binding of GTP is interpreted as evidence that these same effectors interact with and modulate the affinity of the inactive ARF for GTP. A new model for these interactions among ARF, effectors, and GTP is proposed, and a preliminary test in cells is supportive of these observations with relevance to signaling in cells.

ADP-ribosylation factors (ARFs)³ are 21-kDa GTP-binding proteins, ubiquitous in eukaryotes, with roles that include the regulation of a number of steps in vesicular transport, particularly those in and around the Golgi (1–4). In addition to roles in the secretory pathway, ARFs are essential proteins in both mitotic cell growth (5) and independently in the sporulation and respiration processes in the yeast Saccharomyces cerevisiae (6, 7).³ An increasingly diverse collection of immediate downstream effectors of ARF action has been identified in the last few years and currently includes mammalian phospholipase D1 (PLD1) (8–10), arfaptin/POR (11, 12), the yeast Sat protein (13), and five more proteins identified in two-hybrid screens of mammalian cDNA libraries (14).³ These are in addition to the bacterial ADP-ribosyltransferases, cholera toxin, and Escherichia coli heat-labile toxin, which require ARF as cofactor in the covalent modification of the stimulatory regulatory GTPase-activating protein (GAP) (21)-stimulated hydrolysis, respectively. ARF exchange factors (e.g. yeast Gea1/2 (22), mammalian ARNO (23), and cytohesins (24)) share a conserved sequence motif, referred to as the Sec7 homology motif, and the ability to speed the off-rate of GDP from ARF proteins. In addition to these exchange factors, an activity has been described in crude Golgi membrane preparations with the ability to increase the binding of GTP to the slowly hydrolyzing analog GTPγS to ARF without increasing the rate of GDP release. This protease-sensitive ARF-activating protein has proven resistant to purification and has not been characterized further (25). In addition to these specific protein interactions, the binding of GTP to ARFs is generally promoted by lipids, e.g. phosphatidylcholine or phosphatidylethanol (26). In contrast, other lipids, particularly phosphatidylinositol 4,5-bisphosphate (PIP₂), bind to ARFs, promote the release of GDP, and stabilize the nucleotide-free protein (27). Surprisingly, the subsequent removal of PIP₂ allows GTP to bind at a higher stoichiometry than was supported prior to exposure to high concentrations of PIP₂ (27).

In contrast to other families of GTPases, the extent of in vitro binding of GTP to ARFs is very low (typically <10%) (26, 28), and this binding is highly dependent on the presence of a hydrophobic environment, which can be supplied for in vitro assays by either lipids or detergents. A mixture of 3 mM dimyristoyl-1,2-phosphatidylcholine (DMPC) and 2.5 mM sodium cholate is commonly used (26, 28). An amphipathic α-helix at the N terminus of ARFs, absent in other GTPases, acts as an inhibitory domain that both limits GTP binding and confers the dependence on lipids (29).

Because of its role in GTP binding, phospholipid dependence, membrane binding, and potentially effector interactions, the N-terminal α-helix has been studied extensively (29–32). This isolated domain was first shown to be capable of assuming an α-helical structure in solution, but only when exposed to a hydrophobic environment (29). Later, this N-terminal domain of the protein was shown, by solution of the x-ray crystal structure, to form a helix in the intact protein when bound to GDP (33). This helix is held tightly in place by a series of hydrophobic interactions, provided by leucine and phenylalanine residues in the N-terminal domain, with a hydrophobic region of the protein core. Deletion of the N-terminal 17 residues by mutagenesis results in a protein that lacks ARF activity (29), but that binds GTP at a high stoichiometry and independently of added lipids or detergents. ARFs are N-myristoylated at the N-terminal glycine, and the first dozen or so residues are thought to provide further energies that assist in the anchoring of N-myristoylated ARF to membrane surfaces.

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³ The abbreviations used are: ARFs, ADP-ribosylation factors; PLD, phospholipase D; GAP, GTPase-activating protein; GTPγS, guanosine 5′-3′-O-(thio)triphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; DMPC, dimyristoyl-1,2-phosphatidylcholine; CTA, cholera toxin A subunit; it, E. coli heat-labile toxin A subunit; BSA, bovine serum albumin; GST, glutathione S-transferase; MBP, myelin basic protein.

² M. Cavenagh, C. Zhang, and R. A. Kahn, unpublished observations.

³ Boman, A. L., Zhang, C. J., Zhu, X., and Kahn, R. A. (2000) Mol. Biol. Cell, in press.
(32, 34, 35). Thus, the N terminus and myristate are proposed to be critical for ARF activities, as they are needed for the association and possibly orientation of the protein at the membrane interface. Because of its apparent role in membrane binding, it is generally thought that the N-terminal helix is not involved in protein-protein interactions. However, this conclusion is challenged by both functional biochemical data (30) and results of two-hybrid assays with newly discovered ARF-binding proteins (14).  

We speculated that interaction with protein factors may involve the N-terminal helix and in so doing relieve the inhibition of GTP binding. One prediction from this model is that the presence of the protein should promote a higher binding stoichiometry than observed in the presence of lipids. We sought to test this prediction using purified recombinant proteins. In so doing, we found that effectors can increase the binding stoichiometry of purified ARF by up to 25-fold, to 1 mol of GTP bound per mol of ARF protein. Further investigation of the GTP-binding properties of ARF proteins led to the surprising conclusions that ARFs exist in at least two, slowly inter-converting conformations and that one of these has a very low affinity for GTP. Also surprising is the conclusion that protein effectors of ARF signaling must interact specifically with this low affinity state to convert it into the higher affinity state. We believe that these results identify a novel feature of ARF interactions with effectors and reveal an even more complex picture of the actions of this family of GTPases with likely consequences to its actions in cells.

**EXPERIMENTAL PROCEDURES**  

**Materials**—[35S]GTP·S, [3H]GDP, 1,2-dideoxyinosin-glycero-3-phosphoryl[3H]choline, and [adenine-2,8-14C]NAD were purchased from NEN Life Science Products. Cholera toxin and cholera toxin A subunits (CTAs) were purchased from List Biological Laboratories, Inc. Dowex AG 1-X2 (100–200 mesh in the hydroxyl form) was obtained from Bio-Rad. Amylose resin was purchased from New England Biolabs Inc. All other reagents and chemicals were obtained from Sigma. All experiments shown or described were repeated at least twice with similar results.

**GTPyS Binding Assay**—Binding of GTPyS to ARF was determined by the method described by Kahn and Gilman (26) and Kahn (28). ARF was incubated at 30 °C in binding buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 0.5 mM MgCl₂ (low Mg²⁺ condition) or 1.25 mM MgCl₂ (high Mg²⁺ condition), 3 mM DMPC, 0.1% sodium cholate, and 100 μg/ml BSA) and 10 μM GTPyS ([35S]GTP·S, 2500 cpm/pmol). Duplicates of 10-μl samples were taken and diluted into 15 μl of TNMD buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM sodium cholate, 20 μg/ml phosphatase-free BSA, 0.1% sodium cholate, and 0.3 μg/ml Hi5 membrane protein). The reaction was performed at 37 °C for 15 min and stopped by the addition of 1 ml of chloroform/methanol/HCl (50:50:0.3) followed by 350 μl of 1x HCl and 5 mM EDTA. A portion of the aqueous phase (500 μl) was analyzed for the release of [3H]choline. A blank was determined as the amount of product formed in the absence of any PLD, and this was subtracted from each sample. PLD assay was then expressed as the amount of choline released per min/μg of membrane protein.

**Cell Transformation and Indirect Immunofluorescence**—Normal rat kidney cells were grown in cell culture medium as described previously (41). GGA1 was overexpressed in these cells after transfection with pcDNAs-GGA1 using Fugene 6 (Roche Molecular Biochemicals) according to manufacturer’s protocol. The open reading frame encoding full-length human GGA1 was subcloned into pcDNA3 (Invitrogen) at unique and unique XhoI and EcoRI sites. Expression of a single 78-kDa product was confirmed by Western blotting using GGA1-specific rabbit polyclonal antibody R78716 (15), raised against the recombinant protein.

**RESULTS**

Effectors Increase the Amount of GTPyS Bound to ARF at Equilibrium—To test the prediction that an ARF effector could increase the binding of GTP to ARF, we first used LTA, as it has been shown to bind directly to ARF, has been well studied and characterized, and is available in large amounts as a recombinant protein (18). As described previously (28), in our standard nucleotide binding assay (1 μM ARF, 3 mM DMPC, 0.1% cholate, 20 mM HEPES, pH 7.4, 1 mM dithiothreitol, 100 mM sodium chloride, 1 mM EDTA, and 0.5 mM MgCl₂), purified recombinant human ARF3 bound [35S]GTPyS at only low stoichiometry (~4% in the experiment shown in Fig. 1, open cir-
Effectors Increase GTP Binding to ARF

Fig. 1. ARF3 binds GTPγS at a higher stoichiometry after interacting with LTA. Specific binding of [35S]GTPγS to ARF3 (1 𝜇M) was determined after various times of incubation at 30 °C in binding buffer (with DMPC/sodium cholate) with low micromolar free Mg2+ either alone (open circles) or in the presence of 10 𝜇M LTA (closed circles). At the indicated times, 10-μl samples were taken, and specifically bound nucleotide was determined by rapid filtration as described under “Experimental Procedures.” Data represent the mean ± S.E. of duplicate determinations.

Effectors Increase GTP Binding to ARF—A dependence of GTP binding on a hydrophobic environment, provided in the form of lipid vesicles, detergent(s), or mixed micelles, has been noted since ARFs were first found to bind guanine nucleotides (26). This dependence is more marked in the N-myristoylated proteins, but is evident also in non-acylated ARF preparations. The effect of removing the DMPC/sodium cholate mixture from the binding reaction was investigated. As previously reported, equilibrium binding of GTPγS to human ARF3 was higher in the presence of 3 mM DMPC with 2.5 mM sodium cholate (DMPC/sodium cholate) (Fig. 3, closed symbols) than in their absence (open symbols) for both N-myristoylated (triangles) and non-acylated (squares and circles) proteins. Enhanced binding in the presence of POR1ΔΔN is evident in Fig. 3, and this effect can clearly be seen regardless of the presence of DMPC/sodium cholate or the N-myristoylation of ARF3. In the data shown in Fig. 3, the maximal effect of POR1 was only ~40%, as the protein preparation was several weeks old. Thus, neither N-myristoylation nor the presence of a hydrophobic environment is required for the enhancement of activation by effectors.

We next examined the effects of free magnesium ions on this effect, as this has been shown to have profound effects on GTP binding to ARF proteins. The use of a metal chelator (EDTA) to buffer the free metal ion concentration in nucleotide binding assays allows stable low levels of magnesium to be achieved. The concentration of free magnesium in our buffer (containing 0.5 mM MgCl2 and 1 mM EDTA) is in the low micromolar range,
whereas that found in mammalian cell cytosol is thought to be in the high micromolar range. Increasing the free metal concentration by either omitting the EDTA or adding MgCl₂ to excess over EDTA caused a slowing in the time required to reach equilibrium, yet the same binding stoichiometry was ultimately achieved in the presence of POR1ΔN (Fig. 4). At the higher concentrations of magnesium, the off-rate of GDP was decreased markedly (see below and Fig. 5), leading directly to slower rates of GTP₆S binding, as described previously (36).

Note that this is not a true on-rate for GTP binding, as it is limited by the off-rate of GDP. On-rates for binding to nucleotide-free ARF have been reported only once before (36), as the protein is very unstable in this apo form.

Effect of POR1ΔN on GTP₆S Binding to ARF3 Is Dose-dependent—When the concentration of ARF3 was held constant at 1 μM and that of POR1ΔN was varied between 0 and 8 μM, the extent of binding of GTP₆S increased with increasing POR1ΔN (Fig. 4). Similar results were obtained when LTA was used as the effector (data not shown). We have achieved 100% binding of GTP₆S to ARF3 at a ratio of 1:1 (LTA/ARF), but not with a lower ratio. These results are consistent with the conclusions that the two proteins form a stable 1:1 complex and that each is fully active. Storage of ARF effectors for weeks at 4 °C or for months at −80 °C led to a decrease in the maximal activity attainable.

GDP Binding Is Not Altered by Effectors—The time course and magnitude of the increase in stoichiometry with effectors were the same whether the ligand used was [γ³²P]GTP or [³⁵S]GTP₆S (data not shown). In contrast, although [³H]GDP bound ARF3 alone at a higher stoichiometry (50–80%) than did either of the triphosphates, no effect of LTA or the other effectors was observed on GDP binding in the presence of DMPC/sodium cholate and low magnesium. A small (no more than 50% increase) but consistently observed increase in GDP binding was observed when assayed at the higher magnesium concentration (data not shown) with LTA, but not with the other effectors. Thus, the dramatic increase in binding was highly specific to the activating guanine nucleotide triphosphates.

**Fig. 2.** GTP₆S binding to ARF3 is also increased by other effectors. Specific binding of [³⁵S]GTP₆S to ARF3 (1 μM) was determined after various times of incubation at 30 °C in low Mg²⁺-containing binding buffer (with DMPC/sodium cholate) either with ARF alone (open circles) or with the addition of a 5 μM concentration of one of the following effectors: MKLP1 (open triangles), GGA1 (closed squares), POR1ΔN (open squares), or LTA (closed circles). At the indicated times, 10-μl samples were removed, and bound nucleotide was determined by rapid filtration as described under “Experimental Procedures.”

**Fig. 3.** Enhancement of GTP₆S binding to ARF3 promoted by effectors is not dependent on either DMPC/sodium cholate or N-myristoylation. Specific binding of [³⁵S]GTP₆S to ARF3 was determined after various times of incubation at 30 °C in low Mg²⁺-containing binding buffer in either the absence (open symbols) or presence (closed symbols) of DMPC/sodium cholate. Binding was determined in the presence of 1 μM ARF3 alone (squares), ARF3 with 5 μM POR1ΔN (circles), 1 μM myrARF3 alone (inverted triangles), or myrARF3 with 5 μM POR1ΔN (triangles). At the indicated times, 10-μl samples were removed, and bound nucleotide was determined by rapid filtration as described under “Experimental Procedures.”
GTP and GTP\(_\gamma\)S and was not seen for the binding of GDP.

Effects of ARF-binding Proteins on Nucleotide Off-rates—An indication of the mechanism by which effectors increase the level of GTP binding to ARF was sought using kinetics of the nucleotide exchange reaction. An increase in the equilibrium level of bound ligand could result from an increased affinity for ligand, which in turn could be caused by either an increase in the rate of association or a decrease in the rate of dissociation. Because under all conditions tested in vitro the binding of nucleotide is limited by the rate of dissociation of GDP, a change in the equilibrium level of bound ligand could result from an increased affinity for ligand, which in turn could be caused by either an increase in the rate of association or a decrease in the rate of dissociation. Because under all conditions tested in vitro the binding of nucleotide is limited by the rate of dissociation of GDP, a change in the rate of dissociation may first reveal itself as an increase in the rate of binding of GTP\(_\gamma\)S. Note that both bovine brain and recombinant E. coli-expressed ARF proteins were previously shown to purify as a 1:1 complex with GDP (36). The rate of GDP dissociation was determined by preincubating \(^3\)H\)GDP with ARF3 until it reached equilibrium; 30–80\% stoichiometry was achieved within 60 min. Off-rates were then determined by measuring the amount of labeled GDP remaining bound after the addition of an excess of unlabeled GDP in the presence and absence of effectors (LTA and POR1-MBP). As shown in Fig. 5, POR1-MBP had no effect on the rate at which GDP dissociated from ARF3. The same data were replotted using a semilog axis (Fig. 5, inset) and used to determine dissociation rates of GDP at the lower magnesium concentration: 0.035 and 0.033 min\(^{-1}\) in the absence and presence of POR1-MBP, respectively, at low magnesium concentration and 0.0030 and 0.0031 min\(^{-1}\) at high magnesium concentration. Despite being slowed markedly when the concentration of free magnesium was raised, there was still no effect of POR1-MBP on the rate. Similar results were obtained with LTA (data not shown). The off-rates of GDP were also determined using an alternate protocol in which the effector was included in the preincubation step. In this case, only the excess unlabeled GDP was added to begin the experimental phase. Again, the rates of GDP dissociation were unaltered by the inclusion of either POR1-MBP or LTA (data not shown). Thus, these effectors did not act as ARF exchange factors or guanine nucleotide-dissociating stimulators to speed the release of bound nucleotides.

Fig. 4. Binding of GTP\(_\gamma\)S to ARF3 promoted by effectors is dose-dependent. Specific binding of \(^{35}\)S\)GTP\(_\gamma\)S was determined after ARF3 (1 \(\mu\text{M}\)) was incubated with various concentrations of POR1\(\Delta\)N in low Mg\(^{2+}\)-containing (A) and high Mg\(^{2+}\)-containing (B) binding buffer in a total volume of 100 \(\mu\text{l}\). Duplicate 10-\(\mu\text{l}\) samples were removed, and bound nucleotide was determined by rapid filtration as described under “Experimental Procedures.” The molar ratios of ARF3 and POR1\(\Delta\)N are 1:0 (open circles), 1:0.2 (closed circles), 1:0.5 (open triangles), 1:1 (closed diamonds), 1:4 (closed triangles), 1:6 (open squares), and 1:8 (closed squares).
The other likely mechanism for effectors to increase GTP binding to ARFs was via a decrease in the off-rate of GTP (or GTPyS). Using the same protocol used to determine GDP off-rates, we also determined GTPyS off-rates. However, no changes were observed in the initial rates of GTPyS dissociation when POR1-MBP or LTA was included (data not shown). Thus, the rates of nucleotide dissociation from ARF3 were not changed by the binding of these effectors to ARF.

Equilibrium Binding of GTP to ARF—As shown in Fig. 1 (open circles), the extent of GTPyS binding to ARF3 alone (1 μM) reached a plateau of ~0.04 mol of GTPyS bound per mol protein that was stable for many hours. The concentration of ligand used in such experiments was usually 10 μM, well in excess of the published binding constant for GTPyS under these conditions (50 nM) (36). Because we know that the ARF3 protein preparation used was capable of binding nucleotides at a 1:1 stoichiometry, the causes of the low binding and mechanism by which effectors can reverse it were investigated.

Half-maximal binding of ARF1 (Fig. 6A, open inverted triangles) or ARF3 (data not shown) was achieved at a concentration of 1.6 μM GTPyS. This apparent KD for GTPyS binding is very close to the Ka for GTP and GTPyS in the ARF-dependent, cholera toxin-catalyzed ADP-ribosylation of Go (1–5 μM) (16). When POR1-MBP was included in the ligand binding assay, a higher level of binding was evident (Fig. 6A, closed inverted triangles). Although a higher level of GTPyS binding was reached, half-maximal binding was seen with about the same concentration of ligand: in this case, 1.7 μM. The concentration dependence of GTPyS on binding stoichiometry, described above with POR1 (Fig. 6A), was then repeated with LTA (Fig. 6B). In this case, we observed a more complex curve that was resolved into two different apparent binding affinities with half-maximal concentrations of GTPyS of 0.92 and 8.4 μM. Thus, presumably through direct interaction of the two proteins, LTA caused ARF to assume a conformation with a binding site that could be filled with GTPyS, although with a lower affinity than seen with ARF alone. As shown in Fig. 6A, increasing the concentration of ligand to 100 μM did not increase the binding to ARF1 (alone) beyond ~5%. The specific activity of the radiolabeled ligand became too low to accurately measure binding above ~100 μM. Because we know that the preparation of ARF1 is capable of binding GTP at a 1 mol/mol stoichiometry (in the presence of LTA), we conclude that the binding site for GTP must exist in (at least) two different states, one with an apparent affinity of ~1 μM and one with an affinity (>100 μM) too low to measure using nitrocellulose filter trapping of bound ligand. The consequence of added effectors is predicted then to convert the lower affinity binding sites into the higher affinity state (Fig. 6A, closed inverted triangles). POR1-MBP was capable of converting only 50–80% of the sites to a higher affinity state, and the affinity was indistinguishable from that seen for the 4–5% of the ARF3 that bound GTP in the absence of effectors. In contrast, LTA was able to convert 100% of the sites to a higher affinity state, but with an apparent Ka for GTPyS of only 8.4 μM (Fig. 6B).

A Role for the N Terminus?—Because the lipid independence and high stoichiometric binding seen with effectors are reminiscent of the consequences of deletion of the amphipatic N terminus of ARF, we compared the binding of GTPyS to the Δ17ARF1 mutant in the presence and absence of effectors with that to wild-type ARF1 or ARF3. No differences were seen between wild-type ARF1 and ARF3. As shown in Fig. 6A, the GTPyS concentration dependence of Δ17ARF1 was very similar to that seen for full-length ARF1 or ARF3 (data not shown) or for ARF1 in the presence of POR1, with a Ka in this experiment of 1.3 μM. POR1 had no effect on the concentration dependence of or level of equilibrium binding to the truncation mutant. Thus, an interaction between effectors and the N-terminal helix of ARFs is suggested by the similarities in consequences between deletion of the N-terminal helix and the addition of effectors.

Assaying Effector Binding in Solution—We took advantage of the previously described change in intrinsic fluorescence of ARF proteins that accompanies the binding of activating guanine nucleotide triphosphates (26) to develop an assay for GTP binding that occurs in solution. A comparison between the time-dependent change in intrinsic tryptophan fluorescence (open symbols) in the presence (triangles) and absence (circles) of LTA and the binding of [35S]GTPyS (closed symbols) is shown in Fig. 7. Note the similarities in magnitude of the effects in the two assays and similarity in time dependence. It took longer to reach equilibrium in each assay as compared with previous data because these assays were performed at room temperature (21 °C) instead of the usual 30 °C. These data further support the conclusion that binding of effectors to ARFs in solution alters the nucleotide-binding properties of the
GTPase and are inconsistent with the hypothesis that the filter trapping assay was producing artifactual results.

**CTA Binding Increases the Activation of ARF, Which in Turn Activates CTA**—We next investigated whether the complex of effector ARF—GTPγS, achieved using the protocols described above, was enzymatically active. As shown in Table I, preincubation of CTA with ARF3 led to an increase of 18-fold in the ADP ribosyltransferase activity of the cholera toxin, a value similar to the increase in bound GTPγS. Similar results were observed when the partially N-myristoylated ARF3 preparation was used; but a smaller -fold increase was seen, as myrARF3 alone had a higher activity than the non-acylated protein. Together, these data reveal that the bacterial toxins increased both the binding of GTPγS and activation of ARF.

These results support the conclusion that effectors can promote the activation of ARFs through a positive feedback mechanism to increase the amount of activated ARF, which in turn is functional to interact with and activate downstream effectors.

**ARF, Activated by POR1ΔN, Is Not Free to Activate PLD1**—We then compared the ability of ARF, activated by preincubation with GTPγS, with that of ARF, activated by preincubation in the presence of GTPγS and POR1ΔN, to increase PLD1 activity. As shown in Table II, in the absence of any preincubation, we observed only an ~2-fold stimulation of PLD1 activity by ARF. When the same amount of ARF had been exposed to GTPγS for 6 h, there was a robust 77-fold increase in PLD activity over that without prior activation. A parallel incubation revealed that preincubated ARF bound GTPγS to 60% occupancy. When POR1ΔN was included in the preincubation and the amount of activated (GTPγS-bound) ARF was markedly increased, the effect of ARF on the PLD activity was almost completely eliminated. We conclude that the binding of POR1ΔN by activated ARF precludes it from productive interaction with PLD1. Furthermore, these data suggest that the ARF that was activated by exposure to POR1ΔN is not free to rapidly dissociate and interact with PLD1, at least under these conditions.

**GGA1 Increases the Binding of ARF to Golgi Membranes**—As a simple test of the model that effectors can increase ARF activation in cells, we investigated whether overexpression of an ARF-binding protein could increase the binding of ARF to Golgi membranes. Because of the lipid dependence and low binding stoichiometry, it has been difficult to isolate ARF proteins with bound nucleotides as indicators of the degree of
activation in live cells. Instead, we took advantage of the translocation of ARF to membranes upon activation and used this phenomenon as an indicator of the activation status of ARF in the cell. This is most evident at Golgi membranes (43).

**FIG. 7.** Time course and relative extents of enhanced binding of GTP\(^\gamma\)S to ARF3 by LTA are similar whether monitored by radioligand binding or intrinsic fluorescence. ARF3 (1 \(\mu\)M) was incubated for the times indicated at 21°C in low Mg\(^2\)\(^+\) containing binding buffer with (triangles) or without (circles) LTA (7 \(\mu\)M). Reactions were performed in parallel, with one set used to determine changes in intrinsic fluorescence (open symbols) and the other used to determine specific binding of radionucleotide (closed symbols) as a function of time. Concentrations of all reactants were the same, except the latter included \(^{35}\)S-GTP\(^\gamma\)S (2500 cpm/pmol). Duplicates were taken at the indicated times, and specific binding was determined by filtration. Fluorescence was determined with an excitation wavelength of 290 nm, and emission was recorded at 345 nm as described under “Experimental Procedures.”

**Table I**

| Preincubation | Additions | ADP-ribosyltransferase activity of CTA increases by preincubation with ARF3 |
|---------------|-----------|--------------------------------------------------------------------------------|
| No            | CTA       | 0.34                                                                           |
| No            | CTA + ARF3| 1.2 0.86                                                                       |
| Yes (CTA only)| ARF3      | 1.6 1.3 1.5                                                                     |
| Yes (CTA + ARF3)| 0       | 15.8 15.5 18.0                                                                 |
| No            | CTA       | 1.1                                                                           |
| No            | CTA + myrARF3 | 4.1 3.0                                                                         |
| Yes (CTA only)| myrARF3   | 4.3 3.2 1.1                                                                     |
| Yes (CTA + myrARF3) | 0     | 29.1 28.0 9.3                                                                  |

**Table II**

PLD activities were determined either without or with a 6-h preincubation at 30°C of ARF3 (10 \(\mu\)M) in binding buffer and with or without POR1ΔN (10 \(\mu\)M). Preincubated samples were then diluted 10-fold into the PLD assay such that concentrations of reactants were the same as those in the non-preincubated set. PLD assay was performed in a 15-min reaction as described under “Experimental Procedures.”

**Discussion**

A number of effector proteins were found to have the ability to increase the equilibrium level of GTP binding to ARF proteins. Equally surprising was the observation that the low stoichiometry of GTP binding to ARF could not be driven higher by increasing the ligand concentration to up to 100 \(\mu\)M unless effectors were present. These and related observations led to two surprising conclusions: (i) that ARF exists in two different states in solution, one with higher apparent affinity (1 \(\mu\)M) for GTP and (the predominant) one with low affinity (\(>100\ \mu\)M) for GTP, and (ii) that several ARF effectors interact with an inactive form of ARF and convert it into the higher affinity state, which binds GTP more readily and which in turn promotes the binding of ARF to effectors. In other words, effectors may have distinct “positive feedback” and downstream effector functions. This prediction was tested in intact cells, and preliminary data supporting it are reported. The results and conclusions described in this study appear more consistent with a role for ARFs in a proofreading function, perhaps similar to that of elongation factor Tu in protein elongation, rather than a simple on-off switch, like that of \(G_s\) in activation of adenyl cyclase.
and ARF (antibody 1D9) as described under "Experimental Procedures." Cells that overexpress GGA1 also show brighter Golgi-localized staining of ARF. Two representative fields are shown, each containing normal and transfected cells.

Reports of substoichiometric ligand binding are quite common in the GTPase literature, and the reasons are typically assumed to be partial protein denaturation, suboptimal conditions, or subcellular ligand binding, or subcellular ligand concentrations (e.g. to allow high specific activities to be achieved for the ligand). However, the stoichiometry of GTP binding to ARF proteins occurs for protein preparations that bind 1 mol of GDP/mol of protein (36). The failure of GTP at concentrations up to 100 μM to drive the binding of ARF to form the active complex cannot be readily explained and led us to propose the existence of a low affinity state for ARF proteins. The physical nature of the low affinity state is not known, but the characteristics are very similar to those of the "comatose state" of Gα subunits, first observed by Sternweis. In each case, the GTPase exists in two different conformational states, only one of which is capable of rapid equilibrium binding of GTP. The rate of interconversion between these states must be very slow, relative to the rate of GTP binding. The two states are essentially kinetically uncoupled, otherwise binding in the presence of high concentrations of GTP (relative to the low affinity state) should drive the binding to approach 100%, but it does not (Fig. 6A).

What do we know of the low affinity state? It cannot be a partially denatured preparation, unless we also posit that it retains the ability to bind GDP in this form. It is a very stable form of the protein, as ARF preparations can be stored for weeks at 4 °C with little change in binding properties. It is also stable at 30 °C, as we can allow ARF to incubate with GTP·S for several hours before adding effectors and still achieve the same end point seen when effectors are added at t₀. The lack of effect of BSA (included in binding buffers to decrease "nonspecific" adsorption to plasticware) and Sat1Δ5 and a likely 1:1 LTA:ARF3-GTP complex are suggestive of the specificity of this role for effectors. It is formally possible that the low affinity state, with GDP bound, may bind to a set of effectors distinct from those that bind to the activated ARF protein, similar to the Ran GTPase. What makes the low affinity state particularly interesting from a cell regulation perspective is the ability of effectors or lipids to alter the percentage of ARF in the rapidly exchangeable conformation.

Four of the proteins previously shown to bind ARF in a GTP-dependent fashion were found to possess the ability to increase the binding of GTP to ARF3, although to different extents (Fig. 2). It appears that the ability to increase the level of GTP binding to ARFs at equilibrium is a common feature of effectors for this GTPase. Only LTA was able to support 100% occupancy of the nucleotide-binding site on ARF3 with GTP. Why the other proteins cannot support full occupancy is not clear at this time. The other three proteins, POR1ΔN, GST-GGA1–145–639, and GST-MKLP1–649–960, are truncation mutants and fusion proteins. Thus, at this time, we cannot distinguish between different effectors having different maximal activities and the consequences of truncations or fusion constructs. Only the deletion mutant of yeast Sat1, termed Sat1Δ5, was deficient in this activity among proteins that bind ARF in a GTP-dependent fashion. Sat1Δ5 retains function in yeast cells and GAP activity in vitro, but could be inactive in our assay as a result of deletion of residues important to this activity. The positive feedback role for effectors brings to three the distinct but interactive roles for effectors, along with downstream mediators of ARF and cofactors for ARF GAPs (44).

Current models for ARF activation include an essential role for its translocation from a soluble GDP-bound form to a membrane-associated state upon GTP binding. This membrane association is increased by N-myristoylation and is thought both to orient the GTPase in two-dimensional space and to increase interactions between ARF and membrane-bound effectors, e.g. PtdIns14, N-Myristoylation of ARF is a cotranslational process that is essential to its function in yeast, as mutation of the acceptor site, glycine 2, to alanine is a null allele (6). The myristate is proposed to insert into the lipid bilayer with the amphipathic N-terminal helix lying along the bilayer and interacting with polar lipid head groups (32), as previously proposed for Src (45) and recoverin (46). This intimate association with membrane lipids is thought to explain the lipid and/or detergent dependence for GTP binding to ARFs, first described in 1986 (26). The ability of effectors to alter the nucleotide-binding properties of ARF was found to be independent of N-myristoylation (Fig. 3). Surprisingly, LTA supported full occupancy of the GTP-binding site in the absence of lipids or detergents. We infer from this that the hydrophobic N terminus (and myristate when present) is more effectively shielded from the aqueous environment by binding to LTA than by interactions with lipids or detergents. We cannot exclude the possibility that the effect of lipids/detergents to increase GTP binding to ARF is to weakly promote conversion to the high affinity state, the same process proposed for effectors. It is interesting to note that a phospholipid, PIP₃, has been shown to have the ability to modulate the guanine nucleotide binding of ARF; and after exposure to relatively high concentrations of PIP₃, ARF was able to bind GTP at a higher stoichiometry (20%) than without prior exposure to PIP₂, (5%) (27). This specific lipid interaction involves regions of the ARF protein (the "positive patch" described by Amor et al. (33)) distinct from those that are involved in the myristate-driven membrane anchoring. Thus, lipids are likely determinants of the amount of ARF in the high affinity state. Another surprising conclusion from these results is that instead of requiring a membrane for effective interactions between ARF and its binding partners, the effector-ARF-GTP trimeric complex may be fully functional in solution.

The results described above (e.g. Table II) raise the possibility that one ARF effector present in excess at a cellular location could sequester the GTPase in a stable complexed form and decrease the chances for bifurcations in signaling at the level of the GTPase. If the binding of ARF to effector is lower affinity, it raises the possibility that one effector could conceivably promote the activation of ARFs, which would then be free to dissociate and activate a variety of other effectors. The latter

5 P. C. Sternweis, unpublished observations.
appears inconsistent with tight regulation of an essential and highly conserved signaling molecule; but with so many different potential effectors known, it will surely be a point of increasing interest. In efforts to begin to discriminate between these two possibilities, we devised a preliminary test. We simply asked if the ARF-GTP G-S complex produced by incubation with POR1ΔN is free to dissociate and activate another effector, PLD1.

As shown in Table II and previously reported (8–10), ARF3 is a potent activator of PLD1. Without prior activation of ARF3, we saw only about a doubling of the PLD1 activity. This is the result of the short time (15 min) used in the assay with consequent limited amounts of ARF3 binding GTP G-S and the fact that the conditions used in the PLD assay are not optimal for nucleotide exchange on ARF proteins. When ARF3 was activated in a preincubation reaction, a much more robust (>75-fold) stimulation of PLD1 activity was observed. However, the inclusion of POR1ΔN in the pre-activation step of ARF3 with GTP G-S almost completely eliminated this increase in PLD activity. These data indicate that PLD1 and POR1 cannot simultaneously bind productively to ARF3, as previously reported (47). Although this may appear to suggest that the activated ARF is not free to dissociate in the active state and to interact with other effectors, such a conclusion cannot be drawn until we know more about the binding constants of active ARF for the different effectors and the relative abundance of each. The formation of relatively stable signaling complexes will have important implications for models of ARF signaling, but must await further biochemical characterization of each of the binding interactions already described as well as likely ones to be described later.

Does either the proposed low affinity state of ARF or the interaction of this species with effectors have counterparts in live cells that are of physiological importance? We cannot be certain yet, but the fact that they exist at all is suggestive of relevance to cell regulation. The findings that high stoichiometric binding of GTP to ARF3 was observed in the presence of both low and high micromolar concentrations of free magnesium and that this held true for N-myristoylated ARF preparations are consistent with the effects observed in a cellular context. We devised a simple test of the model, looking for evidence of increased ARF activation with overexpression of an effector, GGA1, and found evidence that supported the prediction. As shown in Fig. 8, those cells that expressed elevated levels of GGA1 also displayed increased amounts of ARF staining at the Golgi. In work to be reported elsewhere,3 we observed that GGA1 localizes to Golgi membranes in a brefeldin A-dependent manner. Because brefeldin A is an inhibitor of some ARF exchange factors (25, 48), such a result is suggestive of an ARF dependence on the GGA1 localization. In addition, the finding that increased levels of GGA1 promote the binding of ARFs to Golgi membranes is consistent with the feed-forward model for ARF activation in the presence of effectors described above. We cannot yet conclude that the mechanism of increased ARF binding to Golgi membranes in cells overexpressing GGA1 is accomplished through the positive feedback mechanism implied by our model, but no other model for GTPase activation makes such a prediction.

The other question related to biological relevance is the relative roles of ARF exchange factors versus positive feedback by effectors. We cannot say if one is more important than the other, as we do not yet understand in any one case what the instigating factor is in ARF activation. The abundance of specific ARF exchange factors and effectors may determine both the degree of GTP binding and the extent to which the activated ARF is free to bind other effectors present on the same membranes.

We describe an added level of complexity in the activation of ARF by effectors that requires the postulation of a low affinity state of ARF that binds specifically to “downstream” effectors. It is not particularly surprising that these effectors bind to the inactive GDP-bound form of ARF, as activation of GTPases only increases their affinity for their binding proteins. For example, the binding of GTP to G G-α only increases its affinity for its effectors, adenylyl cyclase, by a factor of 10; and the GDP-bound G G-α is able to stimulate activity to the same maximal extent (15). What is surprising is the ability of the effector to modulate ARF instead of the other way around. Although not addressed in these studies, it is possible that other regulatory GTPases could have similar interactions with effectors, with similar implications for their cellular mechanisms of activation and signaling.

The complex interactions among ARFs, nucleotides, and effectors appear more consistent with a role for ARFs in proof-reading essential steps in vesicular transport or other cellular functions. Such a model has been proposed before for the role of elongation factor Tu in protein elongation and is in contrast to that of heterotrimeric G proteins. In the latter case, it is thought to be important to have both fine temporal regulation and signal amplification. By homology to other GTPase families in the Ras superfamily and from genetic studies in yeast, it is likely that ARFs will ultimately be found to have >10 different immediate downstream effectors. To unravel these signaling pathways and to determine the biological relevance of each in different contexts will require much more detailed biochemical studies with the determination of binding affinities and rate constants for each of the proposed interactions as well as evolving models of the regulatory role(s) played by this family of GTPases.

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Effectors Increase the Affinity of ADP-ribosylation Factor for GTP to Increase Binding

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