Dual Interaction of ADP Ribosylation Factor 1 with Sec7 Domain and with Lipid Membranes during Catalysis of Guanine Nucleotide Exchange*

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Sec7 domains catalyze the replacement of GDP by GTP on the G protein ADP-ribosylation factor 1 (myrARF1) by interacting with its switch I and II regions and by destabilizing, through a glutamic finger, the β-phosphate of the bound GDP. The myristoylated N-terminal helix that allows myrARF1 to interact with membrane lipids in a GTP-dependent manner is located some distance from the Sec7 domain-binding region. However, these two regions are connected. Measuring the binding to liposomes of functional or abortive complexes between myrARF1 and the Sec7 domain of ARNO demonstrates that myrARF1, in complex with the Sec7 domain, adopts a high affinity state for membrane lipids, similar to that of the free GTP-bound form. This tight membrane attachment does not depend on the release of GDP induced by the Sec7 domain but is partially inhibited by the uncompetitive inhibitor brefeldin A. These results suggest that the conformational switch of the N-terminal helix of myrARF1 to the membrane-bound form is an early event in the nucleotide exchange pathway and is a prerequisite for a structural rearrangement at the myrARF1-GDP/Sec7 domain interface that allows the glutamic finger to expel GDP from myrARF1.

ADP ribosylation factors (ARFs)† are small G proteins of the Ras superfamily involved in intracellular trafficking (1, 2). The best studied member of this family is ARF1. Its crystal structure has been determined in several forms (3–5), and its function in the formation of transport vesicles has been well established by reconstitution studies (6, 7). ARF1 controls the binding to Golgi membranes of coatomer, a large (700 kDa) cytoplasmic complex, which upon oligomerization deforms the lipid bilayer and induces the formation of “COPI”-coated vesicles (6, 7). In addition, ARF1 activates phospholipase D (8, 9). The molecular mechanism by which ARF1 recruits coatomer to the membrane is poorly understood but is based on the ability of ARF1 to interact directly, simultaneously, and in a GTP-dependent manner both with its protein target and with lipid membranes. This dual interaction can be ascribed to two regions of ARF1. The classical switch I and II regions of the “Ras-like” core domain of ARF1 are probably the main determinants of its interaction with coatomer (10), whereas the interaction of ARF1 with membrane lipids involves a region specific to ARF1: the N-terminal α-helix, which is amphipathic and myristoylated (11–14). [Δ17]ARF1, a truncated form of ARF1 that lacks the N-terminal helix, is still able to interact with coatomer but remains soluble in the GTP-bound state and hence cannot promote the membrane recruitment of coatomer (15, 16).

The exchange of GDP for GTP on ARF proteins is catalyzed by a class of guanine nucleotide exchange factors (GEFs) that share a conserved catalytic domain of ∼200 residues, named the Sec7 domain (17–19). The Sec7 domain is found in proteins of variable size and domain organization (2). In the simplest case the Sec7 domain is flanked by a coiled-coil region and a PH domain. Recent structural and site-directed mutagenesis studies have led to a determination of the mechanism by which Sec7 domains interact with the core domain of ARF1 and promote the release of GDP (5, 20–23). Sec7 domains are characterized by a groove that contains mainly exposed hydrophobic residues and that binds the switch I and II regions of ARF1 (5, 20–23). This interaction positions an essential glutamate residue of the Sec7 domain, which lays on one edge of the groove, near the Mg2+- and β-phosphate region of the guanine nucleotide-binding site of ARF1 (5, 23). By its long negatively charged side chain this “glutamic finger” probably destabilizes GDP by displacing Mg2+ and the β-phosphate, allowing very rapid nucleotide exchange on ARF1 (23). In the recent crystal structure of nucleotide-free [Δ17]ARF1 complexed with the Sec7 domain of Gea2p, the carboxylate group of the Glu finger occupies nearly the position of the β-phosphate of the expelled GDP and is coordinated to Lys30 of ARF1, a residue that interacts with the β-phosphate of bound nucleotide (5).

Minimalistic studies with [Δ17]ARF1 and Sec7 domains have thus given a simple picture of the mechanism by which Sec7 domains induce the dissociation of bound GDP. However, catalysis of GDP/GTP exchange on authentic ARF1 (hereafter abbreviated myrARF1) is more complex than on [Δ17]ARF1 because the reaction is correlated with a change in the interaction of the G protein with membrane lipids. The substrate (myrARF1-GDP) is mostly soluble, whereas the product (myrARF1-GTP) is tightly attached to membrane lipids. This increase in membrane affinity is due to a change in the membrane exposure of the N-terminal helix of myrARF1. In the GDP-bound state, the helix is buried in a hydrophobic pocket at the surface of the core domain of ARF1 (3, 4), whereas in the GTP-bound state the hydrophobic pocket is eliminated (5) and the N-terminal helix is fully available for membrane interaction (13, 14). In addition to the membrane insertion of the

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† The abbreviations used are: ARF, ADP-ribosylation factor; ARNO-Sec7, Sec7 domain of ARNO; BFA, brefeldin A; GEF, guanine nucleotide exchange factor; [YSMD]ARNO-Sec7, mutated-form of ARNO-Sec7 carrying the following mutations: F190Y,A191S,S198D,P208M; PAGE, polyacrylamide gel electrophoresis.
myristate, this direct protein/lipid interaction allows myrARF1-GTP to be strongly attached to the membrane surface (13). At which step of the nucleotide exchange reaction does the switch of the N-terminal helix occur? The reaction proceeds through the formation of a series of ARF-Sec7 domain complexes in which the nucleotide-binding site is initially occupied by GDP, then vacant, and finally occupied by GTP. In these transient complexes, what is the state of the N-terminal helix? Is it held in the hydrophobic pocket on the core domain of ARF1 as in free ARF1-GDP, or does it interact with membrane lipids as in free ARF1-GTP?

In this paper, we have studied the binding to lipid vesicles of full-length myristoylated ARF1 (myrARF1) in complex with the Sec7 domain of ARNO, a GEF active in vitro on ARF1 and on other members of the ARF family including ARF6 (18, 24). We have taken advantage of the fact that the ARF1-Sec7 domain complex can be “frozen” at different stages of the nucleotide exchange reaction pathway, upon removal of GDP (25), mutagenesis of the essential glutamic finger of the Sec7 domain (23), or addition of the competitive inhibitor, brefeldin A (26). We show that in the complex between nucleotide-free myrARF1 and ARNO-Sec7, the key intermediate of the nucleotide exchange pathway, myrARF1 interacts tightly with membrane lipids like free myrARF1-GTP. Analysis of the membrane interaction of abortive complexes in which GDP remains bound to myrARF1 suggests that the tight membrane interaction of myrARF1 in complex with the Sec7 domain is not a consequence of Sec7-induced GDP dissociation on myrARF1. Rather, our data support the idea that the interaction of the N-terminal helix of ARF1 with membrane lipids is an early event in the Sec7-domain catalyzed nucleotide exchange reaction that is required for all subsequent steps including destabilization of GDP by the glutamic finger of the Sec7 domain. Together with previous kinetics studies (25, 27) and with the crystal structure of the soluble ARF1-Sec7 domain complex (5), these results lead to a model for coordinated conformational changes at the myrARF1/lipid membrane interface and at the myrARF1/Sec7 domain interface during catalysis of guanine nucleotide exchange.

EXPERIMENTAL PROCEDURES

Materials—Brefeldin A, alkaline phosphatase (from bovine intestinal mucosa), and all lipids, including azolectin (a mixture of soybean lipids) were purchased from Sigma. Unlabeled nucleotides were from Roche Molecular Biochemicals. [3H]GDP and all chromatography columns were from Amersham Pharmacia Biotech.

Proteins—The expression in Escherichia coli and purification of full-length myristoylated ARF1 (myrARF1), [17]ARF1 (residues 18–181 of ARF1) and the Sec7 domain of ARNO (residues 52–242 of ARNO) have been described elsewhere (12, 13, 18). MyrARF1 was purified from bacteria coexpressing bovine ARF1 and yeast N-myristoyltransferase in three steps: (i) precipitation at 35% saturation ammonium sulfate of the supernatant obtained after bacteria lysis; (ii) DEAE-Sepharose chromatography; and (iii) MonoS chromatography. The expression in E. coli of ARNO-Sec7 in the absence of lipid vesicles was analyzed by gel filtration on a superose 12 HR 10/30 column as described for the corresponding complexes with [17]ARF1 (23, 25, 26). MyrARF1-GDP (40 μM) was in a buffer containing 1 mM MgCl2 was first labeled with [3H]GDP by incubation for 15 min at room temperature with tracer [3H]GDP (final concentration, 10 μCi/ml). Labeled myrARF1-GDP (10 μM) was then incubated in buffer B for 15 min at room temperature with a stoichiometric amount of ARNO-Sec7 (either wild-type or mutant). For the interaction with wild-type ARNO-Sec7, the sample was supplemented with alkaline phosphatase (13 μg/ml, 100 units/ml). For the interaction with [E156K]ARNO-Sec7, the sample was supplemented with 2 mM EDTA (1 μM free Mg2+). For the interaction with [F190Y-A191S-S198D-P208M]ARNO-Sec7, 1 mg/ml azolectin vesicles, and 30 μg/ml alkaline phosphatase (150 units/ml) in buffer B. After 20 min at room temperature, membrane-bound proteins were recovered by centrifugation (400,000 × g, 15 min, 4 °C) and washed once with buffer A supplemented with 2 mM EDTA to remove residual alkaline phosphatase. The sample was centrifuged again, and the pellet was finally resuspended in buffer A. For fluorescence measurements, the sample was diluted in buffer A at a final concentration of 0.1 mg/ml azolectin vesicles in a 7-mm-diameter cylindrical quartz cell (13). The cuvette was continuously stirred with a small magnetic bar and thermostated at 25 °C. Fluorescence was recorded at 340 nm (bandwidth, 20 nm) upon excitation at 297.5 nm (bandwidth, 5 nm) and sampling rate of 1.0 s. The signal resulting from vesicles light scattering was subtracted. At the time indicated, GDP or GTP (final concentration, 50 μM) was added from concentrated stock solutions. The mixing time in the cuvette was about 1 s.

Gel Filtration—The formation of complexes between myrARF1 and wild-type ARNO-Sec7 or [E156K]ARNO-Sec7 or [F190Y-A191S-S198D-P208M]ARNO-Sec7 in the absence of lipid vesicles was analyzed by gel filtration on a superose 12 HR 10/30 column as described for the corresponding complexes with [17]ARF1 (23, 25, 26). MyrARF1-GDP (40 μM) in a buffer containing 1 mM MgCl2 was first labeled with [3H]GDP by incubation for 15 min at room temperature with tracer [3H]GDP (final concentration, 10 μCi/ml). Labeled myrARF1-GDP (10 μM) was then incubated in buffer B for 15 min at room temperature with a stoichiometric amount of ARNO-Sec7 (either wild-type or mutant). For the interaction with wild-type ARNO-Sec7, the sample was supplemented with alkaline phosphatase (13 μg/ml, 100 units/ml). For the interaction with [E156K]ARNO-Sec7, the sample was supplemented with 2 mM EDTA (1 μM free Mg2+). For the interaction with [F190Y-A191S-S198D-P208M]ARNO-Sec7, 0 or 50 μM BFA was added. The sample (210 μl) was loaded on the column and eluted at a flow rate of 0.5 ml/min with buffer B. When indicated, the elution buffer was supplemented with 2 mM EDTA (for the interaction with [E156K]ARNO-Sec7) or with 50 μM BFA (for the interaction with [F190Y-A191S-S198D-P208M]ARNO-Sec7. Fractions (300 μl) were collected and two samples of 60 μl were used for SDS-PAGE analysis and [3H]GDP counting.

RESULTS

Stimulation of Futile [3H]GDP/GDP Exchange on myrARF1 by the Sec7 Domain of ARNO Requires Lipid Membranes—Monitoring the rate of futile [3H]GDP/GDP exchange on myristoylated ARF1 catalyzed by the Sec7 domain of ARNO (ARNO-Sec7) eliminates two protein/lipid interactions that occur when full-length ARNO catalyzes GDP/GTP exchange on myrARF1; the tight binding of myrARF1-GTP to the bilayer and the binding of ARNO to phosphoinositides through its PH domain. Because ARNO-Sec7 is fully soluble and ARNO-Sec7-GDP interacts only weakly with membrane lipids, one might expect that ARNO-Sec7 would catalyze [3H]GDP/GDP exchange on myrARF1 in the absence of lipid vesicles. However, Fig. 4A shows that ARNO-Sec7 is very inefficient in catalyzing [3H]GDP/GDP exchange on myrARF1 in solution. In contrast, a 7-fold stimulation was observed when the same experiment was performed in the presence of a phosphoinositide bilayer.
brane binding of myrARF1 and ARNO-Sec7 was observed when 7.5, 100 mM KCl, 1 mM MgCl₂, and 1 mM dithiothreitol) supplemented cause of its conversion to the GTP-bound form (Fig. 2A, lanes 13 and 14 with lanes 5 and 6). EDTA, which chelates magnesium and hence weakens the affinity of GDP for the nucleotide-binding site of myrARF1, has a similar effect (data not shown). These results suggest that the complex between nucleotide-free myrARF1 and ARNO-Sec7 displays a high affinity for membrane lipids.

Like myrARF1-GTP, the membrane-bound complex between nucleotide-free myrARF1 and ARNO-Sec7 interacts with membrane lipids through the N terminus of ARF1. No cotranslocation of ARNO-Sec7 and ARF1 to azolectin vesicles could be detected when myrARF1-GDP was replaced by [Δ17]ARF1-GDP (data not shown). This was expected because the complex between nucleotide-free [Δ17]ARF1 and ARNO-Sec7 is soluble and can be isolated by gel filtration (23, 25). Thus, both [Δ17]ARF1 and myrARF1 are able to form a nucleotide-free complex with ARNO-Sec7, but the complex with [Δ17]ARF1 is soluble like isolated [Δ17]ARF1-GDP or [Δ17]ARF-GTP, whereas the complex with myrARF1 is tightly bound to membrane lipids, like myrARF1-GTP. It should be noted that when myrARF-GDP and ARNO-Sec7 were incubated with alkaline phosphatase in the absence of lipid vesicles and then loaded on a gel filtration column, no complex between the two proteins could be detected (data not shown). Therefore, the complex between nucleotide-free myrARF and ARNO-Sec7 is so lipophilic that it can form only in the presence of lipids.

To determine whether specific interactions with membrane lipids are required for the formation of the complex between nucleotide-free myrARF1 and ARNO-Sec7, sedimentation experiments were performed with artificial vesicles of defined lipid composition (Fig. 2B). With neutral vesicles containing 50% phosphatidylethanolamine and 50% phosphatidylglycerol, about 70% of ARNO-Sec7 and myrARF1 were found associated with vesicles after incubation in the presence of alkaline phosphatase. With 5% phosphatidylserine and 5% phosphatidic acid in the vesicles, the amount of myrARF1 and ARNO-Sec7 bound to the lipid vesicles was slightly enhanced. This suggests that the complex between nucleotide-free myrARF1 and ARNO-Sec7 interacts with membrane lipids mainly through hydrophobic interactions and that the contribution of electrostatic interactions with anionic lipids is weak. By its hydrophobic nature and the essential role of the N terminus of ARF1, the membrane interaction of nucleotide-free myrARF1 in complex with ARNO-Sec7 closely resembles that of myrARF1-GTP (13).

To verify that the cotranslocation of myrARF1 and ARNO-Sec7 to vesicles in the presence of alkaline phosphatase corresponded to the formation of a stoichiometric complex between nucleotide-free myrARF1 and ARNO-Sec7, sedimentation experiments were performed with a constant amount of myrARF1-GDP (3 μM) and varying amounts of ARNO-Sec7. Fig. 3A shows that the amount of ARNO-Sec7 in the lipid pellet was carried out in the presence of 2 mg/ml azolectin vesicles (Fig. 1B). Thus, apart from being essential for the stabilization of myrARF1 in the GTP bound state, membrane lipids are also essential for the catalysis of GDP release on myrARF1 by the Sec7 domain of ARNO. This is in agreement with a previous study on an unidentified ARF exchange factor from bovine retina (27) and suggests that productive interaction between myrARF1 and ARNO-Sec7 can occur only at the surface of lipid membranes.

Cotranslocation of myrARF1 and ARNO-Sec7 to Lipid Vesicles—The effect of lipid vesicles on the association of myrARF1 with the Sec7 domain of ARNO was assessed by sedimentation experiments. MyrARF1-GDP or ARNO-Sec7 or a stoichiometric mixture of the two proteins was incubated with azolectin vesicles in an isotonic buffer containing 1 mM MgCl₂. After centrifugation, the amount of protein in the pellet and in the supernatant was determined by scanning Coomassie-stained polyacrylamide gels. In the presence of 0.5 mg/ml azolectin vesicles, myrARF1-GDP displayed weak membrane binding (Fig. 2A, lanes 1 and 2), in agreement with previous studies (12, 13). Under the same conditions, ARNO-Sec7 was totally soluble (Fig. 2A, lanes 3 and 4). When the same experiment was repeated with a stoichiometric mixture of the two proteins, a significant amount (20%) of ARNO-Sec7 was found in the lipid pellet, and the amount of membrane-bound myrARF1 doubled (Fig. 2A, lanes 5 and 6). If ARNO-Sec7 displayed no preference for membrane-bound myrARF1 versus membrane-free myrARF1, it should not affect the membrane partitioning of myrARF1. Therefore, the synergy between the two proteins for their binding to lipid vesicles suggests that a complex between myrARF1 and ARNO-Sec7 displays a higher “affinity” for lipid membranes than isolated myrARF1-GDP and isolated ARNO-Sec7.

The Membrane-bound Complex between myrARF1 and ARNO-Sec7 Is Devoid of Nucleotide and Interacts with Membrane Lipids through the N Terminus of ARF1—To determine whether myrARF1 in the membrane-bound complex with ARNO-Sec7 has lost its nucleotide, as is usually the case for functional complexes between GEFs and G proteins, sedimentation experiments were repeated in the presence of excess GDP or GTP. With 100 μM GDP, no enhancement in the membrane binding of myrARF1 and ARNO-Sec7 was observed when the two proteins were incubated together (Fig. 2A, lanes 7 and 8). With 100 μM GTP, ARNO-Sec7 was found soluble, whereas myrARF1 became totally membrane-bound, as expected because of its conversion to the GTP-bound form (Fig. 2A, lanes 9 and 10). Thus, the synergy between myrARF1 and ARNO-Sec7 for their binding to lipid vesicles observed in the absence of added nucleotide probably reflects the formation of a membrane-bound complex between myrARF1 and ARNO-Sec7, in which the nucleotide-binding site of myrARF1 is vacant. However, even when carried out in the absence of added nucleotide, these experiments were performed under conditions that do not favor the stabilization of the nucleotide-free complex between myrARF1 and ARNO-Sec7. Indeed, the stoichiometric GDP that was initially associated with myrARF1 could antagonize the formation of the nucleotide-free complex with ARNO-Sec7. Therefore, the experiment was repeated in the presence of a catalytic amount of alkaline phosphatase which hydrolyzes GDP as it is released from myrARF1. Strikingly, under these conditions myrARF1 and ARNO-Sec7 bound almost completely to azolectin vesicles (Fig. 2A, compare lanes 13 and 14 with lanes 5 and 6). EDTA, which chelates magnesium and hence weakens the affinity of GDP for the nucleotide-binding site of myrARF1, has a similar effect (data not shown). These results suggest that the complex between nucleotide-free myrARF1 and ARNO-Sec7 displays a high affinity for membrane lipids. Figure 1 shows that the amount of ARNO-Sec7 in the lipid pellet
saturated at about 2.5 μM, indicating a roughly 1:1 stoichiometry between myrARF1 and ARNO-Sec7. We then determined whether, in this 1:1 complex with ARNO-Sec7, nucleotide-free myrARF1 was functional, i.e. able to bind GDP or GTP very rapidly. To test this, we used the tryptophan fluorescence of ARF1, which is extremely sensitive to the conformation of the protein (13, 28). We first prepared the membrane-bound complex between myrARF1 and ARNO-Sec7 by incubating stoichiometric amounts of myrARF1-GDP and ARNO-Sec7 with azolectin vesicles and alkaline phosphatase as in Figs. 2 and 3A. Membrane-bound proteins were recovered by centrifugation, washed with buffer to remove residual alkaline phosphatase, and finally diluted at a final concentration of 0.1 mg/ml azolectin vesicles in a quartz cuvette. The fluorescence of the sample was continuously recorded upon the addition of 50 μM GDP or GTP. As shown in Fig. 3B, a fluorescence increase was observed upon the first addition of GTP, whereas a decrease was observed upon the first addition of GDP. In both cases the signal was too fast to be resolved and a second addition of nucleotide had no effect on the fluorescence signal. This demonstrates the ability of nucleotide-free myrARF1 in complex with ARNO-Sec7 and at the surface of azolectin vesicles to bind GDP very rapidly with GDP or GTP. These results also indicate that in the complex with ARNO-Sec7, nucleotide-free myrARF1 displays a fluorescence level that is intermediate between the fluorescence of myrARF1-GDP and that of myrARF1-GTP. Similar observations were made for the soluble complex between nucleotide-free [Δ17]ARF1 and ARNO-Sec7 (data not shown).

Sec7-induced GDP Dissociation on myrARF1 Is Not Required for the Membrane Association of the myrARF1-ARNO-Sec7 Complex—To determine whether, in the complex between nucleotide-free myrARF1 and wild-type ARNO-Sec7, the tight interaction of myrARF1 with membrane lipids was a consequence of the dissociation of GDP induced by ARNO-Sec7, we used a mutated form of ARNO-Sec7 (E156K) that lacks the glutamic residue involved in the destabilization of GDP on ARF1. [E156K]ARNO-Sec7 interacts with [Δ17]ARF1-GDP in solution but does not promote GDP release (23). The interaction is observed only at low Mg2+ level (1 μM), suggesting a steric hindrance between the mutated residue 156 of ARNO-Sec7 and Mg2+, which is associated with GDP in the nucleotide-binding site of ARF1 (23). We first noticed that in solution, and under conditions where a 1:1 complex between [Δ17]ARF1-GDP and [E156K]ARNO-Sec7 could be isolated by gel filtration (1 μM free Mg2+), no interaction between myrARF1-GDP and [E156K]ARNO-Sec7 could be detected (data not shown). We then examined whether the two proteins could interact in the presence of azolectin vesicles. Fig. 4 shows that myrARF1-GDP and [E156K]ARNO-Sec7 cotranslocated to azolectin vesicles when the magnesium concentration was lowered to 1 μM. This effect was not abolished by the addition of an excess of GDP in contrast to what was observed with wild-type ARNO-Sec7 (Fig. 4; see also Fig. 2A, lanes 7 and 8). This suggests that an abortive complex between myrARF1-GDP and [E156K]ARNO-Sec7 forms at low Mg2+ and that in this complex, myrARF1-GDP has a higher affinity for membrane lipids than isolated myrARF1-GDP. It should be noted that the extent of cotranslocation of [E156K]ARNO-Sec7 with myrARF1-GDP was lower as compared with the effect observed for the complex between nucleotide-free myrARF1 and wild-type ARNO-Sec7 (compare Figs. 2 and 4). However, at the concentration of proteins used (3 μM) and given the moderate affinity (~1 μM) of [E156K]ARNO-Sec7 for [Δ17]ARF1-GDP (23), only about 60% of total protein was expected to be in the form of complex. Taken together, the results of these gel filtration and sedimentation assays suggest that tight interaction of myrARF1 with membrane lipids while interacting with ARNO-Sec7 does not depend on the catalytic activity of the Sec7 domain.

The Abortive myrARF1-GDP-Sec7 Domain Complex Blocked by BFA Exhibits a Moderate Affinity for Membrane Lipids—The fungal metabolite BFA provides another tool to study the interplay between the interactions of myrARF1-GDP with the Sec7 domain and with membrane lipids. Gel filtration and
kinetics studies with [17]ARF1 have shown that BFA acts as an uncompetitive inhibitor of Sec7 domain-catalyzed nucleotide exchange on ARF1 by stabilizing an abortive ARF1-GDP-Sec7 domain complex (26). The sensitivity of Sec7 domains to BFA can be modulated by specific mutations in a region that overlaps the ARF1-binding site (26, 29). We have generated a structural rearrangements at the interface between the core domain of myrARF1 and the Sec7 domain (26), this is another argument for an interplay between this interface and the in

We first determined by gel filtration analysis whether [YSMD]ARNO-Sec7 either in the absence or in the presence of 50 μM BFA, and the mixture was loaded on a superose-12 column equilibrated with the same concentration of BFA (Fig. 5A). In the absence of BFA, the two proteins eluted in two peaks, corresponding to the elution profiles of isolated [YSMD]ARNO-Sec7 and myrARF1-GDP. In contrast, with 50 μM BFA about 30% of myrARF1 coeluted with [YSMD]ARNO-Sec7. This fraction remains in the GDP-bound form, because the elution profile of [3H]GDP matches the elution profile of myrARF1 as determined by SDS-PAGE. Therefore, BFA promotes the formation of an abortive complex between myrARF1-GDP and [YSMD]ARNO-Sec7 in solution. Strikingly, this complex is the only soluble complex between myrARF1 and a Sec7 domain that we have been able to detect by gel filtration (see above). We then looked for the effect of lipid vesicles on the formation of the abortive complex between BFA, myrARF1-GDP, and [YSMD]ARNO-Sec7. Stoichiometric amounts of [YSMD]ARNO-Sec7 and myrARF1-GDP were incubated with azolectin vesicles in the presence of an excess of GDP and with or without BFA (Fig. 5B). In the absence of BFA, [YSMD]ARNO-Sec7 was mostly soluble. With increasing concentration of BFA, [YSMD]ARNO-Sec7 became partially (~40%) membrane-bound. This membrane association was strictly dependent on the presence of myrARF1-GDP. Therefore, BFA induces the formation of an abortive complex between myrARF1-GDP and [YSMD]ARNO-Sec7 at the membrane surface. However, the synergy between the two proteins for their binding to lipid vesicles was modest. Indeed, the membrane partitioning of myrARF1-GDP was only slightly increased, reaching a maximal value of 40% at saturating concentration of BFA. Taken together, the results of sedimentation and gel filtration assays suggest that in the complex between myrARF1-GDP and [YSMD]ARNO-Sec7 blocked by BFA, myrARF1 does not acquire a high affinity state for membrane lipids but rather displays the moderate membrane partitioning of free myrARF1-GDP. Because BFA might block structural rearrangements at the interface between the core domain of myrARF1 and the Sec7 domain (26), this is another argument for an interplay between this interface and the interface of myrARF1 with membrane lipids.
DISCUSSION

Interaction of myr-ARF1 with membrane lipids is mediated by its myristoylated and amphipathic N-terminal helix (11–14). Because [Δ17]ARF1 lacks this helix, it has been an exquisite tool for biochemical and structural studies of the ARF1/Sec7 domain interface, including the delineation of the interacting surfaces (21, 23), the identification of the glutamic finger in the Sec7 domain (20), and the determination of the mechanism by which some Sec7 domains are inhibited by BFA (26). Studies using [Δ17]ARF1 have culminated in the determination by Goldberg of the crystal structures of [Δ17]ARF either bound to a GTP analog or in complex with the Sec7 domain of Gea2p (5). However in a recent paper, Pacheco-Rodriguez et al. (30) have questioned the validity of this latter crystal structure. They claimed that the N terminus of ARF1 must be involved in the interaction of ARF1 with GEFs, because the rate of GDP/GTP exchange catalyzed by cytohesin or its Sec7 domain is affected by mutations in the N-terminal helix. Here we show that throughout the nucleotide exchange reaction pathway, the N terminus of myrARF1 interacts with membrane lipids as a prerequisite to catalysis of guanine nucleotide exchange by the Sec7 domain. These results are in perfect agreement with what can be inferred from the crystal structure of the complex between nucleotide-free [Δ17]ARF1 and Gea2p-Sec7 (5, 31).

In Fig. 6A, sequential intermediates of the nucleotide exchange reaction have been schematized. The whole switch region in the core domain of myrARF1 is represented as a bold trace. This includes the classical switch I and II regions, which face the cytosol and interact with the Sec7 domain (5, 20–23) and a β-hairpin formed by two strands (β2 and β3) and a connecting loop (α3), which crosses the whole core domain of myrARF1. On the membrane side of myrARF1, the N-terminal helix is shown either bound to a hydrophobic pocket of the core domain of ARF1 or laid on the surface of the lipid bilayer. These are probably the only stable states for such a myristoylated and amphipathic helix. First, it is important to note the major difference between the “substrate” (myrARF1-GDP) and the “product” (myrARF1-GTP) of the reaction, namely that mem-

![Image](http://example.com/image1.png)
brane exposure of the helix is coupled to the position of the β-hairpin (5). Hence myrARF1-GDP (state 1 in Fig. 6A) is mostly soluble because loop λ3 does not prevent (and in fact contributes to) the interaction of the N-terminal helix with the core domain of ARF1 (3, 4). In contrast, myrARF1-GTP (state 5 in Fig. 6A) is tightly attached at the membrane surface because translation of the β-hairpin has eliminated the hydrophobic pocket for the helix, thereby freezing the helix in a membrane-bound state (5, 13).

Because myrARF1-GDP and its GEFs are found mostly in the cytosol, one could imagine a reaction scheme in which GDP/GTP exchange on myrARF1 would occur in the cytosol and would be followed by the translocation of free myrARF1-GTP to the membrane. However, all the results presented in this study as well as previous kinetics studies (25, 27) strongly favor a model in which all steps of the exchange reaction occur at the surface of the lipid membrane. First, the Sec7 domain promotes GDP release on myrARF1-GDP only in the presence of lipid vesicles (Fig. 1). Second, myrARF1 does not form a nucleotide-free complex with ARNO-Sec7 in solution. However, in the presence of lipid vesicles and under conditions that favor the stabilization of the nucleotide-free state, a 1:1 functional complex between myrARF1 and ARNO-Sec7 can be isolated on vesicles (Figs. 2A and 3). Like myrARF1-GTP, this complex forms even with neutral lipid vesicles (Fig. 2B). These results strongly suggest that in this nucleotide-free complex, the N-terminal helix of ARF1 interacts with membrane lipids as in myrARF1-GTP. This is exactly what can be inferred from the crystal structure of nucleotide-free [Δ17]ARF1 in complex with Gea2p-Sec7 (5, 31). In this structure, the hydrophobic pocket of ARF1 that serves as a binding site for the N-terminal helix in the GDP-bound form is occupied by loop λ3. If we extrapolate this structure to myrARF1 (state 4 in Fig. 6), it is clear that the nucleotide-free state can be reached only if the N-terminal helix interacts with membrane lipids, otherwise either a steric clash would occur or hydrophobic residues of the helix would be exposed to solvent. In summary, not only the dissociation of GDP but also the translation of the β-λ3-β3 hairpin and the tight interaction of the N-terminal helix of ARF1 with membrane lipids occur in the pathway between the cytosolic myrARF1-GDP form (state 1 in Fig. 6) and the nucleotide-free complex with the Sec7 domain (state 4 in Fig. 6).

Surprisingly, the tight membrane association of myrARF1 with membrane lipids is not a consequence of the dissociation of GDP induced by the glutamic finger of the Sec7 domain. Indeed, even the catalytically inactive form [E156K]ARNO-Sec7 interacts with myrARF1-GDP only in the presence of lipid membranes (Fig. 4). The interpretation of this result is somehow biased by the fact that in the complex with [E156K]ARNO-Sec7, the nucleotide-binding site of myrARF1-GDP is not exactly as in free myrARF1-GDP, because only the latter contains Mg2++. However, the cotranslocation of myrARF1-GDP with [E156K]ARNO-Sec7 to lipid vesicles suggests a coupling between the membrane interaction of the N-terminal helix of ARF1 and the interaction of ARF1 with the Sec7 domain, regardless of the catalytic activity of the Sec7 domain. How can the membrane-binding surface of myrARF1 (the N-terminal helix) be connected to its cytosolic face (the switch I and II regions), which interacts with the Sec7 domain? Again, the answer is given by the translation of the β-λ3-β3 hairpin. This hairpin, whose sliding along the central β-sheet of the protein can either create or eliminate a hydrophobic pocket for the N-terminal helix, is flanked by the switch I and II regions that form the binding surface for the Sec7 domain (5). We had previously proposed a docking model for the interaction of ARF1-GDP with a Sec7 domain (23). Although this model nicely anticipated the relative orientation of the two interacting surfaces, the crystal structure of nucleotide-free [Δ17]ARF1 in complex with Gea2p-Sec7 revealed that the switch I and II regions undergo dramatic rearrangements to form a larger interacting surface with the groove of the Sec7 domain (5).

Therefore, we propose a sequential model in which, thanks to a reasonable complementarity between the switch I and II regions of myrARF1-GDP and the groove of the Sec7 domain, the two proteins first form a “docking” complex of low affinity (states 3 and 6 in Fig. 6A). Then, the switch I and II regions reorganize to yield larger contacts with the groove of the Sec7 domain (state 4). This transition leads to the destabilization of the bound GDP by the glutamic finger whose carboxylate group becomes ideally positioned to displace the β-phosphate group. However, destabilization of GDP is not required *per se* for the reorganization of the myrARF1-Sec7 domain interface (Fig. 6C). Instead, the critical determinant for this transition is the lipid membrane. The switch I and II regions can reorganize only in concert with the translation of the β-hairpin (5). Because this translation is not possible when the N-terminal helix is bound to the protein core, any interaction in solution between myrARF1-GDP and the Sec7 domain is a dead end (state 6 in Fig. 6A). However, myrARF1-GDP is not completely soluble but binds moderately to lipid membranes (12). This weak membrane interaction, which requires the myristate, dictates the rate at which myrARF1-GDP can be activated (Refs. 12 and 27; see also Fig. 1). We suggest that, at the membrane surface, the N-terminal helix of myrARF1-GDP flickers between a “closed” conformation and an “open” (membrane-bound) conformation (state 2 in Fig. 6A). This flickering opens a time window for the rearrangements of the switch I and II regions of ARF1 and the translation of the β2-β3 hairpin that are induced by the Sec7 domain. This provides a reasonable model for the essential role of the weak membrane binding of myrARF1-GDP in the nucleotide exchange reaction (12, 25, 27).

In our model, destabilization of the N-terminal helix by membrane lipids allows Sec7-induced conformational changes on the switch I and II region of ARF1. An alternative model would be that once the N-terminal helix of myrARF1-GDP swings out from the core domain of ARF1-GDP and interacts with the lipid membrane, the whole switch region would reorganize spontaneously before the binding of the Sec7 domain. However, we do not favor this hypothesis. First, [Δ17]ARF1-GDP and ARF1-GDP adopt the same structure (5), indicating that removal of the N-terminal helix does not induce by itself the switch to a “GTP-like” conformation. Second, the uncompetitive mechanism of BFA inhibition (26) favors a model in which the reaction starts with a docking complex, i.e. a complex in which myrARF1-GDP keeps the features of the free form, including a moderate affinity for membrane lipids (states 3 and 6 in Fig. 6). In contrast to other complexes with myrARF1, the abortive complex between myrARF1-GDP and [YSMD]ARNO-Sec7 blocked by BFA can be observed both in solution and on the lipid vesicles (Fig. 5). By blocking rearrangements at the myrARF1-GDP/Sec7 domain interface (26), BFA would also inhibit rearrangements at the ARF1/lipid interface (Fig. 6B).

It must be noted that the general features of the scheme shown in Fig. 6A also applies for the nonmyristoylated form of full-length ARF1 that was used by Pacheco-Rodriguez et al. (30). Indeed, the presence of the amphipathic helix makes translation of the β-hairpin very unfavorable in solution. Therefore, as for myrARF1 but in contrast to [Δ17]ARF1, the presence of lipid membranes is required for efficient catalysis of nucleotide exchange by GEFs and for the stabilization of the GTP-bound form (11, 25). However, nonmyristoylated ARF1 is a very bad substrate for GEFs because the lack of myristate
reduces dramatically the ability of the helix to be destabilized by the lipid membrane and to remain tightly associated with it throughout the reaction (25). In other words, the lack of myristoylation does not exempt ARF1-GDP from taking a “membrane route” but makes this route much harder. As compared with myrARF1 or Δ17ARF1, nonmyristoylated ARF1 is therefore not the ideal form for exploring the ARF/GEF interface. Any mutation in the N-terminal helix could have an effect on the rate of nucleotide exchange simply because of its effect on the ability of the helix to interact with membrane lipids or with the core domain of ARF1 or to adopt a solvent exposed conformation. Hence this type of analysis alone cannot be used to demonstrate a direct interaction of the N-terminal helix with a GEF without first verifying the effect of the mutation on ARF1/membrane interaction.

The fully soluble Sec7 domain of ARNO was used here to focus only on the membrane interaction of myrARF1 during the exchange reaction. Because myrARF1-GDP can be activated only at the surface of a lipid membrane, the membrane localization of a Sec7 domain through an adjacent region such as a PH domain provides a dramatic catalytic advantage (18, 25, 32). Therefore, the mechanisms by which GEFs for ARFs are translocated to a given membrane will determine when and on which compartment the large pools of ARF proteins will be used and turned on (33, 34).

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