The Amino Terminus of ADP-ribosylation Factor (ARF) 1 Is Essential for Interaction with G\textsubscript{s} and ARF GTPase-activating Protein* 

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The role of the amino terminus in the actions of ADP-ribosylation factor 1 (ARF1) was examined by comparing wild type ARF1, a 13-residue NH\textsubscript{2}-terminal deletion mutant ([\textDelta]13ARF1), and a 17-residue NH\textsubscript{2}-terminal deletion mutant ([\textDelta]17ARF1). The amino-terminal 13 residues of ARF1 are required for cofactor activity in the ADP-ribosylation by cholera toxin when G\textsubscript{s} is the substrate. This is in marked contrast to the finding that cofactor activity is the same for wild type and [\textDelta]13ARF1 when agmatine is substrate (Hong, J.-X., Haan, R. S., Tsai, S.-C., Moss, J., and Vaughan, M. (1994) J. Biol. Chem. 269, 9743-9747). These data support the conclusion that ARF1 interacts with both cholera toxin and G\textsubscript{s} and that the amino terminus of ARF1 is required specifically for binding G\textsubscript{s}. Surprisingly, this result also clearly revealed that the two principal assays for ARF activity, cofactor activity for cholera toxin using either G\textsubscript{s} or agmatine as substrates, used for over 10 years in different laboratories, can yield quite different results.

While both NH\textsubscript{2}-terminal deletion mutants failed to support the ADP-ribosylation of G\textsubscript{s} by cholera toxin, [\textDelta]13ARF1, but not [\textDelta]17ARF1, inhibited the activity of the wild type protein. The GTPase activity of [\textDelta]13ARF1 was activated to a small extent by ARF GTPase-activating protein (GAP), whereas that of [\textDelta]17ARF1 was unaffected. We conclude that residues 14-17 are involved in the interaction of ARF with both cholera toxin and ARF GAP.

The co-purifying nucleotides, nucleotide exchange kinetics, and dependence of exchange on phospholipids for the mutant proteins were all different from the wild type ARF1 proteins. The importance of monitoring the nucleotide binding to ARF proteins under the conditions used in the ARF assay and expressing ARF activities as specific activities, normalized to GTP binding sites, particularly when comparisons between different proteins or preparations are made, is discussed.

ADP-ribosylation factors (ARF)\textsuperscript{1} are a family of GTP-binding proteins that include the ARFs and the structurally related ARF-like (ARL) proteins (1, 2). Several members of the ARF family are ubiquitous and highly conserved in both structure and function in eukaryotes (2). They are essential in Saccharomyces cerevisiae (ARF1 and ARF2) (3) and in Drosophila melanogaster (arflike) (4). ARF proteins have been implicated as regulators of a number of steps in both the exocytic and endocytic transport pathways (5, 6) and as an activator of phospholipase D activity (7, 8).

ARF was originally purified as the protein cofactor for cholera toxin-catalyzed ADP-ribosylation of G\textsubscript{s}, the G-protein that regulates activation of adenylate cyclase and is the major cellular substrate for cholera toxin. This covalent modification of G\textsubscript{s} results in the persistent activation of adenylate cyclase, leading to the imbalances in salt and fluid retention associated with cholera. By reconstituting this reaction with purified components, ARF was found to be a required cofactor and was first purified from membrane using the activation of G\textsubscript{s} as a quantitative assay for ARF (9, 10). The purified protein allowed the cloning of the first ARF cDNA (11). Parallel studies originally focusing on the mechanism of action of cholera toxin led to the development of an ARF assay which employs the arginine analogue, agmatine, as substrate for cholera toxin (12). Using this assay ARF was first purified from soluble sources and led to the cloning of bovine ARF3 (13). ARF1 and ARF3 proteins are 96% identical and apparently indistinguishable in these two assays. As these assays each quantify cholera toxin cofactor activity and have yielded very similar results over the past 10 years it has been assumed that they are interchangeable. However, this assumption has never been examined directly.

ARF1 is a ubiquitous and abundantly expressed, N-myristoylated, GTP-binding protein that co-purifies with tightly bound GDP (10). The binding of GTP is required for expression of ARF activity, using either G\textsubscript{s} or agmatine as ADP-ribose acceptors. The binding of GTP, or the slowly hydrolyzable GTP analog GTP\textsuperscript{\gamma}S, to ARF in vitro is highly dependent on phospholipids (10, 14) and is sensitive to the concentrations of divalent metals and detergents. Under conditions which promote the formation of ARF-GTP, GTP stimulates the ADP-ribosylation of G\textsubscript{s} by cholera toxin more than 50-fold. Thus, stimulation of the ADP-ribosylation of G\textsubscript{s} by cholera toxin is a highly specific and quantitative biochemical assay for the activated protein, ARF-GTP. However, because of the enormous differences in nucleotide binding observed under different conditions the use of specific activities, i.e. normalizing cofactor activity to ARF-GTP determined under identical assay conditions, is particularly important when comparing different ARF proteins or preparations. Modification of agmatine by cholera toxin is also dependent on ARF and GTP but fewer studies are available which correlate the binding of the activating nucleotide with cofactor activity.

Among the distinguishing structural features of ARF proteins is a 14–16-amino acid extension at the amino terminus, evident after alignment with other Ras superfamily members. A mutant lacking the first 17 amino acids, termed [\textDelta]17ARF1, lost both dependence on phospholipids for GTP binding and all activity in the cofactor assay, using G\textsubscript{s} as substrate (15). Cons-

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1 The abbreviations used are: ARF1, human ADP-ribosylation factor 1; [\textDelta]17ARF1, ARF1 with the 17 amino-terminal residues deleted; [\textDelta]13ARF1, ARF1 with the 13 amino-terminal residues deleted; ARF GAP, ARF GTPase-activating protein; DMPC, 1,2-dimyristoyl phosphatidylcholine; GTP\textsuperscript{\gamma}S, guanosine 5'-O-(3-thiotriphosphate); HPLC, high performance liquid chromatography.
versely, replacing the amino terminus of Drosophila Arf1 with the 17 amino-terminal residues of ARF1 was sufficient to bellow both ARF activity and increased dependence on phospholipids for GTP binding on the chimera (15). Thus both loss of function and gain of function were tightly linked to the presence of the amino terminus.

The conclusion that the amino terminus of ARF1 is an essential functional domain has been challenged recently by Hong et al. (16). Another NH2-terminal mutant, in which the 13 NH2-terminal amino acids were deleted, [Al3]ARF1, was found to stimulate the cholera toxin-catalyzed transfer of ADP-ribose to agmatine to a similar extent (5-10 fold over cholera toxin alone) as did ARF1. Further, this activity was independent of added GTP. These data were interpreted as evidence that the amino terminus of ARF is not critical for activity (16).

These data have motivated us to re-examine the role of the NH2-terminus in ARF activities by directly comparing the two NH2-terminal deletion mutants and wild type protein in ARF and related assays. One of two explanations for the different conclusions seemed likely. Either residues 14-17 are critical for ARF activity or the assays used by the different laboratories are different in some fundamental way. Surprisingly, we conclude that these two assays of cofactor activity yield very different results as a result of the different cholera toxin substrates utilized. We also conclude that residues 14-17 are not critical for ARF (G1) activity but are implicated in the binding of ARF-GTP to both ARF GTP and cholera toxin.

EXPERIMENTAL PROCEDURES

Proteins—Recombinant ARF1 (14), [Al7]ARF1 (15), recombinant G, (17) and bovine brain G, (18) were prepared as described elsewhere. [Al3]ARF1 was prepared by polymerase chain reaction amplification using a sense primer that inserts an NdeI site and an initiating methionine residue immediately 5' of residue 14 and an antisense primer that adds a BamHI site 6 base pairs 3' of the stop codon. The resulting open

| Protein     | ARF activity | GAP activity | GDP dissociated | GTPyS bound |
|-------------|--------------|--------------|-----------------|-------------|
| ARF1        | 0.007 ± 0.001 | 0.013 ± 0.005 | 0.005 ± 0.001  | 0.168 ± 0.013 |
| [Al3]ARF1   | 0.013 ± 0.001 | 0.038 ± 0.005 | 0.030 ± 0.005  | 1.02 ± 0.03  |
| [Al7]ARF1   | 0.030 ± 0.005 | 1.06 ± 0.002  | 0.020 ± 0.001  | 0.007 ± 0.001 |

TABLE I

Comparison of activities of ARF1, [Al3]ARF1, and [Al7]ARF1

FIG. 1. Effect of [Al3]ARF1 and ARF1 on cholera toxin catalyzed ADP-ribosylation of Gs. Cholera toxin catalyzed ADP-ribosylation of Gs in the presence of 2 μM ARF1 (squares), 2.5 μM [Al3]ARF1 (triangles), 24 μM [Al7]ARF1 (inverted triangles), or no further addition (circles) was determined as described under "Experimental Procedures." Data are the average of duplicate samples which differed by less than 10%.

FIG. 2. Time course of GTPyS binding to ARF1, [Al3]ARF1, and [Al7]ARF1. GTPyS binding was determined for ARF1 (circles), [Al3]ARF1 (triangles), and [Al7]ARF1 (inverted triangles) in the presence (filled symbols) or absence (open symbols) of DMPC. GTPyS binding to ARF1 (open squares) and [Al3]ARF1 (filled squares) was also determined in the presence of 0.0005% SDS and 5 mM MgCl2 (squares) as described under "Experimental Procedures." All proteins were 1 μM except [Al7]ARF1 which was 1.1 μM. Insert, magnified scale showing ARF1 data in more detail.
reading frame was subcloned into the pET3C vector before transfection into BL21(DE3) cells (14). Bacteria containing a vector directing expression of [Δ13]ARF1 were also generously provided by Dr. Martha Vaughan (16). Both proteins were purified as described for ARF1 (19) with yields of 25 and 40 mg/liter of bacterial culture, respectively. Thermal cycle DNA sequencing was performed using the Promega kit (fmol Sequencing System, Promega Corp., Madison, WI) to confirm sequences of each mutant. As expected, the [Δ13]ARF1 proteins behaved identically in all respects.

HPLC—Proteins were heated to 95 °C for 10 min and the released nucleotide was separated from the denatured protein by centrifugation in a Centricon 30 filtration apparatus. Filters prepared from 2–20 nmol of protein were fractionated using a Whatman analytical 10-μm Partisil SAX column equilibrated in 7 mM ammonium phosphate, pH 3.8 (buffer A) at a flow rate of 2 μl/min. The chromatograms were developed in a gradient from 100% buffer A to 100% 250 mM ammonium phosphate, pH 4.75, 500 mM KCl, over 30 min and monitored by absorption at 254 nm. To verify peak positions and to estimate recoveries, filtrates of samples to which known amounts of GTP and GDP were added prior to sample preparation were also fractionated. Between 0.5 and 1.0 mol of nucleotide was recovered/mol of ARF in each instance.

GTP\(\gamma\)S Binding—Binding reactions contained 1 μM ARF and 10 μM GTP\(\gamma\)S (~10,000 cpm/pmol) in exchange buffer of 25 mM HEPES, pH 7.4, 100 mM NaCl, 0.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, and, where indicated, 3 mM L-D-mimerythylphosphatidylcholine (DMPC) and 0.1% sodium cholate as previously described (10). Where indicated, binding was also determined in 50 mM KP\(_2\), pH 7.4, 5 mM MgCl\(_2\), 20 mM dithiothreitol, 0.003% sodium dodecyl sulfate, 0.5 mM ATP, and 50 μg/ml bovine serum albumin (16). Samples (10 μl) were taken at the indicated times, and binding was determined by rapid filtration on nitrocellulose filters as described elsewhere (20).

Nucleotide Dissociation—ARF (1–4 μM) was incubated with carrier-free \(^{32}\)P-GDP (25 Ci/mmol, 50 μCi/ml) for 2–3 h in exchange buffer to radioactively label the nucleotide binding site. Dissociation was determined after a 10-fold dilution of the ARF-GDP into a exchange buffer containing 1 mM GDP. Samples (10 μl) were withdrawn at 10–15 time points at 0–120 min. The data were fit to a single exponential decay equation using the Marquardt algorithm implemented by Fig. P ( Biosoft) or Ultrafit (Biosoft), and the calculated constants are presented ± standard errors.

ARF GAP—The ARF GAP assay was performed as previously described (21) in the presence of 1 mg/ml phosphoinositides, 10–80 mM ARF-GTP as substrate, and 10–87 μg/ml of partially purified ARF GAP from bovine brain (21). ARF GAP specific activity (ARF-GTP hydrolyzed min/mg GAP/ml) was calculated as previously described (21).

ARF Assay—The ARF/GTP assay was performed as described previously, using 0.5 μM chola toxin, 0.25 μM recombinant G\(_{x}\), and the indicated concentrations of ARF proteins (10). This assay measures the ability of ARF to stimulate the chola toxin catalyzed corvonal incorporation of ADP-ribose from [α\(^{32}\)P]NAD into G\(_{x}\). G\(_{x}\) subunits are required in the assay and are supplied as a preparation from bovine brain containing a small amount of ARF as a contaminant. ADP-ribose incorporation in the absence of added recombinant ARF was subtracted as background. Parallel GTP\(\gamma\)S binding to ARF was performed using the same conditions but in the absence of G\(_{x}\). ARF activity was then normalized to the amount of GTP\(\gamma\)S bound under the same conditions and specific activity was expressed as fmol ADP-ribose-G\(_{x}\)/pmol GTP\(\gamma\)S bound/20 min.

Miscellaneous—[α\(^{32}\)P]GTP, [\(^{35}\)S]GTP\(\gamma\)S, [\(^{32}\)P]NAD, and [\(^{3}H\)GDP, and [\(^{32}\)P]NAD were purchased from DuPont-NEN. L-D-mimerythyl phosphatidylcholine, phosphoinositides (catalog no. P-6023), G\(_{x}\), NAD, ATP, and Triton X-100 were purchased from Sigma. Sodium cholate was obtained from Fluka. GTP\(\gamma\)S, pyruvate kinase, and phospho(eno1)pyruvate were obtained from Boehringer Mannheim. Protein concentrations were determined using the Amido Black assay (22).

**RESULTS AND DISCUSSION**

To test the hypothesis that residues 2–13 or 14–17 are critical determinants of ARF activity we compared wild type, [Δ13]ARF1, and [Δ17]ARF1 in the ARF cofactor assay, using G\(_{x}\) as substrate. Neither [Δ13]ARF1 nor [Δ17]ARF1 had any activity in this assay. A time course for ADP-riboseylation of G\(_{x}\) is shown in Fig. 1, and specific activities normalized to ARF-GTP formed are presented in Table I. In the presence of 0.5 μM ARF, ADP-riboseylation increased with time over a 20 min incubation (Fig. 1). In contrast, in the presence of [Δ13]ARF1, tested at concentrations ranging from 2.5 to 38 μM, no incorporation of ADP-ribose into G\(_{x}\) was detected (Fig. 1). This is thought to result from inhibition by [Δ13]ARF1 of the small amount of ARF contaminating the G\(_{x}\) preparation. Consistent with this interpretation, 2 μM [Δ13]ARF1–GTP and 6 μM [Δ13]ARF1–GTP inhibited activity of 0.12 μM wild type ARF1 by 95 and 100%, respectively (not shown). [Δ17]ARF1 also had no detectable activity (see Table I) (15), and up to 7 μM [Δ17]ARF1 did not significantly inhibit activity of the wild type protein. The differences in the two deletion mutants in the ability to inhibit wild type ARF1 activity is discussed further below. We conclude that the 13 NH\(_{2}\)-terminal amino residues of ARF1 are essential for activity in this assay.

The amino terminus has previously been shown to affect nucleotide exchange (15). Under the conditions of the ARF assay, which include the presence of DMPC/cholate mixed micelles, [Δ13]ARF1 and [Δ17]ARF1 bound 1 mol of GTP\(\gamma\)S/mol protein (Table I and Fig. 2). If phospholipid was excluded from the binding mixture, the stoichiometry was still 0.7–0.85 mol of GTP\(\gamma\)S bound/mol ARF. In contrast, in the presence of phos-
pholipid, wild type ARF1 bound only 0.02–0.1 mol of GTPγS/mol protein (range of six experiments). In the absence of phospholipid, the wild type protein bound 5–10-fold less GTPγS (≈0.01 mol/mol; Fig. 2, range of three experiments). The two mutants had similar GDP dissociation rates (Table I) which were more than 10-fold faster than the GDP dissociation rate from ARF1. These effects of the amino terminus to decrease stoichiometry of GTPγS binding and increase dependence on phospholipids are evidence that the amino terminus is involved in conformational changes that accompany GTP binding.

The rapid and complete exchange of guanine nucleotide on [Δ13]ARF1, described above and seen in Fig. 2, are in marked contrast with the conclusion reached in Hong et al. (16) that nucleotides that purify with [Δ13]ARF1 do not exchange. As a direct test, GTPγS binding under the conditions of the agmatine assay used by Hong et al. (16) that nucleotides that purify with [Δ13]ARF1 do not exchange. In contrast, GDPγS was determined with no addition of partially purified ARF GAP (filled triangles and inverted triangles) as described under “Experimental Procedures.” Data are expressed as GDP formed/GTP present at the start of the reaction (GTPγS).

The differences in bound nucleotides in the different ARF preparations and slow nucleotide exchange rates measured under conditions used in the agmatine assay likely explain the lack of dependence of activity on added GTP, reported in Hong et al. (16). We estimate that the amount of ARF1.GTP and [Δ13]ARF1.GTP present in the assay employed by Hong et al. (16) differ by 100–1000 fold. The need to express ARF activities as specific activities, normalized to the active protein (ARF-GTP), when comparing different proteins or preparations is clear from these results.

In spite of the differences in exchange rates and co-purifying nucleotides we reach the startling conclusion that the cofactor assays yield different results when testing the [Δ13]ARF1 mutant, depending on the substrate for cholera toxin employed. The clear qualitative difference is that this mutant is active in the ARF/agmatine assay but not in the ARF/G, assay. Having considered the effects of bound nucleotides and nucleotide exchange on specific activities we are left with only the differences in substrates as the likely explanation for this marked difference. Thus, we conclude that the amino terminus of ARF1 is required for interaction with G, but is not required for the binding of cholera toxin.

The conclusion that G, and cholera toxin have distinct binding sites on ARF is further supported by the observation that [Δ13]ARF1, but not [Δ17]ARF1, inhibits the ADP-riboseylation of G, by ARF1 (see above). This observation is explained if residues 14–17 of ARF1 are involved in the binding of cholera toxin. The [Δ13]ARF1 mutant can bind to the toxin but not G, thus blocking the productive interaction of wild type ARF1 with toxin and G,.

We also analyzed the nucleotides that had co-purified with the different purified, recombinant proteins (Fig. 3). More than 85% of the nucleotide bound to ARF1 was GDP. In contrast, 93 and 90% of the nucleotide bound to [Δ13]ARF1 (Fig. 3) and [Δ17]ARF1 (not shown), respectively, was GTP. The differences in co-purifying nucleotides likely reflect differences in nucleotide affinities found for the different proteins as all three proteins lack intrinsic GTPase activity. The wild type protein has a greater affinity for GDP than for GTP, with GDP dissociation rates 10-fold greater than GDP dissociation rates. In contrast, GDP dissociates 10-fold faster than does GTPγS from [Δ17]ARF1 (Table I and unpublished observation). Thus, NH2 truncations promote the formation of ARF-GTP.

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These same mutants were used to test whether the amino terminus was required for interactions of ARF with ARF GAP. ARFs are unique among GTP-binding proteins in having no intrinsic GTPase activity (10). GTP hydrolysis by ARF requires the presence of a GTPase activating protein, ARF GAP (21). Like the wild type protein, neither [Δ13]ARF1 (Fig. 4) nor [Δ17]ARF1 (not shown) had detectable GTPase activity. Both mutants were found to be very poor substrates for ARF GAP (Table I). GAP stimulated GTPase activity of [Δ13]ARF1 was 1.2% that of ARF1 (Fig. 4 and Table I) and ARF GAP did not significantly activate the GTPase activity of [Δ17]ARF1. The
addition of up to 24 μM [Δ13]ARF-GTP had no effect on GAP-stimulated ARF1 GTPase activity. Therefore, the small stimulation of [Δ13]ARF1 GTPase activity was likely the result of a low affinity interaction with ARF GAP. These data demonstrate that the amino terminus is required for interaction of ARF1 with ARF GAP but also identify residues 14–17 as playing a small but detectable role in the interaction.

In summary, the NH₂-terminal 13 residues of ARF1 are required for activity in the ARF/G₁ assay and for interaction with G₁. We speculate that residues 14–17 are involved in the interaction of ARF1 with both cholera toxin and ARF GAP, based on differences in activities observed between the [Δ13]ARF1 and [Δ17]ARF1 mutants. These results also demonstrate the need to monitor both the nucleotides bound and the rates of nucleotide exchange on ARF proteins when making quantitative comparisons between different ARF proteins or preparations. The dramatic effects of a variety of agents (most notably phospholipids, detergents, and divalent metals) on the rates of nucleotide exchange can lead to differences between preparations of greater than 1000-fold in the amount of active protein present and can confound even qualitative comparisons. The different cofactor assays have been used previously in attempts to define the site of ARF binding. It was originally suggested that activated ARF binds G₁ (10), but the fact that it increases cholera toxin activity in the absence of G₁ led to the conclusion that ARF binds the toxin (12). An attractive feature of the above results is the conclusion that both previous conclusions appear to have been correct. It will be very interesting to see if ARF plays a similar "coupling" role in promoting coatamer association with membranes or in activation of phospholipase D.

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