Effects of Arfaptin 1 on Guanine Nucleotide-dependent Activation of Phospholipase D and Cholera Toxin by ADP-ribosylation Factor*

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Arfaptin 1, a ~39-kDa protein based on the deduced amino acid sequence, had been initially identified in a yeast two-hybrid screen using dominant active ARF3 (Q71L) as bait with an HL-60 cDNA library. It was suggested that arfaptin 1 may be involved in Golgi functions, since the FLAG-tagged protein was associated with Golgi membranes when expressed in COS-7 cells and could be bound to Golgi in vitro in an ADP-ribosylation factor (ARF)- and GTPγS-dependent, brefeldin A-inhibited fashion. Arfaptin 2, found in the same two-hybrid screen as arfaptin 1, is 60% identical in amino acid sequence and may or may not have an analogous function. We now report some effects of arfaptin 1 on ARF activation of phospholipase D and cholera toxin ADP-ribosyltransferase. Arfaptin 1 inhibited activation of both enzymes in a concentration-dependent manner and was without effect in the absence of ARF. Two ARF1 mutants that activated the toxin, one lacking 13 N-terminal amino acids and the other, in which 73 residues at the N terminus were replaced with the analogous sequence from ARL1, were not inhibited by arfaptin, consistent with the conclusion that arfaptin interaction requires the N terminus of ARF. This region has also been implicated in phospholipase D activation, but whether the two proteins interact with the same structural elements in ARF remains to be determined. Arfaptin inhibition of the action of ARF5 and ARF6 was less than that of ARF1 and ARF3; its effects were less on nonmyristoylated than myristoylated ARFs. Arfaptin effects on guanine nucleotide binding by ARFs were minimal whether or not a purified ARF guanine nucleotide-exchange protein was present. These findings indicate that arfaptin acts as an inhibitor of ARF actions in vitro, raising the possibility that it has a similar role in vivo.

ADP-ribosylation factors (ARF)

ADP-ribosylation factors (ARF)1 (1), ubiquitous ~20-kDa GTP-binding proteins, are present in all eukaryotic cells from Giardia to mammals and are essential for vesicle formation at Golgi, endosomal, and probably nuclear membranes. Six mammalian ARFs have been identified by cDNA cloning. Based on deduced amino acid sequence, phylogenetic analysis, and gene structure, they have been divided into three classes: class I, ARF1, -2, and -3; class II, ARF4 and -5; class III, ARF6 (1). By definition, all ARFs stimulate cholera toxin (CTA) ADP-ribosyltransferase activity. In addition, all ARFs activate a specific phospholipase D (PLD) that can serve as an effector in cellular signal transduction (2, 3). Whereas, the domain necessary for CTA activation resides in the C-terminal portion of the ARF molecule, activation of PLD is a function of an N-terminal region (4). The identification and isolation of ARF GTPase-activating proteins, termed ARF GAPs (5–7), and GEPs, or guanine nucleotide-exchange proteins (8–10), have extended our understanding of ARF action and its regulation. ARF GAP accelerates the hydrolysis of GTP bound to ARF, yielding inactive ARF-GDP. ARF GAP catalyzes the replacement of bound GDP by GTP to produce active ARF-GTP. It has been recognized relatively recently that several proteins initially characterized in other contexts exhibit ARF GAP activity. These include proteins with Sec7 domains, such as p200 from bovine brain (11), yeast Gea1 and Gea2 (12), and human ARNO (13) and cytohesin 1 (14).

Arfaptin 1, a ~39-kDa protein based on the deduced amino acid sequence, was identified in a yeast two-hybrid screen using dominant active ARF3(Q71L) as bait with an HL-60 cDNA library (15). The recombinant arfaptin2 bound tightly to both myristoylated and nonmyristoylated ARF1 and ARF3 but much less to ARF5 and ARF6. The native arfaptin, immunoprecipitated from an HL-60 cell lysate, behaved as a molecule of ~44 kDa on gel electrophoresis (15). The physiological role(s) of arfaptins remains to be defined. It was suggested that arfaptin 1 may be involved in Golgi functions based on observations that the FLAG-tagged protein was associated with Golgi membranes when expressed in COS-7 cells and could be bound to Golgi in vitro in an ARF- and GTPγS-dependent, brefeldin A-inhibited fashion (15). Arfaptin 2, which has 60% amino acid identity to arfaptin 1, may or may not have an analogous function. Both of the proteins were phosphorylated to a limited extent by protein kinase C (15). A Rac-binding protein termed POR1, which may have a role in Rac-dependent membrane ruffling, is identical to the C-terminal 303 amino acids of arfaptin 2 (15).

To understand better the arfaptin-ARF interaction, we investigated some effects of arfaptin 1 on ARF activation of CTA and PLD, and on ARF guanine nucleotide exchange. To begin to define regions of the ARF molecule that influence its interaction with arfaptin, native and recombinant ARFs (with and

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‡ The abbreviations used are: ARF, ADP-ribosylation factor; mARF or rARF, myristoylated or nonmyristoylated recombinant ARF, respectively; GDPγS, guanosine 5’-γ-thiodiphosphate; GTPγS, guanosine 5’-γ-thiotriphosphate; CTA, cholera toxin A subunit; GEP, guanine nucleotide-exchange protein partially purified from rat spleen cytosol (9); GAP, GTPase-activating protein; PLD, phospholipase D; GST, glutathione S-transferase.

‡ For simplicity, arfaptin 1 is referred to as arfaptin in this report.
without myristoylation) were studied as well as chimeric molecules that include portions of the ARF amino acid sequence along with that from human ARL1, an ARF-like protein that is 56% identical in sequence to human ARF1 (4) and which, unlike ARFs, does not activate cholera toxin or rescue *Saccharomyces cerevisiae* with the lethal arf1 arf2 double deletion (16).

**EXPERIMENTAL PROCEDURES**

Materials—GST-arfaptin 1 (15) was added to assays as the fusion protein (~67 kDa) in 50 mM Tris-HCl, pH 8.0, 10 mM glutathione. Amounts of vehicle were equal in all assays. Mixed ARF preparations (1 and 3 after DEAE and Ultrogel AcA 54 chromatography), native ARF1, ARF3, and ARF5 were purified from bovine brain cytosol (11, 17, 18). Recombinant myristoylated mARF1, mARF3, and mARF5 and nonmyristoylated rARF1, rARF5, and rARF6 were synthesized in *Escherichia coli* (19). Preparation of Δ13ARF1 (20) and ARF/ARL chimeric proteins (4) is published. ARF GEP was purified from rat spleen cytosol through the heparin-agarase step (9). ARF-dependent phospholipase D was partially purified from bovine brain membranes (21). Sources of other materials are published (8, 9).

**Assay of ARF Activity**—ARF activity was assayed by three methods, guanine nucleotide binding, CTA activation, and PLD activation. All data reported are means of values from duplicate assays and have been replicated twice or more except those in Table IV.

To assay nucleotide binding, ARF was incubated at 37°C with or without arfaptin and either [35S]GTP-S or [3H]GDP for the indicated time. Protein-bound nucleotide was collected on nitrocellulose for radioassay (10).

For assay of ARF activation of CTA, ARF was incubated with or without arfaptin for 40 min at 37°C with 10 μM GTP-S or 100 μM GDP in a 50-μl volume, then placed in an ice bath. After addition of CTA, [14C]NAD and agmatine in a volume of 250 μl, samples were incubated for 60 min at 30°C and [14C]ADP-ribosylagmatine was collected for radioassay (8).

The activity of partially purified ARF-dependent PLD was assayed by a published method (4). Briefly, mixed lipids with choline[methyl-3H]dipalmitoyl phosphatidylcholine were added to PLD, ARF, and GTP-S with or without arfaptin and incubated for 1 h at 37°C before addition of CHCl₃/CH₃OH/HCl, followed by centrifugation. [3H]choline in the aqueous phase was quantified by liquid scintillation spectroscopy.

**RESULTS**

**Effects of Arfaptin on Stimulation of Cholera Toxin ADP-ribosyltransferase Activity by Native, Recombinant, and Mutant ARFs**—In an effort to define the functions of arfaptin 1, its in vitro effects on some known ARF activities were first tested. Stimulation of cholera toxin ADP-ribosyltransferase activity by native ARF3 (8) or ARF5 (18) was assayed with or without arfaptin. As shown in Fig. 1, inhibition of ARF activity was dependent on, but not linearly proportional to, the concentration of arfaptin. The activities of 10 pmol of native ARF1 and ARF3 were similar and were similarly inhibited ~30% by 10 pmol and 60–70% by 40 pmol of arfaptin (Table I). The activities of recombinant ARF proteins myristoylated or nonmyristoylated, synthesized in *E. coli* were variable and much lower than those of native proteins. Inhibition, which was dependent on arfaptin concentration, was apparently somewhat less for ARF5 and ARF6 than for ARF1 and ARF3 (Table I).

CTA activity was enhanced by ARL73/ARF, which contains the first 73 amino acids of ARL1 and the last 108 of ARF1, but arfaptin did not interfere with its activity (Table I). The considerably greater activation by rARF1Δ13 (ARF1 lacking the N-terminal 13 amino acids) likewise was not prevented by arfaptin. These data are consistent with the possibility that arfaptin interactions require the N terminus of ARF. ARF3ARL, a recombinant protein containing the first 73 amino acids of human ARF1 and the last 108 of human ARL1, did not activate CTA as previously reported (4).

**Effects of Arfaptin on Stimulation of Phospholipase D by Recombinant and Mutant ARFs**—In the absence of added ARF, partially purified PLD from bovine brain exhibited a low level of activity, probably due to ARF contamination (Table II), a possibility consonant with its inhibition by arfaptin. The same recombinant ARF preparations used for the experiments in Table I stimulated PLD activity from 10- to 25-fold (Table II). In these assays, mARF3 and mARF5 were clearly more active than nonmyristoylated forms. The dramatic activation by mARF3 was virtually completely inhibited by arfaptin, which was less effective against myristoylated rARF3 and also nonmyristoylated rARF5 and rARF6 preparations (Table II). ARF activity of PLD required the N-terminal domain of the ARF molecule as shown earlier (4). The chimeric ARF73/ARL, containing the N-terminal 73 amino acids of ARF1, increased activity 14-fold and was clearly inhibited by arfaptin (Table II). ARL73/ARF with the C-terminal region of ARF1 and the N-terminal 73 amino acids of ARL1 as reported (4) did not activate PLD nor did the mutant rARF1Δ13, which lacks 13 amino acids at the N terminus, confirming that the N terminus...
of the ARF molecule is important for PLD activation as well as for arfaptin inhibition of CTA activation by ARF (Table I). Whether arfaptin and PLD interact with the same structural determinants in ARF remains to be determined.

**Effect of Arfaptin on GTPγS and GDP Binding to ARF3**—Replacement of ARF-bound GDP by GTP to produce the active ARF-GTP (in the presence of 2 mM MgCl₂) is very slow in the absence of an ARF GEP. Although exchange can be accelerated by decreasing MgCl₂ to <1.0 mM (9), it is known that the higher MgCl₂ concentration enhances stability of ARF-nucleotide complexes and that at low MgCl₂ concentrations ARF is rather unstable. Using 0.7 or 3.5 mM MgCl₂, the time course of [³⁵S]GTPγS binding to ARF3 was determined with or without arfaptin (Fig. 2). At the lower MgCl₂ concentration, the initial rate of binding was 10-fold that at 3.5 mM MgCl₂. Arfaptin increased the amount of [³⁵S]GTPγS bound after longer incubations, perhaps by stabilizing ARF, but the initial rate of binding was not markedly accelerated. At 3.5 mM MgCl₂ [³⁵S]GTPγS binding to ARF3 was much slower (Fig. 2), and arfaptin had little effect. Replacement of ARF-bound [³⁵S]GDP with GDP from the medium was assayed without and with arfaptin at two concentrations of MgCl₂ (Fig. 3). At 0.1 mM MgCl₂ [³⁵S]GDP binding to ARF3 was only 30% higher than it was at 3.4 mM MgCl₂. Binding was slow, and the rate was approximately constant for 80 min. Replacement binding of GDP was <20% greater with arfaptin than without it at both MgCl₂ concentrations (Fig. 3).

**Effect of MgCl₂ Concentration and Arfaptin on GTPγS Binding to mARF3**—Stable binding of GTPγS to mARF5 required relatively high concentrations of MgCl₂ (Fig. 4). Binding was maximal with 2–5 mM MgCl₂ and much lower with 1 mM MgCl₂ (in the presence of 1 mM EDTA). Arfaptin 1 increased GTPγS binding 4-fold with 1 mM MgCl₂, but only ~40% with 2–3 mM MgCl₂. The enhancement by arfaptin 1 (Figs. 2 and 4) could reflect slowing of nucleotide dissociation as a result of ARF-GTPγS interaction with arfaptin.

**Effect of Arfaptin on Release of Bound [³⁵S]GTPγS from ARF3—**ARF3 (15 pmol) with 2.4 pmol of [³⁵S]GTPγS bound was incubated for 40 min at 37°C with or without arfaptin 1 and several concentrations of GDP or GTPγS (Table III). Differences in [³⁵S]GTPγS bound differed <10% under all conditions, although binding was consistently slightly higher in all samples that contained arfaptin 1. Thus, arfaptin 1 displayed little, if any, ARF GEP activity.

**Effect of Arfaptin on [³⁵S]GTPγS or [³⁵S]GDP Binding to ARF3 in the Presence of GEP—**To determine whether arfaptin interfered with GEP stimulation of nucleotide binding, ARF3 was incubated with GEP with or without arfaptin 1 and

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**TABLE II**

**Effect of arfaptin on activation of phospholipase D by recombinant and mutant ARFs**

| ARF protein (pmol) | Phospholipase D activity (pmol/h) |
|-------------------|----------------------------------|
|                   | None                             |
|                   | 10 pmol arfaptin 1               |
|                   | 40 pmol arfaptin 1               |
|                   | No arfaptin                      |
| mARF3 (210)       | 36                               |
| rARF3 (220)       | 36                               |
| rARF5 (240)       | 42                               |
| mARF6 (210)       | 48                               |
| ARF3/ARL (400)    | 49                               |
| ARL3/ARF (400)    | 3.1                             |
| rARF1A13 (200)    | 4.0                             |

^a In parentheses, activity relative to that without arfaptin 1 = 100.

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**DISCUSSION**

Arfaptin inhibited the actions of all classes of ARFs on CTA and PLD activities. However, ARF1 and ARF3 (class I) were more sensitive than ARF5 (class II) and ARF6 (class III) to its inhibitory effects (Tables I and II). Studies on the interaction of recombinant human ARF1, -3, -5, and -6 with immobilized GST-arfaptin fusion proteins previously revealed an extent of stable association decreasing in that order with negligible bind-
Effects of Arfaptin on ARF Activities

FIG. 4. Effect of MgCl₂ concentration and arfaptin 1 on GTP·S binding to mARF5. mARF5 (2 pmol in its presence. 2.33 pmol were bound in the absence of arfaptin and 2.54 pmol with arfaptin. After incubation for 40 min without unlabeled [³²S]GTP·S, 1 mM EDTA, and 1 mM MgCl₂ for 40 min at 37 °C then cooled on ice. Arfaptin 1 alone did not bind GTP·S.

TABLE III
Effect of arfaptin 1 on release of [³²S]GTP·S bound to ARF3

| Nucleotide (µM) | With GTP·S | With GDP |
|-----------------|------------|----------|
|                 | No arfaptin | Plus arfaptin | No arfaptin | Plus arfaptin |
|                 | pmol       | pmol      | pmol       | pmol |
| 1               | 2.24       | 2.53      | 2.26       | 2.54 |
| 5               | 2.16       | 2.50      | 2.23       | 2.50 |
| 10              | 2.23       | 2.58      | 2.18       | 2.62 |
| 25              | 2.20       | 2.51      | 2.25       | 2.40 |

TABLE IV
Effect of arfaptin on [³²S]GTP·S or [³H]GDP binding to ARF3 in the presence of GEP

| Arfaptin (µM) | [³²S]GTP·S bound | [³H]GDP bound |
|---------------|------------------|---------------|
|               | pmol             | pmol          |
| 0             | 4.4 (100)        | 5.3 (100)     |
| 10            | 5.1 (116)        | 5.9 (111)     |
| 40            | 5.3 (120)        | 6.0 (113)     |

* In parentheses, bound nucleotide relative to that in the absence of arfaptin 1 = 100.

Arfaptin 1, 15 pmol) were added to 80 µl of solution to achieve final concentrations of 0.48 mM EDTA and 3.18 mM MgCl₂ without or with arfaptin (60 pmol). After incubation for 40 min at 37 °C with the indicated concentrations of unlabeled GTP·S or GDP, proteins were collected on nitrocellulose for radioassay. At the beginning of the final 40-min incubation, bound [³²S]GTP·S was 2.45 pmol without and 2.43 pmol with arfaptin. After incubation for 40 min without unlabeled nucleotide, 2.35 pmol were bound in the absence of arfaptin and 2.54 pmol in its presence.

TABLE V
Effect of arfaptin on CTA activation by ARFs with and without GEP

| ARF protein (µM) | ARF activity |
|------------------|--------------|
|                  | pmol [³²C]ADP-ribosylagmatine/h |
|                  | ARF1–3* (40) | 12.5 (86) |
| + ARF GEP        | 27.0         | 23.4 (87) |
| ARF5 (15)        | 3.1          | 1.4 (45)  |
| + ARF GEP        | 4.3          | 2.0 (46)  |

* Mixture of predominantly ARFs 1 and 3. In parentheses, activity relative to that without arfaptin 1 = 100.

Myristoylation was demonstrated as not essential for ARF3 interaction with arfaptin 1 in the yeast two-hybrid screen, although its influence was not directly assessed (15). In the functional studies reported here, no consistent effect of myristoylation on the inhibitory action of arfaptin was observed (Tables I and II). The activity of CTA was not inhibited by arfaptin in the absence of ARF, and the effect of arfaptin on the very low activity of PLD in the absence of ARF is probably due to ARF contamination of the PLD preparation (data not shown). Percentage inhibition of PLD activation by arfaptin was, in general, greater than that of CTA activation. It may be relevant that the N-terminal 13 amino acids of ARF that were required for PLD activation were also required for arfaptin inhibition of cholera toxin activation (Tables I and II). Interpretation of the difference is complicated, however, by the major differences in assay conditions. It is necessary also to take into account the differences in effects of specific phospholipids on GTP binding by individual ARFs that can influence markedly the rate at (and extent to) which each becomes activated. Differences in behavior of the chimeric proteins are seemingly more straightforward in determining the structural requirement for interaction of ARF and arfaptin. As expected, ARF73ARL did not activate CTA but did activate PLD (4), and its action on the latter was inhibited by arfaptin (Table II). Neither ARFL73ARF nor rARF1315 significantly activated PLD in these experiments as reported (4). Both did, however, activate CTA, and arfaptin had no effect on their action, consistent with the suggestion of Kanoh et al. (15) that the N terminus of ARF is important for its interaction with arfaptin.

No GEP-like activity of arfaptin was detected. Nor was there any evidence that arfaptin 1 interfered with the action of a GEP partially purified from rat spleen. Whether nucleotide binding or release was measured, the amount of GTP·S or GDP bound to several ARF preparations was consistently slightly
higher in the presence of arfaptin 1 than in its absence. This was true also when GEP was added to accelerate guanine nucleotide exchange. Since this effect of arfaptin 1 was greater with longer times of incubation, it could reflect stabilization of the ARF protein as a result of its association with arfaptin 1.

Because arfaptin was discovered using the yeast two-hybrid system with a presumably GTP-liganded ARF mutant as bait, ARF was apparently able to interact with arfaptin in these cells (15). In addition, arfaptin in HL-60 cell cytosol was recruited by ARF to Golgi membranes in a GTP$^\gamma$S-dependent and brefeldin A-sensitive manner (15). It remained unclear, however, from the prior studies how its interaction with arfaptin would affect the ability of ARF to activate either PLD or cholera toxin. The experiments reported here established that, even though activation of PLD and cholera toxin involves two different domains of ARF (4), arfaptin interferes with both processes. Based on these data, it would appear that once ARF recruits arfaptin to a membrane, dissociation of ARF from arfaptin would need to occur prior to activation of phospholipase D.

The present findings demonstrate that arfaptin is a potent inhibitor of ARF actions on CTA and PLD in vitro, but has minimal effects on GEP-catalyzed guanine nucleotide binding to ARF. Among the questions arising from this work are how the association of ARF with arfaptin modifies its interaction with GAPs and the hydrolysis of bound GTP. ARF plays a major role in the regulation of vesicular trafficking through the Golgi, and it will be important to demonstrate whether and how this is modified by arfaptin. These and other questions are subjects of current study.

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