Isolation of an amino-terminal deleted recombinant ADP-ribosylation factor 1 in an activated nucleotide-free state

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ABSTRACT ADP-ribosylation factors (ARFs) are ~20-kDa guanine nucleotide-binding proteins that activate cholera toxin ADP-ribosyltransferase in vitro and participate in intracellular vesicular membrane trafficking. ARFs are activated when bound GDP is replaced by GTP and inactivated by hydrolysis of bound GTP to yield ARF-GDP. Usually, ARFs are isolated in an inactive GDP-bound state and require addition of GTP along with detergent or phospholipid for activity. Purified mutant recombinant ARF1 lacking the first 13 amino acids (rA13ARF1-P) stimulated cholera toxin activity essentially equally with or without added GTP (and phospholipid or detergent), at least in part due to the presence of bound nucleotides, which later were identified as GTP and GDP. Nucleotide-free rA13ARF1 (rA13ARF1-F), prepared by dialysis against 7 M urea, was active without added GTP in the absence of SDS but inactive without added GTP in its presence. Renaturation of rA13ARF1-F in the presence of GTP, ITP, or GDP yielded, respectively, rA13ARF1-GTP and rA13ARF1-ITP, which were active, and rA13ARF1-GDP, which was inactive. Effects of phospholipids and detergents on nucleotide exchangeability evaluated as effects on activity of rARF1 and rA13ARF1-F differed. With rA13ARF1-F, 100 μM ITP and 100 μM GDP were essentially equally effective in the presence of cardiolipin or SDS. The finding that rA13ARF1 differs from rARF1 in the effects of phospholipids and detergents on nucleotide binding is consistent with the conclusion that the ARF amino terminus plays an important role in nucleotide binding and its specificity as well as the molecular conformation and associated activity.

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requires phospholipid and/or detergent for demonstration of high-affinity GTP binding and optimal enhancement of CTAcatalyzed ADP-ribosylation (21–24). Here we report that a mutant rARF1 with the first 13 amino acids deleted (rΔ13ARF1) can be purified (rΔ13ARF1-P) with GTP and GDP bound in a fully active state, independent of added nucleotide (25). After dialysis against 7 M urea, the renatured nucleotide-free rA13ARF1 (rA13ARF1-F) maintained the activated state in the absence of detergents or phospholipids.

MATERIALS AND METHODS

Ammonium acetate was purchased from Advanced Biotechnologies (Columbia, MD); 3-hydroxypicolinic acid was from Aldrich; acetonitrile was from Baxter Health Care Co. (Musk-egon, MI); and trifluoroacetic acid was from Pierce. Sources of other materials have been published (25).

rARF1 and rA13ARF1 proteins were synthesized and purified as described (25). For HPLC analysis of ARF-bound nucleotide (26), a WAX column (weak anion exchange column from DuPont, Zorbax Bio series) was used; absorbance at 280 nm was recorded.

For preparation of nucleotide-free rA13ARF1, the protein was diluted to a concentration of 100 μg/ml with KEND buffer (10 mM potassium phosphate, pH 7.4/1 mM EDTA/100 mM NaCl/1 mM dithiothreitol) and dialyzed at 4°C against 1 liter of 7 M urea in KEND buffer, with changes of buffer every 10–12 hr for 250–300 hr until protein (rA13ARF1-F) was nucleotide-free as verified by HPLC. The protein was renatured by dialysis for 24 hr at 4°C against two changes of 500 ml of KEND buffer without 7 M urea. To rebind nucleotide, rA13ARF1-F was renatured by dialysis against 500 ml of KEND buffer containing 100 μM GTP, 100 μM ITP, or 100 μM GDP, at 4°C for 24 hr with two changes of buffer, followed by dialysis against 500 ml of KEND buffer without nucleotide at 4°C for 24 hr with two changes of buffer to yield rΔ13ARF1-GTP, rARF1-ITP, or rA13ARF1-GDP, respectively.

ARF activation of CTACatalyzed ADP-ribosyltransferase formation was assayed in a total volume of 150 μl containing 1 μl of CTAC, 15 μg of ovalbumin, 50 mM potassium phosphate (pH 7.4), 5 mM MgCl2, 10 mM agmatine, 20 mM dithiothreitol, 200 μM NAD, 0.05 μCi of [adenosine-14C]NAD (1 Ci = 37 GBq), and, as indicated, phospholipid, detergent, and nucleotides. Assays (in duplicate) were terminated after 1 hr at 30°C and ADP-ribosyltransferase was isolated for radioassay (27).

RESULTS AND DISCUSSION

All preparations of rΔ13ARF1 and rARF1, synthesized in E. coli and purified on Ultrogel AcA54, appeared to be >90% pure as judged by Coomassie blue staining and immunoblot analysis with anti-sARFII antibodies after SDS/PAGE (data

Abbreviations: ARF, ADP-ribosylation factor; r, recombinant; CTAC, cholera toxin A subunit; GTP[y-S], guanosine 5’-[γ-thio]triphosphate; GDP[bS], guanosine 5’-[β-thio]diphosphate; MALDI, matrix-assisted laser desorption.
FIG. 1. Activation of CTA by rARF1, rΔ13ARF1-P, or rΔ13ARF1-F without added nucleotide. Assays in duplicate with the ARF preparation as indicated containing 1 μg of CTA without (○) or with (◇) 0.003% SDS in 150 μl were incubated at 30°C for 1 hr. Experiments were replicated two to four times.

not shown). Without added nucleotide, rΔ13ARF1-P produced almost the same stimulation of CTA-catalyzed ADP-ribosylagmatine formation with or without 0.003% SDS, whereas rARF1 was inactive (Fig. 1). In the presence of SDS, rΔ13ARF1-P activity was also similar with added guanosine 5'-[γ-thio]triphosphate (GTP[yS]) or guanosine 5'-[β-thio]diphosphate (GDP[βS]), whereas rARF1 had significant activity only in assays with GTP[yS] (25). After removal of bound nucleotides, rΔ13ARF1-F was active without SDS but was inactive in its presence (Fig. 1). In the presence of SDS, activity of rΔ13ARF1-F was dependent on GTP[yS] concentration and was half-maximal at ~80 μM (Fig. 2).

The negative ion matrix-assisted laser desorption (MALDI) mass spectrum of nucleotides from rΔ13ARF1-P (Fig. 3) had strong signals at m/z 442 and 522, corresponding to quasi molecular ions [M-1] of GDP and GTP, respectively. Due to the nature of MALDI, conclusions about relative quantities of each nucleotide cannot be easily drawn from comparison of signals. In addition, some degradation of GTP to GDP and GDP to GMP occurred during parallel preparation of standard nucleotides for analysis.

In the presence of SDS, ITP was almost as effective as GTP in supporting rΔ13ARF1-F activity, whereas it was markedly less so with rARF1 (Fig. 4). In the presence of cardiolipin, however, 100 μM ITP was almost as effective as GTP for stimulation of CTA activity by rARF1, rARF5, or rARF6 (Table 1). Differences in the behavior of rΔ13ARF1-F and rARF1 with 100 μM GTP or 100 μM ITP were assessed in the presence of several detergents and phospholipids (Table 2). With GTP, rΔ13ARF1-F was similarly active with cardiolipin or SDS or without additions; dimyristoylphosphatidylcholine/cholate, phosphatidylinositol/cholate, and Tween 20 were less effective, in that order. With ITP, rΔ13ARF1-F activity was similar in SDS and cardiolipin or without additions and was much lower in other conditions. For rARF1 with GTP or ITP (Table 2), cardiolipin was clearly much more effective than other additions.

rΔ13ARF1-GTP, rΔ13ARF1-GDP, and rΔ13ARF1-ITP were prepared to determine whether any of them exhibited the behavior of rΔ13ARF1-P. In the CTA assay with SDS, the activities of rΔ13ARF1-GTP, rΔ13ARF1-ITP, and rΔ13ARF1-P were similar (Table 3); rΔ13ARF1-GDP was clearly much less active.

FIG. 2. Effect of GTP[yS] and GDP[βS] on CTA activation by rΔ13ARF1-P and rΔ13ARF1-F. Assays (in duplicate) containing 0.003% SDS, nucleotide as indicated, and 1 μg of rΔ13ARF1-P (○) or rΔ13ARF1-F (◇) were carried out as described in Fig. 1. The experiment was replicated twice.

FIG. 3. MALDI negative ion mass spectrum of nucleotides from rΔ13ARF1. Peaks at m/z 522 and 442 are quasimolecular ions (M-H) of GTP and GDP, respectively. Peak labeled M is the matrix-related adduct ion (sodium picolinate-H). Protein was diluted to 100 μg/ml and dialyzed against 500 ml of 20 mM ammonium acetate at 4°C, for 24 hr with two changes. After concentration, the protein was denatured at 95°C for 10 min and discarded after centrifugation. Supernatant containing nucleotides was concentrated, diluted with distilled water, and concentrated at least twice to remove residual ammonium acetate before mass spectrometry. MALDI mass spectra were obtained by using a Kratos Kompact MALDI III mass spectrometer (Manchester, U.K.), equipped with a 337-nm nitrogen laser. 3-Hydroxypicolinic acid dissolved in acetonitrile/0.1% trifluoroacetic acid, 1:1 (vol/vol), was used as matrix solution. A mixture (0.6 μl) of nucleotide solution from 300 to 500 μg of rΔ13ARF1-P and matrix solution was applied to the sample slide and dried in air. To improve mass resolution in the low mass range of interest, the mass analyzer was used in the reflectron negative ion mode at an accelerating voltage of ~20 kV.
Addition of GTP increased the activity of rΔ13ARF1-GDP only slightly, as, presumably, only part of the bound GDP was exchanged for GTP during the reaction. Similarly, addition of GDP decreased only partially the activity of rΔ13ARF1-GTP, rΔ13ARF1-ITP, or rΔ13ARF1-P. In SDS, activity of rARF1 by ITP was much less than by GTP (Table 3 and Fig. 4).

Native ARFs require GTP binding for optimal stimulation of CTA-catalyzed ADP-ribosylation (22–24). Recombinant ARFs synthesized in Escherichia coli have usually been isolated in an inactive GDP-bound state (21, 24). A recombinant ARF6 fusion protein with maltose-binding protein at the amino terminus was, however, isolated in an active GTP-bound form (26). We have reported (25) the isolation of rΔ13ARF1 and another amino-terminal mutant ARF1 (rPKA14ARF1) in an activated form. After removal of bound nucleotides, these proteins were dependent on GTP for activity when assayed in the presence of SDS (25).

Table 3. Effects of rΔ13ARF1 with nucleotide rebound on CTA activity

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>rARF1-F</th>
<th>rARF1-F</th>
<th>rARF1-F</th>
<th>rARF1-F</th>
<th>rARF1-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP</td>
<td>2.3 ± 0.1</td>
<td>9.7 ± 0.7</td>
<td>1.5 ± 0.1</td>
<td>7.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>9.6 ± 0.4</td>
<td>10.7 ± 0.1</td>
<td>7.6 ± 0.3</td>
<td>8.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>IDP</td>
<td>9.5 ± 0.4</td>
<td>8.4 ± 0.3</td>
<td>7.3 ± 0.4</td>
<td>7.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>10.0 ± 0.6</td>
<td>11.0 ± 0.4</td>
<td>8.3 ± 0.5</td>
<td>9.1 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>ADP-ribosyltransferase activity, nmol/hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nucleotides extracted from rΔ13ARF1-F have now been identified as GTP and GDP by MALDI mass spectrometry (Fig. 3) and HPLC (data not shown). To what extent the GDP was generated from GTP during extraction and analysis is unclear. Nucleotide bound to rARF1 was all apparently GDP (data not shown). Randazzo et al. (28) reported that most of the nucleotide bound to rΔ13ARF1 was GTP, based on HPLC analysis. As rARF1-F was equally active with or without nucleotides and SDS, its bound nucleotide was apparently not exchanged under these conditions and thus its structure or conformation clearly differed from that of rARF1. Although the nucleotide-free rΔ13ARF1-F in the absence of detergent or phospholipid still had high activity without GTP, it became GTP-dependent when SDS was added; i.e., it seemingly adopted an active structure or conformation, like rΔ13-

ADP-ribosyltransferase activity, nmol/hr

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Detergent</th>
<th>rΔ13ARF1-F</th>
<th>rΔ13ARF1-F</th>
<th>rΔ13ARF1-F</th>
<th>rΔ13ARF1-F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Basal</td>
<td>GTP</td>
<td>ITP</td>
<td>Basal</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>SDS</td>
<td>12.0 ± 0.7</td>
<td>9.0 ± 0.7</td>
<td>7.0 ± 0.5</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>DMPC</td>
<td>Cholate</td>
<td>2.2 ± 0.31</td>
<td>5.3 ± 0.11</td>
<td>2.7 ± 0.17</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>PI</td>
<td>Cholate</td>
<td>1.4 ± 0.03</td>
<td>4.3 ± 0.05</td>
<td>2.4 ± 0.16</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Tween 20</td>
<td>1.2 ± 0.07</td>
<td>3.8 ± 0.21</td>
<td>2.8 ± 0.16</td>
<td>1.2 ± 0.04</td>
</tr>
</tbody>
</table>

Assays contained 1 μg of CTA, 0.8 μg of rΔ13ARF1-F or rARF1, and 100 μM of the indicated nucleotide, with cardiolipin (1 mg/ml), 3 mM dimyristoylphosphatidylcholine (DMPC), phosphatidylglycerol (PG) (1 mg/ml), 0.2% sodium cholate, 0.3% Tween 20, and/or 0.003% SDS, as indicated. Data are the mean ± one-half the range of values from duplicate assays. Experiments were replicated twice.

### Table 1. Effects of nucleotides on the activity of rARF1, rARF5, and rARF6

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>rARF1</th>
<th>rARF5</th>
<th>rARF6</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP</td>
<td>1.7 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>GTP</td>
<td>9.8 ± 0.1</td>
<td>9.9 ± 0.6</td>
<td>9.3 ± 0.8</td>
</tr>
<tr>
<td>IDP</td>
<td>1.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>ITP</td>
<td>7.9 ± 0.5</td>
<td>8.1 ± 0.6</td>
<td>8.0 ± 0.7</td>
</tr>
</tbody>
</table>

Assays (in duplicate) containing cardiolipin (1 mg/ml) with 0.8 μg of ARF protein and 100 μM GDP, 100 μM GTP, 100 μM IDP, or 100 μM ITP were incubated at 30°C for 1 hr. Data are the mean of values from duplicate assays ± one-half the range. The experiment was replicated twice.

**Fig. 4.** Effects of nucleotides on rΔ13ARF1 and rARF1 stimulation of CTA activity. Assays (in duplicate) containing 1 μg of ARF protein and the indicated concentration of GTP (○), GDP (■), ITP (▲), or IDP (▲) were carried out as described for Fig. 1. The experiment was replicated twice.

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