Characterization of a GDP Dissociation Inhibitory Region of ADP-ribosylation Factor Domain Protein ARD1*

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ADP-ribosylation factors (ARFs) are ∼20-kDa guanine nucleotide-binding proteins initially identified by their ability to stimulate cholera toxin ADP-ribosyltransferase activity and later recognized as critical components in intracellular vesicular transport and phospholipase D activation. ARF domain protein 1 (ARD1) is a member of the ARF family that differs from other ARFs by the presence of a 46-kDa amino-terminal extension. We previously reported that this extension acts as a GTPase-activating protein for the ARF domain of ARD1 (Vitale, N., Moss, J., and Vaughan, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1941–1944). Both GTP binding and GTP hydrolysis are necessary for physiological function of guanine nucleotide-binding proteins, and the rates of GDP/GTP exchange and GTPase activity are critical in the activation/deactivation cycle. Dissociation of GDP from the ARF domain of ARD1 was faster than from ARD1 itself (both proteins synthesized in Escherichia coli). Using deletion mutations, it was demonstrated that the 15 amino acids directly preceding the ARF domain were responsible for decreasing the rate of GDP dissociation. By site-specific mutagenesis it was shown that hydrophobic amino acids in this region were particularly important in stabilizing the GDP-bound form of ARD1. It is suggested that, like the amino-terminal segment of ARF, the equivalent region in ARD1, located between the GTPase-activating protein and ARF domains, may act as a GDP dissociation inhibitor.

Although roles of G proteins3 are enormously diverse, these GTPases all operate by a fundamentally similar mechanism (1). When GTP occupies its guanine nucleotide-binding site, the protein can interact with and modify the activity of a downstream target protein. Hydrolysis of GTP causes the dissociation of the G protein-target complex and thus terminates the "active state" of the G protein. Cells regulate the ratio of active and inactive G proteins by modulating the rates of GDP release and GTP hydrolysis (GTPase activity).

ARD1 is a 64-kDa protein that contains a 18-kDa carboxy-terminal ADP-ribosylation factor (ARF) domain (p3) and a 46-kDa amino-terminal domain (p5) (2). ARF proteins have been implicated in vesicular membrane trafficking in several intracellular compartments, including endoplasmic reticulum, Golgi, endosomes, and nuclear envelope (reviewed in Ref. 3). ARFs are ∼20-kDa proteins that exhibit no detectable GTPase activity (4); the ratio of GTP/GDP bound appears to be governed by guanine nucleotide-exchange proteins (GEFs) and GTPase-activating proteins (GAPs) (3). ARF GPs (5–12) and GAPs (13–16) have been both purified and cloned. Inhibition of cytosolic and Golgi-associated GEs by the fungal fatty acid metabolite brefeldin A (BFA) has been reported (5–7, 9); both BFA-insensitive and BFA-sensitive cytosolic GEs have been isolated (8, 9). There is genetic as well as biochemical evidence that both BFA-sensitive and BFA-insensitive ARF GPs contain a domain similar to a part of Sec7 (9–12), a protein necessary for intra-Golgi transport.

It was recently reported that the ARF domain of ARD1 binds specifically GDP and GTP, whereas the amino-terminal domain does not (17). Using recombinant proteins, it was shown that the amino-terminal p5 domain of ARD1 stimulates hydrolysis of GTP bound to the ARF domain p3 and consequently appears to be the GAP component of this bifunctional protein (18). The stimulatory effect of the p5 domain on the GTPase activity of p3 was specific, because GTP hydrolysis by other members of the ARF family was not increased (16). The presence of an intrinsic GAP domain is an apparently unique phenomenon for monomeric guanine nucleotide-binding proteins, because GTPase activity of other members of the Ras superfamily is increased by a separate GAP molecule, which interacts with the effector region of the protein (17). Using chimeric proteins, we demonstrated that the GAP domain of ARD1 similarly interacts with the effector region of the ARF domain of ARD1 and thereby stimulates GTP hydrolysis (17).

Nucleotide hydrolysis and product dissociation are both regulated steps in the GTPase cycle. Because their rates determine when and for how long the G protein is active, it is important to precisely understand these mechanisms. We reported that GDP/S dissociation from p3 was faster than from ARD1 (18). Accordingly, in the presence of certain phospholipids (i.e. brain phosphatidylincholine plus phosphatidylglycerol bisphosphate plus phosphatidylethanolamine or phosphatidylserine) more GTP was bound to p3 than to ARD1 (17). These results suggested that p5 may influence GDP dissociation as well as GTP hydrolysis. We report here that 15 amino acids from the carboxy-terminal domain of p5 positioned before the ARF domain p3 inhibit GDP release but not GTP-S release. Results of site-directed mutagenesis suggest that hydrophobic amino acids within this region are critical in reducing the rate of GDP dissociation. This region therefore appears to have a function similar to that of the amino-terminal segment of ARF proteins.

EXPERIMENTAL PROCEDURES

Materials—Bovine thrombin was purchased from Sigma. TLC plates were purchased from VWR Scientific, and glutathione-Sepharose beads
were from Pharmacia Biotech Inc. PCR reagents and restriction enzymes, unless otherwise indicated, were from Boehringer Mannheim. The sources of other materials have been published (7, 17–19).

Preparation of Recombinant Fusion Proteins (p3, p5, and p8)—For large scale production of fusion proteins (18), 10 ml of overnight culture of E. coli XLmutS competent cells (Stratagene) were transformed with the vector. Ultra competent cells (Stratagene) were transformed with plasmids pGEX5G/LIC/N393p8 or pGEX5G/LIC/N393p8. Sequences of mutual N387 fragments were confirmed by automated sequencing (Applied Biosystems, 373 DNA Sequencer) using the primers 3′-TTATCCACTACCTATTAGGG-3′ and 5′-GCTAGTTATTGCTAGCCG-3′. Fusion proteins were expressed and purified as described for ARD1 proteins.

Construction and Expression of Mutated Form of N387ARD1—For site-directed mutagenesis of N387ARD1, a modification of the unique site elimination mutagenesis procedure of Deng and Nickoloff (23) was used. 25 pmol of a 5′-phosphorylated selection primer and 25 pmol of a 5′-phosphorylated mutagenic primer were simultaneously annealed to 750 ng of ND387ARD1-pGEX-5G/LIC in 20 μl of 10 mM Tris acetate (pH 7.5)10 mM magnesium acetate/50 mM potassium acetate by heating for 5 min at 100 °C and cooling 5 min on ice, followed by incubation at room temperature for 30 min. The selection primer 5′-CTGGTGAAGGTGGACGGTGTCACAACTGTCG-3′ and the mutagenic primer 5′-GCCCAGCATCATGCGTACTTTTACAAAG-3′ changed Val25 to Arg. The mutagenic primer 5′-ATGGTCACTCGTCTAAGAGATAATC-3′ changed Phe6 to Arg. The mutagenic primer 5′-AACAGCCTTCAATGTTGCTTCC-3′ changed Arg8 to Leu. The mutagenic primer 5′-AAGGATAATCGCCGTCACATTGGC-3′ changed Val12 to Ile12 and Arg. The mutagenic primer 5′-ATGGTCACTGCTACAAGAGATAATC-3′ changed Pro14 to Gly. The mutagenic primer 5′-ATGGGTTATTGCTACCGG-3′ changed Val15 to Leu. The mutagenic primer 5′-CAACTTGGACCACCTTACGG-3′ changed Lys21 to Ala. The mutagenic primer 5′-ATGGTCACTCGTCTAAGAGATAATC-3′ changed Phe6 to Arg. The mutagenic primer 5′-AAAGATGACGAGGACCGTACAACTGTCG-3′ and the mutagenic primer 5′-ATGGTCACTCGTCTAAGAGATAATC-3′ changed His12 to Arg.

Primers were extended with T7 DNA polymerase, and the new strands were ligated with T4 DNA ligase for 1 h at 37 °C (final volume, 30 μl). Plasmids were then digested for 2 h at 37 °C with 20 μl of Scnl (final volume, 60 μl), 4-μl samples were used to transform 90 μl of Epicurian coli XL1-Blue competent cells (Stratagene). Plasmids N387(V2R), N387(F4R), N387(N6L), N387(V10R), N387(I12R), N387(P14G), N387(R15A), and N387(K15A), and N387(F4R,V10R,I12R) were purified from XLmutS competent E. coli for each sample. Sequences of the mutant ARD1 constructs were confirmed by automated sequencing (Applied Biosystems, 373 DNA Sequencer) using the primers 5′-TTATCCACTACCTATTAGGG-3′ and 5′-GCTAGTTATTGCTAGCCG-3′. Large scale production of N387ARD1 was carried out as described for the deleted mutant of ARD1.

Release of [3H]GDP5′ or [3H]GTP from Recombinant Proteins—(300 pmol) were incubated for 30 min at 30 °C in 450 μl of 20 mM Tris (pH 8.0), 10 mM dithiothreitol, 2.5 mM EDTA, with 0.3 mg/ml bovine serum albumin and 1 mg/ml cardiolipin and then incubated for 40 min in the same medium plus 10 mM MgCl2 and 3 μM of [3H]GDP5′ (2 × 105 cpm; total volume, 500 μl) or [3H]GTP (1.5 × 105 cpm; total volume, 500 μl) before the addition of p3 or deleted/mutated-forms (or water) in 100 μl of 10 mM Tris (pH 8.0), 2.5 mM EDTA. After incubation for 5 min at 30 °C, samples (60 μl) were transferred to nitrocellulose filters, which were washed five times with 1 ml of 25 mM Tris (pH 8.0). 0.5 mM MgCl2/100 mM NaCl before radioassay in a liquid scintillation counter (19). To calculate zero time values for dissociation curves, radioactivity bound to filters in the absence of protein was subtracted from the total with proteins. The remainders of the mixtures were immediately added to an equal volume of reaction buffer containing 2 mM of GDP5′ or GDP. Samples taken after 5, 15, 30, 45, 60, 75, 90, 105, and 120 min at 30 °C for quantification of bound radioactivity, as described for the zero time samples. Errors bars smaller than the symbols are not shown.

GTP7′ Binding Assay—GTP7′ binding to purified recombinant

GATGATTGGACAAAAATGG-3′ (differences from the original clone are underlined) and the reverse primer 5′-GAATTCGGGAGATCCTACTTTGCG-3′ (italicized sequence is a BamHI restriction site). The forward primer introduced a SacII restriction site (italicized sequences) and an initiation codon in-frame (bold sequences) in the deleted mutants N387p5, N383p5, and N383p5, which were cloned in-frame in SacII- and BamHI-digested pGEX-5G/LIC expression vector. Ultra competent cells (Stratagene) were transformed with plasmids pGEX5G/LIC/N383p8 or pGEX5G/LIC/N383p8. Sequences of mutual N387 fragments were confirmed by automated sequencing (Applied Biosystems, 373 DNA Sequencer) using the primers 5′-TTATCCACTACCTATTAGGG-3′ and 5′-GCTAGTTATTGCTAGCCG-3′. Fusion proteins were expressed and purified as described for ARD1 proteins.

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ARD1 Dissociation Inhibitory Region

RESULTS AND DISCUSSION

Although there was no significant difference between p8 (ARD1) and p3 (ARF domain of ARD1) in the amount of [β, α-35S]GDP bound at zero time (Fig. 1B), [β-35S]GDP release (in the presence of large excess of [35S]GDP) was much slower from p8 than from p3 as seen earlier (18), consistent with the notion that the ARD1 amino-terminal extension p5 may act to inhibit nucleotide release. To define more precisely the region of p5 involved, we generated four mutants of ARD1 with amino-terminal deletions (Fig. 1A). GDP-[β-35S] dissociation from the four mutants (NΔ88p8, NΔ200p8, NΔ304p8, NΔ387p8) approximated that of the intact p8 (Fig. 1B). The shortest deletion mutant protein NΔ387p8, which is equivalent to p3 with 15 amino acids from ARD1 added at the amino terminus, retained the dissociation behavior of ARD1. It was concluded that these 15 residues are apparently involved in stabilizing the inactive GDP form of the ARF domain of ARD1.

When synthesized separately in Escherichia coli and incubated together, the non-ARF domain of ARD1 p5 reduced the rate of GDP/S release from the ARF domain p3 (Fig. 2B) to approximately that of ARD1 (18). Physical interaction between p5 and p3, expressed separately in E. coli, had also been demonstrated by gel filtration and using glutathione S-transferase fusion proteins (18). In agreement with the hypothesis that the 15 carboxyl-terminal amino acids of p5 act as an inhibitor of GDP/S dissociation from p3, three carboxyl-terminal deletion mutants of p5 (Fig. 2A) had no effect on GDP release from p3 (Fig. 2B). Surprisingly, however, three amino-terminal deletion mutants of p5 (Fig. 2A) were also ineffective in reducing GDP/S dissociation from p3. In large excess (up to 10 times p3), NΔ88p5 or NΔ200p5 had no effect on GDP/S release from the ARF domain of ARD1 (data not shown). There are a number of possible reasons for this apparent discrepancy. A domain involved in interaction with p5 may be located in the amino terminus of p5. Incorrect folding of the deleted mutants may also be an explanation. We favor yet another explanation in which the functional interaction between p3 and the part of p5 that slows nucleotide dissociation requires very precise alignment of the two domains to allow the latter to stabilize the GDP form of p3. Consistent with this possibility is the finding that deletion mutants p5NΔ88 and p5NΔ200 can physically interact with p3 and also that addition of NΔ200p5 and CΔ191p5 together with p3 did not reproduce the effect of p5 on GDP/S release from p3 (data not shown).

Fig. 1. Effect of amino-terminal deletion on dissociation of GDP/S from ARD1. A, ARD1 contains a 46-kDa amino-terminal domain (p5) and a 18-kDa ARF domain (p3). Deletion of 88, 200, 304, and 387 amino acids from ARD1 yielded NΔ88p8, NΔ200p8, NΔ304p8, and NΔ387p8. B, 300 pmol of p3, ARD1, or the amino-terminal deletion mutants of ARD1 with [β-35S]GDP bound were incubated with water (100 μl) for 5 min. Release of bound [β-35S]GDP/S was monitored for 120 min as described under “Experimental Procedures.” The data are representative of at least three independent protein preparations.

Fig. 2. Effect of amino- and carboxyl-terminal deletion mutants of p5 on dissociation of GDP/S from p3. A, deletion of 88, 200, or 304 amino acids from the amino terminus of p5 yielded NΔ88p5, NΔ200p5, and NΔ304p5; deletion of 69, 191, or 293 amino acids from the carboxyl terminus of p5 yielded CΔ69p5, CΔ191p5, and CΔ293p5. Molecular mass of each protein product is indicated. B, release of [β-35S]GDP/S from p3 was monitored for 120 min. The data are representative of at least two independent protein preparations.
ARF in regulating nucleotide dissociation (24–26), based on analysis of nucleotide binding and release from amino-terminal deletion mutants of ARF1. We suggest that the carboxyl terminus of p5 corresponding to the amino terminus of ARF may also play this role in ARD1. In favor of this hypothesis are the facts that p5 increased GDP:S release from a chimeric ARF1 protein with an amino-terminal deletion (NA15ARF1(39–45p3)), which possesses the binding site for p5, but not from the chimeric protein with the amino terminus intact (ARF1(39–45p3)) (17), suggesting that the carboxyl end of p5 and the amino terminus of ARF may have analogous locations in the molecules and therefore perform analogous functions in regulating nucleotide dissociation.

GDP:S release from NΔ393p8 was significantly faster than that from ARD1 or NΔ387p8 but also significantly slower than that from p3 (Fig. 3). These results suggested that amino acids 1–6 of the 15 carboxyl-terminal residues of p5 participate in stabilizing the GDP:S-bound form of the ARF domain of ARD1 but also that residues 7–15 have a similar function. Accordingly, removal of 11 residues dramatically affected GDP:S release, because rates of dissociation from NΔ398p8 and p3 were similar (Fig. 3).

GDP:S or GDP dissociated faster from p3 than from p8 or NΔ387p8 (Figs. 1F, 3, and 4). However, GTP:S dissociated slightly faster from p8 or NΔ387p8 than from p3 (Fig. 4). In fact, dissociation curves from NΔ387p8 for both GDP and GTP:S approximated those from p8 or ARD1. These results suggested that the 15 residues preceding p3 may specifically inhibit GDP dissociation and slightly increase GTP:S dissociation. Note that identical results were obtained with [3H]GDP or [35S]GDP:S (Figs. 1, 3, and 4).

Partial deletion of amino acid sequence can have subtle but adverse effects on overall protein structure. Therefore, to reduce the possibility that the observed differences between NΔ387p8, NΔ393p8, and NΔ398p8 in GDP release could arise from slight structural changes that could perturb the three-dimensional organization of the ARF domain, mutant proteins were constructed with single amino acid replacements that should cause minimal disturbance of global protein structure. Specifically, hydrophobic amino acids between residues 1 and 15 were individually changed to arginine, asparagine was replaced by leucine, proline was replaced by glycine, and lysine was replaced by alanine (Table I).

Whereas the mutation (V2R) had no effect on GDP:S release, GDP:S dissociated significantly faster from NΔ387–F4R:p8 than from NΔ387p8 (Fig. 5A). In fact, GDP:S dissociation from NΔ387:F4R:p8 approximated that from NΔ393p8 (Figs. 3 and 5A), suggesting that the accelerated GDP:S release from NΔ398p8 (relative to NΔ387p8) might be due to removal of Phe. Replacement of two other hydrophobic residues, Val10 and Ile12, significantly increased the rate of GDP:S dissociation, whereas replacement of Asn with the hydrophobic L (present in ARF1; Table I) slightly slowed GDP:S release.

**TABLE I**

| Protein          | Amino-terminal sequence | Mean hydrophobicity | Dissociation rate (koff) |
|------------------|-------------------------|--------------------|--------------------------|
| NΔ387p8          | MVTFKDKNVRHIGPK         | 0.08               | 0.023 ± 0.001 (1000)     |
| NΔ387p8(V2R)     | MVTFKDKNVRHIGPK         | 0.24               | 0.024 ± 0.002 (105)      |
| NΔ387p8(F4R)     | MVTFKDKNVRHIGPK         | 0.37               | 0.031 ± 0.002 (134)      |
| NΔ387p8(N8L)     | MVTFKDKNVRHIGPK         | 0.05               | 0.015 ± 0.001 (78)       |
| NΔ387p8(V10R)    | MVTFKDKNVRHIGPK         | 0.36               | 0.029 ± 0.002 (126)      |
| NΔ387p8(112R)    | MVTFKDKNVRHIGPK         | 0.38               | 0.032 ± 0.002 (139)      |
| NΔ387p8(14G)     | MVTFKDKNVRHIGPK         | 0.07               | 0.028 ± 0.001 (121)      |
| NΔ387p8(K15A)    | MVTFKDKNVRHIGPA         | 0.02               | 0.022 ± 0.003 (96)       |
| NΔ387p8(F4R,10R,112R) | MVTFKDKNVRHIGPK     | 0.87               | 0.038 ± 0.003 (165)      |
| ARF1             | MSSIFANFLPRKGLFGK        | 0.34               | 0.020 ± 0.002           |

*In parentheses, the rate relative to that of NΔ387p8 = 100 is indicated.

^ARF1 GDP:S dissociation rate in the presence of cardiolipin appears slightly lower than that reported for GDP (25) in the presence of dimyristoyl-phosphatidylcholine/cholate (0.020 versus 0.027).
(Fig. 5B). However, substitution of Asn by Leu, as well as that of Ile by Arg, did not modify the GTPγS dissociation rate (data not shown). The mutation K15A had no effect on GDPβS release (Fig. 5B). Interestingly, the mutant NA387(P14G) also exhibited an increased rate of GDPβS dissociation, suggesting that this proline may contribute to correct positioning of hydrophobic residues. These results, therefore, are consistent with the view that hydrophobic residues in this region of ARD1, as in the amino terminus of ARF, stabilize the GDP-bound form of the ARF domain, probably by interacting with the hydrophobic core in the tertiary structure of ARF (27). Despite significant differences between the corresponding sequences in ARD1 and ARF1, hydrophobic residues are numerous in both (Table I). Other ARF proteins share the same characteristic α-helical amino-terminal extension. It is notable that the ARF6 amino-terminal region is four amino acids shorter than ARF1, ARF2, and ARF3 but does contain the critical hydrophobic residues. It would be interesting to compare the rates of GDP release among different ARFs to understand more precisely how this domain stabilizes the GDP-bound form of the ARF motif.

We used functional assays to monitor conformational integrity of the deletion and site-specific mutants. Binding of GTPγS to ARF requires a strict positioning of residues involved in the nucleotide-binding pocket and is responsible for the conformational switch that activates ARF proteins. No significant differences in GTPγS binding among ARD1, NA387p8, and single site-specific mutants of NA387p8, NA393p8, NA398p8, and p3 were observed (Table I), suggesting no difference in folding of the guanine nucleotide-binding site of these recombinant proteins. Activation of cholera toxin-catalyzed ADP-ribosylating formation by ARFs requires binding of GTP followed by interaction with the bacterial toxin. The site of interaction with cholera toxin has been localized in the carboxyl-terminal region of ARF (24, 26, 28), and the ability of ARD1 mutants to activate CTA should represent a good indicator of conformational integrity. Removal of 387 residues from the amino terminus of ARD1 reduced CTA activation by about 28% (Table I), suggesting that the large amino-terminal extension contributes to maintain the native conformation of the protein. Accordingly, further deletion of the amino terminus reduced the ability of NA393p8, NA398p8, and p3 to activate CTA by ~ 39, 62, and 63%, respectively (Table I). Because GTPγS binding was not affected (Table I), we assume that the amino-terminal extension of ARD1, specifically the segment that influences nucleotide release, is important for correct folding of the region of the protein that interacts with cholera toxin.

In the crystal structure of ARF1, Phe6, Leu8, and Phe9 reside in the hydrophobic core of the molecule (27, 29). We suggest that residues Phe391, Val397, and Ile399 in ARD1 play the same role as Phe6, Leu8, and Phe9 in ARF1. Because single mutation of Phe4, Val10, or Ile12 in NA387p8 significantly affected GDPβS dissociation (Figs. 5, A and B), we generated a triple mutant NA387(F4R,V10R,I12R)p8 in which critical hydrophobic amino acids were replaced by arginines. Whereas GTPγS dissociation rate was not modified (data not shown), the rate of GDPβS release was faster than those from single-mutants and even faster than that from p3 (Fig. 5C and Table I). At zero time, the triple mutant protein also had ~58% more GDPγS bound than did NA387p8; GDPβγS binding was found to 3.93 ± 0.04% of the former and 2.84 ± 0.02% of the latter (Fig. 5C). Accordingly, we found that NA387(F4R,V10R,I12R)p8 bound more GTPγS (~35%) and was a better activator (~30%) of cholera toxin-catalyzed ADP-ribosylation than was NA387p8 (Table II).

Lower hydrophobicity of the amino-terminal sequences of NA387p8 was apparently correlated with a greater rate of GDP release (koff, Table I). We cannot, however, completely rule out the possibility that disruption of the α-helical structure (Table I) also affected GDP release from ARD1, although the mutant protein NA387p8(V2R), which had virtually no amino-terminal α-helical structure, differed very little from NA387p8 in rate of GDP release (Table I). These results suggest that hydrophobic amino acids in this region are crucial to stabilize the GDP-bound form of the ARF domain of ARD1, because replacement by the positively charged arginine increased GDPβS dissociation. The most important difference between ARFs and the other ~20-kDa GTPases is precisely the presence of an amino-terminal α-helix (αA) and the connecting loop (L1). Both of these sequences have no counterpart in Ras family members (30) but do appear in the sequences of G protein α-subunits (31). In accord with our findings is the report that charged residues constitute the site that provides the signal directly from rho to opsin to transduce to activate GDP release (32).

The amino-terminal segment is critical to ARF1 functions both in vivo and in vitro (reviewed in Ref. 3). Therefore, involvement of the amino-terminal α-helix of ARF1 in membrane affinity, nucleotide-induced conformational change, and effector interaction has been suggested (33, 34). Our results are consistent with the view that an equivalent region may play the same role in ARD1. Thus, as in ARF1, residues 389–402 in

| Protein | GTPγS binding | ARF activity |
|---------|---------------|--------------|
| NA387p8 | 0.956 ± 0.024 | 1.29 ± 0.03 |
| NA387p8(V2R) | 0.959 ± 0.012 | 0.91 ± 0.06 |
| NA387p8(F4R) | 0.980 ± 0.017 | 0.91 ± 0.04 |
| NA387p8(N9L) | 0.949 ± 0.006 | 0.93 ± 0.02 |
| NA387p8(V10R) | 0.979 ± 0.056 | 0.93 ± 0.06 |
| NA387p8(I12R) | 0.975 ± 0.021 | 0.95 ± 0.02 |
| NA387p8(P14G) | 0.979 ± 0.017 | 0.97 ± 0.08 |
| NA387p8(K15A) | 0.934 ± 0.017 | 0.93 ± 0.03 |
| NA387p8(F4R,V10R,I12R) | 1.297 ± 0.006 | 1.22 ± 0.05 |
| NA393p8 | 0.955 ± 0.020 | 0.79 ± 0.06 |
| NA398p8 | 0.962 ± 0.013 | 0.49 ± 0.04 |
| p3 | 0.977 ± 0.035 | 0.48 ± 0.03 |

*Significantly different (p < 0.01) from GTPγS binding to NA387p8 (unpaired t tests).
ARD1 appear to function as a GDP dissociation inhibitory region. In addition, in ARD1, there is a proline in position 388 that may demarcate this region from the rest of p5 and perhaps contribute to the functional alignment with p3.

To our knowledge, no ARF guanine nucleotide dissociation inhibitor proteins have been reported. Although this could be due to technical failure, we might propose that the amino-terminal segment of ARF and the equivalent region in ARD1 can serve as intrinsic modulators of guanine nucleotide dissociation, and thus association. Further study of these regions in ARDs may represent sites of interaction for ARF GEPs. Indeed, in ARF proteins and in ARD1, the guanine nucleotide dissociation inhibitor site is analogous to a site in Go subunits of heterotrimeric G-proteins (31). It is thus tempting to speculate that ARF GEPs increase GDP dissociation by a mechanism similar to that of the receptors with seven membrane-spanning helices. Positively charged amino acids in the third intracellular loop are directly responsible for acceleration by these receptors of guanine nucleotide exchange on heterotrimeric G proteins (35). We are currently investigating the possibility that the amino termini of the ARFs are involved in the activity of ARF GEPs.

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