Interaction of the GTP-binding and GTPase-activating Domains of ARD1 Involves the Effector Region of the ADP-ribosylation Factor Domain

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ARG-ribosylation factors (ARFs) are a family of 20-kDa guanine nucleotide-binding proteins and members of the Ras superfamily, originally identified and purified by their ability to enhance the ADP-ribosyltransferase activity of cholera toxin and more recently recognized as critical participants in vesicular trafficking pathways and phospholipase D activation. ARD1 is a 64-kDa protein with an 18-kDa carboxyl-terminal ARF domain (p3) and a 46-kDa amino-terminal extension (p5) that is widely expressed in mammalian tissues. Using recombinant proteins, we showed that p5, the amino-terminal domain of ARD1, stimulates the GTPase activity of p3, the ARF domain, and appears to be the GTPase-activating protein (GAP) component of this bifunctional protein, whereas in other members of the Ras superfamily a separate GAP molecule interacts with the effector region of the GTP-binding protein. p5 stimulated the GTPase activity of p3 but not of ARF1, which differs from p3 in several amino acids in the effector domain. After substitution of 7 amino acids from p3 in the appropriate position in ARF1, the chimeric protein ARF1(39-45p3) bound to p5, which increased its GTPase activity. Specifically, after Gly40 and Thr45 in the putative effector domain of ARF1 were replaced with the equivalent Asp and Pro, respectively, from p3, functional interaction of the chimeric ARF1 with p5 was increased. Thus, Asp25 and Pro39 of the ARF domain (p3) of ARD1 are involved in its functional and physical interaction with the GTPase-activating (p5) domain of ARD1. After deletion of the amino-terminal 15 amino acids from ARF1(39-45p3), its interaction with p5 was essentially equivalent to that of p3, suggesting that the amino terminus of ARF1(39-45p3) may interfere with binding to p5. These results are consistent with the conclusion that the GAP domain of ARD1 interacts with the effector region of the ARF domain and thereby stimulates GTP hydrolysis.

ARD1, a 64-kDa protein, contains a 46-kDa amino-terminal domain and an 18-kDa carboxyl-terminal ADP-ribosylation domain and thereby stimulates GTP hydrolysis.

The abbreviations used are: ARD, ARF domain protein; ARF, ADP-ribosylation factor; BSA, bovine serum albumin; CTA, cholera toxin A subunit; DTT, dithiothreitol; GAP, GTPase-activating protein; GEP, guanine nucleotide-exchange protein; GSH, glutathione; GST, glutathione S-transferase; GDPθS, guanosine 5′-O-2-thiodiphosphate; GTPθS, guanosine 5′-3-O-thiotriphosphate; LM, low-melting; PC, phosphatidylcholine; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; PIP2, phosphatidylinositol 4,5-bisphosphate; PAGE, polyacrylamide gel electrophoresis.
were from Pharmacia Biotech Inc. PCR reagents and restriction enzymes, unless otherwise indicated, were from Boehringer Mannheim. Sources of other materials have been published (16, 23, 24).

Preparation of Recombinant Fusion Proteins (p3, p5, and p8)—Fusion proteins, synthesized using a ligation-independent cloning method (22), were used in this study. The supernatants from bacteria transformed with silver-stained after SDS-PAGE (23). After cleavage by bovine thrombin, glutathione-S-transferase was removed with glutathione- Sepharose beads and thrombin with benzamidine-Sepharose 6B (26).

The proteins were further purified by gel filtration through Ultrigel AcA 54 and Ultrigel AcA 34. Purity was confirmed by silver staining after SDS-PAGE (23). Recombinant ARF1 was expressed and purified as described (27). Amounts of purified proteins were estimated by a dye binding assay (28) and by SDS-PAGE using bovine serum albumin as standard.

Construction and Expression of Chimeric Forms of p3 and ARF1—The 5′ end of the ARF domain of ARD1 (p3), from pGEX-5G/LIC (1) containing the p3 cDNA (EMBL Data Bank 1993, accession number L04510), was amplified by PCR using the forward primer 5′-CTCGACCATCTCCAGGAGG-3′ (italicized sequence indicates SacI restriction site) and the reverse primer 5′-CTTAGAGCCCGTTTACATTTCAAACAGA-3′ (italicized sequence is an EcoRI restriction site); differences from the original clone are underlined. The reverse primer introduced two replacement bases and an EcoRI restriction site. The 3′ end of p3 from pGEX-5G/LIC/p3 was amplifying the pCRII vector, by PCR using the forward primer 5′-GAATTCCTGCACCATTTCAACATGTGGTT-3′ (italicized sequence is an EcoRI restriction site); differences from the original clone are underlined and the reverse primer 5′-GGATCCAGCTGCGGTCCCTG3′ (italicized sequence is a BamHI restriction site). The forward primer introduced five replacement bases and an EcoRI restriction site. The PCR fragments were extracted from LM-agarose gel, purified by phenol/chloroform precipitation, and subcloned into pCR®II vector using the TA cloning kit (Invitrogen) according to the manufacturer’s instructions. The mutated 5′ fragment was excised with SacI and EcoRI. The mutated 3′ fragment was excised with EcoRI and BamHI. The two fragments were ligated in-frame through their EcoRI restriction sites. The resulting fragment was ligated in-frame to the SacI- and BamHI-digested pGEX-5G/LIC expression vector. Ultracompetent cells (Stratagene) were transformed with the plasmid pGEX-5G/LIC/p3(24-30ARF1). The sequence of the mutated p3 was confirmed by automatic sequencing (Applied Biosystems 373 DNA sequencer) using the primer 5′-CTCGACCATCTCCAGGAGG-3′ (p3(24-30ARF1) fragment was excised from the pCR®II vector. Fusion protein was expressed and purified as described for the non-mutant p3.

Construction of ARP1 (replicating 43 amino acids) from the pBlue-script (p7T/Nde) expression vector containing the ARF1 cDNA (GenBank™ 1992, accession number M84326) was amplified by PCR using the forward primer 5′-GGCGAAGCATTGAGGACATCTCCG-3′ (italicized sequence is an NdeI restriction site) and the reverse primer 5′-GGATTCATCCTCGTAAAGCTTGAAGG-3′ (italicized sequence is an EcoRI restriction site); differences from the original clone are underlined. The reverse primer produced the EcoRI restriction site. The resulting fragment was then ligated into the NdeI- and EcoRI-digested pGEX-5G/LIC expression vector. Ultracompetent cells (Stratagene) were transformed with the plasmid pGEX-5G/LIC/p3(24-30ARF1). The sequence of the mutated p3 was confirmed by automatic sequencing (Applied Biosystems 373 DNA sequencer) using the primer 5′-CTCGACCATCTCCAGGAGG-3′ (p3(24-30ARF1) fragment was excised from the pCR®II vector. Fusion protein was expressed and purified as described for the non-mutant p3.

Construction and Expression of ARF1—For site-directed mutagenesis of ARF1, a modification of the unique site-elimination mutagenesis procedure described by Deng and Nickoloff (29) was used. 25 pmol of a 5′-phosphorylated selection primer and 25 pmol of a 5′-phosphorylated mutated primer were simultaneously annealed to 600 ng of pARF1T7Nde 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 for 5 min on ice followed by incubation at room temperature for 30 min. The selection primer 5′-CTGGTACGTGAGCCGCGTACACAGT-3′ changed a SacI restriction site in the new 5′-end of the ARF domain of ARD1 (p3), from pGEX-5G/LIC (1) restriction site. The 3′ replacement bases and an EcoRI restriction site. The resulting fragment was then ligated into the NdeI- and EcoRI-digested pGEX-5G/LIC expression vector. Ultracompetent cells (Stratagene) were transformed 40 μl. Plasmids were then digested for 2 h at 37°C with 20 units of SacI (final volume, 30 μl). Plasmids were digested for 2 h with 3 units of NdeI (final volume, 60 μl). 4-μl samples were used to transform 10 μl of Epicurian Col XLI-Blue competent cells (Stratagene). Plasmids ARF1 (G40D), ARF1 (T45P), and ARF1 (G40D, T45P) were purified with Miniprep Wizard (Promega) from bacteria grown overnight in 2 ml of 2YT broth with 100 μg/ml ampicillin. Samples (500 ng) of the plasmids were digested with 20 units of SacI for 3 h at 37°C. 4-μl samples were used to transform 40 μl of XLmutS competent cells (Stratagene). Colonies were selectively screened by digestion with MluI, and the presence of the mutations was confirmed by automatic sequencing (Applied Biosystems 373 DNA sequencer) using the primer 5′-CTCGACCATCTCCAGGAGG-3′ (p3(24-30ARF1) fragment was excised from the pCR®II vector. Fusion protein was expressed and purified as described for the chimeric ARF1 (39-45p3).

Construction and Expression of 5′ARF1(39-45p3)—The plasmid p7T/Nde/ARF1(39-45p3) described above was amplified by PCR in the presence of Pfu DNA polymerase (Stratagene) with the forward primer 5′-CTCGACCATCTCCAGGAGG-3′ and the reverse primer 5′-CTCGACCATCTCCAGGAGG-3′ (italicized sequence is a BamHI restriction site). The forward primer produced the deletion of 45 bases from the beginning of the coding region of ARF1(39-45p3). The PCR fragment was extracted from LM-agarose gel, purified, and subcloned into the blunt end 5′F1 restriction site of pCR-Script™ SK(+) according to the manufacturer’s instructions (Stratagene) for transformation into XLI-Blue MRFK supercompetent cells. Because of the orientation of the PCR product in 20 positive colonies (checked by PCR and restriction enzyme digestion) did not allow expression under the T7 promoter, the 5′ARF1(39-45p3) fragment was excised from the pCR-Script™ SK(+) vector with SacII and BamHI and was ligated in-frame to the SacII- and BamHI-digested pGEX-5G/LIC expression vector. Ultracompetent cells (Stratagene) were transformed with the plasmid pGEX-5G/LIC/5′ARF1(39-45p3). The sequence of the ARF1 deletion mutant was confirmed by automatic sequencing (Applied Biosystems 373 DNA sequencer) using the primer 5′-CTCGACCATCTCCAGGAGG-3′. GST-5′ARF1(39-45p3) fusion protein was expressed and purified as described above. Glutathione S-transferase was removed (23) and the protein was further purified by gel filtration through columns of Ultrogel AcA 54 (1.5 × 30 cm) and Frager Aca 34 (1.5 × 30 cm) before storage in small portions at −20°C.

Interaction Domain of ARD1 GAP—Bacterial pellets were dispersed in 5 ml of cold phosphate-buffered saline, pH 7.4, with 20 μg/ml trypsin inhibitor, 5 μg/ml each leupeptin and aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride. Lysozyme (10 mg in 5 ml) was added. After 30 min at 4°C, cells were disrupted by sonication and centrifuged (Sorvall SS34, 16,000 rpm, 4°C, 20 min). The supernatant was applied to a column (1.5 × 40 cm) of DEAE, eluted with a linear gradient of 0 to 500 mM NaCl (60 ml each column) and further purified by DEAE chromatography on a column (1.5 × 40 cm) of DEAE, eluted with a linear gradient of 0 to 500 mM NaCl (60 ml each column). Fractions that had both high ARF activity and high purity were pooled and further purified by DEAE chromatography on a column (1.5 × 40 cm) of DEAE, eluted with a linear gradient of 0 to 500 mM NaCl (60 ml each column) and gel filtered on Ultrigel AcA 34 (1.5 × 30 cm) before storage in small portions at −20°C.
centration of MgCl₂. Each point is the mean of three determinations with the smallest than the symbol for each value. These results were repeated twice. Inset, p3 (0.4 μg), p5 (1 μg), and p8 (1.4 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membrane.

[α-32P]GTP binding in an overlay assay was carried out in the presence of 1 mg/ml cardiolipin. Identical results were obtained with three different protein preparations.

FIG. 1. Binding of GTP to recombinant ARD1 and ARD1 domains. Binding of 3 μM [35S]GTP·pS to 30 pmol of p3 (●, 0.54 μg), p5 (●, 1.38 μg), or p8 (●, 1.92 μg) was assessed using a rapid filtration technique. The medium contained 1 mM EDTA and the indicated concentration of MgCl₂. Each point is the mean of three determinations with the smaller than the symbol for each value. These results were repeated twice. Inset, p3 (0.4 μg), p5 (1 μg), and p8 (1.4 μg) were subjected to SDS-PAGE and transferred to nitrocellulose membrane.

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GTP·pS Binding Assay—GTP·pS binding to purified recombinant ARD proteins was assessed using a rapid filtration technique. Samples were incubated for 30 min at 30 °C in 20 mM Tris (pH 8.0), 10 mM DTT, 2.5 mM EDTA with 0.3 mg/ml BSA and 1 mg/ml cardiolipin, and then for 40 min at 30 °C in the same medium plus 10 mM MgCl₂ (as indicated) and 3 μM [35S]GTP·pS (~10⁶ cpm; total volume, 150 μl). Where indicated, cardiolipin was replaced by another lipid or detergent in the binding buffer. Samples (70 μl) were then transferred to nitrocellulose filters in a manifold (Millipore) for rapid filtration followed by washing five times each with 1 ml of ice-cold buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 5 mM MgCl₂). Dried filters were dissolved in scintillation fluid for radioassay.

Assay of GTPase Activity—Samples were incubated for 30 min at 30 °C in 20 mM Tris, pH 8.0, 10 mM DTT, 2.5 mM EDTA with 0.3 mg/ml BSA and 1 mg/ml cardiolipin, and then for 40 min at 30 °C in the same medium with 0.5 μM [α-32P]GTP (3000 Ci/mmol) and 10 mM MgCl₂ (total volume, 120 μl). After addition of p5 or vehicle (40 μl), incubation at room temperature was continued as indicated for 10 min to 2 h (final volume, 160 μl) before proteins with bound nucleotides were collected on nitrocellulose (24). Bound nucleotides were eluted in 250 μl of 2 M formic acid, of which 3–4-μl samples were analyzed by TLC on polyethyleneimine-cellulose plates (19) and 240 μl was used for radioassay to quantify total nucleotide. TLC plates were subjected to autoradiography at ~80 °C for 18–28 h. Total amounts of labeled nucleotides (GTP + GDP) bound to p5, p8, or GST-p5 after incubation with GST-p5, whether quantified by radioassay of the formic acid solution, or by phosphorimaging (Molecular Dynamics) after TLC, were not significantly different under any conditions.

Assay of CTA-catalyzed ADP-ribosylagmatine Formation—p3 or p8 was incubated for 30 min at 30 °C in 40 μl of 20 mM Tris, pH 8.0, 10 mM DTT, 2.5 mM EDTA with 0.3 mg/ml BSA and 1 mg/ml cardiolipin before addition of 20 μl of solution to yield final concentrations of 100 μM GTP·pS or GTP and 10 mM MgCl₂. Where indicated, p5 was then added for 30 min. Components needed to quantify ARF stimulation of cholera toxin-catalyzed ADP-ribosylagmatine formation were then added in 70 μl to yield final concentrations of 50 mM potassium phosphate (pH 7.5), 6 mM MgCl₂, 20 mM DTT, 0.3 mg/ml ovalbumin, 0.2 mM ladanine-14-CNAD (0.05 μCi), 20 mM agmatine, 1 mg/ml cardiolipin, and 100 μM GDP or GTP with 0.5 μg of cholera toxin (30). Where indicated, cardiolipin was replaced by another lipid or detergent. After incubation at 30 °C for 1 h, samples (70 μl) were transferred to columns of AG1-X2 equilibrated with water and eluted with five 1-ml volumes of water. The eluate, containing [14C-ADP-ribosylagmatine, was collected for radioassay.

Release of [35S]GDP·pS from Recombinant Proteins—Samples (450 μl) were incubated for 30 min at 30 °C in 20 mM Tris (pH 8.0), 10 mM DTT, 2.5 mM EDTA with 0.3 mg/ml BSA and 1 mg/ml cardiolipin and then for 40 min in the same medium plus 10 mM MgCl₂ and 3 μM [35S]GDP·pS (2 × 10⁷ cpm/500 μl) before addition of p5 (or water) in 0.2 reaction volume. After incubation for 15 min at 30 °C, samples (60 μl) were transferred to nitrocellulose filters that were washed five times with 1 ml of 25 mM Tris, pH 8.0, 5 mM MgCl₂, 100 mM NaCl before radioassay in a liquid scintillation counter (24). 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 differences of the mixtures were immediately diluted with an equal volume of reaction buffer containing 2 mM GDP·pS. Samples (120 μl) were 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.

RESULTS

Specificity of Guanine Nucleotide Binding to ARD1—Recombinant proteins representing the ARF domain (p3), the non-ARF domain (p5), and the entire ARD1 protein (p8) were subjected to SDS-PAGE and transferred to nitrocellulose for assessment of their ability to bind GTP. Fig. 1, inset, provides visual evidence that p3 and p8 bound [α-32P]GTP, whereas the non-ARF domain, p5, did not. Binding of GTP·pS to p3 and p8 was equivalent and was dependent on magnesium concentration (Fig. 1). Maximal binding to both p3 and p8 was observed with 5 and 10 mM MgCl₂ (in the presence of 2.5 mM EDTA) and was less at 20 mM (Fig. 1). Maximal binding of GTP·γS to ARF1
TABLE I
Effects of detergents and phospholipids on GTPγS binding by p3 and p8

| Detergent/lipid | GTPγS bound | 80 pmol of p3 | 80 pmol of p8 |
|-----------------|-------------|---------------|---------------|
| Brain PC + PIP₂ + PE | 0.506 ± 0.021 | 0.105 ± 0.021 |
| Cardiolipin | 0.904 ± 0.011 | 0.995 ± 0.029 |
| Phosphatidylserine (200 μM) | 0.292 ± 0.032 | 0.095 ± 0.019 |
| Phosphatidylinositol (200 μM) | 0.195 ± 0.024 | 0.124 ± 0.011 |
| Phosphatic acid (200 μM) | 0.168 ± 0.031 | 0.099 ± 0.021 |
| PIP₂ (100 μM) | 0.085 ± 0.021 | 0.078 ± 0.015 |
| PC (200 μM) | 0.135 ± 0.020 | 0.086 ± 0.023 |
| Triton X-100 (0.1%) | 0.155 ± 0.018 | 0.098 ± 0.031 |
| Cardiolipin, PE | 0.062 ± 0.009 | 0.053 ± 0.005 |
| Lubrol PX (0.1%) | 0.045 ± 0.007 | 0.061 ± 0.011 |
| Tween 20 (0.3%) | 0.164 ± 0.011 | 0.152 ± 0.023 |

TABLE II
Effect of different detergents and phospholipids on CTA activation by p3 and p8

| Detergent/lipid | ARF activity | 900 pmol of p3 | 250 pmol of p8 |
|-----------------|--------------|---------------|---------------|
| Brain PC + PIP₂ + PE | 0.54 ± 0.14 | 1.55 ± 0.11 |
| Cardiolipin | 4.32 ± 0.15 | 4.51 ± 0.14 |
| Phosphatidylserine | 2.61 ± 0.11 | 1.21 ± 0.08 |
| Phosphatidylinositol | 1.02 ± 0.11 | 0.85 ± 0.15 |
| Phosphatic acid | 0.95 ± 0.06 | 0.75 ± 0.09 |
| PIP₂ | 0.62 ± 0.23 | 0.54 ± 0.05 |
| PC | 0.54 ± 0.05 | 0.42 ± 0.11 |
| PE | 0.82 ± 0.03 | 1.21 ± 0.08 |
| Dimyristoyl PC/cholate | 1.46 ± 0.09 | 1.01 ± 0.05 |
| Triton X-100 | 0.39 ± 0.02 | 0.40 ± 0.08 |
| Lubrol PX | 0.23 ± 0.06 | 0.31 ± 0.03 |
| Tween 20 | 4.31 ± 0.15 | 4.69 ± 0.09 |

and ARF3 (in the presence of 1 mM EDTA) occurred in the very narrow range of 0.5–1 mM MgCl₂ (17).

The specificity of guanine nucleotide binding was assessed by adding unlabeled nucleotide together with 4 μM (32)GTPγS in the binding buffer. ATP, TTP, and CTP (up to 100 μM) failed completely to compete with GTPγS for binding to p3 and p8, whereas 100 μM GTP or GDP decreased [32]GTPγS binding to p3 or p8 almost 80% (Fig. 2). The fact that GDP and GTP competed similarly for binding is in agreement with the previous observation (31) that at high Mg⁺⁺ concentrations ARFs have similar affinities for GDP and GTP.

Phospholipid Requirement for GTPγS and CTA Activity of ARDI—It has been reported that certain phospholipids or detergents, in the presence of Mg⁺⁺, can promote guanine nucleotide exchange and activate ARF (31, 32). In the absence of GEP, phospholipids differed markedly in their effects on GTPγS binding to ARF (17). As shown in Table I, in the presence of several detergents GTPγS binding to either p3 or p8 was very low (i.e., <0.2%), whereas with certain phospholipids, especially cardiolipin, binding was considerably higher and was similar for p3 and p8. On the other hand, with the mixture of brain PC (saturated and unsaturated PC), PIP₂, and PE, GTPγS binding to p3 was five times that by p8 (Table I).

Like other members of the ARF family, ARD1 activates CTA ADP-ribosyltransferase activity (1, 23). As it was for GTPγS binding (Table I), cardiolipin was the most effective phospholipid for activation of CTA (Table II). Despite supporting relatively low GTPγS binding, however, the detergent Tween 20 enhanced CTA activity induced by p3 or p8 to levels comparable to those with cardiolipin (Table II).
Each experiment was performed at least four times. Data are means of duplicates.

**Interaction Domain of ARD1 GAP**

### Activation by p5 of GTP Hydrolysis by p3

We had reported that the 64-kDa ARD1 (p8) exhibited significant GTPase activity, whereas the ARF domain (p3), synthesized as a recombinant protein in *E. coli*, did not (23). Addition of p5 (the ARD amino-terminal domain) increased hydrolysis of GTP bound to p3 in a concentration-dependent manner (Fig. 3A). The maximal effect was observed with a ratio of 2 mol of p5/mol of p3 and half-maximal hydrolysis with equimolar concentrations of p5 and p3. The reaction proceeded at an essentially constant rate until it stopped at 60 min (Fig. 3B). It appears that hydrolysis of GTP bound to p3 may have stopped when p5 became limiting (with equimolar p3 and p5), because p5 apparently interacts physically with p3-GDP as well as with p3-GTP (Ref. 23 and Fig. 3) and only a small fraction of p3 (~2%) had bound GTP. Indeed, addition of p5 at 60 min maintained GTP hydrolysis (see legend to Fig. 3).

**Identification of the Specific p5-binding Site on p3**—Although p5 acted as a GAP for p3, it was not effective with other ARF proteins (22). To identify the specific interaction domain in p3, we compared the amino acid sequences of p3 and other ARFs. Residues 24–30 in p3 are very different from the equivalent region in other Ras superfamily members, in which it has been shown to interact with GAP. Therefore, we prepared two chimeric proteins by exchanging these sequences in ARF1 and p3 (Fig. 4A). There was no significant difference between these and the nonchimeric proteins in GTP/S binding (data not shown) or in GTP-dependent CTA activation (Table III), consistent with the conclusion that GTP-binding properties and CTA activation were not impaired by the mutations. The chimeric p3 protein with ARF1 sequence, termed p3(24–30ARF1), was not a substrate for p5 (Fig. 4B) and did not interact with immobilized GST-p5 (Fig. 5B). On the other hand, p5 effectively hydrolyzed GTP bound to the chimeric ARF1(39–45p3) protein (Fig. 4, A and B).

Stimulation of CTA activity by p3 was similar with GTP and GTP/S, although it was much lower than the stimulation induced by ARF1 (Table III). As reported (23), addition of p5 to p3 reduced CTA activation with GTP but not with GTP/S (Table III). In the presence of GTP, p5 had no effect on the stimulation of the CTA activity by the chimeric p3(24–30ARF1), whereas it reduced stimulation by ARF1(39–45p3) (Table III). These results are consistent with the conclusion that p5 decreased the activity of the chimeric ARF1(39–45p3) by accelerating the hydrolysis of bound GTP.

Physical interaction between ARFs and the non-ARF p5 domain was assessed by gel filtration under the same conditions that p3 and p5 after incubation together, were eluted together (23). Similarly treated p3 and p5(24–30ARF1) did not coelute (data not shown), whereas ARF1(39–45p3) and p5 did (Fig. 5A, peak I; noninteracting ARF1(39–45p3) and p5 were also detected (Fig. 5A, peaks II and III).

Interactions between p5 and ARF1, p3, ARF1(39–45p3), or p3(24–30ARF1) were additionally evaluated using recombinant fusion GST-p5 bound to GSH-Sepharose beads that were then incubated with the indicated ARF protein. Proteins attached to the beads or interacting with them were eluted with GSH and separated by SDS-PAGE. The ARF domain p3 clearly interacted with GST-p5 (Fig. 5B, lane 1), whereas ARF1 did not (Fig. 5B, lane 2). GST-p5 also interacted with ARF1(39–45p3) but not

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**Fig. 4.** Effect of p5 on GTPase activity of chimeric ARF1 and p3 proteins. A, sequences of indicated amino acids from ARF1, p3, ARF1(39–45p3), and p3(24–30ARF1). The native LGEIVTT sequence of ARF1 was replaced with QDEFMQP from p3 to create ARF1(39–45p3), and the QDEFMQP sequence of p3 was replaced with LGEIVTT from ARF1 to create p3(24–30ARF1). B, effect of p5 concentration on GTP hydrolysis by p3, ARF1, and chimeric proteins. 2 μg of p3 or p3(24–30ARF1) or 1 μg of ARF1 or ARF1(39–45p3) with [α-32P]GTP bound were incubated with the indicated amount of p5 for 60 min before bound nucleotides were separated by TLC. GAP activity, i.e., the decrease in bound GTP due to incubation with p5, is presented as GTP/GTP0 × 100, where GTP and GTP0 are the amounts of GTP bound after incubation with and without p5, respectively. Data are means of duplicates ± one-half the range. Error bars smaller than symbols are not shown. Each experiment was performed at least four times.

**Table III**

| Recombinant protein added | ARF activity |
|---------------------------|--------------|
| p3 or ARF | p5 | GTP | GTP/S |
| nmol/h | nmol/h |
| p3 | 0 | 4.01 ± 0.06 | 3.93 ± 0.02 |
| p3 | + | 2.38 ± 0.05a | 3.85 ± 0.03 |
| p3(24–30ARF1) | 0 | 3.93 ± 0.09 | 3.88 ± 0.01 |
| p3(24–30ARF1) | + | 3.78 ± 0.07 | 3.85 ± 0.08 |
| ARF1 | 0 | 4.76 ± 0.23 | 4.48 ± 0.11 |
| ARF1 | + | 4.77 ± 0.08 | 4.33 ± 0.09 |
| ARF1(39–45p3) | 0 | 4.76 ± 0.08 | 4.59 ± 0.04 |
| ARF1(39–45p3) | + | 3.24 ± 0.08a | 4.51 ± 0.10 |

a With unpaired t tests, significantly different (p < 0.005) from activity in the presence of GTP/S.
equilibrated and eluted (0.2 ml/min) with 20 mM Tris, pH 8.0, 1 mM beads (200 g left. The same results were obtained in two independent experiments. gels followed by silver staining. Positions of standards (kDa) are on the left. These findings have been replicated three times with independent preparations. Differences with unpaired t tests between p5-stimulated GTP hydrolysis by ARF1(T45P) and ARF1(G40D,T45P) were significant (*, p < 0.007; **, p < 0.01).

To identify more precisely the amino acids involved, two single amino acid replacement mutants of ARF1 were made with the amino acid of p3 placed in the equivalent ARF position to generate ARF1(G40D) and ARF1(T45P). Although ARF1(G40D), like ARF1, was not a substrate for p5, ARF1(T45P) was (Fig. 6). Moreover, the effect of p5 was significantly greater (p < 0.01) with the double mutant ARF1(G40D,T45P), suggesting a synergistic effect of the double mutation (Fig. 6). ARF1(G40D,T45P) also interacted with recombinant fusion GST-p5 immobilized on GSH-Sepharose beads, whereas ARF1(G40D) and ARF1(T45P) did not (Fig. 7, lanes 1–3). The lower band (−19 kDa) in Fig. 7, lane 3, is apparently a contaminant, variable amounts of which copurified with the double mutant ARF1(G40D,T45P).

Effect of the Amino-terminal Region of ARFs—We reported that p5 stabilized the ARF domain in the inactive GDP-bound form by inhibiting GDP release as well as by promoting GTP hydrolysis (23). Indeed, when p5 was added to p3, dissociation of GDP bound to p3 was effectively decreased (Fig. 8A). GDP dissociation from ARF1(39-45p3) as well as from ARF1, however, was completely unaffected by p5 (Fig. 8B), indicating that even when physically interacting with ARF1(39-45p3) (Fig. 5B, lane 4) p5 did not induce all of the effects that it had on p3.

To investigate a possible effect of the amino terminus of the mutant ARF1(39-45p3), the first 15 amino acids were deleted to create ∆15ARF1(39-45p3). Hydrolysis of the GTP bound to ∆15ARF1(39-45p3) was stimulated by p5 more effectively than was that of GTP bound to ARF1(39-45p3), suggesting that the amino-terminal part in ARF1(39-45p3) might to some extent interfere with its interaction with p5 (Fig. 9). Indeed, the effect of p5 on GTP hydrolysis by ∆15ARF1(39-45p3) approximated that on p3 (Fig. 9). In addition, ∆15ARF1(39-45p3) binding to GST-p5 immobilized on GSH-Sepharose beads exceeded binding of ARF1(39-45p3) (Fig. 7, lane 4).

GDP dissociation from ∆15ARF1(39-45p3) was faster than from ARF1 or ARF1(39-45p3) (Fig. 8, B and C). When p5 was

To identify more precisely the amino acids involved, two single amino acid replacement mutants of ARF1 were made with the amino acid of p3 placed in the equivalent ARF position to generate ARF1(G40D) and ARF1(T45P). Although ARF1(G40D), like ARF1, was not a substrate for p5, ARF1(T45P) was (Fig. 6). Moreover, the effect of p5 was significantly greater (p < 0.01) with the double mutant ARF1(G40D,T45P), suggesting a synergistic effect of the double mutation (Fig. 6). ARF1(G40D,T45P) also interacted with recombinant fusion GST-p5 immobilized on GSH-Sepharose beads, whereas ARF1(G40D) and ARF1(T45P) did not (Fig. 7, lanes 1–3). The lower band (−19 kDa) in Fig. 7, lane 3, is apparently a contaminant, variable amounts of which copurified with the double mutant ARF1(G40D,T45P).

Effect of the Amino-terminal Region of ARFs—We reported that p5 stabilized the ARF domain in the inactive GDP-bound form by inhibiting GDP release as well as by promoting GTP hydrolysis (23). Indeed, when p5 was added to p3, dissociation of GDP bound to p3 was effectively decreased (Fig. 8A). GDP dissociation from ARF1(39-45p3) as well as from ARF1, however, was completely unaffected by p5 (Fig. 8B), indicating that even when physically interacting with ARF1(39-45p3) (Fig. 5B, lane 4) p5 did not induce all of the effects that it had on p3.

To investigate a possible effect of the amino terminus of the mutant ARF1(39-45p3), the first 15 amino acids were deleted to create ∆15ARF1(39-45p3). Hydrolysis of the GTP bound to ∆15ARF1(39-45p3) was stimulated by p5 more effectively than was that of GTP bound to ARF1(39-45p3), suggesting that the amino-terminal part in ARF1(39-45p3) might to some extent interfere with its interaction with p5 (Fig. 9). Indeed, the effect of p5 on GTP hydrolysis by ∆15ARF1(39-45p3) approximated that on p3 (Fig. 9). In addition, ∆15ARF1(39-45p3) binding to GST-p5 immobilized on GSH-Sepharose beads exceeded binding of ARF1(39-45p3) (Fig. 7, lane 4).

GDP dissociation from ∆15ARF1(39-45p3) was faster than from ARF1 or ARF1(39-45p3) (Fig. 8, B and C). When p5 was
added, release of GDP was slowed to a rate comparable to that from ARF1(39-45p3) (Fig. 8C). These results are consistent with other evidence that the amino-terminal part of ARFs may be important in regulating nucleotide dissociation and that p5 may also play this role in ARD1.

DISCUSSION

The unusual GTPase activity of ARD1 has made possible the identification of a region specifically involved in both functional and physical interaction between the guanine nucleotide-binding domain (p3) and the GAP domain (p5) of ARD1. A major difference between the heterotrimeric and monomeric guanine nucleotide-binding proteins of the Ras superfamily is the much lower intrinsic GTPase activity of the latter. The Ga subunits of trimeric G proteins, as described by Bourne and colleagues (33), contain a GTP-binding core (“Ralph”) common to the monomeric G protein family but differ by the presence of an insertion of 110–140 amino acids (“Gail”) at a position corresponding to loop 2 in p21Gld. Studies of chimeric recombinant proteins (33) and crystal structure (34) established that this inserted domain is responsible for the GTPase activity of the Ga subunit. Numerous proteins that enhance the GTPase activity of members of the monomeric G proteins have now been identified (for review, see Ref. 35). ARF proteins lacking detectable intrinsic GTPase activity (13), and several groups have recently reported the purification or cloning of ARF GAPs (19–22).

ARD1 exhibits significantly greater GTPase activity than other members of the Ras superfamily (23). Although its GTP-binding domain (p3) appeared not to possess GTPase activity, addition of the amino-terminal domain (p5) expressed as a recombinant protein promoted hydrolysis of GTP bound to p3. Amino acids Asp$^{25}$ and Pro$^{30}$ of the ARF domain of ARD1 (p3) were crucial for both the functional and physical interaction between the two domains of ARD1. This region is equivalent to the extended effector loop in Ras, which is known to interact with Ras-GAP and neurofibromin (36). Moreover, based on the three-dimensional structure of ARF (37) and computer analysis, amino acids 24–30 in p3 are part of a β sheet that is believed to be involved in dimerization in ARF crystals (37). Without excluding the existence of other interaction sites, it is reasonable to conclude that this region is involved in the interaction between the two domains of ARD1.

Based on crystal structure and studies showing that GAPs interact with the effector domain of members of the Ras superfamily (36, 37), the corresponding sequences of ARD1 and ARF1 were exchanged. Our data clearly show that replacing the LGEIVTT sequence in ARF1 with QDEFMQP (found in ARD1) conferred on it the ability to hydrolyze bound GTP in response to p5. The single replacement of Thr$^{45}$ by Pro in ARF1 transformed ARF1 into a substrate for p5. We postulate that Pro creates a curve in the β sheet structure that might place the conserved Glu (positions 41 in ARF1 and 26 in p3) in correct position for interaction with p5. The double mutant ARF1(G40D,T45P) provided evidence that Asp influences the interaction with p5, as physical and functional interactions of the double mutant were greater than those of ARF1(T45P).p5, however, hydrolyzed GTP bound to ARF1(39-45p3) better than GTP bound to ARF1(G40D,T45P), suggesting that glutamine(s) and hydrophobic residues in this region could also be involved in the interaction with p5.

The low stoichiometry of GTP binding by the ARF domain of ARD1 and by ARD1 itself is typical of recombinant ARFs (32, 38, 39) and may be due in part to the absence of N-myristoylation, which is a predominant determinant of the phospholipid-induced transition of ARF to the active ARF-GTP conformation (40). It may also be related to the fact that among the nucleotide-binding motifs that are otherwise identical, the CAT sequence of ARFs is replaced by DAR in ARD (1). The physiological significance of the effect of magnesium concentration on nucleotide affinities may be questioned as the intracellular...
concentrations of Mg\(^{2+}\) lipid vesicles due to the presence of p5. Indeed, a two-stage serine), may reflect differences in interactions with phospho-

Therefore, p5 acts as an negative regulator of the activity of ARD1, whereas Gail is centrally involved in both turning the Go switch on (GDP release followed by GTP binding) and turning it off (GTP hydrolysis). These aspects seem likely to be important in the as yet unidentified biological function of the ARF-related protein ARD1.

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**FIG. 9. Effect of p5 on GTPase activity of p3, ARF1, and ARF1 mutants.** 1 μg (~50 pmol) of recombinant p3 or ARF proteins with [α-32P]GTP bound was incubated with the indicated amount of p5 for 60 min before bound nucleotides were separated by TLC. GAP activity is expressed as GTP/GTPo × 100. Data are means of duplicates ± one-half range. Error bars smaller than symbols are not shown. Similar results were obtained from at least three different protein preparations.