The ADP-ribosylation Factor (ARF)-related GTPase ARP binds to the ARF-specific Guanine Nucleotide Exchange Factor Cytohesin and Inhibits the ARF-dependent Activation of Phospholipase D*

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ADP-ribosylation factor-related protein (ARP) is a membrane-associated GTPase with remote similarity to the family of ADP-ribosylation factors (ARF). In a yeast two-hybrid screen designed to identify proteins interacting with ARP, we isolated a partial cDNA of the ARF-specific guanine nucleotide exchange factor mSec7-1/cytohesin encoding its N terminus and most of the Sec7 domain (codons 1–200). ARP and ARP-Q79L (GTPase-negative ARP) exhibited a higher affinity to mSec7-1-(1–200) than ARP-T31N (nucleotide exchange-defective ARP) in the two-hybrid assay. Similarly, full-length [35S]mSec7-1/cytohesin was specifically adsorbed to glutathione-Sepharose loaded with glutathione S-transferase (GST)-ARP-Q79L, GST-ARP, or GST-ARP-T31N, the latter exhibiting the lowest binding affinity. Overexpression of ARP-Q79L, but not of ARP-T31N, in COS-7 cells reduced the fluorescence from co-expressed green fluorescent protein fused with mSec7-1/cytohesin or mSec7-2/ARNO in plasma membranes as detected by deconvolution microscopy. Recombinant ARP and ARP-Q79L, but not ARP-T31N, inhibited the phospholipase D (PLD) activity stimulated by mSec7-2/ARNO and ARP in a system of isolated membranes. Furthermore, transfection of HEK-293 cells with ARP or ARP-Q79L, but not ARP-T31N, inhibited the muscarinic acetylcholine receptor-3 induced PLD stimulation and translocation of ARF from cytosol to membranes. These data suggest that the GTP-bound form of ARP specifically binds mSec7-1/cytohesin, and that ARP may be involved in a pathway inhibiting the ARF-controlled activity of PLD.

ADP-ribosylation factors (ARF)* are Ras-related GTPases that regulate vesicular trafficking pathways in eukaryotic cells

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† The abbreviations used are: ARP, ADP-ribosylation factor; ARF, ARF-related protein; mSec7, mammalian Sec7; PLD, phospholipase D; PKC, protein kinase C; M₃ receptor, muscarinic acetylcholine receptor 3; GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-ace-tate; GPtS, guanosine 5’-O-(3-thio)-triphosphate; PtdEtoH, phosphatidylethanol; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; GFP, green fluorescent protein; PCR, polymerase chain reaction.
in Ref. 29). Thus, we assumed that the interaction of ARP with a mammalian Sec7 isoform might modulate the ARP-mediated signal transduction, and have employed the assay of M3 receptor- or ARP-stimulated PLD activity (11, 30) as a detection system in order to characterize the potential role of ARP in this pathway. Evidence is presented here suggesting that ARP-GTP inhibits the activation of PLD by ARP in isolated membranes, inhibits the M3 receptor-mediated stimulation of PLD in intact cells, and also produces a subcellular re-distribution of co-expressed mSec7-1/cytohesin or mSec7-2/ARNO. Thus, ARP may be involved in a signaling pathway inhibiting the ARP-controlled activity of PLD.

MATERIALS AND METHODS

Plasmids

For subcloning of cDNAs, suitable restriction sites were used or introduced by PCR with primers containing the desired site. All PCR-generated constructs were controlled by sequencing.

**ARP and ARL4 Constructs**—ARP-T31N, ARP-Q79L, ARL4-T34N, and ARL4-Q79L mutants were generated by oligonucleotide-directed mutagenesis (Ref. 31; Muta-Gene phagemid in vitro mutagenesis kit, Bio-Rad) from the rat ARP or ARL4 cDNA (Refs. 19 and 20; accession nos. X78603 and X77235). Wild-type and mutant ARP cDNAs were subcloned as described (19) into pGEX-2TK (Pharmacia, Freiburg, Germany) for preparation of recombinant proteins, or into pCMV for expression in mammalian cells.

**Two-hybrid Vectors**—For assay of the yeast two-hybrid interaction, the plasmids pBTM116 and pVP16 were used (32). The plasmids pLexA-ARP, pLexA-ARP-T31N, pLexA-ARP-Q79L, pLexA-ARL4, and pLexA-ARL4-T34N, and pLexA-ARL4-Q79L were constructed by inserting a PCR-generated fragment of the ARP, ARP-T31N, ARP-Q79L, ARL4, ARL4-T34N, and ARL4-Q79L cDNA into the EcoRI site of the pBTM116 plasmid. The resulting plasmids express ARP as a fusion protein with the DNA binding domain of LexA connected by a short linker (aminoo acid sequence: EFR5GRSSSSST). The plasmids pLexA-Rab6 and pLexA-lamin (33) were gifts of D. B. Goud (Institut Pasteur, Unité de Génétique Somatique, Paris, France). pVP16-mSec7(1–52) and pVP16-mSec7(1–150) were generated by subcloning of the respective cDNA fragments (35) into the BamHI/NcoI sites of pVP16.

**mSec7 Constructs**—Plasmids of mSec7-cytohesin and mSec7-2/ARNO cDNA subcloned into pGEX-KG or the GFP expression vector pEGFP-C1 (CLONTECH) were gifts from Dr. N. Brose (Max Planck ARNO cDNA subcloned into pGEX-KG or the GFP expression vector (Pharmacia), and an exponentially growing culture was induced with 0.1 mM isopropyl-D-thiogalactoside. After incubation at room temperature for 4–6 h, cells were lysed and centrifuged at 12,000 χ g for 10 min. GST fusion proteins were isolated by adsorption to glutathione-Sepharose beads (Pharmacia) for 2 h at 4 °C. In some experiments (Fig. 4), recombinant ARP fusion proteins were loaded with GTP (ARP, ARP-Q79L) or GDP (ARP-T31N) by incubation for 2 h at 4 °C. Thereafter, the beads were washed with phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.7 mM KH2PO4 (pH 7.4)), and fusion proteins were eluted with buffer containing 30 mM glutathione in 100 mM Tris buffer (pH 8.0), or by treatment of the Sepharose beads with thrombin (1 unit/100 μg of fusion protein). Recombinant ARF1 was prepared by transfection of SF9 cells with cDNA subcloned in the vector pACGLT, and isolated by adsorption of the GST fusion protein to glutathione-Sepharose.

**Preparation of Recombinant Proteins**

**Escherichia coli DH5α** was transformed with cDNAs of ARP, ARP-T31N, ARP-Q79L, mSec7-1/cytohesin, or mSec7-2/ARNO subcloned in the pGEX vector (Pharmacia), and an exponentially growing culture was induced with 0.1 mM isopropyl-D-thiogalactoside. After incubation at room temperature for 4–6 h, cells were lysed and centrifuged at 12,000 χ g for 10 min. GST fusion proteins were isolated by adsorption to glutathione-Sepharose beads (Pharmacia) for 2 h at 4 °C. In some experiments (Fig. 4), recombinant ARP fusion proteins were loaded with GTP (ARP, ARP-Q79L) or GDP (ARP-T31N) by incubation for 2 h at 4 °C. Thereafter, the beads were washed with phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.7 mM KH2PO4 (pH 7.4)), and fusion proteins were eluted with buffer containing 30 mM glutathione in 100 mM Tris buffer (pH 8.0), or by treatment of the Sepharose beads with thrombin (1 unit/100 μg of fusion protein). Recombinant ARF1 was prepared by transfection of SF9 cells with cDNA subcloned in the vector pACGLT, and isolated by adsorption of the GST fusion protein to glutathione-Sepharose.

**Binding of mSec7-1/Cytohesin to Glutathione-Sepharose Loaded with GST-ARP**

[35S]Methionine-labeled mSec7-cytohesin was prepared by in vitro translation with a kit (TNT T7 quick coupled transcription/translation system) from Promega (Madison, WI) according to instructions of the manufacturer. Samples of 4 μl of the reticulocyte lysate were added to 10 μl of glutathione-Sepharose beads loaded with approximately 50 μg of recombinant GST-ARP, GST-ARP-Q79L, or GST-ARP-T31N, and were incubated for 2 h at 4 °C in a total volume of 100 μl. The beads were washed four times with Tris buffer (50 mM, pH 7.5) containing 1% Nonidet P-40, 1 mM EDTA, and 500 mM NaCl, and were boiled with Laemmli buffer. Eluted proteins were separated by SDS-polyacrylamide gel electrophoresis (12% gels) and detected by autoradiography.

**Deconvolution Microscopy**

COS-7 cells were seeded at 7 × 10^4 cells/cover-slip and transfected with 2.5 μg of the plasmids harboring the GFF-mSec7 and ARP constructs with the aid of LipofectAMINE (Life Technologies, Inc.). After 30 h of culture, cells were fixed with 3.7% formaldehyde for 20 min, washed, and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Images were obtained with a cooled CCD camera (Photometrics, Tucson, AZ) with exposures of 0.2 and 0.4 s and HQ fluorescein filters from Chroma (Brattleboro, VT). Microscope automation and image analysis were performed with ISEE Inovision (Durham, NC) and Adobe Photoshop software (Mountain View, CA). For each cell, a series of images spaced 4 μm apart through the vertical axis was obtained. Out of focus light was removed using the nearest neighbor deconvolution algorithm (38). Background was subtracted and contrast was stretched with the Adobe Photoshop software.

**Assay of GTP Binding**

Guanine nucleotide binding to recombinant GTPases was assayed by a previously described procedure (39, 24) with minor modifications. Samples of 15 pmol of GTPase were incubated at 37 °C in a buffer containing 4 μM [35S]GTPγS (4 × 10^6 cpm/sample), 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM imidazole, 5.1 mM MgCl2, 10 mM diethio- ritol, 40 μg of bovine serum albumin, 3 μg dityrosylphosphatidylycholine, and 0.1% (w/v) sodium cholate in a total volume of 100 μl. The incubation was terminated by addition of 1 ml of ice-cold buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 25 mM MgCl2. The samples were filtered through nitrocellulose membranes (Sartorius GmbH, Göttingen, Germany; pore size 0.2 μm) and washed four times with 1 ml of fresh buffer. Radioactivity on the filters was determined by scintillation counting in a water-compatible scintillation mixture (Ready Protein, Beckman, Fullerton, CA).

**Cell Culture and Transfection**

HEK-293 cells overexpressing M3 receptors were grown in Dulbecco’s modified Eagle’s medium/F-12 medium as described previously (11) and were transfected by incubation with co-precipitates of calcium phosphate and DNA from the different plasmids. The transfection efficiency (50–80%) was controlled by co-transfection with pSVβ-gal (Promega) and histochemical detection of β-galactosidase activity. All assays were performed 48 h after transfection. Overexpression of ARP was confirmed by immunoblotting of whole cell lysates as described (19).

**Yeast Strains and Media**

Yeast strains, plasmids, and library for the yeast two-hybrid screen were obtained from Dr. B. Goud. The genotype of the Saccharomyces cerevisiae reporter strain L40 is MATα trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA::LacZ (32). Yeast strains were grown at 30 °C in rich medium (1% yeast extract, 2% Bacto-peptone, 2% glucose) or in synthetic minimal medium with appropriate supplements.

**Two-hybrid Screen**

The yeast reporter strain L40, which contains the reporter genes lacZ and HIS3 downstream of the binding sequences for LexA, was transformed with pLexA-ARP-T31N or pLexA-ARP-Q79L and a mouse embryo pVP16 DNA library (34) with the lithium acetate method (35), and was subsequently treated as described (32). Double transformants were plated to synthetic medium lacking histidine, leucine, tryptophan, uracil, and lysine. The plates were incubated at 30 °C for 3 days. His+ colonies were patched on selective plates and assayed for β-galactosidase activity by a filter assay (36). Plasmid DNA was prepared from colonies displaying a HIS+ lacZ+ phenotype by electrophoresis of HB101 cells and used to re-transform the L40 strain containing pLexA-ARP, pLexA-ARP-T31N, pLexA-ARP-Q79L, pLexA-ARL4, pLexA-Rab6, and pLexA-lamin, respectively, to test for specificity. For assay of β-galactosidase activity, transformants were grown in histidine-containing medium, and were lysed and assayed as described (37).
Assay of PLD in Membranes

Membranes of HEK-293 cells were prepared by nitrogen cavitation and differential centrifugation as described previously (30). PLD activity in membranes was assayed as described (3) with the following modifications. For generation of a micellar lipid preparation, [3H]phosphatidylcholine was mixed with PtdIns(4,5)P$_2$ in a molar ratio of 8:1, dried, and resuspended in a buffer containing 50 mM HEPES (pH 7.5), 3 mM EGTA, 80 mM KCl, and 1 mM dithiothreitol. The suspension was sonicated on ice, and samples containing the membranes with the indicated con- structs were grown in histidine-containing medium in suspension, lysed, and β-galactosidase activity was assayed and normalized for protein concentration. The data represent means ± S.D. of three experiments. β-Galactosidase activity from cells co-transfected with pLex Constructs of ARL4, ARL4-T34N, ARL4Q79L, or Rab6, and the indicated VP16 constructs was undetectable (shown only for mSec7(1–200)).

Assay of PLD in Intact Cells

Cellular phospholipids were labeled by culture of cells in the presence of [3H]oleic acid (2 μCi/ml) for 20–24 h. Thereafter, cells were washed twice with a buffer containing 118 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM glucose, and 15 mM HEPES (pH 7.4). The cells were treated as indicated with 1 mM carbachol or 100 nM phorbol 12-myristate 13-acetate (PMA) in the presence or absence of ethanol (400 mM) for 30 min at 37 °C. Total phospholipids and [3H]PtdEtOH were isolated as described previously (11–13). Formation of [3H]PtdEtOH is expressed as percentage of total labeled phospholipids.

Assay of ARF Translocation

Translocation of ARF in HEK-293 cells was assayed with a previously published procedure (12). Cells were stimulated with carbachol for 10 min, and were permeabilized with 10 μM digitonin for 15 min. After centrifugation of cells (15,000 × g, 10 min), the ARF content of the supernatant was assayed as described by immunoblotting. Antiserum against recombinant ARF1 was provided by Dr. J. B. Helms (University of Heidelberg, Heidelberg, Germany). Densitometry of bands corresponding to ARF was performed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Specific Binding of ARP-GTP to mSec7-1/Cytohesin—In a yeast two-hybrid screen designed to identify proteins interacting with ARP, we isolated a cDNA clone that induced growth of lacZ-positive colonies on histidine-free agar plates. The clone harbored 626 nucleotides of the mouse mSec7-1/cytohesin cDNA (accession no. AF051337) fused to the VP16 activation domain. Cytohesin belongs to a small family of cytosolic adapter proteins with nucleotide exchange-accelerating activity for ARF (24, 27–29). As is illustrated in Fig. 1A, the clone induced growth of colonies on histidine-free agar plates when co-transfected with pLex-ARP or with pLex-ARP-Q79L (GTPase-negative mutant); growth of...
colonies co-transfected with pLex-ARP-T31N (exchange-defective mutant) was much slower. When transferred to nitrocellulose filters, these colonies tested positive for the lacZ reporter (data not shown). Similarly, in cells grown in suspension in histidine-containing medium (Fig. 1B), pLex-ARP and pLex-ARP-Q79L induced a significant β-galactosidase activity that was higher than that of pLex-ARP-T31N transfectants. Thus, the protein encoded by the partial mSec7-1/cytohesin cDNA appears to bind ARP-GTP with a higher affinity than ARP-GDP. mSec7-1/cytohesin (1–200) also exhibited some interaction with lamin C (Fig. 1, A and B). However, full (mSec7-1–200) or partial (mSec7-1–150) deletion of the Sec7 domain suppressed the interaction with ARP but not with lamin C (Fig. 1B). Thus, lamin C appears to interact with the coiled-coil domain of cytohesin, whereas the Sec7 domain seems necessary for the specific interaction with ARP. Furthermore, no interaction was observed between mSec7-1-200 and the GTPases ARL4 or Rab6.

In order to further demonstrate the interaction of ARP with mSec7-1/cytohesin, we used glutathione-Sepharose loaded with recombinant GST-ARP constructs, and assayed the binding of full-length 35S-labeled mSec7-1/cytohesin prepared by in vitro translation. As is illustrated in Fig. 2A, recombinant GST-ARP-Q79L adsorbed a significant portion of the tracer, whereas no binding was detected to GST alone or to an unrelated protein (Bcl2). In addition, Sepharose loaded with GST-ARP-Q79L failed to adsorb an unrelated labeled protein, Bcl2 (Fig. 2A), ruling out nonspecific stickiness of the GTPase. An additional band at approximately 40 kDa, which was also specifically adsorbed, appears to be incompletely translated mSec7-1/cytohesin; according to its size, this product should contain the full Sec7 domain. Fig. 2B illustrates the quantitative evaluation of a series of experiments comparing the different ARP constructs. The highest binding was found with the GTPase-negative mutant ARP-Q79L. The exchange-defective mutant ARP-T31N bound significant, but much lower, amounts of mSec7-1/cytohesin than ARP-Q79L. It should be noted that the wild-type ARP employed in this experiment carried GTP and GDP at a ratio of approximately 1:3 (19). Thus, these data are consistent with the conclusion that mSec7-1/cytohesin specifically binds the GTP-loaded ARP with a higher affinity than the GDP-loaded form.

**Subcellular Localization of mSec7 Isoforms in Cells Co-transfected with ARP**—In order to demonstrate the interaction...
of ARP with Sec7 domain proteins in a system of intact mammalian cells, COS-7-cells were co-transfected with ARP and GFP-tagged mSec7-1/cytohesin or mSec7-2/ARNO. Since we assumed that the specific interaction of the proteins might produce a re-distribution of the mSec7 isoforms, the subcellular localization of the GFP fluorescence was analyzed by deconvolution microscopy. Deconvolution microscopy eliminates most light from portions of the specimen not in the focal plane, thereby allowing a more precise localization of proteins in cells. When cells were co-transfected with GFP-mSec7-1/cytohesin or GFP-mSec7-2/ARNO plus the bland pCMV vector, an essentially continuous labeling of the cellular periphery, i.e. of the plasma membrane, was observed, in addition to labeling of intracellular compartments (panels a and d). In contrast, when cells were co-transfected with ARP-Q79L (panels b and e), the GFP fluorescence disappeared almost completely from the plasma membrane (GFP-mSec7-1/cytohesin, panel b) or from large portions of it (GFP-mSec7-2/ARNO, panel e). As is illustrated in panels c and f of Fig. 3, the nucleotide exchange-defective mutant ARP-T31N failed to remove the GFP fluorescence from the plasma membrane. The transfections were repeated three times, and in each experiment 5–7 cells per treatment were examined. In all cells examined, ARP-Q79L produced a marked reduction of the GFP fluorescence associated with the plasma membrane as compared with cells transfected with bland vector or ARP-T31N.

Lack of Effect of mSec7-1/Cytohesin and mSec7-2/ARNO on Guanine Nucleotide Exchange of ARP—In order to test the possibility that mSec7 isoforms alters the guanine nucleotide exchange rate of ARP, binding of [35S]GTP to recombinant ARP was assayed in the presence of mSec7-1/cytohesin or mSec7-2/ARNO. Both mSec7 isoforms failed to alter the rate of binding of [35S]GTP to ARP preloaded with either GDP or unlabeled GTP versus [35S]GTP-S to ARP preloaded with either GDP or unlabeled GTP (t1/2 = 20 min, data not shown). In contrast, mSec7-1/cytohesin and mSec7-2/ARNO produced the expected acceleration of guanine nucleotide exchange on (D17)ARF (t1/2 = 2.5 min in the presence versus >30 min in the absence of the mSec7 isoforms). Addition of recombinant ARP failed to affect the stimulatory effect of mSec7-1/cytohesin on nucleotide exchange of (D17)ARF (data not shown).

Inhibitory Effect of ARP on the mSec7-2/ARNO-stimulated PLD Activity in Isolated Membranes—It has previously been shown that ARP stimulates the activity of PLD (3, 4). This effect can be demonstrated in an in vitro system of isolated membranes by addition of mSec7-2/ARNO, ARP, and GTP versus [35S]GTP to recombinant ARP-Q79L and to a somewhat lesser extent by ARP, but not by ARP-T31N. In these experiments, the recombinant proteins were preloaded with GDP (ARP, ARP-Q79L) or GDP (ARP-T31N). Boiling of ARP-Q79L prevented its inhibitory effect on PLD activity (data not shown). These data are consistent with the conclusion that ARP-GTP may interfere with the ARP-
controlled activity of PLD.

ARP Inhibits the M₃ Receptor-mediated Stimulation of PLD Activity in HEK Cells—In order to define the involvement of ARP in PLD regulation in a system of intact cells, we employed HEK-293 cells transiently transfected with the various ARP constructs. As was shown previously (11, 12), PLD is activated by M₃ receptor stimulation with carbachol; this effect can be enhanced by overexpression of mSec7-2/ARNO or ARF (data not shown). The effects of ARP appeared antagonistic to those of the M₃ receptor agonist, carbachol. As is illustrated in Fig. 5A, transfection of HEK-293 cells with ARP or GTPase-negative ARP-Q79L markedly inhibited the stimulatory effect of carbachol on PLD activity. In contrast, the nucleotide exchange-defective mutant ARP-T31N failed to affect the stimulatory effect of carbachol. Furthermore, neither basal nor PMA-stimulated PLD activity was altered by any of the ARP constructs (Fig. 5B).

ARP Binds to Cytohesin and Inhibits PLD Activation

FIG. 6. Inhibition of the carbachol-induced translocation of ARF in HEK-293 cells by ARP. HEK-293 cells overexpressing M₃ receptors were transfected with blank plasmid (pCMV), ARP, GTPase-negative mutant ARP-Q79L, or nucleotide exchange-defective mutant ARP-T31N. After 48 h, the cells were stimulated with carbachol (1 mM) for 10 min, and were subsequently permeabilized with digitonin in the presence or absence of GTPγS as indicated for 15 min at 37 °C. Release of ARF into the cytosol was assayed by immunoblotting of the supernatant. Panel A, immunoblots from a representative experiment. Panel B, quantitative evaluation of six independent transfections. The data were normalized for ARF release in the absence of stimuli and are expressed as means ± S.E.

DISCUSSION

The present data indicate that the ARF-related GTPase ARP specifically inhibits the PLD activation by ARF and its nucleotide exchange factor mSec7-2/ARNO in isolated membranes.
The effect was produced by the GTPase-negative mutant ARP-Q79L and not by the exchange-defective ARP-T31N, consistent with the conclusion that it is the activated, GTP-bound form of ARP that exerts the effects. Furthermore, overexpression of ARP or ARP-Q79L inhibited the stimulatory effect of carbachol on PLD in HEK-293 cells. In contrast, ARP failed to inhibit the PMA-stimulated PLD, apparently dissociating PKC and ARP-mediated stimulation of the enzyme. This result is consistent with our previous finding that inhibition or down-regulation of PKC suppressed the effect of PMA, but not that of carbachol, on PLD stimulation in HEK-293 cells, and that PKC-mediated stimulation of PLD is ARF-independent (11, 30). Thus, it is suggested that ARP may be involved in a signaling pathway that inhibits the ARF-controlled activation of PLD by the M3 receptor.

The inhibitory effect of ARP on the ARF-stimulated PLD activity may be explained by an interaction of ARP with ARF guanine nucleotide exchange factors, which are required for conversion of ARF to its GTP-bound, activated form (24, 27, 28). Indeed, we observed specific binding of GTP-bound ARP to the nucleotide exchange factor mSec7-2/cytohesin in two independent assays of protein interaction. Furthermore, GTP-bound ARP altered the subcellular distribution of both mSec7-1/cytohesin and mSec7-2/ARNO. However, we failed to detect an inhibitory effect of ARP on the mSec7-1/cytohesin-stimulated guanine nucleotide exchange of ARF in a solution of recombinant proteins. This failure of ARP to inhibit nucleotide exchange might be due to lack of a necessary factor that is not present in the incubation of recombinant, soluble proteins. Alternatively, ARP may inhibit activation of ARF by mSec7 proteins indirectly, i.e. by a spatial separation of ARF and its nucleotide exchange factors, an effect that cannot be produced in a system of isolated proteins in solution.

Binding of ARP to mSec7-1/cytohesin appears to depend on a portion of the Sec7 domain (amino acids 150–200), since a truncated construct (mSec7-1(1–150)) failed to induce β-galactosidase in the two-hybrid assay. According to the crystal structure of the Sec7 domain (40), this portion of the domain harbors the helices G and H, and two conserved motifs considered to mediate the contact with ARP (41). However, the molecular basis of the interaction between ARP and mSec7 proteins as observed here appears to be quite different from that of the interaction between ARF and mSec7. ARP has recently been shown to bind to mSec7-2/ARNO by hydrophobic residues in its switch I region (also designated effector loop) and by a lysine (Lys-73) in the switch II region (41, 42). In ARP, the following residues are placed by serine (Val-43), threonine (Ile-46), or glutamate (Lys-73). Furthermore, the affinity of ARP and ARPKQ79L to mSec7-1/cytohesin was markedly higher than that of exchange-defective ARP-T31N, distinct from what was found for the interaction of ARF with mSec7. The failure of mSec71/cytohesin to alter the nucleotide exchange of ARP is consistent with these differences between the mSec7/ARP and the mSec7/ARF interaction.

From the present results, a hypothesis might be deduced suggesting that ARP prevents the translocation of mSec7 proteins and consequently of ARF to cellular compartments harboring PLD. Two isoforms of PLD with different regulatory characteristics and subcellular localization have been identified so far (14–18). Because of its considerably higher sensitivity to ARF, PLD1 appears to be the isoform responsible for the effects described here. PLD1 is believed to be located predominantly in intracellular compartments (16), but has recently been shown to be translocated to the plasma membrane in response to stimulation of RBL-2H3 cells (43). Furthermore, ARF-sensitive PLD is abundant in plasma membranes from liver (44). Thus, a scenario in which ARP removes mSec7 proteins from the plasma membrane (Fig. 3), thereby preventing activation of PLD1, or of a yet unidentified isoform, seems possible. Alternatively, the possibility cannot be excluded that removal of mSec7 proteins from the plasma membrane of cells overexpressing ARP is a phenomenon unrelated to the inhibition of PLD activity.

It has to be noted that the above suggested role of ARP in regulating PLD activity is based on experiments with isolated proteins and membranes, and with cells overexpressing ARP. This experimental strategy is required because, in the absence of knowledge on the signaling mechanism “upstream” of ARP, it is impossible to activate the endogenous ARP. Thus, it still remains to be shown that the endogenous ARP affects PLD in a “wild-type” cell. Furthermore, it should be noted that ARP, in addition to the effect on PLD, may exert additional, cellular effects through interaction with mSec7 proteins. Since mSec7-1/cytohesin was also identified as a protein interacting with the intracellular domain of integrins (25), it seems that its functions are pleiotropic.

ARP is expressed in most tissues, with somewhat higher protein levels in testis, kidney, and liver. Thus, its pattern of expression does not suggest a tissue-specific function. Furthermore, a data base search revealed the existence of a Drosophila homolog (expressed sequence tag DM1327692 encoding a partial sequence of 112 codons; 74% of amino acids identical with human ARP), and an open reading frame in the S. cerevisiae genome (YPL051, GenBank accession no. U39205) encoding a homologous sequence (47% identical amino acids). Both sequences exhibit the characteristics of the mammalian ARP, in particular the insertion between PM1 and PM2 and an aromatic amino acid (tyrosine or phenylalanine) instead of glycine in position 2. The existence of a yeast homolog suggests that ARP is essential for a unicellular organism, and that it exerts a basic cellular function.

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