Arfaptin 1 forms a complex with ADP-ribosylation factor and inhibits phospholipase D

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Abstract ADP-ribosylation factors (ARFs) regulate coatomer assembly on the Golgi as well as recruitment of clathrin adapter proteins and are therefore involved in vesicle budding from the Golgi and vesicular transport. They are also regulators of phospholipase D (PLD) activity. Arfaptin 1 is an ARF binding protein that inhibits PLD activation, vesicular trafficking and secretion. In the present report, we show that arfaptin 1 interacts with 'high speed' membranes independently of ARF. However, addition of myristoylated ARF3 (myrARF3) increases the association of arfaptin 1 with the membranes, suggesting that arfaptin 1 and ARF form a complex on the Golgi. Utilizing several deletion mutants of arfaptin 1 it is shown that the association of arfaptin 1 with myrARF3 is mediated via two binding sites on arfaptin 1. These two domains are needed for arfaptin 1 inhibition of PLD activation by myrARF3 in vitro.

Key words: ADP-ribosylation factor; Arfaprin; Phospholipase D inhibition

1. Introduction

ADP-ribosylation factors (ARFs) play a key role in COPI (coatomer) assembly on the Golgi and maintenance of trafficking through the Golgi [1]. They are also involved in the recruitment of adapter proteins to membranes for the assembly of clathrin-coated vesicles [2]. Inactivation of ARF by brefeldin A, an inhibitor of guanine nucleotide exchange on ARF, abolishes trafficking from the Golgi, secretion and finally leads to a collapse of the Golgi into the endoplasmic reticulum [1]. ARF is also a well established regulator of phospholipase D (PLD) in vitro [3,4]. PLD hydrolyzes phosphatidylcholine to phosphatidic acid and free choline. In the presence of a primary alcohol, a transphosphatidylation reaction, in which the lipid moiety is transferred to the alcohol instead of water, takes place [5]. The cloning of the ARF-dependent PLD1 isozyme and its localization to the Golgi [6] raised the possibility that PLD1 mediates some of the effects of ARF in this organelle.

Arfaptin 1 is a 39 kDa class I ARF binding protein that associates with Golgi upon binding to active guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS)-liganded ARF3 [7]. We have recently focused our research on the intracellular role of arfaptin 1. It was revealed that in vivo arfaptin 1 inhibits PLD activity resulting in impaired intracellular trafficking and secretion [8]. While the physiological role of arfaptin 1 is beginning to be understood, little is known about the biochemical mechanisms behind these effects. Therefore, in the present study we concentrated on understanding the interaction of arfaptin 1 with ARF3 and Golgi, and its inhibitory effect on PLD activation by ARF.

We show here that association of arfaptin 1 with 'high speed' membranes is independent of ARF. However, it is greater and saturable in the presence of ARF. These data suggest that ARF and arfaptin 1 participate in formation of a complex on the Golgi and we propose that another docking protein may be involved. The mode of interaction of arfaptin 1 with ARF was further studied. Employing seven glutathione *S*-transferase (GST) fusion deletion mutants of arfaptin 1 we identified two ARF3 binding sites on arfaptin 1, one at amino acids 64–116 and the other in the last 76 amino acids. We have previously observed that arfaptin 1 inhibits the stimulation of PLD by ARF [9]. We tested the involvement of the two ARF binding sites in the inhibition by arfaptin 1 of ARF-dependent PLD activation, and found that both sites were needed.

2. Materials and methods

2.1. Materials

All organic solvents were fine grade and obtained from Fisher. Phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylinositol 4,5-biphosphate (PIP_2), bovine serum albumin (BSA) and isopropyl β-D-thiogalactopyranoside were purchased from Sigma. Horseradish peroxidase-labeled horse anti-mouse antibody was from Vector. Sepharose-glutathione beads, nitrocellulose membranes and enhanced chemiluminescence reagents were purchased from Amersham. Dithiothreitol (DTT), guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS) and GTPγS were purchased from Boehringer Mannheim. [2-Palmitoyl-9,10-^{3}H]phosphatidylcholine and [^{35}S]GTPγS were products of NEN. Acrylamide:bisacrylamide was purchased from National Diagnostics. DNA polymerase and DNA ligase were obtained from Promega.

2.2. Preparation of GST-fused deletion mutants of arfaptin 1

All mutants were prepared by polymerase chain reaction with 5′*Bam*HI and 3′*Eco*RI restriction sites. N-terminal deletion mutants were created using the 3′ (antisense) oligonucleotide primer 5′-CCGGAATTCTTACTGTTCTTCAAGCCAAGA-3′. For 5′ (sense) the following primers were used: 5′-CGCGGATCCCAAGCACATAAGAAAAATAT-3′ for arfaptin 1 (266–341), 5′-CGCGGATCCGAGACTCTTCTTGGGGCCATT-3′ for arfaptin 1 (197–341), 5′-CGCGGATCCCGTGGCTCAAGAACTGTGGAC-3′ for arfaptin 1 (117–341) and 5′-CGCGGATCCGGAGCATTTCAAGGTGGCCAG-3′ for arfaptin 1 (64–341). The mutant arfaptins comprised amino acids 266–341, 197–341, 117–341 and 64–341, respectively. C-terminal deletion mutants were created using the 5′ (sense) oligonucleotide primer

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Abbreviations: ARF, ADP-ribosylation factor; ATP, adenosine triphosphate; BSA, bovine serum albumin; GDPβS, guanosine-5′-*O*-(2-thiodiphosphate); GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; myrARF3, myristoylated ARF3; PKC, protein kinase C; PLD, phospholipase D; GST, glutathione *S*-transferase

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5′-CGCGGATCCATGGCTCAAGAATC-TCCCAA 5′ primer. The following oligonucleotides were used for 3′ (antisense) primers: 5′-CCGGAATTCACCTTGAAATGCTCCTGCTTC-3′ for arfaptin 1 (1–68), 5′-CCGGAATTCCACCAAAGTGTTCACACTAGC-3′ for arfaptin (1–204) and 5′-CCGGAATTCATATTTTTCCTTATGTGC-TTG-3′ for arfaptin (1–272). These mutant arfaptins comprised amino acids 1–68, 1–204 and 1–272, respectively. The mutants were subcloned into pGEX-4T vector in the *Bam*HI/*Eco*RI site. The plasmids were introduced into *Escherichia coli* (DH5α) and GST fusion proteins were prepared from isopropyl β-D-thiogalactopyranoside-induced bacteria. **We were unable to obtain and express mutant for amino acids 1–120**. The sequences of all polymerase chain reaction products were confirmed by the Cancer Center DNA Sequencing Laboratory at Vanderbilt University.

2.3. Purification of GST fusion proteins and myrARF3

GST fusion proteins were purified from transformed *E. coli* (DH5α) as described [10], except that before affinity chromatography, the bacterial lysate was dialyzed against phosphate-buffered saline (PBS) (2.68 mM KCl, 1.47 mM KH_2PO_4, 8.05 mM Na_2PO_4, 137 mM NaCl) for 24 h at 4°C. After purification, all the GST fusion proteins that were used for binding assays were first dialyzed against PBS for 24 h at 4°C to remove glutathione. Recombinant myristoylated ARF3 (myrARF3) was prepared as previously described [7].

2.4. In vitro PLD assay

In vitro PLD assays were performed using a lysate from COS-7 cells transfected with rat liver PLD1 (rPLD1) as described [11]. GST-arfaptin 1 was first incubated with myrARF3 and the PLD preparation in reaction buffer for 15 min on ice. Then 1-butanol and phospholipid vesicle substrate containing [^{3}Hpalmitoyl]phosphatidylcholine were added and the reaction allowed to proceed at 37°C for 30 min. PLD activity was measured by the formation of [^{3}H]phosphatidylbutanol.

2.5. Cell fractionation

HT 1080 fibrosarcoma cells were maintained in DMEM, 10% fetal calf serum, 10 U/ml penicillin and 10 μg/ml streptomycin. Before fractionation, the cells were serum-deprived for 18 h in DMEM, 0.1% BSA. They were then washed with PBS and scraped in fractionation buffer (100 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$ and 0.5 mM EDTA in 50 mM HEPES pH 7.2). The cells were broken by 30 min cavitation under 350 psi nitrogen and 40 s sonication. Unbroken cells and large debris were removed by 15 min centrifugation at $750\times g$. The recovered supernatant was centrifuged at $12\,600\times g$ for 20 min to remove plasma membranes. A 'high speed' membrane fraction was separated from the cytosol by a final centrifugation at $38\,600\times g$ for 75 min and resuspended in fractionation buffer. The supernatant was used as a source for cytosol.

2.6. ARF3 loading with GTPγS and binding assay

Loading myrARF3 with [^{35}S]GTPγS was carried out by incubating 1.3 μM ARF3 in loading buffer (25 mM HEPES pH 7.4, 100 mM NaCl, 25 mM KCl, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.3 mg/ml BSA) containing 6.25 mg/ml PC and 10 μM [^{35}S]GTPγS (4 μCi/nmol). After 30 min at 37°C, $MgCl_2$ was added to a final concentration of 15 mM. For binding assays 0.9 μM GSTfusion proteins in a final volume of 500 μl were immobilized on glutathione-Sepharose by 1 h incubation at room temperature in PBS containing 60 mg/ml BSA. Following washing with buffer (25 mM Tris pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) the beads were incubated with 1.3 μM [^{35}S]GTPγS-loaded myrARF3 for 1 h at room temperature, unless otherwise described. The beads were then washed with the same buffer and radioactivity associated with the beads was measured by scintillation spectroscopy.

3. Results

3.1. ARF and arfaptin 1 form a complex on 'high speed' membranes

Arfaptin 1 has previously been shown to associate with the GTPγS-bound form of ARF1 and interact with Golgi membranes [7]. In the present study, the association of arfaptin 1 with 'high speed' membranes of HT 1080 cells was studied. GST-arfaptin 1 was incubated with this membrane fraction in the absence and presence of cytosol (as a source of ARF). Surprisingly, a significant amount of GST-arfaptin 1 associated with the membranes in the absence of cytosol, suggesting that arfaptin 1 interacts with the membranes independently of added ARF (Fig. 1A). In contrast, arfaptin 1 did not associate with plasma membrane preparations from these cells (data not shown). To investigate whether the association was due to ARF and Golgi present in the membrane preparations, the translocation of GST-arfaptin 1 to the membranes was assayed in the absence of cytosol and with GTPγS or GDPβS (Fig. 1B). The nucleotides were included because arfaptin 1 only binds ARF when it is in the GTPγS (or GTP)-liganded form [7]. The figure shows that the association of GST-arfaptin 1 with the membranes was not abolished when GDPβS was added to the reaction, illustrating that arfaptin 1 is capable of interacting with the membranes independently of ARF. However, the association of arfaptin 1 was greater in the presence of GTPγS indicating an additional role for ARF, as shown previously [7].

Further evidence for the involvement of ARF in the association of arfaptin 1 with the membranes from an experiment in which they were incubated with a constant amount of GST-

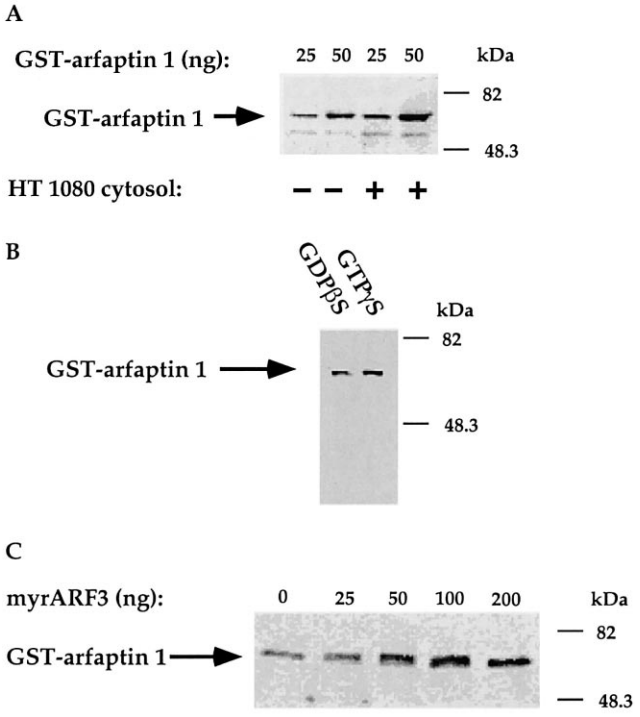

Fig. 1. Interaction of arfaptin 1 with 'high speed' membranes from HT 1080 cells. A: Translocation of 25 and 50 ng GST-arfaptin 1 was assayed with 20 μg 'high speed' membranes in the absence or presence of 20 μg cytosol. The translocation buffer contained 20 μM GTPγS. Association with the membranes after 45 min incubation at 37°C was assayed by immunoblotting the membranes with mouse anti-GST antibody and then horseradish peroxidase-labeled horse anti-mouse antibody and utilizing enhanced chemiluminescence. The position of a GST-arfaptin 1 standard is shown by the arrow. B: Association of 20 ng GST-arfaptin 1 with 20 μg 'high speed' membrane fraction was measured with 20 μM GTPγS or GDPβS. C: The translocation assay was repeated with 20 ng GST-arfaptin 1, 40 ng GST-ARF3, 20 μg Golgi, 20 μg cytosol and the indicated amounts of myrARF3.

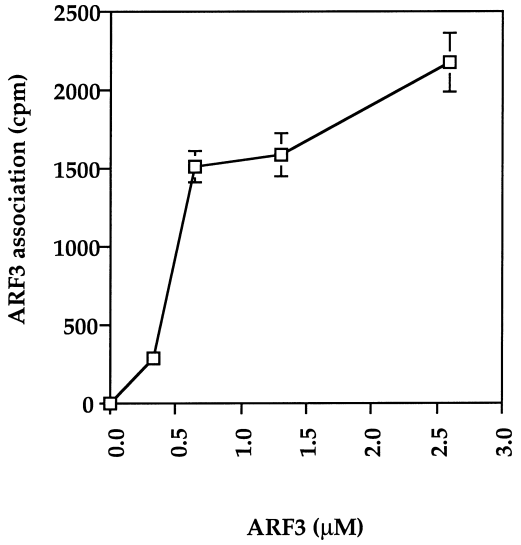

Fig. 2. Association of ARF3 with arfaptin 1. Interaction of arfaptin 1 with ARF was assayed with various concentrations of myrARF3. GST-arfaptin 1 was immobilized on Sepharose-glutathione by incubating 0.5 ml of 0.9 μM GST-arfaptin 1 in PBS, 60 mg/ml BSA for 1 h at room temperature. ARF3 was loaded with [^{35}S]GTPγS by incubating 1.3 μM myrARF3 with 10 μM [^{35}S]GTPγS (4 μCi/nmol) in loading buffer containing PC for 30 min at 37°C after which $MgCl_2$ was added to a final concentration of 15 mM. ARF3 was diluted in loading buffer to the indicated concentrations and incubated with the immobilized GST-arfaptin 1 for 1 h at room temperature. The beads were washed with wash buffer and binding was measured by the radioactivity associated with the immobilized arfaptin 1. Data are expressed as the mean of fold binding of three independent experiments performed in duplicate ± S.E.M.

arfaptin 1 and increasing concentrations of myristoylated ARF3 (myrARF3) in the presence of GTPγS and cytosol (Fig. 1C). GST-arfaptin 1 associated with the membranes in the absence of ARF, however the addition of myrARF3 dose-dependently increased the amount of arfaptin 1 present.

3.2. Association of ARF3 with arfaptin 1 is mediated through two sites on arfaptin 1

The association of ARF3 with arfaptin 1 was further characterized. We first defined the interaction of GST-arfaptin 1 with ARF and then used deletion mutants of arfaptin 1 to localize the ARF binding sites on arfaptin 1. myrARF3 was loaded with [^{35}S]GTPγS and then diluted to various concentrations that were allowed to interact with glutathione-immobilized GST-arfaptin 1. After incubation for 1 h, free myrARF and excess GTPγS were washed from the beads and the association of arfaptin 1 with myrARF3 was measured by the radioactivity associated with the beads. As shown in Fig. 2, the association of myrARF with arfaptin 1 was concentration-dependent and largely monophasic.

In order to identify the ARF binding site(s) of arfaptin 1 we prepared seven deletion mutants of arfaptin 1 fused to GST at its N-terminus or C-terminus. The mutants were subcloned into pGEX-4T vector in the *Bam*HI/*Eco*RI site. The various mutants are detailed in Fig. 3A. They were used to identify myrARF3 binding sites on arfaptin 1 by immobilizing them on Sepharose-glutathione and incubating with [^{35}S]GTPγS-loaded myrARF3. Fig. 3B illustrates that arfaptin 1 (1–68) possessed no binding site for ARF since the background binding to GST alone was 0.2 relative to full length arfaptin 1 fused to GST. Consistent with this, arfaptin 1 (64–341) showed full binding. However, full binding of ARF could not be attributed to a single site since all the other mutants showed partial binding. One binding site was localized to residues 266–341 at the C-terminus since arfaptin 1 (266–341) showed binding, as did arfaptins 1 (197–341), (117–341) and (64–341). On the other hand arfaptin 1 (1–204) and (1–172) which lack the C-terminal site also showed binding. The second site was localized to residues 64–116. This was because arfaptin 1 (64–341) showed full binding whereas arfaptin 1 (117–341) showed only partial binding. In summary, the findings with all the mutants supported the existence of two binding sites, in the 64–116 and 266–341 amino acid sequences.

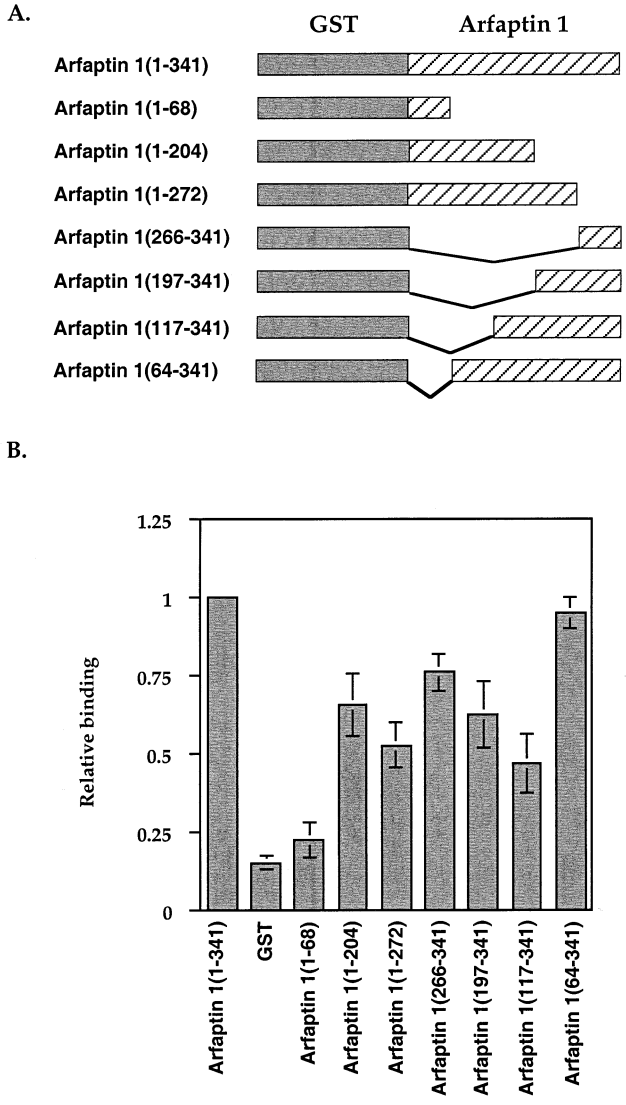

Fig. 3. The association of arfaptin 1 with ARF3 is mediated through two ARF3 binding sites on arfaptin 1. A: Seven deletion mutants of arfaptin 1 were prepared as described in Section 2. B: Full length 0.9 μM GST-arfaptin 1 and its deletion mutants were immobilized on Sepharose-glutathione and then allowed to interact with 1.3 μM [^{35}S]GTPγS-loaded myrARF3. After 1 h at room temperature and removing excess mARF3 by extensive washing with wash buffer, the radioactivity associated with the beads was measured by scintillation spectrometry. Data are expressed as the mean of fold binding in comparison to full length arfaptin 1 in six independent experiments performed in duplicate ± S.E.M.

3.3. Inhibition of PDL activity by arfaptin 1 deletion mutants

ARF proteins are a well established regulators of PLD [2,3,12–15]. Overexpression of arfaptin 1 has been shown to decrease PLD-activation in PMA-stimulated HT 1080 cells (B.-T. Williger, W.-T. Ho and J.H. Exton, submitted for publication) and NIH 3T3 cells [8]. Arfaptin 1 has also been reported to inhibit the effect of several ARF isoforms on PLD partially purified from bovine brain [9]. We therefore tested whether the inhibitory of arfaptin 1 on PLD required one or both ARF binding sites. Fig. 4A illustrates that arfaptin 1 caused a concentration-dependent inhibition of the effect of myrARF3 on PLD activity in lysates from COS-7 cells overexpressing rPLD1. Next we assayed the inhibitory effect of the arfaptin 1 deletion mutants on the stimulation of rPLD1 by myrARF3. The data of Fig. 4B shows clearly that, of all the mutants, only arfaptin 1 (64–341) that contained both binding sites was able to inhibit the effect of ARF on the enzyme. All the other mutants, which contained only one ARF binding site, were completely without effect.

4. Discussion

ARF is an important regulator of Golgi functions, but the mechanisms regulating ARF or its effects are poorly understood. ARF interacts with Golgi mainly when it is in its GTP-bound form [1], making its exchange factors and GTPase activators important regulators by governing its activation status. Myristoylation may also play a role in regulation of ARF association with the Golgi. The cloning of arfaptin 1, and ARF binding protein [7], provided another potential regulator of ARF effects and of the secretory pathway. We show here that arfaptin 1 forms a complex with ARF3 in 'high speed' membranes and the arfaptin 1 interacts with ARF3 through two distinct sites. Upon interaction with ARF through both of the binding sites, arfaptin 1 inhibits PLD activity.

Arfaptin 1 was observed to associate with the 'high speed' membrane fraction to a greater extent in the presence of myrARF3. The binding was saturable in that increasing myrARF3 from 100 to 200 ng did not cause further arfaptin 1 binding. Nevertheless, some membrane binding was still evident in the absence of myrARF3 and was not abolished by GDPβS, which inhibits the interaction of arfaptin 1 with ARF. We propose that the interaction or arfaptin 1 with the 'high speed' membranes involves Golgi membranes. We believe that this is the case because of the enhancement of the association by myrARF3, which is known to be localized to Golgi membranes [16]. The limited ARF-independent interaction of arfaptin 1 with the membranes is in agreement with the low presence in highly purified Golgi membranes from rat liver (Fig. 6 and 7 in [7]). These latter results suggest a direct, ARF-independent, interaction of arfaptin 1 with the Golgi. Therefore, it seems possible that arfaptin 1 forms, together with ARF, a complex on the Golgi by interacting with an unknown docking protein. However, overexpression of arfaptin 1 results in inhibition of vesicular trafficking involving the Golgi [8].

To determine the site(s) at which ARF bound to arfaptin 1, we examined the interaction of various arfaptin 1 mutants with ARF3. Analysis of the various mutants revealed the presence of one ARF binding site between amino acids 64–116, and another between amino acids 266–341. The existence

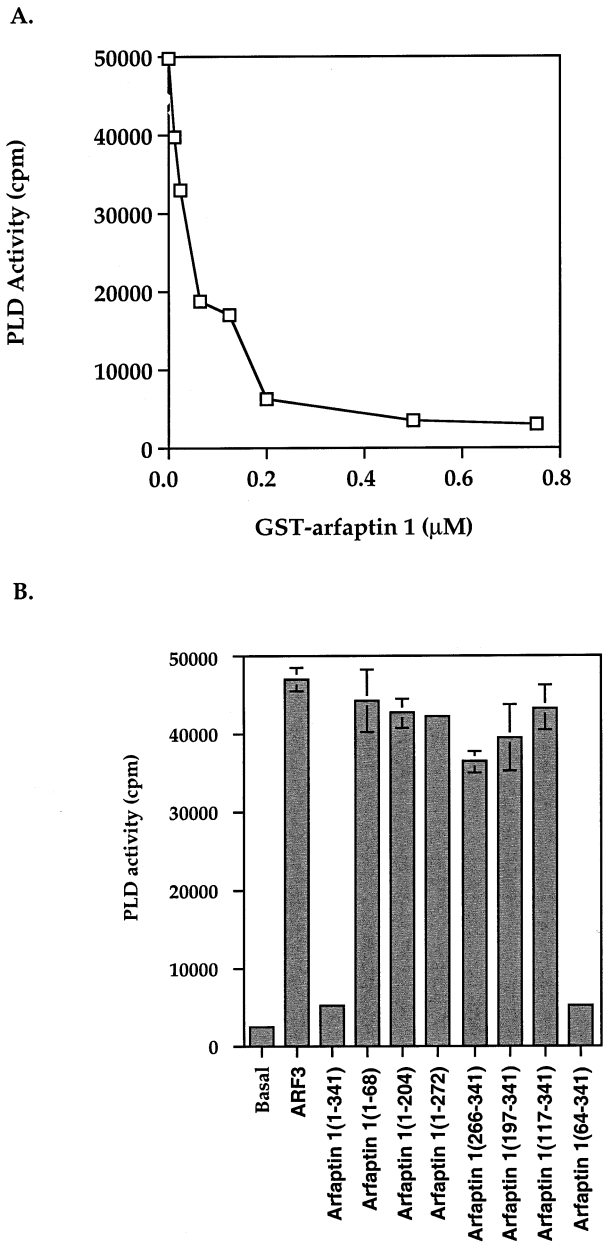

Fig. 4. GST-arfaptin 1 inhibits ARF3-dependent PLD1 activation only in the presence of the two ARF3 binding sites. rPLD1-enriched membrane preparations from COS-7 cells (5 μg/reaction), 3 μM myrARF3 were incubated with (A) various concentrations of GST-arfaptin 1 or (B) 0.9 μM of each GST-fused deletion mutant of arfaptin 1, for 15 min on ice to allow interaction of the various components. A mixture containing PE/PIP_2/[^{3}H]PC (16:1.4:1) and 1-butanol (final concentration of 0.3%) was added and the reaction mixture was incubated for 30 min at 37°C. PLD activity was determined by the formation of [^{3}H]PtdBut. Data presented are representative of three independent experiments performed in triplicate ± S.E.M.

of two binding sites is supported by the significantly higher binding of arfaptin 1 (64–341), which was the only mutant that contained both binding sites. This mutant bound ARF3 to the same extent as wild type arfaptin 1. Interestingly, this mutant was the only one which fully inhibited the activation of rPLD1 by ARF3, suggesting that interaction with ARF3 through both of its binding sites is needed for arfaptin 1 to fully inhibit PLD1 activity. Since studies with purified pro-

teins have indicated that ARF interacts directly with PLD [14,15], it is likely that arfaptin 1 interferes with that interaction and prevents the activation of PLD by ARF3.

Tsai et al. [9] have recently shown that the first 13 N-terminal amino acids of ARF are essential for arfaptin 1 inhibition of its activation of cholera toxin ADP-ribosyltransferase. The same sequence has been implicated in the activation of PLD by ARF [9]. This suggests that arfaptin 1 binds to the N-terminus of ARF and thus blocks its interaction with PLD. An important issue is whether or not the activity of arfaptin 1 is regulated in the cell through phosphorylation or another mechanism. Interestingly, protein kinase C has been reported to phosphorylate arfaptin 1 [7], but it is not known if this alters its activity.

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