Arfaptin 1, an ARF-binding protein, inhibits phospholipase D and endoplasmic reticulum/Golgi protein transport

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Abstract Class I ADP-ribosylation factors (ARFs) are essential for coatomer and clathrin coat assembly and vesicular transport in the Golgi apparatus. However, little is known about the in vivo regulation of ARF actions. Recently we cloned arfaptin 1, a 39 kDa protein that binds active, GTPγS-liganded ARF and translocates with it to Golgi membranes. Here we show that phorbol ester-stimulated phospholipase D (PLD) activity is inhibited in arfaptin 1-overexpressing NIH 3T3 cells and that arfaptin 1 inhibits ARF activation of Golgi-associated PLD. Since PLD activity is thought to play a role in regulating vesicular transport in the secretory pathway, we determined the rate of glycosylation of vesicular stomatitis virus glycoprotein as a measure of protein transport from the endoplasmic reticulum through the Golgi apparatus. Arfaptin 1 overexpression was found to decrease the rate of this reaction approximately two-fold. These data suggest that arfaptin 1 is a regulator of ARF action in the Golgi apparatus.

1. Introduction

The important role of ADP-ribosylation factor (ARF) in recruitment of COPI (coatomer) and clathrin coat proteins to the Golgi apparatus is well established [1–5]. Inhibition of ARF with brefeldin A (BFA), a fungal metabolite that inhibits guanine nucleotide exchange on ARF, totally blocks vesicle formation and secretion and leads to collapse of the Golgi very rapidly after addition [4,6]. ARF is also a regulator of phospholipase D (PLD) [7–9]. Furthermore, recent studies have associated the formation of phosphatidic acid (PA) by PLD with vesicle budding on the Golgi [10–13]. For example, addition of PLD stimulates secretory vesicle budding from Golgi membranes [12] and induces coatomer binding to Golgi membranes [10], whereas 1-butanol and ethanol, inhibitors of PLD-catalyzed PA formation, impair this [12]. Furthermore, in cells expressing high PLD activity, the requirement for ARF during coat assembly on Golgi membranes is replaced [10]. These results suggest that PLD mediates some of the effects of ARF on the Golgi.

Arfaptin 1 is a class I ARF-binding protein [14] that was shown to associate with the GTPγS-bound form of ARF-1 and then to interact with Golgi membranes [14]. In vitro arfaptin 1 inhibits ARF-dependent PLD activation [15]. In a recent study we showed that ARF and PLD are involved in PKC stimulation of matrix metalloproteinase-9 (MMP-9) secretion from HT 1080 cells (B.-T. Williger, W.-T. Ho, S. Gharacholou and J.H. Exton, submitted). In the present study, we investigated the effects of arfaptin 1 overexpression in NIH 3T3 cells. In cells stably transfected with arfaptin 1, we found that PLD activity was greatly inhibited compared to control cells. Furthermore, transport of vesicular stomatitis virus glycoprotein (VSV-G) from the endoplasmic reticulum (ER) through the Golgi apparatus was attenuated in cells overexpressing arfaptin 1. We also show that arfaptin 1 can inhibit the stimulation of Golgi-associated PLD activity by ARF. These results suggest that arfaptin 1 and PLD regulate vesicular transport in the secretory pathway.

2. Materials and methods

2.1. Materials

Essentially fatty acid-free bovine serum albumin (BSA), Tween 20, sodium dodecyl sulfate and phorbol 12-myristate 13-acetate (PMA) were products of Sigma. All organic solvents were of fine grade and obtained from Fisher. Horseradish peroxidase-labeled goat anti-rabbit antiserum was purchased from Vector. Enhanced chemiluminescence kit was purchased from Amersham. Non-fat dry milk was obtained from Bio-Rad. Lipofectamine, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and all other supplements for growth media were purchased from Gibco BRL. All radioactive materials were products of NEN/Dupont. Protogel (acrylamide:bisacrylamide solution) was purchased from National Diagnostics. pTK-Hyg and pTRE plasmids were obtained from Clontech. GST-arfaptin 1 was purified from pGEX-2T/arfaptin 1-transformed *Escherichia coli* (DH5α) as described before [16], except that before the affinity chromatography the bacterial lysate was dialyzed against phosphate-buffered saline overnight at 4°C. Recombinant myrARF3 was purified as previously described [14].

2.2. Cell culture and production of arfaptin 1-overexpressing cells

All cells were maintained in DMEM, 10% FCS, 10 U/ml penicillin and 10 μg/ml streptomycin. Before fractionation or PLD assay the cells were serum-deprived for 18 h in DMEM, 0.1% BSA. For PLD assays, 1 μCi/ml [^{3}H]myristic acid was included. Arfaptin 1-overexpressing NIH 3T3 cells were produced by cotransfection with pTK-Hyg and either arfaptin 1 in the pTRE vector (Clontech) or pTRE alone as a control using lipofectamine according to the manufacturer's instructions. Selection was carried out in hygromycin B for 6 weeks.

2.3. In vivo and in vitro PLD assays

Serum-deprived and labeled cells were washed with DMEM, 0.1% BSA. Following 20 min preincubation in DMEM, 0.1% BSA, 0.3% 1-butanol, the cells were stimulated with PMA at the concentrations described in the figure legends. After 20 min, the cells were washed with PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 8.05 mM Na_2PO_4, 137 mM NaCl) including 0.1% BSA, scraped with 1 ml ice-cold CH_3OH and transferred into glass tubes. $CHCl_3$ and 0.1 N HCl were then added to a final ratio of 1:1:1. The lipid-containing lower phase was collected, dried and dissolved in 30 μl CH_3OH, $CHCl_3$

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Abbreviations: ARF, ADP-ribosylation factor; PLD, phospholipase D; ER, endoplasmic reticulum; VSV-G, vesicular stomatitis virus glycoprotein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PMA, phorbol 12-myristate 13-acetate; PtdBut, phosphatidylbutanol; BSA, bovine serum albumin; MMP-9, matrix metalloproteinase-9

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(1:1). The sample was loaded on a thin layer chromatography plate which was developed in the lower phase of H_2O, ethylacetate, acidic acid and iso-octane (100:110:20:50 respectively). Tritiated phosphatidylbutanol (PtdBut) was measured after scraping the band corresponding to a PtdBut standard (Avanti Polar Lipids). In vitro PLD assays were done as described [7] with the exception that before the assay GST-arfaptin 1 was incubated with myrARF3 in reaction buffer for 15 min on ice and then 1% butanol, phospholipid vesicle substrate and 50 μg Golgi-enriched membranes were added.

2.4. Vesicular stomatitis virus glycoprotein trafficking assay

Infection of cells with VSV, pulse-chase labeling of VSV-G, and digestion of VSV-G with endoglycosidase H (Endo H) were carried out as described [17]. VSV-G was visualized by phosphorimaging. Exposures were chosen with maximum intensity (100% black) between 10^2 and 10^3. Minimum intensity (0% black) was set to 1/100 of this value and intensities between were linearly assigned gray scale values from 0 to 100%.

2.5. Cell fractionation

Serum-deprived cells were washed twice with phosphate-buffered saline and scraped with fractionation buffer (100 mM KCl, 5 mM NaCl, 1 M $MgCl_2$ 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 cell membranes. Golgi-enriched membranes were 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.

3. Results

3.1. Overexpression of arfaptin 1 in NIH 3T3 cells

Arfaptin 1 overexpression inhibits secretion of MMP-9 in HT 1080 cells (B.-T. Williger, W.-T. Ho, S. Gharacholou and J.H. Exton, submitted). In this study we investigated the role of arfaptin 1 in NIH 3T3 cells. The cells were cotransfected with pTK-Hyr and either empty pTRE or pTRE-arfaptin 1 as described in Section 2. Selection was maintained for 6 weeks in hygromycin B and then expression of arfaptin 1 was assayed. Serum-deprived cells were fractionated and cytosol and Golgi-enriched membranes were separated by SDS polyacrylamide gel electrophoresis. The proteins were immunoblotted with anti-arfaptin 1. Fig. 1 shows that expression of arfaptin 1

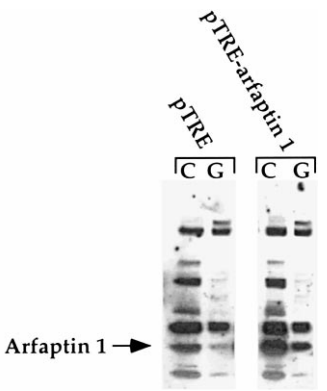

Fig. 1. Expression of arfaptin 1 in transfected cells. NIH cells were transfected with pTK-Hyg and pTRE or pTRE containing arfaptin 1. After selection for 6 weeks, the cells were serum-deprived for 18 h and then fractionated as described in Section 2. Cytosol or Golgi-enriched membranes (15 μg) were separated on 10% polyacrylamide gels and were immunobltoted with rabbit anti-arfaptin 1 polyclonal antibodies. Immunoreactive bands were visualized after incubation with horseradish peroxidase-labeled goat anti-rabbit serum and reagent (C, cystosol; G, Golgi-enriched membrane).

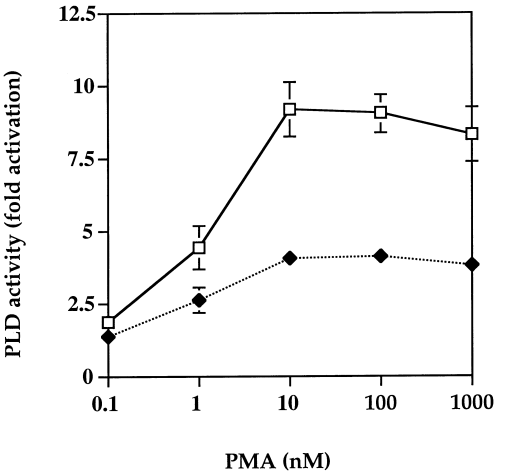

Fig. 2. Effects of arfaptin 1 on PLD activity in vivo. [^{3}H]Myristic acid-labeled control cells (□) and arfaptin 1-overexpressing cells (■) were preincubated for 20 min with 0.3% 1-butanol in DMEM, 0.1% BSA and then stimulated with the indicated concentrations of PMA for 20 min. PLD activity was measured by the production of [^{3}H]PtdBut as described in Section 2. Data are means ± S.E.M. of three experiments.

was significantly increased in both cytosol and Golgi membranes from cells transfected with pTRE-arfaptin 1 versus cells transfected with empty vector (pTRE).

3.2. Inhibition of PMA stimulation of PLD in arfaptin 1-overexpressing cells

As noted above, ARF has been shown to activate PLD and has been implicated in the regulation of Golgi function. Previous findings have shown that arfaptin 1 inhibits PLD activity in vitro [15] and in HT 1080 cells (B.-T. Williger, W.-T. Ho, S. Gharacholou and J.H. Exton, submitted). In light of these data, we compared PLD activity in arfaptin 1-overexpressing and control cells stimulated with PMA. Cells were labeled overnight with [^{3}H]myristic acid, treated with 1-butanol for 20 min, and stimulated with various concentrations of PMA. PLD activity measured by the formation of [^{3}H]PtdBut was decreased in the cells overexpressing arfaptin 1 (Fig. 2).

3.3. Arfaptin 1 overexpression attenuates VSV-G trafficking

It has previously been shown that inhibition of ARF activation by BFA impairs trafficking through the Golgi apparatus and secretion [6]. Furthermore, it has been shown that this treatment leads to coatomer redistribution from the Golgi to the cytosol [2,4], suggesting that the impairment of trafficking and secretion is due to inhibition of vesicle formation. We therefore studied vesicle trafficking in arfaptin 1-overexpressing NIH 3T3 cells. For this purpose, we measured the transport of VSV-G from the ER through the Golgi apparatus in arfaptin 1-overexpressing cells compared with control cells. We used a pulse-chase protocol to measure the kinetics of the disappearance of the ER- and early Golgi-localized (Endo H) sensitive form of VSV-G [17]. Overexpression of arfaptin 1 slowed the glycosylation of VSV-G as it is transported from the ER through the Golgi apparatus (Fig. 3A). This is shown quantitatively in Fig. 3B by determining the amount of radioactivity in the Endo H-sensitive form of VSV-G after different chase times.

3.4. Arfaptin 1 inhibits ARF-stimulated PLD activity in Golgi membranes

Although arfaptin 1 has been shown to inhibit ARF-stimulated PLD partially purified from rat brain membranes [15], we thought that it was important to demonstrate this for the PLD activity associated with Golgi membranes. Golgi-enriched membranes were prepared from HT-1080 cells as described in Section 2. Fig. 4 shows that myristoylated ARF3 produced a 3-fold stimulation of PLD activity in the presence of guanosine 5′,3-*O*-(thio)triphosphate (GTPγS). Increasing concentrations of arfaptin 1 produced complete inhibition of the effect of ARF3.

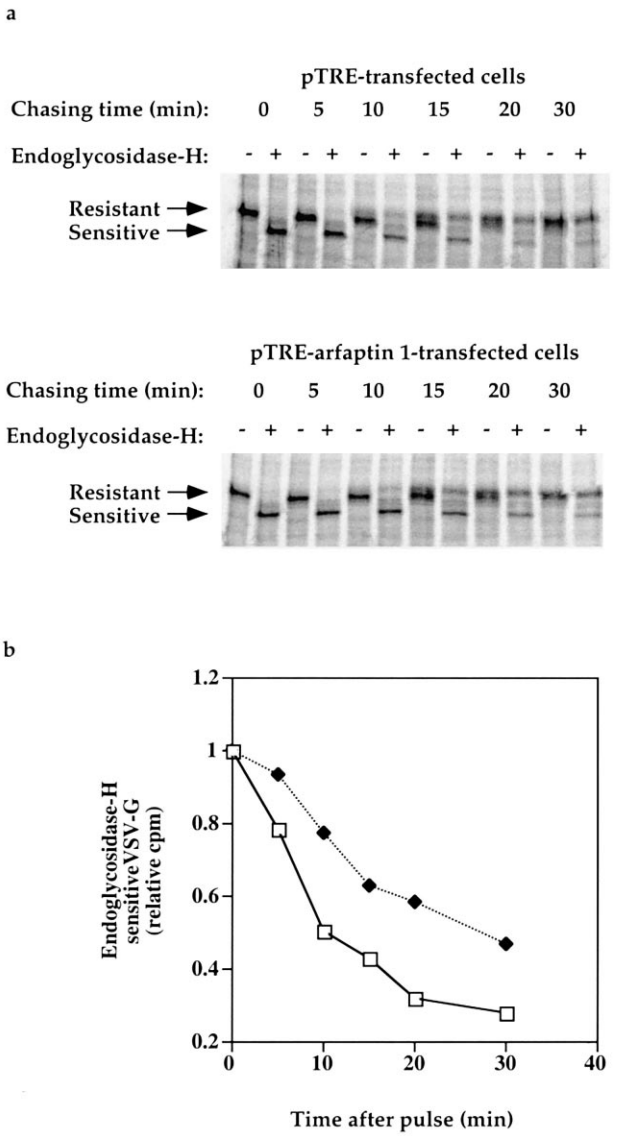

Fig. 3. Effects of arfaptin 1 on VSV-G trafficking in cells. A: Control cells (□) and arfaptin 1-overexpressing cells (■) were infected with VSV. After incubation for 3.75 h, the cells were pulsed with 1 mCi/ml of [^{35}S]methionine/cysteine for 5 min and the Endo H-resistant VSV-G form was chased by sampling the cells at the indicated times. Endo H-resistant and -sensitive forms of VSV-G, measured as described in [17] are indicated. B: VSV-G trafficking was calculated after measuring band densities. Data presented as the relative disappearance of the Endo H-resistant form.

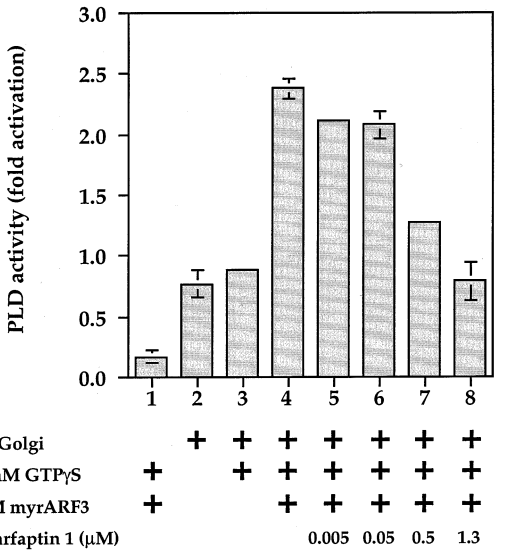

Fig. 4. Effects of ARF and arfaptin 1 on Golgi-associated PLD activity. Golgi-enriched membranes (50 μg) were prepared from HT 1080 cells as described in Section 2. They were incubated with 0.3 μM myristoylated ARF3 and 0.83 mM GTPγS with or without the indicated concentrations of GST-arfaptin 1. PLD activity was assayed in vitro using [^{3}H]palmitoyl phosphatidylcholine as described in [7].

4. Discussion

The present findings support previous observations that arfaptin 1 is an inhibitor of ARF actions in vitro including PLD activity (Fig. 4) [15]. Nevertheless, the intracellular role of arfaptin 1 is unclear. In this study we report that overexpression of arfaptin 1 in NIH 3T3 cells inhibited PMA-stimulated PLD activity. This result suggests that ARF could be involved in the action of PMA on PLD. This could arise because PMA modulates ARF activation or activity. Alternatively, the effect could be due to the demonstrated synergistic interaction between PKC and ARF in the regulation of PLD [18,19]. The idea that PKC could modulate ARF activation or activity is an attractive one, but clearly needs more support.

The other major observation was that VSV-G trafficking from the ER and through the Golgi was inhibited in arfaptin 1-overexpressing cells. This observation is supported by another study showing that arfaptin 1 overexpression results in inhibition of MMP-9 secretion (B.-T. Williger, W.-T. Ho, S. Gharacholou and J.H. Exton, submitted). These findings indicate that arfaptin 1 has inhibitory activity in vivo consistent with its in vitro effects. The effects of arfaptin 1 on VSV-G trafficking and on MMP-9 secretin were not as great as those of BFA, which produced complete inhibition [6] (B.-T. Williger, W.-T. Ho, S. Gharacholou and J.H. Exton, submitted). This probably reflects the fact that whereas fully inhibitory concentrations of BFA could be produced by addition, completely inhibitory concentrations of arfaptin 1 could not be achieved by expression.

An intriguing possibility is that the inhibitory action of arfaptin 1 on Golgi function is due in part to its inhibition of ARF-mediated activation of PLD. As noted above, the inhibition of PLD activity has been previously demonstrated for partially purified brain PLD in vitro [15] and is also shown for Golgi-associated PLD in the present study (Fig. 4). Inhibition of PMA stimulation of PLD is also observed in vivo

(Fig. 2) (B.-T. Williger, W.-T. Ho, S. Gharacholou and J.H. Exton, submitted), although the basis for the latter effect is unclear (see above). There is also evidence that PLD plays a role in PMA-stimulated secretion since this inhibited by 1-propanol through the transphosphatidylation reaction to a much greater extent than 2-propanol [20]. In view of the evidence that ARF regulates PLD activity in Golgi membranes [10,12,21] and that PLD could play a role in Golgi function [10–13] and secretion [20], it seems reasonable to propose that arfaptin 1 exerts part of its effect on vesicle trafficking and secretion via inhibition of PLD activity.

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