Arfaptin 1 inhibits ADP-ribosylation factor-dependent matrix metalloproteinase-9 secretion induced by phorbol ester in HT 1080 fibrosarcoma cells

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Abstract Matrix metalloproteinase-9 (MMP-9) is a collagenolytic enzyme secreted by cancer cells and involved in invasiveness and metastasis. Its secretion from human fibrosarcoma HT 1080 cells is markedly enhanced by phorbol 12-myristate 13-acetate (PMA) and abolished by brefeldin A, an inhibitor of ADP-ribosylation factor (ARF) activation. These results support a role for ARF in PMA-stimulated MMP-9 secretion. Overexpression of arfaptin 1, a 39 kDa ARF-binding protein that inhibits in vitro activation of cholera toxin ADP-ribosyltransferase and phospholipase D (PLD) by ARF, inhibited PMA-stimulated MMP-9 and PLD activation. These data are in agreement with previous results demonstrating a significant role for PLD in regulating MMP-9 secretion.
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Key words: ADP-ribosylation factor; Arfaptin; Phospholipase D; Phosphatidic acid; Matrix metalloproteinase

1. Introduction

Class I ADP-ribosylation factors (ARFs) are 20 kDa small G proteins that are important regulators of COPI (coatomer) and clathrin coat assembly on the Golgi apparatus [1–5]. In order to exert their effects, ARFs need to be in their GTP-bound active form, and to be N-terminally myristoylated to enable their interaction with membranes [5]. The fungal metabolite brefeldin A (BFA) inhibits large, but not small [6–8], guanine exchange factors (GEFs) for ARF and leads to inhibition of bud formation on the Golgi and consequently impaired secretion [4,9]. Thus, ARFs are essential for maintaining regular Golgi functions. ARFs are also regulators of phospholipase D (PLD) [10,11], suggesting that PLD might mediate some of the effects of ARFs. Indeed, several studies implicate PLD and phosphatidic acid (PA), the product of phosphatidylcholine hydrolysis by PLD, in Golgi functions [12–15]. Arfaptin 1 is a 39 kDa ARF-binding protein [16] that attenuates the effects of ARF on cholera toxin ADP-ribosyltransferase and PLD [17–19] and on the trafficking of vesicular stomatitis virus glycoprotein (VSV-G) from the endoplasmic reticulum (ER) and through the Golgi [18]. Arfaptin 1 exhibits no GEF activity or GTPase activating protein activity towards ARF [17] and its mode of action is unclear.

In this study, we investigated the role of ARF in the secretion of matrix metalloproteinase-9 (MMP-9) (also known as gelatinase B and 92 kDa type IV collagenase) from the human fibrosarcoma HT 1080 cell line. MMP-9 is a collagenolytic enzyme which plays a major role in hydrolysis of the extracellular matrix and the invasion of cancer cells into the circulation and to secondary sites [20,21]. First we established that MMP-9 secretion requires active ARF and could be used for studies of ARF in the cellular level. It was shown that BFA dose-dependently inhibited secretion of MMP-9. Furthermore, increased MMP-9 secretion was found in cells overexpressing ARF3 and decreased secretion in those overexpressing an inactive ARF3 mutant. Taken together these findings provide strong support for a role of ARF in phorbol 12-myristate 13-acetate (PMA)-induced MMP-9 secretion. Finally, we studied the role of arfaptin 1 in regulating MMP-9 secretion. Overexpression of arfaptin 1 significantly reduced PLD activity and MMP-9 secretion. These changes agree with previous data implicating PLD activity and PA production in the induction of MMP-9 secretion [22]. These results suggest that arfaptin 1 may be a regulator of some in vivo effects of ARF and may therefore affect regulated secretory pathways.

2. Materials and methods

2.1. Materials

Essentially fatty acid-free bovine serum albumin (BSA), Tween 20, PMA, sodium dodecylsulfate (SDS) and rabbit anti-actin antibody were products of Sigma. All organic solvents were of fine grade and obtained from Fisher. FuGENE6 was a product of Roche. Horseradish peroxidase-labeled goat anti-rabbit antiserum was purchased from Vector. Enhanced chemiluminescence (ECL) kit was purchased from Amersham. Non-fat dry milk was obtained from Bio-Rad. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and all other supplements for growth media were purchased from Invitrogen. [9,10-^{3}H]Myristic acid was a product of Perkin Elmer

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Abbreviations: ARF, ADP-ribosylation factor; BFA, brefeldin A; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FCS, fetal calf serum; GEF, guanine nucleotide exchange factor; MMP-9, matrix metalloproteinase-9; PA, phosphatidic acid; PBS, phosphate-buffered saline; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PtdBut, phosphatidylbutanol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VSV-G, vesicular stomatitis virus glycoprotein

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Life Science. Polyacrylamide 10% gels, zymogram gels, zymography renaturing buffer and developing buffer were products of Novex.

2.2. Plasmids and vectors

pcDNA3-ARF3 and pcDNA3-G2A/Q71L-ARF3 were prepared as described before [16].

2.3. Cell culture and transfection

Cells were maintained in DMEM, 10% FCS, 10 U/ml penicillin and 10 mg/ml streptomycin (growth medium) at 37°C and in 10% CO_2 atmosphere. For experiments, 6×10^5 cells were seeded in 60 mm plates and then allowed to grow for 24 h. Prior to experiments, cells were serum-deprived for 18 h in DMEM, 0.1% BSA. For transfection, cells were seeded at density of 4.5×10^5 in 60 mm plates for 24 h. Transfections were carried out in growth medium using FuGENE6 according to the manufacturer's instructions. After 18 h, the medium was replaced with fresh growth medium and 10 h later cells were serum-deprived as detailed above.

2.4. MMP-9 secretion and activity assay

Before assay, the cell medium was replaced with fresh DMEM, 0.1% BSA containing 100 nM PMA. In experiments involving inhibition with BFA, it was added 30 min before induction with PMA. Medium samples were collected after 7.5 h and loaded with non-reducing sample buffer (2% SDS, 10% glycerol in 62.5 mM Tris pH 6.8) on zymogram gels. Before developing, gels were rinsed for 30 min in renaturing buffer and then 30 min in developing buffer at room temperature. Gels were incubated in fresh developing buffer for 18 h at 37°C. MMP-9 activity was indicated by the clear 92 kDa band that appeared after staining with Coomassie brilliant blue and removing excess dye by an 18 h rinse in water. Gels were dried and scanned and then the image was inverted (clear to black and black to clear).

2.5. In vivo PLD assay

Serum-deprived cells were labeled for 18 h with 1 μCi/ml [^{3}H]myristic acid and 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, unless otherwise described, 100 nM PMA for 20 min. Cells were washed with phosphate-buffered saline (PBS) (2.68 mM KC1, 1.47 mM KH_2PO_4, 8.05 mM Na_2PO_4, 137 mM NaC1), 0.1% BSA, scraped with 1 ml ice-cold CH_3OH and transferred into glass tubes. $CHC1_3$ and 0.1 N HC1 were added to a final ratio of 1:1:1. The lipid-containing lower phase was collected, dried and dissolved in 30 μl CH_3OH, $CHC1_3$ (1:1). The samples were loaded on a thin-layer chromatography plate which was developed in the upper phase of H_2O, ethylacetate, acetic acid and iso-octane (100:110:20:50 respectively). Tritiated phosphatidylbutanol (PtdBut) was measured after scraping the band corresponding to the PtdBut standard (Avanti Polar Lipids).

2.6. Cell lysis and immunoblotting

Overnight serum-deprived cells in 60 mm plates were washed with PBS and then lysed with 200 μl lysis buffer (1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 100 mM NaF, 10 mM Na-pyrophosphate, 2.5 mM Na_3VO_4 in PBS). The soluble fraction was separated by 30 min centrifugation in a bench top microcentrifuge at 14 000 rpm at 4°C. Protein samples (15 μg) were analyzed after SDS–polyacrylamide gel electrophoresis (SDS–PAGE) by immunoblotting with rabbit anti-arfaptin 1 [16] diluted 1:7500 in PBS, 0.5% Tween 20 and 5% dry milk powder and with horseradish peroxidase-labeled secondary antibody. Immunoblots with rabbit anti-actin antibody were carried out according to the manufacturer's instructions. Immunoreactive bands were visualized by the ECL reaction.

3. Results

3.1. BFA inhibits MMP-9 secretion induced by PMA

BFA inhibits large ARF-GEFs and causes disruption of the Golgi [4,6–9,23]. Upon treatment with BFA Golgi-dependent trafficking and secretion are abolished. Therefore, BFA inhibition may be used as a marker of Golgi-dependent trafficking. PMA-induced MMP-9 secretion from HT 1080 cells was dose-dependently inhibited by BFA. Inhibition was detectable with 10 ng/ml and 100 ng/ml produced total inhibition (Fig. 1). Since no MMP-9 secretion was detected in the absence of PMA [19] BFA alone was not tested.

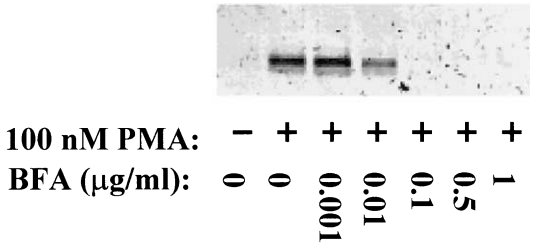

Fig. 1. BFA inhibits MMP-9 secretion in a dose-dependent manner. Overnight serum-deprived cells were incubated with fresh DMEM, 0.1% BSA containing the indicated BFA concentrations and, after 30 min, PMA was added to 100 nM. After 7.5 h, medium samples (5 μl) were assayed for MMP-9 activity. The data shown are representative of three independent experiments.

3.2. MMP-9 secretion is enhanced by overexpression of ARF3 and inhibited by overexpression of inactive ARF3

ARF3 is a member of the class 1 family of ARFs that have been shown to regulate Golgi function [5,23]. Evidence suggesting a possible involvement of the ARF pathway in secretion of MMP-9 came from a previous study illustrating a major role for PLD, a downstream effector of ARF, in MMP-9 secretion [22]. In the present study we investigated the effect of ARF itself on MMP-9 secretion. Overexpression of wild-type ARF3 in HT 1080 cells increased PMA-induced MMP-9 secretion by 46 ± 4.7% (Fig. 2, mean value from scans of three independent experiments). We also overexpressed G2A/Q71L-ARF3 in the cells. This mutant form of ARF3 is inactive because it cannot be myristoylated and does not associate with Golgi [3,24]. It inhibited PMA-induced MMP-9 secretion by 22 ± 1.9% (Fig. 2, mean value from scans of three independent experiments), presumably by competing with endogenous ARF. The effect of constitutively active ARF3 mutant Q71L-ARF3 [16] on MMP-9 secretion was not tested since overexpression of this protein was associated with a marked decline in cell viability. This was not observed with wild-type or inactive ARF3.

3.3. Examination of PLD activity in cells overexpressing arfaptin 1

As was demonstrated before, arfaptin 1 inhibits PLD activation by ARF in vitro [18]. However, there is no previous report of the intracellular role of arfaptin 1 or of its effect on PLD activity in stimulated cells. In view of the evidence that ARF activates the PLD1 isozyme [25] that is present in Golgi [26] and that PLD or its product PA can regulate Golgi functions [12–15], we tested for changes in PLD activity in cells overexpressing arfaptin 1. As shown in Fig. 3, in cells overexpressing arfaptin 1, the effect of PMA on PLD was inhibited by approximately 30%, a similar inhibition to that observed on MMP-9 secretion shown later in this report.

3.4. Arfaptin 1 downregulated MMP-9 secretion induced by PMA

Previously, it was shown that PLD regulates MMP-9 secretion and PA induces it in HT 1080 cells [22]. In light of these results and those shown herein regarding the effect of ARF3

overexpression on MMP-9 secretion, and the in vivo (Fig. 2) and in vitro [18] inhibition of PLD activity by arfaptin 1, we investigated the effect of arfaptin 1 on MMP-9 secretion from HT 1080 cells. As shown in Fig. 4A, transient transfection of HT 1080 cells with increasing amounts of plasmid encoding arfaptin 1 caused a large expression of the protein. This inhibited the effect of PMA on MMP-9 secretion when examined in terms of amount of plasmid transfected (Fig. 4B) or of time of MMP-9 sampling (Fig. 4C). Maximal inhibition (33 ± 3.8%, mean value from three independent experiments) was observed in cells transfected with 800 ng/60 mm plate. Finally, the effect of arfaptin 1 on MMP-9 secretion is specific. This was concluded since overexpression of arfaptin 1 did not reduce the level of other proteins in the cells (Fig. 4D).

4. Discussion

The present report provides for the first time evidence that ARF is required for the stimulatory effect of PMA on MMP-9 secretion in HT 1080 cells. Overexpression of inactive ARF3 inhibited MMP-9 secretion from PMA-induced HT 1080 cells whereas wild-type ARF3 markedly increased it. BFA also had a strong inhibitory effect on MMP-9 secretion at comparatively low concentrations. These data illustrate the requirement of active ARF in order to achieve MMP-9 secretion. BFA inhibits large ARF-GEF activity [6–8,23] and thus blocks the activation of ARF in the Golgi and causes complete dissolution of this organelle [4,9,27]. Inactive G2A/Q71L-ARF3 also inhibited MMP-9 secretion, presumably by competing with the endogenous ARF3. Interestingly, since the association of the G2A/Q71L-ARF3 mutant with the membrane is greatly impaired, this result suggests that the competition takes place in the cytosol and not at the membrane. The difference between the partial inhibition of MMP-9 secretion caused by overexpressing G2A/Q71L-ARF3 and the full inhibition observed with BFA is presumably due their different mechanisms of inhibiting ARF action.

Establishing MMP-9 secretion as a cellular model system for the activity of ARF allowed us to approach an important question, namely, the intracellular role of arfaptin 1. In vitro, arfaptin 1 inhibits the activation of PLD1 by ARFs [17]. However, in vitro systems do not always reflect cellular events. To explore an in vivo role for arfaptin 1, we overexpressed it in HT 1080 cells. This resulted in inhibition of PMA-stimulated PLD activity (30%) and MMP-9 secretion (33%). These results suggest a possible role for arfaptin 1 in regulation of secretory pathways.

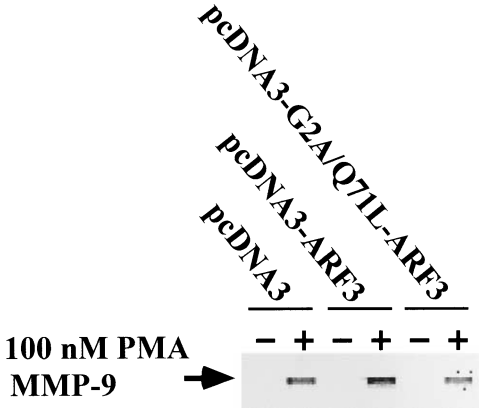

Fig. 2. Effects of wild-type and inactive mutant ARF3 on MMP-9 secretion in PMA-induced cells. HT 1080 cells transiently transfected with either pcDNA3, pcDNA3-ARF3 or pcDNA3-G2A/Q71L-ARF3 were serum-deprived for 18 h in DMEM, 0.1% BSA. The medium was replaced with fresh DMEM, 0.1% BSA containing 100 nM PMA. Following 7.5 h incubation, 5 μl medium samples were assayed for MMP-9 activity. The data shown are representative of three independent experiments.

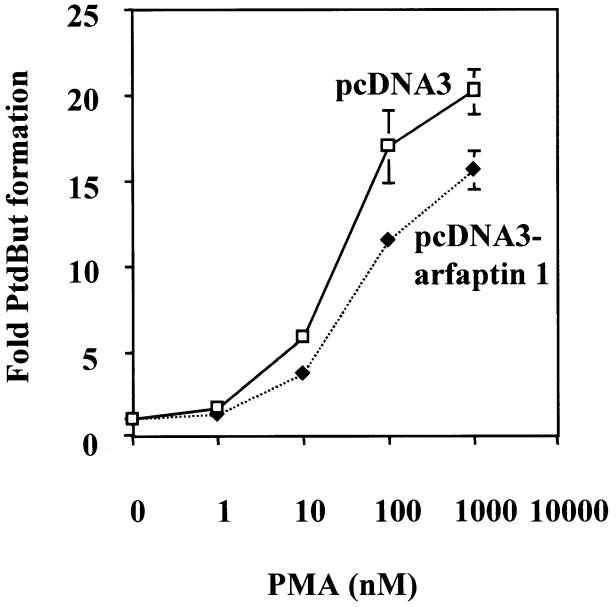

Fig. 3. Arfaptin 1 overexpression inhibits PLD stimulation in PMA-treated HT 1080 cells. Cells were transfected with 800 ng/60 ml plate of either pcDNA3 (open squares) or pcDNA3-arfaptin 1 (solid squares) and then labeled with 1 μCi/ml [^{3}H]myristic acid in DMEM, 0.1% BSA. After 18 h, the cells were washed and preincubated with DMEM, 0.1% BSA, 0.3% 1-butanol. Following 20 min incubation, the cells were stimulated with the indicated concentrations of PMA for 20 min. The lipids were then fractionated and [^{3}H]PtdBut formation was measured as described in Section 2. [^{3}H]PtdBut values were normalized by dividing measured cpm by total cpm in the lipid fraction, and plotted as fold increases over control (without PMA). Data are expressed as the means ± S.E. of three independent experiments performed in duplicate. $P < 0.05$ with respect to cells transfected with pcDNA3.

In addition, arfaptin 1 inhibits the activation of PLD1 by ARFs [17]. However, in vitro systems do not always reflect cellular events. To explore an in vivo role for arfaptin 1, we overexpressed it in HT 1080 cells. This resulted in inhibition of PMA-stimulated PLD activity (30%) and MMP-9 secretion (33%). These results suggest a possible role for arfaptin 1 in regulation of secretory pathways.

Another interesting question is the involvement of PLD in regulation of Golgi functions. In a previous study reported elsewhere [22], the effect of PMA on MMP-9 secretion was observed to be inhibited by 1-propanol to a much greater extent than 2-propanol, implying the involvement of PLD. This was further supported by the stimulatory effect of dioctanoyl PA [22]. In the present study, arfaptin 1 was shown to inhibit PMA-dependent PLD stimulation (Fig. 3) and MMP-9 secretion (Fig. 4). Moreover, arfaptin 1 was previously implicated in inhibition of VSV-G secretion [19]. Thus, we suggest that arfaptin 1, ARF and PLD operate in concert in regulation of Golgi functions and regulated secretion. It appears that the role of arfaptin 1 is to regulate and fine-tune the effects of ARF on the Golgi including those that are mediated by PLD.

An issue of interest is the mechanism(s) by which PMA stimulates MMP-9 secretion. Previous evidence [22] has shown that the effect of PMA is mediated by protein kinase C (PKC), but the target(s) of this kinase is unknown. Because

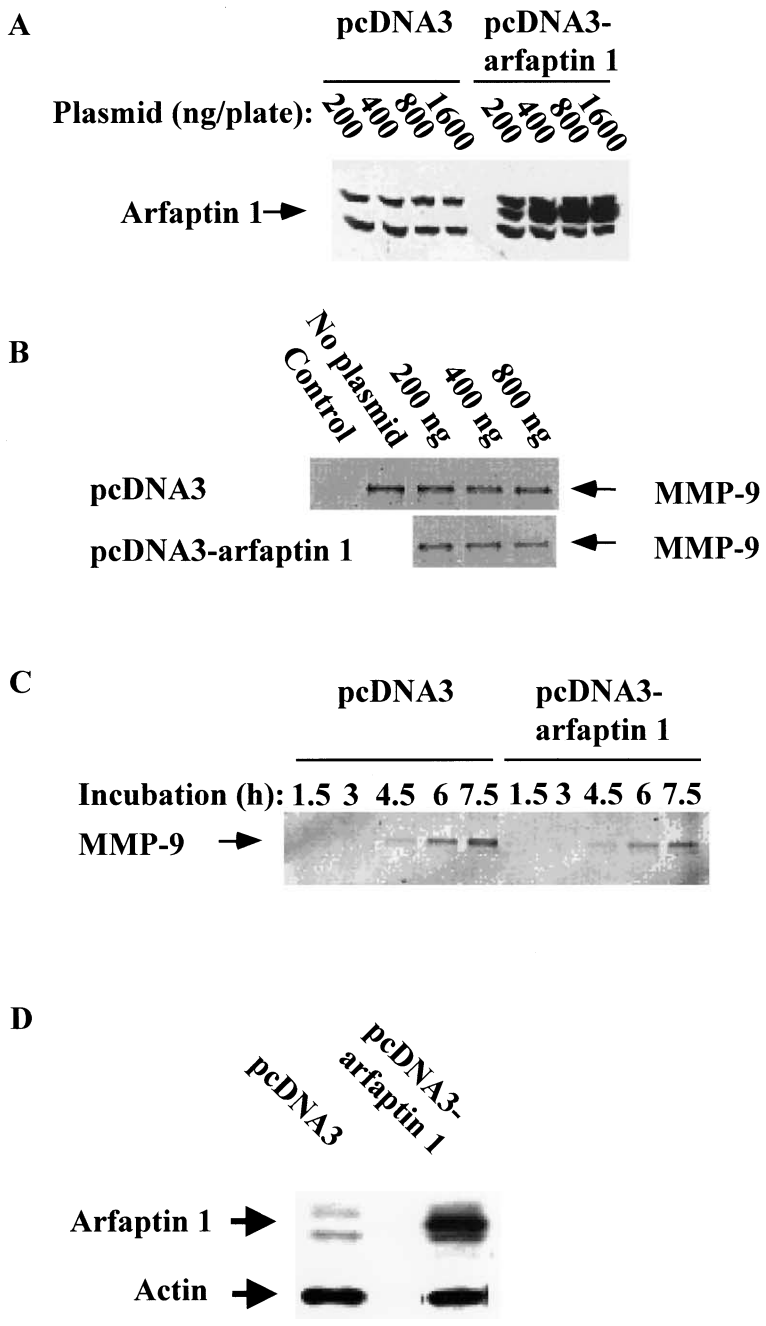

Fig. 4. Arfaptin 1 overexpression inhibits MMP-9 secretion. HT 1080 cells were transfected with either pcDNA3 or pcDNA3-arfaptin 1 using FuGENE6 as described in Section 2. After 24 h, cells were serum-deprived in DMEM, 0.1% BSA for 18 h. A: Cells transfected with the indicated amounts of plasmid were lysed as described in Section 2 and whole cell lysates were subjected to SDS–PAGE and immunoblotting with rabbit anti-arfaptin 1. Immunoreactive bands were visualized using the ECL reaction. B: Cells transfected with the indicated amounts of DNA were incubated with fresh DMEM, 0.1% BSA and 100 nM PMA. After 7.5 h, 5 μl medium samples were assayed for MMP-9 activity as described in Section 2. C: Cells transfected with either pcDNA3 or pcDNA3-arfaptin 1 (800 ng/60 ml plate) were incubated with fresh DMEM, 0.1% BSA containing 100 nM PMA. Medium samples collected at the indicated time points were analyzed for MMP-9 secretion. D: Cells transfected with either pcDNA3 or pcDNA3-arfaptin 1 (800 ng/60 ml plate) were lysed as described in Section 2 and whole cell lysates were subjected to SDS–PAGE and immunoblotting with rabbit anti-actin or rabbit anti-arfaptin 1. Immunoreactive bands were visualized using the ECL reaction. The data shown are representative of three independent experiments.

the steps involved in the processing and translocation of MMP-9 are complex and involve many proteins, it will take more work to define the site of action of PKC.

In summary, in this study, we further characterized MMP-9 release from HT 1080 cells and its regulation by PLD, ARF and arfaptin 1. The cellular system used herein could also be used as a model to explore the regulation of secretion. With this system we utilized several approaches to demonstrate the involvement of ARF and PLD in this mechanism and, in addition, we obtained evidence for the role of arfaptin 1.

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