Arfaptins Are Localized to the trans-Golgi by Interaction with Arl1, but Not Arfs*†‡

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Arfaptins (arfaptin-1 and arfaptin-2/POR1) were originally identified as binding partners of the Arf small GTPases. Both proteins contain a BAR (Bin/Amphiphysin/Rvs) domain, which participates in membrane deformation. Here we show that arfaptins associate with trans-Golgi membranes. Unexpectedly, Arl1 (Arl-like 1), but not Arfs, determines the trans-Golgi association of arfaptins. We also demonstrate that arfaptins interact with Arl1 through their BAR domain-containing region and compete for Arl1 binding with golgin-97 and golgin-245/p230, both of which also bind to Arl1 through their GRIP (golgin-97/RanBP2/Imh1p/p230) domains. However, arfaptins and these golgins show only limited colocalization at the trans-Golgi. Time-lapse imaging of cells overexpressing fluorescent protein-tagged arfaptin-1, exogenously expressed in COS-7 cells, was localized to the Golgi apparatus (5), and inhibits phospholipase D (12, 13). On the other hand, arfaptin-2/POR1 interacts with all Arf proteins examined (14, 15) and an Arl-like protein, Arl1 (15, 16), regulates cytoskeletal rearrangements at the cell periphery induced by Arf6 and Rac1 (6), and modifies aggregation of polyglutamine-expanded huntingtin (17, 18). Thus, arfaptins appear to function at diverse cellular locations. However, there has been only one report about the subcellular localization of arfaptins; Kanoh et al. (5) described that FLAG-tagged arfaptin-1, exogenously expressed in COS-7 cells, was localized to the Golgi: in immunofluorescence analysis, its perinuclear staining disappeared upon incubating cells with brefeldin A (BFA).

In this study, we first examined the subcellular localization of endogenous arfaptin-1 and arfaptin-2 and attempted to characterize their membrane recruitment by Arfs. However, we found that association of arfaptins with the trans-Golgi membranes is not determined by Arfs, but instead by Arl1.

**MATERIALS AND METHODS

Antibodies and Reagents—Preparation and affinity-purification of polyclonal rabbit antibodies against Arl1 and ARF-related protein 1 (ARFRP1) were described previously (19). Monoclonal mouse antibody to the Shiga toxin B fragment (StxB) was purified from culture supernatant of a hybridoma clone (13C4) obtained from ATCC. Sources of other antibodies used in the present study were as follows: polyclonal rabbit anti-TGN46, a kind gift from Minoru Fukuda, Burnham Institute (20); polyclonal rabbit anti-golgin-97, a kind gift from Nobuhiro Nakamura, Kyoto Sangyo University (21); polyclonal goat anti-arfaptin-1, Santa Cruz Biotechnologies; polyclonal rabbit anti-arfaptin-2, Zymed Laboratories Inc.; monoclonal mouse anti-arfaptin-2, Abnova; monoclonal mouse anti-GM130, anti-γ-adaptin, anti-golgin-245, and anti-Arf3, BD Biosciences; monoclonal mouse anti-Arf1, Stresggen; monoclonal mouse anti-actin, Chemicon; mono-
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clonal rat anti-HA, Roche Applied Science; AlexaFluor-conjugated secondary antibodies, Molecular Probes; horseradish peroxidase-conjugated secondary antibodies, Jackson ImmunoResearch Laboratories. Recombinant StxB was purified as described previously (22, 23). BFA and nocodazole were purchased from Sigma-Aldrich. ML7 and Y-27632 were purchased from Calbiochem and Wako Pure Chemical Industries, respectively.

Plasmids—Construction of expression vectors for C-terminally HA-tagged Arl1 and Arfs was described previously (19, 22, 24–26). Expression vectors for N-terminally HA-tagged arfaptin-1 and arfaptin-2 were constructed by subcloning the corresponding human cDNAs covering the entire coding sequence into the pcDNA3-HAN vector (27). Expression vectors for C-terminally EGFP-tagged Arl1, and N-terminally EGFP-tagged arfaptin-1, arfaptin-2, and golgin-97 were constructed by subcloning the corresponding cDNAs into pEGFP-N3 and pEGFP-C1 (Clontech), respectively. Expression vectors for N-terminally mCherry-tagged arfaptin-2 and golgin-97 were constructed by subcloning the corresponding cDNAs into pcDNA3.1-mCherryN (28) (a kind gift from Roger Tsien, UCSD). Bacterial expression vectors for full-length arfaptin-1 and arfaptin-2 (and their fragments) fused to glutathione S-transferase (GST) were constructed by subcloning the corresponding cDNAs into pGEX6P-1 (GE Healthcare Bioscience). An expression vector for the GGA1-GAT domain fused to GST was described previously (24). The StxB expression vector (23) was a kind gift from Ludger Johannes, Institut Curie.

Cell Culture, siRNA-mediated Knockdown, Immunofluorescence Analysis, and Time Lapse Recording—Culture of HeLa cells and transfection of expression plasmids were performed as described previously (19, 29). Preparation of pools of siRNAs for Arl1 and ARFRP1 was described previously (22). A pool of siRNAs targeted at the mRNA region of human arfaptin-1 (nucleotide residues 1–1,024, when the A residue of the initiation Met codon is assigned as residue 1) or arfaptin-2 (nucleotide residues 91–901), or an siRNA pool for the 3′-untranslated region of human Arf1 (PCR-amplified using a set of primers: 5′-CTCTCACTCCTCTTGCCTCTC-3′ and 5′-GGCTGCTCTGAAGGGTGATAG-3′) or Arf3 (PCR-amplified using a set of primers: 5′-CAGACAGCCCTAAACAGCAC-3′ and 5′-CAGAGGAGGGTAAACCAGTC-3′) was prepared using a BLOCK-iT RNAi TOPO transcription kit and a BLOCK-iT Dicer RNAi kit (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen) and incubated for 24 h. The transfected cells were then transferred to a new culture dish and incubated for 48 h (for arfaptin siRNA) or 24 h (for Arf siRNA). The cells were then subjected to a second siRNA transfection and incubated for 24 h. The cells were transferred to a new culture dish containing coverslips and further incubated for 24 h (for arfaptin siRNA) or 48 h (for Arf siRNA), and processed for immunofluorescence and immunoblot analyses. For time-lapse recording, cells were incubated in HEPES-buffered modified Eagle’s medium and placed on a microscope stage that had been preincubated at 37 °C. The cells were observed using FV1000D on an inverted microscope IX81 (Olympus). Images were acquired sequentially every 1 s. Movies correspond to three frames per second.

StxB Uptake Assay—Cells were incubated with StxB (a final concentration of 4.25 μg/ml) at 4 °C for 50 min. After a medium change, the cells were incubated at 37 °C for 10–30 min, and processed for immunofluorescence analysis with anti-StxB antibody.

Pull-down Assays—Pull-down assays to detect interactions of arfaptins with Arfs, Arl1, or arfaptins were performed as previously described for detecting interaction of the GAT domain of the GGA protein with Arfs (24, 30). Briefly, a recombinant GST fusion (full-length, N-terminal portion, or C-terminal portion of arfaptin-1 or arfaptin-2, or the GGA1-GAT domain) was incubated with lysates prepared from HeLa cells transfected with an expression vector for a candidate interacting protein (C-terminally HA-tagged Arf1, Arf3, Arf5, Arf6, or Arl1; or N-terminally HA-tagged arfaptin-1 or arfaptin-2) and pulled down with glutathione-Sepharose beads. Proteins bound to the beads were subjected to immunoblot analysis with anti-HA antibody.

RESULTS

Localization of Arfaptins to the trans-Golgi—Early studies showed that exogenously expressed arfaptin-1 and arfaptin-2 are found in perinuclear region (5, 17, 18). As exogenously expressed arfaptin-1 in the perinuclear region was redistributed into the cytoplasm upon treating cells with BFA (5 μg/ml, 10 min), Kanoh et al. (5) suggested that it was localized to the Golgi apparatus. However, in order to understand the role of arfaptins in membrane traffic, it is important to determine whether arfaptins are associated with the cis- or trans-face of the Golgi. Therefore, we first compared localization of endogenous arfaptin-1 and arfaptin-2 with that of GM130, a cis-Golgi protein, and TGN46, a TGN protein. Immunofluorescence analysis of HeLa cells showed that staining for both arfaptin-1 (Fig. 1A) and arfaptin-2 (supplemental Fig. S1A) was found in the perinuclear region. The staining for arfaptin-1 or arfaptin-2 showed significant overlap with TGN46 (a-a′; also see supplemental Fig. S2), while limited overlap with GM130 (b-b′; also see supplemental Fig. S2), suggesting that both arfaptin-1 and arfaptin-2 associate primarily with the trans-Golgi/TGN. To unequivocally show trans-Golgi localization of arfaptins, we treated HeLa cells with nocodazole to fragment the Golgi structure; the Golgi fragments consist of mini-stacks and are suitable to determine the cis and trans polarity (31). In the fragmented Golgi structures, the staining for arfaptin-1 or arfaptin-2 overlapped significantly with the TGN46 staining (Fig. 1B and supplemental Fig. S1B, a-a′; also see supplemental Fig. S2). In contrast, the arfaptin staining was juxtaposed with the signal for GM130 (b-b′; also see supplemental Fig. S2). These observations indicate that both arfaptin-1 and arfaptin-2 associate predominantly with the trans-Golgi/TGN.

Association of Arfaptins with Golgi Membranes Is Insensitive to BFA and Independent of Arfs—We next examined effects of BFA on the Golgi localization of arfaptin-1 and arfaptin-2. BFA is an inhibitor of guanine nucleotide exchange factors for Arfs; treating cells with this drug causes dissociation of not only Arf itself but also Arf effector coat proteins, such as the COPI and
AP-1 complexes, from Golgi membranes (32, 33). For example, treatment of HeLa cells with 5 μg/ml BFA for 2 min redistributed γ-adaptin, an AP-1 subunit, from the perinuclear region to the cytoplasm (Fig. 2, compare A and B). In striking contrast, the 2-min BFA treatment appeared not to cause significant change in the distribution of arfaptin-1 (A′ and B′) or arfaptin-2 (A″ and B″). However, after 10 min of BFA treatment, both arfaptin-1 and arfaptin-2 disappeared from the perinuclear Golgi region. The time course of the change in the distribution of arfaptins upon BFA treatment suggests that

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FIGURE 1. Association of arfaptin-1 with the trans-Golgi. HeLa cells left untreated (A) or treated with 5 μg/ml nocodazole for 2 h (B) were doubly stained for arfaptin-1 (a and b) and either TGN46 (a′) or GM130 (b′).

FIGURE 2. BFA-insensitive, Arf-independent Golgi-association of arfaptins. HeLa cells were incubated with 5 μg/ml BFA for the indicated time periods and triply stained for γ-adaptin (A–D), arfaptin-1 (A′–D′) and arfaptin-2 (A″–D″).

AP-1 complexes, from Golgi membranes (32, 33). For example, treatment of HeLa cells with 5 μg/ml BFA for 2 min redistributed γ-adaptin, an AP-1 subunit, from the perinuclear region to the cytoplasm (Fig. 2, compare A and B). In striking contrast, the 2-min BFA treatment appeared not to cause significant change in the distribution of arfaptin-1 (A′ and B′) or arfaptin-2 (A″ and B″). However, after 10 min of BFA treatment, both arfaptin-1 and arfaptin-2 disappeared from the perinuclear Golgi region. The time course of the change in the distribution of arfaptins upon BFA treatment suggests that

FIGURE 3. Binding of arfaptins to Arl1. A, binding of arfaptins to GTP-bound Arl1. Lysates of HeLa cells expressing C-terminally HA-tagged Arl1(WT), Arl1(Q71L), or Arl1(T31N) were pulled down with GST, GST-GGA1(GAT), GST-arfaptin-1, or GST-arfaptin-2 as indicated, and analyzed by immunoblot for HA as described under “Materials and Methods.” B, BAR domain-containing C-terminal region of arfaptins is responsible for Arl1 binding. Lysates of HeLa cells expressing Arl1(Q71L)-HA were pulled down with a GST fusion protein of full-length, N-terminal portion or C-terminal portion of arfaptin-1 or arfaptin-2 and analyzed by immunoblot for HA. C, schematic representation of the structures of human arfaptin-1 and arfaptin-2. Regions of constructs used in the experiment shown in B are indicated.

FIGURE 4. Colocalization of arfaptin-1 with Arl1. A, HeLa cells left untreated (a–d) or treated with 5 μg/ml nocodazole for 2 h (b–d′) were doubly stained for arfaptin-1 (a′ and b′) and Arl1 (a′ and b′). B, HeLa cells were incubated with 5 μg/ml BFA for the indicated time periods and triply stained for Arl1 (a–d), arfaptin-1 (a′–d′), and arfaptin-2 (a″–d″).
Arfaptins remain associated with Golgi membranes and are redistributed concomitant with disintegration of the Golgi apparatus. These observations together are suggestive of BFA-insensitive, Arf-independent association of arfaptins with Golgi membranes. To corroborate the Arf-independent Golgi association of arfaptins, we next examined whether the arfaptin localization to the Golgi is affected by depleting Arf1 and/or Arf3, which are class I Arfs that function at the TGN and/or endosomes (34, 35) and can bind to arfaptins (14) (also see supplemental Fig. S3C). As shown in supplemental Fig. S3B, the localization of arfaptins were not significantly altered in cells treated with siRNAs for Arf1 or Arf3 alone, or their combination, as compared with control cells treated with siRNAs for LacZ, although the Arf proteins were almost completely depleted by the siRNA treatment (supplemental Fig. S3A).  

**Arl1-dependent Recruitment of Arfaptins onto trans-Golgi Membranes**—The above data suggested Arf-independent association of arfaptins with Golgi membranes. We therefore set out to reevaluate the interaction of arfaptins with Arfs. As shown in supplemental Fig. S3C, both arfaptin-1 and arfaptin-2, as well as the GAT domain of GGA1 (used as a positive control) (24, 30), interacted with Arf1, Arf3, Arf5, and Arf6 in the GST-pulldown assay; note that the upper one of the two Arf bands in each lane represents the full-length Arf protein, whereas the lower one is the protein translated from the second methionine (residue 18 for Arf1, Arf3, and Arf5, and residue 14 for Arf6). We next examined the interaction of arfaptins with Arl1, because we previously showed localization of Arl1 to the trans-Golgi/TGN (22) and others reported that Arl1 interacts with arfaptin-2 in their yeast two-hybrid assays (15, 16), although the interaction has not been further characterized. In the GST-pulldown assay, wild-type Arl1 and its GTP-locked mutant, Arl1(Q71L), but not its GDP-bound mutant, Arl1(T31N), interacted with both arfaptin-1 and arfaptin-2. None of the Arl1 proteins interacted significantly with the GGA1-GAT domain (Fig. 3A). When the arfaptin protein was separated into two parts (Fig. 3C), the C-terminal region encompassing the BAR domain (amino acid residues 109–341) was shown responsible for the Arl1 interaction (Fig. 3B). Thus, arfaptins bind to Arl1 in a GTP-dependent manner through their BAR domain-containing region. 

4 H. Takatsu and K. Nakayama, unpublished results.
We then compared the localization of Arl1 with that of arfaptins. Both arfaptin-1 (Fig. 4A, a-a") and arfaptin-2 (supplemental Fig. S1A, c-c") showed significant colocalization with Arl1 in the Golgi region (also see supplemental Fig. S2). The colocalization was highlighted on fragmented Golgi structures induced by treating cells with nocodazole (Fig. 4B, b-b" and supplemental Fig. S1B, c-c"). When cells were treated with BFA, arfaptins, and Arl1 were redistributed from the Golgi region to a cytoplasmic pattern with essentially the same kinetics (Fig. 4B); after 2 min of BFA treatment Arl1 and arfaptins were still found in the Golgi region, and after 10 min of treatment they all showed cytoplasmic patterns. Therefore, the time courses of redistribution of Arl1 and arfaptins upon BFA treatment parallel with each other, but contrast with the time course of γ-adaptn redistribution (Fig. 2A), indicating that Arl1 and arfaptins remain associated with Golgi membranes after BFA treatment.

To directly examine whether Arl1 determines the Golgi localization of arfaptins, we depleted cells of Arl1 by siRNA treatment (Fig. 5A); we previously showed that siRNA treatment results in almost complete depletion of Arl1 and concomitant dissociation from Golgi membranes of golgin-245 (22), which is a well-characterized effector of Arl1 and is recruited onto the TGN by interacting with Arl1 through its GRIP (golgin-97/RanBP2/Imh1p/p230) domain (15, 19, 36–38). As shown in Fig. 5B, the Arl1 siRNA treatment abolished signals for Golgi-associated arfaptin-1 and arfaptin-2 (b-b"). Furthermore, exogenous expression of HA-tagged Arl1 restored the Golgi association of arfaptin-1 and arfaptin-2, as well as that of golgin-245 (Fig. 5C, b-b" and d-d"). By contrast, knockdown of ARFRP1 (used as a negative control) did not significantly affect the arfaptin localization (Fig. 5B, d-d"). These results together indicate that arfaptin-1 and arfaptin-2 are recruited onto Golgi membranes through interaction with Arl1.

**Arfaptin-1 and Arfaptin-2 Can Form a Heterodimer, but Independently Associate with Golgi Membranes**—A previous x-ray structural analysis showed that arfaptin-2 forms a dimer to constitute a crescent-like BAR domain (9). These domains have been implicated in sensing curvature and remodeling of...
membranes (10, 11). Because the above data showed that both arfaptin-1 and arfaptin-2 exhibit Arl1-dependent association with Golgi membranes, we then asked whether they can form a heterodimer. As shown in supplemental Fig. S4A, a GST fusion protein of arfaptin-1 or arfaptin-2 pulled down both arfaptin-1 and arfaptin-2, indicating that arfaptins can form both homodimers and heterodimers. When separated into two parts (see Fig. 3C), the C-terminal region encompassing the BAR domain was shown to be responsible for both the homotypic and heterotypic interactions (supplemental Fig. S4A).

The potential to form a heterodimer leads to a possibility that Golgi association of arfaptin-1 and arfaptin-2 is interdependent. To address this possibility, we examined localization of arfaptin-2 in cells depleted of arfaptin-1 by siRNA treatment, and vice versa. As shown in supplemental Fig. S4B, arfaptin-1 and arfaptin-2 were specifically depleted by treating cells with corresponding siRNAs. It is noteworthy that depletion of arfaptin-1 did not change the amount of arfaptin-2, and vice versa, indicating that arfaptin-1 or arfaptin-2 does not affect each other's intracellular stability. When examined by immunofluorescence microscopy, treatment of cells with arfaptin-1 siRNAs abolished the Golgi-associated staining for arfaptin-1, but did not detectably affect the arfaptin-2 staining (supplemental Fig. S4C). Likewise, arfaptin-2 siRNAs did not affect Golgi-association of arfaptin-1. Thus, it is unlikely that Golgi-association of arfaptin-1 and arfaptin-2 is interdependent, even though they have the potential to form a heterodimer.

We also asked whether depletion of arfaptin-1 and/or arfaptin-2 affect the Golgi association of Arl1. As shown in supplemental Fig. S4D, the Arl1 localization was not visibly altered when cells were depleted of arfaptin-1 or arfaptin-2 alone, or their combination. Furthermore, the arfaptin depletion did not affect the localization of golgin-245 (supplemental Fig. S4E). Overall, these observations demonstrate that arfaptins are downstream effectors of Arl1.

**Similar, but Incompletely Overlapping, Localization of Arfaptins and Golgins—** Well-characterized effector proteins of Arl1 include the GRIP domain-containing golgins, including golgin-97 and golgin-245/p230 (37, 38). These proteins are recruited onto trans-Golgi membranes through interaction with Arl1 (37, 38), although these golgins localize to distinct microdomains of the trans-Golgi and have been implicated in transport of distinct cargo proteins (39–42). Therefore, we then compared the localization of arfaptin-1 and arfaptin-2 with that of these golgins. As shown in Fig. 6A and supplemental Fig. S1A, both arfaptins and golgins were found in the perinuclear region characteristic of the Golgi apparatus. However, arfaptin-1 (Fig. 6A, a–a') and arfaptin-2 (supplemental Fig. S1A, d–d') showed incomplete overlap with golgin-245 (also see supplemental Fig. S2). Essentially the same results were obtained with golgin-97 in place of golgin-245 (Fig. 6A, b–b' and supplemental Fig. S1A, e–e'; also see supplemental Fig. S2). The incompletely overlapping localization of arfaptins and golgins is more evident, when the Golgi was fragmented by treating cells with nocodazole (Fig. 6B and supplemental Fig. S1B). These observations suggest that arfaptins and golgins are associated with distinct microdomains of the trans-Golgi, although both types of proteins are recruited by Arl1.
Exogenous Expression of Arfaptin Displaces Golgins from the Golgi—Over the course of our experiments, we noticed that endogenous golgins were displaced from Golgi membranes when arfaptin-1 or arfaptin-2 was exogenously expressed in cells (Fig. 7). Specifically, in cells with moderate expression levels of EGFP-tagged arfaptin-1 (B and E) or arfaptin-2 (C and F), golgin-245 was not found in the Golgi region (B’, C’, E’, and F’); cells indicated by asterisks; also see G and H). In contrast, TGN46 (B’, C’, and G) and Arl1 (E’, F’, and H) remained localized in the Golgi region, indicating that the Golgi structure or Arl1 association with Golgi membranes per se was not affected. Expression of EGFP as a control did not affect the golgin-245 localization (A’ and D’; cells indicated by asterisks). One possible explanation for these observations is that exogenously expressed arfaptin sequestered endogenous Arl1, which is required for membrane recruitment of golgins.

Arl1 and golgins have been implicated in retrograde transport of the Shiga toxin B fragment (StxB) from endosomes to the Golgi (22, 41, 42). As exogenously expressed arfaptin displaces golgins, one can speculate that retrograde transport of StxB is disturbed by exogenous arfaptin expression. This was the case: as shown in Fig. 8, by 30 min incubation at 37 °C, extracellularly applied StxB did not reach the perinuclear Golgi region in cells with exogenous expression of EGFP-arfaptin-1 or arfaptin-2 (E-E’ and F-F’; cells indicated by asterisks), whereas it was able to reach there in non-transfected cells (E-E’ and F-F’; cells without asterisk) and in cells expressing EGFP as a control (D-D’, cells indicated by asterisks). Next, we asked whether the inhibition of StxB transport is simply due to the displacement of golgins in the arfaptin-expressing cells, or whether instead arfaptins play a regulatory role in the transport process together with golgins. To answer this question, we examined StxB transport in cells depleted of arfaptins. As shown in supplemental Fig. S5, knockdown of neither arfaptin-1 nor arfaptin-2 had a significant effect on the retrograde transport of StxB; by 30 min of incubation at 37 °C, StxB was transported to the perinuclear Golgi region in cells depleted of both arfaptins, as in control cells. It is therefore likely that arfaptins could participate in transport processes, other than StxB retrograde transport involving GRIP domain-containing golgins.

Arfaptins Induce Vesicular and Tubular Intermediates from the Golgi—We next examined the dynamic distributions of Arl1 and arfaptins in living cells by expressing these proteins tagged with a fluorescent protein. When EGFP-tagged Arl1 (Fig. 9A and supplemental Video S1) was expressed in HeLa cells and subjected to time-lapse recording, the protein was found to be associated with typical Golgi-like structures, but its localization appeared to be static. In considerable contrast, mCherry-tagged arfaptin-2 (Fig. 9B and supplemental Video S2) or arfaptin-1 (not shown) exhibited dynamic changes in its distribution; in particular, arfaptin-2 was often found on dynamic vesicular and tubular structures emanating from the Golgi region. The observation of arfaptin-positive tubular structures is consistent with a previous in vitro study showing that arfaptin-2 can deform liposomes, often by causing them to form tubules (10). When Arl1-EGFP and mCherry-arfaptin-2 were coexpressed, they were colocalized on the Golgi structures. Furthermore, Arl1-EGFP was often found along the mCherry-arfaptin-2-positive tubular structures (Fig. 9C and supplemental Video S3). In contrast to the case of arfaptins, vesicular and tubular profiles were much less frequently observed when mCherry-tagged golgin-97 was expressed alone (Fig. 9D and supplemental Video S4) or in combination with Arl1-EGFP (Fig. 9E and supplemental Video S5).

Counting vesicular and tubular intermediates de novo formed within a defined time interval in single cells revealed that Arl1-positive vesicular and tubular structures were more prominent when Arl1-EGFP and mCherry-arfaptin-2 were coexpressed than when Arl1-EGFP was expressed alone or in combination with mCherry-golgin-97 (Fig. 9F, compare green bars). On the other hand, the frequency of the formation of mCherry-arfaptin-2-positive vesicular and tubular structures was not significantly changed whether it was expressed alone or
in combination with Arl1-EGFP (Fig. 9F, red bars). These observations suggest that exogenous expression of arfaptin-2 induces vesicular and tubular intermediates from the Golgi. However, Arl1 does not affect the arfaptin-2-driven tubule formation, though Arl1 does recruit arfaptin-2 onto Golgi membranes.

Myosin II is involved in fission of Rab6-dependent transport vesicles at the Golgi apparatus (43); inhibition of myosin II activation by a mixture of ML7 (a myosin light chain kinase inhibitor) and Y-27632 (a Rho kinase inhibitor), inhibition of myosin II motor activity by blebbistatin, and treatment with myosin II siRNA all lead to formation of long tubules connected to the Golgi. We therefore asked whether this is also the case with the formation of vesicular and tubular intermediates involving Arl1 and arfaptins. As compared with control cells (Fig. 10A and supplemental Video S6), cells treated with ML7 and Y-27632 formed prominently long tubules positive for mCherry-arfaptin-2 (Fig. 10B and supplemental Video S7). In addition, signals of Arl1-EGFP were often observed along the arfaptin-2-positive tubules. It is worth noting that these tubules underwent infrequent fission. This observation brings out the involvement of BAR domain-containing arfaptins in membrane deformation and suggests the potential involvement of myosin II in fission of arfaptin-driven tubules.

**DISCUSSION**

Arfaptins were originally identified by their interaction with Arf small GTPases but little is known about their functions. In the present study, we showed that both arfaptin-1 and arfaptin-2 associate with the trans-Golgi/TGN. Our analyses using BFA and siRNAs for Arfs suggested that their trans-Golgi association is not determined by Arfs, and we eventually found that an Arf-like small GTPase, Arl1, is responsible for the trans-Golgi recruitment of arfaptins through interacting with their BAR domain-containing region.

About a decade ago, two groups reported that arfaptin-2 is able to interact with Arl1 as well as Arfs in their two-hybrid assays (15, 16), but since then the Arl1-arfaptin interaction has not been further characterized. Our time-lapse analyses show that exogenous overexpression of arfaptin is able to induce vesicular and tubular intermediates from the Golgi region, although the observation may not represent a physiological cellular event. By contrast, exogenous expression of Arl1 neither...
induced such intermediates nor enhanced the arfaptin-mediated tubule formation, even though it is required for membrane recruitment of arfaptins. Thus, the active vesicle and tubule formation in cells overexpressing arfaptin suggests that, like other BAR domain-containing proteins (10, 11), arfaptins may have the ability to deform membranes or amplify and stabilize the membrane curvature. We think that Arl1 provides a platform that makes the membrane competent to form vesicles or tubules by recruiting arfaptins, yet it is dispensable for maintaining the membrane association of arfaptins and allowing membrane deformation once they have been recruited. In view of the fact that BAR domain proteins by themselves are able to deform liposome membranes in vitro (10, 11), and that BAR domain proteins other than arfaptins often have membrane-interacting modules, such as PH and PX domains and amphipathic α-helices (11), we hypothesize that Arl1 may have a regulatory role in triggering membrane deformation by recruiting cytosolic arfaptins, which do not contain such modules.

Inhibition of membrane fission by inhibiting myosin II activation exaggerates the arfaptin-positive tubular structures, supporting a role for BAR domain-containing arfaptins in membrane deformation. This observation is reminiscent of in vitro tubulation of liposomes induced by BAR domain proteins (10, 11). Even though the BAR domain proteins may deform membranes and stabilize the membrane curvature to induce tubules, they are unable to cause membrane fission. Thus, fission of budding membranes requires proteins other than BAR domain proteins. For example, other BAR domain proteins are known to interact with dynamin and components of the actomyosin system (44, 45). As arfaptin-2/POR1 is known to regulate reorganization of the actin cytoskeleton (6, 8), it will be interesting for future studies to address how Rac1 participates in the arfaptin-mediated membrane deformation.

To date, Arl1 has been implicated in membrane traffic through its interaction with GRIP domain-containing golgins, including golgin-97 and golgin-245/p230 (46). These golgins have been proposed to serve as tethering factors (47, 48). Both arfaptins and golgins are recruited onto trans-Golgi membranes through interacting with Arl1, and exogenous expression of arfaptins appears to perturb the function of golgins; this blocks StxB retrograde transport, probably by sequestering endogenous Arl1. However, knocking down arfaptins has no apparent effect on StxB transport, which is under the regulation of Arl1 and golgins. The most plausible explanation for these results is that arfaptins and GRIP domain-containing golgins are involved in distinct transport processes. In support of this, these golgins localize to distinct microdomains of the trans-Golgi and have been implicated in transport of distinct cargoes (39–42). Another possibility is that arfaptins may play a regulatory role in, but be dispensable for, the Arl1-mediated transport by triggering deformation of membranes. In either case, the identification of specific cargo molecules under the regulation of Arl1 and arfaptins is an important issue to be addressed in future studies.

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