Roles of ARFRP1 (ADP-ribosylation factor-related protein 1) in post-Golgi membrane trafficking

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Summary

ADP-ribosylation factor (ARF)-related protein 1 (ARFRP1) is a small GTPase with significant similarity to the ARF family. However, little is known about the function of ARFRP1 in mammalian cells, although knockout mice of its gene are embryonic lethal. In the present study, we demonstrate that ARFRP1 is associated mainly with the trans-Golgi compartment and the trans-Golgi network (TGN) and is an essential regulatory factor for targeting of Arl1 and GRIP domain-containing proteins, golgin-97 and golgin-245, onto Golgi membranes. Furthermore, we show that, in concert with Arl1 and GRIP proteins, ARFRP1 is implicated in the Golgi-to-plasma membrane transport of the vesicular stomatitis virus G protein as well as in the retrograde transport of TGN38 and Shiga toxin from endosomes to the TGN.

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Key words: ARFRP1, Arl1, Golgin, TGN38, Shiga toxin, VSVG

Introduction

ADP-ribosylation factors (ARFs) constitute a family of small GTPases belonging to the Ras superfamily that play essential roles in intracellular membrane trafficking. In addition to ARFs, there exists a subfamily of small GTPases with sequence similarity to ARFs, referred to as the Arl (ARF-like) subfamily (Pasqualato et al., 2002). Little is known about the function of the Arl proteins, yet Arl1 has been relatively well characterized. Arl1 is localized to the Golgi complex (Lowe et al., 1996; Schurmann et al., 1994) and recruits a subset of golgins (golgin-97 and golgin-245/p230/tGolgin-1/GCP230) onto Golgi membranes through binding directly to the GRIP domain at their C termini (Burd et al., 2004; Gangi Setty et al., 2003; Lu and Hong, 2003; Panic et al., 2003a; Panic et al., 2003b). Additional Arl1 effectors have also been reported, including Arfaptin, MLKP1 and the GARP/VFT tethering complex (Lu et al., 2001; Panic et al., 2003b; Van Valkenburgh et al., 2001). Overexpression of mutant forms of Arl1 has also been shown to affect the structure and function of the Golgi (Lu et al., 2001; Van Valkenburgh et al., 2001).

It has been shown that the GRIP domain of golgin-245 fused to green fluorescent protein (GFP) is incorporated into Golgi-derived tubulovesicular carriers (Brown et al., 2001; Gleeson et al., 1996; Gleeson et al., 2004). Moreover, overexpression of the interacting domains of golgin-245 and MACF1, which is one of binding partners of golgin-245, blocks transport of the GPI-anchored protein from the trans-Golgi network (TGN) to the plasma membrane (Kakinuma et al., 2004). However, GRIP domain-containing proteins have also been suggested to play a role in the endosome-to-TGN transport (Yoshino et al., 2003). In addition, Lu et al. have very recently demonstrated that Arl1 and golgin-97 are required for transport from early/recycling endosomes to the TGN (Lu et al., 2004).

Mammalian ARFRP1 (ARF-related protein 1), previously designated ARP (Schurmann et al., 1995), is a membrane-associated 25 kDa GTPase with 33% amino acid identity to ARF1. Arl3p, a yeast counterpart, shares 43% amino acids identity with mammalian ARFRP1 (Huang et al., 1999). ARFRP1 and Arl3p are unusual among the ARF/Arl family of small GTPases because they lack a glycine residue at position 2 that is required for *N*-myristoylation. It has recently been shown that the Golgi targeting of Arl3p requires its N-terminal acetylation by the NatC *N*-acetyltransferase and an integral Golgi membrane protein, Sys1p (Behnia et al., 2004; Gangi Setty et al., 2004). Moreover, hSys1, a human orthologue of yeast Sys1p, has been shown to control the Golgi localization of ARFRP1 (Behnia et al., 2004).

The functional importance of ARFRP1 is underscored by the finding that targeted disruption of the *Arfrp1* gene in mice resulted in embryonic lethality at the gastrulation stage and apoptosis of ectodermal cells (Mueller et al., 2002). Studies in yeast have shown that Arl3p is required for recruitment of Arl1p and the GRIP domain-containing protein, Imh1p/Sys3p, onto the Golgi (Gangi Setty et al., 2003; Panic et al., 2003b). However, the function of mammalian ARFRP1 has not been explored at the molecular level. Here, we demonstrate that ARFRP1 is required for recruitment of Arl1 and GRIP domain-containing proteins onto Golgi membranes and is involved in

the anterograde transport from the Golgi to the plasma membrane, as well as in the retrograde transport from endosomes to the Golgi.

Materials and Methods

Plasmids

The entire coding sequence of the human Arl1 or ARFRP1 cDNA was cloned into the mammalian expression vector pcDNA3 (Invitrogen) or its derivative, pcDNA3HAC, which has a sequence for a C-terminal HA-tag (Hosaka et al., 1996). For better exogenous expression, the Arl1 cDNA was subcloned into the modified mammalian expression vector, pcDNA4HisMaxHAC, which is a derivative of pcDNA4HisMax lacking the N-terminal Xpress-tag sequence and containing a C-terminal HA-tag sequence. The Arl1(T31N), Arl1(Q71L), ARFRP1(T31N) and ARFRP1(Q79L) mutants were generated using a Quick change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instruction. Vectors for EGFP-tagged vesicular stomatitis virus G protein (VSVG) tsO45 (Presley et al., 1997) and for FLAG-tagged TGN38 (Yoshimura et al., 2004) were kindly provided by Jennifer Lippincott-Schwartz (National Institutes of Health, USA), and Shin-ichiro Yoshimura and Nobuhiro Nakamura (Kanazawa University, Japan), respectively. The entire coding sequence for EGFP-VSVG tsO45 was transferred into pcDNA3 as described previously (Kasai et al., 1999).

Antibodies and reagents

Anti-Arl1 and anti-ARFRP1 antisera were raised in rabbits against a synthetic peptide, (C)DEAMEWLVETLKSRQ (Lowe et al., 1996) and (C)VVRNVHRPPRQRDIT, respectively, conjugated to keyhole limpet hemocyanin. The antisera were affinity-purified using the corresponding immunized peptide immobilized on Sulfolink beads (Pierce). Polyclonal rabbit antibodies against TGN46 (Kain et al., 1998) and golgin-97 (Yoshimura et al., 2004) were kindly provided by Minoru Fukuda (Burnham Institute, CA, USA), and Shin-ichiro Yoshimura and Nobuhiro Nakamura, respectively. Monoclonal mouse antibody against galactosyltransferase (GalT) (GTL2) was prepared as previously described (Kawano et al., 1994). Monoclonal mouse antibodies against γ-adaptin (100.3) and the FLAG epitope (M2) were purchased from Sigma and those against GFP (JL-8), GM130 (clone 35), p230/golgin-245 (clone 15), GGA3 (clone 8) and EEA1 (clone 14) from BD Biosciences. Monoclonal rat anti-HA antibody (3F10) was from Roche Diagnostics. Polyclonal rabbit antibody against β-COP and monoclonal mouse antibody against cation-independent mannose 6-phosphate receptor (CI-MPR) (2G11) were from Affinity Bioreagents. Alexa Fluor 488-conjugated secondary antibodies were from Molecular Probes, and Cy3-, Cy5- and peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Cy3-conjugated Shiga toxin 1 (Stx1) (Miyake et al., 2000; Shin et al., 2004) was a generous gift from Naoko Morinaga (Chiba University, Japan).

Cell culture, transfection and immunofluorescence analysis

HeLa cells were grown in minimum essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells grown to ~30% confluency were transfected with the indicated plasmids using a FuGENE6 reagent (Roche Diagnostics) according to the manufacturer's instruction. After 20 hours, the transfected cells were fixed with 3% paraformaldehyde and processed for immunofluorescence staining as described previously (Shin et al., 1997).

Experiments involving Stx1 internalization or antibody internalization were carried out in a manner similar to that described previously (Shin et al., 2004). Briefly, HeLa cells expressing HA-tagged ARFRP1 alone or in combination with FLAG-tagged TGN38 were grown on glass coverslips and incubated for 50 minutes with Cy3-conjugated Stx1 or with anti-FLAG M2 antibody at 19.5°C or on ice, respectively. The cells were then extensively washed with PBS and incubated at 37°C for the indicated periods of time in normal medium. The cells were then fixed and stained with monoclonal rat anti-HA antibody followed by Cy3-conjugated anti-rat and Alexa Fluor 488-conjugated anti-mouse secondary antibodies.

For the VSVG transport assay, vectors for HA-tagged ARFRP1 and GFP-tagged VSVG tsO45 were cotransfected into HeLa cells using the FuGENE6 reagent as described above. The cells were incubated at 40°C overnight, then shifted to 32°C and incubated for indicated periods of time. The cells were then processed for immunofluorescence staining as described above. The immunofluorescence staining was visualized using an LSM Pascal confocal microscope (Carl Zeiss) or an Axiovert 200 MAT microscope (Carl Zeiss).

Immunoblotting

Total cellular proteins were boiled in SDS electrophoresis sample buffer and separated by SDS-PAGE under reducing conditions. The separated proteins were electroblotted onto PVDF membrane and immunoblotting was carried out by the method described previously (Shin et al., 1999).

Immunoelectron microscopy

HeLa cells stably expressing GFP-CI-MPR (Waguri et al., 2003) were fixed with 4% paraformaldehyde–0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 minutes at 4°C. Procedures for immuno-gold labeling on cryo-thin sections were described previously (Waguri et al., 1999).

Biotinylation of cell surface VSVG

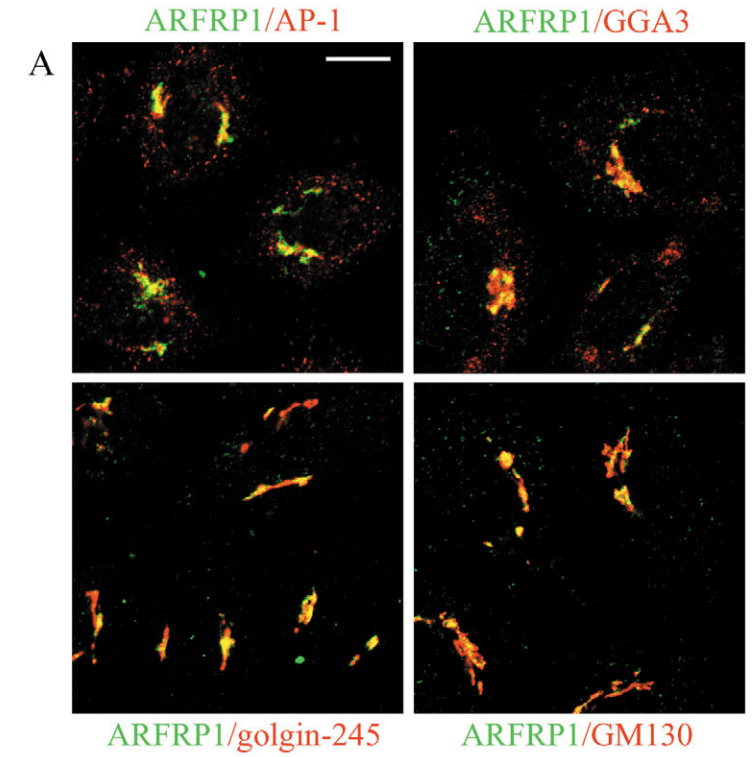
HeLa cells grown on a 10-cm dish were transfected with an expression vector for C-terminally HA-tagged ARFRP1(WT), ARFRP1(Q79L) or ARFRP1(T31N), or an empty vector in combination with that for GFP-tagged VSVG tsO45 using a FuGENE6 reagent, and incubated at 40°C overnight, then at 32°C for 1 hour. Cell surface proteins were biotinylated using modified protocols of Ying et al. (Ying et al., 2003) and Daniels and Amara (Daniels and Amara, 1998). Briefly, cells were washed three times with ice-cold PBS(+) (containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH 7.4) and incubated with 1 mg/ml Sulfo-NHS-LC-biotin (Pierce) in PBS(+) for 30 minutes on ice to biotinylate cell surface proteins. The reaction was quenched by washing the dish twice with ice-cold PBS(+) containing 100 mM glycine and 0.3% (w/v) bovine serum albumin, and subsequently washed twice with PBS(+). The cells were then lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100) containing a Complete™ protease inhibitor mixture (Roche). The lysates were centrifuged at maximum speed for 20 minutes at 4°C in a microcentrifuge to remove cellular debris and insoluble materials. The supernatant was incubated with streptavidin-agarose beads (Pierce) overnight at 4°C with constant rotation. The beads were washed three times with lysis buffer, twice with high-salt wash buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Triton X-100) containing Complete™ and once with 50 mM Tris-HCl, pH 7.5. The proteins were eluted from the beads by boiling in 30 μl of SDS sample buffer, separated by 10% SDS-PAGE, and analyzed by immunoblotting as described previously (Shin et al., 1999). The intensity of resulting bands was quantified using Image Gauge software (LAS-3000, Fuji Photo Film).

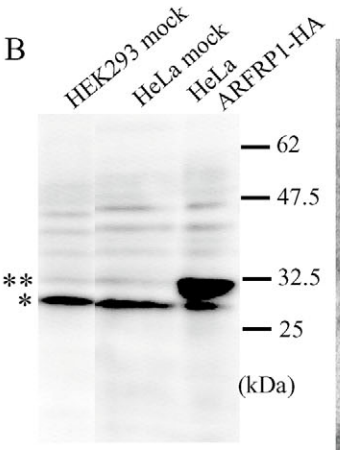
Results

Localization of ARFRP1 to the trans side of the Golgi complex

Although previous studies have indicated that ARFRP1 is associated with the Golgi and the plasma membrane (Behnia et al., 2004; Schurmann et al., 1995), the exact subcellular localization of ARFRP1 has not been determined. To determine the localization of endogenous ARFRP1, we raised antibodies against ARFRP1 (see Materials and Methods). In immunoblot analysis, the affinity-purified antibodies specifically detected endogenous ARFRP1 and exogenously expressed ARFRP1-HA in cell lysates (Fig. 1B), whereas no specific band was detected with preimmune serum (data not shown). The bands disappeared when the antigen peptide was included in the blot incubation with the antibodies (data not shown), indicating that the antibodies specifically recognize ARFRP1. The localization of endogenous ARFRP1 overlapped with GM130 (a cis/medial-Golgi marker), golgin-245 (a trans-Golgi and TGN marker) and the γ-adaptin subunit of the AP-1 complex and GGA3 (TGN markers) in the perinuclear region (Fig. 1A).

To substantiate the immunofluorescence data, we then analyzed the ARFRP1 localization by immunoelectron microscopy (Fig. 1C). We used HeLa cells stably expressing a GFP-tagged CI-MPR construct, GFP-CI-MPR, because the fusion protein mainly localizes in the TGN (Waguri et al., 2003). When cryo-thin sections from the cells were double-labeled with anti-ARFRP1 and anti-GFP antibodies, ARFRP1





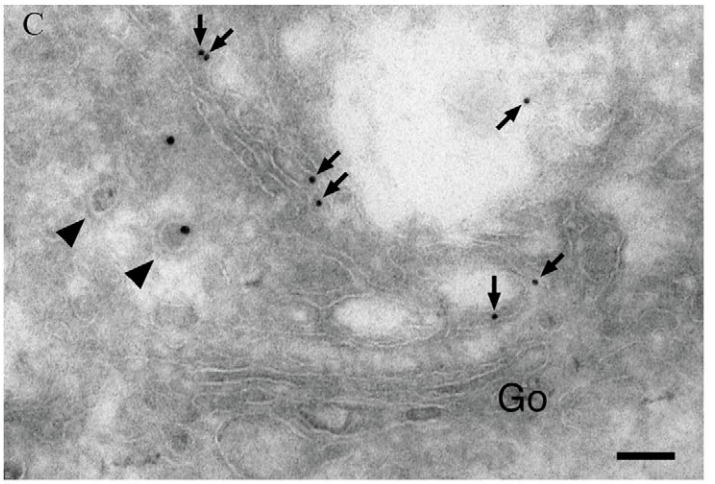


Fig. 1. Localization of endogenous ARFRP1. (A) HeLa cells were fixed and double-stained for ARFRP1 and either the AP-1 γ-adaptin subunit, GGA3, golgin-245 or GM130 followed by Cy3-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG. Bar, 20 μm. (B) Immunoblot analysis for ARFRP1 in mock transfected HEK293 and HeLa cells, and HeLa cells transfected with HA-tagged ARFRP1. An asterisk indicates the positions of endogenous ARFRP1 and a double asterisk indicates exogenously expressed ARFRP1-HA. (C) Cryo-thin sections of HeLa cells expressing GFP-CI-MPR were double-labeled with mouse anti-GFP and rabbit anti-ARFRP1 (arrows) antibodies, which were then detected by two secondary antibodies conjugated with 15 nm- and 10 nm-colloidal gold particles, respectively. Arrowheads indicate two clathrin-coated vesicles, one of which contains a GFP-CI-MPR signal. Go, Golgi stack. Bar, 0.1 μm.

(arrows) was detected preferentially on vesicular-tubular membrane profiles on one side of the Golgi where the GFP-CI-MPR-positive structures and clathrin-coated vesicles (arrowheads) were found. This result indicates that ARFRP1 is localized on the trans side of the Golgi complex and the TGN.

In immunofluorescence analyses, although the anti-ARFRP1 antibodies sometimes stained some punctate structures throughout the cytoplasm, which disappeared by co-incubation of the antibodies with the antigen peptide, they did not significantly overlap with punctate endosomal structures positive for the AP-1 complex (γ-adaptin; Fig. 1A) or with those for other endosomal markers, including Lamp-1 (late endosome/lysosome) or EEA1 (early endosome) (see Fig. S1 in supplementary material). Because our attempts to identify the ARFRP1-positive endosome-like structures by immunoelectron microscopy were unsuccessful, we did not address this issue further.

We then examined the effects of brefeldin A (BFA) on the membrane association of the endogenous ARFRP1. This drug is an inhibitor of guanine-nucleotide exchange factors for ARFs and has been a useful tool in studying membrane trafficking (Jackson, 2000; Klausner et al., 1992). ARFs and coat proteins, including the AP-1 complex, dissociate from Golgi membranes within 2 minutes of BFA treatment, whereas dissociation of Arl1 and its effectors, golgin-97 and golgin-245, requires more than 5 minutes of treatment (Gleeson et al., 1996; Lowe et al., 1996; Lu and Hong, 2003; Van Valkenburgh et al., 2001). As shown in Fig. 2, the majority of ARFRP1 and golgin-245 remained associated with Golgi membranes even after 5 minutes of BFA treatment, although AP-1 dissociated within 2 minutes. After 10 minutes treatment, ARFRP1 was still associated with the Golgi in some populations of cells. Notably, golgin-245 also remained associated with the ARFRP1-positive Golgi-like structures in these cells. Thus, in response to BFA treatment, ARFRP1 dissociates from the Golgi apparatus with kinetics similar to those of golgins and Arl1 (Gleeson et al., 1996; Lowe et al., 1996; Lu and Hong, 2003; Van Valkenburgh et al., 2001), suggesting a functional coupling of these proteins.

Expression of a GDP-bound mutant of ARFRP1 causes mislocalization of a subset of TGN proteins

To explore the potential role of ARFRP1 in regulating Golgi structure and function, we generated mutants of ARFRP1 that were expected to exist preferentially in either a GDP- or a GTP-bound state. Such mutants were made in a similar manner to mutants of other Ras-like small GTPases, such as Rabs and ARFs (Chavrier and Goud, 1999; Dascher and Balch, 1994; Zhang et al., 1994). ARFRP1(T31N) is expected to be restricted to the GDP-bound form and to function as a dominant-negative mutant. However, ARFRP1(Q79L) is expected to be restricted mainly to the GTP-bound active form, owing to impaired GTPase activity. At moderate levels of expression in HeLa cells, the ARFRP1(Q79L) mutant was associated predominantly with Golgi membranes, and was found to a lesser extent in the cytoplasm than was ARFRP1(WT) (see Fig. 4A), whereas ARFRP1(T31N) was mostly cytoplasmic (see Fig. 4B,C).

To investigate the function of ARFRP1 in the Golgi, we examined whether overexpression of ARFRP1(T31N) affected the localization of several Golgi marker proteins. It was shown that the overexpression of ARFRP1(T31N) causes the disappearance of the TGN localizing proteins, such as GRIP domain-containing golgins, golgin-97 and golgin-245 and TGN46 but not the cis/medial Golgi marker GM130 (Behnia

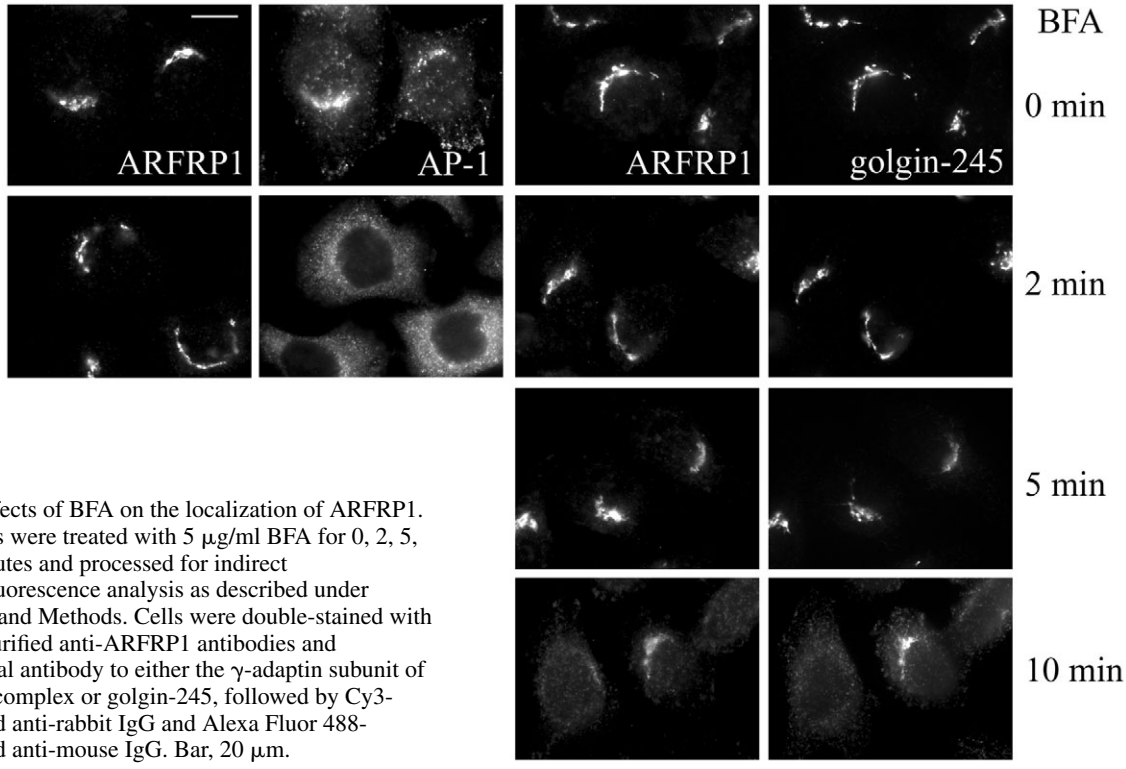


Fig. 2. Effects of BFA on the localization of ARFRP1. HeLa cells were treated with 5 μg/ml BFA for 0, 2, 5, or 10 minutes and processed for indirect immunofluorescence analysis as described under Materials and Methods. Cells were double-stained with affinity-purified anti-ARFRP1 antibodies and monoclonal antibody to either the γ-adaptin subunit of the AP-1 complex or golgin-245, followed by Cy3-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG. Bar, 20 μm.

et al., 2004) (Fig. 3A,B and Fig. S2 in supplementary material). In particular, TGN46 appeared to accumulate in peripheral punctate structures reminiscent of endosomes (Fig. 3B). By contrast, the localization of the trans-Golgi marker, GalT (Fig. 3C), was unaffected by the ARFRP1(T31N) overexpression. More intriguingly, the overexpression of ARFRP1(T31N) affected the TGN localization of AP-1 and CI-MPR (Fig. S2 in supplementary material) but not that of GGA3 (Fig. 3D). Thus, it seems likely that ARFRP1(T31N) does not disrupt the TGN structure itself but does affect the localization of a subset of TGN-localizing proteins. However, association of AP-1 with early/recycling endosomes or association of CI-MPR with late endosomes was not affected by the ARFRP1(T31N) overexpression (Fig. S2 in supplementary material). Furthermore, neither EEA1 (an early endosomal marker) nor Lamp-1 (a late endosome/lysosome marker) was mislocalized by the ARFRP1(T31N) overexpression (our unpublished data). In contrast to ARFRP1(T31N), ARFRP1(Q79L) affected the localization of none of the cis-, medial- or trans-Golgi proteins, or TGN proteins examined (our unpublished data).

ARFRP1 functions upstream of Arl1 and GRIP proteins

Since ARFRP1(Q79L) does not bind directly to the GRIP domain (our unpublished data) but GTP-bound Arl1 does bind to it (Gangi Setty et al., 2003; Lu and Hong, 2003; Panic et al., 2003a; Wu et al., 2004), we examined whether ARFRP1 is required for the Golgi localization of Arl1 and GRIP domain-containing proteins. As expected, upon moderate levels of ARFRP1(T31N) expression, both endogenous Arl1 and golgin-97 disappeared from the Golgi region (Fig. 3A and Fig. S2E in supplementary material). By contrast, similar levels of expression of ARFRP1(WT) or ARFRP1(Q79L) did not affect the localization of Arl1, golgin-97 or golgin-245 (our unpublished data).

Recent studies in yeast have indicated that Arl3p functions upstream of Arl1p (Behnia et al., 2004; Gangi Setty et al., 2004). We reasoned that, if this was also the case in mammalian cells, Arl1(Q71L) could restore mislocalization of golgins induced by the ARFRP1(T31N) expression. As a control, the ARFRP1(Q79L) overexpression did not affect the Golgi localization of exogenously expressed Arl1(WT) or endogenous golgin-97 (Fig. 4A), whereas ARFRP1(T31N) released Arl1(WT) and golgin-97 from Golgi membranes (Fig. 4B). By striking contrast, when Arl1(Q71L) was coexpressed with ARFRP1(T31N), not only the exogenously expressed Arl1 mutant but also endogenous golgin-97 remained associated with Golgi membranes in spite of the ARFRP1(T31N) overexpression (Fig. 4C). These observations together indicate that ARFRP1 is an upstream regulator for the targeting of Arl1 and golgins onto Golgi membranes.

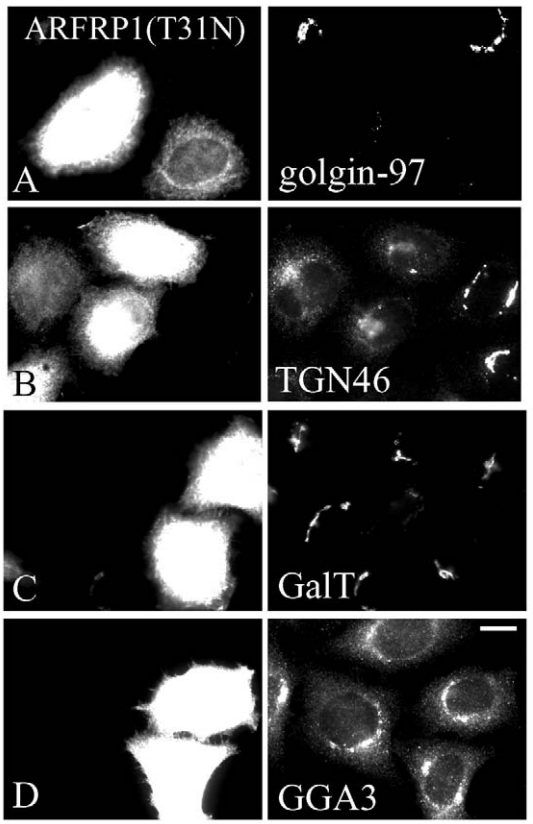


Fig. 3. Localization of Golgi proteins in cells overexpressing ARFRP1(T31N). HeLa cells expressing HA-tagged ARFRP1(T31N) (A-D; left column) were fixed and double-stained (right column) for HA and either golgin-97 (A), TGN46 (B), galactosyl transferase (GalT; C) or GGA3 (D) followed by Cy3-conjugated anti-rat IgG and Alexa Fluor 488-conjugated anti-rabbit IgG (A,B) or anti-mouse IgG (C,D). Bar, 20 μm.

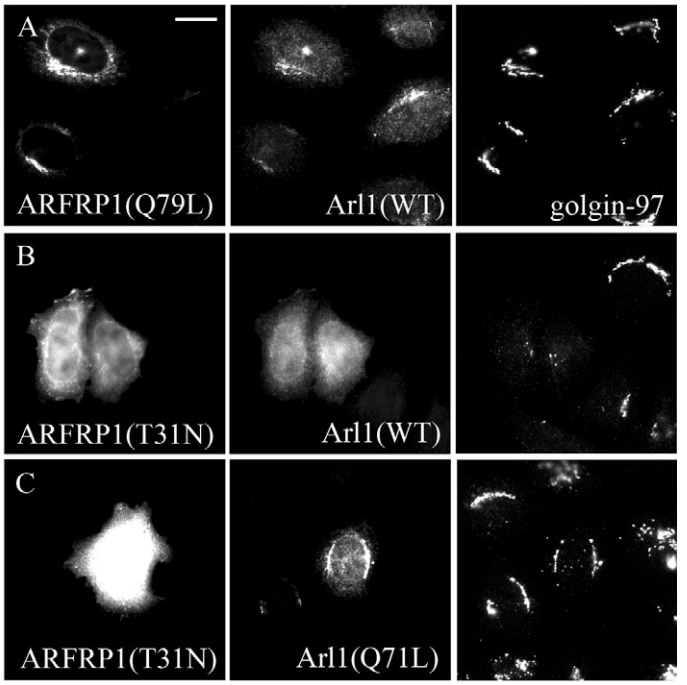


Fig. 4. Arl1(Q71L) suppresses redistribution of golgin-97 induced by ARFRP1(T31N) expression. HeLa cells co-expressing a combination of either HA-tagged Arl1(WT) (A,B) or Arl1(Q79L) (C) and either FLAG-tagged ARFRP1(Q79L) (A) or ARFRP1(T31N) (B,C) were triple-stained for HA (middle column), FLAG (left column) and golgin-97 (right column) followed by Alexa Fluor 488-conjugated anti-rat IgG, Cy3-conjugated anti-mouse IgG and Cy5-conjugated anti-rabbit IgG. Bar, 20 μm.

Involvement of ARFRP1 in the endosome-to-Golgi retrograde transport

Affinity chromatography of yeast cytosol with immobilized Arl1p-GTP has revealed its interaction with the GARP/VFT complex (Panic et al., 2003b), which is implicated in the retrograde transport from endosomes back to the late Golgi (Conibear and Stevens, 2000).

As described above, TGN46 appeared to accumulate in peripheral punctate structures, as it disappeared from the TGN region upon the ARFRP1(T31N) overexpression. Rat TGN38, an orthologue of human TGN46, is known to cycle between the TGN and the plasma membrane at a very low rate (Ladinsky and Howell, 1993; Molloy et al., 1994; Reaves et al., 1993). Therefore, we speculated that the redistribution of TGN38/TGN46 induced by ARFRP1(T31N) also resulted from a block in its retrograde transport. To address this issue, we performed an antibody uptake experiment. Namely, exoplasmically FLAG-tagged TGN38 was coexpressed with either ARFRP1(WT), ARFRP1(Q79L) or ARFRP1(T31N) in HeLa cells, and its retrograde transport was analyzed by following extracellularly applied anti-FLAG antibody (Fig. 5). In cells overexpressing ARFRP1(WT) or ARFRP1(Q79L), a large fraction of the anti-FLAG antibody internalized for 1 hour was found to reach the Golgi as evident from its significant overlap with β-COP (Fig. 5A, upper and middle panels, respectively). By marked contrast, in cells overexpressing ARFRP1(T31N), the internalized antibody was barely detectable in the Golgi region and was found to associate predominantly with punctate endosome-like structures (Fig. 5A, lower panels). We quantified the immunofluorescence results by estimating the percentage ratio of the overlapping area of β-COP and anti-FLAG antibodies versus the total β-COP-positive Golgi area. As shown in Fig. 5B, the overlapping area of anti-FLAG and anti-β-COP antibodies was only 12% in cells overexpressing ARFRP1(T31N), whereas it was over 40% in cells overexpressing ARFRP1(WT) or ARFRP1(Q79L). Thus, the disappearance of TGN38/TGN46 from the TGN by the ARFRP1(T31N) overexpression appears to be due, at least in part, to a block in its retrograde transport from endosomes.

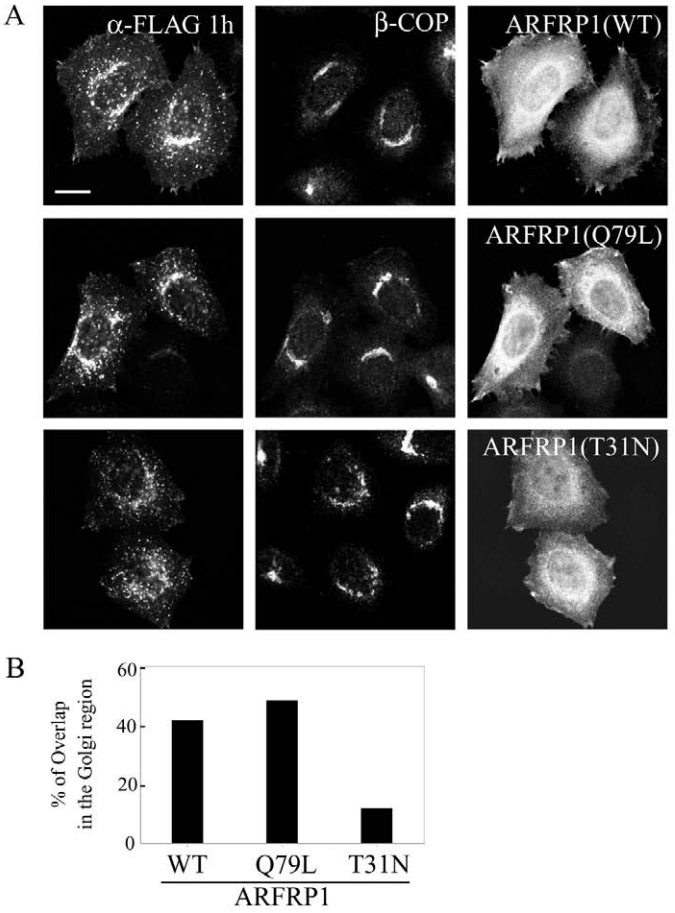


Fig. 5. Retrograde transport of TGN38 in cells expressing ARFRP1 mutants. (A) HeLa cells co-expressing FLAG-TGN38 and either HA-ARFRP1(WT), HA-ARFRP1(Q79L) or HA-ARFRP1(T31N) were incubated with mouse monoclonal anti-FLAG M2 antibody on ice for 50 minutes, washed, and further incubated at 37°C for 60 minutes. The cells were then fixed and stained with rat anti-HA and rabbit anti-β-COP antibodies followed by Alexa Fluor 488-conjugated anti-rat, Cy3-conjugated anti-mouse and Cy5-conjugated anti-rabbit secondary antibodies. Bar, 20 μm. (B) The efficiency of the retrograde transport of TGN38 was estimated by calculating the ratio of the perinuclear overlapped area of the TGN38- and β-COP-positive structures versus the total area of the perinuclear β-COP-positive structures. In each case, the areas were estimated for over 10 cells.

We then examined whether ARFRP1 is involved in the retrograde transport of Shiga toxin (Stx) from endosomes to the TGN, because an in vitro study suggested that Arl1 and GRIP domain proteins are implicated in the retrograde transport of the Stx B fragment from endosomes to the Golgi complex (Lu et al., 2004) and because TGN38 and Stx1 transit the same compartment, early/recycling endosomes, en route to the Golgi complex (Mallet and Maxfield, 1999; Shin et al., 2004).

To this end, HeLa cells overexpressing ARFRP1(T31N) or ARFRP1(Q79L) were first incubated with Cy3-conjugated Stx1 at 19.5°C for 50 minutes to accumulate the fluorescent toxin at endosomes, then chased at 37°C for 60 minutes to allow its further transport to the Golgi. As shown in Fig. 6, the transport of Stx1 from endosomes to the Golgi complex was significantly blocked in ARFRP1(T31N)-expressing cells but not in ARFRP1(Q79L)-expressing cells (compare cells expressing the ARFRP1 mutant with non-transfected cells surrounding them). This was because a significant fraction of Stx1 became colocalized with GalT after 60 minutes chase at 37°C in cells overexpressing ARFRP1(Q79L), but most of the fraction remained associated with punctate endosomal structures throughout the cytoplasm in the ARFRP1(T31N)-overexpressing cells. Taken together with the FLAG-TGN38 and Stx1 internalization data, it seems likely that ARFRP1 regulates the endosome-to-TGN retrograde transport by functioning upstream of Arl1 and GRIP domain-containing proteins.

Inhibition of anterograde transport from the TGN by ARFRP1(T31N)

Arl1 and GRIP domain-containing proteins have also been suggested to participate in the anterograde transport of VSVG from the Golgi complex to the plasma membrane (Lu et al., 2001; Yoshino et al., 2003). Therefore, we next examined the

effects of the ARFRP1 mutants on VSVG transport. For this purpose, cells expressing a combination of GFP-tagged VSVG tsO45 and either ARFRP1(WT), ARFRP1(Q79L) or ARFRP1(T31N) were incubated at 40°C overnight, to accumulate the temperature-sensitive mutant of VSVG in the endoplasmic reticulum, then incubated at 32°C for 30 minutes or 1 hour to chase the VSVG transport. After 30 minutes chase at 32°C, VSVG reached the Golgi in cells overexpressing ARFRP1(WT), ARFRP1(Q79L) or ARFRP1(T31N) (Fig. 7, left panels). Thus, transport of VSVG from the endoplasmic reticulum to the Golgi appeared to be unaffected by the overexpression of wild-type or mutant ARFRP1. After a 1-hour chase, a certain population of VSVG was transported to the cell surface in cells overexpressing ARFRP1(WT) or ARFRP1(Q79L) (Fig. 7A, right upper and middle panels, respectively). By striking contrast, transport of VSVG from the Golgi to the cell surface was significantly inhibited by ARFRP1(T31N) overexpression; even after the 1-hour chase, the majority of VSVG-GFP was retained in the Golgi and other intracellular structures (see below).

The efficiency of the VSVG transport to the cell surface was estimated by biotinylation of surface proteins. HeLa cells were cotransfected with VSVG-GFP and either HA-tagged ARFRP1(WT), ARFRP1(Q79L) or ARFRP1(T31N) and incubated overnight at 40°C followed by incubation at 32°C for 1 hour. After biotinylation of surface proteins, the biotinylated proteins in the cell lysates were recovered with streptavidin-agarose beads and subjected to immunoblotting with an anti-GFP antibody to detect VSVG-GFP (Fig. 7B, top panel). The total cell lysates were also analyzed by immunoblotting with anti-GFP and anti-HA antibodies to confirm the expression levels of VSVG-GFP (middle panel) and wild-type and mutant ARFRP1-HA (bottom panel), respectively. Despite the fact that the amount of biotinylated VSVG was not significantly changed in cells expressing ARFRP1(WT) or ARFRP1(Q79L) as compared with mock transfected cells (–), it decreased by ~50% in cells expressing ARFRP1(T31N) (Fig. 7B,C). It thus appears that active ARFRP1 is implicated in the anterograde transport from the TGN as well as in the retrograde transport to this compartment.

Intriguingly, in the majority of cells expressing ARFRP1(T31N), tubulovesicular structures carrying VSVG were often found to accumulate (Fig. 7A, insets 1 and 2). This observation makes it probable that the formation of vesicular-tubular carriers for VSVG from the TGN is not disturbed by ARFRP1(T31N) overexpression but rather their targeting to or fusion with the plasma membrane is inhibited (see Discussion).

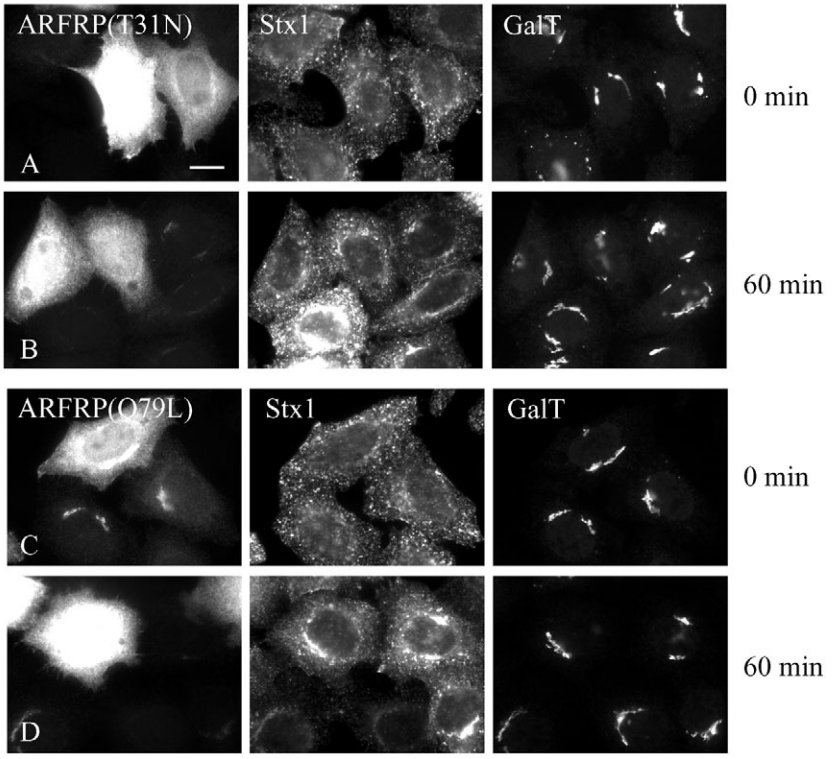


Fig. 6. Stx1 transport in cells expressing ARFRP1 mutants. HeLa cells expressing HA-tagged ARFRP1(T31N) (A,B) or ARFRP1(Q79L) (C,D) were incubated with Cy3-conjugated Stx1 at 19.5°C for 50 minutes, washed, and directly fixed (A,C) or shifted to 37°C for 60 minutes (B,D) then fixed, and stained with rat anti-HA and mouse anti-GalT antibodies followed by Alexa Fluor 488-conjugated anti-rat and Cy5-conjugated anti-mouse IgGs. Bar, 20 μm.

Discussion

On the basis of the data presented here, we are able to draw the following three conclusions as to the biological properties of ARFRP1. First, ARFRP1 is localized predominantly on the trans-side of the Golgi complex or the TGN. Secondly, ARFRP1 is required for recruitment of Arl1 and GRIP domain-containing proteins, which are effectors of Arl1, to Golgi membranes. Finally, ARFRP1 plays roles in the TGN-to-plasma membrane transport as well as in the endosome-to-TGN transport as an upstream regulator of Arl1 and the GRIP proteins. To our knowledge, this is the first report showing evidence for a cellular function of ARFRP1 and its functional link to Arl1 and GRIP domain proteins in mammalian cells.

Our immunofluorescence and immunoelectron microscopic analyses have shown that ARFRP1 associates mainly with the trans-Golgi and the TGN. Overexpression of the ARFRP1(T31N) mutant causes redistribution of some TGN-localizing proteins, such as golgin-97, golgin-245, AP-1 and TGN46, supporting the role of ARFRP1 in the trans-Golgi/TGN. However, the ARFRP1 mutant appears not to affect the Golgi structure itself. Furthermore, our data indicate that ARFRP1 functions upstream of Arl1 and GRIP proteins. First, the ARFRP1(T31N) overexpression causes redistribution of not only Arl1 but also golgin-97 and golgin-245 from the Golgi region. Second, the dissociation of golgin-97 from Golgi membranes induced by the ARFRP1(T31N) overexpression can be suppressed by simultaneous overexpression of Arl1(Q71L). These observations are in good agreement with the previous data in yeast showing that Arl3p is required for the recruitment onto the Golgi of Arl1p and Imh1p/Sys3p, the

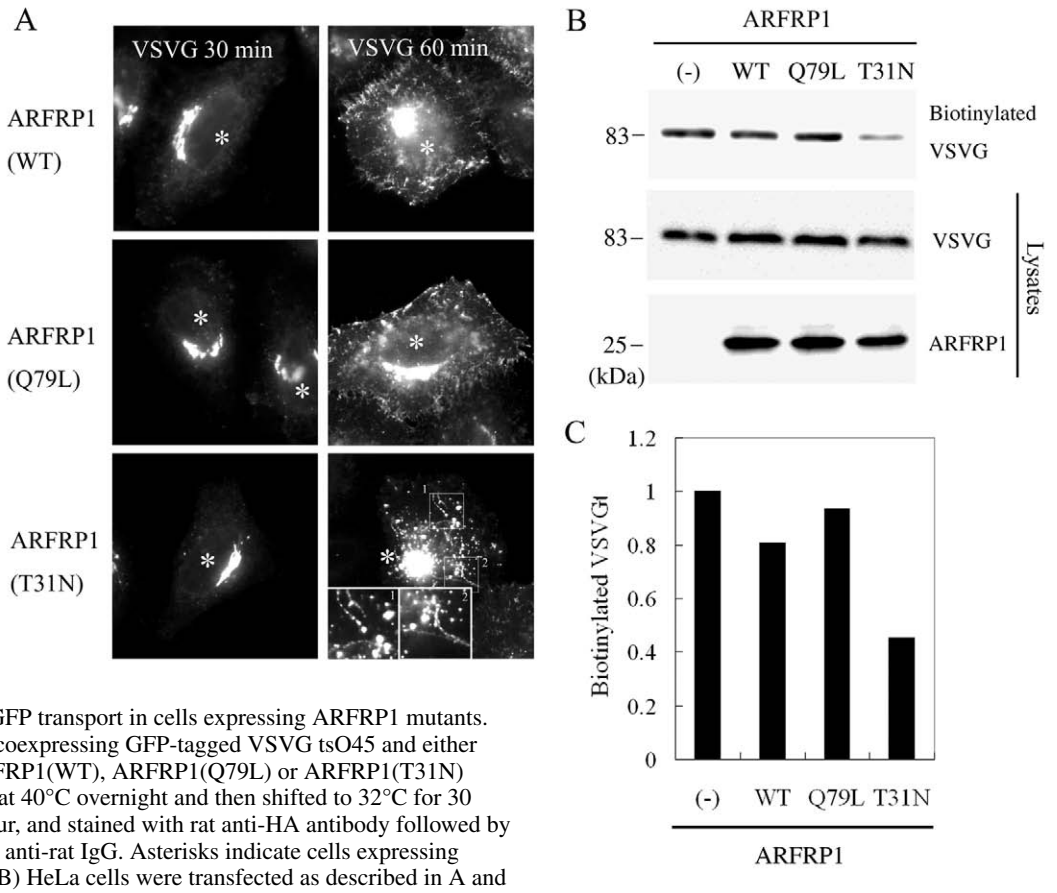


Fig. 7. VSVG-GFP transport in cells expressing ARFRP1 mutants. (A) HeLa cells coexpressing GFP-tagged VSVG tsO45 and either HA-tagged ARFRP1(WT), ARFRP1(Q79L) or ARFRP1(T31N) were incubated at 40°C overnight and then shifted to 32°C for 30 minutes or 1 hour, and stained with rat anti-HA antibody followed by Cy3-conjugated anti-rat IgG. Asterisks indicate cells expressing ARFRP1-HA. (B) HeLa cells were transfected as described in A and incubated at 40°C overnight and then shifted to 32°C for 1 hour. The cell surface proteins were then biotinylated as described in Materials and Methods. Biotinylated surface proteins recovered with streptavidin-agarose beads (top panel) and total cell lysates (one twentieth amount of input; middle and bottom panels) were subjected to immunoblotting with anti-GFP (top and middle panels) or anti-HA antibody (bottom panel). (C) The band intensity in the top panel in B was estimated by Image Gauge software. This is representative of two independent experiments.

sole yeast GRIP protein (Gangi Setty et al., 2003; Panic et al., 2003b).

We have also shown that overexpression of ARFRP1(T31N), but not that of ARFRP1(Q79L), results in a block in retrograde transport of TGN38 and Stx1 from the cell surface to the Golgi and causes their accumulation at punctate endosome-like structures. Moreover, endogenous TGN46 is redistributed to the peripheral punctate structures upon ARFRP1(T31N) expression (Fig. 3B). These observations are in line with recent studies suggesting that GRIP domain-containing golgins and Arl1 participate in trafficking from endosomes to the TGN (Lu et al., 2004; Yoshino et al., 2003) and with previous yeast data indicating that Arl1p genetically and biochemically interact with components of protein machineries that function in the endosome-to-late Golgi trafficking pathway, such as the Ric1p/Rgp1p complex, Ypt6p and the GARP/VFT complex (Bensen et al., 2001; Panic et al., 2003b). GRIP domain-containing golgins have been suggested to be tethering molecules on trans-Golgi/TGN membranes (Barr and Short, 2003; Gillingham and Munro, 2003; Lu and Hong, 2003; Lu et al., 2004) in mammalian cells. Furthermore, Imh1p/Sys3p, the sole GRIP domain-containing golgin in yeast, has been suggested to function in vesicle docking in the endosome-to-late Golgi retrograde trafficking (Tsukada et al., 1999). Taken together, it seems likely that, by functioning upstream of Arl1 and golgins, ARFRP1 regulates the transport of carrier intermediates from endosomes to the TGN.

In addition to its effect on retrograde transport to the TGN, we have found that ARFRP1(T31N) inhibits the anterograde transport of VSVG from the TGN. In agreement with our finding, previous studies have indicated that overexpression of the Arl1(Q71L) mutant and that of the GRIP domain from golgin-245 perturb the TGN-to-plasma membrane trafficking of VSVG (Lu et al., 2001) (Yoshino et al., 2003). Altogether, ARFRP1, Arl1 and GRIP domain-containing golgins may have a role in the TGN-to-plasma membrane transport of VSVG. Data obtained from previous yeast studies are compatible with the roles of ARFRP1 and Arl1 in the anterograde transport from the TGN; namely, a temperature sensitive *arl3* mutant and a *arl3* null mutant showed enhanced cold sensitivity of cell growth and retarded processing of alkaline phosphatase and carboxypeptidase Y (CPY) (Bonangelino et al., 2002; Huang et al., 1999), and deletion of the *ARL1* gene also results in mis-sorting of CPY (Bonangelino et al., 2002).

However, the defect in the anterograde transport might be a secondary effect of depletion of some essential factors from the TGN. For example, the mis-sorting of CPY often results from the impaired recycling to the late Golgi of the CPY receptor,

Vps10p (Cereghino et al., 1995; Conibear et al., 2003; Conibear and Stevens, 2000). Defects in recycling of some SNAREs could also cause a block in the anterograde transport from the TGN, because it has been shown that exocytic v-SNAREs, such as Snc1p and Snc2p, cycle between the Golgi and plasma membrane (Grote et al., 2000; Gurunathan et al., 2000; Lewis et al., 2000; Salem et al., 1998). In this context, it is interesting to note that we have found an accumulation of VSVG in tubulovesicular structures in the ARFRP1(T31N)-expressing cells (Fig. 7). Taken together, it is tempting to speculate that the block in the VSVG transport from the TGN by ARFRP1(T31N) is not due to a defect in the formation of carrier intermediates but rather to a defect in their targeting to or fusion with the plasma membrane.

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