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The distribution and translocation of the G protein ADP-Ribosylation Factor 1 in live cells is determined by its GTPase activity

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SUMMARY

ADP-ribosylation factors (ARF) are small G proteins that play key roles in vesicular transport processes. We have studied the distribution of ARF1 in live cells using chimeras of ARF1 mutants (wild type (wt) ARF1; Q71L-ARF1 (reduced GTPase); T31N (low affinity for GTP); and Δ^{N}wt (deletion of amino acids 2-18)) with green fluorescent protein (GFP). Confocal microscopy studies showed that the wt and Q71L proteins were localized in the Golgi and cytoplasm. The Δ^{N}wt and the T31N mutants were exclusively cytoplasmic. The behavior of the wt and Q71L proteins was studied in detail. About 15% of wt-ARF1-GFP was bound to the Golgi. Bound wt-ARF1-GFP dissociated rapidly after addition of Brefeldin A (BFA). This process did not appear to be a consequence of BFA-induced disappearance of the Golgi. Photobleaching recovery showed that essentially all the ARF-GFP was mobile, although it diffused very slowly. In contrast, about 40-50% of the Q71L mutant was found in the Golgi, and its rate of dissociation in the presence of BFA was slow and biphasic. Q71L-ARF1-GFP diffused more slowly than the wt. We conclude that ARF1 proteins exist in a dynamic equilibrium between Golgi-bound and cytosolic pools, and that the translocation of ARF in live cells requires the hydrolysis of GTP by the Golgi-bound protein.

Key words: ADP-ribosylation factor, ARF, Green fluorescent protein, FRAP, Brefeldin A

INTRODUCTION

G proteins are activated by the binding of GTP and are inactivated by the hydrolysis of the bound GTP to GDP. Many in vitro and cell-free studies on the kinetics of GTP binding and hydrolysis have been carried out to characterize the mechanisms by which these proteins are regulated (Gibbs et al., 1984; Florio and Sternweis, 1985; Brandt and Ross, 1985, 1986; Neal et al., 1988; Weiss et al., 1989). However, it has been very difficult to study the state of activation and the actual rate of GTP hydrolysis of individual G proteins in intact cells. The binding of GTP to Ras has been studied in depth thanks to the existence of an inactivating antibody that blocks Ras GTPase activity, which allows the isolation of the GTP-bound species to determine the state of activation of the protein (Gibbs et al., 1984; Hattori et al., 1987). No antibodies of these characteristics are available for other members of the Ras family.

The ADP-ribosylation factors are a family of small GTP-binding proteins of molecular weight of about 20 kDa originally discovered because of their property to act as a cofactors for the ADP-ribosylation of Gs by cholera toxin (Kahn and Gilman, 1984). Six different, highly conserved ARF genes have been identified in mammals (Kahn et al., 1991; Tsuchiya et al., 1991). Several interesting biological roles for ARF have been proposed, including the regulation of exocytosis, endocytosis, ER-to-Golgi transport, integrity of the Golgi structure (see the reviews of Boman and Kahn, 1995, and Moss and Vaughan, 1995) and the regulation of phospholipase D activity (Brown et al., 1993). An important characteristic of most ARF proteins is that the inactive, GDP-bound protein is soluble whereas the activated, GTP-bound protein is membrane bound (Regazzi et al., 1991; Helms et al., 1993; Houle et al., 1995; Cavenagh et al., 1996). Changes in the subcellular localization of ARF proteins correlate with changes in their state of activation (Rumenapp et al., 1995; Shome et al., 1997; Karnam et al., 1997). Therefore, changes in the subcellular distribution of ARF proteins are likely to reflect changes in the state of activation of the protein.

In order to study the dynamics and translocation of ARF proteins in live cells, we have made fusion DNA constructs of wt and several ARF1 mutants, namely Q71L, T31N and a deletion mutant lacking amino acids 2-18 of the N terminus (Δ^{N}wt) with a red-shifted variant of GFP (Yang et al., 1996). These ARF1 mutants were selected on the basis of their functional properties. The GTPase activity of Q71L-ARF1 is significantly reduced such that the protein behaves partially as a constitutively activated mutant (Tanigawa et al., 1993; Teal

et al., 1994, Zhang et al., 1994). The T31N mutant has very low affinity for GTP and has been shown to behave as a constitutively inactive or dominant negative mutant in in vitro assays (Dascher and Balch, 1994). Finally, the Δ^Nwt mutant is missing an important N-terminal sequence (Kahn et al., 1992) and cannot be N-myristoylated. Using confocal laser scanning microscopy to visualize these fusion constructs, we have studied the intracellular localization and dynamics of the translocation of ARF1 after photobleaching and after the addition of brefeldin A (BFA). Our studies demonstrate that ARF1 exists in an equilibrium between cytosolic and Golgi-bound pools and that the rate-limiting step for the redistribution of ARF1 is the hydrolysis of bound GTP. Using the dissociation of membrane-bound ARF1 as the main parameter, we have calculated the rate of GTP hydrolysis by ARF1 and the steady-state concentrations of active, GTP-bound, and inactive, GDP-bound, ARF1 in intact cells.

MATERIALS AND METHODS

Construction of ARF1-GFP fusion constructs

The cDNAs for human wt-ARF1 and Q71L-ARF1 were generously supplied by Dr Richard Kahn (Emory University). These cDNAs were amplified by PCR using 5′-AC*GAATTC*TCCACCTGTCCACAAGCA-3′ and 5′-AT*GGATCC*TTCCGGAGCTGATTGG-3′ as the 5′ (*Eco*RI site) and 3′ (*Bam*HI site) primers, respectively. T31N-ARF1 was generated from wtARF1 by sequential PCR using 5′-GT*GAATTC*TCCACCTGTCCACAAGC-3′ and 5′-AT*GGATCC*TCGTTCACTTCTGGT-3′ as the 5′ and 3′ end primers, respectively, and 5′-TCGTG*T*TCTTCCCCTGCAGCAT-3′ and 5′-GCTGCAGGGAAGAACACGAT-3′ as the two bridging primers. The Δ^Nwt-ARF1 mutant was generated from wt-ARF1 cDNA using 5′-GC*GAATTC*ATGATCCTCATGGTGGGC-3′ (*Eco*RI site) as the 5′-end primer. The various purified human ARF1 PCR products were digested with *Eco*RI and *Bam*HI and subcloned in the pEGFPN1 vector (Clontech). *E. coli* (DH5-α) cells were transformed with the ligated vector and plated onto LB agar plates containing kanamycin. Plasmid DNA was prepared from these bacteria. The existence of the fusion DNA construct was confirmed by agarose gel electrophoresis of uncut and double digested (*Bam*HI and *Eco*RI) purified DNA. All constructs were verified by sequencing.

Expression of various ARF1-GFP fusion proteins in mammalian cells

Rat fibroblasts overexpressing the human insulin receptor (HIRcB cells) were used to express the various ARF1-GFP chimeras. HIRcB cells were routinely grown on coverslips (31 mm diameter) in 35 mm dishes in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum. After overnight culture, medium was removed and cells were washed once with serum-free DMEM/F12. Cells were transfected in fresh, serum-free DMEM/F12 (1 ml) containing 10 μl/ml lipofectamine and 0.5 μg/ml of the appropriate DNA. 6 hours after the addition of DNA, an additional 1 ml media containing 20% FBS was added to the plates. Cells were used 24-48 hours after transfection.

Western blotting of ARF1-GFP fusion proteins

The expression of the fusion proteins in transfected HIRcB cells was confirmed by western blotting. Cells growing in 100 mm diameter plates were transfected with the appropriate fusion construct of ARF1-GFP and allowed to become about 90% confluent. Cells were extracted in a solubilizing buffer (50 mM Hepes, 150 mM NaCl, 100 mM NaF, 1 mM sodium vanadate, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 1.5% cholate, 1 mM PMSF, 10 μg/ml leupeptin and 400 μg/ml soybean trypsin inhibitor and pH 7.45) and proteins were separated by SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes, which were subsequently probed with anti-GFP and anti-ARF antibodies. Membranes were then incubated with secondary antibody conjugated to HRP and proteins were visualized using a chemiluminescence detection system.

Microscopy of HIRcB cells expressing various ARF1-GFP fusion proteins

All experiments involving confocal laser scanning microscopy were carried out at room temperature (22° to 24°C) using a Molecular Dynamics 2001 confocal microscope equipped with a 60× oil-immersion objective and a live cell chamber. During the entire experiment, the cells were bathed in phenol-red-free DMEM/F12, containing 15 mM Hepes. Samples were excited at 488 nm with a krypton-argon laser source (3% of maximal intensity). Emitted light was passed through a 510 nm band pass filter and detected with a photomultiplier tube. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss induced by photobleaching (FLIP) experiments were performed with the same confocal microscope except that the laser was used at 100% intensity (488 nm) for bleaching before image acquisition.

Microfluorimetry

These experiments were performed using an Olympus inverted microscope equipped with an oil-immersion 100× objective. Cells were illuminated by light from a mercury arc lamp passing through a wide-band FITC cube. An adjustable diaphragm was used to select the Golgi region. Emitted light was collected by a photomultiplier tube powered by a Dual Channel ratio Fluorometer (Biomedical Instrumentation Group, University of Pennsylvania). The signal was then collected and displayed using the X-chart module of the PULSE program (HEKA Electronik) on a PowerPC Macintosh computer. The data were further analyzed using IgorPro.

Materials

wt-ARF1 and Q71L-ARF1 cDNAs and monoclonal antibody 1D9 (ARF-specific) were a gift of Dr Richard Kahn (Emory University). A galactose transferase-GFP construct (GALTase-GFP) used to label Golgi structures was a gift of Dr Jennifer Lippincott-Schwartz (NIH). BODIPY-C5-ceramide was purchased from Molecular Probes. ARF proteins were cloned in pEGFP-N1 supplied by Clontech. Monoclonal and polyclonal antibodies also supplied by Clontech were used to determine the levels of expression of the fusion proteins. BFA was from Sigma Chemicals.

RESULTS

Localization of ARF1 to the Golgi requires GTP binding and an intact N terminus

Four different ARF1-GFP fusion constructs were transfected into HIRcB cells as described above. Fusion constructs were designed such that GFP was fused to the C terminus of ARF1. This strategy was chosen based on previous reports that suggest that the integrity of the N terminus is essential for the activity of ARF proteins (Kahn et al., 1992). The expression of ARF1-GFP constructs was confirmed by western blotting using specific anti-GFP antibodies. As shown in Fig. 1E, all the immunoreactivity found in these cells corresponded to a band of about 47 kDa, consistent with the expected molecular weight of an ARF1 (21 kDa)-GFP (27 kDa) chimera. The absence of an immunoreactive band of 27 kDa suggests that the level of free GFP expression in these cells is negligible.

Transfected cells were studied with a confocal microscope

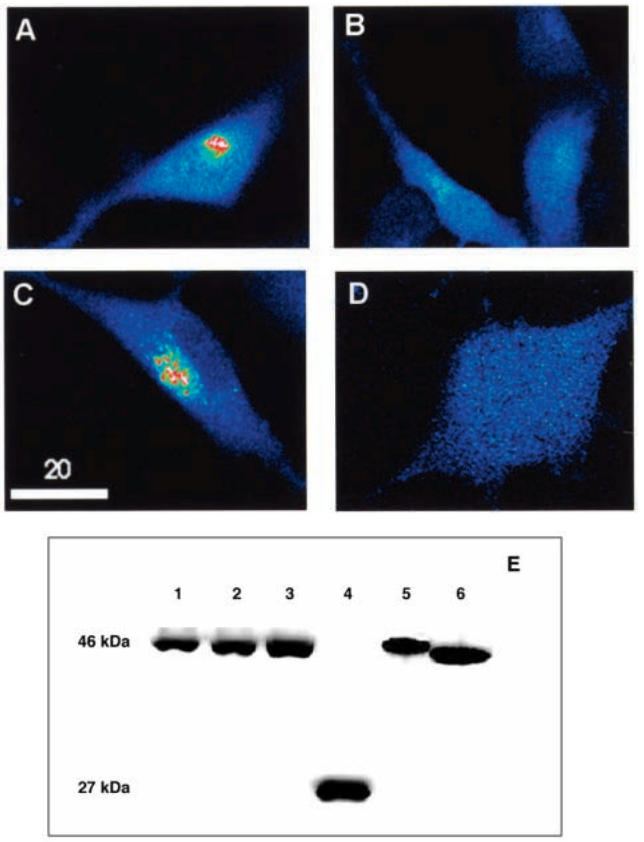

Fig. 1. Expression of various ARF1-GFP fusion proteins in HIRcB cells. Cells were transfected with fusion DNA constructs and 24 hours later the cells were scanned in a confocal microscope with a 60× objective. Notice the localization of the fusion proteins to the Golgi in cells expressing (A) wt ARF1-GFP and (C) Q71L ARF1-GFP. Cells expressing (B) the T31N mutant or (D) the Δ^N wt mutant show exclusively cytoplasmic distribution of the ARF1-GFP chimera. (E) The expression of the various fusion constructs was confirmed by western blotting. wt-ARF1-GFP (lane 1), Q71L-ARF1-GFP (lane 2), T31N-ARF1-GFP (lane 3), pEGFP (cells transfected with the original vector, lane 4), wt-ARF6-GFP (included as an additional control, lane 5) and Δ^Nwt-ARF1-GFP (lane 6).

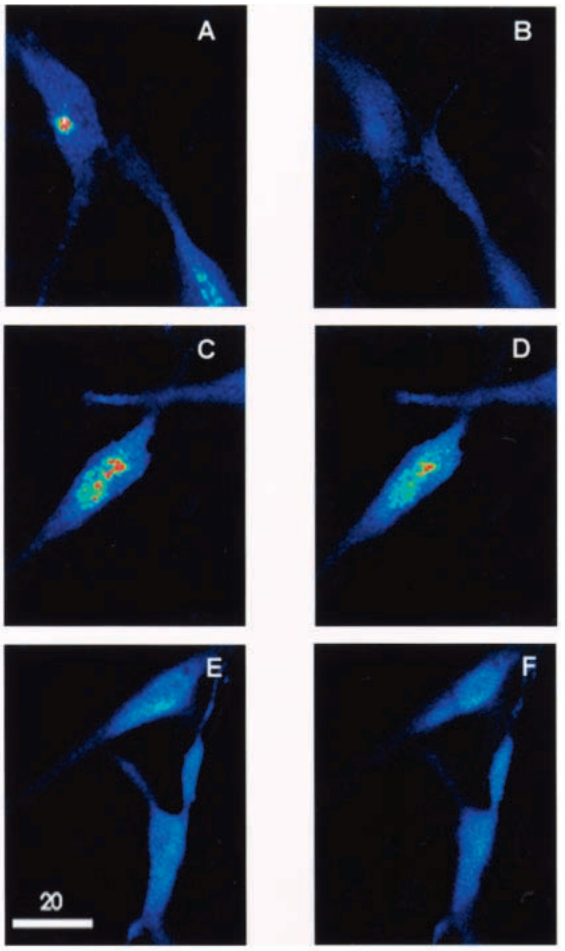

Fig. 2. Effect of brefeldin A (BFA, 5 μg/ml) on HIRcB cells expressing the ARF1-GFP fusion proteins. Transfected cells were scanned (A,C,E) just prior to the addition of BFA or (B,D,F) 5 minutes after the addition of BFA. Notice that in cells expressing wt-ARF1-GFP, addition of BFA causes an almost complete dissociation of ARF1-GFP from the Golgi (A,B), while in cells with Q71L-ARF1-GFP, 40-50% of ARF1-GFP is still in the Golgi (C,D). The addition of BFA does not seem to have any effect on cells expressing T31N-ARF1-GFP (E,F).

24-48 hours after transfection (Fig. 1). Cells expressing wt-ARF1-GFP showed intense fluorescence localized to a region that was identified as the Golgi by 3-D reconstruction of the confocal data and by co-localization with C5-ceramide, a lipid that selectively labels Golgi cisternae. A significant fraction of wt-ARF1-GFP was found dispersed throughout the cytoplasm (Fig. 1A). Cells transfected with Q71L-ARF1-GFP showed a similar pattern in the distribution of the fusion protein (Fig. 1C) although, in this case, the fluorescence associated to the Golgi was significantly more prominent. In contrast, cells transfected with the T31N-ARF1-GFP and Δ^Nwt-ARF1-GFP constructs did not show Golgi localization of the fluorescent tag (Fig. 1B,D). Occasionally, a small fraction of the T31N mutant was found bound to the Golgi, but this was an exceptional finding. The Δ^Nwt mutant was never found associated to Golgi structures. These data demonstrate that, as predicted by previous studies done in cell-free systems, GTP binding and an intact N terminus are required for the binding of ARF proteins to membranes.

The distribution of ARF1 is a function of its GTPase activity

According to the current view of the mode of action of ARF proteins, activated ARF·GTP complexes and inactive ARF·GDP exist in a steady state determined by the rate of activation of ARF and the rate of hydrolysis of ARF-bound GTP. Since ARF·GTP complexes are membrane-bound whereas inactive ARF·GDP complexes are localized in the cytosol, it should be possible to determine the state of activation of ARF by measuring the ratio of particulate to cytosolic ARF. To calculate the ratio of Golgi-bound to cytosolic ARF1, the total amount of fluorescence in the Golgi and in the cytoplasm was determined from confocal images using the Molecular Dynamics ImageSpace software package. The ratio of Golgi-bound to cytosolic wt-ARF1 was estimated as 0.16±0.008 (n=5). This ratio should be a function of the GTPase activity of ARF. Thus, the ratio of Golgi-bound to cytosolic ARF should be significantly greater for GTPase-

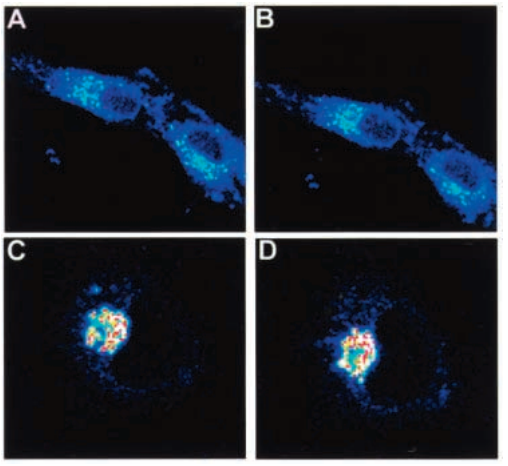

Fig. 3. BFA (5 μg/ml) does not cause any apparent structural changes to the Golgi even 10 minutes after exposure to the drug. (A,B) HIRcB cells were labeled with BODIPY-C_5-ceramide (0.5 μM) overnight. Cells were then scanned in a confocal microscope with a krypton-argon laser at 514 nm. (A) Cells just before the addition of BFA. (B) Cells scanned 10 minutes after the addition of BFA. (C,D) Cells were transfected with GALTase-GFP and scanned as described in the text just prior to and 10 minutes after addition of BFA, respectively.

deficient mutants of ARF. The results obtained for the Q71L-ARF1-GFP chimera (0.51±0.022 (*n*=5)) confirm this prediction. Also, the model predicts that, assuming that the activation rates of wt-ARF1 and mutant ARF1 are the same, the ratio of Golgi-bound to cytosolic ARF should follow an inverse relationship to the ratio of the GTPase activities of both proteins. This conclusion is also supported by the data: the ratio of membrane-bound to cytosolic ARF of the GTPase-deficient mutant is about 3-fold greater whereas the in vitro GTPase activity of the wt protein is 3-fold larger (Tanigawa et al., 1993; Teal et al., 1994).

Brefeldin A causes the dissociation of ARF1-GFP from the Golgi

The fungal metabolite brefeldin A (BFA) is a toxin that causes a relatively rapid redistribution of Golgi structures into the endoplasmic reticulum (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989). The cellular target of this toxin appears to be an ARF guanine nucleotide exchange factor that catalyzes the exchange ARF-bound GDP for GTP and thereby activates ARF (Donaldson et al., 1992; Helms and Rothman, 1992; Randazzo et al., 1993).

According to the current view of the mode of action of BFA, the addition of the drug to cells should inhibit the activation of

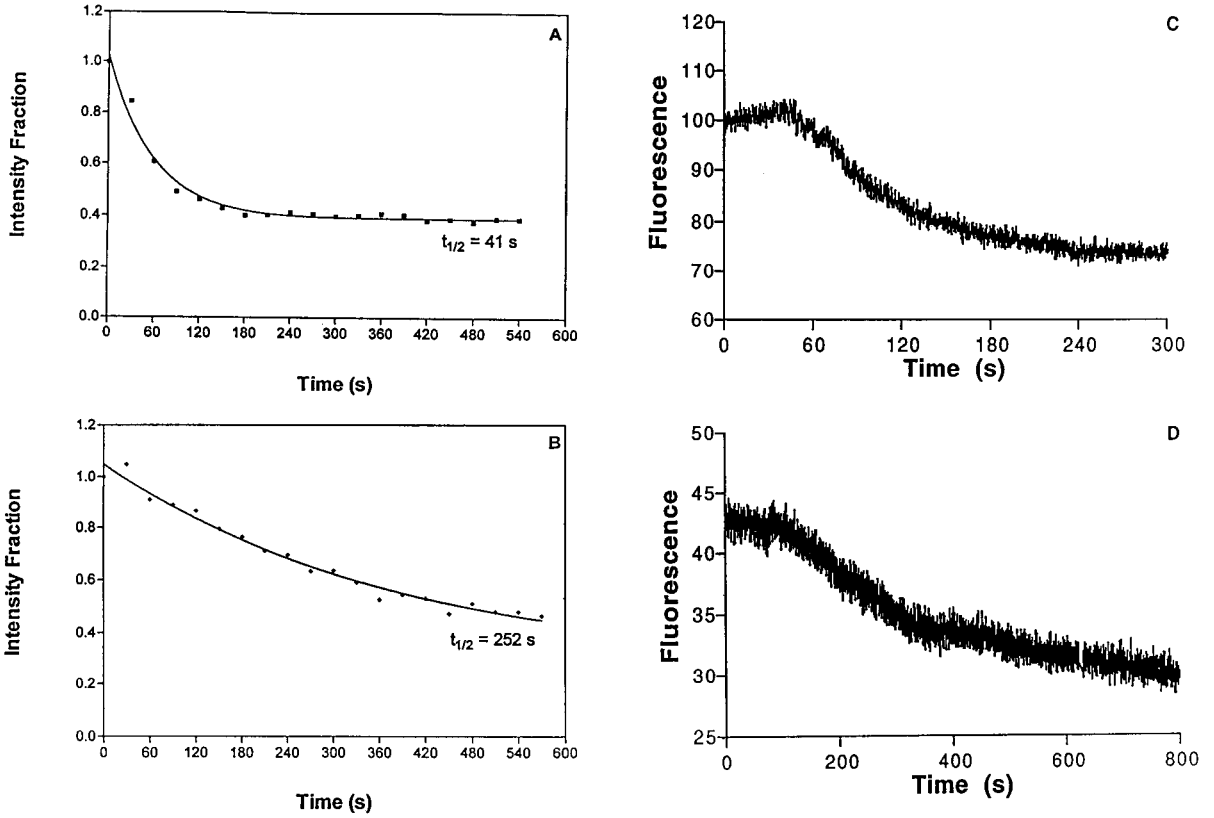

Fig. 4. The kinetics of dissociation of ARF1-GFP from the Golgi after addition of BFA (5 μg/ml) was determined using (A,B) confocal microscopy and (C,D) microfluorometry . For confocal studies, cells were scanned in a confocal laser microscope every 30 seconds. BFA was added to the cells after the second or third scan. Following the completion of the scans, the fluorescent intensity in the Golgi was measured using Molecular Dynamics' ImageSpace software. The initial intensity was the fluorescence of the Golgi in the scan immediately before the addition of BFA. Further data analysis and plots were made using Graphpad Software's Prism package. For microfluorometry, cells were imaged with an Olympus microscope and fluorescent intensity from the Golgi of the cells was collected using a photomultiplier tube interfaced with the microscope and a PowerMac computer. After obtaining a stable baseline, BFA (5 μg/ml) was added and the changes in fluorescence intensity of the Golgi were measured. IgorPro was used to analyze the data and create the plot. (A,C) The time course of dissociation of wt-ARF1-GFP; (B,D) data obtained with Q71L-ARF1-GFP.

ARF without affecting the rate of GTP hydrolysis by ARF proteins. Thus, if, as suggested by the data presented above, ARF proteins are in an equilibrium between activated, membrane-bound and inactive, cytosolic pools, the addition of BFA to cells and the subsequent blockade of the ARF activation pathway should result in the disappearance of membrane-bound ARF1-GFP from the Golgi.

Fig. 2A shows an image of a cell transfected with wt-ARF1-GFP. Fig. 2B shows an image of the same cell 5 minutes after the addition of BFA (5 μg/ml). As shown, wt-ARF-GFP dissociated from the Golgi very rapidly after addition of BFA. However, the effect of BFA was much less dramatic for cells expressing the Q71L construct. In these, about 40-50% of the fluorescence remained bound to the Golgi 5 minutes after the addition of BFA (compare Fig. 2C,D). As expected, BFA did not alter the localization of T31N-ARF1-GFP (Fig. 2E,F).

In parallel experiments, cells labeled with either BODIPY-C5-ceramide or GALTase-GFP were also exposed to BFA and examined with the confocal microscope. Short-chain ceramides are preferentially localized to the Golgi of cells, as shown in Fig. 3A (Ralston, 1993). Addition of BFA had no obvious effects on the distribution of the labeled lipid even 10 minutes after exposure to the drug (Fig. 3B). Since short-chain ceramides also label the *trans*-Golgi system, the stability of the Golgi after addition of BFA was determined using GALTase-GFP as well. Fig. 3C,D shows the distribution of GALTase-GFP in the Golgi immediately prior to (Fig. 3C) and 10 minutes after (Fig. 3D) the addition of BFA. Therefore, the disappearance of ARF1-GFP fluorescence from the Golgi after the addition of BFA is the consequence of the dissociation of ARF and not the result of a BFA-dependent disruption of the Golgi membrane. Given that wt-ARF1 dissociates much more rapidly than the Q71L mutant, these data support a model according to which the rate of disappearance of the fluorescent chimeras from the Golgi after addition of BFA reflects the rate of hydrolysis of GTP by the corresponding ARF1-GFP construct.

A detailed kinetic study of the dissociation of ARF-GFP from the Golgi was performed. These experiments were repeated at least 4 times. Two typical time courses are shown in Fig. 4A. In these experiments, scans were recorded at 20-second intervals. BFA was added after the second or third image was collected and the time of addition of BFA was taken as t=0 for all subsequent calculations. To determine the rate of dissociation of ARF from the Golgi, the total fluorescence intensity of the Golgi was calculated for all images in each stack and analyzed as a function of time. As shown in Fig. 4A, the dissociation of wt-ARF1-GFP is rapid

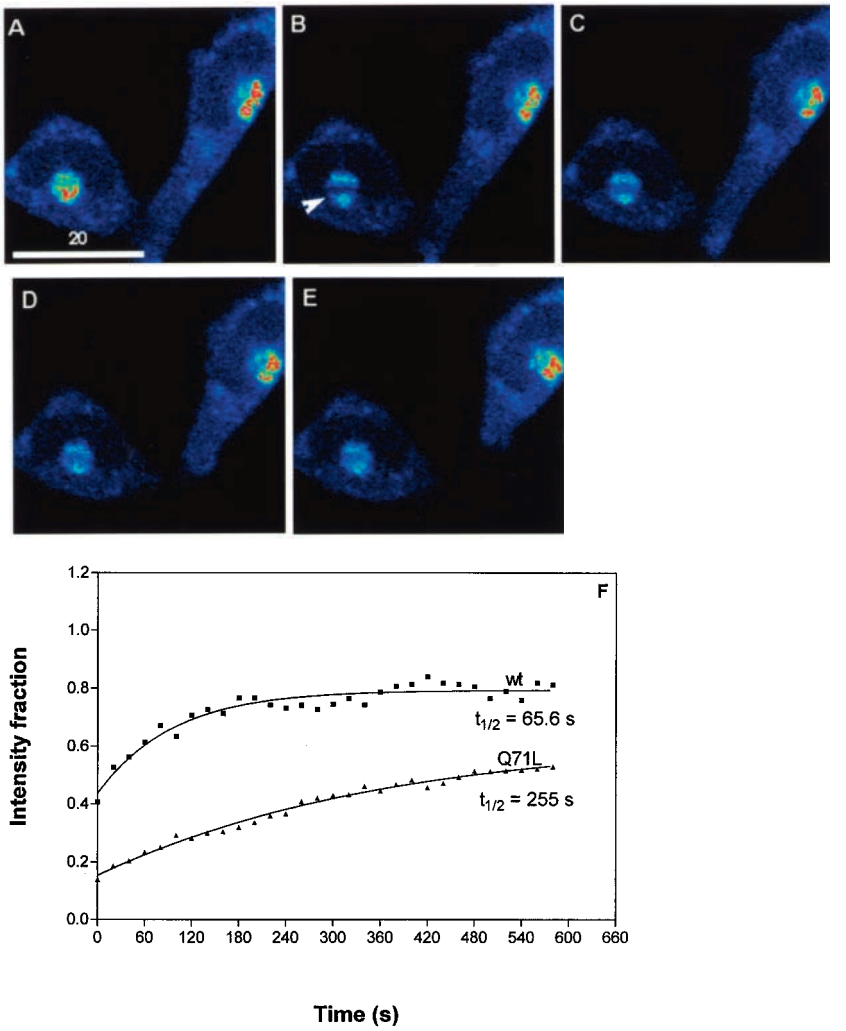

Fig. 5. (A-E) Photobleaching and recovery in Golgi of cells expressing Q71L-ARF1-GFP. (A) Cells just prior to the bleach of the Golgi. (B) Cells were bleached where indicated by the arrow and scanned immediately (<10 seconds) after bleaching. (C-E) Cells scanned 1 minute, 4 minutes and 7 minutes after bleaching. The kinetics of recovery of ARF1-GFP in the Golgi following photobleaching (FRAP) of Golgi is shown in F. Cells expressing ARF1-GFP chimeric proteins were photobleached at maximal laser intensity. Immediately (<10 seconds) after the bleach cells were scanned every 20 seconds at 3% laser intensity. The fluorescent intensity in the bleached area of the Golgi (before and after bleaching) was measured using Molecular Dynamics software. Data analysis and plots were done using Graphpad Prism. The intensity fraction refers to the ratio of the intensity at any given time to the intensity prior to photobleaching.

and appears to follow first-order kinetics ($k=1.7\times10^{-2}\ s^{-1}$). The apparent rate constant of dissociation of Q71L-ARF1-GFP was found to be much slower. A fit to a first-order exponential yields a first-order rate constant of $2.75\times10^{-3}\ s^{-1}$ (Fig. 4B). Additional measurements were done using microfluorometric techniques. In these studies, the total fluorescence intensity of the Golgi was determined using a photomultiplier tube attached to a fluorescence microscope equipped with a 100× objective. After collection of sufficient data to obtain a baseline, BFA was added to the cells and the fluorescence intensity of the Golgi was monitored constantly for an additional period of time. The data obtained in these experiments are shown in Fig. 4C and 4D. The rates calculated from these experiments were consistent with those obtained using confocal microscopy. As suggested by the confocal microscopy data, the dissociation of wt-ARF1-GFP from the Golgi followed first-order kinetics, with a rate constant of $1.86(\pm0.031)\times10^{-2}\ s^{-1}$ (n=4). However, the dissociation of the Q71L mutant showed biphasic kinetics, with rate constants of $3.6(\pm0.8)\times10^{-3}\ s^{-1}$ and $4.65(\pm0.6)\times10^{-4}\ s^{-1}$ (n=4), respectively. The first phase of this process correlates very well with the apparent rate of dissociation of Q71L-ARF1-GFP from the Golgi as measured with the confocal microscope. The slower phase was not observed in confocal microscopy studies.

Fluorescence recovery after photobleaching experiments

Fluorescence recovery after photobleaching (FRAP) experiments were carried out by scanning a line using the full intensity of the laser beam and following the recovery of the bleached fluorescence as a function of time by scanning the specimen at low laser intensity (Fig. 5). In most experiments, a 1 μm line was used. Diffusion coefficients were determined according to the equation: $D=3r^2\gamma/4t_{\frac{1}{2}}$ where r is the half-width of the bleach line, $t_{\frac{1}{2}}$ is the half-life of the recovery and γ is a correction factor for bleach depth (Feldman et al., 1981). Representative kinetic data are shown in Fig. 5F. The mobile fraction of ARF was calculated from the ratio of the fluorescence intensities for regions within and without the bleaching zone, before photobleaching and after recovery. This ratio was 70-110%, suggesting that nearly all of the wt-ARF1-GFP and Q71L-ARF1-GFP chimeras are mobile in Golgi membranes.

The calculated diffusion coefficient for the wt-ARF1-GFP fusion protein was $1.1(\pm0.06)\times10^{-10}\ cm^2\ s^{-1}$ (n=4). This number is much smaller than that calculated by Cole et al. (1996) for Golgi membrane proteins ($2\text{-}5\times10^{-9}\ cm^2\ s^{-1}$). This finding suggests that Golgi-bound ARF proteins do not diffuse freely on the Golgi membrane. The lack of mobility of Golgi-bound ARF can be interpreted in several different ways. For

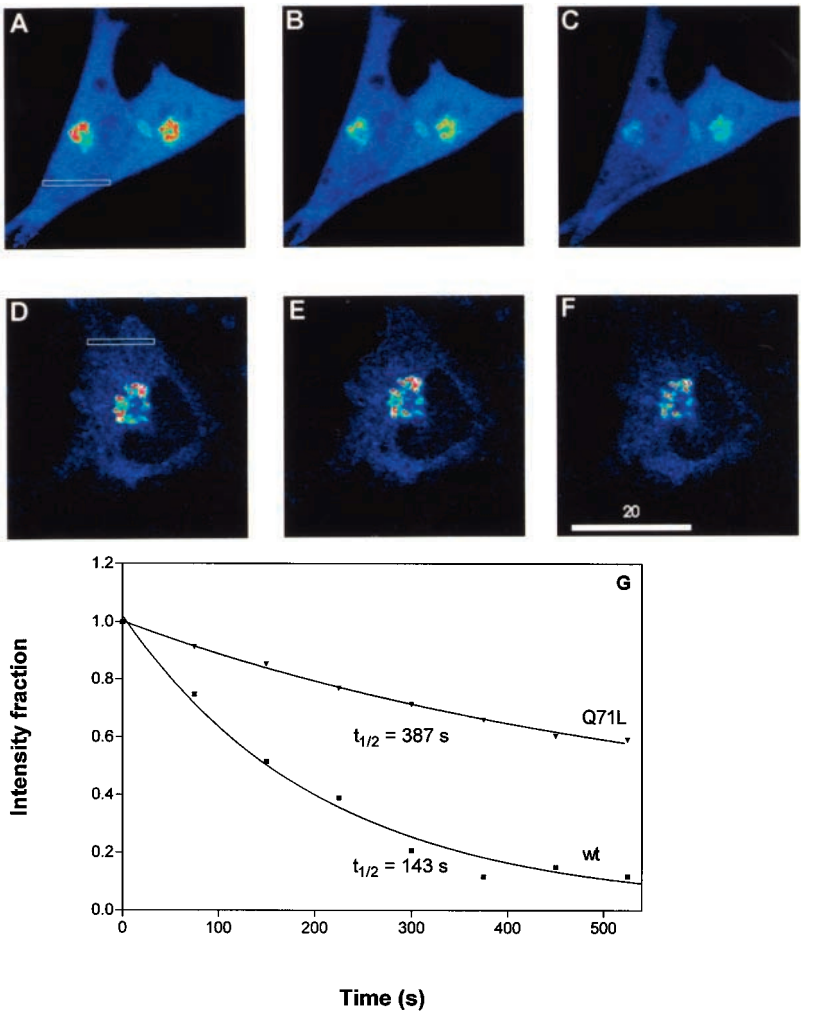

Fig. 6. Photobleaching of the cytoplasm induces fluorescence loss from the Golgi. The pictures show the fluorescence loss from the Golgi of a cell expressing wt-ARF1-GFP (A-C). The white box represents the bleached area. (A) Scan done just before the first bleach; (B) scan recorded after 3 bleaches; (C) scan after 6 bleaches. Notice that bleaching the cytoplasm leads to the disappearance of fluorescence from the Golgi. As a control, we also repeated the same type of analysis with a cell expressing GALTase-GFP (D-F), a resident Golgi protein. In this case, the loss of fluorescence from the Golgi is much slower (about 80% of fluorescence still in Golgi after 6 bleaches) than in cells expressing wt ARF1-GFP. The kinetics of the loss of fluorescence from the Golgi of cells transfected with wt- and Q71L-ARF1-GFP is shown in G. The fluorescent intensity in the Golgi was measured 1 minute after each bleach. The ratio of this intensity to the Golgi intensity just prior to bleaching was calculated and plotted as a function of time. The intensities were measured using Molecular Dynamics software. Further data analysis was done using Graphpad's Prism.

instance, the Golgi of HIRcB cells could be extensively fragmented, such that ARF cannot laterally diffuse between stacks. However, if this were the case, then the mobile fraction of GALTase-GFP, a protein that has been shown to diffuse very rapidly in the Golgi, would be expected to be very small. We tested this model by determining the ratio of the fluorescence in areas within and without the bleached regions, before photobleaching and after recovery, as described above. Our data show that approximately 106±18% (n=5) of the GALTase-GFP construct diffuses on the Golgi. Thus, Golgi fragmentation cannot explain the slow diffusion of ARF. Therefore, the most likely explanation of our observations is that ARF binds to the Golgi as part of a large macromolecular complex with very limited mobility.

The diffusion coefficient of the Q71L-ARF1-GFP chimera was estimated to be $4.2(\pm0.56)\times10^{-11}$ cm^2 s^{-1} (n=5). This figure is approximately 3-fold smaller than the diffusion coefficients calculated for the wild-type protein. Given that these proteins differ in a single amino acid substitution, the fact that the Q71L mutant diffuses much more slowly strongly suggests that GTP hydrolysis plays an important role in the mobility of membrane-bound ARF. In agreement with this conclusion, the ratio of the diffusion coefficients of these proteins is approximately equal to the inverse of the ratio of their GTPase activities. Therefore, we conclude that the recovery of fluorescence in the Golgi requires the hydrolysis of ARF-bound GTP followed by the dissociation of the protein and its subsequent reactivation by the Golgi membrane.

Fluorescence loss in photobleaching (FLIP) studies

The FLIP technique is a modification of FRAP in which a selected area in a cell is repeatedly photobleached and changes in fluorescence in a adjacent area are monitored to study the dynamics of the protein of interest. The data obtained with BFA and the FRAP experiments shown above suggest that all the Golgi-bound ARF-GFP chimeras are mobile. In order to confirm this, we bleached an area in the cytoplasm and imaged the entire cell immediately after the bleach. This process was repeated several times at 1 minute intervals. Control FLIP experiments were done using a GALTase-GFP construct which resides exclusively in the Golgi and does not equilibrate with a cytosolic pool (Cole et al., 1996). Fig. 6 shows the effect of bleaching an area in the cytoplasm of a cell transfected with wt-ARF1-GFP (Fig. 6A-C) and a second cell transfected with GALTase-GFP (Fig. 6D-F). The white rectangular box in Fig. 6A and D represents the bleached area. Images of the cells were taken prior to bleaching (Fig. 6A,D), after the 3rd bleach (i.e. 3 minutes after the start of the experiment; Fig. 6B,E) and after the 6th bleach (6 minutes; Fig. 6C,F). The fluorescence associated with the Golgi in cells expressing wt-ARF1-GFP decays very rapidly due to bleaching of the cytoplasm. The bleaching of the Golgi-resident protein GALTase was very significantly slower, as expected for a protein that does not equilibrate with a cytoplasmic pool. This observation demonstrates that Golgi-bound wt-ARF1-GFP is in a rapid equilibrium with a cytoplasmic pool of ARF1-GFP and confirms the hypothesis that essentially all of the ARF chimeras are mobile.

A kinetic profile of the loss of fluorescence from the Golgi due to the FLIP phenomenon after repeated cytoplasm bleaching is shown in Fig. 6G. Comparing the rate of fluorescence loss from the Golgi of the cells expressing the wt-ARF1-GFP chimera to that of cells transfected with the Q71L mutant, it is apparent that the wt construct is released from the Golgi much more rapidly. The wt-ARF1-GFP chimera leaves the Golgi about three times more rapidly than Q71L-ARF1-GFP. Once again, this ratio is comparable to the GTPase activity of both proteins.

DISCUSSION

We have made DNA fusion constructs of wt and three mutants of ARF1 with a humanized, red-shifted version of green fluorescent protein of *A. victoria.* These chimeras have been expressed in a mammalian cell line and the dynamics of their distribution has been studied by diverse imaging techniques. Two of the chimeric proteins were found to localize in the Golgi (wt and Q71L). These results are consistent with the immunological studies of ARF1 localization (Stearns et al., 1990; Hosaka et al., 1996) and thus demonstrate that the fusion of the fluorescent protein at the C terminus of ARF1 does not affect its subcellular distribution. A mutant with greatly reduced affinity for GTP failed to localize in the Golgi (Fig. 1B). We conclude, therefore, that GTP binding is essential for the localization of ARF in the Golgi. Likewise, as shown in Fig. 1D, an N-terminal deletion mutant of ARF1 also failed to localize in the Golgi. This suggests that this region (probably because of the N-myristoylation site of ARF1) is also required for membrane binding. These data are consistent with the observation that N-myristoylation is required for the interactions of ARF with target proteins, such as phospholipase D (Brown et al., 1993).

It has been shown by numerous studies in vitro that ARF proteins bind membranes when activated (Regazzi et al., 1991; Houle et al., 1995; Cavenagh et al., 1996). The imaging data

Table 1. Comparison of the properties of wild-type and GTPase-deficient ARF1 proteins

Parameter	wt-ARF1	Q71L-ARF1	Ratio wt/Q71L
GTPase activity*	$k=1.1\times10^{-2}$ s^{-1}	$k=4.0\times10^{-3}$ s^{-1}	2.75
Ratio Golgi-bound/soluble ARF-GFP	0.16	0.51	0.32
BFA-induced dissociation	$k=1.9\times10^{-2}$ s^{-1}	$k_1=3.6\times10^{-3}$ s^{-1}, $k_2=4.6\times10^{-4}$ s^{-1}	5.28
Diffusion coefficients	1.1×10^{-10} cm^2 s^{-1}	4.2×10^{-11} cm^2 s^{-1}	2.68

*In vitro first-order rate constant for the hydrolysis of ARF-bound GTP at 30°C, calculated from the data of Teal et al. (1994). These figures were obtained with a Q71I-ARF1 mutant rather than with the Q71L-ARF1 mutant used in the studies reported here.

reported here agrees with that conclusion and shows that ARF1 binds exclusively to Golgi membranes. We have calculated the levels of activation of ARF1 by determining the ratio of membrane-bound to cytosolic ARF-GFP. This calculation assumes that Golgi-bound and cytosolic ARF establish a steady-state distribution. The FLIP and FRAP data shown above demonstrate that ARF1 does indeed exist in such a state. First, FLIP experiments demonstrate that bleaching a region in the cytoplasm rapidly and completely eliminated Golgi-bound wt-ARF1-GFP fluorescence. Second, the ratio of the fluorescence intensities in two different regions of the Golgi (one bleached and one unbleached) was about the same before bleaching and after recovery of fluorescence, suggesting that Golgi-bound ARF has re-equilibrated with the remaining intracellular ARF pools during the FRAP experiment. Based on this assumption, we have calculated that about 15% of ARF1 is activated at steady state. Since the distribution between membrane-bound and cytosolic ARF should be a function of the relative rates of activation and inactivation of ARF, the model also predicts that the fraction of membrane-bound GTPase-deficient ARF mutants should be significantly greater. Our data demonstrate that this is the case. The level of activation of the Q71L mutant was found to be about 40%. These data are consistent with the reduced rate for GTP hydrolysis reported for these mutants (Tanigawa et al., 1993; Teal et al., 1994; Zhang et al., 1994).

Figs 2 and 4 show that, upon exposure to BFA, ARF dissociates from the Golgi relatively rapidly and that this dissociation precedes the disruption of the Golgi membrane, as judged by the data obtained with C5-ceramide and GALTase-GFP (Fig. 3). The mechanism of action of BFA has been studied in some detail in vitro and it appears to be related to the inhibition of ARF activation (Donaldson et al., 1992; Helms and Rothman, 1992). The in vivo data presented here support the hypothesis that BFA's main action is the inhibition of ARF activation. Two separate processes must occur during the dissociation of ARF from the Golgi. First, bound GTP must be hydrolyzed and, second, bound ARF·GDP complex must leave the membrane. The first-order rate constant of the disappearance of fluorescence from the Golgi reflects the slowest of these two steps. The 4-fold difference observed between the rate of release of wt-ARF1-GFP and that of the Q71L mutant suggests that GTP hydrolysis is the rate-limiting step for the dissociation of ARF.

The diffusion of the ARF chimeras on the Golgi was remarkably slow. The diffusion coefficients of Golgi proteins reported by Cole et al. (1996) are about $2\text{-}5\times10^{-9}$ cm^2 s^{-1}. In contrast, the wt-ARF1-GFP chimera diffuses at about 1×10^{-10} cm^2 s^{-1}, a figure that is 20-50 times smaller than that of GALTase. There are two processes that contribute to the recovery of ARF-GFP fluorescence on the Golgi: (i) the diffusion of fluorescent protein from unbleached areas of the Golgi and (ii) the inactivation (by GTP hydrolysis) and dissociation of Golgi-bound ARF followed by the re-activation (by release of GDP and binding of GTP) and association of cytosolic ARF. If the diffusion of fluorescence from unbleached areas was the dominant factor, the diffusion coefficient of the GTPase-deficient Q71L mutant and the wild-type constructs should be approximately the same. However, they are not; the GTPase-deficient mutant diffuses three times more slowly. A simple comparison of these diffusion coefficients with the rates of GTP hydrolysis and the rates of dissociation of ARF1 from the Golgi (see Table 1) immediately suggests that the rate-limiting step in the diffusion of ARF1 is the hydrolysis of GTP. Thus, we conclude that ARF proteins do not diffuse freely on the surface of the Golgi. Since the mobile fraction of GALTase in these cells is close to 100%, we conclude that ARF1.GTP is tethered to a large, relatively immobile complex on the surface of the Golgi membrane and does not come off until after the hydrolysis of GTP has occurred (Rothman, 1994).

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