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Active ADP-ribosylation Factor-1 (ARF1) Is Required for Mitotic Golgi Fragmentation*,S

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Abstract

In mammalian cells the Golgi apparatus undergoes an extensive disassembly process at the onset of mitosis that is believed to facilitate equal partitioning of this organelle into the two daughter cells. However, the underlying mechanisms for this fragmentation process are so far unclear. Here we have investigated the role of the ADP-ribosylation factor-1 (ARF1) in this process to determine whether Golgi fragmentation in mitosis is mediated by vesicle budding. ARF1 is a small GTPase that is required for COPI vesicle formation from the Golgi membranes. Treatment of Golgi membranes with mitotic cytosol or with purified coatomer together with wild type ARF1 or its constitutive active form, but not the inactive mutant, converted the Golgi membranes into COPI vesicles. ARF1-depleted mitotic cytosol failed to fragment Golgi membranes. ARF1 is associated with Golgi vesicles generated *in vitro* and with vesicles in mitotic cells. In addition, microinjection of constitutive active ARF1 did not affect mitotic Golgi fragmentation or cell progression through mitosis. Our results show that ARF1 is active during mitosis and that this activity is required for mitotic Golgi fragmentation.

The Golgi apparatus is a membrane-bound organelle that serves as a central conduit for protein and lipid modification, processing, trafficking, and secretion in all eukaryotic cells. The central and unique feature of the Golgi is a stack of flattened cisternal membranes with dilated rims (1). The stack carries out post-translational processing of newly synthesized proteins as they pass through this organelle after assembly in the endoplasmic reticulum (ER)[2] (2). Processing enzymes, which include those involved in modifying bound oligosaccharides, are arranged across the stack (in the cis to trans direction) in the order in which they function (3). In animals, stacks are often interconnected to form a ribbon that is localized adjacent to the nucleus. Despite its complicated morphology and function, the Golgi apparatus is dynamic, capable of rapid disassembly and reassembly during mitosis (4) or upon drug treatment (5–8). Mitotic fragmentation of the Golgi apparatus was first observed in the early 1900s. One particularly good example is the study by Ludford in 1924

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SThe on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

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[2]The abbreviations used are: ER, endoplasmic reticulum; ARF1, ADP-ribosylation factor-1; BFA, Brefeldin A; IC, interphase cytosol; MC, mitotic cytosol; plk, Polo-like kinase-1; PNS, postnuclear supernatant; WT, wild type; NRK, normal rat kidney; TGN, trans-Golgi network; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

(9), in which the Golgi was described as forming “rodlets” that were scattered throughout the dividing cell and that, at a late stage in cell division, re-associated to form two separate Golgi complexes, one in each daughter cell. Studies using electron microscopy showed fragmentation of the characteristic stack-like organization of the Golgi at the onset of mitosis, and Golgi fragments were subsequently distributed to each daughter cell (10, 11). In the late 1990s, when green fluorescent proteins and live cell imaging techniques became available, it was shown that early in mitosis, during prophase, the Golgi undergoes extensive fragmentation along the cell cycle progression (12–15). During cytokinesis, the dispersed Golgi fragments are converted into larger Golgi fragments that finally re-form the pericentriolar ribbon structure.

Several reasons have been presented to explain the biological significance of mitotic Golgi fragmentation. The first concerns the mechanism of Golgi partitioning between the two daughter cells during the cell cycle. The distribution of Golgi membranes over a larger space of the cell is expected to aid in the even distribution of this organelle into the daughter cells (4). The second hypothesis concerns the release of “mitotic” components stored on the Golgi during interphase, which are important in the promotion of cytokinesis (16, 17). For example, Nir2, a Golgi-associated protein in interphase cells, has been shown to move to the cleavage furrow and is essential for cytokinesis (18). Thus, in addition to directly providing membranes for cleavage furrow invagination (19), it is likely that the Golgi mediates the coordinated release of proteins required for cytokinesis and other cytoplasmic events as the cell progresses through mitosis.

The underlying mechanisms mediating Golgi disassembly during mitosis are so far unclear. One possibility is that disassembly is achieved through a similar mechanism as brefeldin A (BFA) treatment, a fungal metabolite known to redistribute the Golgi membranes, at least partially, into the ER. BFA exerts its effects by inactivating the ADP-ribosylation factor-1 (ARF1) recruitment to Golgi membranes. ARF1 is a small GTPase that is membrane-associated in its active GTP-bound form and recruits coatomer onto the membrane to form COPI vesicles. Inactivation of ARF1 in turn prevents recruitment of coatomer (and hence the formation of COPI vesicles) and leads to the redistribution of Golgi membranes to the ER (6). The central idea of this hypothesis is that mitosis phenocopies BFA treatment in terms of Golgi breakdown by inactivation of ARF1. Using live cell imaging, it has been shown that transiently over-expressed ARF1-GFP disappears from the Golgi region in the early stages of mitosis and that the expression of the constitutively active ARF1(Q71L) mutant blocks mitotic Golgi disassembly as well as cell progression through mitosis (15, 17, 20), suggesting that ARF1 needs to be inactivated in order to disassemble the Golgi. However, whether the endogenous ARF1 is inactivated during mitosis is so far unclear.

Another explanation for the mitotic Golgi disassembly is the continuous budding of COPI vesicles. Isolated Golgi membranes, when treated with mitotic cytosol, undergo extensive fragmentation that mimics mitotic Golgi fragmentation seen *in vivo*. The fragmentation is mediated, in large part, by the continued budding of COPI vesicles (21). Budding requires ARF1, suggesting that ARF1 is active. Consistently, budding is also enhanced in the presence of GTPγS, which is known to activate ARF1. However, GTPγS also activates other GTPases (such as Rab proteins) and impairs cargo uptake in the early stage of COPI vesicle formation (22). So far there is no direct evidence that ARF1 itself is involved in mitotic Golgi fragmentation. Here we have examined the role of ARF1 in mitotic Golgi fragmentation using a variety of techniques. Our results show that ARF1 is active during mitosis and that this activity is required for mitotic fragmentation of the Golgi.

EXPERIMENTAL PROCEDURES

Reagents

All reagents were from Sigma, Roche Diagnostics, or Calbiochem, unless otherwise stated. The following antibodies were used: monoclonal antibodies against ARF1 (1D9, Abcam), GM130, and Gos28 (Transduction Laboratories), GRASP65 (F. Barr), β-COP (M3A5, T. Kreis), α-tubulin (K. Gull); polyclonal antibodies against ARF (D. Shields and D. Sheff), β-COP (EAGE, T. Kreis), GM130 (MLO7, M. Lowe), GRASP65 (23), α-mannosidase II (K. Moremen), Golgin-84 (A. Satoh), rat serum albumin (G. Warren), and TGN38 (24). Secondary antibodies for immunofluorescence and for Western blotting were from Molecular Probes and Jackson ImmunoResearch, respectively. BFA was obtained from Invitrogen and Epicenter Biotechnologies and applied to the tissue culture medium at 5 μg/ml final concentration. Nocodazole (Sigma) was prepared as 0.2 mg/ml stock in Me_2SO and used at 0.5 μg/ml in the medium. Hoechst 33342 was purchased from Invitrogen.

ARF1 Mutagenesis, Expression, and Purification

cDNA constructs for wild type (WT) and the constitutively active mutant (Q71L) ARF1 were obtained from J. Rothman (25, 26). ARF1-inactive mutant (T31N) (26) was made by site-directed mutagenesis and confirmed by DNA sequencing. For preparation of myristoylated ARF1 proteins, BL21(DE3) Gold bacterial cells were co-transformed with a second plasmid encoding the yeast *N*-myristoyltransferase (27). Briefly, ARF1 expression was induced in the presence of 50 μM myristic acid. Myristoylated ARF1 proteins were enriched by 35% saturation of ammonium sulfate followed by purification on a DEAE-Sepharose column as described previously (28). Fractions containing myristoylated ARF1 eluted between 50 and 150 mM NaCl were pooled, buffer exchanged, and concentrated on an AmiconUltra-10 filter device (Millipore) in 50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 1 mM dithiothreitol, 2 μM GDP, to a concentration of 2–5 mg/ml, and aliquots were stored at −80 °C. SDS-PAGE analysis showed that ARF1 is purified to over 90% homogeneity (supplemental Fig. 1*A*). Western blot analysis using a monoclonal antibody against ARF1 confirmed this result. This antibody, however, has lower affinity to ARF1(Q71L) compared with wild type or the T31N mutant (supplemental Fig. 1*B*).

In Vitro Budding Assay and Analysis

Golgi membranes were purified from rat liver (29). Interphase (IC) and mitotic (MC) cytosols were prepared from HeLa S3 cells (21). Cdc2 kinase (cdc2/cyclin B1) and plk kinase were expressed and purified as described, and kinase activity was measured (23). Coatomer complex was purified from rabbit cytosol (30). Golgi budding assay was performed as described previously (22, 31). Briefly, Golgi membranes (200 μg) were mixed with 10 mg of IC or MC or with purified coatomer (100 μg), recombinant myristoylated ARF1 (50 μg), 1 mM GTP, and an ATP-regenerating system (10 mM creatine phosphate, 1 mM ATP, 20 μg/ml creatine kinase, 20 ng/ml cytochalasin B), in assay buffer (50 mM Tris-HCl, pH 7.4, 0.2 M sucrose, 50 mM KCl, 20 mM μ-glycerophosphate, 15 mM EGTA, 10 mM $MgCl_2$, 2 mM ATP, 1 mM GTP, 1 mM glutathione, and protease inhibitors), in a final volume of 1000 μl. After incubation for 20 min at 37 °C, 250 mM KCl in cold assay buffer was added to stop the reaction and to release the vesicles. In some experiments 50 μg/ml BFA (Invitrogen) was included. To separate the membranes from the soluble proteins, samples were centrifuged in a TLA55 rotor at 55,000 rpm for 60 min. To analyze the membranes by equilibrium gradients, reactions were directly loaded onto a step gradient comprised of 1.0 ml 0.5 M, 1.5 ml 0.8 M, 2 ml 1.2 M, 2 ml 1.4 M, and 2 ml 1.6 M sucrose in assay buffer containing 250 mM KCl. Membranes were centrifuged to equilibrium at 200,000 × *g*, 4 °C for 3 h in a near vertical rotor (NVT65, Beckman Instruments, 50,000 rpm). COPI-coated vesicles typically peaked at 1.2–1.3 M sucrose, whereas uncoated Golgi

remnants peaked at about 0.8 M sucrose. Fractions were diluted 3-fold with a buffer without sucrose, and membranes in each fraction were pelleted by centrifugation in a Beckman TLA55 rotor at 55,000 rpm for 60 min followed by Western blotting analysis. Membrane samples were also fixed and processed for EM. Vesicles were defined as round profiles of ~70 nm in diameter. The percentage of membranes in cisternae was determined by the intersection method (23). The statistical significance of the quantitated results was accessed by Student's *t* test.

ARF1 was depleted from mitotic cytosol on a DEAE-Sepharose column used for ARF1 purification following the method described previously (32). Briefly, mitotic cytosol was loaded onto the column, and three fractions were collected as follows: ARF-depleted pool 1 that did not bind to the column; ARF-enriched pool 2 that bound to the column but was eluted with a buffer containing 65 mM KCl; and ARF-depleted pool 3 that contained proteins eluted with a buffer containing 500 mM KCl. Pools 1 and 3 were mixed and concentrated by on a Centricon filter device to the original concentration as ARF1-depleted mitotic cytosol.

Cell Culture, Synchronization, and Subcellular Fractionation

NRK cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). To enrich NRK cells in M phase, subconfluent cells (~75% confluent) were washed by phosphate-buffered saline and incubated in growth medium supplemented with 0.5 μg/ml nocodazole for 15 h (Sigma). Mitotic cells were collected by shake-off (33, 34). Typically, the mitotic index was about 80% when examined under a fluorescent microscope according to the condensation of the chromosomes stained with Hoechst 33258. To avoid unspecific pharmacological effects of nocodazole on mitotic cells, NRK cells were treated for 14 h with 2.5 μg/ml aphidicolin (Calbiochem) to enrich cells in G_1/S phase. Six to nine hours after removal of the drug, mitotic cells were collected by shake-off. Confluent interphase (nonsynchronized) cultures were cleaned by the shake-off method to remove mitotic cells, washed with phosphate-buffered saline, and harvested using a cell scraper. Normally 8 × 15-cm dishes of NRK cells were used for mitotic cell collection and two dishes for interphase collection. The mitotic index of interphase cells was lower than 1%. For BFA treatment, mitotic cells were treated with 5 μg/ml BFA in growth medium at 37 °C for 10 min before the collection of the cells.

Spinner HeLa cells were cultured in RPMI 1640 medium supplemented with 7.5% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells grown in suspension were synchronized by incubation in growth medium with 0.5 μg/ml nocodazole for 20–22 h until the mitotic index reached 95% or higher. Synchronized and nonsynchronized HeLa cells were collected by centrifugation. 200 ml of HeLa cells in suspension culture were used for mitotic or interphase cell collection.

NRK or spinner HeLa cells were washed with homogenization buffer (0.25 M sucrose, 1 mM EDTA, 1 mM magnesium acetate, 10 mM HEPES-KOH, pH 7.2, and protease inhibitors) and resuspended in 800 μl of homogenization buffer by pipetting (4 times blue tip, 6 times syringe). Cells were cracked with a ball bearing homogenizer as monitored under a microscope by trypan blue exclusion to a breakage of 75–80%. The homogenate was centrifuged for 10 min at 1000 × *g*, 4 °C. The post-nuclear supernatant (PNS) was removed and subjected to ultracentrifugation in a TLA55 rotor at 120,000 × *g* for 1 h. The supernatant (cytosol) was removed, and the membranes in the pellet were resuspended in homogenization buffer. Equal volume fractions of the PNS, cytosol, and membrane fractions were analyzed by SDS-PAGE and Western blotting. Protein bands were quantitated using

the NIH ImageJ software. The PNS was also fractionated on a sucrose gradient as described above, and fractions were analyzed by Western blotting.

Microinjection and Immunofluorescence Analysis

Capillary microinjection was performed with a semiautomatic system consisting of a Transjector 5246 and a Micromanipulator 5171 (Eppendorf). NRK cells grown on glass coverslips were treated for 14 h with 2.5 μg/ml aphidicolin (Calbiochem) to enrich cells in G_1/S phase. Eight hours after removal of the drug, the cells were injected with 1.2 mg/ml ARF1(Q71L) together with 2 mg/ml mouse IgG (Sigma) as an injection marker. The cells were incubated for 60–90 min at 37 °C and fixed and permeabilized for 10 min in methanol at −20 °C. For brefeldin A treatments, the injected cells were incubated in growth medium for 30 min followed by a 5-min incubation in the presence of 5 μg/ml brefeldin A. The cells were then labeled with polyclonal antibodies against GM130 or β-COP followed by goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes). The co-injected mouse IgGs were stained with goat anti-mouse Alexa Fluor 594 antibodies (Molecular Probes). DNA was stained with Hoechst 33342, and the cells were mounted in Moviol 4-88 (Calbiochem). Fluorescence analysis was performed using a Plan-Apochromat 63×/1.4 DIC objective (Zeiss) and an Axiovert 200 M microscope (Zeiss). Images were captured with an Orca-285 camera (Hamamatsu) and the software package Openlab 4.02 (Improvision).

RESULTS

Golgi Fragmentation Requires ARF1 Activity

To test whether mitotic Golgi fragmentation requires ARF1 activity, we used a well characterized system for COPI-vesicle formation *in vitro*. The incubation of highly enriched rat liver Golgi membranes with coatomer and myristoylated ARF1 leads to budding of COPI vesicles (22, 31, 35). Three forms of myristoylated ARF1, the wild type (WT), the constitutively active ARF1(Q71L), or the inactivated mutant ARF1(T31N) (26), were expressed in bacteria and purified following the established methods (28). We first examined whether treatment of Golgi membranes with these purified ARF1 proteins, in the presence of purified coatomer, could lead to Golgi fragmentation. As shown in Fig. 1, treatment of Golgi membranes with purified WT ARF1 and coatomer led to extensive fragmentation of Golgi membranes (Fig. 1*A*). Quantitation of the results showed that about 43% of membranes were vesiculated in the presence of WT ARF1, a significant increase compared with treatment with coatomer alone (Fig. 1*I*). A similar result was obtained with the constitutively active form ARF1(Q71L) (Fig. 1*B*), a mutant restricted to the GTP-bound form, with 41% of membranes in vesicles (Fig. 1*B*). In contrast, no fragmentation was observed when the Golgi membranes were treated with the inactive form ARF1(T31N) (Fig. 1*C*), a mutant that has a preferential affinity for GDP compared with the wild type protein (26). Only about 13% of membrane in vesicles was observed, similar to untreated membranes (12%) (Fig. 1*I*). Detailed examination of EM images at higher magnification indicated that most of the vesicles were coated in the presence of WT ARF1 or ARF1(Q71L) (data not shown). Incubation of purified Golgi membranes with purified coatomer did not significantly fragment the membranes (Fig. 1*E*), with about 12% of membrane in vesicles (Fig. 1*I*), suggesting that ARF1 activity is required for the vesiculation. This effect is because of the ARF1 protein added, because analysis of purified Golgi membranes by Western blotting showed that essentially no endogenous ARF1 proteins were associated with the membrane under the experimental conditions (data not shown).

To mimic mitotic conditions, we added purified mitotic kinases cdc2 (in complex with cyclin B1) and polo-like kinase (plk). These kinases mimic mitotic cytosol to phosphorylate most of the Golgi proteins known to be mitotically phosphorylated, such as GRASP65 and GM130 (23, 36, 37) which are involved in mitotic Golgi disassembly. In the presence of mitotic kinases, fragmentation of the Golgi by WT (Fig. 1*F*) and active ARF1(Q71L) (Fig. 1*G*) was significantly enhanced. The number of vesicles increased about 2-fold compared with ARF1 and coatomer, resulting in 80% of the total membranes fragmented into COPI vesicles (Fig. 1*I*). In contrast, the inactive ARF1(T31N) did not fragment Golgi membranes even in the presence of mitotic kinases (Fig. 1*I*), suggesting that ARF1 is not inactivated under mitotic conditions, at least not in this cell-free assay.

Active ARF1 Associates with Fragmented Golgi Membranes

It has been shown that the active GTP-bound form of ARF1 associates with membranes, recruits coatomer, and promotes vesicle budding. Upon GTP hydrolysis, ARF1 is inactivated and disassociates from the membrane (26, 38). To test whether ARF1 is associated with the membranes after mitotic fragmentation of the Golgi membranes, we pelleted the membranes, including the Golgi remnants and vesicles, by high speed centrifugation followed by analysis of ARF1 by Western blotting. As shown in Fig. 2*A*, total membranes were pelleted efficiently, as indicated by GRASP65 and Gos28, two Golgi-associated membrane proteins. Significant amounts of WT ARF1 and ARF1(Q71L) were found in the membrane pellets that contained COPI vesicles indicated by the *β*-COP subunit, whereas no signal was detected in the pellet with the inactive ARF1(T31N). Because recruitment of ARF1-GTP to the membrane is essential for COPI vesicle budding, this strongly suggests that ARF1 is active under mitotic conditions; without ARF1 activity, no vesicles were generated. Coatomer was also recruited onto the membrane by WT ARF1 or ARF1(Q71L), but not the inactive ARF1(T31N) (Fig. 2*A*, *lanes 3* and *6 versus 9*), which is consistent with the ARF1 results. As *β*-COP was largely found in the pellet fraction with active ARF1 (Fig. 2), it indicates that most of the vesicles are coated, which further suggests that ARF1 is maintained in the GTP-bound form. The pattern of ARF1 association with membranes was not changed in the presence of mitotic kinases cdc2 and plk (Fig. 2*B*), with ARF1(WT) and ARF1(Q71L) largely associated with membranes, and with no ARF1(T31N) in the membrane fraction. This result shows that ARF1 remains active in the presence of these mitotic kinases.

The membrane binding property of ARF1 was then tested in the presence of BFA, a chemical compound that is known to inactivate ARF1 (39). BFA abolished the membrane binding property of wild type ARF1 but not the Q71L mutant (Fig. 2*C*, *lane 6*). This result confirms that only active ARF1 is associated with Golgi membranes under the experimental conditions.

To determine whether the ARF1-associated Golgi fragments are COPI vesicles, we separated the Golgi membranes treated with ARF1, coatomer, and mitotic kinases on a 0.5–1.6 M sucrose gradient. After centrifugation to equilibrium, membrane-bound proteins in each fraction were collected by high speed centrifugation. Analysis by Western blotting revealed that the Golgi remnants were found in fractions 2–3, as indicated by GRASP65 (Fig. 3*A*), which is consistent with the density of Golgi membranes, equivalent to 0.8 M sucrose (Fig. 3*B*) (35). The coated vesicle fraction peaked at fraction 8, as indicated by the *β*-COP subunit of the coatomer, which is consistent with the reported density of coated COPI vesicles, equivalent to 1.2 M sucrose (Fig. 3*B*) (22, 31). With WT ARF1, the peak (fractions 7–9) was broader than with the constitutively active ARF1 (fractions 8 and 9) (Fig. 3*A*), suggesting that uncoating of COPI vesicles occurs more frequently for WT ARF1 than that for ARF1(Q71L) during the reaction and/or the centrifugation. Consistent with this result, the peak for *β*-COP was also broader with WT ARF compared with ARF1(Q71L)

(Fig. 3*A*). The signal for WT ARF1 appeared stronger compared with ARF1(Q71L) because the antibody we used has a higher affinity to WT ARF1 and ARF1(T31N) compared with ARF1(Q71L) (supplemental Fig. 1*B*). The inactive form of ARF1 was not found to be associated with membranes in any fraction; there was also no *β*-COP signal detected associated with membranes (Fig. 3*A*). Analysis of nonmembrane-bound proteins in each fraction revealed that soluble ARF1 remained on the top of the gradient (Fig. 3*A*, *sup*), consistent with the result that ARF1(T31N) does not bind to the membrane and does not promote vesicle budding (Fig. 1). This result indicates that active ARF1 is associated with COPI vesicles under mitotic conditions. Besides ARF1 and coatomer, several membrane and secretory proteins were enriched in the vesicle-containing fractions. These include the Golgi SNARE protein Bet1 (Fig. 3*C*) and syntaxin 6 (not shown), Golgi enzymes *α*-mannosidase I and II, Golgi structural protein Golgin-84 and Giantin, and the secretory protein rat serum albumin (Fig. 3*C*).

To further confirm that the membranes in *β*-COP concentrated fractions are COPI vesicles, we pooled fractions 1–3 and 7–9. Membranes in each pool were collected by centrifugation and analyzed by EM. As shown in Fig. 3*D*, fractions 1–3 contained the Golgi remnants, TGN membranes (indicated by the dense lipoproteins), and other vesicular-tubular structures. Fractions 7–9 contained essentially pure COPI vesicles (Fig. 3*E*), with a uniform size of about 70 nm in diameter. Coat proteins were visible on the vesicles at higher magnifications (Fig. 3*F*). Further measurements showed that the size of the vesicles generated by ARF1 and coatomer treatment (69.9 ± 7.4 nm, $n = 50$) was identical to those generated by mitotic cytosol treatment (72.1 ± 6.0 nm, $n = 50$). This size is consistent with the previous reports (40). Taken together, these results show that active ARF1 is associated with COPI vesicles even when the Golgi membranes are mitotically fragmented.

ARF1-dependent Golgi Fragmentation upon Treatment with Mitotic Cytosol

To ensure that the results we obtained using purified mitotic kinases represent mitotic conditions, we examined the fragmentation of Golgi membranes when treated with mitotic cytosol or with interphase cytosol as a control. If mitotic Golgi fragmentation is because of COPI vesicle budding, which requires ARF1 activity, Golgi membranes should be fragmented upon treatment with mitotic cytosol treatment. Alternatively, no COPI vesicles should be generated if fragmentation of the Golgi during mitosis is because of inactivation of ARF1 (17). As shown in Fig. 4, when Golgi membranes were treated with interphase cytosol, the stacks remained intact, with only a few vesicles, which is similar to untreated Golgi membranes (Fig. 4*D*). However, when treated with mitotic cytosol, the Golgi membranes were fragmented to tubular and vesicular structures. Quantitation showed that about 50% of the membranes were found in vesicles (Fig. 4*D*) after treatment with mitotic cytosol, which is significantly higher than interphase cytosol treatment (22%). We then analyzed ARF1 association with vesicles by sucrose equilibrium centrifugation and Western blotting. The result showed that at least a fraction of ARF1 was associated with COPI vesicles that were enriched in fraction 8 (Fig. 4, *E* and *F*), as indicated by *β*-COP. This is consistent with the result described in Fig. 3*A* using purified proteins and shows that fragmentation of Golgi membranes upon mitotic cytosol treatment requires ARF1 activity. As noted in the figure legend, a higher molecular weight form of *β*-COP showed up when purified Golgi membranes were treated with cytosol (Fig. 4) or in living cells (supplemental Fig. 3), especially under mitotic conditions. It is not clear whether this is because of phosphorylation of *β*-COP (41), or a different *β*-COP isoform (42), or ubiquitination because the migration shift is about 8 kDa. Our recent study indicated that a Golgi protein is ubiquitinated during mitosis, which is required for the subsequent Golgi reassembly (43). The nature of this modification and its effect on COPI vesicle uncoating and/or fusion are worthwhile to explore.

To determine whether ARF1 activity is required for mitotic Golgi fragmentation, we depleted ARF1 from the mitotic cytosol using a DEAE-Sepharose column following a procedure described previously (32). The depletion efficiency for ARF1 was high (Fig. 4*G*, *lanes 3* and *4 versus 1* and *2*). ARF1 depletion did not affect other proteins tested, such as *β*-COP and *α*-tubulin (Fig. 4*G*). ARF1-depleted mitotic cytosol failed to fragment the Golgi membranes, whereas replenishment of wild type ARF1 or the Q71L mutant, but not the T31N mutant, restored the activity of the cytosol and led to fragmentation of the Golgi membranes to a similar extent as adding back the ARF1-containing fraction collected during the depletion procedures (Fig. 4*H*). This result showed that ARF1 activity in the mitotic cytosol is required for fragmentation of the Golgi membranes.

ARF1 Remains Active during Mitosis in Cells

Next, we tested whether ARF1 remains active or is inactivated in mitotic cells. Interphase or mitotic NRK cells (collected by nocodazole block and shake-off) were subjected to subcellular fractionation. Membranes from the PNS or post-chromosomal supernatants were pelleted by ultracentrifugation at 150,000 × *g* for 60 min. Proteins in the membrane and the cytosol (supernatant) were analyzed by Western blotting. Under interphase conditions, about 22% of ARF1 was associated with membranes (Fig. 5, *A* and *B*), indicating that this fraction of ARF1 is active in interphase cells. Under mitotic conditions, about 60% of ARF1 was found in the membrane fraction, which is significantly higher than under interphase conditions. To ensure that this result was not an unspecific pharmacological effect caused by nocodazole treatment, we collected mitotic NRK cells by shake-off. NRK cells were first enriched in G_1/S phase by aphidicolin block and released. Cells that entered mitosis were collected by shake-off and analyzed by a similar method described above. A significant amount of ARF1 was associated with the membranes in mitotic cells, which was larger than the amount in interphase cells (supplemental Fig. 2). A similar result was obtained when a different cell line, HeLa cells, was used (data not shown). Taken together, these results indicated that a significant amount of ARF1 is active in mitotic cells.

We then performed the subcellular fractionation experiment using mitotic cells treated with BFA, which is known to inactivate ARF1 (39). Treatment of mitotic cells with BFA for 10 min was sufficient to remove essentially all ARF1 from the membrane fraction (Fig. 5*C*). Because the Golgi was already fragmented prior to BFA treatment, this result indicated that the membrane-associated active ARF1 in mitotic cells could be inactivated, but ARF1 inactivation is not a cause of mitotic Golgi fragmentation.

We then used sucrose gradients to analyze whether the membranes that ARF1 is associated with are COPI vesicles. As shown in supplemental Fig. 3, Golgi membranes were found on the top of the gradient, which peaked in fractions 3 or 4, as indicated by the Golgi matrix protein GM130 or the Golgi t-SNARE Gos28 (supplemental Fig. 3). The Golgi v-SNARE Bet1, however, showed an additional peak in fraction 7 together with *β*-COP especially under mitotic conditions, suggesting that some of this protein was recruited into COPI vesicles. ARF1 was found in two peaks under both interphase and mitotic conditions as follows: fractions 3–4, which contained Golgi membranes; and fractions 7–9, which contained COPI vesicles with the density of about 1.2 M sucrose (supplemental Fig. 2) and indicated by *β*-COP. This is consistent with our result using purified components (Fig. 3, *A* and *B*) and further shows that ARF1 exists as the membrane-bound active form in mitotic cells and is required for mitotic Golgi fragmentation.

Microinjection of the Constitutive Active ARF1 Does Not Block Mitotic Golgi Fragmentation

Our data described above showed that ARF1 is active during mitosis and that this activity is required for mitotic Golgi fragmentation. To test the effect of ARF1(Q71L) on mitotic Golgi fragmentation in tissue culture cells, we microinjected purified ARF1(Q71L) into NRK cells enriched in G_2 phase 60 min before the cells were about to enter mitosis. Cells were fixed as soon as they entered mitosis and stained for GM130. Microinjection of ARF1(Q71L) caused partial disassembly of the Golgi in interphase cells (Fig. 6*A*); however, this did not seem to affect cell progression into mitosis, as indicated by the condensed chromosomes in the metaphase plate and the round cell shape. In most prometaphase and metaphase cells we observed (22 cells of 26 counted), the Golgi was fragmented (Fig. 6, *B* and *C*). Later in telophase, the Golgi reformed in both daughter cells (Fig. 6*D*). To demonstrate that the microinjected ARF1(Q71L) was functional, injected interphase cells were treated for 5 min with BFA. In 190 injected cells of 191 counted, the COPI coat proteins still decorated the Golgi (Fig. 6*E*, *β-COP*), indicating that ARF1(Q71L) could not be inactivated by BFA treatment, which is consistent with the result described in Fig. 2*C*. In the same experiment, *β*-COP was stripped off the Golgi membrane in noninjected neighboring cells (Fig. 6*E*). The results suggest that constitutively active ARF1 has no significant effect on the mitotic fragmentation of the Golgi apparatus or on the mitotic progression of the observed cells.

DISCUSSION

We have used several biochemical and cell biological approaches to determine the contribution of ARF1 to the disassembly process of the Golgi apparatus at the beginning of mitosis. Our results show that ARF1 remains active during mitosis and that this activity is required for mitotic Golgi fragmentation. A previous study has shown that vesicle budding is involved in mitotic fragmentation of Golgi membranes in a cell-free system (21). However, this study used mitotic cytosol, and the role of COPI vesicle formation was not confirmed. In this study, we chose to use the well established *in vitro* COPI vesicle budding assay using purified ARF1 and coat proteins and confirmed the role of COPI vesicle budding in mitotic Golgi fragmentation. We used the mitotic kinases cdc2 and plk to mimic mitotic conditions. Previous studies have shown that these two mitotic kinases mimic mitotic cytosol by modifying most of the mitotically phosphorylated Golgi proteins so far identified, including GRASP65 (23, 36), GM130 (37), RII*α* regulatory subunit of cAMP-dependent protein kinase (44), Golgin-67 (45), and Nir2 (18). These kinases were also shown to mimic mitotic conditions for mitotic Golgi fragmentation (23, 46, 47). In the presence of mitotic kinases, the Golgi was fragmented (Fig. 1, *A* and *B*), and the amount of ARF1 bound to membranes remained high (Fig. 2), showing that ARF1 is not inactivated in the presence of mitotic kinases. This evidence was further strengthened using mitotic cytosol, which fragmented the Golgi membranes into vesicles much more efficiently than interphase cytosol. This activity was abolished when ARF1 was depleted from the cytosol and was restored when recombinant active ARF1 was replenished (Fig. 4). Therefore, our *in vitro* experiments provided strong evidence that ARF1 activity is required for mitotic Golgi disassembly.

We then showed that this conclusion is also true in cells. Fractionation of interphase and mitotic NRK cells showed that a fraction of ARF1 is associated with COPI vesicles and Golgi membranes during mitosis (Fig. 5 and supplemental Fig. 2), suggesting that this population of ARF1 is active during mitosis. Compared with interphase cells, the membrane-bound portion is more prominent in mitotic cells (Fig. 5 and supplemental Fig. 2), suggesting that ARF1 remains active during mitosis. Furthermore, using sucrose gradient centrifugation, we confirmed that at least some of the ARF1-bound membranes are COPI vesicles (supplemental Fig. 3). Therefore, we conclude that mitotic Golgi fragmentation

utilizes a different mechanism than the effect caused by BFA. Our study combined a variety of techniques to study the role in ARF1 in mitotic Golgi disassembly, both *in vitro* and *in vivo*, and provided strong evidence that mitotic Golgi fragmentation requires ARF1 activity and COPI vesicle budding. Further study on the machinery governing disassembly and reassembly of the Golgi apparatus is essential for understanding the mechanism of Golgi inheritance in mammalian cells.

Another study based on live cell imaging to localize ARF1 tagged with GFP in mitotic cells (15, 17) showed that the distribution of ARF1-GFP on Golgi membranes and in the cytosol changed as the cells entered mitosis, with the pool bound to the Golgi decreasing and the pool in the cytosol increasing (17). However, it is not clear whether the diffused pattern of ARF1 during mitosis consists of soluble cytosolic ARF1,or if ARF1 is bound to Golgi-derived vesicles, because the size of these vesicles is well below the spatial resolution of light microscopy (14, 15). Another piece of evidence is the fact that overexpression of ARF1(Q71L) blocks mitotic Golgi fragmentation (17). ARF1(Q71L) was overexpressed by transient transfection over a long time period. Because ARF1(Q71L) directly affects membrane trafficking, it is unclear whether mitotic Golgi fragmentation is directly blocked by ARF1(Q71L) or by an unspecific effect caused by its chronic overexpression. In fact, when we microinjected purified ARF1(Q71L) protein into cells, mitotic Golgi fragmentation and cell cycle progression through mitosis appeared essentially unchanged (Fig. 6). Because in this experiment the presence of ARF1(Q71L) in the cell was reduced to 60 min, this result provided more direct evidence that ARF1 is not inactivated in the progress of mitotic Golgi fragmentation. In addition, using a biochemical approach combined with electron microscopy, our result showed for the first time that ARF1 is bound to vesicles when Golgi membranes are disassembled in the presence of wild type or active ARF1, but not the inactive ARF1 mutant. This result was further confirmed using mitotic cytosol and synchronized cells. Our results showed that at least a portion of the endogenous ARF1 is associated with the membranes even when the Golgi is fragmented, and this association is abolished when cells were treated with BFA (Fig. 5). ARF1 itself has a very low intrinsic GTPase activity, and GTP hydrolysis by ARF1 requires the GTPase-activating proteins (ARF-GAP) (48, 49). The distribution and activity of ARF-GAP proteins during mitosis have not yet been investigated. In addition, we observed a significant migration shift of β-COP on the gel, especially under mitotic conditions (Fig. 4 and supplemental Fig. 3), which might be due to the modification of this protein. Whether this modification inhibits vesicle uncoating and thus fusion during mitosis is an intriguing possibility. Nevertheless, our results strongly suggest that ARF1 is not inactivated during mitosis and that active ARF1 is required for mitotic Golgi fragmentation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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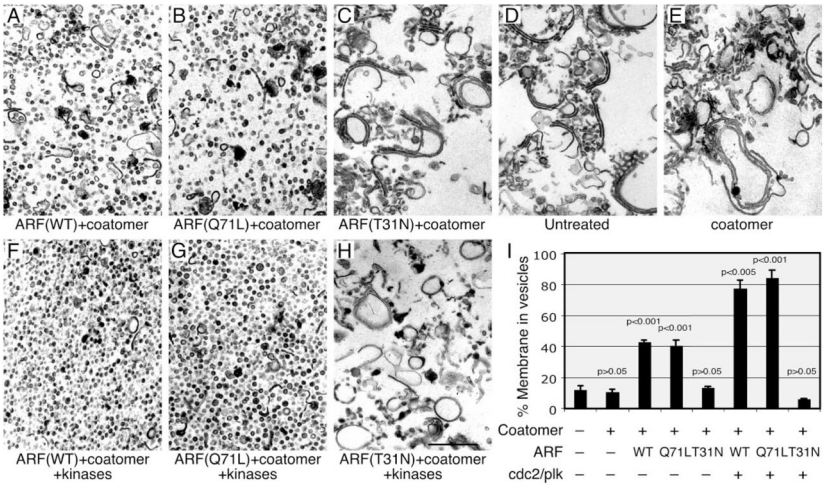

FIGURE 1. ***In vitro*** **Golgi fragmentation using purified coatomer and ARF1**

EM photographs showing fragmentation of purified Golgi membranes purified ARF1, coatomer, and kinases. Purified rat liver Golgi membranes were treated with wild type ARF1 and coatomer (*A*), constitutively active ARF1(Q71L) and coatomer (*B*), or with inactivated ARF1(T31N) and coatomer (*C*) at 37 °C for 20 min. Untreated (*D*) or coatomer alone (*E*) were used as controls. In experiments, purified cdc2 and plk were included (*F–H*). Membranes were fixed and processed for EM. *Bar*, 0.5 μm. *I*, quantitation of *A–H*, by the intersection method, to estimate the percentage of membrane in vesicles. Results represent the mean of three independent experiments ± S.E. To access the statistical significance, the result for treatment with coatomer alone was compared with that of no treatment; results for ARF1 WT, Q71L, and T31N mutants in absence of kinases were compared with that of treatment with coatomer alone; and results for WT, Q71L, and T31N mutants in the presence of kinases compared with those without mitotic kinases, respectively. Note that the WT and the active Q71L mutant of ARF1 fragmented the Golgi but the inactive mutant did not. Fragmentation was enhanced by the presence of purified cdc2 and polo-like kinases (kinases).

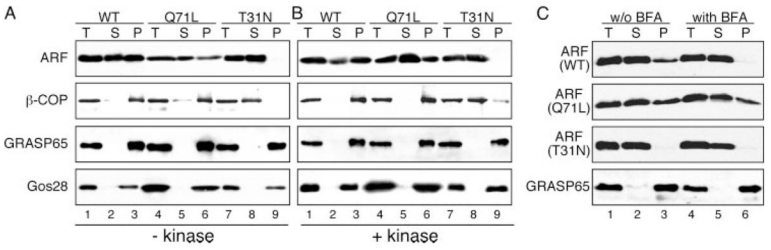

FIGURE 2. Association of activated ARF1 with membranes after the budding assay

Purified rat liver Golgi membranes were incubated with either wild type ARF1, constitutively active ARF1(Q71L), or with inactive ARF1(T31N) and coatomer, in the presence of (*B*) or absence of (*A*) purified mitotic kinases cdc2 and plk, at 37 °C for 20 min followed by isolation of the total membranes by centrifugation. Equal fractions (by volume) of the total (*T*, the reactions before centrifugation), the supernatant (*S*, the soluble fraction), and the pellet (*P*, the membranes) were analyzed by Western blotting for ARF1 and the membrane-associated proteins GRASP65 and Gos28. Note that a large proportion of wild type and active ARF1(Q71L) proteins were found in the pellets (*lanes 3* and *6*) but not the inactive ARF1(T31N) mutant (*lane 9*). Note that addition of kinases did not reduce the amount of ARF1 bound to the membrane fractions. *C*, only active ARF1 can bind to Golgi membrane. Purified ARF1 was incubated with Golgi membranes as in *A* except that BFA was added in some reactions. Note that ARF1 WT and Q71L can bind to Golgi membranes in the absence of BFA (*lane 3*), and only Q71L can bind to membranes in the presence of BFA (*lane 6*).

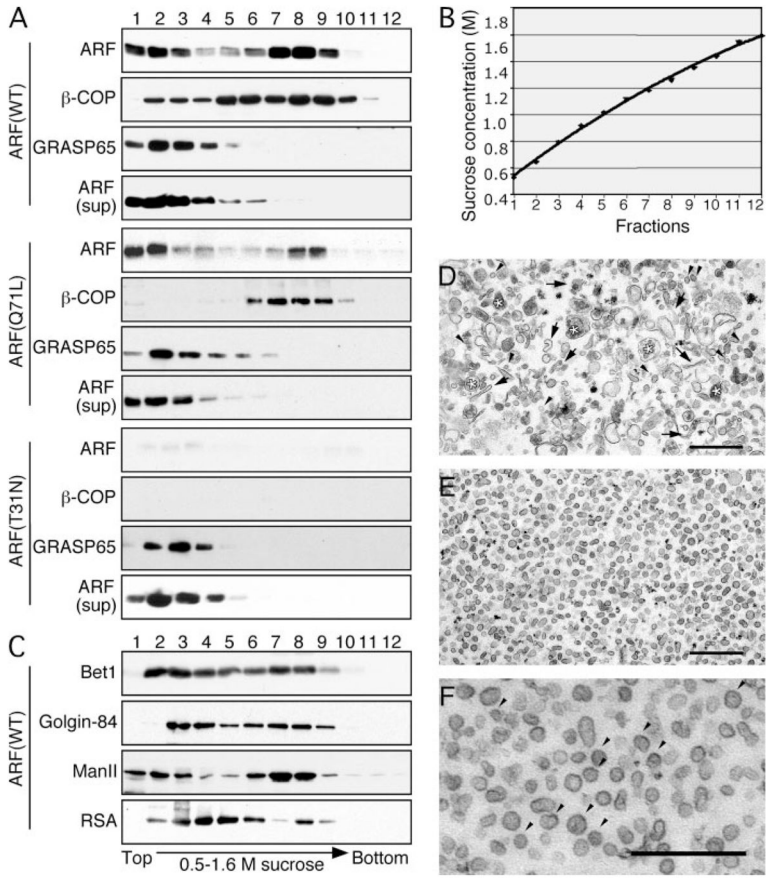

FIGURE 3. ARF1 binds to COPI vesicles

Purified rat liver Golgi membranes were treated with wild type ARF1 in the presence of purified coatomer and mitotic kinases at 37 °C for 20 min. Reactions were fractionated by equilibrium centrifugation on a sucrose gradient (0.5–1.6 M). Membranes in each fraction were pelleted, and equal fractions (by volume) were analyzed by Western blotting for ARF1, *β*-COP, and GRASP65 and shown in *A*. ARF1 (*sup*) refers to ARF1 in the supernatant after membrane pelleting. Sucrose concentrations were measured and shown in *B*. Membrane and cargo proteins in samples from *A* using wild type ARF1 were further analyzed by Western blotting and are shown in *C*. *ManII*, *α*-mannosidase II; *RSA*, rat serum albumin. Membranes in fractions 1–3, which contained Golgi remnants, and in fractions 7–9, which contained COPI vesicles, were collected and processed for EM. EM images are shown in *D* and *E*, respectively. Golgi remnants are indicated by *arrows* and uncoated COPI vesicles by *arrowheads* (*D*). Lipoprotein enriched TGN elements are indicated by *asterisks* (*D*). A large magnification of vesicle enriched fraction is shown in *F* to illustrate the COPI coats, indicated by *arrowheads*. *Bar*, 0.5 *μ*m.

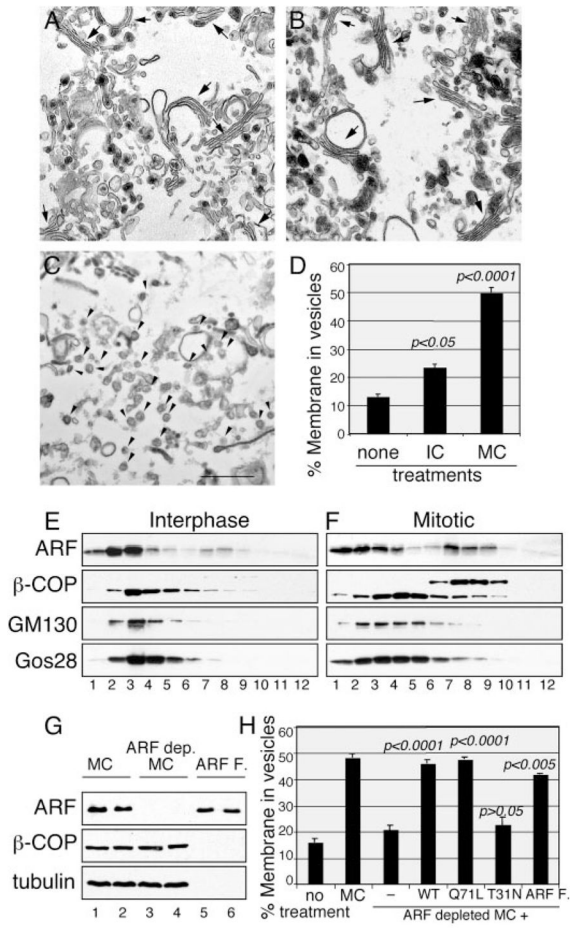

FIGURE 4. Fragmentation of Golgi membranes by mitotic cytosol treatment is ARF1-dependent

Purified Golgi membranes (*A*) were treated with interphase HeLa cell cytosol (*IC*) (*B*) or mitotic cytosol (*MC*) (*C*) at 37 °C for 20 min. Membranes were fixed and processed for EM. Golgi stacks (*A* and *B*) are indicated by *arrows* and COPI vesicles (*C*) by *arrowheads. Bar*, 0.5 μm. *D*, quantitation of *A–C*, by the intersection method, to estimate the percentage of membrane in vesicles. Results represent the mean of three independent experiments ± S.E. Note the increased number of vesicles after treatment with mitotic but not interphase cytosol. *E* and *F*, analysis of protein components of *B* and *C* using sucrose equilibrium gradients. Membranes from *B* and *C* were loaded onto a 0.5–1.6 $_{M}$ (16 – 45%) sucrose gradient followed by ultracentrifugation to equilibrium and fractionated from the top to the bottom. Membranes were pelleted, and equal proportions of membrane-bound proteins in each fraction were analyzed. A higher molecular weight form of *β*-COP appears after treatment with mitotic cytosol and peaks at COPI vesicle-containing fractions 7 and 8 (1.2–1.3 $_{M}$ sucrose). *G*, nontreated mitotic cytosol (*MC*, *lanes 1* and *2*), ARF1-depleted cytosol (*ARF dep. MC*, *lanes 3* and *4*), and ARF1-enriched fraction (*ARF F.*, *lanes 5* and *6*) collected during ARF1 depletion were analyzed by Western blotting for ARF1, *β*-COP, and *α*-tubulin. *H*, mitotic cytosol or ARF1-depleted mitotic cytosol were tested for Golgi fragmentation as in *A* and quantified as in *D*. In some reactions, purified ARF1 proteins or ARF1-enriched fraction (*ARF F.*) were added to ARF1-depleted cytosols, as indicated. Values represent means ± S.E. (n = 5). Statistical significance was analyzed against ARF1-depleted cytosol.

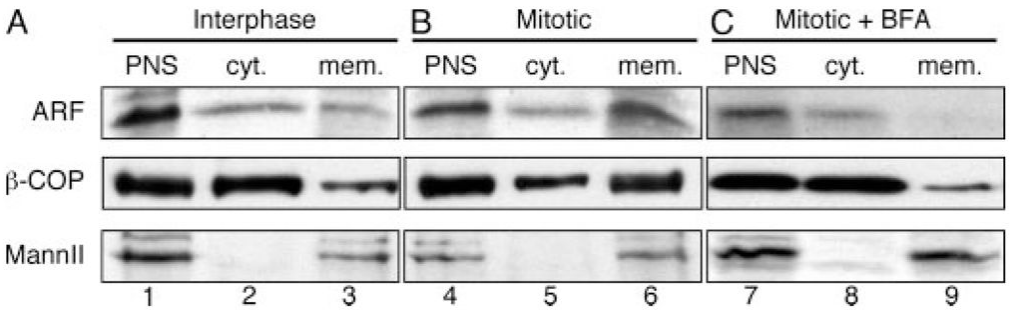

FIGURE 5. ARF1 is associated with membranes during mitosis

Nontreated interphase NRK cells (*A*), mitotic NRK cells synchronized by treatment with nocodazole (*B*), or mitotic NRK cells (as in *B*) treated with BFA for 10 min before harvest (*C*), were homogenized and postnuclear and post chromosomal supernatants centrifuged to separate membranes (P) and cytosol (S). Equal volume proportions of each sample were separated by SDS-PAGE followed by Western blotting for the indicated proteins. Quantitation of the bands showed that about 26% of ARF1 is associated with membranes under interphase conditions (*lane 3*), and about 50% under mitotic conditions (*lane 6*), which is reduced to 11% in the presence of BFA (*lane 9*).

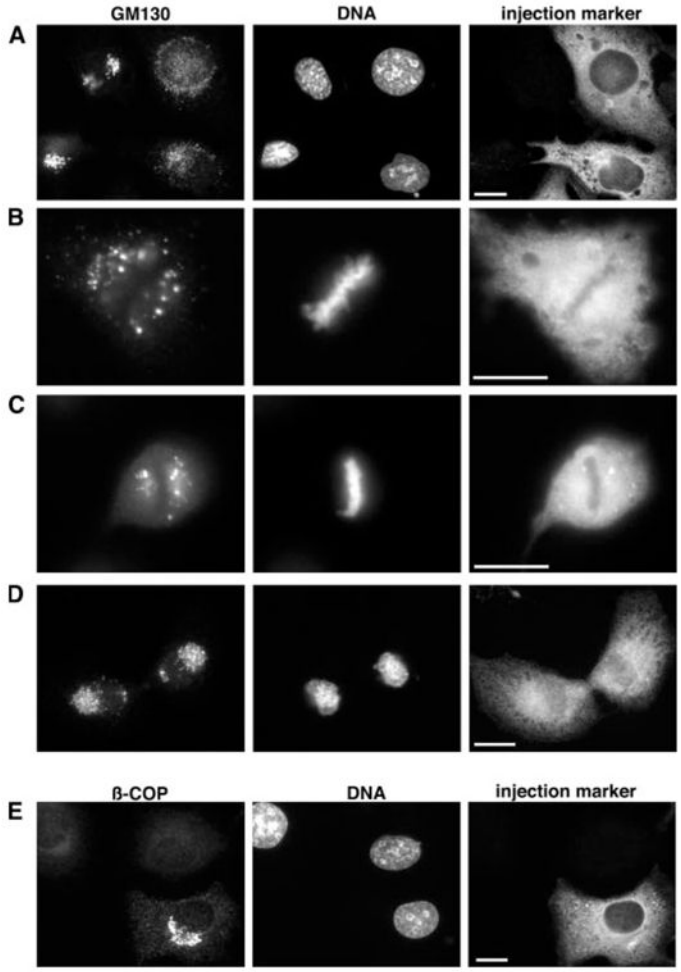

FIGURE 6. Microinjection of ARF1(Q71L) does not block Golgi fragmentation and progression through mitosis

NRK cells enriched in G_2 were microinjected with purified ARF1(Q71L) protein (together with an injection marker) 60 min before entering mitosis. As the cells entered mitosis they were fixed and stained for the Golgi marker GM130 (*left panels*), DNA (*middle panels*), and the injection marker (*right panels*). *A*, interphase. Injection of ARF1(Q71L) led to partial fragmentation of the Golgi. *B* and *C*, prometaphase cells with condensed DNA showed that the Golgi was fragmented. *D*, telophase/late mitosis. Chromosome separation was completed and the injected cell divided into two daughter cells containing a reformed Golgi apparatus. *E*, control. Interphase NRK cells were injected with ARF1(Q71L). After 30 min of incubation in growth medium, they were treated for 5 min with BFA. Note that β-COP is removed from the Golgi in noninjected cells but not in the injected cell, suggesting that the injected ARF1(Q71L) protein remained active in the presence of BFA. *Bar* = 15 μm.