Overexpression of the ARF1 exchange factor ARNO inhibits the early secretory pathway and causes the disassembly of the Golgi complex

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SUMMARY

The small GTPase ARF1 is a key regulator of intracellular membrane traffic. In its active, GTP-bound form, ARF1 is associated with Golgi membranes and promotes the recruitment of the cytosolic coat protein complex, which will result in membrane budding and vesicle formation. ARNO (ARF nucleotide site opener) has been shown to act in vitro as a GTP exchange factor for ARF1. Here, we have investigated the function of ARNO in vivo. By immunofluorescence and cell fractionation, ARNO was found to be mostly cytosolic in HeLa cells. Its overexpression led to a strong inhibition of the secretion of SEAP (secreted form of alkaline phosphatase). Newly synthesized SEAP failed to acquire endoglycosidase H resistance, indicating a block in the early secretory pathway. This effect on secretion was accompanied by a disassembly of the Golgi complex and a redistribution of Golgi resident proteins into the endoplasmic reticulum (ER). On the other hand, ARNO overexpression did not affect the early endocytic pathway. These results show that ARNO functions in vivo in Golgi to ER transport. Its behavior is then consistent with ARNO being an exchange factor for ARF1.

Key words: Small GTPase, Transport vesicle, Golgi, HeLa cell

INTRODUCTION

In eukaryotic cells, transport of proteins along the exocytic and endocytic pathways is mediated by vesicular carriers. The formation of transport vesicles requires the recruitment of cytosolic coat proteins on the membrane of the donor compartment. Clathrin-coated vesicles involved in the early steps of endocytosis and in transport between the trans-Golgi network (TGN) and endosomes are formed with adaptor protein (AP) AP-2 and AP-1 complexes, respectively (see Traub, 1997, for a review). Recently, a third AP complex (AP-3), required for transport to the yeast *S. cerevisiae* vacuole, has been identified (Cowles et al., 1997). Two types of non-clathrin-coated vesicles have been characterized. COPI (coatomer)-coated vesicles have been shown to mediate transport from the endoplasmic reticulum (ER) and/or pre-Golgi compartments to Golgi and possibly intra-Golgi anterograde transport (Orci et al., 1986; Salama and Schekman, 1995). Strong evidence also exists indicating that COPI vesicles are involved in Golgi to ER retrograde transport of proteins bearing ER-retention motifs (Letourneur et al., 1994). COPII-coated vesicles play an essential role in the exit of proteins from the ER (Aridor et al., 1995; Kuehn and Schekman, 1997).

The binding of coat proteins to donor membranes is regulated by small GTPases of the ADP-ribosylation factor (ARF) family. In mammalian cells, the ARF family consists of six ARF proteins and more than ten ARF-like (ARL) proteins (Clark et al., 1993). The best characterized is ARF1, a component of the COPI coat (Kahn et al., 1988; Serafini et al., 1991; Donaldson and Klausner, 1994). ARF1 has been shown to regulate in vitro the assembly/disassembly of COPI coatomer onto Golgi membranes (Serafini et al., 1991; Donaldson et al., 1992a; Orci et al., 1993). ARF1 is also involved in the recruitment of AP-1 complexes on TGN membranes (Stamnes and Rothman, 1993; Traub et al., 1993). In yeast, a protein closely related to ARF1, Sar1, participates in the formation of ER-derived COPII vesicles (d'Enfert et al., 1991; Oka et al., 1991). The function of other ARF and ARL proteins is still unclear. However, good evidence exists that ARF6 regulates some steps of the endocytic pathway, especially the recycling of transferrin receptors from endosomal compartments to plasma membrane (D'Souza-Schorey et al., 1995; Peters et al., 1995).

The first event in COPI recruitment onto membranes is the activation of ARF1. In its inactive conformation, ARF1 is cytosolic and the replacement of GDP by GTP is thought to be coupled to binding of ARF1 to membranes (Donaldson et al., 1992a; Randazzo et al., 1993; Donaldson and Klausner, 1994). The drug Brefeldin A (BFA), which induces the dissociation of coat proteins from membranes and causes a mixing of Golgi and ER compartments, interferes with the GDP/GTP exchange

reaction (Donaldson et al., 1991a,b, 1992b; Helms and Rothman, 1992). An important advance in the understanding of molecular mechanisms of ARF1 activation was the characterization of guanine-nucleotide exchange factors able to catalyze GDP/GTP exchange on yeast and mammalian ARF1 (Peyroche et al., 1996; Chardin et al., 1996). These proteins, termed Gea1/2p (guanine-nucleotide exchange on ARF) and ARNO (ARF nucleotide binding site opener), respectively, share sequence homology with the *S. cerevisiae SEC7* gene product, involved in intra-Golgi transport (Achstetter et al., 1988). The overexpression of yeast ARF1 and ARF2 was shown to suppress the *SEC7* gene product defect (Deitz et al., 1996). In addition, the Sec7 domain of ARNO is sufficient to promote GDP/GTP exchange on ARF1 (Chardin et al., 1996). Gea1 activity is sensitive to BFA, but not that of ARNO, suggesting that ARNO might interact with other(s) protein(s) whose function is altered by BFA. Two other proteins are highly homologous to ARNO (>85% identity): cytohesin-1, a protein expressed in lymphocytes and proposed to be involved in integrin signalling (Meacci et al., 1997), and GRP-1 (for general receptor for phosphoinositides), a major PIP3 binding protein (Klarlund et al., 1997). Another protein of 200 kDa has a BFA-sensitive exchange activity on ARF1 and ARF3 (Morinaga et al., 1997). Finally, a 55 kDa protein displaying a BFA-insensitive ARF1 and ARF3 exchange activity has been purified, but not further characterized (Tsai et al., 1994, 1996).

The aim of this study was to investigate the function of ARNO in vivo. ARNO was transiently overexpressed in HeLa cells and the effects on transport along the biosynthetic/secretory and endocytic pathways and on the morphology of organelles were analyzed. We show that overexpression of ARNO causes a strong inhibition of the early secretory pathway and a BFA-like redistribution of Golgi membranes into the ER.

MATERIALS AND METHODS

Cell culture, infection and transfection procedures

HeLa cells were grown in DMEM medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and penicillin/streptomycin in a 5% CO_2 incubator. Cells were infected with vaccinia virus and transfected with pGEM plasmids bearing various cDNAs as previously described (Martinez et al., 1994, 1997).

Plasmids

The cDNA encoding wild-type ARNO (Chardin et al., 1996) was inserted in pGEM-3 plasmid to generate pGEM-ARNO. The ARNO E156K mutant was obtained by PCR using a primer complementary to the 5′ end of the wild-type ARNO cDNA and the following primer bearing the mutation of aspartic acid at position 156 for a lysine (underlined): 5′ GC GAAGGC CTC CAT CAT CCG GTC AAT TTT CTG GGC CTT TCC GGG T 3′. The PCR product was then cloned in the same vector to generate pGEM-ARNO E156K. The construction of pGEM-SEAP and pGEM-Ii has previously been described (Martinez et al., 1994, 1997).

Antibodies

The following antibodies were kindly donated by the indicated individuals: CTR433, a monoclonal antibody which recognizes a medial Golgi antigen (Jasmin et al., 1989); rabbit anti-Ii antibody (Salamero et al., 1996); mouse antibody against transferrin receptor (White et al., 1992); mouse monoclonal anti-β-COP antibody (Allan

and Kreis, 1986). The monoclonal anti-γ-adaptin antibody (100/3) was purchased from Sigma, the antibody against calf intestine alkaline phosphatase from Tebu (France). ARF1 and ARNO antisera were raised in rabbits against purified recombinant ARF1 and ARNO expressed in *E. coli*. These antibodies were affinity purified on ARF1 and ARNO proteins bound to nitrocellulose. Affinity-purified rabbit anti-Rab1 antibody has been previously described (Saraste et al., 1995).

Immunofluorescence

Cells grown on glass coverslips were processed for immunofluorescence 6 hours after transfection, unless otherwise indicated. They were fixed either with 2.5% paraformaldehyde for 20 minutes, or with methanol for 4 minutes at −20°C, depending on the antibody used, and further incubated with specific antibodies as previously described (Roa et al., 1993). Confocal laser scanning microscopy and immunofluorescence analysis were performed as previously described (Martinez et al., 1997).

Cell fractionation and immunoblotting

To study the partition of endogenous ARNO between membrane and cytosol, confluent HeLa cells in one T75 flask were trypsinized and homogenized with a Dounce homogenizer in the following buffer: 25 mM Hepes, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT, 1 mg/ml glucose and a mixture of protease inhibitors. The post-nuclear supernatant (PNS) fraction was obtained by centrifugation of the cell lysate at 900 *g* for 10 minutes at 4°C. A portion of the PNS was further centrifuged for 15 minutes at 50,000 rpm (100000 *g*), to separate the membrane from the cytosolic fraction. The membrane fraction was resuspended in 1/4 of the original PNS volume. Equivalent amounts of each fraction (about 1/10 of the preparation for PNS, cytosol and membrane) and 3 times the amount of membrane were loaded on a 12% SDS gel and transferred to nitrocellulose. After incubation with affinity-purified anti-ARNO antibody, labelled bands were visualised by the ECL procedure (Amersham).

The same procedure was used to isolate membranes from PNS of transfected cells, which were collected from three 100-mm dishes. One volume of membrane fraction was further treated with 200 volumes of 0.5 M NaCl on ice for 30 minutes and centrifuged at 100,000 *g* for 1 hour. The membrane pellet was then processed for western blot analysis as described above.

Transport of SEAP

Overexpression of the secreted form of alkaline phosphatase (SEAP) was studied in HeLa cells, according to described procedures (Martinez et al., 1994, 1997). Briefly, after a 4 hour 15 minute transfection with pGEM-ARNO, pGEM-ARNO E156K mutant or the empty pGEM-1 vector, together with pGEM-SEAP, cells were depleted of endogenous methionine and cysteine by incubation in RPMI (ICN) lacking these amino acids for 15 minutes, pulsed for 10 minutes in RPMI containing 150 μCi/ml [^{35}S]Promix (Amersham), and chased for various periods of time. At each time point, medium was collected and cells were solubilized in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% BSA) and immunoprecipitated using the anti-alkaline phosphatase antibody. SEAP in the cell fraction was then subjected to endoglycosidase H (endoH) digestion as previously described (Martinez et al., 1994). Samples were analysed by SDS-PAGE on 6%-12% acrylamide gradient gels and autoradiographed.

Glycosylation of Ii

For glycosylation of the MHC class II invariant chains, cells were co-transfected with pGEM-Ii and either the empty pGEM-1 vector, pGEM-ARNO, or pGEM-ARNO E156K for 4 hours 15 minutes and pulsed-labeled as described above. Cells were then chased for 2 hours 30 minutes in the absence or in the presence of 5 μg/ml brefeldin A (BFA; Epicentre Technology). Cell lysates were directly immunoprecipitated with the anti-Ii antibody, or incubated with

agarose-conjugated jacalin beads (Pierce) overnight at 4°C; jacalin-bound material was eluted in 0.8 M galactose as described (Martinez et al., 1997), and immunoprecipitated with the anti-Ii antibody.

Transferrin uptake

The transferrin (Tf) receptor internalization and recycling were studied in HeLa cells plated in 12-well dishes and transfected for 5 hours 30 minutes with pGEM-ARNO, pGEM-ARNO E156K, or with the empty pGEM-1 vector. Cells were incubated with ^{125}I-labelled human Tf (8 μg/ml; approx. 555 μCi/mg). For studying Tf receptor internalization, cells were incubated for 1 hour at 4°C. After extensive washes at 4°C, cell duplicates were incubated in the same medium without radioactivity for different times between 5 and 30 minutes at 37°C. One cell duplicate was acid washed before warming to 37°C to assess the amount of specific ^{125}I-Tf bound to the cells and released by the wash: the amount of radioactivity remaining associated with the cell was taken as the unspecific binding and plotted as the cell-associated ^{125}I-Tf at the 0 time point. At each time point, medium was collected and cells solubilized in 0.1 M NaOH and counted. Internalization was estimated by expressing the percentage of radioactivity found in the cells compared to the total radioactivity found in medium and cells. For receptor recycling experiments, cells were incubated for 11 minutes at 37°C in the presence of ^{125}I-Tf. Following removal of ^{125}I-Tf and extensive washes at 4°C, cells were incubated at 37°C for various times up to 60 minutes and processed as described above. Recycling was estimated as the percentage of radioactivity released in the medium compared to the total found in cells and medium.

RESULTS

Intracellular distribution of ARNO

We first investigated the intracellular distribution of ARNO using a polyclonal antibody raised against the purified recombinant protein expressed in *E. coli*. The affinity-purified antibody recognized in a post-nuclear supernatant of HeLa cells a major species migrating around 47 kDa, which corresponds to the expected molecular mass of ARNO (Fig. 1A,PNS). ARNO was predominantly found in the cytosolic fraction (Fig. 1A,cyt), since no immunoreactive material was detected in the membrane fraction when proportional amounts of membrane and cytosol were loaded on the gel (Fig. 1A,mb,1×). However a faint band was seen when greater amounts of membrane (Fig. 1A,mb,3×) were used, suggesting that a minor fraction of ARNO was membrane-bound. The cytosolic distribution of ARNO was confirmed by indirect immunofluorescence. A weak, diffuse staining was observed in the cytoplasm of HeLa cells (Fig. 1Ba), which was lost when cells were permeabilized prior to fixation (Fig. 1Bb). No obvious staining of membrane, including Golgi and plasma membrane, was detected. In contrast, ARF1 was found in the Golgi region of the cells (Fig. 1Bc), as shown by costaining of cells with CTR433, an antibody against a medial Golgi marker (Fig. 1Bd); a similar staining was observed in prepermeabilized cells (data not shown). The cytosolic distribution of ARNO was confirmed using another cell type, the mouse melanoma cell line M10 (data not shown).

ARNO overexpression dramatically alters the morphology of the Golgi complex

Since ARNO is thought to activate ARF1, a protein involved in
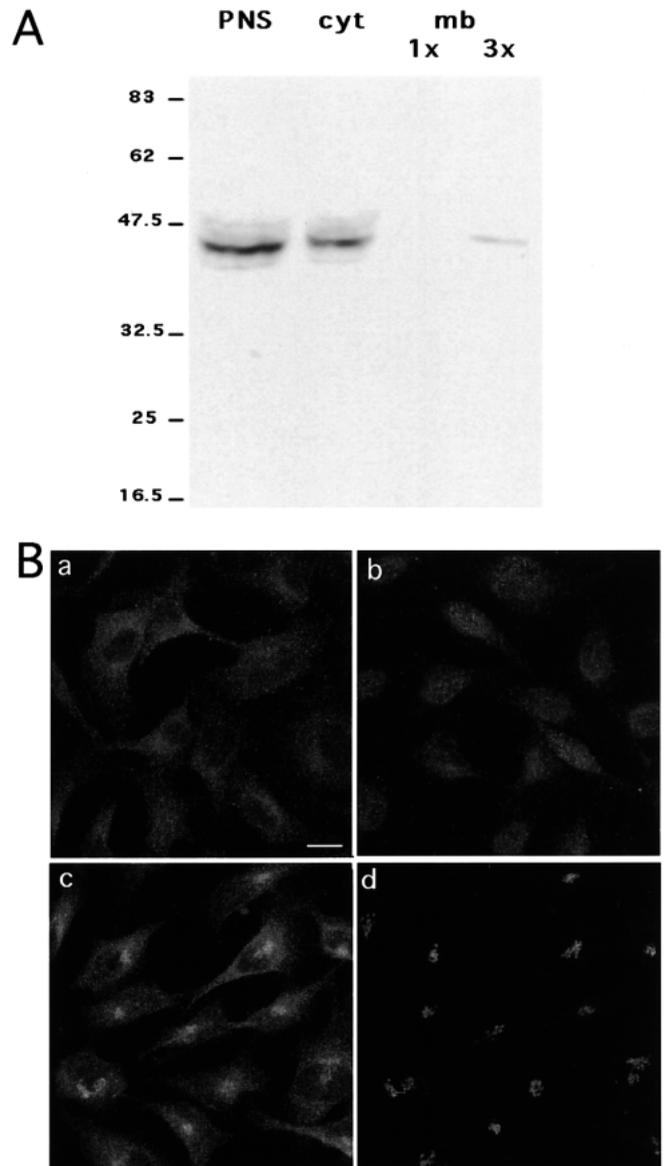
Fig. 1. Endogenous ARNO is a cytosolic protein. (A) A HeLa cell post-nuclear supernatant (PNS) was separated into a cytosol (cyt) and a membrane (mb) fraction. One volume of PNS was directly loaded the gel and four volumes were separated into cytosolic and membrane fraction. The membrane pellet was resuspended in 1/4 of the original PNS volume. Samples of cytosol (1/4 of the original volume) and membranes (1/4, 1× and 3/4, 3× the total volume) were loaded on the gel and submitted to western blot analysis using the affinity-purified anti-ARNO antibody. Molecular mass markers are shown on the left. (B) Indirect immunofluorescence was performed using the affinity-purified anti-ARNO rabbit antibody on HeLa cells non-prepermeabilized (a) or prepermeabilized (b) prior to fixation; in (c) and (d), cells were doubled labeled with an affinity-purified anti-ARF1 antibody (c), and with the monoclonal anti-Golgi antibody CTR433 (d). These pictures represent a compilation of 8 sections obtained by confocal microscopy. Bar, 20 μm.

vesicle transport and membrane traffic at the level of the Golgi complex, we next investigated the effect of ARNO overexpression on the distribution of various Golgi proteins. ARNO and ARNO E156K, an ARNO mutant protein which, in

vitro, has no exchange activity (Cherfils et al., 1998), were overexpressed in HeLa cells using the vaccinia system (Martinez, et al., 1994). Western blot analysis shown in Fig. 2 indicates that the PNS of transfected cells were highly enriched in both the wild type (lane indicated ARNO) and the mutant protein (E156K), as compared to the endogenous level of ARNO detected in cells transfected with the empty vector (0). The level of overexpression of wild type ARNO and of ARNO E156K mutant protein was estimated to be about 20- to 50-fold.

Like endogenous ARNO, overexpressed ARNO was found to be mostly cytosolic (Fig. 3Ab). In addition, no obvious membrane staining was detected when transfected cells were prepermeabilized prior to fixation (data not shown). In cells overexpressing ARNO, the CTR433 antibody gave a diffuse, reticular pattern (Fig. 3Aa). This staining was reminiscent of the one obtained after treatment of cells with BFA (Roa et al., 1993). In contrast, cells overexpressing ARNO E156K (Fig. 3Ad) showed a Golgi apparatus slightly more fragmented than normal, but still concentrated in the perinuclear region of the cells (Fig. 3Ac).

We also investigated the distribution of β-COP, a subunit of the COPI coatomer, and that of γ-adaptin, a component of the AP-1 adaptors, in cells overexpressing ARNO. The distribution of β-COP (Fig. 3Ba), found in non-transfected cells concentrated in the Golgi area, appeared diffuse in cells overexpressing ARNO. As the CTR433 Golgi marker, β-COP was possibly redistributed into the ER. However, the intensity of the signal obtained with the anti β-COP antibody was too weak to ascertain a colocalization of β-COP with ER
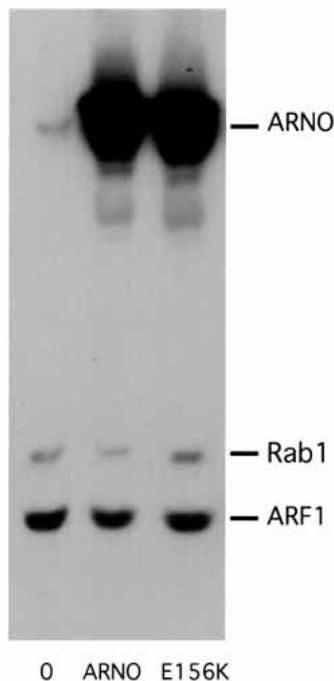
Fig. 2. ARNO content of transfected cells. PNS were prepared from cells transfected for 5 hours with the empty pGEM-1 vector (0), with pGEM-ARNO (ARNO) or pGEM-ARNO E156K (E156K), as indicated at the bottom of the figure, and subjected to western blot analysis using the anti-ARNO, anti-Rab1 and anti-ARF1 antibodies. The corresponding proteins are shown on the right. Rab1 and ARF1 were used as internal controls of this experiment.

structures. The localization of γ-adaptin, normally concentrated in perinuclear and peripheral structures (likely TGN and endosomes, respectively), was also greatly altered in cells overexpressing ARNO. As shown in Fig. 3Bc, γ-adaptin appeared in these cells relocalized into very fine punctate structures spread out into the cytoplasm. In contrast, no marked changes in β-COP and γ-adaptin distribution were observed in cells overexpressing ARNO E156K (data not shown).

ARNO overexpression redistributes Golgi into the ER

The localization of Golgi marker to a diffuse reticular pattern suggests that Golgi enzymes are redistributed to ER in cells overexpressing ARNO. To investigate this further, we co-expressed an ER resident protein together with ARNO. As previously shown (Martinez et al., 1997), the human invariant chain (Ii) of the major histocompatibility complex class II antigen remains associated with the ER when produced in HeLa cells in the absence of α and β chains. In cells expressing both ARNO and Ii, CTR433 was found colocalized with Ii in reticular structures (Fig. 4A left and right, respectively), confirming the redistribution of the Golgi marker CTR433 into the ER upon ARNO overexpression shown in Fig. 3A. It is important to note that virtually 100% of the cells were co-transfected under our experimental conditions and that, in these cells, the distribution of Ii itself was not altered by ARNO overexpression (not shown).

ARNO overexpression strongly inhibits the early secretory pathway

Since the data shown above show a major effect of ARNO overexpression on the morphology of the Golgi complex, we next investigated whether the Golgi function was also altered. SEAP, the secreted form of alkaline phosphatase, was used as a marker of the exocytic pathway and co-expressed with ARNO or ARNO E156K. HeLa cells were pulsed for 10 minutes was virtually absent in the medium of ARNO-transfected cells after a 120 minute chase, whereas it was detected after a 30 minute chase in the medium of cells overexpressing ARNO E156K (Fig. 5A) or of cells transfected with the empty vector (not shown). To determine at which step the secretory pathway was blocked, intracellular SEAP was analysed. Synthesis of SEAP was not affected by ARNO and by ARNO E156K overexpression, as shown in Fig. 5B,C. In contrast, no endoglycosidase H (endoH)-resistant forms of SEAP were detected in cells transfected with the wild-type ARNO, whereas they became visible in lysates of cells overexpressing ARNO E156K after a 30-minute chase. This suggests that SEAP molecules did not reach cis/medial Golgi compartments in cells overexpressing ARNO.

We also noticed that the pattern of endoH-digested forms of SEAP was different in ARNO-expressing cells. In order to better characterize this modification, Ii was used, as a marker unable to move out of the ER. After a chase of 150 minutes, Ii present in lysates of cells expressing ARNO or its mutant was immunoprecipitated and analysed by SDS-PAGE. A shift in the electrophoretic mobility of Ii (Fig. 4B, no treatment) was observed only in cells overexpressing ARNO (lane indicated ARNO), as compared to cells overexpressing ARNO E156K (E156K) or cells transfected with the empty vector (0). The same shift in mobility of Ii was induced in control cells
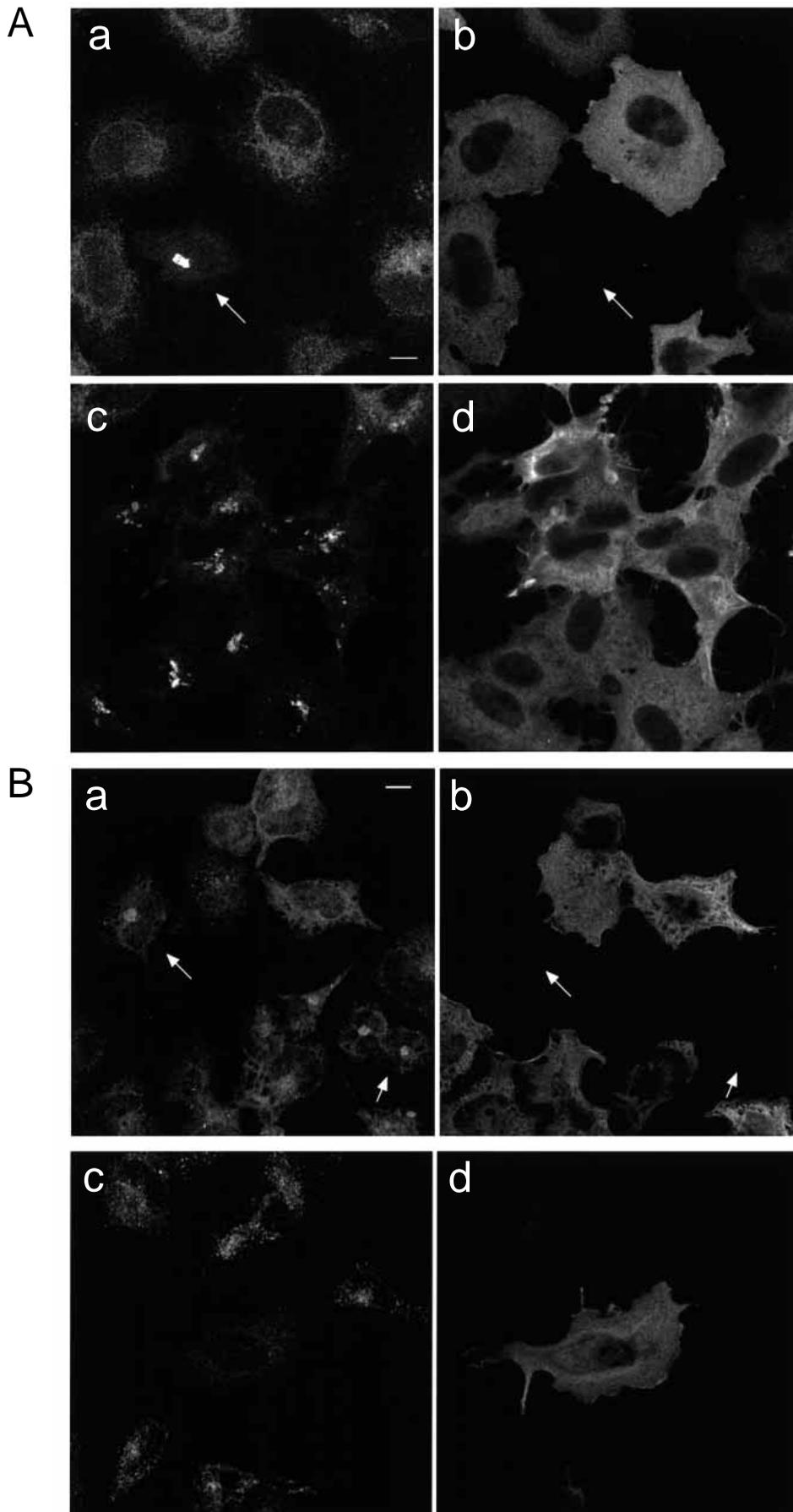
Fig. 3. ARNO overexpression alters the morphology of the Golgi apparatus. Cells were fixed 6 hours after transfection with pGEM-ARNO (Aa,b, Ba-d), or pGEM-ARNO E156K (Ac,d) plasmids. In A, cells were doubled stained with the CTR433 antibody (a,c) and with the affinity-purified anti-ARNO antibody (b,d). In B, cells were double stained with the antibodies against β-COP (a), γ-adaptin (c) and ARNO (b,d). Arrows indicate untransfected cells (right) with normal Golgi marker distribution (left). Bar, 10 μm.

Fig. 4. The MHC class II invariant chain (Ii) is O-glycosylated in cells overexpressing ARNO. (A) Cells were fixed 6 hours after a co-transfection with pGEM-ARNO plus pGEM-Ii plasmids and doubled stained with the CTR433 antibody (left) and an anti-Ii antibody (right). Arrows indicate untransfected cell (right) with normal Golgi marker distribution (left). (B) Cells were co-transfected with pGEM-Ii and either pGEM-ARNO E156K (E156K), pGEM-ARNO plasmids or the empty vector (0) for 4 hours 15 minutes. After a 10-minute pulse and a 2 hour 30 minute chase without (no treatment, jacalin eluate) or with 5 μg/ml BFA (+BFA), cell lysates were immunoprecipitated with the anti-Ii antibody. In the experiment shown on the right

side (jacalin eluate), cell lysates were first incubated with agarose-coupled jacalin and the bound material was eluted with galactose and immunoprecipitated with the anti-Ii antibody. Human Ii cDNA contains two initiation sites, which generate two forms of Ii, p33 and p31 (arrows). The arrowhead points to the major O-glycosylated form of Ii.
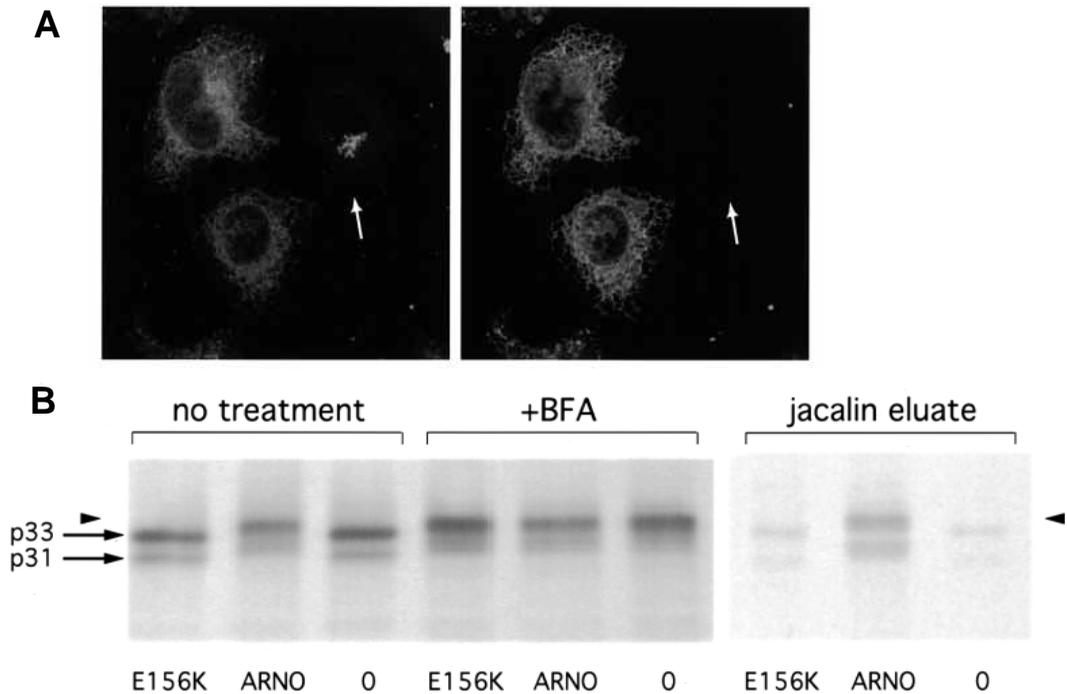
incubated with BFA (Fig. 4B, +BFA, lanes E156K and 0). In contrast, no additional shift of Ii was induced by BFA in cells overexpressing ARNO. As shown for SEAP, Ii did not not acquire endoH resistance (data not shown). In fact, Ii molecules present in cells overexpressing ARNO (Fig. 4B, jacalin eluate) or in cells incubated with BFA (not shown) were retained on jacalin beads, a lectin which specifically binds to the Ser/Thr-GalNAc-Gal(β1-3) motif of O-glycans (see Martinez et al., 1997). Under the same conditions, very low amounts of Ii present in cells transfected with pGEM-ARNO E156K or with the empty vector were retained on jacalin beads (4- to 6-fold less than in pGEM-ARNO-transfected cells, Fig. 4B). These results indicate that the overexpression of ARNO caused a redistribution of Golgi O-glycosylation modifying enzymes into the ER, as does BFA. Such a redistribution also probably accounts for the progressive appearance of SEAP species migrating above the endoH-digested form of the protein (Fig. 5B, thin arrows above the large one, respectively) in cells overexpressing ARNO. In contrast, the N-glycosylation maturation enzymes were not active on SEAP (Fig. 5B) and on Ii, including in the condition of BFA treatment (data not shown), as already shown by Martinez et al. (1997).

ARNO overexpression does not affect the early endocytic pathway

A recent report suggests that ARNO activates GTP exchange in vitro not only on ARF1, but also on ARF6 (Frank et al., 1998), a protein involved in the early endocytic pathway. We therefore studied the distribution of transferrin (Tf) receptor and the uptake of Tf, a ligand internalized from the cell surface and recycled from early endosomes to the surface, in cells

overexpressing ARNO. In contrast to the profound effect of ARNO overproduction exerted on the distribution of Golgi markers, the morphology of endosomes was comparable in cells overexpressing ARNO and in non-transfected cells (see ARNO staining in Fig. 6Aa), as seen after a continuous 30 minute incubation in the presence of TRITC-Tf (Fig. 6Ab). We noticed, however, a cell surface staining slightly more intense in cells overexpressing ARNO. The staining of cells with an antibody against Tf receptor (Fig. 6Ac) revealed no marked effect of ARNO overexpression (Fig. 6Ad) on the overall Tf receptor distribution at the steady state. In addition, both kinetics of ^{125}I-Tf internalization (Fig. 6B, upper panel) and recycling (Fig. 6B, lower panel) were almost identical in cells overexpressing ARNO and in control or ARNO E156K-transfected cells.

Increased level of ARF1 in the membrane fraction of ARNO overexpressing cells

According to current models, active ARF proteins are membrane-bound. We therefore analysed the ARF1 content of membranes isolated from cells overexpressing ARNO, ARNO E156K or from cells transfected with the empty vector. In all three cases, most of ARF1 was found in the cytosolic fraction in HeLa cells (more than 90%, data not shown). However, higher amounts of ARF1 were found in membranes prepared from cells overexpressing ARNO compared to membranes from control cells transfected with the empty vector or with pGEM-ARNO E156K (Fig. 7, lanes ARNO, 0, E156K, respectively). This strongly suggests that ARNO, but not ARNO E156K, was able to promote GTP exchange on ARF in vivo and therefore to increase the level of membrane-bound ARF1.

DISCUSSION

In this study, we have investigated the intracellular localization and function of the ARF1 exchange factor ARNO. We show that endogenous ARNO is predominantly a cytosolic protein. Its transient overexpression strongly inhibits the early secretory pathway and induces a complete disorganization of the Golgi complex as well as a redistribution of Golgi resident enzymes into the ER. These results indicate that ARNO is involved in Golgi traffic and strongly suggest that ARNO is a major exchange factor for ARF1 in vivo.

ARNO is a cytosolic protein

Cytoplasmic staining was obtained by immunofluorescence using affinity-purified anti-ARNO antibody and only a minor part of endogenous ARNO could be detected in the membrane fraction of total cell extracts. In addition, no clear evidence was obtained that ARNO was localized to ARF1-containing compartments, even after transient overexpression of the protein. Like other previously characterized exchange factors for small GTPases (Burton et al., 1994; Walch-Solimena et al., 1997; Horiuchi et al., 1997), ARNO appears to be a soluble protein. A C-terminal pleckstrin homology (PH) domain (Hyvönen et al., 1995), capable of interacting with polyphosphoinositide PIP_2 with an affinity in the 10-100 μM range, is found in ARNO (Chardin et al., 1996). PIP_2 greatly stimulates the exchange activity of ARNO, probably through its PH domain since the exchange activity of ARNO deleted of its PH domain is no longer stimulated by PIP_2 (Chardin et al., 1996). However, the binding of the PH domain to PIP_2-containing vesicles does not affect the intrinsic activity of the catalytic domain of ARNO, suggesting that the interaction between the PH domain and PIP_2 is only involved in the membrane recruitment of ARNO (Paris et al., 1997). The observation that ARNO is mostly cytosolic suggests that the exchange factor interacts only weakly with the membranes, as does its substrate ARF1-GDP (Franco et al., 1996). Since the exchange reaction cannot occur in the cytosol because of the inhibition exerted by the N-terminal helix of ARF1 (Paris et al., 1997), the weak interactions of both ARF1 and ARNO with the membrane may be sufficient to promote ARNO-catalyzed nucleotide exchange on ARF1 in PIP_2-rich sub-domains of membranes. Whether, in addition, ARNO interacts with a specific membrane receptor is unknown. In this respect, it is interesting to note that Gea1p, the yeast ARF1 exchange factor, does not possess a PH domain, nor any obvious membrane-interacting domain (Peyroche et al., 1996).

Inhibition of the early secretory pathway and disassembly of the Golgi complex

ARF1 displays a typical Golgi localization, where its distribution coincides with that of β-COP, γ-adaptin and other Golgi markers (Donaldson et al., 1991b; Stearns et al., 1990). The finding that overexpression of ARNO primarily affects the early secretory pathway is consistent with such a localization and supports the hypothesis that ARNO behaves in vivo as a major exchange factor for ARF1. The lack of effect of ARNO overexpression on the kinetics of transferrin uptake and recycling also suggests that ARF1 is not directly involved in the endocytic pathway. This result is in good agreement with the data showing that overexpression of the wild-type ARF1 and of ARF1 Q71L, the GTPase-deficient mutant, causes no significant changes in transferrin receptor uptake and recycling and in the morphology of endosomes (D'Souza-Schorey et al., 1995; Peters et al., 1995). It should be pointed out, however, that the overexpression of ARF1 Q71L was shown to inhibit fluid-phase endocytosis (Zhang et al., 1994). One possibility is that such an effect is indirect and results from the inhibition of
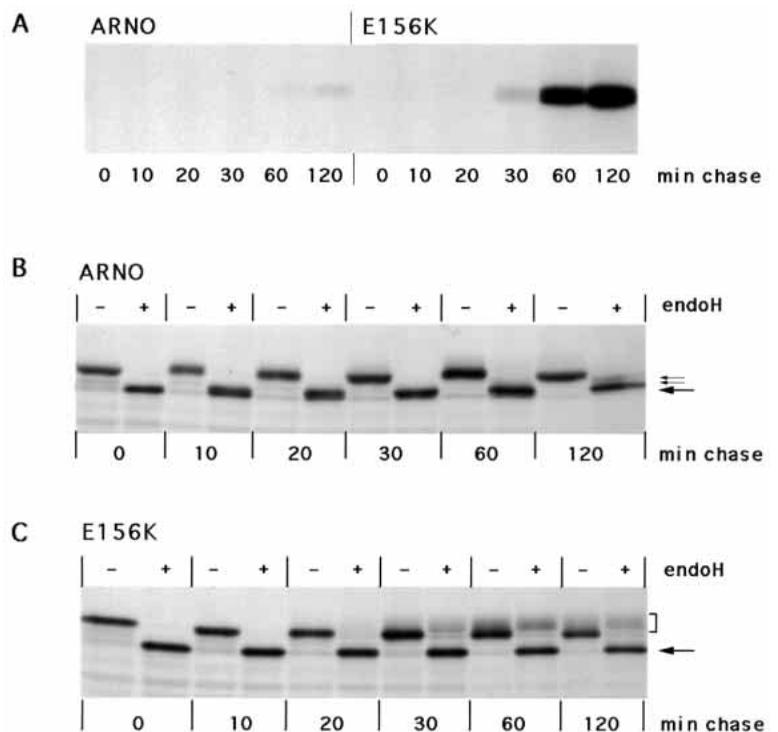

Fig. 5. ARNO overexpression inhibits intracellular transport of SEAP. Cells were co-transfected with pGEM-SEAP and either pGEM-ARNO or pGEM-ARNO E156K plasmids for 4 hour 15 minutes. Cells were then pulsed for 10 minutes and chased for the times indicated on the figure. Medium (A) and cells (B,C) were collected at each time point, immunoprecipitated with the anti-alkaline phosphatase antibody and analyzed by SDS-PAGE and autoradiography. Cell lysate immunoprecipitates were treated (+) or not (−) with endoH. Exposure time of the autoradiograph shown in A was three time longer than those in B and C. The large arrow in B and C indicates the immature form (endoH-digested) of SEAP still associated with the ER, the brackets in C the endoH-resistant products and thin arrows in B the O-glycosylated products.
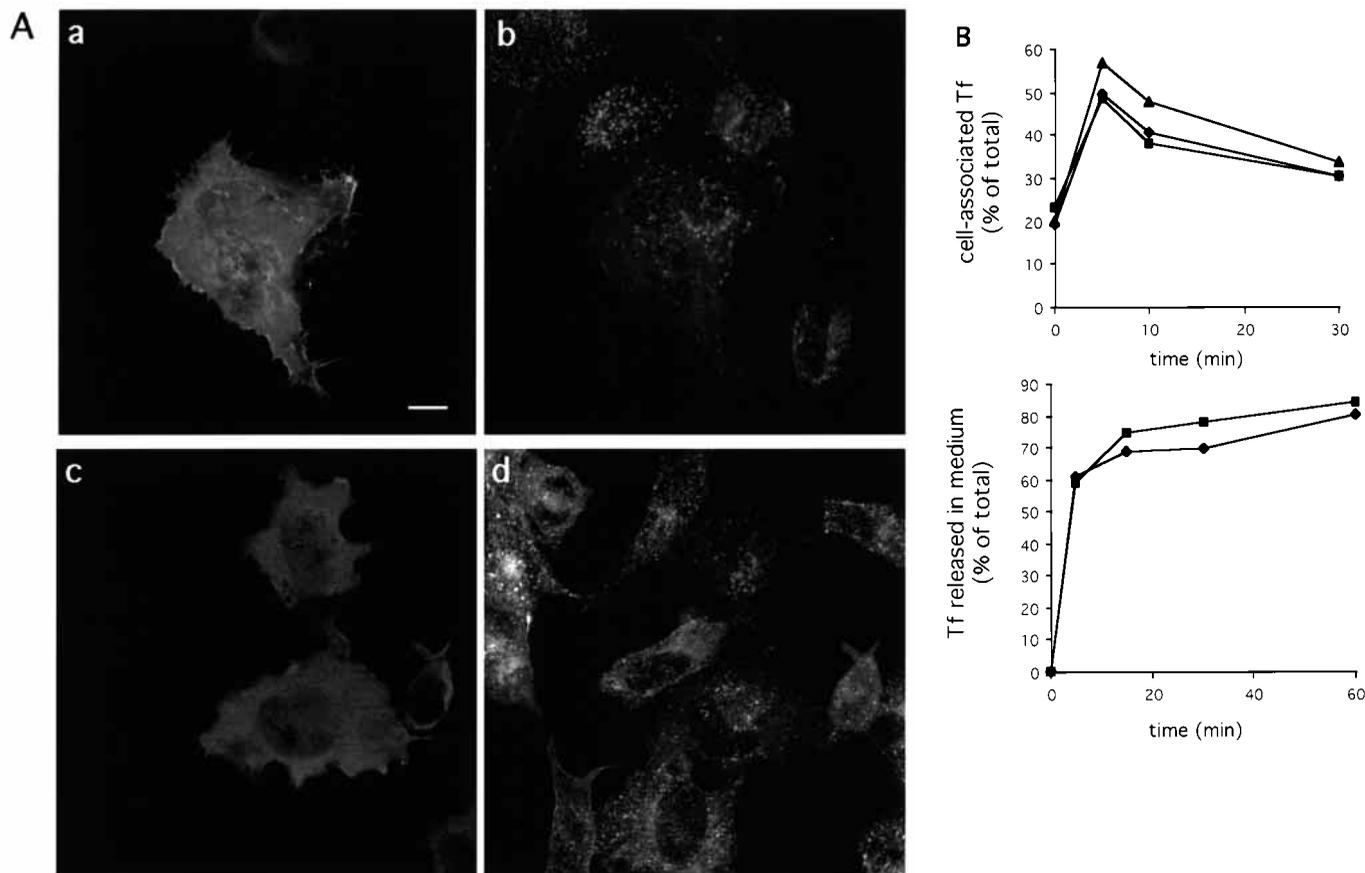
Fig. 6. Overexpression of ARNO does not affect transferrin internalization and recycling. (Aa,b) Cells transfected for 5 hours with pGEM-ARNO plasmid were incubated in the presence of TRITC-coupled transferrin (Tf) for 30 minutes (b), fixed and incubated with the anti-ARNO antibody (a). (Ac,d) Cells transfected for 5 hours 30 minutes with pGEM-ARNO-encoding plasmid were doubled stained with anti-ARNO (c) and anti-Tf receptor antibodies (d); bar, 10 μm. (B) Cells transfected for 5 hours with the empty vector (squares), pGEM-ARNO (diamonds) or pGEM-ARNO E156K (triangles) encoding plasmids were incubated in the presence of ^{125}I-Tf for 1 hour at 4°C (upper panel), or for 11 minutes at 37°C (lower panel) and chased for various times at 37°C. Radioactive Tf present in the cells and released in the medium was counted. Internalization was expressed as the percentage of radioactive material found in the cells compared to total (cells plus medium; upper panel). Recycling was expressed as the percentage of radioactivity released in the medium compared to total (lower panel).

the exocytic delivery of newly synthesized membranes required for fluid-phase endocytosis (Zhang et al., 1994). Evidence now exists that the early endocytic pathway is regulated by another member of the ARF family, ARF6 (D'Souza-Schorey et al., 1995). The lack of effect of ARNO on the endocytic pathway is consistent with the finding that ARNO does not display significant exchange activity for ARF6 (Franco et al., 1998), and with the fact that an ARF6 specific exchange factor has recently been discovered (M. Franco and P. Chavrier, unpublished results). It should also be pointed out that the overexpression of ARF6 has been shown to alter cell morphology by inducing rearrangements of the plasma membrane (D'Souza-Schorey et al., 1995; Peters et al., 1995; Radhakrishna and Donaldson, 1997) and the peripheral actin cytoskeleton (Radhakrishna et al., 1996; D'Souza-Schorey et al., 1997). No such effect was seen in cells overexpressing ARNO, as demonstrated by staining the cells with phalloidin (data not shown). However, a recent report suggests that ARNO is also an exchange factor for ARF6 (Frank et al., 1998). Nevertheless, the in vitro GTP exchange assay described in this paper shows that ARNO is 2- to 5-fold more
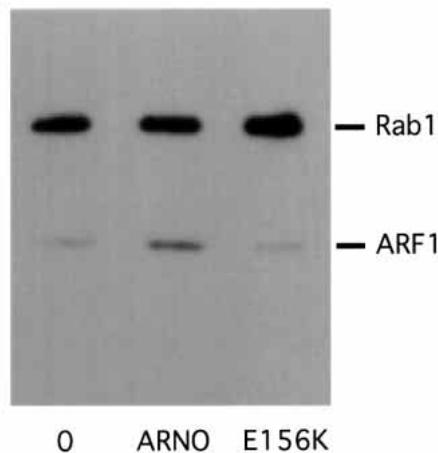
Fig. 7. The level of membrane-bound ARF1 is increased in ARNO-transfected cells. Cell membrane fractions were prepared from the PNS described in Fig. 2, salt-washed and solubilized in sample buffer. Equal amounts were analysed by western blot using the anti-ARF1 and anti-Rab1 antibodies.

active on ARF1 than on ARF6, if one takes into account the portion of active (myristoylated) ARF1 or ARF6 protein used in the test. The authors also conclude that ARF6 is the ARNO target in vivo, based on the fact that a weak staining of membrane ruffles can be observed in cells overexpressing an N-terminally tagged ARNO; however the vast majority of ARNO is clearly cytosolic (Frank et al., 1998). Our present data are fully compatible with ARF1 being the primary target for the exchange activity of ARNO in vivo.

A striking finding of our study is that ARNO overexpression induces a phenotype that resembles that of ARF1 inactivation. Indeed, a redistribution of Golgi membranes into the ER is also induced by BFA treatment (Donaldson et al., 1991a,b), by overexpression of the KDEL receptor (Hsu et al., 1992) or of ARF-GAP (Aoe et al., 1997). In all three cases, this phenotype can be interpreted as a result of ARF1 inactivation, leading to uncoating of Golgi membranes, fusion of the Golgi membranes with the ER and inhibition of ER-to-Golgi transport. BFA is thought to act by inhibiting a GDP/GTP exchange factor for ARF1 (Helms and Rothman, 1992; Donaldson et al., 1992b). The KDEL receptor has recently been shown to recruit ARF-GAP on the Golgi membranes and this interaction probably results in ARF1 inactivation (Aoe et al., 1997, 1998). The most straightforward interpretation of our present results showing that overexpression of ARNO induces the fusion of Golgi membranes with the ER is that increased levels of ARNO lead to the continuous production of β-COP (COPI)-containing vesicles, which finally results in a consumption of membranes of the Golgi complex. This hypothesis assumes that ARF1 itself, shown to be a very abundant protein (Kahn et al., 1988; Cavenagh et al., 1996), is not a limiting component in the formation of vesicles, and that ARF1 cycle, and in particular GTP hydrolysis, occurs normally in cells overexpressing ARNO. It also implies that ARF1/ARNO are mainly involved in the Golgi to ER retrograde transport.

The COPI-coated vesicles are implicated in transport back from the Golgi apparatus of ER-escaped proteins (Letourneur et al., 1994; Orci et al., 1997), and probably in a general membrane recycling pathway between these two compartments (Bannykh and Balch, 1997). We found that Golgi resident proteins, such as Golgi O-glycosidases, were redistributed into the ER. This suggests that Golgi-derived vesicles generated by ARNO overexpression were able to fuse continually with ER membranes. The redistribution of Golgi membranes into the ER probably accounts for the strong inhibition of intracellular transport of an anterograde marker such as SEAP. However, one does not rule out the possibility that COPI-coated vesicles may as well be involved in intra-Golgi anterograde transport (Orci et al., 1997). An overproduction of anterograde vesicles induced by the overexpression of ARNO at early stages after transfection may have been difficult to monitor biochemically and morphologically under the experimental conditions used in this study. The fate of γ-adaptin (AP-1/clathrin)-containing vesicles thought to be generated in cells overexpressing ARNO is unclear. TGN-derived vesicles are expected to fuse with endosomal compartments. Nevertheless, we obtained no clear evidence that the γ-adaptin-positive punctate structures seen in cells overexpressing ARNO contained endosomal markers.

In conclusion, our results strongly suggest that ARNO is an exchange factor for ARF1 in vivo. It remains to be determined what is the exact role of other proteins that also display an exchange activity for ARF1, such as cytohesin-1, p200 and GRP-1 (Meacci et al., 1997; Morinaga et al., 1997; Klarlund et al., 1998). Another important issue will be to dissect at the molecular level the sequence of events that links the activation of ARF1 by ARNO to the recruitment of coat proteins.

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