

Expression of a Dominant Allele of Human ARF1 Inhibits Membrane Traffic In Vivo

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Abstract. ADP-ribosylation factor (ARF) proteins and inhibitory peptides derived from ARFs have demonstrated activities in a number of in vitro assays that measure ER-to-Golgi and intra-Golgi transport and endosome fusion. To better understand the roles of ARF proteins in vivo, stable cell lines were obtained from normal rat kidney (NRK) cells transfected with either wild-type or a dominant activating allele ([Q71L]) of the human ARF1 gene under the control of the interferon-inducible mouse Mx1 promoter. Upon addition of interferon, expression of ARF1 proteins increased with a half-time of 7–8 h, as determined by immunoblot analysis. Induction of mutant ARF1, but not wild-type ARF1, led to an inhibition of protein secretion with kinetics similar to that observed for induction of protein expression. Examination of the

Golgi apparatus and the ER by indirect immunofluorescence or transmission electron microscopy revealed that expression of low levels of mutant ARF1 protein correlated with a dramatic increase in vesiculation of the Golgi apparatus and expansion of the ER lumen, while expression of substantially higher levels of wild-type ARF1 had no discernible effect. Endocytosis was also inhibited by expression of mutant ARF1, but not by the wild-type protein. Finally, the expression of [Q71L]ARF1, but not wild-type ARF1, antagonized the actions of brefeldin A, as determined by the delayed loss of ARF and β -COP from Golgi membranes and disruption of the Golgi apparatus. General models for the actions of ARF1 in membrane traffic events are discussed.

ADP-ribosylation factors (ARFs)¹ are a family of small (21 kD) GTP-binding proteins. ARF was first identified as the protein cofactor required for efficient ADP-ribosylation of the stimulatory regulatory subunit of adenylate cyclase, Gs, by cholera toxin (Kahn and Gilman, 1984, 1986; Kahn, 1989). The ARF family consists of at least 15 structurally related human gene products including the functionally distinct ARF and ARF-like (ARL) proteins (Clark et al., 1993). At least one member of each subfamily is essential for survival; ARF genes in yeast (Stearns et al., 1990a) and *arl* in *Drosophila* (Tamkun et al., 1991). ARF proteins are ubiquitous in eukaryotic cells (Kahn et al., 1988). The ARF family is one of four families in the RAS superfamily based on similarities in molecular weight and

presumptive mechanisms of regulation. However, the ARF proteins exhibit a similar degree of homology to members of both the RAS superfamily and the heterotrimeric G protein family (Sewell and Kahn, 1988). The structural and functional conservation between ARF proteins of divergent species (Sewell and Kahn, 1988; Kahn et al., 1991) suggests fundamental roles for ARF in cellular physiology.

ARF proteins are thought to participate in a number of membrane traffic events. ARFs may regulate traffic through the secretory pathway, based on both genetic (Stearns et al., 1990b) and biochemical data, employing in vitro assays of protein transport (Balch et al., 1992; Kahn et al., 1992; Lenhard et al., 1992). Previous studies have also demonstrated that ARF is associated with Golgi membranes (Stearns et al., 1990b; Donaldson et al., 1991) and Golgi-derived COP-coated vesicles (Serafini et al., 1991). ARF proteins also modulate endosome–endosome fusion in vitro (Lenhard et al., 1992) and nuclear membrane fusion in *Xenopus* egg extracts (Boman et al., 1992). Despite this wealth of information with regard to the in vitro biochemical properties of ARF protein family members, the relationship between these properties and in vivo function of ARF proteins remains un-

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1. *Abbreviations used in this paper:* AAP, ARF-activating protein; ARF, ADP-ribosylation; ARF GAP, ARF GTPase-activating protein; ARL, ARF-like; BFA, brefeldin A; NRK, normal rat kidney.

clear. In addition, the role(s) of specific members of the ARF family in each of these *in vitro* assays has been difficult to ascertain due to the presence of multiple gene products in purified ARF preparations and the extent of structural relatedness among these gene products.

A common feature of all GTP-binding proteins, including ARFs, is the regulated binding and hydrolysis of guanine nucleotides which serve as molecular switches in signaling pathways. Replacement of the glutamine residue in the consensus sequence, DXXGQ, with leucine impairs intrinsic GTP hydrolysis by members of both the G protein (Graziano and Gilman, 1989; Masters et al., 1989) and RAS (Vogel et al., 1988; Gideon et al., 1992) superfamilies. Expression of such mutants has provided insights into regulatory functions of these proteins, including their potential role(s) in oncogenesis (Barbacid, 1987; Landis et al., 1989).

ARF proteins are myristoylated *in vivo* and the cellular actions of ARF proteins involve the reversible association with specific intracellular membranes in myristoylation-dependent and guanine nucleotide-regulated reactions (Balch et al., 1992; Donaldson et al., 1992a; Kahn et al., 1992; Lenhard et al., 1992). Binding of the activating nucleotide, GTP, results in a conformational change in the protein (Kahn and Gilman, 1986) and an increased affinity for phospholipids and membranes (Donaldson et al., 1991; Regazzi et al., 1991; Randazzo et al., 1993). The inactive GDP-bound form of ARF is soluble (Weiss et al., 1989; Randazzo et al., 1993). Recent work from our laboratory and others has documented the presence of an ARF-activating protein (AAP) in crude Golgi fractions that is sensitive to the fungal metabolite, brefeldin A (BFA) (Donaldson et al., 1992b; Helms and Rothman, 1992; Randazzo et al., 1993). Addition of BFA to normal rat kidney (NRK) cells results in the rapid dissociation of ARF and β -COP from the Golgi and subsequent fusion of Golgi elements with the ER (Donaldson et al., 1990, 1991; Lippincott-Schwartz et al., 1991). BFA affects the organization of the Golgi apparatus in a variety of cells in culture and also has effects on endosome recycling (Lippincott-Schwartz et al., 1989; Wood et al., 1991), transcytosis (Hunziker et al., 1991), and cell proliferation in a number of human tumor cell lines (for a recent review of the effects of BFA, see Klausner et al., 1992).

To define more clearly the physiological role(s) of one member of the ARF family, we produced stably transfected cell lines carrying either wild-type ARF1 or the dominant activating mutant of human ARF1, [Q71L]ARF1, under the control of the tightly regulated mouse Mx1 promoter (Hug et al., 1988; Arnheiter et al., 1990). The results suggest that expression of mutant ARF1, but not wild-type ARF1 protein, has distinct effects on the structure and/or function of ER, Golgi apparatus, and endocytic pathway in mammalian cells.

Materials and Methods

Construction of Plasmids

Vector pSS2-2 was constructed by ligation of the 2.5-kb EcoRI-BamHI fragment of the mouse Mx1 upstream regulatory sequence (Hug et al., 1988) and a 1.5-kb BamHI-XbaI fragment of the mouse β -globin gene (including a noncoding part of exon 2, intron 2, and noncoding exon 3 containing the poly A site) into the polylinker region of Sp65. The entire coding region and ~50 bp of the 5' untranslated region of wild-type or mutant human ARF1 was inserted at the BamHI site, which corresponds to the start

site of transcription. The resulting plasmids are referred to as pZC1-1 (wild-type ARF1) and pZC1-3 (mutant ARF1). The mutant ARF was produced by oligonucleotide-directed PCR mutagenesis. Sequencing of the original mutant revealed two mutations resulting in two changes in amino acid sequence: [K38R] introduced by Taq polymerase error and [Q71L] as designed into the oligonucleotide primers. Much of the data shown here were obtained using this double mutant. We have subsequently constructed two independently isolated cell lines capable of expressing the single mutant, [Q71L]ARF1, and confirmed that all of the effects described result from this single point mutation.

Cells and Cell Culture

NRK cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and grown and passaged in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% fetal bovine serum (GIBCO BRL) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were cotransfected with either pSS2-2, pZC1-1, or pZC1-3 and pSV2-*neo* (carrying the selectable marker for resistance to neomycin [G418]; ATCC) at a ratio of 10:1 with lipofectin (GIBCO BRL), according to manufacturer's specifications. Selection and cloning of stable transfectants was performed as previously described (Southern and Berg, 1982). The cell lines used in this study were ZC1-0B (transfected with pSS2-2-lacking insert), ZC1-1A (transfected with the plasmid allowing inducible expression of wild-type ARF1), and ZC1-3B (transfected with the plasmid allowing inducible expression of [Q71L]ARF1). Induction of ARF expression was performed by incubating cells in medium containing 1,000 U/ml α/β rat interferon (Lee Biomolecular, San Diego, CA) for up to 24 h.

Microscopy

For indirect immunofluorescence microscopy, cells were grown on 10-mm glass coverslips for 24–48 h before induction with interferon. Cells were fixed and stained as previously described (Stearns et al., 1990b) with either a mouse monoclonal anti-ARF antibody (ID9, 13 μ g/ml), a polyclonal rabbit anti- α -mannosidase II antibody (gift of Drs. Marilyn Farquhar [University of California, Los Angeles, CA] and Kelley Moremen [University of Georgia, Athens, GA]) (Moremen et al., 1991), a monoclonal anti-protein disulfide isomerase (PDI) antibody (gift of Dr. Jane Cheng [Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD]) (Hasamura et al., 1986), or a polyclonal rabbit anti- β -COP peptide antibody (gift of Dr. Jennifer Lippincott-Schwartz, National Institute of Child Health and Human Development, National Institutes of Health), followed by the appropriate fluorescein isothiocyanate-conjugated secondary antibody (Cappel Labs, Durham, NC). For vital staining, cells were grown on 25-mm glass coverslips for 24–48 h before interferon induction, then stained with the Golgi marker, C₆-NBD-ceramide (Lipsky and Pagano, 1983), as previously described (Rosenwald et al., 1992). Photomicrographs were obtained with a Olympus IMT-2 microscope equipped with a 100 \times lens.

For phase contrast microscopy, cells grown on coverslips were fixed as described (Stearns et al., 1990b) and visualized after treatment with toluidine blue.

Transmission electron microscopy was performed as described previously (Stearns et al., 1990b).

Miscellaneous

Immunoblotting was performed on NRK cell lysates as previously described (Kahn et al., 1988). Briefly, 15–30 μ g protein from total cell lysates was separated by electrophoresis in a 10–20% acrylamide gradient gel, prior to electrophoretic transfer to nitrocellulose. Two ARF antibodies were used in these studies. The monoclonal antibody, ID9, was purified from mouse ascites and used as primary antibody at 1–10 μ g/ml. ID9 is an IgG₁ with specificity for mammalian ARFs 1, 3, 5, and 6. It can detect as little as 0.1 ng ARF per lane but does not bind mammalian ARL proteins (1–3), or yeast ARFs and detects mammalian ARF4 only poorly (~50-fold less reactive than other ARFs). A rabbit polyclonal, No. 1026, was generated against a COOH-terminal peptide derived from ARF1. It has very good specificity for ARF1, and fails to detect ARF3–6 or ARL1–3 (Cavenagh, M., K. Carroll, and R. A. Kahn, unpublished observation). The rabbit serum is used at a 1/1,000 dilution. Details of the production and specificity of these and other ARF antibodies will be found in Cavenagh, et al. (manuscript in preparation).

To analyze the effect of ARF1 induction on protein secretion, cells grown

on 30-mm dishes were re-fed with growth medium with or without 1,000 U/ml α/β rat interferon for up to 24 h. The monolayers were then washed once with Dulbecco's minimal essential media lacking methionine, and incubated in medium containing 5 $\mu\text{Ci}/\text{ml}$ [^{35}S]methionine (cell labeling grade, >1,000 Ci/mmol; Amersham, Arlington Heights, IL) (1 $\mu\text{g}/\text{ml}$ final methionine concentration) and 0.5% fetal bovine serum for 3 h at 37°C. Labeling medium was removed and labeled secreted proteins were precipitated by incubation at 4°C for 30 min after the addition of sodium dodecyl sulfate (0.5% final concentration), bovine serum albumin (50 $\mu\text{g}/\text{ml}$ final concentration), cold methionine (750 $\mu\text{g}/\text{ml}$ final concentration), and trichloroacetic acid (10% final concentration). Precipitates were collected on nitrocellulose filters and washed four times with ice-cold 10% trichloroacetic acid. Filters were allowed to air dry overnight before addition of scintillation fluid. Labeling medium that had not been incubated with cells was subjected to the same precipitation to determine background. Labeled cells were scraped from the dish in 1 ml cold phosphate-buffered saline and subjected to precipitation with trichloroacetic acid as for medium samples.

The uptake of HRP was determined as previously described (Marsh et al., 1987; Bucci et al., 1992). Briefly, cells grown on 60-mm culture dishes treated with or without interferon were incubated with RPMI 1640 medium (serum free) containing 5 mg/ml HRP (type II; Sigma Chemical Co., St. Louis, MO) and 0.1% bovine serum albumin for 60 min. Uptake of HRP was linear with time. The monolayers were then washed with ice-cold Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline containing 0.1% bovine serum albumin. The cells were scraped from the dish and washed several times. Cell lysates were prepared by resuspension in 10 mM Hepes, pH 7.4, 0.2% Triton X-100 followed by multiple passages through a 21-gauge needle. The lysate was centrifuged at 800 g for 10 min and the supernatant was used to determine protein concentration and HRP activity.

Results

Induction of ARF1 Expression by Addition of Interferon

To study the consequences of the overexpression of wild-type ARF1 and the expression of a potentially lethal, dominant allele of ARF1, we created stable cell lines carrying ARF constructs under control of an inducible but tightly regulated promoter, the mouse Mx1 promoter (Hug et al., 1988). The stable cell lines ZC1-0B, ZC1-1A, and ZC1-3B, that carry the parent vector, the vector containing wild-type ARF1, and the vector containing mutant ARF1, respectively, were made as described in Materials and Methods. These cell lines have been growing in continuous culture in the absence of inducer for more than three months.

Addition of interferon did not alter either the growth rate (doubling time = 17 h) or morphology of untransfected NRK cells, cells containing the parent vector, or cells expressing wild-type ARF1. In contrast, the growth rate of ZC1-3B cells, containing the inducible mutant ARF1 construct, was slightly slower than controls (doubling time = 20 h) in the absence of interferon, and proliferation was completely blocked in the presence of interferon. Cell rounding was first observed in ZC1-3B cells after 24 h of induction and by 2–3 d most cells had detached from the culture dish. Those cells remaining adherent were filled with large vesicles or vacuoles.

The levels of ARF proteins expressed in the cell lines were estimated by quantitative immunoblotting of cell lysates with a monoclonal anti-ARF antibody (Fig. 1 A) and with a semi-quantitative, polyclonal anti-ARF1-specific antiserum (Fig. 1 B). Control cells express endogenous ARF1 to a level of ~ 0.03 –0.1% of total NRK cell protein. ARF1 and ARF3 are the major forms of ARF found in NRK cells and are expressed at similar levels while ARF4–6 are below the limits of detection for our antibodies. Thus, endogenous ARF1 is

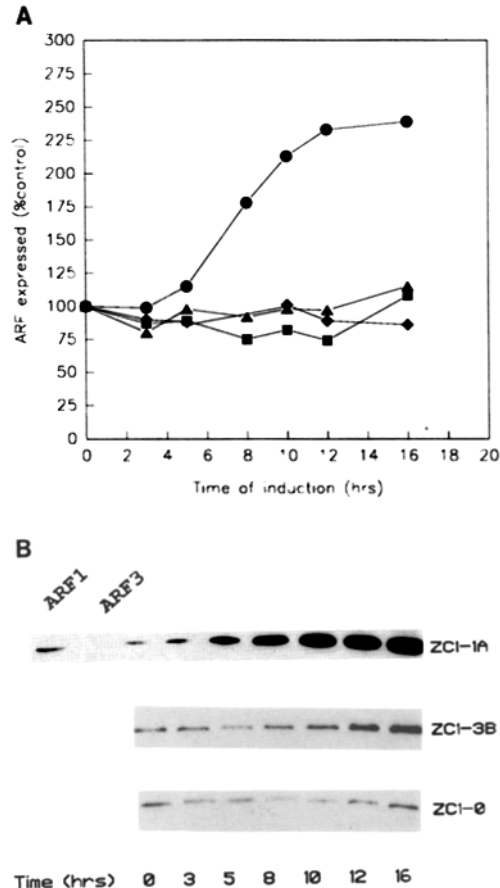


Figure 1. Time course of induction of ARF1 proteins after addition of interferon. (A) NRK (diamonds), ZC1-0B (vector control, triangles), ZC1-3B (mutant ARF1, squares), or ZC1-1A cells (wild-type ARF1, circles) were incubated with interferon and lysates were subjected to quantitative immunoblot analysis using the monoclonal ARF antibody, 1D9, and radioiodinated reporter, as described in Materials and Methods. Quantitation was performed using a PhosphorImager. This experiment was repeated at least five times with similar results. (B) The same three stably transfected cell lines were incubated for various times with interferon and the level of ARF1 expression was assessed using the ARF1-specific polyclonal rabbit antiserum, No. 1026. Purified recombinant ARF1 and ARF3 proteins (25 ng each) were included as controls for specificity. Immunoreactivity was visualized by chemiluminescence, as described in Materials and Methods.

approximately half of the ARF expressed in control cells. Addition of 1,000 U/ml α/β rat interferon led to a time-dependent increase in the level of ARF1 expressed in the transfectants (ZC1-1A; Fig. 1, circles) but no statistically significant change in ARF levels in controls lacking an inducible ARF construct (NRK and ZC1-0B) or in ZC1-3B. The level of expression for at least two independent isolates of each construct was 200–300% ($n = 8$) and 0–25% ($n = 5$; not significantly different from controls) of total endogenous ARF proteins for wild-type and mutant ARF1, respectively (Fig. 1 A). We also determined the time course of protein induction using a specific, polyclonal, anti-ARF1 antibody (Fig. 1 B). This antiserum has about a 10-fold lower affinity for ARF1 but a much greater specificity for ARF1 than does the monoclonal. Visualization of ARF1 ex-

pression in NRK cells with the polyclonal antiserum required the use of a chemiluminescence detection system which is less quantifiable than using radioiodinated secondary or tertiary antibodies. We have found about a 10-fold increase in sensitivity in the chemiluminescence technique over radioiodine detection. Results obtained with the ARF1-specific antibody confirm that the wild-type protein is induced with a half-time of ~7–8 h while induction of the mutant ARF1 takes slightly longer (~10 h). Maximal levels of each ARF1 protein were achieved by 12–16 h.

Taken together, we estimate that wild-type ARF1 is induced to a level of 500–600% of endogenous ARF1 and the ARF1 mutant is expressed to 50–100% the level of endogenous ARF1. This has proven to be a consistent finding. Mutants of ARF proteins are expressed at lower levels than their wild-type counterparts. The reason for this is unclear but may reflect decreased protein half-lives, interference by mutant ARFs with the interferon signaling pathway, or currently unknown consequence of dominant mutant ARF expression. It is not a result of inhibition of general protein synthesis (see below).

Expression of Mutant ARF1 Inhibits Protein Secretion

ARF proteins have been shown to be involved in vesicular traffic through the Golgi apparatus (Donaldson et al., 1991; Serafini et al., 1991; Kahn et al., 1992). To ask if ARF expression affected vesicular traffic, we analyzed protein secretion from cells after induction of wild-type and mutant ARF1 (Fig. 2). We found that upon addition of interferon, there was a time-dependent inhibition of protein secretion, but only from cells expressing the mutant ARF1 protein (Fig. 2, squares), not from cells expressing wild-type ARF1 protein (Fig. 2, circles). The half-time for inhibition of secretion was similar to that observed for induction of ARF1 protein expression as determined by immunoblot analysis (see Fig. 1). Induced ZC1-1A cells, expressing higher levels of wild-type ARF1, were consistently found to have higher rates of protein secretion than controls, but the magnitude of this effect was small and not statistically significant.

Expression of [Q71L]ARF1 Alters the Morphology of Subcellular Organelles

We next examined the location of ARF protein in induced and uninduced cells by immunofluorescence microscopy. Immunolocalization of ARF in control or induced cultures of ZC1-0B (Fig. 3, A and B) or ZC1-1A (Fig. 3, C and D) cells, or uninduced ZC1-3B cells (Fig. 3, E) revealed a predominant perinuclear localization, as previously described for ARF in NIH 3T3 (Stearns et al., 1990b), human adrenal carcinoma (Stearns et al., 1990b), and NRK (Donaldson et al., 1991) cells. Induction of [Q71L]ARF1 protein expression in ZC1-3B cells caused a dramatic increase in the intensity of the perinuclear staining observed with ARF antibodies (Fig. 3, F) and the compartment stained was noticeably larger than that seen in control cells.

As it is known that ARF binds reversibly to Golgi membranes (Stearns et al., 1990b; Donaldson et al., 1991), we next determined that the increase in perinuclear staining in induced ZC1-3B cells reflected changes in the morphology of the Golgi apparatus itself. Immunofluorescence staining

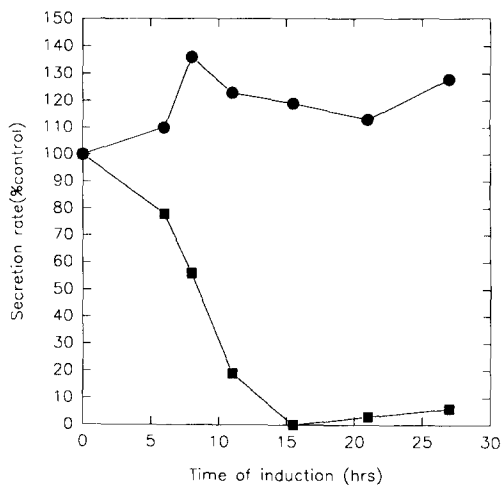


Figure 2. Effect of ARF1 expression on secretion of [³⁵S]methionine-labeled proteins from cells. ZC1-1A (wild-type ARF1, circles) and ZC1-3B (mutant ARF1, squares) cells were incubated with or without interferon for up to 24 h, then with [³⁵S]methionine as described in Materials and Methods. Proteins recovered in the medium were precipitated with trichloroacetic acid and counted. Data is expressed as the percentage of counts secreted in the presence of interferon relative to counts secreted in the absence of interferon (average of 3 determinations).

patterns for the luminal Golgi protein, mannosidase II (Moremen et al., 1991) (Fig. 4, C–F) were very similar to those observed with the ARF antibody in ZC1-1A and ZC1-3B (compare to Fig. 3). C₆-NBD-ceramide, a vital stain for the Golgi apparatus (Lipsky and Pagano, 1983; Rosenwald et al., 1992), also labeled a dramatically increased area around the nucleus in induced ZC1-3B cells (Fig. 4, compare A and B). This increase in Golgi staining in both living and fixed cells was often manifested as a perinuclear “collar.” Staining of all control cells (including NRK, ZC1-0B, and ZC1-1A cells treated with or without interferon and ZC1-3B without interferon induction) with these same probes did not reveal any alterations in the typical perinuclear staining pattern (Fig. 4, A and C–E; and data not shown). The time required to observe changes in morphology of the Golgi apparatus in ZC1-3B cells paralleled the time course of induction of ARF1 expression after addition of interferon (data not shown).

The morphological changes resulting from the expression of mutant ARF1 were also evident in toluidine blue-stained phase contrast images of living or fixed cells (Fig. 5, compare A and B). Time-lapse photomicroscopy of phase contrast images of induced ZC1-3B cells also revealed a close correlation between the time course of induction of mutant protein and changes in Golgi morphology (data not shown). When ZC1-3B cells were viewed using transmission electron microscopy, extensive profusion and vesiculation of membranes in the Golgi region and loss of normal Golgi elements were apparent in induced ZC1-3B cells (Fig. 5 D) but not in induced ZC1-0B (Fig. 5 C) or any of the other control cells (data not shown).

In addition to the obvious changes in the structure of the Golgi apparatus, substantial changes in the ER of induced ZC1-3B cells were also observed. By electron microscopy, the ER, which could be identified by the presence of bound

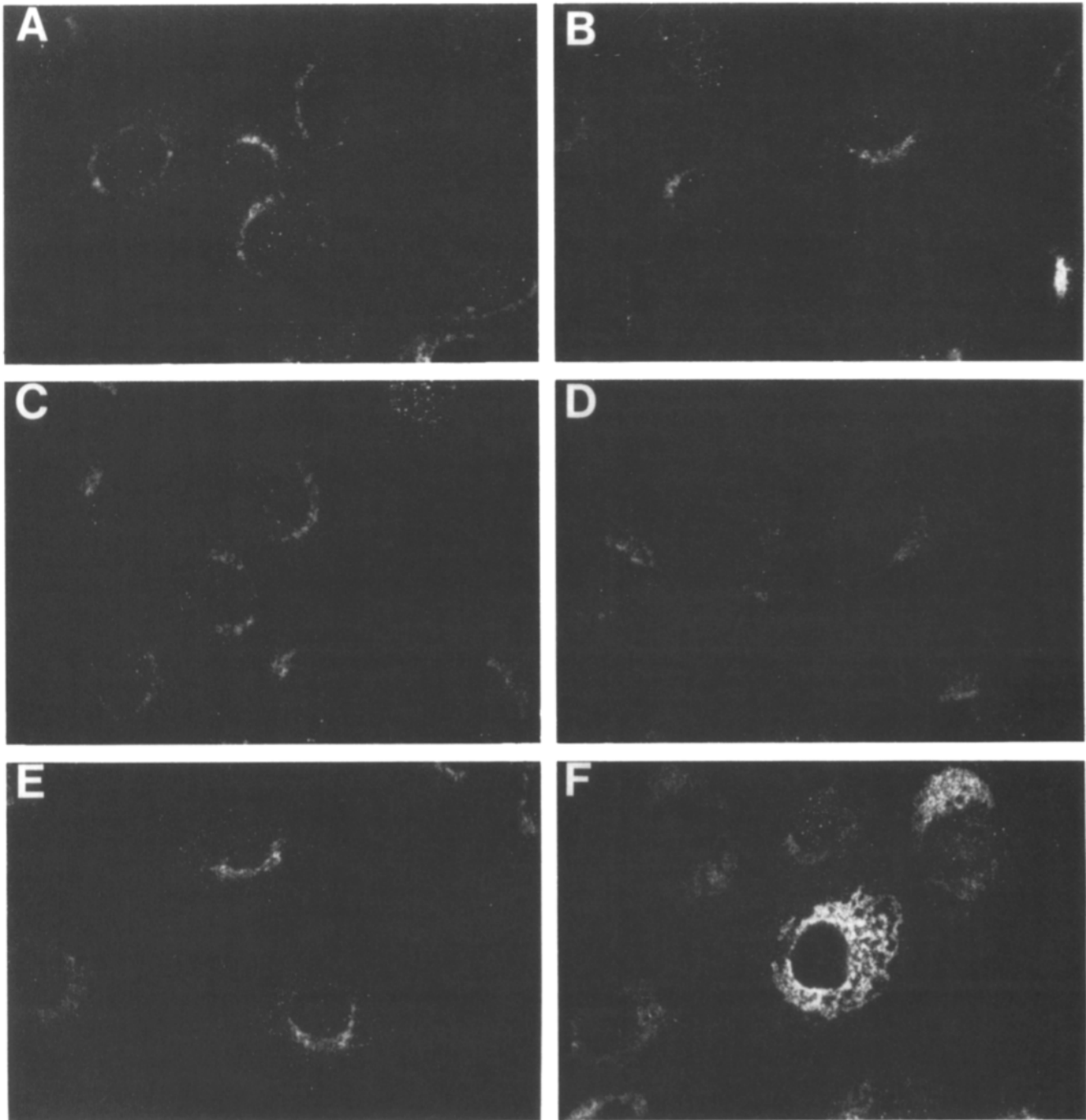


Figure 3. [Q71L]ARF1 expression leads to a nuclear collar of ARF staining. The location of ARF proteins in uninduced (A, C, and E) or interferon-induced (B, D, and F) cultures of ZC1-0B (A and B vector control), ZC1-1A (C and D, wild-type ARF1), and ZC1-3B (E and F mutant ARF1) was determined by indirect immunofluorescence microscopy as described in Materials and Methods. Cells were incubated with interferon for 18 h. Note the presence of the perinuclear collar in F.

ribosomes, appeared swollen (Fig. 5 D). The altered ER structure was quite large in some cases and was occasionally seen to surround other organelles, such as mitochondria. The ER of uninduced ZC1-3B or ZC1-0B cells (Fig. 5 C) was indistinguishable from controls.

The observed change in ER morphology prompted an examination of the immunolocalization of the ER resident protein disulfide isomerase (Hasamura et al., 1986). Typical re-

ticular morphology was seen throughout the cytosol in all control cells (Fig. 4 G) (data not shown). Induced ZC1-3B cells also showed staining in the cytosol but staining was specifically excluded from the Golgi-derived collar (Fig. 4 H). Thus, these two compartments remained clearly distinct in cells which express the mutant ARF protein, although changes in the morphology of both the ER and the Golgi apparatus were apparent by EM.

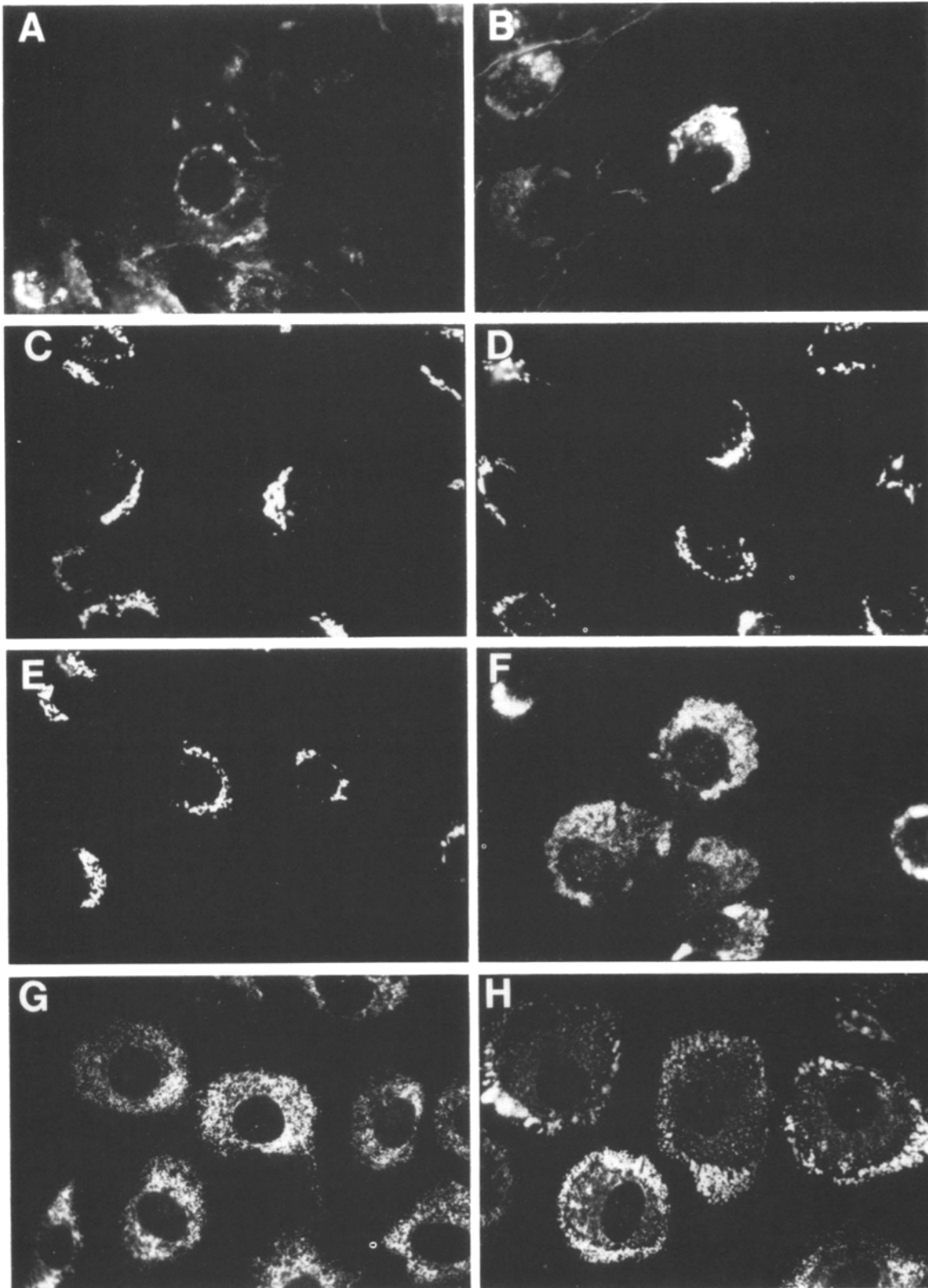


Figure 4. Effect of ARF1 expression on morphologies of the Golgi apparatus and the ER. The distribution of the vital Golgi stain C₆-NBD-ceramide (*A* and *B*), the luminal Golgi protein mannosidase II (*C-F*), and the ER marker, protein disulfide isomerase (*G* and *H*) are shown for ZC1-1A (*C* and *D*, wild-type ARF1) and ZC1-3B (*A*, *B*, and *E-H*, mutant ARF1) cells. Fluorescence images from cells grown in the absence (*A*, *C*, *E*, and *G*) or presence (*B*, *D*, *F*, and *H*) of interferon for 18 h are shown. Only ZC1-3B (mutant ARF1) cells grown in the presence of interferon show alterations at the Golgi apparatus.

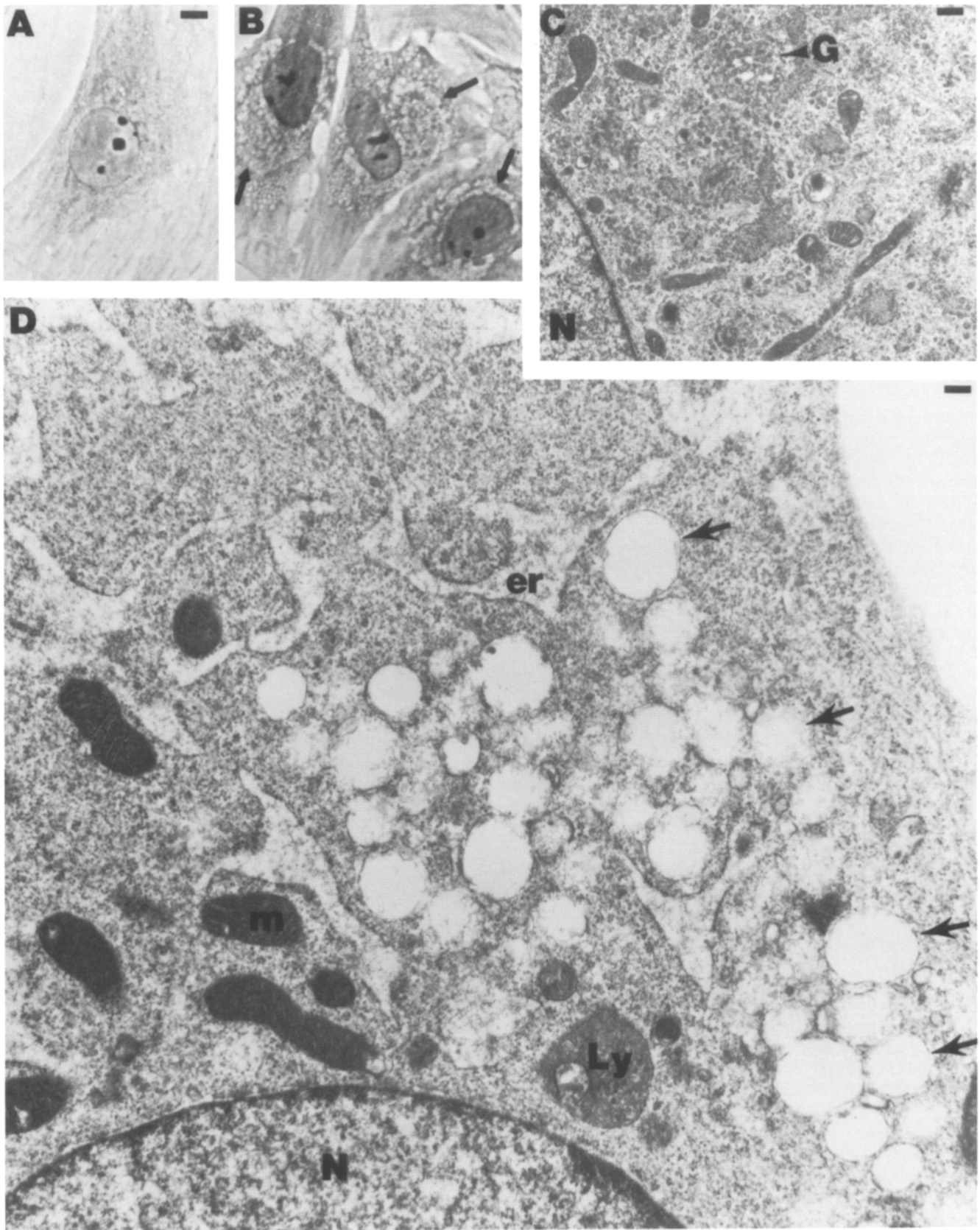


Figure 5. Morphological changes at the ER and Golgi apparatus visualized by phase contrast and transmission electron microscopy. Phase contrast images of toluidine blue-stained fixed ZC1-3B (mutant ARF1) cells in the absence (A) or presence (B) of interferon for 18 h. ZC1-3B cells show extensive enlargement in and vesiculation of the Golgi (B, arrows). Transmission EM images are shown of ZC1-0B (C, vector control) and ZC1-3B (D, mutant ARF1) cells treated with interferon. Induced ZC1-3B cells (D) show extensive vesiculation of membranes in the Golgi region (arrows), loss of normal Golgi elements, and swelling of the ER. G, Golgi apparatus; er, endoplasmic reticulum; N, nucleus; m, mitochondrion; Ly, lysosome). Bars: (A and B) 7 μm ; (C) 0.4 μm ; (D) 0.2 μm .

Expression of Mutant ARF1 Inhibits Fluid-phase Endocytosis

Previous work has demonstrated that ARF proteins, in addition to their role(s) in traffic through the secretory pathway, also participate in endosome–endosome fusion reactions *in vitro* (Lenhard et al., 1992). Therefore, we next determined the effect of ARF1 expression on uptake of the fluid-phase marker, HRP (Fig. 6). Expression of mutant ARF1 protein (Fig. 6, *circles*) inhibited fluid-phase endocytosis, but not wild-type ARF1 protein (Fig. 6, *squares*), or vector control (ZC1-0B, Fig. 6, *triangles*). The time required for inhibition paralleled that required for induction of ARF1 expression by interferon. The uptake of HRP was linear for up to 60 min in these experiments and inhibition was observed as early as 2.5 min after addition of HRP to previously induced cells (data not shown).

Effect of ARF1 Expression on BFA Activity

Incubation of cells with the fungal metabolite, BFA, causes disruption of the Golgi apparatus (Lippincott-Schwartz et al., 1991). This is preceded by release of ARF and β -COP proteins from the Golgi membranes (Donaldson et al., 1991, 1992a). Previous work from our laboratory (Randazzo et al., 1993) and others (Donaldson et al., 1992b; Helms and Rothman, 1992) has shown that AAP, present on Golgi membranes, is sensitive to BFA. This suggests that BFA, which inhibits activation of ARF proteins, and the [Q71L]-ARF1 mutant, which is predicted to lack GTPase activity and promote the active form of ARF1, should act antagonistically. Therefore, we next determined the effect of mutant and wild-type ARF1 expression on the ability of BFA to release β -COP from the Golgi apparatus (Fig. 7). Expression of mutant ARF1 protein, but not wild-type ARF1 protein, delayed the BFA-induced release of β -COP. The BFA-induced release of ARF from Golgi structures was also delayed in cells expressing the mutant ARF protein (data not shown). It should be noted that expression of mutant ARF1 caused expansion of the Golgi compartment, but did not lead to mixing of the Golgi and ER compartments (Fig. 4), as is typically seen upon addition of BFA to cells. Thus it appears that the expression of [Q71L]ARF1 was antagonistic to the activity of BFA.

Discussion

We demonstrated that expression of a dominant allele of human ARF1 in mammalian cells leads to inhibition of both the secretory and endocytic pathways for membrane transport, and results in marked changes in the morphology of several subcellular organelles. In cells expressing mutant ARF1 protein, the time required to see effects on protein secretion, morphological changes in the ER and Golgi, and functional effects on endocytosis all paralleled the time required for induction of ARF1 antigen as determined by immunoblot analysis. The failure of wild-type ARF1, expressed at higher levels than the mutant, to cause these changes argues against any nonspecific consequences resulting from the overexpression of human ARF1. Moreover, changes in gene expression as a result of incubation of cells with the inducer, interferon, did not cause the changes documented here. These results extend previous observations made in cell-free systems and

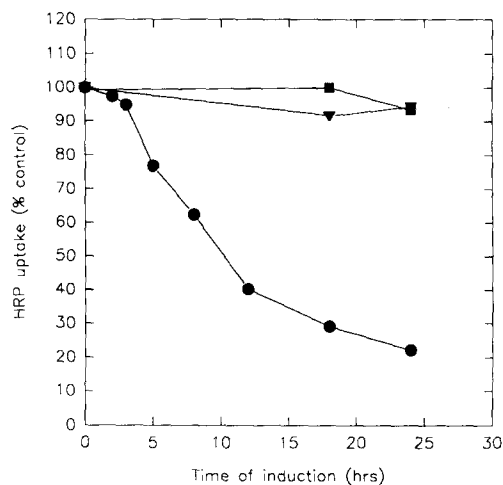


Figure 6. Effect of ARF1 expression on fluid-phase endocytosis. ZC1-0B (vector control, *triangles*), ZC1-1A (wild-type ARF1, *squares*), and ZC1-3B (mutant ARF1, *circles*) cells were incubated with interferon for up to 24 h, then analyzed for their ability to take up the fluid phase marker, HRP, as described in Materials and Methods. The data is expressed as the percent of peroxidase activity taken up in the presence of interferon compared to the absence of interferon. The 100% values were 106 ± 5.5 , 106 ± 5.1 , and $73 \pm 1.5 A_{460}$ U/mg protein for ZC1-0B, ZC1-1A, and ZC1-3B, respectively. Each point is the average of triplicate determinations of uptake. This experiment was repeated four times with nearly identical results.

strongly suggest a central role for ARF proteins as regulators of intracellular vesicle traffic at several steps.

The most dramatic effects of [Q71L]ARF1 expression, visible by both light and electron microscopy, are on the Golgi apparatus. This was not unexpected as ARF proteins have been previously localized to the Golgi in a number of cell types (Stearns et al., 1990b; Donaldson et al., 1991), and have been functionally linked to Golgi function both by genetic (Stearns et al., 1990b) and biochemical tests (Serafini et al., 1991; Kahn et al., 1992). Large changes in the morphology and apparent volume of this compartment accompany the expression of mutant but not wild-type ARF1. These changes were observed with markers of Golgi lipids (NBD-ceramide; Fig. 4 B), luminal proteins (mannosidase II; Fig. 4 F), and cytosolic-associated proteins (β -COP [not shown] and ARF, Fig. 3 F), indicating that the organelle itself has undergone gross morphological changes and not simple redistribution of a single marker. EM reveals the loss of Golgi lamellae, replaced by a profusion of vesicles. Although quantitative analyses of membrane surface to volume ratios were not performed in these studies, it is obvious that the volume of the Golgi (and ER) lumen is expanded enormously in cells expressing mutant ARF.

What is the relationship between the vesicles produced in induced ZC1-3B cells and the previously described (Orci et al., 1986; Malhotra et al., 1989) Golgi-derived, COP-coated vesicles? Interestingly, we did not observe an accumulation of coated vesicles, as might be predicted if the ARF1 mutant simply cannot hydrolyze GTP. It is not clear if either the vesicles observed with mutant ARF1 expression in live cells or the Golgi-derived coated vesicles, observed after GTP γ S treatment of broken cell preparations, represent an inter-

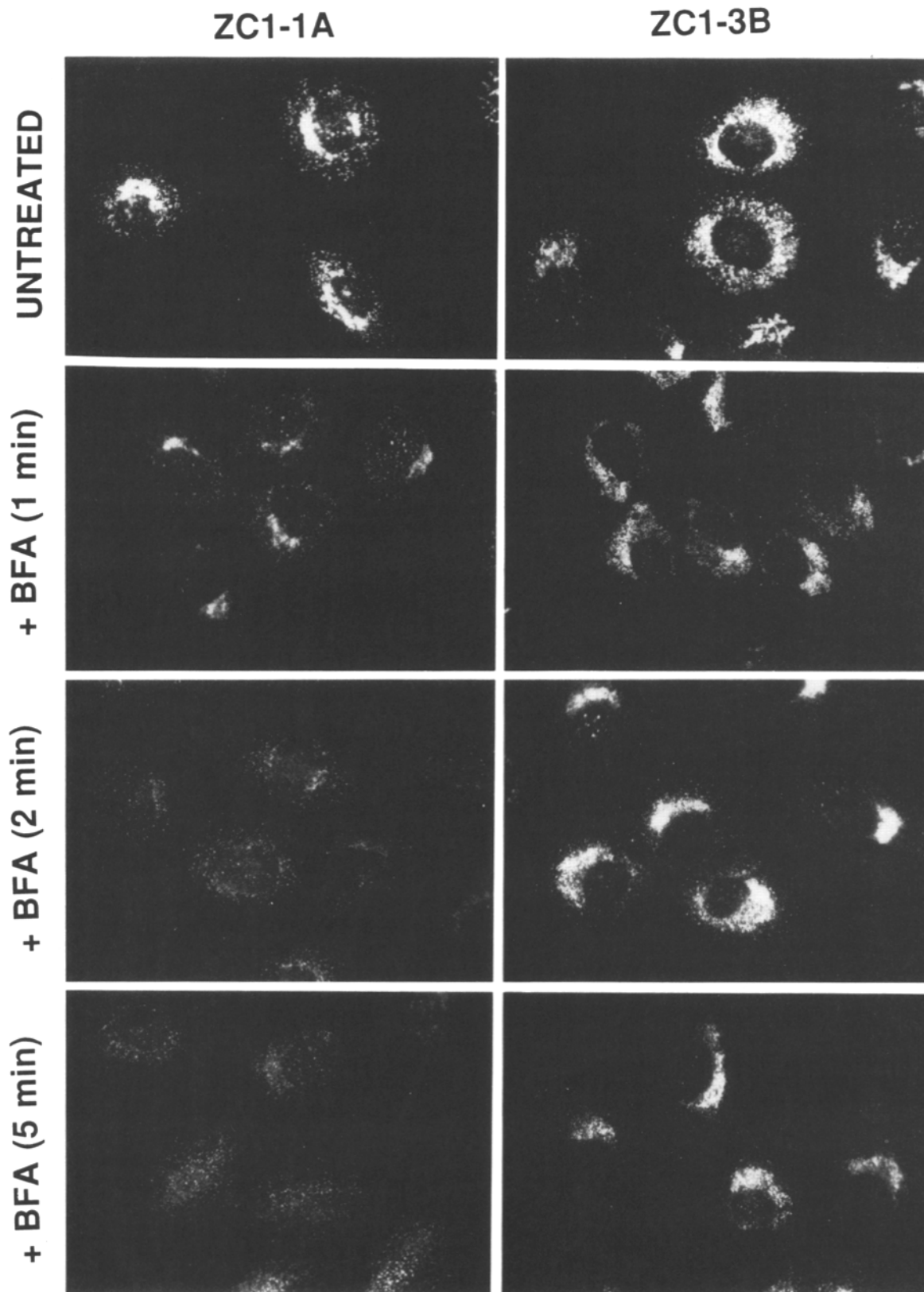


Figure 7. [Q71L]ARF1 expression attenuates the effects of brefeldin A on coatamer binding to the Golgi apparatus. ZC1-1A (wild-type ARF1) and ZC1-3B (mutant ARF1) cells were grown in the presence of interferon for 18 h before the addition of BFA (10 μ M) for 0 (untreated), 1, 2, or 5 min (as indicated) at 37°C. The cells were then fixed and stained by indirect immunofluorescence using the anti- β -COP antibody, as described in Materials and Methods.

mediate in protein transport, as both are fusion incompetent and result from gross changes in Golgi morphology and integrity. Nevertheless, these data from live cells expressing mutant ARF1 provide new information since they were obtained without the use of GTP γ S, which can activate more than one GTP-binding protein. The vesicles shown in Fig. 5 are larger and less uniform in size than the 75–110-nm diameter Golgi-derived vesicles obtained by incubating Golgi preparations in GTP γ S (Serafini et al., 1991). However, the vesicles seen in Fig. 5 are from cells maximally induced for the expression of mutant ARF1. We cannot exclude the possibility of smaller vesicles fusing to form these larger structures. We consider it unlikely that the vesiculated Golgi seen in these cells result from an inability to arrest or regulate the normal budding process. According to at least one model for ARF action in vesicle transport (Serafini et al., 1991), the presence of ARF and associated coat proteins helps target the vesicle and is incompatible with fusion. Thus, fusion of Golgi-derived, COP-coated vesicles to form the larger vesiculated Golgi remnants seen in Fig. 5 appears unlikely. We believe a more likely explanation for the observations reported here is that a properly cycling ARF1 is required for the integrity of the Golgi apparatus itself. We lack detailed information regarding molecular mechanisms for such a role but they may include a structural role in coordinating the assembly or stability of a multi-subunit complex such as a coatomer, or as a regulator of the volume of a membrane-bounded space, perhaps by affecting ion movements, or of intracellular membrane lipids (see below).

The consequences of the expression of [Q71L]ARF1 on the morphology of the ER are also dramatic. Two previous studies (Stearns et al., 1990b; Balch et al., 1992) provide data supporting a role for ARF in ER function or ER–Golgi traffic. In yeast cells made deficient in ARF, the secreted protein invertase accumulated in a core-glycosylated form, similar to that seen in the *sec18-1* mutant, blocked for ER–Golgi transport (Stearns et al., 1990b). Such data indicate a lack of processing of the invertase, which normally occurs in the Golgi compartment, and, thus suggests, an ER–Golgi blockade. Similarly, the *in vitro* transport of vesicular stomatitis virus G protein from ER to Golgi was potently and specifically inhibited by an NH₂-terminal inhibitory ARF1 peptide (Balch et al., 1992). Although no data on the functional integrity of the ER or quantitation of surface to volume ratios were analyzed in these studies, it is clear that the lumen of the ER is substantially enlarged and likely that the function of this compartment is compromised. Indeed, inability of newly synthesized protein or membrane to exit the ER may be responsible for the enlargement of the ER lumen and contribute to loss of Golgi lamellae integrity. It is likely that a common molecular mechanism will be found to explain the expansion in the luminal volume of both the ER and the Golgi compartments.

In contrast to the dramatic changes observed in the ER and Golgi compartments with [Q71L]ARF1 expression, we were unable to detect gross changes in the morphology of the endocytic compartment. No gross changes in the morphology of the early endocytic compartment were observed by visualization of fluorescent markers (data not shown). However, it is clear from our functional studies (shown in Fig. 6) that uptake of HRP, an indicator of fluid phase endocytosis, is inhibited by expression of the mutant ARF1 protein. Studies of

in vitro endosome–endosome fusion suggested that activated (GTP bound) ARF is required for endosome fusion, but inhibits when the active protein is in excess (Lenhard et al., 1992). Based on these observations we speculate that the induced ZC1-3B cells likely contain endosomes that cannot fuse, with the concomitant sequestration of critical components required for endosome formation or fusion. Another possibility is that the inhibition of Golgi function prevents exocytic delivery of new membrane required for prolonged endocytic activity.

We are currently considering two general models to explain the diverse changes accompanying expression of a single ARF mutant in cells that also express several other native ARF proteins. In one model we hypothesize that ARF1 specifically regulates multiple steps in the endocytic and exocytic pathways and that each of the phenotypes identified in our studies result from mutant ARF1 acting at sites where the wild-type protein normally functions. This model leaves open the role for the other ARF proteins (ARF3–6) in the cell. In the alternative model, we hypothesize that [Q71L]-ARF1 can functionally interact with a regulatory protein shared by more than one ARF isoform and in this way affect vesicle traffic at steps normally regulated by distinct ARF proteins. Such regulatory proteins may include the BFA-sensitive AAP or an ARF GTPase-activating protein (ARF GAP). These models are currently being tested by the regulated expression of different ARF isoforms and through biochemical tests of mutant ARF1 proteins in AAP and ARF GAP assays, respectively.

Mutation of the glutamine in the consensus GTP-binding domain, DXXGQ, results in a dominant activated allele of a number of regulatory GTP-binding proteins, but decreased GTP hydrolysis can apparently arise by two distinct mechanisms. The intrinsic GTP hydrolytic rate of [Q227L]G α is reduced \sim 100-fold resulting in a prolonged activated state (Graziano et al., 1989; Masters et al., 1989), while the critical effect of [Q61L]ras is more likely explained by its higher (\sim 50-fold) affinity for GTPase-activating protein (GAP) and failure to hydrolyze GTP when bound to GAP (Vogel et al., 1988; Gideon et al., 1992). Preliminary evidence indicates that ARFs are more ras-like than G protein-like in that a specific GAP activity has been identified which is potently inhibited by the [Q71L] mutant (Randazzo, P. A., and R. A. Kahn, unpublished observation). Other data can also be interpreted as indirect support for the hypothesis that the dominant effects of [Q71L]ARF1 result from its increased level of activation and/or inhibition of ARF GAP activity. First, the purified human ARF1 protein has no detectable intrinsic GTPase activity (Kahn and Gilman, 1986; Weiss et al., 1989) and thus lowering this rate is unlikely to be of biological significance. Second, the corresponding mutation in *S. cerevisiae* ARF1 ([Q71L]ARF1) is a dominant lethal allele but the purified recombinant protein has nearly the same intrinsic GTPase activity as the wild-type protein (Rulka, C., and R. A. Kahn, manuscript in preparation). Third, analysis of bound nucleotides on immunoprecipitated [Q71L]ARF1 in yeast revealed most of the ARF1 protein to have GTP bound while wild-type protein analyzed the same way had exclusively GDP bound (Zhang, C., unpublished observation). We have been unable to document the nucleotides bound to ARF proteins obtained by immunoprecipitation from cultured mammalian cells. Taken together these data make it

very likely that the [Q71L]ARF1 mutant exists in cells as an activated form of ARF which inhibits ARF GAP activity.

Studies of the effects of BFA on cultured cells and enriched membrane fractions have confirmed that ARF proteins function through the regulated cycling of activated (GTP bound) and inactive (GDP bound) proteins which are membrane associated or soluble, respectively. The finding that BFA cannot reverse the activated state but rather, blocks the activation step led to the identification by three different laboratories of the BFA- and trypsin-sensitive AAP. It was predicted, therefore, that expression of a dominant activating allele of ARF1 may appear antagonistic to the actions of BFA. Indeed, as shown in Fig. 7, the actions of BFA were markedly attenuated or delayed by the prior expression of mutant ARF1. In addition, in contrast to the effects of BFA on cells in culture, expression of [Q71L]ARF1 resulted in altered morphology but maintained distinct ER and Golgi compartments (see Fig. 4, F and H). These results further indicate that the mutant ARF1 is not cycling, but is arrested in the activated, membrane-associated form. We would predict that expression of a dominant inactivating allele of ARF would mimic the effects of BFA and cause the retrograde movement of Golgi elements back toward the ER.

The expression of dominant alleles of members of the ARF family under inducible control will likely provide valuable information regarding the functions of these proteins as regulators of distinct steps in the secretory pathway and other cellular processes. The sensitivity of a variety of vesicle budding/fusion reactions to ARF proteins suggests that a common molecular mechanism will ultimately be found. The recent purification of ARF proteins as the cytosolic factor which confers GTP γ S sensitivity to phospholipase D activity (Brown et al., 1993) offers an attractive explanation for the actions of ARF on vesicle formation or fusion. Modulation of bilayer lipids by an ARF-regulated phospholipase D may also explain the sensitivity of ARF activities to different lipids (Kahn et al., 1986). Specific tests of this and other hypotheses are now possible with these and related cell lines.

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