Guanine Nucleotides Modulate the Effects of Brefeldin A in Semipermeable Cells: Regulation of the Association of A 110-kD Peripheral Membrane Protein with the Golgi Apparatus

Julie G. Donaldson, Jennifer Lippincott-Schwartz, and Richard D. Klausner

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

Abstract. The release of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A (BFA) action, preceding the movement of Golgi membrane into the ER. ATP depletion also causes the reversible redistribution of the 110-kD protein from Golgi membrane into the cytosol, although no Golgi disassembly occurs. To further define the effects of BFA on the association of the 110-kD protein with the Golgi apparatus we have used filter perforation techniques to produce semipermeable cells. All previously observed effects of BFA, including the rapid redistribution of the 110-kD protein and the movement of Golgi membrane into the ER, could be reproduced in the semipermeable cells. The role of guanine nucleotides in this process was investigated using the nonhydrolyzable analogue of GTP, GTPγS. Pretreatment of semipermeable cells with GTPγS prevented the BFA-induced redistribution of the 110-kD protein from the Golgi apparatus and movement of Golgi membrane into the ER. GTPγS could also abrogate the observed release of the 110-kD protein from Golgi membranes which occurred in response to ATP depletion. Additionally, when the 110-kD protein had first been dissociated from Golgi membranes by ATP depletion, GTPγS could restore Golgi membrane association of the 110-kD protein, but not if BFA was present. All of these effects observed with GTPγS in semipermeable cells could be reproduced in intact cells treated with AIF4−. These results suggest that guanine nucleotides regulate the dynamic association/dissociation of the 110-kD protein with the Golgi apparatus and that BFA perturbs this process by interfering with the association of the 110-kD protein with the Golgi apparatus.

BREFELDIN A (BFA), a natural product of several different fungi, has profound effects on the structure and function of the Golgi apparatus. At concentrations of 100 nM and higher, it induces the disassembly of the cis-, medial, and trans-Golgi apparatus (Lippincott-Schwartz, 1989, 1990; Fujiwara et al., 1988, 1989), causes both resident and itinerant components of the Golgi apparatus to mix with the endoplasmic reticulum (Lippincott-Schwartz et al., 1989, 1990; Doms et al., 1989), and arrests transport to organelles beyond the Golgi apparatus (Mismi et al., 1986; Oda et al., 1987). When BFA is removed from cells a rapid sorting of Golgi components out of the ER ensues and the Golgi apparatus reforms (Lippincott-Schwartz et al., 1990). Whether these striking effects are all a consequence of a single molecular target of BFA remains to be determined. The specific loss of the Golgi apparatus as a distinct organelle during BFA treatment, however, suggests that BFA interferes with the fundamental mechanism(s) that maintain Golgi complex identity and structure.

The earliest morphologic changes detected by immunofluorescence microscopy when BFA is added to cells, include the apparent swelling of the Golgi complex followed by the appearance of long tubular structures which emanate out of the Golgi region. This begins ~2 min after the addition of BFA. The tubules frequently span large distances (up to 10 μm) in the cell and colocalize with microtubules. Depolymerization of microtubules abrogates their appearance. Tubule formation peaks at ~5 min of exposure to high doses of BFA and is followed by the dispersal of Golgi markers into the ER (Lippincott-Schwartz et al., 1990). That the Golgi complex and ER truly mix during BFA treatment has been demonstrated by the observation that ER resident and retained proteins are processed by Golgi enzymes during BFA treatment (Lippincott-Schwartz et al., 1989; Doms et al., 1989; Ulmer and Palade, 1989).

The ability of BFA to induce these effects might be due to changes in the distribution of and/or function of Golgi structural proteins which regulate transport into and out of the Golgi apparatus. We have recently observed dramatic changes in the distribution of a 110-kD Golgi-associated protein (Allan and Kreis, 1986) during BFA treatment. This pro-
tein appears to associate with the cytoplasmic face of Golgi membranes, although the exact nature of the association and the detailed distribution of the protein in the Golgi region is yet to be defined. Within 30 s of addition of BFA the 110-kD protein is redistributed from Golgi membrane into a diffuse cytoplasmic distribution. This effect precedes all other morphological changes in the distribution of integral membrane components of the Golgi apparatus during BFA treatment (Donaldson et al., 1990). The 110-kD protein also redistributes to the cytosol, in a manner indistinguishable from BFA-treated cells, when cellular ATP levels were depleted by treatment with sodium azide and 2-deoxyglucose (Donaldson et al., 1990). The ability of ATP levels to regulate the distribution of the 110-kD protein suggested that the relationship between the 110-kD protein and Golgi membrane might be dynamically regulated and that BFA might alter this.

Understanding the basis for the effects of BFA will require the ability to examine its molecular target(s) and to define the biochemical processes with which it interferes. While virtually nothing is known about how the separate identities of organelles of the secretory pathway are established and maintained, much biochemical information is beginning to emerge about the components of transport through the secretory pathway. This is largely the result of the development of cell-free and permeabilized cell systems for reconstituting transport (Balch et al., 1984; Simons and Virta, 1987; Beckers et al., 1987) and the identification of specific genes and their products from yeast mutants defective in the secretory process (Novick et al., 1980; Schechter, 1985). Using in vitro Golgi membrane reconstitution assays, several structural proteins have been identified and proposed to be involved in vesicle budding, targeting and fusion of membranes, and required to reconstitute transport in vitro (Melançon et al., 1987; Orci et al., 1989; Malhotra et al., 1989). One interesting characteristic of these proteins is that they appear to exist both in the cytosol and in association with membranes. It has therefore been proposed that these, and other proteins involved in transport through the secretory pathway, may cycle between the cytosol and the cytosolic surface of membranes.

Another set of proteins believed to have regulatory roles in the secretory pathway are low molecular weight guanine nucleotide binding proteins (Bourne, 1988; Balch, 1989; Hall, 1990). A large family of these proteins has been identified and recent work points to the localization of different low molecular weight GTP binding proteins to specific intracellular organelles (Segev et al., 1988; Goud et al., 1988; Nakano and Muramatsu, 1989; Stearns et al., 1990; Goud et al., 1990; Chavrier et al., 1990). It has been proposed that these proteins control the many cyclic processes that must be required for the repetitive events of vescicular transport (Bourne, 1988). Exactly where in this complex process they act, however, has not been established.

To examine the biochemical events associated with the perturbations induced by BFA, we have established a semi-permeable cell system using the method of Simons and Virta (1987) in which we can observe the morphologic consequences of BFA treatment. This system has allowed us to propose that BFA interferes with the cycle of association/dissociation of the 110-kD protein with the Golgi apparatus. In the absence of BFA such cycling is likely to be controlled itself by guanine nucleotides. The association of the 110-kD protein with the Golgi apparatus is maintained by the addition of nonhydrolyzable GTP analogues which block the acute effects of BFA. On the other hand, once the 110-kD protein redistributes to the cytosol upon the addition of BFA, GTPγS cannot reverse this effect. These studies allow us to begin to place the effects of this drug within the emerging biochemical parameters of regulatory events in the secretory pathway.

Materials and Methods

Materials and Cell Culture

BFA was obtained from Sandoz Co. (Basel, Switzerland) and Epicentre Technologies (Madison, WI), was stored at -20°C as a stock solution of 2 mg/ml in methanol, and used at a final concentration of 2 μg/ml in all incubations. GTPγS and other nucleotides were purchased from Boehringer Mannheim Diagnostics, (Houston, TX) and stored as 50 mM stock solutions in 10 mM Hepes/KOH pH 7.0 with 1 mM DTT at -80°C until use. Rhodamine-phalloidin was obtained from Molecular Probes Inc. (Eugene, OR) and used in cell incubations at a dilution of 1:50 of manufacturer's stock. All other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). NRK cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and 0.3% gentamycin at 37°C in 5% CO2. Cells were plated onto 12-mm-diam glass coverslips 24-48 h before experiments.

Antibodies

A rabbit IgG directed against Golgi mannosidase (Man II) (Moremen and Touster, 1985) was generously supplied by Dr. K. Moremen. The mouse monoclonal antibody, M3A5, characterized by Allan and Kreis (1986), which recognizes the 110-kD Golgi peripheral membrane protein, was kindly provided by Dr. T. Kreis. Rhodamine- and fluorescein-labeled goat anti-mouse and goat anti-rabbit IgG were purchased from Organon Teknika-Cappel (Westchester, PA) and used at 1:500 dilution of manufacturer's stock solution.

Preparation of Semipermeable Cells and Drug Treatments

Adapting the procedures of Simons and Virta (1987) and Kobayashi and Pagano (1988), subconfluent NRK cells grown on coverslips were drained of excess moisture by touching the edge of the coverslip to absorbent paper, and placed cell side up on the inverted cover of a 100-mm petri dish. An HATF filter (Millipore Corp., Bedford, MA), presoaked in incubation buffer (25 mM Heps/KOH pH 7.0, 125 mM K acetate, 2.5 mM Mg acetate, 1 mM DTT, and 1 mg/ml glucose) and blotted on filter paper (Whatman Inc., Clifton, NJ) was then overlaid on top of the exposed cell surface. After 1 min at room temperature the filter was gently peeled off the coverslip and 50 μl incubation buffer applied to the cells which were then incubated at 37°C for up to 1 h before fixation and immunofluorescence staining procedures.

For experiments with intact cells, coverslips of NRK cells were incubated in 1 ml culture medium in 12-well cluster dishes with the appropriate drugs for the indicated times at a 37°C incubator with 5% CO2.

Immunofluorescence Microscopy

After the appropriate incubations, cells were then fixed for 10 min at room temperature in 2% formaldehyde in PBS. The cells were then washed with several changes of PBS. To visualize the 110-kD protein with the monoclonal antibody M3A5, it was necessary to permeabilize the cells, after formaldehyde fixation, with methanol at 0°C for 1 min and then place the cells in PBS containing 10% fetal calf serum. Cells were incubated with primary antibodies in PBS/10% fetal calf serum and 0.15% saponin for 1 h, washed free of antibody, and then incubated in the fluorescently labeled secondary antibodies for 1 h. After washing, coverslips were mounted on slides in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL) and viewed with a 63× Planapo lens on a Zeiss microscope equipped with barrier filters to prevent crossover of fluorescein and rhodamine fluorescence.
Figure 1. BFA induces redistribution of Man II in intact and semipermeable cells. NRK cells grown on coverslips were either filter stripped, as outlined in Materials and Methods (SEMIPERMEABLE CELLS) or not filter stripped (INTACT CELLS), and then overlaid with 50 μl incubation buffer containing rhedamine-phalloidin without (Untreated) or with BFA (2 μg/ml) and incubated at 37°C for 5 and 15 min. The coverslips were then drained of excess incubation buffer, fixed in 2% formaldehyde, and stained in the presence of saponin with antibodies to Man II, followed by second antibody labeling with FITC goat anti-rabbit antibody. Rhodamine-phalloidin was observed staining actin filaments in all semipermeable cells, but absent in intact cells. Man II labeled tight perinuclear Golgi structures in untreated intact and semipermeable cells, but after 5 min of BFA, a time when intermediates in the Golgi redistribution can be observed, Man II was observed in a diffuse punctate/reticular pattern, residual Golgi structures and tubular structures emanating from these Golgi fragments (arrows). After 15 min BFA Man II was distributed in a fine punctate pattern in both intact and semipermeable cells. Bar, 10 μm.

Results

Semipermeable Cells Respond Fully to BFA

To more clearly define the biochemical events occurring during BFA treatment, we used the procedure of Simons and Virta (1987) to obtain perforated, semipermeable cells. Our aim was to render the cell sufficiently permeable to exogenous molecules while maintaining cellular morphology and integrity. To ascertain the efficiency and extent of this filter stripping technique, we tested the accessibility of intracellular actin to labeling with exogenously added rhodamine-labeled phalloidin, a small bicyclic peptide that binds with high affinity to F actin filaments (Barak et al., 1980). Intact and filter-stripped cells were incubated for 10 min at 37°C in the presence of rhodamine-phalloidin, drained of excess incubation fluid, fixed in 2% formaldehyde, and then labeled with antibodies to Man II. As shown in Fig. 1, Man II labeling in both the intact and permeabilized cells appeared in perinuclear, ring-like structures characteristic of the Golgi apparatus in NRK cells. No staining of actin with rhodamine-phalloidin was observed in intact cells, but the filter-perforated, semipermeable cells exhibited staining of actin filaments demonstrating that the perforated cells allowed access during the 37°C incubation to small molecules such as rhodamine-phalloidin (1,200 D). Although >95% of the cells on the coverslip were found to be perforated by this filter stripping technique in most assays, similar to efficiencies reported by Simons and Virta (1987), the intensity of the staining exhibited varied somewhat from cell to cell, suggesting that some cells had more or larger holes than others. The efficiency of perforation decreased with confluent and overgrown monolayers. Although these filter-stripped cells were readily permeable to small molecules such as rhodamine-phalloidin (1,200 D) and trypan blue (961 D), they were essentially impermeant to larger molecules, such as antibodies (data not shown), and hence we refer to these cell preparations as semipermeable. This is in contrast to the larger perforations obtained in MDCK cells after filter stripping which allowed access of antibodies into the cytoplasm (Simons and Virta, 1987). Because the holes induced in NRK cells were of limited size, most high molecular mass
maintenance of a functional microtubular network in these
cells as was observed by staining with antibodies to tubulin
(data not shown). Additionally, at this time BFA-induced
Man II punctate/reticular staining was also observed in both
the semipermeable and intact cells, a characteristic pattern
which has been shown by EM and biochemical assays to in-
dicate the residence of Man II within the ER during BFA
treatment (Lippincott-Schwartz et al., 1989). After 15-min
incubation with BFA, all of the Man II staining was now ob-
served in a fine punctate reticular pattern in both intact and
components of the cytoplasm were retained within the cell
and glucose addition to the incubation buffer was sufficient
as an energy source for up to 1 h incubation. Alternatively,
an ATP-regenerating system could be used in place of glu-
cose with similar results.

Brefeldin A treatment caused a change in the staining pat-
tern observed with Man II in both intact and semipermeable
cells (Fig. 1, middle row). After 5-min BFA treatment, the
Man II was no longer visible in a tight perinuclear structure
but displayed the mixture of patterns observed during early
periods of BFA treatment. Some fine tubular structures (Fig.
1, arrows), stained with antibodies to Man II, were observed
emanating out from Golgi-like structures in both intact and
semipermeable cells. These tubular structures are transi-
ent intermediates observed during early BFA incubations
that appear to be dependent upon microtubules (Lippincott-
Schwartz et al., 1990). The presence of these tubular struc-
tures in semipermeable cells treated with BFA attests to the

Figure 2. Redistribution of the 110-kD protein during BFA treat-
ment in semipermeable cells. Semipermeable NRK cells were in-
cubated with BFA (2 μg/ml) for 0 (Untreated), 2, or 15 min at
37°C, before fixation and double-label immunofluorescence stain-
ing with antibodies to the 110-kD protein and Man II. In untreated
semipermeable cells the 110-kD protein overlapped with Man II
labeling of perinuclear Golgi structures, with some additional la-
beling in a small punctuate pattern. After 2 min BFA the 110-kD
protein appeared diffusely scattered throughout the cytoplasm while
the Man II-labeled Golgi structures remained largely intact. Then,
after 15 min, Man II was no longer distributed in a tight Golgi-like
structure but was instead distributed in a punctate/reticular pattern.
Bar, 10 μm.

Figure 3. GTPγS inhibits BFA action in semipermeable cells. Semi-
permeable NRK cells were incubated at 37°C in buffer alone for
10 min (Untreated), with BFA (2 μg/ml) for 10 min, with GTPγS
(1 mM) for 10 min, or pretreated with GTPγS (1 mM) for 10 min
followed by addition of BFA for 10 additional min, and then were
fixed and processed for double-label immunofluorescence as de-
scribed in Materials and Methods. Treatment of cells with BFA for
10 min (second row) resulted in the wide dispersal of the 110-kD
protein and Man II distributed in a punctate reticular pattern. Treat-
ment of cells with GTPγS alone (third row) did not greatly change
the distribution of the 110-kD protein or Man II as compared to un-
treated (first row) and subsequent addition of BFA for 10 min to
cells pretreated with GTPγS (fourth row) did not result in dispersal
of the 110-kD protein or in Man II-labeled Golgi redistribution into
the punctate/reticular pattern. Bar, 10 μm.
Figure 4. AIF₄ inhibits BFA action in intact cells. Intact NRK cells were preincubated at 37°C in media alone for 10 min (Untreated), with BFA (2 μg/ml) for 10 min, with 30 mM NaF, and 50 μM AICl₃ (AIF₄⁻) for 10 min or pretreated with 30 mM NaF and 50 μM AICl₃ (AIF₄⁻) before the addition of BFA (+BFA) for 10 additional minutes. The cells were then fixed and prepared for indirect immunofluorescence. Bar, 10 μm.

Figure 4. AIF₄⁻ inhibits BFA action in intact cells. Intact NRK cells were preincubated at 37°C in media alone for 10 min (Untreated), with BFA (2 μg/ml) for 10 min, with 30 mM NaF, and 50 μM AICl₃ (AIF₄⁻) for 10 min or pretreated with 30 mM NaF and 50 μM AICl₃ (AIF₄⁻) before the addition of BFA (+BFA) for 10 additional minutes. The cells were then fixed and prepared for indirect immunofluorescence. Bar, 10 μm.

semipermeable cells. The Man II staining pattern obtained throughout BFA incubations was the same in intact and semipermeable cells. The presence of phalloidin in the incubation buffer had no effect on the pattern or distribution of Man II during BFA treatment in either intact or semipermeable cells (data not shown).

Since the semipermeable cells were virtually indistinguishable from intact cells with regards to the morphologic changes in the distribution of Golgi resident proteins observed during BFA treatment, we next tested whether other documented changes induced by BFA in intact cells occurred in the semipermeable cells. Within 30 s of BFA treatment in intact cells, a 110-kD Golgi structural protein is released from its association with the Golgi apparatus (Donaldson et al., 1990); this precedes the movement of Golgi membrane into tubular structures extending out from the Golgi apparatus to the periphery, followed at later times by their distribution in a punctate/reticular pattern. The effect of BFA on the association of the 110-kD protein with the Golgi apparatus, and Golgi membrane redistribution in semipermeable cells is shown in Fig. 2. In untreated cells, the 110-kD protein staining pattern largely overlapped with staining for Man II with additional punctate peripheral staining. No difference in the extent of the 110-kD protein association with the Golgi apparatus was observed between semipermeable and intact cells (compare Fig. 2, semipermeable and Fig. 4, intact cells). In contrast, attempts to permeabilize cells with detergent or streptolysin O treatments resulted in a loss of the association of the 110-kD protein with the Golgi complex (data not shown). After 2-min incubation with BFA the 110-kD protein was no longer colocalized with the Golgi apparatus but was instead widely scattered throughout the cytoplasm. At this time Man II-labeled tubular extensions can sometimes be observed extending from the largely intact Golgi apparatus in these perforated cells. By 15 min no identifiable Golgi complex remained, instead Golgi membrane components, as visualized by Man II labeling, were distributed in a punctate/reticular pattern while the 110-kD protein remained widely dispersed throughout the cell. Thus, in the semipermeable cells the sequence of events and morphologic characteristics of BFA-induced disassembly and redistribution of Golgi membrane were similar to those observed in intact cells, with dissociation of the 110-kD protein preceding tubular extensions and dispersal of the Golgi complex.

Pretreatment with GTPyS or AIF₄⁻ Inhibits BFA Action

The involvement of GTP binding proteins in regulating a number of steps of the secretory pathway has been well documented (Bourne, 1988; Balch, 1989; Hall, 1990). Inhibition of transport has been observed in ER to Golgi (Beckers and Balch, 1989) and inter-Golgi transport (Melançon et al., 1987) in the presence of nonhydrolyzable analogues of GTP such as GTPyS. To test whether the BFA-induced redistribution of Golgi membrane was sensitive to guanine nucleotides, semipermeable cells were incubated with 1.0 mM GTPyS at 37°C for 10 min before addition of BFA (Fig. 3). Treatment of semipermeable cells with GTPyS alone did not appreciably affect the morphology of the Golgi apparatus, as visualized by Man II staining or the association of the 110-kD protein with the Golgi apparatus (compare control with GTPyS), although a somewhat more compact Golgi structure was observed. When semipermeable cells were treated with BFA for 10 min the 110-kD protein was widely scattered throughout the cells and most of the Man II-labeled Golgi membrane had redistributed into a more diffuse, punctate pattern characteristic of the ER. In contrast, semipermeable cells that had been pretreated with GTPyS for 10 min showed little change in morphology after a subsequent 10-min incubation with BFA. No dissociation of the 110-kD protein from the Golgi apparatus and no Golgi membrane movement was detected. GTPyS was thus able to protect the cell from BFA treatment. This protection by GTPyS required that the cell be rendered semipermeable by the filter stripping procedure since intact cells preincubated with GTPyS were not resistant to the effects of BFA (data not shown). Extended treatment of semipermeable cells (>1 h) with GTPyS resulted in some fragmentation of the Golgi apparatus but striking maintenance of 110-kD protein association with the Golgi apparatus (data not shown).
GTPT inhibits and reverses the redistribution of the 110-kD protein observed with ATP depletion. Semipermeable NRK cells were untreated, incubated at 37°C with incubation buffer containing DOG/Az for 10 min, pretreated with GTPγS (1 mM) for 10 min before addition of DOG/Az for 10 min (DOG/Az←GTPγS), treated with DOG/Az for 10 min followed by addition of GTPγS and further incubation for 10 min (DOG/Az→GTPγS), or incubated with DOG/Az for 10 min before the addition of BFA for 3 min followed by the addition of GTPγS and further incubation for 10 min (DOG/Az→BFA→GTPγS) before preparation for immunofluorescence. Bar, 10 μm.

The ability of GTPγS to prevent both the BFA-induced dissociation of the 110-kD protein and redistribution of Golgi membrane was partially shared by GMP-PNP, another non-hydrolyzable GTP analogue, but no protection was observed with GTP, GDP, ATP or AMP-PNP (data not shown). Complete protective effects of GTPγS were observed at lower concentrations of 100 and 500 μM in a subset of cells, but in order to protect the maximal number of cells, concentrations of 1 and 2 mM were required. This may reflect a varying degree of permeabilization within the cell population which can also be discerned when monitored by staining with phalloidin (shown in Fig. 1). To assure that the effect observed with GTPγS was due to it being a non-hydrolyzable analogue of GTP, we tested whether GTP, added in 100-fold excess, could abrogate the protection observed with GTPγS.

In cells treated with 100 μM GTPγS alone before BFA treatment for 10 min, ~75% of the cells were protected. In contrast, pretreatment of cells with GTPγS plus 10 mM GTP resulted in protection for only 15% of the cells.

A well-described characteristic of trimeric G proteins is their ability to be activated not only by non-hydrolyzable analogues of GTP such as GTPγS but also by the addition of NaF and AlCl₃ (Sternweis and Gilman, 1982; Gilman, 1987). Although originally thought of as a fluoride effect on G proteins, subsequent discovery of contaminating levels of aluminum in laboratory buffers and media suggested the active species to be AlF₄⁻ (Sternweis and Gilman, 1982). More recently it has been shown that AlF₄⁻ reversibly interacts with GDP bound to G proteins, mimicking the γ-phosphate of GTP (Bigay et al., 1987). Since activation of G proteins can be observed in intact cells treated with AlF₄⁻ the effect of AlF₄⁻ pretreatment is the BFA response of intact cells was examined (Fig. 4). Treatment of cells with 30 mM NaF and 50 μM AlCl₃ for 10 min did not dramatically affect the association of the 110-kD protein with the Man II-stained Golgi structure, as compared to control cells, although a somewhat tighter association and condensing of the Golgi complex and the 110-kD protein was often apparent. Cells pretreated with AlF₄⁻ showed no change in distribution of either the 110-kD protein or Man II after 10 min BFA. In contrast, treatment of control cells with BFA for 10 min resulted in complete release of the 110-kD protein and the redistribution of Man II into the expected punctate/reticular pattern. The ability of AlF₄⁻ to inhibit BFA action is not likely to be due to detrimental effects of NaF on ATP levels in the cell since lowered ATP levels results in the opposite phenotype, that is release of the 110-kD protein from the Golgi apparatus (Donaldson et al., 1990, and see below). When cells were treated with NaF alone, a partial ability to inhibit BFA action was observed which could be clearly enhanced by the addition of AlCl₃, consistent with some low level contamination of the buffers with aluminum (data not shown).

Although GTPγS is known to exert a stabilizing effect on microtubules (Kirchner and Yarbrough, 1981), it is unlikely that the effects we observed with GTPγS and AlF₄⁻ are due to this effect on microtubules for several reasons. The association of the 110-kD protein with the Golgi apparatus is maintained in cells treated with microtubule disruptive agents, such as nocodazole (Allan and Kreis, 1986; Donaldson et al., 1990). Furthermore, addition of BFA to nocodazole-treated cells induces the dissociation of the 110-kD protein from the Golgi fragments (Donaldson et al., 1990), although subsequent Golgi membrane redistribution is inhibited by the absence of microtubules (Lippincott-Schwartz et al., 1990). To further exclude the possibility of involvement of microtubules, we treated NRK cells with nocodazole (20 μg/ml) for 1 h to depolymerize microtubules and found that AlF₄⁻ was able to protect the 110-kD protein from the BFA-induced dissociation even in the absence of microtubules (data not shown).

Experiments designed to test whether GTPγS in semipermeable and AlF₄⁻ in intact cells could reverse the effects of BFA were performed. When cells were pretreated with
Figure 6. AIF₄⁻ inhibits and reverses the redistribution of the 110-kD protein observed with ATP depletion in intact cells. Intact NRK cells were untreated, incubated at 37°C with media containing 50 mM DOG/Az for 10 min, pretreated with 30 mM NaF and 50 μM AIC₁₃ (AIF₄⁻) for 10 min before the addition of DOG/Az for 10 min (DOG/Az→AIF₄⁻), or incubated with DOG/Az for 10 min before the addition of BFA (2 μg/ml) for 3 min, followed by the addition of AIF₄⁻ and subsequent incubation for 10 more minutes (DOG/Az→BFA→AIF₄⁻). The cells were then fixed and prepared for immunofluorescence. Bar, 10 μm.

BFA for 3 min, a time sufficient to release most of the 110-kD protein from the Golgi apparatus, before the addition of GTPγS or AIF₄⁻, no effect of these agents on the BFA response was observed (data not shown). Specifically, the 110-kD protein, once dissociated in the presence of BFA, was not able to reassociate with the disassembling Golgi complex regardless of the modulation of putative GTP-binding proteins by GTPγS or AIF₄⁻. We considered the possibility that the 110-kD protein could not reassociate with the Golgi complex if cells were first treated with BFA because the Golgi complex had already disassembled. To attempt to address this we examined cells that were treated with BFA at 20°C, where the breakdown of the Golgi complex is greatly slowed, but the redistribution of the 110-kD protein is not. Even at this reduced temperature, neither GTPγS nor AIF₄⁻ could reverse the BFA-induced redistribution of the 110-kD protein (data not shown). Thus, the protective effects of GTPγS and AIF₄⁻ were only evident when cells were pretreated with these agents before BFA addition.

GTPγS and AIF₄⁻ Prevent and Reverse the ATP Depletion-induced Dissociation of the 110-kD Protein

In a previous study we demonstrated that depletion of cellular ATP with 50 mM 2-deoxyglucose and 0.05% sodium azide for 5 min caused the dissociation of the 110-kD protein from the Golgi apparatus in a manner similar to BFA. In contrast to BFA treatment, however, the Golgi disassembly did not occur. Removal of 2-deoxyglucose and sodium azide resulted in reassociation of the 110-kD protein with the Golgi complex within 5 min (Donaldson et al., 1990). Since GTPγS treatment appeared to result in a stabilization of the association of the 110-kD protein with the Golgi and resistance to BFA, semipermeable cells were tested to see whether GTPγS also provided protection against the effects of ATP depletion. As shown in Fig. 5, treatment of perforated cells with 2-deoxyglucose and sodium azide (DOG/Az) for 10 min resulted in the release of the 110-kD protein from the Man II-labeled Golgi complex. If however, cells were first pretreated with 1.0 mM GTPγS for 10 min before the addition of DOG/Az for 10 additional min the 110-kD protein remained associated with the Golgi complex. Since removal of DOG/Az from the semipermeable cells by brief washing, and subsequent incubation in buffer containing glucose for 10 min at 37°C resulted in the reassociation of the 110-kD protein with the Golgi apparatus (data not shown), we tested whether GTPγS could reverse the effects of ATP depletion on the 110-kD protein. When semipermeable cells were first treated with DOG/Az for 10 min to completely release the 110-kD protein from the Golgi apparatus and then 1.0 mM GTPγS was added for 20 additional min, the 110-kD protein was observed to largely colocalize with the Man II staining once again. Thus a nonhydrolyzable analogue of GTP could reverse the effect of ATP depletion, causing a reassociation of the 110-kD protein with the Golgi apparatus. Although GTPγS could protect the cell from and reverse the effects of ATP depletion on the association of the 110-kD protein with the Golgi apparatus, if BFA was added to the cells after DOG/Az, GTPγS could not subsequently induce the reassociation of the 110-kD protein with the Golgi apparatus (Fig. 5, fifth row).

As was observed with BFA, the ability of GTPγS to protect from and reverse the effects of ATP depletion was partially shared by GMP-PNP, another nonhydrolyzable GTP analogue, but no protective/reversal was observed with GTP, GDP, ATP, or AMP-PNP (data not shown). Complete protective effects of GTPγS were observed at lower concentrations of 100 and 500 μM in a subset of cells, but in order to protect the maximal number of cells, concentrations of 1 and 2 mM were required.

The same protective and reversal effects were observed with AIF₄⁻ treatment of intact cells, as shown in Fig. 6. As had been observed previously (Donaldson et al., 1990), treatment of intact cells with deoxyglucose and sodium azide for 10 min resulted in the 110-kD protein appearing widely
distributed throughout the cell. In cells pretreated with AlF₄⁻ for 10 min before DOG/Az addition, however, the 110-kD protein remained associated with the Golgi apparatus (Fig. 6, third row), and the staining pattern was indistinguishable from control cells. Thus, AlF₄⁻ was able to protect the 110-kD protein from release during ATP depletion. Additionally, AlF₄⁻ was also able to reverse the effect of ATP depletion since treatment of the cell for 10 min with DOG/Az followed by addition of AlF₄⁻ to the incubation media for 20 min resulted in the reassociation of the 110-kD protein with the Golgi despite the continued presence of DOG/Az (Fig. 6, fourth row). Like GTPγS in semipermeable cells, although AlF₄⁻ could protect the cell from and reverse the effects of ATP depletion on the 110-kD protein's association with the Golgi membrane, if BFA was added to the cells after ATP depletion, AlF₄⁻ could not subsequently induce the reassociation of the 110-kD protein with the Golgi apparatus (Fig. 6, fifth row). This phenomenon, also observed with GTPγS, suggests that although the association of the 110-kD protein with the Golgi apparatus can be manipulated by GTPγS and AlF₄⁻, if the Golgi apparatus is first treated with BFA subsequent addition of these reagents is not able to cause the reassociation of the 110-kD protein with the Golgi apparatus.

**Discussion**

A semipermeable cell system that allows one to observe the dramatic effects of BFA on the structure of the Golgi apparatus offers an expanded ability to manipulate and identify the cellular components involved in maintaining the structure and function of this organelle. A variety of broken cell systems have been developed and used over the past several years to study different aspects of intracellular traffic (Balch et al., 1984; Haselbeck and Schekman, 1986; Balch et al., 1987). The use of nitrocellulose filters to permeabilize cells, developed by Simons and Virta (1987), has proven to be particularly well-suited to the requirements for the study of BFA. Simons and colleagues used this technique with MDCK cells to make large perforations in the cell surface, allowing the ready exchange of cytosolic proteins and antibodies. This technique applied to subconfluent monolayers of NRK cells, using shortened times of filter adherence in our studies, resulted in a population of cells selectively permeable to small molecules. This selective permeabilization was very reproducible; >95% of the cells were permeabilized when assessed by their inability to exclude trypan blue. While actin fibers could be stained with the small molecular mass molecule rhodamine-phalloidin (1,200 D), larger molecules such as antibodies did not readily enter the cells. We therefore refer to these cells as semipermeable to distinguish them from the semi-intact MDCK cells produced by Simons and Virta (1987). We suspect that it is this property of limited permeability of the NRK cells that makes them amenable to the study of the complex changes in the Golgi apparatus induced by BFA. Indeed, all of the changes observed during BFA action in intact cells were observed in the semipermeable cells. The ability of these cells to maintain intracellular function is emphasized by the simple need for glucose in the small volume incubation buffer as an energy source. In the absence of glucose, the cells remain functional if we supply a source of ATP in the form of an ATP-generating system.

We have chosen to focus on the early phase of BFA action (i.e., the dissociation of the 110-kD protein) in our study with semipermeable cells for a variety of reasons. First, the biochemical basis for the redistribution of the 110-kD protein may be a simpler process to examine than the subsequent events of Golgi disassembly, transport, and mixing with the ER. Second, the change in distribution of the 110-kD protein is an excellent candidate for a proximal event that may well underlie subsequent morphological changes of the Golgi apparatus.

Our previous studies suggested that the 110-kD protein might constitutively cycle on and off Golgi membrane. This was supported by the observation that treatment of cells with agents that reduce intracellular levels of ATP cause the 110-kD protein to redistribute from the Golgi apparatus to an apparent cytosolic localization. Restoration of ATP levels reversed this effect (Donaldson et al., 1990). When we examined the effect of ATP depletion on the distribution of the 110-kD protein in semipermeable cells, the same results were observed as in intact cells. The 110-kD protein redistributed off the Golgi apparatus but no subsequent disassembly of the Golgi apparatus, a process that seems to require ATP (see below), occurred.

To further investigate the biochemical properties of the dynamic association/dissociation of the 110-kD protein with the Golgi apparatus we turned to guanine nucleotides, which, via GTP-binding proteins, have been implicated in repetitive or cyclic processes in the cell (Bourne, 1988; Hal, 1990). A growing number of organelle-specific small, GTP-binding proteins have been identified and proposed to play a regulatory role in the secretory pathway (Segev et al., 1988; Goud et al., 1988, 1990; Nakano and Muramatsu, 1989; Stearns et al., 1990; Chavrier et al., 1990). In particular, it has been proposed that transport of proteins between Golgi cisternae, which requires both ATP and cytosol, occurs via a cycle of coating and uncoating of vesicles and this cycle is blocked by GTPγS (Melançon et al., 1987; Orci et al., 1989). We wondered, therefore, whether the cycling of the 110-kD protein between the Golgi apparatus and the cytosol might represent just such a GTP binding protein controlled cyclic process in the secretory pathway.

When we tested the role of guanine nucleotides in our system we found that the association of the 110-kD protein with the Golgi apparatus could be altered in a manner consistent with that association being regulated by one or more GTP binding proteins. Pretreatment of the permeabilized cells with the nonhydrolyzable GTP analogue GTPγS prevented both the BFA and ATP depletion-induced redistribution of the 110-kD protein. Nonhydrolyzable analogues of ATP had no such protective effect. All of the effects of GTPγS seen in the permeabilized cells could be reproduced in intact cells by the addition of AlF₄⁻ (i.e., NaF plus AlCl₃). The ability of AlF₄⁻ to mimic the effects of GTPγS has also been observed in ER to Golgi and inter-Golgi transport assays (Beckers and Balch, 1989; Melançon et al., 1987; Orci et al., 1989).

Our results showing that the order of addition of BFA and GTPγS (or AlF₄⁻) to the permeabilized cells makes a difference in the response of the Golgi apparatus sheds some light on how these agents may be working. It is clear that BFA and GTPγS are not competitive antagonists in this system since the ability of one to influence the effects of the
other is strictly dependent upon the order of addition to the permeabilized cells. Thus, only if cells are pretreated with GTPγS (or AlF₄⁻) is the effect of BFA on the distribution of the 110-kD protein abrogated. If BFA is added first, the subsequent addition of GTPγS (or AlF₄⁻) had no effect on the distribution of the protein. These results are in contrast to the combined effects of ATP depletion and GTPγS. In this situation, GTPγS can both prevent and reverse the effects of ATP depletion on the distribution of the 110-kD protein. One simple interpretation of this is that the effect of ATP depletion on the distribution of the 110-kD protein is actually a manifestation of GTP depletion. In support of this, addition of AlF₄⁻ was also able to both protect and reverse the effects of ATP depletion on the distribution of the 110-kD protein in intact cells.

The results of the studies reported here can be summarized by the model shown in Fig. 7. According to this model, the 110-kD protein is normally undergoing a dynamic cycle in the cell between the cytoplasmic surface of the Golgi apparatus and the cytosol. We propose that this cycle is regulated by one or more GTP binding proteins. When the relevant GTP binding protein(s) is in its GTP state, the 110-kD protein associates with the Golgi apparatus. To be released into the cytosol, the bound GTP must be hydrolyzed. The 110-kD protein remains in the cytosol when the GTP binding protein is in the GDP state and rebinds to the Golgi complex when it returns to the GTP state. We cannot say where the relevant GTP binding protein is and whether it too cycles with the 110-kD protein or remains bound to the Golgi. In the presence of GTPγS or AlF₄⁻, the 110-kD protein is effectively locked onto the Golgi complex. This effect of GTPγS is reminiscent of the block in transport and change in morphology observed in isolated Golgi membranes after GTPγS. Inter-Golgi transport is blocked by GTPγS and the accumulation of coated vesicles is observed (Melançon et al., 1987; Orci et al., 1989).

In the context of the proposed cycle, BFA can be viewed as either causing the release of the 110-kD protein from the Golgi apparatus or preventing its rebinding from the cytosol. Considering the strict dependence of the order of addition of BFA plus either AlF₄⁻ or GTPγS in determining the localization of the 110-kD protein, we favor the interpretation that BFA acts to prevent the association of "free" 110-kD protein with the Golgi apparatus. Thus, if the 110-kD protein is in the cytosol and BFA is present, it can no longer rebind to the Golgi apparatus even when the regulatory GTP binding protein is in the GTP state. On the other hand, if the 110-kD protein is no longer cycling but is locked onto Golgi membrane by the regulatory GTP binding protein, with GTPγS or AlF₄⁻, then BFA has no effect on its distribution.

As stated earlier, we suspect that the redistribution of the 110-kD protein (and perhaps other as yet untested cycling proteins) induced by BFA is both proximal to and necessary for the subsequent changes in the Golgi apparatus induced by BFA. Consistent with this are two observations made in the course of the studies reported here. First, inhibition of the BFA-induced 110-kD protein redistribution results in the abolition of the subsequent effects of BFA on the structure of the Golgi apparatus (i.e., its dispersal into the punctate/reticular pattern). Second, during ATP depletion when the 110-kD protein has redistributed into the cytosol, we have occasionally observed long, tubular structures emanating from the Golgi complex in the absence of BFA (Donaldson, J. G., unpublished observations). It is as if these cells have lost enough ATP to release the Golgi structural protein but still maintain enough ATP to support the formation of Golgi tubules. We are currently testing whether there is indeed a window of ATP depletion that allows us to more consistently observe this phenotype.

Although much more work is needed to relate the different effects of BFA to each other, it is tempting to speculate on the possible relationship between cytosolic proteins and the Golgi apparatus with regards to both the structure and function of this organelle. It has been proposed in isolated Golgi membrane transport studies, that structural proteins cycling between the cytosol and Golgi membrane regulate transport through the Golgi apparatus (Orci et al., 1989). The failure to assemble such structures may underlie the transport block seen in the secretory pathway observed in the presence of BFA (Lippincott-Schwartz et al., 1989; Misumi et al., 1986). Another possible function of these peripheral proteins may be to form an organelle-specific exoskeleton responsible for the maintenance of the structure and identity of the Golgi apparatus. Assembly of such an exoskeleton might inhibit and/or regulate membrane movement out of and perhaps within the Golgi complex (i.e., membrane trafficking might require the selective breakdown of this coat). GTPγS and BFA, therefore, could be seen as two opposite extremes of the loss of regulation of the cycling of this coat. GTPγS, by locking the coat on the Golgi apparatus, would freeze membrane traffic into and out of the organelle. In contrast, the unregulated total loss of such a coat produced by BFA would lead to the unregulated trafficking of membrane out of the Golgi apparatus and the loss of the organelle as an identifiable entity. Whatever our final understanding of these processes proves to be, the utility of pharmacologic agents such as BFA and the ability to study their effects in semipermeabilized or cell-free systems will undoubtedly add to our understanding of the structure and function of organelles.

We are grateful to the Sandoz Company (Basel, Switzerland) and Epicentre Technologies (Madison, WI) for their gifts of brefeldin A, and Dr. K. Moremen (Massachusetts Institute of Technology, Cambridge, MA) for...
supplying antibody to mannosidase II. We thank Dr. T. E. Kreis (European Molecular Biological Laboratory, Heidelberg, Federal Republic of Germany) for generously providing the monoclonal antibody M3A5 against the 110-kD protein and for discussions. We also thank Drs. Juan S. Bonifacino and Victor Hsu for critical reading of the manuscript.

J. G. Donaldson was supported by a National Research Council-National Institutes of Health Research Associateship.

Received for publication 4 October 1990 and in revised form 23 October 1990.

References

Allan, V. J., and T. E. Kreis. 1986. A microtubule-binding protein associated with membranes of the Golgi apparatus. J. Cell Biol. 103:2229-2239.

Balch, W. E. 1989. Biochemistry of interorganelle transport. J. Biol. Chem. 264:16965-16968.

Balch, W. E., B. S. Glick, and J. E. Rothman. 1984. Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. Cell. 39:525-536.

Balch, W. E., K. R. Wagner, and D. S. Keller. 1987. Reconstitution of transport of vesicular stomatitis virus G protein from the endoplasmic reticulum to the Golgi complex using a cell-free system. J. Biol. Chem. 260:749-760.

Barak, L. S., R. R. Yokum, E. A. Nothnagel, and W. W. Webb. 1980. Fluorescence staining of the actin cytoskeleton with 7-nitrobenz-2-oxa-l,3-diazolephallacidin. Proc. Natl. Acad. Sci. USA. 77:980-984.

Beckers, C. J. M., and W. E. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. J. Cell Biol. 108:1245-1256.

Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permissible to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. Cell. 50:523-534.

Bieny, J., P. Deterre, C. Pflüger, and M. Chabre. 1987. Fluoride complexes of aluminum or of beryllium act on G-proteins as reversibly bound analogues of the γ-phosphate of GTP. EMBO (Eur. Mol. Biol. Organ.) J. 6:2907-2913.

Bourne, H. R. 1988. Do GTPases direct membrane traffic in secretion? Cell. 53:669-671.

Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell. 62:317-329.

Domín, R. W., G. Russ, and J. W. Yewdell. 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. J. Cell Biol. 109:61-72.

Donaldson, J. G., J. Lippincott-Schwartz, G. S. Bloom, T. E. Kreis, and R. D. Klausner. 1990. Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus: an early event in brefeldin A action. J. Cell Biol. 111:2295-2306.

Fujikawa, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara. 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. J. Biol. Chem. 263:18545-18553.

Fujikawa, T., K. Oda, and Y. Ikehara. 1989. Dynamic distribution of the Golgi marker thiamine pyrophosphatase is modulated by brefeldin A in rat hepatoma cells. Cell Struct. Funct. 14:605-616.

Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. 56:615-649.

Goud, B., A. Salminen, N. C. Walworth, and P. J. Novick. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. Cell. 53:753-768.

Goud, B., A. Zahrnauer, A. Tavitian, and J. Saraste. 1990. Small GTP-binding protein associated with Golgi cisternae. Nature (Lond.). 345:553-556.

Halt, A. 1990. The cellular functions of small GTP-binding proteins. Science (Wash. DC). 249:635-640.

Haselbeck, A., and R. Schekman. 1986. Interorganelle transfer and glycosylation of yeast invertase in vitro. Proc. Natl. Acad. Sci. USA. 83:2017-2021.

Kirkner, M., and L. R. Yarbrough. 1981. Assembly of tubulin with nucleotide analogs. J. Biol. Chem. 256:106-111.

Kobayashi, T., and R. E. Pagano. 1988. ATP-dependent fusion of liposomes with the Golgi apparatus of perforated cells. Cell. 55:797-805.

Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H. P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell. 60:821-836.

Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from the Golgi to ER. Cell. 56:801-813.

Malhotra, V., T. Sarfattini, L. Orci, J. C. Shepard, and J. E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. Cell. 58:329-336.

Mélançon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Sarfattini, M. L. Glaeser, L. Orci, and J. E. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. Cell. 51:1053-1062.

Misumi, Y., K. MiKi, A. Takatsuki, G. Tamaura, and Y. Ikehara. 1986. Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J. Biol. Chem. 261:11398-11403.

Moremen, K., and O. Touster. 1985. Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 cells. J. Biol. Chem. 260:6654-6662.

Nakano, A., and M. Muramatsu. 1989. A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. J. Cell Biol. 109:2677-2691.

Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell. 21:205-215.

Oda, K., S. Hirose, N. Takami, Y. Misumi, A. Takatsuki, and Y. Ikehara. 1987. Brefeldin A arrests the intracellular transport of a precursor of complement C3 before its conversion site in rat hepatocytes. FEBS (Fed. Eur. Biochem. Soc.) Lett. 214:135-138.

Orci, L., V. Malhotra, M. Amherdt, T. Sarfattini, and J. E. Rothman. 1989. Dissection of a single round of vesicular transport: sequential intermediates for intercisternal movement in the Golgi stack. Cell. 56:357-368.

Schekman, R. 1985. Protein localization and membrane traffic in yeast. Annu. Rev. Cell Biol. 1:115-144.

Segev, N., J. Mulholland, and D. Botstein. 1988. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with secretion machinery. Cell. 52:915-924.

Simons, K., and H. Virta. 1987. Perforated MDCK cells support intracellular transport. EMBO (Eur. Mol. Biol. Organ.) J. 6:2241-2247.

Stearns, T., M. C. Willingham, D. Botstein, and R. A. Kahn. 1990. ADP-ribosylation factor is functionally and physically associated with the Golgi complex. Proc. Natl. Acad. Sci. USA. 87:1238-1242.

Sternweis, P. C., and A. G. Gilman. 1982. Aluminum: a requirement for activation of the regulatory component of adenylate cyclase by fluoride. Proc. Natl. Acad. Sci. USA. 79:4858-4891.

Ulmer, J., and G. Palade. 1989. Targeting and processing of glycoporphins in murine erythroblast leukemia cells: use of brefeldin A as a perturbant of intracellular traffic. Proc. Natl. Acad. Sci. USA. 86:6992-6996.