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A Brefeldin A-like Phenotype Is Induced by the Overexpression of a Human ERD-2-like Protein, ELP-1

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Summary

Brefeldin A (BFA) is a unique drug affecting the molecular mechanisms that regulate membrane traffic and organelle structure. BFA's ability to alter retrograde traffic from the Golgi to the endoplasmic reticulum (ER) led us to ask whether the ERD-2 retrieval receptor, proposed to return escaped ER resident proteins from the Golgi, might either interfere with or mimic the effects of the drug. When either human ERD-2 or a novel human homolog (referred to as ELP-1) is overexpressed in a variety of cell types, the effects are phenotypically indistinguishable from the addition of BFA. These include the redistribution of the Golgi coat protein, β-COP, to the cytosol, the loss of the Golgi apparatus as a distinct organelle, the mixing of this organelle with the ER, the addition of complex oligosaccharides to resident ER glycoproteins, and the block of anterograde traffic. Thus, these receptors may provide signals that regulate retrograde traffic between the Golgi and the ER.

Introduction

The central vacuolar system comprises a complex set of organelles into which newly synthesized proteins are introduced through the endoplasmic reticulum (ER). Specific routes of membrane traffic carry material out of the ER to the cis-Golgi network (CGN), the Golgi stacks, the trans-Golgi network (TGN), and then to a variety of peripheral organelles, including the plasma membrane, endosomes, secretory granules, and lysosomes (Mellman and Simons, 1992). At each of these organelles, decisions are made either to transport or to retain specific molecules. Selective transport and retention underlie the ability of the cell to establish and maintain the individual identities of the separate organelles of this system (Palade, 1975; Pfeffer and Rothman, 1987; Klausner, 1989). Although it is reasonable to assume an essential relationship between membrane traffic and the maintenance of individual organelles within the central vacuolar system, experimental evidence for the existence of such a relationship has only recently been garnered. This evidence derives in part from studies (reviewed in Klausner et al., 1992) with the fungal product brefeldin A (BFA).

When added to cells, BFA exerts a profound effect on many organelles of the central vacuolar system. A stereotyped structural transformation of the Golgi stacks, TGN, endosomes, and lysosomes is induced and characterized by the production and extension of uncoated tubules (Lippincott-Schwartz et al., 1990, 1991; Woods et al., 1991). While membrane traffic patterns within the peripheral organelles of this system are altered (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Low et al., 1991), the effects of this drug on traffic and organelle identity have been best studied and characterized for the Golgi apparatus. Perhaps the most striking initial morphological change is the extension of uncoated tubules that emanate from the stacked cisternae of this organelle. These tubules represent structural intermediates that carry the membrane from the Golgi apparatus to the ER. All anterograde traffic beyond the mixed Golgi–ER system is tightly blocked (Lippincott-Schwartz et al., 1989; Doms et al., 1989). The specific traffic of membrane from the Golgi stacks to the ER was proposed to represent an exaggeration of an intrinsic Golgi to ER recycling pathway (Lippincott-Schwartz et al., 1990). Such a pathway had been postulated to explain the retrieval of resident lumenal ER proteins via the recognition of specific carboxy-terminal tetrapeptide sequences: KDEL in mammals (Munro and Pelham, 1987) and HDEL, DDEL, or ADEL in yeast (Pelham et al., 1988; Pelham, 1990). Compelling genetic evidence for the function of a membrane-bound receptor (the ERD-2 protein) for these sequences has been provided by Pelham and colleagues (Semenza et al., 1990; Lewis et al., 1990).

The ability of BFA to shut off one pathway of membrane traffic out of the Golgi complex (anterograde) while simultaneously enhancing an alternative pathway (retrograde) suggests that the drug interferes with one or more biochemical processes that regulate the balance between these two alternative traffic routes. Recent data demonstrate that a proximal effect of BFA is the inhibition of binding to the Golgi apparatus of cytosolic proteins strongly implicated to participate in membrane traffic (Donaldson et al., 1990; Orci et al., 1991; Klausner et al., 1992). Such proteins include the non-clathrin coat proteins or COPs (Serafini et al., 1991a; Duden et al., 1991) and the low molecular weight, GTP-binding protein, ADP-ribosylation factor (ARF) (Donaldson et al., 1991b; Serafini et al., 1991b). The rapid cycling on and off the Golgi membrane of these proteins is controlled by GTP binding and hydrolysis (Orci et al., 1989; Donaldson et al., 1991a). The addition of BFA prevents their reassociation with the membrane and results in their accumulation in the cytosol. In in vitro transport assays (Balch et al., 1984), the addition of BFA prevents the formation of coated vesicles and enhances the formation of noncoated tubules (Orci et al., 1991). These findings suggest that the binding of cytosolic coat proteins may determine the balance between coat-mediated vesicular and non-coat-mediated tubular traffic. Furthermore, this balance is essential to the steady-state maintenance of the Golgi apparatus as an organelle distinct from the ER.

Whether the effects of BFA are relevant to a more physiologic control of membrane traffic depends upon whether

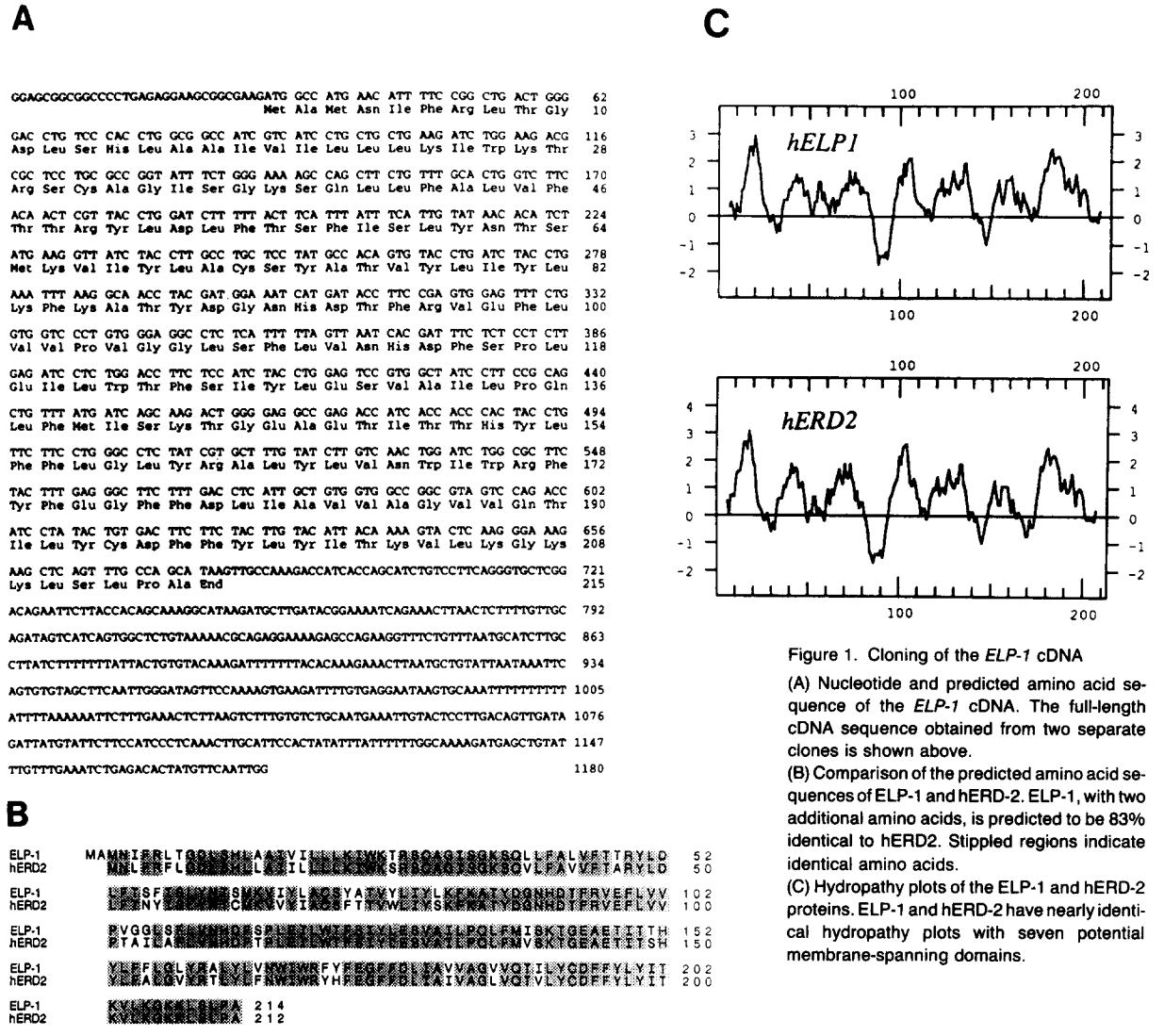

Figure 1. Cloning of the *ELP-1* cDNA

(A) Nucleotide and predicted amino acid sequence of the *ELP-1* cDNA. The full-length cDNA sequence obtained from two separate clones is shown above.

(B) Comparison of the predicted amino acid sequences of ELP-1 and hERD-2. ELP-1, with two additional amino acids, is predicted to be 83% identical to hERD2. Stippled regions indicate identical amino acids.

(C) Hydropathy plots of the ELP-1 and hERD-2 proteins. ELP-1 and hERD-2 have nearly identical hydropathy plots with seven potential membrane-spanning domains.

the cell possesses mechanisms to regulate the binding of cytosolic coat proteins to specific organelles. The recent finding implicating the potential role of trimeric G proteins in the assembly of cytosolic proteins onto the membrane has raised the possibility that the binding of such cytosolic proteins may be a truly regulated process, by analogy to the function of G proteins in signal transduction (Donaldson et al., 1991b). As the characterized activity of trimeric G proteins is regulated by membrane receptors, we were intrigued by the possibility that receptors such as ERD-2 may have signaling activity and may actively function to regulate retrograde traffic from the Golgi apparatus to the ER, rather than passively following a constitutive recycling pathway. Such a regulatory role for a membrane protein suggests that it may belong to a family of proteins, by analogy to other signaling receptors. For these reasons, we embarked upon a search for *ERD-2*-related genes as well as studies to determine whether overexpression of such proteins may either mimic or antagonize the dysregulation of Golgi traffic patterns induced by BFA. In this paper we report the cloning of one such protein, referred to as ELP-1 (ERD-like protein). Overexpression of this protein in a variety of cell types results in a perturbation of the Golgi apparatus that is phenotypically indistinguishable from that produced by treatment with BFA.

Results

Cloning of a Human ERD-2-Related Protein

In attempting to identify ERD-2-related proteins, we designed degenerate oligonucleotide primers corresponding to the most conserved regions among the reported ERD-2-related sequences (see Experimental Procedures). Total RNA from the human erythroleukemia cell line, K562, was reverse transcribed to make first-strand DNA that was then used as substrate for polymerase chain reaction (PCR) amplification. The presence of related sequences within a given amplified band was suggested by restriction digestion and the identification of fragments whose combined sizes added up to a molecular weight greater than

the original PCR product (Buck and Axel, 1991). A fragment of approximately 550 bp, consisting of a mixture of DNA sequences, was the major species generated from oligonucleotides corresponding to sequences within the predicted second and seventh transmembrane domains of ERD-2. We further selected these sequences by amplification with the original 5′ primers and another set of degenerate oligonucleotides, directed against a conserved region in the predicted sixth transmembrane domain. The resulting 420 bp amplified fragment was again shown to contain more than one DNA species. This mixture was then subcloned, and 20 individual colonies were screened by sequencing. While one clone gave rise to the identical sequence for the first reported human ERD-2 homolog (Lewis and Pelham, 1990), which we refer to as hERD-2, two others contained an open reading frame that corresponds to a closely related but novel gene product encoding an ERD-2-like protein, ELP-1.

This partial cDNA sequence was used to probe a human T cell cDNA library. Several clones were isolated, one of which encoded the entire open reading frame and included the poly(A) tail (Figure 1A). Northern blot analysis revealed that this gene encoded a 1.2 kb mRNA species distinct from hERD-2. The DNA sequence predicted a single open reading frame of 214 aa encoding a protein with a predicted molecular size of 24.6 kd. The predicted protein was 83% identical to the hERD-2 protein and had 75% nucleotide identity within the coding region (Figure 1B). The two genes demonstrate essentially no sequence homology within either the 5′ or 3′ untranslated regions. The hydropathy plots of ELP-1 and hERD-2 are strikingly similar (Figure 1C). In contrast to hERD-2, ELP-1 contains a consensus N-linked glycosylation site present at aa 62–64, between the second and third predicted transmembrane domains. Treatment with endoglycosaminidase H (endo H) of metabolically labeled ELP-1, however, suggests that this site is not utilized (data not shown).

Localization of hERD-2 and ELP-1

Transient expression of either hERD-2 or ELP-1 in COS cells was performed using the pCDLSRα expression vector (Takebe et al., 1988). Both proteins were tagged with the 13 aa, Myc-derived epitope to allow recognition of the expressed proteins (Evan et al., 1985). In COS cells, approximately 25% of the total population expressed the introduced proteins, as assessed by indirect immunofluorescence microscopy. Interestingly, more than one pattern of cellular distribution of the ELP-1 protein was observed. Approximately 50% of the expressing cell population stained for ELP-1 in a juxtanuclear, Golgi-like pattern, while the remainder showed a reticular, ER-like pattern with nuclear envelope staining (Figure 2). An identical pattern of distributions was observed when a distinct epitope tag derived from influenza hemagglutinin (Field et al., 1988) was utilized. Expression of hERD-2 in COS cells yielded cell populations with the same mixed pattern (Figure 2). The existence of a second ERD-2-like protein in human cells raises the issue of the function of these two closely related gene products. Whether they function differently, perhaps recognizing distinct ligands, will need to be addressed. It will also be intriguing to determine whether additional ERD-like proteins exist. For the remainder of this study, we focus primarily on the ELP-1 protein.

Using the same expression vector, we observed a similar heterogeneity in the pattern of distribution of ELP-1 after transient transfection into a human cell line, RD-4. In expressing cells, about 80% stained for ELP-1 in a Golgi-like pattern, while the remaining 20% showed an ER-like pattern. Our impression was that cells displaying an ER pattern of ELP-1 appeared to express more protein than the cells in which a Golgi distribution was seen. To test this hypothesis, we treated the transfected RD-4 cells with sodium butyrate, which is known to enhance expression of exogenously introduced genes (Gorman et al., 1983). RD4 cells were transfected with ELP-1 and then treated with or without 5 mM butyrate for 24 hr before the cells were examined by immunofluorescence microscopy. In untreated cells, approximately 20% of the ELP-1-positive cells demonstrated an ER pattern. In contrast, the addition of butyrate resulted in 80% of the expressing cells showing

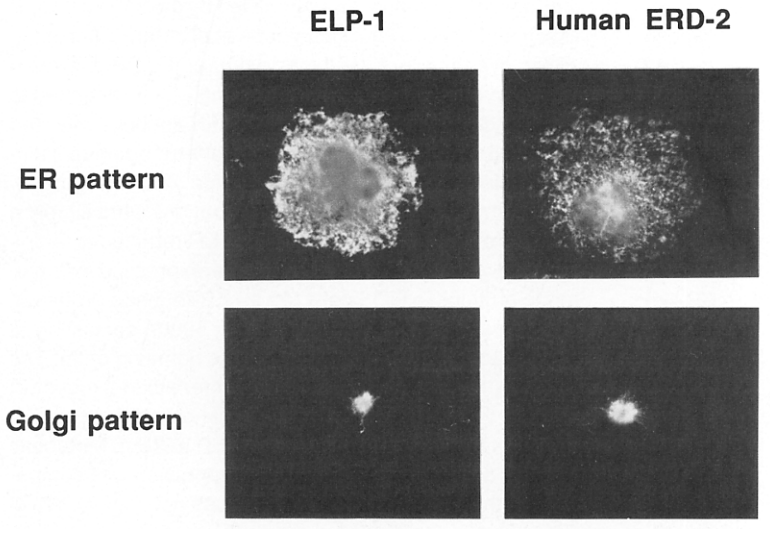

Figure 2. Transient Expression of Either ELP-1 or hERD-2 Results in Cell Populations with Mixed Patterns of Intracellular Distribution

COS cells were transiently transfected with either ELP-1 or hERD-2 and then stained with the anti-Myc antibody followed by fluorescein-labeled donkey anti-mouse antibody. Transfected cells revealed either a diffuse, reticular ER-like pattern or a compact, juxtanuclear Golgi-like pattern.

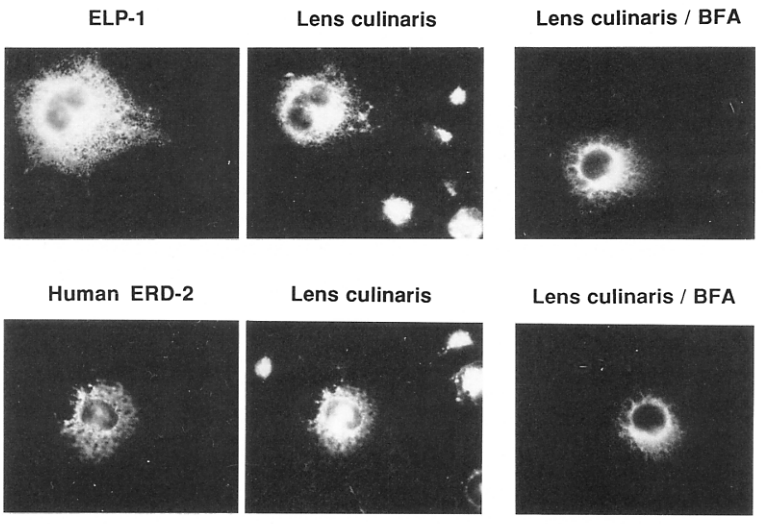

Figure 3. Overexpression of ELP-1 or hERD-2 Redistributes Golgi Markers into the ER

COS cells were transiently transfected with either ELP-1 or hERD-2, fixed, and permeabilized. Cells were double labeled with anti-Myc antibody followed by fluorescein-labeled donkey anti-mouse antibody and rhodamine-labeled lens culinaris lectin. Untransfected cells revealed an intact Golgi apparatus, as assessed by staining with the lens culinaris lectin, while transfected cells with ELP-1 staining in an ER pattern (left column) revealed the redistribution of the Golgi marker to a similar ER-like pattern (center column). For comparison, untransfected cells were treated with BFA and stained with the lens culinaris lectin (right column).

an ER distribution. This shift to an increase in ER distribution correlated with studies by metabolic labeling and immunoprecipitation that revealed a greater than 10-fold increase in protein expression (data not shown). As a further estimate of the level of overexpression achieved, we compared the ELP-1 mRNA levels in the COS transfectants with the endogenous ELP-1. When this is corrected for the fraction of cells expressing the protein, we estimate an 80- to 100-fold increase in mRNA levels in the cell population expressing the introduced gene.

Effect of Overexpressed ELP-1 on the Golgi Apparatus

We were curious why increased expression of ELP-1 in transfected cells seemingly resulted in its staining more of an ER pattern than a Golgi pattern. Perhaps as a result of overexpression the newly synthesized ELP-1 was unable to exit the ER owing to aggregation, misfolding, or an as yet undefined mechanism. In many of the cells expressing ELP-1 in an ER pattern, no coexisting Golgi pattern was observed. This suggested that an alternative explanation of the heterogeneous distribution patterns was that overexpression of this protein may have resulted in a collapse of the Golgi apparatus into the ER, as seen in cells treated with BFA. We utilized the lens culinaris lectin as a Golgi marker (Ridgway et al., 1992) because the available Golgi-specific antibodies gave no staining in COS cells. In these cells, the lens culinaris lectin gave a typical Golgi staining pattern, both in untransfected cells and in cells whose ELP-1 distribution was Golgi-like. In cells expressing ELP-1 in an ER pattern, however, no Golgi-like staining by the lectin was seen. Rather, the lectin gave an ER-like staining pattern (Figure 3). This staining could not be attributed to the lectin recognizing ELP-1, because we have previously shown ELP-1 to be unglycosylated. Furthermore, when hERD-2 (not a glycoprotein) yielded an ER staining pattern, the staining with the lens culinaris lectin was likewise apparently limited to the ER (Figure 3). The same ER distribution of lectin staining could be produced by treatment of cells with BFA. We sought to confirm this finding in RD-4 cells using the trans-Golgi marker, galactosyltransferase (GalTf). As with the lectin, in cells expressing ELP-1 in an ER pattern the pattern of staining with an antibody directed against GalTf (kindly provided by Dr. Eric Berger) was ER-like, in contrast to the Golgi-like pattern observed in control cells or in cells expressing ELP-1 in a Golgi distribution. Again, the pattern of staining for GalTf in the ELP-1-overexpressing RD-4 cells was indistinguishable from that seen after treatment with BFA. Finally, ELP-1 was transiently expressed in NRK cells, which allowed us to follow the fate of the cis-medial Golgi marker, mannosidase II (using an antibody kindly provided by Dr. Kelly Moremen). While only a small percentage (<5%) of these cells displayed an ER pattern, examination of the distribution of mannosidase II also revealed an ER-like pattern and again was indistinguishable from NRK cells treated with BFA (data not shown).

The critical proximal effect of BFA appears to be the prevention of binding of cytosolic coat proteins to target membranes, the best studied of which are the Golgi nonclathrin coat proteins, as exemplified by the 110 kd β-COP (Allan and Kreis, 1986; Donaldson et al., 1990; Orci et al., 1991). We therefore examined whether β-COP showed a diffuse cytosolic pattern in cells having ELP-1 restricted to the ER. Staining of such cells with an antibody directed against β-COP revealed a diffuse pattern, again indistinguishable from that seen upon addition of BFA (Figure 4). To determine whether these redistributions were simply a result of overexpression of either an ER-retained or Golgi-retained membrane protein, we overexpressed two chimeric proteins in COS cells. As an ER-retained protein, a chimera (Tac-E19; Bonifacino et al., 1990), consisting of the extracellular and transmembrane domains of the Tac antigen (the α subunit of the human interleukin-2 receptor) fused to the cytoplasmic tail of the adenovirus E19 protein, was expressed and yielded only an ER pattern. Metabolic labeling confirmed that its level of expression was comparable with that achieved for ELP-1 (data not shown). While

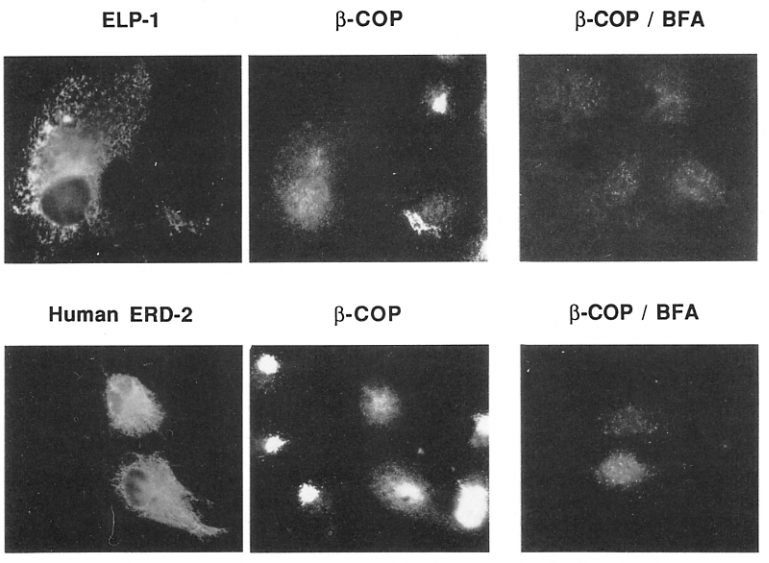

Figure 4. Overexpression of ELP-1 or hERD-2 Leads to the Redistribution of β-COP from Its Golgi Pattern

COS cells expressing ELP-1 or hERD-2 were fixed and permeabilized. Cells were then double-labeled with anti-Myc antibody followed by fluorescein-labeled donkey anti-mouse antibody, and anti-β-COP antibody followed by rhodamine-labeled donkey anti-rabbit antibody. Untransfected cells revealed β-COP staining in a Golgi pattern, while transfected cells with ELP-1 staining in an ER pattern (left column) showed staining of β-COP in a diffuse, hazy pattern (center column). Untransfected cells treated with BFA and stained for β-COP showed a similar diffuse, hazy pattern (right column).

immunofluorescence studies demonstrated an ER pattern for the overexpressed TacE19, in no cell was there any redistribution of either the lens culinaris lectin or β-COP. As a membrane protein retained in the Golgi apparatus, we utilized a chimera (supplied to us by Dr. Caroline Machamer; Swift and Machamer, 1991) consisting of the vesicular stomatitis virus G protein whose transmembrane domain was replaced by the first transmembrane domain of the coronavirus E1 protein. Overexpression of this construct in COS cells yielded a heterogeneous pattern of distribution by immunofluorescence, similar to that of ELP-1, in which some cells displayed a predominantly Golgi pattern, while others displayed a predominantly ER-like pattern of distribution of the introduced protein. In contrast to ELP-1, however, no underlying alteration in the Golgi apparatus of any cell was observed when stained by either lens culinaris lectin or antibody to β-COP (data not shown).

Effects of ELP-1 Overexpression on Secretion and Golgi Processing

The effects of BFA on the structure of the Golgi apparatus are characterized by two biochemical hallmarks. First, proteins cannot leave the mixed ER–Golgi system, resulting in a tight secretion block. Second, the redistribution of Golgi enzymes into the ER results in Golgi-specific carbohydrate processing of ER-retained glycoproteins (Lippincott-Schwartz et al., 1989). To examine the possibility that ELP-1 overexpression results in a secretion block, we first transfected COS cells with lysozyme, a marker protein for secretion. Indirect immunofluorescence staining for lysozyme revealed a mixed population of cells with both ER and Golgi patterns. When cycloheximide was added to these singly transfected cells at 20 μg/ml for 4 hr, virtually all cells were either negative or had a weak Golgi staining for lysozyme, suggesting that lysozyme was being "chased" out of transfected cells. We then coexpressed ELP-1 with lysozyme. Double labeling by indirect immunofluorescence showed that greater than 90% of transfected cells coexpressed both proteins in either colocalized ER or Golgi patterns. The subsequent addition of cycloheximide resulted in the persistent staining of cells with lysozyme in an ER distribution only. All of these cells demonstrated a persistent ER staining pattern of ELP-1 (Figure 5A). Meanwhile, cells with persistent staining for ELP-1 in a Golgi pattern showed no staining for lysozyme (Figure 5A). These results suggested that cells with ELP-1 in an ER pattern blocked secretion of lysozyme, while cells with ELP-1 in a Golgi pattern did not. More exact quantitation of the apparent secretion block was obtained by performing metabolic labeling and pulse-chase experiments. In cells transfected with lysozyme alone, 85% of the newly synthesized marker protein was recovered in the medium after a 4 hr chase. In contrast, in cells doubly transfected with lysozyme and ELP-1, only 35% of the newly synthesized lysozyme was recovered from the medium (Figure 5B). Considering that only about 50% of the doubly transfected cells appeared to overexpress ELP-1 in an ER-like pattern and those were the cells that appeared to retain lysozyme by immunofluorescence, we believe that the level of the secretion block may, in fact, be complete in those cells with apparently disrupted Golgi structures.

We next asked whether the apparent presence of Golgi components in the ER would result in the processing of carbohydrate side chains of ER-retained proteins, as observed with BFA treatment. Tac-E19, an ER-retained protein (Bonifacino et al., 1990), was expressed either alone or with ELP-1 in COS cells. After transfection, cells were metabolically labeled, chased in unlabeled medium, and then immunoprecipitated for Tac-E19. In singly transfected populations, the vast majority of the Tac-E19 remained sensitive to endo H during the chase period. In contrast, in the double transfectants, a fraction of the Tac-E19 increased in apparent molecular weight, and approximately 50% acquired endo H resistance during the chase period. In comparison, addition of BFA resulted in a simi-

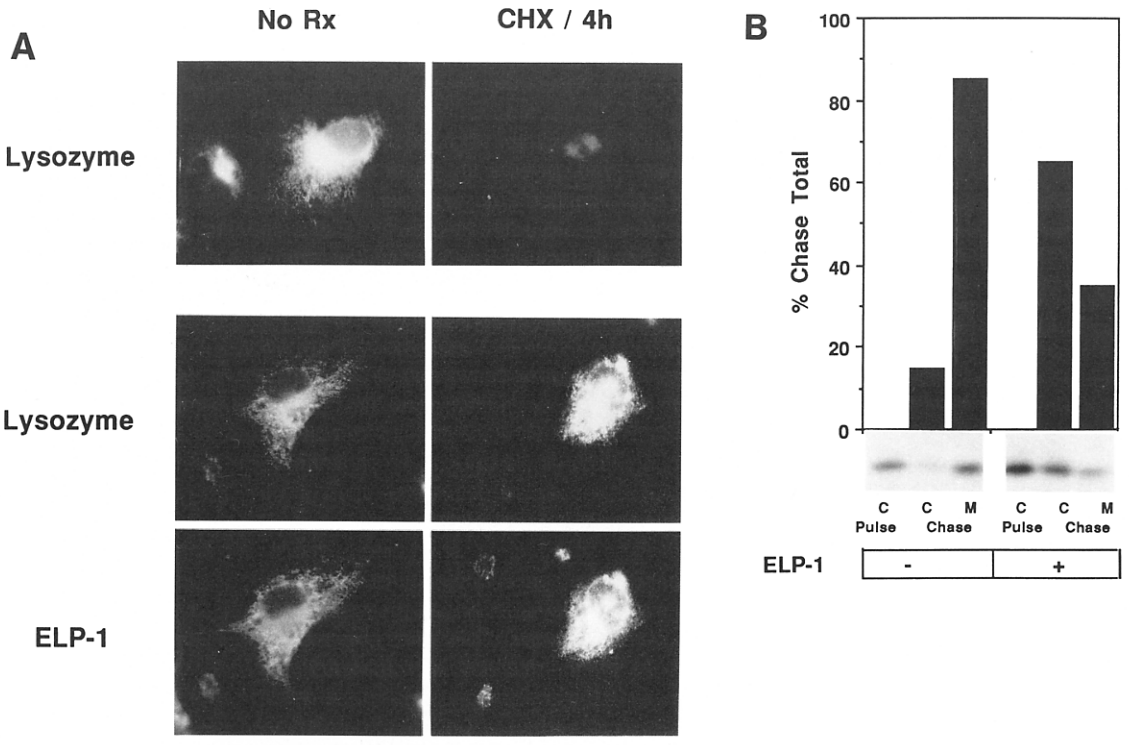

Figure 5. Overexpression of ELP-1 Blocks Secretion

(A) Secretory block assessed by immunofluorescence. Lysozyme was transfected either alone or with ELP-1 into COS cells. Cells were then either not treated (No Rx) or treated with cycloheximide (20 μg/ml) for 4 hr at 37°C (CHX/4h), fixed, and permeabilized. Double labeling was performed with anti-Myc antibody followed by fluorescein-labeled donkey anti-mouse antibody and anti-lysozyme antibody followed by rhodamine-labeled donkey anti-rabbit antibody. Staining in cell populations transfected with lysozyme alone (top row) revealed a mixed ER and Golgi pattern. After treatment with cycloheximide for 4 hr, the few cells that still stained for lysozyme showed a predominantly Golgi staining pattern. Doubly transfected cell populations (bottom two rows) had both lysozyme and ELP-1 staining showing a mixed ER and Golgi pattern. After treatment with cycloheximide, however, most cells that stained for ELP-1 in an ER pattern retained lysozyme staining in an ER pattern, while cells that stained for ELP-1 in a Golgi pattern lost staining for lysozyme.

(B) Biochemical correlation of the secretory block. COS cells were transfected with either lysozyme alone or together with ELP-1, pulsed with ^{35}S-methionine for 30 min, and chased in complete medium for 4 hr. Both the cell lysate (C) and the medium (M) were immunoprecipitated with anti-lysozyme antibody and evaluated by SDS–PAGE. After the chase, 15% of labeled lysozyme remained intracellularly in cells transfected with lysozyme alone, while 65% remained intracellularly in cells transfected with ELP-1 and lysozyme.

lar, but now uniform, acquisition of endo H resistance by the ER-retained protein (Figure 6).

Effect of ER Retention of ELP-1 on the Alteration of the Golgi Apparatus

The results presented thus far do not allow us to determine whether overexpression of ELP-1 results in its retention in the ER, which in turn leads to the disassembly of the Golgi apparatus, or whether ELP-1 must be able to reach the Golgi in order to alter the structure of that organelle. If the latter were true, we reasoned that the BFA effect might be lessened if ELP-1 could be retained in the ER. In an attempt to address this, we constructed two variants of the ELP-1 protein. In the first construct, we introduced the cytoplasmic tail of the adenovirus E19 protein at the carboxyl terminus of ELP-1 (ELP-1/E19). The second variant was identical to the first, but the two lysines at positions −3 and −4 of the E19 tail were replaced with serines (ELP-1/E19s). It has previously been shown that these substitutions abolish the ER retention information of this sequence (Jackson et al., 1990). When ELP-1/E19 was overexpressed in COS cells, only an ER pattern of the expressed protein was seen (Figure 7A). The efficacy of this sequence suggests that the carboxyl terminus of ELP-1 is oriented toward the cytosol. In contrast, the distribution of ELP-1/E19s was like that of ELP-1, yielding some cells that display a Golgi-like pattern and others with an ER-like pattern (Figure 7A). We next compared the fraction of all cells expressing either of these proteins that display a BFA-like distribution of β-COP. In several experiments cells expressing ELP-1/E19 had two to three times fewer cells with a diffuse β-COP distribution than observed in cells expressing either ELP-1 or ELP-1/E19s (Figure 7A). We further assessed the relative ability of these two proteins to induce the Golgi-like processing of the carbohydrate side chains of the ER-retained, Tac-E19 construct. As shown in Figure 7B, the coexpression of ELP-1/E19 with Tac-E19 resulted in little additional processing of Tac-E19 than was seen over 24 hr in cells expressing Tac-E19 alone. In contrast, coexpression of ELP/E19s with Tac-E19 resulted in efficient processing of the Tac chimera, consistent with the ability of this ELP-1 chimera

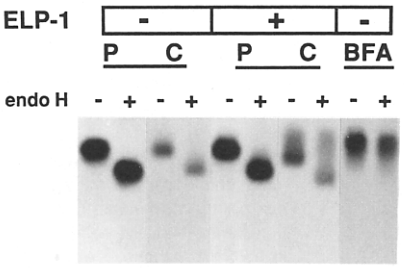

Figure 6. Biochemical Evidence for the Redistribution of the Golgi Apparatus into the ER

COS cells were transfected with either Tac-E19 alone or together with ELP-1, pulsed with ^{35}S-methionine for 3 hr (P), chased with complete medium for 24 hr (C), lysed, immunoprecipitated with anti-Tac antibody (7G7), and evaluated by SDS–PAGE. For BFA treatment, cells transfected with Tac-E19 alone were chased in the presence of BFA (5 μg/ml). While Tac-E19 remained endo H sensitive when transfected alone, cotransfection with ELP-1 revealed that approximately 50% of Tac-E19 attained some degree of endo H resistance during the chase period.

to induce a BFA-like phenotype. In these experiments, cells were expressing comparable levels of either ELP-1 chimeric protein, as assessed by immunoprecipitation.

Endocytosis Continues in Cells Overexpressing ELP-1

We next examined the endocytic system to determine whether internalization and recycling in this peripheral organelle continued in cells whose secretory pathway was disrupted by ELP-1 overexpression. COS cells transfected with ELP-1 were allowed to internalize human diferric transferrin at concentrations selected to assure only receptor-mediated uptake (Lippincott-Schwartz et al., 1991). The steady-state distribution of transferrin within the cells was examined by immunofluorescence microscopy. Neither the pattern nor the apparent amount of internalized transferrin was significantly altered in cells expressing ELP-1 in an ER pattern. If these cells were cycling transferrin, a washout and continued incubation should result in a loss of transferrin from the cells. When this procedure was carried out, no apparent difference was discerned between cells overexpressing ELP-1 and the rest of the population (Figure 8).

Discussion

The most striking findings in this study are the dramatic phenotypic changes associated with ELP-1 overexpression in a variety of cell types. It is particularly interesting to contrast the effects of ELP-1 overexpression with the effects of ERD-2 deletion in Saccharomyces cerevisiae described by Semenza et al. (1990). While deletion of this gene is lethal, the authors were able to examine the consequences of down-regulating the cellular expression of the ERD-2 protein. Such cells accumulate intracellular membranes that may be either the Golgi apparatus or Golgi derived and demonstrate a block in normal transport through the secretory pathway at the level of the Golgi apparatus. These results led the authors to propose that,

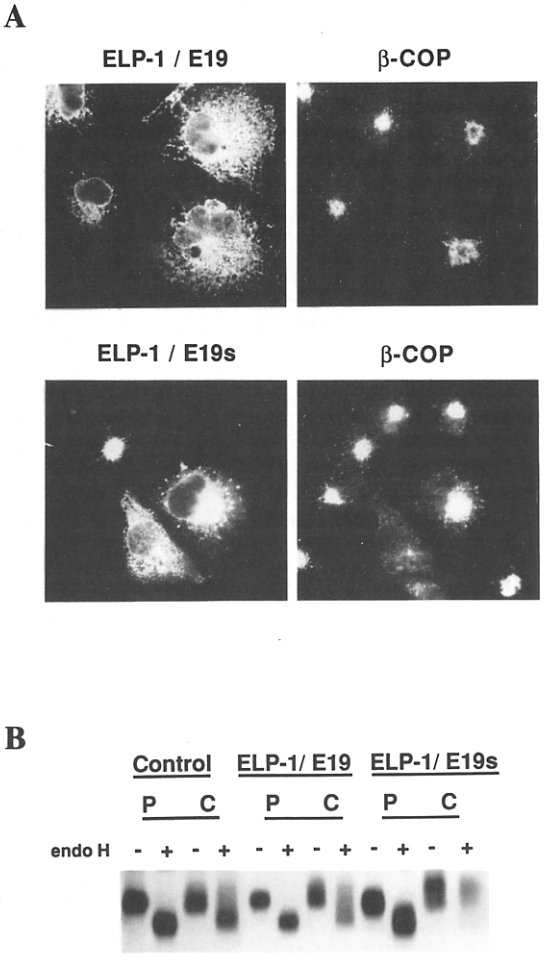

Figure 7. The BFA-like Phenotype Induced by ELP-1 Overexpression Is Diminished by the ER Retention of ELP-1

(A) Effect of ER-retained ELP-1 on β-COP redistribution. ELP-1/E19 or ELP-1/E19s were transfected into COS cells. Cells were fixed, permeabilized, and double labeled with mouse anti-Myc antibody followed by fluorescein-labeled donkey anti-mouse antibody and rabbit anti-β-COP antibody followed by rhodamine-labeled donkey anti-rabbit antibody. ELP-1/E19 was distributed in an ER pattern in all transfected cells, while the expression of ELP-1/E19s was heterogeneous with the transfected cell population exhibiting a mixed ER and Golgi pattern as previously seen in ELP-1 expression. ELP-1/E19 expression resulted in two to three times fewer cells with β-COP redistributed as compared with cells with ELP-1/E19s expression.

(B) Effect of ER-retained ELP-1 on Golgi redistribution into the ER. Tac-E19 was cotransfected with either a control plasmid (ELP-1/E19) or ELP-1/E19s into COS cells, pulsed with ^{35}S-methionine for 3 hr (P), chased with complete medium for 24 hr (C), lysed, immunoprecipitated with anti-Tac antibody (7G7), and evaluated by SDS–PAGE. While both control and cotransfection with ELP-1/E19 resulted in Tac-E19 being mainly endo H sensitive, ELP-1/E19s cotransfection led to almost complete endo H resistance of Tac-E19, consistent with a restoration of the BFA-like phenotype.

apart from retrieval of lumenal proteins bearing the HDEL sequence, ERD-2 functions by regulating the maintenance of the Golgi apparatus, most likely by regulating the retrograde movement of membrane from the Golgi apparatus to the ER. According to this view, in the absence of ERD-2, retrograde traffic would stop. In contrast, our

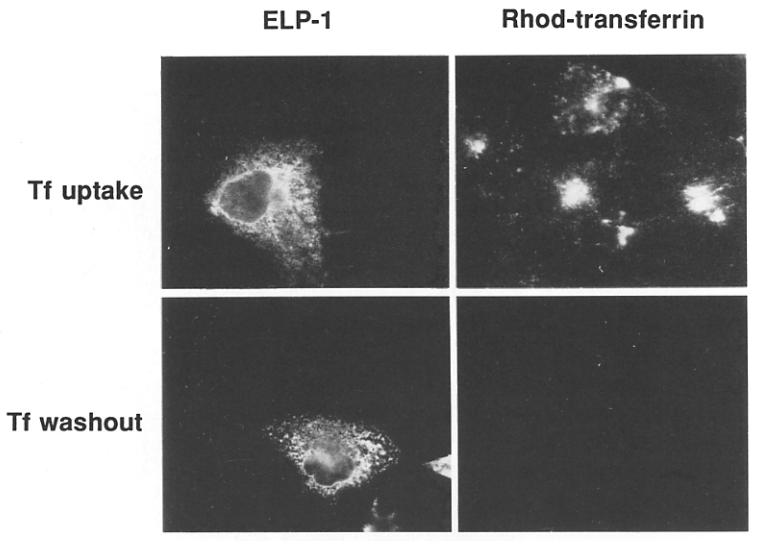

Figure 8. Endocytosis Continues in Cells Overexpressing ELP-1

COS cells were transiently transfected with ELP-1, incubated with rhodamine-labeled transferrin for 2 hr, fixed, permeabilized, and then stained with anti-Myc antibody followed by fluorescein-labeled donkey anti-mouse antibody. Transferrin washout was done by incubating cells in complete medium for 6 hr prior to fixation. Transferrin (Tf uptake) maintained a juxtanuclear distribution in either cells expressing ELP-1 in an ER pattern or untransfected cells. After transferrin washout (Tf washout), rhodamine-labeled transferrin could not be detected in any cells.

results can be explained by proposing that overexpression of ELP-1 results in the opposite effect: hyperactive retrograde traffic resulting in the loss of the Golgi stacks and the mixing of these stacks with the ER, as seen in treatment with BFA.

The Golgi apparatus can be viewed as a steady-state structure maintained by the controlled balance of membrane traffic into and out of it. Based upon the profound alteration in the Golgi complex induced by BFA, we proposed that the balance of this traffic depends on a carefully controlled cycle of association/dissociation of cytosolic coat proteins such as the coatomer (Klausner et al., 1992). If one of the functions of molecules such as ERD-2 and ELP-1 is to influence this coat cycle, the phenotypic changes observed could be explained by the overexpression of these proteins perturbing this coat cycle. Although we do observe a redistribution of β-COP in cells overexpressing ELP-1, we have no direct evidence that ELP-1 actually inhibits coatomer binding and whether this inhibition is a proximal effect in the dysregulation of Golgi membrane traffic that is seen in cells overexpressing ELP-1. Thus, despite the resemblance between BFA addition and ELP-1 overexpression, it is difficult to discern whether these two effects arise via analogous mechanisms. This difficulty is partly due to the inability to examine the acute effects of ELP-1 overexpression or the acute reversal of that overexpression, analogous to the addition or removal of BFA.

That the traffic of hERD-2 can itself be regulated is suggested in a recent study (Lewis and Pelham, 1992) demonstrating that coexpression of hERD-2 with a ligand results in the redistribution of hERD-2 from a Golgi to an ER pattern. These results strongly point to a change in the traffic of the receptor in response to occupancy by a specific ligand. Whether the effects of ELP-1 overexpression reported here require ligand–ELP-1 interaction cannot be determined. It is possible that the phenotypic changes might be explained as the result of reaching a critical level of ligand-independent receptor activation that would mimic the proposed ligand-induced stimulation of retrograde traffic from the Golgi apparatus to the ER. Numerous examples exist in which overexpression of a normal signaling molecule leads to a "constitutively on" state (for example, see review by Yarden and Ullrich, 1988). We believe that the similarity between the phenotypes induced by BFA and ELP-1 overexpression raises the possibility that these two phenomena are mechanistically related. In this regard, it is tempting to speculate on recent data suggesting a role for trimeric G proteins in the regulated binding of BFA-sensitive coat proteins to Golgi membranes (Donaldson et al., 1991b). Perhaps these seven transmembrane receptors, localized mainly to the Golgi apparatus, will prove to transmit local signals that are mediated by interactions with G proteins.

The regulatory model described above predicts that the effect of ELP-1 overexpression is exerted at the level of the Golgi apparatus rather than at the ER. To test this hypothesis, we examined the effects of overexpression of ELP-1, whose carboxyl terminus had been modified to contain either a functional or inactive ER retention motif derived from the adenovirus E19 protein (Jackson et al., 1990). As predicted, the steady-state distribution of ELP-1/E19 was restricted to the ER in all cells expressing the protein. When compared with the inactive retention variant (which was found in both ER and Golgi distribution patterns), the retained ELP-1 produced a markedly diminished BFA-like phenotype. The ELP-1/E19 protein, however, still had some effect. This may be due to a lowered activity of the ELP-1 exerted at the level of the ER. Alternatively, if this protein is able to leave the ER and be retrieved from a post-ER compartment, its BFA-like effects might still be attributed to its passage through the Golgi. Even if the latter occurs, it is clear that the presence of this cytoplasmic sequence results in a steady-state distribution that is greatly biased toward residency in the ER, and this seems to correlate with its diminished BFA-like effect. If ELP-1 must reach the Golgi complex in order to produce the effects reported here, how might this process occur?

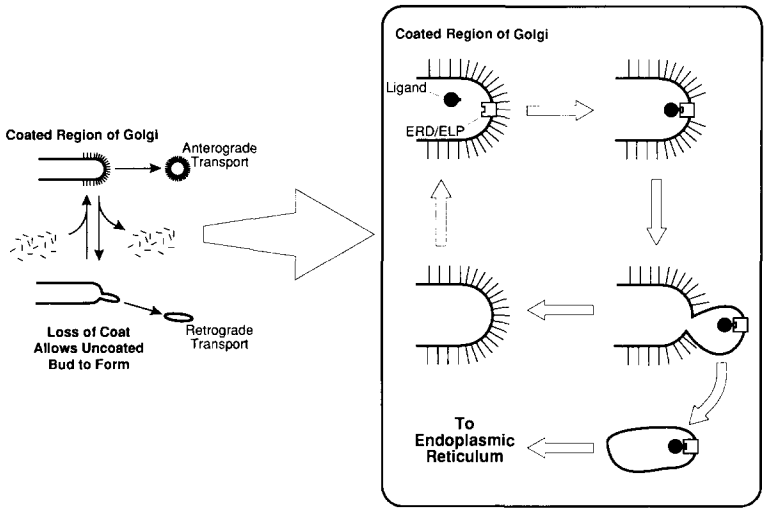

Figure 9. Model for ERD/ELP Regulation of Golgi–ER Traffic

Traffic leading out of the Golgi apparatus (left side of the diagram) can utilize either coated vesicles (anterograde transport) or uncoated tubulovesicular structures (retrograde transport). Ligand binding of ERD/ELP receptors leads to a local loss of Golgi coat protein binding and allows the formation of uncoated tubulovesicular structures (right side of the diagram).

A model can be proposed based on the similarity between the effects of ELP-1 overexpression and the addition of BFA to cells. BFA most likely exerts its effects by preventing the GTP-regulated binding of cytosolic coat proteins to the Golgi apparatus and associated structures (Donaldson et al., 1991a). We can view traffic leading out of the Golgi complex as utilizing either coated vesicles (anterograde traffic) (Rothman and Orci, 1992) or uncoated tubules or tubulovesicular structures (retrograde traffic) (Klausner et al., 1992). In the presence of BFA, the profound inhibition of coat protein binding inhibits the former pathway and greatly exaggerates the latter. We can imagine then that the presence of liganded ERD-2 or ELP-1 in the Golgi might result in a local inhibition of coat protein binding. In the absence of coat, a BFA-like uncoated bud would emerge, carrying the receptor–ligand complex back to the ER (Figure 9). Thus, according to such a model, the level of retrograde traffic, mediated by uncoated buds, would be determined by the level of ER lumenal protein leakage into the Golgi complex.

Aspects of the phenotype first described for BFA are observed in at least three different instances: upon addition of the drug, when ELP-1 is overexpressed, and in an intriguing mutant cell line first isolated by Nakano et al. (1985). In this last instance, a temperature-sensitive mutant of CHO cells, selected by virtue of a conditional secretion block, has been reported to mimic at least some of the effects of BFA on the Golgi apparatus at nonpermissive temperatures (Zuber et al., 1991). Although we must directly address the mechanistic relations underlying these phenomena, we are beginning to gather multiple pieces of evidence that the Golgi apparatus exists as a steady-state structure, maintained at a fine and highly regulated balance. Such a view has implications far beyond this organelle and raises the question of how cellular control over the structure and dynamics of its various organelles is maintained. As useful as the drug BFA may ultimately prove to be in studying membrane traffic, we can, in addition, observe dramatic changes in organelle structure and membrane traffic by altering the level of expression of endogenous proteins. The identification and perturbation of such proteins, with a detailed description of the resulting phenotypic changes, will set the stage for asking more mechanistic questions regarding the biochemical basis of membrane traffic and organelle dynamics.

Experimental Procedures

RNA PCR Cloning

Degenerate oligonucleotides were synthesized (Applied Biosystems) corresponding to conserved amino acid sequences among S. cerevisiae ERD-2 and its Kluyveromyces lactis and human homologs. The following primers were used to generate a fragment of ELP-1:

GGIAT(T/C/A)(T/A)(C/G)IGGIAA(A/G)(T/A)(C/G)ICA(A/G)GTI(C/T)TIT(T/A)(T/C)GC (corresponding to the second hydrophobic region, sense direction).
(A/G)TAIA(A/G/T)(A/G)TA(A/G)AA(A/G)AA(A/G)TC (corresponding to the seventh hydrophobic region, antisense direction).
(A/G)TAIC(G/T)CCA(A/G/T)ATCCA(A/G)TT (corresponding to the sixth hydrophobic region, antisense direction).

RNA PCR (Perkin/Elmer Cetus) was performed according to the manufacturer's guidelines with modifications (Buck and Axel, 1991). First-strand cDNA was generated by adding random hexamers to total RNA from the human erythroleukemia cell line K562 (provided by F. Samaniego) and incubating with reverse transcriptase at 42°C for 15 min, 37°C for 30 min, 99°C for 5 min, and then cooled to 4°C. Oligonucleotides 1 and 2 were then added to the mixture with Taq polymerase, and PCR was performed under the following conditions: 95°C for 2 min (1 cycle); 92°C for 2 min, 45°C for 2 min, 72°C for 2 min (50 cycles); 72°C for 5 min (1 cycle). One microliter of this amplified mixture was then added to 100 μl of fresh PCR mixture using oligonucleotides 1 as the 5′ primers and oligonucleotides 3 as the 3′ primers, and nested PCR was performed with the following parameters: 95°C for 2 min (1 cycle); 92°C for 2 min, 45°C for 2 min, 72°C for 2 min (25 cycles); 72°C for 5 min (1 cycle).

One microliter of this amplified mixture was then used for ligation in the TA cloning system (Invitrogen), and subcloning was performed as recommended by the manufacturer. Positive colonies were analyzed by dideoxy DNA sequencing (Sequenase, US Biochemicals) of their inserts.

cDNA Library Screening

A 450 bp fragment subcloned as described above was found to have

an open reading frame encoding a predicted amino acid sequence 82% identical to hERD-2. This fragment, along with two oligonucleotides within it (CAGGTAGATCAGGTACACTGTGGCAT and CAAAGCACGATAGAGGCCCAGGAAG, both antisense direction) designed to be at least 50% dissimilar to the corresponding sequence in hERD-2, was used to screen a λZap II cDNA library (Stratagene) generated from the human YT T cell line (Gnarra et al., 1990). Triplicate filters (DuPont/NEN) were obtained, and a total of approximately 250,000 plaques was screened. The 450 bp probe was ^{32}P-labeled by random priming (Feinberg and Vogelstein, 1984), and the two oligonucleotides were end labeled (T4 polynucleotide kinase, BRL; 7000 Ci/mmol [γ-^{32}P]ATP, ICN) to a specific activity of ~2 × 10^9 cpm/μg. Hybridization was performed at 42°C for 16 hr by standard methods as described in Maniatis et al. (1982) followed by two washes of 2× SSC/1% SDS at room temperature. The final washes were performed in 0.1 × SSC/1% SDS twice at 65°C for the 450 bp probe or twice at 55°C for the two oligonucleotide probes. Plaques positive for all three probes were purified and subjected to phagemid rescue for subcloning into Bluescript plasmids.

RNA Gel Blot

Total cytoplasmic RNA was prepared from cultured cells lysed by NP-40. Samples containing 10 μg of total RNA were then loaded onto an agarose–formaldehyde gel, separated by electrophoresis, and electroblotted onto nylon filter. Hybridization and washes were performed as described above.

Reagents, Antibodies, and Cells

BFA was obtained from Epicentre Technologies and stored as stock solutions of 5 mg/ml in methanol. Cycloheximide was purchased from Sigma and stored as stock solutions of 10 mg/ml in ethanol.

The following antibodies were used: mouse monoclonal antibody directed against an epitope in Myc (9E10, provided by T. Rouault), rabbit polyclonal anti-lysozyme antiserum (provided by A. Colman), rabbit anti-mannosidase II antiserum (provided by K. Moremen), rabbit polyclonal anti-β-COP antiserum (provided by T. Kreis), mouse monoclonal anti-Tac antibody (7G7, provided by D. Nelson), and rabbit anti-galactosyltransferase antiserum (provided by E. Berger).

Fluorescein-labeled, donkey anti-mouse antibody and rhodamine-labeled, donkey anti-rabbit antibody were purchased from Jackson Immunoresearch. Rhodamine-labeled lens culinaris lectin was obtained from E. Y. Laboratories, and rhodamine-labeled transferrin was purchased from Molecular Probes.

The monkey fibroblast cell line COS, rat kidney cell line NRK, and human rhabdomyosarcoma cell line RD-4 were all grown in complete medium consisting of Dulbecco's modified Eagle's medium (Biofluids) with 8% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 cell incubator.

Plasmids

For the expression of epitope-tagged ELP-1 or hERD-2, oligonucleotides containing the 5′ or 3′ coding sequence of either ELP-1 or hERD-2 were synthesized with flanking restriction sequences. The coding sequence for the Myc epitope (SMEQKLISEEDLN) or the hemagglutinin epitope (YPYDVPDYA) was added to the 3′ end of the 3′ oligonucleotides. ELP-1 was amplified from the cloned cDNA by PCR using the appropriate primers. hERD-2 was amplified by RNA PCR. Human K562 total RNA was incubated with random hexamers and reverse transcriptase and then the appropriate primers were added and amplified with the PCR parameters: 95°C for 2 min (1 cycle); 92°C for 2 min, 55°C for 2 min, 72°C for 2 min (50 cycles); 72°C for 5 min (1 cycle). PCR products for both ELP-1 and hERD-2 were then digested with appropriate restriction enzymes and subcloned into the expression vector pCDLSRα. To generate ELP-1 chimeras, PCR was performed using the ELP-1/Myc construct as the template. The 3′ oligonucleotide contained the last 24 bases encoding the Myc epitope followed by the coding sequence for the E19 cytoplasmic tail: KYKSRRSFIDEKKMP (for ELP-1/E19) or KYKSRRSFIDESSMP (for ELP-1/E19s). All constructs generated by PCR were confirmed by DNA sequencing.

Complementary DNAs coding for lysozyme (provided by A. Colman) and the chimera GM1 (provided by C. Machamer; Swift and Machamer, 1991) were subcloned into the expression vector pCDLSRα. The chimeric Tac-E19 cDNA in the expression vector pCDM8 was kindly provided by J. Bonifacino.

Cell lines were transiently transfected by the calcium-phosphate precipitation method (Graham and Van Der Eb, 1973) with 20 μg of DNA per 100 mm Petri dish for 16 hr, washed once in PBS, and then incubated in complete medium for another 24 hr prior to studies of transfected cells. For NRK and RD-4 cells, 5 mM sodium butyrate was added 10 hr after washing off the DNA precipitates, and cells were incubated in sodium butyrate for 16 hr prior to subsequent studies.

Immunofluorescence Microscopy

Cells were plated onto coverslips and grown in complete medium overnight. For studies on transfected cells, transfections were performed on cells previously plated on coverslips. After appropriate incubations, cells were fixed in 2% formaldehyde in PBS and washed in PBS twice and PBS/10% serum once. Cells were then incubated with primary antibodies in PBS/10% serum/0.2% saponin for 1 hr at room temperature, washed in PBS/10% serum three times, incubated with fluorescently labeled secondary antibody for 1 hr, washed three times in PBS/10% serum and once in PBS, and then mounted onto glass slides with fluoromount G (Southern Biotechnology Associates). Samples were examined under a microscope (Zeiss) through a 63 × oil planapo lens.

Metabolic Labeling, Immunoprecipitation, and Electrophoresis

As previously described (Bonifacino et al., 1990), transfected cells were preincubated in labeling medium (Dulbecco's modified Eagle's medium without methionine, 5% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 20 min at 37°C, labeled with ^{35}S-methionine (ICN Radiochemicals) at 250 μCi/ml for the times indicated, washed, and then chased in complete medium for the times indicated. Cells were removed from plates, pelleted by centrifugation, and then lysed in 1% Triton X-100, 300 mM NaCl, 50 mM Tris–HCl (pH 7.4). Lysates were incubated with antibody-coupled protein A–Sepharose at 4°C. Beads were left either untreated or treated with endo H for 16 hr at 37°C. Samples were then analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

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