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A Fluorescent Lipid Analogue Can Be Used to Monitor Secretory Activity and for Isolation of Mammalian Secretion Mutants

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The use of reporter proteins to study the regulation of secretion has often been complicated by posttranslational processing events that influence the secretion of certain proteins, but are not part of the cellular mechanisms that specifically regulate secretion. This has been a particular limitation for the isolation of mammalian secretion mutants, which has typically been a slow process. To provide a reporter of secretory activity independent of protein processing events, cells were labeled with the fluorescent lipid analogue C_5-DMB-ceramide (ceramide coupled to the fluorophore boron dipyrromethene difluoride) and its secretion was followed by fluorescence microscopy and fluorescence-activated cell sorting. Brefeldin A, which severely inhibits secretion in Chinese hamster ovary cells, blocked secretion of C_5-DMB-ceramide. At high temperature, export of C_5-DMB-ceramide was inhibited in HRP-1 cells, which have a conditional defect in secretion. Using C_5-DMB-ceramide as a reporter of secretory activity, several different pulse-chase protocols were designed that selected mutant Chinese hamster ovary cells that were resistant to the drug brefeldin A and others that were defective in the transport of glycoproteins to the cell surface. Mutant cells of either type were identified in a mutagenized population at a frequency of 10^{-6}. Thus, the fluorescent lipid C_5-DMB-ceramide can be used as a specific marker of secretory activity, providing an efficient, general approach for isolating mammalian cells with defects in the secretory pathway.

INTRODUCTION

The isolation of mammalian cells with specific mutations in mechanisms regulating the secretory pathway has been slow in part due to limitations of the experimental strategies that have been used. All methods employed to date have sought to identify mutant cells unable to export to the cell surface a specific reporter protein (Nakano *et al.*, 1985; Kingsley *et al.*, 1986; Hearing *et al.*, 1989). While these strategies have had some notable successes, proving, for example, that a component of the Golgi coatomer is necessary for transport from the endoplasmic reticulum to the Golgi apparatus in vivo (Guo *et al.*, 1994), they have had two intrinsic limitations. First, since they rely on the use of a single reporter protein, any cells with defects specific to the biosynthesis of the reporter have the phenotype being selected, and most of these would be of limited interest for understanding the secretory pathway. For example, when the receptor for low density lipoprotein (LDL) was used as a reporter, as one would expect, mutants in one of the six complementation groups isolated (*ldlA*) encoded the gene itself (Kozarsky *et al.*, 1986). Such mutants can be eliminated after the initial selection or screen by replica plating and probing with radiolabeled fragments of the receptor gene (Hobbie *et al.*, 1994), but this is time-consuming. Second, selections based upon surface expression of a reporter protein cannot eliminate mutants that efficiently remove the reporter from the cell surface due to changes in endocytosis. In fact, many mammalian secretion mutants identified to date were isolated following selections based on endocytosis defects and are defective for both processes. The difficulty in sep-

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arating mutants with specific defects in exocytic membrane transport from more numerous mutants with defects that alter the biosynthesis or cellular distribution of a single reporter protein is shown by the work of Nakano and colleagues, who isolated secretion mutants from a mutagenized population at an overall recovery frequency of 1 in 10^8 cells by selection for resistance to diphtheria toxin, followed by replica plating, and then screens for secretion of radioactive polypeptides (Nakano *et al.*, 1985).

In an effort to overcome these technical limitations and to accelerate the isolation of mammalian secretion mutants, we explored the use of fluorescent lipid analogues as reporters of secretory activity. Fluorescent analogues of ceramide have been developed in recent years in the laboratory of Dr. R. Pagano and have been widely used as vital stains of the Golgi complex (Lipsky and Pagano, 1983; Pagano, 1990; Pagano *et al.*, 1991). In particular, ceramide coupled to the fluorophore boron dipyrromethene difluoride (BODIPY, or C_5-DMB-Cer) was shown to be bright, resistant to photobleaching, and to stain the Golgi complex more efficiently than other cellular membranes. In this work we show that the transport of C_5-DMB-Cer to the cell surface and its subsequent loss from the cells is a time-dependent process that is blocked by Brefeldin A (BFA), a drug that also blocks secretion (Misumi *et al.*, 1986; Lippincott-Schwartz *et al.*, 1990; Hendricks *et al.*, 1992). In addition, cells with known thermosensitive defects in secretion retain the fluorescent label longer than wild-type cells at the restrictive temperature. Based on these results we concluded that C_5-DMB-Cer is a good reporter of secretory activity. We devised selection protocols using fluorescence-activated cell sorting (FACS) and obtained Chinese hamster ovary (CHO) cell lines with two types of secretion defects: partial or total block in the transport of glycoproteins to the cell surface, and resistance to the effects of BFA on the secretory pathway. Since this method is fast and works well, it is expected that the isolation of mammalian cell lines with defects in secretion may keep pace with other approaches currently employed to understand the secretory pathway.

MATERIALS AND METHODS

Cell Culture CHO K1 cells were cultured at 34°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.35 mM proline, and 5% fetal bovine serum (HyClone). HRP1, a mutant CHO K1 cell line with a temperature-sensitive defect in secretion, was obtained from Dr. R. Draper, (U.T. Dallas) and maintained at 34°C in the same medium. PtK1 cells were cultured at 37°C in minimal DMEM supplemented with nonessential amino acids and 10% bovine calf serum. CHO cells were mutagenized by growth for one doubling time at 34°C in concentrations of ethyl methane sulfonate (EMS) (200–275 μg/ml) such that the survival of cells following mutagenesis was 35–65%. Mutagenized CHO cells were maintained at 33–34°C at 5% CO_2 and 100% humidity.

Labeling of Cells with Fluorescent Lipids

Cells to be labeled with fluorescent lipids were plated so that, on the day of the experiment, cultures were 60–80% confluent. For the labeling medium, C_6-NBD-Cer (nitrobenzoxadiazole coupled to ceramide) or C_5-DMB-Cer (Molecular Probes, Inc., Eugene, OR) was mixed with defatted BSA (Sigma Chemical Co., St. Louis, MO) in serum-free DMEM to give a final concentration of 5 μM for both lipid and BSA initially and 1 μM in later experiments. This labeling medium was vortexed vigorously and allowed to warm to the appropriate temperature. Cells were washed three times with serum-free DMEM, then were incubated for 1 h in 1 ml of the labeling medium for each 2×10^6 cells. Following labeling, the cells were washed three times with serum-free DMEM and were incubated in complete medium for the appropriate time of chase.

Fluorescence Microscopy

Labeling with Fluorescent Ceramide. Cells were grown on coverslips and labeled with fluorescent ceramide (either NBD or BODIPY) as described above. The cells were visualized without fixation with a Zeiss Axioplan microscope equipped with a ×20 dry objective and epifluorescence optics. Labeling with C_6-NBD-Cer results in green fluorescence, whereas labeling with C_5-DMB-Cer results in fluorescence visible with standard filters for both fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) fluorescence (see below), but with the red fluorescence specific for the Golgi complex (Pagano *et al.*, 1990). Black and white photomicrographs were obtained using T-Max 100 film (Eastman Kodak Co., Rochester NY).

Indirect Immunofluorescence. Cells grown on coverslips and treated in various ways (e.g., BFA treatment or infection with influenza virus), were washed twice with phosphate-buffered saline (PBS) and fixed for 10–15 min at room temperature with 3.7% formaldehyde in 100 mM phosphate buffer (pH 7.0). Cells were rinsed in DMEM and then permeabilized for 2–10 min in methanol at −20°C. For all subsequent steps the coverslips were kept in a solution of 1% BSA in PBS at room temperature. Staining with primary antibody (1:1000 dilution of rabbit anti-mannosidase II (a kind gift of Drs. K. Moremen and M. Farquhar) or tissue culture supernatant of monoclonal anti-Japan HA (from cells provided by Dr. T. Braciale) was for 40 min. Staining with fluorescent second antibody, a 1:200 dilution of FITC-goat anti-rabbit (Fisher Biotech, Pittsburgh, PA) or Texas Red-donkey anti-mouse (Southern Biotechnology Associates, Birmingham, AL) was for 30 min. Between antibody incubations the coverslips were washed with BSA-PBS three times for a total of 20 min. After the final wash, the coverslips were mounted on glass slides using a drop of Aqua-Poly/Mount (Polysciences), dried, examined in the Zeiss Axioplan microscope described above under the ×63 objective, and photographed as described above.

Fluorescence-activated Cell Sorting

Cells to be analyzed or sorted by FACS were labeled and chased as described above. At the end of the chase the cells were washed twice with PBS without Ca^{2+} and Mg^{2+}, trypsinized with standard tissue culture trypsin (GIBCO BRL/BRL, Grand Island, NY), resuspended in DMEM containing 10% serum to neutralize the trypsin, and centrifuged at $50 \times g$ for 5–7 min. The cell pellet was resuspended in serum-free DMEM to give a final concentration of $2.5–3.0 \times 10^6$ cells/ml. The cell suspension was then analyzed in a FACStar Plus (Becton-Dickinson) cell sorter operated at a wavelength of 488 or 514 nm (250 mW) with bandpass filters of 515–545 nm, or 564–586 nm, respectively. Data were collected and analyzed using LYSYS 2 software. In preliminary experiments we determined that at 514 nm the separation between positive and negative cells was 10–15% better than the separation of the same cells at 488 nm. Cells to be kept after the sort were collected in DMEM plus serum under sterile conditions.

Preselection of Mutagenized CHO Cells with Diphtheria Toxin

Mutagenized CHO cells were shifted to 41°C for 4 h and then to 39.5°C for 16 h. The cells were treated with trypsin at the same temperature for 5 min to remove diphtheria toxin receptors at the cell surface (Nakano *et al.*, 1985). The trypsin was removed and the cells were incubated at 39.5°C for 30 min to allow any internalized receptors to return to the surface from endocytic compartments and then were treated with trypsin again for 5 min. The trypsin was removed and the cells were incubated an additional 4 h at 39.5°C, a period that we have determined to be long enough to allow cells competent for secretion to regenerate toxin receptors. Diphtheria toxin (100 nM; Berna Products Corp., Coral Gables, FL) was added to the culture medium for three additional hours. This medium was removed, and the cells were washed and incubated for 1 h at 39.5°C in 5 μg/ml of the nontoxic diphtheria toxin mutant, CRM197 (Berna Products Corp.), which binds to any remaining diphtheria toxin receptors, displacing any active toxin present (Naglich *et al.*, 1992). The cells were then shifted to the permissive temperature, 34°C, and plated for growth as single colonies. Survival of this selection occurred at a frequency of 1.25×10^{-5} cells.

Assays for Secretion

Secretion of Endogenous Polypeptides. Candidate mutant cells were plated on 24-well plates so that the day of labeling they were 80–90% confluent. The cells were washed twice with DMEM lacking methionine and cysteine, and were then incubated with 150 μl of this medium containing 30 μCi of Tran^{35}S-label (ICN, Irving CA). After 90 min of labeling, the supernatant was removed, centrifuged for 5 min at 500 × *g* to remove debris, and was mixed with Laemmli sample buffer for gel electrophoresis on 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS). For secretion in the presence of BFA, BFA was included at the indicated concentration in the labeling mix.

Secretion of a Viral Glycoprotein. Duplicate monolayers on 24-well plates were washed free of serum with DMEM and inoculated with 50 to 100 pfu/cell of influenza virus for 30 min on ice. These conditions were routinely assayed by immunofluorescence and found to produce uniform expression of influenza HA in cells of the monolayer after 3 h at 34°C. Following infection, the cells were incubated at 34 or 39.5°C for 3 h to allow production of viral glycoproteins. The cells were then incubated in DMEM lacking methionine and cysteine for 30 min and labeled with 40 μCi of Tran^{35}S-label in 100 μl of the same medium for 10 min. Cells were then chased in 200 μl of complete DMEM for 60 min. During the chase, trypsin at 10 μg/ml was included in the medium for one-half of the samples to cleave the HA arriving at the cell surface into its HA1 and HA2 subunits (Lazarovits *et al.*, 1990; Brewer and Roth, 1991). At the end of the chase the trypsin was neutralized with soybean trypsin inhibitor, the cells were lysed with 50 mM Tris-HCl (pH 8.0) containing 1% NP-40, 0.1% SDS, and 0.1 unit of aprotinin/ml, and the HA was immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Lazarovits *et al.*, 1990; Brewer and Roth 1991). The HA arriving at the cell surface was quantified by densitometry by calculating the fraction (HA1 + HA2)/(HA1 + HA2 + HA0). Resistance of the immunoprecipitated HA to digestion by endoglycosidase H was determined as described previously (Lazarovits *et al.*, 1990). Localization of HA by indirect immunofluorescence was achieved by using the protocols described above for infecting and staining the cells, except that at 3.5 h postinfection the cells were treated with 100 μg/ml cycloheximide in complete DMEM for 20 min (to clear the HA in wild-type cells from the exocytic organelles).

Additional Assays for BFA-resistant Mutants

Permeabilization with Streptolysin O (SLO). The method of Miller and Moore (1991) for permeabilizing cells with SLO was modified slightly and used to allow BFA direct access to the cytosol of wild-type or mutant CHO cells. One or 2 days after seeding on coverslips, cells were rinsed twice in transport buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 78 mM KCl, 4 mM MgOAc) and incubated for 10 min at 37°C in 0.3 ml of the same buffer containing 2 mM ATP with or without 0.2 units/ml SLO (Burroughs-Wellcome, Research Triangle Park, NC). BFA at 0.5 μg/ml or 1.0 μg/ml was added together with SLO where appropriate. To test the integrity of the plasma membrane, control samples were treated with 20 μg/ml ethidium bromide, a membrane-impermeable fluorescent dye used as an indicator of permeabilization. Only when greater than 90% of the cells were permeabilized were experimental samples fixed and stained by indirect immunofluorescence for Golgi coatomer β subunit (β-COP) as described previously (Ktistakis *et al.*, 1991).

Genetic Complementation. Parental cells were transfected with plasmid pCB6HA that encodes the gene for resistance to G418, and mutant cells were transfected with pCB7 that encodes the gene for resistance to hygromycin B (Brewer, 1994). The cells were selected for 2 wk in 400 μg/ml G418 or hygromycin B, respectively, and drug-resistant colonies were isolated and expanded. We determined that the transfection procedure and the expression of the antibiotic-resistance genes did not affect the sensitivity of cells to BFA. Each mutant cell line was fused with the parental cells using 50% polyethylene glycol 1000 in serum-free DMEM for 40 s at room temperature, and, after 24 h in normal medium, cells were grown for 2 wk in medium containing both G418 and hygromycin B. Surviving colonies were tested for sensitivity to BFA by measuring the secretion of endogenous polypeptides as described above.

RESULTS

Labeling of Cells with C_5-DMB-Cer

We investigated the use of C_5-DMB-Cer as a reporter for secretory activity based on its property to (a) enter cells via spontaneous transfer from exogenously provided BSA-lipid sources and (b) to specifically label the Golgi complex before its metabolites are transported to the cell surface (Pagano *et al.*, 1991). To confirm that transport of the C_5-DMB-Cer from the cell was largely through the secretory pathway, cells were labeled with C_5-DMB-Cer for 1 h and then chased for various times in the presence of 5 μg/ml of BFA, a concentration of the drug that completely inhibits protein secretion in CHO cells. Figure 1 shows the distribution of fluorescence in CHO cells chased in the absence or presence of BFA for various periods. As previously reported, for the untreated cells labeling was first detected in the endoplasmic reticulum (ER): it then moved to the Golgi complex, and then it was found on the cell surface before it was lost. BFA retarded the disappearance of the fluorescence in comparison to the control cells and the label remained in an ER-like pattern. Similar results were obtained when using C_6-NBD-Cer, but the difference between control- and BFA-treated cells was less pronounced.

We used FACS to obtain a more quantitative comparison of the fluorescence intensity in cells that were untreated or treated during the chase with BFA. Figure 2 shows a panel of histograms following such an experiment. After a very short chase, the intensity of the two cell populations was identical, but after a 3-h

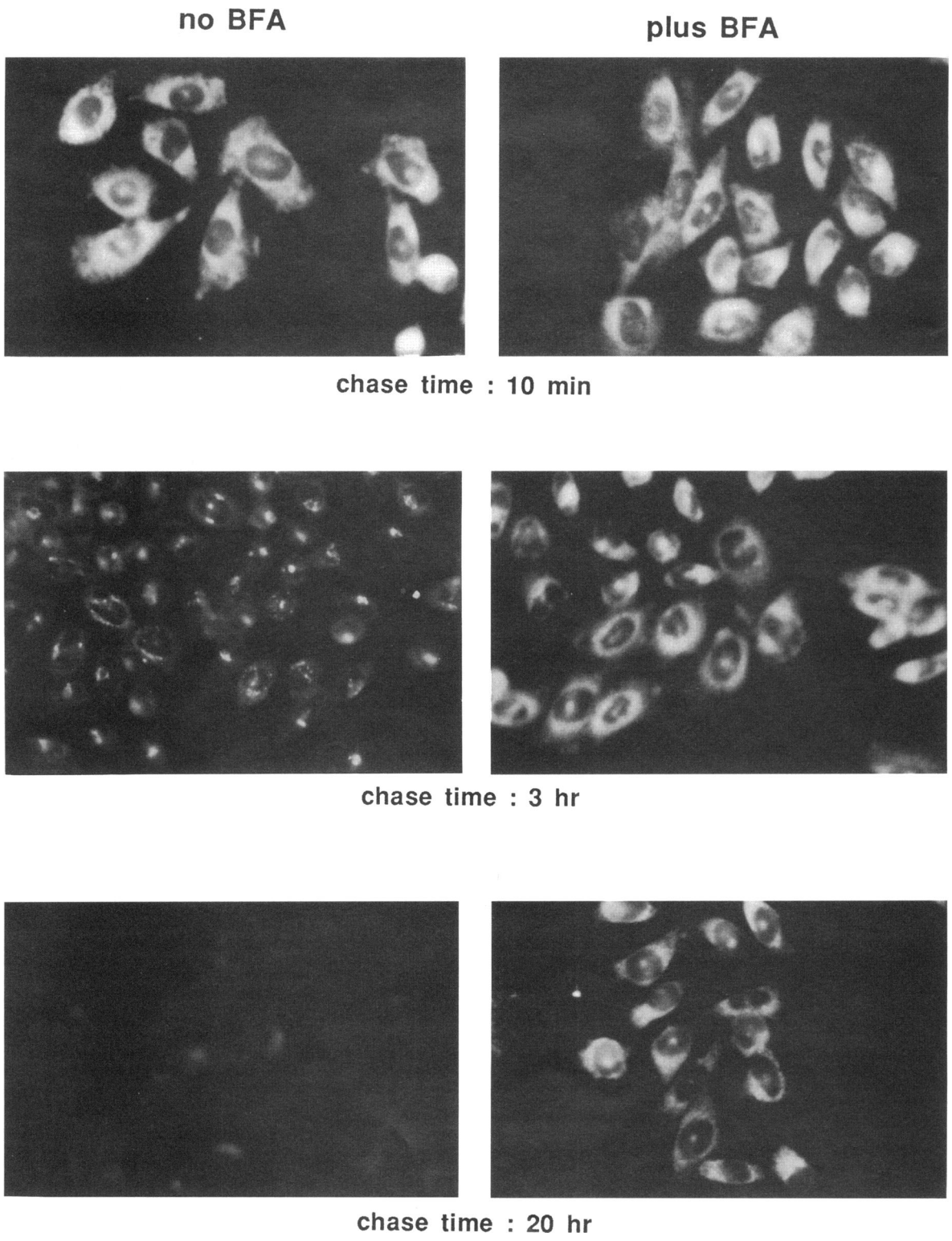

Figure 1. Fluorescence micrographs of CHO cells following C_5-DMB-Cer labeling and a chase in the presence or absence of BFA. CHO cells on coverslips were labeled for 1 h with 5 μM C_5-DMB-Cer-defatted BSA liposomes and chased for 10 min, 3 h, or 20 h in medium with or without 5 μg/ml BFA as indicated. The live cells were photographed using optics for red fluorescence.

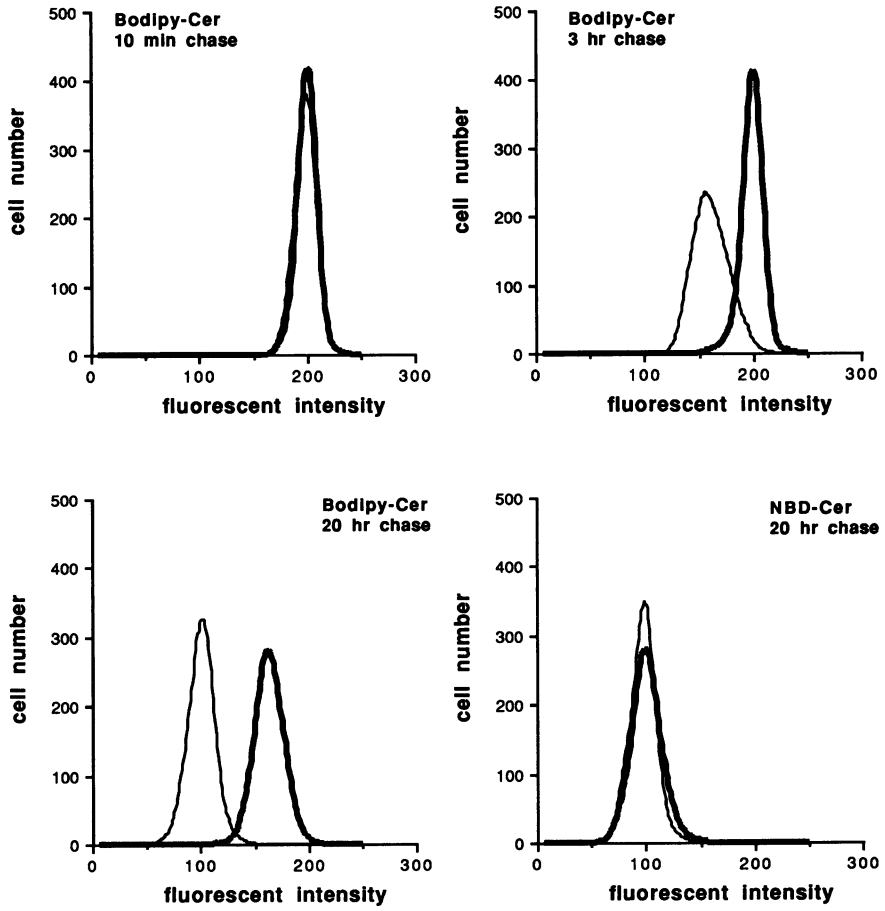

Figure 2. Histograms of cells following labeling with fluorescent lipid and a chase in the presence or absence of BFA. CHO cells were labeled with 5 μM C_5-DMB-Cer-defatted BSA liposomes (BODIPY-Cer) or with 5 μM C_6-NBD-Cer-defatted BSA liposomes (NBD-Cer) and were chased for 10 min, 3 h, or 20 h in the presence (thick lines) or absence (thin lines) of 5 μg/ml of BFA as indicated. Intensity profiles from 10,000 cells were obtained using a FACStar Plus cell sorter with the laser emission at 514 nm.

chase the BFA-treated cells were brighter than the control cells. By 20 h of chase, the cells treated with BFA were 8–15-fold brighter than the control cells, indicating that, in the absence of secretory activity, the cells could not export the fluorescent label. It is interesting that cells labeled with C_6-NBD-Cer and chased for 20 h in the presence or absence of BFA produced identical fluorescence intensity distributions by FACS analysis (Figure 2). This was also true for shorter chase times. Although we did not investigate this difference further, it is probably due to the fact that C_6-NBD-Cer is less specific for the Golgi apparatus and labels the nuclear envelope and mitochondria (Pagano *et al.*, 1991), organelles not affected by BFA.

The Retention of Fluorescence in the Presence of BFA Is Due Specifically to a Block in Transport

Treatment of cells with BFA affects both the secretory pathway as well as late steps in the endocytic pathway (Hunziker *et al.*, 1991; Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991). To investigate the possibility that labeled cells retained their fluorescence due to an effect of BFA on endocytosis, we labeled PtK1 cells with C_5-DMB-Cer. PtK1 cells are resistant to BFA with respect to secretion (Ktistakis *et al.*, 1991) but retain sensitivity to BFA in the endocytic pathway (Lippincott-Schwartz *et al.*, 1991). PtK1 or CHO cells were labeled with C_5-DMB-Cer and then chased for 20 h in the presence or absence of BFA. Whereas CHO cells retained the fluorescence label in the presence of BFA (Figure 3, top panel), after 20 h of chase, untreated PtK1 cells were indistinguishable from PtK1 cells treated with BFA (bottom panel). This quantitative result was confirmed by fluorescence microscopy of single cells, where we observed that all PtK1 cells lost the fluorescent label as a function of chase time in the presence or absence of BFA (unpublished observations). These results suggested that, after C_5-DMB-Cer labeling and chase, the loss of fluorescence was due to secretory activity.

If intracellular retention of C_5-DMB-Cer in the presence of BFA was due to the inhibition of secretion and not some other effect of the drug, such as mixing of Golgi and ER membranes, then a cell line with a temperature-sensitive defect in secretion should also remain fluorescent after a chase at the nonpermissive

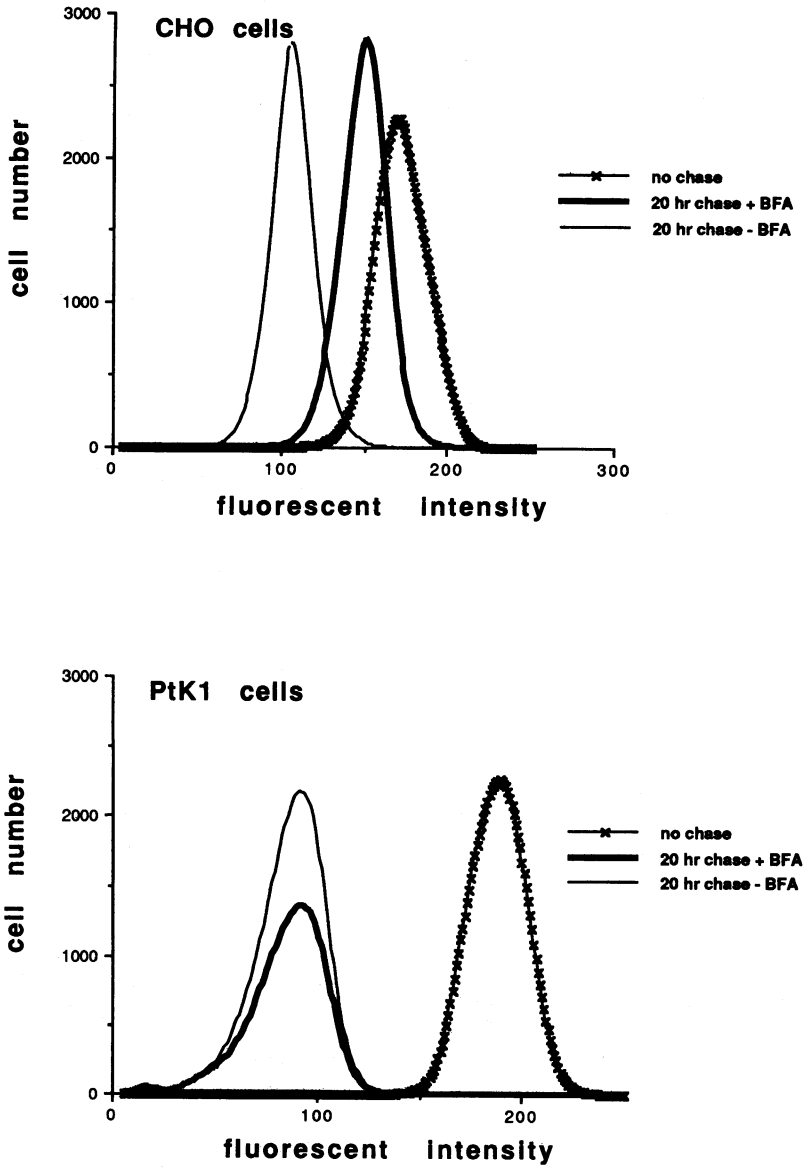

Figure 3. Histograms of cells following labeling with C_5-DMB-Cer and a chase in the absence or presence of BFA. CHO or PtK1 cells were labeled with 5 μM C_5-DMB-Cer-defatted BSA liposomes for 1 h and chased for 0 or 20 h in the absence or presence of 5 μg/ml BFA as indicated. Acquisition of the data was as described for Figure 2, except that 100,000 cells were analyzed.

temperature in the absence of the label. To test this, we used a mutant cell line, HRP1, with a known temperature-sensitive defect in transport. In these cells secretion of a glycoprotein is reduced by 80–90% at the nonpermissive temperature (Wang *et al.*, 1992). HRP1 cells were labeled with C_5-DMB-Cer and chased at the permissive (34°C) or restrictive (39.5°C) temperature. When transport was blocked at 39.5°C the cells were twofold brighter (Figure 4). The differences that we observed in the fluorescence of cells labeled with C_5-DMB-Cer and chased under conditions when secretion was either normal or inhibited suggested that the fluorescent lipid might be used to select cell mutants with defects specifically related to the transport of membrane and should avoid a large background of mutants with defects in other activities of the secretory pathway, such as protein folding, glycosylation, or other posttranslational modifications.

Strategies for Mutant Selection

Mutant CHO cells with defects in the secretion of proteins or CHO cells in which secretion was resistant to the drug BFA were selected following labeling and chase with C_5-DMB-Cer using the following strategies. (1) If BFA was absent during the chase, the majority of the cells would become dimly fluorescent but cells which could not transport membrane to the cell surface should accumulate the fluorescence lipid and be brighter. (2) If BFA was present during the chase,

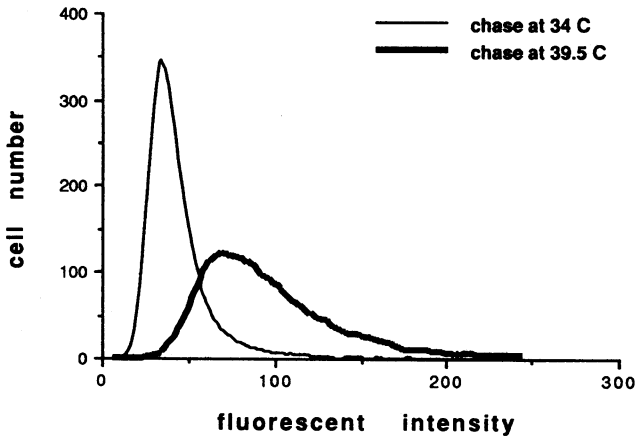

Figure 4. Histograms of secretion-defective HRP1 cells following labeling with C_5-DMB-Cer and a chase at the permissive or restrictive temperature. HRP1 cells were labeled with 5 μM C_5-DMB-Cer-defatted BSA liposomes for 1 h and chased for 20 h at 34 or 39.5°C as indicated.

then the majority of the cells should remain brightly fluorescent but cells which were resistant to BFA should be able to export the fluorescence lipid and become less bright. In preliminary experiments, we determined that neither BFA treatment nor C_5-DMB-Cer labeling alone or in combination had any effect on the subsequent viability of the cells.

We used FACS to isolate both classes of cell mutants following labeling with C_5-DMB-Cer and chases in the presence or absence of BFA. For each isolation we sorted 15–20 million cells mutagenized with EMS, and we kept two populations: Cells at the 0.01% level of fluorescence (either at the bright or the dim end of the spectrum depending on the experiment) or cells between the 0.1 and 0.01% level of fluorescence. The sorted cells were allowed to form colonies, and each colony was individually assayed for the desired phenotype as described under MATERIALS AND METHODS.

Isolation of CHO Cells Resistant to BFA

Cells were labeled with C_5-DMB-ceramide and chased at 39.5°C either for 4 h in 1 μg/ml BFA or for 16 h in 10 μg/ml BFA. Cells from both chase protocols were sorted by FACS, and the dimmest 0.01% of the cells were kept. The resulting colonies were analyzed for secretion and Golgi morphology in the presence of BFA. BFA-resistant cells were selected by both the short and longer chase protocols (Table 1). Varying the concentration of BFA and the chase time did not result in differences in either the frequency of detection of mutants or the phenotype of the mutants. Cells from each of the two sorted populations were next screened by a biochemical assay to identify cells that retained the ability to secrete proteins in the presence of BFA. Cells with this phenotype, designated CHO-BR (*B*refeldin *R*esistant), were identified at a frequency of 8% of the cells tested, and ten cell lines were selected for more detailed study (Table 1). Figure 5 shows a biochemical assay for secretion in the presence of BFA for two of these mutants and for wild-type CHO cells.

Table 1. Characteristics of BFA-resistant CHO cell lines

Cell line	Concentration required to inhibit secretion		Concentration required to inhibit growth		Concentration that affects Golgi in intact cells	Resistant to 1 μg/ml BFA after SLO treatment	Dominant or recessive
	34°C	39.5°C	34°C	39.5°C			
CHO wt	<.01	1	0.1	1	<1	no	NA
K39	3	10	ND	ND	6	ND	D
K40	3	10	ND	ND	6	ND	D
C1.3	0.3	10	3	3	10	yes	D
D1.4	0.3	10	3	3	10	yes	D
D3.1	>1	>20	3	10	>20	yes	D
D3.2	>1	>20	3	10	>20	yes	D
G2.1	>1	>20	3	10	>20	yes	D
I1.3	>1	>20	1	3	>20	yes	D
I2.1	>1	>20	1	3	>20	ND	D
J2.1	>1	20	3	3	>20	yes	D

Values shown are the concentration of BFA in μg/ml required to inhibit secretion or growth by 90% or disperse Golgi mannosidase II into an ER-like pattern as detected by immunofluorescence. Golgi morphology was determined by immunofluorescence with anti-mannosidase II antibody. Sensitivity or resistance to BFA in cells treated with SLO (streptolysin O) was determined by immunofluorescence with anti-β-COP antibody.

ND, not done.

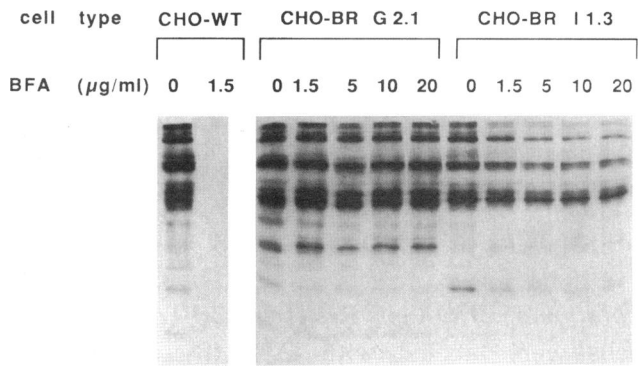

Figure 5. Secretion of proteins by cells selected for BFA resistance. Wild-type (CHO-WT) or cloned BFA-resistant (CHO-BR) cells were grown on 24-well plates and labeled continuously for 90 min with Tran^{35}S-label in the absence or presence of different amounts of BFA as indicated. The labeled proteins present in the culture medium after 90 min were analyzed by SDS-PAGE, and fluorograms of the dried gels are shown.

For CHO-BR G 2.1 cells, secretion of ^{35}S-labeled proteins was unaffected by BFA concentrations much higher than needed to completely inhibit secretion in wild-type cells (CHO-WT). For the other mutant cell line, CHO-BR I 1.3, secretion was partially inhibited by BFA but was not completely blocked even at the highest concentrations of the drug.

The cell lines shown in Table 1 maintained a recognizable Golgi, as judged by indirect immunofluorescence with antibodies to mannosidase II (Figure 6), in concentrations of BFA equal to or greater than that for which resistance of secretion was measured. Whereas the wild-type cells lost a recognizable Golgi after 30 min of BFA treatment (Figure 6, top panels), the mutants maintained a Golgi structure that in some cases looked very similar to that of cells not treated with BFA (Figure 6, bottom panels) and in other cases looked partially affected by BFA (Figure 6, middle panels).

Table 1 summarizes several characteristics of the BFA-resistant cell lines. We have determined that resistance to BFA for at least seven of the CHO-BR mutants was not due to a block in BFA import by permeabilizing the cells with streptolysin O in the presence of BFA. Following such treatment, the mutant but not wild-type CHO cells retained their Golgi complex as shown by indirect immunofluorescence with antibodies to β-COP. These mutant cells were stable, maintaining the BFA-resistant phenotype for more than 3 mo of continuous culture, and varied in their resistance to BFA, ranging from threefold to over 30-fold more resistant than wild-type cells. All ten mutants analyzed by genetic complementation were dominant over the wild-type cells making them good candidates for isolating the gene(s) responsible for the resistance phenotype.

Isolation of CHO Cells with Defects in Secretion

We developed several protocols that allowed us to isolate cells with defects in the secretion of proteins by selecting cells deficient in the secretion of fluorescent lipid. Mutagenized CHO cells were labeled with C_5-DMB-Cer and chased at 39.5°C, then analyzed by FACS, and then a population of bright cells that were unable to export the fluorescent label was collected. In preliminary experiments, we found that a potential problem for this selection was a subpopulation of mutagenized cells that grew slowly at all temperatures and were bright under the conditions of our screen. Presumably many of these cells contained defects in metabolism affecting cellular energy levels. To eliminate these cells, we labeled the mutagenized population with C_5-DMB-Cer, chased at the permissive temperature, and used the cell sorter to isolate the population of cells in the middle 80% of the labeled peak, thereby discarding the brightest 10% and the dimmest 10% of the fluorescent cells. The sorted cells were allowed to recover for 3 d, were labeled and chased at the nonpermissive temperature, and were sorted again as described above. When selection was at very low frequencies (0.01%) we found that the survival of the sorted cells was 1–3% of the cell number recorded by the cell sorter. From the colonies that grew up from this selection, 8–15% proved to have defects in the secretion of polypeptides. The survival of cells at higher frequencies of selection (0.1–0.01% range) was 10-fold better, but cells with defects in protein secretion were rare in this population.

We used a second protocol to enrich for cells with defects in protein secretion before FACS selection. For this, mutagenized cells were treated with trypsin to remove diphtheria toxin receptors (see MATERIALS AND METHODS) and selected for the ability to survive 3-h treatment with 100 nM diphtheria toxin at 39.5°C. The survivors of this selection were labeled with C_5-DMB-Cer, and the most fluorescent 1% of the cells (4.5×10^4 cells) was selected by FACS using the protocol described above. Survival of this sorted population was approximately 15%, giving us a much larger population from which to establish clonal lines for further analysis. Each of these protocols, screening by FACS alone and screening a preselected population, has its advantages (see DISCUSSION).

To separate cells with defects in secretion from the other types of mutants included in the populations selected by each method, we analyzed individual clonal cell lines first for the ability to secrete ^{35}S-labeled proteins into the medium. Cell lines that showed a marked decrease in the amount of protein secreted were then assayed for the ability to trans-

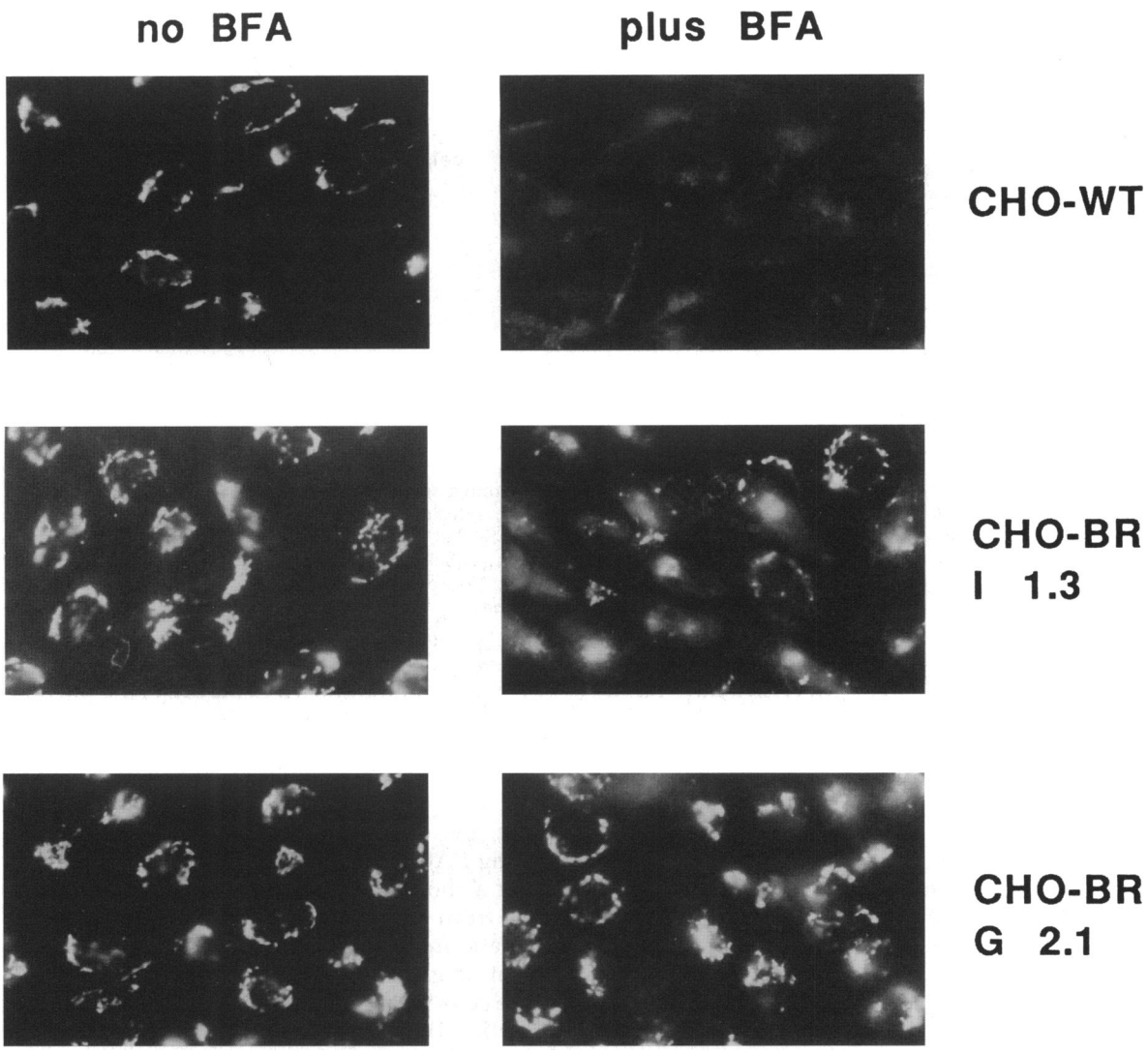

Figure 6. Indirect immunofluorescence of cells selected for resistance to BFA. Wild-type (CHO-WT) or BFA-resistant (CHO-BR) cells were plated on coverslips, treated with BFA for 30 min, fixed, and stained with polyclonal antibodies to mannosidase II. The BFA concentration for CHO-WT was 1.5 μg/ml, and for the two mutants the concentration was 20 μg/ml.

port a viral glycoprotein to the cell surface at the nonpermissive temperature. In this way, we isolated cells which had defects in the secretory pathway at a frequency of 15% from each of the two selected populations. These cells were designated CHO-BEM (*B*ODIPY-Cer *E*xport *M*utants). Complete characterization and genetic complementation analysis of CHO-BEM with previously identified mutant CHO cell lines is in progress (Ktistakis, Kao, Wang, and Roth, unpublished data).

When CHO cells infected with the influenza virus are labeled with Tran^{35}S-label and chased at 39°C, the HA protein appears at the cell surface with a half-time of 20–30 min (Wang *et al.*, 1990). If trypsin is present during the chase, the intact HA band (HA0) is cleaved into two polypeptides (HA1 and HA2) if it is properly folded, or into more polypeptides if folded incompletely (Lazarovits *et al.*, 1990). Therefore, the ratio of HA1 + HA2 to total HA (HA0 + HA1 + HA2) can be used to estimate the extent of HA arrival at the cell surface (Matlin and Simons, 1984). The mobility of the HA1 and HA2 bands, and the presence or absence of other tryptic fragments, can be used as an indicator of the extent of folding of HA (Lazarovits *et al.*, 1990). Furthermore, because HA changes in size as it becomes fully glycosylated and processed, the mobility of HA0 can be used as an indicator of how far this protein has traveled in the exocytic pathway. The extent of HA arrival at the cell surface for some CHO-BEM cell lines isolated after toxin preselection fol-

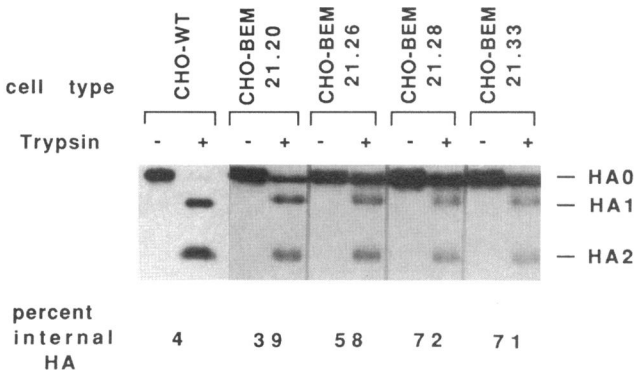

Figure 7. Biosynthesis of influenza HA in cells selected for secretion defects. Wild-type (CHO-WT) or cloned secretion-defective cells (CHO-BEM) were grown on 24-well plates at 41°C for 4 h and then overnight at 39°C. Cells were then infected with influenza virus, labeled for 10 min with Tran^{35}S-label, and chased for 60 min at 39°C in the absence or presence of 10 μg/ml of trypsin as indicated. Labeled HA was immunoprecipitated and resolved by SDS-PAGE. These mutants were selected from a cell population that survived treatment with diphtheria toxin and was subsequently sorted by FACS.

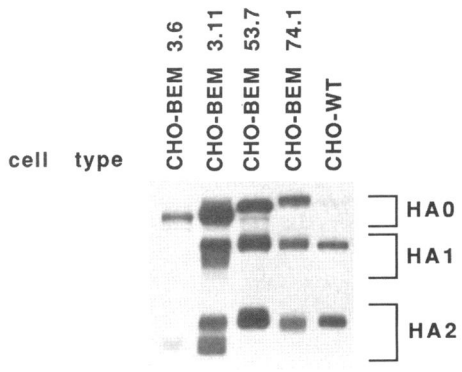

Figure 8. Secretion-defective mutants differ in the rate or extent of intracellular processing of HA. Wild-type (CHO-WT) or temperature-sensitive secretion-defective cells (CHO-BEM) following single cell cloning were grown at the restrictive temperature on 24-well plates, infected with influenza virus, labeled for 10 min with Tran^{35}S-label, and chased for 70 min in the presence of 10 μg/ml of trypsin. Labeled HA was immunoprecipitated and resolved by SDS-PAGE. Notice the different mobility of the HA0 band in the different cell lines.

lowed by C_5-DMB-Cer (see MATERIALS AND METHODS) is shown in Figure 7. Whereas 4% of the protein in wild-type cells was still internal at the end of a 70-min chase, in mutant cells the percentage of internal HA ranged from 39 to 72%. This 10- to 20-fold decrease in HA transport for the CHO-BEM mutants is in the same range as the extent of inhibition of invertase secretion observed for the *sec* mutants in yeast (Novick *et al.*, 1980). CHO-BEM mutants included some with secretory defects at all temperatures and others with thermosensitive defects. Table 2 shows the characteristics of two of these mutants that were defective at both temperatures. Both showed a 2-fold decrease in the rates of acquisition of resistance to endoglycosidase H (endo H) and of arrival at the cell surface but were unaffected in the rate of protein synthesis or in the rate of internalization of HRP.

For all mutants shown in Figure 7, the portion of HA that did arrive at the cell surface was well folded (notice the normal mobility of the HA1 and HA2 bands). Another set of mutants isolated following C_5-DMB-Cer labeling alone is shown in Figure 8. For these, the electrophoretic mobility of the HA0 band during PAGE and the extent of correct folding varied. After a short pulse and 60-min chase, the HA0 recovered from these cell lines had four different electrophoretic mobilities, characteristic of HAs having different degrees of oligosaccharide processing. Since these cells were shown to have decreases in the rate of secretion of all polypeptides, this result is consistent with inhibition of HA transport at different stages of processing and perhaps in different organelles. In addition, the portion of HA that arrived at the cell surface was in some cell lines correctly folded (notice the

Table 2. Characteristics of two CHO-BEM cell lines

	Protein synthesis in 30 min (% of wild type)		Accumulation of HRP in 30 min (% of wild type)		$T_{1/2}$ of arrival of HA at plasma membrane (min)		$T_{1/2}$ of endo H resistance of HA (min)	
Cell line	34°C	39.5°C	34°C	39.5°C	34°C	39.5°C	34°C	39.5°C
WT	100	100	100	100	26	19	10	7
BEM 21.28	98	119	94	82	40	30	18	18
BEM 21.33	102	104	142	127	50	40	19	20

Wild-type or secretion-defective CHO cells were plated at 34°C for 1 day. Half of the samples were then shifted for 4 h at 41°C and then overnight at 39.5°C. The other half were kept at 34°C. Protein synthesis and accumulation of HRp were measured as described previously (Wang *et al.*, 1992). The kinetics of HA transport were determined following influenza infection as described previously (Lazarovits *et al.*, 1990).

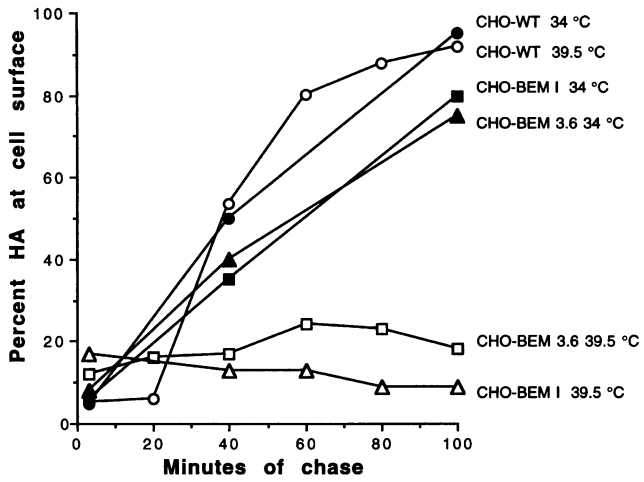

Figure 9. Kinetics of the appearance of HA at the plasma membrane of wild-type and two CHO-BEM cell lines. Cells grown at 34 or 39.5°C for 24 h were infected with influenza virus and labeled as described above. Following chase for the indicated times in the presence of extracellular trypsin, the HA was immunoprecipitated, resolved by SDS-PAGE, and quantified using densitometry. The percent of HA on the cell surface at the end of the chase was determined as described under MATERIALS AND METHODS.

HA1 and HA2 bands for CHO-BEM 74.1), whereas in others it was not (CHO-BEM 3.11). Figure 9 presents the kinetics of arrival of HA to the plasma membrane for two of the most severely impaired mutants. For these cells, the lesion is thermosensitive and the block in transport is almost complete.

The site of intracellular HA accumulation of these mutants was visualized using indirect immunofluorescence. To establish the differences in HA transport between wild-type and mutant cells more clearly, the infected cells were treated for 20 min with 100 μg/ml cycloheximide before fixation. This treatment allows the internal HA that is being transported normally to exit the organelles of the secretory pathway, whereas the HA that is blocked in transport accumulates at the site of the block. Figure 10 shows characteristic examples of two mutants having an ER-like distribution of HA after a chase in cycloheximide long enough for most of the HA in wild-type CHO cells to reach the plasma membrane. Other mutants showed, in addition to ER staining, perinuclear staining for HA, consistent with slower export of HA through the secretory pathway.

The morphology of the Golgi complex was altered for some of the CHO-BEM mutants. Following staining with antibodies to mannosidase II, by immunofluorescence microscopy we have observed two different phenotypes (Figure 11). Some mutants lost a recognizable Golgi at the nonpermissive temperature (CHO-BEM 53.7), whereas others had a larger and more dispersed organelle (CHO-BEM 74.1). The former phenotype is reminiscent of the morphological changes produced by BFA.

Based on a number of morphological and biochemical assays, we have grouped the CHO-BEM cell lines into six different classes as shown in Table 3. With the exception of class III, the rest of the cell lines are temperature-sensitive for growth and for the arrival of HA at the cell surface. Although this grouping is not based on genetic complementation, it serves to show that distinctly different mutant phenotypes have been selected by our screen.

DISCUSSION

We have described a new method for isolating mammalian cells with changes in secretory activity. The usefulness of the genetic approach in understanding secretion is already established from the work in *Saccharomyces cerevisiae* where the large collection of secretory mutants is yielding important information on the components that are involved in this pathway (Pryer *et al.*, 1992). Although it is possible that differences in the basic mechanisms which control vesicle formation and consumption in yeast and mammalian cells might be minor, we expect that in mammals additional mechanisms may exist which couple secretion to signal transduction and perhaps provide different cell types with a more specialized repertoire of control mechanisms. For example, recent pharmacological evidence suggests the involvement of heterotrimeric GTP-binding proteins in the secretory pathway of mammalian cells (Donaldson *et al.*, 1991; Stow *et al.*, 1991; Ktistakis *et al.*, 1992), something not reported in yeast. In addition, because in mammalian cells the morphology of the secretory organelles is better visualized and described, questions involving organelle identity and biogenesis may be more easily answered there. As an outstanding example of this, three mutant CHO cell lines have been isolated in which the onset of a block in secretion is accompanied by a rapid and reversible loss of the Golgi complex (Zuber *et al.*, 1991; Kao and Draper, 1992; Guo *et al.*, 1994). This phenotype, which is reminiscent of the effects of BFA on secretion and on the morphology of the Golgi complex, strongly suggests a coupling of secretory activity and organelle identity (Hurtley, 1992).

Our method of mutant selection involves the use of a fluorescent ceramide analogue as a reporter of secretory activity. These molecules combine an endogenous lipid, ceramide, with a fluorophore moiety (either NBD or BODIPY) and, like their cellular counterparts, are metabolized intracellularly to fluorescent sphingomyelin and glycosylceramide. These metabolites are then transported from the Golgi complex to the cell surface in a process which is inhibited (a) in mitotic cells, (b) by temperatures below 20°C, or

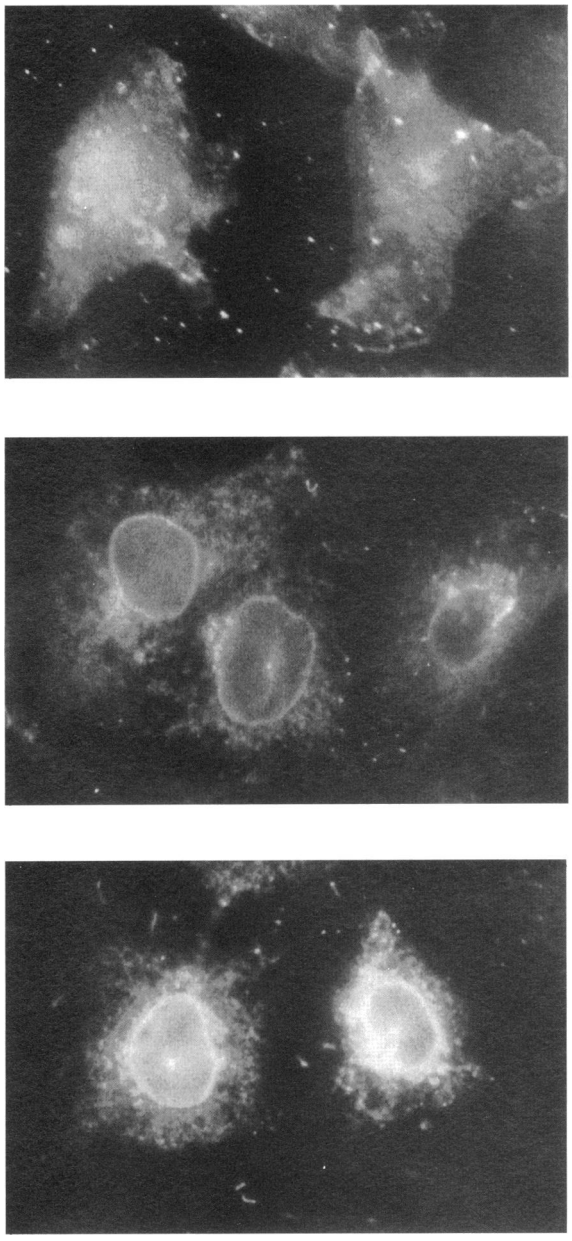

Figure 10. Distribution of HA in mutant CHO cells. Wild-type (CHO-WT) or secretion-defective (CHO-BEM) cells were plated on coverslips, grown for 18 h at 39.5°C, and infected with the Japan influenza virus. After 3.5 h, the cells were treated with cycloheximide long enough to allow HA to leave the ER and Golgi complex and then were stained with antibodies to HA followed by the appropriate fluorescent second antibodies.

(c) by the ionophore monensin (Lipsky and Pagano, 1985; Kobayashi and Pagano, 1989; van Echten and Sandhoff, 1989). Since all these conditions also inhibit the transport of newly synthesized glycoproteins to the cell surface, it is thought that transport of the ceramide metabolites is analogous to exocytosis of glycoproteins (Pagano, 1990). Our results showing that BFA-sensitive cells after a 20-h chase in the presence of BFA retained fluorescence, whereas BFA-resistant cells did not, also suggests that the process of fluorescent lipid transport can be used as an indicator of general secretory activity.

There are advantages for using fluorescent ceramide as a reporter for secretion. (1) Treatment of cells with C_5-DMB-Cer has no effect on viability. (2) The process of C_5-DMB-Cer uptake is by spontaneous transfer at high rates so that mutants that cannot internalize the label are not a background problem during the selection. (3) Since C_5-DMB-Cer labeling is most prominent in the Golgi (Pagano *et al.*, 1991), potential mutants will include those with defects not only in secretion but in Golgi biogenesis and stability as well. (4) Because of the specificity in Golgi labeling, many mutants will have defects in the pathway of secretion

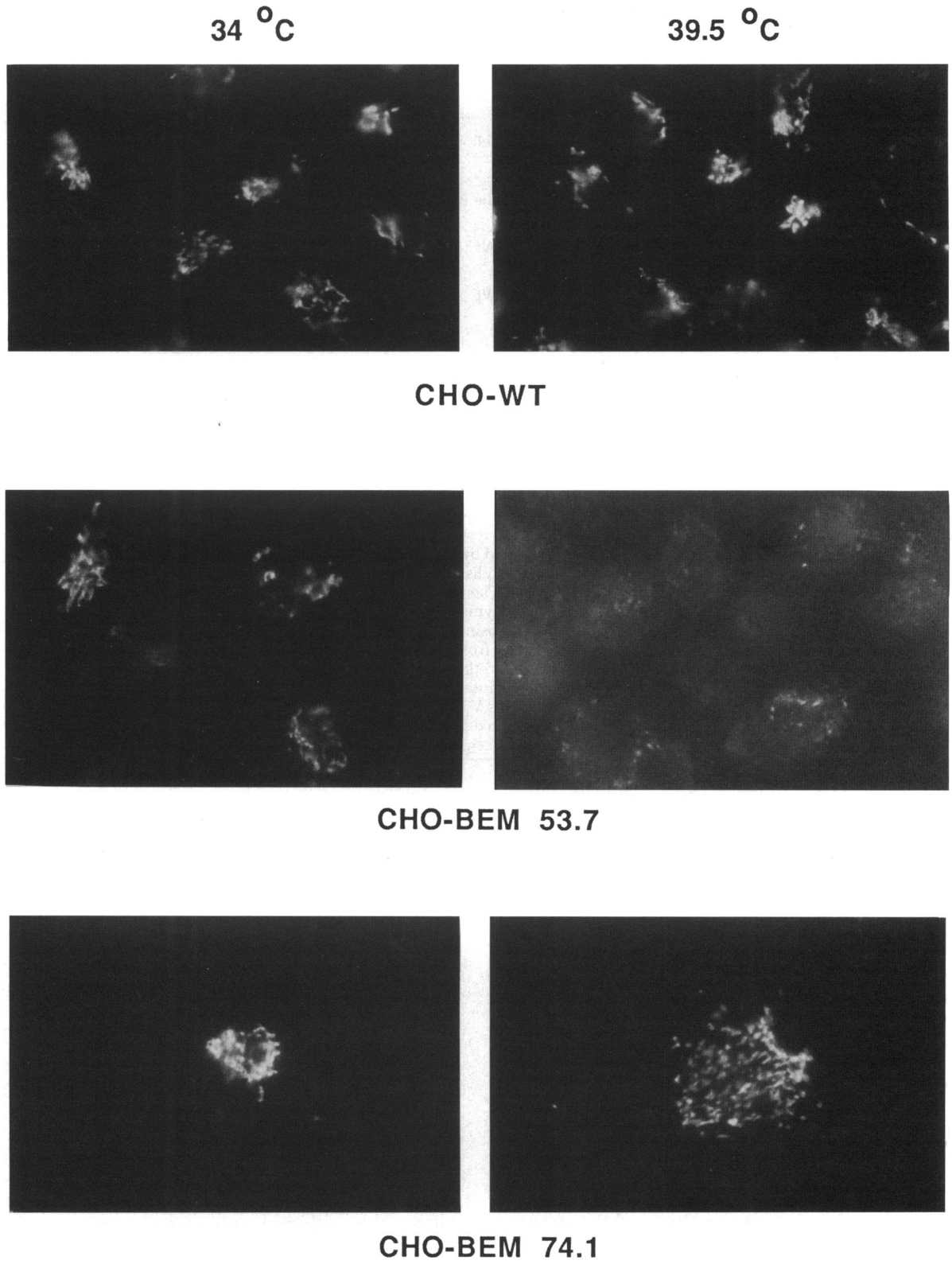

Figure 11. The morphology of the Golgi complex of cells selected for secretion defects. Wild-type or CHO-BEM cells were grown on coverslips for 24 h at the indicated temperatures and then were stained with antibodies to mannosidase II followed by the appropriate second antibody. The photograph of the CHO-BEM 53.7 cells at 39.5°C was overexposed during printing to show the remnants of immunoreactive mannosidase II.

Table 3. Phenotypes of CHO exocytosis mutants at high temperature

	Phenotype at 39.5°C					
		Morphology				
BEM mutant class	Growth	Golgi	Cell	Virus infection	HA processing in Golgi	Surface expression of processed HA
I (3, 37)	None	Wt	Fusiform, die in 2 days	Wt	<10%	<30%
II (53, 72)	Very slow	Disappears	Wt	Wt	Wt	<30%
III (18, 65)	Wt	Wt	Wt	<10%	Wt	<60%
IV (43, 51)	Very slow	Wt	Ruffled edges	Wt	Wt	<30%
V (74)	Very slow	Big, dispersed	Most cells multinucleated	Wt	Wt	<50%
VI (I)	None	Wt	Fusiform, die in 2 days	Wt	None	<30%

Mutant cell lines were always compared to wild-type cells treated in parallel. For each assay, cells were plated at 34°C for 24 h, then were shifted to 41°C for 4 h and to 39.5°C for 16–20 h (for assays of morphology or employing virus infectin) or for 7 days for assays of growth. Golgi morphology was determined by indirect immunofluorescence with antibodies to mannosidase II and by labeling with fluorescent ceramide. Cell morphology was determined by phase-contrast microscopy. Ability to be infected by influenza virus was determined by indirect immunofluorescence with anti-influenza virus antibodies and by pulse chase experiments. Percentages in this assay represent percent of infected mutant cells compared to complete infection of parental cells with the same virus preparation. The extent of HA processing in the Golgi was determined after pulse-labeling HA and chasing 60 min. The percentage of HA immunoprecipitated that had shifted to the slower migrating form having oligosaccharides processed by Golgi enzymes was determined by electrophoresis and autoradiography. The percentage of this processed HA that had become accessible to trypsin in the extracellular medium after 60 min chase is given in the next column to the right. The mutant cell line of class VI was derived from a clone identified during a screen for BFA resistance that failed secondary tests for resistance to the drug, but was observed to be secretion defective. For any assay, phenotypes identical to wild-type control cells are designated Wt. Listed in parentheses below each class of mutant are the actual clone numbers. At 34°C, mutants of Class I, II, and IV have a wild-type phenotype in all assays listed in the table. Class V is wild-type at 34°C except for the presence of a few multinucleated cells. Class III mutants grow very poorly at 34°C, cannot be infected with influenza virus, do not spread well, but have a normal Golgi morphology. Class VI cells are wild-type at 34°C except for oligosaccharide processing, which occurs at 60% of the wild-type rate.

from the Golgi to the cell surface (some of the secretion-defective cells characterized by us so far have defects affecting transport of HA after it becomes resistant to endo H (Wang and Roth, unpublished data). Since the Golgi and *trans*-Golgi network are sites of intracellular sorting, this method might also be used to identify mutants with defects in the sorting machinery. (5) Finally, because the purpose of isolating and studying mutants is ultimately the description of a pathway at the molecular level, it is advantageous that the method of mutant selection could also be used for mutant rescue by genetic complementation. In principle, isolation of cells in which a secretion defective phenotype has been rescued by genetic complementation can be accomplished by first introducing an expression library into the cell line of interest and then by using FACS to select cells with levels of fluorescence diametrically opposite to those of the starting population.

Using C_5-DMB-Cer, we have selected two different categories of mammalian cell mutants, those with general defects in secretion and those which are resistant to BFA and continue to secrete in the presence of the drug. For the secretion-defective mutants, we have used two variations of FACS selection. The first, using FACS alone, does not require endocytosis and should produce some mutants primarily defective in exocytosis. Such cells will be extremely useful, because current methods that block exocytosis in mammalian cells, including BFA, also alter the endocytic pathway. The disadvantage of a single selection by FACS is that mutants must be selected at a frequency of 0.01% of the cell population, conditions under which survival of individual cell colonies was poor. It is possible that sequential FACS selection at higher frequency will allow plating of mutant colonies at higher cell densities, where survival is better. Our second approach, preselecting the mutagenized cell population with diphtheria toxin before the FACS screen, also accomplishes this, although endocytosis mutants will be enriched in this initial selection. Based on many experiments using either approach, we estimate that selecting cells for the inability to secrete BODIPY-ceramide can identify secretory mutants that are

present at a frequency of 1×10^{-6}, which is close to the frequency with which cells having a single mutation should be present in the mutagenized population.

The mutant cell lines that were resistant to BFA were not temperature-sensitive for the phenotype and do not appear to have any noticeable impairment of growth or secretion. These cell lines arose from several independently mutagenized and screened cell populations. They vary in their resistance to BFA and are dominant for the resistance phenotype. Unlike the BFA-resistant epidermoid carcinoma KB cell lines isolated by Seguchi *et al.* (1992) in which the morphology of the Golgi complex is altered, CHO-BR mutants have a normal Golgi morphology. Two other types of cellular mutants to BFA have been reported (Chen *et al.*, 1992; Yan *et al.*, 1993). In both cases, they were selected for the ability to grow in the presence of BFA, and the relationship of these mutants to the CHO-BR mutants is currently not known. We are in the process of identifying the mutant genes using an expression cloning strategy for the CHO-BR cell lines.

The mutant cell lines with defects in secretion (CHO-BEM cell lines) appear to be well suited for further studies at the biochemical and molecular levels. The characteristics of the different mutant phenotypes shown in Table 3 suggest that some of these cell lines arose from mutations in different genes, although we cannot exclude at present that some could contain mutations that affect a single gene to varying extents. It is interesting that the block of transport of HA to the cell surface in the CHO-BEM cell lines was not complete. This is not due to contamination of the mutant population with wild-type cells, because the mutant phenotype was not changed when the mutant cells were subcloned in 96-well plates.

After FACS screening, only a subset of the cells we obtained were subsequently determined to have defects in secretion. Based on the known properties of ceramide metabolism and labeling, two other interesting classes of mutants with defects unrelated to secretion might be selected. Some mutants might have defects in the complex pathway of ceramide metabolism (Hoekstra and Kok, 1992), which uses enzymes that are also involved in the processing of glycoproteins. Mutants in this category might be of interest to laboratories studying glycosylation. Another class of mutants might be those with defects resulting in reduced intracellular cholesterol levels. It has been reported that, following various treatments to deplete cellular cholesterol, very little C_6-NBD-Cer label can be seen on the Golgi complex (Martin *et al.*, 1993). If the same holds for labeling with C_5-DMB-Cer, any selection for dim cells may result in some mutants with defects in cholesterol accumulation. These mutants will be of interest to laboratories studying lipid metabolism and transport.

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