Membrane traffic between secretory compartments is differentially affected during mitosis

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Membrane traffic has been shown to be regulated during cell division. In particular, with the use of viral membrane proteins as markers, endoplasmic reticulum (ER)-to-Golgi transport in mitotic cells has been shown to be essentially blocked. However, the effect of mitosis on other steps in the secretory pathway is less clear, because an early block makes examination of following steps difficult. Here, we report studies on the functional characteristics of secretory pathways in mitotic mammalian tissue culture cells by the use of a variety of markers. Chinese hamster ovary cells were transfected with cDNAs encoding secretory proteins. Consistent with earlier results following viral membrane proteins, we found that the overall secretory pathway is nonfunctional in mitotic cells, and a major block to secretion is at the step between ER and Golgi: the overall rate of secretion of human growth hormone is reduced at least 10-fold in mitotic cells, and export of truncated vesicular stomatitis virus G protein from the ER is inhibited to about the same extent, as judged by acquisition of endoglycosidase H resistance. To ascertain the integrity of transport from the trans-Golgi to plasma membrane, we followed the secretion of sulfated glycosaminoglycan (GAG) chains, which are synthesized in the Golgi and thus are not subject to the earlier ER-to-Golgi block. GAG chains are valid markers for the pathway taken by constitutive secretory proteins; both protein secretion and GAG chain secretion are sensitive to treatment with *n*-ethyl-maleimide and monensin and are blocked at 19°C. We found that the extent of GAG-chain secretion is not altered during mitosis, although the initial rate of secretion is reduced about twofold in mitotic compared with interphase cells. Thus, during mitosis, transport from the trans-Golgi to plasma membrane is much less hindered than ER-to-Golgi traffic. We conclude that transport steps are not affected to the same extent during mitosis.

Introduction

Secretory pathways in eukaryotic cells have been an area of major research efforts because they are fundamental to a large number of cellular processes, including intercellular communication, immune response, and regulation of cell growth. Proteins destined for extracellular space are inserted across the endoplasmic reticulum (ER)¹ membrane by way of signal recognition and the translocation machinery (for reviews, see Meyer, 1988; Siegel and Walter, 1989). Resident proteins are retained in the lumen of the ER by means of specific sequences, whereas other proteins are carried by vesicles from the ER to the cis-Golgi, possibly via an intermediate compartment. This step of transport has been successfully reconstituted *in vitro* in mammalian cells and yeast (Balch *et al.*, 1987; Beckers *et al.*, 1987; Baker *et al.*, 1988), as has transport through the Golgi apparatus (Orci *et al.*, 1989). At the trans-Golgi, proteins are sorted into vesicles targeted for different destinations. At least three routes exist: transport to lysosomes, constitutive secretory vesicles, and regulated secretory vesicles (for reviews see Burgess and Kelly, 1987; Huttner and Tooze, 1989; Moore *et al.*, 1989). The final stage of secretion involves fusion of the secretory vesicles with the plasma membrane. The trans-Golgi-to-plasma membrane portion of the secretory pathway is an area of major efforts and has only

¹ Abbreviations: BiP, heavy chain binding protein; C₆-NBD-Cer, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl D-erythro-sphingosine; CHO, Chinese hamster ovary; DMEM, Dulbecco's minimum essential medium; DMSO, dimethyl sulfoxide; endo H, endoglycosidase H; ER, endoplasmic reticulum; FCS, fetal calf serum; GAG, glycosaminoglycan; GRP 78, glucose regulated protein 78 kDa (BiP); HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; hGH, human growth hormone; HMEM, DMEM + 18 mM HEPES buffer, pH 7.4; mAb, monoclonal antibody; NDET(S), 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris buffer, pH 7.4 (0.1% SDS); NEM, *n*-ethyl-maleimide; PBS, phosphate-buffered saline; SAC, *Staphylococcus aureus* cells; SDS, sodium dodecyl sulfate; TG, truncated VSV G protein; Tris, tris(hydroxymethyl)aminomethane; VSV, vesicular stomatitis virus.

recently been reconstituted (Miller and Moore, unpublished observations).

During cell division, a number of cellular processes are altered, including intracellular protein trafficking (Warren, 1985). Several reports indicate that ER-to-Golgi traffic is largely reduced (Featherstone *et al.*, 1985; Balch *et al.*, 1987) and that stimulated secretion is inhibited during mitosis (Hesketh *et al.*, 1984). Additionally, endocytosis appears to be inhibited during mitosis (Warren *et al.*, 1984), and signal transduction of hormones is altered (Volpi and Berlin, 1989). Concomitant with altered intracellular transport are changes in the morphology and distribution of organelles during cell division. Secretory granules are relocalized during metaphase and telophase (Tooze and Burke, 1987), apparently to allow equal distribution to daughter cells. The Golgi apparatus fragments and becomes dispersed throughout the cytoplasm in metaphase (Lucocq *et al.*, 1987), and in telophase, the scattered elements appear to reassemble in the perinuclear region (Lucocq *et al.*, 1989) of the daughter cells. It is not clear how transport between organelles is inhibited, nor is the mechanism underlying fragmentation of organelles understood. Warren (1989) proposed a mechanism that elegantly explains both the arrest of membrane traffic and organelle disassembly during mitosis: if budding events can continue but fusion events are inhibited, transport between compartments would stop, and organelles would become vesicularized. Confirmation of this proposal and the resolution of this issue requires individual examination of each step in the secretory pathway.

In this report, we use transfected secretory proteins to evaluate individual steps in the secretory pathway of mitotic cells. In particular, we examine the functional state of the trans-Golgi network during mitosis by asking whether constitutive vesicles can bud from the trans-Golgi and fuse with the plasma membrane. We report that, whereas ER-to-Golgi transport is dramatically reduced in mitotic Chinese hamster ovary (CHO) cells, trans-Golgi-to-surface transport is minimally affected. The implications of our results in relation to prior data are discussed.

Results

Overall secretion is reduced in mitotic CHO cells

Previous studies of secretory characteristics during cell division have used viral membrane proteins as markers (Featherstone *et al.*, 1985;

Balch *et al.*, 1987). We asked whether the same restrictions on membrane traffic during mitosis apply to soluble proteins. We cotransfected CHO cells with cDNA encoding the secretory protein human growth hormone (hGH) on a plasmid containing the RSV promoter and with a selectable marker plasmid, pSV2neo, which confers resistance to the antibiotic G418. Colonies were subcloned and assayed by immunoprecipitation. A relatively high-expressing line, CHO.hGH-7, was used for these experiments. The stably transfected cell line secretes hGH with a half-time of ~30 min. Secretion of hGH from mitotic CHO cells was assayed by the use of immunoprecipitation of media and extracts from CHO.hGH-7 cells. Interphase cells were used as a control. A significant portion of hGH is secreted into the media from interphase cells at 30 min; almost all the hGH is released by 2 h (Figure 1, A and B). In contrast, hGH secretion from mitotic cells is dramatically reduced; even after 2 h, very little is recovered in the media. We determined the initial rates of secretion in mitotic and interphase cells (Figure 1B): hGH secretion is reduced ~10-fold in mitotic cells compared with interphase controls (Table 1). The actual inhibition during mitosis could be greater because the amount of secreted hGH from mitotic cells is near the limit of detection; it is thus likely that the overall reduction of hGH secretion during mitosis is >10-fold. ER-to-surface traffic in mitotic CHO cells is hence reduced a minimum of 10-fold.

Because hGH is not glycosylated or sulfated, we could use it only as a marker for the overall secretory pathway. We therefore sought a soluble marker to assay ER-to-Golgi traffic during mitosis.

ER-to-Golgi traffic is inhibited during mitosis

The vesicular stomatitis virus (VSV) G protein is a well characterized marker for constitutive transport through the secretory pathway; however, it is a membrane-anchored protein. It has been shown previously that a truncated VSV G protein (TG) that lacks the C-terminal hydrophobic membrane-spanning region and the small cytoplasmic tail can be secreted slowly from transfected cells (Rose and Bergmann, 1982; Moore and Kelly, 1985). We have transfected this plasmid into CHO cells, as described for hGH. In generating a CHO cell line stably transfected with TG, we are able to measure ER-to-surface traffic and ER-to-Golgi traffic with the use of a soluble protein as a marker. The amount of TG secreted from mitotic cells is

Figure 1. The rate of hGH secretion is dramatically reduced in mitotic cells. (A) An autoradiogram of a 10–18% gradient polyacrylamide gel is shown of double immunoprecipitations of cell extracts and media from CHO cells transfected with a plasmid encoding hGH. Lanes: 1, interphase cell extract after pulse with ^{35}S -Met; 2, interphase cell extract after pulse and 30-min chase; 3, media from interphase cells during 30-min chase; 4, mitotic cell (95% metaphase) extract after pulse only; 5, mitotic cell extract after pulse and 30-min chase; 6, media from mitotic cells during 30-min chase. Molecular weight markers (14, 20, and 24 kDa) are shown on the left. hGH is specifically immunoprecipitated. (B) Line graph demonstrating inhibition of hGH secretion from mitotic cells (○) compared with interphase cells (■) at two chase time points.

minimal compared with interphase controls (Figure 2B); these results confirm that ER-to-plasma membrane traffic is dramatically inhibited during mitosis. TG is an excellent marker for ER-to-Golgi traffic integrity, as it becomes endoglycosidase H (endo H) resistant on entering the Golgi, but remains endo H sensitive in the ER. We further used the CHO cell line stably transfected with a plasmid encoding TG to assay ER-to-Golgi transport during mitosis. Figure 2, A and B, shows a gel from a typical experiment, using interphase cells as a control. Most of the TG remains endo H sensitive in mitotic cells, but becomes resistant with a half-time of

~2 h in interphase cells (Figure 2C). Very little TG is secreted from mitotic cells, but it is secreted from interphase cells with a half-time of slightly over 2 h; all of the secreted TG is endo H resistant (Figure 2, B and C). We estimated the rate of acquisition of endo H resistance in mitotic and interphase cells. We obtained a minimum value for the reduction of ~11-fold (Table 1) in mitotic cells.

An interesting aspect of this experiment is the degradation of TG after long chase periods, particularly in mitotic populations. Although >80% of the TG from interphase populations is quantitatively recovered, <50% of the TG is recovered from mitotic populations. Proteins blocked or retained for long times in the ER enter an alternate degradation pathway (Bonifacino *et al.*, 1989). The fact that more TG is lost from mitotic than from interphase cells, coupled with the failure of mitotic cells to render TG endo H resistant, implies that diversion of protein from the ER to an alternate degradation pathway can occur during mitosis. Thus, ER-to-plasma membrane and ER-to-Golgi transport are dramatically inhibited in mammalian cells undergoing mitosis, although the ER-to-degradation pathway appears to be unhindered.

C₆-NBD-Cer staining is dispersed during mitosis

It has been reported that the Golgi is widely dispersed in mitotic cells (Lucocq *et al.*, 1987). We sought to confirm the results of Kobayashi and Pagano (1989), which demonstrate that N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl D-erythro-sphingosine ($\text{C}_6\text{-NBD-Cer}$) displays a punctate pattern reminiscent of the dispersed Golgi reported by Lucocq *et al.* (1987). We labeled mitotic or interphase CHO cells following published procedure. In agreement with Kobayashi and Pagano (1989), the fluorescent lipid pattern of Golgi staining in interphase cells is tubular and perinuclear; in contrast, the Golgi appears as punctate, widely dispersed staining in mitotic cells (data not shown). The punctate pattern is consistent with morphological studies of mitotic Golgi (Lucocq *et al.*, 1987); the elements appear more dispersed than in interphase cells treated with microtubule depolymerizing agents such as nocodazole or colchicine (Kreiner and Moore, unpublished observations). We conclude that the Golgi apparatus is widely dispersed in the cytoplasm of our mitotic cells. The first step in the secretory pathway, ER-to-Golgi traffic, is dramatically hindered during mitosis; the Golgi thus has no

Table 1. Relative rates of transport in the secretory pathway

Marker	Compartments	Interphase	Mitotic	Ratio of rates
hGH	ER to surface	111.4	<10.9	>10.2
TG, endo H	ER to Golgi	104.0	<9.5	>10.9
GAG chains	Golgi to surface	2.98 ± 0.0745	1.32 ± 0.0176	2.25 ± 0.15

The relative rates in interphase and mitotic cells were calculated using the slope of semilog plots from percentage data vs. time. The mitotic values for hGH and TG are given as maximal values because of their proximity to the limit of detection.

input during mitosis. If function follows form, the dissociation of the Golgi during mitosis would imply that transport out of the Golgi is also inhibited. It is difficult to use the same markers to assay other steps to determine whether the inhibition effect is general, as hypothesized (Warren, 1985, 1989), because protein markers are blocked in the ER during mitosis. We sought a marker that would allow us to directly test the integrity of trans-Golgi-to-surface traffic during mitosis.

GAG chains serve as a reliable marker for secretion

We used sulfated glycosaminoglycan (GAG) chains as a soluble marker to assay trans-Golgi-to-surface traffic in mitotic cells. Cells treated with 4-methylumbelliferyl-beta-D-xyloside utilize the drug as an acceptor for GAG chains, which are sulfated in the trans-Golgi, providing a non-protein marker. Xyloside has been extensively used as an acceptor for GAG chains in studies of proteoglycan synthesis (Roden and Smith, 1966; Schwartz *et al.*, 1974; Lohmander *et al.*, 1979; Harper *et al.*, 1986; Rapraeger, 1989). Although it is well documented that proteoglycans are packaged into both regulated and constitutive secretory vesicles (Moore *et al.*, 1983), we demonstrate here that GAG secretion in intact interphase CHO cells is sensitive to the same factors as is constitutive protein secretion. CHO cells treated with 100 μ M *n*-ethyl-maleimide (NEM) for 10 min before the experiment are impaired in their ability to secrete GAG chains (Figure 3); whereas control cells secrete 85% of the GAG chains within 1 h, NEM-treated cells secrete <6% of the GAG chains. Similarly, cells held at 19°C secrete <17% of the GAG chains in 1 h, and cells held at 0°C secrete <4%. Monensin-treated cells secrete <20% of GAG chains in 1 h. Thus, the secretion of sulfated GAG chains from CHO cells is sensitive to NEM, monensin, and temperature.

GAG secretion is minimally affected in mitotic cells

Based on the morphology of the Golgi and the dramatic inhibition of ER-to-Golgi transport we observe, we expected that GAG-chain transport would be similarly affected during mitosis. The sizes of GAG chains synthesized in mitotic and interphase cells are both in the range of 10–20 kDa. We assayed the time course of GAG secretion in mitotic versus interphase cells, using cetylpyridinium chloride to precipitate the sulfated GAG chains. A typical experiment is shown in Figure 4. Whereas GAG secretion from mitotic cells is somewhat delayed compared with interphase cells, it is not blocked; in fact, the extent of GAG secretion from mitotic cells matches that of interphase controls. This surprising result implies that trans-Golgi-to-plasma membrane traffic of soluble markers is minimally affected during mitosis.

Semilog plots of data from several experiments were used to estimate the rates of GAG-chain secretion in mitotic and interphase cells (Table 1). The initial rate of secretion, which is quite rapid and corresponds to the well-defined constitutive route, is reduced about twofold in mitotic cells compared with interphase cells. Sulfated GAG chains, which are reliable soluble markers for transport from the trans-Golgi to plasma membrane, are secreted from mitotic cells. We conclude that, although ER-to-Golgi traffic is dramatically inhibited during mitosis in mammalian cells, trans-Golgi-to-plasma membrane transport of constitutive cargo is relatively unaffected.

BiP (GRP 78) synthesis is unaffected during mitosis

The differential effect of mitosis on discrete transport steps in the secretory pathway could be explained by the difference in function between compartments. Two simple explanations for a reduction in ER-to-Golgi transport during mitosis are apparent to us. First, a cytoplasmic factor that regulates budding or fusion or both

Figure 2. TG secretion is blocked in mitotic cells and TG remains Endo H sensitive. (A and B) An autoradiogram of double immunoprecipitations, using an antisera against VSV G protein, of extracts (A) and media (B) from TG-transfected CHO cells separated on 12% polyacrylamide gels is shown. Lanes: 1, 2, interphase cells, and 3, 4, metaphase cells after 30-min ^{35}S -Met pulse; lanes 5, 6, interphase cells, and 7, 8, metaphase cells after 2-h chase. Lanes 2, 4, 6, and 8 are +endo H, and 1, 3, 5, and 7 are -endo H. On the left, molecular weight markers at 45 and 66 kDa are shown. On the right, the arrowhead indicates the endo H-sensitive form of TG after digestion, and the full arrow indicates the resistant form. (C) A bar graph demonstrating the acquisition of endo H resistance of TG in mitotic cells (▨) and interphase cells (■) at two chase time points is shown. Some TG is not recovered, and presumed degraded, in mitotic cells at both time points (~50% recovered) and interphase cells at the latter time point (>80% recovered).

between the compartments could be altered in function during certain phases of the cell cycle. Second, a luminal factor in the ER that is

involved in folding or selective retention of misfolded proteins could be altered in level during mitosis. Because heavy chain binding protein (BiP) is a glucose-regulated protein (GRP), and other GRPs are regulated during the cell cycle, it seemed possible that the level of BiP could be increased during mitosis and thereby inhibit the transport of proteins between the ER and the Golgi. To investigate this possibility, we radiolabeled mitotic and interphase CHO cells and immunoprecipitated BiP with a monoclonal antibody (mAb) kindly provided by Dr. David Bole. The level of BiP does not appear to be affected during mitosis (Figure 5); in fact, the intensity of the BiP band in mitotic cells is 71% of interphase level, for an approximately equal number of cells. These experiments indicate that the inhibition of transport out of the ER during mitosis is not likely to be due to elevated levels of BiP.

Discussion

Relative rates of transport in the secretory pathway during mitosis

A comparison of transport rates between ER to surface, ER-to-Golgi, and Golgi to plasma mem-

Figure 3. GAG-chain secretion is sensitive to NEM, monensin, and temperature. Interphase CHO cells were treated with xyloside followed by pulse-labeling with ^{35}S -SO₄. Cells were treated with 100 μM NEM, 10 μM monensin at 37°C, or held at 0°C or 19°C, or no treatment, 37°C (control) for 10 min after the pulse. Media was collected at 15-min intervals under the same conditions, except in the case of NEM, in which the drug was removed after the 10-min pretreatment. The percentage secreted at 15-min intervals is shown in line graph form. Both NEM and monensin substantially reduce GAG secretion; low temperature also blocks transport from the site of sulfation to the surface.

brane is summarized in Table 1. We estimate the rate of transport from ER to surface based on secreted hGH recovered by immunoprecipitation of media samples; the rate of ER-to-Golgi transport is based on the acquisition of endo H resistance in TG, including both secreted and intracellular quantities. The estimates for fold of reduction (Table 1) in transport rates for these steps (ER-Golgi and ER-surface) are minimum values. Because transport from the ER is dramatically reduced during mitosis, the amounts recovered were often near the limit of detection, and these were used as overestimates of the amount of hGH or TG actually recovered. Thus, the actual decrease in transport from ER to Golgi and ER to surface is probably higher than the value listed in Table 1 and quite similar to values reported by Warren (1985) and others.

In contrast to ER-Golgi transport, secretion of GAG chains from the trans-Golgi to plasma membrane is rapid and can be quantitated by a highly sensitive assay for sulfated GAG chains (Figures 3 and 4). Almost equivalent percentages of GAG chains (>85%) are recovered from mitotic and interphase populations; thus, the extent of secretion during mitosis is the same as during G1. The rate of secretion is approximately twofold slower in mitotic cells than in interphase cells. The observed secretion cannot be due to contamination with interphase cells for the following reasons. The population of mitotic cells used for these studies is 97% pure. The total amount of GAG chains synthesized is diminished three- to fourfold in mitotic cells as

Figure 4. Secretion of GAG chains continues during mitosis. Samples of mitotic and interphase CHO cells were labeled with $^{35}\text{S}\text{-SO}_4$ in the presence of xyloside to stimulate the formation of GAG chains. Time points were collected in duplicate and assayed for GAG chains for interphase (■) and mitotic (○) cells. The extent of GAG secretion is comparable between interphase and mitotic populations. Although the initial rate of secretion is reduced about twofold from mitotic cells, it is minimally affected compared with the dramatic inhibition of ER-to-Golgi traffic.

Figure 5. BiP synthesis is not increased in mitotic CHO cells. An equal number (2.5×10^5) of interphase (I) or mitotic (M) CHO cells were labeled 30 min with $^{35}\text{S}\text{-Met}$, extracted with NDETS, and immunoprecipitated with anti-BiP (50 μl). After washes and elution, the samples were run on an 8% polyacrylamide gel. On the left, molecular weight markers are indicated at 66, 97, and 116 kDa. On the right, the arrow indicates the BiP (GRP 78) band. BiP expression is not substantially altered during mitosis.

previously reported (Preston *et al.*, 1985); thus, the 3% contamination with interphase cells could give rise to secretion of no more than 9–12% of GAG chains synthesized. Because we observed secretion of more than 85%, we conclude that the majority of GAG chains secreted is from the mitotic pool. The reduction in GAG chains synthesized could be due to a decrease in xyloside uptake or sulfation of synthesized GAG chains; neither of these possibilities affects our interpretation of the data. The surprising result is that this step of membrane traffic is less affected during cell division than other steps in the secretory pathway.

Intra-Golgi membrane traffic

We were not able to assess directly the effects of mitosis on intercisternal transport within the Golgi apparatus. However, consideration of our data in conjunction with prior reports implies that intra-Golgi traffic may be hindered during cell division. By measuring metabolites of $\text{C}_6\text{-}$

NBD-Cer, Kobayashi and Pagano (1989) have elegantly demonstrated that the rate of transport of C₆-NBD-Cer metabolites from the Golgi to the plasma membrane is inhibited >10-fold during mitosis. Thus, although C₆-NBD-Cer is metabolized in the Golgi during mitosis, the lipid metabolites do not reach the plasma membrane (Kobayashi and Pagano, 1989). We demonstrate that GAG-chain secretion continues during cell division at a rate reduced only twofold. This inhibition is substantially less dramatic than transport of C₆-NBD-Cer from the Golgi to the plasma membrane. One possible explanation for the difference is that the compartment for GAG-chain sulfation is past the site of C₆-NBD-Cer metabolism in the Golgi, i.e., C₆-NBD-Cer metabolism may take place in a Golgi stack that precedes the site of sulfation. If intra-Golgi transport between these two compartments is more severely inhibited during mitosis, it could explain the difference between GAG-chain secretion and C₆-NBD-Cer externalization. Indeed, C₆-NBD-Cer appears to be taken up at the trans-Golgi cisternae that are TPPase positive (R.E. Pagano, personal communication), whereas sulfation may occur in the trans-Golgi network (Kimura *et al.*, 1984; Tooze and Huttner, 1990). Clarification of which Golgi compartments the biosynthetic enzymes are localized in will greatly facilitate resolution of this issue.

Transport steps are differentially affected

The finding that different transport steps are differentially affected during mitosis is not expected. Earlier studies indicate that many transport steps are inhibited, and have led to the hypothesis that organelle vesiculation during mitosis results from a general inhibitor of membrane fusion (Warren, 1985). Indeed, recent studies showed that components mediating fusion of transport vesicles between Golgi stacks may also be involved in ER-to-Golgi transport and in transport between endocytic compartments (Beckers *et al.*, 1989; Diaz *et al.*, 1989; Wilson *et al.*, 1989). Such a generalized fusion mechanism makes it attractive to hypothesize a general inhibitor of membrane fusion during mitosis. Consistent with this hypothesis, it has been recently demonstrated that *in vitro* fusion of endocytic vesicles prepared from animal cells is inhibited by mitotic cytosol from *Xenopus* and that the inhibition can be mimicked in interphase cytosol by the addition of a cdc2 protein kinase homologue (Tuomikoski *et al.*, 1989). The cdc2 protein ki-

nase is known to regulate the interphase-to-mitosis transition in yeast (Dunphy and Newport, 1988) and perhaps could serve as a general regulator of membrane fusion during mitosis. If transport between different compartments utilizes a general mechanism, as recent evidence indicates, and if fusion is regulated by a general inhibitor, then why is constitutive transport between Golgi and the surface less affected than other steps? One possibility is that transport between different compartments may share many basic components, but have distinct biochemical requirements that are compartment-specific. One such component may be calcium. ER-to-Golgi transport is known to require physiological calcium, and, at high calcium concentrations, transport is inhibited (Beckers and Balch, 1989). In contrast, constitutive transport between Golgi and the cell surface shows no dependence on calcium (Miller and Moore, unpublished data). Whether differences in transport between compartments could be simply accounted for by calcium changes during mitosis remains to be determined.

A distinct mechanism for regulating Golgi-to-surface traffic during mitosis may be required to maintain the integrity of the plasma membrane. The plasma membrane is a unique organelle in that it cannot undergo fragmentation. Unlike other organelles, which continue to form vesicles without membrane input during mitosis and thus fragment, the plasma membrane requires a continued supply of vesicles. This distinct requirement may necessitate a unique regulatory mechanism to govern membrane traffic between the trans-Golgi and the plasma membrane.

Recently, it has been shown that transport between different intracellular compartments is also differentially affected in meiotic *Xenopus* eggs. However, the inhibition is precisely the opposite observed here in mammalian cells: ER-to-Golgi transport is minimally affected during mitosis (Ceriotti and Colman, 1989), whereas trans-Golgi-to-plasma membrane transport is specifically inhibited (Leaf *et al.*, 1990). Furthermore, transport of invertase from the ER to Golgi and secretion of invertase occur in mitotic yeast cells (Makarow, 1988). These results imply that not only the mechanisms for regulating membrane traffic during cell division differ from one system to another, but also within the same system the molecular mechanisms regulating distinct transport steps may be different. The intriguing question remaining is how a given cell regulates specific intercompartment-

tal transport, such that one step can be blocked and another continue with little or no perturbation.

Methods

Antisera and reagents

Antisera to hGH for immunoprecipitation was purchased from Antibodies (Davis, CA). Antisera to VSV G was made as previously described. Antisera to GRP 78 (BiP) was kindly provided by Dr. David Bole. Radioisotopes were purchased from Amersham (Arlington Heights, IL); *Staphylococcus aureus* cells (SAC) and endo H were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN); C₆-NBD-Cer was obtained from Molecular Probes (Eugene, OR); thymidine was purchased from Aldrich Chemicals (Milwaukee, WI); Nocodazole, xyloside, monensin, NEM, and most standard chemicals were purchased from Sigma (St. Louis, MO).

Generation of cell lines

CHO cells were plated at a density of 3.0×10^6 cells/10-cm dish 12–16 h before transfection. DNA was precipitated by the use of 94 μ l of 2 M CaCl₂, 30 μ g of the nonselectable plasmid (either pRSVhGH or pRSVTG) and 2 μ g of the selectable plasmid, pSV2neo, brought to a volume of 750 μ l with H₂O. This mixture was slowly added to 750 μ l of 2 $\times$ N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffered saline (2 $\times$ = 42 mM HEPES, 247 mM NaCl, 10 mM KCl, 1.6 mM Na₂PO₄, 2 g/l dextrose, pH 7.05, sterile filtered) with bubbling. The precipitate was allowed to form and incubate at 24°C for 40 min. Cells were rinsed with 10 ml and then 5 ml HEPES buffered saline and the DNA precipitate was layered on the cells. The dishes were floated on water baths at 20°C for 30 min, after which 12 ml Ham's F-12 media with 10% fetal calf serum (FCS) and 25 mM HEPES, pH 7.4, was added. Cells were incubated for 4 h at 37°C under 5% CO₂. Media and DNA were aspirated off, and 1 ml of 25% glycerol in Ham's F-12 with 25 mM HEPES, pH 7.4, was added for exactly 1 min. Cells were rinsed three times with Ham's F-12, and then fed with Ham's F-12 containing 10% FCS. At 48 h posttransfection, cells were split into fresh dishes 1:2 with media containing 1.0 mg/ml G418. Approximately 2 wk after transfection, colonies were picked using cloning rings, expanded, and assayed by immunoprecipitating extracts from radiolabeled cells. Stable transformants were maintained in media containing 0.125 mg/ml G418.

Cell culture and collection of mitotic cells

CHO cells transfected with hGH or TG were used for ER-to-Golgi and ER-to-plasma membrane assays. Non-transfected CHO cells were used for GAG-chain assays. CHO cells were grown in Ham's F-12 with 10% FCS under 5% CO₂ at 37°C. Transfected cell lines were grown in the presence of 0.125 mg/ml G418 (GIBCO). Mitotic cells were produced essentially as described by Balch *et al.* (1987). CHO cells were twice replated at high density, $\sim 7 \times 10^6$ per 10-cm plate. The second time, they were plated in the presence of 5 mM thymidine. After 20 h, the thymidine-containing media were removed and the cells were washed twice and fed with normal media (Zieve *et al.*, 1980). Interphase cells for controls were grown on 10-cm plates and treated identically, except no thymidine was added. Five hours later, Nocodazole was added from a 1000 $\times$ stock in dimethyl sulfoxide (DMSO) to 100 ng/ml final concentration. Inter-

phase controls were given an equal amount of DMSO. After 3 h, the cells were harvested by shaking the plates and the cells collected and pelleted in 15-ml sterile centrifuge tubes. Interphase cells were lifted from plates with the use of 5 mM EDTA in calcium-magnesium-free phosphate-buffered saline (PBS) and pelleted. Populations were assayed by adding 10 μ l of the resuspended cells to 10 μ l of 1% para-formaldehyde in PBS containing 2 μ g/ml Hoechst 33342 nuclear stain. After 10 min, 10 μ l of the fixed, stained cells were mounted on a slide and examined on an Axiophot fluorescence microscope (Zeiss, W. Germany). At least 200 cells were counted, and the percentage of mitotic cells was determined. For mitotic populations, >95% were mitotic; <5% were mitotic in interphase controls. All experimental manipulations of cells were done in suspension in 15-ml tubes (Falcon, Lincoln Park, New Jersey).

C₆-NBD-Cer labeling

Mitotic CHO cells in suspension or interphase cells on coverslips were incubated with a C₆-NBD-Cer/bovine serum albumin complex in DMEM + 18 mM HEPES buffer, pH 7.4 (HMEM), followed by washes in HMEM, as described (Kobayashi and Pagano, 1989).

Radiolabeling

For hGH and TG assays, cells were starved of methionine during the Nocodazole treatment. Cells were labeled for 30–90 min in methionine-free Dulbecco's minimum essential medium (DMEM) with 1 mCi/ml ³⁵S-Methionine. Cells were chased for variable times in media containing methionine and extracted on ice with NDET (1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM tris(hydroxymethyl)-aminomethane (Tris), pH 7.4) with protease inhibitors iodoacetamide (final concentration, 0.3 mg/ml) and phenylmethylsulfonylfluoride (final concentration, 0.3 mg/ml). For GAG assays, cells were starved of sulfate and treated with 1 mM xyloside during the Nocodazole treatment. Cells were resuspended in Buffer A (20 mM HEPES, 10 mM MgCl₂, 110 mM NaCl, 5.4 mM KCl, 0.9 mM NaHPO₄, 0.1% glucose, 1.36 mM CaCl₂, pH 7.2) containing xyloside for 10 min, and then ³⁵S-SO₄ was added to a concentration of 1 mCi/ml. After 5 min, cells were either harvested or chased for variable times. Media were collected at each time point, and cells were harvested with 0.5 ml PBS + 0.5% Triton X-100 after the last chase time point.

Secretion of GAG chains from interphase cells

CHO cells were labeled with ³⁵S-SO₄ as described above. Cells were subjected to treatment with 100 μ M NEM, 10 μ M monensin, 0°C, 19°C, or control (37°C) for 10 min. Media containing 10 μ M monensin or normal media (DMEM) was added and collected at 15-min intervals for 1 h; cells were then harvested as above.

Immunoprecipitation

CHO-hGH cell extracts were immunoprecipitated twice using 10 μ l of the hGH antisera as follows. After extraction, cells were preincubated with 60 μ l of twice (NDET + 0.2% sodium dodecyl sulfate [SDS])-washed SAC for 30 min, then the SAC were removed by centrifugation and the supernatant transferred to a new tube. Antibody was added, and the tubes were rotated for 2 h at 4°C. Then, 60 μ l of SAC was added, and the cells shaken for 30 min. The mixtures were loaded on a 0.5-ml cushion of 30% sucrose and pel-

leted in a microfuge for 15 min. Pellets were resuspended in 100 μ l NDET + 0.2% SDS, transferred to new tubes, and washed twice with 1 ml NDET + 0.2% SDS and once with 1 ml H₂O. The pellet was resuspended in 90 μ l water, and 10 μ l of 10% SDS was added. After 30 min at 37°C, the tubes were centrifuged in a microfuge for 5 min, and the supernatant was diluted to 0.5 ml with NDET. The antiserum was added and the procedure repeated until after the water wash, at which point the pellets were boiled in sample buffer and loaded on 10–18% gradient SDS acrylamide gels for electrophoresis. CHO-TG extracts were immunoprecipitated by the same protocol, but using 2 μ l of the antisera. Disco tritium per sample per immunoprecipitation. After the second immunoprecipitation and washes, the samples were eluted with 45 μ l 0.1 M Na citrate, pH 5.5, containing 1% SDS, by boiling for 3 min. Twenty microliters were treated with endo H, and 20 μ l were sham treated by incubating overnight at 37°C with or without the enzyme. Samples were dried, resuspended in sample buffer, and run on 12% SDS gels. Single immunoprecipitation of BiP was carried out essentially as above, but using NDET + 0.1% SDS and doubling the number of washes. Eluates from the SAC were run on 8% polyacrylamide SDS gels.

Quantitation

Gels were scanned with the use of a laser densitometer (Biomed Instruments). Peaks were weighed on a Mettler balance for quantitation. For endo H experiments, the minus and plus totals were averaged. Pulse-only values were used as a standard for experiments, because some protein was lost during long chases in mitotic cells, possibly because of an alternate degradation pathway (Bonifacino *et al.*, 1989). GAG secretion was calculated by summing the total secreted and intracellular at the end of the experiment and taking each time point as a percentage. Errors were calculated by the use of the standard deviation between duplicate or triplicate samples from each time point.

GAG assays

To 0.5-ml samples, pronase E was added to 1 mg/ml. After 4 h incubation at 37°C, 150 μ l of 10% cetylpyridinium chloride and 100 μ g of chondroitin sulfate was added per sample; the samples were incubated 1 h at 37°C, then filtered through 0.45- μ m Metrical filters on a vacuum manifold. After several washes with 1% cetylpyridinium chloride in 25 mM Na₂SO₄, the filters were dried and counted. All GAG assays were done in duplicate or triplicate.

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