

COP-coated Vesicles Are Involved in the Mitotic Fragmentation of Golgi Stacks in a Cell-free System

Tom Misteli and Graham Warren

Cell Biology Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom

Abstract. Rat liver Golgi stacks fragmented when incubated with mitotic but not interphase cytosol in a process dependent on time, temperature, energy (added in the form of ATP) and cdc2 kinase. The cross-sectional length of Golgi stacks fell in the presence of mitotic cytosol by $\sim 50\%$ over 30 min without a corresponding decrease in the number of cisternae in the stack. The loss of membrane from stacked and single cisternae occurred with a half-time of ~ 20 min, and was matched by the appearance of both small (50–100 nm in diameter) and large (100–200 nm in diameter) vesicular profiles. Small vesicular profiles constituted more than 50% of the total membrane after 60 min of incubation and they were shown to be vesicles or very short tubules by serial sectioning. In

the presence of GTP γ S all of the small vesicles were COP-coated and both the extent and the rate at which they formed were sufficient to account for the production of small vesicles during mitotic incubation. The involvement of the COP-mediated budding mechanism was confirmed by immunodepletion of one of the subunits of COP coats (the coatamer) from mitotic cytosol. Vesicles were no longer formed but highly fenestrated networks appeared, an effect reversed by the readdition of purified coatamer. Together these experiments provide strong support for our hypothesis that the observed vesiculation of the Golgi apparatus during mitosis in animal cells is caused by continued budding of COP-coated transport vesicles but an inhibition of their fusion with their target membranes.

THE segregation of chromosomes during mitosis (or meiosis) in animal cells is accompanied by the partitioning of all other intracellular organelles (Birky, 1983). The strategy adopted to ensure equal partitioning between cells depends on the organelle and the organism. The best-characterized and the one that appears to be most commonly used is that based on a stochastic process. Providing that a mechanism exists to divide the mother cell into two equally sized daughters (Rappaport, 1986; White and Borisy, 1983), the accuracy of partitioning will depend on the copy number of the organelle in the cell. The higher the number, the greater the accuracy (Birky, 1983).

Some organelles, such as mitochondria, exist as multiple copies throughout the cell cycle, and because they are randomly dispersed throughout the cell cytoplasm (Brandt et al., 1974) they should be accurately partitioned at cell division. Other organelles, such as the Golgi apparatus and the endoplasmic reticulum (which includes the nuclear envelope), are present as single copies or at most a few copies (Louvard et al., 1982; Lucocq and Warren, 1987). Mechanisms that break up these organelles must exist if accurate partitioning is to occur by a stochastic process.

The interphase Golgi apparatus, as seen by light or fluorescence microscopy, is a compact juxta-nuclear reticulum, most often in the peri-centriolar region of the cell (Golgi, 1898; Novikoff and Goldfischer, 1961; Louvard et al., 1982). High voltage electron microscopy shows that it comprises discrete Golgi stacks linked together by tubules that connect equivalent cisternae in adjacent stacks (Rambourg and Clermont, 1990; Tanaka et al., 1986). At the onset of mitosis (or meiosis), disassembly occurs in two stages. The first stage, during prophase, generates discrete stacks (Colman et al., 1985) suggesting that the tubules linking stacked cisternae are somehow severed. This severing appears to be linked to the reorganization of the microtubule network since it can be mimicked by the addition of anti-microtubule agents such as nocodazole (Thyberg and Moskalewski, 1985). At about the same time the nuclear envelope breaks down, as does the peripheral ER (Zeligs and Wollman, 1979; Tamaki and Yamashina, 1991; Warren, 1993).

The second stage of disassembly occurs during prometaphase, metaphase and anaphase, and has been studied in most detail in mitotic HeLa cells (Lucocq et al., 1987, 1989; Lucocq and Warren, 1987). It involves fragmentation of the Golgi stacks yielding first clusters of small vesicles and short tubules, and eventually free vesicles distributed at random throughout the mitotic cell cytoplasm. Up to ten thousand Golgi vesicles are produced which would, theoretically, and

Address all correspondence to Dr. Graham Warren, Cell Biology Laboratory, Imperial Cancer Research Fund, P.O. Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom.

in a stochastic manner, provide almost every daughter cell with $50 \pm 1.5\%$ of Golgi membrane (Warren, 1993). An understanding of the vesiculation mechanism would, therefore, provide insight into the process that partitions the Golgi apparatus between the two daughter cells.

The best clue to such a mechanism comes from the many studies showing that there is a general inhibition of membrane traffic at the onset of mitosis (or meiosis) in animal cells (for review see Warren, 1993). Traffic through the Golgi apparatus is inhibited, both *in vitro* (Stuart et al., 1993) and *in vivo* (Collins and Warren, 1992), and resumes during telophase as the Golgi stack is reassembling (Souter et al., 1993). A simple model (Warren, 1985) that links the inhibition of transport through the Golgi apparatus with its disassembly is based on the mechanism of protein transport through the stack (Rothman and Orci, 1992). Vesicles bud from the dilated cisternal rims using coat protomers (COPs)¹ each containing a small GTP-binding protein, ARF (ADP-ribosylation factor) (Serafini et al., 1991a), and coatomer, a complex of seven polypeptides ranging in molecular weight from 20 to 160 kD (Serafini et al., 1991b; Waters et al., 1991). Lateral association of COPs is thought to cause deformation of the membrane leading to budding and eventual pinching off of a COP-coated vesicle. This then docks with the next cisterna towards the trans side of the stack which results in eventual fusion (Söllner et al., 1993). If, at the onset of mitosis, budding of COP-coated vesicles were to continue but their fusion with the next cisterna were to be inhibited, then Golgi cisternae would be converted into vesicles. Consistent with this model is the observation that mitotic Golgi vesicles are 47 ± 2.3 nm in diameter (Lucocq et al., 1987), very similar to the diameter of uncoated transport vesicles (~ 50 nm, Oprins et al., 1993).

To test this model, we have developed a cell-free system using purified Golgi stacks and cytosol generated from mitotic HeLa cells. Here we provide both morphological and biochemical data showing that fragmentation depends on continued budding of COP-coated vesicles.

Materials and Methods

All reagents were of analytical grade or higher and purchased from Sigma Chem. Co. (St. Louis, MO) or BDH unless otherwise stated.

Preparation of Rat Liver Golgi Membranes

Rat liver Golgi stacks were prepared as described by Slusarewicz et al. (1994a), and assayed for β 1,4-galactosyltransferase (GalT) activity as described by Bretz and Stäubli (1977).

Preparation of Mitotic and Interphase Cytosols

Spinner HeLa cells were grown, and cytosols prepared as described by Stuart et al. (1993). Interphase cytosol was prepared in the same way from FT210 cells which were grown in 1l spinner culture to 4×10^5 cells/ml at 32°C in RPMI medium containing 2% sodium bicarbonate (ICRF Cell Production Services) supplemented with 10% FCS, 2 mM glutamine, 100 IU penicillin/ml, 10 mg streptomycin/ml, and essential amino acids. All cell culture reagents were from GIBCO BRL, Uxbridge, U.K.

1. *Abbreviations used in this paper:* ARF, ADP-ribosylation factor; BFA, brefeldin A; COP, coat protomer; cycA, cyclin A; GalT, β 1,4-galactosyltransferase; OkA, Okadaic acid; Stp, staurosporine.

Histone H1 Kinase Assay

Histone kinase activity was measured as described by Felix et al. (1989), except that samples were incubated for 20 min at 32°C.

Fragmentation Assay

Cytosolic proteins were transferred to MEB buffer (50 mM Tris-HCl, pH 7.3, 50 mM KCl, 10 mM MgCl₂, 20 mM β -glycerophosphate, 15 mM EGTA, 2 mM ATP, 1 mM DTT) just before use by application to a 1.5-ml P6-DG (Bio-Rad, Hemel Hempstead, U.K.) spin column. A typical assay consisted of 5 μ l of a 10x concentrated ATP-regenerating system (100 mM creatine phosphate, 10 mM ATP, 0.2 mg/ml creatine phosphokinase), 5 μ l 2 M sucrose in H₂O, 2.5 μ l Golgi stacks (100 μ g/ml final concentration), and 37.5 μ l cytosol (7.5–10 mg/ml final concentration). For electron microscopy experiments the incubations were scaled up threefold. All incubations were carried out in siliconized glass tubes.

In some experiments the ATP-regenerating system was replaced by an ATP-depletion system (Smythe et al., 1989). MBP-cyclin A was prepared as described by Stuart et al. (1993) and used at 50 μ g/ml final concentration. Okadaic acid (OkA, Calbiochem, Nottingham, U.K.) was routinely used at 1 μ M and staurosporine (Calbiochem, Nottingham, U.K.) at 10 μ M from stock solutions of 1 mM in DMSO. GTP γ S was made up freshly in 25 mM Hepes-KOH, pH 7.2 to 2 mM and used at a final concentration of 20 μ M.

Sedimentation Assay

A 15- μ l sample of the incubation was diluted with one volume of MEB buffer and centrifuged at 5,500 rpm (2,000 g_{av}) for 10 min at 4°C in a fixed angle Eppendorf table top centrifuge 5415. 20 μ l of the supernatant was removed and the pellet made up to 20 μ l. Supernatant and pellet were assayed for GalT activity as described in Bretz and Stäubli (1977).

Electron Microscopy

Membranes were collected after incubation by centrifugation at top speed (14,000 g_{av}), for 20 min at 4°C in an Eppendorf table top centrifuge 5413 fitted with a horizontal rotor. The pellets were fixed at room temperature for 30 min with 1% glutaraldehyde (Fluka, Gillingham, U.K.) in 0.1 M KHPO₄-buffer, pH 6.7, 0.2 M sucrose, washed extensively with PBS, postfixed for 30 min with 1% osmium tetroxide, 1.5% cyanoferrate in 0.1 M cacodylate buffer, pH 7.2 or alternatively by the tannic acid (Mallinckrodt Inc., Paris, Kentucky) method according to Simionescu and Simionescu (1976), and then dehydrated and embedded in Epon 812 (Taab Laboratories, Reading, U.K.). Transverse sections through the entire pellet were cut on a Reichert ultramicrotome 2E set to 50–70 nm, picked up on a nickel grid, and stained with 2% uranyl acetate and lead citrate (Roth and Berger, 1982).

For cryo-electron microscopy, membranes were collected and fixed in the same way or alternatively fixed overnight at 4°C in 1% paraformaldehyde in 0.1 M KHPO₄-buffer pH 6.7, 0.2 M sucrose. Pellets were then embedded in 10% gelatin in H₂O, cut into small blocks, and infiltrated with 2.1 M sucrose in PBS overnight at 4°C. Blocks were cut on a Reichert ultramicrotome 2E at -95°C and sections collected on collodion/carbon-coated grids. Sections were stained for 6 min at room temperature in 2% uranyl acetate and contrasted for 20 min with 2% methylcellulose, 0.4% uranyl acetate on ice.

Stereology

Cisternae were defined as membrane profiles with a length more than four times their width, the width being not more than 80 nm. Tubules were defined as profiles with their length smaller than four times their maximal width. Stacks comprised two or more cisternal profiles separated by a gap of no more than 15 nm and overlapping by more than half their length. Small vesicles were defined as circular profiles with a diameter of 50–100 nm, large vesicles were defined as circular profiles with a diameter of 100–200 nm. Open profiles such as broken cisternae were not included in the quantitation.

To determine the relative proportion (using membrane length as the parameter) of each category of membrane, photographs of fields selected in a systematic random fashion at low magnification were taken at a final magnification of 22,250 \times or 52,500 \times . The number of intersections of each membrane structure with the lines of either a 4-mm square grid or a line grid was counted, and the structure assigned to one of the defined membrane categories. The number of intersections of all membranes in the sample was set to 100%, and the fraction of membrane in each category was calculated

as $N_{\text{intersections in category}}/N_{\text{total intersections}}$ and expressed as percentage of total membrane. 25 micrographs were counted for each sample in each experiment and all membrane structures except identifiable contaminations (such as plasma membrane, nuclei, and mitochondria) were counted. Both counting methods were applied to all samples and gave comparable results. All profiles which fitted the definition of a stack were used to determine the number of cisternae in a stack. For each timepoint ~50 stacks were examined.

The length of cisternae was determined by counting the number of intersections of an imaginary line running along the centre of the cisterna with a 5-mm grid or alternatively by measuring the length with a ruler. Both methods gave comparable results. Stacked and free cisternae were measured. For each experiment 120 cisternal profiles were measured.

The surface density of membranes was determined by counting the number of intersections of membrane with grid lines divided by the number of points falling over the lumen of the membrane-bound structure. Only the set of high magnification photographs was used for this value. For each experiment 50 Golgi regions were examined.

For serial sectioning, areas were selected with a random start and photographs were taken at a magnification of 22,250 \times and enlarged three times onto paper and OHP transparency film. 100 vesicular profiles were selected at random and tracked through five consecutive sections starting from the central section by overlaying the paper with the transparency of the adjacent section. The number of consecutive sections in which the profile appeared was scored.

COP-coated vesicles were defined as apparently free vesicular profiles of 50–70-nm diam with a non-clathrin cytoplasmic coat or as buds bearing the same coat. Photographs were printed to a final magnification of 95,000 \times . By counting the number of grid points falling over the lumen of the membrane-bound structures, the total sectioned area of membrane was determined. The number of COP-coated vesicles and buds in the measured area was then counted and the density of COP-coated vesicles/sectioned Golgi area determined. For each experiment 50 Golgi regions were examined.

Immunodepletion of Coatomer

CM1A10 antibody (Orci et al., 1993) was bound to protein G-agarose beads (Calbiochem, Nottingham, U.K.) by cross-linking with dimethyl suberimide and stored as a 50% slurry at 4°C. For depletion, 0.5-ml (10–15 mg/ml) cytosol was incubated for 2 \times 6 h with a change of beads and for an additional 12 h with 25 μ l of the slurry at 4°C with mixing in the presence of a 10 \times ATP-regenerating system as described above. Before use, the beads were washed twice with MEB buffer and dried using a Hamilton syringe.

Western Blotting

30 μ g cytosolic proteins were fractionated on a 15% SDS-polyacrylamide gel, transferred to a Highbond membrane (Amersham, U.K.) by semi-dry blotting in 20 mM Tris, 150 mM glycine, 2% methanol, 0.1% SDS, and the membrane blocked overnight at 4°C in PBS, 5% milk, and 0.1% Tween. Anti-p36 antibody was used at 1:30,000 in PBS, 2.5% milk, 0.1% Tween, incubated for 1 h at room temperature, washed off over 1 h in the incubation buffer and detected with HRP-goat anti-rabbit antibody (Tago Inc., Burlingame, CA; diluted 1:1,000) in the above buffer for 1 h at room temperature. Washing was carried out for 1 h in PBS and the bands visualized using the ECL system (Amersham, U.K.).

Results

Golgi Stacks Fragment in the Presence of Mitotic Cytosol

Stacked Golgi membranes were isolated by forcing minced rat liver through a small-mesh sieve (Hino et al., 1978) followed by fractionation on a sucrose step gradient (Leelavathi et al., 1970; Slusarewicz et al., 1994a). These preparations were routinely enriched 100–150-fold for the *trans*-Golgi marker β 1,4-galactosyltransferase (GalT) (Roth and Berger, 1982) and more than 60% of the cisternal profiles were stacked (see Figs. 1 A and 6 A). The origin of cisternal profiles was confirmed by immuno-gold labeling using antibodies to the *medial*-Golgi marker mannosidase II (Velasco et al., 1993) and the luminal content marker, rat serum albumin (Judah et al., 1989) (data not shown).

Interphase cytosols were prepared from large-scale cultures of unsynchronized HeLa cells grown in spinner culture. Mitotic cytosols were prepared from spinner cells that had been treated for 24 h with nocodazole. This anti-microtubule drug arrested 96–98% of the cells in prometaphase and yielded cytosols that had histone kinase activities 10–20-fold higher than those from unsynchronized cells (Stuart et al., 1993).

When Golgi stacks were incubated with mitotic cytosol for 60 min at 37°C in the presence of ATP and a regenerating system, the morphology of the membranes underwent dramatic changes (Fig. 1). The stacked and single cisternae present before the incubation (Fig. 1 A) disappeared and were replaced by profiles of small (50–100 nm diam, *small arrowheads* in Fig. 1 C) and large (100–200 nm diam, *large arrowheads* in Fig. 1 C) vesicles and remnants of stacked and single cisternae (*arrows* in Fig. 1 C). The small vesicular profiles appeared uncoated and had an average diameter of 54 ± 4 nm ($n = 120$), similar to that reported for mitotic Golgi profiles in HeLa cells (47 ± 2.3 nm; Lucocq et al., 1987). Cisternal remnants ranged in length from 100 nm up to the length of intact, single cisternae.

Fragmentation only occurred under mitotic conditions. Golgi stacks incubated with interphase cytosol underwent very few changes in morphology (Fig. 1 B) compared to the starting membranes (Fig. 1 A). Most of the cisternal profiles were still full length and most were stacked (*arrows* in Fig. 1 B). There was a slight increase in the number of small vesicles (*small arrowheads* in Fig. 1 B) most of which were presumably transport vesicles (Balch et al., 1984).

Preparations of Golgi stacks were contaminated to a small extent with plasma membrane sheets, and, to an even lesser extent, with mitochondria and nuclei. All these contaminations were identified on the basis of their morphology. They provided internal controls and their behavior paralleled events in the cell. Nuclei broke down in the presence of mitotic but not interphase cytosol whereas mitochondria (data not shown) and plasma membranes were not visibly affected by either cytosol (Fig. 1 C, *asterisks*).

Fragmentation did not occur in the absence of cytosol (Fig. 1 D), in the presence of mitotic cytosol incubated on ice (Fig. 1 E), or in the presence of mitotic cytosol but with an ATP-depleting system (Fig. 1 F). In all these cases intact cisternae and stacks were preserved. The absence of ATP caused slight swelling of Golgi membranes and the formation of tubular structures and networks similar to those reported by Cluett et al. (1993) (Fig. 1 F, *asterisks*). Fragmentation was also reduced by lowering the amount of mitotic cytosol (Fig. 1 G). The normal ratio of cytosolic protein to Golgi protein was maintained at 100:1. This is similar to the ratio in the cell since a 100-fold purification of Golgi membranes over homogenate is needed to obtain almost homogeneous preparations of Golgi membranes (Slusarewicz et al., 1994a). Halving this ratio prevented significant fragmentation (Fig. 1 G) suggesting the presence of a limiting factor or factors in mitotic cytosol.

In a complementary, biochemical approach, the fragmentation process was also characterized using a rapid and simple sedimentation assay that exploited the difference in sedimentability between vesicles and stacks. Pilot studies showed that a 10-min spin at 2,000 g_{av} sedimented all but $12 \pm 8\%$ of the GalT activity in Golgi stacks after incubation

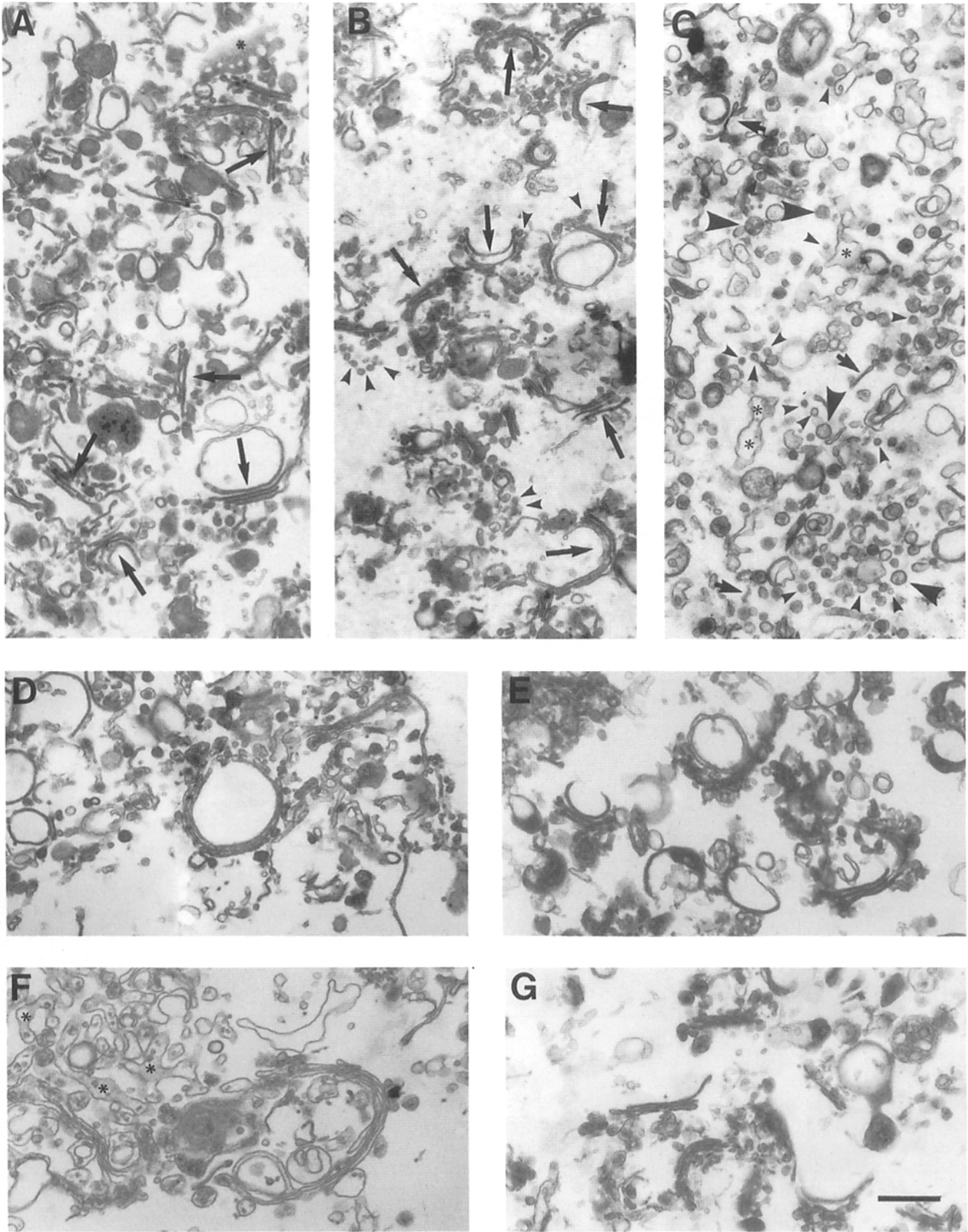


Figure 1. Morphology of Golgi membranes after incubation with interphase or mitotic cytosol. Golgi stacks (A) were incubated for 60 min at 37°C in the presence of interphase (B) or mitotic cytosol (C) with ATP and a regenerating system. Extensive fragmentation in mitotic cytosol (C) yielded large vesicular profiles (*large arrowheads*), small vesicular profiles (*small arrowheads*), and remnants of stacked and single cisternae (*arrows*). En face views of fenestrated cisternae as occasionally seen in Golgi membrane preparations (*asterisk* in

in interphase cytosol, whereas $52 \pm 8\%$ of GalT activity was present in the supernatant after incubation with mitotic cytosol (Fig. 2 C). Assays for the *medial/trans*-Golgi enzyme, N-acetylglucosaminyltransferase I, gave similar results (data not shown).

The GalT activity in the supernatant could be sedimented at higher speed (20 min, $15,000 g_w$) showing that it was associated with small Golgi fragments and had not simply been released into the supernatant (data not shown). Electron microscopy confirmed that the low-speed supernatant was enriched about threefold in both small and large vesicles (*arrowheads* in Fig. 2 A), but did contain some cisternal remnants (*arrow* in Fig. 2 A). The pellet contained mostly cisternal remnants (*arrows* in Fig. 2 B) though a number of vesicles appeared to be trapped (*small arrowheads* in Fig. 2 B). This would help explain why more of the GalT was not present in the supernatant after low speed centrifugation.

The mitotic conversion of Golgi stacks to a less sedimentable form was dependent on time, temperature and ATP (Fig. 2 C). Only 6–12% of the GalT activity was present in the supernatant at zero time, after 60 min on ice, or in the presence of mitotic cytosol but with an ATP-depleting system. Halving the concentration of mitotic cytosol decreased the amount of GalT activity in the supernatant from $52 \pm 8\%$ to $12 \pm 2\%$ showing once again the need for a limiting factor or factors in mitotic cytosol (Fig. 2 C).

Cdc2 Kinase Is Required for Fragmentation

The fragmentation activity was dependent on the action of a kinase (Fig. 3). Addition of $10 \mu\text{M}$ staurosporine (Stp), a broad-range inhibitor of many kinases including *cdc2* kinase (Gadbois et al., 1992), to mitotic cytosol reduced the percentage of GalT in the supernatant from $65 \pm 9\%$ to $21 \pm 14\%$, close to the level for interphase cytosol ($18 \pm 6\%$).

Kinase effects can often be enhanced by the addition of phosphatase inhibitors such as OkA which is a specific inhibitor of protein phosphatases 1 and 2A (Takai et al., 1987). OkA also mimics the mitotic fragmentation of the Golgi apparatus when added to interphase cells (Lucocq et al., 1991). When added to interphase cytosol (to $1 \mu\text{M}$) the percentage of GalT in the supernatant increased from $18 \pm 6\%$ to $82 \pm 2\%$, even higher than the level for mitotic cytosol ($65 \pm 9\%$) (Fig. 3).

Cdc2 kinase can be activated by recombinant cyclin A (*cycA*) and this complex has been shown to inhibit intra-Golgi transport in a cell-free system (Stuart et al., 1993). When added to interphase cytosol (to $50 \mu\text{g/ml}$) the percentage of GalT in the supernatant rose from $18 \pm 6\%$ to $63 \pm 5\%$ (Fig. 3). Addition of both *cycA* and OkA to interphase cytosol raised the level of GalT in the supernatant to $86 \pm 2\%$ similar to that with OkA alone (Fig. 3).

Cyclin A activates *cdk2* kinase (the S phase kinase) as well as *cdc2* kinase (Pagano et al., 1992; Rosenblatt et al., 1992). To show that *cdc2* kinase was responsible for the observed

fragmentation of Golgi stacks, interphase cytosols were prepared from FT210 cells (Th'ng et al., 1990). This cell line has a temperature-sensitive defect in *cdc2* but not *cdk2* kinase. When the cells (or cytosol) are incubated at the non-permissive temperature of 39°C , *cdc2* kinase is irreversibly inactivated (Th'ng et al., 1990). Cytosols were preincubated at either 32°C (the permissive temperature) or 39°C for 20 min followed by cyclin A for an additional 10 min. Golgi stacks were then added and the incubation continued for another 90 min at 34°C , a compromise temperature that supported a reasonable level of fragmentation while preserving most of the *cdc2* kinase activity. Cyclin A activated the histone kinase activity in cytosol preincubated at 32°C by 1.7-fold (Fig. 4 B) and led to fragmentation of the Golgi stacks (Fig. 4 A). The percentage of GalT in the supernatant rose from $30 \pm 9\%$ (for pretreated cytosol not activated by cyclin A) to $56 \pm 6\%$ (Fig. 4 A). The percentage of GalT in the supernatant in the absence of cyclin A ($30 \pm 9\%$) was higher than that observed for interphase HeLa cytosols ($12 \pm 2\%$; Fig. 2 C). This was not a difference in the type of cytosol used but the consequence of increasing the incubation time from 60 to 90 min.

Cyclin A did not activate the histone kinase activity of the FT210 cytosol preincubated at the non-permissive temperature of 39°C , consistent with inactivation of the *cdc2* kinase (Fig. 4 B). There was also no significant fragmentation of the Golgi stacks since the percentage of GalT in the supernatant was almost unchanged ($21 \pm 2\%$ in the absence of cyclin A compared with $23 \pm 3\%$ in its presence) (Fig. 4 A). The capacity to fragment Golgi stacks could be restored to this inactivated cytosol by the addition of purified *cdc2* but not *cdk2* kinase (Fig. 4 C). Similar results were obtained when fragmentation was assayed using electron microscopy (data not shown) and together they confirm the role of the mitotic kinase in the fragmentation process.

Golgi Cisternae Are Converted into Vesicles

The time course of fragmentation was examined both morphologically (Fig. 5) and stereologically (Fig. 6 and Table I). For morphological analysis, single cisternae were defined as membrane profiles with a length more than four times their width, the width being not more than 80 nm, while Golgi stacks were defined as two or more cisternal profiles separated by a gap of no more than 15 nm and overlapping by more than half their length. Before incubation, the Golgi fraction contained $67 \pm 9\%$ of total membrane in the form of single and stacked cisternae (Fig. 6 A). After incubation at 37°C for 60 min, the proportion of cisternal membrane had decreased to $16 \pm 10\%$ in the presence of mitotic cytosol (Fig. 6 A), but only to $52 \pm 11\%$ in the presence of interphase cytosol (Fig. 6 A). The subpopulation of stacked cisternae behaved in the same manner. The percentage of membrane in this population decreased dramatically from $43 \pm 7\%$ to $3 \pm 2\%$ when incubated with mitotic cytosol

A) were absent after mitotic incubation. Contaminating plasma membrane sheets (*asterisks* in C) did not fragment in the presence of mitotic cytosol. Interphase cytosol (B) had little effect on stacked cisternae (*arrows*) and the small vesicular profiles observed were probably transport vesicles (*small arrowheads* in B). In the absence of cytosol (D), in the presence of mitotic cytosol but incubated on ice (E), or with mitotic cytosol depleted of ATP (F), stacks were preserved. Asterisks in F mark tubular networks formed in the absence of ATP. When the concentration of mitotic cytosol was halved (G) fragmentation was also inhibited. All pictures are the same magnification. Bar, $0.5 \mu\text{m}$.

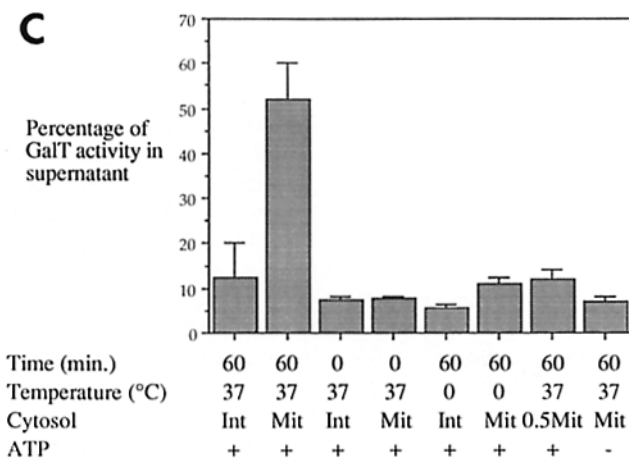
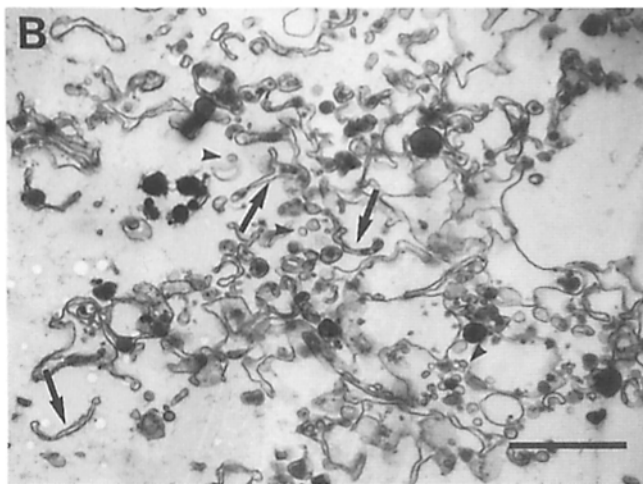
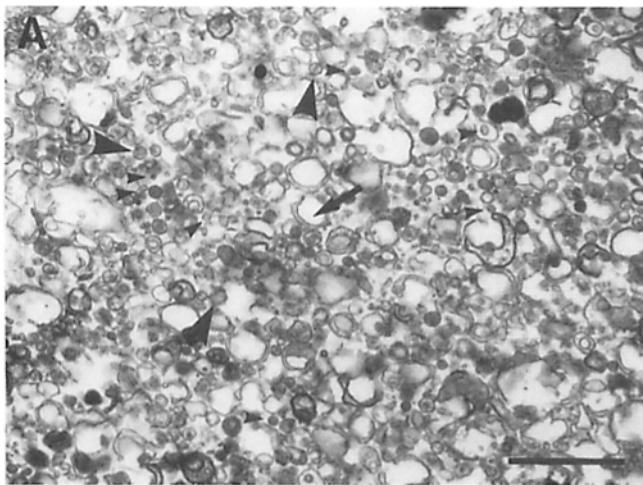


Figure 2. Sedimentability of Golgi membranes after treatment with mitotic or interphase cytosol. (A and B) Golgi stacks were incubated in mitotic cytosol for 60 min at 37°C in the presence of ATP and a regenerating system. After centrifugation at low speed (2,000 g_w for 10 min) to separate vesicles (in the supernatant) from Golgi remnants (in the pellet), the membranes in both the supernatant and pellet were reisolated at higher speed, fixed, and processed for electron microscopy. Note the enrichment of vesicles in the supernatant (*small and large arrowheads* in A) and cisternal remnants in the pellet (*arrows* in B). Some cisternal remnants were visible in the supernatant (*arrow* in A) and some vesicles appeared to be trapped in the pellet (*small arrowheads* in B). Bar, 0.5 μ m.

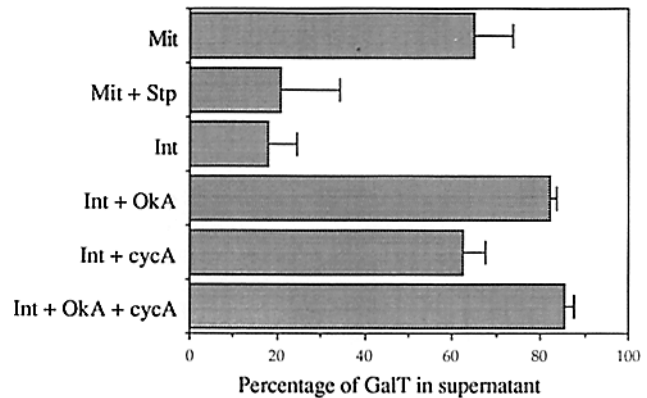


Figure 3. Involvement of kinases and phosphatases in the fragmentation process. Golgi stacks were incubated in mitotic (*Mit*) or interphase (*Int*) cytosol for 60 min at 37°C in the presence of ATP, a regenerating system, and either 10 μ M staurosporine (*Stp*), 1 μ M okadaic acid (*OkA*), 50 μ g/ml cyclin A (*cycA*), or no additions. After centrifugation at low speed to separate vesicles (in the supernatant) from Golgi remnants (in the pellet), both fractions were assayed for GalT activity. Results are presented as the mean of 3–5 experiments \pm SEM.

(Fig. 6 A), but changed only slightly to $37 \pm 12\%$ when incubated with interphase cytosol (Fig. 6 A). The half-time for the loss under mitotic conditions was ~ 20 min for both single and stacked cisternae (Fig. 6, A and C). Single and stacked cisternal profiles (Fig. 5 A) appeared to become shorter over the first 20 min of incubation in mitotic cytosol (Fig. 5 C) and stacks disappeared completely between 20 and 40 min (cf Fig. 5, C and E).

As the cisternal profiles became shorter and disappeared, there was a corresponding increase in the amount of total membrane found in vesicular profiles (Fig. 5, B–F). Before incubation 9 \pm 3% of total membrane was present in a population of small vesicular profiles (circular profiles with a diameter of 50–100 nm) (Fig. 6 B). This increased dramatically to $52 \pm 13\%$ after incubation for 60 min at 37°C with mitotic cytosol (Fig. 6 B and *small arrowheads* in Fig. 5 F), but only to $21 \pm 5\%$ in the presence of interphase cytosol (Fig. 6 B). This increase probably represented the formation of intra-Golgi transport vesicles. A transient population of large vesicular profiles (circular profiles with a diameter of 100–200 nm, *large arrowheads* in Fig. 5 D) also appeared with mitotic cytosol. This population increased from $14 \pm 3\%$ of total membrane at the start of the incubation to $29 \pm 7\%$ at 30 min before decreasing again to $17 \pm 10\%$ at 60 min (Fig. 6 B). Finally, tubular profiles increased 1.7-fold over 60 min of incubation with mitotic cytosol (Table I) though this was not significantly different from the 1.5-fold increase observed in the presence of interphase cytosol (data not shown).

(C) Golgi stacks were incubated under the conditions shown. After centrifugation at low speed to separate vesicles (in the supernatant) from Golgi remnants (in the pellet), both fractions were assayed for GalT activity. Results are presented as the mean of 3–7 experiments \pm SEM. *Int*, interphase cytosol; *Mit*, mitotic cytosol; 0.5 *Mit*, half the normal concentration of mitotic cytosol.

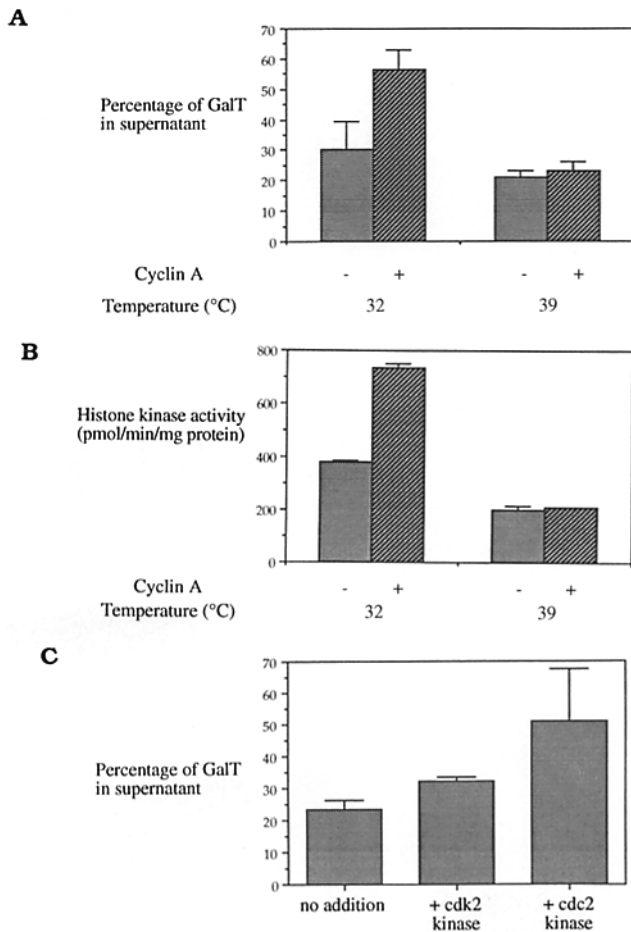


Figure 4. Involvement of *cdc2* kinase in the fragmentation process. (*A* and *B*) Cytosol from FT210 cells was preincubated at the permissive (32°C) or non-permissive (39°C) temperature for 20 min in the presence of ATP and a regenerating system. Incubation was continued at the same temperature for 10 min in the presence or absence of 50 $\mu\text{g/ml}$ cyclin A. After shifting the temperature to 34°C, Golgi membranes were added for an additional 90 min. Samples were centrifuged to separate vesicles (in the supernatant) from Golgi remnants (in the pellet). (*A*) Duplicate samples of both pellet and supernatant were assayed for GalT activity. (*B*) Duplicate samples of the incubation were taken before centrifugation and were assayed for histone H1 kinase activity. Results are presented as the mean of four experiments \pm SEM. (*C*) Cytosol from FT210 cells was preincubated at 39°C for 20 min in the presence of ATP and a regenerating system to inactivate endogenous *cdc2* kinase. Cyclin A (50 $\mu\text{g/ml}$) was then added together with either buffer (no addition), *cdk2* kinase or *cdc2* kinase. The same amounts of *cdc2* and *cdk2* kinase as measured by histone H1 kinase activity were used. After incubation at 39°C for 10 min, the temperature was lowered to 34°C, and rat liver Golgi membranes added for an additional 90 min. After centrifugation at low speed to separate vesicles (in the supernatant) from Golgi remnants (in the pellet), duplicate samples of both pellet and supernatant were assayed for GalT activity. All results are presented as the mean of three experiments \pm SEM.

There was an inverse correlation between the rate of loss of cisternal profiles and the appearance of vesicular profiles (Fig. 6 C). The half-times were identical (~ 20 min) and the loss of total membrane from cisternal profiles (52%) was matched by the increase in large and small vesicular profiles

(45%) (Table I). Taken together these data strongly suggest that vesicular profiles are derived from cisternal profiles.

To confirm the vesicular nature of the vesicular profiles found after 60 min incubation in the presence of mitotic cytosol, serial sectioning was performed at a section thickness close to the diameter of the small vesicles, i.e., ~ 50 nm. 100 vesicular profiles were tracked through five sections starting from the middle section. As shown in Fig. 7, $\sim 60\%$ of the profiles were present in only one or two sections confirming that they originated from vesicles. About 20% of the profiles originated from tubular structures less than 150 nm in length (profiles in three sections) and 10% from structures less than 200 nm in length (profiles in four sections). Only 10% of the profiles originated from structures the length of cisternae (≥ 250 nm).

Vesiculation Occurs in Stacked Cisternae

The loss of membrane from stacks could occur in one of two ways. Cisternae could remain stacked and vesiculate at the edges or they could first unstack and then undergo vesiculation. To distinguish between these two possibilities, the number of cisternae in the stack and the cisternal length were measured during the incubation of Golgi stacks with mitotic or interphase cytosol.

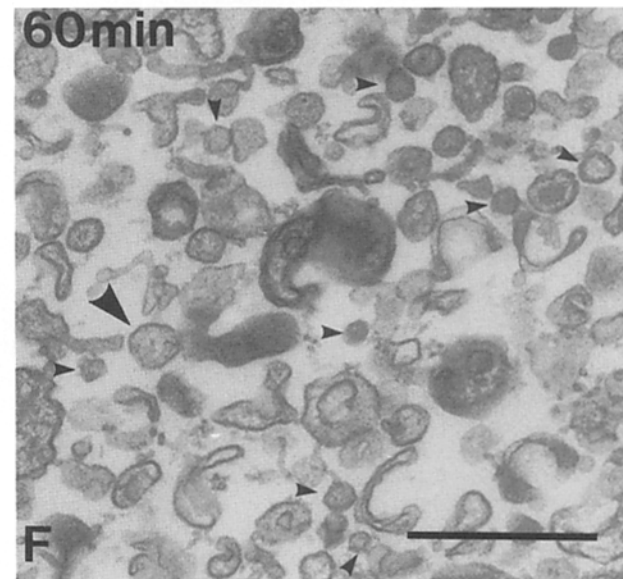
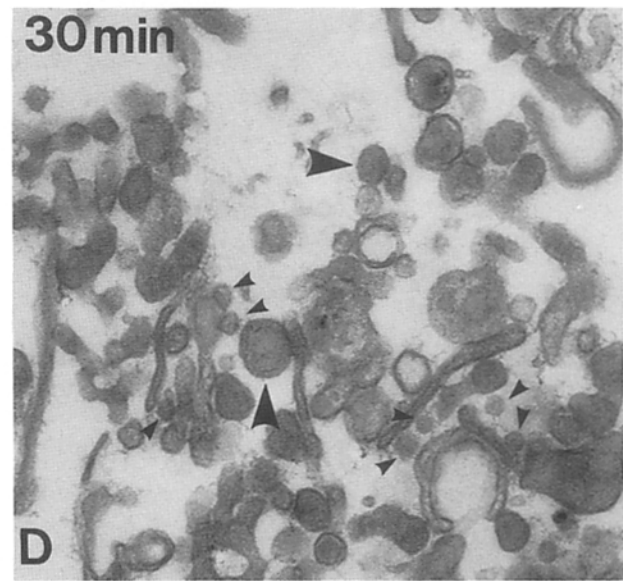
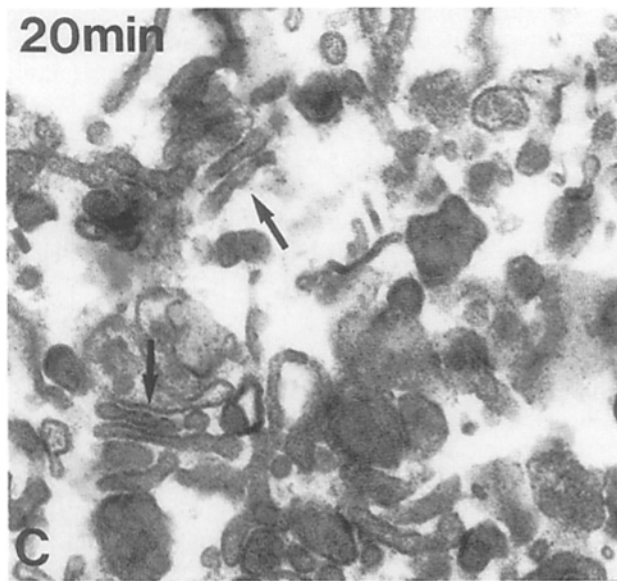
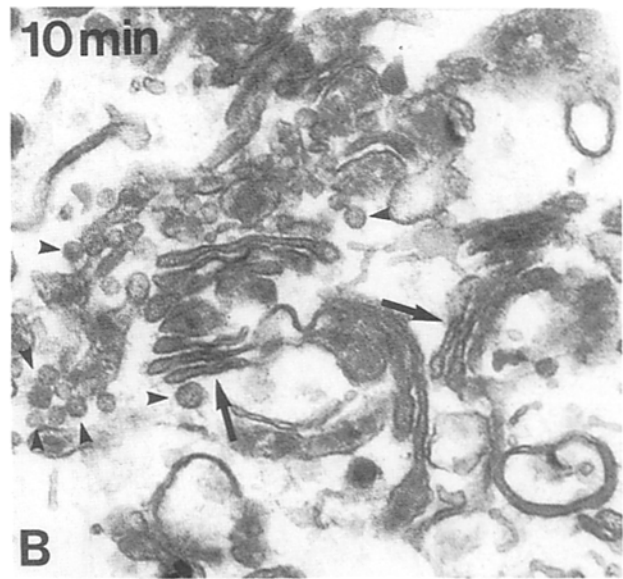
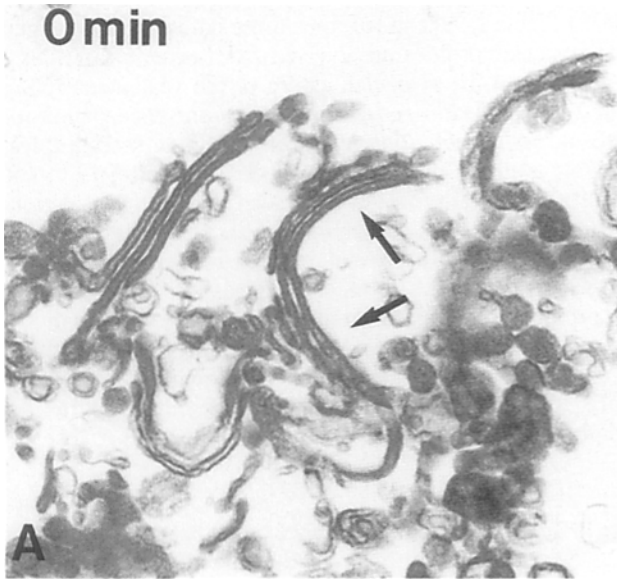
The average number of cisternae in the stack was 2.77 ± 0.81 and this decreased only slightly to 2.52 ± 0.58 after a 30-min incubation with mitotic cytosol (Table II). This decrease of only 9% was in marked contrast to the 49% decrease in average cisternal length (in cross section) from $0.73 \pm 0.30 \mu\text{m}$ to $0.37 \pm 0.14 \mu\text{m}$ (Table II). This change was significant at the $P = 0.0001$ level by the Mann-Whitney test and was not a consequence of membrane swelling since the surface density of Golgi stacks was not affected by the incubation conditions (Table II). The decrease in cisternal length did, however, depend on mitotic cytosol since incubation with interphase cytosol caused a reduction of only 5% in the number of cisternae in the stack and 12% in the average length of the cisternae (Table II). Taken together these data suggest that vesiculation can occur without prior unstacking of cisternae.

COP-coated Vesicles Form in the Presence of Mitotic Cytosol

The small vesicles were very similar in size to Golgi transport vesicles which are formed by a COP-mediated budding mechanism (Orci et al., 1989; Oprins et al., 1993). COP coats are not readily visible in Epon sections so samples after incubation were fixed and processed for cryo-electron microscopy. COP coats were readily visible on many vesicular profiles (*arrowheads* in Fig. 8, *A* and *B*).

Golgi stacks were first incubated in the presence of 20 μM GTP γS so as to prevent uncoating of COP-coated vesicles (Melancon et al., 1987) and allow an estimate of their rate of formation in interphase and mitotic cytosols. After a 20-min incubation, the number of COP-coated vesicles/ μm^2 sectioned Golgi area was 63 ± 23 for interphase and 63 ± 22 for mitotic cytosol (Fig. 8 C). This showed that the budding-rate of COP-coated vesicles was the same in both interphase and mitotic cytosol.

The same incubations were also carried out in the absence of GTP γS and the number of COP-coated vesicles/ μm^2 sec-



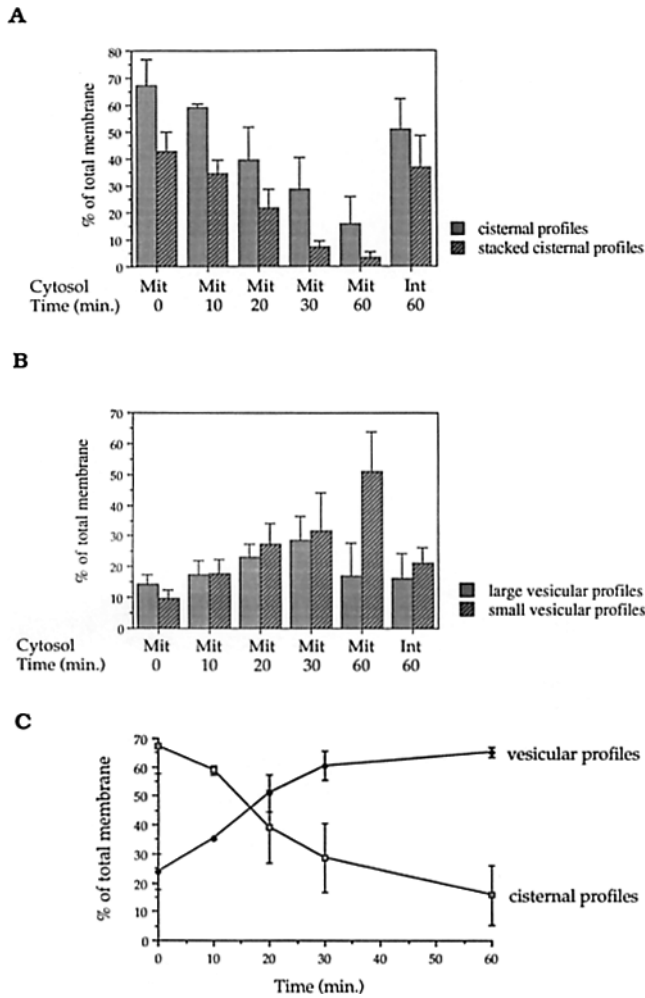


Figure 6. Quantitation of the intermediates during the fragmentation process. Golgi stacks were incubated in mitotic (*Mit*) or interphase (*Int*) cytosol in the presence of ATP and a regenerating system at 37°C for the times indicated, and then fixed and processed for electron microscopy, and quantitated as described in Materials and Methods. (A) The percentage of cisternal and stacked cisternal membrane profiles expressed as a percentage of total membrane. (B) The percentage of small (50–100 nm diam) and large (100–200 nm diam) vesicular profiles expressed as a percentage of total membrane. (C) Time-dependent change in cisternal or vesicular profiles expressed as a percentage of total membrane. Note the inverse correlation. Results are presented as the mean \pm S.D.

Table I. Quantitation of Membrane Intermediates before and after Fragmentation

Percentage of total membrane	Total cisternae	Stacked cisternae	Small vesicles	Large vesicles	Tubules
at 0 min	67.3 \pm 9.5	42.9 \pm 7.2	9.5 \pm 3.0	14.3 \pm 3.2	8.9 \pm 4.0
at 60 min	15.7 \pm 10.3	3.2 \pm 2.0	51.7 \pm 12.7	17.2 \pm 10.5	15.4 \pm 9.0
change	-51.6	-39.7	+42.2	+2.9	+6.5

Summary of the results presented in Fig. 6, A and B at 0 and 60 min of incubation with mitotic cytosol. Values for tubules are the percentage change for each type of membrane.

Figure 5. Morphology of the intermediates during the fragmentation process. Golgi stacks were incubated with mitotic cytosol in the presence of ATP and a regenerating system at 37°C for the times indicated, and then fixed and processed for electron microscopy. Representative fields are shown. Note the decrease in cross-sectional length of the cisternae and stacks (compare *arrows* in A and C) and the loss of stacks between 20 and 40 min. Note the corresponding increase in small (50–100 nm in diameter, *small arrowheads*) and large (100–200 nm in diameter, *large arrowheads*) vesicular profiles over the period of the incubation. Bar, 0.5 μ m.

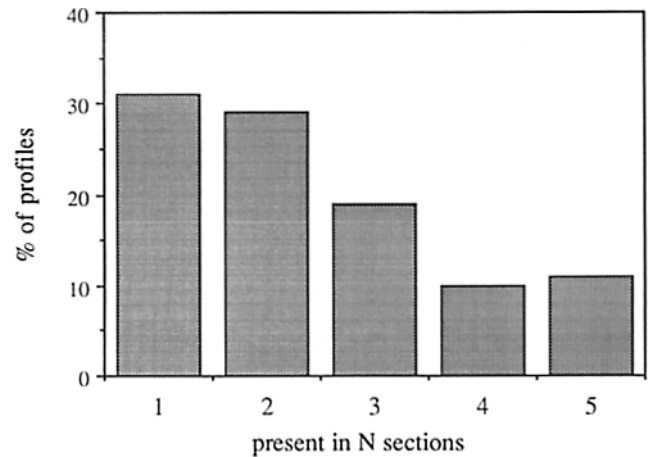


Figure 7. Serial sectioning of vesicular profiles. Golgi stacks were incubated in mitotic cytosol in the presence of ATP and a regenerating system at 37°C for 60 min, and then fixed and processed for electron microscopy. 100 small vesicular profiles were tracked through five serial sections (\sim 50 nm thickness) starting from the middle section and the results are presented as the percentage of total profiles in N sections.

tioned Golgi area was again found to be the same under both conditions (25 ± 2 for interphase, 25 ± 5 for mitotic cytosol) (Fig. 8 C). The difference between these values and those for incubations in the presence of GTP γ S represented the extent of uncoating that would have occurred. Since these differences (38 for interphase, 38 for mitotic cytosol) were the same under both conditions this meant that the uncoating-rate of COP-coated vesicles was the same in both interphase and mitotic cytosol.

The capacity of COP-mediated budding to vesiculate the Golgi was assessed by continuing the incubation in the presence of GTP γ S for a total of 60 min (Fig. 8, A and B). Under mitotic conditions, the population of small vesicles constituted $65 \pm 9\%$ of total membrane and virtually all of the small vesicles were COP-coated (Fig. 8 B). Incubation with interphase cytosol in the presence of GTP γ S had far less effect on Golgi stacks (Fig. 8 A). Though almost all of the small vesicles were COP-coated, quantitation showed that they constituted only $37 \pm 11\%$ of total membrane.

Mitotic Fragmentation Requires Coatamer

To test directly for an involvement of COP-mediated budding

Table II. Change in the Number of Stacked Cisternae and Cisternal Length during Incubation with Interphase or Mitotic Cytosol

	Number of cisternae in stack	Cisternal length (μm)	Surface density
0 min	2.77 \pm 0.81	0.73 \pm 0.30	4.13
Mitotic cytosol			
10 min	2.96 \pm 1.06	0.41 \pm 0.17	3.94
Mitotic cytosol			
30 min	2.52 \pm 0.58	0.37 \pm 0.14	3.56
Interphase cytosol			
30 min	2.62 \pm 1.00	0.64 \pm 0.32	3.71

Golgi stacks were incubated in mitotic or interphase cytosol in the presence of ATP and a regenerating system at 37°C for the times indicated. Membranes were collected by centrifugation and processed for electron microscopy. The parameters were determined as described in Materials and Methods, and the results are presented as the mean \pm SD.

in the fragmentation process, mitotic and interphase cytosols were depleted of one of the COP subunits, coatamer, using the CMLA10 antibody (Orci et al., 1993), coupled to protein-G beads. Immunodepletion was monitored by Western blotting using the monoclonal antibody p36, directed against one of the proteins in the coatamer complex, ϵ -COP (Kuge et al., 1994).

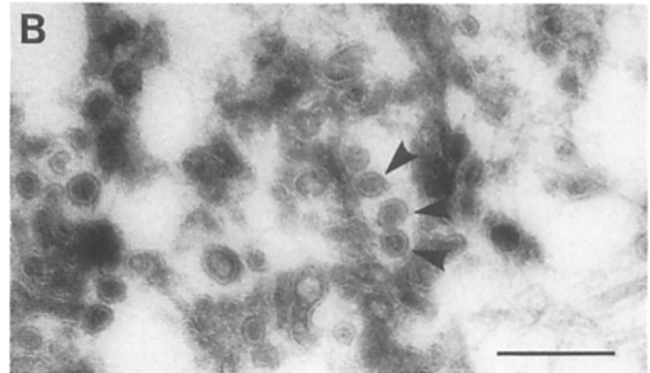
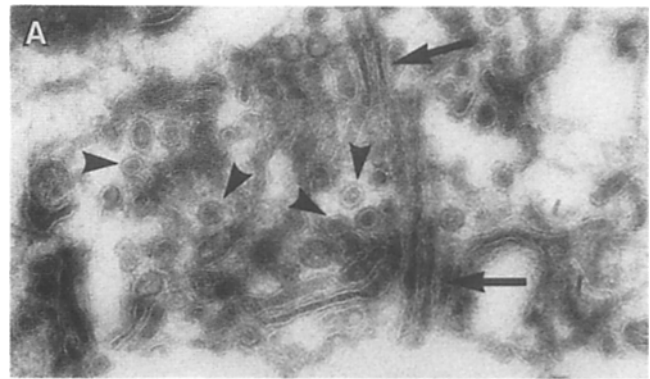
As can be seen in Fig. 9 A, ϵ -COP was present in mitotic and interphase cytosol before immuno-depletion but not after. Quantitation showed that 90–95% of the ϵ -COP (and hence coatamer) had been removed. Coatamer was not removed using protein-G beads alone. Golgi stacks were not fragmented by coatamer-depleted mitotic cytosol (Fig. 9 B). Instead, the cisternae appeared to become unstacked and extensive tubular networks were formed. This effect was reversed by the re-addition of purified coatamer which led to almost complete vesiculation of Golgi stacks (Fig. 9 C). Similar effects were noted using coatamer-depleted interphase cytosol though the fenestrated networks were more compact and the diameter of the tubules appeared to be larger (Fig 9 D). Again, this effect was reversed by the re-addition of purified coatamer which led to single and stacked cisternae at the end of the incubation (Fig. 9 E).

Discussion

Fragmentation of Golgi Stacks in a Cell-free System

We have described a cell-free system in which rat liver Golgi stacks fragment when incubated with mitotic but not interphase cytosol. Earlier we developed a cell-free assay (Stuart et al., 1993) that mimicked the inhibition of intra-Golgi transport that we had observed in vivo (Collins and Warren, 1992). This assay was closely based on that described by Balch et al. (1984) for the vesicle-mediated transfer of the VSV G protein from *cis* to *medial* cisternae. We found, however, that these conditions were not sufficient to fragment Golgi stacks, and two modifications had to be introduced.

The first modification was to change the buffers and salts to those used by Nagakawa et al. (1988) for the disassembly of isolated chicken nuclei. The most important of these new components were β -glycerophosphate and EGTA, both introduced in earlier nuclear disassembly assays (Newport and Spann, 1987) as general phosphatase inhibitors needed to



C

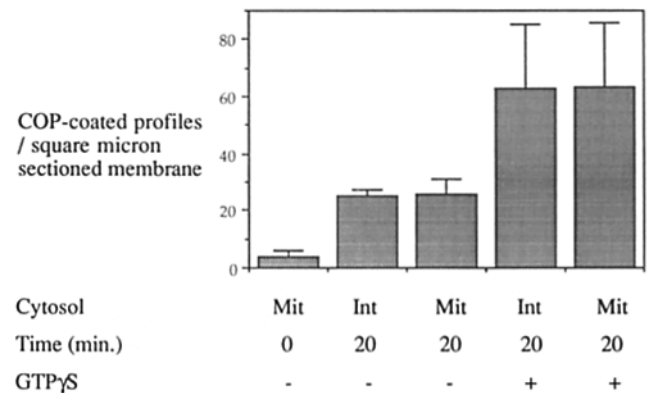


Figure 8. Quantitation of COP-coated vesicles. (A and B) Golgi stacks were incubated in interphase (A) or mitotic (B) cytosol in the presence of 20 μM GTP- γ S, ATP, and a regenerating system at 37°C for 60 min. After fixation, the samples were processed for cryo-electron microscopy. Note that virtually all of the small vesicular profiles are coated (arrowheads) and that stacked cisternal profiles (arrows in A) only remain in the interphase not the mitotic incubation. (C) Similar incubations in interphase (Int) or mitotic (Mit) cytosols were carried out for different times in the presence or absence of 20 μM GTP- γ S and the number of COP-coated vesicles and buds counted/square micron of sectioned membrane. The results presented are the mean of three experiments \pm SEM.

preserve the mitotic state of the cytosol (Maller et al., 1977; Nigg, 1991).

The second modification was to increase the ratio of cytosol to membrane protein from 5:1 (for the transport assay) to 100:1, closer to the ratio in the cell and similar to the

A

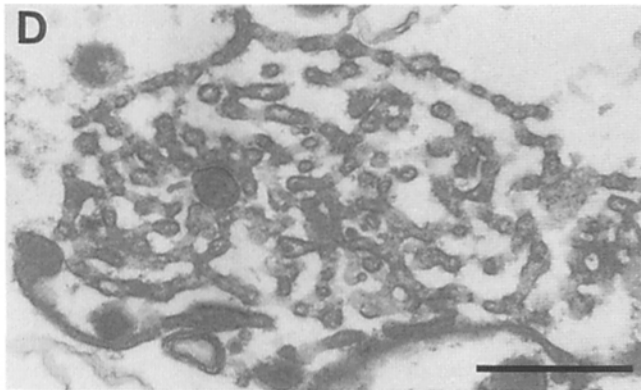
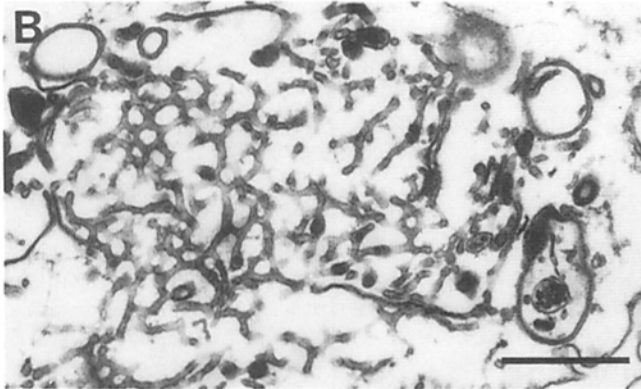
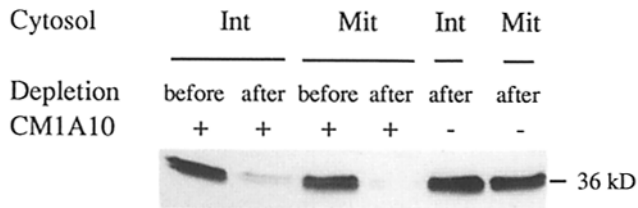
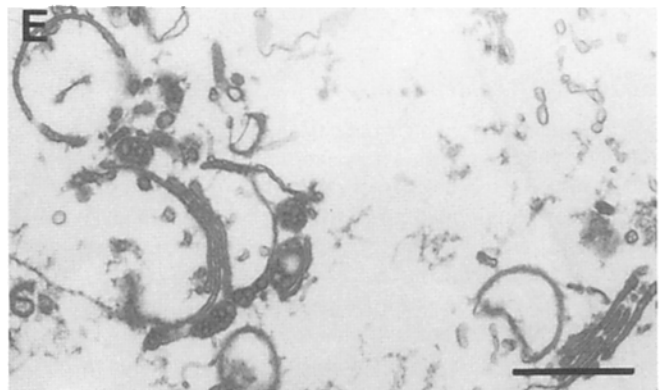
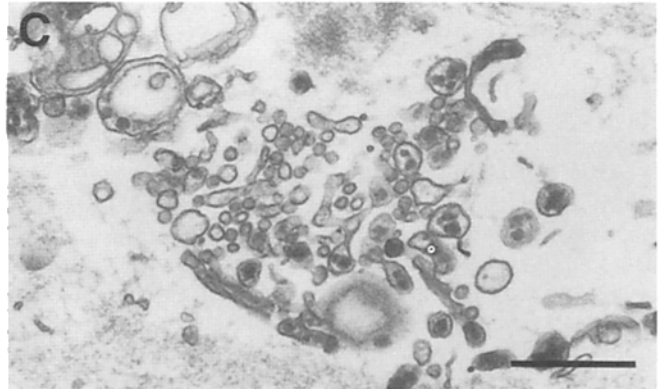


Figure 9. Effect of cytosols depleted of coatomer on the fragmentation process. (A) Interphase (*Int*) or mitotic (*Mit*) cytosols were incubated with either protein-G beads coupled to the CM1A10 anti-coatomer antibody or Protein-G beads alone. After removal of the beads, 50 μ g cytosol was fractionated by SDS-PAGE using a 15% gel, Western blotted, and probed with antibody against coatomer component ϵ -COP (p36). (B–E) Golgi stacks were incubated in the presence of ATP and a regenerating system at 37°C for 60 min in either (B) mitotic cytosol depleted of coatomer, (C) depleted mitotic cytosol supplemented with purified coatomer (to 52 μ g/ml), (D) interphase cytosol depleted of coatomer or (E) depleted interphase cytosol supplemented with purified coatomer (to 52 μ g/ml). Samples were then fixed and processed for electron microscopy. Note that the depleted mitotic cytosol generated highly fenestrated networks and no vesicles, while fragmentation activity was restored after supplementing the depleted cytosol with coatomer. Bar, 1 μ m.



ratios that were found to be necessary for disassembly of the nuclear envelope (Newport and Spann, 1987).

The need for these two modifications shows that there are differences between fragmentation and the inhibition of intra-Golgi transport, a point we shall return to later. For the moment the most important point is that these modifications did not affect the budding rate of COP-coated vesicles. In the presence of interphase cytosol and GTP γ S, 3.2 ± 1.1 COP-coated vesicles and buds were formed/min/ μ m² sectioned Golgi area. In comparison, we can calculate that in the intra-Golgi assay developed by Balch et al. (1984), 2.7 ± 0.1 COP-coated vesicles and buds were formed/min/ μ m² sectioned Golgi area when GTP γ S was present (Melancon et al., 1987). Both values also compare very well with the 3.0 ± 0.2 COP-coated vesicles and buds formed/min/ μ m² sectioned Golgi area measured in permeabilized CHO cells treated with GTP γ S (Orci et al., 1989).

Several lines of evidence strongly suggest that the fragmentation observed both by electron microscopy and the centrifugation assay mimics that observed *in vivo* during prometaphase, metaphase, and anaphase.

First, Golgi stacks underwent fragmentation in the presence of mitotic but not interphase cytosol, and did so in a manner dependent on time, temperature, and the presence of energy which was added in the form of ATP. Second, the kinetics of the fragmentation process compared well with that observed *in vivo*. The half-time for fragmentation in the cell-free system was 20–25 min (Fig. 6 C) and extensive fragmentation had occurred by 40 min (Fig. 5 E). This is the time taken for HeLa cells to traverse prometaphase, metaphase, and anaphase (Zieve et al., 1980). Third, there were three clearly defined end products of fragmentation in the cell-free system: small vesicular profiles (50–100 nm in diameter), large vesicular profiles (100–200 nm in di-

ameter), and short tubules. Each was found in mitotic cells (Lucocq et al., 1987) and the average diameter of the small vesicles (54 ± 4 nm) was very similar to that for the small vesicles found in mitotic Golgi clusters and free in the cytoplasm (47 ± 2.3 nm, Lucocq et al., 1987). Fourth, the observed fragmentation depended on the action of cdc2 kinase which initiates entry of cells into mitosis (Nurse, 1990). Fragmentation was inhibited by staurosporine, a general kinase inhibitor that also inhibits cdc2 kinase and was activated by the addition of recombinant cyclin A to interphase cytosols. Cyclin A complexes with, and activates, both cdc2 kinase and cdk2 kinase, the S phase kinase. Cdc2 kinase was shown to be responsible for the observed fragmentation by pretreating the cytosol from FT210 cells at 39°C, a temperature that destroys the temperature-sensitive cdc2 kinase in this cell line. Fragmentation was inhibited by heat pretreatment and could be restored by adding purified cdc2 but not cdk2 kinase. Fifth and last, fragmentation occurred in the presence of OkA, which, when added to HeLa cells, appears to mimic the mitotic disassembly of the Golgi apparatus (Lucocq et al., 1991). This kinase was not cdc2 kinase since there was no increase in the histone kinase activity above background levels either in vitro (data not shown) or in vivo (Lucocq et al., 1991). This suggests either that the kinase is one acting downstream of cdc2 kinase or is an independent kinase capable of acting on the same targets as cdc2 kinase. Similar suggestions have been made for transport between endocytic vesicles and endosomes (Woodman et al., 1992) and between ER and the Golgi stack (Davidson et al., 1992). Additional work will be needed to distinguish between these two possibilities.

Role of COP-coated Vesicles

Several lines of evidence indicate the involvement of COP-mediated budding in the fragmentation of Golgi stacks. First, the COP-mediated mechanism had the capacity to cope with the flux of membrane from cisternae observed during fragmentation. As measured on Epon sections, small vesicles were produced under mitotic conditions at the rate of $2.5 \pm 0.5/\text{min}/\mu\text{m}^2$ sectioned Golgi area, which is less than the 3.2 ± 1.1 COP-coated vesicles and buds formed/ $\text{min}/\mu\text{m}^2$ sectioned Golgi area in the presence of GTP γ S in either interphase or mitotic cytosol. Second, the COP-mediated budding mechanism has the capacity to fragment most of the added Golgi stacks. Continued incubation in the presence of GTP γ S resulted in more than 60% of the total membrane being present within COP-coated vesicles. Third and most convincing, removal of coatomer from mitotic cytosol, by immunodepletion, completely abolished fragmentation. Re-addition of purified coatomer restored fragmentation activity.

Continued budding of COP-coated vesicles which can uncoat but cannot fuse would best explain the observed fragmentation. The number of COP-coated vesicles and buds formed/ $\text{min}/\mu\text{m}^2$ sectioned Golgi area in the presence of GTP γ S was the same for both interphase (3.2 ± 1.1) and mitotic (3.2 ± 1.2) cytosol showing that the COP-mediated mechanism was neither activated nor inhibited under mitotic conditions. Uncoating was measured by comparing the rate of formation of COP-coated buds and vesicles in the presence and absence of GTP γ S. The difference represents the number of vesicles that would have uncoated during the incubation period. This number was the same for both interphase

and mitotic, suggesting that the site of inhibition occurs after uncoating. Since uncoating seems to take place after the vesicle has docked with the target membrane (Orci et al., 1989), proteins involved in fusion are the most likely components for inactivation (Söllner et al., 1993). Additional work will be needed to pin-point the exact components involved.

Fragmentation occurs without prior unstacking of cisternae. The observed reduction in the cross-sectional length of cisternae was $\sim 50\%$ over the first 30 min of incubation with mitotic cytosol. The reduction with interphase cytosol was only 9% suggesting that mitotic cytosol contains factors that enable COP-coated vesicles to invade the stacked cisternal membranes so as to permit continued budding without prior unstacking. They most likely act to disassemble the matrix that stacks the cisternae (Cluett and Brown, 1992; Slusarewicz et al., 1994b). These factors would not be needed to observe an inhibition of intra-Golgi transport which would help explain the different requirements of the present fragmentation assay and that described earlier to study mitotic inhibition of transport (Stuart et al., 1993). Additional work will be needed to identify and characterize these factors.

Tubular Networks in the Absence of Coatomer

Immunodepletion of coatomer from mitotic cytosol provided conclusive evidence for the involvement of COP-mediated budding in the fragmentation process. The stacked cisternae were, however, converted into extensive tubular networks (Fig. 9 B), similar to the networks formed using depleted interphase cytosol (Fig. 9 D).

It has recently been shown that, in the absence of coatomer, fusion still occurs but is no longer coupled to vesicle budding (Elazar et al., 1994). Golgi stacks fuse with each other, mostly at the cisternal rims (Elazar et al., 1994), though the extent was much less than that reported here (Fig. 9 D). This might be due to different conditions of incubation or to different levels of residual coatomer. We were able to deplete cytosol of 90–95% of coatomer whereas, Orci et al. (1993), using more similar conditions to those reported here, found even 85% depletion gave little evidence of cisternal fusion. Consistent with this interpretation is the observation that brefeldin A (BFA), which almost completely prevents the binding of coatomer to Golgi membranes (Donaldson et al., 1990; Helms et al., 1992; Donaldson et al., 1992) results in the formation of extensive tubular networks very similar to those reported here (Orci et al., 1991).

The formation of such networks depends on uncoupled fusion yet they form in the presence of coatomer-depleted mitotic cytosol. Several lines of evidence suggest that fusion does not occur under mitotic conditions. In mitotic cells from a mast cell line, secretory granules are unable to fuse with the plasma membrane (Hesketh et al., 1984). In vitro, endocytic vesicles will not undergo homotypic fusion in the presence of mitotic cytosol or active cdc2 kinase (Tuomikoski et al., 1989; Woodman et al., 1993). In this paper we have shown that the small vesicles produced by the COP-mediated budding mechanism are the major products of fragmentation, consistent with an inhibition of fusion. Furthermore, we have measured the transport of VSV G protein under these conditions and it was found to be inhibited by more than 90% (data not shown). It could be argued that depletion of coatomer from mitotic cytosol reactivates the fusion process. This seems unlikely since addition of BFA, to prevent

binding of coatomer to Golgi membranes, did not restore fusion activity to mitotic cytosol as measured using the VSV G protein (Mackay, D., and G. Warren, unpublished results).

If the fusion process is inhibited, how then can the networks in depleted mitotic cytosol be explained? One possibility comes from careful examination of the networks formed under mitotic conditions in the absence of coatomer (Fig. 9 B). There was no evidence of central portions of cisternae with few, if any, fenestrations. It was as if all cisternae had become highly fenestrated, a possibility that could be explained by periplasmic fusion (Rothman and Warren, 1994).

Periplasmic fusion is needed to complete the budding of COP-coated transport vesicles. COP coats deform the membrane and palmitoyl-CoA is needed for the final pinching off (Ostermann et al., 1993) which involves fusion between the non-cytoplasmic (periplasmic) surfaces of the cisterna. Periplasmic fusion differs from cytoplasmic fusion events in that specific targeting of membranes is not required. The only two membranes that can fuse are those within the same compartment. It may also be triggered simply by the close apposition of periplasmic surfaces. Though this would normally occur only when mechanical devices such as COP coats deform the membrane, it might also occur spontaneously. If the membranes on the opposite side of a cisterna were to touch, the result would be a fenestration. This would normally be prevented by homotypic fusion between membrane surrounding the fenestration and by the matrix that stacks Golgi cisternae which would prevent opposite sides touching. During mitosis, however, inhibition of homotypic fusion would remove the first constraint on periplasmic fusion and disassembly of the matrix the second. Continued periplasmic fusion would then lead to extensive fenestration generating the highly tubular networks that were observed (Fig. 9 B).

Of course, the ultimate end point of periplasmic fusion would be small membrane fragments. This would help explain the breakdown of the nuclear envelope and ER at the onset of mitosis as well as the first stage of Golgi division, the severing of the tubules linking the Golgi stacks (Rothman and Warren, 1994). It might also explain some of the observed products of Golgi fragmentation including the larger vesicles. Additional work using the cell-free system described here should help us explore this possibility. It should also help us identify those factors that permit the COP-mediated budding mechanism access to membrane in the central regions of Golgi stacks.

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