

Reassembly of Golgi Stacks from Mitotic Golgi Fragments in a Cell-free System

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Abstract. Rat liver Golgi stacks were incubated with mitotic cytosol for 30 min at 37°C to generate mitotic Golgi fragments comprising vesicles, tubules, and cisternal remnants. These were isolated and further incubated with rat liver cytosol for 60 min. The earliest intermediate observed by electron microscopy was a single, curved cisterna with tubular networks fused to the cisternal rims. Elongation of this cisterna was accompanied by stacking and further growth at the cisternal rims. Stacks also fused laterally so that the typical end product was a highly curved stack of 2–3 cisternae mostly enclosing an electron-lucent space. Reassembly occurred in the presence of nocodazole or cytochalasin B but not at 4°C or in the absence of

energy supplied in the form of ATP and GTP. Pretreatment of the mitotic fragments and cytosol with *N*-ethylmaleimide (NEM) also prevented reassembly. GTP γ S and AIF prevented reassembly when added during fragmentation but not when added to the reassembly mixture. In fact, GTP γ S stimulated reassembly such that all cisternae were stacked at the end of the incubation and comprised 40% of the total membrane. In contrast, microcystin inhibited stacking so that only single cisternae accumulated. Together these results provide a detailed picture of the reassembly process and open up the study of the architecture of the Golgi apparatus to a combined morphological and biochemical analysis.

THE Golgi apparatus has a complex architecture. The central feature is the stack of cisternae, each comprising a flattened central portion that is closely apposed to adjacent cisternae in the stack, and a peripheral rim that is often fenestrated and the site of both the budding and fusion of coat protomer (COP)-coated transport vesicles (Weidman et al., 1993). Each face of the stack is apposed to an extensive tubular reticulum termed the *cis*- and *trans*-Golgi networks (Griffiths and Simons, 1986; Hauri and Schweizer, 1992). There are typically several hundred Golgi apparatus in animal cells (Lucocq and Warren, 1987) but they are concentrated in the pericentriolar region by the action of microtubule motors (Corthésy-Theulaz et al., 1992). They become linked together laterally by tubules which connect equivalent cisternae in adjacent stacks generating a bifurcating, ribbon-like structure (Lucocq and Warren, 1987; Rambourg and Clermont, 1990).

The proteins that determine the size and shape of this organelle are unknown, in part because it has been difficult to apply biochemical techniques to take it apart. The available tools are simply too crude. An alternative approach, and the one we have been pursuing, is to exploit the fact that animal

cells divide the Golgi ribbon during mitosis by taking it completely apart and putting it back together again in each daughter cell (Warren, 1993).

At the onset of mitosis, the Golgi ribbon is broken down into discrete stacks (Colman et al., 1985) which undergo fragmentation to yield clusters of vesicles, small tubules, and cisternal remnants (Lucocq et al., 1987). The vesicles are generated by the budding of COP-coated transport vesicles that uncoat but cannot then fuse with the next cisterna in the stack (Misteli and Warren, 1994). This inhibition of fusion is part of a general shutdown of membrane traffic during mitosis in animal cells (Warren, 1993) that, at the level of the Golgi apparatus, leads to vesiculation of the peripheral cisternal rims and part of the central core regions. Most of the core regions, however, appear to fragment by a different pathway involving tubular networks that break down to form the tubules seen in mitotic Golgi clusters (Lucocq et al., 1987; T. Misteli and G. Warren, unpublished results). The mechanism is unknown but may reflect the periplasmic fusion triggered by the breakdown of scaffolds that maintain cisternal structure (Rothman and Warren, 1994). During prometaphase and metaphase, the clusters shed vesicles and tubules that become randomly distributed throughout the mitotic cell cytoplasm (Lucocq and Warren, 1987). Complete fragmentation of the Golgi ribbon in HeLa cells could theoretically generate up to 10,000 fragments (Lucocq et al., 1989), sufficient to ensure accurate partitioning between daughter cells by a stochastic process (Birky, 1983).

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1. *Abbreviation used in this paper:* COP, coat protomer.

Reassembly occurs during telophase within minutes of a fall in the histone kinase activity of the mitotic kinase p34^{cdc2} (Souter et al., 1993). Several hundred clusters grow by accretion of free vesicles that fuse to form stacked cisternae (Lucocq et al., 1989), a process that only takes ~10 min (Souter et al., 1993). These discrete Golgi apparatus then move to the pericentriolar region, most likely along microtubules (Ho et al., 1989), where they congregate and fuse to form the interphase Golgi ribbon (Lucocq et al., 1989).

From the point of view of a biochemist, fragmentation represents homogenization with the added advantage that it occurs in such a way as to permit reassembly of the original organelle. If it were possible to take this homogenate and mimic the reassembly in the test tube, it would eventually be possible to identify those components underlying the structure of the organelle. We have already devised a system to mimic mitotic fragmentation in the test tube (Misteli and Warren, 1994). Here we show that these mitotic fragments, when incubated in interphase cytosol, will reassemble Golgi stacks.

Materials and Methods

Materials

All reagents were of analytical grade or higher and purchased from Sigma Immunochemicals (St. Louis, MO) or BDH (Poole, Dorset, UK) unless otherwise stated.

Golgi Membranes

Rat liver Golgi membranes were prepared as described by Slusarewicz et al. (1994a) and assayed for GalT activity as described by Bretz and Stäubli (1977). Purification ranged from 100 to 130 times over homogenate and the membranes were stored at -80°C at a concentration of 1–1.5 mg/ml.

Mitotic and Interphase Cytosols

Mitotic cytosol from HeLa cells was prepared as described by Stuart et al. (1993) in the absence of either microcystin or okadaic acid. Aliquots were frozen in liquid nitrogen and stored at -80°C . Batches (four to five preparations) were thawed and desalted using P6-DG spin columns into MEB buffer (50 mM Tris-HCl, 50 mM KCl, 20 mM β -glycerophosphate, 10 mM MgCl_2 , and 15 mM EGTA, adjusted to pH 7.3) containing 2 mM ATP and 1 mM glutathione. Aliquots were frozen in liquid nitrogen and stored at -80°C until needed. The protein concentration was typically 10 mg/ml.

Interphase cytosol was prepared as a byproduct of the preparation of rat liver Golgi stacks. After centrifugation of the homogenate, the 0.5 M sucrose load (containing the cytosol and hemoglobin) was centrifuged at 45 Krpm (275 Kg_{av}) for 60 min at 4°C in the Ti50.2 rotor. Ammonium sulphate was added to the supernatant to 40% saturation and, after 30 min stirring at 4°C , the mixture was centrifuged at 8.5 Krpm for 30 min at 4°C in a centrifuge (Beckman J2 21; Beckman Instrs., Inc., Fullerton, CA). The pellet (which now lacked hemoglobin) was resuspended in KHM buffer (25 mM Hepes, pH 7.35, 25 mM magnesium acetate, and 25 mM KCl) and desalted (either immediately or after storage at -80°C) in the same buffer containing 2 mM ATP, 1 mM GTP, and 1 mM glutathione using spin columns as described above. Aliquots were frozen in liquid nitrogen and stored at -80°C .

Fragmentation Assay

For five assays, 800 μl of mitotic cytosol was supplemented with 80 μl of a regenerating system (comprising 200 mM creatine phosphate, 10 mM ATP, 2 mg/ml creatine kinase and containing 0.2 mg/ml cytochalasin B) and 80 μl 2 M sucrose. Golgi membranes were then added (usually 80 μl keeping the cytosol to membrane protein ratio at 100:1) and the mixture incubated for 30 min at 37°C . 200- μl aliquots were transferred to new tubes followed by 3–5 μl 2 M sucrose to act as a cushion. The samples were then centrifuged in the cold room at 15 Krpm (13.1 Kg_{av}) for 15 min in the

horizontal rotor of the Eppendorf centrifuge. The supernatant was carefully removed and discarded.

Reassembly Assay

Rat liver cytosol was desalted once more just before use into KHM buffer containing 2 mM ATP, 1 mM GTP, and 1 mM glutathione. The reisolated membranes were resuspended using 9 μl of this cytosol (5–40 mg/ml) mixed with 1 μl of the regenerating system. Incubations were carried out for up to 60 min at 37°C . The reaction was stopped by the addition of an equal volume of 1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for at least 30 min at room temperature.

Electron Microscopy

The fixed samples were processed and embedded in Epon 812 using standard procedures and ultra-thin sections (50–70 nm) were cut using an ultramicrotome 2E (Reichert Jung, Vienna). Sections were always cut at the same point in the pellet. The homogeneity of the pellet was checked for critical experiments by systematic sectioning through the entire pellet. Serial sections were cut to a thickness of 150 nm. Sections were stained with 2% uranyl acetate and lead citrate and then viewed using an electron microscope (CM10; Philips Electronics Instrs. Co., Mahwah, NJ).

Stereology

Stacked cisternae were defined as two (or more) cisternae that were separated by no more than 15 nm and overlapped by more than 50% of their length. Cisternae ranged from continuous to extensively fenestrated. They had profiles with a length greater than four times their width, the latter not exceeding 30 nm. Fenestrated cisternae were often wider and more translucent but could be distinguished from tubules by the fenestration. Tubules were defined as profiles with a length more than 1.5 times their width, the latter exceeding 30 nm. They were more undulating than cisternae and when they were branched they formed networks. Vesicles had spherical or nearly spherical (length less than 1.5 times their width) profiles and were typically 50–200 nm in diameter. Lipoprotein particles were easily identified by their content and were counted separately when the profiles were not attached to cisternae, tubules, or networks. All other membranes including contaminants such as the plasma membrane were classified as "rest" and generally constituted 5–15% of total membrane.

The relative proportion of each category of membrane was determined as described previously (Misteli and Warren, 1994) except that the final magnification of the pictures was 38.7 K and the space between the lines was 1 or 2 cm depending on the size of the structure being measured.

The length of each cisternal profile was measured by the intersection method (Weibel, 1979) using 0.5 cm grid and placed in one of the following categories: 0–0.24 μm , 0.25–0.49 μm . . . $n-(n+0.24)$ μm . The number in the lowest category was then expressed as a percentage of the total number and plotted against the upper category value. The next number was the sum of the lowest and the next lowest category and was expressed in the same way. This operation was repeated until the last category was reached when the cumulative frequency of length reached 100%. This method is very sensitive to variations in length between different samples and allows the median cisternal length to be extracted from the 50% cumulative value.

Treatments

Incubations were depleted of ATP (and GTP) by addition of hexokinase and glucose (Smythe et al., 1989). Nocodazole was diluted to a final concentration of 20 $\mu\text{g}/\text{ml}$ from a 2 mM stock in DMSO (Zieve et al., 1980). GTP γ S was added to a final concentration of 20 μM (Misteli and Warren, 1994). AIF was generated by adding NaF to 30 mM followed immediately by AlCl_3 to 50 μM (Oprins et al., 1993). Microcystin was diluted to a final concentration of 10 μM from a 1 mM stock in methanol (MacIntosh et al., 1990).

Complete incubations were treated either with a final concentration of 1 mM *N*-ethylmaleimide (NEM) on ice for 15 min before quenching with 2 mM DTT or with a mixture of 1 mM NEM/2 mM DTT (Block et al., 1988).

Results

Mitotic Golgi Fragments

Rat liver Golgi stacks were initially treated with mitotic

cytosol as described previously (Misteli and Warren, 1994). The cytosol was prepared from spinner HeLa cells that had been arrested for one cell cycle (24 h) in the antimicrotubule agent, nocodazole (Zieve et al., 1980; Stuart et al., 1993). The prometaphase cells were broken using a ball bearing homogenizer, in the presence of a cocktail of phosphatase inhibitors, including EGTA, β -glycerophosphate, and microcystin, and used to treat Golgi membranes for 60 min at 37°C in the presence of ATP and a regenerating system.

It soon became clear that the mitotic membranes reisolated on a sucrose cushion after this treatment could not reassemble when incubated with interphase cytosol and two changes were found to be necessary. The first was to reduce the time of incubation from 60 to 30 min. This permitted substantial but not complete fragmentation of the Golgi stacks. The second was to omit microcystin from the buffer in which the mitotic cells were broken. Omission of this specific inhibitor of protein phosphatases 1 and 2A (Cohen and Cohen, 1989; MacIntosh et al., 1990) did not significantly affect the kinetics or extent of fragmentation over the first 30 min of incubation.

Highly purified Golgi stacks are shown in Fig. 1 A. Typically they contained 2–3 cisternae and quantitation showed that 56% of the total membrane was present as cisternae, more than 65% of which were present in stacks. After 30-min incubation in mitotic cytosol lacking microcystin there was an almost complete disappearance of stacked cisternae (Fig. 1 B), the percentage falling from 36% of total membrane to 2% (Fig. 2). The percentage of membrane in all cisternae (both single and stacked) fell by 34% and this was matched by a 13% increase in vesicular profiles (Fig. 1 B, *closed arrowheads*) and a 25% increase in tubules (Fig. 1 B, *open arrowheads*). Tubules represent a new category of membrane that arose out of the realization that tubular networks (Fig. 1 B, *open arrows*) are intermediates on the fragmentation pathway (T. Misteli and G. Warren, unpublished results). They were originally classified as cisternae but can be distinguished by their greater width and morphology (see Materials and Methods for definitions).

The cross-sectional length of cisterna also decreased during the mitotic incubation as cisternae were converted to cisternal remnants. When plotted as a cumulative frequency, the median length of all cisternae (both single and stacked) fell from 0.71 to 0.48 μm after 30 min of incubation (Fig. 3). This 32% decrease in length is reasonably similar to the 49% decrease observed previously using a different stereological procedure (Misteli and Warren, 1994).

Cisternae, vesicular profiles, and tubules constituted 80% of the total membrane with unattached lipoprotein particles (LP), and unidentified membranes and identifiable contaminants such as the plasma membrane (rest) making up the remainder. The amounts of membrane in these two latter categories did not change significantly during the incubation (Fig. 2).

Reassembled Golgi Stacks

Mitotic Golgi fragments were isolated on a sucrose cushion and reincubated with ATP and GTP in the presence of a regenerating system and interphase cytosol. The volume of rat liver cytosol used was critical. The mitotic membranes isolated from a 200- μl incubation had to be resuspended in 10 μl or less. A volume of 25 μl decreased the percentage

of reassembled stacks by 50%. The concentration of protein in the cytosol was less important. Concentrations ranging from 5 to 40 mg protein/ml were equally efficacious.

Rat liver cytosol was used in preference to HeLa interphase cytosol because large amounts could easily be prepared and the percentage of reassembled stacks was higher. The buffer used for fragmentation did not permit reassembly. It was replaced by one lacking the phosphatase inhibitors EGTA and β -glycerophosphate.

Golgi stacks reassembled after a 60-min incubation at 37°C (see Fig. 1 D and 6 A). There were typically two to three cisternae in the reassembled stacks (see Fig. 6 A), though a minority had four to five cisternae (see Figs. 1 D and 5 E) and a few even had six (see Fig. 5 D). The typical reassembled stack had the same number of cisternae as the starting material (see Figs. 1 A and 6 A) but there were striking differences in the size and shape. Their large size was the consequence of a greatly increased cisternal length. The median length of the stacked cisternae was 3.0 μm , 4.2 times longer than that of the starting material (0.71 μm ; Fig. 3). The reassembled stacks were also highly curved and surrounded a space that lacked the amorphous material found in the cytosol and had a lower concentration of membranes (Fig. 1 D, *asterisk*). This electron-lucent space was not completely enclosed by the stacked membranes since serial sections showed at least one opening from which tubules and tubular networks often emanated. Fig. 4 shows sections starting from the point furthest from the opening (*a*) and ending at the opening (*g-i*). The diameter of this electron-lucent space was fairly constant at $\sim 1.2 \pm 0.5 \mu\text{m}$.

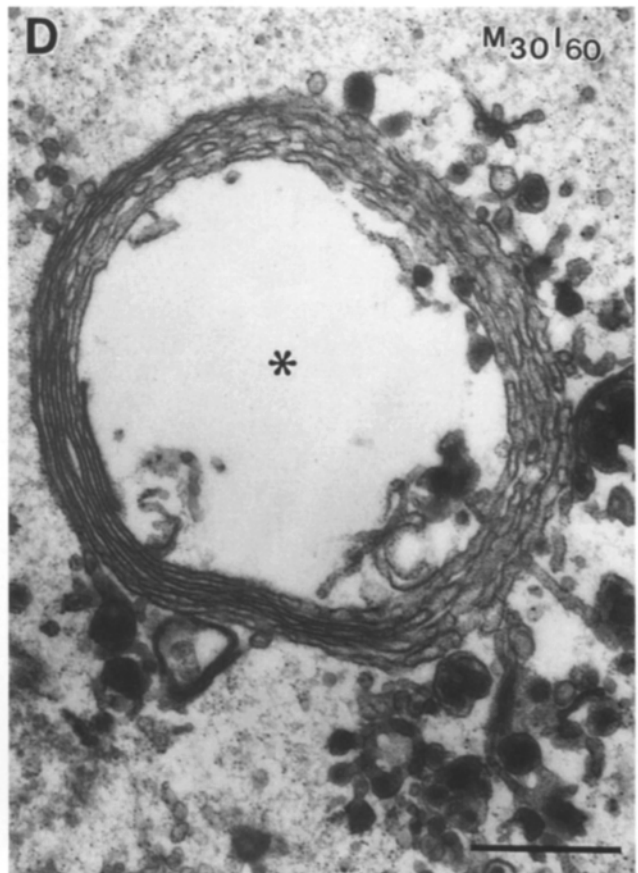
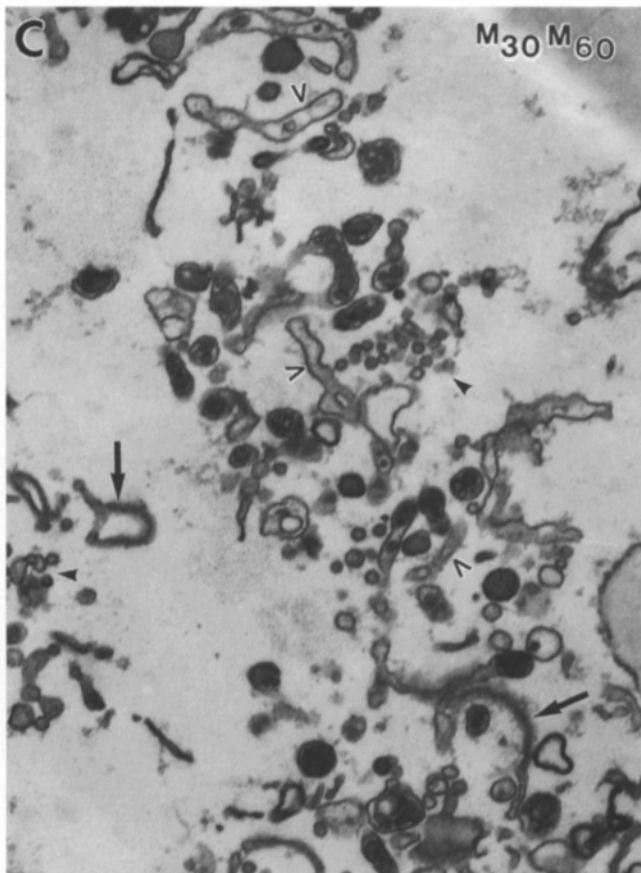
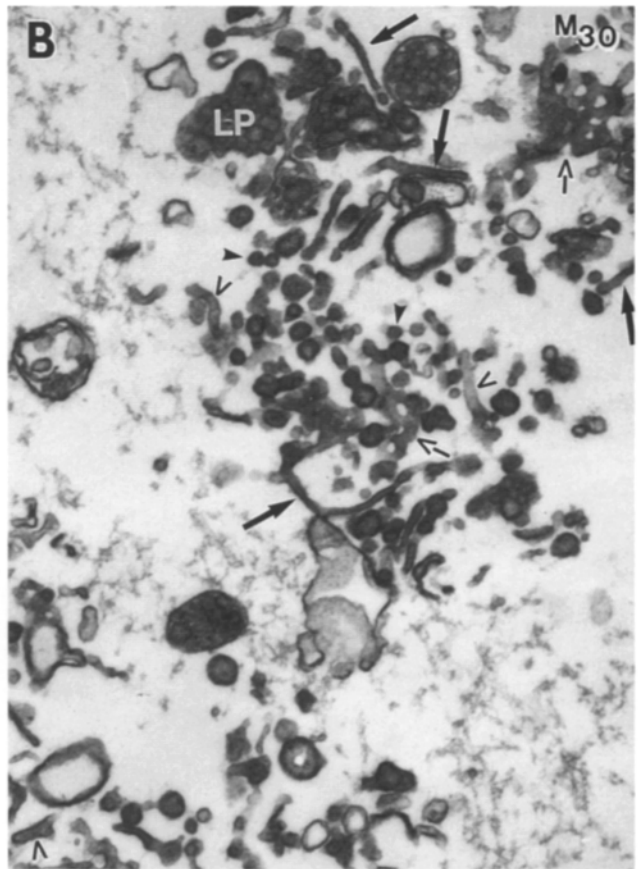
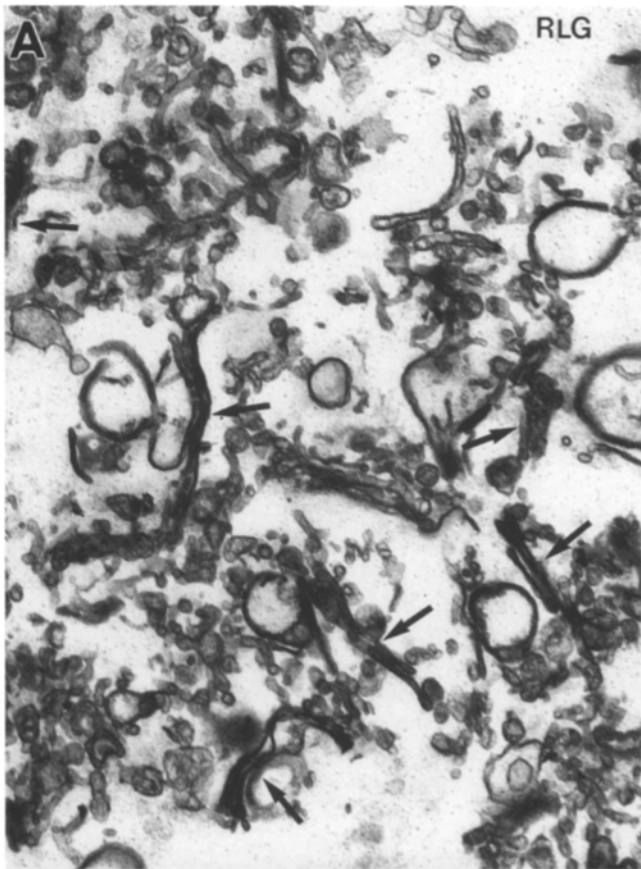
Quantitation showed that reassembly reversed the changes observed during fragmentation (Fig. 2). There was a fall of 15% in vesicular profiles (from 30 to 15%) and 13% in tubular profiles (from 32 to 19%) which was mostly matched by a 19% increase in cisternal membrane (from 22 to 41%). The percentage of stacked cisternae rose from 2 to 21% of total membrane. This was more than half the original value of 36% showing that reassembly is a reasonably efficient process.

Reassembly was dependent upon the presence of interphase and not mitotic cytosol. When the isolated mitotic fragments were incubated with mitotic cytosol no reassembly occurred though the cisternal remnants were slightly longer than those at the start of the incubation (Fig. 1 C) (median length of 0.65 vs. 0.48 μm ; Table I, line 3). This most likely reflects the lower cytosol to membrane protein ratio during reincubation (5 to 1 instead of 100 to 1 during fragmentation; Table I). We have shown previously that a high ratio of mitotic cytosol to membrane is necessary to drive fragmentation to completion (Misteli and Warren, 1994). Lower levels presumably permit some fusion of cisternal remnants.

Intermediates During Reassembly

Samples were fixed at different times during reassembly and processed for electron microscopy. Four intermediates could be discerned and examples of each are presented in Fig. 5. It is important to stress that reassembly was a highly asynchronous process and though most of a particular intermediate was present at the time indicated, it was present at other times as well.

The first intermediate was mostly present after 15 min of



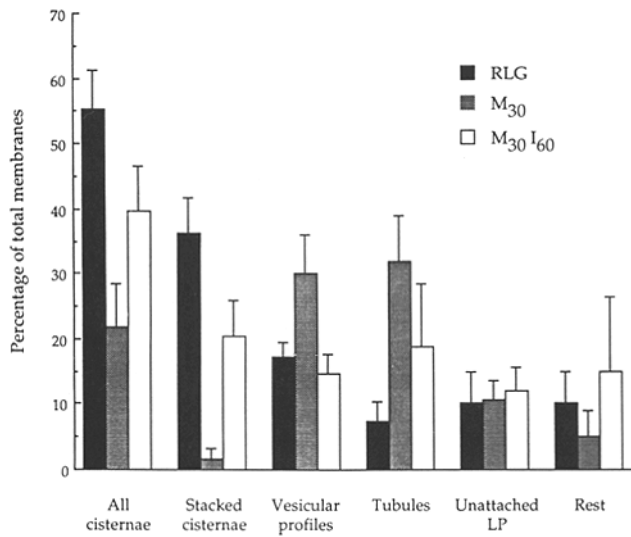


Figure 2. Quantitation of Golgi fragmentation and reassembly. Rat liver Golgi membranes (RLG) were incubated with mitotic cytosol for 30 min (M_{30}) and the fragments reassembled in rat liver interphase cytosol for 60 min ($M_{30}I_{60}$). Membranes were placed in one of six categories as defined in Materials and Methods and the results for membrane in each category expressed as a percentage of the to-

reassemble and comprised a single, curved cisterna with a median length of $0.65 \mu\text{m}$, 1.4 times longer than the mitotic cisternal remnants. The cisternal rims were continuous with small, tubular networks, which, very occasionally, were apposed to the convex face of the cisterna (Fig. 5 A).

The second intermediate, mostly seen at 30 min, comprised a stack of two cisternae, the rims of which were continuous with larger tubular networks (Fig. 5, B and C). Fig. 5 B shows an early example of this intermediate whereas Fig. 5 C shows a later example with more extensive networks. It is worth noting that lipoprotein particles were only found in the tubular networks and almost never in the stacked cisternae. The median cisternal length was now $0.9 \mu\text{m}$, 1.9 times longer than the mitotic cisternal remnants.

The number of cisternae in the stack often remained at two for the rest of the incubation but addition of further cisternae could occur and by 45 min stacks with three and even four cisternae were observed. The example shown in Fig. 5 D is

tal membrane in that category \pm SEM. Unattached LP, profiles enclosing lipoprotein particles not connected to cisternae, or networks; Rest, unidentified membranes and identifiable contaminants such as plasma membrane.

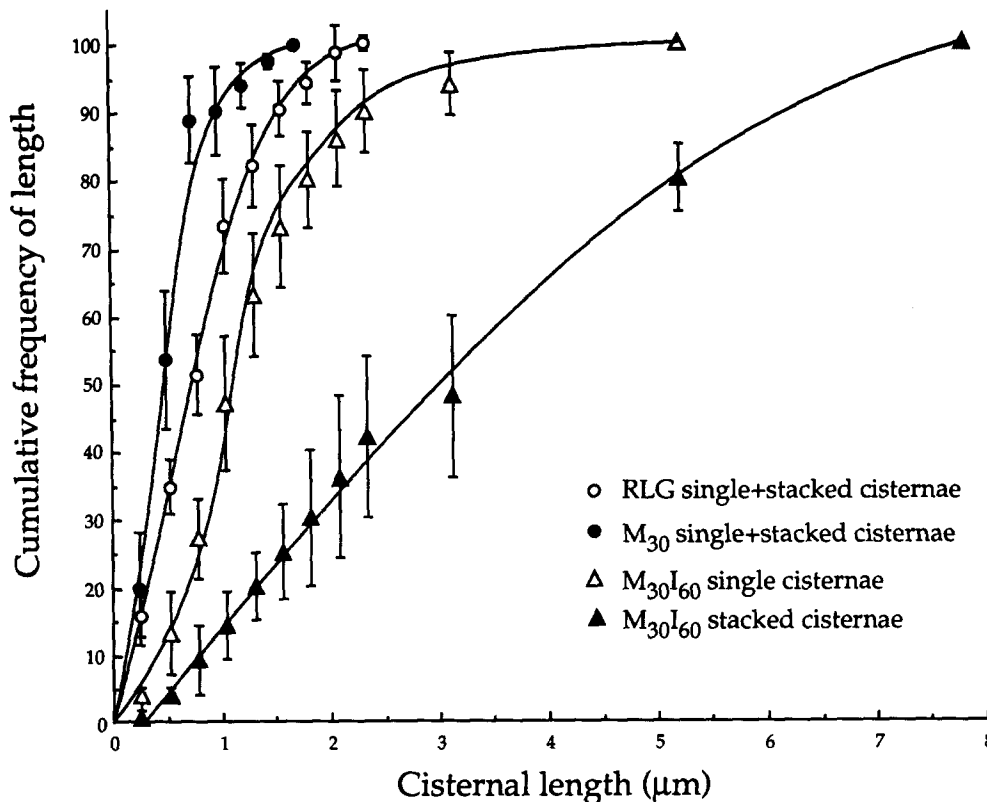


Figure 3. Cumulative frequency of cisternal length after fragmentation and reassembly. The length of cisternal profiles was measured for rat liver Golgi membranes (RLG), the same membranes after incubation with mitotic cytosol for 30 min (M_{30}) or after further incubation with interphase cytosol for 60 min ($M_{30}I_{60}$). Each cisternal length was placed into categories as defined in Materials and Methods such that each value on the y-axis represents the number of cisternae (expressed as a percentage of the total \pm SEM) with a length equal to or less than the cisternal length on the x-axis. The median cisternal length was derived from the 50% cumulative frequency value for each incubation condition: RLG (single + stacked) = $0.71 \mu\text{m}$; M_{30} (single + stacked) = $0.48 \mu\text{m}$; $M_{30}I_{60}$ (single) = $1.0 \mu\text{m}$; $M_{30}I_{60}$ (stacked) = $3.0 \mu\text{m}$.

Figure 1. Morphology of Golgi membranes after fragmentation and reassembly. Rat liver Golgi membranes (A, RLG) were incubated for 30 min at 37°C in the presence of ATP and mitotic cytosol (B, M_{30}). The membranes were reisolated and incubated for a further 60 min at 37°C in the presence of either mitotic cytosol containing ATP (C, $M_{30}M_{60}$) or interphase rat liver cytosol containing ATP and GTP (D, $M_{30}I_{60}$). Samples were fixed and processed for electron microscopy. Note the presence of numerous stacks in A (closed arrows), their absence in B and C, and the typical, large reassembled stack in D that appears to surround an electron-lucent space (asterisk). Note the fragmentation products in B and C that comprise cisternal remnants (closed arrows), tubular networks (open arrows), tubules (open arrowheads), and vesicular profiles (closed arrowheads). LP, Lipoprotein particle. Bar, 500 nm.

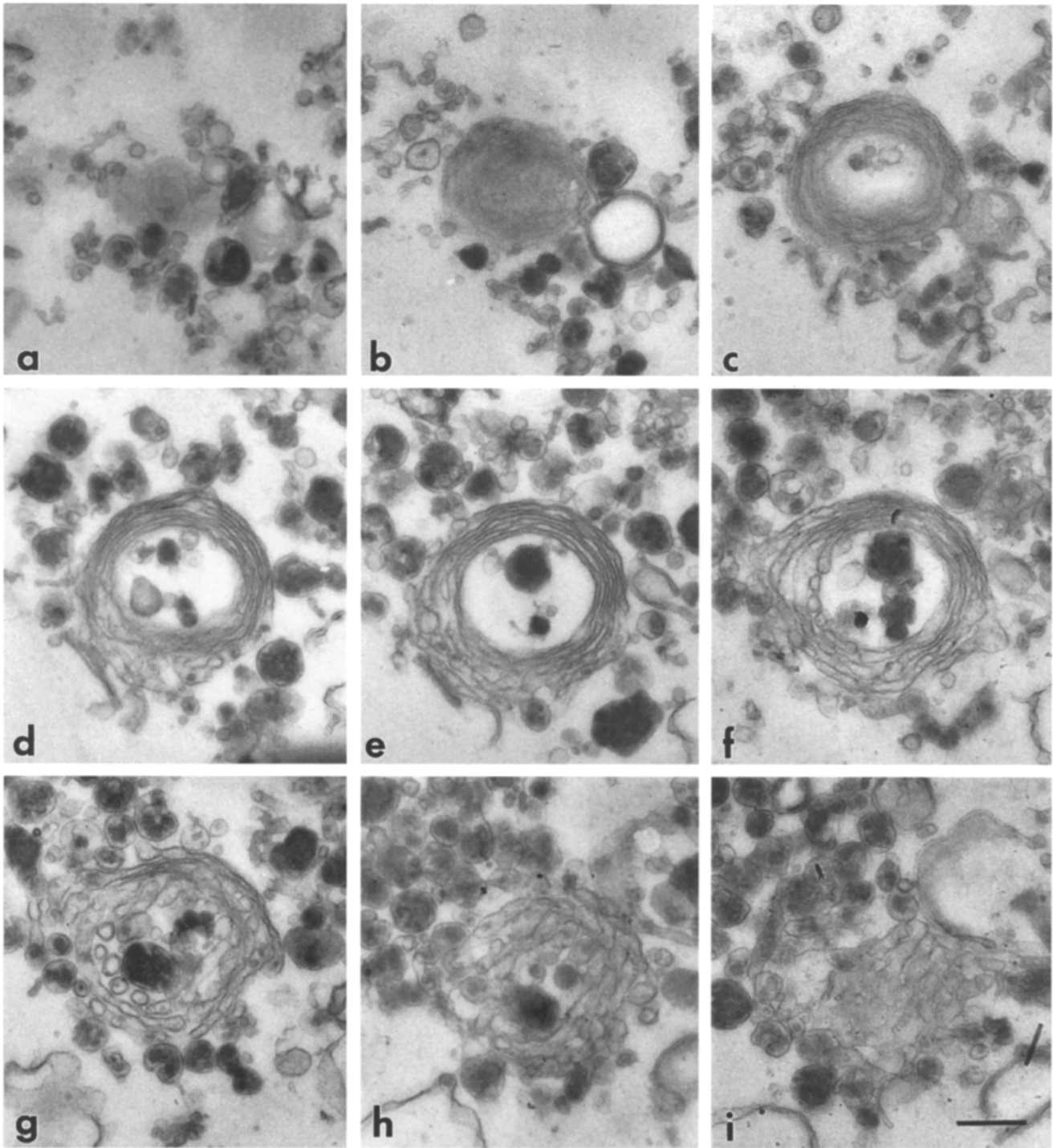


Figure 4. Serial section analysis of reassembled Golgi stacks. Mitotic Golgi fragments were reassembled for 60 min, fixed, processed for electron microscopy, and then serial thin sections (150 nm) were cut. The reassembled stacks were mostly spherical structures with a single opening filled with tubular networks. The sections (a-i) start at the point furthest from the opening that is visible in sections g-i. There are three to four cisternae in the stack. Bar, 500 nm.

exceptional in that it has six cisternae. It did, however, have many of the other characteristic features of the third intermediate. The increasing loss of tubular networks was accompanied by an increase in the fenestrations observed close to the cisternal rims (Fig. 5 D, *thin arrows*). The median cisternal length was now 3.0 μm , which was 6.3 times that of the

mitotic cisternal remnants. This median length was the same irrespective of the number of cisternae in the stack.

The fourth and final intermediate after 60 min of incubation is shown in Fig. 5 E. There was no increase in the number of cisternae and the median length was the same as for the third intermediate. The tubular networks had, however,

Table I. Conditions Affecting the Reassembly of Golgi Stacks

Conditions of disassembly	Conditions of reassembly	End products
1 Standard mitotic*	—	Mitotic fragments ^{‡§}
2 Standard mitotic	Standard interphase	Golgi stacks typically containing 2–4 cisternae and single cisternae [§]
3 Standard mitotic	Standard mitotic [†]	Mitotic fragments containing slightly longer cisternal remnants
4 Standard mitotic	Standard interphase at 4°C	Mitotic fragments
5 Standard mitotic	Standard interphase after energy depletion	Mitotic fragments
6 +Nocodazole	Standard interphase	Golgi stacks typically containing 2–4 cisternae and single cisternae
7 Standard mitotic	+Nocodazole	Golgi stacks typically containing 2–4 cisternae and single cisternae
8 +AIF	Standard interphase	Mitotic fragments containing slightly longer cisternal remnants
9 Standard mitotic	+AIF	Golgi stacks typically containing 2–4 cisternae and single cisternae
10 +GTP γ S	Standard interphase	Mitotic fragments lacking cisternal remnants and tubular networks
11 Standard mitotic	+GTP γ S	Golgi stacks typically containing 2–4 cisternae continuous with extensive tubular networks. No single cisternae
12 +NEM	Standard interphase	Golgi stacks typically containing 2–4 cisternae and single cisternae
13 Standard mitotic	+NEM	Mitotic fragments containing slightly longer cisternal remnants
14 +Microcystin	Standard interphase	Mitotic fragments containing slightly more and longer cisternal remnants
15 Standard mitotic	+Microcystin	No Golgi stacks, only single cisternae

* Rat liver Golgi stacks were incubated in mitotic cytosol containing ATP for 30 min at 37°C.

‡ Comprising cisternal remnants, tubular networks, tubules, and vesicles.

§ Quantitated in Figs. 2 and 3.

|| Reisolated mitotic Golgi fragments were incubated in rat liver interphase cytosol containing ATP and GTP for 60 min at 37°C.

† Except that the volume was 10 times lower.

mostly disappeared and cisternal fenestrations were much less frequent (Fig. 5 E, *thin arrows*).

Conditions for Reassembly

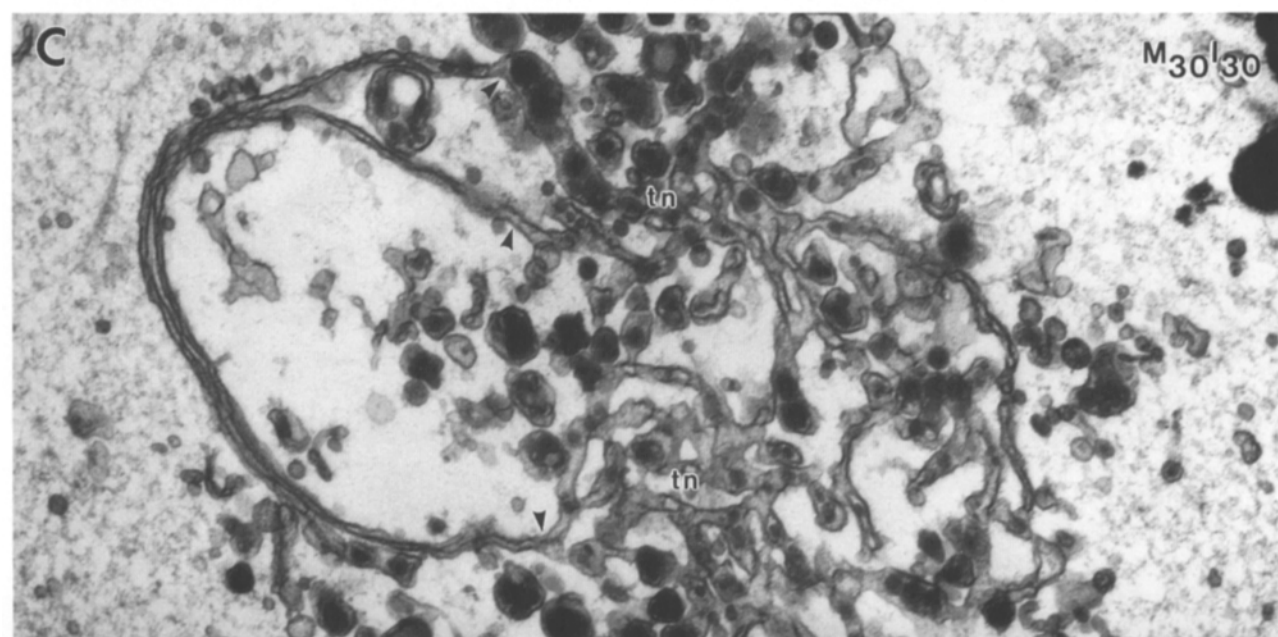
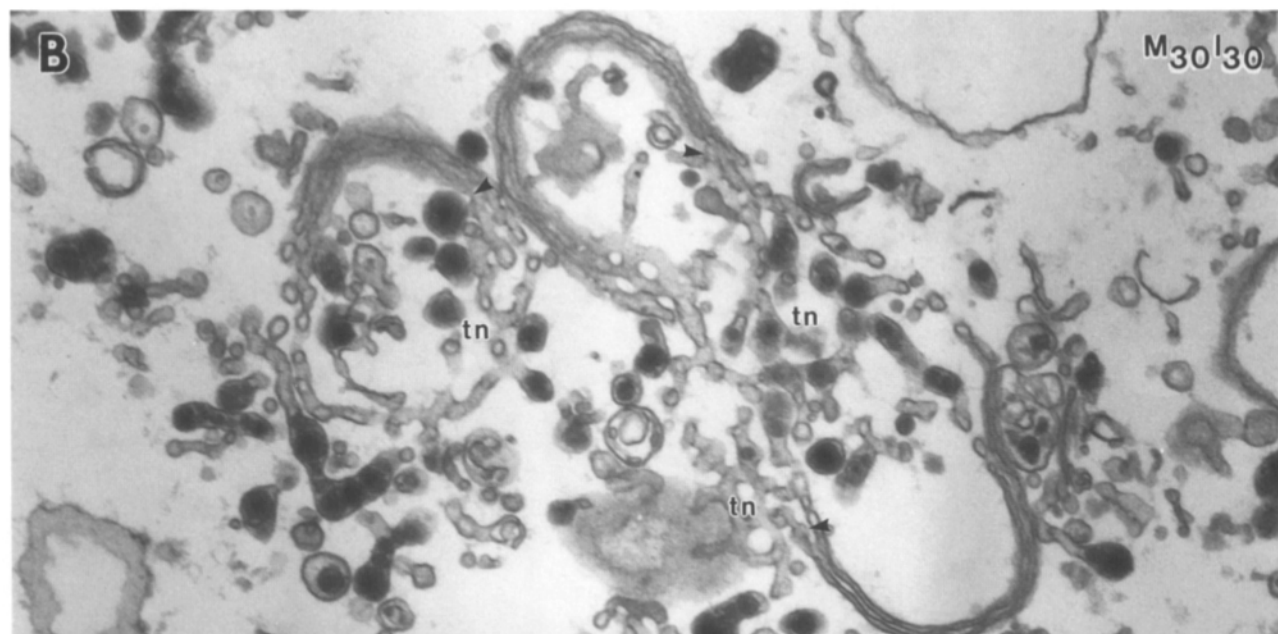
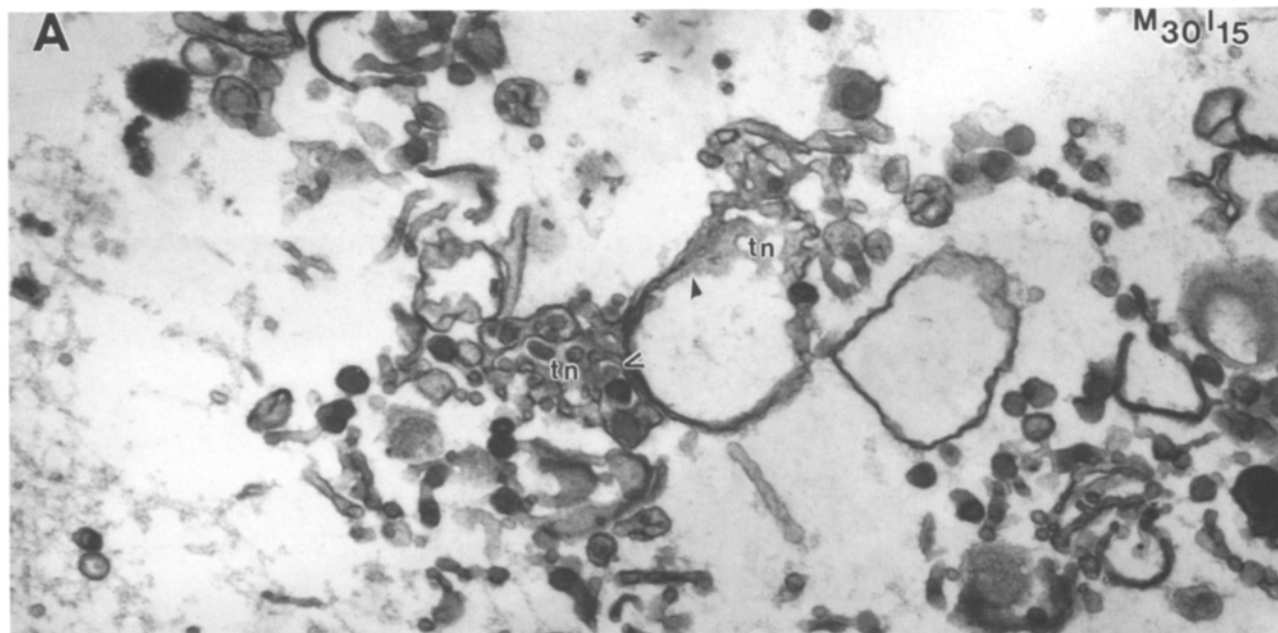
Reassembly did not occur at 4°C (Table I, line 4) or in the presence of an ATP-depleting system (which also depletes GTP) (Table I, line 5). Neither microtubules nor microfilaments appeared to be involved since reassembly was not affected by either nocodazole (Table I, lines 6 and 7) or cytochalasin B. The latter was always present in the standard incubations to prevent gelling of the samples.

AIF acts on heterotrimeric G proteins (Gilman, 1987) and permits budding but not uncoating of COP-coated vesicles (Orci et al., 1989). At a concentration of 50 μ M, it had no effect on the mitotic fragmentation of Golgi stacks but the fragments formed could not participate in the reassembly process (Table I, line 8). GTP γ S acts on rab proteins (Melançon et al., 1987) and ARF (ADP-ribosylation factor; Donaldson et al., 1991) and also causes the accumulation of COP-coated vesicles (Orci et al., 1989). At 20 μ M it had a similar effect to AIF but, in addition, fragmentation continued and went to completion during the subsequent incubation.

Table II. Effect of Microcystin and GTP γ S on Single and Stacked Cisternae

Incubation condition	Single cisternae		Stacked cisternae	
	% total membrane	Median cisternal length μ m	% total membrane	Median cisternal length μ m
None (RLG)	20	0.71	36	0.71
M ₃₀	20	0.48	2	—
M ₃₀ I ₆₀	20	1.0	21	3.0
M ₃₀ I ₆₀ microcystin	33	1.1	0	—
M ₃₀ I ₆₀ GTP γ S	1	—	42	3.0

Rat liver Golgi membranes (RLG) were incubated with mitotic cytosol for 30 min (M₃₀) and the fragments reincubated with interphase cytosol for 60 min in the absence (M₃₀I₆₀) or presence of 10 μ M microcystin (M₃₀I₆₀microcystin) or 20 μ M GTP γ S (M₃₀I₆₀GTP γ S). The percentage total membrane and the median cisternal length were calculated as described in Materials and Methods. The results for RLG, M₃₀, and M₃₀I₆₀ are taken from Figs. 2 and 3 for comparison.



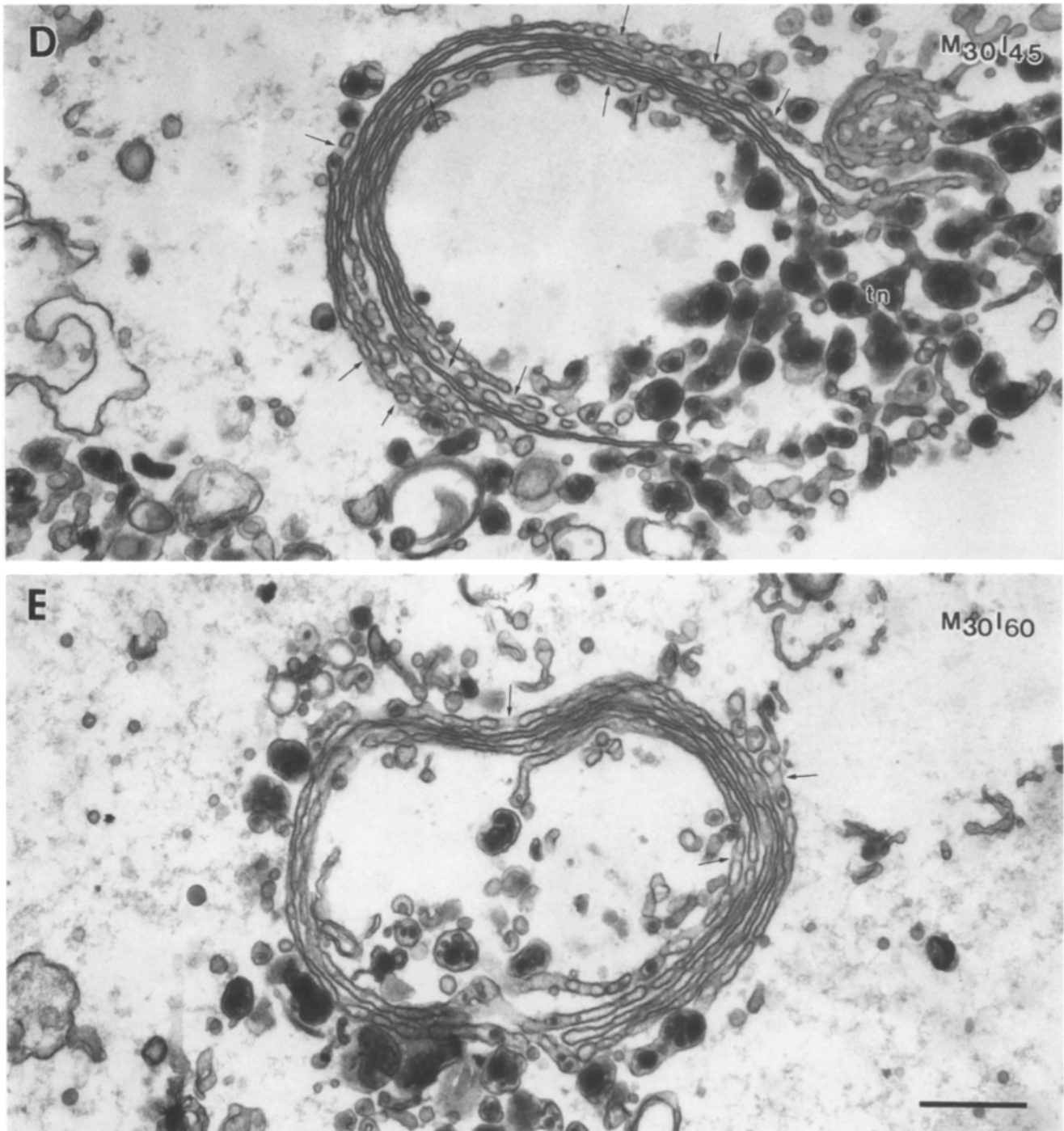
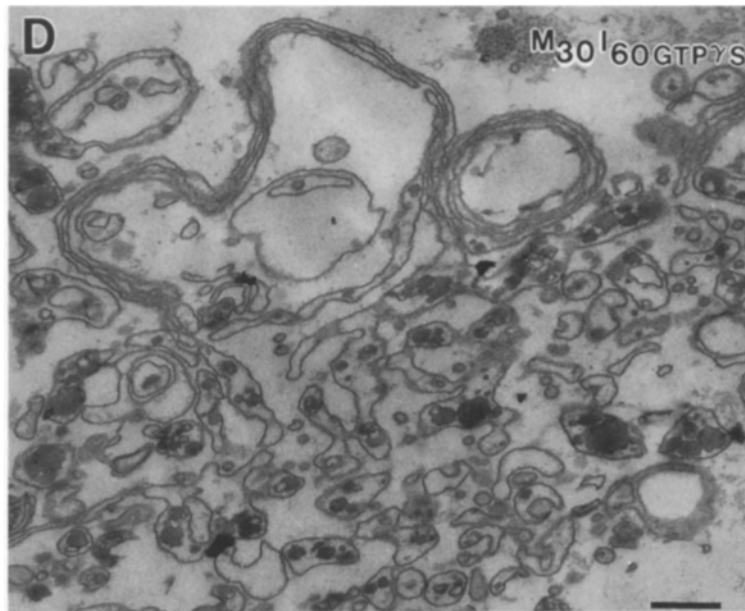
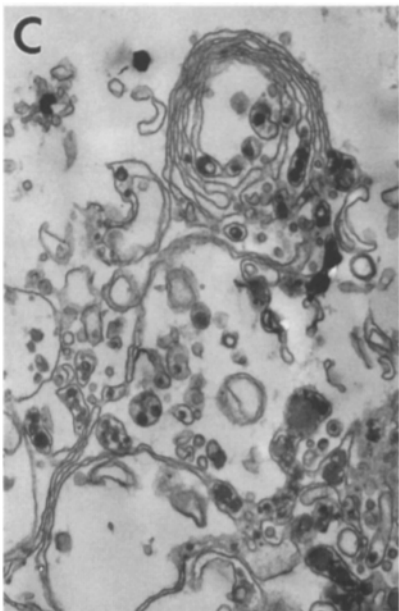
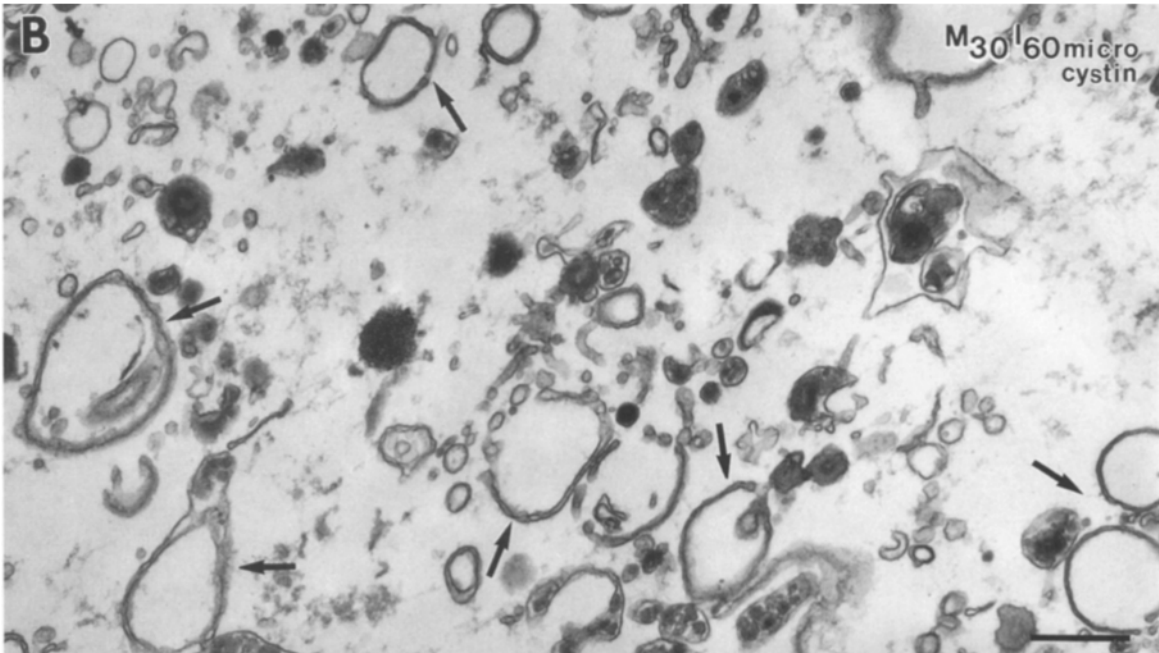
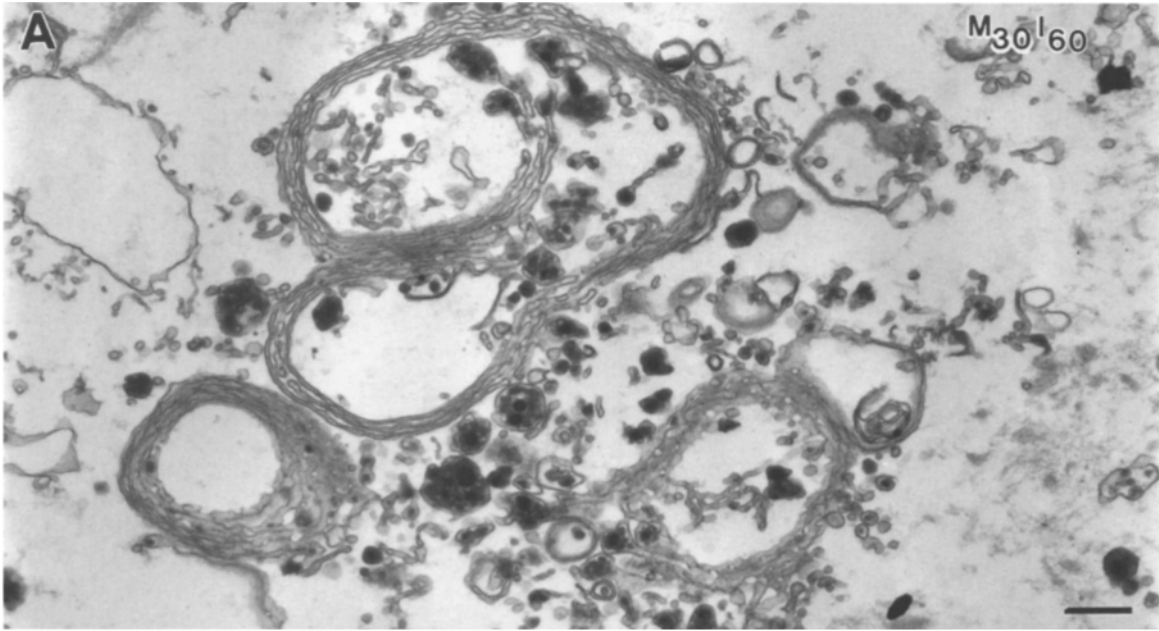


Figure 5. Morphological intermediates on the reassembly pathway. Golgi membranes fragmented for 30 min in the presence of mitotic cytosol were incubated in interphase cytosol for 15 (*A*, *M*_{30I15}), 30 (*B* and *C*, *M*_{30I30}), 45 (*D*, *M*_{30I45}), and 60 (*E*, *M*_{30I60}) min. An example of the intermediate present at each time point is shown. Note the tubular networks (*tn*) which, at 15 min, were small and mostly continuous with the cisternal rims (*A*, *closed arrowhead*), though on rare occasions they were found apposed to one face of the cisterna (*A*, *open arrowhead*). At 30 min they were more extensive and continuous with the cisternal rims (*B* and *C*, *closed arrowheads*). They had begun to disappear by 45 min (*D*) and were mostly gone by 60 min (*E*). Growing single cisternae predominated at early times (*A*) and were replaced by stacks first with two (*B* and *C*) and then more (*D* and *E*) cisternae. Fenestrations became less frequent at later times (compare *D* and *E*, *thin arrows*). Note that the space enclosed by the stacked cisternae (especially prominent in *D*) was mostly devoid of the amorphous material seen in the surrounding cytosol and contained fewer membranes. Lipoprotein particles were restricted to the tubular networks and were absent from the stacked cisternae (*C* and *D*). Bar, 500 nm.



tion of the fragments in interphase cytosol (Table I, line 10).

In marked contrast, neither 50 μM AIF nor 20 μM GTP γ S inhibited the reassembly process when added to the reassembly mixture (Table I, lines 9 and 11). In fact, GTP γ S stimulated reassembly driving it beyond the point at which is normally stopped. This happened even in the presence of the GTP (1 mM) that was normally added to the standard reassembly mixture. As shown in Table II, there was no change in the median cisternal length (3.0 μm) or in the percentage of total membrane incorporated into cisternae (43 vs. 41%). However, the percentage of single cisternae fell from 20 to 1% thereby raising the percentage of stacked cisternae from 21 to 42%. Furthermore, most of the tubules and many of the vesicles were incorporated into tubular networks that were continuous with the stacked cisternal rims (compare Fig. 6 A with C and D).

NEM is a specific inhibitor of the general fusion protein, NSF (NEM-sensitive factor) (Block et al., 1988). When used to pretreat Golgi membranes and mitotic cytosol, NEM had no effect on either fragmentation (data not shown) or subsequent reassembly (Table I, line 12). However, when used to treat the mitotic Golgi fragments in interphase cytosol, reassembly was completely abolished (Table I, line 13). The end products were slightly longer than the starting mitotic fragments suggesting some residual fusion activity. When the interphase cytosol alone was treated with NEM, reassembly was normal. When the mitotic membranes were treated with NEM, reassembly occurred but the stacks that accumulated contained highly fenestrated cisternae (data not shown). These results are consistent with the observation that NSF is found both in cytosol and on membranes (Block et al., 1988).

Microcystin had a dramatic effect on the reassembly process. As mentioned earlier, when present during fragmentation, it prevented subsequent reassembly (Table I, line 14). However, when present during reassembly, it permitted the growth of single cisternae but prevented their stacking (Table I, line 15; Fig. 6 B). The drug had a moderate inhibitory effect on the percentage of cisternal membrane that formed (33 vs. 41% without the drug; Table II) but the single cisternae grew to a median cisternal length of 1.1 μm , very similar to that of single cisternae in incubations lacking the drug (1.0 μm ; Table II).

Discussion

Cell-free systems have proven to be powerful tools in understanding the molecular basis of cellular function. Glycolysis was the first cellular function to succumb to this approach (Büchner, 1897) followed by protein synthesis (Zamecnik, 1969). In the membrane traffic field, the reconstitution of protein translocation across ER membranes (Blobel and Dobberstein, 1975) was followed by cell-free vesicular transport (Fries and Rothman, 1980; Rothman, 1992). Together

with genetic approaches (e.g., Novick et al., 1980), these systems have led to a detailed understanding of how proteins reach the correct organelle after synthesis is initiated in the cell cytoplasm (Nilsson and Warren, 1994; Rothman, 1994). The challenge now is to understand organelle architecture in terms of these proteins. As a first step we need to understand the rules that govern the architecture of cellular organelles.

A useful approach is to exploit the mechanisms used by animal cells to divide many membrane-bound organelles. Both the nuclear envelope and the RER have been reconstituted from mitotic fragments (Forbes et al., 1983; Burke and Gerace, 1986; Balch et al., 1987) and we have now succeeded in reassembling the Golgi apparatus. This success emphasizes yet again the autonomous nature of cellular organelles. The Golgi membranes used in these studies were free of almost all other membrane-bound organelles yet the mitotic fragments contained all the information necessary for reassembly when incubated with interphase cytosol and an energy source.

There were two overlapping phases to the process of Golgi reassembly. The first phase involved the formation of single cisternae by the lateral fusion of cisternal remnants, and the fusion of tubules and vesicles at the cisternal rims. The second phase involved the stacking of these cisternae, further fusion of tubules and vesicles, and lateral fusion of these stacks to form an elongated structure surrounding an electron-lucent space.

Several lines of evidence showed that single cisternae were intermediates and not side products or dead-end products of the reassembly reaction. The first was that single cisternae accumulated in the presence of microcystin which somehow inhibits the stacking mechanism. The percentage of membrane in these single cisternae rose from 20 to 33% and the median length of 1.1 μm was close to that of the single cisternae found in standard incubations (1.0 μm ; Table II). The second line of evidence was that single cisternae were consumed in the presence of GTP γ S, the percentage falling from 20 to 1% of total membrane (Table II). Since single cisternae accumulated in the presence of an inhibitor of reassembly and were consumed in the presence of an activator, they are clearly intermediates in the reassembly process. Single cisternae have been observed *in vivo* during telophase (Zeligs and Wollman, 1979; Lucocq et al., 1989) but their role has not been determined. Microcystin and GTP γ S might prove to be useful tools for this purpose.

During the first phase of reassembly, single cisternae were observed to grow from a median cisternal length of 0.48 to 1.1 μm (Fig. 3; Table II). In the presence of microcystin a similar increase was noted (Table II) and the amount of membrane in single cisternae rose to 33% compared to 20% found in cisternal remnants. This shows that growth was not simply the consequence of lateral fusion of cisternal remnants but the fusion of vesicles and tubules as well. It is not yet clear whether tubules and vesicles alone can initiate cisternal growth in the absence of cisternal remnants though

Figure 6. Effect of GTP γ S and microcystin on the reassembly of Golgi stacks. Mitotic Golgi fragments were incubated for 60 min at 37°C in interphase cytosol in the absence (A) or presence of 10 μM microcystin (B) or 20 μM GTP γ S (C and D). Note the stacked cisternae in the standard incubation compared to single cisternae in the presence of microcystin (arrows in B). Very few single cisternae were left after treatment with GTP γ S and most of the tubules and many of the vesicles were present in the tubular networks attached to the cisternal rims. Bar, 500 nm.

preliminary fractionation experiments suggest that they cannot.

The role played by vesicles and tubules in cisternal growth is suggested by the first intermediate observed during reassembly. This comprises a single, curved cisterna with tubular networks emanating from the cisternal rims. Such networks were never seen in direct continuity with one or other face of the cisterna, though, on rare occasions, they were seen apposed to the convex face (Fig. 5 *A*, *open arrowhead*). This argues strongly that the cisternal rims are the only place where fusion can occur. If fusion immediately confers this property to the fusing membrane so that it in turn can fuse with incoming tubules and vesicles, the result will be a tubular network. Such a process would be akin to the compound fusion of secretory granules that is often observed during regulated exocytosis (Ishikawa, 1965; Amsterdam et al., 1969; Rohlich et al., 1971).

The absence of fusion on either face of the single cisterna most likely reflects the presence of a scaffold which acts as a barrier to fusion. This scaffold might be related to the material found in the intercisternal space and thought to be involved, at least in part, in the stacking of cisternae (see Slusarewicz et al., 1994*b*, for references). It might even be related to the hetero-oligomers containing Golgi enzymes that are thought to be involved in the localization of these enzymes to particular cisternae (Nilsson et al., 1993, 1994). Elements of this scaffold might also be involved in determining the overall shape of the cisterna. Growth of such a scaffold at the edges would flatten the tubular networks at the rims leading to cisternal growth. Further fusion would seal the fenestrations converting the network first to a fenestrated cisterna and then to an unfenestrated one (Rothman and Warren, 1994). This process of defenestration was observed in single and stacked cisternae especially in those regions closest to the tubular networks (compare Figs. 5, *D* with *E*).

Whatever the nature of the scaffold, its growth is clearly self-limiting. The median length of single cisternae at the end of a standard incubation is close to that of the starting material (1.1 vs. 0.71 μm ; Table II) and more than half the cisternae have lengths within the narrow range of 0.8 to 1.5 μm (Fig. 3). The same is true for the single cisternae that accumulate in the presence of microcystin (Table II). Since the Golgi stacks in the starting material likely represent the immediate precursors of the Golgi ribbon found in animal cells, this suggests that one of the dimensions of this precursor, the cisternal length, is controlled during regrowth, perhaps by a scaffold. The underlying mechanism merits further study.

The second phase of reassembly involves the stacking of single cisternae and their further elongation. The first intermediate in this phase comprises two, stacked cisternae with tubular networks that become increasingly more complex with time (Fig. 5, *B* and *C*). The median cisternal length of this intermediate is 0.9 μm , shorter than that of completed single cisternae (1.1 μm). This shows that the two phases are overlapping such that single cisternae can stack before they have completed their growth.

Though stacking frequently stops at this stage in a standard incubation, further stacking can occur and it is not uncommon to find stacks with three to four cisternae. The simplest way to generate such stacks is to combine stacked cisternae with each other or with single cisternae. One other possibility is that the tubular networks themselves fold back

along the convex face of a cisterna to form a new cisterna. Though such tubular networks have been observed (Fig. 5 *A*), they are rare. Furthermore, such a mechanism would generate images of adjacent cisternae connected via tubular networks. Such images have not been seen.

Elongation of stacked cisternae most probably occurs by the fusion of tubular networks in congregating stacks. This mechanism is consistent with images such as those shown in Figs. 5 *B* and 6 *A*. It is also consistent with the observation that the median cisternal length rises to 3.0 μm , 2.7 times longer than that for completed single cisternae (1.1 μm) and 4.2 times longer than the original starting Golgi stacks (0.71 μm) (Table II).

Both single and stacked cisternae appear to enclose an electron-lucent space that is largely devoid of amorphous cytosolic material and membrane structures were only present at a lower concentration than found in the surrounding cytosol. Tubular networks were not observed. It is not entirely clear whether it is a space or a structure. As curved cisternae grow, they might be able to prevent cytosolic material from entering the space they enclose. Alternatively, if it is a structure, the nearest cellular equivalent would be the zone of exclusion that surrounds the interphase Golgi apparatus and from which most other cytoplasmic organelles are excluded (Mollenhauer and Morre, 1978). Its function is unknown. If this structure is the zone of exclusion then it has assumed a shape very different to that in vivo.

Reassembly involves two phases of fusion that probably reflect what happens in vivo. During telophase, Golgi clusters grow by accretion of vesicles that fuse to form discrete stacks. These then congregate in the pericentriolar region where they fuse to form the Golgi ribbon (Lucocq et al., 1989). Both types of fusion are of the same type (homotypic) but are triggered at different times. In vivo, this might reflect the time at which microtubule motors start to work again (Allan and Vale, 1991). Discrete clusters will fuse to form stacks but they cannot fuse with other stacks until they are transported to the pericentriolar region most probably along microtubules (Ho et al., 1989; Corthésy-Theulaz et al., 1992). In vitro, microtubules appear to play no part in the reassembly process. It may be that the concentration of membranes is so high that as soon as cisternae stack they can fuse with other stacks without the need for microtubules and their motors to bring them together. This does not, however, explain why completed single cisternae do not normally fuse with each other unless they are part of a stack. Perhaps stacking increases the likelihood of productive docking and fusion as a consequence of random encounters.

Fusion, at least during the early phase, was sensitive to NEM under conditions that are known to inactivate NSF. Similar results have been obtained for reassembly of the nuclear envelope and ER (Vigers and Lohka, 1991; Newport and Dunphy, 1992). Though NSF has been proposed as a general fusion factor, at least for heterotypic fusion (Rothman, 1994), more recent data suggest that some homotypic fusion reactions use a related protein (Latterich and Schekman, 1994). It will, therefore, be important to show that the effects of NEM on reassembly can be reversed by the addition of recombinant NSF.

The role of GTP-binding proteins has been studied using AIF and GTP γ S. Neither affected the fragmentation process, but when these fragments were incubated in interphase

cytosol, no reassembly occurred. Since both reagents prevent the uncoating of COP-coated vesicles (Melançon et al., 1987; Orci et al., 1989), these vesicles, or some of the components they contain, must be involved in the reassembly process. In the case of GTP γ S, the mitotic fragments continue to break down even when incubated in interphase cytosol. This may reflect a pool of ARF-GTP γ S on the membrane which continues to recruit coatomer and bud COP-coated vesicles. Alternatively, it may reflect the continued fragmentation of the cisternal cores by a COP-independent pathway that requires a different GTP-binding protein.

In marked contrast, neither AIF nor GTP γ S inhibited reassembly. In fact, 20 μ M GTP γ S promoted reassembly to the extent that almost all the single cisternae were incorporated into stacks and most of the tubules and many of the vesicles were incorporated into the tubular networks (Fig. 6, C and D). Rab proteins are involved in membrane fusion processes (Balch, 1992) and recent evidence suggests that the hydrolysis of bound GTP occurs after the membrane fusion event (Stenmark et al., 1994). If rab proteins are involved in the reassembly process, they could form permanently active complexes with GTP γ S that might promote the reassembly process by catalyzing multiple fusion events.

GTP γ S has the opposite effect on the reassembly of the nuclear envelope after mitosis in *Xenopus* eggs (Newport and Dunphy, 1992; Boman et al., 1992a). The cytosolic component responsible for inhibition is a member of the ARF superfamily (Boman et al., 1992b). Its physiological role is still unclear.

It is not yet clear when transport through the Golgi stack resumes. In vivo, the stack reassembles before newly synthesised proteins reach it from the ER suggesting that transport resumes after reassembly (Souter et al., 1993). This interpretation is consistent with the results obtained using both GTP γ S and AIF. When added during reassembly, neither caused significant accumulation of COP-coated vesicles suggesting that transport had not yet resumed.

It is also not yet clear whether the stacks are polarized though preliminary experiments suggest that they are not. Since fusion is thought to be inhibited during fragmentation (Misteli and Warren, 1994), the mitotic Golgi fragments should be a mixed population, each derived from a particular cisterna. The absence of polarized stacks after reassembly would then suggest that different populations of vesicles can fuse with each other though whether fusion is random or the consequence of limited errors has still to be determined. Random fusion would be expected to lead to interconnections between stacked cisternae but these have never been observed. The mechanism that generates cisternae and stacks them would have to be independent of the docking and fusion mechanism. Random fusion does not pose any other problems in practice since work with brefeldin A has clearly revealed the existence of mechanisms to sort out Golgi proteins after they have all been mixed (Lippincott-Schwartz et al., 1990). Both anterograde and retrograde transport through the Golgi stack might be involved in reestablishing polarity immediately after reassembly has occurred. It will be important to reconstruct this event in a cell-free system.

In conclusion we have shown that a complex membrane-bound organelle can be reassembled in the test tube by exploiting cellular division mechanisms. The way is now open to identify the components that determine its architecture.

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