

Microtubule Independent Vesiculation of Golgi Membranes and the Reassembly of Vesicles into Golgi Stacks

Barbara Veit, Jennifer K. Yucel, and Vivek Malhotra

Department of Biology, University of California San Diego, La Jolla, California 92093

Abstract. We have recently shown that ilimaquinone (IQ) causes the breakdown of Golgi membranes into small vesicles (VGMs for vesiculated Golgi membranes) and inhibits vesicular protein transport between successive Golgi cisternae (Takizawa et al., 1993). While other intracellular organelles, intermediate filaments, and actin filaments are not affected, we have found that cytoplasmic microtubules are depolymerized by IQ treatment of NRK cells. We provide evidence that IQ breaks down Golgi membranes regardless of the state of cytoplasmic microtubules. This is evident from our findings that Golgi membranes break down with IQ treatment in the presence of taxol stabilized microtubules. Moreover, in cells where the microtubules are first depolymerized by microtubule disrupting agents which cause the Golgi stacks to separate from one another and scatter throughout the cytoplasm, treatment with IQ causes further breakdown of these Golgi stacks into VGMs. Thus, IQ breaks down Golgi membranes indepen-

dently of its effect on cytoplasmic microtubules. Upon removal of IQ from NRK cells, both microtubules and Golgi membranes reassemble. The reassembly of Golgi membranes, however, takes place in two sequential steps: the first is a microtubule independent process in which the VGMs fuse together to form stacks of Golgi cisternae. This step is followed by a microtubule-dependent process by which the Golgi stacks are carried to their perinuclear location in the cell. In addition, we have found that IQ has no effect on the structural organization of Golgi membranes at 16°C. However, VGMs generated by IQ are capable of fusing and assembling into stacks of Golgi cisternae at 16°C. This is in contrast to the cells recovering from BFA treatment where, after removal of BFA at 16°C, resident Golgi enzymes fail to exit the ER, a process presumed to require the formation of vesicles. We propose that at 16°C there may be general inhibition in the process of vesicle formation, whereas the process of vesicle fusion is not affected.

CYTOPLASMIC microtubules are thought to play a central role in the pericentriolar localization of the Golgi complex in animal cells. This is based on the findings that disruption of cytoplasmic microtubules with drugs such as nocodazole causes fragmentation of the Golgi complex into stacks, which subsequently disperse throughout the cytoplasm (Rogalski and Singer, 1984). Upon removal of microtubule depolymerizing drugs, the microtubule network reforms and the Golgi stacks move along the repolymerized microtubules into the pericentriolar region (Rogalski and Singer, 1984; Ho et al., 1989; Turner and Tartakoff, 1989; Cooper et al., 1990; Kreis, 1990). Thus, a relationship between the state of microtubules and the integrity of the Golgi complex has been defined. Under conditions where microtubules are depolymerized and the Golgi stacks are dispersed throughout the cytoplasm, newly syn-

thesized proteins are still transported through the Golgi cisternae (Iida and Shibata, 1991). Prolonged incubation with nocodazole (18–24 h) arrests cells in mitosis by inhibiting the dynamics of the mitotic spindle (Jordan et al., 1992). The Golgi membranes under these conditions are found to be composed of small vesicular structures and are thought to represent the mitotic form of Golgi (Lucocq et al., 1987a,b, 1989; Warren, 1985, 1989). This observed breakdown of the Golgi stacks into the mitotic form is not a direct effect of microtubule disruption, but rather a corollary of the ensuing mitosis, which is prolonged by the drug's inhibitory effect on mitotic spindle dynamics. Activated cdc2 kinase is believed to be the key player in the events responsible for converting the interphase cell into its mitotic state (for a review see Moreno and Nurse, 1990). Among the observed changes in a cell during mitosis are the breakdown of the Golgi complex into vesicles and a block in vesicular transport (Featherstone et al., 1985; Lucocq et al., 1987a,b). The possible targets of cdc2 kinase that alter the dynamics of the Golgi membranes have yet to be identified.

We have recently reported the identification of a novel nat-

Please address all correspondence to Dr. V. Malhotra, Department of Biology, University of California of San Diego, La Jolla, CA 92093.

ural metabolite, Ilimaquinone (IQ)¹, that disassembles Golgi membranes completely into small vesicles (VGMs for vesiculated Golgi membranes) as revealed by both, electron microscopy and light microscopy (Takizawa et al., 1993). Upon removal of the drug the Golgi membranes reassemble rapidly into the pericentriolar region of the cell. This process is observed in >95% of the cells, is energy dependent, occurs at 37°C and does not require protein synthesis (Takizawa et al., 1993). In the presence of IQ, transport of newly synthesized proteins from ER to the *cis*-VGMs is not affected, however, further transport along the secretory pathway is blocked. Unlike BFA, IQ does not induce retrograde transport of Golgi enzymes into ER. IQ works most efficiently in kidney derived tissue culture cells and less effectively in tissue culture cells such as CHO and HeLa. Intracellular organelles such as the ER, nucleus, mitochondria, and cytoskeletal elements such as actin filaments and intermediate filaments are not affected by IQ.

Microtubules have been implicated in some vesicle fusion events as well as in some steps of the secretory pathway, yet other transport and fusion events occur normally in the absence of polymerized microtubules (Herman and Albertini, 1984; Matteoni and Kreis, 1987; Rindler et al., 1987; De Brabander et al., 1988; Gruenberg et al., 1989; Turner and Tartakoff, 1989; Scheel et al., 1990; Iida and Shibata, 1991). We, therefore, wanted to explore the involvement of cytoplasmic microtubules in the process of VGM production and fusion. We have found that IQ, in addition to vesiculating Golgi membranes, depolymerizes cytoplasmic microtubules. However, IQ causes Golgi membranes to break down into VGMs even in the presence of taxol-stabilized microtubules. Moreover, we provide evidence that if the Golgi complex is first dismantled into stacks with microtubule disrupting agents, IQ treatment further breaks down Golgi stacks into VGMs. Thus, IQ acts independently both on microtubules and Golgi membranes and breaks down Golgi membranes regardless of the state of cytoplasmic microtubules.

Upon removal of IQ, the microtubules repolymerize and the Golgi membranes assemble in the pericentriolar position. During the reassembly process, the VGMs first assemble into stacked cisternae by a process that is independent of cytoplasmic microtubules. These stacks then assemble into a Golgi complex in the pericentriolar region by a microtubule-dependent process. Therefore, while the localization of the Golgi complex to the microtubule organizing center (MTOC) requires polymerized microtubules, the generation of VGMs by IQ, and the reassembly of the VGMs to form stacks of Golgi cisternae occur independently of microtubules.

It has been shown that the transport of proteins from ER to Golgi is blocked at temperatures lower than 16°C, and the transport of proteins from the TGN en route to the cell surface is inhibited at temperatures below 20°C (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Tartakoff, 1986). We have found that while VGM production is inhibited at 16°C, the VGMs fuse and assemble into stacks of Golgi cisternae at 16°C. In contrast, cells pretreated with BFA to redistribute Golgi membranes to the ER do not, upon removal of the drug, reassemble the Golgi apparatus at 16°C, even after 6 h. This seems to corroborate our findings that

1. *Abbreviations used in this paper:* IQ, ilimaquinone; MTOC, microtubule organizing center; OA, okadaic acid; VGMs, vesiculated Golgi membranes.

budding processes (such as those proposed to be needed for ER to Golgi transport for recovery from BFA treatment, and those needed for IQ-mediated vesicle formation) are impaired at 16°C but fusion events proceed relatively normally at the reduced temperature.

Materials and Methods

Reagents, Antibodies, and Cells

Brefeldin A was obtained from Sigma Chem. Co. (St. Louis, MO) and stored as a stock solution of 2 mg/ml in methanol at -20°C. IQ is made up fresh as a stock solution of 5 mg/ml in DMSO. Nocodazole and colcemid are purchased from Calbiochem, La Jolla, CA. Nocodazole is made up at 10 mg/ml in DMSO and stored at -20°C. Colcemid is made up at 10 mM in DMSO and stored at -20°C. Taxol is purchased from Sigma Chem. Co. and stored at 10 mg/ml in DMSO at -20°C. NRK cells were grown in complete medium consisting of DMEM (from GIBCO BRL, Gaithersburg, MD) with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a 5% CO₂ cell incubator.

All drugs were used at the indicated final concentrations in complete tissue culture medium supplemented with 25 mM Hepes, pH 7.4: IQ 30 µM; colcemid 1 µM; taxol 10 µg/ml; nocodazole 100 µg/ml; Brefeldin A 2 µg/ml. When more than one drug was used, final concentrations for each drug were as described.

Rabbit anti- α -mannosidase II was a gift from Dr. M. Farquhar (University of California at San Diego, La Jolla, CA). Fluorescein-labeled goat anti-rat IgG was purchased from Jackson Labs (West Grove, PA). Rat anti-tubulin was purchased from Accurate Chemical & Sci. Corp. (Westbury, NY). Rhodamine-labeled goat anti-rabbit was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Other Procedures

Electron Microscopy. Cells were fixed for 2 h at room temperature in 2% glutaraldehyde and 4% paraformaldehyde, washed extensively in PBS, and then fixed in 2% OsO₄ in PBS for 2 h. The sample was washed in ddH₂O three times and carried through a series of dehydration steps using acetone as described in Tokuyasu (1992). The sample was embedded in spurs, and sections viewed on Philips 300.

Immunofluorescence. Cells were fixed with methanol at -20°C for 5-10 min, and then washed three times with PBS and put in blocking buffer (PBS with 2.5% FBS, 0.2% azide) for 10-15 min at room temperature. Cells were then washed three times with PBS before incubation with primary antibody for 30 min (the primary antibodies were diluted in blocking buffer -1:1,000 for anti-mannosidase II; 1:100 for anti-tubulin). The cells were washed again three times with PBS after removal of the primary antibody, and then were incubated for 30 min with the secondary antibodies (diluted 1:100 in blocking buffer). The cells were washed three times with PBS before being mounted on slides using 90% glycerol in tris buffer pH 8.5 and phenyldiamine (a gift from Dr. M. Yaffe's lab at University of California at San Diego) as an anti bleach agent. Cells were photographed through a Nikon Microphot-FXA microscope using T-Max 3200 asa film.

Results

IQ Depolymerizes Cytoplasmic Microtubules, and the IQ Breaks Down Golgi Membranes in the Presence of Taxol Stabilized Microtubules

For all the experiments described, IQ was used at 30 µM final concentration in complete tissue culture medium supplemented with 25 mM Hepes, pH 7.4. NRK cells were treated with IQ for 30 min at 37°C. The cells were then fixed and stained with antibodies to tubulin and the Golgi specific polypeptide mannosidase II. Under these conditions Golgi membranes break down and are subsequently dispersed throughout the cytoplasm (Fig. 1 C). Additionally, IQ causes depolymerization of cytoplasmic microtubules (Fig. 1 D),

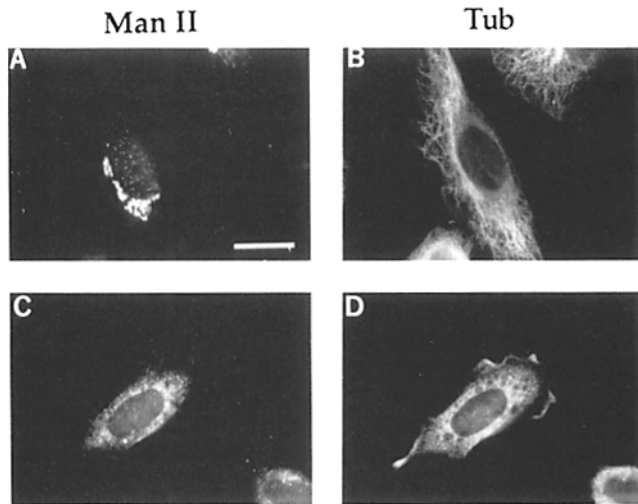


Figure 1. IQ causes both depolymerization of microtubules and dispersal of the Golgi complex. NRK cells were incubated with IQ (C and D) or DMSO as a control (A and B) for 30 min at 37°C. The cells were fixed, permeabilized, and double stained with antibodies to mannosidase II (A and C) and tubulin (B and D). Anti-mannosidase II and anti-tubulin antibodies were revealed by using a goat anti-rabbit antibody conjugated to rhodamine and a goat anti-rat antibody conjugated to fluorescein, respectively. As is evident, the Golgi complex and the cytoplasmic microtubules are completely disassembled by IQ treatment. Bar, 20 μm .

however, intermediate filaments and actin filaments are not affected (data not shown).

As mentioned before, depolymerization of microtubules with agents such as nocodazole or colcemid cause the Golgi complex to disassemble into small stacks which subsequently disperse throughout the cytoplasm. Since this dispersal of the Golgi complex is a direct result of the depolymerization of microtubules, pretreatment of cells with the microtubule stabilizing agent taxol, should inhibit the ability of these drugs to cause dispersal of the Golgi complex. This is demonstrated in NRK cells pretreated for 30 min with 10 $\mu\text{g}/\text{ml}$ taxol before treatment with 100 $\mu\text{g}/\text{ml}$ nocodazole for 90 min in the presence of taxol. As shown in Fig. 2 A, taxol-stabilized microtubules are not depolymerized by subsequent nocodazole treatment. Furthermore, this stabilization of microtubules prevents the dispersal of Golgi stacks normally observed upon treatment with nocodazole (Fig. 2 B). Therefore, stabilization of microtubules with taxol inhibits the nocodazole-mediated disassembly of the Golgi complex into stacks and their subsequent dispersal in the cytoplasm.

IQ treatment also result in microtubule depolymerization. However, unlike other microtubule depolymerizing agents, which cause the disassembly of the Golgi complex into stacks dispersed in the cytoplasm, IQ causes the Golgi complex to vesiculate (Takizawa et al., 1993). It therefore seemed likely that IQ was acting more specifically on the Golgi membranes themselves. Thus, we were interested in testing whether depolymerization of microtubules was requisite for IQ-mediated Golgi membrane vesiculation. To investigate this, NRK cells were preincubated for 30 min with 10 $\mu\text{g}/\text{ml}$ taxol to stabilize microtubules, and then treated with

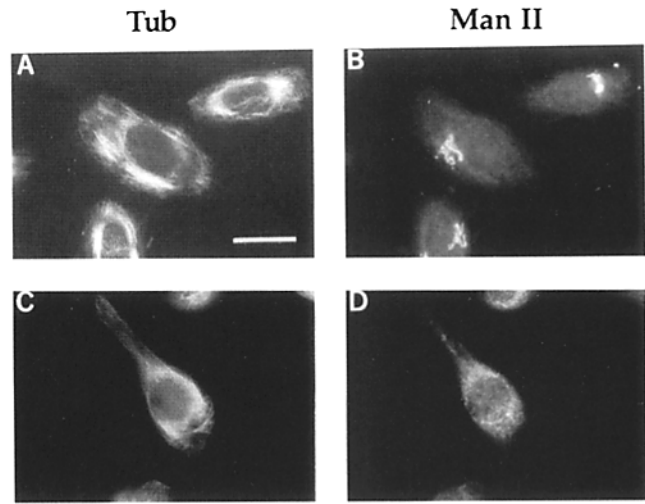


Figure 2. IQ breaks down Golgi membranes in the presence of taxol-stabilized microtubules. NRK cells were incubated with taxol for 30 min at 37°C. The cells were then treated in the presence of taxol with nocodazole for 90 min (A and B) or IQ for 30 min at 37°C (C and D). The cells were fixed, permeabilized, and double-stained with an anti-tubulin antibody (A and C) and an anti-mannosidase II antibody (B and D). Anti-mannosidase II and anti-tubulin antibodies were revealed by using a goat anti-rabbit antibody conjugated to rhodamine and a goat anti-rat antibody conjugated to fluorescein, respectively. Pretreatment of cells with taxol prevents both microtubule depolymerization (A) and the dispersal of the Golgi stacks (B) normally observed by treatment with nocodazole alone. However, while the treatment of cells with taxol prevents microtubule depolymerization by IQ (C), it does not inhibit the IQ-mediated disassembly of the Golgi complex (D). Bar, 20 μm .

IQ in the presence of taxol for 30 min at 37°C. IQ does not depolymerize taxol-stabilized cytoplasmic microtubules (Fig. 2 C), but does, however, still cause Golgi membranes to vesiculate (Fig. 2 D). Thus, IQ breaks down Golgi membranes independent of its effect on microtubules.

Cytoplasmic Microtubules Are Not Required for the Breakdown of Stacks of Golgi Cisternae into VGMs

To further demonstrate that IQ acts on Golgi membranes directly in a manner independent of microtubules the following experiment was performed. NRK cells were pretreated with 1 μM colcemid for 90 min at 37°C. A sample of cells was fixed and double stained with antibodies to mannosidase II and tubulin. This treatment results in depolymerization of cytoplasmic microtubules (Fig. 3 A) and causes the Golgi membranes to disassemble into numerous smaller stacks that are dispersed throughout the cytoplasm (Fig. 3 B). If these cells are then treated with IQ in the presence of colcemid, the Golgi stacks further vesiculate into VGMs (Fig. 3 C) while continued treatment with colcemid alone does not cause any further breakdown of the Golgi stacks (data not shown). These results demonstrate that IQ causes breakdown of Golgi stacks into smaller fragments and provide additional evidence that IQ interacts with Golgi membranes and causes their breakdown regardless of the state of cytoplasmic microtubules.

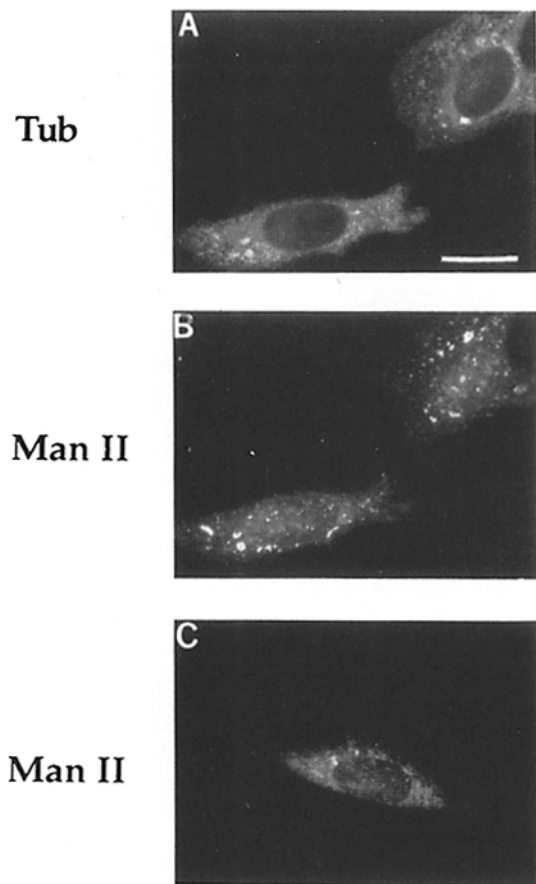


Figure 3. Dispersed stacks of Golgi cisternae break down further upon IQ treatment. NRK cells were treated with the microtubule depolymerizing agent colcemid for 90 min at 37°C. The cells were fixed, permeabilized, and double stained with an anti-tubulin antibody (A) and an anti-mannosidase II antibody (B). Anti-mannosidase II and anti-tubulin antibodies were revealed by using a goat anti-rabbit antibody conjugated to rhodamine and a goat anti-rat antibody conjugated to fluorescein, respectively. Under these conditions the cytoplasmic microtubules depolymerize, and the Golgi complex disassembles into small stacks that are subsequently dispersed throughout the cytoplasm. In parallel, NRK cells were treated with colcemid as above and then subsequently incubated with IQ in the presence of colcemid for 30 min at 37°C (C). The cells were fixed, permeabilized, and stained with anti-mannosidase II antibody followed by a goat anti-rabbit antibody conjugated to rhodamine. As is evident (C), the Golgi stacks break down further upon IQ treatment. Bar, 20 μ m.

The Assembly of VGMs into a Golgi Complex upon Removal of IQ Is in Two Sequential Steps: a Microtubule-independent Step, followed by a Microtubule-dependent Process

We have shown before that upon removal of IQ, the Golgi membranes reassemble in the pericentriolar position (Taki-zawa et al., 1993). Based on these findings, we expected that microtubules would also reassemble after removal of IQ from NRK cells. To test this, NRK cells pretreated with IQ for 30 min at 37°C were washed free of drug, and then incubated at 37°C in drug free medium. After 2 h, at which time the Golgi complex has reformed and localized to the

perinuclear region (Fig. 4 A), the microtubule network is re-established (Fig. 4 B).

The next question we wanted to address was whether polymerized microtubules were required for the reassembly of VGMs to form the Golgi complex. To test this NRK cells were treated with IQ to break down Golgi membranes into VGMs. The cells were then washed extensively to remove IQ. As previously shown (Fig. 4 A), VGMs assemble into a complex of Golgi stacks in the pericentriolar position after 2 h at 37°C. However, if during the recovery from IQ treatment the cells are treated with 100 μ g/ml nocodazole to keep the microtubules depolymerized (Fig. 4 D), the VGMs reassemble to form large discrete Golgi structures that are found dispersed in the cytoplasm (Fig. 4 C). Upon removal of nocodazole, microtubules repolymerize (Fig. 4 F) and these Golgi structures reform into the Golgi complex localized to the pericentriolar region of the cell (Fig. 4 E).

To identify the organization of the assembled form of VGMs in the presence of depolymerized microtubules, a preparation of such cells was examined by electron microscopy. As shown in Fig. 5, the Golgi membranes under these conditions are organized into stacks of cisternae. Therefore, in the absence of cytoplasmic microtubules, VGMs assemble to form stacks of Golgi cisternae but fail to progress further along the assembly pathway to form a complex of Golgi stacks in the pericentriolar position. Thus, our results demonstrate that the assembly of Golgi membranes from VGMs takes place in at least two steps: a microtubule independent step in which stacks of cisternae are formed from the VGMs, followed by a microtubule-dependent process in which these stacks are brought together into the pericentriolar position.

Fusion of IQ Derived VGMs Occurs at 16°C, However Budding to Form VGMs Does Not

It is known that many transport processes are sensitive to low temperatures: transport out of the TGN does not occur at temperatures below 20°C, while ER to Golgi transport is sensitive to temperatures below 16°C (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Tartakoff, 1986). We, therefore, wanted to examine the effect of lowered temperatures on the IQ-mediated vesiculation of Golgi membranes to form VGMs and the subsequent fusion of VGMs, upon removal of the drug, to form Golgi stacks. NRK cells were treated with 30 μ M IQ at 16 and at 20°C. While substantial depolymerization of microtubules occurs after 30 min of IQ treatment at 20°C, neither vesiculation nor any other phenotypic changes in the organization of the Golgi membranes was observed at either 16 or 20°C (data not shown). To examine whether VGMs would fuse at 16°C after removal of the drug, NRK cells were treated with IQ for 30 min at 37°C, and then washed free of the drug. The cells were then incubated at either 16 or 37°C. As shown previously, at 37°C the Golgi membranes completely reassemble in the pericentriolar position after 2 h (Fig. 4 A). However, if cells are incubated at 16°C, the VGMs assemble into discrete structures that are dispersed throughout the cytoplasm. These structures remain dispersed even after 6 h at 16°C after which time microtubules have completely repolymerized (Fig. 6, A and B). Furthermore, cells treated with IQ in the presence of taxol-stabilized microtubules, which are then washed free of IQ and incubated at 16°C, assemble the vesicles into these

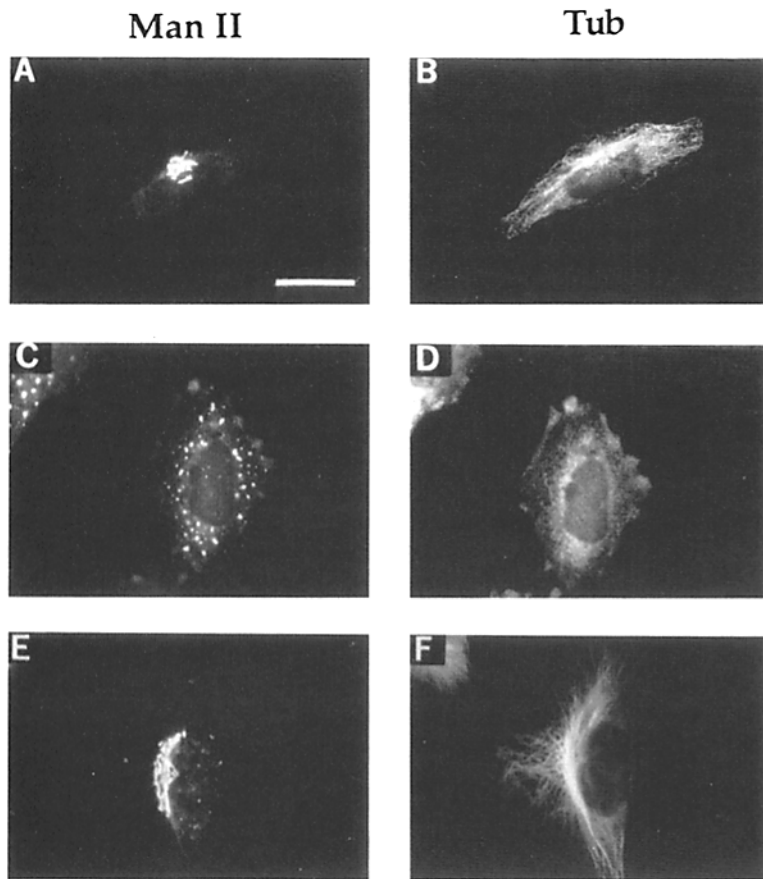


Figure 4. Cytoplasmic microtubules assemble upon removal of IQ, and full reassembly of the Golgi complex requires polymerized microtubules. NRK cells were treated with IQ for 30 min at 37°C. The cells were washed extensively to remove IQ, and then incubated at 37°C in the absence (A and B) or in the presence (C and D) of 100 µg/ml nocodazole for 2 h. In a parallel experiment after 2 h of reassembly in the presence of nocodazole, the cells were washed extensively to remove nocodazole and incubated at 37°C (E and F). The cells were fixed, permeabilized, and double stained with an anti-mannosidase II antibody and an anti-tubulin antibody. Anti-mannosidase II and anti-tubulin antibodies were revealed by using a goat anti-rabbit antibody conjugated to rhodamine and a goat anti-rat antibody conjugated to fluorescein, respectively. Both the Golgi complex and the cytoplasmic microtubules reassemble upon removal of IQ (A and B). If, however, the microtubules are kept depolymerized by nocodazole treatment, the IQ-mediated breakdown products (VGMs) of Golgi membranes assemble to form large discrete structures that remain dispersed in the cytoplasm (C and D). Upon removal of nocodazole, the microtubules repolymerize (F) and these Golgi structures assemble to form a Golgi complex in the pericentriolar position (E). Bar, 20 µm.

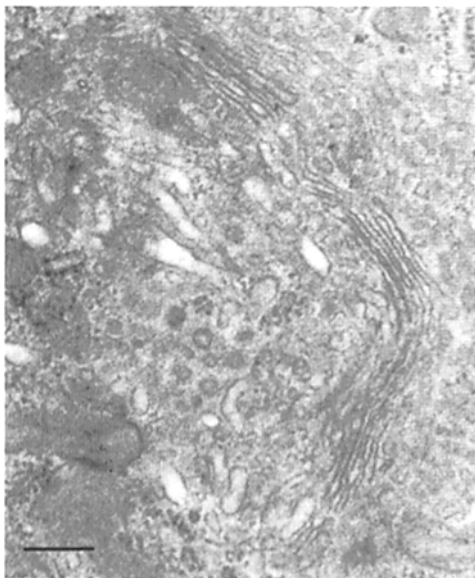


Figure 5. VGMs assemble into stacks of Golgi cisternae in the absence of cytoplasmic microtubules. NRK cells were treated with IQ for 30 min at 37°C, washed extensively to remove IQ, and then incubated at 37°C for 2 h in the presence of nocodazole. The cells were then fixed with glutaraldehyde and processed for electron microscopy. Thin sections reveal the presence of stacks of cisternae in the cytoplasm of such cells. Bar, 0.2 µm.

discrete structures, but fail to further localize the Golgi complex (data not shown). In contrast, cells pretreated with BFA to relocalize Golgi membranes to the ER, and then incubated at 16°C in the absence of BFA, are not capable of reforming the Golgi apparatus even after 6 h (Fig. 6, C and D). These findings further corroborate the notion that budding events (as speculated to be needed for recovery from BFA) and not fusion events (those required for IQ generated vesicles to form cisternae) are inhibited at 16°C.

We wanted to examine the ultrastructure of the Golgi membranes formed at 16°C from IQ generated VGMs. An electron micrograph of these cells (Fig. 7) shows that the structures formed from VGMs at 16°C are composed of stacks of cisternae indicating that at 16°C VGMs are capable of fusing to form stacks of Golgi cisternae, but do not progress further along the assembly pathway. If the cells at this stage are shifted to 37°C, within 60 min the Golgi membranes are found to be clustered in the pericentriolar position (Fig. 8, A–C) indicating that the stacks formed at 16°C are indeed kinetic intermediates that are capable of pericentriolar relocalization.

We wished to monitor the kinetics of Golgi stack formation from VGMs at 16°C. NRK cells pretreated with IQ were subsequently incubated in drug-free medium at either 16 or 37°C. As shown in Fig. 9, VGMs fuse into large structures after 15 min at either temperature while microtubules are still depolymerized (Fig. 9, A–D). It appeared that the struc-

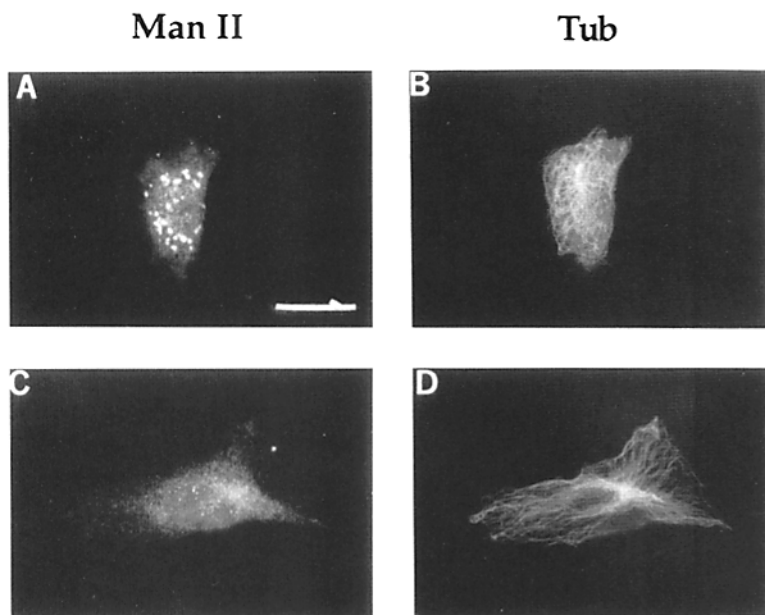


Figure 6. VGMs assemble into stacks of Golgi cisternae at 16°C upon removal of IQ. NRK cells were treated with IQ (A and B) or BFA (C and D) for 30 min at 37°C. The cells were washed extensively to remove drug, and then placed at 16°C for 6 h. The cells were fixed, permeabilized, and double labeled with an anti-mannosidase II antibody and an anti-tubulin antibody. Anti-mannosidase II and anti-tubulin antibodies were revealed by using a goat anti-rabbit antibody conjugated to rhodamine and goat anti-rat antibody conjugated to fluorescein, respectively. In cells treated with IQ, VGMs reassemble into large discrete structures that are dispersed in the cytoplasm, whereas the Golgi membranes in cells treated with BFA display a fully dispersed staining pattern. Bar, 20 μ m.

tures formed at 16°C may on average be somewhat smaller than those formed at 37°C, and additionally, a smaller percentage (75 vs 95%) of the cells showed this phenotype. After 30 min of recovery, cells at 37°C are already beginning to show very large aggregates of Golgi stacks and some pericentriolar clustering (Fig. 9 E), which is probably due to the reorganization of the microtubule network at this temperature (Fig. 9 F). However, cells left at 16°C after 30 min show little if any progression beyond the large structures seen after 15 min (Fig. 9 G), and it is noteworthy that

microtubules are still depolymerized (Fig. 9 H). In fact, after 3 h at 16°C the Golgi membranes are still found in stacks throughout the cytoplasm while the microtubule network is just beginning to display asters (Fig. 10). Therefore, although microtubules do not repolymerize with the same kinetics at the two temperatures, the kinetics of the formation of Golgi stacks from VGMs is approximately the same. Our findings suggest a more general correlation of temperature dependence with regard to budding and fusion. Budding events such as those needed to form VGMs or transport vesicles (the latter has also been proposed as a mechanism for the reestablishment of the Golgi apparatus after BFA treatment) cannot occur at 16°C. However, fusion events such as those needed to form stacks of Golgi cisternae from VGMs are not inhibited at 16°C.

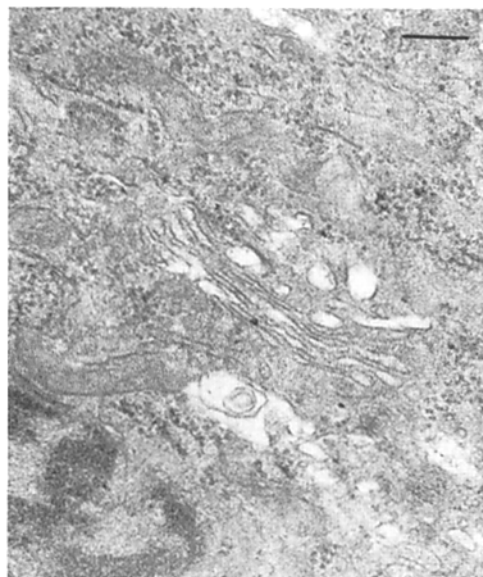


Figure 7. VGMs assemble into stacks of Golgi cisternae indistinguishable from control Golgi stacks, upon removal of IQ at 16°C. NRK cells pretreated with IQ were washed free of drug, and then incubated at 16°C for 6 h. Thin sections were then visualized by electron microscopy. As is evident from this micrograph, under these conditions, the VGMs have reassembled into stacks of Golgi cisternae. Bar, 0.2 μ m.

Discussion

Golgi Breakdown by IQ Is Independent of Cytoplasmic Microtubule Depolymerization

Treatment of cells with microtubule disrupting agents such as nocodazole, dismantles the Golgi complex into small stacks that then disperse throughout the cytoplasm. Upon removal of nocodazole the Golgi stacks reassemble and localize to the pericentriolar region. Based on these findings it has been suggested that microtubules are required for maintaining the stacks in a complex as well as localizing the Golgi complex to the MTOC. Therefore, although IQ depolymerizes cytoplasmic microtubules, previous data suggest that mere disruption of microtubules is not sufficient to cause Golgi membrane vesiculation. Indeed, our findings suggest that the vesiculation of the Golgi apparatus occurs in a microtubule-independent manner. Whether cytoplasmic microtubules are first stabilized with taxol or depolymerized with colcemid, the observed effects of IQ on the Golgi membranes remains the same. Our results, therefore, demonstrate that IQ acts independently on both the Golgi membranes and the cytoplasmic microtubules and that the breakdown of

Man II

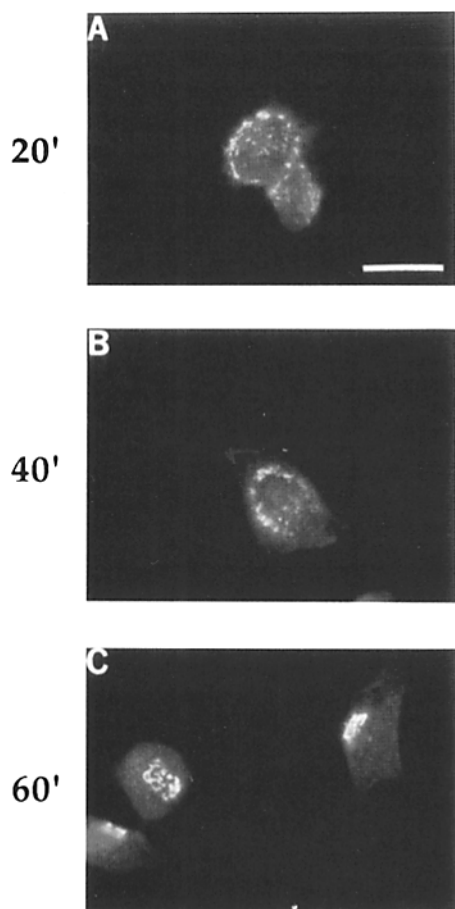


Figure 8. Dispersed stacks of Golgi cisternae formed at 16°C upon removal of IQ are capable of assembly in the pericentriolar region. NRK cells were treated with IQ for 30 min at 37°C, and then allowed to recover for 6 h at 16°C in the absence of drug. The cells were then transferred to 37°C and after 20, 40, and 60 min of incubation, were fixed and visualized by immunofluorescence using an anti-mannosidase II antibody followed by a goat anti-rabbit antibody conjugated to rhodamine. It is evident that after 20 min of incubation at 37°C (A), the Golgi stacks have already begun to form a ring around the nucleus; after 40 min, the stacks are beginning to move to one side of the nucleus (B). After 60 min, the Golgi stacks are found in a tight complex at the pericentriolar position (C). Bar, 20 μm .

Golgi membranes by IQ treatment occurs autonomously with regards to microtubules.

The Reformation of the Golgi Complex Takes Place in Two Steps, a Microtubule-independent and a Microtubule-dependent Step

The reformation and relocation of the Golgi complex after IQ removal takes place in at least two distinct stages that can be detected morphologically. First, the small VGMs fuse with each other to form stacks of Golgi cisternae. This event takes place in the presence of nocodazole and is therefore, microtubule-independent. The second step, in which the stacks come together and localize near the MTOC, only oc-

curs in the presence of polymerized microtubules. The reassembly of Golgi membrane after removal of BFA also takes place in two sequential steps, the microtubule-independent assembly of numerous Golgi stacks followed by the microtubule-dependent clustering of these stacks in the pericentriolar region (Lippincott-Schwartz et al., 1990). Additionally, Okadaic acid (OA) has been shown to cause fragmentation of Golgi complex into cluster of vesicles and numerous isolated vesicular structures (Lucocq et al., 1991). Upon removal of OA, these vesicular structures first assemble into Golgi stacks, which then congregate in the juxtannuclear region of the cells (Lucocq et al., 1991). Warren and colleagues (Lucocq et al., 1989) have shown that in the telophase stage of cell cycle the mitotic Golgi membranes (clusters of vesicles and isolated vesicles generated from interphase Golgi membranes) also first assemble into numerous stacks which then cluster as a complex in the pericentriolar region of each daughter cell. Although the role of microtubules in the Golgi complex assembly pathway after mitosis or upon removal of OA has not been investigated, it is clear that the reassembly occurs in at least two distinct steps. During the assembly process, we expect that VGMs fuse in a homotypic manner: those derived from one cisterna, e.g., *cis*, would fuse with other *cis*-derived VGMs to form a *cis*-Golgi cisterna. In contrast, vesicular protein transport necessitates heterotypic fusion events: vesicles produced from one Golgi cisterna, e.g., *cis*, fuse with the medial cisterna to ensure forward movement of proteins en route to the cell surface during exocytosis. Both VGM fusion to form cisternae, as well as the fusion of Golgi-derived non-clathrin-coated transport vesicles with their target cisternae, are microtubule-independent fusion events. How are vesicles targeted to fuse with other vesicular structures derived from the same maternal cisternae, and how do these homotypic fusion processes differ from the heterotypic fusion processes required for transport? Further, it remains puzzling as to how these scattered VGMs efficiently find their appropriate targets and fuse such that the resulting cisternae retain their identity and form stacks that reestablish their inherent topological polarity.

The second step in the reassembly pathway is the migration of the stacks to the pericentriolar position. This process is inhibited by nocodazole thus implicating a role for microtubules. This is not surprising, since it has been shown before that disruption of microtubules causes the Golgi stacks to scatter and upon reestablishment of the microtubule network these stacks move along repolymerized microtubules to the pericentriolar position (Ho et al., 1989). Furthermore, it has been shown that the *trans*-Golgi cisternae are in a dynamic association with similar cisternae of adjacent Golgi stacks by tubular processes (Cooper et al., 1990). These tubular processes extend from the *trans*-cisterna of one Golgi stack and fuse with the *trans*-cisterna of an adjacent stack. These tubular connections are disrupted by nocodazole suggesting that tubular formation is a microtubule-dependent process. Thus, microtubules are necessary for the clustering of the Golgi stacks and the relocation of the Golgi complex to the nucleus.

How might IQ, which appears to affect microtubules and Golgi membranes independently, be operating? One obvious explanation is that it has (at least) two distinct targets: one on microtubules (or on the monomer form of tubulin thus

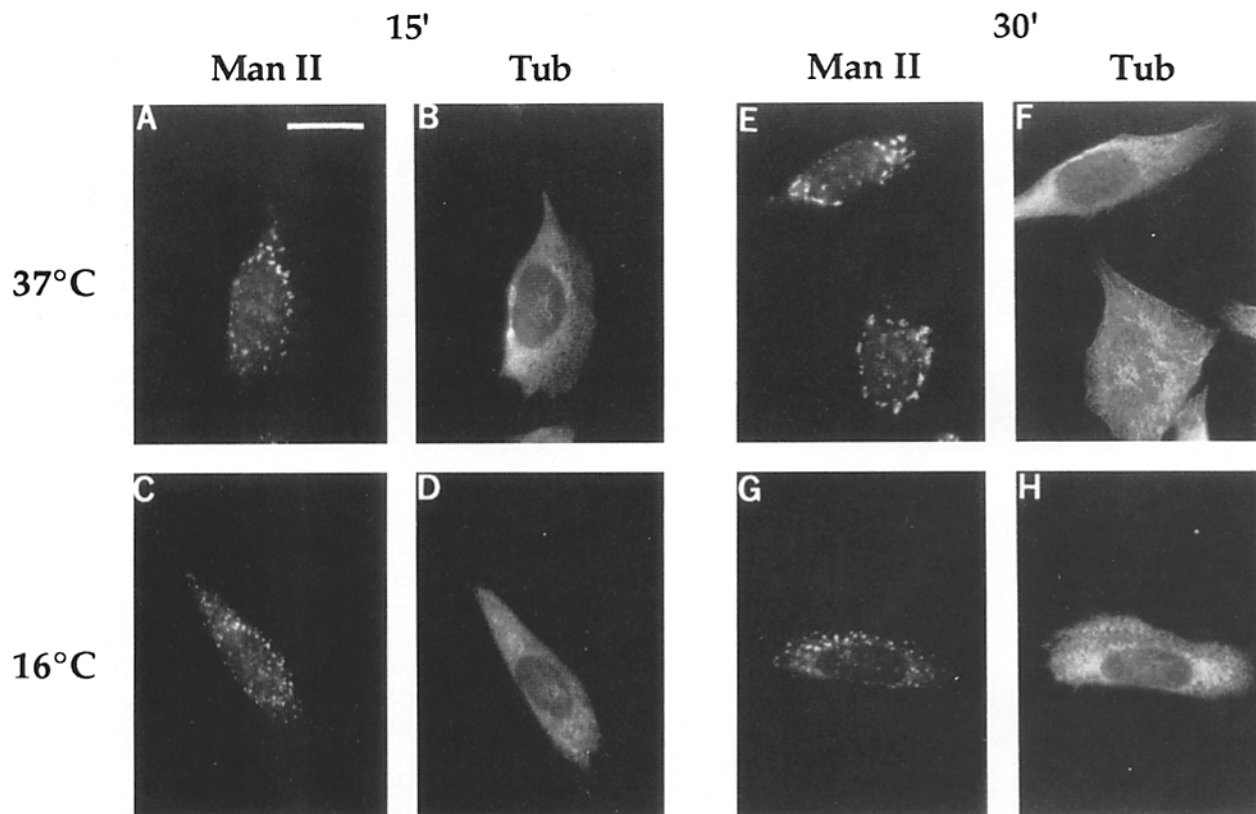


Figure 9. Kinetics of assembly of VGMs into stacks of Golgi cisternae at 16 and at 37°C. NRK cells were treated with IQ to dismantle Golgi membranes into VGMs. The cells were washed extensively to remove drug, and then incubated either at 16 or at 37°C for 15 and 30 min. The cells were fixed, permeabilized, and double stained with an anti-mannosidase II antibody and an anti-tubulin antibody. Anti-mannosidase II and anti-tubulin antibodies were revealed by using a goat anti-rabbit antibody conjugated to rhodamine and a goat anti-rat antibody conjugated to fluorescein, respectively. After 15 min, discrete Golgi structures are evident at both 16 (C) and 37°C (A), while in both cases microtubules are still depolymerized (B and D). After 30 min at 37°C, the Golgi structures are very large and are already beginning to migrate toward the nuclear region (E), a result of the already repolymerized microtubules at this time point (F). However, at 16°C, the microtubules have not repolymerized (H) and the Golgi structures remain dispersed throughout the cytoplasm (G). Bar, 20 μm .

preventing microtubule repolymerization), and one on Golgi membranes themselves triggering vesiculation. Another possible explanation would involve just one target and invokes a protein or protein complex that acts as a scaffold tethering the Golgi apparatus to the MTOC. In this model, a skeleton holding the cisternae in stack form, makes contact with the scaffold apparatus. The scaffold itself interacts with microtubules and tethers the Golgi complex to the MTOC region. Disruption of this scaffold by IQ would signal the

Golgi complex to vesiculate. If this scaffold is part of the fundamental core of the MTOC, the microtubules would also begin to rapidly depolymerize. Stabilizing microtubules with taxol would prevent the depolymerization of cytoplasmic microtubules, however the disassembly of the scaffold would still trigger Golgi membrane vesiculation. Upon removal of the scaffold dismantling drug (IQ), Golgi membranes would be programmed to fuse to form cisternae which reassemble into stacks, however unless microtubules

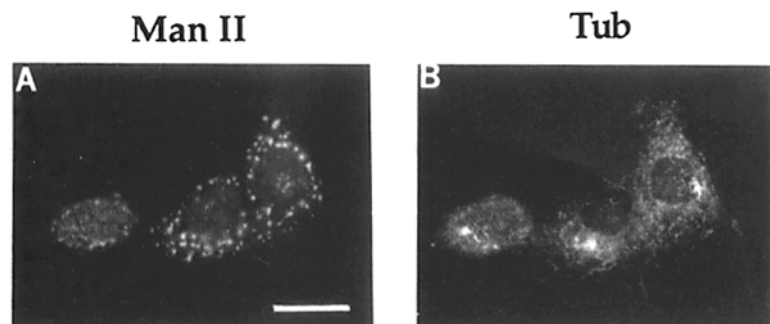


Figure 10. At 16°C, the Golgi stacks formed from VGMs remain scattered throughout the cytoplasm and after 3 h, asters of the microtubule network are just beginning to form. NRK cells pretreated with IQ, and then washed free of drug were incubated for 3 h at 16°C. The cells were fixed, permeabilized, and double stained with an anti-mannosidase II antibody and an anti-tubulin antibody. Anti-mannosidase II and anti-tubulin antibodies were revealed by using a goat anti-rabbit antibody conjugated to rhodamine and a goat anti-rat antibody conjugated to fluorescein, respectively. The Golgi stacks (A) are dispersed in the cytoplasm, and the asters of microtubules (B) are evident. Bar, 20 μm .

are polymerized, the formed stacks cannot unite and relocate to the MTOC. The skeleton would be key in maintaining cisternae in stack form, and the scaffold would tether the unit to the MTOC. Mere disruption of microtubules with drugs such as nocodazole, does not disrupt the reputed scaffolding or the skeleton, but allows the stacks to float away from each other and the MTOC while still maintaining their topological polarity.

VGMs Fuse but Do Not Form at 16°C

Our results demonstrate that IQ cannot vesiculate Golgi membranes into VGMs at 16°C. However, VGMs fuse to form stacks of Golgi cisternae at 16°C. In contrast, if the cells are first treated with BFA, and then washed free of drug and incubated at 16°C, Golgi membranes do not reassemble. Recent studies indicate that the retrograde transport of Golgi enzymes into ER observed upon BFA treatment is dependent upon microtubules (Lippincott-Schwartz et al., 1990). To rule out the possibility that the IQ-derived vesicles assumed an ER fate in the presence of microtubules, we pretreated NRK cells with taxol followed by IQ treatment in the presence of taxol, and allowed them to recover at 16°C in the absence of drug. We found that stacks of Golgi cisternae were still capable of forming under these conditions (evident by staining of cells with the antibody against the medial Golgi specific polypeptide mannosidase II by immunofluorescence, data not shown), while they could not in cells pretreated with BFA. Therefore, in the presence of taxol-stabilized microtubules, VGMs produced by IQ action remain in the cytoplasm and do not relocate into ER. Since it is the generation and not the consumption of IQ vesicles that is inhibited at 16°C, it is tempting to speculate that the 16°C block generally observed in transport processes, is in the inability to form a vesicular structure.

We have found that during the reassembly process at 16°C, Golgi stacks remain scattered in the cytoplasm, even after 6 h after which time the microtubule network has been reestablished. Why don't these stacks move along microtubules to the pericentriolar position of the cells? The migration of these stacks along microtubules requires at least two distinct steps: first, the binding of stacks to microtubules which requires NEM sensitive cytosolic factors and Golgi membrane associated receptors but in vitro, can occur without cytoplasmic dynein (Karecla and Kreis, 1992), and second, the dynein-dependent migration of stacks toward the pericentriolar region (Corthésy-Theulaz, 1992). Perhaps one or both of these steps is temperature sensitive and does not operate efficiently at 16°C. It is known that the dispersal of the Golgi complex into stacks upon depolymerization of microtubules with nocodazole, and the recovery of Golgi complex upon removal of the drug, are multi step processes with different sensitivities to temperature, energy, and ionic perturbations (Turner and Tartakoff, 1989). Our results show that further breakdown of stacks into VGMS and the recovery of stacks from VGMs also have different requirements. While VGMs do not form at 16°C, they are capable of fusing at 16°C and both of these processes are microtubule independent.

The question remains as to how these VGMs are scattered throughout the cytoplasm. It seems improbable that a motor protein carries them along microtubules (or a hitherto uncharacterized cytoskeletal track) distributing them through-

out the cell especially since their dispersal occurs in the absence of polymerized microtubules. A more plausible explanation is that these VGMs do not inherit the ability that the parent Golgi complex has of binding to and moving along microtubules. Instead, the VGMs diffuse independently of microtubules in a random fashion throughout the cell. This leaves a very provocative question unanswered: if Golgi stacks bind to and move along microtubules, but VGMs are incapable of binding to microtubules, at what point during the reassembly of the organelle is the ability to bind to microtubules conferred onto the Golgi membranes?

We thank Kristi Elkins for tissue culture, Drs. John Newport, Douglass Forbes, Mike Yaffe, Scott Emr, and Ron Vale for useful discussions.

This work was supported by National Institutes of Health grant RO1 GM46224 and a Basil O'Connor starter scholars award from the March of Dimes Birth Defect Foundation to V. Malhotra.

Received for publication 26 March 1993 and in revised form 4 June 1993.

References

- Cooper, M. S., A. H. Cornell-Bell, A. Chernjavsky, J. W. Dani, and S. J. Smith. 1990. Tubulovesicular processes emerge from the trans-Golgi cisternae, extend along microtubules, and interlink adjacent trans-Golgi elements into a reticulum. *Cell*. 61:135-145.
- Corthésy-Theulaz, I., A. Pauloin, and S. R. Pfeffer. 1992. Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* 118:1333-1345.
- De Brabander, M., R. Nuydens, H. Geerts, and C. R. Hopkins. 1988. Dynamic behavior of the transferrin receptor followed in living epidermoid carcinoma (A431) cells with nanovid microscopy. *Cell Motil. Cytoskeleton*. 9:30-47.
- Featherstone, C., G. Griffiths, and G. Warren. 1985. Newly synthesized G protein of Vesicular Stomatitis Virus is not transported to the Golgi complex in mitotic cells. *J. Cell Biol.* 101:2036-2046.
- Gruenberg, J., G. Griffiths, and K. E. Howell. 1989. Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with the assay of vesicle fusion in vitro. *J. Cell Biol.* 108:1301-1306.
- Herman, B., and D. F. Albertini. 1984. A time-lapse video image intensification analysis of cytoplasmic organelle movements during endosome translocation. *J. Cell Biol.* 98:565-576.
- Ho, W. C., V. J. Allan, G. vanMeer, T. E. Berger, and T. E. Kreis. 1989. Reclustering of scattered Golgi elements occurs along microtubules. *Eur. J. Cell Biol.* 48:250-263.
- Iida, H., and Y. Shibata. 1991. Functional Golgi units in microtubule-disrupted cultured atrial myocytes. *J. Histochem. Cytochem.* 39:1349-1355.
- Jordan, M. A., D. Thrower, and L. Wilson. 1992. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles; Implications for the role of microtubule dynamics in mitosis. *J. Cell Sci.* 102:401-416.
- Karecla, P. I., and T. E. Kreis. 1992. Interaction of membranes of the Golgi complex with microtubules in vitro. *Eur. J. Cell Biol.* 57:139-146.
- Kreis, T. E. 1990. Role of microtubules in the organization of the Golgi apparatus. *Cell Motil. Cytoskeleton*. 15:67-70.
- Lippincott-Schwartz, J., J. G. Donaldson, A. Schweitzer, E. G. Berger, H. P. Hani, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of Brefeldin A suggests an ER recycling pathway. *Cell*. 60:821-836.
- Lucocq, J., and G. Warren. 1987a. Fragmentation and partitioning of the Golgi apparatus during mitosis in HeLa cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3239-3246.
- Lucocq, J. M., J. G. Pryde, E. G. Berger, and G. Warren. 1987b. A mitotic form of the Golgi apparatus in HeLa cells. *J. Cell Biol.* 104:865-874.
- Lucocq, J. M., E. G. Berger, and G. Warren. 1989. Mitotic Golgi fragments in HeLa cells and their role in the reassembly pathway. *J. Cell Biol.* 109:463-474.
- Lucocq, J. M., G. Warren, and J. Pryde. 1991. Okadaic acid induces Golgi apparatus fragmentation and arrest of intracellular transport. *J. Cell Sci.* 100:753-759.
- Matlin, K. S., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell*. 34:233-243.
- Matteoni, R., and T. E. Kreis. 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. *J. Cell Biol.* 105:1253-1265.
- Moreno, S., and P. Nurse. 1990. Substrates for p34cdc2. In vitro veritas. *Cell*. 61:549-551.
- Rindler, M. J., I. E. Ivanov, and D. Sabatini. 1987. Microtubule-acting drugs lead to nonpolarized delivery of the influenza hemagglutinin to the cell surface of polarized Madin-Darby canine kidney cells. *J. Cell Biol.* 104:231-241.

- Rogalski, A. A., and S. J. Singer. 1984. Association of the elements of the Golgi apparatus with microtubules. *J. Cell Biol.* 99:1092-1100.
- Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest Virus membrane glycoproteins to the cell surface. *Cell.* 38:535-549.
- Scheel, J., R. Matteoni, T. Ludwig, B. Hofflack, and T. Kreis. 1990. Microtubule depolymerization inhibits transport of cathepsin D from the Golgi apparatus to lysosomes. *J. Cell Sci.* 96:711-720.
- Takizawa, P. A., J. K. Yucel, B. Veit, J. D. Faulkner, T. Deerinck, G. Soto, M. Ellisman, and V. Malhotra. 1993. Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite ilimaquinone. *Cell.* 73:1079-1090.
- Tartakoff, A. 1986. Temperature and energy dependence of secretory protein transport in the exocrine pancreas. *EMBO (Eur. Mol. Biol. Organ.) J.* 5: 1477-1482.
- Tokuyasu, K. T. 1992. Effects of washing on positive staining. *Acta Histochem. Cytochem.* 25:143-146.
- Turner, J. R., and A. M. Tartakoff. 1989. The response of the Golgi complex to microtubule alterations: the roles of metabolic energy and membrane traffic in Golgi complex organization. *J. Cell Biol.* 109:2081-2088.
- Warren, G. 1985. Membrane traffic and organelle division. *TIBS (Trends Biochem. Sci.)*. 10:439-443.
- Warren, G. 1989. Mitosis and membranes. *Nature (Lond.)*. 327:17-18.