Kinesin Is the Motor for Microtubule-mediated Golgi-to-ER Membrane Traffic

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Abstract. The distribution and dynamics of both the ER and Golgi complex in animal cells are known to be dependent on microtubules; in many cell types the ER extends toward the plus ends of microtubules at the cell periphery and the Golgi clusters at the minus ends of microtubules near the centrosome. In this study we provide evidence that the microtubule motor, kinesin, is present on membranes cycling between the ER and Golgi and powers peripherally directed movements of membrane within this system. Immunolocalization of kinesin at both the light and electron microscopy levels in NRK cells using the H1 monoclonal antibody to kinesin heavy chain, revealed kinesin to be associated with all membranes of the ER/Golgi system. At steady-state at 37°C, however, kinesin was most concentrated on peripherally distributed, pre-Golgi structures containing βCOP and vesicular stomatitis virus glycoprotein newly released from the ER. Upon temperature reduction or nocodazole treatment, kinesin's distribution shifted onto the Golgi, while with brefeldin A (BFA)-treatment, kinesin could be found in both Golgi-derived tubules and in the ER. This suggested that kinesin associates with membranes that constitutively cycle between the ER and Golgi. Kinesin's role on these membranes was examined by microinjecting kinesin antibody. Golgi-to-ER but not ER-to-Golgi membrane transport was found to be inhibited by the microinjected anti-kinesin, suggesting kinesin powers the microtubule plus end-directed recycling of membrane to the ER, and remains inactive on pre-Golgi intermediates that move toward the Golgi complex.

MICROTUBULES are believed to play an important role in the optimal organization and distribution of organelles and their transport intermediates in higher eukaryotic cells. During interphase in many non-epithelial cell types, these polarized filaments have their slow growing, or "minus," ends localized at the microtubule organizing center (MTOC)[1] near the nucleus, and their fast growing, or "plus," ends radiating outwards toward the cell periphery. Organelles and their transport intermediates use this elaborate network to spatially segregate themselves, translocating toward the minus ends (inward) or plus ends (outward) of microtubules after attaching to them (Schroer and Sheetz, 1991; Bloom, 1992).

The ER and Golgi complex are two examples of organelles whose distribution and dynamics are dependent on microtubules. Together, these organelles function in the generation, processing, and sorting of lipid and protein moving through the secretory pathway. Their subcellular positions and morphologies are remarkably different, however, due in large part to their interaction with microtubules. The ER comprises an extensive array of interconnecting tubules and cisternae that extend throughout the cytoplasm (Lee et al., 1989). Microtubules provide a scaffold for the peripheral, plus end-directed extension of this reticulum (Terasaki et al., 1986; Dabora and Sheetz, 1988; Vale and Hotani, 1988). The Golgi complex, by contrast, consists of compact stacks of cisternae which use microtubules to actively cluster inwards toward the MTOC (Thyberg and Moskalewski, 1985; Ho et al., 1989), where microtubule minus ends are anchored. In the absence of microtubules, Golgi elements disperse throughout the cytoplasm (Rogalski and Singer, 1984; Turner and Tartakoff, 1989). Why the ER and Golgi complex maintain this association with microtubules is not clear, but probably relates to their distinct functions. The ER mediates a diverse set of processes, including protein synthesis, folding, and assembly; lipid biosynthesis and metabolism; detoxification; nuclear compartmentalization; regulation of ion gradients; and membrane transport into the secretory pathway (Sitia and Meldolesi, 1993; Lippincott-Schwartz,

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1. *Abbreviations used in this paper*: BFA, brefeldin A; MTOC, microtubule organizing center; VSV G, vesicular stomatitis virus G.

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1994). The microtubule-facilitated spatial extension of the ER could serve to facilitate these processes. The Golgi complex, by comparison, functions primarily in the receipt, processing and sorting of membrane lipids and proteins arriving from the ER (Mellman and Simons, 1992). The proximity of the Golgi to the MTOC, the hub of the radially arranged microtubules that serve as highways for membrane traffic, is likely to promote delivery of secretory products to specific regions of the plasma membrane (Rogalski et al., 1984), and facilitate communication between the secretory and endocytic pathways (Farquhar, 1991).

Microtubules also have been proposed to play a role in the membrane transport pathways connecting the Golgi and ER. Exchange of lipids and proteins between these organelles is controlled by forward (ER-to-Golgi) and reverse (Golgi-to-ER) membrane transport pathways (Lippincott-Schwartz, 1993). Whereas forward transport is followed by newly synthesized protein and lipid enroute to the cell surface or lysosomes, reverse transport recycles escaped ER resident and other proteins, as well as lipid needed for maintaining the surface area of the ER in the face of forward membrane traffic through the secretory pathway (Pelham, 1991). ER-to-Golgi transport has been shown to involve coatomer-enriched, pleiomorphic transport intermediates (Saraste and Kuismanen, 1984; Plutner et al., 1992; Pepperkok et al., 1993) which often must travel significant distances from peripheral ER sites toward the centrally located Golgi complex (Saraste and Svensson, 1991). This movement has been proposed to be facilitated by microtubules, with transport occurring toward their minus ends (Saraste and Svensson, 1991; Mizuno and Singer, 1994). Golgi-to-ER traffic observed in brefeldin A (BFA)-treated cells, by contrast, has been shown to utilize membrane tubules which extend plus end-directed along microtubules outwards from centrally located Golgi structures (Lippincott-Schwartz et al., 1990; Hauri and Schweizer, 1992). The emerging picture of membrane traffic within the ER/Golgi system is therefore one in which microtubules control both organelle positioning, and transport of vesicular and tubular intermediates. The net effect is to permit the ER and Golgi to be spatially segregated, but functionally integrated compartments of the secretory pathway.

The involvement of microtubules in positioning and translocating membranes of the ER/Golgi system requires the activity of motor proteins that interact with both microtubules and membranes. Two microtubule-stimulated ATPases, kinesin (Brady, 1985; Vale et al., 1985*a*; Scholey et al., 1985; Kuznetsov and Gelfand, 1986) and cytoplasmic dynein (Lye et al., 1987; Paschal et al., 1987; Schroer et al., 1989), along with numerous kinesin-related proteins (Bloom and Endow, 1994) have been demonstrated to mediate microtubule-based motility within cells. While kinesin translocates membranes toward the plus ends of microtubules and the cell periphery (Vale et al., 1985*b*), dynein is known to be involved in membrane movement towards the minus ends of microtubules and the cell center (Paschal and Vallee, 1987). Considerable progress has been made in defining the enzymatic activities of kinesin and dynein, their polypeptide composition and structure, their associated and related proteins, and their interactions with microtubules. Headway has only begun to be made, however, on the subcellular distributions of dynein and kinesin, and their interactions with membrane-bound organelles. Immunolocalization studies have demonstrated that these ATPases are present on membrane-bound organelles in numerous cell types (Hollenbeck, 1989; Pfister et al., 1989; Hirokawa et al., 1990, 1991; Koonce and McIntosh, 1990; Wright et al., 1991; Fath et al., 1994). More specifically, there is evidence for the association of both dynein and kinesin with lysosomes and synaptic vesicles, and of kinesin with mitochondria, Golgi, and pigment and chromaffin granules (Hollenbeck and Swanson, 1990; Rodionov et al., 1991; Lacey and Haimo, 1992; Leopold et al., 1992; Lin and Collins, 1992; Marks et al., 1994; Schmitz et al., 1994). The extent of kinesin's association with ER/Golgi membranes and its regulation on these membranes, however, are issues in need of being addressed.

In the study described here, we examine the distribution and role of kinesin in the membrane structures comprising the ER/Golgi system. We show that kinesin is intimately associated with membranes that constitutively cycle between the ER and Golgi complex and provide evidence that kinesin is the motor for recycling membrane moving from the Golgi to the ER, towards microtubule plus ends.

Materials and Methods

Materials and Cell Culture

Nocodazole was purchased from Sigma Chemical Co. (St. Louis, MO) and was used at concentrations ranging between 1–20 μg/ml. When added to cells at 4°C, subsequent incubation at 37°C resulted in the depolymerization of all microtubules (detected by immunofluorescence) within 5 min of warming. BFA was purchased from Epicentre Technology (Madison, WI) and was used at 1-5 μg/ml. Fluorescein dextran (10 kD, lysine fixable) was purchased from Molecular Probes (Eugene, OR) and used at 0.3 mg/ml.

NRK, Hela, and M1 fibroblasts were grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 150 μg/ml penicillin or 2.5 μg/ml gentamycin at 37°C in 5% CO_2.

Antibodies

Mouse H1 monoclonal IgG1 to kinesin heavy chain has been previously characterized by Pfister et al. (1989) and Hirokawa et al. (1989). Mouse anti-58K-9 monoclonal antibody directed against a microtubule binding Golgi protein has previously been characterized by Bloom and Brashear (1989). Mouse IgG directed against vesicular stomatitis virus G protein (VSV G) was a generous gift of Dr. C. Macamer (Johns Hopkins University, Baltimore, MD). Rabbit IgG directed against Golgi mannosidase II was a kind gift of Dr. K. Moremen (University of Georgia, Athens, GA). Rabbit polyclonal antibodies to ER resident proteins were generously provided by Dr. D. Louvard (Pasteur Institut, Paris, France). Rabbit polyclonal antibody, O_{14}, against human milk galactosyltransferase was kindly provided by Dr. E. Berger (Universität Zürich-Irchel, Zürich, Switzerland). Rabbit IgG antibodies to βCOP were generated by the authors against the EAGE peptide sequence of this protein as described in Duden et al., 1991. Fluorescein and rhodamine-labeled goat anti-rabbit IgGs, and fluorescein and rhodamine-labeled goat anti-mouse IgGs were purchased from Southern Biotechnology (Birmingham, AL). Nonspecific mouse IgG1 used in the microinjection experiments was purchased from Boehringer Mannheim Corp. (Indianapolis, IN).

VSV Infection

Infections with ts045 VSV were performed according to a modification of Bergman (1989). Cells were rinsed twice in RPMI without serum before adding 50–100 pfu/cell of ts045 VSV for 1 h at 32°C. Cells were washed two times in RPMI 1640 without serum to remove unadsorbed virus and then incubated in complete medium for 1 h at 37°C followed by 2 h at 40°C before the start of experiments.

Immunofluorescence Microscopy

Cells were grown on glass coverslips two days before experiments. After appropriate incubations, cells were fixed in 2% formaldehyde in PBS for

10 min at 25°C and then washed twice in PBS containing 10% fetal calf serum. Cells were then incubated in PBS containing antibody, 0.15% saponin, and 10% fetal calf serum for 1 h. After washing to remove unbound antibody, the cells were incubated with fluorescently labeled secondary antibody for 1 h followed by washing in PBS serum. Coverslips were mounted on glass slides in Fluoromount G (Southern Biotechnology) and viewed in a Zeiss IM35 or photo-microscope equipped with barrier filters to prevent cross over of fluorescein and rhodamine fluorescence.

Immunoelectron Microscopy

VSV-infected cells maintained at 40°C were placed on ice for 10 min and warmed to 32°C for 20 min in the presence of nocodazole (20 μg/ml). Confluent monolayers that had undergone these treatments were fixed for 2 d in 8% ρ-formaldehyde in 0.2 M phosphate buffer at pH 7.2. Fixed cells were removed from culture dishes, centrifuged for 5 min at 1,000 rpm in a clinical centrifuge, and infused for 4 h in 2.3 M sucrose + 4% ρ-formaldehyde in PBS. Cell pellets were frozen on aluminum pins in liquid nitrogen, sectioned at a thickness of ~100 nm, and picked up on 100-mesh carbon and formvar coated copper grids. Sections were blocked with 5% BSA in 0.01 M glycine/PBS, and labeled with purified H1 monoclonal antibody to kinesin heavy chain at 40 μg/ml for 30 min. After 3 x 5-min rinses, sections were incubated for 30 min with rabbit anti-mouse IgG, rinsed 3 × 5 min, and floated for 30 min on protein A-conjugated 20-nm gold particles at 10 μg/ml protein. Sections were then fixed in 1% glutaraldehyde in PBS for 5 min, rinsed 3 × 5 min, blocked with 5% BSA in glycine/PBS, and labeled for 30 min with an affinity-purified polyclonal antibody to VSV G protein. After 3 × 5-min rinses, sections were incubated with protein A-conjugated 10-nm gold particles at 10 μg/ml protein for 30 min. All antibodies and protein A-gold conjugates were suspended in 5% BSA/0.01 M glycine/PBS, pH 7.2, while all rinses were in 0.01 M glycine/PBS. Following the final protein A-gold step, sections were rinsed 3 × 5 min, fixed in 1% glutaraldehyde in PBS for 5 min, rinsed 3 × 5 min in glass distilled H_2O, and floated on 2% methylcellulose with 2% uranyl acetate (7:1). As controls, primary antibodies were omitted or sections were labeled with irrelevant primary antibodies.

Results

Localization of Kinesin on Peripheral Pre-Golgi Elements

To determine whether kinesin associates with membranes of the ER/Golgi system, we used the H1 monoclonal antibody, that is specific for kinesin heavy chain (Hirokawa et al., 1989; Pfister et al., 1989), along with markers for the ER, Golgi and intermediate compartment to compare the distributions of these markers within NRK cells. As illustrated in Fig. 1, immunofluorescence staining of cells with the anti-kinesin antibody revealed labeling that was predominantly localized to numerous small vesicle-like structures scattered throughout the cytoplasm. A similar punctate distribution of kinesin staining was observed in a variety of cell types, including Hela, M1, and PtK_1 (not shown, see Pfister et al., 1989). When compared to the distribution of an ER marker in double immunofluorescence labeling experiments (Fig. 1), the punctate kinesin staining appeared quite distinct from the diffuse reticulum labeled by the ER-specific antibody although constellations of kinesin-containing structures frequently showed a pattern of staining resembling the ER. Double-labeling of cells for kinesin and the Golgi-specific enzyme, mannosidase II (man II) (Fig. 1), showed only a small proportion of the punctate kinesin staining in the juxtanuclear Golgi region, with most in peripheral sites. Under steady-state conditions, therefore, kinesin did not obviously associate to a significant extent with either ER or Golgi membranes when assessed by immunofluorescence labeling.

ER-to-Golgi membrane transport has been shown to occur via pleiomorphic vacuolar and tubulovesicular structures (called the intermediate compartment) which form at both central and peripheral ER sites, and translocate into the Golgi region (Saraste and Kuismanen, 1984; Hauri and Schweizer, 1992; Saraste and Svensson, 1991). These structures are coated with the peripheral membrane protein and coatomer subunit, βCOP (Pepperkok et al., 1993; Peter et al., 1993; Lippincott-Schwartz, 1993), which when assembled on membranes with other COPs, is believed to facilitate the formation of ER-to-Golgi transport intermediates (Rothman and Orci, 1992). When the distribution of kinesin was compared to that of βCOP in double-immunolabeling experiments, a significant colocalization of these two markers in numerous vesicle-like structures that were scattered throughout the cytoplasm was observed (see Fig. 1). H1 staining was more highly concentrated in punctate, peripheral structures containing βCOP than on the larger juxtanuclear structures labeled with βCOP. However, after 2 h of nocodazole treatment (which causes microtubules to depolymerize), H1 colocalized with both small and large βCOP-containing structures that were dispersed through the cytoplasm. Addition of BFA for 5 min to the nocodazole-treated cells caused βCOP to dissociate from the peripheral membrane structures, while H1 antibody remained associated with these membranes.

To further characterize the peripheral membrane structures labeled by the anti-kinesin H1 antibody in NRK cells, we compared the distribution of these structures to that of VSV G at various times and conditions after release of VSV G from the ER in cells infected with the temperature sensitive ts045 strain of vesicular stomatitis virus (VSV) (Bergman, 1989). At its restrictive temperature of 40°C, VSV G is unable to fold correctly and therefore cannot exit the ER. Upon warming the temperature to 32°C, however, VSV G folds properly and rapidly exits the ER moving as a synchronous population through the secretory pathway to the plasma membrane.

Immunofluorescence of infected cells that were held at 40°C for 2 h revealed that VSV G to be distributed throughout an extensive anastomosing network characteristic of the ER (Figs. 2 and 3, *top*), indicating a failure of the viral glycoprotein to reach pre-Golgi or Golgi membranes. Under these conditions, double-labeling experiments comparing the distribution of VSV G with kinesin (Fig. 2), or with man II (Fig. 3) demonstrated no significant colocalization. Reducing the temperature to 32°C for 5 min, however, caused a substantial proportion of VSV G to become colocalized with kinesin in numerous small peripheral structures (Fig. 2), which also contained βCOP (Fig. 3). Localization of VSV G in peripheral structures containing kinesin and βCOP was transient, however, with longer incubations at 32°C (i.e., 20 min) resulting in an accumulation of VSV G in juxtanuclear Golgi elements labeled with man II (Fig. 3). In contrast to VSV G, the distribution of kinesin (Fig. 2) and βCOP (not shown) during this time period was not altered, with kinesin remaining predominantly associated with small peripheral structures scattered throughout the cytoplasm, while βCOP localized in both these peripheral pre-Golgi structures and the central Golgi complex.

Strikingly, if cells were incubated in nocodazole at the same time the temperature was lowered to 32°C, much of VSV G failed to redistribute into the central Golgi region and remained associated with the peripheral structures con-

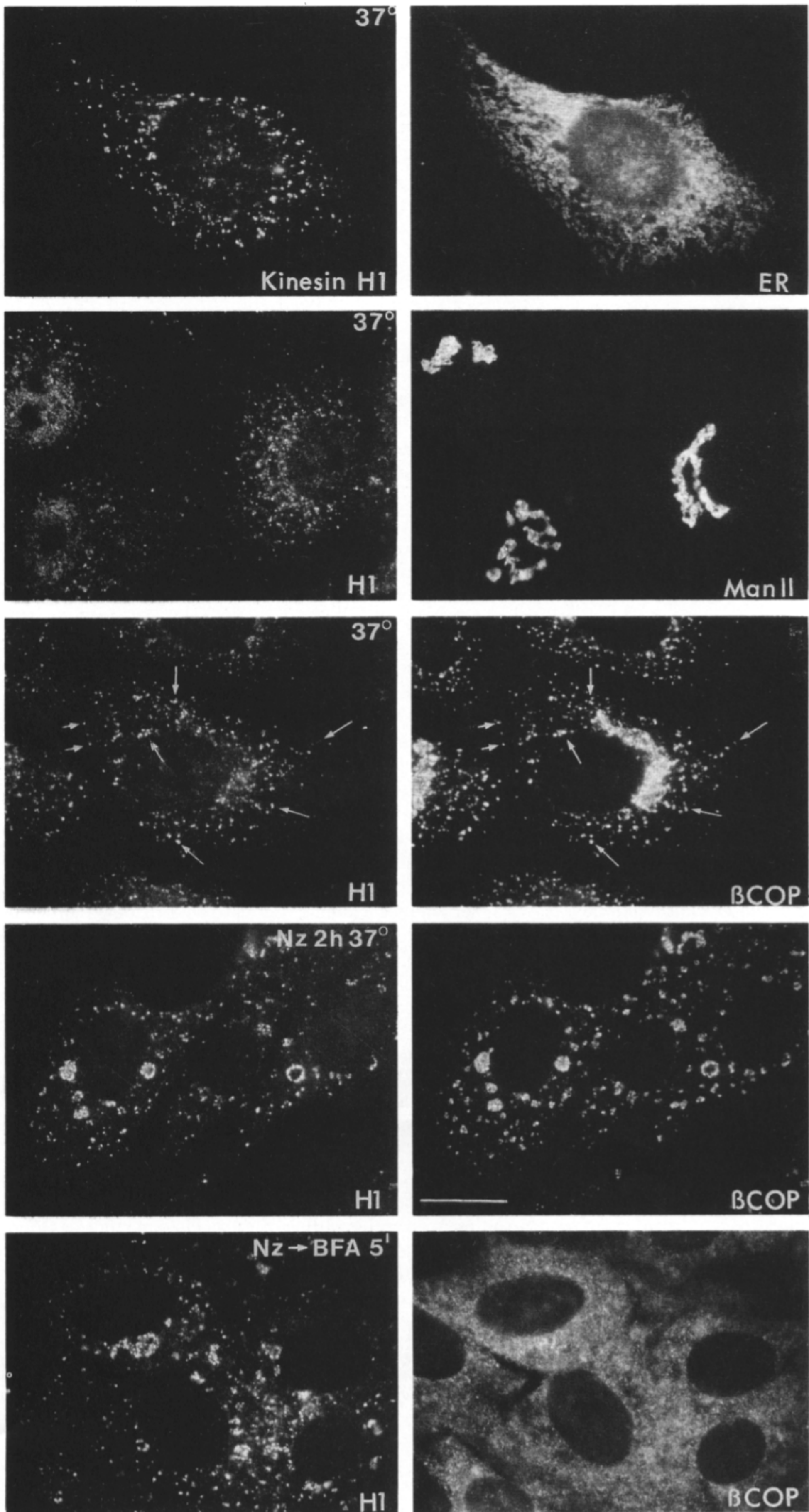

Figure 1. Localization of kinesin on peripheral elements enriched in βCOP: effects of nocodazole and BFA. NRK cells were incubated as indicated in each micrograph before fixation, permeabilization with saponin, and double-labeling with antibodies to kinesin (*H1*) plus marker proteins for the ER, Golgi (*man II*) or Golgi/intermediate compartment (βCOP). Note that in control cells at steady-state, kinesin colocalized only with βCOP in peripheral structures scattered throughout the cytoplasm. Colocalization of kinesin and βCOP was even more extensive in nocodazole-treated cells, but unlike βCOP, kinesin remained membrane-bound in the presence of BFA. Bar, 10 μm.

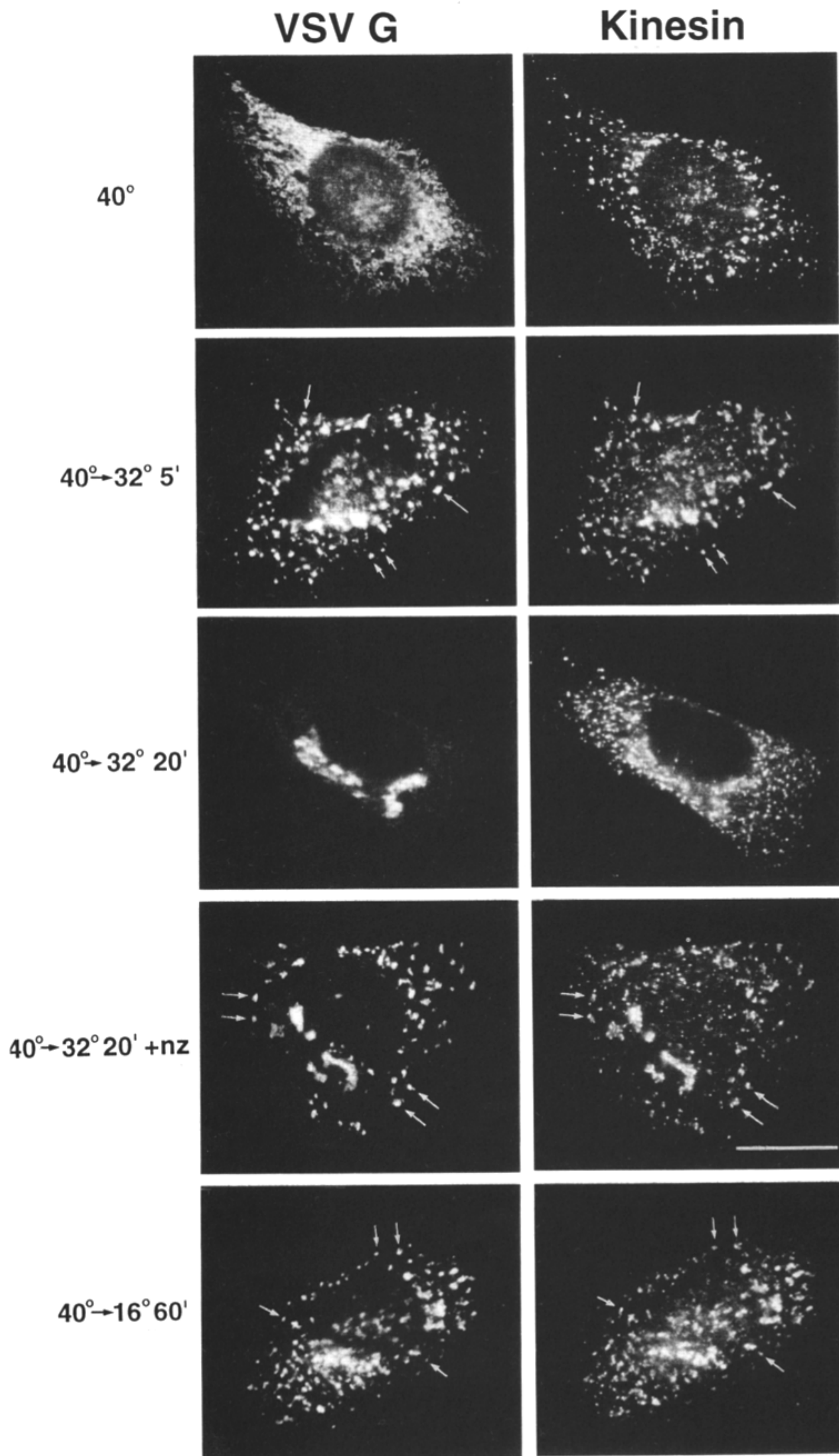

Figure 2. Association of kinesin with ER-to-Golgi transport intermediates carrying VSV G protein: effects of nocodazole and low temperature treatments. NRK cells infected with the temperature-sensitive ts045 strain of VSV were incubated at 40° for 2 h to accumulate VSV G protein in the ER. The cells were then fixed (*top*) or placed on ice for 10 min and then incubated under the following conditions before fixation: 32°C for 5 min; 32°C for 20 min; 32°C for 20 min in the presence of nocodazole; and 16°C for 1 h. Placement of cells on ice for 10 min was performed to ensure the complete depolymerization of microtubules upon warmup of cells in the presence of nocodazole. Cells were then permeabilized with saponin and incubated with antibodies to VSV G protein and kinesin, followed by rhodamine and fluorescein conjugated secondary antibodies. Arrows point to representative peripheral structures where VSV G and kinesin appear colocalized. This occurred shortly after release of VSV G protein from the ER (32°C, 5 min), at 16°C, and at later time points at 32°C when microtubules were disrupted with nocodazole. Bar, 10 μm.

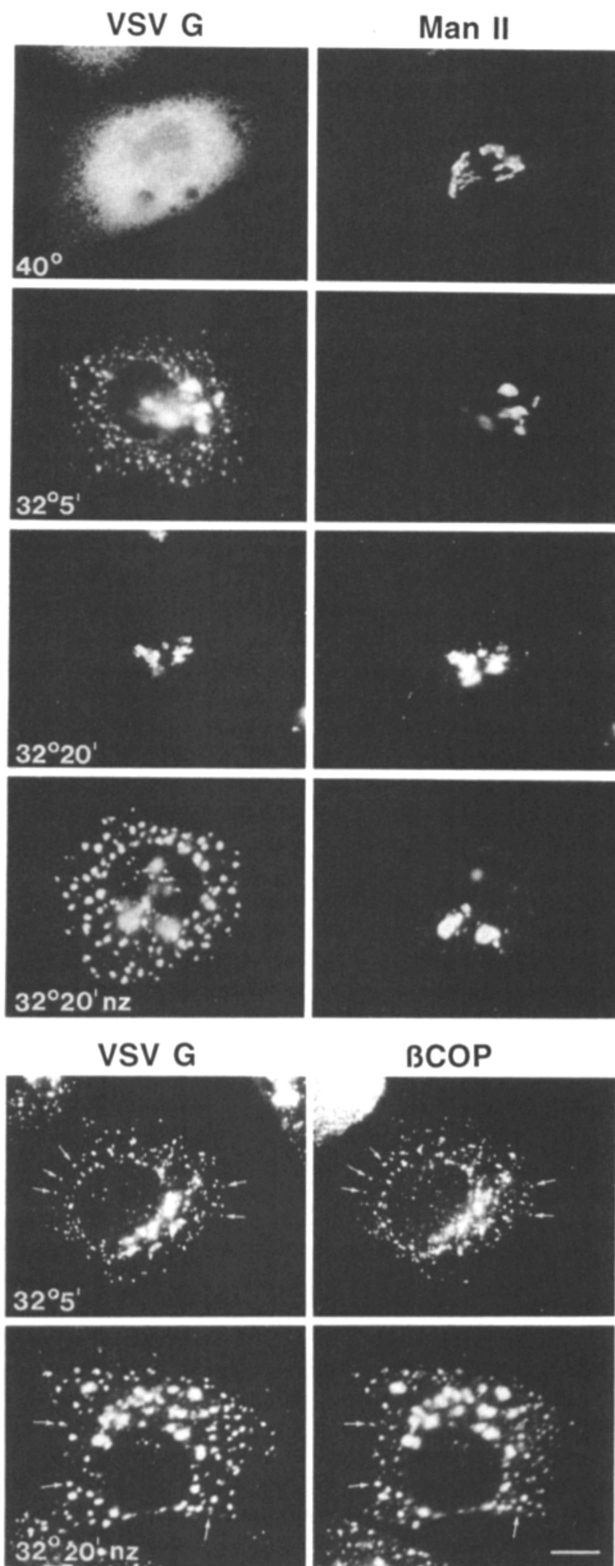

Figure 3. Immunolocalization of VSV G recently released from the ER with Man II and βCOP. NRK cells infected with VSV were incubated at 40°C for 2 h. The cells were then fixed (*top*) or placed on ice for 10 min and then incubated under the following conditions before fixation: 32°C for 5 min; 32°C for 20 min; and 32°C for 20 min in the presence of nocodazole. Cells were then permeabilized with saponin and incubated with antibodies to VSV G protein and man II, or VSV G protein and βCOP, followed by rhodamine and fluorescein conjugated secondary antibodies. Arrows point to representative peripheral structures where VSV G and βCOP appear colocalized. This occurred shortly after release of VSV G protein from the ER (32°C, 5 min), and at later time points at 32°C when microtubules were disrupted with nocodazole. Bar, 10 μm.

taining kinesin and βCOP (Figs. 2 and 3). The pattern of VSV G and kinesin staining under the nocodazole conditions was similar to that in cells shifted from 40 to 16°C for 1 h, which slows ER-to-Golgi transport (Saraste and Kuismanen, 1984). Under these conditions, VSV G and kinesin were colocalized in numerous peripheral structures in addition to central Golgi structures (see Fig. 2).

Taken together, these observations support the hypothesis that kinesin recognized by the H1 monoclonal antibody is associated with peripheral pre-Golgi structures carrying newly synthesized proteins into the Golgi complex. Further support for this premise was obtained from immunoelectron microscopy of virus-infected cells which had undergone a temperature shift from 40 to 32°C in the presence of nocodazole to accumulate VSV G protein in the peripheral structures. Ultrathin cryosections of such cells were then labeled with H1 anti-kinesin, alone, or in combination with anti-VSV G. The antibodies to kinesin and VSV G protein were then detected using 20- and 10-nm colloidal gold probes, respectively.

As shown in Fig. 4, kinesin was localized on pleiomorphic, occasionally swollen membrane-bounded structures that were scattered throughout the cytoplasm, and whose appearance is consistent with that of the ER/Golgi transport intermediates in VSV-infected cells (Schweizer et al., 1990; Lotti et al., 1992). Staining was confined to cytoplasmic, membrane-enriched areas, and was rarely observed on recognizable Golgi stacks, ER, mitochondria, or within nuclei. Moreover, kinesin and VSV G protein were extensively codistributed in cells that had been processed for double immunoelectron microscopy (Fig. 4 *D*). These results support the hypothesis based on light microscopy that kinesin recognized by the H1 monoclonal antibody is associated with a membranous compartment which carries newly synthesized secretory proteins from the ER to the Golgi.

Redistribution of Kinesin onto the Golgi Complex

The finding that kinesin associates with peripheral pre-Golgi elements that translocate inward toward the central Golgi complex (toward the minus ends of microtubules) was surprising, since abundant evidence points to kinesin being a plus end-directed microtubule motor protein (Vale et al., 1985; Scholey et al., 1985). We wondered, therefore, about the fate of kinesin on these structures, and focused on the question of whether kinesin is carried with these peripheral membranes as they move toward and merge with the Golgi complex. To address this question we examined the effect of temperature reduction (2 h at 16°C) and brief warming (37°C for 5 min) on the distribution of kinesin recognized by the H1 antibody. Incubation at 16°C causes newly synthesized secretory products to accumulate in pre-Golgi structures (Saraste and Kuismanen, 1984), while shifting the temperature to 37°C for a brief period results in the synchronous movement of these membrane components into the central

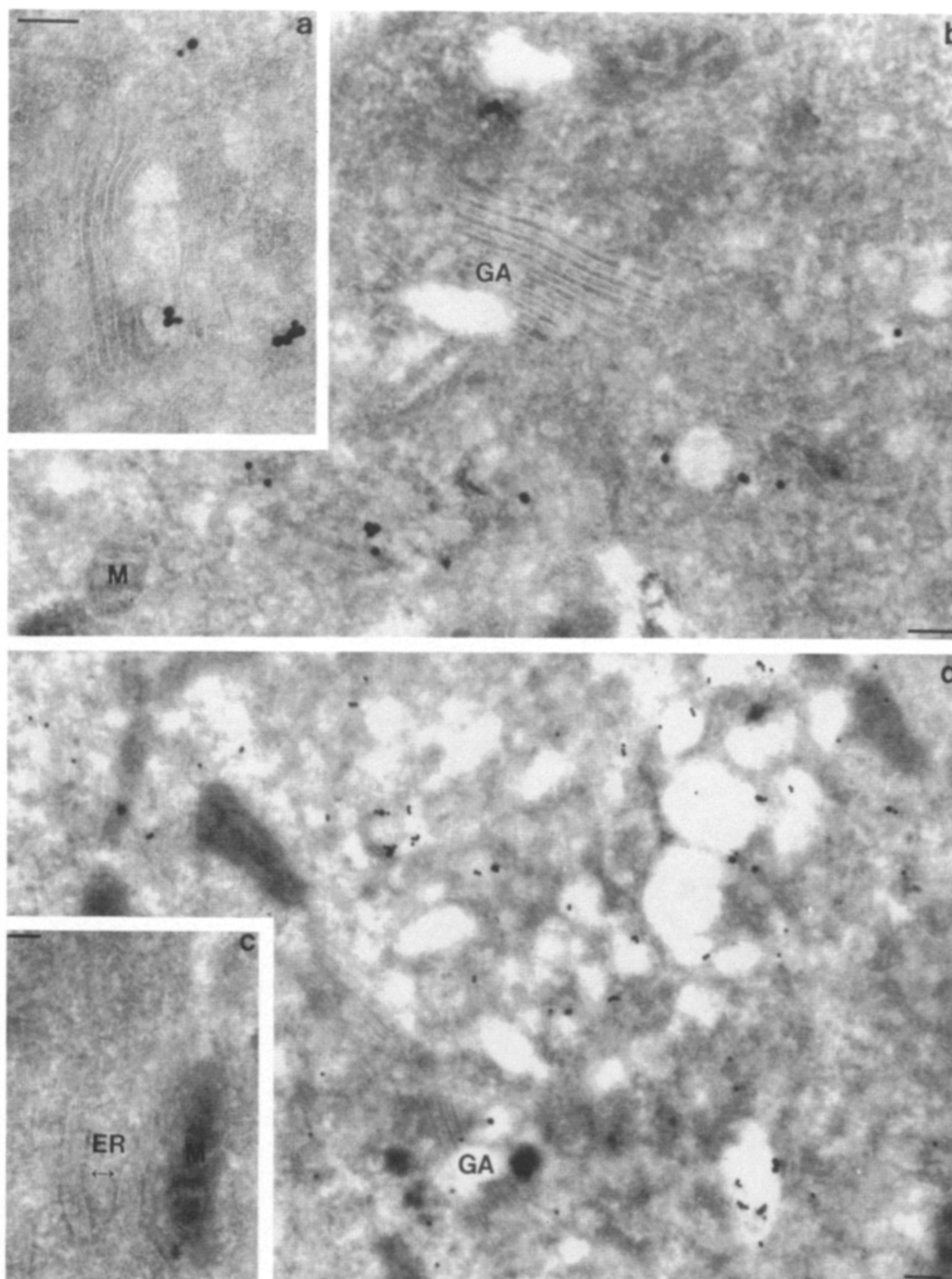

Figure 4. Localization of kinesin and VSV G protein in peripheral pre-Golgi transport intermediates in VSV-infected NRK cells. VSV-infected cells were held at the non-permissive temperature of 40°C for 1 h. The cells were placed on ice for 10 min and then incubated for 20 min at 32°C in the presence of nocodazole. This treatment allowed VSV G protein to accumulate in peripheral pre-Golgi transport intermediate structures. *a*, *c*, and *d* are double-labeled for VSV G protein (10 nm gold) and kinesin (20 nm gold), while *b* is labeled for kinesin alone. Abundant pleiomorphic membranous structures and large, electron-lucent vesicles surrounding Golgi stacks were observed. Note the colocalization of kinesin and VSV G protein in these structures (*a* and *d*). Under the conditions of this experiment, kinesin and VSV G protein were not found in association with Golgi stacks (GA: *a*, *b*, and *d*), ER tubules (ER: *c*) or mitochondria (M: *b* and *c*). Bars, 100 nm.

Golgi region (Lippincott-Schwartz et al., 1990; Hsu et al., 1991). When the distribution of kinesin was examined during such a temperature shift protocol (Fig. 5), a significant proportion of kinesin was found relocated from peripheral pre-Golgi sites typically seen in control cells (*top row*) to the central Golgi region (*second row*).

The distribution of kinesin recognized by H1 could also be shifted onto Golgi structures by long-term nocodazole treatment of cells. As shown in Fig. 5 (*bottom*), after 2 h of nocodazole treatment, kinesin and man II both were associated with numerous large membrane structures that were scattered throughout the cytoplasm. Taken together, these results indicate that kinesin's distribution is not limited to peripheral, pre-Golgi structures, but can be shifted onto or directly adjacent to Golgi membranes with perturbations in membrane traffic, including temperature shift and microtubule disruption.

Localization of Kinesin on Golgi-to-ER Tubules Carrying Membrane from the Golgi to the ER

The presence of kinesin on both pre-Golgi and Golgi membrane, and the failure of BFA and other manipulations (including low temperature and pH) to drive it off membrane raised the possibility that kinesin maintains its steady-state distribution on these structures by constitutively cycling between the ER and Golgi complex. Such a cycling pathway between the ER and Golgi complex has been proposed to be

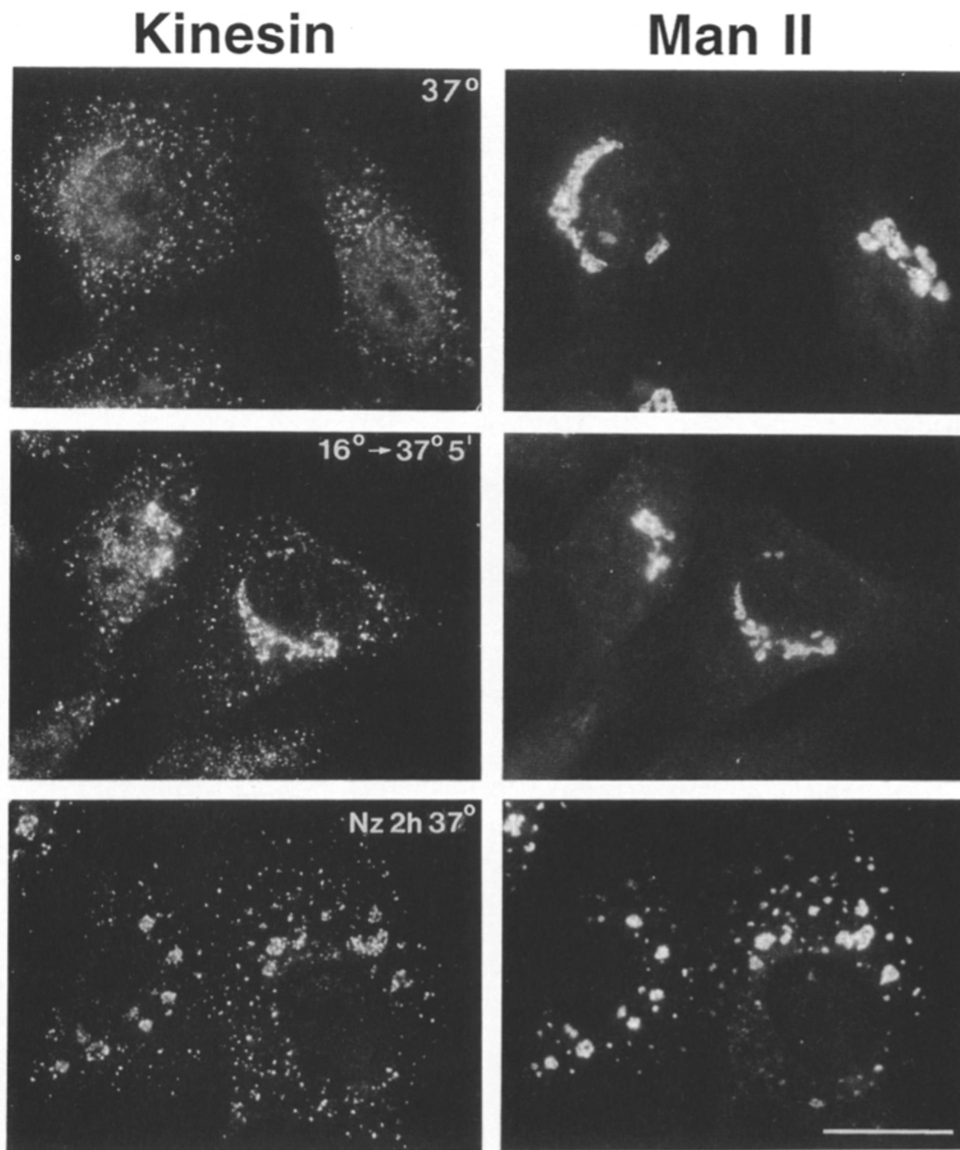

Figure 5. Effect of temperature and nocodazole on the distribution of kinesin and man II. NRK cells were incubated as indicated in each micrograph before fixation, permeabilization with saponin, and double-labeling with antibodies to kinesin and man II followed by rhodamine and fluorescein conjugated secondary antibodies. Note that kinesin accumulated on or near Golgi membranes in cells perturbed by either temperature reduction or nocodazole. Bar, 10 μm.

followed by two proteins, ERGIC 53 and its potential homolog p58 (Saraste and Svensson, 1991; Hauri and Schweizer, 1992), which both reside at 37°C in pre-Golgi structures. Like kinesin, these molecules can be shifted from pre-Golgi structures into the Golgi region by incubation at 16°C or nocodazole treatment.

To investigate whether kinesin is capable of cycling with membrane from the Golgi to the ER, we examined its distribution in cells treated with the drug BFA. BFA treatment dramatically alters the distribution and flow of membrane between the ER and Golgi complex. Within minutes of its addition to most cell types, protein transport into the Golgi is blocked and nearly all Golgi membrane and content flow into the ER via tubular processes which extend microtubule plus end-directed to the cell periphery (Lippincott-Schwartz et al., 1990). As shown in Fig. 6, kinesin recognized by the H1 antibody colocalized with the Golgi enzyme man II in fine tubule processes after 5 min of BFA treatment. In these experiments cells were incubated for 2 h at 16°C and warmed to 37°C for 5 min before adding BFA. The temperature shift had the effect of concentrating kinesin recognized by H1 antibody at the Golgi, while BFA treatment led to movement of Golgi enzymes into Golgi-to-ER tubular processes before their final redistribution into the ER. Upon longer incubations with BFA at 37°C, the distribution of kinesin recognized by H1 appeared first in a dispersed network characteristic of the ER (not shown), and by 30 min had accumulated in discrete peripheral structures, resembling those containing kinesin in control cells (Fig. 6). A similar temporal change in distribution (from Golgi to ER then to discrete peripheral structures) has been observed for the ERGIC-53 intermediate compartment marker after warm-up from 16°C in the presence of BFA (Lippincott-Schwartz et al., 1990; Hauri et al., 1992).

Microinjection of Anti-kinesin Inhibits Golgi-to-ER Transport

The results described to this point suggest that kinesin is associated with membranes which move bidirectionally along

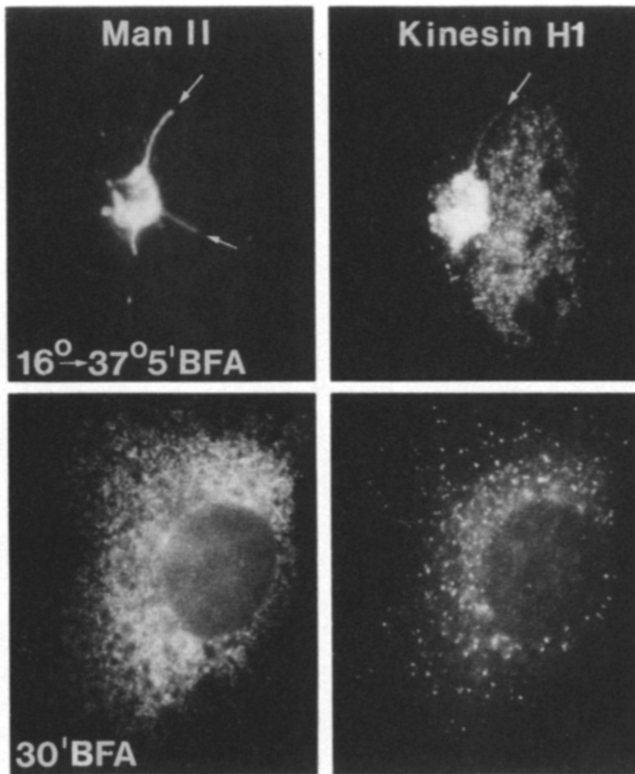

Figure 6. Localization of kinesin on Golgi-to-ER tubule intermediates induced by BFA. NRK cells were incubated at 16°C for 2 h and then warmed to 37°C for 5 min or 30 min in the presence of BFA before fixation. Cells were then permeabilized with saponin and double-labeled with antibodies to kinesin and man II followed by rhodamine and fluorescein conjugated secondary antibodies. Arrows point to Golgi-derived tubules containing kinesin and man II in cells treated with BFA for 5 min. By 30 min of BFA exposure, however, man II and kinesin redistributed to ER and intermediate compartment membranes, respectively. Bar, 10 μm.

microtubules between the ER and Golgi complex. Since kinesin is known to be a microtubule plus end-directed motor, and Golgi-to-ER transport occurs toward microtubule plus ends, we wondered whether kinesin functions as a motor for Golgi-to-ER transport and is functionally inactive on transport intermediates moving in the opposite direction. To test this possibility we performed microinjection experiments using the H1 monoclonal anti-kinesin in cells that were subsequently treated with BFA for 5 min, to ascertain the effects of anti-kinesin on the formation of Golgi-to-ER tubules induced by BFA. As shown by immunofluorescence in Fig. 7, numerous tubular processes labeled with man II antibody (see *small arrows* in *A*) were observed extending out of central Golgi structures after the short BFA treatment in all cells except those which were microinjected with anti-kinesin. Coinjection with fluorescent dextran enabled us to readily identify these cells (Fig. 7 *B*, *large arrows*). In cells injected with anti-kinesin, Golgi-derived tubule processes were absent and man II remained associated with discrete Golgi elements. Upon longer incubation periods with BFA (20 min) (Fig. 7, *C–F*) the majority of man II molecules redistributed into the ER of uninjected or nonspecific IgG-injected cells (*C*), whereas in anti-kinesin injected cells (*E*), the majority of man II staining remained in the Golgi region. Fig. 7, *C* and *E* show the distribution of man II under these conditions, whereas *D* and *F* show the distribution of fluorescent dextran in microinjected cells. The qualitative results described here are documented quantitatively in Fig. 8. Less than 10% of cells microinjected with H1 anti-kinesin showed Golgi-derived tubules after 5 min of BFA treatment, compared to over 90% of uninjected cells or cells injected with control antibody. With longer BFA-treatment (i.e., 20 and 60 min) in uninjected and control injected cells, Golgi enzymes had completely redistributed into the ER, with no Golgi remnant structures apparent. By contrast, in cells injected with H1, the majority of cells showed discrete Golgi remnants at these later time points in the presence of the drug.

Microinjected anti-kinesin antibody did not have any obvious effect on the distributions of man II or βCOP in cells not treated with BFA. Moreover, as shown in Fig. 8, no effect on the reformation of a central Golgi complex by anti-kinesin microinjection was observed during recovery from nocodazole or BFA-treatment. Hence, there was no indication that the anti-kinesin antibody, which inhibited Golgi-to-ER traffic, could interfere with membrane transport in the opposite direction. Collectively, these data imply that bidirectional movement along microtubules of kinesin-enriched membrane between the ER and Golgi occurs in a manner which is synchronized with the cycling of kinesin between functionally active and inactive states. The kinesin motor is evidently active on membranes being transported from the Golgi to the ER, but inactive on membranes moving in the opposite direction toward the Golgi complex.

Discussion

The dynamic features of membranes within the secretory pathway reflect the actions of distinct classes of intracellular components. The most widely recognized class comprises regulatory factors that control the formation of transport vesicles and tubules from donor compartments, and their targeting and fusion to acceptor compartments. Factors such as NEM-sensitive factor (NSF), soluble NSF attachment proteins (SNAP), SNAP receptors, ADP ribosylation factors, coatomer proteins (COP), and rabs collectively control a broad variety of essential membrane-trafficking functions which take place at precise locations in the cell (Beckers et al, 1989; Duden et al., 1991; Serafini et al., 1991*a*,*b*; Balch et al., 1992; Schwaninger et al., 1992; Orci et al., 1993; Söllner et al., 1993; Pepperkok et al., 1993; Peter et al., 1993). In general, however, they do not directly control long range interactions between membrane compartments which communicate with each other by transport intermediates.

It is cytoskeletal elements, particularly microtubules, which are responsible for integrating into a functionally cohesive unit the diverse membrane budding and fusion steps that characterize secretion, and for coordinating interactions between the secretory and endocytic pathways. Microtubules have long been known to serve as highways along which membranes of both pathways travel throughout the cell (Freed and Lebowitz, 1970). They control the structural integrity and intracellular location of the Golgi apparatus (Wehland et al., 1983; Rogalski and Singer, 1984), and provide a scaffold for the elaboration of an anastomosing network of ER tubules (Terasaki et al., 1986; Lee et al., 1989). Paralleling the progress made in understanding membrane

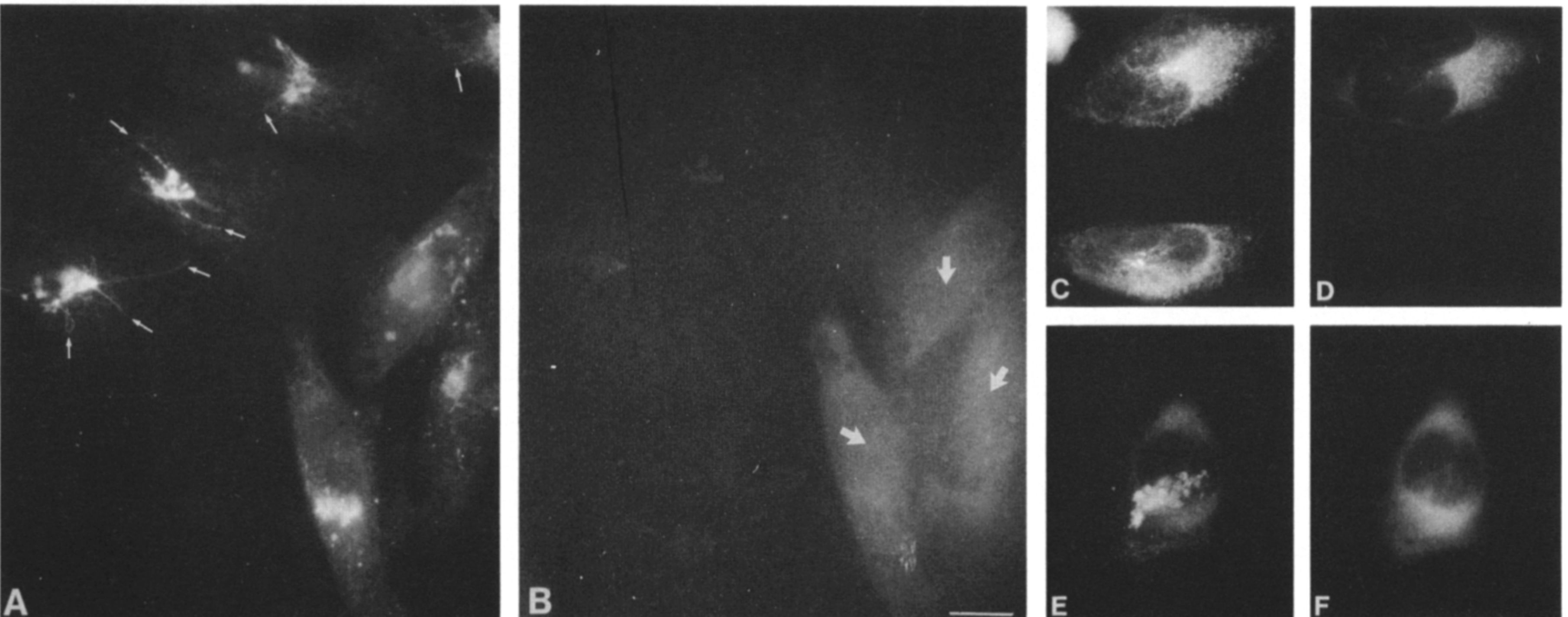

Figure 7. Microinjected anti-kinesin inhibits the formation of Golgi-to-ER tubule processes observed in BFA-treated cells. (*A* and *B*) NRK cells were microinjected with a 6 mg/ml solution of H1 anti-kinesin supplemented with FITC-dextran. The cells were then incubated at 37°C for 30 min before the addition of BFA (1 μg/ml). After 5 min of BFA treatment, cells were fixed, permeabilized with saponin and labeled with man II antibody followed by rhodamine conjugated secondary antibody. *A* shows the distribution of man II (arrows indicate Golgi-to-ER tubule intermediates) in cells that were viewed in rhodamine optics. *B* shows the distribution of fluorescein dextran within microinjected cells (see large arrows) viewed in fluorescein optics. Note that microinjected anti-kinesin inhibited the formation of Golgi-to-ER tubules emerging from the Golgi complex. (*C–F*) Cells microinjected with FITC-dextran plus a 6 mg/ml solution of nonspecific IgG (*C* and *D*) or H1 anti-kinesin (*E* and *F*) followed by incubation with BFA for 20 min. *C* and *E* show the distribution of man II under these conditions, while *D* and *F* show the distribution of FITC dextran in microinjected cells. Note that Man II redistributed into a reticular ER-like pattern after 20 min of BFA treatment in cells injected with the nonspecific IgG (*C*) or in uninjected cells (*C*). In cells injected with anti-kinesin (*E*), however, a significant fraction of Man II remained predominantly in a juxtanuclear position, although some appeared to have redistributed into the ER. Significantly, no tubule processes emerging from Golgi remnants were detected. Bar, 10 μm.

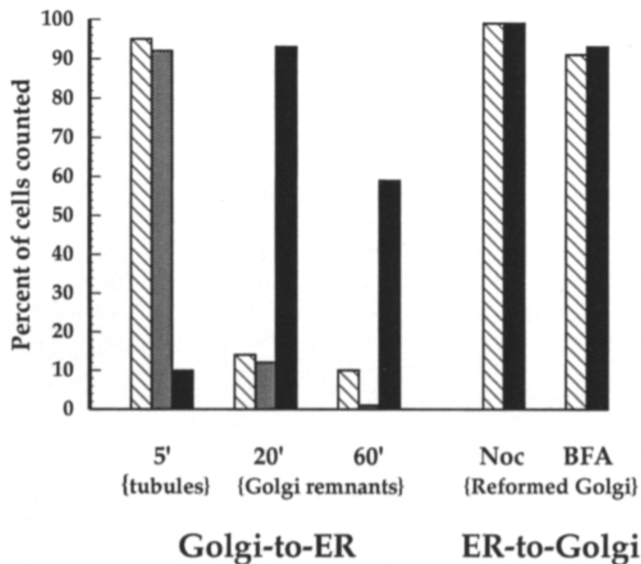

Figure 8. Quantitative effects of microinjected antibodies on bidirectional transport between the ER and Golgi complex. NRK or HeLa cells growing on glass coverslips were microinjected with either of two purified monoclonal IgG1 antibodies. The H1 antibody to kinesin heavy chain (Pfister et al., 1989; Hirokawa et al., 1989) was used at 6 mg/ml, while a control antibody, anti-58K-9, which recognizes a microtubule-binding Golgi protein (Bloom and Brashear, 1989), was used at 5 mg/ml. Another control monoclonal IgG1, which was used for the micrographs shown in Fig. 7 *C* and *D*, yielded quantitative results nearly identical to those obtained using anti-58K-9 (not shown). To examine Golgi-to-ER membrane traffic, cultures were treated with 1–5 μg/ml BFA 30 min after microinjection, and were fixed 5, 20, or 60 min later. Following fixation, the coverslips were processed for immunofluorescence, using polyclonal antibodies to man II or GalTf, to enable detection of Golgi tubules (at the 5-min time point) or Golgi remnants (at the 20- and 60-min time points). Note that in contrast to uninjected cells or cells injected with control antibody, cells injected with anti-kinesin were nearly devoid of Golgi tubules 5 min after exposure to BFA, and typically contained Golgi remnants as long as one hour after the drug was added. Hence, anti-kinesin prevents the extension of tubule processes out of the ER and retards membrane transport from the Golgi to the ER. To examine ER-to-Golgi transport, cultures were microinjected with anti-kinesin, and treated 30 min later with 10 μg/ml nocodazole (*Noc*) or 5 μg/ml BFA. For the nocodazole experiments, cells were incubated with the drug for 1 h, transferred to drug-free medium for an additional hour, and then fixed and stained for immunofluorescence with polyclonal anti-man II. For the BFA experiments, cells were treated with the drug for 90 min, then incubated in BFA-free medium for 30 min, and finally fixed and stained with polyclonal anti-man II. As can be seen, intact Golgi complexes reformed in virtually all of the injected and uninjected cells. Therefore, anti-kinesin did not interfere with ER-to-Golgi membrane traffic (BFA washout), or the formation of a centrally located, intact Golgi from previously dispersed Golgi fragments (nocodazole washout). From 30–200 cells were scored for each category shown in this figure. ▧, uninjected; ▩, anti-58K-9 (control); ■, H1 (anti-kinesin).

budding, targetting and fusion has been a series of equally far reaching advances regarding motor proteins that transport intracellular cargo along the microtubule's surface. Two microtubule-stimulated ATPases, kinesin and cytoplasmic dynein (and possibly other kinesin-related proteins) have been shown to be capable of moving membrane along microtubules. Significant headway has been made toward characterizing the structure and enzymatic activities of these proteins and their interactions with microtubules. However, detailed knowledge of how these motors perform their functions in the secretory pathway has remained elusive.

In this paper we have focused on the role of kinesin in facilitating microtubule-dependent membrane transport within the early secretory pathway. We have found that kinesin is intimately associated with transport intermediates that constitutively cycle between the ER and Golgi complex. These transport intermediates appear to move bidirectionally along microtubules in both minus and plus end directions to reach central and peripheral sites where the Golgi and ER, respectively, reside. Here, we provide data consistent with kinesin having a specific function within this system: to power membrane transport along microtubules in the Golgi-to-ER arm of the cyclic pathway connecting the ER to the Golgi. This is evidently accomplished by a mechanism that enables kinesin to move constitutively in both directions between the ER and Golgi, but in a manner where it is functionally inactive during ER-to-Golgi transport, and active during Golgi-to-ER transport.

Membrane Dynamics of Kinesin within the ER/Golgi System

The first indication for a role of kinesin within the ER/Golgi system came with the surprising observation that membrane-bound kinesin, detected with the H1 monoclonal, at 37°C resides primarily on peripherally distributed ER-to-Golgi transport intermediates moving microtubule minus end-directed toward the cell center. Evidence for this was severalfold. Membrane-bound kinesin colocalized with several markers of these transport intermediates, including βCOP and VSV G recently released from the ER in VSV-infected cells. Movement of these peripheral structures into the central Golgi region could be inhibited by microtubule disruption. Additional immuno-EM experiments showing colocalization of kinesin and VSV G released from ER in peripheral, pleiomorphic membrane structures confirmed the presence of kinesin on ER-to-Golgi transport intermediates.

The fact that kinesin's distribution was not restricted to pre-Golgi intermediates, but could be shifted onto Golgi structures under specific conditions (including after warming cells from 16°C and in nocodazole-treated cells after Golgi elements have fragmented to peripheral sites), argued against kinesin distributing by a mechanism involving membrane association/dissociation at specific sites. Rather, kinesin appeared to be moving with these pre-Golgi membranes as they translocated from peripheral to central sites.

That kinesin was capable of cycling with membrane back to the ER from central Golgi structures was suggested from morphological experiments following recycling intermediates in BFA-treated cells. Golgi-to-ER tubules carrying the Golgi enzyme, man II, as well as kinesin, into the ER could be observed in cells incubated at 16°C for 2 h and then transferred briefly to 37°C in the presence of BFA. At later time points upon warmup from 16°C in the absence or presence of BFA, kinesin was found by immunofluorescence both in peripheral structures, such as those found in control cells, and in the ER as well.

Diverse Roles of Kinesin in Membrane Traffic

Given the presence of kinesin on the membranes which move bidirectionally between the ER and Golgi, we next considered its role on these membranes. A general function of kinesin is to move membranes along microtubules in a plus end-directed manner. Since Golgi-to-ER transport, in contrast to ER-to-Golgi transport, occurs toward microtubule plus ends, we investigated the possibility that kinesin is the motor for Golgi-to-ER traffic. Evidence consistent with this hypothesis was supplied by experiments in which the monoclonal IgG1 anti-kinesin used for the immunolocalization experiments was microinjected into Hela and NRK cells. Anti-kinesin, but not a control IgG1 monoclonal, was found to suppress the formation of Golgi-to-ER tubules in cells that were warmed from 16 to 37°C in the presence of BFA. Moreover, a series of experiments failed to reveal any evidence that microinjected anti-kinesin could impede transport in the opposite direction. In particular, when anti-kinesin was microinjected into cells that had been pretreated with BFA or nocodazole, and the cells were then transferred to drug-free media, reestablishment of a normal appearing, centrally located Golgi complex proceeded with kinetics that were indistinguishable from those observed in uninjected cells.

These results and their implications for kinesin's function are entirely consistent with prior evidence that kinesin is a microtubule plus end-directed motor. For example, kinesin is reportedly responsible for the movement of ER-like tubules along microtubules in vitro (Dabora and Sheetz, 1988; Vale and Hotani, 1988) and the transport of lysosomes (Hollenbeck and Swanson, 1990; Swanson et al., 1992) and pigment granules (Rodionov et al., 1991) from the cell center to the periphery. Kinesin has also been implicated as a motor for anterograde fast axonal transport (a post-Golgi transport phenomenon), which corresponds to the movement of membrane-bounded organelles, principally synaptic vesicles and their precursors, from neuronal cell bodies to axon terminals (Brady, 1985, 1990; Vale et al., 1985*a*,*b*; Hirokawa et al., 1991). In addition, kinesin has been localized on synaptic vesicles, brain microsomes, mitochondria, Golgi and membrane-bounded organelles located in the mitotic spindles of early embryonic cells of the sea urchin (Wright et al., 1991; Leopold et al., 1992; Yu et al., 1992; Marks et al., 1994; Schmitz et al., 1994).

What accounts for the diverse localizations and roles of kinesin reported in these studies? A steadily emerging body of evidence points to the possibility that numerous biochemically and functionally distinct forms of kinesin exist, each specialized for transporting a particular class of membrane. The kinesin molecule comprises two ~124-kD heavy chains and a pair of ~64-kD light chains (Bloom et al., 1988; Kuznetsov et al., 1988). The light chains exist in multiple forms that are assembled by alternative splicing of a single gene (Cyr et al., 1991; Gauger and Goldstein, 1993; Wedaman et al., 1993), and there are at least two separate heavy chain genes which encode similar, but clearly distinct polypeptides (Navone et al., 1992; Niclas et al., 1994). Both the heavy and light chains of kinesin undergo phosphorylation at multiple sites in vivo (Hollenbeck, 1993; Matthies et al., 1993), and exhibit substantial electrophoretic and immunological heterogeneity (Pfister et al., 1989; Wagner et al., 1989; Matthies et al., 1993). There is thus considerable opportunity for the cell to construct kinesin molecules from various types of subunits in multiple states of posttranslational modification which could serve related functions. The H1 monoclonal anti-kinesin used in our study might recognize, therefore, a structurally and functionally distinct subset of these kinesin molecules in the cell. Consistent with this possibility, the H1 antibody labels only a subset of the kinesin heavy chain bands on western blots (Hirokawa et al., 1989; Pfister et al., 1989) and, as shown by immunofluorescence in the experiments described here, labels a characteristic set of intracellular membranes—those involved in ER/Golgi traffic.

Regulation of Kinesin Motor Activity within the ER/Golgi System

The microinjection experiments imply that kinesin is mechanochemically inactive for membrane movement from the ER to the Golgi, but fully functionally for the reverse transport step. Evidently, therefore, the functional activity of kinesin is tightly regulated within the early secretory pathway. Because kinesin is present on membranes that move toward microtubule minus ends, from the ER to the Golgi, regulation does not involve dissociation of the motor from membranes that travel in the "wrong" direction. Instead, we envision a scheme in which the ATPase or microtubule-binding activity of kinesin is suppressed during ER-to-Golgi transport and stimulated during Golgi-to-ER transport. Several mechanisms could accomplish this task. For example, posttranslational alterations of kinesin or associated proteins, such as changes in the phosphorylation state of heavy chains, light chains or both, could modulate the enzymatic properties of kinesin and its ability to bind microtubules (McIlvain et al., 1994). Indeed, there is evidence that phosphorylation of kinesin by protein kinase A suppresses the ability of calmodulin to inhibit the ATPase activity of kinesin (Matthies et al., 1993).

An alternative scheme suggested by the data presented here is regulation of kinesin motor activity by coatomer proteins, which envelop kinesin-containing membranes travelling from the ER to the Golgi, but not the membranes moving back to the ER from the Golgi. Perhaps the coats formed around ER-to-Golgi intermediates by βCOP and the other coatomer subunits physically block kinesin from attaching to microtubules. Not only would this mechanism prevent kinesin from translocating these structures along microtubules, but it would also suppress the ATPase activity of the motor, which is potently stimulated by microtubules (Kuznetsov and Gelfand, 1986). Consistent with this possibility, when COPs are dissociated from membranes with BFA, Golgi membrane now moves plus end-directed along microtubules to the cell periphery (see Figs. 6 and 7).

Illustrated in Fig. 9 is a model which integrates the principal findings of the collective data presented here. The key features of this model are that kinesin cycles constitutively between the ER and the Golgi apparatus, traveling toward the Golgi in an inactive state on pleiomorphic, COP-coated vesicles, and in the reverse direction in a functionally active state on uncoated, tubulovesicular structures. The function of kinesin on the latter structures presumably is to power their efficient return to the ER. This allows selected protein and membrane components which have left the ER to recycle

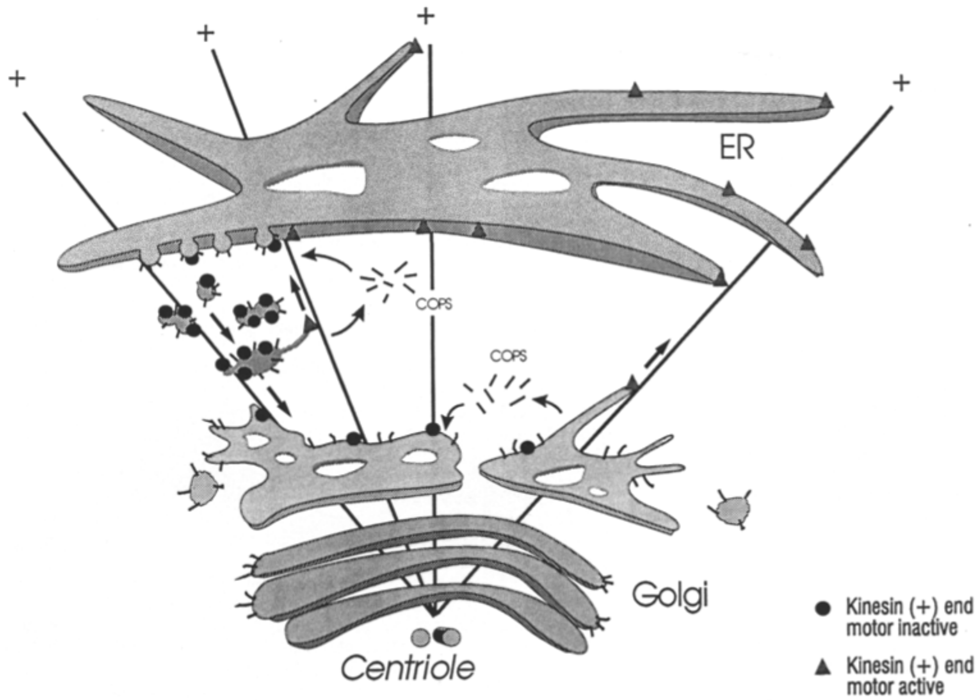

Figure 9. Model for the distribution and dynamics of kinesin within the ER/Golgi system. According to this model, kinesin constitutively cycles between the ER and Golgi. Kinesin would travel toward the central Golgi region in an inactive state on COP-coated intermediates and in the reverse direction in a functionally active state on uncoated tubulovesicular structures. At steady-state the majority of kinesin molecules would associate with ER-to-Golgi transport intermediates. Within the ER, kinesin would remain active as a microtubule plus end-directed motor, enabling the ER to extend peripherally. Membrane association/dissociation of COPs might play a role in regulating kinesin motor activity at pre-Golgi and Golgi sites.

back, allowing the ER to maintain its surface area in the face of continuous outward flow of membrane. The model also envisions kinesin as responsible for moving ER tubules toward microtubule plus ends (Dabora and Sheetz, 1988; Vale and Hotari, 1988). This model and the data from which it is derived underscores the importance of microtubules and their motor proteins for integrating the numerous distinct compartments of the secretory pathway into a functionally cohesive unit. Systematic studies of regulatory factors like NSF and the SNAPS have led to an ever increasingly detailed understanding of the molecular basis of endomembrane budding, targetting and fusion. Likewise, further studies of microtubules, motor proteins, and the factors which regulate their functions are bound to paint a complementary picture of membrane trafficking. This picture will focus on how transport intermediates make their excursions between spatially segregated compartments whose biochemical and functional integrity is thereby ensured.

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