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A possible role for stable microtubules in intracellular transport from the endoplasmic reticulum to the Golgi apparatus

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SUMMARY

The intracellular transport of secretory proteins involves at an early stage the formation of vesicles from transitional elements of the endoplasmic reticulum (ER) containing these proteins and the transfer of these vesicles to the *cis*-face of the Golgi apparatus. We propose that the latter transfer process does not occur by random diffusion, but is instead mediated by tracking along stable microtubules. To test this proposal, we have carried out double immuno-electron microscopic labeling experiments on frozen sections of HepG2 hepatoma cells secreting the protein human serum albumin (HSA). By a cycloheximide treatment protocol, the stage during which the transfer of newly synthesized HSA from the ER to the Golgi apparatus occurs in vivo was determined. Sections of the cells were then double immunolabled using primary antibodies to HSA and to *glu*-tubulin, the latter specifically detecting stable microtubules. We observed a significantly high frequency of HSA-containing structures between the ER and the Golgi apparatus with which stable microtubules were closely associated. These results support the proposal that stable microtubules may play a critical role in directing the transfer process from the ER to the Golgi apparatus.

Key words: secretion, human serum albumin, hepatoma cell, immunoelectron microscopy

INTRODUCTION

The complex pathway taken by soluble secretory proteins inside cells, starting with their synthesis and ending with their release at the cell surface, was laid out some time ago by Palade (1975). In the years since, the efforts of many investigators have concentrated on working out the detailed molecular biology accompanying the structural events of each stage of the pathway. In this paper, we focus on that part of the pathway from the endoplasmic reticulum (ER) to the Golgi apparatus. A soluble secretory protein, after being deposited inside the lumen of the ER, is thought to be transferred within transition vesicles that bud off transitional elements of the ER and then fuse with the *cis*-face of a stack of Golgi saccules (cf. Palade, 1975; Merisko et al., 1986; Lodish et al., 1987). (It is not clear whether the pleiomorphic intermediate compartment characterized by Saraste and Kuismanen (1984, 1992) is physically separated from, or is a part of, the traditionally described transitional elements of the ER (Palade, 1975). For the purposes of this paper, however, we do not need to distinguish between these two possibilities). Inside eukaryotic cells in interphase, the ER with its transitional elements constitutes a highly ramified membrane-bounded organelle that is spread throughout the cytoplasm, whereas the Golgi apparatus is usually a much more compact organelle that is localized near the cell nucleus. These grossly different morphologies and distributions of the ER and Golgi apparatus pose questions about the spatial and ultrastructural features of the ER-to-Golgi transfer process. Do transition vesicles bud from transition elements located anywhere within the broadly dispersed ER, and then eventually find their way by a diffusional process to the *cis*-face of a Golgi stack (which is termed a 'stochastic' process)? Or is the process less random? In particular, do the transition vesicles bud only at confined sites in the ER: for example, perhaps only at transition element sites in the ER that are proximal to the *cis*-faces of Golgi stacks? Furthermore, might the transition vesicles be directed from their sites of origin in the ER to the *cis*-face of the Golgi apparatus, perhaps tracked along cytoskeletal elements, rather than finding their way by random diffusion?

Recently, we obtained evidence supporting the view that ER-to-Golgi transfer is not simply stochastic. We designed and carried out a series of experiments involving cell fusion hybrids (Valtersson and Singer, 1987; Valtersson et al., 1990, 1993). In these experiments, heterokaryons were formed in culture between two types of human cells secreting different soluble proteins. Some time (2.5 hours) before the cells were fused, they were emptied of their respective secretory proteins by treatment with either cycloheximide or puromycin and then, shortly after fusion, the cycloheximide or puromycin was washed out and protein synthesis and secretion was re-initiated in the intact heterokaryons. Each heterokaryon contained the two Golgi apparatuses contributed by the two cells that had undergone fusion. The two Golgi's remained physically

separated near their respective nuclei for some time (~90 minutes) after fusion. We reasoned that if the process of ER-to-Golgi transfer were purely stochastic, then transition vesicles might be rapidly disseminated throughout the heterokaryon, and a given secretory protein might simultaneously enter both Golgi apparatuses of the heterokaryon. But if the ER-to-Golgi transfer was restricted, either by being spatially confined or physically directed, and this restriction was retained after the heterokaryon was formed, then a secretory protein might first enter its homologous Golgi apparatus (that is, that one of the two Golgi's that was contributed by the cell for which that secretory protein was specific). With a number of heterokaryons made from different cell types secreting several different soluble proteins, immunofluorescence observations showed that invariably a secretory protein entered its homologous Golgi apparatus well before it appeared in the heterologous Golgi. After eliminating trivial explanations, we interpreted these results to indicate that the process of ER-to-Golgi transfer was therefore not simply stochastic, but was somehow restricted.

How might such restriction arise? One possibility we considered is that ER-to-Golgi transfer might not occur by diffusion of transition vesicles, but by their directed migration along microtubules. There have been several indications in the literature that elements of the ER can interact with microtubules. Fluorescence microscopy experiments have shown at the light microscopic level of resolution that ER and microtubules are coordinately distributed inside intact cells (Terasaki et al., 1986), and in living cells dynamic reorganizations of ER occur along linear tracks, presumably microtubules (Lee and Chen, 1988). The in vitro experiments of Dabora and Sheetz (1988) strongly suggest that elements of the ER can be translocated and become extended along microtubules. Retrograde movement of elements of the Golgi apparatus to the ER, induced by treatment of cells with brefeldin A, also appear to track along microtubules (Lippincott-Schwarz et al., 1990; Klausner et al., 1992).

On the other hand, there is a substantial body of evidence, reviewed by Rogalski et al. (1984), that the drug-induced depolymerization of microtubules does not appreciably affect the transfer of soluble secretory proteins (cf. Boyd et al., 1982) or membrane integral proteins (Rogalski et al., 1984) from the ER to the Golgi apparatus. This argues against a critical role for microtubules in ER-to-Golgi transfer.

One possibility to reconcile these two lines of apparently conflicting results is that only, or predominantly, stable microtubules might be involved in mediating ER-to-Golgi transfer. In higher eukaryotic cells in interphase, two states of microtubules have been recognized, dynamically unstable and stable (cf. Schulze and Kirschner, 1988). Dynamically unstable microtubules, the preponderant type in most cells, are in rapid dynamic flux, not simply exchanging subunits from their ends, but nearly entirely depolymerizing and repolymerizing in a matter of minutes. Stable microtubules, the minority type in most cells, do not undergo such rapid depolymerization and repolymerization, but instead can persist for times of the orders of hours (Kreis, 1987; Webster et al., 1990). The basis for such stability is not clear, but in higher eukaryotic cells stable microtubules can be distinguished from dynamically unstable ones by the fact that their α-tubulin subunits have a *glu* residue at their carboxyl-terminus, because the *tyr* residue normally at that terminus, present on the unstable microtubules, is enzymatically removed (Argarana et al., 1978; Kumar and Flavin, 1981). Antibodies have been prepared (Gundersen et al., 1984; Kreis, 1987; Wehland and Weber, 1987) that distinguish the *glu*-carboxyl terminus of the α-tubulin chains from the *tyr*-carboxyl terminus, and can thus be used to detect stable microtubules inside cells by immunocytochemical methods.

Stable microtubules appear to be less sensitive to microtubule depolymerizing drugs than are dynamically unstable ones (Kreis, 1987). It is therefore possible that in previous studies, which found the effects of such drugs on ER-to-Golgi transfer to be negligible (Rogalski et al., 1984), the minority of stable microtubules might largely have persisted, and therefore maintained ER-to-Golgi tracking.

In order to investigate whether stable microtubules might indeed be involved in the transfer of soluble secretory proteins from the ER to the Golgi apparatus, we have carried out the immunoelectron microscopic double labeling experiments reported in this paper. As the experimental system, we made use of the cultured human hepatoma cell line, HepG2, secreting human serum albumin (HSA), that we have earlier employed in related studies (Mizuno and Singer, 1993). In order to focus on HSA-containing transitional elements that occur between the ER and the Golgi apparatus, steady-state HepG2 cells were first treated with cycloheximide for 2.5 hours to arrest new protein synthesis and to empty the cells of their previously synthesized HSA (Keller et al., 1986). Upon washing out the cycloheximide and resuming protein synthesis, a more-or-less synchronous wave of transfer of HSA through the HepG2 cells was initiated, which took about 20 minutes to travel from the ER to fill the Golgi apparatus. During this interval, sections of the cells were double immunolabled for electron microscopy for HSA and for *glu*-tubulin. We observed a significant correlation between the distributions of the two colloidal gold-conjugated immunolabels in the same sections, supporting the proposal that stable microtubules might be involved in the process of transfer of transitional elements from the ER to the Golgi apparatus.

MATERIALS AND METHODS

Immunochemical reagents

In our earlier related studies (Mizuno and Singer, 1993) we used a polyclonal rabbit antibody as the primary reagent to immunolabel HSA. In the present studies, to facilitate double indirect immunolabeling, a polyclonal guinea pig anti-HSA antibody was generated and affinity-purified. Microtubules were immunolabeled either with an affinity-purified rabbit antibody to chicken brain tubulin (Heggeness et al., 1978), which labeled total microtubules (consisting mainly of the dynamically unstable type), or with the affinity-purified polyclonal rabbit antibody αT12 (Kreis, 1987), an anti-peptide antibody directed against the 11 carboxyl-terminal amino acids of α-tubulin from porcine brain. This antibody, the generous gift of Dr Thomas E. Kreis, is specific for *glu*-tubulin (Kreis, 1987). As secondary antibody reagents for immunofluorescence microscopy, we used a rhodamine-conjugated goat anti-guinea pig IgG and fluorescein- conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG (Jackson ImmunoResearch, Avondale, PA). For fluorescent labeling with wheat germ agglutinin (WGA), rhodamine-conjugated WGA (Vector Lab Inc., Burlingame, CA) was employed. As secondary reagents for immunoelectron microscopy, goat anti-rabbit IgG conjugated with 5 nm diameter

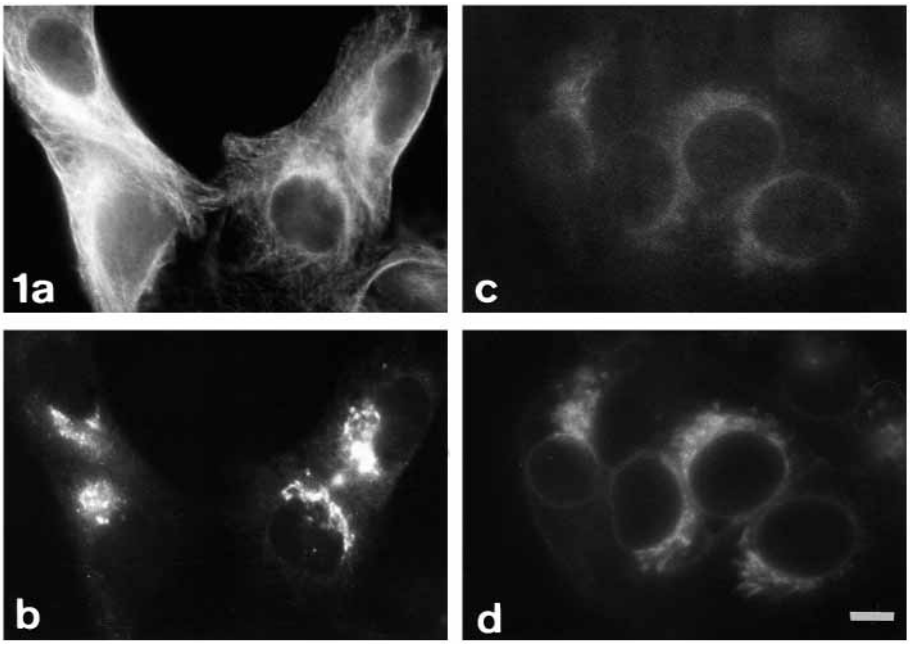

Fig. 1. Double fluorescent microscopic labeling of HepG2 cells in steady-state culture. (a) Indirect fluorescein immunolabeling for total tubulin, paired with (b) indirect rhodamine immunolabeling for HSA. (c) Indirect fluorescein immunolabeling for *glu*-tubulin, paired with (d) rhodamine-conjugated WGA. See text for details. The bar in (d) applies to all panels and represents 10 μm.

colloidal gold and goat anti-guinea pig IgG conjugated with 10 nm diameter colloidal gold (Janssen Life Science Products, Pisctaway, NJ) were used. The 5 nm gold preparation showed some heterogeneity in particle diameter, but there was no difficulty in distinguishing it from the relatively uniform 10 nm gold particles.

Cell culture

Frozen HepG2 cells were thawed and grown on 60 mm plastic culture dishes for one generation to confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and 10% fetal calf serum in 5% CO_2 humidified atmosphere at 37°C. They were then trypsinized, carefully dispersed, and then replated in one of two ways, depending on the nature of the experiments. For the immunoelectron microscopic experiments, the cells were replated in the same medium on 60 mm plastic dishes at 60-80% confluency and then processed within 24 hours after plating, as indicated below. For the immunofluorescence experiments, the cells were replated in the same medium on glass coverslips to about 60% confluency, and then used before 24 hours after plating.

Indirect immunofluorescence microscopy

The cells plated on glass coverslips were washed with serum-free medium, and were either processed directly (steady-state controls), or were incubated with 50 μg cycloheximide in DMEM for 2.5 hours at 37°C. The drug was then rapidly washed out of the cells by 4 changes of serum-free medium. Protein synthesis and secretion were resumed in fresh serum-free medium at 37ºC. At 3, 10, or 21 minutes after the washout of the cycloheximide, samples of the cells were washed twice with phosphate-buffered saline (PBS) pH 7.4, fixed with 3% formaldehyde in PBS for 15 minutes and then permeabilized with 1% Triton X-100 in PBS for 3 minutes. The steady-state controls were similarly fixed and permeabilized. After rinsing in PBS, the fixed and permeabilized cells were reacted for 10 minutes with a mixture of the two primary antibodies used in a given experiment. After washing in PBS, they were incubated with the secondary antibody reagents, and in some experiments, with fluorescein-conjugated WGA. After rinsing, the coverslips were mounted in 90% glycerol, 10% Tris-buffered saline, pH 8.5, and examined with a Zeiss Photomicroscope III, with epi-illumination or Nomarski differential interference-contrast optics at ×63 magnification.

Indirect immunoelectron microscopy

The cells replated on culture dishes were either processed directly (steady-state controls), or were treated with 50 μg/ml cycloheximide for 2.5 hours and then washed free of the drug, as indicated above. At 3, 10, or 21 minutes after resumption of protein synthesis, the cells were fixed with 3% formaldehyde, 0.1% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.4, for 1-2 hours at room temperature. The steady-state control cells were fixed similarly. The cells were scraped from the dishes and processed for cryosectioning as described (Mizuno and Singer, 1993). The specimens were cut in 60-90 nm thick sections with a Reichert OMV-4/FC-4 cryoultramicrotome at −110°C, and the thawed sections were then treated with a mixture of the two primary antibodies for 30-45 minutes and, after rinsing, with a mixture of the two colloidal gold-conjugated secondary antibody reagents for 60 minutes. The sections were then post-stained with neutral and acidic uranylacetate and embedded in polyvinyl alcohol (Tokuyasu, 1989). Electron microscopy was performed with a JEOL-1200 EX or a Phillips EM-300 instrument operated at 60 or 80 kV.

RESULTS

Immunofluorescence microscopy

In order to obtain a low resolution overview of the relative distributions of total microtubules (mostly dynamically unstable ones) as compared to stable microtubules, and to locate these two elements with respect to the Golgi apparatus, the following experiments were carried out. HepG2 cells in the steady-state were double immunolabled with antibodies to total tubulin (Fig. 1a) and anti-HSA antibodies (Fig. 1b); or with anti-*glu*-tubulin antibodies (Fig. 1c) and WGA (Fig. 1d). Although the two kinds of primary antibody reagents used for total tubulin and *glu*-tubulin labeling were not directly comparable, it is clear from the relative fluorescence intensities that stable microtubules (Fig. 1c) represent qualitatively only a fraction of the total microtubules (Fig. 1a). Furthermore, the stable microtubules appear to be most concentrated in the vicinity of the

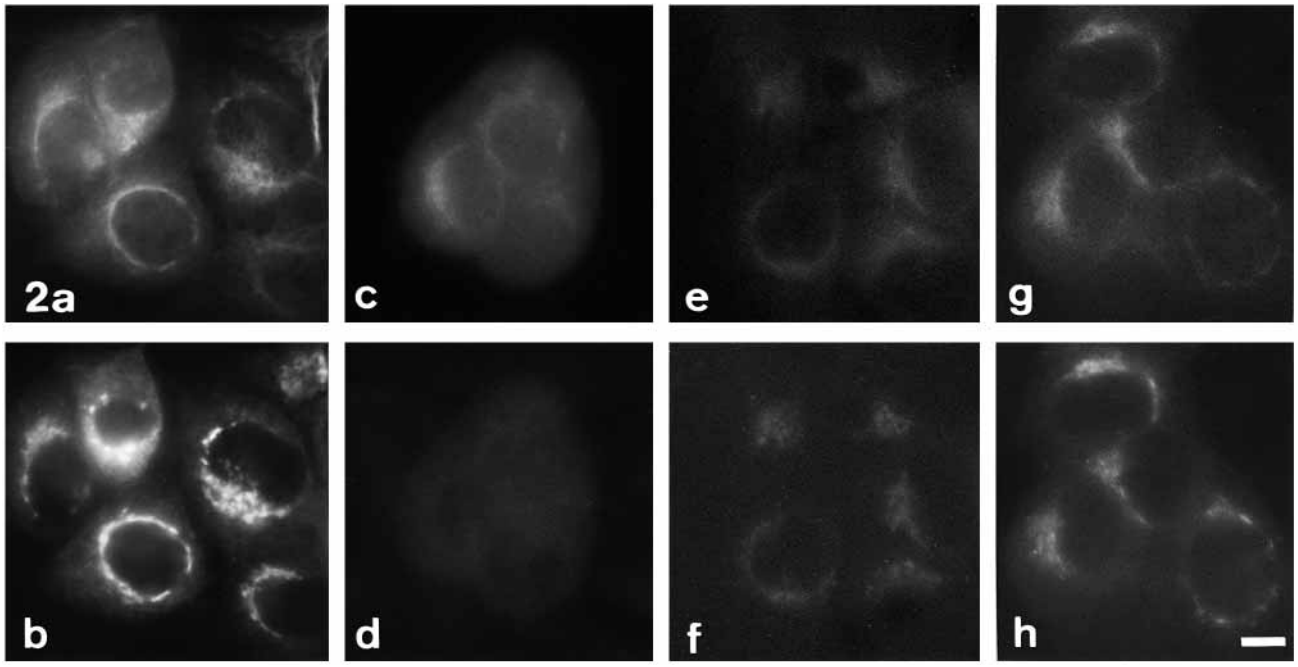

Fig. 2. Indirect double immunofluorescent labeling of HepG2 cells: the effect of the cycloheximide treatment protocol on the distribution of *glu*-tubulin (a,c,e,g) and HSA (b,d,f,h). (a and b) Control cells in the steady-state; (c and d) 3 minutes after washing out the cycloheximide that had previously been present for 2.5 hours; (e and f) 10 minutes after washout; and (g and h) 21 minutes after washout. The bar in (h) applies to all panels and represents 10 μm.

cell nucleus (Fig. 1c) in close proximity to the Golgi apparatus, localized by WGA labeling (Fig. 1d), in the same cell.

Most of the experiments reported in this paper deal with HepG2 cells that were treated with cycloheximide for 2.5 hours at 37°C, and in which, after washout of the cycloheximide and resumption of protein synthesis, the intracellular transport of HSA was followed with time. A representative experiment is shown in Fig. 2, as observed by double immunofluorescence with anti-*glu*-tubulin and anti-HSA antibodies. As a control, HepG2 cells in the steady-state, prior to the cycloheximide treatment, are shown after double immunolabeling for stable microtubules (Fig. 2a) and for HSA (Fig. 2b). With cells that had first been treated with cycloheximide for 2.5 hours, at 3 minutes after washout of the cycloheximide stable microtubules were still detected (Fig. 2c), but the same cells had been emptied of their HSA (Fig. 2d) as a result of the cycloheximide treatment. By 10 minutes after washout, immunolabeling for HSA was now evident (Fig. 2f) corresponding (see below) to newly synthesized HSA present mostly in transitional elements between the ER and Golgi apparatus, the latter being largely still empty at this time. By 21 minutes after washout, immunolabeling for HSA in the Golgi apparatus was now quite prominent (Fig. 2h). Stable microtubules were observed throughout this period (Fig. 2c,e, and g, respectively). The variations in the intensities of the immunofluorescent labeling for stable microtubules from one Figure to the next are probably not significant. While these experiments do not prove that the *same* stable microtubules persisted through the 2.5 hour cycloheximide treatment, they at least allow for that possibility. The stable microtubules clearly did not depolymerize when protein synthesis was turned off.

Immunoelectron microscopy

At the higher resolution of the electron microscope, when HepG2 cells in the steady-state were frozen sectioned and then double immunolabled for total tubulin and for HSA, results such as those shown in Fig. 3 were obtained (compare with the immunofluorescence results in Fig. 1a,b). There was intense labeling for total microtubules immediately surrounding the Golgi stacks (G in Fig. 3) but no labeling within the stacks or between the saccules. Additional labeling for total tubulin was observed in regions away from the Golgi stacks as well. The colloidal gold labels for tubulin were often collected in clusters that were circular in outline, probably corresponding to the labeling of single microtubules (thickened by antibody binding) that were cut in cross-section (cf. open arrow, Fig. 3). Immunogold labeling for HSA was most intense over the Golgi stacks, as expected; sparser labeling for HSA was also observed at other sites. Of most interest for our present purposes, there were a significant number of local areas where the labels for total microtubules and for HSA were in close proximity to one another (long arrows, Fig. 3). These closely proximal distributions usually involved 1 to 3 larger gold labels for HSA and 5 to 10 smaller gold for tubulin. Such local areas of coordinate double labeling were often observed associated with membrane elements of the ER or at the outer rims of the Golgi stacks.

Turning from total microtubules to stable microtubules, we obtained double immunolabeling results with anti-*glu*-tubulin antibodies and anti-HSA antibodies on frozen sections of steady-state HepG2 cells such as those shown in Fig. 4. The intensity of immunogold labeling for the stable microtubules was much less than for total microtubules (compare Fig. 4 with Fig. 3), consistent with the immunofluorescence results in Fig.

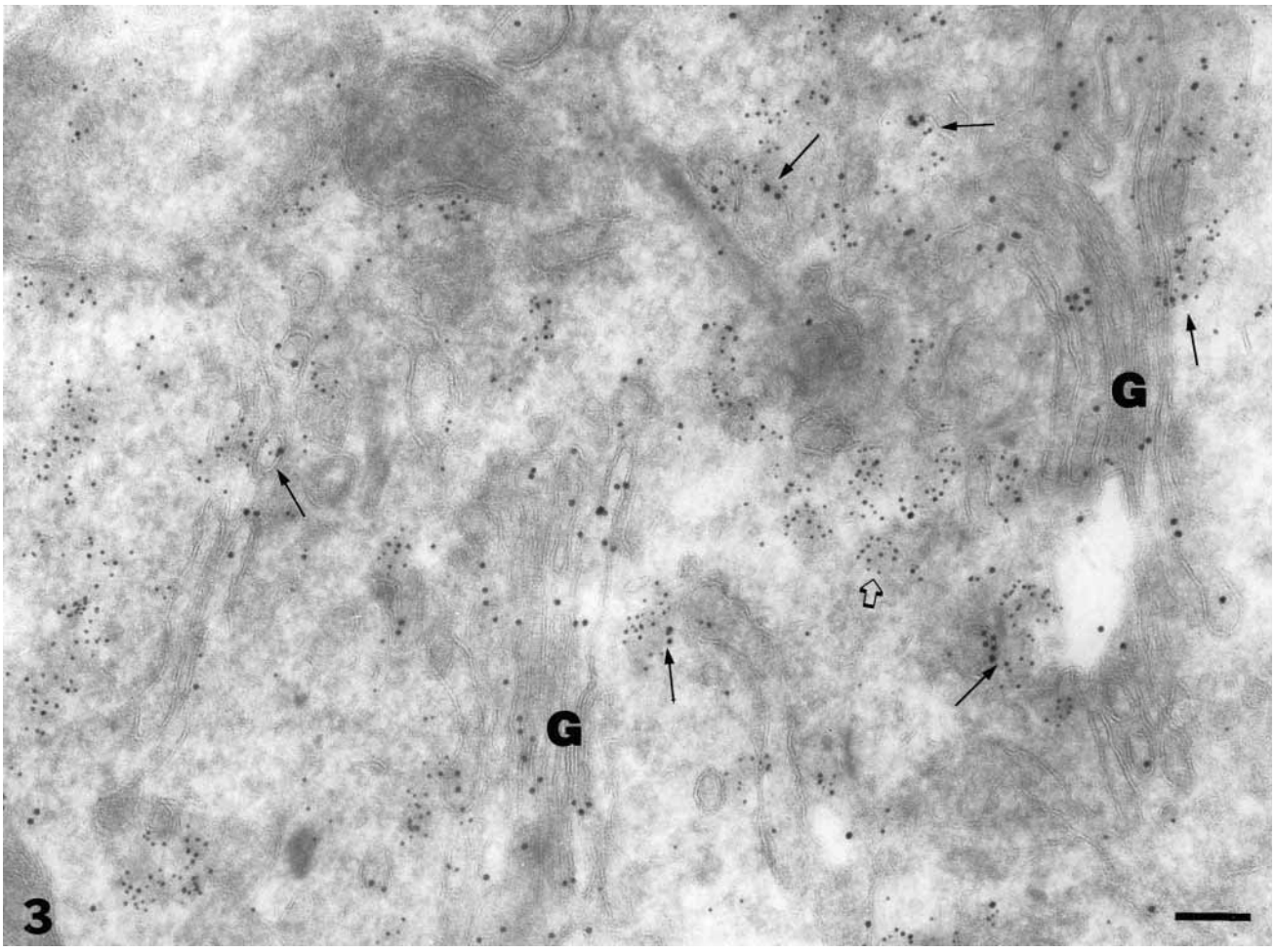

Fig. 3. Double indirect immunogold labeling of a frozen section of a HepG2 cell in steady-state culture using primary antibodies against total tubulin (labeling with 5 nm average diameter gold particles) and against HSA (10 nm gold particles). The 5 nm gold particles are not as uniform in diameter as are the 10 nm particles. The long arrows indicate some of the sites of closely apposed double labeling. The open blunt arrow shows a circular cluster of gold labels for tubulin that probably represents the labeling of a single microtubule in cross-section. G, Golgi apparatus. Bar, 0.1 μm.

1a and c. The two different primary antibodies used in the labeling of total microtubules and stable microtubules are not directly comparable, and therefore do not lend themselves to a direct quantitative analysis of the labeling results. While recognizing this limitation, it is nevertheless of interest that from observations of a number of comparable sections, there were about (5±1) times as many gold labels per section for total microtubules as for stable ones in the general vicinity of the Golgi apparatus. The immunolabels for stable microtubules were often found immediately adjacent to the Golgi stacks (Fig. 4) but not within them. This was the case for Golgi stacks that were close to the nucleus (Fig. 4a) as well as for those near the cell periphery (Fig. 4b). In addition, labeling for stable microtubules was often found in close association with ER membranes (arrowheads, Fig. 4b). Of most direct interest for our present purposes, there were a significant number of sites where the colloidal gold labels for stable microtubules and those for HSA were in close proximity to one another (long arrows, Fig. 4a), some in the area between the nucleus and the Golgi apparatus, which might therefore represent the coordinate labeling of transitional elements of the ER.

We next examine the double immunolabeling results obtained with the HepG2 cells that were first treated with cycloheximide for 2.5 hours. Shortly (3 minutes) after the cycloheximide was washed out, the sections were found to be devoid of labeling for HSA (Fig. 5). In particular, the Golgi apparatus and the region between the nucleus and the Golgi showed no HSA labeling, indicating that the cycloheximide treatment had emptied the cells of their previously synthesized HSA. In these cells the labeling for stable microtubules, however, was just as prominent (Fig. 5) as in the steady-state cells (Fig. 4).

Fig. 5 also demonstrates that the overall distribution of stable microtubules between local areas of the cytoplasm is strikingly non-uniform. (This is also evident in Figs 4 and 6.) Thus, the entire left half of Fig. 5 is nearly completely devoid of labels for the stable microtubules, whereas the adjacent regions immediately surrounding the Golgi apparatus, and between the nucleus and the Golgi show a concentration of labels. Of particular interest is that the region of the cytoplasm adjacent to the nucleus, but directed away from the Golgi apparatus (asterisk in Fig. 5) is devoid of labels. This non-

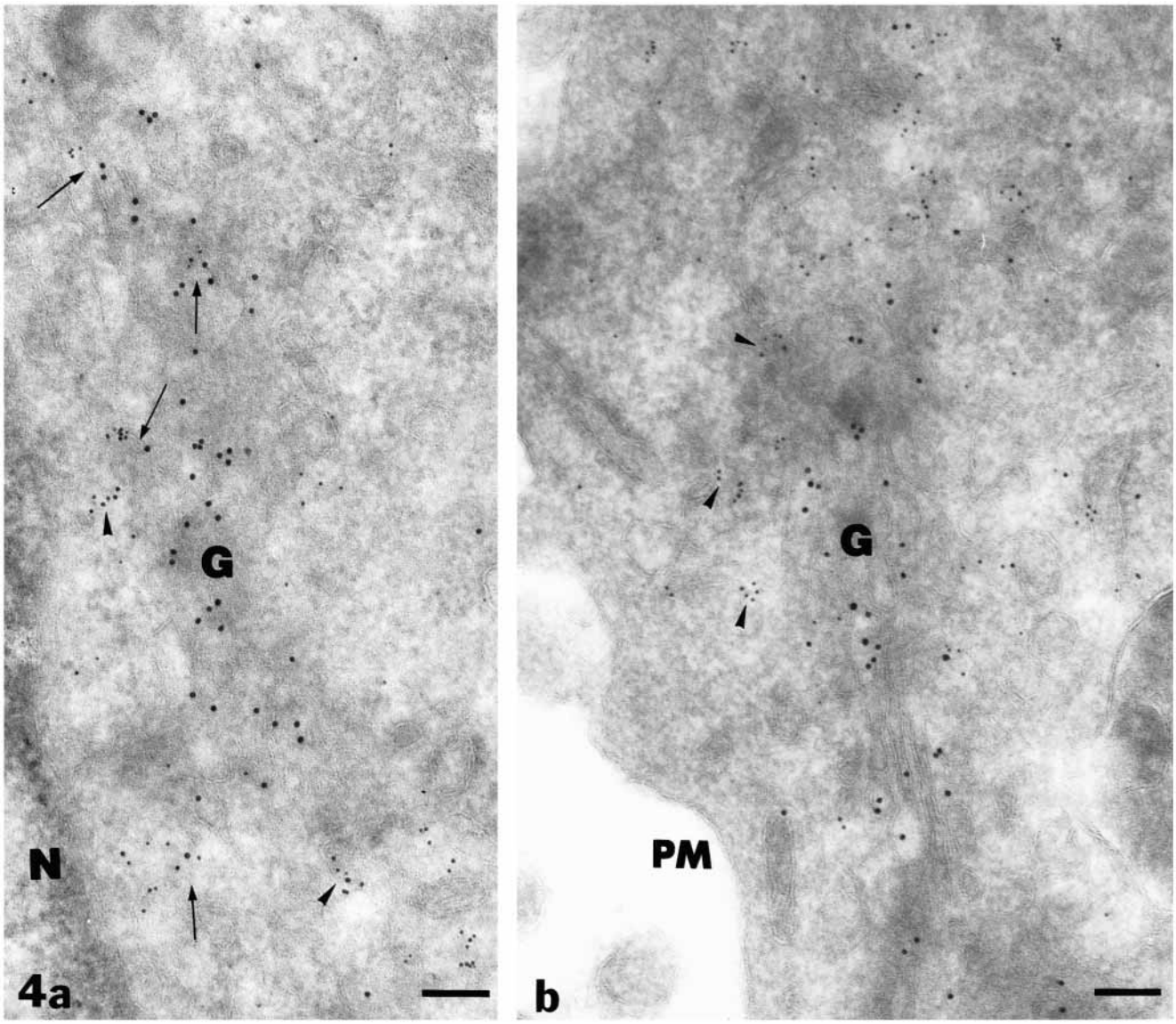

Fig. 4. Two examples of double indirect immunogold labeling of a frozen section of a HepG2 cell in steady-state culture, using primary antibodies against *glu*-tubulin (labeling with 5 nm average diameter gold particles) and against HSA (10 nm gold particles). (a) A region between the nucleus (N) and the Golgi apparatus (G), (b) a region containing a Golgi apparatus near the cell boundary. (PM, plasma membrane). The long arrows indicate some of the sites of closely apposed double labeling, and the arrowheads indicate some of the sites labeled only for *glu*-tubulin. Bars, 0.1 μm.

uniform distribution cannot be accidental, and strongly suggests that specific interactions of the stable microtubules with certain cytoplasmic elements (such as the peripheral elements of the Golgi apparatus, and elements of the ER localized between the Golgi and the nucleus) are acting to produce these sharply non-uniform distributions of these microtubules.

By 10 minutes after washout of the cycloheximide, significant labeling for HSA had reappeared (Fig. 6), but at this time, the newly synthesized HSA had not yet entered the Golgi apparatus (G in Fig. 6a and c) in significant amounts. HSA labeling was sometimes seen near one side of a Golgi stack, as in Fig. 6a and c, or between the nucleus and the Golgi apparatus, as in Fig. 6c. In these locations, HSA labels were often observed in close proximity to the labels for stable microtubules (long arrows, Fig. 6a and c). In other areas, labeling for stable microtubules was found associated with membrane structures that did not show HSA labeling (arrowheads, Fig. 6b and c), which appeared to correspond to transitional elements in the ER. In Fig. 6b, an elongated tubular structure marked at either end by two white arrows contains colloidal gold labels for HSA in the lumen of the tubule (near the arrows), and at some distance from the HSA labels, a cluster of labels for stable microtubules, marked by an arrowhead, associated with the outer surface of the same tubule. The possible significance of this remarkable structure is taken up in the Discussion.

By 21 minutes after washout of the cycloheximide, significant labeling for HSA within the Golgi stacks was found (Fig. 7). Colloidal gold labels for stable microtubules were present

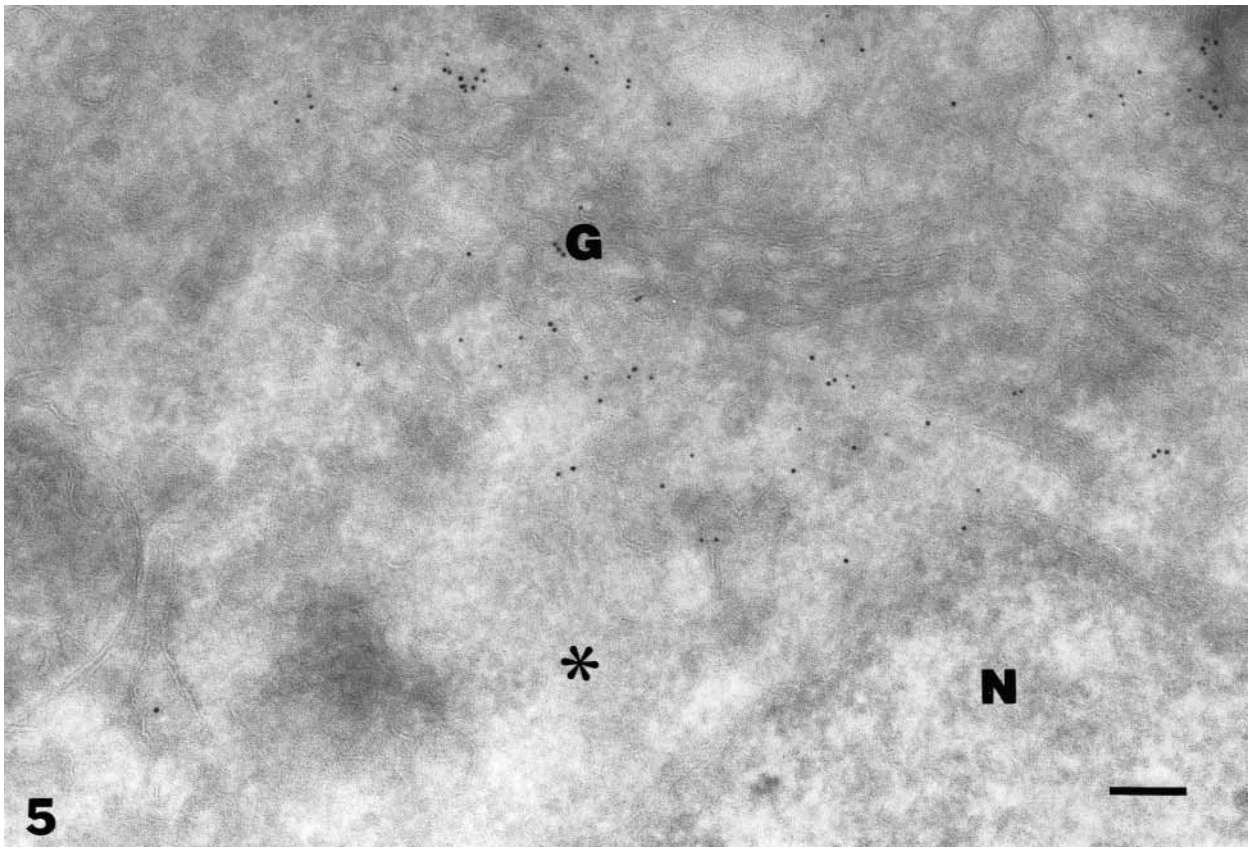

Fig. 5. Double indirect immunogold labeling of a frozen section of a HepG2 cell 3 minutes after washout of the cycloheximide (see Materials and Methods), using primary antibodies against *glu*-tubulin (labeling with 5 nm average diameter gold particles) and against HSA (10 nm gold particles). Only the smaller gold particles are observed, the HSA having been emptied from the cell by the prior 2.5 hours cycloheximide treatment. Note the highly non-uniform distribution of the stable microtubules, which are concentrated around the periphery of the Golgi apparatus (G), and in the region between the Golgi apparatus and the nucleus (N). The left half of the Figure is devoid of labels. In particular, the cytoplasm adjacent to the nucleus, but directed away from the Golgi apparatus (asterisk), is devoid of labels. Bar, 0.1 μm.

around the Golgi apparatus, and in particular, some were associated with vesicles (arrowheads and tie-bar, Fig. 7) that were situated at or near one face, probably the *cis*-face, of the Golgi stack.

DISCUSSION

In the Introduction, the question was raised whether vesicular traffic from the ER to the Golgi apparatus is a stochastic process, or whether it is in some ways restricted or directed. We mentioned our earlier studies with heterokaryons (Valtersson and Singer, 1987; Valtersson et al., 1990, 1993), which suggested to us that the latter is the more likely possibility. In pursuing this idea of a non-random transfer process, we considered whether ER-to-Golgi traffic might be directed along microtubules (Valtersson et al., 1990), or more specifically, along the small fraction of total microtubules that are stable within most cells (Valtersson et al., 1993). As a first test of this possibility, we investigated by immunofluorescence microscopy whether stable microtubules exist in the HepG2 cells that we used in our experiments, and if so, whether the location of stable microtubules in the cell might be consistent with a role in directing ER-to-Golgi traffic. With anti-*glu* tubulin antibodies to detect them, we showed (Fig. 1c) that stable microtubules are indeed present as a small fraction of the total microtubules (Fig. 1a) in the HepG2 cells. Futhermore, the stable microtubules are most concentrated near, and at low resolution are distributed close to, the Golgi apparatus in the cells (Fig. 1d). These results parallel those that other investigators have obtained with other kinds of cultured cells (Gundersen et al., 1984; Wehland and Weber, 1987; Kreis, 1987; Skoufias et al., 1990).

As a more probing test of the possible involvement of stable microtubules in directing ER-to-Golgi traffic, we decided to ask whether at the electron microscopic level of resolution stable microtubules are spatially closely associated with transitional elements of the ER. To do this experimentally, we carried out double immunoelectron microscopic labeling experiments on ultrathin frozen sections of cultured HepG2 hepatoma cells, again using anti-*glu* tubulin antibodies to label stable microtubules, and anti-HSA antibodies to label transitional elements that contained the soluble secretory protein HSA in transit between the ER and the Golgi apparatus. In order to detect such transitional elements specifically, we utilized a cycloheximide treatment protocol (Keller et al., 1986; Mizuno and Singer, 1993), that synchronized HSA transfer and secretion in the HepG2 cells (Fig. 2) and thereby

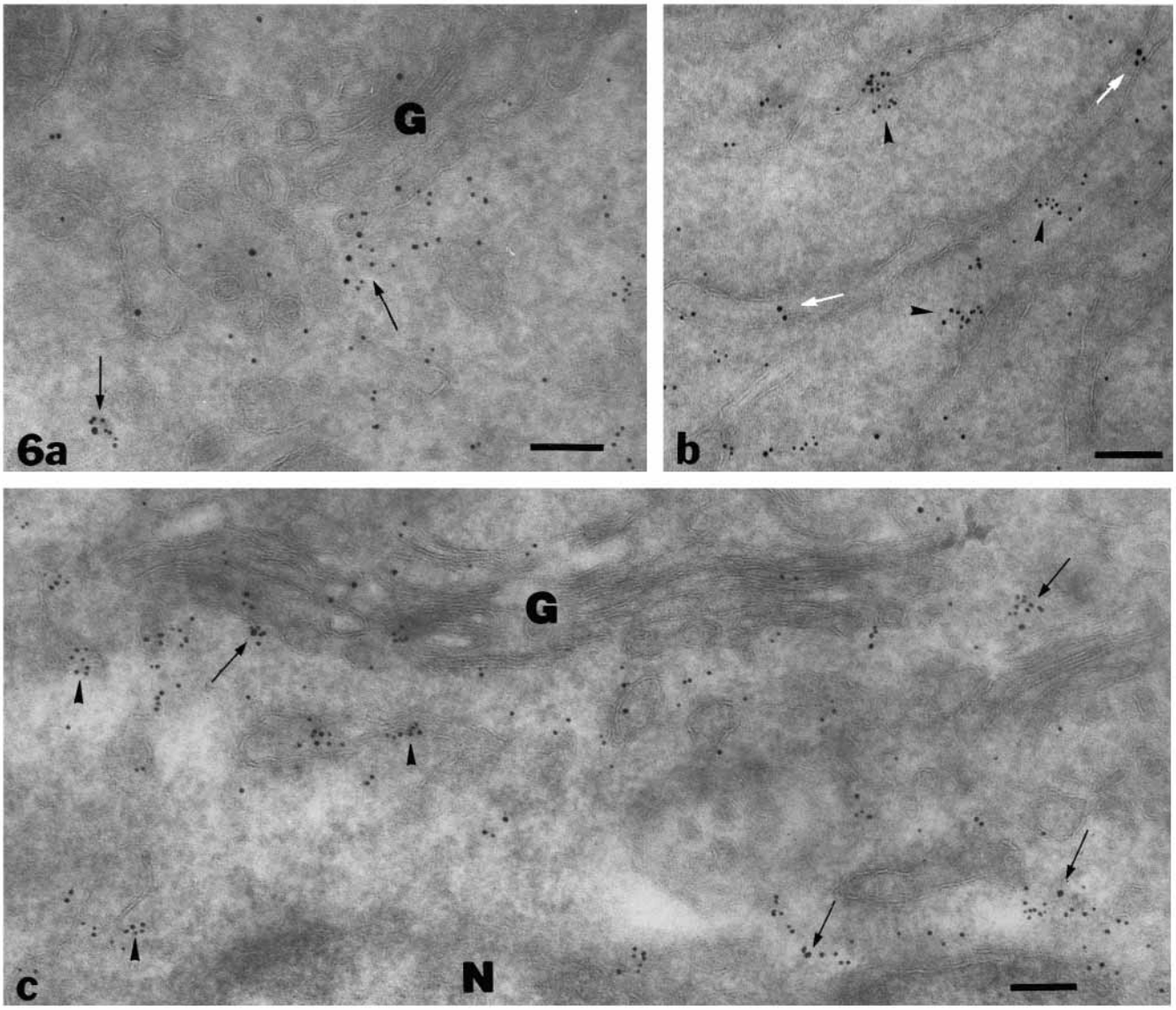

Fig. 6. Three examples of double indirect immunogold labeling of frozen sections of HepG2 cells 10 minutes after washout of the cycloheximide, using primary antibodies against *glu*-tubulin (labeling with 5 nm average diameter gold particles) and against HSA (10 nm gold particles). The long black arrows in (a) and (c) indicate some of the sites of closely apposed double labeling for both HSA and *glu*-tubulin, and the arrowheads in (b) and (c) indicate some of the sites closely apposed to membranous structures of the ER that are labeled only for *glu*-tubulin. In (b) an elongated tubule, its lumen indicated by two white arrows pointing to widely separated HSA labels, is also labeled for *glu*-tubulin at one site on its outer surface(arrowhead). The Golgi apparatuses in (a) and (c) (G), are empty of HSA labels at this time. N, nucleus. Bars, 0.1 μm.

allowed us to define the time interval in which the transfer of the HSA from the ER to the Golgi apparatus took place.

As controls for these double immunoelectron microscopic labeling experiments, we first examined HepG2 cells in the steady-state (Figs 3 and 4), that is, prior to cycloheximide treatment. Labeling for stable microtubules was about 5-fold less intense than for total microtubules in the general region of the Golgi apparatus, confirming the qualitative immunofluorescence results in Fig. 1. In addition, a close proximity of these microtubule labels to those for HSA near the Golgi apparatus was observed with significant frequencies for both total and stable microtubules (long arrows in Figs 3 and 4).

For cells that had been treated with cycloheximide for 2.5 hours, shortly after the cycloheximide was washed out there was no or little colloidal gold immunolabeling for HSA (Fig. 5, compare to Fig. 2d), showing that the HSA that was synthesized prior to the cycloheximide treatment was essentially completely secreted from the cell, and also demonstrating the very low level of non-specific labeling with our technique. The same cycloheximide treatment, however, did not remove the stable microtubules from the region between the nucleus and the Golgi apparatus (Fig. 5). By 10 minutes after protein synthesis was resumed, a substantial intensity of labeling for HSA was now observed (Fig. 6) at a time when the Golgi apparatus was still largely empty. All of the HSA in the cell at this time must therefore have been located either in the ER or

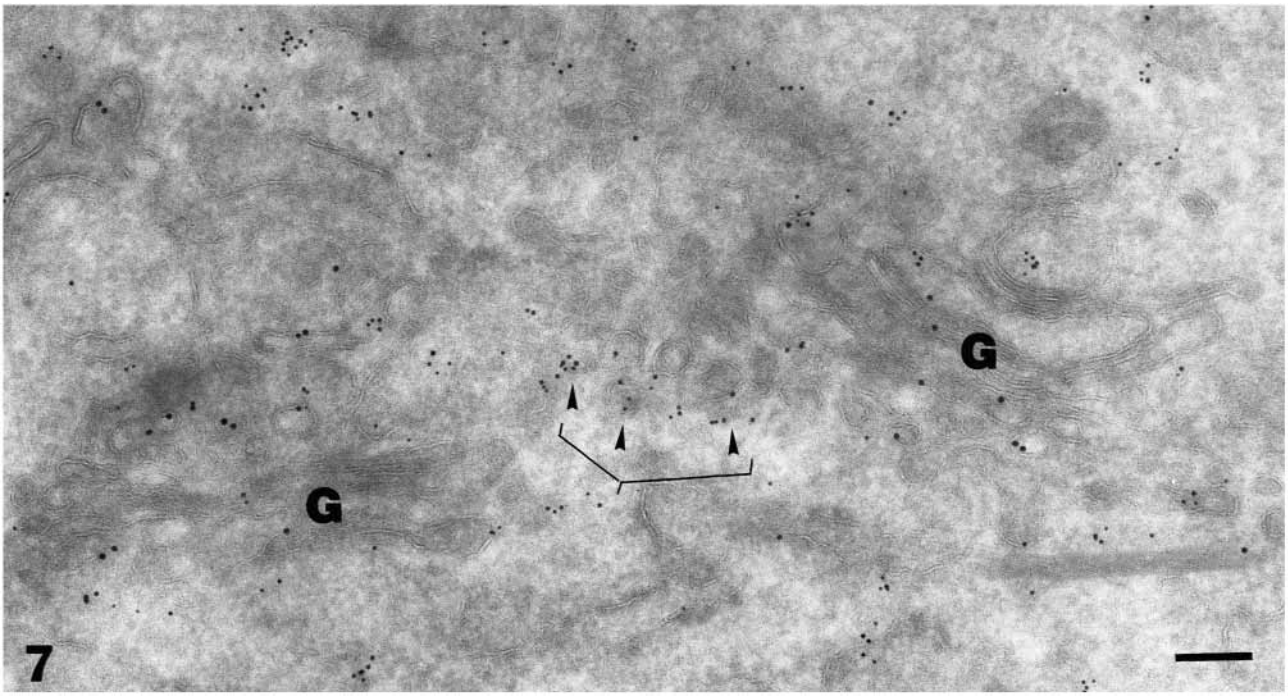

Fig. 7. Double indirect immunogold labeling of a frozen section of a HepG2 cell 21 minutes after washout of the cycloheximide, using primary antibodies against *glu*-tubulin (labeling with 5 nm average diameter gold particles) and against HSA (10 nm gold particles). HSA labeling appears throughout the Golgi apparatuses (G) by this time. The bracketed arrowheads indicate sites near one face of a Golgi stack where *glu*-tubulin labeling is closely apposed to membranous elements, probably at the *cis*-face of the stack. Bar, 0.1 μm.

in transitional elements between the ER and the Golgi apparatus, some near the *cis*-faces of the Golgi stacks (Fig. 6a,c). In close proximity to an appreciable number of such sites of transitional elements labeled for HSA, labeling for stable microtubules was also observed (long arrows in Fig. 6a,c). While there were a considerable number of sites where labeling for either HSA or for stable microtubules was not closely localized to the other, we consider that the relative number of sites of closely proximal double labeling is large enough to be highly significant. This conclusion derives in part from the following detailed consideration of Fig. 6b.

In Fig. 6b, also of a cell 10 minutes after protein synthesis was resumed, a section of an elongated tubule is visible, with immunogold labeling for HSA in the lumen near opposite ends of the tubule (white arrows). The timing of the experiment and the morphology of the tubule identify the tubule as a transitional element between the ER and the Golgi apparatus. A cluster of labels for stable microtubules is seen close to the outer surface of the same tubule (arrowhead) and at some considerable distance from the HSA labels. These observations strongly suggest that a functionally significant association of stable microtubules with transitional elements might exist even though close proximity of the HSA and *glu*-tubulin labels in a section is not observed. Had the tubule in Fig. 6b been cut transversely in successive cross-sections, most of such circular cross-sections would have shown neither HSA nor *glu*-tubulin labels, a few would have shown either one or the other label, but none would have shown both labels. These observations, therefore, suggest that the frequencies with which closely proximal double labeling for HSA and for stable microtubules were indeed observed (long arrows in Figs 4a, 6a, and 6c) are highly significant.

Of additional significance are the instances where single immunolabeling for *glu*-tubulin was observed in close proximity to what appears to be the *cis*-face of the Golgi apparatus (arrowheads, Fig. 4a and particularly Fig. 7), suggesting again the involvement of stable microtubules in the delivery of transition vesicles to the Golgi apparatus, or possibly in the recycling of vesicles emptied of their contents back to the ER (Lippincott-Schwartz et al., 1990).

The close spatial proximities of glu-tubulin and HSA labels cannot simply be adventitious, but rather suggest that stable microtubules are directly or indirectly linked to elements of the ER containing HSA. This conclusion is inferred from the fact that in much of the cytoplasm adjacent to areas of substantial glu-tubulin labeling, there are other areas devoid of glu-tubulin labels (cf. Fig. 5). Such non-uniform local densities of labeling strongly suggest that specific interactions of stable microtubules with elements in the cytoplasm are involved in the observed sequestration of glu-tubulin labels.

The evidence presented in this report is therefore consistent with, but clearly does not prove, the proposal that stable microtubules are involved in the tracking of transitional elements from the ER to the Golgi apparatus, and probably in the recycling pathway back from the Golgi apparatus to the ER. This is not necessarily to say that only stable microtubules are involved, to the exclusion of dynamic ones. However, the many results in the literature cited in the Introduction that show that microtubule-depolymerizing drugs have little or no effect on ER-to-Golgi transfer can be best reconciled with the results in this paper, if stable microtubules, that are more resistant to microtubule-depolymerizing drugs than are the dynamic microtubules (Kreis, 1987; Webster et al., 1990), are the pre-

dominant ones involved in the ER-to-Golgi transfer process. This important point is, however, difficult to test by the methods used in this article. Microtubule-depolymerizing drugs are well known (cf. Rogalski et al., 1984) to cause the normally compact Golgi apparatus to be dispersed into many individual stacks distributed throughout the entire cytoplasm. It would be difficult to find and identify these grossly dispersed stacks with reasonable frequencies in ultrathin sections, and then to ascertain by double immunoelectron microscopic labeling whether there were still HSA-containing transitional elements of the ER associated with intact stable microtubules in their immediate vicinity.

Other kinds of experiments to test for a possible role for stable microtubules in ER-to-Golgi transfer can be suggested. If the microinjection of anti-*glu*-tubulin antibodies, or their Fab fragments, into cycloheximide-treated HepG2 cells, followed by washing out of the cycloheximide and the re-initiation of protein synthesis, was shown by immunofluorescence microscopy to specifically inhibit the appearance of HSA in the Golgi apparatus, this would constitute evidence for a functional role of stable microtubules in the process of ER-to-Golgi transfer. Related microinjection experiments on the transfer of vesicular stomatis virus G-protein, a membrane integral protein, from the ER to the Golgi apparatus have recently shown that the protein β-COP is involved in this transfer (Pepperkok et al., 1993), probably, however, in the vesiculation rather than the transport stage of the process.

There are two ways that stable microtubules could conceivably be involved in ER-to-Golgi transfer. Vesicles may first bud off transitional elements of the ER, and then be tracked along the microtubules to the *cis*-face of a stack of Golgi saccules. Alternatively, ER tubules may first be extended by tracking along the microtubules (Lee and Chen, 1988; Dabora and Sheetz, 1988), and only then might vesicles bud off the tubules in close proximity to the *cis*-face of a Golgi stack. The low voltage electron microscopic observations we have made of ultrathin sections do not allow discrimination between these alternatives.

Finally, the proposal that ER-to-Golgi transfer is a directed process involving stable microtubules implies a requirement for some kind of structural organization of elements of the ER, the Golgi apparatus, and stable microtubules. This may explain why only very gentle cell permeation treatment is required to allow ER-to-Golgi transfer to be retained in in vitro model system experiments (Balch, 1989). In the usual more highly disrupted cell-free systems, in which transfer between fragmented Golgi compartments can still be reconstituted (Rothman et al., 1984), ER-to-Golgi transfer cannot be recovered. This is consistent with the idea that the vesicular transfer between compartments of the Golgi apparatus is a stochastic process (Rothman et al., 1984), whereas vesicular transfer between the ER and the Golgi apparatus is not.

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REFERENCES

Argarana, C. E., Barra, H. S. and Caputto, R. (1978). Release of [^{14}C] tyrosine from tubulinyl-[^{14}C] tyrosine by brain extract. Separation of a carboxy peptidase from tubulin tyrosine ligase. *Mol. Cell. Biochem.* **19**, 17-22.

Balch, W. E. (1989). Biochemistry of interorganelle transport. A new frontier in enzymology emerges from versatile in vitro model systems. *J. Biol. Chem.* **264**, 16965-16968.

Boyd, A. E., III, Bolton, W. E. and Brinkley, B. R. (1982). Microtubules and beta cell function: effect of colchicine on microtubules and insulin secretion *in vitro* by mouse beta cells. *J. Cell Biol*. **92**, 425-434.

Dabora, S. L. and Sheetz, M. P. (1988). The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell* **54**, 27-35.

Gundersen, G. G., Kalnoski, M. H. and Bulinski, J. C. (1984). Distinct populations of microtubules: tyrosinated and non-tyrosinated alpha tubulin are distributed differently *in vivo*. *Cell* **38**, 779-789.

Heggeness, M. H., Simon, M. and Singer, S. J. (1978). Association of mitochondria with microtubules in cultured cells. *Proc. Nat. Acad. Sci. USA* **75**, 3863-3866.

Keller, G.-A., Glass, C., Louvard, D., Steinberg, D. and Singer, S. J. (1986). Synchronized synthesis and intracellular transport of serum albumin and apolipoprotein B in cultured rat hepatocytes as studied by double immunofluorescence. *J. Histochem. Cytochem*. **34**, 1223-1230.

Klausner, R. D., Donaldson, J. G. and Lippincott-Schwartz, J. (1992). Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol*. **116**, 1071-1080.

Kreis, T. E. (1987). Microtubules containing detyrosinated tubulin are less dynamic. *EMBO J*. **6**, 2597-2606.

Kumar, N. and Flavin, M. (1981). Preferential action of a brain detyrosinating carboxypeptidase on polymerized tubulin. *J. Biol. Chem*. **256**, 7678-7686.

Lee, C. and Chen, L. B. (1988). Dynamic behavior of endoplasmic reticulum in living cells. *Cell* **54**, 37-46.

Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H.-P, Yuan, L. C. and Klausner, R. D. (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.

Lodish, H. F., Kong, N., Hirani, S. and Rasmussen, J. (1987). A vesicular intermediate in the transport of hepatoma secretory proteins from the rough endoplasmic reticulum to the Golgi complex. *J. Cell Biol*. **104**, 221-230.

Merisko, E. M., Fletcher, M. and Palade, G. E. (1986). The reorganization of the Golgi complex in anoxic pancreatic acinar cells. *Pancreas* **1**, 95-109.

Mizuno, M. and Singer, S. J. (1993). A soluble secretory protein is first concentrated in the endoplasmic reticulum before transfer to the Golgi apparatus. *Proc. Nat. Acad. Sci. USA* **90**, 5732-5736.

Palade, G. E. (1975). Intracellular aspects of the process of protein secretion. *Science* **189**, 347-358.

Pepperkok, R., Scheel, J., Horstmann, H., Hauri, H. P., Griffiths, G. and Kreis, T. (1993). β-COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex *in vivo*. *Cell* **74**, 71-82.

Rogalski, A. A., Bergmann, J. E. and Singer, S. J. (1984). Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane. *J. Cell Biol*. **99**, 1101-1109.

Rothman, J. E., Urbani, L. I. and Brands, R. (1984). Intercompartmental transport in the Golgi complex is a dissociative process: facile transfer of membrane protein between two Golgi populations. *J. Cell Biol*. **99**, 260-271.

Saraste, J. and Kuismanen, E. (1984). Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell* **38**, 535-549.

Saraste, J. and Kuismanen, E. (1992). Pathways of protein sorting and membrane traffic between the rough endoplasmic reticulum and the Golgi complex. *Semin. Cell Biol*. **3**, 343-355.

Schulze, E. and Kirschner, M. (1988). New features of microtubule behavior observed *in vitro*. *Nature* **334**, 356-359.

Skoufias, D. A., Burgess, T. L. and Wilson, L. (1990). Spatial and temporal co-localization of the Golgi apparatus and microtubules rich in detyrosinated tubulin. *J. Cell Biol*. **111**, 1929-1937.

Terasaki, M., Chen, L. B. and Fujiwara, K. (1986). Microtubules and the endoplasmic reticulum are highly interdependent structures. *J. Cell Biol*. **103**, 1557-1568.

Tokuyasu, K. T. (1989). Use of poly(vinylpyrrolidone) and poly(vinyl alcohol) for cryoultramicrotomy. *Histochem J*. **21**, 163-171.

Valtersson, C. and Singer, S. J. (1987). The transfer of the secretory protein serum albumin to the two Golgi apparatuses in heterokaryons formed by fusion of human hepatoma and fibroblast cell lines. *J. Cell Biol*. **105**, 266 (abstract).

Valtersson, C., Dutton, A. H. and Singer, S. J. (1990). Transfer of secretory proteins from the endoplasmic reticulum to the Golgi apparatus: Discrimination between homologous and heterologous transfer in intact heterokaryons. *Proc. Nat. Acad. Sci. USA* **87**, 8175-8179.

Valtersson, C., Mizuno, M., Dutton, A. H. and Singer, S. J. (1993). Intracellular transport of secretory proteins with intact transient heterokaryons and homokaryons. In *The Legacy of Cell Fusion* (ed. S. Gordon). Oxford University Press (in press).

Webster, D. R., Wehland, J., Weber, K. and Borisy, G. G. (1990). Detyrosination of alpha tubulin does not stabilize microtubules *in vivo*. *J. Cell Biol*. **111**, 113-122.

Wehland, J. and Weber, K. (1987). Turnover of the carboxy-terminal tyrosine of α-tubulin and means of reaching elevated levels of detyrosination in living cells. *J. Cell Sci*. **88**, 185-203.

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