The Golgi complex is organized into three functionally distinct parts: the cis-Golgi network (CGN), the stack of Golgi cisternae (SGC) and the trans-Golgi network (TGN) (Novikoff et al., 1971; Palade, 1975; Farquhar and Palade, 1981; Griffiths and Simons, 1986; Rothman and Orci, 1992; Warren and Malhotra, 1998). The CGN, as the entry face of the Golgi, receives the newly synthesized proteins from the ER for their further transport to the SGC. In addition, the CGN recycles back to the ER the proteins that escaped the ER and components of the machinery of vesicular transport that operates between the ER and the Golgi complex (Dean and Pelham, 1990; Semenza et al., 1990). Forty genes have been identified to date in the transport between ER and the CGN in the yeast (Kaiser et al., 1997), and the metabolic (Jamieson and Palade, 1968), protein (Tisdale et al., 1992; Nuoffer et al., 1994; Pind et al., 1994; Dascher et al., 1994; Dascher and Balch, 1994; Barlowe et al., 1994; Tisdale and Balch, 1996; Rowe et al., 1998), ion (Beckers and Balch, 1989; Beckers et al., 1990), nucleotide (Balch and Rothman, 1985; Balch et al., 1986), and temperature requirements (Saraste et al., 1986) of this transport have been determined. ATP-sensitive steps appear to punctuate the transport of protein between the compartmental boundaries of the ER and CGN (Jamieson and Palade, 1968; Balch et al., 1986). Moreover, depletion of the cytosolic pool of ATP causes the tubulation of Golgi membranes both in vivo and in vitro (Cluett et al., 1993; Banta et al., 1995).

The manipulation of the vesicular transport machineries (Dascher and Balch, 1994; Wilson et al., 1994), the overexpression or manipulation of proteins resident in specific compartments (Hsu et al., 1992; Nilsson et al., 1996; Nelson et al., 1998), and the ability of drugs to disrupt the membrane traffic (Lippincott-Schwartz et al., 1989; Takizawa et al., 1993; Deerinck et al., 1993; Sandoval and Carrasco, 1997; Fujiwara et al., 1998) have been used to dissect the protein transport pathways as well as to define the boundaries, morphology and links between the Golgi compartments and adjacent organelles. These approaches nevertheless often provoked alterations that were extended to part of or to the whole secretory pathway, and sometimes to the entire vacuolar system of the cell (Lippincott-Schwartz et al., 1991; Klausner et al., 1992).

We report here the effect of the controlled depletion of the ATP cellular pool on the integrity of the Golgi compartments. We observe that, when the cellular ATP pool is reduced to 15-25% of the control, the CGN is specifically disassembled by a mechanism involving long tubular structures and microtubules. The energy depletion-insensitive SGC and TGN remain sensitive to BFA. Upon restoration of the energy levels, the CGN membrane proteins are transported out of the ER, and the CGN compartment is slowly reconstituted by a process that is inhibited by BFA.
**MATERIALS AND METHODS**

**Cell culture**

NRK (normal rat kidney) and Vero (Monkey, African green) fibroblasts were cultured for a minimum of 72 hours to 80% confluence on plastic dishes or glass coverslips at 37°C in normal medium (DMEM, 10% fetal calf serum (FCS), 2 mM glutamine, non-essential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin), in an atmosphere of 93% air, 7% CO₂ and 85% humidity.
Protein probes

The development of CC92, the mouse monoclonal IgM antibody (mAb) against the CGN marker membrane protein gp74, as well as the rabbit polyclonal antibodies (pAb) 15C8, against the cis/middle Golgi membrane protein, GMPc-1, and 18B11, against the trans-Golgi membrane protein GMP t-1, has been reported (Yuan et al., 1987; Alcalde et al., 1992, 1994). The mouse mAb P5D4 against the VSV tsO45 G protein and anti-β-COP maD were purchased from Sigma. The rat mAb against tubulin and the polyclonal antibody against sec13 were the gift of Dr J. V. Kilmartin (Kilmartin et al., 1982) and Dr Hong (Tang et al., 1997), respectively. Helix pomatia lectin conjugated to fluoresceine isothiocyanate (FITC) or to horse radish peroxidase (HRPO) was purchased from Sigma. Texas red and fluorescein-conjugated second antibodies were from Cooper-Biomedical Inc. (Malvern, PA).

Depletion and replenishment of the ATP intracellular pools

To lower the ATP, NRK cells were washed three times with warm glucose-pyruvate-free DMEM containing 1% dialyzed FCS and then incubated for the required times in the same medium supplemented with 50 mM 2-deoxy-D-glucose (DOG medium). To restore the ATP levels, the cells were washed three times with warm DMEM medium, before subsequent incubation with DMEM/1% FCS supplemented with 100 mM glucose (GLUC medium). Cellular ATP levels were measured by the luciferase-luciferin procedure (Sigma) on perchloric acid extracts from NRK cells grown on 60 mm plates. Briefly, the cells were washed with PBS, resuspended in 1 ml cold perchloric acid, and upon the acid neutralization with KOH the protein and salt were removed by centrifugation for 10 minutes at 14,000 g in the cold and the ATP levels measured by the luciferin-luciferase procedure in the protein-free supernatant as follows: 1 ml of 25 mM Hepes/10 mM MgCl2/2 mM EDTA, pH 7.75, were mixed

Fig. 3. Effect of energy depletion on the integrity and morphology of the CGN. NRK cells incubated for the times indicated in DOG medium, were double stained for HPBP (A,B,D,E), the ER marker PDI (F) or GMPc-1 (C,G). Images of the cell shown in B is an enlargement of the cell marked with open arrow in A. Tubular extensions of the CGN are marked with solid arrows in A and B. Bars in H indicate the percentage of cells with recognizable CGN (i.e. intact or tubulated CGN as shown in A, B and in Fig. 2A) (□), SGC (as in Fig. 2B) (■) and TGN (as in Fig. 2E) (■) after treatment with 50 mM DOG for the indicated times. Bars in I indicate the percentage of cells stained with FITC-Helix pomatia lectin that showed staining of intact CGN (as in Fig. 2A) (□), tubulated CGN (see A and B) (■) or staining of the ER spread throughout the cytoplasm (see D-E) (■). Bars, 10 μm.

Fig. 4. Effect of energy depletion on the distribution of β-COP. NRK cells incubated in normal medium (A,B), or for 45 minutes in DOG medium (C,D) and then treated for 15 minutes with 10 μg/ml BFA in the same medium (E,F), were double stained for β-COP and GMPc-1. Bar, 10 μm.
CHARACTERIZATION OF HELIX POMATIA BINDING PROTEINS (HPBP)

NRK cells grown on 60 mm plates were scraped in 0.5 ml of 0.1 M Na₂CO₃ pH 11.3, the nuclei and cellular debris removed by centrifugation for 10 minutes at 600 g, and the cellular membranes pelleted by centrifugation for 40 minutes at 4°C at 150,000 g in a TL100 centrifuge (Beckman). The membranes were resuspended in Laemmli buffer, run on SDS-PAGE gels, blotted onto nitrocellulose and the HPBP probed with HRPO-Helix pomatia lectin.

STUDY OF THE TRANSPORT OF THE VSVtsO45 G PROTEIN OUT OF THE ER AND ITS ACQUISITION OF ENDOH RESISTANCE

Vero cells grown to 80% confluency on coverglasses or on 60 mm plates in normal medium were infected for 45 minutes at 31°C with VSVtsO45 (20-30 pfu/cell) in 200 μl DMEM. Infected cells were incubated for 4 hours at 39.5°C with normal medium and then for 45 minutes at 39.5°C in either DME/0.1% FCS (DME/0.1% FCS medium) or in DOG medium before incubation for different time periods at 31°C in DME/0.1% FCS, DOG or GLUC medium. The cellular distribution of the tsO45 G protein was studied by immunofluorescence microscopy using the mAb P5D4 as described below. To study the acquisition of EndoH resistance by the tsO45 G protein the infected cells were labeled for 30 minutes at 39.5°C with [35S]methionine/cysteine (200 μCi/ml; specific activity >1000 Ci/mmol) (Amersham) before their incubation in DMEM or DOG medium at the restrictive temperature. The labeled cells were scraped in 0.5 ml of 50 mM Tris/0.1 M NaCl/1% NP40/2 mM EDTA/0.1 mM PMSF, pH 7.4, centrifuged for 15 minutes at 4°C at 11,000 g and the supernatants mixed with 1 volume of the same buffer without detergent and incubated overnight at 4°C with 10 μl of mAb P5D4 (Sigma) bound to Protein G (Pharmacia). The immunoprecipitates were washed, incubated overnight at 37°C with or without 1 milliunit of EndoH (Boehringer) in 0.1 M sodium phosphate buffer/2% SDS/2 mM β-mercaptoethanol pH 6.1, resolved by SDS-PAGE, and the gels soaked for 1 hour in 1 M sodium salicylate, dried and autoradiographed (Barriocanal et al., 1986).

IMMUNOFLOURESCENCE MICROSCOPY

Single and double immunofluorescence microscopy (IFM) studies were performed on NRK cells grown on coverglasses and fixed and permeabilized with methanol at ~20°C (Barriocanal et al., 1986). The morphology and localization of the CGN and SGC was studied using the Helix pomatia lectin or the mAb CC92. Similar studies of the SGC and TGN were performed with the mAb 15C8, and the mAb 18B11, respectively. To quantify the effects of ATP depletion, BFA, nocodazole and taxol on the distribution of the CGN membrane proteins studied by IFM, groups of two hundred cells grown on separate coverslips and randomly picked under the microscope were studied. The stained cells were examined in an Axiosvert 135M microscope (Zeiss) and photographed separately or simultaneously through Texas red or FITC fluorescence microscopy.

IMMUNOELECTRON MICROSCOPY

Ultrastructural studies were performed on cells fixed, permeabilized and stained with HRPO-Helix pomatia lectin, using the pre-embedding method as previously described (Yuan et al., 1987). Parafomraldehyde was from Merck (Darmstadt, Germany), glutaraldehyde (25% in water) from Fluka, (Buchs, Switzerland), osmium tetroxide from Serva (Heidelberg, Germany) and diaminobenzidine from Sigma.
contrast, neither the SGC (Fig. 3C) nor the TGN (not shown), stained with the anti-GMP_c-1 and GMP_p-1 antibodies, respectively, were affected by the 15 minutes incubation in DOG medium (Fig. 3C). After 30-45 minutes of incubation in DOG medium, the perinuclear reticular structure stained by Helix pomatia disappeared (Fig. 3D,H,I) and a haze of fluorescence was spread throughout most of the area stained with an antibody against the ER marker protein disulphide isomerase (PDI) (Fig. 3E and F). The second CGN marker, gp74, underwent the same redistribution (not shown). Again, no significant changes in the location and morphology of the SGC (Fig. 3G) and TGN (not shown) were observed after a careful quantitative analysis of the effect (Fig. 3H). Longer incubations of 60 to 90 minutes in DOG medium did not affect the reticular staining pattern of the SGC, but staining of the TGN became more diffuse, albeit all the marker protein, GMP_p-1, was localized to the Golgi area (not shown). Incubation of cells in DOG medium thus resulted in the rapid relocation of CGN membrane proteins, a phenomenon that probably reflected the disassembly of this Golgi compartment.

The intact SGC retains the β-COP attached to its membranes following energy depletion

The pattern of CGN disassembly was similar to that of Golgi disassembly described in BFA-treated cells (Lippincott-Schwartz et al., 1989). It has been shown that BFA causes the reversible redistribution of β-COP from Golgi membranes into the cytosol (Donaldson et al., 1990; Orci et al., 1991). To further define the effects of ATP depletion, we studied whether incubation of cells in DOG medium caused redistribution of β-COP from the SGC membranes into the cytosol. When cells were incubated for 45 minutes in DOG medium and double stained for β-COP and GMP_c-1, a significant amount of β-COP remains attached to the Golgi membranes as shown by immunofluorescence microscopy (Fig. 4C,D). This contrasted, therefore, with the effect of BFA which induced the release of all the Golgi-associated β-COP before the redistribution of the Golgi proteins to the ER (Donaldson et al., 1990; Orci et al., 1991). Moreover, treatment of cells preincubated for 45 minutes in DOG with 10 μg/ml BFA for 15 minutes resulted in release of the β-COP associated with the Golgi membranes to the cytoplasm, before complete disassembly of the SGC, a process which in cells incubated with DOG proceeds more slowly than in cells incubated in normal medium (Fig. 4E,F).

Role of microtubules in the disassembly of the CGN

The perinuclear location and integrity of the Golgi complex requires the web of microtubules organized by the centrosome and extended throughout the cytoplasm (Wehland and Sandoval, 1983; Sandoval et al., 1984; Turner and Tartakoff, 1989), as does the vesicle-mediated anterograde and retrograde transport of materials through the secretory pathway (Lippincott-Schwartz et al., 1995; Lippincott-Schwartz and Cole, 1995; Cole et al., 1996; Presley et al., 1997). To test whether low ATP affected the integrity and organization of cytoplasmic microtubules, we incubated NRK cells for 60 minutes in DOG medium. As shown in the fluorescence experiment (Fig. 5A) the cells stained with the anti-tubulin antibody displayed the characteristic web of long cytoplasmic microtubules organized by the centrosome as in normal cells. Therefore, under the conditions that caused CGN disassembly the integrity and organization of the cytoplasmic microtubules were not significantly affected, with the exception of some bundling, thus excluding the possibility that disassembly of the CGN was provoked by extensive microtubule alterations. Moreover, cells treated for 60 minutes with the microtubule polymerization inhibitor nocodazole (Fig. 5B), and then for 45 minutes more with the drug in DOG medium, were able to reconstitute substantially the network of cytoplasmic microtubules after being washed free of nocodazole (data not shown). As described before, addition of nocodazole to cells incubated in DMEM was followed by the rapid relocation of gp74 from the perinuclear reticulum characteristic of the CGN, into punctate structures scattered throughout the cytoplasm (Fig. 5C) (Alcalde et al., 1994).

We next studied the effect of DOG on the HPBP accumulated in the punctate structures. For this purpose cells incubated for 60 minutes in nocodazole were further incubated for 0, 20, 45 or 60 minutes with the drug in DOG medium (Fig. 5D,E,I). We observed that whereas the punctate structures still retained a great part of HPBP a diffuse cytoplasmic fluorescence became visible in a significant number of cells, suggesting some translocation of HPBP from the punctate structures to the ER (Fig. 5D). In these cells the distribution of HPBP and the SGC marker GMPc-1 was strikingly different, the latter being localized to large fragments near the perinuclear area (Fig. 5E) (Alcalde et al., 1994).

It has been reported that reorganization of microtubules into bundles disconnected from the centrosome by incubation of cells with taxol (Fig. 5F) caused a profound change in the Golgi structure: the drug provoked the disruption of the Golgi into large fragments that became associated with the microtubule bundles (Wehland et al., 1983). To further investigate whether disruption of the microtubule organization had any effect on the redistribution of HPBP induced by ATP depletion, cells were incubated for 3 hours with 10 μM taxol and the incubation with the drug continued in DOG medium for 0, 20 or 45 minutes before cells were fixed, permeabilized and stained for HPBP and GMP_c-1. Disruption of microtubule organization severely retarded, but did not block, the HPBP redistribution (Fig. 5G,I). Following 20 minutes incubation of taxol-treated cells in DOG medium, most cells retained the HPBP in large perinuclear structures that were also stained with the SGC marker GMP_c-1 (compare Fig. 5G and H). Even after 45 minutes incubation with taxol in DOG medium, roughly half of the cells still retained HPBP in the reticular perinuclear structures characteristic of the CGN. Interestingly, many of these structures displayed still the tubule-like expansions (Fig. 5G; arrows) observed in cells incubated for 15 minutes in DOG medium without drug (Fig. 3A,B). It is likely, therefore, that the taxol-induced microtubule rearrangement and the resulting Golgi and ER reorganization may affect the biogenesis and function of the tubules involved in the redistribution of the CGN membranes.

Reassembly of the CGN upon energy repletion

The process of CGN reconstitution is illustrated in Fig. 6, in which cells incubated for 45 minutes in DOG medium were washed free of DOG and incubated in GLUC medium for periods between 15 minutes and 2 hours. The double fluorescence experiment shows that after 15 minutes of incubation in GLUC medium, the HPBP were redistributed
from a diffuse staining pattern to large vesicle-like structures (i.e. pre-CGN elements), which were often arranged in a semicircle around the intact SGC (Fig. 6A,B,C). Reconstruction of the CGN was a relatively slow process; for completion it required incubations of more than 90 minutes in GLUC medium for most cells (D-I).

To further study the interaction between the structures precursor of the CGN and the SGC, we compared the cellular distributions of pre-CGN elements and of the elements derived from the SGC disrupted with nocodazole. Cells were preincubated for 45 minutes in the same medium with 20 µM nocodazole before being washed free of DOG and subsequent incubation for 90 minutes with nocodazole in GLUC medium. In contrast to the cells incubated without nocodazole, the cells incubated with the anti-microtubule drug were unable to completely reconstruct the continuous reticulum of the CGN in the vicinity of the nucleus, and the HPBP were localized to large pleomorphic structures scattered throughout the cytoplasm (Fig. 6J). Interestingly, most of these structures were found to codistribute and to be shaped in the same fashion as the SGC fragments produced by nocodazole treatment (Fig. 6F,G). This observation again, suggests that the CGN and the SGC elements interact in an unknown manner during the process of CGN reconstruction.

gp74 codistributes with COPI and COPII shortly after replacing the DOG medium by GLUC medium

Recent studies have documented the involvement of COPII coated vesicles in the anterograde movement of membranes out of the ER (Barlowe et al., 1994; Campbell and Schekman 1997; Gaynor and Emr, 1997) and of COPI in retrograde transport (Scheel et al., 1997; Orci et al., 1997). In addition, COP I appears to replace COPII within the tubulovesicular clusters
Membrane flow through Golgi compartments

that form the intermediate compartment, to prime the budding of vesicles that are targeted to the CGN (Aridor et al., 1995; Bannykh et al., 1996; Rowe et al., 1996; Scheel et al., 1997; Orci et al., 1997) (for reviews see Kuehn and Schekman, 1997; Schekman and Mellman, 1997). To further characterize the structures involved in CGN reconstitution, we compared the distribution of gp74, sec 13 (i.e. COPII) and β-COP (COPI) in cells cultured in normal medium and in cells preincubated for 45 minutes in DOG medium prior to their incubation in GLUC medium for 15 minutes. A large amount of COPI and COPII was localized to the Golgi and perinuclear area of cells incubated in normal medium, but whereas sec 13 was also
found in scattered punctuate structures outside the Golgi area, β-COP was more uniformly distributed throughout the cytoplasm (compare Figs 4A and 7B). Incubation of cells for 45 minutes with DOG did not alter significantly the patterns of COPI (compare Fig. 4A and C) and COP II (compare Fig. 7B and D) distribution found in cells incubated in normal medium. Double fluorescence experiments revealed that, in cells preincubated for 45 minutes in DOG medium and then incubated for 15 minutes in GLUC medium, the major part of the structures involved in the redistribution of gp74 and in the reconstruction of the CGN contained sec 13 (Fig. 7E,F) and β-COP (Fig. 7G,H). Somehow, the overlapping between gp74 and β-COP was more complete than between gp74 and sec 13, since a significant number of the sec 13-positive punctuate structures was not stained by gp74. The same observations were made when the experiment was repeated and the cells stained for HPBP instead of gp74 (not shown).

**Transport of the VSV tsO45 G protein through the secretory pathway is restored by energy repletion**

To study the effect of energy repletion on the transport of proteins out of the ER and through the Golgi, we infected Vero cells with the temperature-sensitive mutant vesicular stomatitis virus (VSV) and studied the cellular distribution of the tsO45 G protein and HPBP in cells incubated for 45 minutes at 39.5°C (i.e. restrictive temperature) in DOG medium and then transferred to 31°C (permissive temperature) and incubated for different time periods in DOG or GLUC medium. The double fluorescence study of the tsO45 G protein and the HPBP in cells incubated for 45 minutes at 39.5°C in DOG medium (Fig. 8A,B), showed that both infected and uninfected cells displayed the homogeneous cytoplasmatic distribution of HPBP characteristic of cells that suffered the disassembly of the CGN. In contrast, in cells subsequently incubated for 20 minutes at 31°C in GLUC medium, both the tsO45 G protein and the HPBP were found to co-localize in punctuate structures often arranged in a semicircle at one pole of the nucleus (Fig. 8C,D). Extension of the incubation in GLUC medium to 3 hours resulted in their transport to a compact perinuclear structure, probably the Golgi (Fig. 8E and F) since the HPBP were retained in that whereas some of the the tsO45 G protein molecules were transported to the cell periphery, as shown by the staining of the plasma membrane (Fig. 8E, arrows). The effect of the incubation at 31°C in GLUC medium on the redistribution of the tsO45 G protein, was consistent with the resume of the protein transport between the ER and Golgi and strongly suggested that the viral and the HPBP were transported out of the ER through the same pathway.

Monitoring of the acquisition of EndoH resistance by the tsO45 G protein, an event that marks the transport of glycoproteins through the SGC (Fig. 8G) showed that most of the newly synthesized viral protein became resistant to the glycosidase between 1 and 2 hours after transfer of the cells incubated in DMEMS from 39.5°C to 31°C. Incubation of cells for 45 minutes at 39.5°C in DOG medium and then at 31°C in DOG medium completely inhibited the acquisition of EndoH resistance by the protein. Interesting, much of the protein recovered from cells incubated for 45 minutes at 39.5°C in DOG medium and then incubated for 10 hours at 31°C in GLUC medium, remained sensitive to EndoH. The slow acquisition of EndoH resistance and the rapid transport of the tsO45 G protein to the cell surface after transfer of cells to the permissive temperature and GLUC medium, may reflect different sensitivities of the protein transport and the glycosylation systems to changes in the ATP levels. Alternatively, though less likely, the tsO45 G protein might be transported to the cell surface by a path that excludes the stack of Golgi cisternae.

**Ultrastructure of NRK cells incubated in DOG medium before and after their transfer to GLUC medium**

To better define the CGN membrane protein distribution in NRK cells with low ATP levels, we examined cells incubated in DOG medium by electron microscopy. Cells incubated for the times indicated in DOG medium were fixed, permeabilized, and labeled with HRPO-Helix pomatia lectin using the preembedding immunoperoxidase procedure. After incubation of the cells for 15 minutes in DOG medium, staining was often localized within the Golgi to a few convoluted tubular structures area and, less often, to long smooth tubules (Fig. 9A and inserts). Moreover, after incubation in DOG medium for 30 to 45 minutes the peroxidase-staining virtually disappeared, a phenomenon similar to that observed in NRK cells treated with BFA and stained for the medial-Golgi marker mannosidase II (Lippincott-Schwartz et al., 1989), and numerous stain-free SGC were localized to the vicinity of the nucleus (Fig. 9B).

To further characterize the structures involved in CGN reconstruction, cells incubated for 45 minutes in DOG medium were incubated for time periods between 15 minutes and 3 hours in GLUC medium (Fig. 9C-F). Among the HRPO-Helix pomatia stained structures observed in cells incubated for 30 minutes in GLUC medium, we found a significant number of ER cistermae with the characteristic ribosomes (inserts in Fig. 9C,D) attached to their surface, an indication that the HPBP and/or the CGN enzymes involved in O-glycosylation were translocated to the ER of the ATP depleted cells. The peroxidase staining was also localized to cisterna-like structures that emerged as finger-like extensions from large uncoated vesicles localized to the Golgi area (Fig. 9D, and lower insert). Finally, cells incubated in GLUC medium for 90 minutes or longer accumulated the HRPO staining product in tubulovesicular elements often lining one of the sides of the SGC that remained stain-free (Fig. 9E,F). Serial sections of these cells (Fig. 9E,F) showed that the reconstruction of the CGN along the Golgi ribbon was not uniform.

**Differential effects of ATP depletion and BFA on the trafficking of CGN and SGC membrane proteins**

Previous studies performed with cells stained for gp74 showed that, after treatment with BFA, the reticular CGN was replaced by a punctate staining that appear to reflect an engagement of the CGN membrane glycoprotein in an idle cycle of continuous entry and exit from the ER mediated by vesicles (Fig. 10A) (Alcalde et al., 1994). The localization of gp74 to punctuate structures was in contrast with the homogenous distribution of the SGC marker, GMPc-1, throughout the cytoplasm (Fig. 10B; a quantitative analysis of the experiment is shown in P). To study whether the SGC of cells incubated in DOG medium remained BFA sensitive, cells preincubated for 45 minutes in DOG medium were further incubated with 10 μg/ml BFA for
30 or 60 minutes in the same medium before being fixed and stained with antibodies against gp74 (Fig. 10C) and GMPc-1 (Fig. 10D). Counting of the cells displaying distinct GMPc-1-positive perinuclear structures, such as those shown in Fig. 10D, demonstrated that though the SGC was disassembled significantly more slowly in cells with low ATP levels it remained sensitive to BFA (Fig. 10P).

To further characterize the pathway of transport of CGN membranes out of the ER, the distribution of gp74 and the SGC marker GMPc-1 was investigated in cells preincubated for 45 minutes in DOG medium and then treated for 30 minutes with 10 µg/ml BFA in GLUC medium. It can be seen that the gp74 reappeared in punctuate structures scattered throughout the cytoplasm (Fig. 10E,P), a pattern of distribution similar to that observed when BFA was added to cells incubated in normal medium (compare A and E), whereas in most of the cells the SGC marker GMPc-1 showed the homogeneous distribution expected for its redistribution from the SGC to the ER (Fig. 10F,P). When cells were incubated for 45 minutes with DOG and then for 60 minutes with BFA in the same medium, replacement of GLUC for DOG also resulted in reappearance of gp74 in punctuate structures (Fig. 10G,H), however, the number of these was significantly less than when BFA was added together with GLUC (Fig. 10E,F), the difference being probably due to the prolonged incubation of the cells in DOG medium. As expected, in cells preincubated for 60 minutes with BFA in normal medium and then incubated for 60 minutes with the drug in DOG, both the gp74 and the GMPc-1 were homogeneously distributed throughout the cytoplasm, indicating that they were retained in the ER (Fig. 10I,P). Moreover, in cells washed-free of the drug and transferred to GLUC medium, both gp74 and GMPc-1 were localized to the same cytoplasmic structures (Fig. 10K to M), suggesting that they were transported out of the ER by the same pathway. The size and frequent arrangement of these structures in a semicircle around the Golgi area was in fact reminiscent of the structures that housed the CGN membrane proteins after the substitution of DOG for glucose in the culture medium and their release from the ER (Fig. 6A). Finally, in cells incubated for 60 minutes with BFA in normal medium before incubation for 60 minutes with BFA in DOG medium and then incubated for 60 minutes in BFA-free DOG medium, the gp74 was uniformly distributed throughout the cytoplasm whereas the GMPc-1 was localized to punctuate and elongated structures clustered near the nucleus, indicating that GMPc-1 was transported out of the ER that retained the gp74 (Fig. 10N,O,P).

**DISCUSSION**

The studies reported here extend previous observations that ATP-sensitive steps punctuate protein transport between the ER and CGN (Jamieson and Palade, 1968; Balch and Rothman, 1985; Balch and Keller, 1986), and that depletion of the ATP cellular pool causes the tubulation of Golgi membranes both in vivo and in vitro (Cluett et al., 1993; Banta et al., 1995). Immunofluorescence microscopy studies show that incubation of cells with DOG in glucose- and pyruvate-free medium results in rapid and complete redistribution of membrane proteins normally restricted to the CGN. Redistribution is specific in that only the CGN membrane proteins redistribute to the ER (see below), whereas the SGC and TGN membrane proteins are retained in apparently intact Golgi elements. This is in contrast with the indiscriminate redistribution of membrane proteins from Golgi to the ER produced by BFA, redistribution that resulted in complete disassembly of the Golgi complex. Nevertheless, since we have studied only the redistribution of HPBP and gp74, the conclusion that all the CGN membrane proteins undergo the same redistribution in ATP-depleted cells must await definitive confirmation from independent studies using other CGN membrane markers.

Immunoutrastructural studies using HRPO-conjugated Helix pomatia show that after incubation of cells for 15 minutes in DOG medium, the typical CGN reticular structure in the vicinity of the nucleus is replaced by convoluted tubular structures as well as by long smooth tubules that are more rarely seen. Longer incubations with DOG result in localization of the CGN membrane markers HPBP and gp74 to a delicate reticulum that extends throughout the cytoplasm and is stained by the ER marker PDI as shown by immunofluorescence microscopy. Immunoelectron microscopy studies of these cells, however, did not show clear ER staining with HRPO-Helix pomatia lectin, probably for the same technical reasons that impeded the visualization of the Golgi proteins translocated to the ER of NRK cells treated with BFA (Lippincott-Schwartz et al., 1989).

The staining of ER cisternae with HRPO-Helix pomatia lectin and the localization of HPBP and gp74 to the structures that transport the VSVtsO45 G protein out of the ER upon washing the cells free of DOG and their incubation for 15 minutes in GLUC medium, further support the possibility that the CGN membrane markers are translocated to the ER upon ATP depletion.

The specific redistribution of CGN membranes that follows the ATP depletion strongly suggests that within the Golgi complex the CGN is a separate compartment with distinct boundaries and which bears a distinct membrane flow. The massive exchange of membranes between the ER and the CGN (Wieland et al., 1987) and their retrieval by recycling, probably determine much of the size and structure of the CGN, and may explain its sensitivity and distinct response to ATP depletion.

The disassembly of the CGN and the lack of response of the SGC membranes to ATP depletion may be explained by the retention of COPI by the SGC membranes of the cells treated with DOG, a fact which is in contrast with the rapid release of COPI from the Golgi membranes in BFA-treated cells (Donaldson et al., 1990).

As with BFA, the redistribution of CGN proteins in ATP-depleted cells is mediated by long tubular structures. It is noteworthy that whereas the tubules promoted by lowering ATP levels have been shown to be substrates for the rebinding of vesicle-associated coat proteins (Cluett et al., 1993), the tubules extended in response to BFA appeared to form after the release of COPI components from the Golgi membranes (Donaldson et al., 1990). These observations suggest that the tubules formed after DOG or BFA treatment may be different structures and suggest that they may have different functions.

The transport of the SGC marker, GMPc-1, out of the ER after washing free of BFA the cells cultured in DOG medium, indicate that levels of ATP as low as 15-25% below the normal level do not inhibit completely the transport out of the ER mediated by vesicles, and shows that vesiculation is not always suppressed under conditions that favor tubulation (Klausner et al., 1992; Cluett et al., 1993).
Fig. 7. Transport of gp74 out of the endoplasmic reticulum is mediated by COPI/COPII positive vesicles. NRK cells incubated in normal medium (A,B) or for 45 minutes in DOG medium (C,D) prior to incubation for 15 minutes in GLUC medium (E-H) were fixed-permeabilized and double stained for gp74 and for sec 13 (i.e. COPII) or β-COP (COPI) as indicated in the panels. The cells in G were directly stained with Texas red-conjugated gp74. Fluoresceine- and Texas red-conjugated second antibodies were used to stain the cells shown in the remaining panels. Enclosed in squares in E to H are the areas enlarged in the inserts. When photographed the cells shown in H were underexposed to eliminate the fluorescence due to cytoplasmic β-COP pool (see Fig. 4A) and facilitate the study of its co-distribution with the membrane protein gp74. Arrows in inserts mark double-stained vesicles. Bar, 11.2 μm.

Fig. 8. HPBP and VSVts045G protein are co-transported out of the ER by the same vesicles. Vero cells incubated for 4 hours at 39.5°C upon infection with the tsO45 VSV were further incubated for 45 minutes at 39.5°C in DOG medium and then for the indicated time periods at 31°C in DOG or in GLUC medium. Cells were fixed permeabilized with methanol and double stained for the tsO45 G protein and HPBP as indicated in the panels. In E the VSVts045 G protein localized to the plasma membrane is marked with arrows. Bars, 8.4 μm. Kinetics of acquisition of EndoH resistance by the tsO45 G protein. tsO45 VSV infected Vero cells were labeled with [35S]methionine/cysteine and incubated as described in G. Incubations in DMEMS medium (green arrows, a, c-e) in DOG medium (red arrows, b,f,g) and in GLUC medium (blue arrows, b,h-j) were stopped at the indicated times and the EndoH resistance of tsO45 G protein (samples a-j) analyzed as described in Materials and Methods.
**Fig. 9.** Distribution of HPBP in ATP-depleted and in ATP-replenished cells studied by EM. NRK cells incubated in DOG medium for 15 minutes (A), or for 45 minutes (B) prior to subsequent incubation for 45 minutes (C,D) or for 90 minutes (E,F; serial sections) in GLUC medium. The cells were processed and stained with HRPO-Helix pomatia lectin using the pre-embedding procedure. Arrows in A mark Helix-pomatia-stained convoluted tubular structures and cisterna remnants in the vicinity of the centrosome (Ct) and nucleus (N). Two of these HRPO-stained convoluted structures and a long smooth tubule are shown in the inserts. Arrows in B mark stain-free SGC. Arrows in C mark four cisterna with the characteristic spiny surface of the RER. Three of these RER cisterna are shown at large magnification in the insert of C and in the upper insert of D. Arrows in D mark stained vesicles with finger-like expansions, one of these structures is shown at large magnification in the lower insert. (E and F) Stretches of a Golgi ribbon (arrows) displaying different extents of CGN reconstitution on the cis-side (E,F). Bars: A, 0.43 μm; A, inserts, 0.17 μm; B, 0.34 μm; C, 0.6 μm; D, 0.33 μm; D, insert, 0.15 μm; E and F, 0.67 μm.

**Fig. 10.** ATP depletion and BFA treatment affect differently the trafficking of CGN and SGC membranes. BFA interferes with the pathway of CGN reconstitution. NRK cells incubated 30 minutes with 10 μg/ml BFA in normal medium (A,B), or for 45 minutes in DOG medium prior to their treatment for 30 minutes with 10 μg/ml BFA in DOG (C,D) or GLUC medium (E,F). After a 45 minute incubation in DOG medium, cells were also treated for 60 minutes with BFA in DOG medium prior to their treatment for 30 minutes with BFA in GLUC medium (G,H). Cells were also incubated for 60 minutes with 10 μg/ml BFA in normal medium before their incubation with the drug in DOG medium for 60 minutes (I,J), and subsequent incubation for 30 minutes in drug-free GLUC medium (K-M) or for 60 minutes in DOG medium (N,O). The cells were fixed, permeabilized, and stained for gp74 and GMP c-1 as indicated in the panels. Bars: A-D and I-J, 16 μm; E-H and K-O, 7.5 μm. Bars in P show in the described experiments the percentage of cells in which gp74 (■) and GMP c-1 (□) were not relocated to the ER and were displayed as punctuate or perinuclear reticular structures.
We find that low ATP and BFA cause the specific retention of CGN and SGC membrane proteins, respectively, in the ER. Their different effects probably reflect differences in the mechanisms of anterograde and/or retrograde transport that determine the distribution of those proteins between the ER and the Golgi.

The labeling of the vesicles that transport gp74/HPBP out of the ER with antibodies against COPI and COPII, and the evidence that COPI appears to replace COPII coat on the surface of the membranes of the tubulovesicular clusters that constitute the intermediate compartment (Rowe et al., 1996), strongly suggests that after their release from the ER the CGN membrane proteins traffic through the same pathway described for secretory proteins (Schweitzer et al., 1990; Hauri and Schweitzer, 1992). The localization of CGN and SGC membrane proteins to the same large structures that encircle the perinuclear area that host the Golgi upon removal of BFA and the replacement of GLUC for DOG medium, shows that at some point, their pathways from the ER to the area in which the Golgi is reconstructed converge. With regard to this further research is necessary to establish the origin of the large uncoated vesicles that appear to mother the CGN cisternae.

With respect to the question of whether the CGN and SGC can be independently assembled, at least under the experimental conditions here used, the answer is no. Neither the CGN nor the SGC were reconstituted when the membrane components of the other compartment were retained in the ER. It was interesting, however, that reconstitution of the CGN upon restoration of ATP levels was conditioned by the structural integrity and distribution of the SGC elements. Thus, in untreated cells with intact perinuclear SGC, the CGN was completely reconstructed, to the extent that the newly assembled elements exactly matched the intricate laced morphology of the SGC. In contrast, in cells treated with nucodazole and as result with the SGC broken into fragments dispersed throughout the cytoplasm, the HPBP as well as gp74 were always localized to structures that exactly matched the shape and distribution of the SGC fragments, with independence of their size or distribution. These results and the ability of BFA to impede the assembly of the CGN by retaining the components of the SGC in the ER, suggest that some interaction between the CGN and SGC elements must exist during the process of Golgi reconstruction.

Finally, the protocol used here to specifically disassemble the CGN may be helpful to study the ultrastructure and function of this part of the Golgi apparatus.

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