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The recycling pathway of protein ERGIC-53 and dynamics of the ER-Golgi intermediate compartment

Judith Klumperman[1,*], Anja Schweizer[2,*], Henrik Clausen[3], Bor Luen Tang[4], Wanjin Hong[4], Viola Oorschot[1] and Hans-Peter Hauri[2,‡]

[1]Department of Cell Biology, School of Medicine, Utrecht University, 3584 CX Utrecht, The Netherlands
[2]Department of Pharmacology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland
[3]School of Dentistry, Faculty of Health Sciences, DK-2200 Copenhagen, Denmark
[4]Institute of Molecular and Cell Biology, National University of Singapore, Republic of Singapore
*The first two authors contributed equally to this study
‡Author for correspondence (e-mail: hauri@ubaclu.unibas.ch)

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SUMMARY

To establish recycling routes in the early secretory pathway we have studied the recycling of the ER-Golgi intermediate compartment (ERGIC) marker ERGIC-53 in HepG2 cells. Immunofluorescence microscopy showed progressive concentration of ERGIC-53 in the Golgi area at 15°C. Upon rewarming to 37°C ERGIC-53 redistributed into the cell periphery often via tubular processes that largely excluded anterograde transported albumin. Immunogold labeling of cells cultured at 37°C revealed ERGIC-53 predominantly in characteristic β-COP-positive tubulo-vesicular clusters both near the Golgi apparatus and in the cell periphery. Concentration of ERGIC-53 at 15°C resulted from both accumulation of ERGIC-53 in the ERGIC and movement of ERGIC membranes closer to the Golgi apparatus. Upon rewarming to 37°C the labeling of ERGIC-53 in the ERGIC rapidly returned to normal levels whereas ERGIC-53's labeling in the *cis*-Golgi was unchanged. Temperature manipulations had no effect on the average number of ERGIC-53 clusters. Density gradient centrifugation indicated that the surplus ERGIC-53 accumulating in the ERGIC at 15°C was rapidly transported to the ER upon rewarming. These results suggest that the ERGIC is a dynamic membrane system composed of a constant average number of clusters and that the major recycling pathway of ERGIC-53 bypasses the Golgi apparatus.

Key words: ER-Golgi intermediate compartment, ERGIC-53, β-COP, Immunoelectron microscopy, Protein recycling

INTRODUCTION

Protein transport from the rough endoplasmic reticulum (ER) to the Golgi apparatus involves complex membrane structures whose organization remains controversial (for reviews see Hauri and Schweizer, 1992, 1997; Mellman and Simons, 1992; Saraste and Kuismanen, 1992; Hong and Tang, 1993; Pelham, 1995; Bannykh and Balch, 1997; Farquhar and Hauri, 1997). We refer to these membranes as ER-Golgi intermediate compartment (ERGIC). Four different views of the ERGIC have been expressed. In one it is considered a subcompartment physically connected with the ER and including the *cis*-Golgi (Sitia and Meldolesi, 1992; Krijnse-Locker et al., 1994). Another view is that the ERGIC is physically continuous with the first fenestrated cisterna of the Golgi stack, also referred to as the *cis*-Golgi network (Huttner and Tooze, 1989; Hsu et al., 1991; Mellman and Simons, 1992). In a third view the ERGIC is regarded as a transient structure composed of ER-derived transport vesicles that fuse with one another and eventually with the first *cis*-Golgi cisterna (Saraste and Svensson, 1991; Lippincott-Schwartz, 1993; Bannynkh and Balch, 1997). Finally, the ERGIC may be a distinct organelle separated from ER and Golgi apparatus by vesicular transport steps (Pelham, 1989).

The membranes of the ERGIC are enriched in the 53 kDa membrane protein ERGIC-53/p58 (Schweizer et al., 1988; Schindler et al., 1993; Lahtinen et al., 1996), a mannose-selective lectin (Arar et al., 1995; Itin et al., 1996) that is required for efficient secretion of cathepsin C (Vollenweider et al., 1998) and the maintenance of appropriate levels of coagulation factors V and VIII in the blood (Nichols et al., 1998). At the ultrastructural level ERGIC-53 is present in tubulo-vesicular membrane profiles predominantly near the *cis*-side of the Golgi stacks (Schweizer et al., 1988, 1990; Saraste and Svenson, 1991; Lotti et al., 1992; Bannykh et al., 1996). Previous morphological studies have indicated that the subcellular distribution of ERGIC-53 is affected by temperature manipulations. Lowering the culture temperature from 37°C to ~15°C led to its concentration in the Golgi area (Lippincott-Schwartz et al., 1990; Schweizer et al., 1990; Saraste and Svensson, 1991). Upon rewarming to 37°C ERGIC-53 rapidly acquired a more widespread distribution in the cytoplasm suggesting a membrane recycling pathway (Lippincott-Schwartz et al., 1990; Tang et al., 1995b). The

presence in ERGIC-53 of a C-terminal dilysine ER retrieval signal, KKFF (Schindler et al. 1993; Itin et al., 1995a), interacting with COPI coat proteins (Kappeler et al., 1997; Tisdale et al., 1997), supports the notion that ERGIC-53 recycles between ER and post-ER compartments.

The precise recycling route taken by ERGIC-53 is unknown. A dilysine signal per se does not determine the recycling route of a protein. The efficiency of retrieval may depend on the sequence context of the dilysine motif, and it was proposed, therefore, that retrieval may be initiated from multiple positions along the exocytic pathway (Jackson et al., 1993). Because antibodies to ERGIC-53/p58 in addition to the ERGIC also labeled the first *cis*-cisterna of the stacked Golgi (Schweizer et al., 1988, 1990; Chavrier et al., 1990; Hendricks et al., 1991) the major site of recycling has been suggested to be the *cis*-Golgi (Saraste and Svensson, 1991). Although the general conclusion that ERGIC-53 recycles in the early secretory pathway of transfected cells is convincing (Kappeler et al., 1997), it has not been formally excluded that the temperature induced redistribution of ERGIC-53 reflects a dynamic feature of the ERGIC itself rather than the recycling of its marker protein.

Protein recycling in the early secretory pathway was initially described for soluble ER resident proteins possessing a C-terminal KDEL, HDEL, or related tetrapeptide signal. These proteins are retrieved from post-ER sites by the KDEL receptor (Lewis and Pelham, 1992) that is predominantly localized to the *cis*-Golgi and the ERGIC (Tang et al., 1993; Griffiths et al., 1994). Major recycling of KDEL proteins occurs from the *cis*-Golgi (Conolly et al., 1994) although the capacity to retrieve escaped KDEL-proteins extends to the *trans*-Golgi (Griffiths et al., 1995; Miesenböck and Rothman, 1995; Majoul et al., 1996). Protein cycling may also contribute to quality control of oligomeric proteins in the early secretory pathway. For instance, unassembled histocompatibility class I molecules cycle between ER and post-ER membranes (Hsu et al., 1991).

The elucidation of recycling routes in the early secretory pathway is hampered by the fact that recycling proteins are by definition also present in the anterograde pathway. Therefore, recycling studies often rely on experimental conditions that selectively induce the retrograde pathway. For example, it is generally assumed that brefeldin A (BFA)-induced changes of the Golgi apparatus reflect the Golgi-to-ER retrograde pathway in an exaggerated form (Klausner et al., 1992). Within minutes of adding BFA to cells the Golgi apparatus transforms into tubules and vesicles that, at least in part (De Lemos-Chiarandini et al., 1992; Hendricks et al., 1992; Hidalgo et al., 1992) fuse with the ER (Doms et al., 1989; Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989). However, this BFA-induced retrograde pathway has been visualized with resident Golgi proteins not known to recycle in untreated cells while cycling proteins accumulate in the ERGIC in response to BFA as shown by morphological approaches (Lippincott-Schwartz et al., 1990; Saraste and Svensson, 1991; Alcalde et al., 1994; Rios et al., 1994; Tang et al., 1995a) as well as subcellular fractionation (Itin et al., 1995b; our unpublished observations).

The present study was undertaken to unravel the recycling pathway of ERGIC-53 using its temperature-dependent redistribution as a means to synchronize this process. Both by immunoelectron microscopy and subcellular fractionation we unexpectedly found that the major recycling pathway of ERGIC-53 to the ER does not involve the stacked *cis*-Golgi but is mediated by membrane tubules extending from the ERGIC itself. These results suggest that the ERGIC plays a major role in sorting retrograde from anterograde protein traffic.

MATERIALS AND METHODS

Antibodies

The following antibodies were used: mAb G1/93 against human ERGIC-53 (Schweizer et al., 1988), mAb G1/296 against the ER marker p63 (Schweizer et al., 1993, 1995), mAb A1/118 against the *cis*/medial Golgi protein GPP130 (Linstedt et al., 1997), polyclonal rabbit antibodies against the rough ER marker protein disulfide isomerase (PDI, bovine) and mAb ID3 recognizing both PDI and calreticulin (kind gifts from Stephen Fuller, EMBL, Heidelberg/Germany), a rabbit polyclonal antiserum against the *trans*-Golgi marker human galactosyltransferase (a kind gift from Eric Berger, University of Zürich, Zürich/Switzerland; Roth and Berger, 1982), mAbs M3A5 and MAD against β-COP of COP1 vesicles (kind gifts from Thomas Kreis, University of Geneva, Geneva/Switzerland; Allan and Kreis, 1986; Duden et al., 1991; Pepperkok et al., 1993), different rabbit polyclonal antibodies against the EAGE peptide of β-COP (kindly provided by Thomas Kreis, Jennifer Lippincott-Schwartz, Suzanne Pfeffer, and Hans-Dieter Söling), affinity-purified polyclonal rabbit antibodies and a mouse mAb against bovine KDEL receptor (Tang et al., 1993, 1995a), polyclonal sheep antibodies against human albumin (ANAWA Trading, Dübendorf, Switzerland), and FITC- and TRITC-labeled secondary antibodies against mouse or rabbit antibodies (Cappel, West Chester, PA). A mAb against BAP31, a major type 1 membrane protein of the rough ER, was generated using an established mAb technique (Hauri et al., 1985; Schweizer et al., 1988). A Vero cell membrane fraction enriched in ERGIC-53 (Schweizer et al., 1991) was mixed with Freund's adjuvant and injected into Balb/C mice. After fusion of spleen and PA1 myeloma cells, the culture supernatants were screened for reactivity to the ERGIC fraction by a dot blot assay. BAP31 was identified by peptide sequencing of the affinity-purified protein.

Cell culture, temperature shift experiments, and perturbation with AlF_4^-

HepG2 cells were cultured in MEM supplemented with 10% FCS, 100 i.u./ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml fungizone. The cells were subcultured at confluency and split 1:3. For immunofluorescence experiments the cells were grown in poly-lysine-coated 8-well multichamber slides. For the temperature shift experiments the cultures were tightly enrapped in Parafilm and immersed in a water bath at exactly 15°C for up to 3 hours. Rewarming was done by rapidly replacing the medium with 37°C pre-warmed fresh medium and immediate transfer of the dishes to a 37°C incubator. In some instances rewarmed cultures were returned to the 15°C water bath after a 5 minute rewarming period for up to 3 hours before fixing and processing. To block the recycling of ERGIC-53 in the ERGIC, AlF_4^- (30 mM NaF and 50 μM $AlCl_3$) was added to the complete culture medium for 20 or 30 minutes prior to processing of the cells for immunofluorescence microscopy.

Study of newly synthesized albumin

HepG2 cells seeded in multi-chamber slides were treated with 50 μg/ml cycloheximide in DMEM for 2.5 hours at 37°C. Thereafter, the drug was rapidly washed out by four rinses with serum-free medium at 37°C and protein synthesis was resumed in fresh serum-free medium at 37°C (Mizuno and Singer, 1993). At various times after cycloheximide washout the cells were fixed and processed for double immunofluorescence microscopy with antibodies against albumin and

ERGIC-53. We found the same kinetics of reappearance of albumin as described by Valtersson et al. (1990).

Immunofluorescence microscopy

HepG2 cells were grown to approximately 50% confluency. After the temperature manipulations the cells were rinsed twice with PBS, fixed with 3% formaldehyde (made from para-formaldehyde), permeabilized with 0.1% saponine in PBS and processed for single or double immunofluorescence microscopy (Schweizer et al., 1988). The specimens were examined with a Reichert Polyvar microscope equipped with epifluorescence and photographed with a Konica FT-1 camera using Ektachrome 400HC slide films. All the necessary control experiments were performed to ascertain specificity of all the antibody reactions.

Immunoelectron microscopy

HepG2 cells were fixed by adding a volume of 2% acrolein and 0.2% glutaraldehyde in 0.2 M sodium phosphate (pH 7.4, prewarmed to 37°C) equal to the volume of medium on the cells. The cells were left in the fixative for 2 hours at RT and thereafter rinsed thrice in PBS before scraping the cells from the plates in 1 ml PBS. After three washes in 0.15% glycine in PBS the cells were resuspended in 10% gelatine in PBS from which 1 mm^3 blocks were cut at 4°C. The blocks were impregnated with 2.3 mM sucrose overnight and frozen in liquid N_2. Cryosections were prepared, and single- or double-labeled with different antibodies according to the Protein A-gold method (Slot et al., 1988, 1991). A semi-quantitative analysis of the subcellular distribution of ERGIC-53 under the different experimental conditions was performed by a double blind assay using cells double-immunolabeled for ERGIC-53 and galactosyltransferase. At low magnification an area of the grid was selected by morphological criteria. The electron beam was placed in one corner of the selected field and at a magnification of ×20,000 a random area was scanned by moving the grid in one direction. All gold particles at a distance of maximally 30 nm of a membrane were taken into account and ascribed to the compartment over which they were located. The number of gold particles found over a specific compartment was expressed as a percentage of the total number of gold particles counted. The total scanned area was calculated by determining the *x*,*y* coordinates of the position of the grid at the beginning and the end of a track. At least three such areas were scanned per sample. The number of membrane clusters per cell profile was determined in 20 randomly encountered cells double-labeled for ERGIC-53 and β-COP, of which the nucleus was within the plane of the section. 120 clusters were counted in control cells and 137 in cells cultured for 3 hours at 15°C. To calculate the number of intersections and cross-points per cluster a television screen was connected to the JEOL CX100 electron microscope. A transparent overlay with horizontal and vertical lines at a distance of 1 cm was placed on the screen. For each condition 25 membrane clusters were analysed in cells double labeled for ERGIC-53 and β-COP by projecting the area of interest to the screen at a magnification of ×15,000. The number of intersections is indicative for membrane length, whereas the number of cross-points over a certain area reflects the relative surface area (Weibel, 1979).

Subcellular fractionation

HepG2 cells were homogenized by repeated passages through a ball-bearing homogenizer with a clearance of 20 μm in 120 mM NaCl/5 mM KCl/25 mM $NaHCO_3$, pH 7.4, containing protease inhibitors. A postnuclear supernatant was prepared and applied to the top of a 12 ml linear Nycodenz gradient (13-29%, with a 35% cushion). After centrifugation at 25,500 rpm for 3 hours in a TST 28.17 rotor (Kontron, Zurich, Switzerland), the gradients were fractionated from the bottom and each fraction was assayed for density, the ER marker BAP31, the *cis*-Golgi marker *N*-acetylgalactosamine (GalNAc)-transferase activity (Roth et al., 1994; Schweizer et al., 1994) and KDEL-receptor protein (Tang et al., 1993, 1995a), which in HepG2 cells is predominantly localized in the *cis*-Golgi (our unpublished observation), the *cis*/medial Golgi marker protein GPP130 (Linstedt et al., 1997), and the *trans*-Golgi marker enzyme galactosyltransferase (Verdon and Berger, 1983). The amounts of ERGIC-53, BAP31, GPP130 protein and KDEL-receptor were determined by densitometry after transfer from SDS-PAGE gels and immunoblotting.

RESULTS

Distribution of ERGIC-53 in HepG2 cells cultured at 37°C

The human hepatoma cell line HepG2 was selected for the present study since it expresses relatively high levels of ERGIC-53 which facilitated the morphological analysis. Immunofluorescence microscopy of HepG2 cells cultured at 37°C showed the well-known distribution of ERGIC-53 in the Golgi area and in punctate structures in the more peripheral cytoplasm (Fig. 1A; Schweizer et al., 1988). Some ERGIC-53 was also associated with the ER as indicated by a fine-dotted outline of the nuclear membrane.

Immunogold labeling of ultrathin cryosections revealed the majority of labeling for ERGIC-53 in characteristic convoluted tubular and vesicular membrane clusters (Fig. 2B,C). The average number of clusters per cell profile amounted to 6.0±0.9 (mean ± s.d., *n*=20 cell profiles). The clusters were similar in appearance irrespective of whether they were localized near a Golgi apparatus (Fig. 2B) or in a 'non-Golgi' region (Figs 2A,C, 3B,C). Semi-quantitative analysis of ERGIC-53 labeling showed that ~55% of total label was found in membrane clusters close to the Golgi apparatus and ~40% in the 'non-Golgi' region of the cell (Table 1; all ERGIC-53-positive membrane clusters that, at least in the plane of the section, were not in the immediate neighborhood of a Golgi stack were considered to be in the 'non-Golgi' region). Only 4% of the overall label was associated with the stacked Golgi apparatus (Table 1) where it was exclusively present in the first recognizable *cis*-cisterna (Fig. 2A).

The tubulo-vesicular clusters were often observed in close apposition to rough ER elements as shown by double labeling for PDI and grp 55. Label for these ER markers in the clusters was nearly absent. In rare instances, budding profiles of the rough ER stained positive for ERGIC-53 (Fig. 2C), whereas in these ultrathin sections the level of ERGIC-53 immunoreactivity in the ER itself was below detection level (Fig. 2B,C). Many of the vesicular and budding profiles of the tubulo-vesicular clusters carried an electron-dense coat that was morphologically distinct from clathrin (e.g. Fig. 3A). Double-immunogold labeling showed the presence of β-COP in a subset of these coats, both in ERGIC-53-positive clusters near the Golgi (Fig. 3A) as well as in 'non-Golgi' areas (Fig. 3B-D).

Overall this ultrastructural analysis showed that the ERGIC consists of a multitude of tubulo-vesicular clusters all of which include β-COP-coated membrane elements.

Concentration of ERGIC-53 close to the Golgi apparatus at 15°C

When HepG2 cells were cultured at 15°C and analyzed by immunofluorescence microscopy the ERGIC-53 was seen to undergo a characteristic redistribution as already described for other cells (Lippincott-Schwartz et al., 1990; Schweizer et al.,

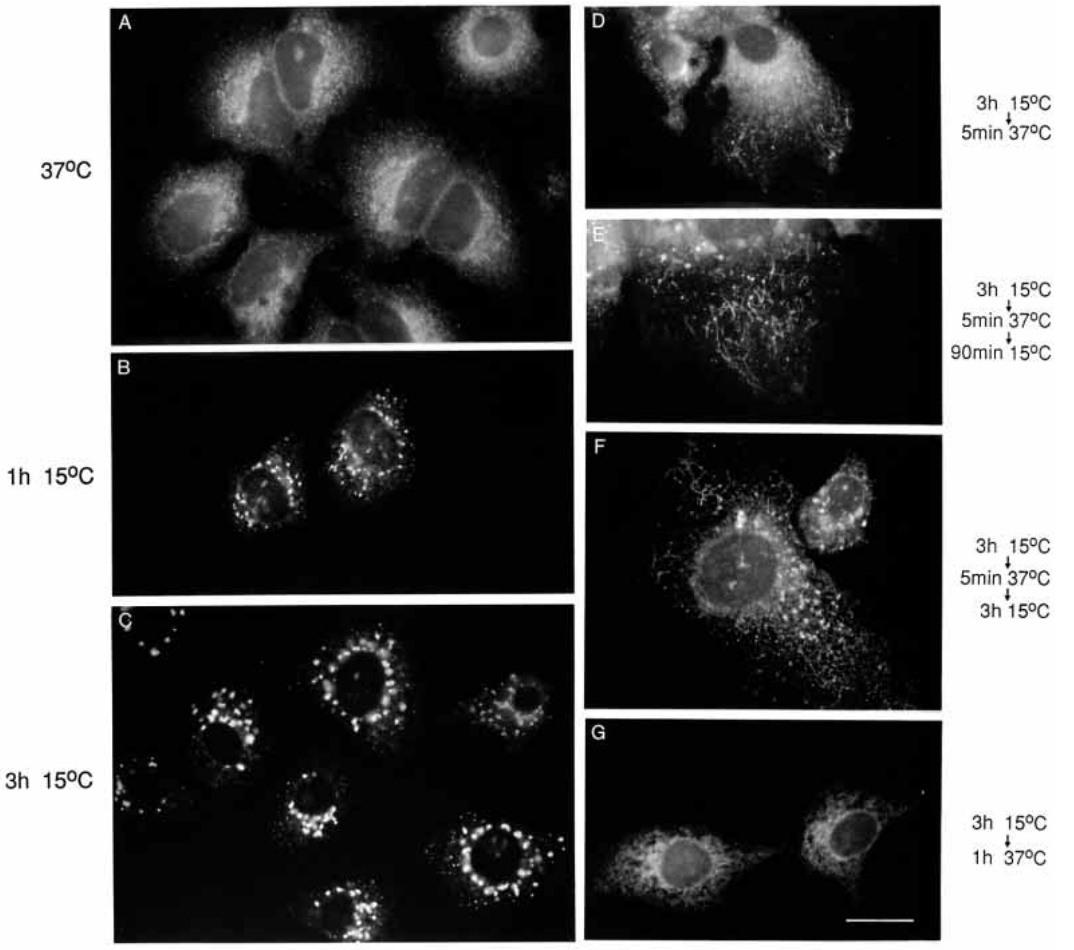

Fig. 1. Temperature-dependent redistribution of ERGIC-53 visualized by immunofluorescence microscopy. HepG2 cells were cultured as indicated, fixed with para-formaldehyde, permeabilized with saponin and incubated with a mAb against ERGIC-53 followed by a FITC-labeled goat anti-mouse antibody. Bar, 10.8 μm.

1990). ERGIC-53 progressively concentrated in large dots mostly in the Golgi area (Fig. 1A-C). This process was maximal after 3 hours (Fig. 1C), at which time the labeling for ERGIC-53 was dominant close to galactosyltransferase-positive Golgi elements (Fig. 4A,B). In addition, some label persisted in the cell periphery as smaller dots.

Semi-quantitative analysis of the immunogold labeling of ERGIC-53 also showed progressive concentration in the Golgi area after 3 hours at 15°C. Under these conditions ~76% of ERGIC-53 labeling was found in tubulo-vesicular clusters near the Golgi apparatus and only ~21% in clusters in 'non-Golgi' regions (Table 1). The number of these clusters per cell profile in cells cultured for 3 hours at 15°C was 6.9±0.8 (mean ± s.d., n=20 cell profiles) and hence unchanged as compared to 37°C. The clusters observed at 15°C were morphologically similar to those seen at 37°C (Fig. 5) and labeled positive for β-COP (Fig. 5A). In addition, the ER marker PDI/grp55 remained virtually absent from the clusters after the 15°C treatment (Fig. 5B). The morphology of the Golgi apparatus of some but not all cells was slightly changed at 15°C. The *cis-trans* topology and the average number of cisternae per stack (i.e. 5 to 6) were maintained, but the cisternae were relatively shorter. Moreover, at 15°C the Golgi stacks sometimes comprised up to 4 galactosyltransferase positive cisternae (Fig. 5C) versus one to two at 37°C (not shown). Importantly, ERGIC-53 labeling of the *cis*-Golgi was not increased after 3 hours at 15°C (Table 1).

Quantification of the immunolabeling (Table 2) showed a twofold statistically significant (P<0.05) increase of gold particles in individual ERGIC clusters when the temperature was lowered to 15°C. In addition, the mean number of intersections per cluster (a measure for membrane length) was increased. However, the mean number of gold particles per intersection (a measure for ERGIC-53 density per membrane length) did not significantly change, indicating that two processes are responsible for the increased labeling of the ERGIC at 15°C: Recruitment of ERGIC-53 from the rough ER (see subcellular fractionation data, below) and enlargement of total membrane area per cluster.

Combined with the immunofluorescence results these data indicate that some clusters had moved closer to the Golgi apparatus at 15°C. Overall these experiments suggest that the redistribution of ERGIC-53 at 15°C seen by immunofluorescence microscopy is due to both accumulation of ERGIC-53 in the ERGIC clusters and progressive concentration of a majority of individual ERGIC-membranes near the Golgi apparatus.

Redistribution of ERGIC-53 into the cell periphery after rewarming to 37°C

If HepG2 cells were maintained at 15°C for 3 hours followed by 5-10 minutes rewarming to 37°C, long tubular processes were seen by immunofluorescence microscopy to emanate from the Golgi area into the cell periphery (Fig. 1D). The

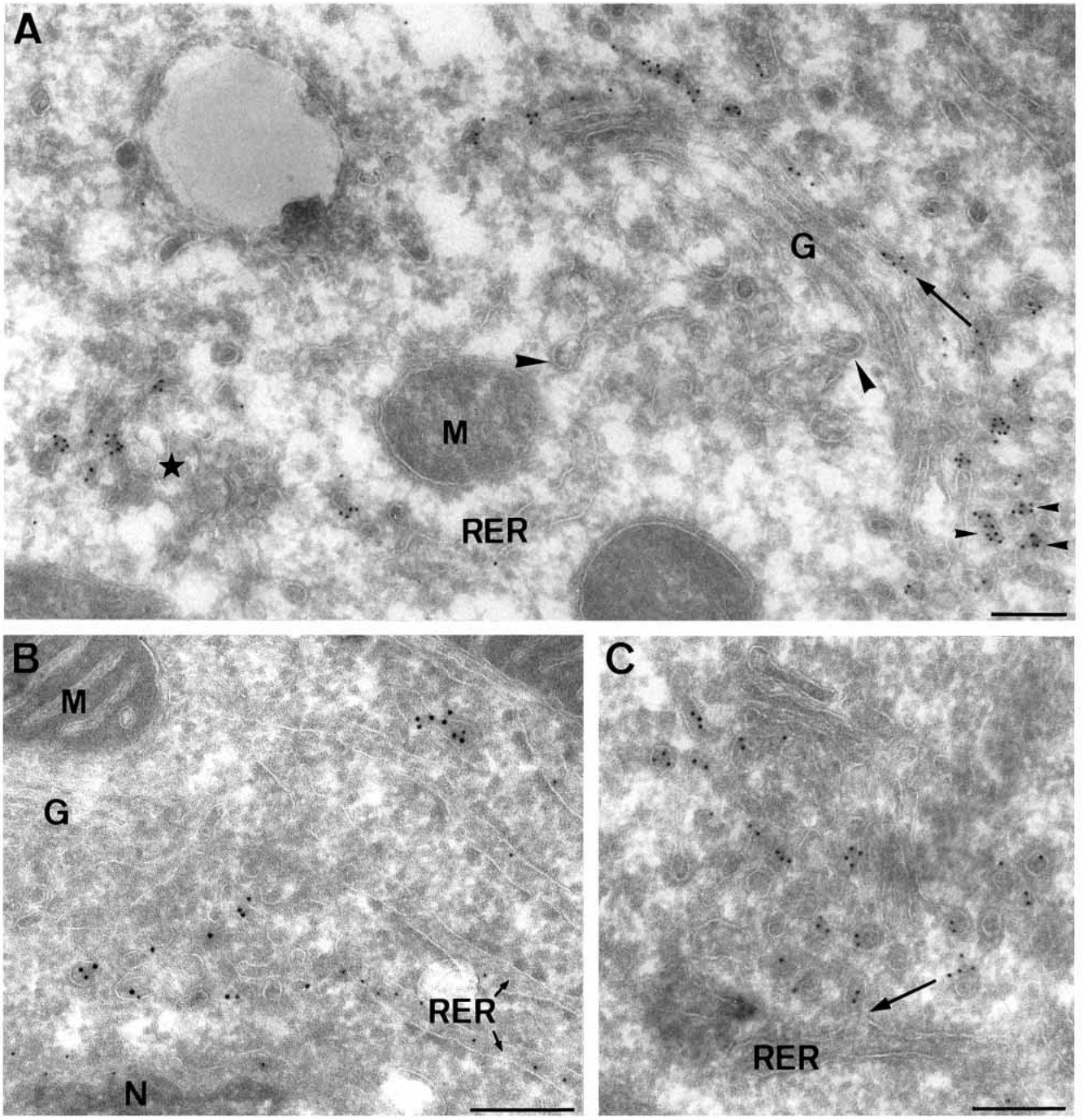

Fig. 2. Immunogold labeled ultrathin cryosections showing the subcellular distribution of ERGIC-53 in HepG2 cells cultured at 37°C. (A) Within the Golgi (G) ERGIC-53 is restricted to the *cis*-most cisterna (arrow). The TGN, in which clathrin-coated membranes are visible (big arrowheads) is devoid of ERGIC-53 label. ERGIC-53 is predominantly localized in ERGIC membranes closely associated with the Golgi (small arrowheads) and in ERGIC clusters at some distance from the Golgi apparatus (*) in close vicinity to the rough ER. (B) Double-immunolabeling of the rough ER resident protein PDI (5 nm gold) and ERGIC-53 (10 nm gold). Note that the localization of ERGIC-53 and PDI is mutually exclusive. (C) ERGIC-53-labeled 'budding profile' (arrow) of a rough ER element in the vicinity of a characteristic tubulo-vesicular ERGIC cluster. M, mitochondrion. N, nucleus. Bars, 200 nm.

processes could be preserved by recooling the cells to 15°C (Fig. 1E,F). The tubules often had a necklace appearance. These features are reminiscent of an early BFA effect on the Golgi apparatus. However, various Golgi markers including giantin (Linstedt and Hauri, 1993), GPP130 (Linstedt et al., 1997) and galactosyltransferase did not form any tubules during rewarming (not shown). ERGIC-53-positive tubules are not ER elements because they did not co-label for PDI (Fig. 4C,D) or p63 (not shown). Upon longer incubations at 37°C ERGIC-53 dispersed progressively to an ER-like pattern.

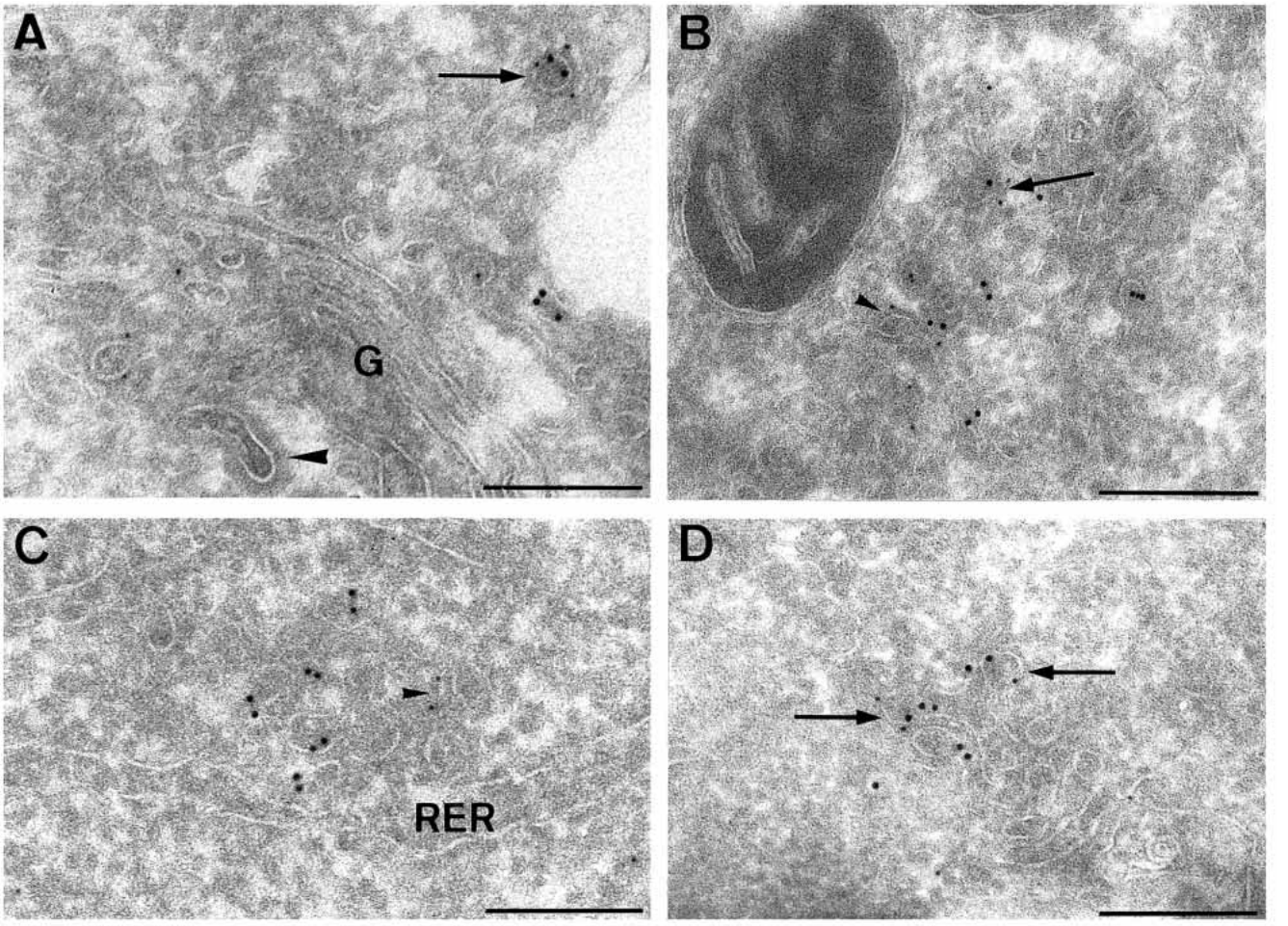

Fig. 3. Double-immunolabeling of β-COP (5 nm gold) and ERGIC-53 (10 nm gold) in cryosections of HepG2 cells cultured at 37°C. (A) ERGIC-53 is present in a β-COP-coated vesicular profile (arrow) near the Golgi apparatus (G). The COP-coated vesicle is morphologically well distinguishable from a clathrin coated vesicular element (arrowhead). (B-D) Gallery of electron micrographs showing the distribution of β-COP and ERGIC-53 in ERGIC clusters not associated with a Golgi stack in the plane of the section. Note that β-COP is associated with ERGIC membranes that do (B,D, arrows) or do not co-label (B,C, small arrowheads) with ERGIC-53. M, mitochondrion. Bars, 200 nm.

Although some cells showed an ER-like pattern already after 10 minutes, spreading of ERGIC-53 was maximal 30-60 min after rewarming (Fig. 1G). The appearance of a nuclear ring of immunofluorescence (Fig. 1G) and partial overlap of the ERGIC-53 pattern with that of PDI (Fig. 4E,F) suggested entry of (a fraction of) ERGIC-53 into the rough ER. It is important to note that all the immunofluorescence experiments with temperature-manipulated cells gave the same results irrespective of the presence or absence of cycloheximide (not shown) and hence these localization studies reflect changes of the steady state distribution of ERGIC-53.

Semi-quantitative immunoelectron microscopy revealed that 5 minutes after rewarming there was no significant increase in ERGIC-53 labeling of the first *cis*-cisterna of the Golgi apparatus (Table 1). In the electron microscope it is more difficult to see tubules than by immunofluorescence since one looks at ultrathin sections instead of whole cells. Moreover, in ultrathin sections tubules can only be recognized when they are cut longitudinally. Nevertheless, long ERGIC-53-positive (but PDI/calreticulin-negative) tubules could sometimes be observed shortly after rewarming (Fig. 6). The average number of β-COP-positive ERGIC clusters per cell profile was unchanged during the rewarming period (not shown).

Collectively, our morphological analysis of the rewarming experiments suggests that the retrograde pathway of ERGIC-53 from ERGIC to ER bypasses the Golgi apparatus and is mediated at least in part by tubular processes.

Segregation of newly synthesized albumin and ERGIC-53 in the ERGIC

If the ERGIC-53-positive tubules observed in the rewarming experiments indeed reflect a retrograde pathway one would expect that the tubules exclude secretory proteins travelling through the ERGIC in an anterograde direction. To address this issue we studied the passage of newly synthesized albumin through the ERGIC. HepG2 cells were treated with cycloheximide for 2.5 hours to chase all the intracellular albumin into the culture medium (Valtersson et al., 1990). Thereafter cycloheximide was rapidly washed out and the cells were recultured in fresh medium for 10 minutes at 37°C during which time albumin was synthesized de novo but did not yet enter the stacked Golgi (Mizuno and Singer, 1993). The cells

Table 1. Relative distribution of ERGIC-53 in HepG2 cells

	Incubation			
	37°C (*n*=4) [G: 1181]	3 hours 15°C (*n*=3) [G: 1568]	3 hours 15°C+ 5 minutes 37°C (*n*=4) [G: 1201]	3 hours 15°C + 60 minutes 37°C (*n*=4) [G: 1062]
Area				
ERGIC (near Golgi)	54.9±6.6	76.2±3.4	63.4±4.6	56.2±4.6
ERGIC (periphery)	40.4±3.3	21.2±3.5	33.0±3.5	38.3±6.0
cis-Golgi	4.6±0.8	2.6±0.2	3.5±1.5	5.5±2.4

Ultrathin cryosections of HepG2 cells were labeled with mAb G1/93 against ERGIC-53 followed by a rabbit anti-mouse secondary antibody and Protein A-gold. The numbers represent the percentage of gold particles found over the indicated area (mean ± s.d.).

n, number of grid areas scanned per sample; G, total number of gold particles encountered.

were then cultured at 15°C for 3 hours to further accumulate newly synthesized albumin in the ERGIC. Double immunofluorescence microscopy of these cells indeed showed considerable colocalization of ERGIC-53 and albumin (not shown). When such cells were rewarmed for 5-10 minutes they formed the expected ERGIC-53 tubules that did not significantly co-label with anti-albumin (Fig. 7A,B).

ERGIC-53-containing tubules can also be seen in cells treated with trimeric G-protein activator AlF_4^- (Kahn, 1991) in the presence of which COPI coats are locked to membranes (Melançon et al., 1987). AlF_4^- leads to the accumulation of ERGIC-53 in the ERGIC (Kappeler et al., 1997). Tubules can still be formed under these conditions but retrograde transport is blocked. To test if AlF_4^--induced tubules also exclude newly synthesized albumin, cycloheximide treated and washed out HepG2 cells were directly incubated with AlF_4^--containing medium for 30 minutes and subjected to double immunofluorescence analysis. Although AlF_4^- eventually blocked albumin synthesis it did not do so until about 10 minutes after its addition to intact cells (not shown) allowing enough albumin to be resynthesized for the immunofluorescence analysis. As in the rewarming experiment, the ERGIC-53-positive tubules were negative for albumin (Fig. 7C,D). These data support the notion that the ERGIC tubules reflect the retrograde pathway.

Since coatomer has been implicated in mediating retrograde transport from post-ER compartments back to the ER (Letourneur et al., 1994), we tested by double immunofluorescence microscopy whether the ERGIC-53-positive tubules appearing after rewarming from 15°C or by $AlF4^-$ treatment at 37°C carry β-COP. Fig. 8 shows that the tubules do not co-stain for β-COP under either condition.

Table 2. Quantification of ERGIC-53 immunoreactivity in ERGIC clusters of HepG2 cells cultured at 37°C or at 15°C

	Parameter		
Culture condition	Gold particles per cluster	Intersections per cluster	Gold particles per intersection
37°C	11.2±1.5	65.1±10.7	0.23±0.03
15°C (3 hours)	22.9±4.5	85.0±13.4	0.28±0.03

Quantification was performed on ultrathin cryosections double-labeled for ERGIC-53 and β-COP. The latter labeling together with morphological appearance of β-COP-labeled membrane structures was used to identify the ERGIC clusters. 280 gold particles and 1,627 intersections were counted in cells cultured at 37°C. 573 gold particles and 2,124 intersections were counted in cells cultured at 15°C. Numbers are means ± s.e.m. (*n*=25 for both culture conditions). At 15°C the labeling density of ERGIC-53 per cluster increases, as does the amount of membranes per cluster. The labeling density of ERGIC-53 per membrane length does not significantly change.

Recycling of ERGIC-53 analyzed by subcellular fractionation

In a complementary approach to study the temperature-dependent recycling of ERGIC-53 we used subcellular fractionation. A recently established Nycodenz gradient method allows the separation of ER, ERGIC, and *cis*-medial Golgi membranes from HepG2 postnuclear supernatants (Kappeler et al., 1997; our unpublished observatons). Fig. 9 shows the distribution on a Nycodenz gradient of the rough ER marker BAP31, the *cis*-Golgi marker GalNAc-transferase (Roth et al., 1994; Schweizer et al., 1994), the (in HepG2 cells) mostly *cis*-Golgi localized KDEL receptor, the *cis*-medial Golgi marker GPP130, and the *trans*-Golgi marker galactosyltransferase. At 37°C ERGIC-53 codistributed with ER as well as with *cis*/medial Golgi markers. A third ERGIC-53 peak comigrating with the *trans*-Golgi marker galactosyltransferase originated from the ERGIC membranes. Evidence that ERGIC membranes migrate to this position comes from the observation that this peak became the dominating one under conditions that led to the accumulation of ERGIC-53 in the ERGIC including BFA and $AlF4^-$ (our unpublished observations). The distribution of ERGIC-53 on Nycodenz gradients appears somewhat different from the (nonquantitative) impression obtained by immunofluorescence microscopy, where the dominant signal for ERGIC-53 was associated with the ERGIC. This difference is most likely due to the fact that the concentration of ERGIC-53 in the ER is lower than in the ERGIC but that the ER is a much larger organelle.

A 3 hour incubation at 15°C led to a twofold increase of ERGIC-53 in the ERGIC position at the expense of ERGIC-53 in the ER position (Fig. 9) suggesting that the accumulation of ERGIC-53 in the ERGIC seen by electron microscopy indeed resulted from ongoing ER-to-Golgi transport. Within 10 minutes after rewarming, ERGIC-53 increased in the ER position and decreased in the ERGIC position. 30 minutes after rewarming the original distribution of ERGIC-53 was reestablished. This redistribution indicates recycling to the ER upon rewarming. Collectively, these biochemical data confirm and extend our morphological observations. They identify the ER as the compartment from which ERGIC-53 is recruited when it accumulates in the ERGIC at 15°C, and to which ERGIC-53 recycles upon rewarming.

DISCUSSION

The recycling pathway of ERGIC-53

This study provides the first detailed analysis of the recycling route of an endogenous membrane protein of the early secretory pathway of mammalian cells. Only the combined analysis by immunofluorescence, immunoelectron microscopy, and

subcellular fractionation provided a comprehensive picture of the recycling pathway. Immunofluorescence microscopy showed progressive concentration of ERGIC-53 near the Golgi apparatus at 15°C and its redistribution to an ER pattern upon rewarming. The redistribution revealed a tubular retrograde pathway. Due to its limited resolution this method did not indicate, however, to what extent the recycling pathway involved the stacked *cis*-Golgi. Surprizingly, our immunogold analysis showed that the major recycling pathway from the ERGIC clusters bypasses the stacked *cis*-Golgi as indicated by the fact that at no time after rewarming the labeling for ERGIC-53 in the first *cis*-Golgi cisterna was increased. Since the total surface of ERGIC-53-positive ERGIC membranes exceeds that of the first *cis*-Golgi cisterna it appears unlikely that we would have missed a major recycling event involving the *cis*-Golgi even if it was rapid. A previous ultrastructural analysis of the recycling pathway of the rat homologue of ERGIC-53, p58, was interpreted to indicate recycling via the stacked Golgi (Saraste and Svensson, 1991). However, this study was done with the peroxidase technique that precludes a quantitative analysis. Another study using permeabilized cells suggested that p58 may in part recycle before its arrival in the *cis*-Golgi (Aridor et al., 1995). Since this study relied on light microscopic evidence, it did not exclude the possibility that p58 enirely recycles through the stacked *cis*-Golgi. Clearly, the present and previous (Schweizer et al., 1988, 1990; Chavrier et al., 1990) immunogold labeling experiments indicate that the first fenestrated *cis*-Golgi cisterna also has some recycling potential for ERGIC-53, but we conclude that the recycling route of this protein largely bypasses the stacked Golgi. In line with this conclusion is the observation that it takes several hours for an N-glycosylation site variant of ERGIC-53 to attain maximal *cis*-Golgi-dependent glycosylation in transfected Lec1 cells (Kappeler et al., 1997).

The temperature-dependent redistribution of ERGIC-53 by immunofluorescence and immunoelectron microscopy as well as subcellular fractionation support the model illustrated in Fig. 10. At 37°C ERGIC-53 concentration is highest in characteristic tubulo-vesicular membrane clusters close to the Golgi apparatus and farther out in the cell periphery. Hence the ERGIC defined by ERGIC-53 is the sum of these tubulo-vesicular clusters. ERGIC-53 is also present in the first *cis*-cisterna of some but not all Golgi stacks and in the ER. In the ER it was detectable by immunofluorescence microscopy and subcellular fractionation but was below the detection limit of the immuno-gold procedure. Lowering the temperature to 15°C induces a progressive concentration of ERGIC membranes near the Golgi apparatus and leads to a 2-fold accumulation of ERGIC-53 in these clusters most likely because protein exit

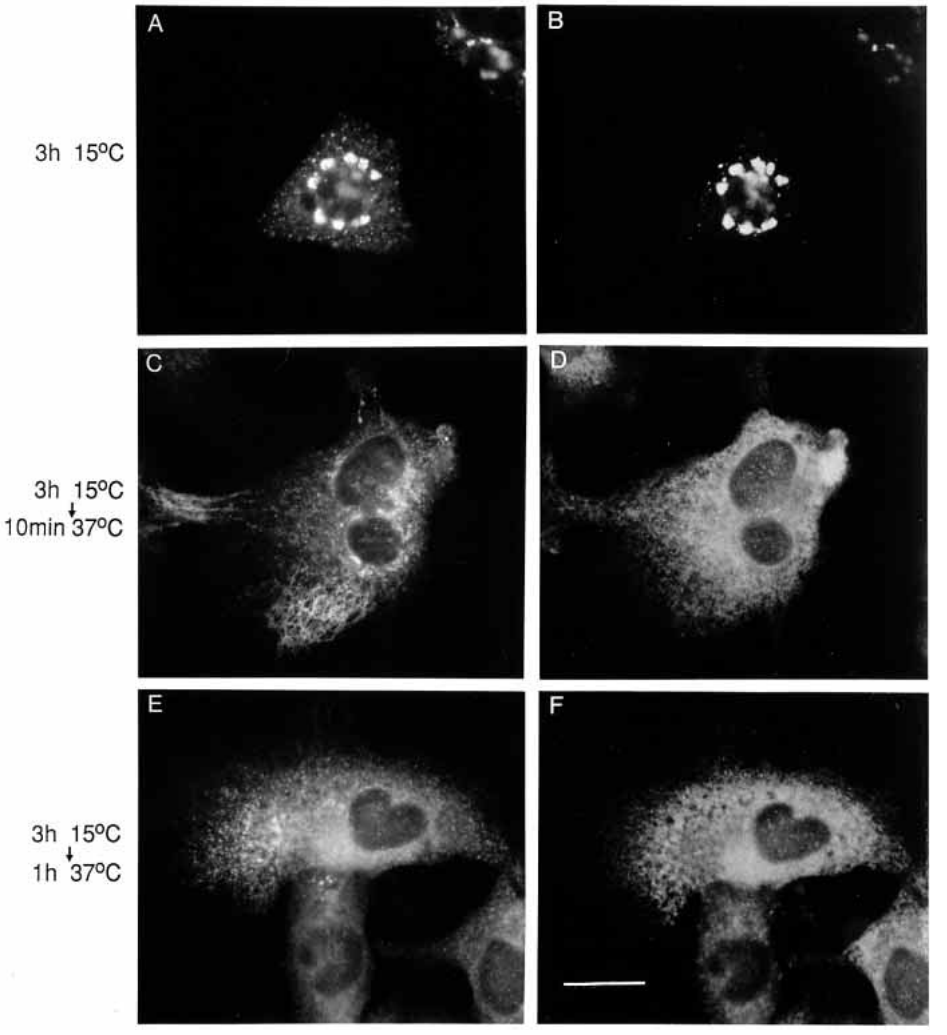

Fig. 4. Temperature-dependent changes of ERGIC-53's localization in relation to the Golgi apparatus and the ER visualized by double immunofluorescence microscopy. HepG2 cells were incubated as indicated, fixed with para-formaldehyde, permeabilized with saponin and sequentially incubated with a mAb against ERGIC-53, FITC-labeled goat anti-mouse, a polyclonal antibody against galactosyltransferase, and rhodamine-labeled goat anti-mouse (A,B), or sequentially incubated with a mAb against ERGIC-53, FITC-labeled goat anti-mouse, a polyclonal antibody against PDI, and rhodamine-labeled goat anti-mouse (C-F). Shown is the localization of ERGIC-53 in the FITC channel (A,C,E) and of galactosyltransferase (B) and PDI (D,F) in the rhodamine channel. Note the extensive apparent co-localization of ERGIC-53 and galactosyltransferase at 15°C (A,B) and that ERGIC-53 tubules do not co-stain for the ER marker PDI (C,D). Bar, 10.8 μm.

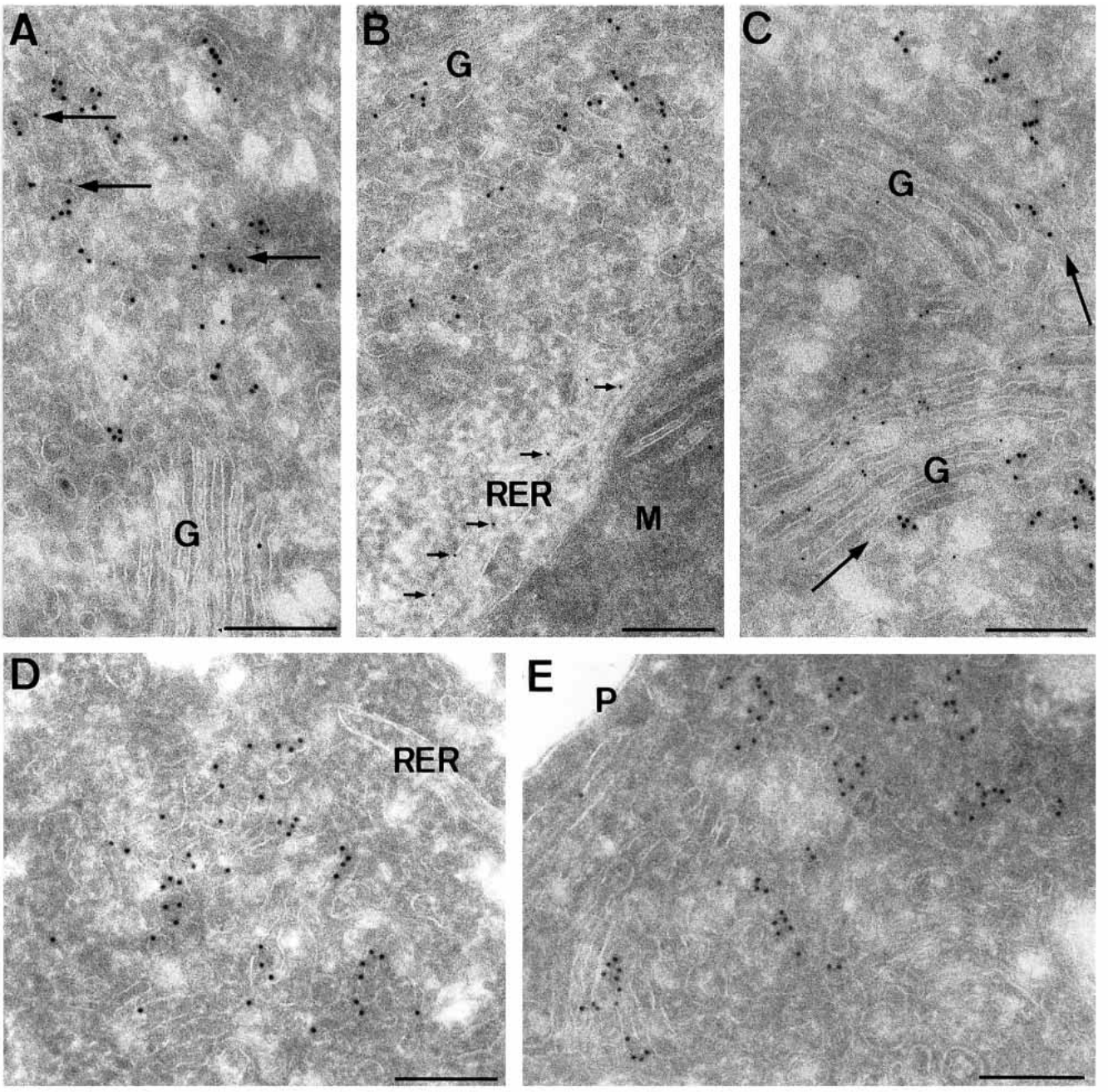

Fig. 5. Immunogold-labeled HepG2 cells after a 3 hour incubation at 15°C showing the distribution of ERGIC-53 in Golgi-associated (A-C) and peripheral (D,E) ERGIC clusters. (A) Double immunolabeling of β-COP (5 nm gold) and ERGIC-53 (10 nm gold) in an ERGIC cluster in close vicinity to a Golgi stack (G). The arrows point to β-COP-coated and ERGIC-53-positive membranes. (B) Double immunolabeling of PDI (5 nm gold) and ERGIC-53 (10 nm gold). PDI labeling is restricted to the rough ER (small arrows). (C) Double immunolabeling of galactosyltransferase (5 nm gold) and ERGIC-53 (10 nm gold). The galactosyltransferase label extends from the *trans*- to the medial-Golgi, whereas ERGIC-53 label is undetectable beyond the *cis*-most cisterna (arrows). (D,E) ERGIC-53 clusters at the periphery of the cell. P, plasma membrane. M, mitochondrion. Bars, 200 nm.

from the ERGIC is blocked while protein import from the ER is less temperature sensitive as already reported for vesicular stomatitis virus (VSV)-G protein (Schweizer et al., 1990; Lotti et al., 1992). Rewarming to 37°C leads to recycling of ERGIC-53 directly from the ERGIC to the ER by means of tubular extensions that appear within 5 to 10 minutes and to the original distribution of the clusters within the cytoplasm.

Organisation of the ER-to-Golgi anterograde pathway

It is now generally accepted that the ERGIC is an intermediate way station for anterograde transport of proteins from ER-to-Golgi (Schweizer et al., 1990; Saraste and Svensson, 1991; Plutner et al., 1992; Hammond and Helenius, 1994; Griffiths et al., 1995; Aridor et al., 1995). In contrast, the relationship of the

ERGIC to ER and Golgi has remained unclear (Pelham, 1995). Concerning the identity of the ERGIC the following major observations of the present study are most relevant: (1) The average number of ultrastructurally identifiable ERGIC-clusters per cell is constant irrespective of the temperature manipulation. (2) All the clusters appear equivalent in respect to the presence of the coatomer subunit β-COP as well as their temperature-dependent accumulation of ERGIC-53 although more marker proteins will have to be studied to know to what extent peripheral and juxta-Golgi ERGIC clusters are identical. (3) ERGIC clusters are mobile, at least in part, moving slowly to the Golgi area at 15°C and apparently to the periphery shortly after rewarming. (4) ERGIC clusters do not seem to fuse with one another upon rewarming. (5) The major recycling pathway of ERGIC-53 from ERGIC to ER does not involve the stacked Golgi.

How can our data be accomodated with the different ERGIC models? In the CGN model (Huttner and Tooze, 1989) the ERGIC and the first *cis*-Golgi cisterna are thought to be in continuity and constitute one compartment (Mellman and Simons, 1992). Although both the ERGIC and the *cis*-Golgi display COPI-coated areas, the distribution and mobility of (a fraction of) the ERGIC clusters argue against the notion that all ERGIC clusters and the first *cis*-cisterna of the Golgi stack are connected by membrane continuities. Moreover, at 15°C there is accumulation of ERGIC-53 in the ERGIC clusters but not in the first cisterna of the Golgi stacks indicating qualitative differences of the two membrane structures. It is clear, however, that the *cis*-Golgi is also engaged in protein recycling as indicated by the fact that the KDEL receptor is mainly localized in the stacked *cis*-Golgi (Tang et al., 1993; Griffiths et al., 1994) and KDEL-tagged reporter proteins progress into the *cis*-most Golgi cisterna (Conolly et al., 1994). Similarly, some di-lysine motif tagged proteins can recycle via the Golgi as indicated by Golgi-specific carbohydrate modifications (Jackson et al., 1993). Our data argue, however, that the ERGIC and the first *cis*-cisterna of the Golgi stack are separate membrane entities. While it is unlikely that peripheral clusters and the stacked *cis*-Golgi are connected, the possibility remains that so far undedected connections exist between juxta-Golgi ERGIC clusters and *cis*-Golgi which would, however, not lead to massive diffusion of ERGIC-53 into the *cis*-Golgi cisterna.

In the ER-subcompartment model the ERGIC is considered to be physically connected to the rough ER (Sitia and Meldolesi, 1992). In support of this notion, Krijnse-Locker et al. (1994) have found frequent membrane continuities from the rough ER all the way to the *cis*-Golgi including the ERGIC in hepatitis virus-infected permeabilized mouse L-cells and, in conjunction with a biochemical assessment of the site of O-glycan initiation, they concluded that vesicular transport is not required for protein transport from rough ER to the first *cis*-Golgi cisterna. We cannot confirm such extensive membrane continuities within the early secretory pathway of HepG2 cells and our data on the site of O-glycan initiation (Schweizer et al., 1994) are also at variance with those of Krijnse-Locker et al. (1994). In a few instances we observed short ERGIC-53-labeled protrusions of ER elements that resemble the classical transitional elements of the rough ER described in pancreatic acinar cells while the ERGIC clusters themselves correspond to the β-COP-positive 'peripheral elements' (Jamieson and Palade, 1967; Oprins et al., 1993). Considering their paucity we think that the ERGIC-53-positive ER protrusions are COPII-coated budding vesicles rather than permanent continuities. This is in line with the conclusion of Stinchcombe et al. (1995) who found that ER-to-Golgi transport of a horseradish peroxidase-transferrin receptor chimera occurs by vesicles in HEp-2 cells cultured at 37°C and by immunofluorescence studies on the transport of VSV-G protein in living cells (Presley et al., 1977; Scales et al., 1997).

According to the most popular current model, the maturation

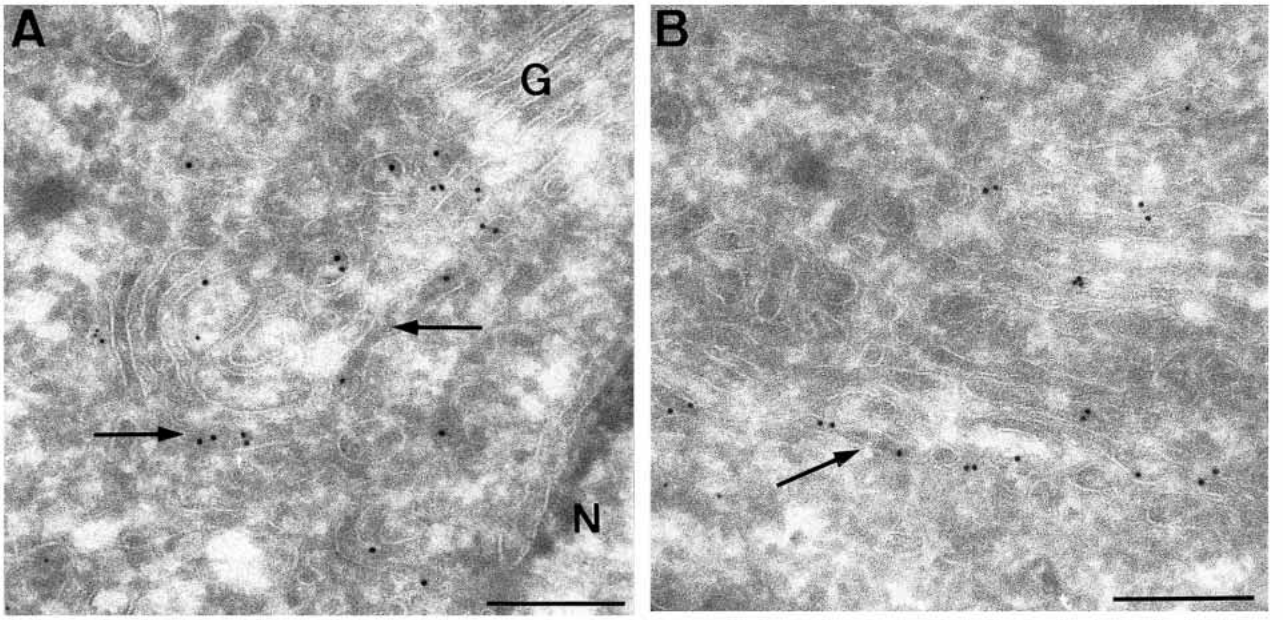

Fig. 6. Double immunogold labeling of ERGIC-53 (10 nm gold) and PDI (5 nm gold) in HepG2 cells cultured for 3 hours at 15°C followed by 5 minutes at 37°C. (A,B) ERGIC-53 is present in typical tubular membranes (arrows), sometimes in close vicinity to a Golgi stack (G). N, nucleus. Bars, 200 nm.

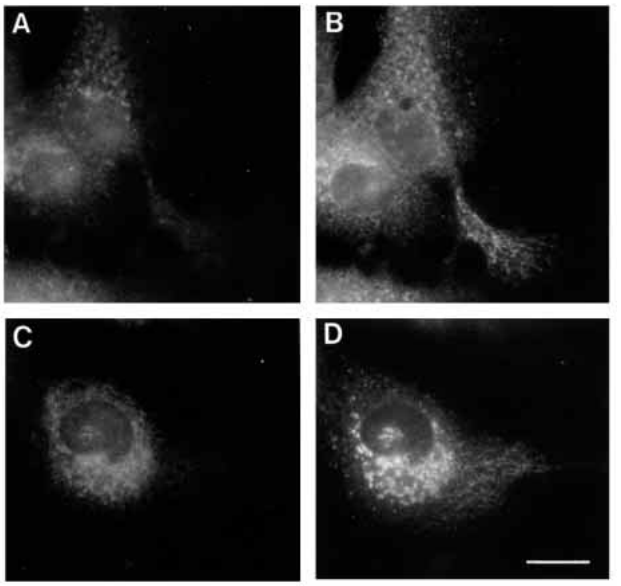

Fig. 7. Segregation of newly synthesized albumin and ERGIC-53 in the ERGIC. (A,B) HepG2 cells were cultured for 2.5 hours in medium containing 50 μg/ml cycloheximide to clear the secretory pathway of albumin. Thereafter, the drug was rapidly washed out by serum-free medium and protein synthesis was resumed in serum-free medium at 37°C. 10 minutes after cycloheximide wash out the cells were incubated for 3 hours at 15°C to accumulate newly synthesized albumin and persisting ERGIC-53 in the ERGIC. Following 5 minutes rewarming to 37°C, the cells were fixed with para-formaldehyde and processed for double immunofluorescence microscopy using a rabbit antiserum to localize albumin (A) and mAb G1/93 to localize ERGIC-53 (B). (C,D) HepG2 cells were cultured for 2.5 hours in 50 μg/ml cycloheximide. After washing out the drug, the cells were cultured in AlF_4^--containing medium to accumulate newly synthesized albumin and persisting ERGIC-53 in the ERGIC. The cells were processed for double immunofluorescence microscopy to localize allbumin (C) and ERGIC-53 (D). Note co-localizing dots while ERGIC-53 tubules largely exclude albumin. Bar, 8.8 μm.

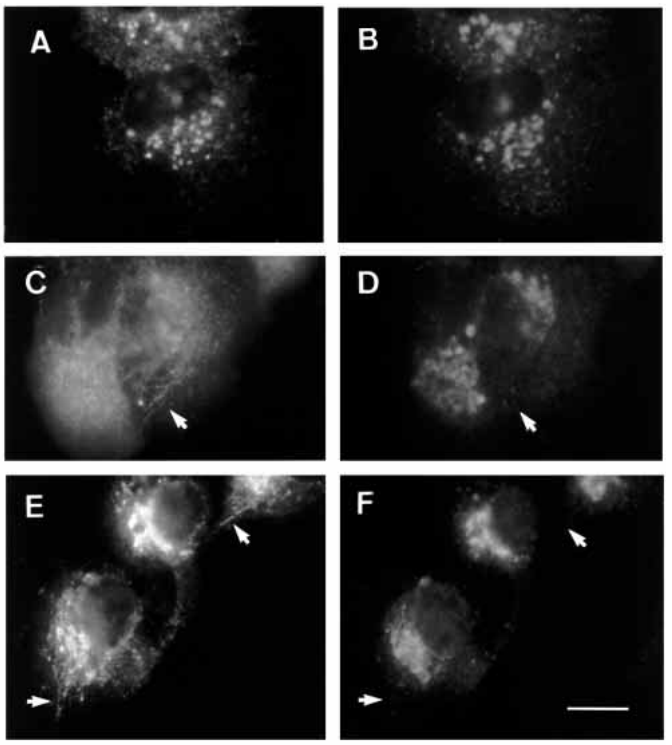

Fig. 8. ERGIC tubules do not co-stain with anti-β-COP. HepG2 cells were cultured at 15°C for 3 hours without rewarming (A,B), or followed by rewarming to 37°C for 10 minutes (C,D). Alternatively, the cells were treated with AlF_4^- at 37°C for 20 minutes (E,F). After fixation and permeabilisation the cells were double labeled for ERGIC-53 (A,C,E: fluorescein channel) and β-COP (B,D,F; rhodamine channel). Note that ERGIC-53 positive tubules appearing after rewarming or by AlF_4^- treatment do not co-label for β-COP (arrows). Bar, 14 μm.

model, the ERGIC is a transport intermediate that forms by fusion of transport vesicles and translocates to the Golgi region where it fuses with and matures to the *cis*-Golgi (Saraste and Kuismanen, 1992; Lippincott-Schwartz, 1993; Bannykh and Balch, 1997). This notion initially comes from a study on p58 (Saraste and Svensson, 1991), in which, however, neither the number of ERGIC clusters nor the postulated entry of p58 into the Golgi apparatus was quantified at the ultrastructural level. Recent elegant studies with VSV-G/green fluorescent protein hybrid proteins in living cells showed that ER-to-Golgi transport involves highly dynamic elements that are larger than transport vesicles (Presley et al. 1997; Scales et al., 1997). These elements can form at peripheral sites in the cytoplasm and rapidly move to the central Golgi area. A straightforward interpretation of these experiments is that the GFP-labeled structures represent ERGIC elements moving form ER-exit sites to the Golgi where they fuse with one another to form the *cis*-Golgi. It should be noted, however, that the resolution of immunofluorescence microscopy is insufficient to solve this issue, as documented in the present study. Moreover, by studying a non-cycling anterograde transported protein a dissociative process occurring in the ERGIC may not become apparent at the light microscope level. Indeed our immunogold quantitation indicates such a dissociative process in the ERGIC by which most of the ERGIC-53 does not continue to be transported to the stacked Golgi but directly returns to the ER.

Collectively, our data support the notion that the ERGIC is a mobile transport intermediate and indicate two dissociative transport steps, i.e. ER-to-ERGIC and ERGIC-to-Golgi. Studies with permeabilized cells showed that ER-to-ERGIC transport is mediated by COPII-coated vesicles (Aridor et al., 1995). In line with this notion, exit of ERGIC-53 from the ER requires a di-phenylalanine motif that in vitro interacts with the COPII component sec23 (Kappeler et al., 1997). The mechanism of ERGIC-to-Golgi protein transport is less clear but may involve COPI-coats (Pepperkok et al., 1993; Griffiths et al., 1995; Scales et al., 1997) although COPI is also involved in retrograde transport (Cosson and Letourneur, 1994; Letourneur et al., 1994; Aridor er al., 1995). Our observations that: (1) ERGIC-53-containing clusters do not appear to fuse upon rewarming from 15°C; (2) their number remains constant; and (3) a substantial fraction of ERGIC-53 remains in ERGIC elements after rewarming can be accomodated with both the maturation and the stable compartment model. According to the maturation model ERGIC clusters would be cleaved into an ERGIC-53-enriched retrograde and an ERGIC-53-depleted anterograde element. The anterograde elements would fuse with one another to form the

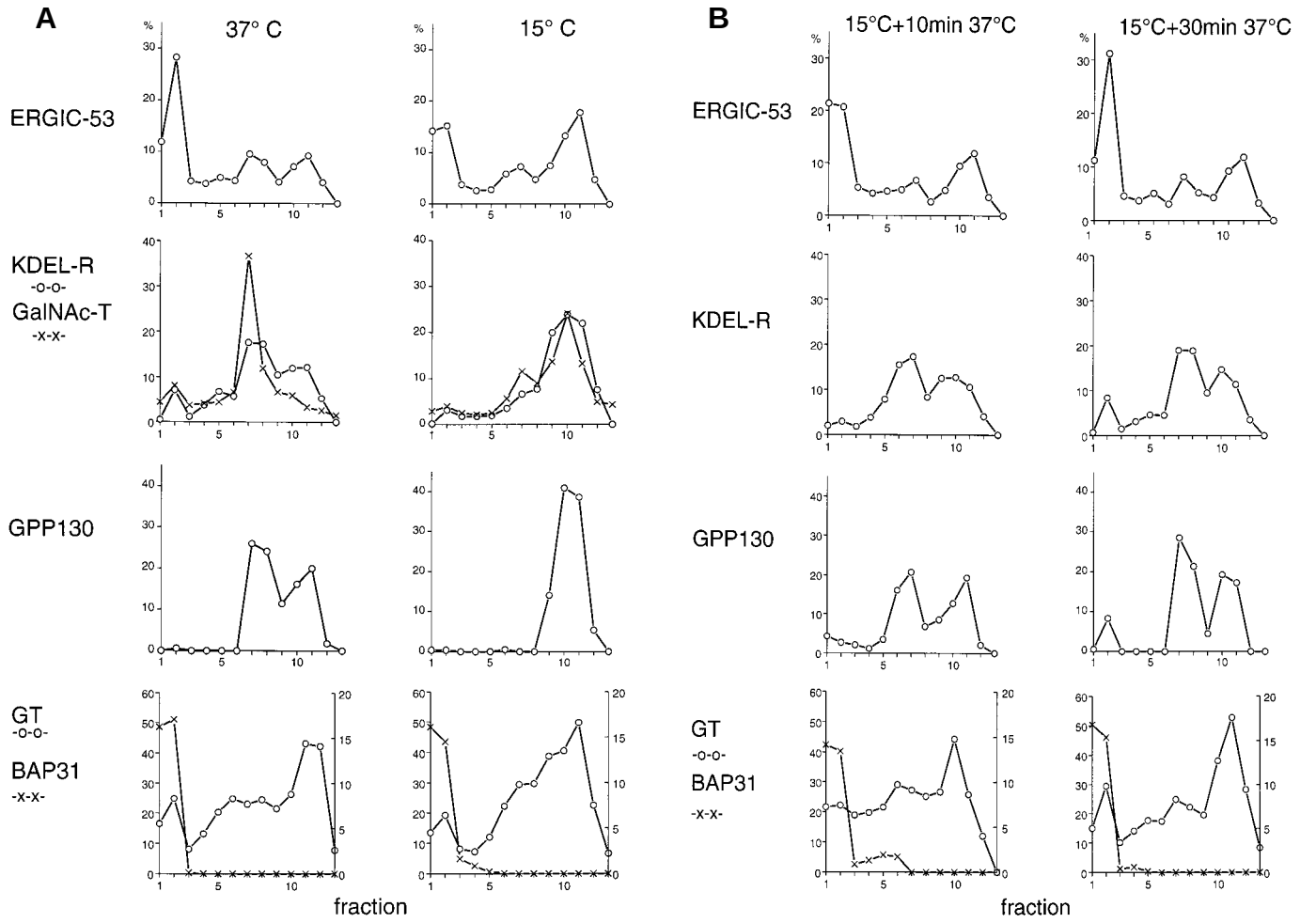

Fig. 9. Recycling of ERGIC-53 studied by subcellular fractionation. Postnuclear supernatants of HepG2 cells treated as indicated were fractionated on Nycodenz gradients. Fractions were collected from bottom (fraction 1) to top (fraction 13). Total enzymatic, radiochemical or densitometric activity in the gradient was set to 100%. Shown is the distribution of ERGIC-53 protein (ERGIC-53), the ER marker protein BAP31, the *cis*-Golgi markers KDEL-receptor (KDEL-R) and GalNAc transferase activity (GalNAcT), the *cis*/medial Golgi protein GPP130, and the *trans*-Golgi marker galactosyltransferase activity (GT).

cis-Golgi while the retrograde elements would recycle ERGIC-53 to the ER. Very rapid recycling of ERGIC-53 back to the ER and its accumulation in ERGIC elements that reform from the ER would explain the apparent incomplete depletion of ERGIC clusters from ERGIC-53 as well as the constant number of ERGIC-53 clusters. However, our data and those obtained by recent time-lapse imaging studies with VSV/G-protein chimeric proteins are equally compatible with a stable compartment model in which ERGIC-53-clusters are permanently existing, highly mobile entities that shuttle forth and back to deliver cargo to the *cis*-Golgi when they approach a Golgi stack and to accept cargo when they are near the ER. Simultaneous visualisation of ERGIC-53 and an anterograde marker in living cells will be required to differentiate between these two alternative models.

ERGIC as a sorting organelle of the early secretory pathway

The ERGIC is the first way station of protein sorting in anterograde and retrograde directions in the secretory pathway. Protein sorting may in fact be the major function of the ERGIC which makes less demand on membrane recycling from the Golgi apparatus. Our rewarming experiments suggest that sorting into the retrograde pathway involves at least in part tubules that largely exclude anterograde transported albumin. Similar tubules were also present in unperturbed cells cultured at 37°C but they tended to be shorter and less frequent. It is likely that membrane tubules of the ERGIC develop along microtubules. Such a tubulation may be powered by the plus-end directed microtubule motor kinesin which has indeed been localized to the ERGIC (Lippincott-Schwartz et al., 1995). Because these authors assumed that the ERGIC is an only transiently existing transport intermediate that fuses with the *cis*-Golgi and hence would move in a microtubule minus-end direction toward the cell center, the authors were surprized to find the microtubule plus-end directed motor kinesin associated with these elements. They concluded therefore that kinesin constitutively cycles between the ER and Golgi and that during its travel to the Golgi on ERGIC clusters it remains inactive. Our data offer a simpler interpretation of these data. We propose that the ERGIC-associated kinesin is active and

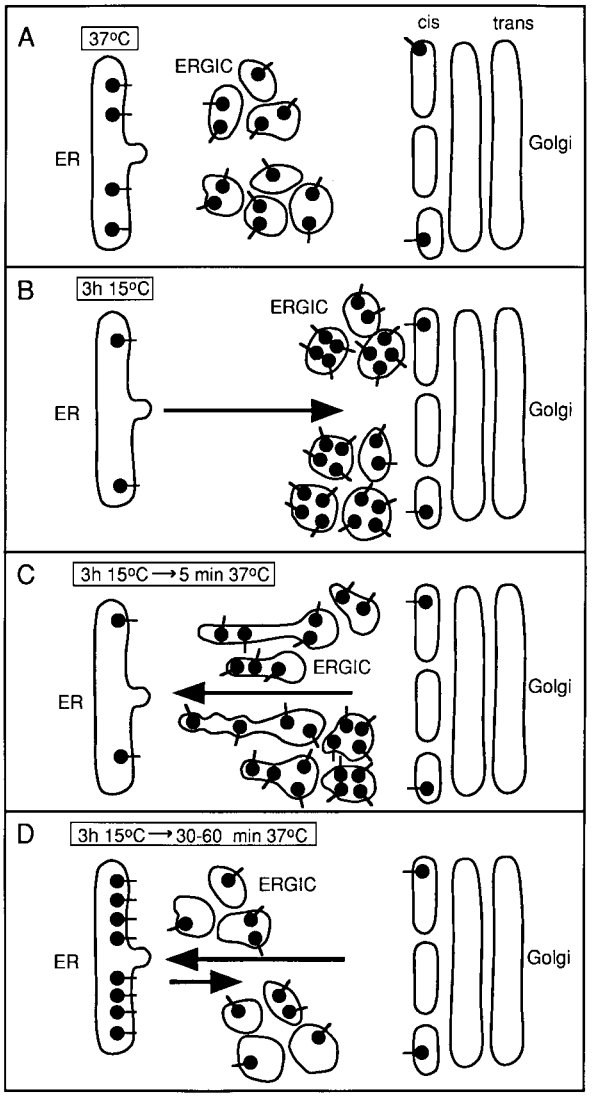

Fig. 10. Model of ERGIC-53 recycling and dynamics of the ERGIC. In cells cultured at 37°C ERGIC-53 (lollipop symbol) is present in ER, ERGIC, and *cis*-Golgi with highest concentration in tubulovesicular clusters constituting the ERGIC. Note, however, that the total amount of ERGIC-53 is higher in ER than in ERGIC (not indicated; see Fig. 9). Lowering the culture temperature to 15°C leads to a twofold accumulation of ERGIC-53 in the ERGIC at the expense of ERGIC-53 in the ER. Concomittantly the ERGIC clusters move closer to the Golgi apparatus. Upon rewarming for 5-10 minutes ERGIC-53-containing tubules appear indicating the retrograde pathway from ERGIC to ER and the original distribution of ERGIC clusters is reestablished. Note that the major recycling pathway does not involve the *cis*-Golgi and that the number of ERGIC clusters is unchanged during temperature manipulation (see Discussion).

responsible for retrograde tubule formation from the ERGIC. In a stable compartment model one could envision that a balance between plus-end directed motor activity by kinesin and minus-end directed activity by dynein (Presley et al., 1997) determines the position of the ERGIC clusters in between ER and Golgi. Reduced temperature may tip the balance toward dynein activity and thereby lead to the observed accumulation of ERGIC clusters near Golgi stacks in cells cultured at 15°C.

Is the retrograde transport entirely mediated by tubules? Collective evidence suggests that sorting of ERGIC-53 into the retrograde pathway is COPI-dependent. First, ERGIC-53 carries a functional di-lysine retrieval signal (Schindler et al., 1993; Itin et al., 1995b) interacting with COPI in vitro (Kappeler et al., 1997; Tisdale et al., 1997). Second, the ERGIC is a major site of COPI in the cell (Oprins et al., 1993; this study). Third, brefeldin A which is known to dissociate COPI from membranes (Klausner et al., 1992) leads to an accumulation of ERGIC-53 in the ERGIC as revealed by subcellular fractionation (our unpublished observation). Interestingly, the ERGIC-53-positive tubules did not appreciably co-stain for β-COP. Assuming that the tubules bind to microtubules this would make sense, since a coat can be expected to disturb the interaction. ERGIC-53 may directly mediate microtubule binding as it has been reported that a dilysine signal can bind β-tubulin in vitro (Dahllof et al., 1991). Based on these findings we propose three different scenarios for retrograde transport. First, COPI may only be involved in the intitial budding step of tubule formation. Upon coatomer dissociation ERGIC membranes would interact with microtubules (in part) mediated by ERGIC-53 itself and thereby form retrograde tubules powered by kinesin. Second, there may be two different retrograde pathways: one mediated by tubules that is COPI-independent and a another one mediated by COPI-coated vesicles. Third, COPI may function as a matrix for ERGIC integrity and to transiently retain ERGIC-53 in these elements preventing its further anterograde transport.

Tubules appear to also mediate retrograde transport of GFP-tagged KDEL-receptor from the Golgi to the ER in untreated cells (Sciaky et al., 1997) suggesting that tubule formation from the ERGIC and *cis*-Golgi may mechanistically be related. A notable difference is the extent of tubulation seen under different tubule-inducing conditions in HepG2 cells including rewarming from 15°C, as well as treatment with AlF_4^- or deoxy-glucose/azide that is considerably larger for ERGIC (defined by ERGIC-53) than *cis*-Golgi (defined by endogenous KDEL-receptor) (our unpublished observation). Whether this reflects a difference of tubulation potential or is simply due to the fact that ERGIC-53 constitutively recycles whereas the endogenous KDEL-receptor recycles in a ligand induced manner is currently unknown.

The ERGIC shares a number of features with endosomes. Like the ERGIC, endosomes have a high tendency to tubulate (Hopkins et al., 1990; Tooze and Hollinshead, 1991), are mobile within the cytoplasm (Mateoni and Kreis, 1987), can recruit COPI proteins (Whitney et al., 1995; Gu et al., 1997), and appear to be predominantly involved in protein sorting. Remarkably, the di-lysine intracellular targeting signal of ERGIC-53 can also act as an endocytosis signal suggesting mechanistic similarities of protein sorting (Kappeler et al., 1994; Itin et al., 1995c). The similarity also extends to the controversy over whether the two membrane structures are permanently or only transiently existing entities which is intimately related to the problem that they apparently lack non-cycling marker proteins. This controversy can be expected to continue until the machineries that define the individual transport steps have been characterized. Moreover, simultaneous recording of anterograde and retrograde transport markers by the green fluorescent protein technology in living cells and by immunoelectron microscopy in fixed cells will be required for further progress.

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