The Golgi Apparatus Maintains Its Organization Independent of the Endoplasmic Reticulum

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Under artificial conditions Golgi enzymes have the capacity to rapidly accumulate in the endoplasmic reticulum (ER). These observations prompted the idea that Golgi enzymes constitutively recycle through the ER. We have tested this hypothesis under physiological conditions through use of a procedure that captures Golgi enzymes in the ER. In the presence of rapamycin, which induces a tight association between FKBP (FK506-binding protein) and FRAP (FKBP-rapamycin-associated protein), an FKBP-tagged Golgi enzyme can be trapped when it visits the ER by an ER-retained protein fused to FRAP. We find that although FKBP-ERGIC-53 of the ER-Golgi intermediate compartment (ERGIC) rapidly cycles through the ER (30 min), FKBP-Golgi enzyme chimeras remain stably associated with Golgi membranes. We also demonstrate that Golgi dispersion upon nocodazole treatment mainly occurs through a mechanism that does not involve the recycling of Golgi membranes through the ER. Our findings suggest that the Golgi apparatus, as defined by its collection of resident enzymes, exists independent of the ER.

INTRODUCTION

In mammalian cells the Golgi apparatus is composed of dozens of connected stacks of cisternae that are anchored to the peri-centriolar region. Each stack is compartmentalized by the *cis*-*trans* (early-late) distribution of the different Golgi-specific enzymes (Farquhar and Palade, 1981). Integral membrane proteins and proteins that are secreted from the cell are translated into the endoplasmic reticulum (ER), where they are properly folded, and transported to the Golgi apparatus. Within Golgi membranes proteins are sequentially modified by the polarized array of Golgi enzymes and then transported to their respective destinations. Brefeldin A (BFA), a fungal metabolite that blocks ER-Golgi transport (Fujiwara *et al.*, 1988), induces the relocation of Golgi enzymes into the ER (Lippincott-Schwartz *et al.*, 1989). Preventing ER exit through the overexpression of a dominant-negative form of sar1, a GTPase that recruits the COPII coat to ER exit sites (Barlowe *et al.*, 1994), also relocates Golgi enzymes into the ER (Storrie *et al.*, 1998; Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). These studies demonstrate that Golgi enzymes have the capacity to quickly redistribute into the ER under artificial conditions, but they may not depict what happens normally, when the organization of the secretory pathway is not perturbed. Nonetheless, based on these findings, it was proposed that Golgi enzymes constitutively cycle through the ER (Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). Further investigation through use of dominant-negative sar1 reagents has revealed that ER recycling is a general property of all Golgi proteins (Miles *et al.*, 2001). Implicit to this idea is the dependence of Golgi function on the perpetual retrieval of

Golgi components from the ER, which opposes the status of the Golgi as an independent organelle.

The dynamics of the Golgi apparatus are determined by the collective behavior of its resident enzymes. Thus, the relationship between the Golgi and the ER can be ascertained by determining the rate at which Golgi enzymes associate with the ER. Previously, researchers have used fluorescence recovery after photobleaching techniques to achieve this (Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). However, in these studies conclusions were based on the analysis of very few cells ($n = 3$ cells; Miles *et al.*, 2001). Moreover, in some studies a clearly detectable ER-specific pool of a fluorescently tagged Golgi reporter was required in order to measure ER recycling with this method (Zaal *et al.*, 1999; Miles *et al.*, 2001). In our experiments Golgi enzymes are only detected in the ER if highly overexpressed. Because the behavior of highly overexpressed Golgi enzymes may not accurately emulate that of endogenous enzymes, we made a point to execute the experiments presented in this report under more physiological expression levels. We have investigated the ER recycling of resident Golgi proteins through use of a procedure that captures Golgi enzymes in the ER (Pecot and Malhotra, 2004). This method exploits the conditional interaction of two proteins and thus allows the dynamics of Golgi enzymes to be observed without photobleaching cells or disrupting the secretory pathway. The FK506-binding protein (FKBP) and the FKBP-rapamycin-associated protein (FRAP) only interact in the presence of rapamycin, a small molecule. Rapamycin specifically binds to FKBP (Wiederrecht *et al.*, 1991) and FRAP binds to the FKBP-rapamycin complex (Brown *et al.*, 1994; Sabatini *et al.*, 1994). In our procedure, FKBP is fused to a Golgi enzyme, and FRAP is attached to an ER-retained protein. If the FKBP-tagged Golgi enzyme ever visits the ER it can be trapped there in the presence of rapamycin by the ER-FRAP chimera (Figure 1A). We have shown previously that a Golgi enzyme (sialyl-transferase) fused to FKBP can be trapped quickly and efficiently in the ER of BFA-treated cells via this method (Pecot and Malhotra, 2004). Through use of this procedure

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Figure 1. Scheme of the ER-trapping procedure and description and localization of trapping constructs. (A) Diagram of the trapping procedure. FKBP is fused to a Golgi enzyme and FRAP is fused to an ER protein. When the FKBP-Golgi chimera visits the ER, it can be captured there in the presence of rapamycin. (B) Description and localization of trapping constructs. FKBP-E53 is made up of FL ERGIC-53 fused to 2× FKBP coding regions, GFP, and the prolactin signal sequence (PrtSS). For the construction of M2-FKBP the Golgi localization domain of mouse α -mannosidase II was fused to the FKBP coding region and GFP. ST-FKBP consists of the Golgi localization domain of sialyl-transferase appended to the FKBP coding region and GFP. Ii-FRAP is an ER-retained version of the invariant chain protein fused to the FKBP-rapamycin binding domain of FRAP and the HA epitope. All constructs were designed so that FKBP and FRAP domains are in the lumen of the Golgi or ER. In HeLa cells expressing Ii-FRAP FKBP-E53, M2-FKBP, and ST-FKBP (green, GFP) localize to the ERGIC, early Golgi, and late Golgi, respectively, and remain separate from ER-specific Ii-FRAP (red, HA).

we demonstrated that Golgi membranes remain segregated from the ER during mitosis in mammalian cells (Pecot and Malhotra, 2004). Here we use the ER-trapping method to investigate the relationship between the Golgi apparatus and the ER in nondividing cells.

MATERIALS AND METHODS

Antibodies

The monoclonal HA antibody (HA.11) was purchased from Covance (Madison, WI), and monoclonal Sec31A antibody was purchased from Transduction Laboratories (Lexington, KY). Secondary antibodies were purchased from Molecular Probes (Eugene, OR): Rhodamine Red anti-mouse, and Alexa fluor 647 Hoechst was also purchased from Molecular Probes.

Cloning and Constructs

ST-FKBP and Ii-FRAP were previously described (Pecot and Malhotra, 2004). GFP-ERGIC-53 was a gift from Dr. Hauri (Ben-Tekaya *et al.*, 2005). One tandem repeat of full-length FKBP was cloned into the BglII restriction site of GFP-ERGIC-53, generating GFP-FKBPx2-ERGIC-53 (FKBP-E53). M2-FKBP was constructed by cloning the first 100 amino acids of mouse α -manno-

sidase II (base pairs 88-307) into the HindIII and EcoRI restriction sites of pEGFP-N3. The entire FKBP coding region was then cloned into the EcoRI and BamHI restriction sites of this vector, leaving a C-terminal GFP tag.

Cell Culture. HeLa cells (ATCC, Manassas, VA) were grown at 37°C with 7% CO₂ in DMEM supplemented with 10% FBS, and 2 mM L-glutamine.

De-convolution Microscopy. Images were captured with a Nikon Eclipse TE 2000-U inverted microscope (Melville, NY) and a Photometrics CoolSNAP HQ camera (A. G. Heinz, Tucson, AZ). Image acquisition was run by MetaMorph software (Universal Imaging, West Chester, PA), and images were de-convolved by AutoDeblur and AutoVisualize 9.3 software (AutoQuant Imaging, Watervliet, NY). In general, using a 60× objective lens (NA 1.4), 21 optical sections per cell spaced by 0.1 μ m were taken. The best optical section for each set of data were used for publication. Exposure times were set such that the camera response was in the linear range for each fluorophore.

Transfections. HeLa cells were split into 6-cm plates the night before (600–900K cells/plate). In the morning the cells were between 80 and 95% confluent. Transfections were performed in the morning via Lipofectamine, 2000 Reagent (Invitrogen, Carlsbad, CA). Twenty microliters of Reagent plus 10 μ g of total DNA (FKBP-E53, M2-FKBP, or ST-FKBP + Ii-FRAP) was added to each 6-cm plate, and transfections were allowed to proceed for at least 6 h. In

Figure 2. Li-FRAP does not accumulate in ERGIC elements at 15°C. HeLa cells expressing Li-FRAP (red, 1st panel) and FKBP-E53 (green) were incubated at 37 or 15°C for 3 h. At 15°C ERGIC elements proliferate and accumulate Sec31 (red, 2nd panel) but not Li-FRAP. FKBP-E53 was visualized by GFP fluorescence and Li-FRAP with an α HA antibody, and Sec31 was labeled with a monoclonal α Sec31 antibody.

the evening the cells were split onto coverslips in 6-cm plates and allowed to attach for 12–18 h.

ER-Trapping Procedure. After transfection, cells were treated with media only, media + cycloheximide (CHX, 100 μ g/ml) + BFA (10 μ g/ml), or media + CHX + BFA + rapamycin (Rap, 200 nM) for 10 min at 37°C. At this point coverslips were fixed to determine the percentage of cells that had completely redistributed M2-FKBP into the ER. Cotransfected cells expressing high amounts of Li-FRAP were counted (~400 cells/experiment). The remaining coverslips were washed three times with PBS and incubated for 2 h at 37°C with media + CHX, or media + CHX + Rap. The coverslips were then fixed (4% formaldehyde in PBS) for 10 min at room temperature, followed by treatment with blocking buffer (2% FBS, 0.1% TX-100, and 0.05% NaN_3 in PBS) for 30 min at room temperature. Li-FRAP was labeled with a monoclonal HA antibody (1:1K in PBS, 1 h room temperature). All secondary antibodies

(Molecular Probes) were used at a dilution of 1:2K in PBS (20 min room temperature). After antibody staining, cotransfected cells were visualized by fluorescence microscopy (GFP constructs were visualized via GFP fluorescence). ER trapping was determined in cells expressing large amounts of Li-FRAP. Only the complete redistribution of M2-FKBP into the ER was counted as trapping. Approximately 300 cells were scored per experiment (3 independent experiments).

15°C Experiment. HeLa cells expressing Li-FRAP and FKBP-E53 were incubated at 37 or 15°C for 3 h in sealed 24-well plates with culture medium containing 25 mM HEPES. The cells were then fixed and prepared for fluorescence microscopy as described above.

Recycling Experiments. HeLa cells were transfected as described above. Transfected cells were treated with media + CHX (100 μ g/ml), or media +

Figure 3. Recycling of FKBP-E53. (A) Stages of FKBP-E53 recycling. HeLa cells expressing FKBP-E53 (green) and Li-FRAP (red) were treated with cycloheximide $\pm$ rapamycin for assorted periods of time. FKBP-E53 recycles to the ER in III stages. By stage II the entire pool of FKBP-E53 has been converted into the ER. FKBP-E53 was visualized by GFP fluorescence and Li-FRAP was stained with an α HA antibody. (B) Time course of FKBP-E53 recycling. Cells were treated as in A, and the percentage of cotransfected cells displaying each stage of FKBP-E53 recycling was determined at various time points. After 30 min in the presence of rapamycin FKBP-E53 has recycled to the ER in nearly all cotransfected cells.

Figure 4. M2-FKBP can be quickly and efficiently trapped in the ER in the presence of BFA. (A) BFA-induced M2-FKBP trapping in the ER. HeLa cells coexpressing M2-FKBP (green) and Ii-FRAP (red) were treated with BFA (to fuse Golgi membranes with the ER) and cycloheximide (CHX) $\pm$ rapamycin (Rap) for 10 min. After BFA treatment M2-FKBP colocalizes extensively with Ii-FRAP in the ER. Cells were then washed and incubated with fresh media and CHX $\pm$ Rap for 2 h. With CHX only, after BFA release M2-FKBP relocates to the peri-centriolar region. However, in the presence of Rap M2-FKBP is trapped in the ER bound to Ii-FRAP after BFA is washed out. M2-FKBP was visualized by GFP fluorescence, and Ii-FRAP was stained with an α HA antibody. (B) Efficiency of ER trapping. Cells were treated as in A, and the percentage of cells displaying the entire pool of FKBP in the ER was determined after BFA release in the presence or absence of Rap. This value was normalized to the percentage of cells in which BFA completely redistributed M2-FKBP into the ER. In the presence of Rap $\sim 80\%$ of cotransfected cells in which BFA worked to completion displayed absolute trapping of M2-FKBP in the ER after BFA release.

CHX + Rap (200 nM) for various periods of time. At designated time points the cells were fixed and prepared for fluorescence microscopy (as described above). Cotransfected cells expressing high amounts of Ii-FRAP were scored for the recycling of Golgi chimeras to the ER. Only complete relocation into the ER was counted as recycling. Approximately 200 cells were analyzed per experiment with M2-FKBP (at least 3 independent experiments), ~ 55 cells per experiment with FKBP-E53 (at least 2 independent experiments), and ~ 80 cells per experiment with ST-FKBP (2 independent experiments).

Quantitation of Partial Trapping for Recycling Experiments. Recycling experiments were performed as described above. The percentage of cotransfected cells displaying complete Golgi localization (No Trapping), complete ER localization (Complete Trapping), weak partial ER localization (weak ER staining), or strong partial ER localization (strong ER staining) for M2-FKBP or ST-FKBP after 8 h or 16 h in the presence of CHX $\pm$ Rap was determined.

Nocodazole Experiments Cells expressing Ii-FRAP and M2-FKBP were treated with media + CHX (100 μ g/ml), media + CHX + nocodazole (Noc, 1 μ M), or media + CHX + Noc + Rap (200 nM) at 37°C for 1.5–2 h. Cells were prepared for fluorescence microscopy as described above, except that Hoechst (1:25K) was added to the secondary antibody incubation. The effects of ER trapping on the appearance of Noc-induced Golgi fragments were observed

in cotransfected cells expressing large amounts of Ii-FRAP. Cells were scored based on their having or not having small Golgi fragments. Approximately 100 cells were analyzed per experiment (4 independent experiments).

RESULTS

Generation and Expression of ER-trapping Constructs

Our strategy was to compare the rate at which Golgi enzymes associate with the ER to that of a protein known to rapidly cycle through the ER. To accomplish this we fused the FKBP coding region to the Golgi localization domain of mannosidase II (M2, M2-FKBP) and to full-length ERGIC-53 (E53, FKBP-E53; Figure 1B). M2 is a resident enzyme of early Golgi cisternae (Dunphy *et al.*, 1981), and E53 resides in the ERGIC (Schweizer *et al.*, 1988). E53 rapidly cycles through the ER as it is a cargo receptor for the transport of glycoproteins between the ER and ERGIC (Hauri *et al.*, 2000). For this reason E53 is an excellent molecule with which to com-

Figure 5. Golgi enzymes do not constitutively cycle through the ER. (A) M2-FKBP does not recycle through the ER. HeLa cells coexpressing M2-FKBP and Ii-FRAP were treated with cycloheximide (CHX) $\pm$ rapamycin (Rap) for assorted periods of time. After 16 h in the presence of Rap 90% of cotransfected cells display a clearly visible Golgi-specific pool of M2-FKBP. (B) Phenotypes of M2-FKBP during recycling experiments. Cells expressing Ii-FRAP and M2-FKBP (green) were treated as in A, and the following patterns for M2-FKBP were observed to varying degrees: No Trapping (complete Golgi localization), Complete Trapping (complete ER localization), Weak-ER (partial trapping, weak ER staining), and Strong-ER (partial trapping, strong ER staining).

pare the ER association of Golgi enzymes. When expressed in HeLa cells M2-FKBP and FKBP-E53 localize to the Golgi and ERGIC, respectively (Figure 1B). The GFP-E53 construct, from which FKBP-E53 is derived, was previously constructed and utilized to investigate the dynamics of the ERGIC in living cells and is thus a proven marker of the ERGIC (Ben-Tekaya *et al.*, 2005). Ii-FRAP and ST-FKBP (Figure 1B) have been previously described (Pecot and Malhotra, 2004). Briefly, the FKBP-rapamycin binding domain of FRAP (Chen *et al.*, 1995) was fused to an ER-retained version of the invariant chain protein (Iip33; Schutze *et al.*, 1994; Ii-FRAP), and the Golgi localization domain of sialyl-transferase (a late Golgi enzyme) was fused to FKBP and GFP (ST-FKBP). Iip33 cannot be detected in post-ER compartments with biochemical methods (Schutze *et al.*, 1994) or by fluorescence microscopy (Schutze *et al.*, 1994; Figure 1B), indicating that it does not leave the ER. In addition, unlike Sec31 Ii-FRAP does not accumulate

in proliferating ERGIC elements at 15°C (Figure 2). At this temperature ERGIC elements undergo proliferation (Hauri and Schweizer, 1992; Saraste and Kuismanen, 1992) and accumulate molecules that cycle between the ER and Golgi (Schweizer *et al.*, 1990; Jackson *et al.*, 1993; Tang *et al.*, 1993; Nickel *et al.*, 1997; Hay *et al.*, 1998) including components of ER exit sites (Hammond and Glick, 2000).

FKBP-E53 Rapidly Cycles through the ER

To determine the kinetics with which FKBP-E53 cycles through the ER, FKBP-E53 and Ii-FRAP were coexpressed in HeLa cells. The cells were then treated or not with rapamycin for assorted periods of time in the presence of cycloheximide to prevent contamination from newly synthesized proteins. Cycloheximide has been shown to inhibit the peptidyl prolyl isomerase activity of the FKBP protein (Christner *et al.*, 1999), but does not hinder the efficiency of ER trapping in our experiments. At designated time points the cells were fixed and the localization of FKBP-E53 was observed by fluorescence microscopy. We discovered that the recycling of FKBP-E53 to the ER takes place in three stages (Figure 3A). Stage I is depicted by the loss of a compact peri-nuclear structure and the formation of an ER-like reticular pattern. More than 90% of observed cells display this phenotype after 5 min (Figure 3B). In stage II, the entire visible pool of FKBP-E53 has been converted into a reticular ER pattern. At this point the cycling of FKBP-E53 into the ER is complete as there is no longer evidence of any other structure by de-convolution microscopy. After 15 min, more than 60% of the observed cells exhibit this staining for FKBP-E53 (Figure 3B). Interestingly, although at this time FKBP-E53 is completely in the ER, it has not yet spread throughout the entire ER network, as observed by Ii-FRAP staining. Stage III signifies the complete dispersal of FKBP-E53 throughout the ER network. In 30 min approximately half of the observed cells are stage III, and by 1 h nearly all the cells display this phenotype (Figure 3B). These results confirm that FKBP-E53 rapidly cycles through the ER and that the ER-trapping procedure is capable of capturing such proteins.

In BFA-treated Cells M2-FKBP Is Rapidly and Efficiently Captured in the ER in the Presence of Rapamycin

After establishing the rate of FKBP-E53 recycling we tested whether M2-FKBP, when in the ER, can be rapidly and proficiently trapped there in the presence of rapamycin. HeLa cells coexpressing M2-FKBP and Ii-FRAP were treated with BFA to fuse Golgi membranes with the ER. After 10 min with a high concentration of BFA (10 μ g/ml) M2-FKBP can be completely relocated into the ER where it colocalizes with Ii-FRAP (Figure 4A). The effects of BFA are reversible, and when the BFA-treated cells are washed and incubated

Table 1. Percentage of cotransfected HeLa cells

Experiment	No. trapping	Partial trapping			Total
		Complete trapping	Weak ER staining	Strong ER staining	
CHX + Rap 8 h (M2-FKBP)	74 $\pm$ 7	9 $\pm$ 0	7 $\pm$ 2	10 $\pm$ 5	17 $\pm$ 7
CHX + Rap 16 h (M2-FKBP)	88 $\pm$ 1	4 $\pm$ 2	5 $\pm$ 3	3 $\pm$ 2	8 $\pm$ 1
CHX + Rap 16 h (ST-FKBP)	72 $\pm$ 10	2 $\pm$ 1	20 $\pm$ 10	6 $\pm$ 1	26 $\pm$ 10

Figure 6. Nocodazole-induced Golgi dispersion does not occur via the recycling of Golgi membranes through the ER. (A) Diagram of the current model for nocodazole (Noc)-dependent Golgi fragmentation, and the expected effect of the ER-trapping procedure under these circumstances. Currently it is thought that Golgi proteins recycle through the ER via a constitutive pathway. From peri-centriolar Golgi stacks Golgi proteins are transported to the ER in small vesicles that do not require microtubules for movement. Inside the ER Golgi proteins move to ER Exit Sites (ERES) and exit the ER in membrane bound transport carriers. The transport carriers fuse together, forming a Golgi stack that is subsequently directed to the peri-centriolar region via a microtubule dependent process. In the presence of Noc, which depolymerizes microtubules, Golgi proteins continue to recycle through the ER. However, Golgi stacks that form from the fusion of transport carriers harboring Golgi proteins fail to move to the peri-centriolar region. This results in Golgi fragmentation in the form of numerous dispersed, individual Golgi stacks that reside near ERESs. When cells are treated with Noc in the presence of rapamycin, recycling M2-FKBP should be captured in the ER by Ii-FRAP. This would prevent Golgi fragmentation as visualized by M2-FKBP. (B) The ER-trapping procedure does not prevent the formation of Noc-induced Golgi fragments. HeLa cells expressing M2-FKBP (green) and Ii-FRAP (red, bottom panel) were treated with Noc and cycloheximide (CHX) $\pm$ rapamycin (Rap) for 2 h. Under these circumstances M2-FKBP was found in punctate structures adjacent to ERES, as visualized with an antibody against Sec 31 (red, top panel). In cells coexpressing M2-FKBP and Ii-FRAP Golgi fragmentation was not prevented in the presence of Rap. M2-FKBP was visualized by GFP fluorescence and Ii-FRAP was observed through staining with an α HA antibody. (C) Quantitation of Noc-induced Golgi fragmentation in HeLa cells coexpressing M2-FKBP and Ii-FRAP in the presence and absence of Rap. Cotransfected cells were treated with Noc and CHX $\pm$ Rap for 1.5–2 h. In the presence of Rap the formation of small Golgi fragments is inhibited in a small percentage of cells, indicating that Golgi fragmentation mainly occurs through a mechanism that does not involve ER recycling.

with fresh media at 37°C for 2 h, M2-FKBP returns to the peri-centriolar region (Figure 4A). However, if rapamycin and BFA are added together for 10 min, M2-FKBP remains trapped in the ER bound to Ii-FRAP after BFA release (rapamycin bound to FKBP cannot be washed out; Figure 4A). To establish the efficiency of ER trapping, we determined the

percentage of cotransfected cells containing the *entire* pool of M2-FKBP trapped in the ER after BFA release in the presence of rapamycin. Because in many cells BFA treatment failed to completely redistribute M2-FKBP into the ER, it was necessary to normalize the trapping efficiency to the percentage of cells in which BFA worked to completion in

each experiment. This lessened the probability of recording false-negative results by adjusting for cells that were scored negative for trapping because of an incomplete ER redistribution of M2-FKBP. We found that, of the cotransfected cells in which BFA completely relocated M2-FKBP into the ER, ~80% had trapped the entire pool of M2-FKBP in the ER after BFA release in the presence of rapamycin (Figure 4B). These results, combined with the data obtained for FKBP-E53 demonstrate that the ER-trapping procedure is efficient and capable of capturing rapidly recycling proteins, thereby justifying its use as a method to determine if Golgi enzymes constitutively cycle through the ER.

Golgi Enzymes Are Stably Associated with Golgi Membranes

If Golgi enzymes recycle through the ER then, in the presence of rapamycin, our Golgi enzyme reporters (M2-FKBP, ST-FKBP) should get captured there overtime in cells expressing Ii-FRAP. HeLa cells coexpressing M2-FKBP and Ii-FRAP were treated with cycloheximide in the presence or absence of rapamycin for various periods of time. As a positive control BFA-trapping experiments were performed in parallel. At specific time points the cells were fixed and the localization of M2-FKBP was determined by fluorescence microscopy. The percentage of cotransfected cells at each time point in which M2-FKBP had recycled to the ER was determined, and the results are presented as a graph in Figure 5A. Recycling was deemed complete when the *entire* visible Golgi-pool of M2-FKBP was converted into the ER. Based on previous reports, it was expected that M2-FKBP would completely recycle after a couple of hours (Zaal *et al.*, 1999; Miles *et al.*, 2001). However, after 16 h in the presence of rapamycin, 90% of the observed cells still displayed a clearly visible Golgi-specific pool of M2-FKBP (Figure 4A, Table 1). Similar results were recorded for cells coexpressing Ii-FRAP and ST-FKBP (Table 1). When M2-GFP (lacking FKBP coding region) was substituted for M2-FKBP in recycling experiments, no background recycling was observed (data not shown). Time points longer than 16 h could not be taken because long-term cycloheximide treatment induces apoptosis.

Although complete recycling was not observed in the vast majority of cells, it is still possible that a significant amount of our Golgi reporters relocate to the ER in our recycling experiments. We therefore quantified the percentage of cotransfected cells displaying partial trapping of M2-FKBP or ST-FKBP in the ER after 8 or 16 h of treatment with rapamycin (Figure 5B, Table 1). Partial trapping was scored as weak (weak ER staining) or strong (strong ER staining) and was normalized for background at time $t = 0$. After 8 h 17% of cells coexpressing Ii-FRAP and M2-FKBP displayed partial ER trapping of M2-FKBP (7% weak and 10% strong), 9% of the cells had completely trapped M2-FKBP in the ER, and 74% of the cells had no visible ER staining for M2-FKBP (Table 1). After 16 h in the presence of rapamycin 8% of cotransfected cells exhibited partial trapping of M2-FKBP (5% weak and 3% strong), 4% displayed complete ER trapping of M2-FKBP, and 88% showed no visible ER pool of M2-FKBP (Table 1). Similar results were observed for cells coexpressing Ii-FRAP and ST-FKBP (Table 1). The small population of cotransfected cells displaying partial ER trapping of our Golgi reporters strongly indicates that these constructs do not recycle through the ER. However, it may be difficult to detect Golgi proteins in the ER because it is a much larger compartment, and so we cannot rule out that small portions of the reporters relocate to the ER over time. The partial trapping that is observed in our experiments

may be attributed to missorting of our reporters back to the ER overtime or an incomplete block in protein synthesis. These findings demonstrate that early and late Golgi enzymes remain stably associated with Golgi membranes.

Nocodazole-induced Golgi Dispersion Does Not Result from the Recycling of Golgi Membranes through the ER

In cells treated with Noc, a microtubule de-polymerizing agent, the Golgi apparatus becomes fragmented into individual stacks of cisternae that are dispersed throughout the cell (Thyberg and Moskalewski, 1985; Cole *et al.*, 1996). It is thought that this fragmentation occurs via the recycling of Golgi membranes through the ER and their reemergence at areas adjacent to ER exit sites (Cole *et al.*, 1996; Storrie *et al.*, 1998; Figure 6A). We have tested this idea through use of the ER-trapping procedure. If it is true that Noc induces the fragmentation of Golgi membranes through ER recycling, then trapping Golgi enzymes in the ER should prevent the appearance of the scattered Golgi fragments (Figure 6A). If the ER-trapping procedure cannot prevent the formation of small Golgi elements then Noc-induced Golgi dispersion must transpire through an alternative mechanism. HeLa cells expressing M2-FKBP and Ii-FRAP were treated with Noc (1 $\mu\text{g}/\text{ml}$), and at assorted time points the cells were fixed and analyzed by fluorescence microscopy. After 1.5 h the typical Golgi-pattern represented by M2-FKBP began to breakdown into small, scattered elements that localized near ER exit sites (Figure 6B). When rapamycin was added with Noc and cycloheximide for 1.5–2 h, the appearance of small scattered fragments was prevented in ~14% of cotransfected cells (Figure 6C). In the vast majority of cells (86%) small Golgi elements were clearly visible, indicating their appearance was not due to ER recycling (Figure 6, B and C). Our results suggest that the initial fragmentation and dispersal of the Golgi apparatus in response to Noc treatment primarily occurs through a mechanism that does not involve the recycling of Golgi membranes through the ER.

DISCUSSION

How does the Golgi apparatus maintain its organization amid the constant flux of traffic through the secretory pathway? Recent reports suggest that Golgi proteins constitutively cycle through the ER at a rapid rate, implying that the Golgi undergoes constant biogenesis from the ER (Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). Exposing cells to BFA or dominant-negative forms of sar1 inhibits protein traffic out of the ER (Fujiwara *et al.*, 1988; Storrie *et al.*, 1998) and induces the accumulation of Golgi enzymes in the ER (Lippincott-Schwartz *et al.*, 1989; Storrie *et al.*, 1998; Miles *et al.*, 2001; Ward *et al.*, 2001), suggesting the existence of a Golgi-ER recycling pathway. However, both reagents may act nonspecifically due to the large amounts that must be added to cells to observe the desired effects. In support of this, BFA-mediated Golgi recycling requires microtubules (Lippincott-Schwartz *et al.*, 1990), while sar1 induced recycling is microtubule independent (Storrie *et al.*, 1998).

Fluorescence recovery after photobleaching techniques have been applied to demonstrate the rapid ER cycling of Golgi enzymes under physiological conditions (Zaal *et al.*, 1999; Miles *et al.*, 2001; Ward *et al.*, 2001). However, as was discussed earlier few cells can be examined with this method, and in some reports the cells that are examined must overexpress the observed Golgi reporter in the ER (Zaal *et al.*, 1999; Miles *et al.*, 2001). We feel that these limitations make it difficult to make conclusions about the behavior of endogenous Golgi enzymes. Researchers have

also utilized the temperature-sensitive features of the vesicular stomatitis virus G-protein (VSVG) to show that Golgi proteins recycle to the ER under normal conditions (Cole *et al.*, 1998). The thermosensitive form of VSVG (VSVGtsO45) contains a temperature-sensitive mutation that causes it to aggregate and be retained in the ER at temperatures above 39.8°C (Gallione and Rose, 1985). Golgi proteins that have been fused with VSVGtsO45 have been shown to accumulate in the ER under these conditions (Cole *et al.*, 1998), providing evidence for their constitutive recycling to the ER. However, it has been reported that at temperatures sufficient to arrest VSVGtsO45 in the ER, the budding of COPII-coated vesicles from the ER is prevented (Aridor *et al.*, 1999) and general protein transport is inhibited (Trucco *et al.*, 2004), demonstrating that these conditions do not represent a physiological situation. Furthermore, this study excluded Golgi enzymes and focused instead on Golgi-associated proteins known to behave dynamically as a result of their function in membrane trafficking (KDEL Receptor, TGN-38).

Because of the difficulty in accurately studying the behavior of Golgi enzymes, the relationship between the Golgi and the ER has been a contentious topic. For this reason, we developed the ER-trapping procedure (Figure 1A), which represents a simple, straightforward method to observe the association of Golgi enzymes with the ER under physiological circumstances. Previously we established that a late Golgi reporter (ST-FKBP) can be efficiently trapped in the ER when it visits there (Pecot and Malhotra, 2004). Here we have done the same for an early Golgi marker (M2-FKBP; Figure 4, A and B). We then determined if these reporters relocate to the ER over time. We discovered that although the entire pool of an ERGIC-53 reporter (FKBP-E53) is rapidly captured in the ER (30 min, Figure 3, A and B), our early and late Golgi markers remain stably associated with Golgi membranes (Figure 5, Table 1). Partial relocation of these proteins into the ER occurred in only a small percentage of cells (Figure 5B, Table 1), indicating that they do not constitutively cycle through the ER.

In this study we have also investigated the mechanism underlying Noc-induced Golgi fragmentation. Prior reports have claimed that Golgi fragmentation under these conditions occurs via the constitutive recycling of Golgi membranes through the ER (Cole *et al.*, 1996; Storrie *et al.*, 1998; Figure 6A). Based on these findings, it was also suggested that ER recycling is responsible for Golgi inheritance during cell division (Zaal *et al.*, 1999; Altan-Bonnet *et al.*, 2006), when Golgi membranes breakdown during spindle formation and reassemble in daughter cells (Warren, 1993). We have demonstrated that the appearance of Noc-dependent Golgi fragments cannot be prevented by trapping Golgi proteins in the ER (Figure 6, B and C), suggesting that fragmentation occurs through an alternative mechanism. These results support our prior finding that Golgi membranes remain segregated from the ER during mitosis in mammalian cells (Pecot and Malhotra, 2004).

We have demonstrated that both early and late Golgi enzymes remain stably associated with Golgi membranes and do not constitutively cycle through the ER. On the basis of this finding, we propose that the Golgi apparatus is a stable compartment that does not rely on the ER for its constant biogenesis. The results presented in this report combined with work regarding the fate of Golgi membranes during cell division (Jesch and Linstedt, 1998; Rossanese and Glick, 2001; Axelsson and Warren, 2004; Pecot and Malhotra, 2004) indicate that the Golgi apparatus remains independent from the ER throughout the life of the cell.

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