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Evidence that the entire Golgi apparatus cycles in interphase HeLa cells: sensitivity of Golgi matrix proteins to an ER exit block

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We tested whether the entire Golgi apparatus is a dynamic structure in interphase mammalian cells by assessing the response of 12 different Golgi region proteins to an endoplasmic reticulum (ER) exit block. The proteins chosen spanned the Golgi apparatus and included both Golgi glycosyltransferases and putative matrix proteins. Protein exit from ER was blocked either by microinjection of a GTP-restricted Sar1p mutant protein in the presence of a protein synthesis inhibitor, or by plasmid-encoded expression of the same dominant negative Sar1p. All Golgi region proteins examined lost juxtanuclear Golgi apparatus–like distribution as scored by conventional and confocal fluorescence microscopy in response to an ER exit block, albeit with a differential dependence on Sar1p concentration. Redistribution of GalNAcT2 was more sensitive to low $Sar1p^{dn}$ concentrations than giantin or GM130. Redistribution was most rapid for p27, COPI, and p115. Giantin, GM130, and GalNAcT2 relocated with approximately equal kinetics. Distinct ER accumulation could be demonstrated for all integral membrane proteins. ER-accumulated Golgi region proteins were functional. Photobleaching experiments indicated that Golgi-to-ER protein cycling occurred in the absence of any ER exit block. We conclude that the entire Golgi apparatus is a dynamic structure and suggest that most, if not all, Golgi region–integral membrane proteins cycle through ER in interphase cells.

Introduction

The Golgi apparatus occupies a central position within the secretory pathway and is located as a compact juxtanuclear structure in typical animal cells. It provides the organellar framework in which proteins and lipids that originate in the ER can undergo a series of posttranslational events including glycosylation and sorting. These events are ordered cis, medial to trans, entry to exit, across the stacked Golgi region membranes. Unlike the ER, the Golgi apparatus itself has no capacity to synthesize de novo either proteins or lipids. In a fundamental sense, the Golgi apparatus must be derived from the ER where the integral membrane components of the Golgi apparatus are synthesized. Evolutionarily, the Golgi apparatus may have originated as an outgrowth of the nuclear envelope (for review see Storrie et al., 2000). The ER and nuclear envelope are continuous.

Several lines of evidence indicate that Golgi region proteins cycle both within the Golgi apparatus itself and also between the Golgi apparatus and ER, perhaps as a reflection of the evolutionary relationship between the two organelles. One mechanism is coatomer protein I (COPI)* coat protein–dependent transport. The COPI protein complex consists of coatomer, a cytosolic complex, and arf-1, a small GTPase active in the recruitment of coatomer to membranes. COPI-coated vesicles bud from all levels of the Golgi apparatus and COPI is required for intra-Golgi apparatus transport in vivo (Pepperkok et al., 1993). Functional COPI-derived vesicles or related transport intermediates can be isolated or generated in vitro and have relatively high levels of Golgi region–resident enzymes (Love et al., 1998; Lanoix et al., 1999) and KDEL receptor (Sönnichsen et al., 1996), consistent with these being recycling intermediates. Inhibition of COPI function through expression of mutated arf-1 or microinjection of antibodies to COPI blocks recycling of both the KDEL receptor and ERGIC53 from the Golgi apparatus to ER (Girod et al., 1999). The KDEL receptor recognizes a COOH-terminal K(H)DEL motif present in many lumenal, soluble ER proteins. The

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*Abbreviations used in this paper: BFA, brefeldin A; CHX, cycloheximide; COPI, coatomer protein I; GFP, green fluorescent protein.

K(X)KXX motif present on many membrane proteins of the ER binds to COPI in vitro, and mutations that abolish binding result in the loss of reporter molecules to the cell surface (Cosson and Letourneur, 1994). Likewise, the transport from the Golgi apparatus to the ER of internalized proteins such as cholera toxin or *Pseudomonas* exotoxin containing COOH-terminal KDEL or KDEL-like sequences is blocked by microinjected antibodies to COPI (Majoul et al., 1998; Jackson et al., 1999). COPI-dependent transport appears to mediate both intra-Golgi apparatus recycling of Golgi glycosyltransferases and the transport of rapidly shuttling proteins such as the KDEL receptor and ERGIC53 between the Golgi apparatus and ER (for review see Storrie et al., 2000).

Our work reveals the existence of a second COPI-independent Golgi region transport mechanism which cycles "resident" proteins from the Golgi apparatus to the ER (Storrie et al., 1998; Yang and Storrie, 1998; Girod et al., 1999; Storrie et al., 2000). The first inklings of a pathway came from experiments in which Golgi apparatus scattering in response to microtubule depolymerization was studied with respect to the kinetics of individual Golgi region protein redistribution (Cole et al., 1996; Yang and Storrie, 1998). The observed kinetics were consistent with individual Golgi region proteins cycling to generate scattered Golgi region stacks, perhaps de novo, at or about peripheral ER exit sites. This apparent de novo formation of scattered Golgi region stacks could be blocked by the introduction of a GTP-restricted mutant Sar1p protein (Storrie et al., 1998). Sar1p is required for COPII-dependent budding from the ER. When ER exit is inhibited in interphase cells, Golgi region–resident glycosyltransferases such as GalNAcT2, GalT, SialylT, and Mann II accumulate, albeit slowly, in the ER (Storrie et al., 1998; Girod et al., 1999; Zaal et al., 1999; Seemann et al., 2000; Todorow et al., 2000). This ER accumulation is COPI-independent and rab6-dependent as indicated by the effect of arf-1 and rab6 mutations and COPI inhibitory antibodies (Girod et al., 1999). That Golgi apparatus–resident proteins normally cycle to the ER in a COPI-independent pathway was supported further by the finding that the transport of Shiga-like toxin from the Golgi apparatus to the ER is similarly rab6-dependent and COPI-independent (Girod et al., 1999). This finding is particularly important as toxin transport can be studied in wild-type cells in the absence of any ER exit block. These results and the COPI-dependent cycling of KDEL receptor and ERGIC53 to the ER suggest that the entire Golgi apparatus in interphase cells is a metastable structure that is continuously being assembled and disassembled by a combination of COPI-independent and -dependent mechanisms.

Protein cycling, though, may not be a general property of the Golgi apparatus. The proposed cis Golgi matrix protein GM130 (Golgi matrix polypeptide of 130 kD; Nakamura et al., 1995) and other golgins interact strongly with each other and also with the "tethering" protein p115 (Waters et al., 1992) and the integral membrane protein giantin (Sönnichsen et al., 1998). The findings imply stability to the association of such proteins with each other or membrane. Conceivably, these proteins generate a cycling-insensitive Golgi matrix and, in fact, experimental evidence indicating a cycling-resistant GM130 complex has been presented (Seemann et al., 2000). Seemann et al. (2000) find that the integral membrane protein, giantin, and the peripheral membrane proteins, GM130 and GRASP65, do not cycle in response to microinjection of the ER exit block protein, GTP-restricted Sar1p^{dn}, at a stock concentration of ~0.7 mg/ml. Sar1p^{dn} is a dominant negative mutant of the small GTPase required for the recruitment of COPII coat proteins to the ER membrane (Aridor et al., 1995). Moreover, in brefeldin A (BFA) washout experiments, these authors find that the formation of a juxtanuclear Golgi "matrix" complex is insensitive to microinjected Sar1p^{dn}. BFA is a drug that disperses many Golgi region proteins to the ER (for review see Klausner et al., 1992). On the basis of these experiments, Seemann et al. (2000) propose that a cycling-insensitive cis Golgi matrix nucleates the assembly of the glycosyltransferase-containing Golgi apparatus stack.

In the present work, we compared, in HeLa cells, the effect of an ER exit block on the distribution of "resident" Golgi region enzymes and putative matrix–interacting proteins such as giantin, GM130, and p115. We emphasized the plasmid-driven expression of the ER exit block protein, Sar1p^{dn}, as a tool because it provides a strong and constantly produced source of the protein. The distributions of 12 different Golgi region proteins were characterized. All were found to lose their juxtanuclear distribution in response to the ER exit block, albeit with a differential dependence on Sar1p^{dn} concentration. Perhaps, as expected for putative Golgi matrix proteins, the redistribution of giantin and GM130 required higher levels of Sar1p^{dn} than that of a Golgi glycosyltransferase. Accumulation in the ER occurred for all integral membrane proteins, even giantin. The kinetics of redistribution were at least as fast for matrix proteins as glycosyltransferases. Redistribution occurred in wild-type cells expressing normal levels of Golgi region proteins and in cells microinjected with the Sar1p^{dn} protein in the presence of a protein synthesis inhibitor. The juxtanuclear distribution of organellar proteins was stable in the long-term presence of protein synthesis inhibitors, suggesting that maintenance of a juxtanuclear Golgi apparatus requires protein and presumably lipid cycling per se, rather than replacement of degraded components. We conclude that the entire Golgi apparatus may well cycle in interphase mammalian cells.

Results

Our goal was to provide evidence for or against the hypothesis that the Golgi apparatus in interphase cells is a dynamic structure, many, if not all, of whose proteins cycle between a compact, juxtanuclear Golgi apparatus and peripheral organelles, most prominently the ER. To address this question, we compared the effect of a GTP-restricted, Sar1p^{dn}-induced ER exit block on the distribution of proteins normally located either in the cis Golgi apparatus/cis Golgi network or the cisternal Golgi stack/TGN. In some experiments the Sar1p^{dn} was directly microinjected into the cells. This had the advantage that experiments could be done in the presence of a protein synthesis inhibitor providing direct evidence that any Golgi region protein redistribution was of preexisting protein. In other experiments, the pSARA^{dn} plasmid–driven expression of Sar1p^{dn} was used as a tool because it provides a strong and constantly produced source of the

protein, albeit with a time lag of ~30 min based on previous experiments (Storrie et al., 1998). Protein distributions were assessed using immunofluorescence or green fluorescent protein (GFP)-conjugated reporter molecules. As a functional assay of Golgi region protein redistribution, the in situ glycosylation state of a newly synthesized marker protein in the ER of pSARA^{dn}-expressing cells was characterized. Photobleaching was used to characterize the equilibration of a GFP-conjugated Golgi glycosyltransferase between the Golgi apparatus and ER pools as an additional independent approach. With photobleaching, redistribution to the ER can be investigated in the absence of any ER exit block.

Golgi glycosyltransferases and putative Golgi matrix proteins are dissimilar in the concentration of Sar1p^{dn} required to induce relocation

Our first experiment was to compare the dependence of putative Golgi matrix and Golgi glycosyltransferase relocation on Sar1p^{dn} concentration in the presence of the protein synthesis inhibitor, cycloheximide (CHX). Example proteins were chosen: giantin and GM130 as representative putative Golgi matrix proteins and GalNAcT2 as a representative Golgi glycosyltransferase. Giantin and GM130 interact to form a complex (Sönnichsen et al., 1998) and may generate a stable, cis-located Golgi matrix (Seemann et al., 2000). GalNAcT2 is distributed across the entire Golgi stack (Röttger et al., 1998) and has been shown previously to relocate to the ER in response to a Sar1p^{dn}-induced ER exit block (Storrie et al., 1998, 2000; Girod et al., 1999). GalNAcT2 was expressed stably as a GFP fusion protein and either endogenous giantin or GM130 were localized in the same cells by immunofluorescence using a Cy3-conjugated second antibody, and in the case of microinjected cells (asterisks) Cascade blue dextran was the coinjection marker. All three Golgi region proteins were located, in the absence of an ER exit block, in a compact juxtanuclear distribution characteristic of the Golgi apparatus in HeLa cells (Fig. 1, noninjected cells). In cell populations microinjected with increasing concentrations of Sar1p^{dn} in the presence of CHX, an increasing proportion of the microinjected cells showed loss of juxtanuclear Golgi apparatus fluorescence for the respective protein (Fig. 1, A–C, GalNAcT2 and giantin shown qualitatively). At a concentration of 1.45 mg/ml Sar1p^{dn} stock protein, ~60% of the microinjected cells stained for giantin or GM130 exhibited extensive protein relocation 6 h postinjection (Fig. 1 C). The redistribution of both giantin and GM130 was indistinguishable in its Sar1p^{dn} concentration dependence. At the end of the 6 h incubation, both had accumulated in somewhat granular staining structures located throughout the cytoplasm. In striking contrast, GalNAcT2 in the same microinjected cells redistributed to an obvious ER-like distribution, as indicated by nuclear rim staining and web-like distribution at a lower Sar1p^{dn} concentration. In ~80% of cells microinjected with 0.15 mg/ml Sar1p^{dn}, GalNAcT2 no longer had a juxtanuclear distribution, but rather was distributed to the ER. We conclude that both matrix and glycosyltransferases are distributionally dynamic in response to an ER exit block, albeit with a differential sensitivity to Sar1p^{dn} dosage. As the amount of protein microinjected per cell varied, the variable response of individual microinjected cells was not surprising. The distribution of GalNAcT2-GFP was virtually identical whether observed as the inherent fluorescence of the GFP conjugate or after antibody staining with polyclonal antibodies directed

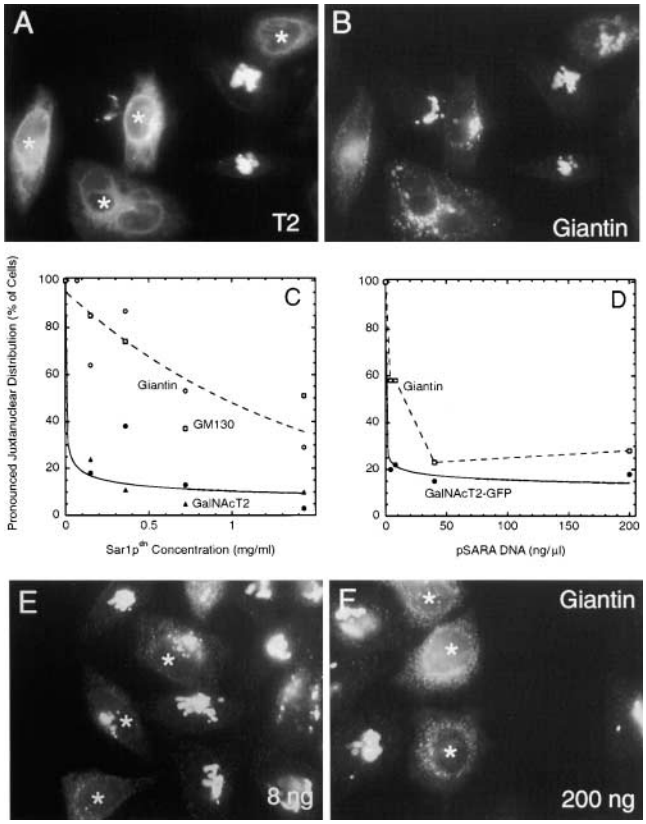

Figure 1. **Concentration dependence of the Sar1p^{dn} induced redistribution of GalNAcT2, giantin, and GM130.** HeLa cells stably expressing GalNAcT2-GFP were microinjected with either various stock concentrations of Sar1p^{dn} protein (A–C) or pSARA^{dn} plasmid (D–F), incubated for 6 h at 37°C, and then fixed. In the case of cells microinjected with Sar1p^{dn}, the postinjection incubation was in the presence of CHX. Following fixation, cells were stained for either GM130 or giantin by immunofluorescence. In A and B, the Sar1p^{dn} stock concentration was 0.15 mg/ml and the micrographs show cells double labeled for GalNAcT2-GFP and giantin. Asterisks over the cell nuclei indicate microinjected cells. Noninjected control cells are unmarked. Note that at this concentration GalNAcT2 had redistributed into an ER distribution in all four microinjected cells shown. Giantin, however, often displayed a more mixed distribution; all microinjected cells exhibited at least a partial diffuse cytoplasmic distribution for giantin with the left-hand cell exhibiting a full redistribution of giantin, and the other three injected cells also showing some concentrated juxtanuclear staining indicative of residual Golgi apparatus. In C, the incidence of microinjected cells displaying an overall pronounced juxtanuclear distribution for GalNAcT2, giantin, or GM130 was scored relative to the Sar1p^{dn} stock concentration as described previously (Girod et al., 1999). Two sets of data points are shown for GalNAcT2 because it was scored separately in giantin- and GM130-stained cells. In D, the incidence of microinjected cells displaying an overall pronounced juxtanuclear distribution for GalNAcT2 or giantin was scored relative to the pSARA^{dn} stock concentration. In E and F, the pSARA^{dn} stock was either 8 ng or 200 ng/μl. At the low pSARA^{dn} concentration, a mixed pattern of giantin redistribution was common, whereas at the high concentration little incidence of juxtanuclear giantin staining was seen. The end giantin distribution was more granular than the continuous ER-like GalNAcT2 distribution. A coinjection marker was used to distinguish microinjected cells independently of phenotype. The average diameter of a HeLa nucleus is 10 μm.

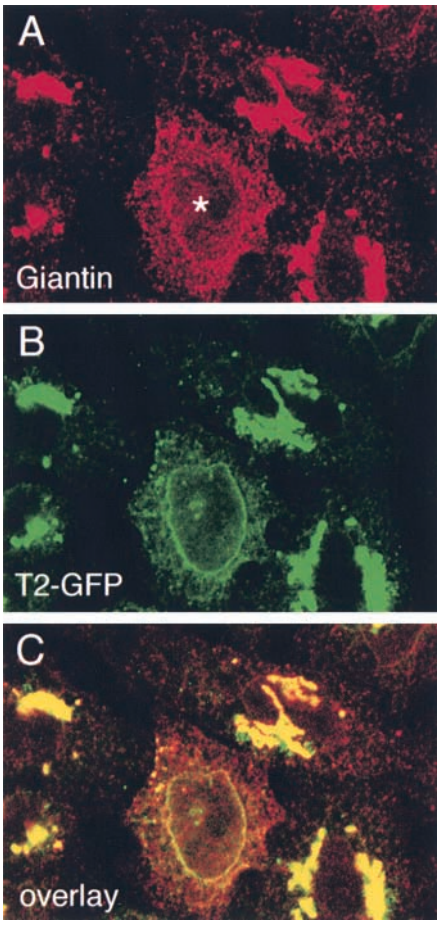

Figure 2. **Confocal fluorescence microscopy colocalizes giantin with GalNAcT2 in the ER of cells expressing pSARA^{dn}.** HeLa cells stably transfected with GalNAcT2-GFP were microinjected with pSARA^{dn}. After a 6 h expression period, cells were fixed and stained for giantin using a Cy3-conjugated second antibody. A, giantin; B, GalNAcT2-GFP; C, overlay. As shown by the overlay, giantin and GalNAcT2 appeared to extensively colocalize to the nuclear envelope and elsewhere within the cytoplasm. Such localizations are characteristic of the ER. Asterisk (A) indicates microinjected cell.

against full-length GFP (unpublished data), indicating that the intrinsic fluorescence of the GFP was a bona fide indicator of reporter protein distribution.

We next determined the dependence of giantin and GalNAcT2 redistribution on the concentration of microinjected pSARA^{dn} plasmid. We had postulated that the plasmid-driven expression of Sar1p^{dn} should be a more effective tool in producing relocation of Golgi region proteins. As shown in Fig. 1, D–F, this proved to be the case, and at high pSARA^{dn} concentrations both GalNAcT2 and giantin redistributed to a similar extent. By fluorescence, the distribution of GalNAcT2 and giantin were similar following a 6-h incubation of cells microinjected with a high concentration of pSARA^{dn} plasmid. Both appeared to have a distribution suggestive of accumulation in the ER. To provide further evidence of ER accumulation, giantin was colocalized relative to GalNAcT2-GFP by confocal fluorescence microscopy. GalNAcT2-GFP accumulates in the ER in correspondence with protein disulfide isomerase in response to an ER exit block (Storrie et al., 2000). As shown by confocal fluorescence microscopy in Fig. 2, giantin in pSARA^{dn}-microinjected cells appeared to rim the cell nucleus in a somewhat discontinuous pattern and to be present in general correspondence to GalNAcT2-GFP at both the nuclear rim and in the cytoplasm. There was extensive overlap between the two in computer overlays. As further evidence and as a biochemical assay of ER accumulation of the reference protein, GalNAcT2, a Golgi apparatus–specific O-glycosylation enzyme, the in situ glycosylation state of an artificial substrate, CD8, fused with the ER retention domain of glucuronyltransferase was characterized. This chimeric protein localizes to the ER and is a potential substrate for in situ O-glycosylation by redistributed Golgi region enzymes. When Sar1p^{dn}-microinjected CD8 glucuronyltransferase cells were reacted with rhodamine-conjugated Helix pomatia lectin, a lectin specific for O-linked terminal α-GalNAc, considerable ER-specific staining was observed 6 h after pSARA^{dn} microinjection (Fig. 3, compare microinjected cells [asterisks] with noninjected cells [arrowheads]), a result indicative of in situ ER activity of Golgi apparatus–specific O-glycosylation enzymes. Note that Helix pomatia–specific staining was restricted to a juxtanuclear, Golgi apparatus–like distribution in the control, non-pSARA^{dn}–injected cells.

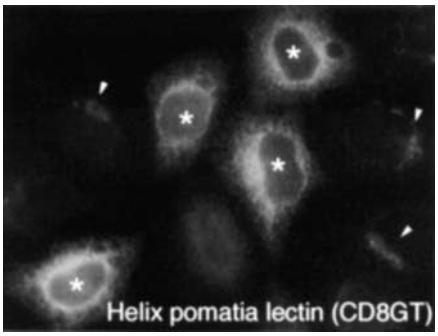

Figure 3. **Golgi apparatus–specific O-glycosylation occurs within the ER of Sar1p^{dn}-microinjected cells.** Cells (asterisks) were microinjected with Sar1p^{dn} and at the end of 6 h, fixed and stained with rhodamine-conjugated Helix pomatia lectin. The target cell population stably expresses an artificial ER-localized O-glycosylation substrate, CD8, fused with the ER retention domain of glucuronyltransferase. Helix pomatia lectin is a lectin specific for O-linked terminal α-GalNAc. In the microinjected cells, distinct staining of the nuclear envelope and into the cytoplasm was apparent. In the noninjected cells (arrowheads), a distinct and fainter concentration of staining was apparent in the juxtanuclear Golgi complex–like region.

Golgi glycosyltransferases and putative Golgi matrix proteins are similar in the kinetics of Sar1p^{dn}-induced relocation

Next, we examined the kinetics of relocation of a series of Golgi matrix–localized proteins, including putative matrix proteins and glycosyltransferases, in response to a high concentration of pSARA^{dn}-induced ER exit block. The first example protein was p27, a protein known to shuttle between the cis Golgi apparatus and the ER (Füllekrug et al., 1999). In the pSARA^{dn}-injected cells (Fig. 4 A, asterisks), the distribution of p27 was much more disperse even after only 2 h of plasmid expression. Little to no juxtanuclear concentration of p27 was apparent. Instead, low intensity, granular cyto-

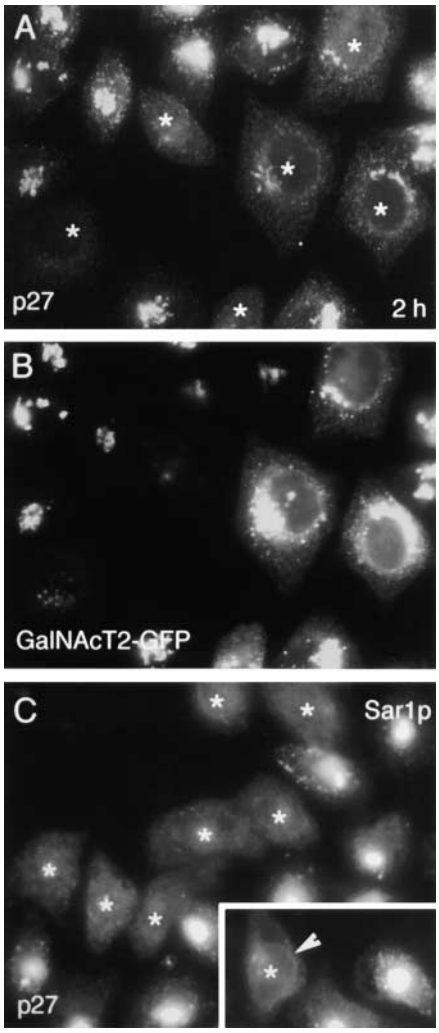

Figure 4. **Rapid redistribution of p27 in cells expressing pSARA^{dn}.** HeLa cells stably expressing GalNAcT2-GFP were microinjected with pSARA^{dn} (A and B). At the end of a 2 h pSARA^{dn} expression period, cells were fixed and stained for p27 using a Cy3-conjugated second antibody. p27 (A) appeared to lose juxtanuclear Golgi apparatus–like localization faster than GalNAcT2 (B) in the same cells. Wild-type HeLa cells were microinjected with Sar1p^{dn} in the presence of CHX and incubated for 6 h postinjection (C). In these wild-type cells, p27 had completely lost juxtanuclear Golgi apparatus–like localization, and in favorable cells p27 staining appeared to localize to the nuclear envelope, e.g., arrowhead, inset. Asterisks indicate microinjected cells.

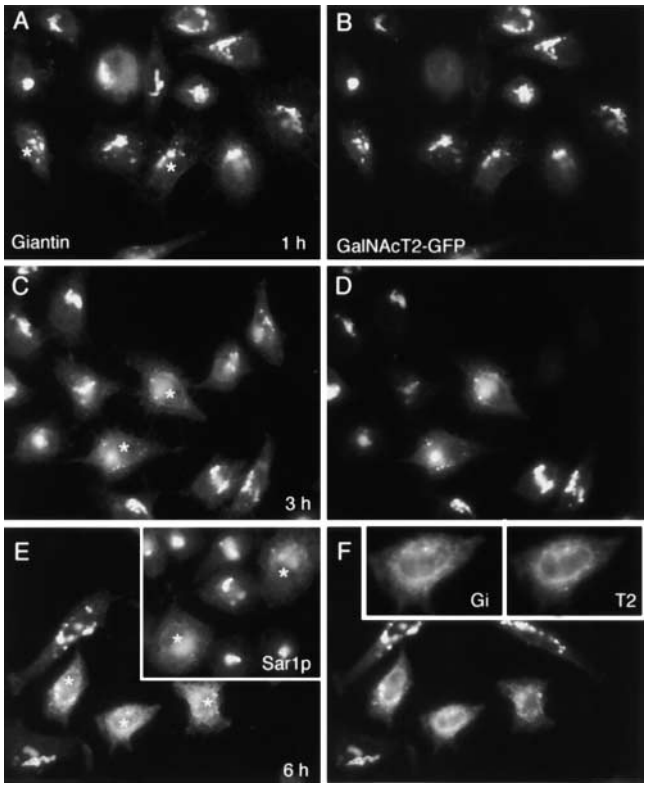

Figure 5. **Similar redistribution kinetics for giantin and GalNAcT2-GFP in cells expressing pSARA^{dn}.** HeLa cells stably transfected with GalNAcT2-GFP were microinjected with pSARA^{dn} in the absence of CHX. After 1 h (A and B), 2 h (C and D), or 6 h (E and F) expression period, cells were fixed and stained for giantin using a Cy3-conjugated second antibody. Giantin appeared to lose juxtanuclear Golgi apparatus–like localization with the same kinetics as GalNAcT2. As shown in 3.7× higher magnification inset to F, redistributed giantin and GalNAcT2-GFP similarly rimmed the nuclear envelope. As shown in the inset to E, giantin similarly redistributed in wild-type HeLa cells microinjected with Sar1p^{dn} in the presence of CHX. Asterisks indicate microinjected cells.

plasmic staining was observed. If anything, the redistribution of the cis Golgi matrix protein p27 was faster than that of GalNAcT2-GFP in the same cells. As shown in Fig. 4 B, considerable juxtanuclear GalNAcT2 remained after 2 h of plasmid expression. However, cytoplasmic GalNAcT2 fluorescence did increase considerably in the injected cells and ER-like nuclear rim staining was apparent at higher magnifications. Cytoplasmic accumulation was due predominantly to redistribution of components from juxtanuclear Golgi apparatus. As shown explicitly in Fig. 4 C, p27 lost its juxtanuclear distribution in wild-type HeLa cells microinjected with 1.7 mg/ml Sar1p^{dn} and maintained in the presence of CHX to inhibit protein synthesis. With favorable focus, p27 fluorescence appeared to rim the nucleus (Fig. 4 C, inset, arrowhead). Nuclear rim staining is characteristic of an ER accumulation. This and similar experiments are also important as they demonstrate redistribution in the absence of any Golgi region protein overexpression, i.e., relocation is not a consequence of spillover of overexpressed protein into an abnormal pathway. To investigate the kinetic effect of an ER exit block on a putative Golgi matrix–interacting protein localized toward the cis Golgi complex, the pSARA^{dn} plasmid-induced redistribution of giantin was compared with that of GalNAcT2-GFP. As shown in Fig. 5, A–F, the loss of compact, juxtanuclear distribution for giantin in pSARA^{dn}-expressing cells appeared similar to the slow redistribution kinetics of GalNAcT2-GFP. A similar loss of juxtanuclear giantin concentration was seen also in wild-type HeLa cells microinjected with 1.7 mg/ml Sar1p^{dn} protein and maintained in the presence of CHX (Fig. 5 E, inset). As shown in Fig. 5 F, inset, at 3.7-fold higher magnification, both giantin and GalNAcT2 at 6 h pSARA^{dn} expression showed similar rim staining of the nucleus, a characteristic trait of ER accumulation.

To further assess whether there were kinetic differences between Golgi region cisternal elements in response to an

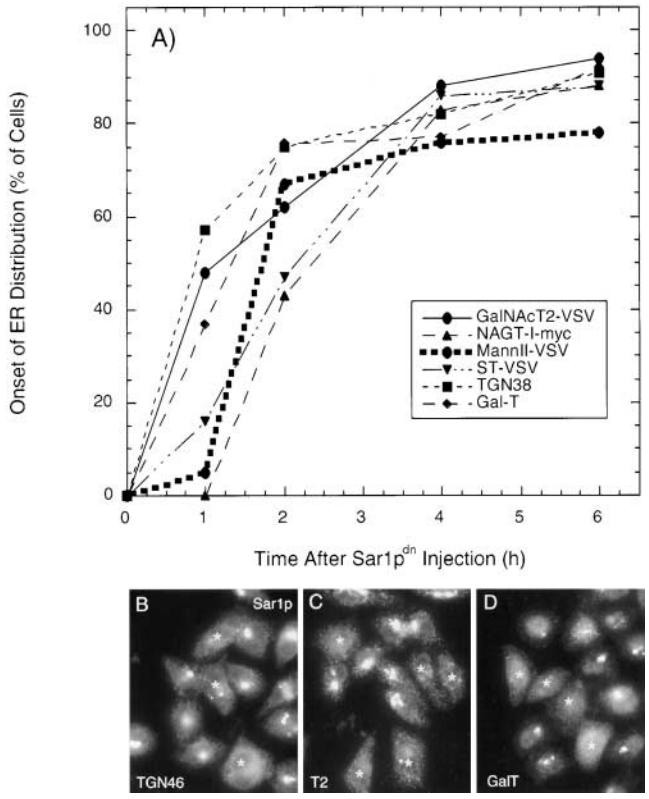

Figure 6. **Quantification and demonstration of the appearance of a series of Golgi apparatus cisternal and TGN proteins in the ER.** In A, HeLa cells stably overexpressing a series of cisternal-to-TGN proteins were microinjected with Sar1p^{dn} in the presence of CHX and appearance in the ER quantified as described previously (Girod et al., 1999). In B–D, TGN46 (B), GalNAcT2 (C), and GalT (D) in wild-type HeLa cells microinjected with 1.7 mg/ml Sar1p^{dn} in the presence of CHX were localized 6 h post-injection. Asterisks indicate microinjected cells.

ER exit block, the distributions of six different integral membrane proteins of medial Golgi complex to TGN were quantified in stably transfected HeLa cells microinjected with 1.7 mg/ml Sar1p^{dn} in the presence of CHX. These cells overexpress Golgi region proteins and therefore provide a strong signal. Cells were scored semiquantitatively for onset of ER distribution of marker protein (onset of nuclear rim staining and web-like cytoplasmic staining) and predominantly juxtanuclear (i.e., Golgi complex) distribution. As shown in Fig. 6, the two medial proteins, NAGT-I-myc and MannII-VSV, were slower in initial ER appearance and loss of juxtanuclear Golgi complex concentration than the trans-to-TGN protein, GalT. However, a second trans-to-TGN protein, ST-VSV, was also slow in initial ER appearance. The redistribution of GalNAcT2-VSV, a protein that is initially distributed across the entire Golgi stack, and TGN38, a classic marker of the TGN, appeared relatively fast. The differences, although small, support at least in part the outcome of experiments on the kinetics of Golgi apparatus redistribution in response to microtubule depolymerization (Yang and Storrie, 1998), and also provide partial explanations for the previous data. To test whether the cisternal Golgi apparatus proteins redistributed in wild-type cells, the distribution of endogenous TGN46, GalNAcT2, and GalT was determined in nontransfected HeLa cells microinjected with 1.7 mg/ml Sar1p^{dn} in the presence of CHX. A similar loss of juxtanuclear Golgi complex staining was observed in all cases 6 h postinjection (Fig. 6, B–D). However, little, if any, distinct fluorescence signal could be detected about the nuclear envelope as definitive evidence of ER localization. We attribute this to insufficient signal to detect wild-type levels of Golgi apparatus proteins upon their dilution into the ER.

Golgi glycosyltransferases cycle in the absence of an ER exit block

The above experiments indicate eight different cis-to-TGN integral Golgi apparatus membrane proteins plus GM130 and giantin cycle in response to an ER exit block. To provide direct evidence that such recycling is not an artifact of the ER exit block, the equilibration of GalNAcT2-GFP between two pools, the Golgi apparatus and ER, was characterized by a photobleaching approach. As shown in Fig. 7 A, inset, GalNAcT2-GFP fluorescence in cells stably expressing high levels of the protein can be detected at high intensities in association with the juxtanuclear Golgi apparatus and at low intensities over the cytoplasm in association with the ER. The ER fluorescence was not due to newly synthesized GalNAcT2-GFP as it did not chase out of the ER with incubation of the cells in the presence of CHX (unpublished data; no ER exit block). To test whether the two GalNAcT2-GFP pools were in equilibrium with one another, GalNAcT2-GFP fluorescence over the cytoplasm was photobleached in the presence of CHX and the same cell was then imaged at 10 min intervals in the presence of drug. As shown qualitatively in Fig. 7 A, inset, complete photorecovery was seen. Quantitatively, recovery had a half time of ~15 min, indicating that indeed GalNAcT2-GFP normally is transported in a fairly rapid manner between the Golgi apparatus and ER (Fig. 7 A). This half time was fourfold faster than that for the loss of GalNAcT2-GFP–marked Golgi matrix mass (average Golgi matrix pixel number per cells) in response to an ER exit block ($t_{1/2}$ = 1 h; Fig. 7 B). The kinetics for FRAP are similar to those reported by Zaal et al. (1999). As an additional control that GalNAcT2-GFP had accumulated in the ER, a continuous structure, in response to the ER exit block, HeLa cells following a 6 h pSARA^{dn} expression period were photobleached over approximately one-half the cell area and imaged at 1 min intervals. In these cells, the Golgi apparatus had redistributed, presumably to the ER. Extensive photorecovery was seen over a 4 min time period over the bleached area (unpublished data), the expected result for an interconnected ER network.

GM130 relocates with similar kinetics to GalNAcT2

To test if an ER exit block results in the relocation of other cis Golgi matrix proteins with similar kinetics to that of a cisternal Golgi matrix enzyme, HeLa cells were microinjected with pSARA^{dn} and the distribution of endogenous putative matrix or coat proteins observed relative to GalNAcT2-GFP. As shown in Figs. 8–10, the concentrated juxtanuclear distribution of each of the three peripheral proteins decreased decidedly in a time-dependent manner. COPI coat protein relocated rapidly and close to complete loss of juxtanuclear fluorescence was observed after 1h (Fig.

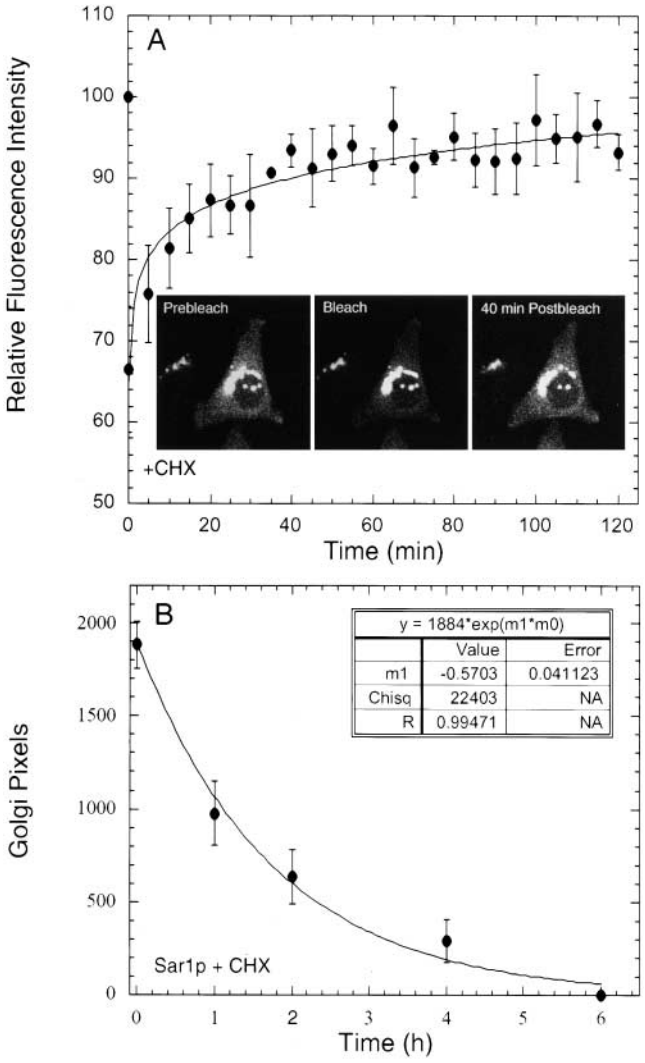

Figure 7. **Kinetics of GalNAcT2-GFP transfer between the Golgi apparatus and ER from photobleaching (A) or quantification of Golgi pixel mass (B).** In A, fluorescence intensity over the ER region of individual cells was quantified before and after a photobleach in the presence of the protein synthesis inhibitor CHX. In this experiment, there is no ER exit block. The irregular line function of the ZEISS LSM510 microscope software was used to encircle the ER and the ER was then photobleached. Inset, qualitative evidence that the bleach is selective for the ER. The data are for three cells and the average intensity and standard deviation are shown. About 35% of the ER-localized GFP was photobleached in each case. A partial photobleach rather than complete was chosen to minimize possible photodamage. Quantitatively, recovery of ER fluorescence was best fit by a single exponential function and had a half-time of 15 min. To image the less intense ER contribution, the Golgi apparatus was imaged to oversaturation. In B, loss of fluorescence from the Golgi apparatus following Sar1p^{dn} microinjection in the presence of CHX was analyzed based on mass action kinetics. Golgi area was scored by using the irregular line tool of the NIH Image program to enclose the juxtanuclear concentrated GalNAcT2-GFP pixels in ~10 cells/time point. The total pixel number within the enclosed area was then quantified for 10 cells. Pixel number was used rather than summed fluorescence intensity because the Golgi apparatus had been imaged to oversaturation in the micrograph set in order to image the less intense ER contribution (see Fig. 6). The decrease in Golgi pixels with fluorescent signal was described well by a first-order rate constant (KGolgi-ER = $-0.5703\ h^{-1}$; R = 0.99). The half-time for depletion is 1 h. Fluorescence appearance in the ER is described well by a first-order process with no depletion term, indicating that ER exit in the presence of Sar1p is not significant (unpublished data). The error bars in B indicate standard error of the mean.

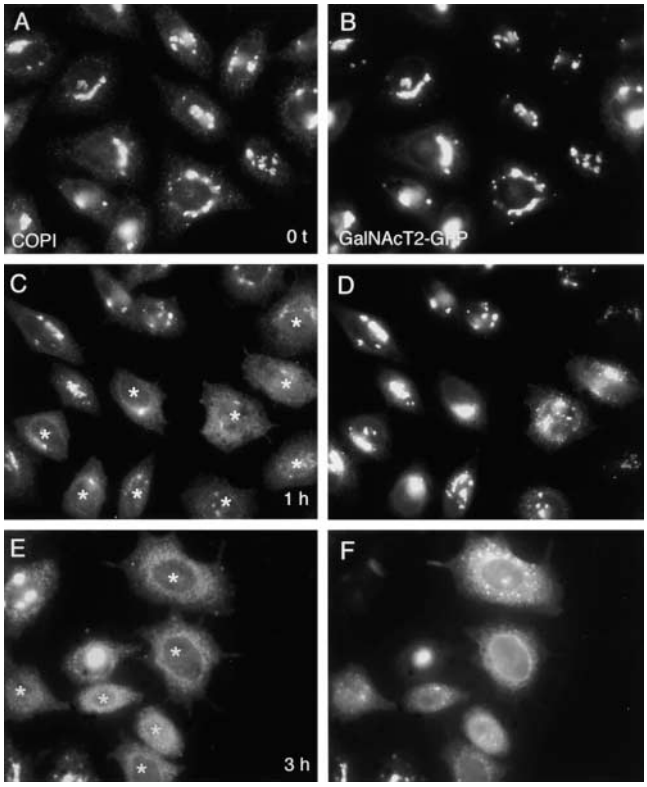

Figure 8. **COPI loses its juxtanuclear Golgi region localization faster than GalNAcT2-GFP in pSARA^{dn}-expressing cells.** GalNAcT2-GFP HeLa cells were microinjected with pSARA^{dn} (asterisks) and fixed at 0 t and 1 and 3 h. Cells were stained for COPI using a Cy3 second antibody.

8). Much of this relocation may be to the cytosol. COPI exists as a membrane-associated coat and as soluble coatomer. p115 lost juxtanuclear localization at least as fast as GalNAcT2-GFP (Fig. 9). GM130 relocated with roughly the same kinetics as GalNAcT2-GFP (Fig. 10). To better characterize the site of relocation of these peripheral proteins in HeLa cells, the distribution of GM130 was determined in GalNAcT2-VSV–tagged HeLa cells (Röttger et al., 1998) microinjected with 150 ng/μl stock concentration pSARA^{dn}, incubated for 6 h, and then fixed with methanol. Methanol fixation better preserves microtubules than formaldehyde fixation and as such should give a better preservation of the ER. The distribution of GM130 in the injected cells (Fig. 11 A, asterisks) was predominantly diffuse with some local concentrations. The diffuse labeling may well be cytosolic. GalNAcT2 in the same cells displayed a distinctly ER-like distribution (Fig. 11 B). We conclude that putative Golgi matrix proteins including GM130 relocate, likely in part to the cytosol, with roughly similar kinetics to GalNAcT2 in response to a strong ER exit block.

As indicated by the normal distribution of Golgi region proteins in non-Sar1p^{dn}–microinjected cells in the presence of CHX, the effect of an ER exit block upon Golgi matrix protein distribution must be a consequence of a block of protein cycling rather than the failure to replenish a short-lived protein(s). This was further supported by the observation that the distribution of GalNAcT2-VSV was normal in Sar1p^{dn}-microinjected cells in the presence of 5 μg/ml eme-

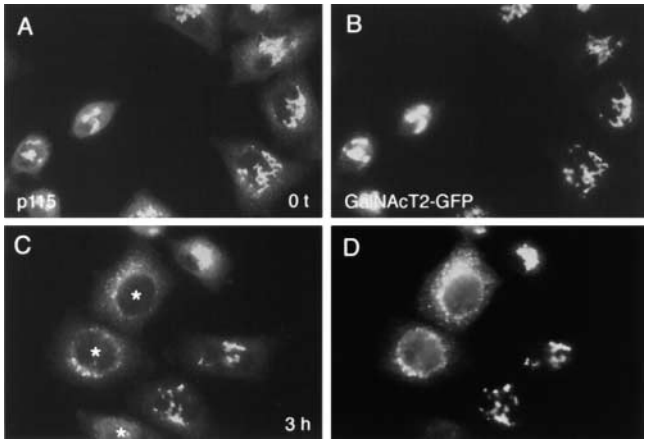

Figure 9. **p115 loses its juxtanuclear Golgi region localization in a similar manner to GalNAcT2-GFP in pSARA^{dn}-expressing cells.** GalNAcT2-GFP HeLa cells were microinjected with pSARA^{dn} (asterisks) and fixed at 0 t and 1, 3, and 6 h. Cells were stained for p115 using a Cy3 second antibody. Only the 0 t and 3 h time points are shown.

tine, a concentration sufficient to inhibit >99% of cytoplasmic protein synthesis in HeLa cells (Perlman and Penman, 1970; unpublished data).

Sar1p^{dn} inhibits the juxtanuclear accumulation of both Golgi glycosyltransferases and putative Golgi matrix proteins in BFA washout experiments

Others have provided evidence that after BFA treatment a Golgi matrix consisting in part of giantin and GM130 provides a subsequent nucleus for Golgi apparatus reassembly in a BFA washout experiment (Seemann et al., 2000). We have tested this possibility in HeLa cells microinjected with both moderately high (0.72 mg/ml) and high (2.85 mg/ml) stock concentrations of Sar1p^{dn}. A 30-min exposure of HeLa cells to BFA produced three different distributions for Golgi region proteins. GalNAcT2 was clearly redistributed to the ER (Fig. 12 A). Giantin exhibited an ER-associated distribution, granular, but in clear correspondence to nuclear envelope and web-like distribution of GalNAcT2 in the cytoplasm (Fig. 12 B). GM130 and p27 were distinctly different in distribution and exhibited a scattered, granular distribution (Fig. 12 C, GM130 shown). To test whether these scattered granular structures were nuclei for Golgi apparatus assembly or BFA-arrested intermediates in protein cycling, we probed the effect of Sar1p^{dn} on Golgi apparatus reassembly following BFA washout. As shown in Fig. 12 H, little GalNAcT2, p27, or GM130 accumulated juxtanuclearly during a BFA washout in cells microinjected with 2.85 mg/ml Sar1p^{dn}. Rather GalNAcT2 stayed predominantly ER in distribution with some accumulation into scattered punctate structures (Fig. 12 D). We attribute these to possible arrest at ER exit sites. Endogenous p27 was distributed similarly (Fig. 12 G) and GM130 on the whole exhibited a similar distribution to GalNAcT2. However, in the case of endogenous GM130, no distinct ER web–like or nuclear rim staining could be detected. This may reflect antibody strengths or expression levels. In some cells, both GalNAcT2 and

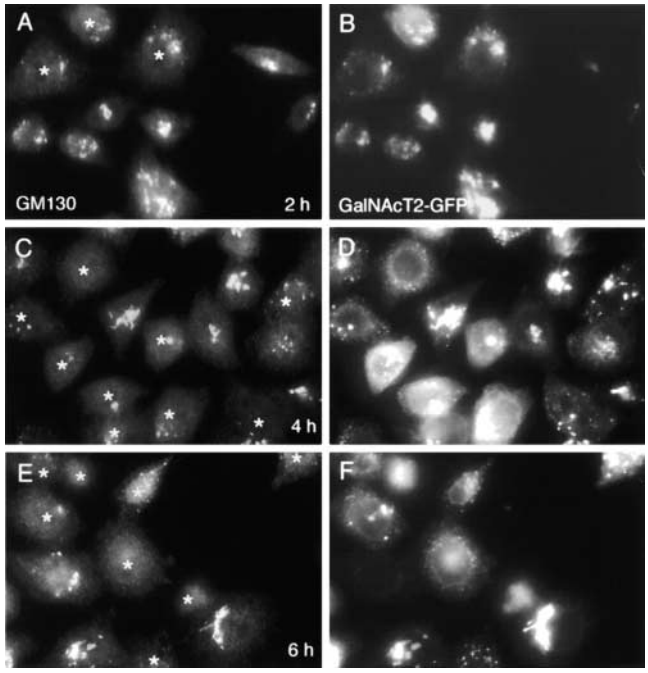

Figure 10. **GM130 loses its juxtanuclear Golgi region localization in a similar manner to GalNAcT2-GFP in pSARA^{dn}-expressing cells.** GalNAcT2-GFP HeLa cells were microinjected with pSARA^{dn} (asterisks) and fixed at 2, 4, and 6 h. Cells were stained for GM130 using a Cy3 second antibody.

GM130 could be seen, especially at low Sar1p^{dn} concentrations, to exhibit a juxtanuclear concentration (Fig. 12, D and F, insets). Giantin showed less tendency to accumulate juxtanuclearly than GM130 (Fig. 12, E and H). We conclude at high Sar1p^{dn} levels that both Golgi complex glycosyltransferases and putative matrix components are sensitive to an ER exit block.

Discussion

We assessed the response of 12 different Golgi matrix proteins to an ER exit block (Table I) as a test of the hypothesis

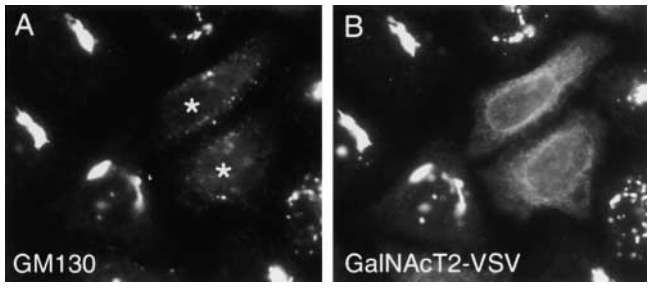

Figure 11. **GM130 has a mixed diffuse and granular distribution in HeLa cells microinjected with pSARA^{dn} and fixed with methanol.** HeLa cells stably expressing GalNAcT2-VSV were microinjected with 150 ng/μl stock concentration pSARA^{dn}, incubated for 6 h, and fixed with −20°C methanol to give better preservation of microtubules than with formaldehyde fixation. Microinjected cells were identified by the ER distribution of GalNAcT2 after antibody staining. GM130 has a diffuse to granular distribution in the microinjected cells. A, GM130; B, GalNAcT2. Asterisks indicate microinjected cells.

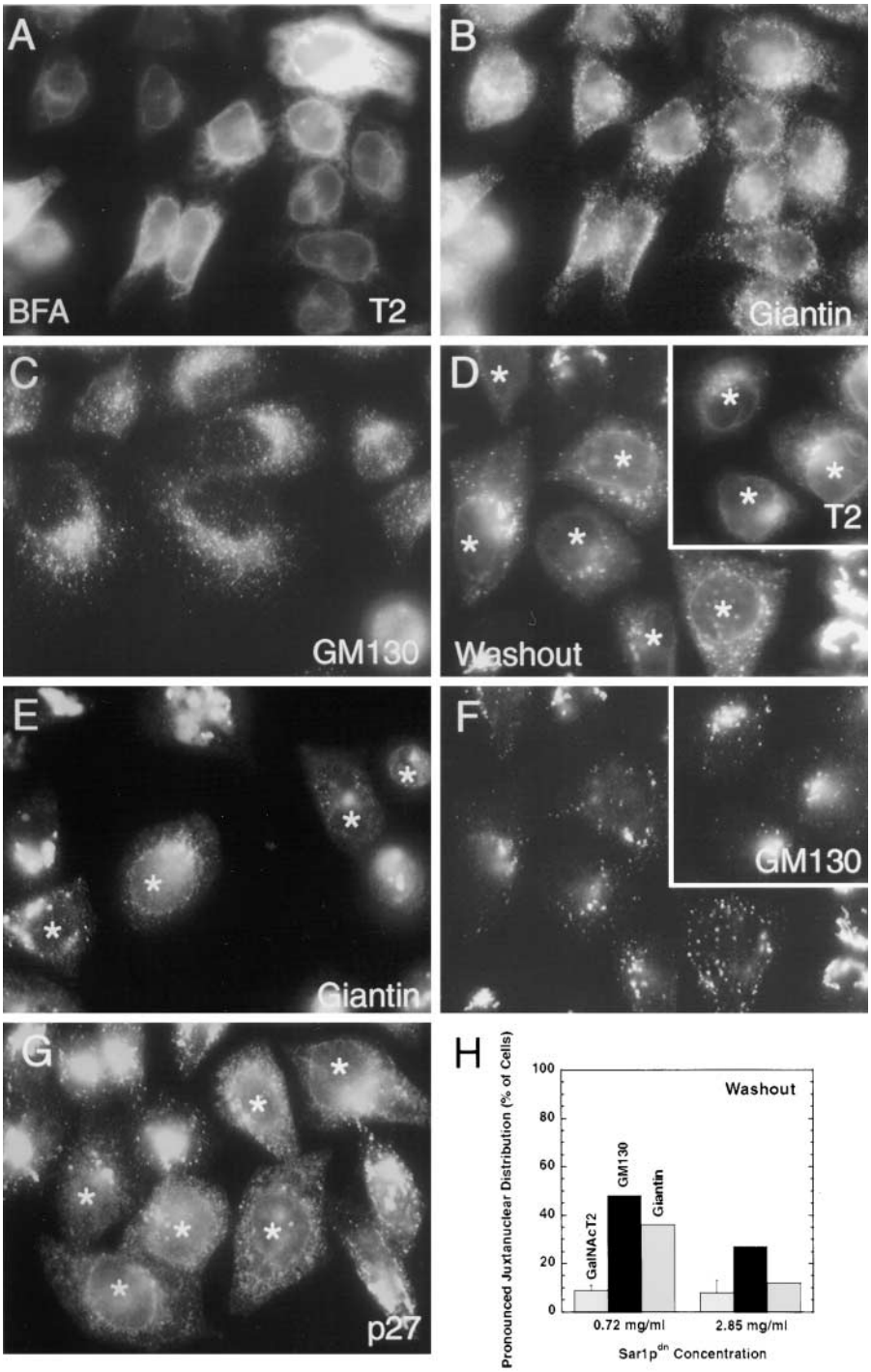

Figure 12. **Sar1p^{dn} blocks the juxtanuclear accumulation of Golgi region proteins in a BFA washout protocol.** HeLa cells stably expressing GalNAcT2-GFP were exposed to BFA for 30 min in the presence of CHX, fixed, and then stained. A, GalNAcT2-GFP; B, giantin in same cells, double labeling; C, GM130. HeLa cells stably expressing GalNAcT2-GFP were microinjected with either 0.72 or 2.85 mg/ml Sar1p^{dn}, incubated with BFA for 30 min in the presence of CHX, and then incubated for an additional 2 h in the presence of CHX after BFA washout. D, GalNAcT2-GFP, 0.72 mg/ml Sar1p^{dn}; E, giantin, 2.85 mg/ml Sar1p^{dn}; F, GM130, double labeling of same cells shown in D; G, p27, 2.85 mg/ml Sar1p^{dn}; H, quantification of fluorescence distributions after BFA washout, scoring as described previously by Girod et al. (1999). Asterisks indicate microinjected cells.

that the entire Golgi apparatus cycles in interphase mammalian cells. The proteins chosen spanned the entire range of cis-to-TGN and included examples of both putative Golgi matrix proteins and Golgi matrix glycosyltransferases. In general, distributions were scored in HeLa cells stably overexpressing the protein of interest; this provided a stronger signal upon dilution of the protein into the cytoplasm/ER. Most protein distributions were assessed also in wild-type HeLa cells. Protein exit from the ER was blocked either by pSARA^{dn}-encoded expression of the Sar1p^{dn} protein or by direct microinjection of the Sar1p^{dn} protein in the presence of a protein synthesis inhibitor. Three key observations emerged from these experiments. First, both putative Golgi matrix proteins and Golgi glycosyltransferases lost their juxtanuclear localization in response to an ER exit block, albeit with differential dependence on Sar1p^{dn} levels. The redistribution of GalNAcT2 was more sensitive to low Sar1p^{dn} concentrations than that of giantin and GM130. Second, the relocation of putative Golgi matrix proteins was at least as rapid, if not more so, than that of Golgi glycosyltransferases, no matter if cis or trans. In the case of "matrix," some integral membrane proteins such as giantin redistributed in a pattern very suggestive of the ER and others, peripheral membrane proteins such as GM130, may relocate at least in

part to the cytosol. For the coat protein, COPI, this was apparently the case. Third, Golgi apparatus reassembly following BFA washout was sensitive to an ER exit block irrespective of whether the distribution of a putative Golgi matrix protein or a Golgi glycosyltransferase was assessed. Distinct ER accumulation could be demonstrated for all integral Golgi region membrane proteins, even giantin. ER-accumulated Golgi region proteins were functional. Moreover, photobleaching experiments indicated that Golgi-to-ER protein cycling occurred in the absence of an ER exit block. In sum, these observations suggest that the entire Golgi apparatus is a dynamic structure.

We deliberately choose to emphasize fluorescence assays to assess phenotype because they permitted quantitative surveys across target cell populations. In all cases, the pSARA^{dn} plasmid or Sar1p^{dn} protein was introduced into the target cell by microinjection. Through the use of a fluorescent coinjection marker, we could score whether a cell was microinjected independent of phenotype. This permitted an objective assessment that pSARA^{dn} was more effective in redistributing Golgi region proteins than microinjected Sar1p^{dn}. Moreover, coinjection marker brightness provided an indication of how much Sar1p^{dn} had been transferred into the individual cell. This was particularly important as a semiquantitative predictor of Sar1p^{dn} effect on the distribution of putative matrix proteins. Electron microscope–based assays (e.g., Seemann et al., 2000) are probably best applied to pSARA^{dn}-microinjected cells where there is little variability in phenotype from cell to cell with respect to the distribution of either a putative matrix protein or a Golgi glycosyltransferase. Our results are an extension of and consistent with previous results (Storrie et al., 1998; Girod et al., 1999; Zaal et al., 1999; Seemann et al., 2000; Todorow et al., 2000) that individual Golgi glycosyltransferases cycle to the ER. Clearly, as indicated by the greater granularity of redistributed giantin than GalNAcT2, our data are insufficient to prove that each protein is fully intermixed with other Golgi proteins within the same element of ER. In our opinion, this may well reflect the technical limitations of immunofluorescence; in our hands, two antibodies to the same ER protein fail to give identical distributions in fixed cells (Storrie et al., 1994). We note that even GalNAcT2 in the ER can show concentrations of fluorescence (see Fig. 1 B; Girod et al., 1999). As Sar1p redistributed GalNAcT2-GFP has a uniform ER distribution in living cells (unpublished data), we conclude that preservation of structure during fixation and antibody staining is a persistent problem.

Unlike Seemann et al. (2000), we find that putative Golgi matrix components such as giantin, GM130, and p115 are sensitive to a GTP-restricted ER exit block, albeit at a high Sar1p^{dn} level. This is an important result and was observed both for the disassembly of a juxtanuclear Golgi apparatus and its reassembly following dispersal of many Golgi matrix proteins to the ER through BFA treatment. Sensitivity was essentially complete at high Sar1p^{dn} concentrations with the pSARA^{dn} plasmid being the most effective vehicle for the introduction of Sar1p^{dn} and producing subsequent matrix protein redistribution. The kinetics of redistribution of GM130, a peripheral membrane protein, at these pSARA^{dn} concentrations was virtually identical to that of GalNAcT2. GM130 redistribution was not to an obvious ER-like pattern. Much of the distribution was diffuse, presumably cytosolic, and granular concentrations were observed. Granularity in the distribution of giantin, an integral membrane protein, appeared to overlap with ER. In agreement with Seemann et al. (2000), we found redistribution of Golgi glycosyltransferases to the ER was more sensitive to Sar1p^{dn} than putative matrix proteins. Seemann et al. (2000) present no concentration dependence data and no quantification of phenotype. We find that at the same GTP-restricted Sar1p^{dn} protein concentration used by Seemann et al. (2000) that both giantin and GM130 had redistributed in ~50% of the microinjected cells. The images shown by Seemann et al. (2000) are of single microinjected cells. Like Seemann et al. (2000), we find that BFA disperses Golgi glycosyltransferases into the ER more effectively than putative Golgi matrix components. We found that in BFA-treated cells that giantin was dispersed into a somewhat discontinuous ER-associated pattern and GM130 distributed into a granular pattern much like that reported by Seemann et al. (2000). Unlike Seemann et al. (2000), we found during Golgi apparatus reassembly in BFA washout experiments, particularly at high Sar1p^{dn} concentrations (2.85 mg/ml), that giantin, GM130, and p27 distributed with GalNAcT2. The GM130 fluorescence signal was insufficient to say where dilute GM130 might be located. Only concentrated GM130 could

Table I. **Response of Golgi apparatus–associated proteins to an ER exit block**

Protein	Associated with putative matrix	Initial localization	End localization[a]	Kinetics of redistribution vs. GalNAcT2
p27	No	Cis	ER	Faster
giantin	Yes	Cis	ER	Similar
NAGT-I	No	Medial/trans	ER	Slower
GalNAcT2	No	Cis-trans	ER	Not applicable
Mann II	No	Medial/trans	ER	Slower
SialylT	No	Trans/TGN	ER	Slower
GalT	No	Trans/TGN	ER	Similar
TGN38	No	TGN	ER	Similar
TGN46	No	TGN	ER	Similar
COPI	No	Cis concentrated	Cytoplasm	Faster
p115	Yes	Cis concentrated	Cytoplasm	Similar
GM130	Yes	Cis concentrated	Cytoplasm	Similar

[a]In the case of endogenous proteins, immunofluorescence staining can be readily assigned to the Golgi apparatus where the proteins are concentrated. However, in Sar1p^{dn} ER exit–blocked cells, the proteins are diluted as they redistribute and their end location within the cytoplasm may be difficult to assign.

be visualized and these concentrations corresponded to concentrations of GalNAcT2. GalNcT2, giantin and p27 were found in predominantly ER-like distributions during BFA washout in the presence of Sar1p^{dn}. This is particularly interesting as p27 with BFA treatment displayed a granular distribution similar to GM130. These results suggest that in BFA-treated cells p27 was cycling between a pre-Golgi apparatus intermediate structure and the ER. This granular BFA intermediate also could be an ER subdomain such as ER exit sites. With BFA washout in the presence of Sar1p^{dn}, the cycling protein was now blocked in a continuous ER distribution. In other words, the putative pre-Golgi structure is not a stable intermediate.

Currently, two contrasting hypotheses of Golgi apparatus assembly in mammalian cells contend with one another. The one hypothesis, de novo Golgi apparatus assembly from the ER, has been advanced on the basis of experiments in which Golgi apparatus assembly is seen at scattered peripheral sites in nocodazole-treated cells in which the microtubule network is depolymerized (e.g., Cole et al., 1996; Yang and Storrie, 1998; Storrie et al., 1998) or concentrated GFP chimeric proteins are used to study Golgi apparatus distribution in mitotic cells (Zaal et al., 1999). The other hypothesis, nucleated assembly, postulates that a stable, vesicular-associated Golgi matrix nucleates the downstream assembly of the glycosyltransferase rich Golgi stack. This has been advanced on the basis of the experiments of Seemann et al. (2000) reporting that a Golgi matrix is stable to an ER exit block. Our experiments certainly cast doubt on this contention. The other experimental basis has been microsurgery experiments in which cytoplasm is separated from juxtanuclear Golgi complex and Golgi apparatus reassembly in the separated cytoplasm is observed only when at least a few percent of proteins, such as giantin, have been predispersed into the cytoplasm before the microsurgery (Pelletier et al., 2000). These experiments are important. They certainly indicate that little in the way of Golgi region components are found in the normal interphase ER. This conclusion is also supported by the morphometric distribution of GalNAcT2-VSV in HeLa cells (Röttger et al., 1998; Storrie et al., 1998). In our opinion, it is unlikely that there is a preformed Golgi "matrix" at each site of Golgi apparatus assembly in the nocodazole-treated cell. However, at present in the case of the nocodazole-treated cell system, there are no data regarding the order of "matrix" component assembly at the peripheral sites versus Golgi glycosyltransferase assembly into a "new" Golgi stack. Such data are needed. In our opinion, Golgi apparatus assembly may well be an ordered process. Conceivably, the difference between these two hypotheses, de novo versus nucleated assembly, may be kinetic in the sense that the assembly of a cis Golgi region structure containing giantin and GM130, for example, may well precede the assembly of a glycosyltransferase-rich Golgi stack in all cases. If this is true, then the question becomes what is the minimal initial physical state, free or membrane associated, of GM130, for example, as Golgi apparatus assembly begins. Additional experimentation will be necessary to define the dynamics of GM130 and other "matrix" proteins in vivo in the absence of any ER exit block and how they complex. The development of a reversible Sar1p^{dn} block would greatly facilitate experiments to address Golgi apparatus assembly questions in vivo.

An individual transport step, the transport of Golgi glycosyltransferases from the Golgi apparatus to the ER, may be faster than we previously thought on the basis of ER exit block experiments alone (Storrie et al., 1998; Girod et al., 1999). Consistent with previous data (Storrie et al., 1998; Girod et al., 1999; Zaal et al., 1999; Seemann et al., 2000), we found that the loss of Golgi complex mass (juxtanuclear GalNAcT2–positive pixels) in response to an ER exit block was relatively slow, with a half-time of ~1 h. To our surprise, we found that in photobleaching experiments in the absence of an ER exit block that the half-time for ER transport (photorecovery) for the same protein, GalNAcT2-GFP, was 0.25 h. Zaal et al. (1999) report similarly fast Golgi apparatus to ER transport times in photobleaching recovery experiments in the absence of an ER exit block. Although the Golgi mass assay and the photobleaching Golgi apparatus to ER fluorescence transfer assay do not measure the same parameter, the photobleaching results suggest that the rate of Golgi apparatus to ER transport step may well be more rapid than we had thought previously. Zaal et al. (1999) show through more extensive analysis a mean roundtrip Golgi glycosyltransferase cycling time of 84 min with a Golgi complex residence time of ~57 min. We do not address overall cycling times and Golgi complex residence times here. A mean cycling time of 84 min is consistent with our observed slow loss of Golgi apparatus mass, i.e., pixel number.

In conclusion, our data support the hypothesis that the entire Golgi apparatus is a dynamic structure. The experimental results cannot be explained on the basis of the metabolic instability of a Golgi apparatus component. The juxtanuclear Golgi apparatus appears stable to almost complete inhibition of protein synthesis for at least several hours. We conclude that it is likely the entire Golgi apparatus cycles in interphase mammalian cells with much of this cycling for integral membrane proteins being between the Golgi apparatus and ER. Peripheral Golgi apparatus–associated membrane proteins such as COPI and GM130 also relocate and are presumably in dynamic equilibrium with soluble cytoplasmic pools. Finally, we give caveat emptor caution to the reader that, however much the major conclusion of a dynamic equilibrium Golgi apparatus is clear from our data, the data are insufficient to give a full set of detailed conclusions regarding the localization of proteins under all conditions. We are at the limit of what fluorescence localization can reveal. Other approaches will be necessary to fully resolve the nature of various granular staining structures.

Materials and methods

Cell culture

Wild-type HeLa cells were grown in DME supplemented with 10% fetal bovine serum under standard tissue culture conditions. HeLa cells stably expressing tagged Golgi apparatus proteins were maintained in the presence of 0.45 mg/ml of G-418 sulfate. HeLa cells localizing CD8 to the ER through a fusion of the protein to the ER retention portion of glucuronyltransferase (clone 1:6:1) were a gift from Dr. Nilsson (European Molecular Biology Laboratory, Heidelberg, Germany).

Microinjection of pSar1p^{dn} CMUIV (pSARA^{dn} [H79G] and Sar1p^{dn} [H79G])

Purified plasmids were microinjected into cell nuclei with minor modifica-

tions of previous procedures (Storrie et al., 1998). Typical plasmid concentrations were between 140 and 200 ng/μl, although in some experiments, plasmid concentrations as low as 4 ng/μl were used. Sar1p^{dn} protein at an injection concentration of 0.2–2.85 mg/ml was microinjected directly into the cytoplasm of cells incubated in the presence of 100 μg/ml CHX to inhibit protein synthesis during microinjection. Typical Sar1p^{dn} protein concentrations were 1.7–2.85 mg/ml. Protein-injected cells were subsequently maintained in the presence of CHX during postmicroinjection culture. Generally a coinjection marker (70 kD, aldehyde fixable, Cascade blue dextran [Molecular Probes]) at a stock concentration of 3.33 mg/ml was used as a coinjection marker.

BFA treatment and washout

HeLa cells were treated with BFA essentially as described by Seemann et al. (2000). To disperse Golgi region proteins, cells cultured on coverslips were incubated with 5 μg/ml BFA for 30 min at 37°C in the presence of CHX. To washout BFA, coverslips were transferred sequentially between a series of four tissue culture dishes containing 2 ml each of room temperature CO_2-independent microinjection media (GIBCO BRL) supplemented with CHX and then transferred to warmed dishes containing 37°C complete culture media supplemented with CHX. The subsequent incubation period for Golgi apparatus reformation was 2 h. To investigate the effects of microinjected Sar1p^{dn} on Golgi apparatus reformation, cells were microinjected with 0.72 or 2.85 mg/ml Sar1p^{dn} in the presence of CHX and then incubated with BFA as described above.

Antibodies

Affinity-purified rabbit polyclonal antibodies directed against the VSV-G epitope (CPYTDIEMNRLGK; Kreis, 1986) or p27 have been described previously (Röttger et al., 1998; Füllekrug et al., 1999). Rabbit polyclonal antibodies recognizing human GalT polypeptide have been described by Watzele et al. (1991). Rabbit polyclonal antibodies directed against GM130 were a gift from Dr. Francis Barr (Max Planck Institute, Martinsried, Germany). Sheep polyclonal antibodies directed against TGN46 were a gift from Dr. Vas Ponnambalam (University of Dundee, Dundee, UK). Rabbit polyclonal antibodies directed against full-length GFP were purchased from Molecular Probes. 9E10 mouse monoclonal antibody (mAb) directed against a myc peptide (Evan et al., 1985) was diluted from an ascites preparation for most purposes. CM1A10 mAb directed against β′-COPI and 4C4 mAb directed against UDP-N-acetylgalactosylamine:polypeptide N-acetylgalactosaminyltransferase-2 (GalNAcT2) have been described previously (Palmer et al., 1993; Röttger et al., 1998). mAb directed against giantin (Lindstedt and Hauri, 1993) and mAb directed against rat TGN38 were gifts from Dr. Hans-Peter Hauri (Biozentrum, University of Basel, Switzerland) and Dr. George Banting (Liverpool, UK), respectively. Clone 35 mAb directed against GM130 and clone 46 mAb directed against p115 were purchased from Transduction Laboratories. Cy3- or Cy2-conjugated donkey anti–rabbit, –mouse, or –sheep IgG antibodies were obtained from Jackson ImmunoResearch Laboratories.

Conventional and live cell confocal fluorescence microscopy

Microinjected cells were fixed standardly with formaldehyde and permeabilized with Triton X-100 as described previously (Storrie et al., 1998). In some experiments, cells were instead fixed with −20°C methanol for 4 min. With this fixation protocol, no further permeabilization treatment was necessary for antibody labeling. Optimal visualization of the accumulation of Golgi region proteins in the ER of microinjected cells frequently required overexposure of the fluorescence intensity present in juxtanuclear Golgi apparatus of noninjected cells.

For live cell confocal microscopy, cells were viewed with a ZEISS LSM 510 microscope. Cells were maintained on the microscope stage at 37°C in an FCS2 chamber (Bioptechs) or in a small aluminum slide chamber in complete DME medium that had been preequilibrated in a CO_2 incubator. The small chamber was heated by conduction through the immersion oil from a heated objective. This heating maintains the cells under immediate observation at 37°C. GFP was excited using the 488-nm line of the argon laser. In FRAP experiments, ZEISS software was used to bleach either a rectangular area or an irregular area corresponding to the ER by repeated scanning at high laser intensity. Cells were observed at 1–10 min intervals following the photobleach. Quantification was similar to that of Zaal et al. (1999).

Image processing and analysis of fluorescence intensity

All quantification of fluorescence intensity was done using gray scale images and the public domain software NIH Image v1.62 (developed at the National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image) as described previously (Storrie et al., 1998). Protein distributions were scored in at least 30 cells per concentration or time point as described previously (Girod et al., 1999).

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