Dynamic Nucleation of Golgi Apparatus Assembly from the Endoplasmic Reticulum in Interphase HeLa Cells

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Models of Golgi apparatus biogenesis and maintenance are focused on two possibilities: one is self-assembly from the endoplasmic reticulum, and the other is nucleation by a stable template. Here, we asked in three different experimental situations whether assembly of the Golgi apparatus might be dynamically nucleated. During microtubule depolymerization, the integral membrane protein p27 and the peripheral Golgi protein GM130, appeared in newly formed, scattered Golgi elements before three different Golgi apparatus cisternal enzymes, whereas GRASP65, a medial peripheral Golgi protein, showed, if anything, a tendency to accumulate in scattered Golgi elements later than a cisternal enzyme. During Golgi formation after brefeldin A washout, endoplasmic reticulum exit of Golgi resident enzymes could be completely separated from that of p27 and GM130. p27 and GM130 accumulation was onto newly organized perinuclear structures, not brefeldin A remnants, and preceded that of a cisternal enzyme. Reassembly was completely sensitive to guanosine 5'-diphosphate-restricted Sar1p. When cells were microinjected with Sar1pWT DNA to reverse a guanosine 5'-diphosphate-restricted Sar1p endoplasmic reticulum-exit block phenotype, GM130 and p27 collected perinuclearly with little to no exit of a cisternal enzyme from the endoplasmic reticulum. The overall data strongly indicate that the assembly of the Golgi apparatus can be nucleated dynamically by GM130/p27 associated structures. We define dynamic nucleation as the first step in a staged organelle assembly process in which new component association forms a microscopically visible structure onto which other components add later, e.g. Golgi cisternae.

Key words: brefeldin A, Golgi apparatus, nocodazole, organelle assembly, Sar1p

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The Golgi apparatus plays a crucial role in cells of higher organisms in the transport of secretory vesicles and membrane proteins (1). Proteins and lipids that originate in the endoplasmic reticulum (ER) undergo a series of post-translational modifications in the Golgi apparatus. Three-dimensional reconstructions from electron micrographs of the Golgi apparatus reveal a series of flattened cisternae surrounded by a number of spherical membrane vesicles (2,3). The Golgi cisternal membranes are arranged in a stacked order: cis-Golgi (entering face), medial Golgi and trans-Golgi (exit face). Despite its complicated morphology and function, the Golgi apparatus is dynamic, capable of rapid disassembly and reassembly during mitosis (4), drug treatment (5–8), or in response to mutational blocks (9–16). Glycoprotein and glycolipid processing enzymes and peripheral proteins of the golgin family are often used to mark different portions of the Golgi apparatus during organelle disassembly/reassembly.

Currently two different models compete to explain mechanistic formation of the Golgi apparatus. In one model, Golgi apparatus is viewed as an autonomous organelle; a stable template/scaffold is needed to nucleate the assembly of the dynamic Golgi membranes. Evidence for stable template/scaffold model comes from several lines of experiments. Electron microscopy of mammalian cells treated with brefeldin A (BFA), a fungal metabolite which inactivates Arf1 (17), reveals the presence of residual Golgi-related tubulovesicular clusters around the nucleus, termed Golgi remnants (e.g. 14,18). Detergent extraction of isolated Golgi apparatus fractions results in a Triton X-100 insoluble residue that includes a protein named GM130 for Golgi matrix protein of 130 kDa (19,20). This and related members of the golgin family may form a stable Golgi matrix. When a guanosine-5'-diphosphate (GTP)-mutant of Sar1p protein is injected into NRK or HeLa cells at a low concentration, GM130 and GRASP65 remain juxtanuclearly collected, whereas cisternal Golgi enzymes redistribute to the ER (14,15). During mitosis, cell fractionation studies indicate Golgi-derived vesicles remain distinct from the ER, suggestive of an autonomously dividing organelle (21,22). The other model emphasizes de novo formation of the Golgi apparatus from ER. It conceptualizes the Golgi apparatus as a dynamic steady-state organelle whose maintenance and biogenesis depend on dynamic membrane input and output flow. Evidence for the second model comes from several different experimental approaches. When mammalian cells are treated with BFA, the Golgi apparatus disassembles and Golgi enzymes redistribute to the ER (6). After BFA
washout, the Golgi apparatus reforms as Golgi proteins are exported from the ER. During microtubule depolymerization by nocodazole, Golgi proteins redistribute to ER exit sites, where they assemble apparently de novo into Golgi ministacks (23–25). Time lapse studies in yeast indicate that Golgi apparatus forms de novo in wild-type yeast cells at ER exit sites (26), whereas time lapse studies in mammalian cells suggest that the Golgi apparatus collapses into the ER during mitosis (27). Overexpression of guanosine-5’-diphosphate (GDP)-restricted Sar1p to block ER exit leads to redistribution of Golgi matrix proteins towards the ER (16,28).

Some Golgi components, specifically peripheral matrix proteins, e.g. GM130 and GRASP65, remain associated with Golgi remnants around the nucleus after BFA-treatment. Based on the stable template model, these BFA remnants may seed the formation of the higher order architecture of the Golgi apparatus in interphase mammalian cells (14). Recently, the stability and importance of Golgi matrix/scaffolding components have been challenged on the basis of three kinds of observations. After expression of GDP-restricted Sar1p, Golgi matrix proteins disperse like cisternal Golgi enzymes towards the ER (16,28). As shown by Ward et al. (16), dispersed GM130 may be present in subdomains of the ER, i.e. transitional ER/ER exit sites. Moreover, any matrix associations are likely dynamic; Golgi matrix proteins, GM130 or GRASP65, do not permanently associate with Golgi membranes as demonstrated in FRAP (fluorescence recovery after photobleaching) experiments (16). Finally, a temperature-sensitive mutant of CHO cells lacking GM130 is able to assemble a functional Golgi apparatus juxtanuclearly (30).

In this study, we investigated a variation on the stable Golgi hypothesis, i.e. the hypothesis that Golgi apparatus assembly might be a dynamically nucleated process. Specifically, we asked whether structures marked by peripheral proteins such as GM130 and integral membrane proteins such as p27 might have the kinetic properties expected of a dynamically formed nucleation site for Golgi apparatus biogenesis. Dynamic nucleation is defined here as an early stage in an assembly process in which a newly formed complex acts as the core onto which subsequent assembly occurs. We tested three experimental situations. In the first, taking nocodazole-induced microtubule depolymerization as a tool, we characterized early protein accumulation at scattered sites using a range of Golgi markers from cis to trans. By performing pairwise comparisons, we determined the relative order of appearance of the marker proteins at scattered sites and found that GM130 and p27 appeared first. In the second situation, we then examined Golgi assembly following BFA-washout and found that at slightly reduced temperatures, GM130 and p27-positive structures accumulated at perinuclear sites, distinct from Golgi remnants, before detectable exit of cisternal enzymes from the ER. In our last approach, we examined abortive Golgi reassembly in microinjected cells in which the phenotypic effect of GDP–Sar1p induced ER exit block was at least partially reversed by overexpressing wild-type Sar1p. Here, the only stage of Golgi reassembly supported was the formation of perinuclear GM130/p27-positive structures. Our data, overall, suggest that Golgi assembly is a staged process in which early structures marked by both peripheral and membrane proteins dynamically nucleate assembly of the cisternal membrane stack. Dynamic nucleation of Golgi assembly provides a possible experimental reconciliation of stable and de novo models of Golgi formation.

Results

We chose four classes of Golgi-specific proteins as markers to study the process of Golgi assembly from the ER in HeLa cells. The first class was glycosyltransferases/glycosidases (α-mannosidase II (Mann-II), α-2,6-sialyltransferase (ST), and N-acetylgalactosaminytransferase-2 (GalNAcT2)), all of which are type II integral membrane proteins of the cisternal Golgi apparatus (31–33). GM130, GRASP65 and GRASP55 were chosen to represent peripheral Golgi-matrix proteins; these two proteins are predominantly located at the cis- and medial-Golgi membranes, respectively (20,34,35). Giantin, which occupies a relatively unique place among the Golgi proteins with respect to its size (400 kDa), is localized to cis-Golgi membranes with a cytoplasmic domain of at least 350 kDa (36). p27, representing the p24 family of rapidly recycling Golgi proteins, is a type I integral membrane protein and mainly localized over the cis-Golgi (37). Finally, to mark ER exit sites, we chose mammalian Sec13p, a protein in the COPII complex (38). GalNacT2, ST and Mann-II were expressed stably as tagged fusion proteins and the other endogenous proteins were localized in the same cells by immunofluorescence using Cy3 and Cy5-conjugated secondary antibodies.

De novo assembly of the Golgi apparatus during microtubule depolymerization occurs first through GM130/p27-positive structures

In interphase cells, all of the Golgi proteins and p27 showed a predominantly compact juxtanuclear distribution (e.g. GalNAcT2 and GM130) (Figure 1). Golgi formation at scattered peripheral sites during microtubule depolymerization is the apparent result of de novo Golgi apparatus assembly (13,23,25). To elucidate possible stages in Golgi assembly, cells were fixed after progressive time intervals in the presence of the drug, nocodazole, to depolymerize microtubules and Golgi markers were localized by fluorescence. As shown in Figure 1C,D (arrowheads), after 30 min of nocodazole addition, a number of scattered fluorescence structures positive for GM130 but negative for GalNAcT2 started appearing. With time, the juxtanuclear Golgi apparatus disappeared, while the number of cytoplasmic
patches increased at different rates for GM130 vs. GalNAcT2. By 240 min, all Golgi proteins were found in overlapping fluorescent patches scattered throughout the cytoplasm (Figure 1G,F). To assess staged Golgi formation quantitatively, we then scored the extent of divergence of GM130 from cisternal glycosyltransferases/glycosidases. The number of structures positive for GM130 but negative for glycosyltransferases/glycosidases peaked 1–2 h after the addition of nocodazole; 40–60% of the GM130-positive structures showed no detectable staining for GalNAcT2, ST, or Mann II (Figure 2A). In contrast, when peripheral structures positive for glycosyltransferases/glycosidases were scored for colocalization with GM130, 90% or more scored positive for GM130.

To determine whether the early GM130-positive peripheral structures were also positive for Golgi-related membrane proteins, we scored next for the relationship between GM130 and the membrane proteins p27, GalNAcT2, and giantin. Comparison of the distribution of GM130 and p27, a p24 family member, showed a high congruence; 80% of the peripheral structures were positive for both after

Figure 1: GM130 is more rapidly found in nocodazole scattered structures than GalNAcT2. HeLa cells expressing GFP-tagged GalNAcT2 were incubated in the absence (A, B) and presence of 10 μM nocodazole at 37 °C for 30 min (C, D), 60 min (E, F), and 240 min (G, H). GalNAcT2-GFP was visualized using green channel. Cells were stained for immunofluorescence microscopy using mouse monoclonal anti-GM130 antibodies (red channel). Arrowheads in D point to examples of cytoplasmic patches that are positive for GM130 but not for GalNAcT2. Bar = 30 μm.
30 min of nocodazole treatment and 85–90% were positive for both at all other time points. On the other hand, p27 diverged greatly from GalNAcT2 initially, with 60% of the peripheral p27 structures being negative for GalNAcT2 after 30 min of nocodazole addition (Figure 2B). In comparison, at all time points, any peripheral structure positive for GalNAcT2 was also positive for p27. Giantin showed at most 20% divergence from GalNAcT2 while GalNAcT2 diverged little from giantin (Figure 2B). GRASP55, a matrix component of the medial-Golgi apparatus (35,39), showed maximally less than 25% divergence from GalNAcT2. Interestingly, GalNAcT2 showed a maximum divergence of 30% from GRASP55, a result inconsistent with GRASP55 having a nucleating role in GalNAcT2 distribution. At all time points, peripheral GM130-positive structures were proximal to ER exits, as indicated by Sec13p staining (data not shown). Kinetically, GM130/p27-positive structures appear to seed subsequent Golgi assembly. In summary, GM130 and p27 were found in peripheral structures early on, and in cisternal membrane proteins later.

**Newly formed GM130/p27-positive structures are the first stage in Golgi formation following BFA washout**

The above results indicated that the assembly of GM130 and p27-positive structures preceded the addition of glycosyltransferases/glycosidases in de novo assembly of the Golgi apparatus. We now take a second approach in which cells were treated with BFA to characterize further these findings. BFA rapidly blocks protein transport through the secretory pathway (5) with many Golgi proteins being found in the ER (6). To initiate Golgi reassembly, BFA washout was typically done at 34 °C. BFA washouts at 34 °C rather than 37 °C gave more distinct kinetic stages for Golgi reassembly (data not shown). As expected, upon BFA addition, GalNAcT2 dispersed into the ER network, with occasional cells having residual juxtanuclear concentrations of GalNAcT2 (Figure 3A, arrowheads). In comparison with GalNAcT2, GM130 was concentrated more frequently in the juxtanuclear area with only a few of the cells showing completely scattered, granular GM130 distribution (Figure 3B, arrows). These granular GM130-positive structures around the nucleus are BFA remnants (14–16,18). p27 exhibited clustering of small punctate structures in areas where GM130-positive structures were concentrated; the overall distribution of p27 after BFA treatment was more reticular than the distribution of GM130 (Figure 3C).

After 30 min of BFA washout at 34 °C, there was little exit of GalNAcT2 from ER, with occasional punctuate GalNAcT2 fluorescence, suggesting the occurrence of GalNAcT2 ER exit in some cells (Figure 3D). These occasional punctuate structures were positive for both GM130 and p27. Interestingly, as shown in Figure 3E,F, GM130 and p27 showed less juxtanuclear concentration after 30 min of BFA washout at 34 °C, indicating that the juxtanuclear concentrations (BFA remnants) observed before BFA washout were not stable. Overall, GM130 and p27 were found in larger patches, which were collected perinuclearly and colocalized for both. In a similar manner, the peripheral scaffolding proteins, GRASP55 and GRASP65, which displayed granular distributions similar to that of GM130 after BFA treatment, showed perinuclearly collected, large patches that exhibited extensive continuity in 1-μm-thick confocal microscope slices (Figure 4A,B). There was general but not complete overlap between GRASP55 and GRASP65 with the GRASP55, medial Golgi apparatus, being somewhat less perinuclearly collected (Figure 4D). In the confocal slices, GalNAcT2 showed little exit from the ER (Figure 4C). After 60 min of BFA washout at 34 °C, GalNAcT2, GM130, GRASP55,

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**Figure 2:** Quantification of the early appearance of GM130 (A) and p27 (B) in scattered Golgi structures during microtubule depolymerization. To assess the relative contribution of GM130 and p27 vs. cisternal enzymes to early stages of Golgi formation, the divergence of GM130 and p27 vs. cisternal enzymes was determined. The frequency of structures positive for GM130 or p27 but negative for a given glycosyltransferase/glycosidase and vice versa was scored in 30 cells for each of three experiments. The results were expressed in percentage terms and then averaged. Error bars are plus and minus standard deviation of the mean for three experiments. Bar = 30 μm.

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GRASP65, and p27-positive structures were all found in the same structures, exhibiting a patchy distribution about the cell nucleus (GalNAcT2, GM130 and p27 shown) (Figure 3G–I). As expected, after 120 min of BFA washout, all three proteins collected juxtanuclearly with no apparent punctate structures, indicative of complete Golgi assembly (data not shown). We infer from these observations that GalNAcT2, after exiting the ER, assembles onto GM130- and p27-positive structures and these structures then collect juxtanuclearly. The assembly process does not appear to be onto a preexisting stable perinuclear scaffold, as previously suggested by Seemann et al. (14). Any possible Golgi remnants appeared unstable.

To test whether the initial redistribution of perinuclear punctate p27-positive structures following BFA washout were sensitive to ER exit block, we disrupted ER exit sites by expressing GDP-restricted mutant of Sar1p (T39N), in BFA-treated cells (Figure 5). When cells expressed Sar1pT39N, GalNAcT2 stayed in the ER, showing a reticular ER pattern with a nuclear rim (Figure 5A, asterisks). In cells expressing GDP-restricted Sar1p, p27 showed a more dispersed distribution, with some juxtanuclear rimming and with very little scattered granular staining (Figure 5B). The juxtanuclear rim staining suggests that p27 accumulates in the ER during the exit block (see also (28)). The appearance of GM130-positive structures in GDP-restricted Sar1p expressing cells was as previously described (28), GM130 being delocalized with no apparent juxtanuclear accumulation (Figure 5C). In addition, in cells expressing GDP-restricted Sar1p, ER exit sites were disrupted, as indicated by cloudy distribution of Sec13p in microinjected cells as opposed to the distinct appearance of ER exit sites in non-microinjected cells (Figure 5D, inset shown with arrowheads).

In summary, we observed that the distribution of p27 and GM130 was sensitive to ER exit block. Our results...
indicate that p27- and GM130-containing Golgi remnants are not stable structures, and it is likely that these Golgi remnants do not play a seeding role in assembly of the Golgi apparatus.

A partial wild-type Sar1p reversal of an ER-exit indicates that GM130-positive structures are an early stage in Golgi assembly

As a third test of the nature of early stages in Golgi apparatus formation, we used wild-type Sar1p to partially reverse Golgi apparatus disassembly induced by the expression of GDP-restricted Sar1p (Sar1pT39N mutation). The expression of GDP-restricted Sar1p redistributes GM130 and other Golgi proteins towards the ER (16,28). After 6 h of expression, GM130 is found in a scattered, granular pattern in HeLa cells while GalNAcT2 has redistributed into a full ER-like pattern (28). We expressed Sar1pT39N for 6 h from a stock plasmid DNA concentration of 10 ng/μL to redistribute GM130 and GalNAcT2, then microinjected the same cells with plasmid DNA encoding wild-type Sar1p from a stock concentration of 600 ng/μL (60:1 molar ratio, WT:GDP plasmid) with the aim of reversing the ER exit block. After 18 h expression of Sar1p wild-type protein, we processed cells to determine the distribution of GM130, GalNAcT2 and Sec13p (Figure 6A–C). GM130 in sequentially microinjected cells (asterisks) exhibited redistribution towards its original compact juxtanuclear structure as indicated by the formation of punctate structures around the nucleus (Figure 6A). GalNAcT2 in the same cells exhibited an ER-like distribution with a distinct nuclear rim fluorescence. Little, if any, exit of GalNAcT2 had occurred from the ER (Figure 6B). The distribution of GM130 and GalNAcT2 in sequentially injected cells is very similar to that observed 30 min after BFA washout at 34°C. Sec13p in sequentially injected cells was associated with scattered punctate structures with no juxtanuclear concentration. In wild-type HeLa cells, Sec13p was associated with scattered sites and juxtanuclear clusters (28,37,38,40). The absence of Sec13p-containing juxtanuclear clusters in sequentially microinjected cells suggests that the ER exit block reversal is at most very partial. This is entirely consistent with the failure of GalNAcT2 to exit from the ER to any appreciable extent.

Discussion

In the interphase mammalian cell, Golgi apparatus proteins cycle continuously between the organelle and the ER. During mitosis, the organelle both disassembles and reassembles. In sum, the Golgi apparatus may well be a dynamically self-organizing organelle. Here, we asked a
central question regarding assembly of such a dynamically self-organizing organelle: Is assembly in vivo a staged process in which nucleation itself might be dynamically self-organizing? We took a kinetic approach to the analysis of:

- microtubule depolymerization induced reassembly of Golgi apparatus at scattered sites;
- Golgi reassembly following BFA washout;
- partial phenotypic reversal of a GDP-restricted Sar1p ER-exit block by overexpression of wild-type Sar1p.

We found in all three cases that structures marked by proteins such as GM130 and p27 appeared early in Golgi assembly to which cisternal membrane components such as glycosyltransferases and glycosidases were subsequently added. Moreover and importantly, these early structures appeared to be themselves self-organizing rather than stable, pre-existing structures. De novo formation of nocodazole-treated cells was particularly clear. That assembly in each case was ER-dependent was indicated strongly by inhibition by an ER exit blockade (present work, see also (13,15,23,28)). Hence, we conclude that Golgi assembly can be separated into distinct stages in vivo and is seeded by a process of dynamic self-organization.

In support of self-organizing nucleation, we found little to no evidence in the nocodazole-treated, microtubule-depolymerized cell of pre-existing peripheral concentrations of GM130 or p27 to which Golgi assembly could be anchored. Instead the development of scattered punctate GM130 or p27 staining was time dependent upon drug addition. Moreover, it was rapid relative to that of scattering of the cisternal enzymes: sialyl transferase, GalNacT2 or mannosidase II, membrane proteins residing in different portions of the Golgi stack. The lack of pre-existing scattered concentrations of p27 is in complete agreement with the previous experiments of Füllekrug et al. (37) in which ER exit sites as marked by Sec13 were scattered about the cytoplasm, whereas p27 was concentrated juxtanuclearly with a low frequency of nearby rather than scattered punctate staining. Similarly during BFA washout,

**Figure 5:** Expression of GDP-restricted Sar1p (T39N) blocks the juxtanuclear accumulation of GalNAcT2, p27 and GM130 in BFA washout experiments. HeLa cells were microinjected with pSar1p T39N at a concentration of 50 ng/µL, incubated for 2 h at 37 °C in the presence of BFA, and then incubated for an additional 1 h at 34 °C in the absence of BFA. GalNAcT2-GFP was visualized using the green channel. Cells were stained for immunofluorescence microscopy using rabbit polyclonal anti-p27 antibodies (Cy5 channel), mouse monoclonal anti-GM130 antibodies (red channel) or rabbit polyclonal anti-Sec13p antibodies (red channel). Asterisks in A and D indicate microinjected cells. Microinjected cells were identified using a Cascade blue dextran co-injection marker (blue channel). Arrowheads in A and D (inset) point to nonmicroinjected cells. Bar = 30 μm.
cisternal enzyme accumulation was not onto pre-existing Golgi remnants marked by juxtanuclear concentration of granular GM130 staining, but rather appeared to be onto perinuclear structures marked by both GM130 and p27, which only became obvious following drug removal. As these structures formed, particularly at 34°C, little to no exit of GalNAcT2 from the ER occurred. Finally, partial wild-type Sar1p reversal of an ER-exit block phenotype was marked by the formation of perinuclear GM130/p27-positive structures. GalNAcT2 was retained in the ER. Overall, we infer that GM130/p27-positive structures play a dynamic, self-organizing role in the subsequent complete assembly of the Golgi apparatus.

Our results suggest that assembly of early Golgi structures is driven by both peripheral and integral membrane proteins and their probable interactions. The choice of GM130 and p27 as marker proteins was a matter of convenience. GM130 was chosen as a marker protein because it is one of the more intensively studied and the earliest example of peripheral, matrix proteins of the cis-Golgi apparatus (20) and it belongs to the golgin family of proteins (41,42). GRASP55 and GRASP65, Golgi apparatus stacking proteins, were also examined and behaved on the whole similarly to GM130. Contrary to the expectation for essential matrix proteins, recent analysis indicates that mammalian cells deficient in detectable GM130 have normal, functional Golgi apparatus (30). p27 (gp27) is a member of the p24 family of proteins which normally shuttle between the ER and Golgi apparatus and are thought to be cargo receptors (37,43). However, the fact that these two proteins, GM130 and p27, had coincident distributions under all conditions of Golgi apparatus assembly is of some importance as it suggests that peripheral proteins such as GM130 do not drive membrane organization by first forming a membrane-independent scaffold (see e.g. (14)) but rather interact coincidentally with membrane proteins to produce early Golgi intermediates. The very partial assembly of Golgi apparatus with the sequential expression of GDP-restricted Sar1p followed by the injection of a 60-fold excess of plasmid DNA encoding wild-type Sar1p is likely consistent with our prior evidence for Sec13, a COPII component, aggregating to perhaps form inclusion bodies in HeLa cells expressing GDP-restricted Sar1p (28). By fluorescence microscopy, we saw little evidence for the formation of wild-type Sec13 distribution in the sequentially injected cells. We know of no similar examples in the literature of such sequential injection experiments with mutant and wild-type Sar1p encoding DNAs.

We can only speculate for now as to whether apparent self-organizing, in vivo ‘nucleation’ of Golgi assembly is a physiologically normal process. For lower eukaryotes, evidence from such organisms as Toxoplasma gondii indicates that the single Golgi stack present is inherited by lateral extension followed by medial fission (44) rather than by dynamic nucleation as part of a sequential, staged assembly process. For mammalian cells, numerous inves-

Figure 6: Wild-type Sar1p partially reverses ER-exit block. HeLa cells were microinjected with 10 ng/µL Sar1p T39N and incubated for 6 h at 37°C to block ER exit. The same cells were then microinjected with 600 ng/µL pSar1p wild-type and incubated for an additional 18 h at 37°C to rescue cells from ER exit block. Cells were stained for immunofluorescence microscopy using either mouse monoclonal anti-GM130 antibodies (Cy5 channel) or alternatively with rabbit polyclonal anti-Sec13p antibodies (Cy5 channel). GalNAcT2-GFP was visualized using green channel. Asterisks in A and C indicate sequentially microinjected cells. Bar = 30 µm.
Drug treatments

HeLa cells were incubated at 37 °C with 10 µM nocodazole for various time periods as previously described (25). HeLa cells were treated with BFA as previously described (14,15,28). For BFA washout, coverslips were transferred sequentially between four tissue culture dishes containing 2 mL of room temperature CO₂-independent microinjection media (Gibco BRL, Rockville, MD). For Golgi reformation, coverslips were then transferred to 34 °C dishes containing complete culture media.

Antibodies and preparation of cells for immunofluorescence microscopy

GM130 antibodies were purchased from BD Biosciences (San Diego, CA). mAbs directed against giantin (36) were a gift from Dr. H.-P. Hauri (Biozentrum, University of Basel, Switzerland). Affinity purified antibodies directed against p27 (37) were a gift from Dr. T. Nilsson (EMBL, Heidelberg, Germany). Rabbit polyclonal antibodies recognizing the human homolog of yeast Sec13p (38) were a gift from Dr. W. Hong (Institute of Molecular and Cell Biology, National University of Singapore, Singapore). Sheep polyclonal antibodies recognizing GRASP65 (34) were affinity-purified, non-cross-reactive secondary antibodies recognizing GRASP65 (34). Non-cross-reactive secondary antibodies (Cy2, Cy3, Cy5) raised in donkeys were purchased from Jackson Immunoresearch (West Grove, PA). Cells were grown on 11-mm round glass coverslips in complete medium for 24–48 h before use. After appropriate drug treatments and microinjection, cells were fixed with formaldehyde and permeabilized with either 0.1% saponin or 0.5% Triton X-100 as described previously (28). Coverslips were mounted in Mowiol.

Cells were observed with either wide field microscopes (Zeiss Axiovert 100 or Zeiss Axiovert 200) or a laser-scanning microscope (Zeiss LSM 510Meta) fitted with a 63X/1.4 NA objective and selective fluorescence filter sets. Wide field micrographs were taken with either a Roper CoolSNAP HQ 1392 × 1040 pixel CCD camera or a QImaging Retiga EXi 1360 × 1036 pixel CCD camera controlled by IPLab software (Scanalytics, Fairfax, VA). Confocal micrographs were taken with a LSM 510 zoom factor of 2.9 to yield voxel dimensions of 0.1 µm × 0.1 µm XY plane, 1.0 µm Z axis.

Microinjection of pSar1pT39N and pSar1pWT

pSar1pT39N and pSar1pWT were previously prepared in pCMUIV as described in Stroud et al. (28) and Storrie et al. (13), respectively. Plasmid purifications were performed with a Qiagen Maxi Prep DNA purification kit (Qiagen, Valencia, CA). Purified plasmids were microinjected into cell nuclei as described previously (13). In single microinjection experiments, Cascade blue dextran (70 kDa, aldehyde fixable; Molecular Probes, Eugene, OR) was used as a coinjection marker at a concentration of 0.2 mg/mL. In sequential

Materials and Methods

Cell culture

HeLa cells, either wild-type or stably expressing tagged Golgi apparatus proteins (GalNAcT2-GFP, SialyIT-VSV, and mannosidase II-VSV), were cultured under standard tissue culture conditions as described (15).

Investigators have shown that cisternal proteins cycle between the organelle and the ER (13–16,23,27,28) and that the distribution of Golgi matrix proteins like GM130 is dependent on active ER exit sites (16,26,28,45). However, the distribution of Golgi matrix proteins are at least under some conditions less dynamic than cisternal Golgi enzymes (14,15). This may well indicate a stable rather than dynamic nucleus for organelle assembly. On the other hand, these experiments all use the GTP-restricted, active mutant of Sar1p. At low expression levels, this mutant does not disrupt ER exit sites as the GDP-restricted mutant does (16,28). Moreover, dissection experiments in which the cell is separated into cytoplasm- and karyoplast-containing peripheral ER and juxtanuclear Golgi apparatus, respectively, suggest that interphase ER normally contains insufficient Golgi components to support downstream organelle biogenesis (46). Nevertheless, in sum, accumulating evidence does suggest that the interphase mammalian Golgi apparatus is in dynamic equilibrium with the ER. Whether the same may be true during mitosis and exactly how mitosis reorganizes the ER remains quite controversial (see for example (22,27,47,48)). In vitro work on Golgi assembly from mitotic extracts does suggest that Golgi assembly can be separated into stages (see (49–51)). In summary, it is fair to state that an in vivo staged/dynamic assembly relationship between the ER and Golgi apparatus is likely to reveal important aspects of Golgi apparatus biogenesis.

The similarity of our conclusions to the parallel, independent and very recently published studies of Puri & Linstedt (52) should be noted. These authors conclude from drug wash-out experiments that the Golgi apparatus in interphase mammalian cells has the capacity for biogenesis from the ER. Moreover, they conclude that GM130 is a fast ER exiting protein. In their experiments, either BFA plus H89 or clofibrate treatment was used to completely disperse Golgi apparatus proteins into the ER. Golgi apparatus reassembly was then tracked by fluorescence microscopy and cell fractionation. Assembly appeared to be de novo, with assembly of GM130-positive structures preceding that of cisternal membrane protein-positive structures. Clearly the details and exact systems used by this laboratory and by Puri & Linstedt differ. Our studies place more emphasis on the probable self-organizing role of GM130/p27 marked structures as intermediates that likely dynamically seed Golgi apparatus assembly. In proposing a model of dynamic seeding of Golgi apparatus assembly, we suggest what is an experimental resolution to conflicts between a stable Golgi core and simple ER derived Golgi assembly models.
Quantification of marker proteins

We define divergence as the frequency of structures that are, for example, positive for GM130 or p27 but negative for a given marker protein or vice versa. Fluorescent micrographs were printed with a black and white Apple laser printer (Laser Writer 16/1600) and Golgi protein-positive scattered punctate structures were marked on the paper with a pen, excluding the immediate juxtanuclear Golgi complex. The distributions of each protein were then examined side by side to determine the extent of correspondence/noncorrespondence. A fluorescent structure negative for noncorresponding Golgi proteins, pSar1pT39N was microinjected at a stock concentration of 10 ng/µL and then incubated with BFA as described. pSar1pWT stock concentration for sequentially microinjected cells was 600 ng/µL (60:1 molar ratio, WT:T39N mutant plasmids).

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