

Dispersal of Golgi matrix proteins during mitotic Golgi disassembly

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Summary

During mitosis, the mammalian Golgi disassembles into numerous vesicles and larger membrane structures referred to as clusters or remnants. Following mitosis, the vesicles and clusters reassemble to form an intact Golgi in each daughter cell. One model of Golgi biogenesis states that Golgi matrix proteins remain assembled in mitotic clusters and then serve as a template for Golgi reassembly. To test this idea, we performed a 3D-computational analysis of mitotic cells to determine the extent to which these proteins remain in mitotic clusters. As a control we used brefeldin A-induced Golgi disassembly which causes dispersal of Golgi enzymes, but leaves matrix proteins in

remnant structures. Unlike brefeldin A-treated cells, in which matrix proteins were clearly sorted from non-matrix proteins, we observed extensive dispersal of matrix proteins in metaphase cells with no evidence of differential sorting of these proteins from other Golgi proteins. The extensive disassembly of matrix proteins argues against their participation in a stable template and supports a self-assembly mode of Golgi biogenesis.

Key words: Golgi apparatus, Biogenesis, Golgin, GRASP, Template, De novo

Introduction

The mammalian Golgi apparatus mediates processing and sorting reactions in the secretory pathway. Its behavior is particularly intriguing in that although it has a complex structure, it undergoes substantial structural transformations in every cell cycle. During interphase, Golgi membranes consist of stacked cisternae. Each stack contains approximately four cisternae and there are several hundred of these stacks per cell. Each stack is also linked laterally to other stacks to form a ribbon-like membrane system positioned adjacent to the microtubule organizing center.

At mitosis, the entire structure disassembles and then reassembles in each daughter cell. However, the actual extent of mitotic Golgi disassembly is controversial, and this has led to opposing models regarding the mechanism by which Golgi membranes reassemble in each daughter cell post-cytokinesis. One model suggests the use of a pre-existing template (Seemann et al., 2000; Seemann et al., 2002), whereas the other predicts reformation by self-assembly of the breakdown products (Miles et al., 2001; Ward et al., 2001; Bevis et al., 2002).

The template model derives from work on two sets of Golgi-localized proteins, the golgins and GRASPs (Golgi Re-Assembly Stacking Proteins). Golgins have extensive heptad repeats predicted to form elongated coiled-coil domains on the cytoplasmic side of the Golgi membrane. GRASPs share significant sequence identity and are anchored to the cytoplasmic side of the Golgi membrane by myristoylation. One of the golgins, GM130, was discovered in a detergent insoluble extract of purified Golgi membranes (Nakamura et al., 1995). Importantly, this insoluble material yielded a pattern in electron microscope images reminiscent of stacked cisternae

(Slusarewicz et al., 1994). On this basis the material was called the Golgi 'matrix' and GM130 was termed a 'matrix' protein.

Other golgins and the GRASPs are termed matrix proteins for two reasons. The first is based on interactions among these proteins. GM130 interacts directly with GRASP65 and indirectly with giantin (Barr et al., 1997; Dirac-Svejstrup et al., 2000). GRASP55 may function in a parallel complex as it binds golgin-45 (Short et al., 2001). The second is based on the behavior of these proteins versus other Golgi proteins during treatment of cells with brefeldin A (BFA). BFA treatment induces redistribution of most Golgi-localized proteins to the ER, but giantin, GM130, GRASP65 and GRASP55 end up in membranes called BFA remnants that are distinct from the ER (Seemann et al., 2000). As BFA-induced Golgi disassembly is reversed upon drug washout, this finding is consistent with the view that these proteins remain in intact assemblies that then mediate reassembly upon drug washout. In fact, giantin, GM130, GRASP65 and GRASP55 are each required for Golgi stacking as measured using an *in vitro* assay (Barr et al., 1997; Shorter et al., 1999; Shorter and Warren, 1999).

The mitotic Golgi disassembly/reassembly reaction offers a critical and physiologically relevant test case for this model. Most work suggests that mitotic Golgi disassembly proceeds by vesiculation and yields Golgi proteins in an abundant population of small vesicles and in some larger membranes variously termed vesicle clusters or cisternal remnants (Lucocq et al., 1989; Misteli and Warren, 1995; Shima et al., 1997; Jesch and Linstedt, 1998; Jesch et al., 2001; Jokitalo et al., 2001). The cisternal remnants are hypothesized to constitute the Golgi template. Opposed to this is evidence suggesting that during mitosis the Golgi collapses into, and partitions with, the

ER (Zaal et al., 1999). If the entire Golgi is in the ER, then post-mitotic Golgi biogenesis must occur *de novo*. Interestingly, this latter work used Golgi enzymes rather than any golgin or GRASP as marker proteins. It was subsequently observed that during BFA-induced Golgi collapse, Golgi enzymes move to the ER whereas golgins and GRASPs accumulate in BFA remnants. Therefore, as suggested in several reviews (Nelson, 2000; Short and Barr, 2000; Rossanese and Glick, 2001; Jesch, 2002; Shorter and Warren, 2002), it might be possible that during mitosis redistribution to the ER is the mode for enzymes whereas golgins and GRASPs remain in mitotic clusters.

This is important because the 'matrix' template model makes a strong prediction with respect to the distribution of golgins and GRASPs. By this view mitotic Golgi clusters are the partitioning units of the Golgi (Seemann et al., 2002). They correspond roughly to the number of stacks per cell and contain golgins and GRASPs in an assembled state that will serve as template for post-mitotic Golgi reassembly. Hence, golgins and GRASPs should remain behind in mitotic Golgi clusters, whereas other Golgi markers may move into the vesicle fraction, or even into the ER. We tested this prediction by quantitative measurement of sorting of golgins and GRASPs from other Golgi proteins during mitotic Golgi breakdown.

Materials and Methods

Cell culture and staining

Growth medium for NRK cells (Dulbecco's modified Eagles medium, Life Technologies, Grand Island, NY, USA) and HeLa cells (Minimal essential medium, Sigma-Aldrich, St. Louis, MO, USA) contained 10% fetal bovine serum and 100 IU/ml penicillin-streptomycin. Where indicated, the growth medium was supplemented with 10 μ g/ml BFA (Sigma). For immunofluorescence, cells were grown on 12-mm glass coverslips, treated where indicated, and either fixed in methanol (-20°C for 10 minutes) or 3% paraformaldehyde (20 minutes at room temperature). Paraformaldehyde-fixed cells were stained as previously described (Jesch and Linstedt, 1998). After three washes with PBS, the cells were incubated for 30 minutes in blocking buffer (PBS containing 2.5% calf serum and 0.1% Tween-20), followed by consecutive 30-minute incubations in primary and secondary antibodies diluted in blocking buffer. Each antibody incubation was followed by five washes with PBS. The antibodies and their dilutions were: mouse anti-GPP130 at 1:200 (Linstedt et al., 1997); mouse anti-giantin at 1:100 (Linstedt and Hauri, 1993); rabbit anti-GPP130 at 1:500 (Puri et al., 2002); rabbit anti-giantin at 1:500 (Puthenveedu and Linstedt, 2001); rabbit anti-GM130 at 1:500 (Puthenveedu and Linstedt, 2001); rabbit anti-p115 at 1:500 (Puthenveedu and Linstedt, 2001); rabbit anti-GRASP65 at 1:2000 (Sutterlin et al., 2002); rabbit anti-GRASP55 at 1:2000 (a gift from Vivek Malhotra, UCSD, La Jolla, CA); mouse anti-TGN38 at 1:100 (Affinity BioReagents, Golden, CO, USA); mouse anti-mannosidase II at 1:10,000 (Sigma); fluorescein-labeled goat anti-mouse at 1:200 (Zymed, San Francisco, CA, USA); and rhodamine-labeled goat anti-rabbit at 1:200 (Zymed). Some experiments used a HeLa cell line stably expressing N-acetylgalactosaminyltransferase 2 tagged with GFP (Storrie et al., 1998).

Image analysis

Microscopy was performed using either a conventional fluorescence microscope (Linstedt et al., 1997) or a DeltaVision system (Applied Precision, Issaquah, WA, USA) essentially as previously described (Jesch et al., 2001). In the latter case a 100 \times , 1.35 NA oil immersion

objective yielding a pixel dimension of 0.0671 μm was used. Sections, 15 for interphase and 40 for metaphase cells, were taken at a step size of 0.3 μm . Images were deconvolved using SoftWorx (Applied Precision). Deconvolved manually cropped sections were assembled into a 3D image using Matlab (Mathworks, Natick, MA, USA). The background value subtracted from each voxel was the most common voxel value outside the cell multiplied by a factor of 1.54 and 1.22 for the fluorescein and rhodamine channel, respectively. These factors were the average of the ratio of the most common voxel value inside the cell to the most common voxel value outside the cell determined from the 3D data set of 10 interphase and metaphase cells that were stained with secondary antibody only. Threshold values were manually chosen for sections 4, 7 and 11 for interphase cells and sections 10, 20 and 30 for metaphase cells. Using the original gray scale image as the standard, the sections were thresholded to maximize inclusion of discernable objects and minimize inclusion of non-discernable objects. The average of the three values was then used as the threshold for that cell. Threshold values varied from 0.0001 to 0.003% of the maximum intensity. Object finding was done on the thresholded image using 26-neighbor connectivity and the fraction of total fluorescence in objects was calculated. Given that mitotic clusters may be compartmentalized (Shima et al., 1997), we determined the percentage of mitotic clusters that were positive for a given pair of markers by considering objects identified in one channel to be positive for a marker in another channel only if object edges overlapped.

Results

To determine the extent of matrix marker recovery in mitotic Golgi clusters we used a 3D analysis, because 2D projections of 3D data, particularly when using the maximum value of all aligned Z-axis pixel values instead of their sum, severely under-represent dispersed fluorescence (Jesch et al., 2001). The analysis was performed by collecting a stack of images by deconvolution microscopy. Total fluorescence above background, as well as fluorescence attributed to objects, was then analyzed in three dimensions. To reduce experimental bias and to account for possible variation between cells, a statistical analysis was performed on results from multiple cells for each condition and each cell was chosen solely on the basis of its DNA staining. Also, endogenous markers for matrix and non-matrix proteins were used to avoid the various problems associated with transgene expression. Finally, NRK cells were chosen because the presence of mitotic Golgi clusters was most apparent in these cells. If it occurs, this would increase our ability to demonstrate selective retention of matrix markers.

The deconvolved images demonstrated presence of matrix and non-matrix markers in both punctate and hazy staining, previously shown to correspond to mitotic Golgi clusters and mitotic Golgi vesicles, respectively (Jesch et al., 2001). Note that the non-resolvable, or hazy, fluorescence was significantly above background and was equally present for fluorescence derived from GFP coupled to a Golgi marker (see below). For the purpose of illustration, Fig. 1 presents representative total fluorescence patterns of deconvolved optical sections as 2D projections summed along the Z-axis. In this case, interphase and metaphase NRK cells were costained for non-matrix marker mannosidase II and matrix marker GM130. For untreated interphase cells, each antibody yielded specific staining of the Golgi ribbon and the staining patterns were markedly coincident (Fig. 1A-C). In contrast, in untreated metaphase cells, both mannosidase II and GM130 yielded a

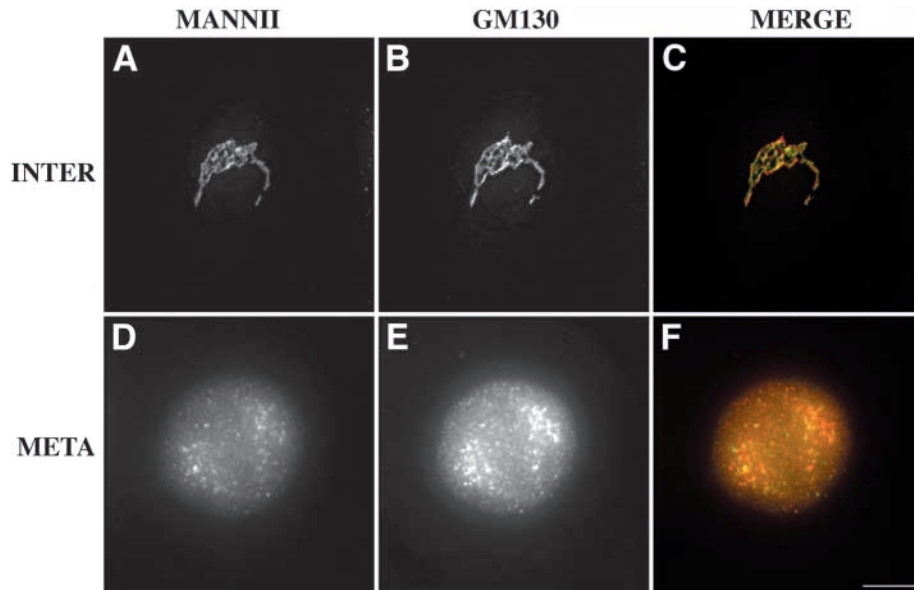


Fig. 1. Comparison of a matrix protein and a non-matrix protein in interphase and metaphase cells. Interphase (A-C) and metaphase (D-F) NRK cells were costained for mannosidase II (A,D) and GM130 (B,E). Single-channel images are also overlaid (C,F) with mannosidase II in green and GM130 in red. The images shown are 2D projections from the deconvolved 3D data set using summation of pixel values in each plane. Bar: 10 μ m.

mixture of hazy and punctate staining, indicating that each marker was present in vesicular haze as well as mitotic Golgi clusters (Fig. 1D-F). Note that the marker staining of punctate structures was mostly adjacent rather than coincident (see below). Similar results were obtained for matrix markers giantin (Fig. 2A), GRASP65 (Fig. 2B) and GRASP55 (Fig. 2C), as well as a non-matrix marker, GPP130 (Fig. 2D).

Identification of the NRK mitotic Golgi clusters in three dimensions indicated that there were, on a per cell basis, 203 ± 54 (s.d., $n=60$) clusters and these were an average of 1 μ m in diameter. Importantly, the fraction of total fluorescence comprised by mitotic Golgi clusters was at most 10% for any

marker tested (Fig. 3). Thus, although mitotic Golgi clusters in NRK were larger than those previously reported in other cell types (Shima et al., 1997; Jesch et al., 2001), our analysis indicated that these clusters only represented a small fraction of the mitotic Golgi. Furthermore, the fact that the extent of marker recovery was similar for matrix and non-matrix proteins indicates a striking absence of sorting based on category of marker protein. The only exceptions were for GRASP55 and p115, which were actually recovered to an even lower extent in mitotic Golgi clusters. For p115, this can be explained as it is a peripheral membrane protein that partially dissociates from membranes during mitosis (Levine et al., 1996). Whether GRASP55 actually dissociates remains to be tested. Despite its lipid anchor, this may be possible, as there is evidence that lipid-anchored GRASP65 rapidly cycles on and off the Golgi membrane during interphase (Ward et al., 2001).

Even though the recovery of Golgi markers in clusters was only 10%, this was higher than the 2% previously observed for GPP130 in metaphase HeLa cells (Jesch et al., 2001). In fact, we simultaneously analyzed this marker and several others in both cell types and found that, independent of marker tested, and independent of fixation conditions, mitotic Golgi clusters account for 2% and 10% of HeLa and NRK Golgi, respectively. Identical results in HeLa were also obtained for analysis of a

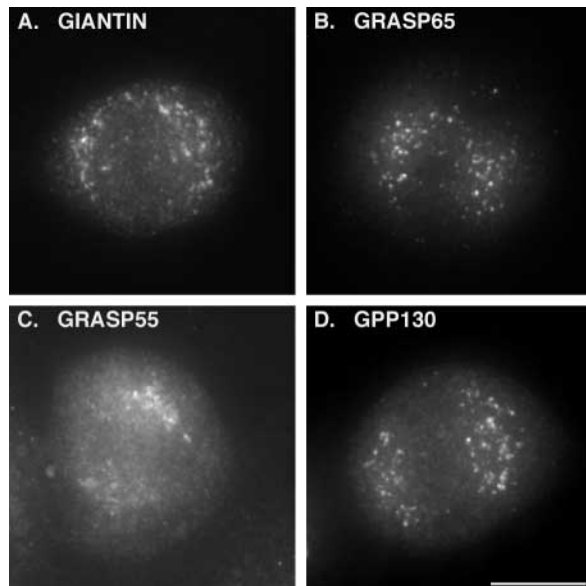


Fig. 2. Presence of Golgi markers in mitotic Golgi clusters and vesicular haze. Metaphase NRK cells were stained using antibodies against giantin (A), GRASP65 (B), GRASP55 (C) and GPP130 (D). Images shown are 2D projections using summation of pixel values. Bar: 10 μ m.

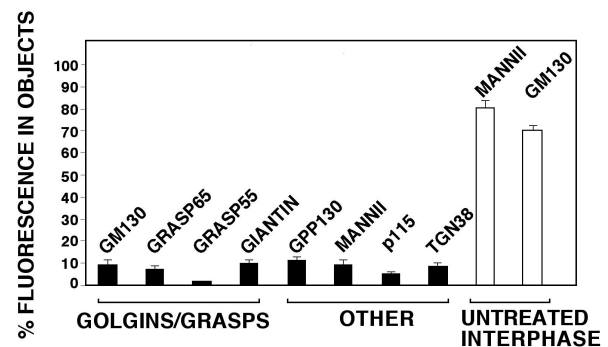
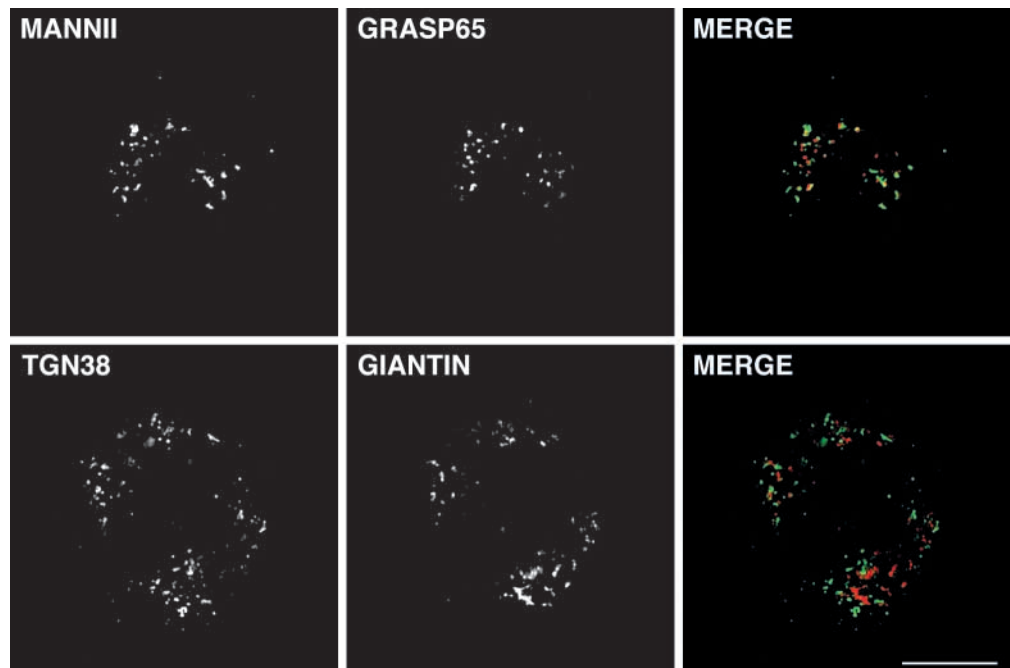


Fig. 3. Matrix proteins are extensively dispersed in vesicular haze rather than selectively retained in mitotic Golgi clusters. Untreated interphase (open bars) and metaphase NRK cells were stained for different proteins as indicated, and the percentage fluorescence in objects of each was calculated as in Materials and Methods (\pm s.e.m.). The results are averages from 10 cells per condition.

Fig. 4. Coincidence of Golgi proteins in mitotic Golgi clusters. Metaphase NRK cells were costained with antibodies against mannosidase II and GRASP65 and a merged image is shown with mannosidase II in green and GRASP65 in red. Metaphase cells were also costained with antibodies against TGN38 and giantin, and the merge shows TGN38 in green and giantin in red. All images shown are summed projections that were thresholded to isolate mitotic Golgi cluster staining from vesicular haze. Note that markers were frequently adjacent in clusters rather than truly coincident. Bar: 10 μ m.



stably expressed Golgi marker visualized on the basis of a GFP tag, arguing against inaccuracies because of staining procedures or background determination. Thus, Golgi breakdown varies depending on cell type, but at least for these two cell types, it is extensive for all categories of marker. This includes the trans-Golgi network as TGN38 was also mostly present in hazy staining with only 10% recovered in NRK mitotic Golgi clusters.

Interestingly, most mitotic Golgi clusters appeared to contain each Golgi marker. Mannosidase II was present in 92% of the GM130 positive clusters ($n=411$), 90% of the giantin positive clusters ($n=146$), and 88% of the GRASP65 clusters ($n=147$). Tests of other pairs of markers in clusters also yielded remarkably coincident patterns. However, within each cluster, marker staining was often adjacent rather than precisely coincident as is evident in metaphase cells costained for mannosidase II and GRASP65 (Fig. 4A-C). Adjacent staining was most noticeable in clusters costained for an early and late Golgi marker – such as giantin and TGN38, respectively (Fig. 4D-F) – consistent with previous work suggesting marker compartmentalization within individual clusters (Shima et al., 1997).

Non-matrix Golgi markers redistribute to the ER upon BFA treatment, whereas matrix markers become localized in BFA remnants (Seemann et al., 2000). Consistent with this, after BFA treatment of interphase cells, mannosidase II staining collapsed from a ribbon pattern (Fig. 5A) to an ER pattern (Fig. 5C), and GM130 staining went from Golgi ribbon (Fig. 5B) to the punctate pattern of BFA remnants (Fig. 5D). Quantitative analysis showed that GM130, GRASP55 and GRASP65 were clearly enriched in BFA remnants relative to other markers (Fig. 5E). Nevertheless, matrix marker recovery in BFA remnants was less than 50% and, in the case of giantin, only 5% of the signal was in BFA remnants.

These experiments with BFA make two points. First, a quantitative analysis of Golgi marker behavior in response to

BFA, which had not been previously performed, revealed greater complexity than previously appreciated. Not only is recovery in BFA remnants incomplete, but also, not all golgins/GRASPs respond in a similar manner. As a further example, golgin 84, a protein which we could not quantitatively assess because of lack of antibodies, redistributes to the ER and not to BFA remnants (Diao et al., 2003). These complexities cast doubt regarding the stability of a Golgi matrix comprised of golgins and GRASPs. Second, the assay we used to test for selective retention of matrix markers in mitotic Golgi clusters is clearly capable of detecting sorting of proteins between larger ‘intact’ golgin/GRASP structures and dispersed structures. Therefore, the low and uniform recovery of all Golgi proteins, including matrix markers, in mitotic Golgi clusters is strong evidence for a self-assembly rather than a stable template-mediated mode of post-mitotic Golgi reassembly.

Discussion

Our test of the matrix protein model for Golgi biogenesis focused on a key prediction. Sorting during disassembly may allow dispersal and/or ER redistribution of other Golgi markers, but matrix proteins should be left in a primarily assembled state in remnant Golgi structures (Seemann et al., 2000). The presence of a stable template contrasts with de novo Golgi biogenesis in which structural elements can undergo complete disassembly followed by reassembly. It also fits with previous non-quantitative observations showing accumulation of golgins and GRASPs in BFA remnants (Seemann et al., 2000), and their presence in mitotic Golgi clusters (Shima et al., 1997). However, our quantitative assessment indicated that mitotic Golgi disassembly was accompanied by substantial dispersal of golgins and GRASPs rather than concentration in mitotic Golgi clusters. In NRK and HeLa, mitotic Golgi clusters accounted for at most 10% and 2% of any marker tested, respectively. There was also no indication that golgins

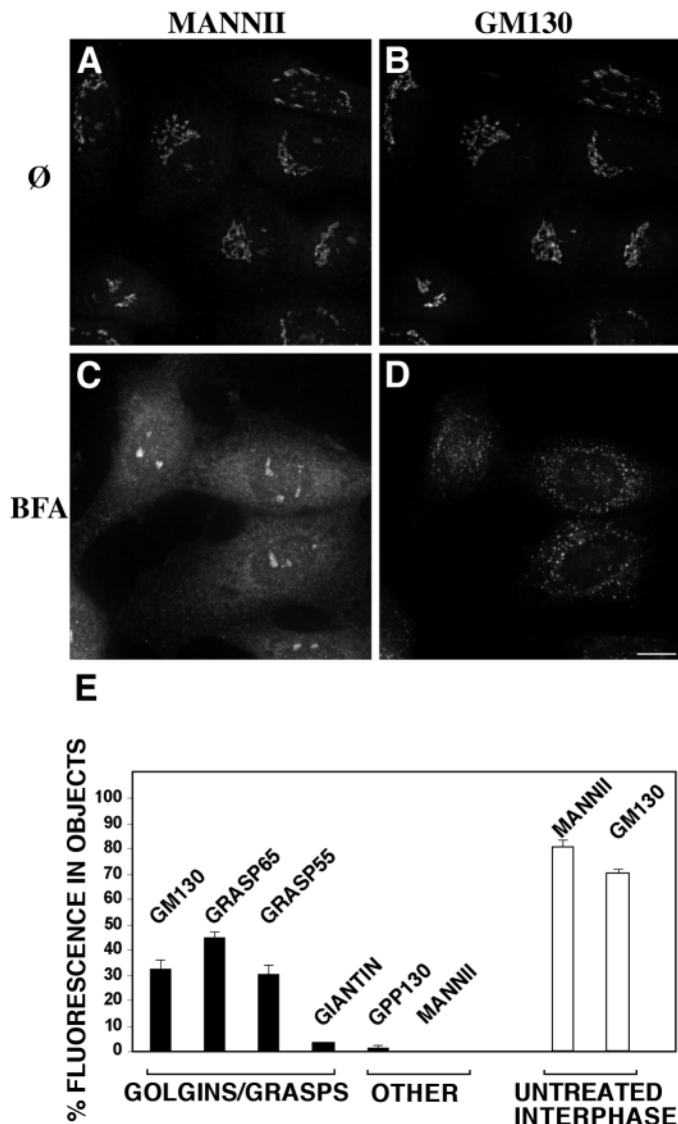


Fig. 5. Matrix proteins are selectively, but incompletely, retained in BFA remnants. NRK cells, either untreated (A,B) or treated with BFA for 30 minutes (C,D), were costained for mannosidase II (A,C) and GM130 (B,D). Summed projections are shown. Note that for an unexplained reason, nucleolar-like staining was observed when the anti-mannosidase II antibody was used to stain interphase cells, particularly at the exposure setting necessary to visualize BFA-treated cells. This non-specific staining was excluded in quantitative analyses. Bar: 10 μ m. (E) Untreated interphase (open bars) and BFA-treated NRK cells were stained for different proteins as indicated, and the percentage fluorescence in objects of each was calculated as in Materials and Methods (\pm s.e.m.). The results are averages from 10 cells per condition.

and GRASPs are sorted differently from other categories of Golgi proteins. Even BFA remnants, which did show accumulation of golgin and GRASPs relative to other proteins, at most accounted for 50% of these markers. This finding is more consistent with BFA-induced redistribution involving cycling of golgins and GRASPs through the ER followed by their subsequent accumulation in post-ER structures such as the ERGIC (Miles et al., 2001; Ward et al., 2001).

Based on these observations, it can be concluded that if matrix proteins act as a template for post-mitotic Golgi assembly, it must be a dynamic template capable of considerable self-assembly. However, the evidence with respect to the actual role of matrix proteins is mixed. Two golgins, golgin-45 and golgin-84, have a profound role in Golgi biogenesis as their knockdown causes Golgi breakdown (Short et al., 2001; Diao et al., 2003). Inhibition of transport causes Golgi collapse; so it is not yet clear whether the effects observed after golgin-45 and golgin-84 knockdown are because of transport or structural defects. Note that golgin-84 is not present in BFA remnants and therefore it is arguably not a matrix protein (Diao et al., 2003). Required roles for other golgins and GRASPs are either unknown or unlikely. The principle matrix protein, GM130, as well as giantin, appears to be dispensable for Golgi biogenesis (Puthenveedu and Linstedt, 2001; Kondylis and Rabouille, 2003; Vasile et al., 2003). It may be that, at least in the case of giantin and GM130, instead of playing a role as a template, these proteins facilitate trafficking in cells in which they are expressed. If this is the case, the in vitro Golgi stacking reaction may have created dependencies for these proteins, and maybe other components, which are not required in vivo. It is noteworthy that a major factor leading to the designation of matrix proteins as such, is their presence in BFA remnants. That is, matrix proteins may not represent a functional grouping, but rather a grouping based on targeting characteristics. If matrix proteins localize to BFA remnants by cycling through the ER rather than by staying intact in a matrix (Miles et al., 2001; Ward et al., 2001), then it is certainly possible that they may mediate diverse functions, only some of which are required for Golgi biogenesis.

Mitotic Golgi clusters represent a small fraction of the Golgi (Zaal et al., 1999; Jesch et al., 2001) (this study), are not selectively enriched in any particular known Golgi component (this study), and exhibit dynamic behavior suggesting that they are not stable but rather have rapid interchange with dispersed Golgi vesicles (Jesch et al., 2001). At the EM level, mitotic Golgi clusters do not exhibit any distinguishable features of Golgi structure. Nevertheless, mitotic Golgi clusters exhibit evidence of Golgi subcompartmentalization (Shima et al., 1997) (this study). Inhibition of vesicle docking and fusion at mitosis may underlie a Golgi breakdown reaction that preserves the sorting reactions that take place to maintain the Golgi apparatus during interphase. Mitotic Golgi clusters may represent incomplete breakdown and/or incomplete reassembly. Although it seems reasonable that these structures participate in post-mitotic Golgi reassembly, there is no compelling evidence that they mediate the process. Rather, it is probable that, in accordance with recent work (Bevis et al., 2002; Puri and Linstedt, 2003) the Golgi apparatus is capable of self-assembly.

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