

Golgi duplication in *Trypanosoma brucei*

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Duplication of the single Golgi apparatus in the protozoan parasite *Trypanosoma brucei* has been followed by tagging a putative Golgi enzyme and a matrix protein with variants of GFP. Video microscopy shows that the new Golgi appears de novo, near to the old Golgi, about two hours into the cell cycle and grows over a two-hour period until it is the same size as the old Golgi. Duplication of the endoplasmic reticulum (ER) export site

follows exactly the same time course. Photobleaching experiments show that the new Golgi is not the exclusive product of the new ER export site. Rather, it is supplied, at least in part, by material directly from the old Golgi. Pharmacological experiments show that the site of the new Golgi and ER export is determined by the location of the new basal body.

Introduction

The Golgi apparatus has the same basic structure in all eukaryotic cells, comprising flattened cisternal membranes with dilated rims, most often arranged in the form of a stack. In animal cells, each face of the stack is bounded by tubular networks: the cis, or entry, face of this network is termed the cis Golgi network (CGN); the trans, or exit, face is termed the TGN (Ladinsky et al., 1999). The Golgi apparatus processes the entire output of newly synthesized proteins and lipids it receives from the ER and prepares them for delivery to their final destinations (Mellman and Warren, 2000).

The unique architecture of the Golgi apparatus raises the question of its biogenesis. How is this complex structure duplicated, and then partitioned during mitosis and cell division? Partitioning has been most studied and is aided by dispersal of Golgi components. In many plants and fungi, Golgi stacks are dispersed throughout the cell cycle (Drionich and Stachelin, 1997; Rossanese et al., 1999), but in interphase animal cells, several hundred stacks are linked together in a ribbonlike structure near to the nucleus and often the centrosomes (Rambourg et al., 1987). Dispersal is a necessary

prelude to partitioning, but the mechanism is still being debated (Zaal et al., 1999; Jesch et al., 2001; Jokitalo et al., 2001; Shorter and Warren, 2002; Axelsson and Warren, 2004; Pecot and Malhotra, 2004).

Golgi duplication is less well understood though several models have been put forward (Munro, 2002). The first model is templated biogenesis, in which the old Golgi is thought to act as a scaffold for the new. This model has its origins in EM images that appear to show elongated Golgi stacks dividing into two (Domozych et al., 1981; Benchimol et al., 2001). Though static images cannot indicate the direction of events, we have recently been able to confirm this sequence by exploiting the simplicity of the protozoan parasite *Toxoplasma gondii* and its amenability to genetic manipulation. The single Golgi apparatus in these organisms was tagged using GFP variants and the elongation and medial fission followed by video microscopy. The structural intermediates of the dividing Golgi were corroborated by three-dimensional EM reconstructions (Pelletier et al., 2002).

The other model is de novo biogenesis, in which new Golgi appear at new ER export sites. This model has been nicely demonstrated in *Pichia pastoris*, a budding yeast with stacked Golgi cisternae located next to ER export sites (Bevis et al., 2002). There are sufficiently few Golgi to make it possible to visualize the process of new Golgi synthesis. The results clearly show that new Golgi appear at new ER export

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Abbreviation used in this paper: CGN, cis Golgi network.

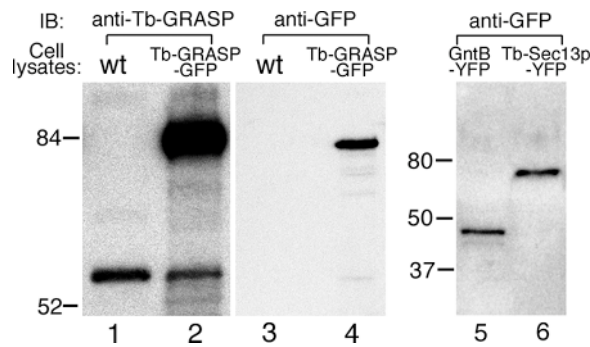


Figure 1. Protein characterization of *T. brucei* markers. Whole cell lysates (5×10^6 cells in each lane) from wild-type (wt) parasites (lanes 1 and 3) and those stably expressing Tb-GRASP-GFP (lanes 2 and 4), GntB-YFP (lane 5), or Tb-Sec13p-YFP (lane 6) were fractionated by SDS-PAGE and processed for immunoblotting. Polyclonal antibodies to Tb-GRASP revealed a single 55-kD protein in wild-type parasites (lane 1) and an additional 84-kD protein in parasites stably expressing Tb-GRASP-GFP (lane 2), which also reacted with polyclonal antibodies to GFP (lane 4). Cells stably expressing GntB-YFP or Tb-Sec13p-YFP each yielded a single species of protein recognized by anti-GFP antibodies (lanes 5 and 6, respectively).

sites. They do not appear as outgrowths of preexisting Golgi. The new Golgi can also appear in the daughter cell, suggesting that duplication and partitioning can be coupled processes.

An implied feature of de novo biogenesis is that the new Golgi components are funneled through the new ER export site. However, there is no evidence for this finding. Some or even all of the components could be funneled through the old Golgi using the new ER export site as a positional marker.

This possibility was raised by the studies reported here on another protozoan parasite, *Trypanosoma brucei*, the causative agent of sleeping sickness in man and Nagana in ruminants. This parasite also has a single Golgi that is duplicated during the cell cycle, and examination of fixed images had suggested that duplication might be a templated process (Duszenko et al., 1988; Field et al., 2000). Using video microscopy, we show that the new Golgi appears de novo next to the new ER export site. Nevertheless, photobleaching studies show that the old Golgi plays an important role, supplying some components to the new, suggesting that the two models for duplication are more similar than initial inspection might suggest.

Results

Tb-GRASP

To study Golgi duplication in *T. brucei*, the behavior of a Golgi matrix protein, belonging to the GRASP family, was followed. In mammals, the GRASP proteins have been implicated in the stacking of Golgi cisternae (Barr et al., 1997; Linstedt, 1999) and other regulatory events at the onset of mitosis (Colanzi et al., 2003). The *T. brucei* homologue Tb-GRASP is most similar to GRASP55 (36.3% similarity, 26.9% identity; Shorter et al., 1999).

Antibodies raised to Tb-GRASP identified a single protein of 55 kD in immunoblots of whole cell lysates (Fig. 1,

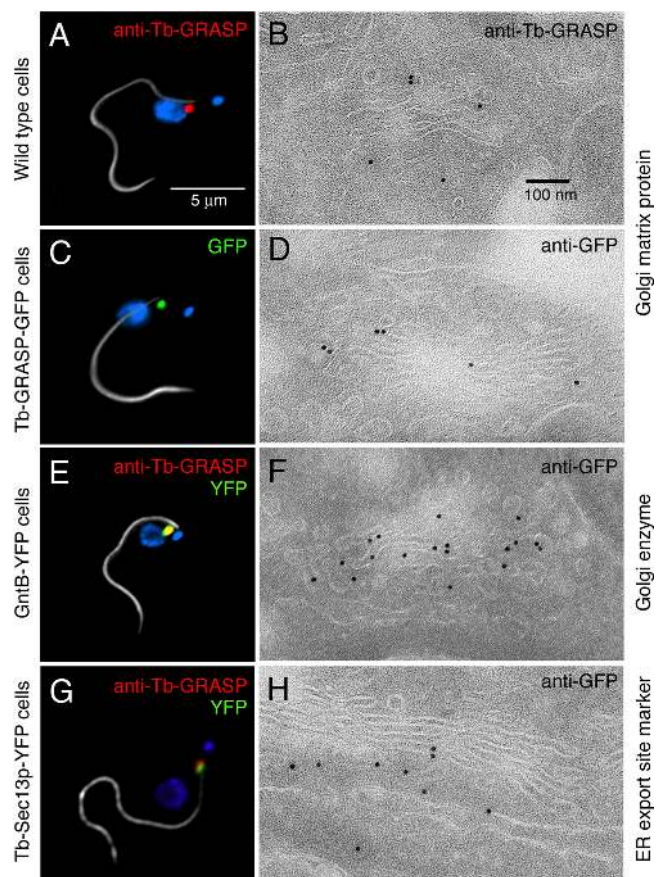


Figure 2. Characterization of markers for *T. brucei* Golgi and ER export sites. (A, C, E, and G) Fluorescence microscopy of fixed cells early in the cell cycle showing a single Golgi structure (red, observed using antibodies to Tb-GRASP [A, E, and G], or green, fluorescence from Tb-GRASP-GFP [C]), a single flagellum (gray; using anti-PAR), a single nucleus (blue; DAPI), and a single kinetoplast (smaller blue structure containing mitochondrial DNA; DAPI). Note that the putative Golgi enzyme marker GntB-YFP (E, green) and the matrix marker Tb-GRASP (E, red) colocalized (yellow), whereas the ER export site marker Tb-Sec13p-YFP (G, green) labeled a structure closely associated with the Golgi (G, red). (B, D, F, and H) Immunolabeling of wild-type parasites (B), cells stably expressing Tb-GRASP-GFP (D), GntB-YFP (F), or Tb-Sec13p-YFP (H) using antibodies to Tb-GRASP (B) or GFP (D, F, and H). Antibodies were visualized using 10 nm protein A-gold. Note that the matrix (GRASP) and putative enzyme (GntB) markers were localized to the Golgi stack, whereas the ER export site marker (Tb-Sec13p) was localized between the Golgi stack and the associated ER.

lane 1) and labeled, by immunofluorescence microscopy, one or a few structures near the kinetoplast, the single mass of mitochondrial DNA, in the posterior region of the cell (Fig. 2 A). These structures were shown to be Golgi by immunogold labeling (Fig. 2 B), and quantitation showed that the density of labeling over the Golgi stacks was >3.5 -fold that of the rest of the cell (Table I).

The full-length Tb-GRASP was fused to GFP and stable lines selected. Antibodies to Tb-GRASP showed an additional protein of 84 kD, expressed at about fivefold the level of the endogenous protein in wild-type cells (Fig. 1, lane 2). Antibodies to GFP recognized this but not the endogenous protein (Fig. 1, compare lanes 3 and 4). Fluorescence microscopy showed that the structures revealed by Tb-GRASP-

Table I. Quantitation of immunogold labeling of *T. brucei* markers

Gold particle count	Anti-Tb-GRASP		Anti-GFP	
	Wild-type cells	Tb-GRASP-GFP cells	GntB-YFP cells	Tb-Sec13p-YFP cells
Golgi area ^a	2.9 ± 0.6	5.6 ± 1.8	11.8 ± 4.2	
ER export area ^b				14.6 ± 3.2
Other parts of the cell	0.8 ± 0.7	1.1 ± 0.9	1.6 ± 1.3	3.0 ± 1.7

Immunogold labeling in Fig. 2 (B, D, F, and G) was quantitated, and the results are presented as the number of gold particles per $\mu\text{m}^2 \pm \text{SD}$ ($n = 15$ cells).

^aThe Golgi area was defined as the region containing Golgi stacks and associated vesicles.

^bThe ER export area was defined as the region between the cis-cisterna of the Golgi stack and the associated ER.

GFP were the same as those labeled by antibodies to Tb-GRASP (Fig. 2 C). Immuno-EM confirmed this location (Fig. 2 D) and showed that the density of labeling for Tb-GRASP-GFP over the Golgi stacks was >5-fold than that of the rest of the cell (Table I), validating its use as a marker for studies of Golgi biogenesis.

Duplication of Golgi and ER export sites

Video fluorescence microscopy was used to follow the Golgi during one cell cycle, which lasts 7–8 h. During G1, each cell contained a single Golgi structure, as shown in the still images in Fig. 3 A and Video 1 (available at <http://www.jcb.org/cgi/content/full/jcb.200311076/DC1>). Then, a small structure appeared de novo, which grew over the next ~ 2 h until it was about the same size as the old Golgi. Later on, toward the end of the cell cycle, more Golgi appeared, near to the preexisting Golgi. Most of these Golgi disappeared just before the mother cell underwent cytokinesis. The function of these additional Golgi is unclear. A similar excess of Golgi has been observed before in the blood-

stream form of *T. brucei* (Field et al., 2000) and was also observed near the end of the cell cycle in *T. gondii* (Pelletier et al., 2002).

The biogenesis of ER export sites was followed next. These ER export sites are closely associated with the Golgi (McConville et al., 2002) as they are in *T. gondii* (Hager et al., 1999) and *P. pastoris* (Bevis et al., 2002). Parasite cells expressing the Sec13p export site marker (Payne et al., 2000; Bevis et al., 2002; Tb-Sec13p: 37.7% similarity, 28.6% identity to *P. pastoris* Sec13p), tagged with YFP, emphasized this tight association with the Golgi by both immunofluorescence microscopy (Fig. 2 G) and immuno-EM (Fig. 2 H). The density of gold labeling over the ER export site was >4.5-fold that over the rest of the cell (Table I). Antibodies to GFP recognized a single protein of ~ 70 kD in immunoblots of whole cell lysates from parasites expressing Tb-Sec13p-YFP (Fig. 1, lane 6). The tight association between the Golgi and the ER export sites persisted throughout the cell cycle as shown using parasites stably expressing Tb-Sec13p-YFP and Tb-GRASP-CFP (Tb-GRASP fused to

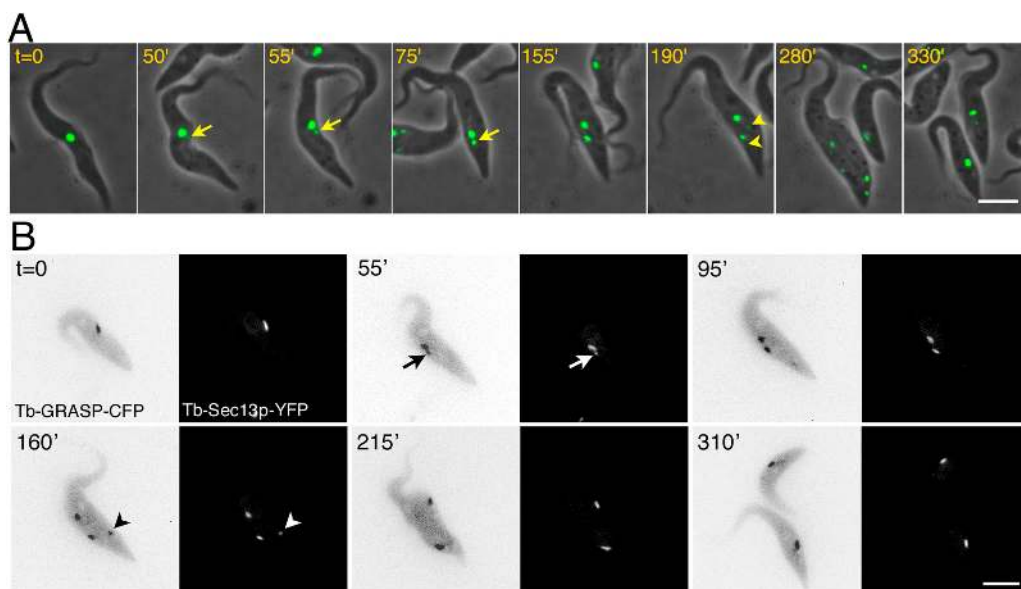


Figure 3. **Biogenesis of Golgi and the ER export site.** (A) Cells stably expressing the Golgi marker Tb-GRASP-GFP were imaged during one cell cycle, and the fluorescence and phase images were merged. Selected images are shown to illustrate the growth of the new Golgi (arrows) and the subsequent appearance of smaller Golgi (arrowheads). Note that the new Golgi is always situated closer to the stumpy (posterior) end of the cell than the old. The complete image sequence is available as Video 1 (available at <http://www.jcb.org/cgi/content/full/jcb.200311076/DC1>). (B) Cells stably expressing both Tb-GRASP-CFP (left) and Tb-Sec13p-YFP (right) were imaged during one cell cycle (Video 2). Selected time points illustrate the coincident assembly of Golgi and ER export sites during duplication (arrows) and subsequent generation of additional, smaller structures (arrowheads). Bars, 5 μm .

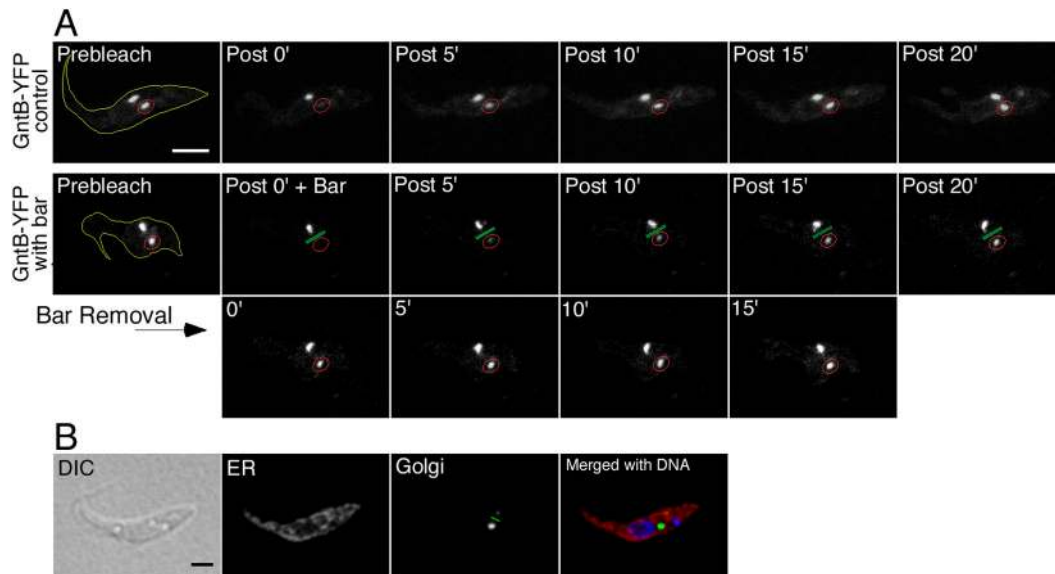


Figure 4. **Identifying the source of new Golgi components using FRAP analysis.** (A) The new Golgi (red circles) in cells stably expressing the putative Golgi enzyme GntB-YFP was photobleached and allowed to recover in the absence (top) or presence (middle) of a bleaching bar (green; 10-s bleaching, 50-s rest) placed equidistant between the old and the new Golgi. The bleaching bar was then removed (bottom) to allow recovery. (B) The same cells were fixed and labeled for ER using antibodies to BiP (ER). Comparison with the DIC image (DIC) shows that the ER pervades the entire cell cytoplasm. The green bar between the old and new Golgi (Golgi) is representative of that used for repetitive photobleaching. Note that it overlies a small portion of the ER (Merged with DNA). Bars, 5 μ m.

cyan fluorescent protein). As shown in the still images (Fig. 3 B) and Video 2 (available at <http://www.jcb.org/cgi/content/full/jcb.200311076/DC1>), the new ER export sites appeared at exactly the same time as the new Golgi (Fig. 3 B, arrows), and this association continued even when additional Golgi appeared (Fig. 3 B, arrowheads) later in the cell cycle.

Photobleaching experiments

The role played by the old Golgi in the synthesis of the new was tested by photobleaching experiments. Tb-GRASP-GFP could not be used because it was found to exchange rapidly through the cytoplasm. Similar observations have been made for the GFP-tagged versions of mammalian GRASP65 (Ward et al., 2001). Instead, a membrane spanning protein was used, a putative Golgi enzyme (GntB), identified by its similarity to a UDP-GlcNAc:Thr/Ser mucin-type polypeptide α -GlcNAcTransferase from *Dictyostelium discoideum* (Wang et al., 2003; West, 2003; West et al., 2004). A fusion of the NH₂-terminal retention domain to YFP (GntB-YFP) was shown to localize specifically to the Golgi by both immunofluorescence microscopy (Fig. 2 E) and immuno-EM (Fig. 2 F). The Golgi labeling density was >7-fold than that of the rest of the cell (Table I). GntB-YFP colocalized with Tb-GRASP at all stages of the cell cycle (Fig. 2 E and not depicted). Antibodies to GFP recognized a single protein of \sim 45 kD in immunoblots of whole cell lysates from parasites expressing GntB-YFP (Fig. 1, lane 5).

Photobleaching was performed when the new Golgi was \sim 50–80% of the size of the old and recovery occurred within 20–25 min (Fig. 4 A, top). Recovery was quantitated at each time point by measuring the fluorescence in the new Golgi and expressing it as a fraction of the total fluorescence

(in the new and old Golgi). This approach corrected for the gradual diminution in fluorescence, which varied from 5 to 30% during the 30-min observation period. Expressing this fraction as a percentage of the prebleach value allowed data from different cells to be averaged so as to derive statistically meaningful results. Fig. 5 A (blue diamonds) shows the recovery curve for the new Golgi after photobleaching. Cycloheximide experiments show that some of the fluorescence during recovery derives from new synthesis, whereas the rest derives from newly synthesized GntB-YFP in the process of folding up in the ER (unpublished data). The important question was the route being taken. Was the newly assembled protein moving directly from the ER to the new Golgi, or was it moving via the old Golgi?

To test this question, the new Golgi was photobleached, and the region between the old and the new was repetitively photobleached (10 s of bleach followed by 50 s of rest). As shown in Fig. 4 A (middle) and quantitated in Fig. 5 A (red squares), this process slowed both the rate and extent of fluorescence recovery in the new Golgi, consistent with delivery from the old. Removal of the bar led to full recovery of fluorescence in the new Golgi (Fig. 4 A, bottom; and quantitated in Fig. 5 B, green triangles), showing that this photobleaching regime had no irreversible, toxic effects on Golgi duplication.

Importantly, the position of the photobleaching bar was crucial. When moved to the other side of the new Golgi (but at the same distance from it), there was no effect on the recovery of the new Golgi (Fig. 5 A, yellow triangles). There was also no effect when the bar was placed on the other side of the old Golgi and over other parts of the cytoplasm (unpublished data). There remained the possibility that the ER was mediating this transfer of material between the two

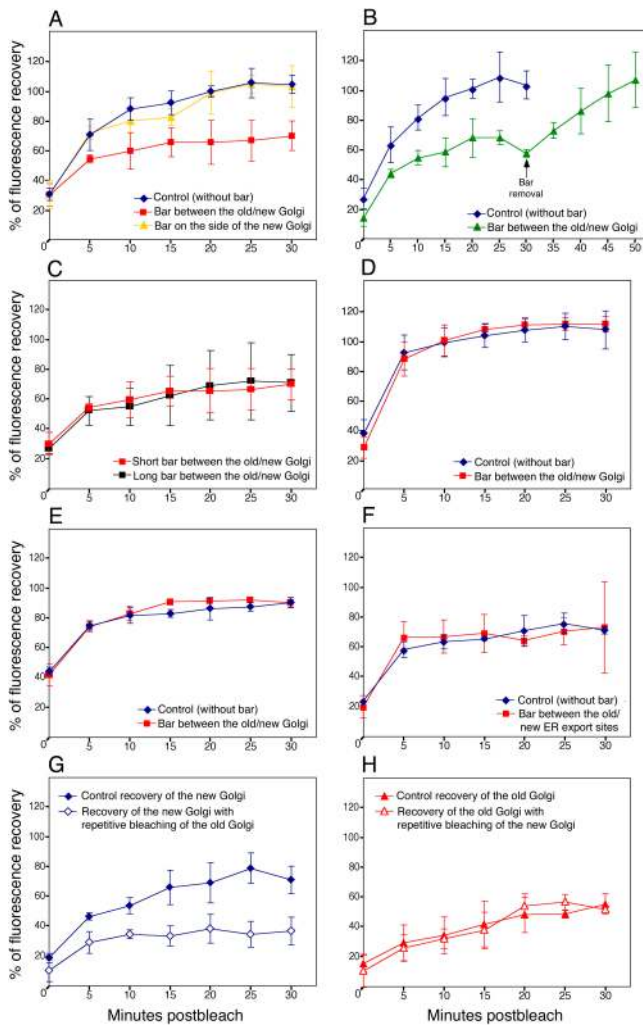


Figure 5. Quantitation of recovery after photobleaching. (A) Recovery after photobleaching the new Golgi. Note that the rate and extent was delayed in the presence of the photobleaching bar (red squares) when compared with the control (blue diamonds), but not when the bar was placed at the same distance on the other side of the new Golgi (yellow triangles). (B) Recovery after removal of the photobleaching bar. Removal of the bar at 30 min (arrow) allowed full recovery of the new Golgi. (C) A longer photobleaching bar, transecting the entire cell (black squares), did not inhibit recovery of the new Golgi more than the normal bar which was $\sim 1/3$ the width of the cell (red squares). (D) Recovery after photobleaching the old Golgi was not affected by a bar placed between the two Golgi. (E) Recovery of the fully grown new Golgi (the same size as the old) was not affected by a photobleaching bar between the two Golgi. (F) Bleaching the new ER export site in parasites stably expressing Tb-Sec13p-YFP. Recovery was not affected by the presence of the photobleaching bar. (G) Repetitive photobleaching of the old Golgi inhibited recovery of the new. (H) Repetitive bleaching of the new Golgi did not affect recovery of the old. Results in A–F are presented as the mean \pm SD ($n = 5$), and results in G and H are presented as the mean \pm SD ($n = 3$). Fluorescence recovery was calculated as a percentage of the prebleach value either corrected (A–E) or not (G and H) for photobleaching during the course of the experiment (see Materials and methods).

Golgi and that the photobleaching bar was interrupting this flow. Because the ER in this parasite pervades the entire cell cytoplasm (Bangs et al., 1993; Fig. 4 B using an antibody to the ER chaperone, BiP), we reasoned that a longer pho-

to-bleaching bar transecting the cell should have a greater inhibitory effect than the shorter bar. As shown in Fig. 5 C, a threefold increase in the length (and hence intensity) of the photobleaching bar had no additional inhibitory effect on the recovery of the new Golgi. Together, these data suggest a direct, focused flow of material between the two Golgi that does not involve the ER.

Photobleaching the old Golgi led to recovery that was not affected by the photobleaching bar (Fig. 5 D), showing that the net flow was from old to new. This flow stopped once the new Golgi reached the same size as the old because the photobleaching bar then had no effect on the recovery of the new Golgi (Fig. 5 E). To further show that the old Golgi was indeed the source of material needed for recovery of the new Golgi after bleaching, the recovery of the new Golgi was monitored while the old Golgi was repetitively photobleached, and vice versa. Recovery of the new Golgi was inhibited by repetitive photobleaching of the old (Fig. 5 G), whereas recovery of the old Golgi was not affected by photobleaching of the new (Fig. 5 H). Note that the lower percentage recoveries were the consequence of not being able to correct for the gradual diminution in fluorescence during the course of these experiments (see Materials and methods). Together these data suggest a net flow from the old to the new Golgi, but only during the growth phase.

Experiments were also performed using cells stably expressing both Tb-Sec13p-CFP and GntB-YFP. This procedure allowed the ER export sites to be monitored during repetitive photobleaching and to show that they were not being targeted. This experiment eliminated the possibility that the slower recovery in Fig. 5 A was the consequence of inadvertent repetitive bleaching of the new ER export site (not depicted). Conversely, when cells expressing only Tb-Sec13p-YFP were used to photobleach the new ER export site and monitor recovery in the absence or presence of repetitive photobleaching, no difference was observed (Fig. 5 F). This finding argues strongly that the route taken to assemble new ER export sites is not the same as that for new Golgi, at least for the proteins being studied.

Role of the basal bodies

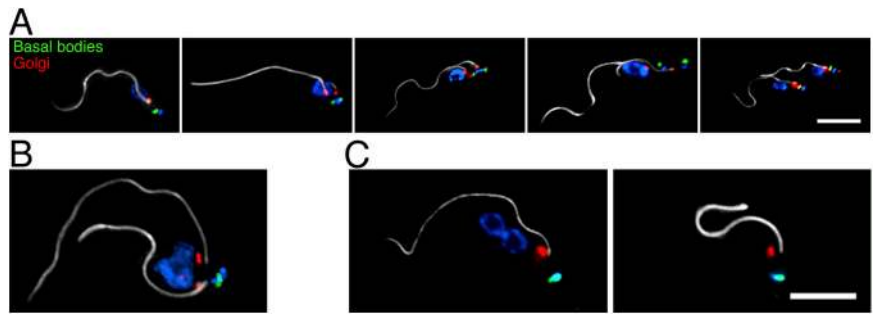
Earlier work suggested a relationship between the Golgi and the basal bodies, the centrosomes in these parasites (Field et al., 2000). Basal bodies nucleate the flagella that originate in the flagellar pocket and run the length of the organism (Gull, 1999). They are also tightly associated with the kinetoplast and drive its segregation during mitosis (Robinson and Gull, 1991; Fig. 6 A). Duplication of the basal bodies was closely followed by the appearance of a new Golgi near to the new basal body, and this relationship was maintained through the rest of the cell cycle (Fig. 6 A). Measurements showed that the growing new Golgi was $1.40 \pm 0.36 \mu\text{m}$ ($n = 53$) from the old Golgi and always situated toward the more posterior end of the mother cell.

Basal body and kinetoplast separation can be inhibited by anistomicin-p (Robinson and Gull, 1991), and this also slowed the separation of the old and new Golgi. This result is readily seen by comparing parasites at the same cell cycle stage (Fig. 6, compare A, extreme right panel, with B). Nuclear separation can be inhibited by rhizoxin (Robinson et

Figure 6. The site of new Golgi synthesis is determined by the new basal body.

(A) Fixed parasites at various stages of the cell cycle were labeled for Golgi (red; anti-Tb-GRASP), basal bodies (green; YL1/2 antibody), flagella (gray; anti-PAR), and DNA (blue; DAPI). Note that the anti-PAR antibodies only label the exposed part of the flagella (outside the flagellar pocket) so that there is a gap between the base of the flagella and the basal bodies. (B) Parasites were treated with anistomicin-p to prevent basal body segregation and subsequent cell division.

(C) Parasites were treated with rhizoxin to prevent nuclear separation, and so generate a daughter parasite with two nuclei (left) and a daughter with none (a "zoid;" right). Note the strict spatial relationship between the Golgi and basal bodies at all cell cycle stages (A) and after drug treatments (B and C). Bars, 5 μ m.



al., 1995), which leads to zoids lacking a nucleus but still containing one of the two basal bodies. Again the Golgi followed the basal body (Fig. 6 C). These pharmacological studies complement and confirm the conclusions of earlier ones using TbRAB31, a putative Rab protein, as a Golgi marker (Field et al., 2000).

Discussion

Using video fluorescence microscopy and Golgi proteins tagged with variants of GFP, it has been possible to follow the duplication of the Golgi apparatus in the protozoan parasite *T. brucei*. A matrix protein and a putative enzyme were chosen for study because in mammals there is evidence that these two types of Golgi protein can behave differently during certain phases of the Golgi life cycle (Seemann et al., 2000, 2002). The GRASP family of matrix proteins has been implicated in cisternal stacking and other signaling events. A single homologue was identified by searching the *T. brucei* genome that was most similar to mammalian GRASP55 and had the same domain structure: a predicted NH₂-terminal myristic acid that aids membrane binding; a highly homologous NH₂-terminal region (50% identity) that in mammals binds to the coiled-coil matrix protein GM130 (Barr et al., 1998); and a poorly conserved COOH-terminal region, rich in serines and prolines, which in mammals is thought to mediate mitotic regulation (Shorter and Warren, 2002). Immunolabeling at the EM level showed that Tb-GRASP was localized to the Golgi apparatus. The same pattern of labeling was seen in parasites stably expressing Tb-GRASP tagged with variants of GFP at the COOH terminus at all stages of the cell cycle. This finding validated its use for studies of Golgi biogenesis.

Golgi enzymes are poorly conserved across species, but comparison with *D. discoideum* revealed a putative GlcNAc-Transferase that was termed GntB (Wang et al., 2003; West, 2003; West et al., 2004). These proteins are 32% identical and 56% similar over a stretch of ~280 amino acids that constitutes the catalytic domain, based on comparison with the distantly related animal polypeptide α -GalNAc-Transferases. A UDP-GlcNAc:Thr polypeptide GlcNAc-Transferase-like activity has been detected in extracts of *T. brucei* (Dieckmann-Schuppert et al., 1994). GntB has the same domain structure as all other Golgi enzymes: an NH₂-terminal membrane-spanning domain that should act as a retention

signal, followed by a luminal catalytic domain (Munro, 1998). When attached to variants of GFP, the NH₂-terminal domain did indeed act as a retention domain, localizing the fusion protein to the Golgi apparatus and validating its use as a membrane marker of the *T. brucei* Golgi.

The results are clearly consistent with de novo biogenesis. The new Golgi appears near to the old Golgi and grows until it is the same size. The same pattern was observed for both Tb-GRASP and GntB. There was no evidence for templated biogenesis. From the earliest time at which the new Golgi could be discerned (~1% of the size of the old Golgi), it appeared to be separate from the old. There was also no evidence that the old Golgi grew and then "shed" a new Golgi, though a sufficiently small Golgi might be below the resolution of the methods used.

The de novo appearance of the new Golgi raised the question as to the source of its membrane components. The most obvious possibility was the new ER export site because in *P. pastoris*, there is evidence that new ER export sites appear just before the new Golgi (Bevis et al., 2002). This possibility was tested in *T. brucei* using the homologue of the Sec13p COPII coat component tagged with GFP variants. Immuno-EM localized this construct to the region between the ER and the Golgi, which is consistent with labeling of ER export sites. Coexpression of tagged Tb-Sec13p with either tagged Tb-GRASP or GntB showed close association at all stages of the cell cycle. Even time intervals of 1 min were not sufficient to resolve any difference in the appearance of the new Golgi and ER export site.

Another possible source was the old Golgi. In some experiments, there did appear to be movement of fluorescent material from the old to the new Golgi, but the observations were not consistent and the material could not be resolved using the available methods. Therefore, an indirect approach was taken, using a photobleaching bar to interrupt any transfer that might be occurring, or directly bleaching the old Golgi to deplete the source of material. Tagged GntB was used because it can only move from one Golgi to the other via membranes. Tagged GRASP proteins can move through the cytoplasm. Photobleaching the new Golgi led to rapid recovery that was slowed by repetitively photobleaching the old Golgi or the region between the old Golgi and the new. Photobleaching the old Golgi led to recovery that was not affected by the photobleaching bar or repetitive photobleaching of the new Golgi, showing that the net

movement was from old to new. Timing was important. Inhibition of recovery was observed when the new Golgi was growing, but once it had reached full size, the photobleaching bar had no effect. The position of the photobleaching bar was also critical. When placed on the other side of the new Golgi, but at the same distance from it, no inhibition was observed. Nor was any inhibition observed when the bar was placed over other regions of the cytoplasm.

Together, these data argue that membrane components are moved from the old to the new Golgi, starting about two hours into the cell cycle and cease about two hours later, once the new Golgi has grown to the same size as the old. The route taken is presently unclear. It seems unlikely that transfer occurs via the ER because increasing the length of the photobleaching bar had no additional inhibitory effect on the recovery of the new Golgi. This finding occurred despite the fact that the bar transected the cell, so that it could now interrupt more of the potential flow of material from old to new Golgi mediated by the ER. In addition, photobleaching the new ER export site led to recovery that was not affected by the photobleaching bar, showing that the route taken, at least by the TbSec13p, is different from that taken by GntB. Together, these data suggest that transfer is not mediated via the ER but directly between the two Golgi. One attractive possibility is that transfer is mediated by elements of the cytoskeleton, especially because the basal bodies play a role in determining the site of the new Golgi and ER export site. Experiments are currently underway to examine the possibility that either microtubules or actin filaments might mediate the transfer. It is particularly striking that the movement of membrane components from the old to the new Golgi is reminiscent of the biogenesis of vacuoles in budding yeast (Tang et al., 2003). In this organism, tubules and vesicles move from the mother vacuole to the daughter vacuole in the bud in a process that depends on actin filaments and a myosin V-type motor.

The nature of the transfer carriers that mediate new Golgi growth is presently unknown. One attractive idea is that they are COPI vesicles because these can carry Golgi enzymes in mammalian cells (Lanoix et al., 2001; Martinez-Menarguez et al., 2001). Furthermore, in the cisternal maturation model for Golgi transport, retrograde moving COPI vesicles carrying enzymes remodel cisternae so that they mature (Glick and Malhotra, 1998). At the earliest step, COPII vesicles from the ER fuse with retrograde COPI vesicles to form the first cisterna. It is easy to imagine that these same vesicles could be transferred to the new ER export site, to meet outgoing COPII vesicles, and form the first new cisterna that then matures. Future work needs to focus on the precise components that the old Golgi and the new ER export site supply to the new Golgi, and on the signals that initiate and terminate the duplication process.

There is also a need to rationalize these data with those obtained in other organisms. For *P. pastoris*, one would simply need to postulate that the new Golgi that appear near to the new ER export sites are supplied, in part or in whole, by preexisting Golgi. This possibility was raised by the authors and is apparent in some of the presented figures and videos (Bevis et al., 2002). For *T. gondii*, it is more difficult, but there are similarities because in templated biogenesis the old

Golgi supplies the new, just as we now suggest it does for de novo biogenesis. The main difference is that the new Golgi appears initially to be an extension of the old rather than an independent entity. This finding in turn leads to a requirement for a fission mechanism to divide the elongated Golgi into two. Only further work will determine whether or not this fission mechanism uniquely defines another mode of Golgi duplication.

Materials and methods

Cell culture and transgene expression

Procyclic *T. brucei rhodesiense* Y Tat1.1 cells were used throughout the experiments. Cells were grown and transfected as described previously (Fantoni et al., 1994).

DNA constructs

T. brucei GRASP (TRYP11.0.004609_3) and Sec13p (TRYP10.0.001920_17) were obtained from the Sanger Institute website at http://www.sanger.ac.uk/Projects/T_brucei/. Sequencing of the *T. brucei* genome was accomplished as part of the Trypanosoma Genome Network with support by the Wellcome Trust. The coding sequences were fused to the NH₂ terminus of GFP by cloning the full-length coding sequence into the pXSGFPM3FUS vector (Bangs et al., 1996; Marchetti et al., 2000). The NH₂-terminal 100 amino acids of GntB (GenBank/EMBL/DBJ accession no. AY304297) that contains the spanning domain and flanking regions necessary and sufficient for Golgi targeting were cloned into the same GFP expression vector. YFP and CFP fusions to these proteins were made by replacing GFP with EYFP or ECFP (CLONTECH Laboratories, Inc.) in the same expression vectors.

Antibody production

Full-length Tb-GRASP was fused to GST and fusion protein expressed and purified according to manufacturer's instructions (Amersham Biosciences). Purified protein was used to immunize rabbits and to affinity purify the serum.

Immunofluorescence assays

T. brucei cells were fixed and permeabilized with cold methanol at -20°C and blocked with 3% BSA before antibody staining. YL1/2 antibody (abcam) was used at 1:500 to stain the basal bodies. A monoclonal anti-PAR antibody (Ismach et al., 1989) was used at 1:10,000 to stain the flagellum. The anti-Tb-GRASP was used at 1:1,000 to stain the Golgi. To stain the ER, cells were fixed and permeabilized using 4% PFA followed by 0.25% Triton X-100 at RT, and then quenched and stained with a polyclonal anti-BiP antibody (Bangs et al., 1993) at a 1:1,000 dilution. Fixed cells were observed using an upright microscope (model Axioplan2; Carl Zeiss MicroImaging, Inc.) equipped with a CCD camera (model Orca-II; Hamamatsu) and a Plan-Apochromat 100× 1.4-NA DIC objective. Images were acquired and processed using Openlab software (Improvision). To image the flagellum, serial optical sections 0.3 μm apart were acquired, deconvolved, and superimposed for a two-dimensional presentation.

Live cell imaging

T. brucei cells were plated on the surface of agarose gels prepared in cultivation medium and air dried briefly to limit cell motility. The gel was placed in an imaging dish (MatTek) with the cell side facing the coverslip. Cells were imaged at 28°C in a CO₂ perfusion chamber using an inverted microscope (model Axiovert 100M; Carl Zeiss MicroImaging, Inc.) equipped with a CCD camera (model Orca-100; Hamamatsu) and a Plan-Apochromat Ph3 100× 1.4-NA objective. For simultaneous imaging of CFP and YFP, we used an inverted microscope (model IX-70; Olympus) equipped with a TILL Imago QE cooled CCD camera and a 60× 1.45-NA Plan-Apochromat objective. Images were acquired and processed using TILL image software.

Immunoelectron microscopy

Y Tat1.1 cells and those stably expressing Tb-GRASP-GFP, GntB-YFP, or Tb-Sec13p-YFP were fixed with 4% PFA and 0.1% glutaraldehyde and further processed for cryosectioning. Sections were probed with anti-GRASP or an affinity-purified polyclonal antibody against GFP (Seedorf et al., 1999), followed by protein A-gold and quantified using standard stereological methods.

FRAP analysis

FRAP analysis was performed using a confocal microscope (model LSM510; Carl Zeiss Microimaging, Inc.) equipped with a C-Apochromat 40 × 1.2-NA objective and a Tempcontrol 37–2 digital system for temperature control. YFP-expressing cells were photobleached using a 514-nm laser at full power output (30 mW) for 50 repeats (~10 s). Fluorescence intensity was measured using the histogram function available in the LSM510 software, and all fluorescence readings were adjusted by subtracting the background fluorescence. In Fig. 5 (A–F), the fluorescence recovery was calculated for each time point by measuring the fluorescence in either the new or old Golgi and expressing the result as a fraction of the total fluorescence (in the new and old Golgi). This number was then expressed as a percentage of the prebleach value, and the results from five experiments were combined to obtain the mean ± SD. In Fig. 5 (G and H), where both the old and the new Golgi were bleached, the fraction could not be calculated, so the fluorescence at each time point was expressed as a percentage of the prebleach value for the Golgi being monitored. The results from three experiments were used to obtain the mean ± SD.

SDS-PAGE and Western blotting

Protein gel electrophoresis was performed using 12% SDS-PAGE gels. For Western blotting, proteins were transferred onto nitrocellulose membranes, and antigens were detected using the ECL system (Amersham Biosciences).

Online supplemental material

Two videos are provided as online supplemental material. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200311076/DC1>.

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