Mitotic disassembly of the Golgi apparatus in vivo

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SUMMARY

Populations enriched in prophase cells were obtained either by using a cell line with a temperature-sensitive mutation in the mitotic kinase, p34cdc2, or by treating cells with olomoucine, an inhibitor of this kinase. Both methods resulted in efficient and reversible block of the cells at the G2/M boundary. After cells were released from the cell cycle block, the morphological changes to the Golgi apparatus were characterised using both quantitative conventional electron microscopy and immuno-gold microscopy. The early mitotic phases were divided into six stages (G2 to pro-metaphase) based on the morphology of the nucleus. During prophase the cross-sectional length of Golgi stacks decreased prior to unstacking. At the same time, small vesicular profiles, typically 50-70 nm in diameter, accumulated in the vicinity of the stacks. The disappearance of Golgi stacks was accompanied by the transient appearance of tubular networks. By the time cells entered prometaphase, the stacks had completely disassembled and only clusters consisting of Golgi vesicles and short tubular elements were left. When cells were released from the G2/M boundary and pulsed briefly with [AlF4]− to prevent uncoating of transport vesicles, vesicular profiles with a morphology reminiscent of COP-coated vesicles appeared. These vesicular profiles were either associated with Golgi stacks or, at later stages, with clusters, but were formed at all stages of disassembly. Together these results provide further support for our model that continued budding of vesicles from the rims of Golgi cisternae is at least partly responsible for the disassembly of the Golgi apparatus.

Key words: Golgi, mitosis, FT210, olomoucine

INTRODUCTION

The Golgi apparatus in animal cells is a ribbon-like structure comprising discrete stacks of cisternae linked by tubules that connect equivalent cisternae in adjacent stacks (Rambourg et al., 1987). Dynein-microtubule motors ensure that the Golgi apparatus resides in the peri-centriolar region, as a compact, reticular structure (Corthesy-Theulaz et al., 1992).

When animal cells enter mitosis, membrane traffic ceases and the architecture of the Golgi apparatus alters dramatically (Warren, 1993). The single copy organelle is first converted into several hundred Golgi vesicle clusters and, in a second step, into thousands of free Golgi vesicles which become randomly distributed throughout the mitotic cytoplasm (Lucocq and Warren, 1987; Lucocq et al., 1989; Zeligs and Wollman, 1979). Division of the mother cell into two, equally-sized daughters then ensures nearly equal partitioning by a stochastic process (Birky, 1983). During telophase, the fragmentation process is reversed (Lucocq et al., 1989) and membrane traffic resumes (Warren, 1993). By the time newly-synthesised proteins from the ER reach the Golgi apparatus, it has completely reassembled in each daughter cell (Souter et al., 1993).

The breakdown of the Golgi apparatus begins during prophase and most likely involves the severing of the tubules connecting adjacent stacks (Colman et al., 1985; Rothman and Warren, 1994). At the level of light and fluorescence microscopy, the single copy of the Golgi apparatus is seen to break down into multiple, discrete structures that assume a more peri-nuclear distribution (Burke et al., 1982; Cajal, 1914). These discrete stacks then undergo complete fragmentation generating up to 300 vesicle clusters, termed Golgi clusters (Lucocq and Warren, 1987). These were shown to be derived from the Golgi apparatus by their content of resident Golgi enzymes and markers (Lucocq et al., 1987, 1989; Lucocq and Warren, 1987; Pypaert et al., 1993). In HeLa cells, the clusters are up to 1 μm in diameter and comprise aggregates of vesicular profiles (47 nm in diameter), large vesicular profiles (up to 250 nm in diameter) and tubular profiles (Lucocq et al., 1987). Statistical analysis showed that there was an inverse correlation between the volume density of Golgi clusters and the number of free vesicles. In other words, the clusters shed free vesicles into the surrounding cytoplasm and a total of up to ten thousand vesicles could be released (Lucocq et al., 1989). This shows that the clusters are themselves intermediates on the disassembly pathway and that free vesicles are the major, final product of Golgi disassembly.

A simple model based on the known inhibition of intra-Golgi transport (Collins and Warren, 1992; MacKay et al., 1993; Stuart et al., 1993) can largely explain this vesiculation process. COP-coated vesicles, involved in both anterograde (Rothman, 1994) and retrograde (Letourneur et al., 1994) transport, mediate traffic within the Golgi apparatus. At the...
onset of mitosis we proposed that transport vesicles would continue to bud from Golgi cisternae but could no longer fuse with their target membrane (Warren, 1985). Since the membrane lost through vesicle budding is normally compensated by fusion of an incoming vesicle, the necessary consequence of such an inhibition would be the accumulation of transport vesicles and a shortening of the cisternal length. Cisternae would also need to be unstacked, to make the membrane accessible to the budding mechanism.

Evidence in favour of this model has been obtained using a cell-free system that mimics the fragmentation of the Golgi apparatus (Misteli and Warren, 1994). Purified rat liver Golgi stacks, incubated with mitotic HeLa cytosol, yielded fragments indistinguishable from those seen in vivo in mitotic Golgi clusters (Lucocq et al., 1987, 1989; Lucocq and Warren, 1987; Pypaert et al., 1993). As predicted by the model, COP-coated vesicles were shown to bud at the same rate under interphase and mitotic conditions and the coatomer subunit of COP coats was shown to be essential for the production of mitotic Golgi vesicles. Furthermore, the cross-sectional length of cisternae decreased as vesicles accumulated. More recently we have used this cell-free system to show that there is an additional pathway for fragmentation that involves conversion of Golgi membranes into tubular networks. At least some of these networks break down into smaller fragments by a mechanism that does not depend directly on the COP-mediated budding mechanism (Misteli and Warren, unpublished).

It has proven difficult to study the disassembly of the Golgi apparatus in vivo at high resolution. Cells have to be synchronised to obtain sufficient numbers for analysis by electron microscopy and all synchronisation methods yield mitotic populations that are depleted of cells undergoing prophase (Klevecz, 1972), the stage when Golgi disassembly occurs. The earliest stage that can be studied in detail is pro-metaphase and, by that time, the Golgi apparatus has already been converted into clusters of vesicles (Lucocq et al., 1987, 1989; Lucocq and Warren, 1987).

We have overcome this problem by focusing on the mitotic kinase, p34\textsuperscript{cd2}, which triggers entry into mitosis (Nurse, 1990). The FT210 cell line has a temperature-sensitive mutation in the gene encoding this protein so that cells arrest at the G2/M boundary when grown at the non-permissive temperature. A shift to the permissive temperature permits re-synthesis of the mitotic kinase and reasonably synchronous entry into mitosis (Minoe et al., 1986; Th'ng et al., 1990). Other cell types have been synchronised using olomoucine, a purine analogue that inhibits the mitotic kinase and arrests cells at the G2/M boundary (Abraham et al., 1994; Glab et al., 1994; Vesely et al., 1994). Washing away the drug triggers entry of these cells into mitosis.

Here, these two novel systems have been used to study the earliest stages of Golgi disassembly in vivo. The results provide further support for our model of Golgi disassembly and, in addition, provide a detailed kinetic picture of the disassembly process.

**MATERIALS AND METHODS**

**Materials**

All reagent were of analytical grade or higher and purchased from Sigma or BDH unless otherwise stated. All electron microscopy reagents and accessories were purchased from Agar Scientific (Stansted, UK) unless otherwise indicated.

**Cell culture and synchronisation**

**FT210 cells**

FT210 cells (obtained from Dr John Th’ng, University of California, Davis, CA) were grown at 32°C in RPMI medium with 0.2% bicarbonate, supplemented with 10% FCS, 200 mM glutamine, 50 i.u./ml penicillin, 50 mg/ml streptomycin and 1% non-essential amino acids in an atmosphere of 5% CO\textsubscript{2}/95% air. Cells were grown in suspension to a density of 3-20\times10\textsuperscript{5} cells/ml and split every 2-3 days. For G2/M arrest, 4\times10\textsuperscript{5} cells were removed from the growing suspension culture, centrifuged for 2 minutes at 600 g, the supernatant removed and resuspended in 10 ml of RPMI medium prewarmed to 39.5°C. Cells were grown for at least 18 hours and never more than 25 hours at 39.5°C in an atmosphere of 5% CO\textsubscript{2}/95% air. To release cells into M-phase, cells were centrifuged in the same way and resuspended in RPMI medium at 32°C and grown at this temperature for the desired period of time.

**Myc-NAGT I-HeLa cells**

Myc-NAGT I cells (Nilsson et al., 1993) were grown at 37°C in DMEM medium (Gibco) supplemented with 10% FCS, 200 mM glutamine, 1% non-essential amino acids, and 400 \mu g/ml genetin in an atmosphere of 5% CO\textsubscript{2}/95% air. For G2/M arrest, cells were grown for 24 hours in 2.5 \mu g/ml aphidicolin (Sigma) to prevent entry into S phase. The cells were then washed three times with fresh medium and grown for 8 hours in normal medium. This was replaced with medium containing 200 \mu g/ml olomoucine and cells were grown for 10 hours at 37°C. To release cells into M-phase, the medium was removed and the cells washed 5 times in fresh medium, before incubation for the appropriate period of time.

**FACS analysis**

Approximately 5\times10\textsuperscript{5} cells were removed from the tissue culture dish, centrifuged at 600 g for 2 minutes at room temperature, the pellet washed in 1 ml PBS, and centrifuged again in the same way. The supernatant was removed and the cells fixed by dropwise addition of ice-cold 70% ethanol while vortexing. Cells were fixed for at least 30 minutes and were often left overnight at 4°C. These samples were further processed by the FACS Laboratory, ICRF, London. Pellets were washed three times in PBS, treated with 1 mg/ml RNAsese for 15 minutes at room temperature, the RNAsese washed out by one rinse in PBS, and cells resuspended in propidium iodide (50 mg/ml). FACS analysis was performed on a Becton-Dickinson FACScan 440 machine using a long pass filter. Typically 1.5\times10\textsuperscript{4} cells were scored. Fluorescence intensity values corresponding to G1, S, and G2/M-phases were set based on routine standard values.

**Fluorescence microscopy**

Approximately 2\times10\textsuperscript{5} cells were collected by centrifugation for 30 seconds at 14,000 g at room temperature in an Eppendorf table top centrifuge 5414. The medium was removed and the pellet resuspended in 15 ml of 3% paraformaldehyde, 0.2% Triton X-100, 2 \mu g/ml Hoechst 33258, and left on ice for 10 minutes. A sample was examined by epi-fluorescence microscopy using the FTS10 filter and a 20x oil-immersion lens on a Zeiss Axiohot microscope. The various mitotic stages between pro-metaphase and telophase could be identified based on their characteristic chromosome pattern.

**Electron microscopy**

Cells were either fixed on the dish (myc-NAGT I-HeLa cells) or collected by centrifugation at 2,000 g\textsubscript{av}, for 2 minutes at room temperature in an Eppendorf table top centrifuge 5413 and then fixed (FT210 cells). Fixation was for 3 hours with 2% paraformaldehyde,
0.2% glutaraldehyde (Fluka AG, Buchs, Switzerland), 0.2 M sucrose in 0.1 M Na-phosphate buffer, pH 7.2, at room temperature. Myc-NAGT I-HeLa cells were scraped using a rubber policeman and collected in an Eppendorf tube. Cells were pelleted at room temperature for 15 minutes at 14,000 g in the Eppendorf centrifuge fitted with a horizontal rotor. The pellets were washed three times in PBS and postfixed in 1% osmium tetroxide, 1.5% cyanoferrate in 0.1 M cacodylate buffer, pH 7.2, or, alternatively, by the tannic acid method according to Simionescu and Simionescu (1976), then dehydrated and embedded in Epon 812. Transverse sections through the entire pellet were cut on a Reichert ultramicrotome 2E set to 50-70 nm, picked up on a nickel grid and stained for 6 minutes with 2% uranyl acetate and for 2 minutes with lead citrate (Roth and Berger, 1982).

**Cryo-electron microscopy and immuno-gold labelling**

For cryo-electron microscopy, cells were collected and fixed in the same way as above, embedded in 10% gelatin in H2O, cut into small blocks and infiltrated with 2.1 M sucrose in PBS overnight at 4°C. Blocks were cut on a Reichert ultramicrotome 2E at ~95°C and sections collected on collodion/carbon coated copper grids. The grids were incubated at room temperature in a moist chamber for 10 minutes on a drop of 50 mM NH4Cl in PBS and non-specific binding pre-blocked by incubation with 0.5% fish skin gelatin in PBS (IPBS) for 7 minutes. Sections were labelled with 9E10 antibody at a 1:100 dilution in IPBS for 20 minutes, washed on six drops of IPBS over 15 minutes, and incubated with rabbit anti-mouse antibody coupled to 10 nm gold (Biocell, Cardiff, UK). Sections were washed for 30 minutes in PBS and for 30 minutes in water before staining with 2% uranyl acetate for 7 minutes at room temperature followed by incubation on a drop of 1.5% methylcellulose for 10 minutes on ice. Methylcellulose was removed using a filter paper and the grids air-dried. Sections were observed in a Phillips CM10 electron microscope.

**Stereology**

A Golgi cisterna was defined as a membrane profile with a cross-sectional length more than four times its width, the width being not more than 60 nm. A Golgi stack comprised two or more cisternal profiles separated by a gap of no more than 15 nm and overlapping by more than half their cross-sectional length. A Golgi area was defined as a Golgi stack associated vesicular and tubular profiles including the intercisternal space but not the inter-vesicular, cytoplasmic space. A Golgi cluster in mitotic cells was defined as a group of 4 or more vesicular profiles no further apart from each other than one profile diameter. A vesicle was defined as a membrane profile with a cross sectional length no more than 1.5 times its cross sectional width. A tubule was defined as a membrane profile with a cross-sectional length more than 1.5 times but less than 4 times its cross-sectional width.

Samples were inspected at a low magnification (typically 3,900 to 6,600) where the cell nucleus could easily be observed. Cells were staged according to the criteria listed in Table 1 and the positions on the grid stored on the automatic position finder of the Phillips CM10 electron microscope. After about 50 positions were collected, photographs of entire cells were taken at a final magnification of ×13,000 and the Golgi area in the same cell was usually photographed at a final magnification of ×38,750. At least 10 cells were analysed for each stage and for each experiment. Volume densities were determined by the point-hit method (Weibel et al., 1969) and were measured in relation to the cytoplasmic volume. The reference volume density (Vcytoplasrn) was determined as the ratio of grid intersections falling over the cytoplasmic space (Pc), excluding all cellular organelles, to the number of grid intersections falling over the entire cell including all cellular organelles and, in later stages, chromosomes. To determine the average diameter of cells, the longest diameter and the longest diameter perpendicular to this were measured and the average taken. The ratio of the two diameters was used as a measure of how spherical the cells were. To determine the volume density of the Golgi area in relation to the cytoplasmic volume the grid intersections falling over the Golgi area (Pg) and the number of grid intersections falling over the cytoplasmic space (Pc) were counted on low magnification photographs. Since this was done on the same photograph, the ratio could be determined directly as:

\[
\frac{V_{\text{Golgi}}}{V_{\text{cytoplasm}}} = \frac{P_g}{P_c}.
\]

The size of the square lattice grid was such that more than 200 grid intersections were counted over the cytoplasmic space in each case. The volume densities of Golgi structures (Vstack/Vcytoplasrn, Vvesicle/Vcytoplasrn, Vtubules/Vcytoplasrn) were determined on high magnification photographs by counting the number of grid intersections falling over the corresponding class of profiles (Ps) and the grid intersections falling over the entire Golgi area (Pg). The volume density relative to the cytoplasmic space was then calculated as:

\[
\frac{V_s}{V_{\text{cytoplasm}}} = \left( \frac{V_s}{V_{\text{Golgi}}} \right) \times \left( \frac{V_{\text{Golgi}}}{V_{\text{cytoplasm}}} \right) = \left( \frac{P_s}{P_{\text{Golgi}}} \right) \times \left( \frac{V_{\text{Golgi}}}{V_{\text{cytoplasm}}} \right).
\]

For each sample at least 10 Golgi areas were analysed. To determine the cisternal length, high magnification photographs (×52,500) were used. The length of the cisternal profile was measured by a point-hit method (Weibel et al., 1969). Photographs were overlaid with a square lattice grid of 10 nm and the number of intersections of the grid with an imaginary line running along the centre of the cisternal profile was counted. The average number of intersections with each profile was determined and the length L in metres calculated by:

\[
L = n_{av} \times (d/M),
\]

\(n_{av}\) being the number of intersections of cisternal profiles with the grid, \(d\) being the distance between grid lines in metres, and \(M\) being the final magnification. To determine the number of cisternae in a stack, photographs were taken at a final magnification of ×52,500 and the number of stacked cisternae counted by visual inspection.

**RESULTS**

**Synchronisation of FT210 cells**

FT210 cells were accumulated at the G2/M boundary by a 24 hour incubation at the non-permissive temperature of 39.5°C. FACS analysis of fixed samples treated with propidium iodide showed that more than 85% of the cells had G2 levels of DNA (Fig. 1B) compared with 30% of the cells grown at the permissive temperature (Fig. 1A). Fluorescence microscopy using the DNA intercalating agent Hoechst 33258 showed that almost all of the arrested cells had large, diffusely stained nuclei, typical of G2 cells (data not shown).

Arrested cells were then shifted to the permissive temperature and progression through mitosis was monitored both by FACS analysis (Fig. 1C-E) and fluorescence microscopy (Fig. 2). Cells with a G1 DNA content appeared within 4 hours (Fig. 1D) and more than 60% of the cells had progressed through mitosis within 6 hours (Fig. 1E). Cells maintained at the non-permissive temperature did not progress through mitosis over the same time period (Fig. 1F). Analysis of the DNA pattern using Hoechst 33258 gave a more detailed picture of cells after release. A wave of pro-metaphase cells peaked about 2 hours after shift to the permissive temperature, followed by a wave of metaphase cells at 3-4 hours and telophase cells at 4-6 hours (Fig. 2). The lack of an anaphase wave most likely reflects the short duration of this phase.
To determine the exact stage of individual cells during the earliest phases of mitosis, advantage was taken of the detailed knowledge of the steps in chromosome condensation and nuclear envelope breakdown during the early phases of mitosis (Gerace and Burke, 1988). Electron microscopic examination of nuclear morphology in samples taken 1-2 hours after shift to the permissive temperature permitted six stages between the G2/M boundary and metaphase to be accurately defined. A rep-

**Fig. 1.** FACS analysis of FT210 cells at the permissive and non-permissive temperatures. FT210 cells were grown at (A) the permissive temperature of 32°C or (B-F) for 24 hours at the non-permissive temperature of 39.5°C before shifting to 32°C for (B) 0 hours, (C) 2 hours, (D) 4 hours, (E) 6 hours, or (F) kept at 39.5°C for 7 hours. The DNA content was determined on samples fixed with ethanol and treated with propidium iodide before FACS analysis. Note that the number of cells counted varies from panel to panel.

**Fig. 2.** Progression of FT210 cells through mitosis after release from the temperature block. FT210 cells, grown for 24 hours at 39.5°C, were shifted to the permissive temperature for the indicated periods of time and samples analysed after fixation and staining with Hoechst 33258. At each time point the percentage of cells in a particular phase was determined using epi-fluorescence microscopy; ○, pro-metaphase; ■, metaphase; ▲, anaphase; □, telophase.

### Staging the early phases of mitosis

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Division of the Golgi apparatus

A representative image of a nucleus at each of these six stages is presented in Fig. 3 and the criteria used to define each stage are detailed in Table 1. It should be noted that the time intervals between these stages may vary from stage to stage. They therefore provide an accurate sequence of events rather than an accurate time course.

Disassembly of the Golgi apparatus in FT210 cells

The morphology of the Golgi apparatus was observed in cell sections staged using the morphology of the nucleus and representative images for each of the stages are presented in Fig. 4.

In stage I cells (G2; Fig. 4A), Golgi stacks typically contained 3 to 5 cisternae with a cross-sectional width of 1 to 2 μm. The cisternae on either side of the stack were often fenestrated. Stacks were frequently found in close proximity to each other but membranous connections were not observed. Tubular profiles, with a cross-sectional width of more than 50 nm,
were seen on either or both sides of Golgi stacks. They likely represent the cis and trans Golgi networks. When sections were stained with tannic acid (Simionescu and Simionescu, 1976) to highlight cytoplasmic coats, most of the vesicular profiles which did not appear to be connected to cisternae were found to lack coats. Occasionally, what appeared to be COP-coated and clathrin-coated vesicles or buds were seen in the Golgi area.

In stage II cells (early prophase; Fig. 4B), the stacks had a shorter cross-sectional length but still consisted typically of 3-5 cisternae. In the vicinity of the stack, vesicular/bud profiles accumulated, most of which were 50-100 nm in diameter and apparently devoid of a coat.

In stage III cells (mid-prophase; Fig. 4C), the Golgi area had increased in size and contained more than one and often as many as 5-6 shortened stacks. A large number of small, vesicular profiles or buds, and tubular profiles, some of which appeared to be interconnected, were associated with the stacks. Shortened stacks were present in most but not all Golgi areas.

In stage IV cells (mid-prophase; Fig. 4D), most Golgi areas contained vesicular/bud and tubular profiles together with very short, stacked cisternae. Those areas lacking stacks contained clusters with 5 to about 20 vesicular/bud profiles together with tubular profiles. On occasion, these clusters contained a few, large vesicular profiles with diameters in excess of 100 nm.

In stage V cells (late prophase/prometaphase; Fig. 4E), the clusters had a smaller cross-sectional diameter and the density of the vesicular/bud profiles within the clusters was lower. Cisternal profiles were absent from the clusters and other large fragments were seen only rarely.

Finally, in stage VI cells (prometaphase; Fig. 4F), the Golgi area was even smaller with clusters typically containing fewer than 10 vesicular/bud profiles. In many cells, clusters were not detected at all, most likely reflecting the dispersal of free vesicles throughout the mitotic cell cytoplasm at this stage of disassembly.

**Disassembly of the Golgi apparatus in myc-NAGT I-HeLa cells**

Since the Golgi apparatus loses its characteristic morphology during fragmentation, it was important to show that the vesicular/bud and tubular profiles identified as Golgi fragments were indeed derived from the Golgi apparatus and not, for example, from the disassembling nuclear envelope or ER. To this end, cryo-sections of FT210 cells were labelled with antibodies to the resident Golgi enzymes mannosidase II and β1,4-galactosyltransferase, followed by secondary antibody or Protein A, coupled to gold. The level of labelling was, however, too low to permit unambiguous identification of the fragments.

The level of labelling was increased by using a HeLa cell line stably expressing a myc-tagged version of N-acetylgalcosaminyltransferase I (NAGT I) (Nilsson et al., 1993). This enzyme is expressed at 4 times the level of the endogenous enzyme (Nilsson et al., 1993) and gave adequate levels of labelling when detected with the 9E10 monoclonal antibody to the myc epitope (Evan et al., 1985). The cells were synchronized using a recently-described drug, olomoucine, which specifically inhibits the cyclin-dependent kinases, p34cdc2 and p34cdc2 (Glab et al., 1994; Vesely et al., 1994; Abraham et al., 1994). Olomoucine therefore blocks cells at both the G1/S and G2/M boundaries. To arrest cells at the G2/M boundary, they were first arrested at the G1/S boundary using the specific inhibitor, aphidicolin, released for 8 hours in normal growth medium to permit progression through S phase, and then incubated with olomoucine. FACS analysis showed that a 10 hour incubation with olomoucine sufficed to arrest more than 85% of the cells at the G2/M boundary. After removal of the drug by washing, the cells passed through the different phases of mitosis with similar kinetics to those observed for FT210 cells after release from the temperature block, as judged by FACS analysis and fluorescence microscopy (data not shown). Released HeLa cells were, therefore, fixed at the same time as for FT210 cells, processed for Epon embedding and staged using nuclear morphology in exactly the same way as for FT210 cells.

The results, presented in Fig. 5, show that disassembly

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**Table 1. Criteria for staging cells between G2 and pro-metaphase**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Position in the cell cycle</th>
<th>Morphological characteristics</th>
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<tbody>
<tr>
<td>I</td>
<td>G2</td>
<td>The interior of the nucleus was amorphous and homogeneously stained; the outline of the nuclear envelope was smooth though in some cells, occasional, small indentations or folds were seen; the nucleus was predominantly elongated in shape; nucleoli and coiled bodies were visible; peripheral chromatin was occasionally stained.</td>
</tr>
<tr>
<td>II</td>
<td>Early-prophase</td>
<td>The nucleus was rounded; the outline of the nuclear envelope was wavy with small folds; faint foci of denser chromatin were observed at the periphery of some nuclei.</td>
</tr>
<tr>
<td>III</td>
<td>Mid-prophase</td>
<td>Large ridges and folds were seen in the nuclear membrane and some nuclear pore complexes were absent; there were discrete centres of condensed chromatin.</td>
</tr>
<tr>
<td>IV</td>
<td>Mid-prophase</td>
<td>The nuclear envelope was ruffled and there were some gaps; most of the nuclear pore complexes were absent; there were a larger number of discrete centres of condensed chromatin.</td>
</tr>
<tr>
<td>V</td>
<td>Late prophase/prometaphase</td>
<td>Large nuclear envelope fragments were often closely associated with fully-condensed chromosomes.</td>
</tr>
<tr>
<td>VI</td>
<td>Pro-metaphase</td>
<td>Condensed chromosomes were mostly clustered in the central region of the cell and were not associated with fragments of the nuclear envelope.</td>
</tr>
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</table>

Fig. 4. Stages in the disassembly of the Golgi apparatus. After shift to the permissive temperature for 105 minutes, FT210 cells were fixed and processed for Epon embedding. Sections were stained using the morphology of the nucleus (Table 1 and Fig. 3) and representative images of the Golgi area at each of these stages (I through VI; A through F) are shown. Note the shortening of the Golgi stacks (s) as mitosis proceeds, and the appearance of both Golgi clusters (c) and vesicles (arrows). Bar, 1.0 μm.
Fig. 5. Immuno-gold labelling of myc-NAGT I-HeLa cells after release from an olomoucine block. Myc-NAGT I-HeLa cells were arrested at the G2/M boundary using olomoucine (see Materials and Methods), then washed and incubated for a further 105 minutes before processing for cryo-electron microscopy. Sections were staged using the morphology of the nucleus (Table 1 and Fig. 3) and representative images of the Golgi area at three stages (I, III and IV) are shown. Note that labelling was restricted to the Golgi area (G). At early times, labelled stacks (arrows in A) became increasingly shorter (arrows in B). At later times Golgi clusters comprising both tubules and vesicles were labelled (arrows in C surround a putative Golgi cluster). Nu, nucleus. Bars: 0.5 μm (A) and 0.2 μm (B and C).
followed the same time course and involved the same intermediates as found in FT210 cells (Fig. 4). In stage I cells (G2; Fig. 5A), the resident Golgi enzyme, myc-NAGT I, was exclusively found in Golgi stacks and this labelling persisted as the stacks became shorter (Fig. 5B, stage III). In contrast, virtually no labelling was associated with vesicular/bud profiles at these early stages. During the later stages (IV to VI), Golgi clusters comprising tubules and vesicles or buds were labelled for myc-NAGT-I (Fig. 5C, stage IV).

Quantitative description of Golgi disassembly in FT210 cells

Since immuno-gold labelling had shown that Golgi fragments had been correctly identified by morphology alone, it was possible to use Epon-embedded samples to quantify the changes to the disassembling Golgi apparatus. Epon embedding gives better morphological preservation of structure and better contrast than cryo-sections. As a quantitative parameter the volume occupied by the Golgi apparatus and its fragmentation products was measured in relation to the cytoplasmic volume. This was possible since the overall cell volume and the ratio of cytoplasmic volume to cell volume did not change significantly over the period of disassembly (Table 2). The changes in Golgi volume relative to the cytoplasmic volume (the volume density) were quantitated during disassembly using a point-hit method (Weibel et al., 1969) and the results are presented in Table 3.

The volume density of the Golgi area increased almost three-fold from stage I to stage III and then decreased by stage V to almost half the original value. This pattern was largely caused by the changes to Golgi vesicles/buds which increased in volume density three-fold from stages I to III and then fell back to just below the original value by stage V. A similar pattern was observed for tubular profiles though their volume density was only about a third of the vesicle/bud values at all stages. In contrast, the volume density of stacks decreased two-fold from stage I to stage II and was virtually undetectable by stage IV. This suggests that, during the early stages of disassembly, Golgi stacks are converted into vesicles and tubules which occupy a larger volume. The subsequent decrease in volume density of the Golgi area would be explained by shedding of these vesicles and tubules into the surrounding mitotic cytoplasm.

Other changes to the Golgi stacks were also quantitated and the results are presented in Table 4. Over the first three stages, the average number of cisternae in a section through a Golgi stack dropped by only 12%, from 3.72±0.78 to 3.28±0.78 (P<0.0001). In contrast, the cross-sectional length of stacked cisternae decreased by 45%, from 0.67±0.26 to 0.37±0.06 (P<0.01). These results corroborate those obtained using a cell-free system, which showed that cisternal membrane was removed without prior unstacking of the cisternae (Misteli and Warren, 1994). Table 4 also shows that the stacked cisternal length continued to decrease slightly through stages IV and V without prior unstacking of cisternae. It should, however, be noted that the sampling was biased towards stacks in these samples since stacks were not abundant at later stages (see Table 3).

Role of COP-coated vesicles during the disassembly process

Using a cell-free system, the mitotic conversion of Golgi stacks into vesicles was shown to depend upon the mechanism that generates COP-coated vesicles (Misteli and Warren, 1994). If this is also true in vivo, then the vesicles that accumulate during the early stages of disassembly should be the product of this same mechanism. COP-coated vesicles uncoat rapidly after formation and the coat subunits are recycled back to the membrane for further rounds of vesicle budding. This means that the percentage of vesicles coated at any one time would be expected to be very small and this was indeed the case for the mitotic vesicles/buds that accumulated (Fig. 4). To show that

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<tr>
<th>Table 2. Cell parameters during the early mitotic phases</th>
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</thead>
<tbody>
<tr>
<td><strong>Mitotic Phase</strong></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>V&lt;sub&gt;cytoplasm&lt;/sub&gt;/V&lt;sub&gt;cell&lt;/sub&gt; (µm&lt;sup&gt;3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Average cell diameter (µm)</td>
</tr>
<tr>
<td>Spherical index</td>
</tr>
</tbody>
</table>

Results were obtained from low magnification micrographs from two experiments and represent means ± s.d. For each value 10 cells were examined. The average cell diameter was determined from the longest diameter and the longest diameter perpendicular to it. The spherical index represents the ratio of the two measured diameters.

<table>
<thead>
<tr>
<th>Table 3. Volume density of the Golgi apparatus in FT210 cells between G&lt;sub&gt;2&lt;/sub&gt; and pro-metaphase</th>
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</thead>
<tbody>
<tr>
<td><strong>Mitotic Phase</strong></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Golgi area</td>
</tr>
<tr>
<td>Stacks</td>
</tr>
<tr>
<td>Vesicles</td>
</tr>
<tr>
<td>Tubules</td>
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</table>

Results are from two experiments and represent the mean volume density (×10<sup>-2</sup>) ± s.d. Results for stage VI cells are not included because the dispersal of vesicles made it difficult to obtain unbiased estimates.
these vesicles were once coated, FT210 cells were treated with [AlF₄]⁻ to prevent uncoating (Melancon et al., 1987; Oprins et al., 1993). FT210 cells were released from the temperature block for 100 minutes and then treated for 7 minutes with [AlF₄]⁻. This pulse was sufficient to generate enough coated vesicles for detection by electron microscopy without detectable effect on the morphology of the Golgi apparatus in interphase cells (data not shown). In order to visualise the COP coats, the cells were fixed, stained en-bloc with tannic acid and embedded in Epon. It should be noted that [AlF₄]⁻ slightly accelerated the disassembly of the Golgi apparatus.

Fig. 6 (arrows) shows that small vesicular profiles and buds with a fuzzy coat were found at all stages of the disassembly process. The vesicular profiles had a diameter of 50-100 nm and were particularly abundant in stages II through V. Only in stage VI were fewer coated vesicles and buds observed perhaps as a consequence of their dispersal throughout the mitotic cell cytoplasm.
Division of the Golgi apparatus

Tubular networks as intermediates during the disassembly process

Using a cell-free system to study the disassembly of the Golgi apparatus, we have recently shown that there is an additional pathway for fragmentation that involves conversion of Golgi membranes into tubular networks. Some of these networks then break down in a manner that does not depend directly upon the COP-mediated budding mechanism (Misteli and Warren, unpublished).

Table 4. Cisternal length and average number of cisternae in Golgi stacks in FT210 cells between G2 and pro-metaphase

<table>
<thead>
<tr>
<th>Mitotic Phase</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Stage V</th>
<th>Stage VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisternal length (µm)</td>
<td>0.67±0.26</td>
<td>0.44±0.15</td>
<td>0.37±0.12</td>
<td>0.24±0.06</td>
<td>0.31±0.06</td>
<td>Not determined</td>
</tr>
<tr>
<td>Number of cisternae in the stack</td>
<td>3.72±0.78</td>
<td>3.31±1.00</td>
<td>3.28±0.78</td>
<td>3.00±0.57</td>
<td>2.5±0.57</td>
<td>Not determined</td>
</tr>
<tr>
<td>Sample size</td>
<td>(37)</td>
<td>(63)</td>
<td>(69)</td>
<td>(21)</td>
<td>(12)</td>
<td></td>
</tr>
</tbody>
</table>

Results are from photographs obtained in two experiments and the values are expressed as means ± s.d.

Fig. 7. Tubular networks as intermediates during the disassembly process. After shift to the permissive temperature for 105 minutes, FT210 cells were fixed and processed for Epon embedding. (A) stage III cell, (B) stage IV cell. Note the interconnected tubular profiles (bounded by open arrows) and small vesicular profiles (arrowheads). A stack is indicated with a filled arrow. Nu, nucleus. Bar, 0.5 µm.
To determine whether these networks were intermediates during fragmentation in vivo, we examined sections of FT210 cells that had been released from the temperature block. Close examination showed that many tubular profiles were, in fact, interconnected, forming tubular networks. In most cases these networks were small and occurred either within or close to Golgi clusters. In a few cases these networks were extensive structures (Fig. 7). The fact that they were only found in stage III (Fig. 7A) and stage IV (Fig. 7B) cells suggests that they are intermediates in the disassembly process.

DISCUSSION

In an earlier study we devised a cell-free system to mimic the fragmentation of Golgi stacks using mitotic cytosol (Misteli and Warren, 1994). We showed that fragmentation was triggered by the mitotic kinase, p34<sup>cdc2</sup>, and there was an inverse correlation between the loss of cisternal membrane and the increase in vesicles and tubules. One unexpected observation was that the decrease in cisternal length occurred without prior unstacking, at least during the early stages. Though this did not disagree with the model, we had proposed that separation of cisternae would precede their consumption by transport vesicles (Warren, 1985). Using the same cell-free system we have, more recently, shown that there is an additional fragmentation pathway that does not require transport vesicles. Golgi membranes are converted into tubular networks which then fragment further (Misteli and Warren, unpublished).

To show that these unexpected results were not an artefact of the cell-free system, we decided to look in vivo at the earliest stages of Golgi disassembly. Earlier work to address this problem had been hampered by the low yields of prophase cells obtained with the commonly-used synchronisation protocols. In unsynchronised populations they constitute less than 1% of the cells and the shake-off procedure, the least stressful way of isolating enriched populations of mitotic cells, favours later mitotic stages. During shake-off and subsequent isolation by centrifugation, prophase cells continue to proceed through mitotic phases. During shake-off and subsequent isolation by centrifugation, prophase cells continue to proceed through mitosis so that they are under-represented in the isolated populations (Klevcez, 1972). Other synchronisation procedures, using a variety of drugs, also proved to be ineffective in enriching for prophase cells (E. Souter and G. Warren, unpublished results).

In this study we have been able to exploit the properties of a mutant cell line and a new drug. FT210 cells have a temperature-sensitive mutation in the mitotic kinase, p34<sup>cdc2</sup>, such that, at the non-permissive temperature, the protein is degraded and the cells arrest at the G2/M boundary. Shift to the permissive temperature permits resynthesis of p34<sup>cdc2</sup> which triggers fairly synchronous entry into mitosis. The overall time taken to complete mitosis is about four times longer than usual most likely due to the need to synthesis new p34<sup>cdc2</sup>. The asynchronous entry into mitosis most likely reflects the different amounts of time the arrested cells spend at the G2/M boundary. Those that spend the longest are likely to have the lowest levels of p34<sup>cdc2</sup> and therefore require longer to synthesise the amounts needed to trigger entry.

Olomoucine is a purine analogue that was isolated in a screen for kinase inhibitors. It preferentially inhibits both the S phase kinase, p34<sup>cdc2</sup> and the mitotic kinase p34<sup>cdc2</sup>, which explains why it arrests cells at both the G1/S and G2/M boundaries. To circumvent the G1/S block in our experimental system, cells were synchronised at the G1/S boundary using aphidicolin, allowed to undergo replication in the absence of any drugs, and only then was olomoucine added to inhibit the mitotic kinase and arrest cells specifically at the G2/M boundary. Olomoucine competes with ATP for binding to the mitotic kinase and does so with very high affinity (L. Meijer, personal communication). This makes it difficult to remove by washing and probably explains why the cells only progress slowly through mitosis once the drug is removed.

Using these two methods we were able to obtain sufficient prophase cells to explore the earliest steps in the Golgi disassembly process. The asynchronous entry into mitosis precluded the use of time as the means of staging the cells but this problem was overcome by careful examination of events leading to the disassembly of the cell nucleus. Six stages from G2 to pro-metaphase were identified and used to order the events in Golgi disassembly. The results obtained confirm those using the cell-free system. Specifically, the stacks of cisternae shortened prior to unstacking during the early stages. Second, this decrease in cisternal length was accompanied by an accumulation of vesicular/bud and tubular profiles in the vicinity of the stacks. The Golgi origin of these fragmentation products was confirmed by immuno-gold labelling of a stably expressed resident Golgi enzyme, myc-NAGT I. Third, when uncoating was prevented by the addition of [AlF<sub>4</sub>]<sup>-</sup>, there was an increase in the number of coated vesicles with a morphology reminiscent of COP-coats. This suggests that transport vesicles are involved in the fragmentation process. Fourth, tubular networks were transiently observed during mid- to late prophase suggesting that the additional fragmentation pathway observed in vitro also exists in vivo.

In conclusion we have characterised two methods that can be used to study morphological changes during the early phases of mitosis and have used these methods to show that the fragmentation pathways for Golgi disassembly observed in vitro can also be observed in vivo. Since both pathways rely on an inhibition of membrane fusion for their expression, the next step is to identify the inhibited fusion component(s).

We thank Dr John Th'ng (University of California, Davis) for the FT210 cell line; Tommy Nilsson (EMBL, Heidelberg) for the myc-NAGT I-HeLa cell line; Derek Davis for FACS analysis; Rose Watson for assistance with electron microscopy and photography; Catherine Rabouille and Francis Barr for critical reading of the manuscript; Kathy Wilson (Johns Hopkins University, Baltimore) for helpful comments and especially Laurent Meijer (Station Biologique, Roscoff) for samples of olomoucine, advice, and for communication of results prior to publication.

REFERENCES


