Sec16A defines the site for vesicle budding from the endoplasmic reticulum on exit from mitosis

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Accepted 26 August 2010
Journal of Cell Science 123, 4032-4038
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doi:10.1242/jcs.076000

Summary

Mitotic inhibition of COPII-dependent export of proteins from the endoplasmic reticulum results in disassembly of the Golgi complex. This ensures ordered inheritance of organelles by the two daughter cells. Reassembly of the Golgi is intimately linked to the re-initiation of ER export on exit from mitosis. Here, we show that unlike all other COPII components, which are cytosolic during metaphase, Sec16A remains associated with ER exit sites throughout mitosis, and thereby could provide a template for the rapid assembly of functional export domains in anaphase. Full assembly of COPII at exit sites precedes reassembly of the Golgi in telophase.

Key words: COPII, Golgi, Mitosis

Introduction

The inheritance of organelles during mitosis is essential to ensure the proper organisation and function of successive cell generations. The Golgi is a single copy organelle and as such is dispersed throughout the cytoplasm before inheritance to ensure distribution between both daughter cells (Wei and Seemann, 2009). This is achieved through two means, the dispersion of structural components of the Golgi complex to a peripheral distribution (Lucocq and Warren, 1987; Shima et al., 1997; Seemann et al., 2002) and absorption of other components with the ER [such as Golgi glycosylation enzymes (Storrie et al., 1998; Zaal et al., 1999)]. Many resident glycosylation enzymes of the Golgi complex cycle slowly through the ER during interphase (Storrie et al., 1998). Export of secretory cargo from the ER is inhibited during mitosis (Prescott et al., 2001) and therefore these enzymes accumulate in the ER and are inherited via equal partitioning of this compartment. In other cases, separation of the ER and Golgi through mitosis is essential to ensure segregation of key signalling components that would become activated if localised together (Bartz et al., 2008).

The integrity of the Golgi complex depends on ongoing transport from the ER (Ward et al., 2001). ER export (for a review, see Hughes and Stephens, 2008) is mediated by the COPII coat, which assembles at sites on the ER membrane known as transitional ER (tER). COPII assembly is directed by the Sar1 GTPase, which, following activation by Sec12, directs sequential recruitment of Sec23–Sec24 and Sec13–Sec31 (i.e. biased binding to a predefined site) more accurately describes the organisation of ERES seen in mammalian cells (Heinzer et al., 2008). Notably, this model does not include the principle human orthologue of Sec16A interacts with COPII proteins [at least Sec23 (Bhattacharya and Glick, 2007; Inuma et al., 2007) and Sec13 (Hughes et al., 2009)] and could therefore act in directing the assembly of COPII on the transitional ER membrane before vesicle formation. In interphase, this probably serves to optimise COPII assembly at ERES. Computational modelling of COPII assembly has shown that such directed recruitment of Sar1, Sec23–Sec24 and Sec13–Sec31 (i.e. biased binding to a predefined site) more accurately describes the organisation of ERES seen in mammalian cells (Heinzer et al., 2008). Notably, this model does not include Sec16 itself, but intriguingly, the model initiates from a naked membrane with no prior membrane localisation of COPII proteins. This situation is analogous to that occurring when cells exit mitosis with COPII proteins Sar1, Sec23–Sec24, and Sec13–Sec31 localised to the cytosol.

Using quantitative 4D imaging of cells, we show that Sec16A remains associated with the ER membrane throughout mitosis and defines the localisation for the re-initiation of COPII-dependent budding on exit from mitosis.
Results and Discussion
We generated a stable HeLa cell line expressing Venus–Sec16ΔN, a construct that lacks the N-terminal 1008 amino acids of Sec16A, yet behaves in an indistinguishable manner to transiently transfected fluorescent protein (FP)-tagged Sec16A or to endogenous Sec16A (supplementary material Fig. S1). We were unable to generate stable cell lines expressing FP-Sec16A by plasmid transfection. Lentiviral transduction of FP–Sec16A was precluded by persistent recombination of the parent vector during cloning. Importantly, Venus–Sec16ΔN includes both the central conserved domain and upstream sequences shown to be necessary for membrane association and targeting to ERES (Ivan et al., 2008; Hughes et al., 2009). Specifically, it colocalises exactly with endogenous Sec16A and localises adjacent to Sec24C and Sec31A labelling, as is seen for endogenous Sec16A (Hughes et al., 2009); its expression does not alter the expression of endogenous Sec16A, and it rescues the phenotypes of decreased ERES number and Golgi fragmentation caused by suppression of endogenous Sec16A (supplementary material Fig. S1). We visualised Venus–Sec16ΔN-expressing HeLa cells throughout mitosis in 4D using spinning disk confocal microscopy. This approach provides excellent 3D spatial information over time without significant photobleaching. Fig. 1A (enlarged in Fig. 1B) shows that Venus–Sec16ΔN remained associated with defined puncta throughout mitosis [fluorescence and differential interference contrast (DIC) images, see also supplementary material Movie 1]. During fluorescence recovery after photobleaching (FRAP) experiments, Venus–Sec16ΔN showed similar dynamics to full-length GFP–Sec16A (Hughes et al., 2009) with t_{1/2}≈8 seconds (Fig. 1C). Images and further quantification of these experiments is shown in supplementary material Fig. S2. The rate of cycling was not significantly affected by the transition from interphase to mitosis. This suggests that membrane association of Sec16 is not subject to mitotic regulation. However, it does not preclude mitotic inhibition of interactions of Sec16 with downstream COPII components (Altan-Bonnet et al., 2006). Although the mechanisms underlying this inhibition remain unknown, these data also reinforce previous data showing that Sar1, Sec23–Sec24, Sec13–Sec31 all lie downstream of Sec16 (Ivan et al., 2008; Hughes et al., 2009).
In fixed cells expressing Venus–Sec16AΔN, other COPII proteins, Sec24C (Fig. 1D) and Sec31A (Fig. 1E) were largely redistributed to the cytosol, as has been described previously (Farmaki et al., 1999; Stephens, 2003). Several Sec16A-positive puncta were apparent that do not show adjacent labelling of Sec24C (Fig. 1D) or Sec31A (Fig. 1E). We sought to validate these findings by accumulating cells in mitosis using a series of small molecule inhibitors. Monastrol is a cell-permeable inhibitor of the mitotic kinesin Eg5 (Mayer et al., 1999); its mode of action results in the accumulation of cells with monopolar spindles in mitosis. BI2536 is an inhibitor of polo-like kinase 1, which also arrests cells in mitosis (Steegmaier et al., 2007). UA62784 inhibits the function of CENP-E, thereby preventing proper chromosome congression in metaphase (Henderson et al., 2009). Application of each of these three inhibitors led to accumulation of cells in mitosis that showed clear localisation of Venus–Sec16AΔN to puncta, but loss of Sec24C and Sec31A to a cytosolic distribution (supplementary material Fig. S3). Labelling for other markers [giantin and galactosyltransferase (GalT)] confirmed Golgi dispersal. These data confirm our findings from unperturbed cells during mitosis.

Similarly to Venus–Sec16AΔN, endogenous Sec16A localises to defined puncta throughout mitosis (Fig. 2). This is in stark contrast to previous findings using other COPII proteins, which redistribute to the cytosol during metaphase (Farmaki et al., 1999; Stephens, 2003; Altan-Bonnet et al., 2006). Double labelling with antibodies specific for the Sec31A subunit of the COPII coat (Fig. 2) showed that, as has been previously described, Sec31A remained localised to puncta during interphase and prometaphase. At metaphase (when chromosome are tightly aligned on the metaphase plate) Sec31A was largely lost from discrete puncta (Fig. 2, see boxed enlargements); however, as we have described previously (Stephens, 2003), fixation of cells for immunolabelling enhances the appearance of punctate labelling. Notably these remaining puncta of Sec31A often do not align adjacent to Sec16A and therefore we would not characterise these as functional ERES (see Hughes et al., 2009).

To validate our findings in living cells without potential artefacts from cell fixation, we imaged cells stably expressing both Venus–Sec16AΔN and mRuby–Sec23A. A lentivirus expressing mRuby–Sec23A was constructed by switching the fluorescent protein tag from EYFP–Sec23A, which we have previously successfully used for similar experiments (Stephens, 2003). Control experiments showed that mRuby–Sec23A localised as expected to puncta perfectly aligned with Sec24C and Sec31A but offset from endogenous Sec16A (data not shown). Furthermore, in cells either singly expressing mRuby–Sec23A or doubly transfected cells coexpressing both Venus–Sec16AΔN and mRuby–Sec23A the structure of the Golgi (giantin labelling) and the localisation of GalT (as a marker of the efficiency of ER-to-Golgi transport) in cells were indistinguishable from that of non-transfected cells (data not shown). The rate of mitotic progression was also unaffected by stable transfection of either mRuby–Sec23A alone or of both Venus–Sec16AΔN and mRuby–Sec23A (data not shown). We observed that although Venus–Sec16AΔN remained associated with tER membranes throughout mitosis (Fig. 3A), mRuby–Sec23A dissociated from ERES during metaphase (Fig. 3B and supplementary material Movie 2). Contrast enhancement showed some detectable punctate localisation of mRuby–Sec23A, which could not easily be distinguished from background (Fig. 3C, arrow). These objects did not lie adjacent to Venus–Sec16AΔN-labelled puncta and therefore would not be defined as bona fide ERES (Hughes et al., 2009). Furthermore, FRAP analysis showed that,

Fig. 2. Endogenous Sec16 and COPII subunit localisation during cell division. Fixed cells were labelled to detect endogenous Sec16A and the COPII subunit Sec31A at various stages of cell division. DAPI labelling of DNA was used to define the mitotic state of cells as indicated and 3D stacks of images were acquired to image the entire depth of the cells. Scale bars: 10 μm.
unlike during interphase, these mRuby–Sec23A-positive structures in metaphase did not recover fluorescence after photobleaching (HH and DJS unpublished observations) indicating that they are not functionally the same as ERES. They might represent clusters of Sec13p-labelled membrane tubules as previously observed by immunoelectron microscopy (Farmaki et al., 1999). We speculate that the punctae are more likely to be vesicular-tubular remnants resulting from juxtanuclear ERES clustering and Golgi breakdown.
Automated quantification of multiple experiments (automatically detecting and counting all ERES in total of 15 dividing cells) confirmed these observations (Fig. 3D). The number of discrete objects labelled with Venus–Sec16AΔN decreased by around 70% as cells enter metaphase. The loss of mRuby–Sec23A from discrete puncta was more pronounced, with <10% detectable objects per cell remaining. Our quantification method was based purely on intensity and so will include any sufficiently bright object within the field of view including mitotic ERES remnants (Farmaki et al., 1999). This, along with the increase in ERES association of Sec23A in cells expressing Venus–Sec16AΔN means that these data overestimate the number of discrete mRuby–Sec23A-labelled objects. The standard deviations of five averaged time sequences (Fig. 3E) further validated these observations and revealed a clear statistically detectable difference in numbers of Venus–Sec16AΔN and mRuby–Sec23A puncta. Frame-by-frame quantification (Fig. 3F,G) showed that we frequently did not observe any visible Sec23A-labelled puncta in metaphase cells (as indicated by the blue bars on these graphs). By contrast, we have never observed cells during metaphase that do not contain obvious Venus–Sec16AΔN puncta. Automated quantification of the intensity of objects revealed that although they are smaller, the mean intensity of individual spots was unchanged through mitosis (not shown).

The number of Venus–Sec16AΔN puncta increased on exit from metaphase (around 100 minutes from the start of the time series) and preceded an increase in mRuby–Sec23A-labelled objects. This is consistent with the idea that Sec16A precedes Sec23A during COPII assembly. Indeed, on exit from mitosis, we observed that mRuby–Sec23A-labelled puncta emerged in close apposition to Venus–Sec16AΔN-labelled structures (Fig. 4A, arrows highlight close juxtaposition of Venus–Sec16AΔN and mRuby–Sec23A, see supplementary material Movie 2). This also preceded re-formation of the Golgi, which occurs in late telophase (Fig. 4B, arrows highlight reforming Golgi clusters, see supplementary material Movie 3). Golgi structures appeared to emerge in the vicinity of the centrosomes, consistent with a role for microtubules in directing the spatial organisation of the reforming of the Golgi ribbon (Ho et al., 1989; Corthesy-Theulaz et al., 1992) as well as increasing the efficiency of the ER export and ER-to-Golgi transport (Watson et al., 2005; Palmer et al., 2009).

Our data show that Sec16A marks the transitional ER throughout mitosis and is coincident with the reappearance of COPII puncta on exit from metaphase. These data are entirely consistent with our previous findings that siRNA-mediated depletion of Sec16A results in a decrease in the number of ERES within a cell (Watson et al., 2006). By directing ERES assembly, Sec16A ensures that...
intracellular architecture of the cell is restored quickly and effectively on exit from mitosis. Two major outstanding mechanisms remain to be defined—how Sec16A itself is targeted to the ERES membrane and how Sec16A is uncoupled from other COPII proteins during mitosis.

Materials and Methods

Generation of cdNA constructs and generation of stable cell lines
cDNAs encoding both Venus fluorescent protein (Nagai et al., 2002) and Sec16ΔN were amplified by PCR and subcloned via pGEM-T vector. Venus was excised with BglII-SfiI for insertion into a modified HIV-1-based self-inactivating pSEW sin lentiviral vector backbone lacking the WPRE region (kindly provided by Giles Cory, University of Exeter, Exeter, UK) via BamHI-SfiI. pHRSINCEPPT-SEW-Venus was further digested via BamHI-SfiI for insertion of Sec16ΔN (excised from pGEM via BglII-SfiI). Virus preparation was undertaken using HEK293T cells in a 10 cm culture dish at 80% confluence. 40 μg vector construct, alongside packaging vectors pMDG 2 (10 μg) and p8.91 (30 μg) were combined in 5 ml Optimem. A 1:1 solution of DNA:PEI (2 mM in Optimem) was incubated for 20 minutes before adding dropwise to washed cells. The cells were incubated at 37°C, 5% CO₂ for 4 hours before replacing with complete DMEM. 48 hours after the transfection, viral particles were harvested and added to HeLa cells at 70% confluence. Cells were left to grow for 2 weeks post-confluence, Venus-expressing colonies were determined by light microscopy, picked and replated to produce a clonal Venus-Sec16ΔN stable cell line. The cDNA sequence encoding mRuby (Kredel et al., 2009) underwent codon usage adaptation before gene synthesis (MWG Eurofins) and cloning into pBluescript II SK(+). mRuby was excised with SalI-NotI for insertion into plLVX-Puro (Clontech) to generate plLVX-mRuby. A cDNA encoding human Sec32A was amplified by PCR, gel purified and subcloned via pGEM-T vector (excised with Apal-XbaI) and cloned into plLVX-Puro-mRuby. Lentivirus preparation followed the Clontech Lenti-x. mRuby-Sec23A-expressing cells were selected using puromycin.

Cell synchronisation
Cell synchronisation was achieved using a double thymidine block. Cells grown to 40% confluence were subject to 2 mM thymidine for 16 hours. Cells were washed three times with PBS, and incubated for 8 hours in complete medium, before repeating the block and wash-out. Cells undergo mitosis 12 hours after second wash-out.

Mitotic inhibitors
Inhibitors were incubated in complete medium at the following concentrations; 100 μM Monastrol, 100 mM B 2536, 500 mM UA 62784 for 6 hours after the second wash-out before PFA fixation.

Imaging of fixed cells
PFA-fixed immunolabelled cells were imaged using Leica TCS-SP4 AOBS scanning confocal microscope and processed using Photoshop 6.0 (Adobe, Uxbridge, UK) and montages generated using Adobe Illustrator (Adobe). To provide the highest degree of accuracy, Nyquist sampling theorem was applied to ascertain the correct voxel size for sampling the image. This translates to 11.2 samples per μm or a maximum distance of 89 nm pixel width. To adjust to this criteria, a higher pixel number (1024×1024) and zoom factor (5×4) was used. For Venus–Sec16AΔN, the calculated pixel size used was 43×43×130 (x, y, z in nm). Antibodies were sourced as follows: mouse monoclonal Sec31A and p50Glu (Transduction labs, BD Labs, Montgomery, TX), rabbit polyclonal antibodies against Sec24C and Sec31A ΔN was imaged using a 514 nm laser with a 1.3 NA Plan-Apochromat objective (1.3 NA Plan-Apochromat objective). Images were acquired with a Hamamatsu (Welwyn Garden City, UK) with a Solent Scientific (Portsmouth, UK) incubator set to 37°C and a Ultraview ERS spinning disk confocal microscope with live cell imaging mode.

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