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Cdc2 Kinase-dependent Disassembly of Endoplasmic Reticulum (ER) Exit Sites Inhibits ER-to-Golgi Vesicular Transport during Mitosis[D] [V]

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We observed the disassembly of endoplasmic reticulum (ER) exit sites (ERES) by confocal microscopy during mitosis in Chinese hamster ovary (CHO) cells by using Yip1A fused to green fluorescence protein (GFP) as a transmembrane marker of ERES. Photobleaching experiments revealed that Yip1A-GFP, which was restricted to the ERES during interphase, diffused throughout the ER network during mitosis. Next, we reconstituted mitotic disassembly of Yip1A-GFP–labeled ERES in streptolysin O-permeabilized CHO cells by using mitotic L5178Y cytosol. Using the ERES disassembly assay and the anterograde transport assay of GFP-tagged VSVGts045, we demonstrated that the phosphorylation of p47 by Cdc2 kinase regulates the disassembly of ERES and results in the specific inhibition of ER-to-Golgi transport during mitosis.

INTRODUCTION

The first step of anterograde transport from the endoplasmic reticulum (ER) to the Golgi apparatus is the recruitment of the cytosolic coat complex (COP II) to specialized sites of ER export, called ER exit sites (ERES) or transitional ER (tER), where cargo proteins are actively sorted and concentrated in COP II-coated vesicles (Hobman *et al*., 1998; Hong, 1998; Kaiser and Ferro-Novick, 1998; Stephens *et al*., 2000; Muniz *et al*., 2001; Aridor *et al*., 2001; Barlowe, 2002). Morphologically, this compartment was originally defined as a ribosome-free ER subdomain that is continuous with the rough ER and contains protrusions resembling budding vesicles (Merisko *et al*., 1986; Orci *et al*., 1991; Rossanese *et al*., 1999; Shugrue *et al*., 1999). Because ERES are starting points for ER-to-Golgi transport, the balance of which influences Golgi morphology, the biogenesis of ERES is thought to be closely coupled to Golgi biogenesis (Ward *et al*., 2001; Bevis *et al*., 2002). Thus, ERES are maintained as an important subdomain of the ER, not only for the sorting/budding of proteins from the ER but also for Golgi biogenesis. However, less is known about the mechanism by which ERES maintain their distinct morphological identity as subdomains within the general ER or how their formation/disassembly is regulated during the cell cycle. Few studies have examined either ERES dynamics within the cell or the biochemical requirements for ERES formation.

Time-lapse imaging has been used to study the dynamic behavior of ERES by using fluorescence-tagged cytosolic COP II components as probes, such as in *Pichia pastoris* or Chinese hamster ovary (CHO) cells by using Sec13-GFP (Hammond and Glick, 2000; Bevis *et al*., 2002), or in HeLa cells by using Sec23A-YFP (Stephens, 2003). These studies revealed that ERES are long-lived compartments that move slowly on the ER network and with apparently restricted mobility. Despite the limited movement of individual ERES, fusion, fission, or de novo formation of ERES can be seen in interphase HeLa cells (Stephens, 2003). The cell cycle-dependent accumulation of ERES also has been studied in mammalian cells and yeast. Immunofluorescence studies using anti-Sec13 antibodies revealed that ERES grew in number during interphase but that Sec13 dissociated from ERES during mitosis in mammalian cells (Farmaki *et al*., 1999; Hammond and Glick, 2000). In addition, Sec23A also was confirmed to be predominantly cytosolic during mitosis (Stephens, 2003). These observations suggest that during mitosis, the ERES may be disrupted, leading to the dissociation of COP II components Sec13 or Sec23A. Observations using membrane protein markers of ERES should reveal more precisely the behavior of ERES, because such markers do not dissociate from the ER membranes during mitosis.

In this study, we used Yip1A, a transmembrane marker of ERES, rather than a cytosolic marker to observe the dynamics of ERES directly. Yip1A interacts with the Sec23/Sec24 complex of mammalian COP II and is involved in ER-Golgi transport (Tang *et al*., 2001; Heidtman *et al*., 2003). From time-lapse imaging of ERES in CHO-YIP cells, constitutively expressing Yip1A fused to green fluorescent protein (GFP) (Yip1A-GFP), we found that during mitosis the number of ERES decreased significantly and that Yip1A-GFP became

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Abbreviations used: ERES, endoplasmic reticulum exit site(s); NEM, N-ethylmaleimide; SLO, streptolysin O, TB, transport buffer; TC, transport complex(es); VSVG, vesicular stomatitis virus G protein.

free to diffuse throughout the ER network. We next reconstituted mitotic disassembly of ERES in streptolysin O (SLO)-permeabilized CHO-YIP cells by using mitotic cytosol prepared from L5178Y cells. We showed that phosphorylation of p47, a cofactor of the N-ethylmaleimide (NEM)-sensitive AAA-ATPase p97, by Cdc2 kinase causes the disassembly of ERES and results in the specific inhibition of ER-to-Golgi transport during mitosis.

MATERIALS AND METHODS

Materials and Antibodies

Nocodazole and cytochalasin D were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were from Wako Pure Chemicals (Kyoto, Japan). The following antibodies were used: rabbit mouse anti-α-tubulin antibody (Sigma-Aldrich), mouse anti-Cdc2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-Sec13 antibody from Drs. Yoshio Misumi and Yukio Ikehara (Fukuoka University, School of Medicine, Fukuoka, Japan). GFP-tagged VSVGts045 was a gift from Dr. Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD). Cdc2 protein kinase composed of Cdc2 and cyclin B1 was purchased form New England Biolabs (Beverly, MA). All other antibodies (anti-p47 antibody) and proteins (p97, p47, and nonphosphorylatable version of p47) were prepared as described by Uchiyama *et al.* (2002, 2003).

Synchronization of L5178Y Cells at Mitotic Phase

L5178Y cells were synchronized at the mitotic phase by the method described in Matsukawa *et al.* (1985), with slight modification. Briefly, L5178Y cells were grown at 37°C in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), penicillin-streptomycin-fungizone (Cambrex Bio Science Walkersville, Walkersville, MD). Exponentially growing cells were treated with 2.5 mM thymidine for 12 h to inhibit DNA synthesis. Blocking was reversed by addition of 0.1 mM deoxycytidine. After 2 h, the cells were incubated with 0.1 μg/ml colcemid for 6–10 h until the mitotic index was >90%.

Preparation of L5178Y Cytosol

Cytosol was prepared from unsynchronized or mitotic L5178Y cells as described in Kano *et al.* (2000b,c). Typically, a protein concentration of 2.7–3.5 mg/ml was obtained, which was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Stable or Transient Expression of Yip1A-GFP or Sec24D-GFP

cDNA of mouse Yip1A or of human Sec24 member D (Sec24D) (Stephens *et al.*, 2000) was cloned upstream of EGFP cDNA in the EGFP-N1 or EGFP-C1 mammalian expression vector (BD Biosciences Clontech, Palo Alto, CA), respectively. The construct was introduced into CHO-K1 cells by LipofectAMINE Plus (Invitrogen, Carlsbad, CA). Positive clones stably expressing Yip1A-GFP were selected in complete medium containing 300 μg/ml geneticin (Invitrogen).

Immunofluorescence and Time-Lapse Microscopy

For staining with the anti-Sec13 antibody, cells were first fixed with 3% paraformaldehyde for 30 min at room temperature, permeabilized with 0.2% Triton X-100 for 20 min, and then blocked with transport buffer (TB; 25 mM HEPES-KOH, pH 7.4, 115 mM potassium acetate, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM EGTA) containing 5% skim milk. For staining with anti-α-tubulin, cells were permeabilized and fixed with methanol:acetone [1:1 (vol/vol)] at 0°C for 6.5 min and then blocked with TB containing 5% skim milk. After blocking, cells were incubated with primary antibodies for 2 h and subsequently with fluorescence-conjugated secondary antibodies for 1 h. The samples were viewed with a 100× Plan-NEOFLUAR oil Immersion objective on a confocal microscope, LSM 510 (Carl Zeiss, Jena, Germany). Time-lapse imaging was performed using the same confocal microscope system. CHO-YIP cells were placed in a temperature-controlled chamber on the stage of the LSM 510. Time-lapse sequences of images were acquired every 2–5 s by scanning the specimen with a low power laser (3%). The image sequences were processed by NIH Image software (Wayne Rasband Analytics, National Institutes of Health).

Estimation of ERES Integrity in Semi-intact Cells (ERES Disassembly Assay)

Semi-intact CHO-YIP cells were prepared and the efficiency and extent of SLO-mediated permeabilization was evaluated as described in Kano *et al.* (2000b). The semi-intact CHO-YIP cells were incubated under various conditions with an ATP-regenerating system (1 mM ATP, 8 mM creatine kinase, 50 μg/ml creatine phosphate), 1 mM GTP, 1 mg/ml glucose, and L5178Y mitotic or interphase cytosol (3.5 mg/ml protein concentration) at 32°C for various periods of time. Reactions were stopped with the addition of TB containing ATP-regenerating system, and the cells were examined with a confocal microscope or subjected to the ERES disassembly assay. Briefly, CHO-YIP cells were imaged using an LSM510 (Carl Zeiss) with a 100× Zeiss Plan-NEOFLUAR oil immersion objective numerical aperture 1.4. A Z-series was taken at 2-μm intervals to define each ERES. Typically, 10 to 12 sections were required to cover the majority of the ERES in each cell. We found that the ERES in CHO cells have a uniform size and brightness. The average area covered by a punctate structure labeled with Yip1A-GFP was 0.78 μm^2 (an average radius of ~1.0 μm). To quantify the number of ERES in each cell, we counted the number of ERES (defined as structures larger than 0.7 μm^2) and then obtained the average number of ERES (/μm^2, total number of ERES per total areas of each cell) in each cell. Three independent experiments were performed, and means and standard deviations were calculated (n = 30–50).

Immunodepletion and Cdc2 Kinase Assay

Cdc2 was depleted from mitotic cytosol as described in Kano *et al.* (2000a) by using $p13^{suc1}$ conjugated to agarose (Upstate Biotechnology, Lake Placid, NY) as a substitute for anti-Cdc2 antibody coupled with protein G-Sepharose. The kinase activity of Cdc2 was quantified using a MESACUP Cdc2 kinase assay kit (Medical &Biological Laboratories, Nagoya, Japan).

Fluorescence Loss in Photobleaching (FLIP) Experiment

CHO-YIP cells, grown on glass-bottom dishes (Iwaki, Chiba, Japan), were placed on the stage of an LSM 510 confocal microscope (Carl Zeiss). In FLIP experiments, the boxed area spanning the cell was repetitively photobleached (30 scans) with high laser power (50%). After the bleaching, an image of the whole cell was acquired by scanning with low laser power (3%). In 10-s intervals, the bleaching cycle was repeated. We normally performed fourteen cycles of bleaching. Fluorescence intensity in the selected area was quantified using the LSM 510 software package (Carl Zeiss).

Transport Assay of VSVGts045-GFP in Permeabilized Cells

The gene encoding VSVGts045-GFP was introduced into CHO-K1 cells by transfection by using LipofectAMINE Plus (Invitrogen) and incubated at 39.5°C for 24 h. SLO was added to the transfected cells on ice for 4 min. After extensive washing with phosphate-buffered saline (PBS), the cells were incubated with prewarmed TB containing 3 μg/ml propidium iodide at 32°C for 4 min to permeabilize the plasma membrane. Propidium iodide is an impermeable dye that stains DNA when cells are efficiently permeabilized. The cells were then incubated with L5178Y cytosol (3–4 mg/ml), an ATP-regenerating system, GTP (1 mg/ml), and glucose (1 mg/ml) at 32°C for 40 min. Fluorescence microscopy showed that none of VSVGts045-GFP was transported from the ER during SLO treatment. After incubation, the cells were fixed by treatment with 1% formaldehyde in TB and viewed by confocal microscopy. We counted two sets of permeabilized cells, one in which VSVGts045-GFP remained at the ER network (N_{ER}) only, and a second in which it was localized to the Golgi apparatus (N_G). We could distinguish ER-staining cells from Golgi-staining cells easily because the former show a typical ER network pattern, and the latter show stacked structures at the perinuclear region of the cells. When both ER and Golgi were illuminated by VSVGts045-GFP, we counted those cells as cells, in which VSVGts045-GFP was localized to the Golgi apparatus. Then, we calculated the percentage of cells in which the transport from the ER to the Golgi was inhibited as $100 \times N_{ER}/(N_{ER} + N_G)$. The value of $100 \times N_{ER}/(N_{ER} + N_G)$ for ER-to-Golgi transport was considered to be 100% when the cells were incubated in the presence of mitotic cytsol/ATP and was considered to be 0% when the cells were incubated in the presence of interphase cytosol/ATP. The efficiency of the transport was calculated under various experimental conditions as shown in the graph. For all experiments, three independent transport assays were performed, and the means and standard deviations were plotted.

RESULTS

Yip1A Is an Appropriate Marker of ERES

To investigate the dynamic behavior of ERES in mammalian cells, we first created CHO cells (CHO-YIP) that stably expressed Yip1A fused to GFP (Yip1A-GFP). As shown in Figure 1A, the majority of the Yip1A-GFP fluorescence accumulated as bright punctate structures throughout the ER network, although more diffuse fluorescence of Yip1A-GFPs also was observed on the ER network and nuclear envelope (Figure 1A, Yip1A-GFP, and B). ERES are defined by the presence of budding COP II vesicles, so COP II components, such as Sec13 or Sec24D, are well-characterized markers of

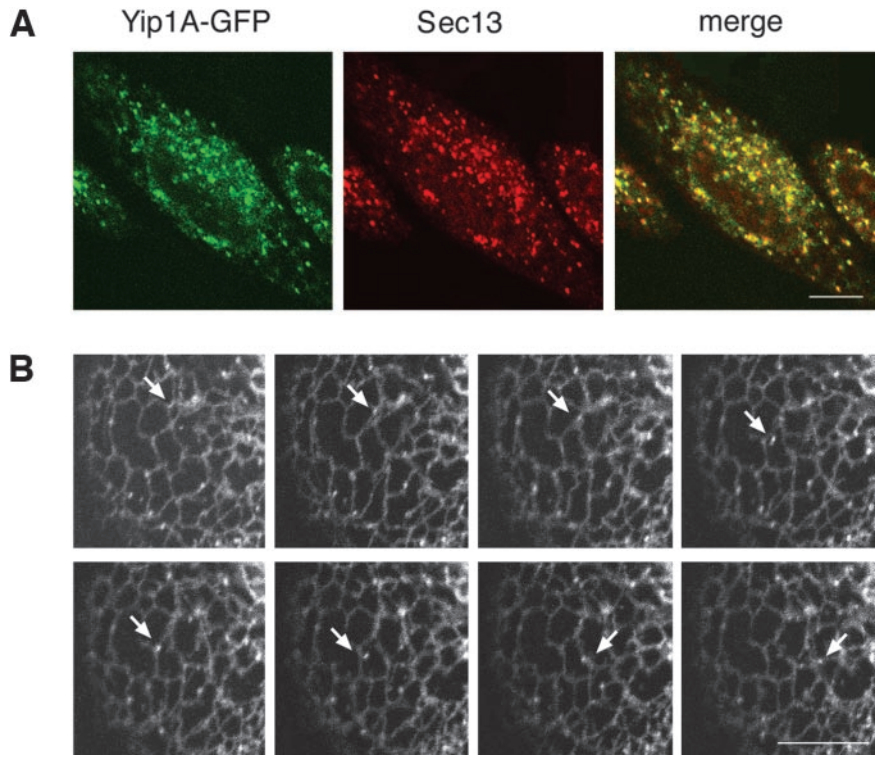

Figure 1. Dynamic behavior of Yip1A-GFP–labeled ERES in CHO-YIP cells. (A) Colocalization of Yip1A-GFP and Sec13 within punctate structures. Bar, 10 μm. (B) Dynamics of ERES labeled with Yip1A-GFP. ERES (arrow) moved along ER tubules and occasionally jumped onto other ER tubules. Images were taken every 4 s. To define the spatial relationship between the ER network and ERES, the fluorescence images of the ER network/ERES were enhanced. Bar, 10 μm.

these structures (Hammond and Glick, 2000; Stephens *et al.*, 2000). Approximately 96% of the punctate structures labeled with Yip1A-GFP fluorescence overlapped with similar punctate structures revealed by indirect immunofluorescence by using an antibody against Sec13 (Figure 1A). We compared the localization of Sec13 in wild-type CHO cells and in CHO-YIP cells. The number and the localization of ERES were similar in wild-type CHO cells and in CHO-YIP cells, suggesting that overexpression of Yip1A-GFP did not alter the integrity of COP II-coated ERES (unpublished data). In addition, nocodazole treatment has been reported to induce the enlargement of COP II-labeled structures in mammalian cells (Hammond and Glick, 2000). Treatment of CHO-YIP cells with 10 μg/ml nocodazole at 37°C for 1 h to disrupt microtubules resulted in the enlargement of Yip1A-GFP–labeled structures in both the perinuclear and peripheral regions of the cell (Supplemental Figure 1). These results support the notion that the Yip1A-labeled structures are ERES.

Otte *et al.* (2001) have shown that in yeast, Yip1p is efficiently incorporated into COP II vesicles in vitro and have supposed that it must recycle between the ER and a post-ER compartment in vivo. As such, we cannot rule out the possibility that the Yip1A-GFP-labeled structures represent transport vesicles that are produced after the loss of the COP II coats. A report in Stephens *et al.* (2000) provides some insight into this issue, by visualizing simultaneously the dynamics of COP II and COP I, as well as COP II and VSVGts045-GFP (GFP-tagged, temperature-sensitive mutant of vesicular stomatitis virus G protein), a well-known secretory transport marker, in living cells. The study demonstrated that the majority of the transport complexes (TC) containing VSVGts045-GFP were not coated with COP II coat protein (Sec24D) while in transit to the Golgi. As mentioned above, we found that nearly all of the Yip1A-GFP–labeled compartments colocalized with the COP II marker protein Sec13, suggesting that the Yip1A-GFP–labeled compartments were unlikely to be TC but rather that they represented ERES.

In addition, we have investigated the colocalization of Yip1A-GFP and VSVGts045-T7 (T7-tagged VSVGts045) at various time points after shift of the temperature. VSVG-T7 was transfected to CHO-YIP cells, and then the cells were cultured at 39.5°C for 24 h to accumulate VSVGts045-T7 in the ER. We transferred the cells to 32°C and incubated them for a further 0, 2.5, 5.0, 7.5, or 10 min (Supplemental Figure 2). The cells were then fixed and subjected to indirect immunofluorescence analysis by using an anti-T7 antibody. Immediately after transfer to 32°C (0 min), VSVGts045-T7 localized to the ER network throughout the cytoplasm but overlapped very little with Yip1A-GFP–labeled structures. Two and one-half minutes after transfer, the fluorescence from Yip1A-GFP–labeled structures began to overlap with that arising from immunodetection of VSVGts045-T7. At later time points, the Yip1A-GFP and VSVGts045-T7 signals were observed to dissociate from one another. A time-course analysis of the percentage of the Yip1A-GFP–labeled structures that colocalized with VSVGts045-T7 indicated that VSVGts045-T7 seemed to associate transiently with Yip1A-labeled structures (within ~2–3 min) (Supplemental Figure 2). We confirmed that stable expression of Yip1A-GFP in CHO-YIP cells did not alter the kinetics of delivery of VSVGts045-T7 to the Golgi (Supplemental Figure 3). The transient colocalization of cargo and Yip1A-GFP in the early steps of ER-to-Golgi transport suggested that the Yip1A-GFP–labeled compartments are ERES rather than TC, which are produced from ERES after loss of COP II coats.

Dynamic Behavior of ER Exit Sites in CHO-YIP Cells

Under a confocal microscope with time-lapse imaging, the dynamic behavior of Yip1A-labeled structures was particularly evident in the periphery of CHO-YIP cells. In inter-

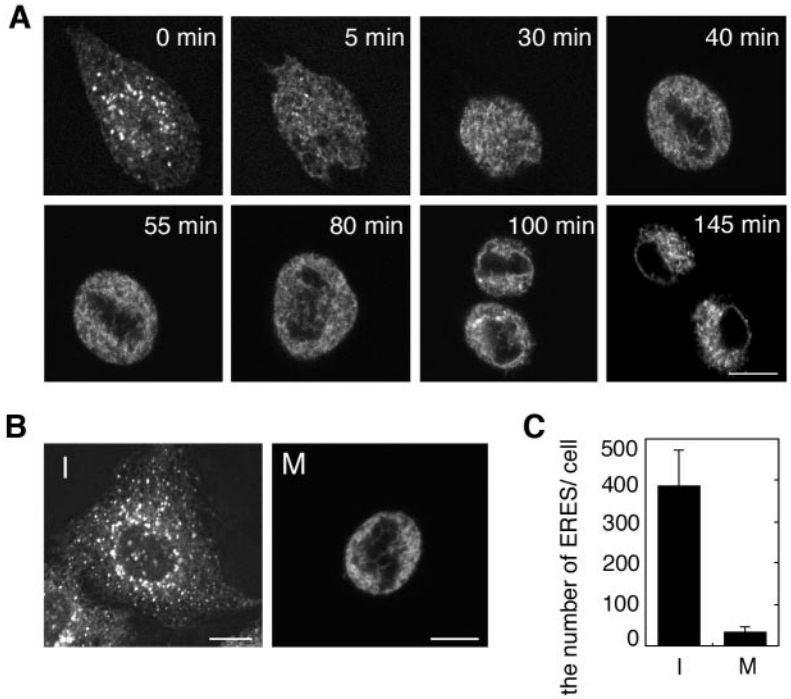

Figure 2. Cell cycle-dependent disassembly/reassembly of ERES in CHO-YIP cells. (A) A single CHO-YIP cell was viewed with a confocal microscope from the end of interphase to the end of cell division. ERES diffused to the ER network during mitosis and reassembled at the beginning of interphase. ERES are shown as bright punctate structures. Incubation times are indicated in each panel. Bar, 10 μm. (B) CHO-YIP cells during interphase (I) or mitosis (M) viewed with a confocal microscope. Bar, 10 μm. (C) The number of ERES in an interphase cell (I) or a mitotic cell (M) was counted, and the means and the standard deviations were plotted. Ten to 12 sections were required to cover most ERES in each cell.

phase cells, the bright punctate structures moved along the ER network and occasionally seemed to be discharged from one ER tubule to another (Figure 1B and Movie 1). Although the structures often fused with one another, they maintained a constant average size through shrinkage after fusion (Figure 1B and Movie 1). To compare the dynamic behavior of Yip1A-labeled structures with that of COP II-labeled structures in living cells more extensively, we expressed Sec24D-GFP in wild-type CHO cells and compared its dynamic behavior with that of Yip1A-GFP (Movies 2 and 3). To obtain quantitative data, we counted the number of fluorescence-labeled structures within a limited area (<2 μm in diameter) during time periods of up to 15 min, as described in Stephens *et al.* (2000). Consequently, we rarely observed long-range (> 2 μm) movement of either chimeric fusion protein, even in the periphery of the cells expressing them. The fraction of the fluorescent structures that exhibited long-range (>2 μm) movement was similar (~8.01 or 8.15%, with 100% representing total number of fluorescent punctate structures in for Yip1A– or Sec24D-GFP–expressing cells, respectively).

Furthermore, we have coexpressed both Yip1A-YFP and Sec24D-CFP in wild-type CHO cells and compared their dynamic behavior by using time-lapse confocal microscopy. Dual visualization of Yip1A-YFP-labeled and Sec24D-CFP-labeled structures revealed that they were localized very close to one another. These structures were observed to move in concert, probably along microtubules (Movies 4 and 5), because their long-range movement was not observed after treatment of the cells with the microtubule disrupting reagent nocodazole (unpublished data).

Together, we found that 1) the dynamic behavior of Yip1A-labeled components was indistinguishable from that of ERES, as revealed by the well-characterized COP II marker; and 2) the movements of both components were confined to a limited area. From these findings and the results of the colocalization experiments described in the previous text, we conclude that the Yip1A-GFP–labeled structures are ERES.

Yip1A-GFP Diffuses throughout the ER Network at the Onset of Mitosis

The ERES markers Sec13 and Sec23A have been reported to exhibit a diffuse distribution at the onset of mitosis, indicating substantial dissociation of these cytosolic proteins from the ERES (Hammond and Glick, 2000; Stephens, 2003). In CHO-YIP cells, we observed similar morphological changes in the ERES: the bright punctate pattern of Yip1A-GFP fluorescence disappeared and gave way to a diffuse signal in the ER network (Figure 2, A and B, and Movie 6). We next counted the number of ERES in single interphase or mitotic CHO-YIP cells. The approximate number of ERES decreased from 400 in an interphase cell to ~20 in a mitotic cell (Figure 2C). We used FLIP in areas of the cell lacking ERES in interphase or mitotic cells (Figure 3A, rectangular regions of interest [ROIs]) and compared the extent of photobleaching of whole cells. In mitotic cells, the relative fluorescence intensity after photobleaching decreased to <10% of the initial intensity (Figure 3B, M). In contrast, in interphase cells the relative fluorescence decreased to 70% of the initial intensity (Figure 3B, I), leaving most of the ERES fluorescence unaffected. This result indicated that there are two pools of Yip1A-GFP: a mobile pool in the ER and a relatively immobile pool in the ERES.

Together, we conclude that the lateral diffusion of Yip1A-GFP is more restricted in ERES than in other regions of the ER and that at the onset of mitosis this restriction is lost and Yip1A-GFP is released to move throughout the ER network.

Biochemical Requirements for the Maintenance of the ERES in Semi-intact Cells

To examine the factors that affect the maintenance of preexisting ERES, we incubated semi-intact cells under various conditions and then subjected them to the ERES disassembly assay (see *Materials and Methods*). The number of ERES in the presence of interphase cytosol and ATP was similar to that observed in intact cells (Figure 4A, interphase cytosol, and B, intact I and semi-intact I). In the presence of AMP-PNP, a

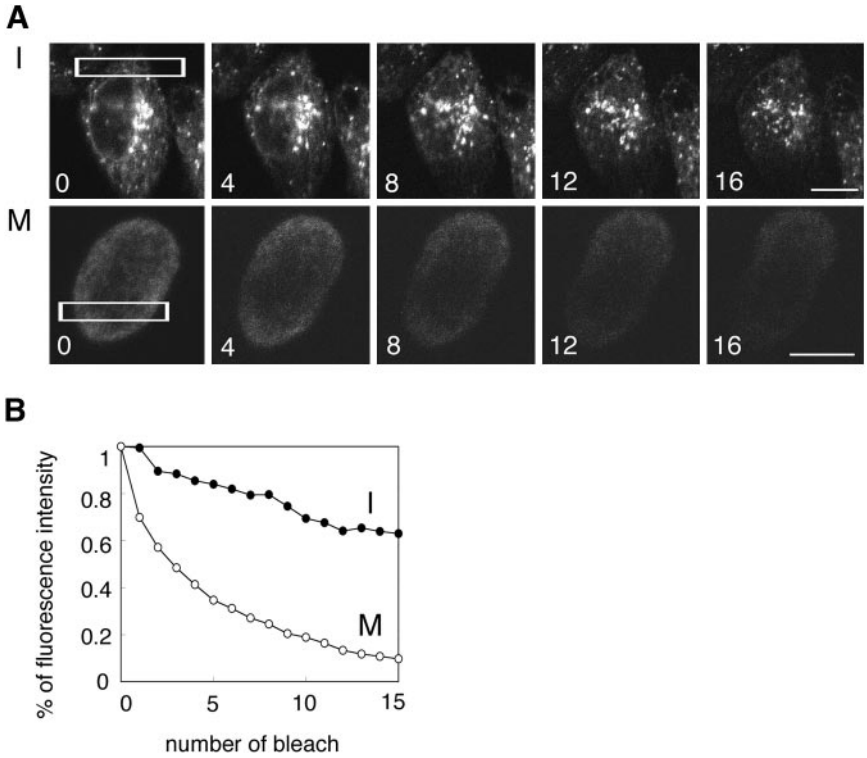

Figure 3. Diffusion of Yip1A-GFP during mitotic phase. (A) FLIP experiments in CHO-YIP cells. A boxed area spanning an interphase cell (I) or a mitotic cell (M) was repetitively photobleached with high laser power, and images were collected as described in *Materials and Methods* after each cycle of bleaching. The numbers of bleaching cycles are indicated at the lower left corner of the images. Bar, 10 μm. (B) Fluorescence intensities of the whole interphase cell (closed circle, I) or the whole mitotic cell (open circle, M) are plotted against number of photobleach cycles.

nonhydrolyzable ATP homologue, most of the ERES disappeared, resulting in reduced numbers of ERES per unit area (unpublished data). These results suggest that hydrolysis of ATP is required to maintain ERES and are consistent with data showing that recruitment of COP II and budding of VSVG from the ER required ATP (Rowe *et al*., 1996; Aridor *et al*., 1998; Aridor and Balch, 2000).

Next, we examined the effects of mitotic L5178Y cell cytosol on the maintenance of preexisting ERES. In the presence of ATP, the cytosol caused the disassembly of ERES (Figure 4A, mitotic cytosol) and reduced their number significantly (Figure 4B, semi-intact I and semi-intact M). The diffuse distribution of Yip1A-GFP in the ER network was similar to that observed in intact mitotic cells (Figure 2). We also investigated the effect of Cdc2 kinase, an important regulator of the cell cycle, on ERES disassembly. Immunodepletion of Cdc2 kinase from mitotic cytosol maintained ERES architecture (Figure 4B, Cdc2 dep). Cdc2 depletion was confirmed by Western blot analysis (unpublished data), and the loss of Cdc2 kinase activity in the depleted cytosol was monitored by the Cdc2 kinase assay (Figure 4B, right column). Furthermore, we examined whether addition of Cdc2/cyclin B into Cdc2-depleted mitotic cytosol could restore the inhibition. As shown in Figure 4B (Cdc2 dep + Cdc2), the disassembly of Yip1A-GFP occurred by addition of Cdc2/cyclin B (72 U/reaction mixture). MEK1 is another kinase reported to be involved in the disassembly of the Golgi apparatus during mitosis (Acharya *et al*., 1998; Colanzi *et al*., 2000; Kano *et al*., 2000a; Cha and Shapiro, 2001). Incubation of semi-intact CHO-YIP cells with mitotic cytosol in the presence of PD98059, a specific inhibitor of MEK1, did permit the disassembly of ERES (unpublished data).

Given that Yip1A-GFP labeled ERES were disrupted in a Cdc2 kinase-dependent manner, we examined whether the dissociation of Sec13, a COP II marker, from ERES also was Cdc2 kinase dependent. We incubated semi-intact CHO-YIP cells in the presence of interphase cytosol, mitotic cytosol (M), mock-depleted mitotic cytosol, Cdc2-depleted mitotic cytosol, or Cdc2-depleted cytosol plus recombinant Cdc2/cyclin B proteins at 32°C for 40 min, and quantified the loss of the COP II marker (Sec13p) from ERES by indirect immunofluorescence, by using an anti-Sec13 antibody. As has been shown for Yip1A-GFP, the number of ERES visualized by detection of Sec13 decreased in the presence of mitotic cytosol (Figure 4C, semi-intact M). This decrease was inhibited in the presence of Cdc2-depleted mitotic cytosol (Figure 4C, Cdc2 dep) and was restored by addition of Cdc2/cyclin B (Figure 4C, Cdc2 dep + Cdc2), consistent with the results obtained using Yip1A-GFP as a marker for ERES (Figure 4B).

Together, these results suggest that the disassembly of ERES by mitotic cytosol is regulated by Cdc2 kinase.

Mitotic Cytosol Inhibits the Anterograde Transport from the ER to the Golgi

In intact cells, anterograde transport is inhibited during mitosis (Featherstone *et al*., 1985; Jesch and Linstedt, 1998; Mackay *et al*., 1993; Farmaki *et al*., 1999). Given that ERES disassemble in a Cdc2 kinase-dependent manner in the presence of mitotic cytosol, the anterograde transport from the ER to the Golgi also might be affected under the same conditions in semi-intact cells. To test this, we examined the transport of VSVGts045-GFP from the ER to plasma membrane via the Golgi apparatus in semi-intact CHO cells. Using the transport assay for VSVGts045-GFP, we found that VSVGts045-GFP was transported to the Golgi and the plasma membrane in the presence of interphase cytosol (Figure 5A, I cytosol). On the contrary, in the presence of mitotic cytosol, VSVGts045-GFP remained at the ER, suggesting that mitotic cytosol blocked vesicular budding from the ER (Figure 5A, M cytosol). As has been observed in the case of mitotic cytosol-induced disassembly of ERES (Figure 4), the block of anterograde transport also was dependent on

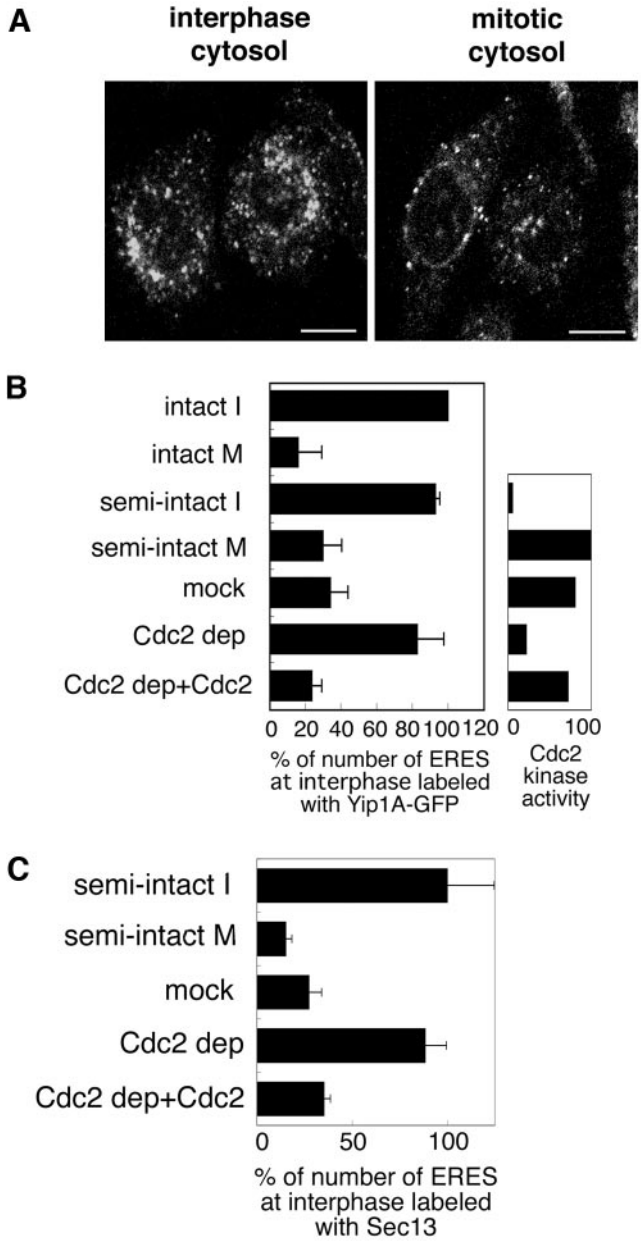

Figure 4. Biochemical requirements for ERES disassembly by mitotic cytosol. (A) Semi-intact CHO-YIP cells were incubated with interphase or mitotic cytosol with an ATP-regenerating system at 32°C for 20 min and then viewed by a confocal microscope. Bar, 10 μm. (B) Number of ERES in interphase cells (intact I) or mitotic cells (intact M) was calculated using the same method used for the ERES disassembly assay. Semi-intact CHO-YIP cells were incubated with interphase cytosol (semi-intact I), mitotic cytosol (semi-intact M), mock mitotic cytosol (mock), Cdc2-depleted mitotic cytosol (Cdc2 dep), or Cdc2-depleted mitotic cytosol plus 72 U of Cdc2/cyclin B (Cdc2 dep + Cdc2). After the incubation, the cells were subjected to the ERES disassembly assay. Cdc2 kinase activity in each reaction mixture (means from two independent measurements) is shown in the right hand column, in which 100% represents the value of Cdc2 kinase activity in mitotic cytosol. Three independent assays were performed and the means and standard deviations are plotted in the graph. (C) Semi-intact CHO-YIP cells were incubated as described above. After incubation, we quantified the loss of COP II marker (Sec13) at ERES by indirect immunofluorescence by using an anti-Sec13 antibody; 100% represents the number of Sec13-positive ERES in the presence of interphase cytosol.

the presence of active Cdc2 kinase in mitotic cytosol (Figure 5B, Cdc2 dep and Cdc2 dep + Cdc2). We conclude that the activation of Cdc2 kinase in mitotic cytosol causes the disassembly of ERES and the corresponding inhibition of anterograde transport from the ER to the Golgi in semi-intact cells.

Phosphorylation of p47 Triggers the Disassembly of ERES by Mitotic Cytosol

The disassembly of ERES by mitotic cytosol in vitro should be regulated by Cdc2 kinase-mediated phosphorylation. One of the candidate target proteins of this phosphorylation activity is p47, a cofactor for p97 that mediates fusion of Golgi membranes (Kondo *et al.*, 1997). p47 was reported to play a crucial role in the de novo formation of ERES in vitro (Roy *et al.*, 2000) and the formation of an ER network from microsomal membrane vesicles (Hetzer *et al.*, 2001). More recently, Uchiyama *et al.* (2003) found that Ser^{140} of p47 was selectively phosphorylated by Cdc2 kinase and that the phosphorylation was involved in Golgi disassembly during mitosis. From these results, we hypothesized that p47 also was required for the maintenance of ERES and that the disassembly of preexisting ERES is controlled by phosphorylation of p47, in a Cdc2-dependent manner.

To test this, we first investigated whether a mutant of p47 that cannot be phosphorylated (S140A, referred to as p47NP) inhibited the disassembly of ERES by mitotic cytosol. We prepared recombinant p97, p47, and p47NP as described in Uchiyama *et al.* (2003). The ERES disassembly assay was performed on semi-intact cells incubated with mitotic cytosol and ATP in the presence of p97 (20 μg/ml) and p47 (22 μg/ml), or p97 (20 μg/ml) and p47NP (22 μg/ml). The mitotic disassembly of ERES was partially blocked in the presence of p97 and p47 (Figure 6A, Yip1A-GFP). However, in the presence of p97 and p47NP, disassembly was completely inhibited (Figure 6A, Yip1A-GFP, M + p97/p47NP). We also examined the effect of p97/p47NP on the dissociation of Sec13 from ERES. As shown in Figure 6A (Sec13), mitotic cytosol-induced dissociation of Sec13 was substantially inhibited by p97/p47NP and partially inhibited by p97/p47WT. These results suggested that the Cdc2 kinase-dependent phosphorylation of p47 plays a crucial role in the disassembly of preexisting ERES. We assumed that the complete inhibition of the disassembly in the presence of mitotic cytosol with p97/p47NP would preserve anterograde transport of VSVGts045-GFP. However, anterograde transport was only partially restored under these conditions (Figure 6A, VSVG-GFP). Other factors, such as microtubule-dependent motor proteins, probably contribute to the specific block of anterograde transport under mitotic conditions.

If functional ERES are directly involved in anterograde transport of VSVGts045-GFP, transport should be inhibited under conditions that perturb ERES integrity but leave other transport processes intact. To confirm this, we examined the effect on both ERES integrity and anterograde transport of antibodies against p47, which have been reported to be necessary for the de novo formation of ERES (Roy *et al.*, 2000). The polyclonal antibody inhibits the fusion of mitotic vesiculated Golgi membranes in vitro (Rabouille *et al.*, 1998). We found that anti-p47 antibody caused substantial disassembly of ERES in semi-intact cells and concomitantly inhibited transport (Figure 6B, I + anti-p47). Coincubation of anti-p47 with p47 recombinant protein inhibited the effects of these antibodies on ERES disassembly or transport (Figure 6B, I + quenched anti-p47). In contrast, unrelated antibody against EEA1, which is a marker protein of early endosomes, had no effect on the disassembly and the trans-

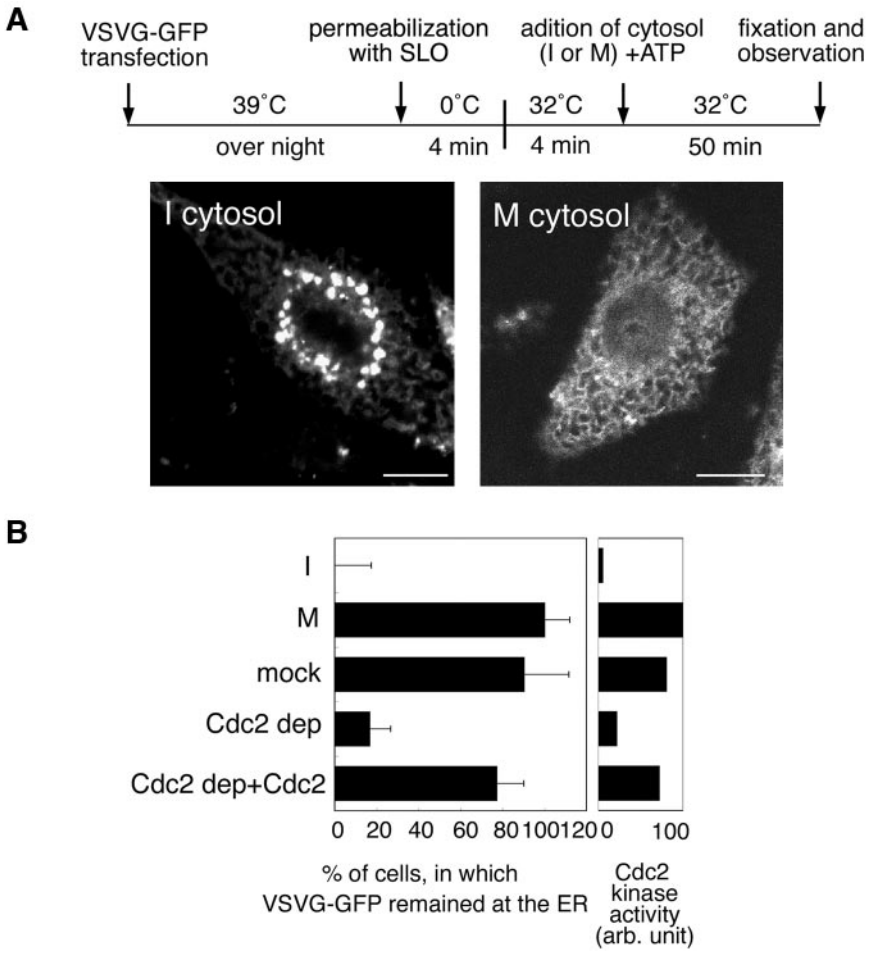

Figure 5. Effect of interphase and mitotic cytosol on the ER-to-Golgi transport of VSVGts045-GFP (A) and the Cdc2 kinase-dependent inhibition of the transport (B). (A) VSVGts045-GFP was transfected into CHO cells, and transport assays were performed as described in *Materials and Methods* in the presence of interphase (I) or mitotic (M) cytosol. The cells were fixed and observed by a confocal microscope. Bar, 10 μm. (B) Extent of transport from the ER to the Golgi was measured in semi-intact cells in the presence of interphase cytosol (I), mitotic cytosol (M), mock mitotic cytosol (mock), Cdc2-depleted mitotic cytosol (Cdc2 dep), or Cdc2-depleted mitotic cytosol plus 72 U of Cdc2/cyclin B (Cdc2 dep + Cdc2). Vesicular transport from the ER to the Golgi was assayed and the mean percent of cells, in which VSVGts045-GFP remained at the ER, was calculated and shown in the left-hand column. Three independent assays were performed, and the means and the deviations are plotted in the graph. Cdc2 kinase activity in each reaction mixture (means from two independent measurements) is shown in the right hand column, with 100% representing the value of Cdc2 kinase activity in mitotic cytosol.

port (Figure 6B, I + anti-EEA1). It also is possible that the antibodies might affect the integrity of the Golgi apparatus and the ER during the transport assays. However, we did not observe any morphological changes in the Golgi or the ER during the transport assay. Similar results were obtained when we examined the dissociation of Sec13 (Figure 6B, Sec13).

These data show that p47 is involved in the maintenance of ERES and that the extent of disassembly of ERES is correlated with inhibition of anterogade transport, even in the presence of interphase cytosol.

DISCUSSION

One of the aims of this study was to visualize cell cycle-dependent morphological changes of ERES by using a transmembrane protein marker, instead of cytosolic COP II components. In addition, we wanted to establish a reconstitution system to investigate the biochemical requirements of mitotic disassembly of ERES in semi-intact mammalian cells. In general, it is difficult to observe the precise morphology of organelles during mitosis in mammalian cells by using a light microscope due to the cells' round shape. Using the semi-intact cell system, we can more easily observe the disassembly of "preexisting" ERES in the presence of mitotic cytosol, and we are able at the same time to investigate the effects of exogenous antibodies or drugs on the disassembly process.

Dynamic Behavior of ERES Visualized with Yip1A-GFP in Intact Interphase Cells

The shape, size, localization, and dynamics of Yip1A-GFP–labeled structures were indistinguishable from those of ERES that had been visualized by COP II marker proteins. Furthermore, we observed an enlargement of Yip1A-GFP–labeled structures by nocodazole that was similar to that observed when COP II markers are used to monitor ERES location and integrity during nocodazole treatment (Hammond and Glick, 2000). Thus, we conclude that Yip1A-GFP is an appropriate marker for ERES location and integrity in mammalian cells.

In CHO-YIP cells, Yip1A-GFP–labeled ERES seem to have a similar size and fluorescence intensity (roughly proportional to the concentration of Yip1A-GFP), as has been reported previously in the case of *P. pastoris* ERES visualized with Sec13-GFP (Bevis *et al.*, 2002). By time-lapse microscopy, we observed that when larger ERES form by fusion, they undergo shrinkage or division, leading to ERES of a constant average size (Movies 1 and 2).

We found that ERES have a relatively long half-life and that they move along the ER network, occasionally protruding from the ER tubules and moving from one ER tube to another (Figure 1B and Movie 1). To our knowledge, this is the first examination of the dynamic behavior of ERES in mammalian cells by using the transmembrane marker Yip1A.

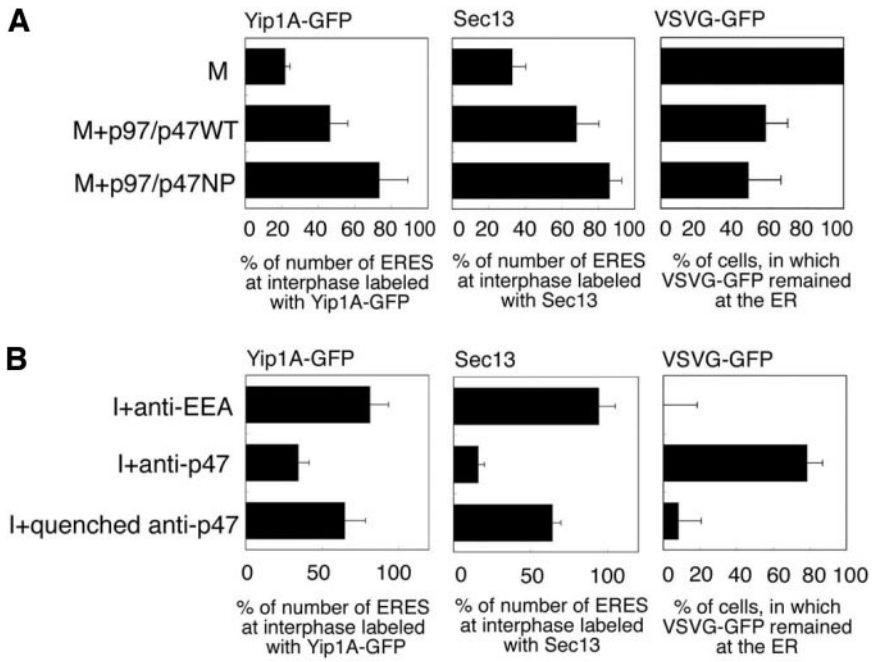

Figure 6. Phosphorylation of p47 is a trigger for ERES disassembly and the inhibition of ER→Golgi transport, and the integrity of ERES is essential for the transport. (A) Semi-intact cells were incubated with mitotic cytosol (M), mitotic cytosol in the presence of p97/p47 wild-type (M + p97/p47WT) or mitotic cytosol in the presence of p97/nonphosphorylatable version of p47 (M + p97/p47NP), and the cells were subjected to the ERES disassembly assay (Yip1A-GFP), immunostaining of Sec13 (Sec13), and the ER→Golgi transport assay (VSVG-GFP). Mean percentage of number of ERES were calculated and are shown. Similarly, the mean number of cells, in which VSVGts045-GFP remained at the ER, was calculated. We confirmed that addition of recombinant proteins p97, p47, and nonphosphorylatable version of p47 did not decrease the Cdc2 kinase activity in mitotic cytosol. (B) Semi-intact cells were incubated with interphase cytosol in the presence of anti-EEA1 antibody (I + anti-EEA1), anti p47 (I + anti-p47), or anti-p47 antibody quenched with recombinant p47 proteins (I + quenched anti-p47), and the cells were subjected to the ERES disassembly assay (Yip1A-GFP), immunostaining of Sec13 (Sec13), and the ER→Golgi transport assay (VSVG-GFP). Three independent assays were performed and the means and deviations are plotted in the graph.

The observation that Yip1A-GFP shows restricted diffusion when localized to ERES is intriguing (Figure 3). Cytosolic factors such as microtubules or actin filaments might be involved in the localization. The evidence in favor of a role of the cytoskeleton in the integrity of Yip1A-labeled ERES might depend on the experimental conditions. As described above, treatment of CHO-YIP cells with nocodazole (10 μg/ml nocodazole at 32°C for 1 h) induced the enlargement of ERES (Supplemental Figure 1). However, treatment with a smaller quantity of this agent (preincubated cells on ice for 15 min, followed by incubation with 2.5 μg/ml nocodazole on ice for 15 min, and then subsequently at 37°C for 15 min to disrupt microtubules only, not the Golgi apparatus) had no effect on ERES integrity in intact or semi-intact cells (unpublished data). In addition, cytochalasin D (25 μM) had no effect on ERES integrity. The mechanism of the restricted diffusion of Yip1A is an interesting issue to be addressed in the future.

Mitotic Disassembly of ERES Induced by Cdc2 Kinase-dependent Phosphorylation of p47

Several reports have suggested that cytosolic marker proteins (Sec13 and Sec24A) dissociate from the ERES during mitosis (Hammond and Glick, 2000; Stephens, 2003). However, because these proteins are cytosolic, it was difficult to know whether the integrity of the ERES structure was disrupted during mitosis. Using Yip1A-GFP, we have shown morphologically that one of the membrane components of the ERES is released from the ERES into the bulk ER network during mitosis (Figure 2 and Movie 6). Based on the results of the photobleaching experiments (Figure 3), we showed spectroscopically that Yip1A-GFP diffuses throughout the ER network at the onset of mitosis. In addition, we found that ER-to-Golgi transport was inhibited under conditions where Yip1A-GFP showed a diffuse distribution (Figures 5 and 6). These morphological and functional data suggest that ERES are substantially disassembled during mitosis.

We reconstituted mitotic disassembly of ERES in semi-intact CHO-YIP cells by using mitotic L5178Y cytosol/ATP and found that the disassembly was dependent on the activation of Cdc2 kinase, a master kinase regulating mitosis (Figure 4, B and C). Additionally, we found that the phosphorylation of p47 by Cdc2 kinase was essential for the disassembly of ERES in semi-intact cells (Figure 6A). p47, a cofactor of AAA-ATPase p97, is phosphorylated by Cdc2 kinase, leading to inability of binding to Golgi membranes (Uchiyama *et al*., 2003). It is notable that p47 plays an important role in the de novo formation of the smooth membrane network or ERES from low-density microsomes (Lavoie *et al*., 1999; Roy *et al*., 2000) and ER network from DilC18-labeled membrane vesicle fractions of *Xenopus* eggs (Hetzer *et al*., 2001). As shown in Figure 6A, in the presence of p97/p47NP with mitotic cytosol, the disassembly of ERES was inhibited, although it was partially blocked even in the presence of p97/p47WT. If all exogenous p97/p47WT was phosphorylated by Cdc2 in mitotic cytosol, complete disassembly of ERES should be induced in the presence of p97/p47WT. The partial inhibition might result from the excess addition of p97/p47WT. Because addition of p97/p47NP substantially inhibited the disassembly of ERES, we conclude that activation of Cdc2 kinase at the onset of mitosis induces the phosphorylation of p47. This phosphorylation is one of the triggers for disassembly of ERES during mitosis.

Mitotic Inhibition of ER-to-Golgi Transport by ERES Disassembly

Several lines of evidence support the idea that the disassembly of ERES is directly coupled to the block in ER-to-Golgi transport. First, mitotic cytosol inhibited the ER-to-Golgi transport of VSVGts045-GFP (Figure 5A). This inhibition seems not to be due to the depolymerization of microtubules by mitotic cytosol, because we have found that the cytosol had little effect on microtubule integrity (unpublished data). Second, in the presence of mitotic cytosol and recombinant p97/p47NP complex, ERES disassembly was inhibited, but

transport was only partially restored (Figure 6A, M + p97/p47NP). The incomplete restoration of ER-to-Golgi transport indicates that other factors in addition to mitotic ERES disassembly contribute to the specific block of anterograde transport. Cdc2 kinase in mitotic cytosol might regulate the activity of multiple microtubule motors (Dillman and Pfister, 1994; Dell *et al*., 2000), and dysfunction of motors and disassembly of ERES during mitosis might inhibit the ER-to-Golgi transport in living cells in a coordinated manner.

Third, we have shown that transport is inhibited even in the presence of interphase cytosol under conditions that induce ERES disassembly (Figure 6B). The polyclonal antibodies against p47 used in this study were confirmed to inhibit the reassembly of the mitotic vesicular Golgi membranes, probably due to inhibition of p97/p47/tSNARE (syntaxin5) complex formation (Rabouille *et al*., 1998; Roy *et al*., 2000). Interphase cytosol caused the disassembly of ERES and concomitantly inhibited transport in the presence of these antibodies (Figure 6B, I + anti-p47), but the use of unrelated antibodies, such as those against EEA1, a marker protein of early endosomes, did not produce this effect (Figure 6B, I + anti-EEA). It is surprising that addition of anti-p47 induced ERES disassembly because the majority of p47 is located within the nucleus during interphase (Uchiyama *et al*., 2003). This result indicates that a small amount of cytosolic p47 during interphase is sufficient to maintain ERES integrity and Yip1A assembly at ERES. Under these conditions, we did not observe the accumulation of any transport intermediates; VSVGts045-GFP remained within the ER network (Figure 5A). This indicates that budding of COP II vesicles was blocked due to disassembly of the ERES through regulation of p47 function. Recently, Dalal *et al.* (2004) reported that transport of VSVGts045 from the ER to the plasma membrane through the Golgi apparatus is unaffected by the expression of p97(E578Q), a mutant of p97 that is deficient in ATP hydrolysis, although transport ceased when the ER had become fully vacuolated due to overexpression of the mutant protein. On the other hand, inhibition of the function of the p97 cofactor p47 by anti-p47 antibodies resulted in the blockade of VSVGts045-GFP transport at the ER. Because the activity of p47 is closely associated with that of p97, our result may seem to be inconsistent with those of Dalal. A possible explanation might be that the ATPase activity of p97 is not involved in ERES disassembly. Muller *et al.* (1999) demonstrated that a mutant of NSF (a fusion protein belonging to the AAA ATPase family) deficient in ATPase activity, allowed efficient fusion of isolated Golgi membranes in vitro. In addition, an ATPase deficient mutant of Cdc48p, a yeast homologue of p97, was functional in cell cycle progression and membrane fusion (Rouiller *et al*., 2000).

Together, we have demonstrated that assembly of Yip1A at ERES is involved in ER-to-Golgi transport in mammalian cells and that phosphorylation/dephosphorylation of p47 regulated the cell cycle-dependent assembly/reassembly of Yip1A at ERES.

The perturbation of the balance between anterograde and retrograde transport is closely coupled to the morphological changes of the Golgi apparatus. Recently, we found that anterograde transport of GFP-tagged galactosyltransferase was inhibited in the presence of mitotic cytosol, whereas retrograde transport was unaffected by this treatment in semi-intact CHO cells (unpublished data). Together with our results, at the onset of mitosis, the activation of Cdc2 kinase induces the phosphorylation of p47 and results in the disassembly of the ERES. The disassembly causes the inhibition of anterograde transport, whereas retrograde transport continues. This would accelerate the Golgi disassembly at the onset of mitosis. There are two controversial models of the Golgi morphology during mitosis; Golgi apparatus fragments into vesicles and remains separated to the ER (Axelsson and Warren, 2004; Pecot and Malhotra, 2004), or the Golgi components relocate to the ER (Zaal *et al*., 1999). Although the fate of the vesicles from the Golgi apparatus remains to be elucidated, ERES integrity would be indirectly involved in morphological changes of the Golgi during mitosis.

Several important questions remains to be solved at this time such as identification of what anchors Yip1A to the ERES and why the membrane fusion process is required for the assembly of ERES. Our reconstitution system, based on semi-intact cells coupled with GFP visualization techniques, will allow us to dissect the cell cycle-dependent disassembly and reassembly of ERES and to investigate the biochemical requirements for each process.

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