Cell Cycle-regulated, Microtubule-independent Organelle Division in *Cyanidioschyzon merolae*

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Mitochondrial and chloroplast division controls the number and morphology of organelles, but how cells regulate organelle division remains to be clarified. Here, we show that each step of mitochondrial and chloroplast division is closely associated with the cell cycle in *Cyanidioschyzon merolae*. Electron microscopy revealed direct associations between the spindle pole bodies and mitochondria, suggesting that mitochondrial distribution is physically coupled with mitosis. Interconnected organelles were fractionated under microtubule-stabilizing condition. Immunoblotting analysis revealed that the protein levels required for organelle division increased before microtubule changes upon cell division, indicating that regulation of protein expression for organelle division is distinct from that of cytokinesis. At the mitochondrial division site, dynamin stuck to one of the divided mitochondria and was spatially associated with the tip of a microtubule stretching from the other one. Inhibition of microtubule organization, proteasome activity or DNA synthesis, respectively, induced arrested cells with divided but shrunk mitochondria, with divided and segregated mitochondria, or with incomplete mitochondrial division restrained at the final severance, and repetitive chloroplast division. The results indicated that mitochondrial morphology and segregation but not division depend on microtubules and implied that the division processes of the two organelles are regulated at distinct checkpoints.

INTRODUCTION

For proper function and inheritance of eukaryotic cells, the number and morphology of organelles need to be controlled (reviewed in Yaffe, 1999a; Miyagishima *et al.*, 2003a; Osteryoung and Nunnari, 2003). Although the division of organelles plays a direct and essential role in this control, how cells regulate organelle division is not clearly understood. Previous studies have demonstrated similarities between the mechanisms of mitochondrial and chloroplast division, because FtsZ, plastid/mitochondrion dividing (PD/MD) rings and dynamin are, respectively, involved in placement, constriction and severance during the organelle division processes (Nishida *et al.*, 2003; Miyagishima *et al.*, 2003b). Mitochondria in higher eukaryotes lack FtsZ and undergo frequent fission and fusion (reviewed in Yaffe, 1999b), and so, too, in higher plants (Arimura *et al.*, 2004). The placement of chloroplast and primitive mitochondrial division implicates the bacterial remnant of FtsZ, because FtsZ forms a ring around the inside of the future division site (Beech *et al.*, 2000; Takahara *et al.*, 2000; Nishida *et al.*, 2003). The constriction is thought to be driven by plastid dividing (PD) or mitochondrion dividing (MD) rings, which are observed as electron-dense rings on both the outside and inside of most chloroplasts or plastids and some primitive mitochondria, and in the intermembrane space of some algal chloroplasts (reviewed in Kuroiwa *et al.*, 1998; Miyagishima *et al.*, 2003a).

Although the molecular components of these rings have not yet been identified, the outer PD ring has been shown to be a bundle of 5-nm filaments (Miyagishima *et al.*, 2001a). In mitochondria in higher eukaryotes, the MD ring and FtsZ are possibly degraded or replaced by unknown mechanisms. The final severance is mediated by dynamin-related proteins in virtually all mitochondria and chloroplasts (Miyagishima *et al.*, 2003b; Nishida *et al.*, 2003). Dynamin polymerizes to form a ring or spiral around the membrane tube and mediates fission of the membrane (reviewed in Orth and McNiven, 2003). Conventional dynamin is involved in the vesicle fission of endocytosis and other membrane remodeling processes in coordination with cytoskeletal elements. Certain dynamin-related proteins are involved in mitochondrial fission (reviewed in van der Bliek, 1999). However, it is still a matter of debate whether dynamin itself can drive the membrane fission force.

Organelle distribution is mediated by microtubules and/or microfilaments in most eukaryotes. In animal cells, microtubule-associated motor proteins are mainly responsible for mitochondrial transport (Nangaku *et al.*, 1994; Pereira *et al.*, 1997). In fission yeasts, microtubule polymerization rather than motor proteins mediates mitochondrial distribution (Yaffe *et al.*, 2003). In budding yeasts, microfilaments but not microtubules mediate mitochondrial positioning and movement (reviewed in Boldogh *et al.*, 2001). In higher plants, microfilaments drive the movement of mitochondria, whereas microtubules control mitochondrial positioning (Van Gestel *et al.*, 2002). In the primitive alga *Cyanidioschyzon merolae*, the cytoskeletal organization itself has yet to be characterized; indicated by the apparent absence of conventional actomyosin by cytological analysis (Suzuki *et al.*, 1995), lack of myosin genes, and failure to detect any ex-

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pression of the conventional actin gene (Matsuzaki *et al.*, 2004).

Each *C. merolae* cell contains one mitochondrion and one chloroplast. When *C. merolae* cell division is synchronized by the light–dark cycle, divisions of these organelles also are synchronized (Suzuki *et al.*, 1994). Previous studies have characterized the FtsZs, namely, CmFtsZ1 and CmFtsZ2 for chloroplast and mitochondrial division, respectively (Takahara *et al.*, 2000), and the dynamins, namely, CmDnm1 and CmDnm2 for mitochondrial (Nishida *et al.*, 2003) and chloroplast (Miyagishima *et al.*, 2003b) division, respectively, involved in these processes. The protein levels of CmFtsZ1, CmFtsZ2 and CmDnm2 are drastically increased during cell division, whereas that of CmDnm1 is not. CmDnm1 is constantly present throughout the cell cycle and forms patches in the cytoplasm before mitochondrial division, when it is dynamically recruited to the constricted mitochondrial division site (Nishida *et al.*, 2003).

In this study, we characterized the association of microtubule organization with mitochondria, the spatial interaction of dynamin with microtubules at the mitochondrial division site, and the cell cycle regulation of mitochondrial and chloroplast divisions by inhibiting DNA synthesis, microtubule organization, or proteasome activity in *C. merolae*.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

C. merolae 10D-14 strain (Toda *et al.*, 1998) was used in this study. Cells were cultured and synchronized as described previously (Suzuki *et al.*, 1994) with minor modifications. For synchronization, a cell culture maintained in Allen's medium (Suzuki *et al.*, 1994) was diluted with 9 volumes of the growth medium, pH 4.6, which was a modified version of Allen's medium and contained 0.3 M sorbitol, 5 mM NH_4NO_3 , 0.4 mM CaCl_2 , 1 mM MgSO_4 , 1 mM K_2HPO_4 , 0.5 mM citric acid, 2 μM CuSO_4 , and a 3/1000 volume of P4 metal solution (0.7 mM FeCl_3 , 0.18 mM MnCl_2 , 80 μM ZnSO_4 , 20 μM CoCl_2 , 10 μM Na_2MoO_4 , and 3 μM Na_2EDTA), and was adjusted to pH 4.6 with H_2SO_4 or KOH. For 5-fluorodeoxyuridine (5FdU) treatment, a 1/10,000 volume of 100 mg/ml 5FdU solution in water was added to give a final concentration of 10 mg/ml at ~6 h before cell division. For microtubule antagonists, up to a 1/100 volume of 33.3 mM nocodazole, 10 mM paclitaxel, 20 mM propyzamide, 20 mM trifluralin, or 20 mM oryzalin stock solution dissolved in dimethyl sulfoxide (DMSO) was added; colchicine was added directly to give a final concentration of 20 mM. Synchronized cells were treated 2 h before cell division. For camptothecin treatment, a 1/50,000 volume of 10 mg/ml camptothecin solution in DMSO was added more than 6 h before cell division. For MG132 and epoxomicin treatments, a 1/500 volume of 50 mM MG132, benzoyloxycarbonyl-leucyl-leucyl-leucinal (Peptide Institute, Osaka, Japan) or a 1/200 volume of 10 mM epoxomicin (Peptide Institute) solutions in DMSO was added ~3 h before cell division.

Antibodies

For generation of anti-elongation factor 2 (EF2) antiserum, the coding region for the amino acid residues from 169 to 769 out of the predicted 846-amino acid sequence of EF2 protein was amplified by PCR by using the following primers CTGGATCCGAGGAGGAGGAAATGTACC and CTTGAAAGCTTAGAGTGGCGTCCCGCAC. The DNA fragment was cloned into pQE80 vector by restriction digestion and ligation in *Escherichia coli* strain XL1Blue. The recombinant protein was produced as fusion protein with a 6-histidine tag at the N terminus and purified by nickel affinity column. The purified protein was injected in mouse to immunize.

The other antisera used have all been characterized previously: anti-CmFtsZ1 and anti-CmFtsZ2 (Takahara *et al.*, 2000), anti-CmDnm1 (Nishida *et al.*, 2003), anti-CmDnm2 (Miyagishima *et al.*, 2003b), and anti-CmEF-Tu(mt) (Nishida *et al.*, 2004). An anti- α -tubulin antibody was commercially purchased (Molecular Probes, Eugene, OR). Immunoblotting was performed as follows. Twenty micrograms of a total protein extract from *C. merolae* was separated by 12.5% SDS-PAGE and then blotted onto polyvinylidene difluoride membrane. After blocking with blocking reagent (Pierce Chemical, Rockford, IL), the membranes were incubated with primary antisera or antibodies at dilutions of 1/200 for anti-CmFtsZ1, 1/1000 for anti-CmFtsZ2, 1/1000 for anti-CmDnm1, 1/500 for anti-CmDnm2, 1/1000 for anti-EF2, and 1/500 for anti- α -tubulin. The secondary antibodies, namely, alkaline phosphatase-conjugated goat anti-rat, anti-rabbit, or anti-mouse IgG (Kirkegaard and Perry

Laboratories, Gaithersburg, MD), were each used at a dilution of 1/1000 and detected using an AP-conjugate substrate kit (Bio-Rad, Hercules, CA).

Cell Fractionation under Microtubule-stabilizing Conditions

Cells were harvested by centrifugation at 3000 rpm for 5 min, suspended in microtubule stabilizing buffer (0.4 M sorbitol, 20 mM Tris, 8 mM EGTA, pH 7.2 adjusted by HCl, 2 mM MgSO_4 , 2 mM KCl, 20 mM dithiothreitol [DTT], 50 μM paclitaxel, and protease inhibitor cocktail). The cells were incubated at 42°C for 20 min and collected by centrifugation. Cells were then washed once and suspended in lysis buffer [1.2 M sorbitol, 10 mM Tris, 4 mM EGTA, pH 7.2 adjusted by HCl, 2 mM MgSO_4 , 2 mM KCl, 1 mM DTT, 50 μM paclitaxel, 0.1% (wt/vol) bovine serum albumin, 10 mg/1 DNase 1, and protease inhibitor cocktail]. Ethanol-washed and equilibrated corn starch [20% (wt/vol)] was added to achieve efficient homogenization. Cells were homogenized in a tight fitting Dounce homogenizer by 50 strokes at room temperature. The homogenate was diluted with an equal volume of lysis buffer and centrifuged at 100g for 3 min to remove corn starch. The supernatant was mixed with an equal volume of loading buffer (0.6 M sorbitol, 10 mM Tris, 4 mM EGTA, pH 7.2 adjusted by HCl, 2 mM MgSO_4 , 2 mM KCl, 1 mM DTT, 10 μM paclitaxel, and protease inhibitor cocktail) containing 10% (wt/vol) iodixanol. The cell lysate was then separated by discontinuous iodixanol density gradient. Four milliliters of 40%, 8 ml of 25%, and 8 ml of 15% iodixanol in loading buffer was layered in this order from the bottom of the centrifugation tube. Up to 15 ml of the cell lysate was layered on the top and then centrifuged at 141,000g for 30 min at 18°C. The band formed at the 25–40% interface was collected as interconnected organelles (ICOs). The fraction was diluted by an equal volume of loading buffer containing 400 mM NaCl, incubated on ice for 30 min, centrifuged at 30,000 $\times$ g for 5 min, and washed twice with loading buffer containing 20% iodixanol and 200 mM NaCl to prepare a salt-washed ICOs pellet.

Microscopy

Cell fixation and immunofluorescence microscopy were performed as described previously (Nishida *et al.*, 2004). The anti- α -tubulin antibody was used at a dilution of 1/100. Highly cross-adsorbed goat anti-mouse IgG conjugated with Alexa 488 was used as the secondary antibody at a dilution of 1/1000.

Transmission electron microscopy was performed as described previously (Miyagishima *et al.*, 2001b). Briefly, a cell pellet was rapidly frozen in liquid propane chilled in liquid nitrogen, transferred to dried acetone containing 1% osmium tetroxide (OsO_4) at -80°C , embedded in Spurr's resin, cut into 90-nm serial thin sections, and stained with uranyl acetate and lead citrate. Sections were examined with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

RESULTS

Protein Levels during Microtubule Organelle Division and throughout the Cell Cycle

Previous studies have indicated that the protein levels of CmDnm2 (Miyagishima *et al.*, 2003b) and CmFtsZ1 and CmFtsZ2 (Takahara *et al.*, 2000) are drastically increased during cell division, whereas those of CmDnm1 are not (Nishida *et al.*, 2003). To elucidate how such changes are coordinated in a cell cycle-dependent manner, synchronous cultures were harvested every 2 h from 23 to 41 h after the onset of synchronization, or every 1 h from 33 to 39 h, during which synchronized cell division is completed. The samples were then subjected to immunoblotting analyses with antisera against the four organelle division proteins and α -tubulin. The level of CmDnm1 was apparently constant throughout the cell cycle (Figure 1, D1). FtsZ1 (Figure 1, Z1) and FtsZ2 (Figure 1, Z2) began to increase at 31 or 33 h, reached a peak at 35 h, and then drastically decreased at 41 h. CmDnm2 (Figure 1, D2) occurred at 34 h, reached a peak at 35 h, decreased at 39 h, and had disappeared at 41 h. The anti- α -tubulin antibody detected a band of ~48 kDa, which is consistent with the predicted size of *C. merolae* α -tubulin (our unpublished data). α -Tubulin showed faintly at 34 h, gradually increased until 38 h, and had disappeared at 41 h (Figure 1, Tub).

Characterization of Microtubule Organization in Association with Mitochondria

To characterize the cell cycle of *C. merolae* in association with organelle division, we simultaneously visualized microtu-

Figure 1. Immunoblotting analysis of proteins for microtubules and organelle division throughout the cell cycle. Cells were harvested at the indicated time points after the onset of incubation from the same synchronous culture. Twenty micrograms of total protein was loaded per lane and detected using the antisera or antibodies indicated on the left. Tub, α -tubulin; D1, CmDnm1; D2, CmDnm2; Z1, CmFtsZ1; Z2, CmFtsZ2.

bule organization and mitochondria. Indirect immunofluorescence microscopy was performed for α -tubulin and EF-Tu(mt), which was previously shown to be a mitochondrial matrix marker (Nishida *et al.*, 2004). Chloroplasts of fixed *C. merolae* cells were observed as bright spots under a phase

contrast microscope. DNA was visualized by 4,6-diamidino-2-phenylindole (DAPI) staining. In Figure 2, images of the labeled cells are aligned in the order cell division progressed. At the initiation of cell division, a single pole or astral of microtubules occurred at one end of the mitochondrion (Figure 2A, Tub and Merged). When the chloroplast began to elongate (Figure 2B, PC/Mito), the microtubules were stretched along the mitochondrion (Figure 2B, Tub and Merged). When the mitochondrion was elongated and the chloroplast began to be constricted as its nucleoids were almost separated (Figure 2C, PC/Mito and DAPI), a second pole of microtubules was formed at the other end of the mitochondrion (Figure 2C, Tub and Merged). As the chloroplast constriction proceeded, the mitochondrion expanded and moved to fill the space created (Figure 2D, PC/Mito), the nucleus broadened (Figure 2D, DAPI) and the microtubules became organized around the mitochondrion, whereas the poles were attached to the edge of the nucleus as well as both ends of the mitochondrion (Figure 2D, Merged). As the mitochondrion began to constrict, the two poles were enclosed (Figure 2. E and F), the nucleus was condensed (Fig-

Figure 2. Microtubule organization in association with mitochondria in *C. merolae*. Images of fixed and immunolabeled cells are aligned vertically (A–K) according to the predicted order of cell cycle progression. Images of mitochondria (red) visualized with an anti-CmEF-Tu(mt) antiserum and an Alexa 555-conjugated secondary antibody are merged with phase contrast images (PC/Mito). DNA is visualized by DAPI staining (DAPI). Microtubules are visualized with an anti- α -tubulin antibody and an Alexa 488-conjugated secondary antibody (Tub). The images of the mitochondria (red), DNA (blue), and microtubules (green) are then merged (Merged). Bar, 1 μ m.

Figure 3. Direct association between spindle pole bodies and mitochondria observed by transmission electron microscopy. (A) Section showing a spindle from pole to pole. Electron-dense spindle poles are located at both ends of the spindle. (B) A spindle pole body is directly associated with the mitochondria. At one end of the elongated spindle, a spindle pole body can be seen to be composed of two electron-dense layers that pinch the nuclear envelope. The outer layer is associated with the tip of one of the divided mitochondria. (C) Fourfold magnification of B. cp, chloroplast; m, mitochondria; asterisk, spindle; arrowhead, nuclear envelope; arrow, association between mitochondria and spindle pole body. Bars, 200 nm (A and B), 50 nm (C).

ure 2, E and F, DAPI), and the microtubules stretched toward the mitochondrial constriction site (Figure 2E, Tub and Merged). The microtubules from one of the two poles tended to remain at the constriction site (Figure 2F, Tub and Merged). When mitochondrial division was completed (Figure 2G, PC/Mito), a thick spindle was formed between the two poles (Figure 2G, Tub) that were still anchored to the divided mitochondria (Figure 2G, Merged). As the spindle elongated (Figure 2, H–I, Tub), nuclear division occurred (Figure 2, H–I, DAPI). After nuclear and mitochondrial segregation was accomplished, cytokinesis occurred as the cell division site was constricted (Figure 2J). After cell division, the microtubules became disorganized (Figure 2K, Tub) and then disappeared (Figure 2L, Tub). Throughout the cell division, the microtubule poles were always associated with the ends of the mitochondria (Figure 2, Merged).

Direct Interaction between Mitochondria and Spindle Poles

To further investigate the spatial interaction between mitochondria and microtubules, dividing cells were examined by transmission electron microscopy. A spindle ran through the dividing cell and seemed to be a bundle of microtubules (Figure 3A, asterisk). At both ends of the spindle, electron-dense components were seen as spindle poles. In another section, the spindle pole was revealed to be a pair of closed layers, with the nuclear envelope in between (Figure 3C, arrowheads). The tip of one of the divided mitochondria was directly associated with one of the layers (Figure 3C, arrow).

Copurification of Interconnected Organelles and Stabilized Microtubules

Before the cell fractionation study, we had raised an antiserum against translational EF2, which was thought to be an essential protein for translation and abundant in cytosol while interacting with ribosomes. Immunoblot analysis using the antiserum detected an ~95-kDa band from the total cell lysate of *C. merolae* (Figure 4A), which is nearly consistent with the predicted size of EF2 in *C. merolae*.

To assess the physical interaction of microtubules to the organelles, cell fractionation was performed under microtubule-stabilizing conditions. Although a previous method for the isolation of dividing chloroplast from *C. merolae* (Miyagishima *et al.*, 1999) does not highly preserve the intactness of mitochondria, gentler homogenization is adopted in this study. For microtubule stabilization, certain concentrations of paclitaxel were added to all buffers used, and chilling of the lysate was avoided until homogenization was completed. After being separated by the iodixanol density gradient, mitochondria, chloroplasts, and nucleus were found in the same fraction connected to each other. These ICOs were then salt washed and analyzed by immunoblotting. Although most of EF2, an abundant cytosolic protein, was excluded from ICOs (Figure 4B, EF2), EF(mt), a mitochon-

Figure 4. Characterization of the antiserum against EF2 and cell fractionation under microtubule-stabilizing condition. (A) Immunoblot analysis on total protein of *C. merolae* by using newly raised antiserum against EF2. Forty micrograms of total protein was separated by 5–20% gradient SDS-PAGE and blotted. Molecular weight size marker was indicated on the right. (B) Forty micrograms of total protein (Total) and salt-washed ICOs (ICO) were detected by immunoblotting by using the antibodies or antisera indicated on the left. EF2, anti-EF2 antiserum; EF(mt), anti-EF-tu (mt) antiserum; HC, anti-HC antiserum; α Tub, anti- α -tubulin antibody; Dnm1, anti-CmDnm1 antiserum.

Figure 5. Effects of 5FdU, oryzalin, or MG132 treatment on organelle division and microtubule organization. Synchronized cells in the modified media were treated with 5FdU, oryzalin, MG132, or a 1/500 volume of dimethyl sulfoxide as a control. Cells were fixed at 2 h after the onset of cell division and immunostained to visualize microtubules and mitochondria. Bars, 1 μ m.

drial translation elongation factor (Nishida *et al.*, 2004), was rich in ICOs [Figure 4B, EF(mt)]. A smaller amount of HC, a chloroplast nucleoid protein (Kobayashi *et al.*, 2002), also was detected in ICOs [Figure 4B, HC(pt)]. Comparable amounts of α -tubulin as well as CmDnm1 were detected from total cell lysate and ICOs (Figure 4B, α Tub and Dnm1).

Effects of Microtubule Disruption, Defective DNA Duplication, and Proteasome Inhibition on Organelle Division

To further analyze the relationships among microtubule organization, organelle division, and the cell cycle, several drugs were applied to *C. merolae*. We first modified the contents of the growth medium, because the conventional medium for *C. merolae* is adjusted to pH 1.5, which may be unsuitable for certain chemical treatments. In our modified medium at pH 4.6 (see *Materials and Methods*), the synchronization of cell division, microtubule organization, and cell morphology seemed similar to those in conventional medium. Using the modified medium, several drugs known to be microtubule antagonists were tested at various concentrations in synchronized cultures. Whereas practically permissive concentrations of nocodazole, colchicines, propyzamide, and paclitaxel did not arrest the cell division of *C. merolae*, 40 μ M oryzalin and 160 μ M trifluralin caused the accumulation of division-arrested cells with two chloroplasts per cell. When microtubules were immunostained, oryzalin-treated cells contained dispersed signals throughout the cytoplasm instead of fibrillar microtubules (Figure 5, Microtubule). Division of mitochondria in oryzalin-treated

cells was completed (Figure 5, Mitochondria), although the mitochondria were apparently smaller than those in control cells or shrinking. Under oryzalin treatment, the mitochondrial and chloroplast divisions only occurred once, even after overnight incubation.

Inhibition of nuclear DNA synthesis by aphidicolin results in cell division arrest with an increase in the number of chloroplasts per cell in *C. merolae* (Itoh *et al.*, 1996), and certain concentrations of the nucleotide analog 5FdU (Miyagishima *et al.*, 1999) and camptothecin, a specific inhibitor for DNA topoisomerase 1, can induce a similar effect. In this study, we further studied the effects on mitochondrial division and microtubule organization. Whereas control cells contained divided chloroplasts, mitochondria, and spindles (Figure 5, Control), cells treated with 10 mg/ml 5FdU contained divided chloroplasts and elongated, but undivided, mitochondria (Figure 5, 5FdU). In addition, mitochondria-associated microtubules were observed but the spindle was not (Figure 5, Microtubule). Overnight incubation resulted in repetitive chloroplast division to produce aberrant cells containing four chloroplasts per cell, whereas single enlarged mitochondria were constricted but undivided (Figure 6). In these cells, microtubules were extremely developed into two large, spoke-like astrals, whereas a bundled spindle was not formed (Figure 6).

Because cell cycle transition requires the specific degradation of certain proteins, inhibition of the proteasome-dependent proteolysis causes cell cycle arrest in many other organisms. We wanted to arrest the cell cycle of *C. merolae* with treatments of MG132, an inhibitor for proteasome and ca-

Figure 6. Aberrant cell morphology induced by 5FdU treatment. A cell found after overnight incubation in the presence of 5FdU. Bar, 1 μ m.

thepsin K, or epoxomycin, a specific inhibitor for proteasome. By adding 100 μ M MG132 or 50 μ M epoxomycin in the modified media added at 3 h before the onset of cell division, *C. merolae* cells apparently arrested at a certain stage of cell cycle. Although MG132 forms aggregations in the media, MG132 causes an apparently similar effect to that of epoxomycin on cell cycle arrest in *C. merolae*. MG132 was mainly used in this study because of its availability. MG132-treated cells contained divided and segregated chloroplasts and mitochondria (Figure 5, MG132). Bundled spindles were formed (Figure 5, Microtubules), whereas nuclear segregation was rarely observed (our unpublished observation). To further clarify the effects of inhibiting DNA duplication, microtubule organization, or proteasome activity on the cell cycle and/or the organelles division in *C. merolae*, cells were simultaneously treated with camptothecin and oryzalin or MG132 and quantitatively analyzed for the effects on the organelles division (Table 1). Camptothecin, which is an inhibitor for DNA topoisomerase 1 and caused a similar effect to 5FdU on the cell cycle of *C. merolae*, was used in this study as tighter and more specific inhibitor on DNA duplication than 5FdU. As shown in Table 1, camptothecin treatment accumulated cells with undivided mitochondria in either presence or absence of the other inhibitors, whereas oryzalin and/or MG132 treatment accumulated cells with divided mitochondria only in the absence of camptothecin. Although combination of camptothecin and oryzalin reduced the frequency of cells with divided chloroplast, this is presumably because of the weakened cell activity rather than the specific inhibition of chloroplast division, as expanded incubation increased the frequency of cells with divided chloroplast (our unpublished observation).

Cell Cycle-dependent Recruitment of CmDnm1

Because mitochondrial division and segregation is supposed to be strictly regulated in cell cycle-dependent manner, we inquired whether the localization of CmDnm1, a dynamin-related protein that is recruited from cytoplasmic patches to the mitochondrial division site for the final severance of mitochondrial division (Nishida *et al.*, 2003), depends on cell cycle. When cells were treated with camptothecin, immunolocalization signals for CmDnm1 dispersed among cytoplasm, whereas mitochondrial division had not yet occurred (Figure 7A, Camptothecin). When cells were treated with MG132, CmDnm1 predominantly localized to a mitochondrial division site or the edge of one of the two divided mitochondria (Figure 7A, MG132). When cells were treated with oryzalin, CmDnm1 also formed foci, although some were dissociated from divided mitochondria (Figure 7A, Oryzalin). Frequency of these localizations was quantified in Table 2. In addition, combination of camptothecin with oryzalin or MG132 was performed. Camptothecin treatment restrained CmDnm1 from the accumulation in >90% of cells regardless of the presence of oryzalin or MG132.

Spatial Association between Microtubules and Dynamin Complexes at the Mitochondrial Division Site

Because the microtubule organization was shown to be in direct association with mitochondria, and the oryzalin treatment seemed to stimulate CmDnm1 dissociation from the divided mitochondria, we investigated whether the mitochondrial division apparatus and/or CmDnm1 complex also were associated with microtubules by simultaneously immunolabeling against α -tubulin and CmDnm1. Before recruitment, no significant association was observed between

Table 1. Effects of inhibitors on the organelles division

Inhibitors	State of chloroplast (%)				State of mitochondria (%)		
	one	dividing	divided	irregular	one	dividing	divided
Control	90.7	3.7	5.7	0	94.0	3.3	2.7
Cmp	18.7	17.7	58.3	5.3	25.3	70.0	4.7
Orz	26.0	5.7	67.7	0.7	21.0	18.0	61.0
MG	24.7	4.7	70.0	0.7	23.0	18.0	59.0
Cmp/Orz	11.3	43.3	44.3	1.0	14.0	80.0	6.0
Cmp/MG	23.7	14.3	56.0	6.0	26.3	67.0	6.7

Cmp, camptothecin; Orz, oryzalin; and MG, MG132.

Numbers are percentage of cells ($n \geq 300$). Cells were fixed at 6 h after the onset of cell division of synchronous culture and immunostained with anti-EF-tu(mt) antiserum to visualize mitochondria. Note that cells in Figure 5 were fixed at 2 h after the onset of cell division.

A

B

Figure 7. Effects of camptothecin, oryzalin, or MG132 treatment on CmDnm1 localization. (A) Synchronized cells in the modified media were treated with camptothecin, oryzalin, or MG132. Cells were fixed and immunostained to visualize mitochondria (red) and CmDnm1 (green). Bar, 1 μ m. (B) Mitochondria (red), microtubules (green), and CmDnm1 (blue) were immunostained simultaneously in a typical dividing cell treated with 1/500 volume of dimethyl sulfoxide in the modified medium as a negative control. The phase contrast image is shown as merged images with mitochondria (P.C./mito).

microtubules and CmDnm1 at the cytoplasmic patches (our unpublished observation). When CmDnm1 was recruited to the mitochondrial division site and the mitochondrial severance was subsequently accomplished, CmDnm1 stuck to

one side of the divided mitochondria, whereas a microtubule from a pole at the tip of one of the divided mitochondria ran along the mitochondria and was elongated to reach the site of the division of the other divided mitochondria,

Table 2. Effects of inhibitors on CmDnm1 localization

Inhibitors	CmDnm1 localization (%)		
	dispersed	division site	dissociated
Control	100.0	0	0
Cmp	99.7	0.3	0
Orz	31.7	38.3	30.0
MG	37.3	57.3	5.3
Cmp/Orz	92.0	3.7	4.3
Cmp/MG	93.3	6.3	0.3

Cmp, camptothecin; Orz, oryzalin; and MG, MG132.

Numbers are percentage of cells ($n \geq 300$). Cells were fixed at 6 h after the onset of cell division of synchronous culture and immunostained with anti-EF-tu(mt) antiserum and anti-CmDnm1 antiserum.

where CmDnm1 was accumulated (Figure 7B). Similar results were obtained for 29 of 30 cells in which the mitochondria-associated microtubules and CmDnm1 accumulation could be simultaneously observed.

DISCUSSION

We have shown that increases in the protein levels of CmFtsZ1, CmFtsZ2, and CmDnm2 precede microtubule changes upon cell division. These observations suggest that protein expression for organelle division apparatuses and that for the mitotic apparatus are distinctly regulated in *C. merolae*. On the other hand, that CmDnm1 was constantly present throughout the cell division cycle, whereas CmFtsZ1 and CmFtsZ2 were just faintly detected during interphase suggests that such organelle division proteins are posttranslationally regulated. Because FtsZs are thought to be involved in the placement of organelle division and dynamins are involved in the final severance (Miyagishima *et al.*, 2003b; Nishida *et al.*, 2003), each step of organelle division processes are possibly under distinct regulation, at least in *C. merolae*.

Microtubules and/or microfilaments generally play major roles in cellular morphogenesis, motility, and transport. However, there is no conventional actomyosin system in *C. merolae* (Takahashi *et al.*, 1995; Matsuzaki *et al.*, 2004), and the current results indicate that the microtubules in *C. merolae* are only organized during cell division, indicating that *C. merolae* do not require such conventional cytoskeletal systems for housekeeping activities. Furthermore, constriction of the dividing cell did not seem to involve microtubules. As well, *C. merolae* lacks septins, which are involved in contractile rings in some organisms. These observations suggest that there are alternative cytoskeletal systems that may be more suitable for small simple cells such as *C. merolae* and that have taken the place of the conventional cytoskeletons.

C. merolae is an acidophilic alga that prefers a low pH and usually grows well at pH 1.5–2.5. For this and other reasons, many drug treatments are not adaptable for this alga (Yagisawa *et al.*, 2004), although the targeted proteins are certainly present. In this study, *C. merolae* was successfully grown and synchronized in our modified medium at pH 4.6. We also ensured that cells grown in the modified medium seemed normal in their microtubule organization as well as cell morphology (our unpublished observations). This may be due to the increased osmotic pressure in the presence of

sorbitol. Nevertheless, many microtubule antagonists did not work under these conditions, which may be due to the cell permeability or specialized features of *C. merolae* microtubules. Only oryzalin and trifluralin were found to disorganize microtubules in *C. merolae*, and such dinitroanilines are effective microtubule antagonists for organisms that reside under conditions similar to those for acidothermophilic algae.

Immunofluorescence microscopy revealed that the microtubules were apparently organized at both ends of the mitochondria and that the formation and elongation of spindles were completely coupled with mitochondrial segregation to the daughter cells. The spindle poles in yeast also associate with mitochondria and segregate them to opposite ends of the cell (Yaffe *et al.*, 2003). The results of the current study indicate that such a mechanism is widespread among eukaryotes. Electron microscopy further revealed that *C. merolae* contained distinctive electron-dense components at both ends of the spindle. These were similar to the spindle pole body in yeast because they were composed of double layers that pinched the nuclear envelope from both the inside and outside. At the same time, we found that the tip of one of the divided mitochondria was directly associated with the outer layer. Such an association would directly couple the action of nuclear division with that of mitochondrial segregation. The results of our study suggest that the spindle pole bodies and their association with mitochondria represent a basic mechanism for the segregation of nuclei and mitochondria in eukaryotes.

In our cell fractionation study, mitochondria and chloroplasts are found in the same fraction. This is probably not due to incomplete separation, because narrower ranged discontinuous density gradient did not separate these organelles (our unpublished data). Furthermore, one nucleus, one or two mitochondria, and one or two chloroplasts were associated to each other in most cases when observed under fluorescence microscopy (our unpublished observation). It is likely that in vivo organelle interactions were preserved in the purified ICOs. Homogeneity of the ICOs was ensured by the absence of EF2, which is thought to be abundant in cytosol, and part of them interact with ribosomes and consequently with endoplasmic reticulum. α -Tubulin also was detected in ICOs, suggesting the physical interaction of ICOs with stabilized microtubules. In contrast, we did not detect α -tubulin from the same fraction when cells were lysed without microtubule stabilization (our unpublished data); supposedly, only polymerized microtubules interact with the organelles. Combined with the observation that the spindle pole body is associated with dividing mitochondrion while it locates in the nuclear envelope, microtubules were possibly responsible for the connection between mitochondria and nuclei during cell division in *C. merolae*.

Because both ends of the mitochondrion are directly anchored to the spindle, completion of mitochondrial division is thought to be required before spindle elongation. Inhibition of DNA duplication by 5FdU or camptothecin resulted in repetitive chloroplast division and blocking of mitochondrial severance, but it did not result in constriction. It has been proposed that chloroplast division is free from checkpoint control that would inhibit the progression of mitosis and cytokinesis (Itoh *et al.*, 1996). However, cell division was arrested when spindle formation failed due to oryzalin treatment, whereas chloroplast and mitochondrial divisions occurred once. These results suggest that G₂-arrest induced by 5FdU or camptothecin enhances the initiation of chloroplast division while suppressing mitochondrial severance and that G₂-M transition stimulates mitochondrial severance

while suppressing the initiation of chloroplast and mitochondrial divisions. In addition, mitochondrial segregation as well as division occurred in the presence of MG132, whereas spindles formed but did not elongate enough to segregate nuclei. This indicates that mitochondrial division does not require microtubules, whereas mitochondrial morphology and segregation depend on microtubules. Because the effect on the organelle division of camptothecin dominated over that of MG132 or oryzalin when simultaneously added, the major effects on the organelle division of these inhibitors are probably the consequences of the cell cycle arrests at different steps.

CmDnm1 localization also was shown to be cell cycle dependent. When DNA duplication was inhibited by camptothecin, CmDnm1 was dispersed among cytoplasm and mitochondrial division did not occur. In contrast, CmDnm1 localized to the mitochondrial division site or at the tip of divided mitochondria when arrested by MG132. Although combination of camptothecin with MG132 slightly increased the cells with CmDnm1 accumulation, this is probably because a small part of cells could overcome the inhibition by camptothecin and arrested only by MG132. Because mitochondrial division mediated by dynamin-related proteins has been shown in diverse eukaryotes (reviewed in Osteryoung and Nunnari, 2003), it is possible that CmDnm1 is one of the mediators for cell cycle-dependent mitochondrial division in *C. merolae*. In addition, dissociated CmDnm1 foci from mitochondria were shown in many of oryzalin-treated cells. This indicates that the CmDnm1 complex itself is stable without association to mitochondria or microtubules. It is likely that disorganization of the CmDnm1 complex is also cell cycle dependent. At the same time, CmDnm1 remaining with the divided mitochondria seemed to be stimulated by the existence of microtubules, because MG132-treated cells contain divided mitochondria associated with microtubules as well as CmDnm1. This implies a physical interaction between microtubule and the CmDnm1 complex at mitochondrial division sites. Indeed, we showed that CmDnm1 at one tip of divided mitochondria also was associated with a tip of a microtubule. On the other hand, CmDnm1 recruitment to the mitochondrial division site has been shown to be independent of microtubules. Only assembled CmDnm1 complexes at a mitochondrial division site might interact with microtubules. One hypothesis to give physiological meaning for such an interaction is that the formation of the CmDnm1 complex ensures completed mitochondrial division. Interaction of the microtubules with the complex might act as one of the checkpoints for spindle formation and elongation, because disorganization of CmDnm1 apparently is restrained until spindle elongation and mitochondrial segregation. As a consequence, mitochondrial segregation driven by spindle elongation follows the completion of mitochondrial division. It is still uncertain whether such cell cycle regulation on organelle division may be active in other organisms whose organelles are numerous and changing in number per cell. Nonetheless, our study consistently showed that each step of organelle division may be distinctly regulated, and this information should stimulate future investigation into the molecular mechanisms regulating organelle division.

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