Cleavage of Mcd1 by Caspase-like Protease Esp1 Promotes Apoptosis in Budding Yeast

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Over the last decade, yeast has been used successfully as a model system for studying the molecular mechanism of apoptotic cell death. Here, we report that Mcd1, the yeast homology of human cohesin Rad21, plays an important role in hydrogen peroxide-induced apoptosis in yeast. On induction of cell death, Mcd1 is cleaved and the C-terminal fragment is translocated from nucleus into mitochondria, causing the decrease of mitochondrial membrane potential and the amplification of cell death in a cytochrome *c*-dependent manner. We further demonstrate that the caspase-like protease Esp1 has dual functions and that it is responsible for the cleavage of Mcd1 during the hydrogen peroxide-induced apoptosis. When apoptosis is induced, Esp1 is released from the anaphase inhibitor Pds1. The activated Esp1 acts as caspase-like protease for the cleavage of Mcd1, which enhances the cell death via its translocation from nucleus to mitochondria.

INTRODUCTION

Cohesin is a conserved protein complex that regulates sister chromatid cohesion and ordered chromosome segregation. It is also involved in chromosome condensation and DNA break repair (Michaelis *et al.*, 1997). Mcd1/Rad21 is a cohesin subunit, and homologues are found from yeast to human (Guacci *et al.*, 1997). The fission yeast cohesin Rad21 is a cell cycle-regulated nuclear phosphoprotein that repairs double-strand DNA breaks, and it is essential for mitotic growth (Birkenbihl and Subramani 1992). In budding yeast (*Saccharomyces cerevisiae*), Mcd1 is required for chromosome morphogenesis from S phase through mitosis, and it functions in both sister chromatid cohesion and chromosome condensation (Guacci *et al.*, 1997; Michaelis *et al.*, 1997).

During mitotic cell cycle, proteolytic cleavage of the Mcd1 at the metaphase–anaphase transition promotes loss of cohesin, followed by its dissociation from the chromatids (Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Uhlmann *et al.*, 1999). Esp1 and Pds1 are the key regulators of this process. Esp1, a separin or separase, is inactivated by forming a complex with its inhibitor Pds1 (called securin) before anaphase (Ciosk *et al.*, 1998; Uhlmann *et al.*, 1999). During metaphase, degradation of Pds1 mediated by a Cdc20-specified multisubunit ubiquitin ligase termed anaphase-promoting complex/cyclosome (APC/C^{Cdc20}) releases Esp1, which cleaves cohesin Mcd1/Rad21, thereby releasing the sister chromatids (Cohen-Fix *et al.*, 1996; Uhlmann *et al.*, 1999; Nasmyth *et al.*, 2000).

In addition to its function in chromatid cohesion, recent studies show that cohesin plays a role in apoptosis (Chen *et al.*, 2002; Pati *et al.*, 2002). Human Rad21 (hRad21) is found as a nuclear caspase target. Induction of apoptosis by diverse stimuli causes the cleavage of hRad21. The cleaved C-terminal product of hRad21 is translocated from the nucleus to cytoplasm and acts as a nuclear signal for apoptosis. RAD21 is also found to be overexpressed in prostate (Porkka *et al.*, 2004) and breast cancer cells (Atienza *et al.*, 2005). In *Caenorhabditis elegans*, apoptotic nuclei, as indicated by 4',6-diamidino-2-phenylindole (DAPI) staining, were observed in the gonads of adult *evl-14/pds-5* and *scc-3* mutants (Wang *et al.*, 2003), suggesting a role of these cohesion-related genes in apoptotic cell death. Apoptotic cell death is also reported in mutation of yeast Pds5, a cohesion-related protein (Ren *et al.*, 2005).

In recent years, yeast has been used successfully as a model system for studying apoptosis (Madeo *et al.*, 2004). The yeast genome encodes many proteins of the basic molecular machinery executing cell death in mammals, including homologues of apoptosis-inducing factor 1 (AIF1) (Wissing *et al.*, 2004), caspases (Madeo *et al.*, 2002), endonuclease G (Büttner *et al.*, 2007), and HtrA2/Omi (Fahrenkrog *et al.*, 2004). In addition, mitochondria play an important role in cell death both in mammals and yeast (Wang 2001; Eisenberg *et al.*, 2007).

Here, we report that the yeast Mcd1 is cleaved upon induction of apoptosis by hydrogen peroxide (H₂O₂). The cleaved C-terminal fragment of Mcd1 is further cleaved into smaller fragment and then translocated from nucleus into mitochondria. The translocation causes the decrease of mitochondrial membrane potential ($\Delta\Psi_M$) and amplification of cell death in a cytochrome *c*-dependent manner. We further demonstrate that Esp1, a caspase-like protease, is responsible for the cleavage of Mcd1 during H₂O₂-induced apoptosis in yeast.

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Abbreviations used: $\Delta\Psi_M$, mitochondrial membrane potential; APC/C, anaphase-promoting complex or cyclosome; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; ROS, reactive oxygen species; zVAD-fmk, *N*-benzoylcarbonyl-Val-Ala-Asp fluoromethyl ketone.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

Yeast strains used in this study are derivatives of MDY506 (MATa ADE2 ade5 can1R CYH2s his7-2 leu1-d lys2-1 met13-d trp1-63 tyr1-1 ura3-13) and MDY507

(MAT α *ade2 ADE5 CAN1s cyh2R his7-1 leu2 lys2-2 met13-c trp1-63 tyr1-2 ura3-1*). Plasmids of MCD1-GFP, MCD1-6HA, and the temperature-sensitive *mcd1-1* mutant were provided by Dr. V. Guacci (Guacci *et al.*, 1997) and integrated into MDY506 and MDY507. N-terminal 6HA tagging of Mcd1 was constructed according to Guedener *et al.* (2002) and Gauss *et al.* (2005). The *yca1* deletion was introduced by polymerase chain reaction (PCR)-mediated gene replacement (Wach *et al.*, 1994), replacing the complete sequence of YOR197W with a G418 marker. Plasmid of ESP1 overexpression was provided by Dr. K. Nasmyth (Uhlmann *et al.*, 2000). Plasmids for making Esp1-deg and Pds1-deg were purchased from EUEOSCARF (Frankfurt, Germany; <http://web.uni-frankfurt.de/fb15/mikro/euroscarf/>), and the two strains were constructed according to Sanchez-Ziaz *et al.* (2004). Mcd1 C-terminal truncations were constructed according to Gelperin *et al.* (2005). Different truncations were first amplified by PCR, using MCD1-GFP plasmid as the template. The PCR products were then integrated into vector BG1805 (kindly provided by Dr. E. Grayhack, University of Rochester School of Medicine and Dentistry, Rochester, NY) (Gelperin *et al.*, 2005). Yeast strain BY4742 and its derived deletion strains (*aif1* Δ , *cyc1* Δ and *nuc1* Δ) was purchased from Open Biosystems (Huntsville, AL).

Cells were all grown at 30°C, except where noted in the text. For overexpression, cells were first cultured in YPRaf medium (1% yeast extract, 2% peptone, and 2% Raffinose) overnight to reach 1×10^8 cells/ml, and then they were transferred to YPG medium (1% yeast extract, 2% peptone, and 2% galactose) for minimum of 1 h. Apoptosis was induced by 5 mM H₂O₂ (Madeo *et al.*, 1999).

Spotting and Survival Assay

For spotting assay, cells were cultured in liquid medium as desired. Cells were then diluted to 2.5×10^6 cells/ml. Four fivefold series dilutions were made, and 5 μ l of each dilution was plated on a YPG plate. Cells were grown at 30°C for 2 d. Survival assay was conducted according to Mason *et al.* (2005). Survival rate was calculated as number of colonies divided by the number of colonies in its corresponding control.

Fluorescence Microscopy

For fluorescence microscopy, cells were collected by centrifuge, rinsed with phosphate-buffered saline (PBS), and mounted on a coverslip with anti-fading medium (0.1 M propyl gallate and 50% glycerol in PBS) containing 0.5 μ g/ml DAPI. For mitochondrial staining, cells were collected at log phase and washed one time with PBS. Cells were resuspended in 100 μ l of PBS containing 2 μ M MitoFluor 589 (Invitrogen, Carlsbad, CA) or 1 μ M MitoTracker Red (Invitrogen). Cells were mounted on a coverslip, and they were checked immediately. A Nikon TE300 inverted microscope, equipped with a Cascade 650 cooled monochrome digital camera (Roper Scientific, Trenton, NJ) was used for image acquisition.

Western Blot Analysis

Cells were collected by centrifugation and lysed in lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS, and protease inhibitor cocktail. Glass beads (200 μ m) were added, and they were vortexed vigorously. Sample was boiled and spun down, and the supernatant was used to run the SDS-gel. Lysates were separated on 10% SDS-polyacrylamide gels, and they were transferred to polyvinylidene difluoride membranes. The membranes were then blocked in 4% nonfat milk and incubated with anti-hemagglutinin (HA) antibody (clone 3F10; Roche Diagnostics, Indianapolis, IN). The membranes were then incubated anti-immunoglobulin G horseradish peroxidase. For caspase inhibitor analysis, 200 μ M of a pan-caspase inhibitor zVAD-fmk or 50 μ M of Caspase 1 and 8 were added to the cells for 1 h before the addition of H₂O₂.

Annexin V Staining

Yeast cells were collected and washed twice with sorbitol buffer (0.8 M sorbitol and 2% potassium acetate, pH 7.0), resuspended in sorbitol buffer containing 10 mM dithiothreitol for 10 min, and then digested with 0.4 mg/ml Zymolyase for 30 min. Cells were harvested and resuspended in 50 μ l of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, and 1.2 M sorbitol, pH 7.4). Then, 4 μ l of Annexin V conjugate was added to the cell suspension and incubated for 20 min at room temperature in the dark. Cells were rinsed with binding buffer, mounted under a coverslip with anti-fading medium containing 0.5 μ g/ml DAPI, and examined with epifluorescence microscopy.

RESULTS

H₂O₂ Treatment Causes Mcd1 Translocated from Nucleus into Mitochondria

H₂O₂ has been shown to cause apoptosis in mammalian cells and yeast (Madeo *et al.*, 2002). To study the potential role of cohesin in yeast apoptosis, H₂O₂ was used to induce apo-

Figure 1. H₂O₂ treatment causes Mcd1 translocated from nucleus to mitochondria. (A). Mcd1-GFP cells were cultured to 2×10^7 cells/ml, treated with 5 mM H₂O₂ for 1 h, and stained with DAPI. Control was the same cells without the addition of H₂O₂. (B). Mcd1-GFP cells were cultured to 2×10^7 cells/ml, treated with 5 mM H₂O₂ for 1 h, and stained MitoFluor 589. Control was the same cells without the addition of H₂O₂.

ptosis in a yeast strain carrying a green fluorescent protein (GFP)-tagged MCD1. When the MCD1-GFP strain was cultured in the absence of H₂O₂, Mcd1-GFP was exclusively localized in the nucleus (Guacci *et al.*, 1997; Figure 1A). When H₂O₂ was added to the culture, Mcd1-GFP was “knocked out” from nucleus into cytoplasm, forming small GFP spots, similar to the size of mitochondria (Figure 1A). Counterstaining with DAPI showed that the GFP spots in the cytoplasm contained DNA. To confirm that the Mcd1-GFP colocalizes with mitochondria, MitoFluor 589, a mitochondrial-specific dye, was used to stain mitochondria. As shown in Figure 1B, the GFP spots colocalized with MitoFluor, indicating that the Mcd1-GFP is indeed in mitochondria.

H₂O₂ Treatment Causes Mcd1 Fragmented

To further study the potential role of Mcd1 in yeast apoptosis, Mcd1 was tagged with a 6HA tag at its carboxy terminus. The HA-tagged Mcd1 strain was incubated in the presence of H₂O₂, and Western blot was used to detect the level of Mcd1 using anti-HA antibody. As shown in Figure 2A, the protein level decreased as the incubation time increased. Whereas the Mcd1 level decreased when treated with H₂O₂, no cleaved fragment was detected using antibody against C-terminal-tagged HA. Microarray analysis showed no significant change of *MCD1* expression when treated with H₂O₂ (Zhang, unpublished data), suggesting that the Mcd1

Figure 2. H_2O_2 treatment causes Mcd1 fragmentation. (A) Western blot using antibody against C-terminal-tagged Mcd1-HA, after treatment with H_2O_2 for 1, 3, 5, and 7 h. WT, wild type treated with H_2O_2 ; WT + zVAD, wild type treated with H_2O_2 and the pan-caspase inhibitor zVAD-fmk; yca1, yca1 deletion treated with H_2O_2 . Tubulin, β -tubulin was used as the loading control. (B) Western blot using antibody against N-terminal-tagged Mcd1-HA, after treatment with H_2O_2 for 1, 3, 5, and 7 h.

decrease was likely caused by Mcd1 cleavage. Mcd1/Rad21 has been reported to be cleaved by caspases in human cells undergoing apoptosis in response to diverse stimuli (Chen *et al.*, 2002; Pati *et al.*, 2002). When a pan-caspase inhibitor (zVAD-fmk) was added before the addition of H_2O_2 , no apparent decrease of Mcd1 level was observed (Figure 2A), suggesting that caspase or caspase-like protease may be responsible for the Mcd1 decrease. We then knocked out YCA1, which encodes the only identified caspase-like protease in yeast, to see whether Yca1 is involved. Although yca1 Δ delayed, if not abolished apoptosis in yeast (Madeo *et al.*, 2002), no effect was observed on the decrease of Mcd1 in the yca1 Δ background (Figure 2A), suggestive of other enzyme being responsible for the decrease of Mcd1.

To further test the possible cleavage of Mcd1, we inserted an HA tag at the N terminus of Mcd1. Western blot was conducted in the same condition as the C-terminal-tagged strain. Two obvious bands were detected using the anti-HA antibody: a 48-kDa band (Figure 2B, arrowhead) and a 83-kDa band (Figure 2B, arrow). Note that the top band (Figure 2B, asterisk) was likely the phosphorylated form of Mcd1 (Uhlmann *et al.*, 2000). The 48-kDa band was similar to that cleaved by Esp1 during mitotic division (Uhlmann *et al.*, 1999). The presence of the 48- and 83-kDa bands suggests that there should have a C-terminal band of Mcd1, which has a molecular mass of ~113 kDa. It was likely that the C-terminal fragments were unstable and further digested into small fragments by the N-end rule pathway (Rao *et al.*, 2001); therefore, they could not be detected when using the HA antibody at C terminus. Please note that the molecular mass of Mcd1 shown here is bigger than what is predicted (www.yeastgenome.org). This difference has been reported previously (Uhlmann *et al.*, 1999; 2000). It is unclear the cause(s) of the difference. Apparently, the addition of the HA tag increases the retardation in gel migration in addition to the actual increase in molecular mass (Uhlmann, personal communication).

H_2O_2 Causes the Translocation of the Truncated Mcd1 C-Terminal Fragment into Mitochondria

It was clearly seen that the Mcd1 was translocated from nucleus to mitochondria in the presence of H_2O_2 (Figure 1).

Figure 3. Diagram of the series truncations of C-terminal Mcd1, and the vector (BG1805) used for overexpression.

The translocated Mcd1 was most likely a C-terminal fragment of Mcd1, because 1) the GFP observed in Figure 1 was tagged at the C terminus of Mcd1, and 2) Mcd1 was shown to be cleaved at the presence of H_2O_2 (Figure 2). Because no C-terminal fragments could be detected, possibly due to its small size, a series of C-terminal truncations of Mcd1 (Figure 3) were created and overexpression of the different truncations were tested. No mitochondrial target sequence in Mcd1 was predicted using MultiLoc (Hoeglund *et al.*, 2006) and other software. The truncations were therefore determined arbitrarily, starting with 20 amino acids from C terminus of Mcd1, except for Mcd1-C95aa, which has a relatively high probability of mitochondrial location (Table 1). The predicted molecular masses of the C-terminal truncations range from 2 to 11 kDa. The plasmid carrying GFP only was used as a control. Overexpression of the various C-terminal Mcd1 truncations revealed that all truncations localized in cytoplasm in the absence of H_2O_2 . When treated with H_2O_2 , the GFP signal remained in cytoplasm, except for truncation Mcd1-C40aa, which formed small GFP dots around edge of the cells (Table 1; Figure 4A), similar to the endogenous Mcd1-GFP when treated with H_2O_2 (Figure 1). MitoTracker staining revealed that these small spots colocalized with mitochondria (Figure 4B), indicating Mcd1-C40aa was translocated from cytoplasm into mitochondria at the presence of H_2O_2 .

Overexpression of the Mcd1 C-Terminal Truncation Leads to More Cell Death

To determine whether Mcd1-C40aa truncation plays a role in H_2O_2 -induced apoptosis, plating assay (Mason *et al.*, 2005) was used to compare the survival rate of wild-type and Mcd1-C40aa-overexpressed cells. As shown in Figure 5A, the survival rate of Mcd1-C40aa overexpressed cells decreased significantly, compared with the wild type, or cells carrying the GFP vector. This result suggests that the small fragment of C-terminal Mcd1 amplifies cell death induced by H_2O_2 .

Plating assay using diploid strains of MCD1/MCD1 (wild type) and MCD1/mcd1^{ts} was also used to test the role of Mcd1 in apoptosis. The MCD1/mcd1^{ts} is used as a way to underexpress MCD1, because MCD1 is an essential gene and null mutant is inviable. As shown in Figure 5B, underexpression of MCD1 (only one copy of functional MCD1) increased cell's survival rate. A similar result was obtained by using a Mcd1 heterozygous knockout strain (MCD1/mcd1 Δ) from Open Biosystems (data not shown).

Table 1. Mcd1 C-terminal truncations and their cellular localizations before and after treatment with H₂O₂

	C0aa	C20aa	C40aa	C60aa	C80aa	C95aa	C100aa
Predicted location (probability score)	N/A	Extra C (0.72)	Extra C (0.93)	Cyto (0.97)	Cyto (0.97)	Mito (0.59)	Cyto (0.97)
Observed location							
– H ₂ O ₂	Cyto	Cyto	Cyto	Cyto	Cyto	Cyto	Cyto
+ H ₂ O ₂	Cyto	Cyto	Mito	Cyto	Cyto	Cyto	Cyto

Predicted location and probability score were according to Hoeglund *et al.* (2006). Cyto, cytoplasm; Extra C, extracellular; Mito, mitochondria; N/A, not applicable.

Overexpression of Mcd1 C-Terminal Truncation Causes the Decrease of $\Delta\Psi_M$

Previous studies show that mitochondria play an important role in the regulation of apoptotic cell death. The collapse of $\Delta\Psi_M$ is an irreversible hallmark of early apoptosis (Karbowski and Youle 2003). To further investigate the role of the Mcd1-C40aa truncation in H₂O₂-induced apoptosis, the Mcd1-C40aa truncation was overexpressed with or without the presence of H₂O₂, and the $\Delta\Psi_M$ was examined using MitoTracker Red CMRos (Invitrogen), which stains mitochondria in a $\Delta\Psi_M$ -dependent manner (Pozniakovsky *et al.*, 2005). The MitoTracker specifically stains mitochondria

when the $\Delta\Psi_M$ is high, but stains in a diffused pattern when $\Delta\Psi_M$ is lost. As shown in Figure 6, when treated with H₂O₂, ~70% of the wild-type cells showed a diffused MitoTracker

Figure 4. H₂O₂ treatment causes Mcd1 truncation (Mcd1-C40aa) translocated into mitochondria. (A). Wild-type cells overexpressing Mcd1-40Caa were cultured to 2×10^7 cells/ml, then treated with 5 mM H₂O₂ for 1 h. Cells were stained with DAPI and imaged using an epifluorescence microscope. (B). Wild-type cells overexpressing Mcd1-40Caa were cultured to 2×10^7 cells/ml, and then they were treated with 5 mM H₂O₂ for 1 h. Cells were stained with MitoTracker Red and imaged using an epifluorescence microscope.

Figure 5. Survival rate of cells overexpressing Mcd1-C40aa (A), and cells of WT/WT versus WT/mcd1-ts (B) after treatment with H₂O₂. (A). Cells of wild type (WT), of overexpressing Mcd1-C40aa (40aa-GFP), and of overexpressing GFP only (WT-GFP) were treated with H₂O₂ for 0.5 h. Then, 1000 cells were plated on YPDA plates for 2 d. Survival rate was calculated as number of colonies on the plate divided by the number of colonies in its corresponding control (WT). (B). Diploid cells of wild type (*MCD1/MCD1*) and heterozygous *MCD1* mutation (*MCD1/mcd1^{ts}*) were cultured to 2×10^7 cells/ml, and then they were treated with 5 mM H₂O₂ for 0.5, 1, or 1.5 h. Cells (1000) were plated on YPDA plates for 2 d. Survival rate was calculated as number of colonies on the plate divided by the number of colonies in its corresponding control (no H₂O₂ treatment).

Figure 6. Overexpression of Mcd1-C40aa in the presence of H₂O₂ causes decrease of mitochondrial membrane potential. Wild-type cells and cells overexpressing Mcd1-C40aa were cultured to 2×10^7 cells/ml, and then they were treated with 5 mM H₂O₂ for 1 h. Cells were then stained with MitoTracker Red for mitochondria membrane potential.

staining, but almost all Mcd1-C40aa overexpressed cells showed a diffused staining, indicating that overexpression of Mcd1-C40aa caused more $\Delta\Psi_M$ loss.

When Mcd1 was underexpressed using the diploid *MCD1/mcd1^{ts}* strain (see also previous section: overexpression of the Mcd1 C-terminal truncation leads to more cell death) in the presence of H₂O₂, cells with diffused MitoTracker staining decreased almost 50% compared with the diploid wild type (*MCD1/MCD1*), suggesting that the knockdown of Mcd1 protects $\Delta\Psi_M$ loss from H₂O₂ treatment.

Cell Death Amplified by the Mcd1 C-Terminal Fragment Is Dependent on Cyc1 But Not Aif1 and Nuc1

Mitochondrial proteins such as apoptosis-inducing factor, endonuclease G, and cytochrome *c* play important roles in apoptosis via their release from mitochondria to nucleus or to cytoplasm in mammalian and yeast (Wang 2001; Wissing *et al.*, 2004; Büttner *et al.*, 2007). We next asked whether the translocation of Mcd1 C-terminal fragment into mitochondria is related to the function of mitochondrial proapoptotic proteins. The Mcd1-C40aa truncation was overexpressed in the knockout strains of *CYC1*, *AIF1*, and *NUC1*, separately. Spotting and plating assays were used to analyze the survival rate for each strain. As shown in Figure 7, overexpres-

sion of Mcd1-C40aa caused more cell death in *nuc1Δ* and *aif1Δ* mutants, similar to the overexpression of Mcd1-C40aa in wild type, suggesting that the cell death amplified by the overexpression is independent of Aif1 and Nuc1. In *cyc1Δ* strain, however, survival rate was increased when Mcd1-C40aa was overexpressed, indicating that Cyc1 is required for the cell death amplified by the C-terminal fragment of Mcd1.

Mcd1 Was Cleaved by the Caspase-like Protease Esp1 in H₂O₂-induced Cell Death

Esp1 is a separase that cleaves the bonds between sister chromatids during metaphase-to-anaphase transition (Uhlmann *et al.*, 1999, 2000). Sequence comparison revealed that the *S. cerevisiae* Esp1 has a similar sequence to human caspase-1 at their conserved proteolysis catalytic sites (Uhlmann *et al.*, 2000). To see whether Esp1 is responsible for cleaving Mcd1p during H₂O₂-induced apoptosis, Esp1 was overexpressed under a *GAL1* promoter. After 6 h of overexpression, ~20% of cells showed markers of apoptotic cell death (Figure 8A). Interestingly, when Esp1 was overexpressed in a MCD1-GFP strain, Mcd1-GFP was translocated from nucleus into mitochondria, similar to H₂O₂-treated cells (Figure 8B).

Contrary to the overexpression of Mcd1-C40aa, in which H₂O₂ is required for the translocation of Mcd1 fragment to mitochondria, overexpression of ESP1 caused Mcd1-GFP translocated into mitochondria without the addition of H₂O₂. One possibility is that overexpression of ESP1 causes production of reactive oxygen species (ROS) itself; therefore, no external H₂O₂ is needed. To test this possibility, we used dihydrorhodamine 123 to detect ROS in ESP1-overexpressing cells (Ren *et al.*, 2005). Our result revealed that ~20% of the cells were ROS positive after 6 h of ESP1 overexpression. When *N*-tert-butyl- α -phenylnitrone (PBN), a ROS scavenger, was added, the ROS-positive cells decreased from 20% to ~5%. The percentage of cells with Mcd1-GFP translocated to mitochondria also dropped dramatically, compared with cells without PBN treatment. These results suggest that ROS plays a major role in translocating the Mcd1 fragment from cytoplasm to mitochondria.

A previous report (Uhlmann *et al.*, 2000) suggests that Esp1 resembles the function of caspase-1. If this were the case, caspase-1 inhibitor should block the decrease of Mcd1 when treated with H₂O₂. Caspase-1 and caspase-8 inhibitors were used to test this possibility. As shown in Figure 8C, caspase-1 inhibitor inhibited the decrease of Mcd1, similar to the pan-caspase inhibitor zVAD-fmk (Figure 2A). Caspase-8 inhibitor slowed down the decrease, but it did not totally block the decrease (compare with the wild type in Figure 2A). This result suggests that a caspase-1-like protease, possibly Esp1, is responsible for the cleavage of Mcd1 in H₂O₂-induced apoptotic cell death.

To further test the function of Esp1 as a caspase-like protease, mutational analysis of Esp1 was performed using a heat-inducible Esp1-degron strain (Sanchez-Diaz *et al.*, 2004). Western blot revealed that the Esp1 was completely depleted after 1 h of induction at 37°C (data not shown). As shown in Figure 8D, at 24°C, when Esp1 was not depleted, Mcd1-GFP was cleaved and translocated into mitochondria at presence of H₂O₂. When shifted to 37°C for 1 h, Mcd1-GFP remained exclusively inside the nucleus, similar to the control cells. No obvious Mcd1 decrease was observed in the presence of H₂O₂ when Esp1 was depleted (Figure 8E), further confirming the role of Esp1 in the cleavage of Mcd1.

Pds1 deletion was also performed to test the cleavage of Mcd1 by Esp1. Pds1 is an anaphase inhibitor and associated

Figure 7. Cell death caused by overexpression of Mcd1-C40aa is dependent on Cyc1 but not Aif1 and Nuc1. Mcd1-C40aa was overexpressed in WT (BY4742), *aif1Δ*, *nuc1Δ*, and *cyc1Δ* strains. Cells were then treated with H₂O₂ for 1 h. Survival rate was measured by spotting assay (A) and plating assay (B).

with the Esp1. At early anaphase, Pds1 is degraded by the APC, releasing Esp1. The released Esp1 then acts as a separin for the cleavage of cohesin, causing the separation of sister chromatid (Cohen-Fix *et al.*, 1996; Agarwal and Cohen-Fox, 2002). We speculate that Esp1 has to be released from Pds1 in order for the cleavage of Mcd1 during H₂O₂-induced apoptosis. As shown in Figure 8E, when Pds1 was depleted by degron, Mcd1-GFP was translocated from nucleus into mitochondria in the absence of H₂O₂, further suggesting that Esp1 is responsible for the cleavage of Mcd1.

DISCUSSION

Mitochondria play a central role in cell death of both mammals (Wang 2001) and yeast (Eisenberg *et al.*, 2007). On induction of apoptosis, mitochondria release several mitochondrial proteins, such as cytochrome *c*, endonuclease G, and apoptosis-inducing factor to promote cell death, by either activating caspases, or by neutralizing cytosolic inhibitors of cell death. In mammalian cells, it has been shown that the Bcl-2 family proteins are critical regulators of mitochondrial apoptosis (Jiang and Wang 2004). Some Bcl-2 proteins (antiapoptotic), such as Bcl-x_L inhibit cell death, whereas others (proapoptotic) promote apoptosis. Interestingly, both antiapoptotic and proapoptotic Bcl-2 proteins are directly targeted to mitochondria, in which they inhibit or promote the release of apoptotic proteins from mitochondria. One of the well-characterized Bcl-2 proteins is the mammalian Bid, which localizes in cytoplasm in living cells (Li *et al.*, 1998; Luo *et al.*, 1998). On induction of cell death, Bid is cleaved by caspase-8, producing an 11-kDa N-terminal and a 15-kDa C-terminal fragment. The truncated C-terminal fragment (tBid) is translocated from cytosol to mitochondria, causing the release of cytochrome *c*. In yeast, little is known about the upstream mechanism and regulation of mitochondrial apoptosis. Our current study addresses this issue. Similar to the mammalian Bid, yeast co-

hesin protein, Mcd1 is cleaved by Esp1, a caspase 1-like protease, upon induction of apoptosis. The truncated C-terminal fragment of Mcd1 is translocated from nucleus to mitochondria, causing the amplification of cell death in a cytochrome *c*-dependent manner. Notably, the translocation of the Mcd1 fragment to mitochondria requires apoptosis stimulus, such as H₂O₂. This explains why no cell death occurs during a normal cell cycle, when Mcd1 is also cleaved by Esp1 at the stage of metaphase/anaphase transition. It remains unknown how the truncated Mcd1 is translocated to mitochondria. In tBid, recent studies in both mammals (Lutter *et al.*, 2000) and yeast (Gonzalez *et al.*, 2005) indicated that the translocation is dependent on cardiolipin, a phospholipid associated with mitochondrial membrane (Hoch 1998). It is also not clear, especially in yeast, how the cell death is affected by the release of cytochrome *c*. A study Silva *et al.* (2005) suggests that the release of cytochrome *c* is involved in activation of caspase in yeast. It is also possible that the release of cytochrome *c* can halt the electron transfer, which in turn causes the loss of mitochondria membrane potential and increase the ROS production.

Esp1, also called separase, is a cysteine protease-related caspase. Separase is thought to be a repressed protease, cleaving only a few substrates and in a very controlled manner. During a normal cell cycle, Esp1 is bound and inactivated by Pds1, an inhibitory protein (Yanagida 2000). At metaphase/anaphase transition, the securin Pds1 is degraded by APC, releasing Esp1. Here, we showed a second role of Esp1, acting in an apoptosis-specific manner. This provides direct evidence of the caspase function of the separase Esp1 (Figure 9). Furthermore, Mcd1 may not be the only substrate of Esp1. A recent report (Sullivan *et al.*, 2001) shows that Esp1 also cleaves a kinetochore/spindle protein called Slk19. A search of *S. cerevisiae* genome database yielded 31 putative substrates of Esp1, including Mcd1 (Uhlmann *et al.*, 2000).

Figure 8. Esp1 is responsible for the cleavage of Mcd1 during H₂O₂-induced apoptosis. (A) Overexpression of *ESP1* caused apoptotic cell death as measured by Annexin V staining. Two adjacent cells, one cell showing Annexin V positive (top) and one cell negative (bottom). The negative PI staining also confirmed the cell death is apoptotic. (B) Overexpression of *ESP1* caused Mcd1-GFP translocated from nucleus to mitochondria. (C) Western blot showing the decrease of Mcd1 when treated with H₂O₂ was blocked by caspase 1, but not caspase 8. β -Tubulin was used as the loading control. (D) When treated with H₂O₂, Mcd1 failed to be translocated into mitochondria, when Esp1 was depleted by the degron. (E) When treated with H₂O₂, Mcd1 failed to be cleaved, after Esp1 was depleted by degron. Note that Mcd1 was measured only in 1- and 3-h time points after the addition of H₂O₂, because the cells became unhealthy after that due the deletion the essential gene *ESP1*. (F) In the absence of H₂O₂, Mcd1 was translocated into mitochondria when Pds1 was depleted by the degron.

The hRad21, homologue of yeast Mcd1, has also been reported to be a nuclear target of caspase-3 and caspase-7. hRad21 is cleaved upon induction of apoptosis via diverse stimuli, producing an ~65-kDa C-terminal fragment, which

is then translocated to cytoplasm. Overexpression of the C-terminal fragment amplifies apoptotic cell death. It is not clear how cell death is enhanced by the cytoplasm-located C-terminal fragment of hRad21. In yeast, our study indicates

Figure 9. Esp1 has dual functions, as a separase during normal cell cycle and as a caspase-like protease in H₂O₂-induced apoptosis.

that the C-terminal fragment is much smaller, possibly by the further degradation ubiquitin-proteasome pathway (Uhlmann *et al.*, 2000). The much smaller fragment is translocated to mitochondria, rather than cytoplasm. The apparent differences between human and yeast, however, should not underestimate the importance of using yeast as a model system to elucidate the pathways and regulation of apoptosis.

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