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Phosphoinositide 3-OH Kinase/Protein Kinase B Inhibits Apoptotic Cell Death Induced by Reactive Oxygen Species in *Saccharomyces cerevisiae*[1]

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Apoptosis is a common mode of programmed cell death in multicellular organisms. However, the recent observation of yeast cell death displaying the morphology of apoptosis has suggested the presence of an ancestral cell death machinery. Here we examined apoptotic features induced by reactive oxygen species (ROS) in yeast. *Saccharomyces cerevisiae* show typical apoptotic features upon exposure to ROS: membrane staining with annexin V and DNA fragmentation by the TUNEL assay. The detection of apoptotic features in yeast strongly support the existence of molecular machinery performing the basic pathways of apoptosis. The phosphoinositide 3-OH kinase (PI3K)/protein kinase B (PKB) signaling pathway has been shown to prevent apoptosis in a variety of cells. It is therefore of interest to determine whether the PI3K/PKB signaling pathway is capable of protecting yeast from apoptosis induced by ROS. We determined that PI3K/PKB is capable of significantly inhibiting ROS-evoked apoptosis in yeast. These results suggest that yeast may provide a suitable model system in which to study the apoptotic signaling pathway elicited by a variety of stimuli.

Key words: apoptosis, phosphoinositide 3-OH kinase, protein kinase B, reactive oxygen species, *Saccharomyces cerevisiae*.

Apoptosis is an active form of cell death with an important role in development and homeostasis in multicellular organisms (*1*). Apoptosis allows the rapid removal of unwanted or damaged cells that could inflame surrounding cells with their cytoplasmic contents. In contrast, during necrosis, a form of cell death that results from overwhelming cellular injury, cells undergo lysis, releasing their cytoplasmic contents.

Apoptotic cells are characterized by a set of distinct morphological changes. An early marker of apoptosis is the translocation of phosphatidylserine (PS) from the cytoplasmic membrane to the outer leaflet on the cell surface (*2*). DNA is fragmented between the nucleosomes and the condensed chromatins. Cells break up and form apoptotic bodies, which are rapidly phagocytosed and digested by macrophages (*3*).

Reactive oxygen species (ROS), byproducts of respiration in aerobic organisms, are highly reactive and can modify intracellular molecules. ROS are, consequently, formed in most organisms exposed to molecular oxygen and appear to play crucial roles in apoptosis. ROS play an important role in the induction of apoptosis in mammalian cells (*4, 5*) for the removal of damaged cells. The recent observations of *Saccharomyces cerevisiae* cell death displaying a morphology of apoptosis by ROS have suggested the presence of an ancestral cell death machinery (*6–8*).

The phosphoinositide 3-OH kinase (PI3K)/protein kinase B (PKB) signaling pathway has been shown to mediate protection from apoptosis induced by external stimuli in a variety of cell types (*9, 10*). In addition, PI3K/PKB plays an essential role in the induction of glutathione S-transferase (GST), which decreases oxidative stress in H4IIE hepatoma cells (*11*). It is, therefore, of interest to determine whether the PI3K/PKB signaling pathway is capable of protecting yeast cells from apoptosis induced by ROS.

We present evidence of apoptotic features induced by ROS in *S. cerevisiae*, indicating that the formation of ROS is a key event in the original apoptotic mechanism. We also report that active PI3K and PKB are capable of significantly inhibiting ROS-evoked apoptosis.

MATERIALS AND METHODS

Cell Culture and Treatments—Yeast cells (YR98:MATα *ade2 his3-Δ200 leu2-3, 112/lys2-Δ201 ura3-52*) were grown in YPD medium (1% yeast extract, 2% peptone, 2% dex-

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Abbreviations: ROS, reactive oxygen species; TUNEL, TdT-mediated dUTP nick end labeling; PI3K, phosphoinositide 3-OH kinase; PKB, protein kinase B; PS, phosphatidylserine; PIP_2, phosphatidylinositol 3,4-bisphosphate; IRS-1, insulin receptor substrate protein-1; MKK3, mitogen kinase kinase 3; MEKK3, mitogen-activated protein kinase/extracellular signal regulated kinase kinase; DTT, dithiothreitol; SP, spheroplast; AO/EtBr, acridine orange/ethidium bromide; FACS, fluorescence-activated cell sorter; TdT, terminal deoxynucleotidyl transferase; PBS, phosphate buffered saline; PI, propidium iodide; H_2DCF, 2′,7′-dichlorofluorescein; UA, ursolic acid; BH3, BCR homology 3.

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trose), stopped at the exponential phase, and washed three times with sterilized distilled water. To identify apoptotic signaling pathways, chemicals such as wortmannin, LY-294002, and phosphatidylinositol 3,4-bisphosphate (PIP_2) were used.

Spheroplasts Preparation—Yeast cells were washed with spheroplast buffer (1 M sorbitol, 50 mM Tris, pH 7.4, 2 mM $MgCl_2$, 10 mM DTT and 10 mM sodium azide; denoted SP buffer), and collected by centrifugation at 1,000 ×*g* for 10 m (*12*). The pellet was dissolved in SP buffer containing 100 U/mg lyticase (Sigma Chemical, St. Lois, USA) and incubated at 30°C for 30 m.

Fluorescent Microscopy by Acridine Orange/Ethidium Bromide Staining—Cell death was studied morphologically using a fluorescent dye that intercalates DNA. Acridine orange stains DNA bright green, allowing visualization of the nuclear chromatin pattern. Apoptotic cells have condensed chromatin that is uniformly stained. Ethidium bromide stains DNA orange, but is excluded by viable cells (*13*). Cells (1×10^5) were stained with 1.5 µl of AO/EtBr solution [acridine orange in PBS (100 µg/ml)/ethidium bromide in PBS (100 µg/ml) = 1:1, v/v], mixed gently, and then examined through a fluorescence microscope (AHPT-514, Olympus) under blue light.

Fluorescence-activated Cell Sorter (FACS) Analysis by Annexin V Staining—Exposed PS was detected by reaction with FITC-coupled annexin V (Annexin-V-Fluos Staining Kit, Boehringer Mannheim, Germany). Spheroplasts were treated with 0 mM, 1 mM, 10 mM, or 100 mM H_2O_2, and harvested at various times. The H_2O_2-treated spheroplasts were washed with SP buffer and centrifuged at 100 ×*g* for 5 m. The pellet (1×10^6 cells) was resuspended in 100 µl of staining-solution [Annexin V-fluorescein labeling reagent: propidium iodide = 1:1 (v/v)] and incubated for 10 m at room temperature. Exposed PS was measured by FACS analysis (FACS Calibur-S System, Becton Dickinson).

Detection of DNA Fragmentation by TUNEL Assay—For the TdT-mediated dUTP nick end labeling (TUNEL) test, DNA ends were labeled using an *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Germany). Spheroplasts (1×10^6) fixed with 4% paraformaldehyde were washed with SP buffer and incubated in permeablization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 m on ice (*6*). The Spheroplasts were washed twice with SP buffer, incubated in 50 µl of TUNEL reaction mixture for 1 h at 37°C, and washed twice with SP buffer. DNA fragmentation was observed under a confocal fluorescent microscope (MRC-1024, Bio-Rad).

Measurement of Intracellular Free Radicals—Yeast cells (7×10^7) were washed three times with phosphate buffered saline (PBS). Then, they were treated with 50 µM dichlorodihydrofluoresein diacetate, and incubated for 1 h at room temperature (*7, 14*). Relative concentrations of free radicals were measured by a fluorescence spectrophotometer (LS 50B, Perkin Elmer). The bandwidths for excitation at 488 nm and emission at 525 nm were 10 and 5 nm, respectively.

Protein Kinase B and MEKK3 Assays—Protein extracts were obtained from spheroplasts suspended in lysis buffer (50 mM tirs, pH 7.5, 150 mM NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 µg/ml each protease inhibitors, aptotinin, leupeptin, and pepstatin A). At specific time points following treatment with H_2O_2 and/or PI3K inhibitors, the suspension was extracted with glass beads. The extract was clarified by centrifugation for 10 min at 5,000 ×*g*. Nonradioactive PKB and MEKK assays were performed as described previously (*15, 16*). Fluorescein (fluorescein isthiocynate was conjugated in the amino terminus of the peptide) IRS-1 (^{30}RKRSRKESYS^{39}) for PKB substrates and MKK3 (^{183}SGYLVDSVAKTIDA^{196}) for MEKK substrate oligopeptides were purchased from Sigma Chemical (St. Lois, USA). Briefly, 0.5 µg of fluorescein-conjugated oligopeptide was incubated with 10 µl of protein extract in 20 µl of protein kinase reaction buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, 1.3 mM $CaCl_2$, and 1 mM ATP) at 30°C for 1 h. The reaction was stopped by heating at 95°C for 10 m. The phosphorylated peptide was separated in a 0.8% agarose gel at 100 V for 25 m.

RESULTS

Hydrogen Peroxide Induces Apoptotic Cell Death in S. Cerevisiae—The effect of H_2O_2 on cell death in *S. cerevisiae* was detected through various morphological changes. We examined cell death in response to various concentrations of H_2O_2 in *S. cerevisiae* cultures growing exponentially on YPD by AO/EtBr staining (Fig. 1). Treatment with 100 mM H_2O_2 for 2 h mostly induced cell death (Fig. 1D). The pattern of yeast cell death correlates with incubation times with H_2O_2 (data not shown). Madeo *et al* (*6*) has suggested that oxygen stress elicits apoptotic cell death based on various experimental evidence including TUNEL test and Transmission Electron Microscopy (TEM) assay in yeast. In contrast, evidence has been presented that H_2O_2 evokes necrotic cell death in yeast (*17, 18*).

To evaluate whether H_2O_2 induces apoptotic or necrotic cell death, we tested the translocation of PS from the inner leaflet of the membrane to the outer leaflet of the membrane using annexin V. In contrast to mammalian cells, yeast cells contain approximately 10% PS on the cell surface (*19*). It was difficult to detect the translocation of PS by fluorescent microscopy. We, accordingly, detected H_2O_2-induced translocation of PS by measuring the fluorescent intensity of annexin V by FACS analysis (Fig. 2). To examine the translocation of PS in yeast, various concentrations of H_2O_2 were added to spheroplasts. Simultaneously, cells stained by annexin V increased in proportion to incubation time and H_2O_2 concentration. When *S. cerevisiae* were treated with 100 mM H_2O_2 for 4 h, annexin V staining did not increase compared to that after 1 h incubation, indicating that PS translocation is completely ensured within 1 h (Fig. 2). Although apoptotic cell death was complete 2 h after treatment with 100 mM H_2O_2 based on the results of AO/EtBr staining (Fig. 1D), the translocation of PS as shown by annexin V staining occurred within 1 h (Fig. 2D). It is possible that PS translocation occurs at an early stage on apoptotic cell death. There was no significant difference found between untreated- and H_2O_2 treated-spheroplasts in PI staining (data not shown).

To establish further that H_2O_2 elicits apoptosis in yeast, we employed DNA fragmentation detection using the TUNEL assay. At 4 h incubation with 50 mM H_2O_2, TUNEL staining was very weakly observed (data not shown). After 2 h incubation with 100 mM H_2O_2, TUNEL staining was also weakly observed (data not shown). Therefore, we increased the incubation time with 100 mM H_2O_2 to 4 h, after

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which the spheroplasts were clearly stained by the TUNEL assay (Fig. 3). Chromatin in *S. cerevisiae* has a higher-order structure and lacks linker DNA between the nucleosomes (*20*). Therefore, DNA fragmentation was observed weakly by TUNEL staining and the fragmentation was delayed. This finding is consistent with the results of Madeo *et al.* (*7*). Taken together with the observations in the annexin V experiment, these results confirm that cell death measured by AO/EtBr staining is apoptotic cell death rather than necrotic cell death.

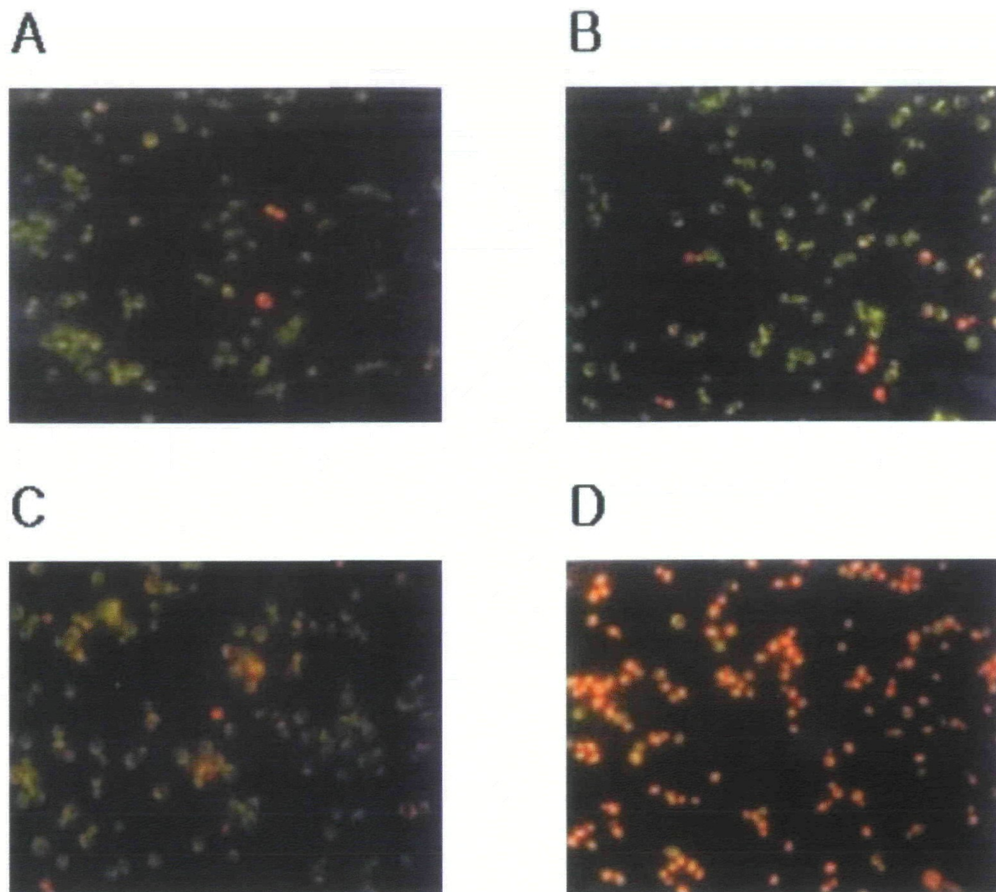

Fig. 1. **Cell death in response to the extracellular addition of various concentrations of H_2O_2 in *S. cerevisiae* as determined by AO/EtBr staining.** *S. cerevisiae* cultures on YPD were treated with 0 mM (A), 10 mM (B), 50 mM (C), or 100 mM H_2O_2 (D) for 2 h. The results shown are each representative of at least 20 independent experiments.

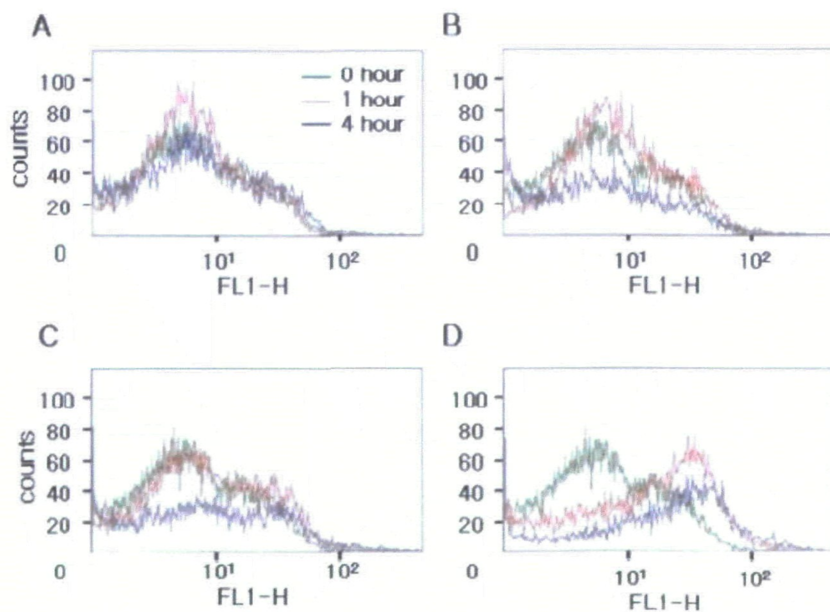

Fig. 2. **H_2O_2 induces apoptosis in spheroplasts of *S. cerevisiae* as determined by measuring the translocation of PS.** The detection of PS translocation was performed by annexin V staining using FACS analysis. Spheroplasts were exposed to 0 mM (A), 1 mM (B), 10 mM (C), or 100 mM H_2O_2 (D) for various times. Results are representative of five independent experiments.

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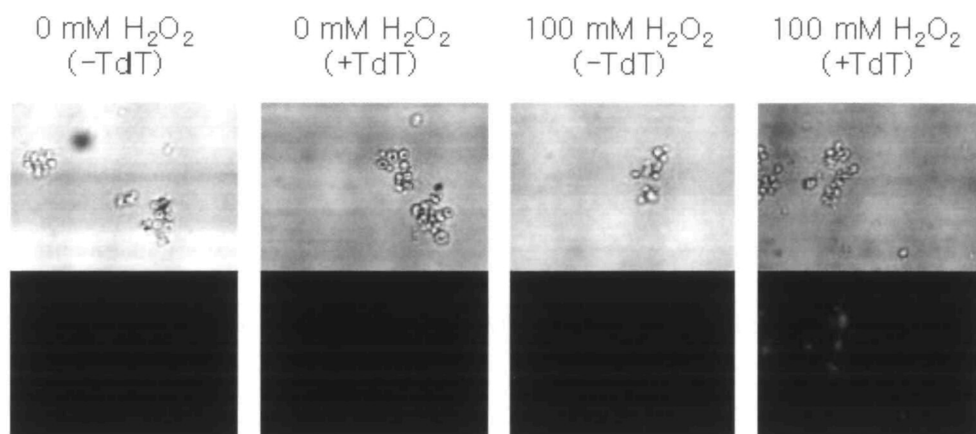

Fig. 3. **DNA fragmentation in *S. cerevisiae* exposed to 100 mM H_2O_2.** DNA fragmentation was assessed in paraformaldehyde-fixed cells by TUNEL staining using laser confocal microscopy. TdT indicates terminal deoxynucleotidyl transferase. The data are representative of three independent experiments.

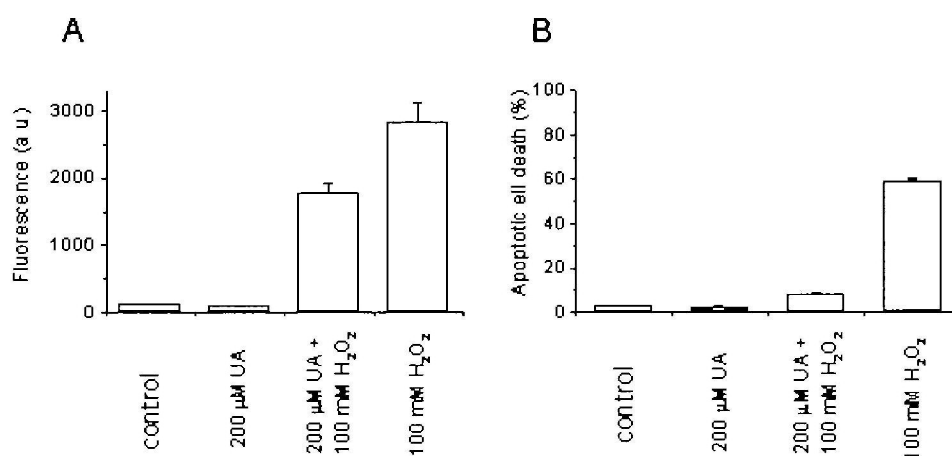

Fig. 4. **Ursolic acid blocks the production of intracellular radicals and apoptotic cell death elicited by H_2O_2 in *S. cerevisiae*.** (A) Intracellular radicals were measured by the oxidation of DCF. (B) The proportion of apoptotic cells was calculated from the number of cells with apoptotic nuclear chromatin as determined by AO/EtBr staining divided by the total number of counted cells. Fluorescence at 525 nm emission was measured with 488 nm excitation 5 s after the addition of H_2O_2. Apoptotic cell death was measured 2 h after the addition of H_2O_2. Ursolic acid (UA) was added 30 m before the addition of 100 mM H_2O_2. Values are the average ± standard deviation of nine determinations.

Ursolic Acid as an Antioxidant Blocks H_2O_2-Evoked Apoptosis in S. cerevisiae—The formation of ROS in yeast was measured by the H_2O_2-dependent oxidation of 2′,7′-dichlorofluorescein (H_2DCF). The oxidation of H_2DCF was significantly augmented by the extracellular addition of various amount of H_2O_2 for 1 h (data not shown). When 200 µM ursolic acid was added 30 m before the addition of 100 mM H_2O_2, there was a significant, (about 23-fold) enhancement of H_2DCF compared with the control (Fig. 4A). Interestingly, the addition of 200 µM ursolic acid reduced the oxidation of H_2DCF by about 30% compared to 100 mM H_2O_2 (Fig. 4A). To investigate whether ursolic acid also blocks H_2O_2-elicited apoptosis, cell death was observed by AO/EtBr staining. Treatment with 100 mM H_2O_2 for 1 h resulted in a rate of cell death of 58.5%. In contrast, incubation with ursolic acid followed by the addition of H_2O_2 for 1 h resulted in a cell death rate of only 8.1% (Fig. 4B). These results suggest that ursolic acid reduces intracellular free radicals produced by the external addition of H_2O_2, thus inhibiting apoptosis in yeast.

PI 3K/PKB Inhibits Apoptosis Induced by H_2O_2 in S. cerevisiae—The ability of both PI3K and PKB to suppress apoptotic cell death has been described in a variety of cells (*10, 11*). The PI3K/PKB signaling pathway is also involved in the regulation of cell responses in yeast (*21*). We, therefore, examined whether PI3K/PKB blocks H_2O_2-evoked apoptosis in *S. cerevisiae*. Phosphatidylinositol 3,4-bisphosphate (PIP_2) as an activator of PI3K was added to yeast cultures at 2 h before the addition of 100 mM H_2O_2. As shown in Fig. 5A, the presence of PIP_2 significantly reduced apoptotic cell death induced by 100 mM H_2O_2 in a PIP_2 concentration–dependent manner. In addition, we examined the effects of wortmannin and LY294002 on 10 mM H_2O_2-elicited apoptosis in *S. cerevisiae*. Wortmannin showed a synergistic effect with 10 mM H_2O_2 in apoptotic cell death (Fig. 5B). LY294002 had an effect similar to that of wortmannin (data not shown).

The activation of PI3K appears to elicit PKB activity by phosphorylation (*22*). We, therefore, examined the relationship between the activity of PKB and H_2O_2-evoked apoptosis. However, the activity and substrate proteins for PKB have not yet been reported in yeast. First, we measured the activity of PKB and identified the putative PKB substrate proteins in yeast (Fig. 6). The consensus sequence of the PKB substrate proteins was identified (**XXRXRXXS/TXX**; the underlined residue is a hydrophobic amino acid, Ref. *23*). To determine whether yeast contain PKB activity, we employed a nonradioactive protein kinase assay method using the fluorescein IRS-1 peptide ([30]RKRSRKESYS[39]), which is known to be a PKB substrate in mammalian cells (*16*). As shown in Fig. 6A, the IRS-1 peptide was phosphorylated by the yeast cell lysate, showing that the lysate con-

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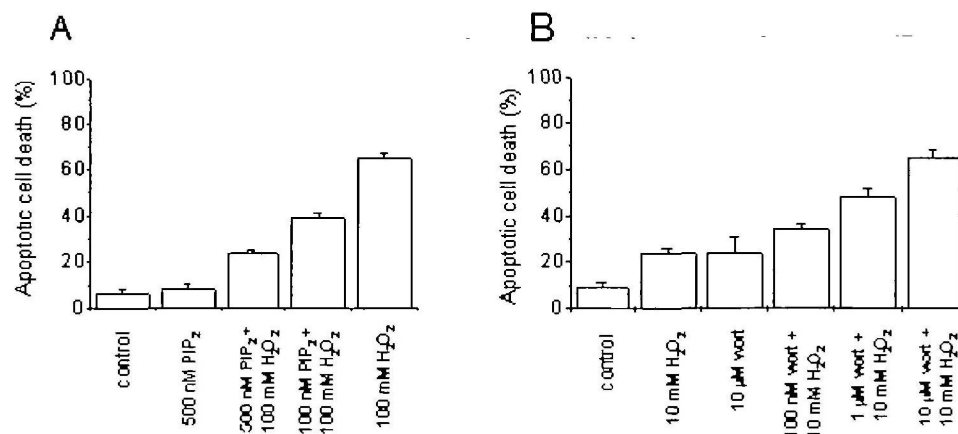

Fig. 5. **PI3K/PKB suppresses the induction of apoptosis elicited by H_2O_2 in *S. cerevisiae*.** (A) PIP_2 inhibited apoptotic cell death. PIP_2 was added 2 h before treatment with 100 mM H_2O_2. (B) Wortmannin (wort) enhanced H_2O_2-evoked apoptosis. Wortmannin was added 30 m before the addition of 10 mM H_2O_2. Values are the average ± standard deviation of six determinations.

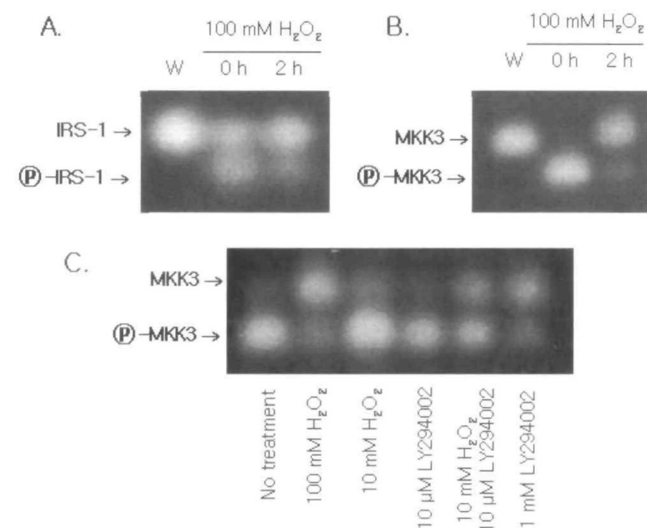

Fig. 6. **Phosphorylation of IRS-1 and MKK3 peptides by yeast protein lysate.** (A) IRS-1 peptide, as a PKB substrate, was phosphorylated by yeast protein lysate (10 mg protein/ml). (B and C) MKK3 peptide, as a MEKK3 substrate, was phosphorylated by yeast protein lysate (1 mg protein/ml). LY294002 was added 30 m before the addition of H_2O_2 (2 h) or before cell lysis. W and Ⓟ indicate treatment with water and phosphorylation of the peptide, respectively. No treatment in (C) means the addition of a protein lysate obtained from untreated yeast.

tains PKB activity. We also observed the inhibition of PKB activity by treatment of H_2O_2 (100 mM, 2 h) (Fig. 6A).

MEKK3 is an important protein kinase that is phosphorylated by PKB in mammalian cells. If MEKK3 or MEKK3-related activity is detected in yeast, then a down-regulation of PKB should be observed, providing an indirect method for measuring PKB activity as well. For the MEKK3 assay, we used another fluorescein peptide, MKK3 ([189]**S**VAK**T**MDA[196]; bold indicates phosphorylation sites), known to be a MEKK3 substrate in mammalian cells. The peptide was phosphorylated by yeast lysates and also inhibited by H_2O_2 treatment (Fig. 6B), indicating that MEKK3 acts as an authentic down regulator of PKB in yeast. LY294002 inhibited the phosphorylation of the MKK3 peptide (Fig. 6C), possibly due to the inhibition of MEKK3 activity. Wortmannin also blocked the activities of PKB and the MEKK3 (data not shown). Taken together, these results confirm that the PI3K/PKB signaling pathway is involved in cell survival and in the suppression of apoptotic cell death in yeast.

DISCUSSION

Recent studies have demonstrated that yeast, a unicellular organism, possess apoptotic machinery (*6–8, 24, 25*). Even if the complete genomic sequences of *S. cerevisiae* show no homology regions with the major apoptotic regulators described for mammalian cells, such as the Bcl-2 family and caspases, Komatsu *et al.* (*24*) recently reported that *Schizosaccharomyces pombe* contain Rad9, which has a BH3-like region and interacts with the anti-apoptotic protein Bcl-2.

Apoptotic features of yeast in terms of morphological changes are somewhat different from those in mammalian cells. For example, the translocation of PS occurs in yeast (Fig. 2) as in mammalian cells, but DNA fragmentation is more complicated in yeast cells (*6*). In spite of the induction of apoptosis by treatment with 100 mM H_2O_2 for 2 h, DNA fragmentation at that time was weak, although it occurred by 4 h incubation (Fig. 3). An examination of genomic DNA by agarose gel electrophoresis did not reveal a DNA ladder pattern (Ref. *5*, data not shown). As mentioned earlier, this might be due to the highly ordered structure of chromatin and the lack of linker DNA in yeast (*6, 20*).

The extracellular addition of ROS also triggers apoptotic cell death in a variety of cells including yeast cells (*4–8*). The addition of 200 µM ursolic acid reduced both intracellular ROS levels and the rate of H_2O_2-induced apoptotic cell death (Fig. 4). Ursolic acid has been reported increase the activities of both catalase and superoxide dismutase (SOD), resulting in the removal of ROS in a variety of cell types (*26, 27*). Ursolic acid might enhance the activities of catalase and SOD in mammalian cells as well as in yeast. Our finding that there is an accumulation of ROS and induction of apoptosis indicates that *S. cerevisiae* may undergo apoptosis or apoptosis-like cell death.

Recently, apoptosis signal-regulating kinase 1 (ASK1) was shown to interact directly with GST and suppress apoptosis evoked by oxidative stress (*28*). In addition, ROS enhance the induction of GST, in which PI3K/PKB serves as the essential pathway (*11*). Hence, PI3K/PKB may play

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a critical role in reducing oxidative stress-induced apoptosis in mammalian cells. However, although there is information that yeast cells also contain PI3K activity (*21*), there is no evidence concerning an anti-apoptotic effect in yeast. It was observed that both wortmannin and LY294002, structurally unrelated PI3K inhibitors, significantly enhanced apoptotic cell death by low concentrations of H_2O_2 (Fig. 5B). In addition, PIP_2, a PI3K activator, inhibits apoptosis induced by high concentrations of H_2O_2 (Fig. 5A). PI3K inhibits apoptotic cell death by inhibiting caspase 3 (*29*), delaying the onset of p53-mediated apoptosis (*30*), and enhancing bad phosphorylation (*31*) in mammalian cells (*32*). In yeast, however, there is limited information as to whether PI3K suppresses apoptotic cell death and how PI3K regulates apoptotic cell death. Our observations suggest that the PI3K/PKB signaling pathway plays a significant role in mediating ROS-elicited apoptotic cell death in yeast as in mammalian cells (*11, 28*).

PI3K enhances the activities of PKB and MEKK3 in yeast, indicating that yeast also contains PKB- and MEKK3-related proteins (Fig. 6). Based on the consensus sequence of the PKB substrate protein (XX**R**X**R**XX**S/T**XX), we inspected several putative PKB substrate proteins (^{64}YD**R**L**R**PL**S**YP^{73} in CDC42; ^{71}YD**R**L**R**PL**S**YP^{80} in Rho 1; ^{68}YE**R**L**R**PF**S**YS^{77} in Rho 2; ^{77}FD**R**L**R**SL**S**YS^{86} in Rho 3; and ^{134}YS**R**L**R**PL**S**YT^{143} in Rho 4) in yeast (*33*). The results suggest that CDC42 and/or the Rho protein family are involved in PI3K/PKB signaling pathways in yeast. Several biochemical studies have reported that protein kinases encoded by *STE11* and *STE7* are structurally related to mammalian protein kinases: STE11 is a MEKK3 homolog (41% identity); STE7 is a MKK3 homolog (36% identity) (*34*). MKK3 as a substrate for MEKK3, a PKB substrate protein, contains conserved phosphorylation sites (^{189}**S**VAK**T**MDA^{196}; bold letters indicate sites phosphorylated by MEKK3). The sequence is highly conserved in yeast STE7 (^{359}**S**IAD**T**FV^{365}). These kinases might act in the order PKB→CDC42 or Rho protein→STE11→STE7 in yeast as in mammalian cells (*35, 36*). Our results suggest that yeast also contains the PI3K/PKB signaling pathway, which negatively regulates ROS-elicited apoptotic cell death through a protein phosphorylation cascade in yeast.

The specific apoptotic cell death machinery in yeast might be different from that in multicellular organisms, although apoptotic cell death has been identified in several unicellular organisms (*6–8*). Our results indicate that *S. cerevisiae* contain apoptotic cell death machinery, which suppresses apoptosis through the PI3K/PKB signaling pathway. The observations clearly suggest that yeast may provide a suitable model system for use in understanding apoptotic signaling pathway evoked by a variety of stimuli. There is limited information that any genes and/or proteins are involved in the regulation of apoptotic cell death other than Rad9 protein in yeast (*24*). Therefore, further studies are required to explore and identify the apoptotic machinery and to characterize the regulatory mechanism of PI3K/PKB involved in the blockage of apoptosis in *S. cerevisiae*.

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