Schizosaccharomyces pombe Cells Deficient in Triacylglycerols Synthesis Undergo Apoptosis upon Entry into the Stationary Phase*

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Triacylglycerols (TAGs) are important energy storage molecules for nearly all eukaryotic organisms. In this study, we found that two gene products (Phl1p and Dga1p) are responsible for the terminal step of TAG synthesis in the fission yeast Schizosaccharomyces pombe through two different mechanisms: Phl1p is a phospholipid diacylglycerol acyltransferase, whereas Dga1p is an acyl-CoA:diacylglycerol acyltransferase. Cells with both dag1* and plh1* deleted (DKO cells) lost viability upon entry into the stationary phase and demonstrated prominent apoptotic markers. Exponentially growing DKO cells also underwent dramatic apoptotic when briefly treated with diacylglycerols (DAGs) or free fatty acids. We provide strong evidence suggesting that DAG, not sphingolipids, mediates fatty acids-induced lipoapoptosis in yeast. Lastly, we show that generation of reactive oxygen species is essential to lipoapoptosis.

Triacylglycerols (TAGs) are important energy storage molecules that can be found in almost all eukaryotes. In mammals, TAG synthesis plays essential roles in a number of physiological processes, including intestinal fat absorption, energy storage in muscle and adipose tissue, and lactation. It also contributes to pathological conditions such as obesity and hypertriglyceridemia (1). TAG synthesis through both the glycerol-3-phosphate pathway and the monoacylglycerol pathway is acyl-CoA-dependent. The transfer of an acyl group from acyl-CoA to diacylglycerols (DAGs) catalyzed by the enzyme diacylglycerol acyltransferase (DGAT) is regarded as the only committed reaction in TAG synthesis in the glycerolipid pathway, because DAG is diverted from membrane glycerolipid biosynthesis (2). Two distinct mammalian DGAT genes have been identified recently. DGAT1 was cloned based on its sequence homology to genes involved in sterol esterification (3, 4).

DGAT2 was identified by its homology to a DGAT isolated from the fungus Mortierella ramanniana (5, 6). Other acyl-CoA-dependent TAG-synthesizing enzymes are likely present but are yet to be identified. In addition, acyl-CoA-independent TAG synthesis was also shown to exist in eukaryotes. A DAG transacylase, which synthesizes TAG from two DAGs, was purified from rat intestinal microsomes, and its activity was comparable to that of DGAT (7).

Four genes, i.e. DGA1, LRO1, ARE1, and/or ARE2, have been found to encode proteins capable of synthesizing TAG in the budding yeast Saccharomyces cerevisiae (8–11). Dga1p is highly homologous to mammalian DGAT2, whereas Lro1p encodes a protein with significant sequence similarity to the mammalian enzyme lecithin cholesterol acyltransferase. Dga1p utilizes acyl-CoA to esterify DAG, whereas Lro1p transfers an acyl group from a phospholipid molecule to the sn-3 position of DAG. Dga1p and Lro1p mediate the bulk of TAG synthesis; however, in their absence, 2–4% of normal TAG synthesis could still be detected. It was later determined that Are1p and Are2p, two acyl-CoA sterol acyltransferases in yeast, are responsible for this residual activity. When all four genes are deleted simultaneously, synthesis of both sterol esters and TAG is completely blocked. However, no obvious growth defects were detected in the budding yeast cells completely free of either TAG or neutral lipids. This is rather surprising, because neutral lipids have long been regarded as a safe depot for polar and potentially toxic lipids such as fatty acids, DAG, or sterols. A recent study has proven that synthesis of TAG prevents fatty acids-induced lipotoxicity in mammalian cells (12).

The fission yeast S. pombe, similar to the budding yeast, is genetically tractable and equipped with a rich repertoire of molecular tools and a completely sequenced genome (13, 14). Although the fission and budding yeasts are as divergent from each other as each from the mammals, S. pombe has been shown to have greater similarity to mammals at least in certain steps of cell division and in aspects of stress signaling (15). The enzymes and pathways of lipid metabolism, their physiological significance, and their resemblance to mammalian systems are largely unexplored in S. pombe. In this work, we describe the identification of two genes, plh1* and dag1*, that encode enzymes that are responsible for the bulk of TAG synthesis in the fission yeast. We provide convincing evidence that fission yeast cells defective in TAG synthesis undergo apoptosis upon entry into the stationary phase. The important role of DAG in the induction of lipoapoptosis is also investigated.

EXPERIMENTAL PROCEDURES

Yeast Strains, General Techniques, and Reagents—S. pombe strains MBY257 (h–, his3-D1, ade6-M210, leu1–32, ura4-D18) and MBY266 (h+, his3-D1, ade6-M210, leu1–32, ura4-D18) were used in this study.
(16). Growth media (YES and EMM) and basic genetic, cell, and biochemical techniques were used according to a previous report (17).

Transformation of yeast was performed with electroporation, followed by prototrophic selection (18). Yeast extract, Yeast Nitrogen Base, Bacto-peptone, and Bacto-agar were from Difco Laboratories; β-d-glucose, β-d-galactose, and β-araffinose were from Sigma. N-Acetylsphingosine (C2-sphingosine) was from US Biological. [1-14C] oleoyl-CoA, 1-stearoyl-2-[1-14C] arachidonoyl-sn-glycerol, 1-palmitoyl-2-[1-14C] oleoyl phosphatidylethanolamines (PEs), and [9,10-(3H) oleic acid] were from Amersham Biosciences. An in situ cell death detection kit and Annexin-v-fluos were from Roche Applied Science.

After incubation, cells were analyzed for viability and DNA fragmentation. DiC, DAG and ceramide were dissolved in Me2SO. The working concentrations for DAG were 0.1, 0.2, or 0.3 μM, whereas for ceramide they were 10 or 20 μM (23, 24).

Nile Red Staining—Cells were grown to early stationary phase, washed with deionized H2O two times, and incubated with 1 μg/ml Nile Red (1 mg/ml in acetone stock). Fluorescence images were obtained with a Leica DMLB microscope (25).

Detection of Apoptotic Markers—All assays that follow were performed as previously described (26). For 4′,6-diamidino-2-phenylindole (DAPI) staining, cells were fixed with 3.7% formaldehyde for 10 min, washed once with PBS containing 1% Nonidet P-40 and twice with PBS, and then stained with DAPI. Cells were viewed using a Leica DMLB microscope. For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, cells were permeabilized with 0.1% Triton X-100 in 1× PBS for 1 h, digested with zymolase, washed with PBS, incubated in a permeabilization solution (0.1% Triton in 0.1% sodium citrate) for 2 min on ice, washed twice with PBS, and incubated with 50-μl TUNEL mixtures for 1 h at 37 °C. Cells were washed with PBS twice and then viewed using a Leica DMLB microscope. For annexin V staining, cells were washed in sorbitol buffer (1.2 M sorbitol, 0.5 mM MgCl2, potassium phosphate, pH 6.8), digested with zymolase for 2 h at room temperature, harvested, washed in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, 1.2 μM sorbitol), pelleted, and resuspended in binding buffer. 2 μl of annexin-FITC and 2 μl of propidium iodide were added to a 38-μl cell suspension and then incubated for 20 min at room temperature. The cells were harvested, suspended in binding buffer, and applied to microscopic slides.

Production of reactive oxygen species (ROS) was detected by dihydroethidium (Sigma), which was used at 5 μg/ml cell culture. After incubation for 10 min, cells were viewed under a Leica DMLB microscope through a Texas Red filter. The free radical spin trap reagent 3,3,5,5-tetramethylpyrroline-1-oxide (TMPO) was used at 125 μM cell culture. Cells were pretreated with TMPO for 2 h before lipids were added.

In Vivo Assay of Oleate Incorporation—The incorporation of [3H] oleate into TAG was used as a measurement of DAG esterification essentially as described previously (9). Briefly, Cells were cultured in YES medium without amino acid supplements for 24–27 h, and those cells were used to seed MBY266 and MBY257. Cells were harvested, washed once with distilled water and plated in triplicates on YES agar. Colonies were scored after 3 days of incubation at 30 °C.

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serum albumin, 150 μM DAG, 8 mM MgCl2, 150 μM phosphatidylethanolamine (PS) phosphatidylethanolamines (PEs) liposomes (1:1 molar ratio), and 50 μM oleyl-CoA. All the assays were conducted at room temperature for 25 min. For PDAT assay, oleyl-CoA was omitted while 14C phosphatidyethanolamines were added in liposomes at different concentration (0, 15, 30, 45, and 60 μM). For DGAT assay, 14C oleyl-CoA was added at different concentrations (0, 5, 20, 25, and 50 μM). In the diacylglycerol transacylase assay, 14C DAG was added at 0, 7.5, 15, 35, and 70 μM, whereas MgCl2 and bovine serum albumin were omitted. In control assays, all components were the same except microsomes were removed. Reactions were stopped by the addition of 6 ml of chloroform/methanol (2:1). Phase separation was induced by the addition of 1.2 ml of water. 1 μl of [3H]cholesterol and 15 μg of triolein were added as an internal standard and carrier, respectively. The lipid-containing phase was dried with nitrogen, and the lipids were dissolved in 100 μl of chloroform for spotting on TLC plates. The plates were developed in hexane/diethyl ether/acetic acid (70:30:1), and TAG was quantified by scintillation counting.

**Diacylglycerol Kinase Assay**—The assay was conducted as described in the Biotrak assay reagents system (Amersham Biosciences). Wild type and DKO yeast cells were grown in YES medium to mid-log phase and then treated with medium containing 0.8 mM palmitate or oleate. Cells were collected at different time points (0, 30, 60, and 120 min). DAG was extracted with other lipids and quantified through a phosphorylation reaction catalyzed by a bacterial DAG kinase.

**RESULTS**

*Identification of plh1* and *dga1* in *S. pombe*—We searched the fission yeast genome database for homologous sequences to human DGAT1 (hDGAT1), human DGAT2 (hDGAT2), and the budding yeast LRO1 using tBLASTX. A sequence with significant homology to hDGAT2 (40% identity at protein level) was identified and named *dga1* (GeneDB systematic name: SPCC1235.15). In addition, as previously reported, an open reading frame highly homologous (45% identity at protein level) to the budding yeast LRO1 was found in the fission yeast genome and named *plh1* (for Pombe LRO1 Homolog 1, GeneDB systematic name: SPBC776.14) in this study. A few open reading frames showing limited homology to DGAT1 were also found, but they are unlikely to play a role in TAG synthesis as suggested by a previous report (9). *plh1* predicts a protein of 623 amino acids, with a putative transmembrane domain near its N terminus. Phlp1 also has a conserved serine lipase motif HS/M/L/G between amino acids 292 and 296. *dga1* encodes a 349-residue protein with at least one transmembrane domain. The region of the putative glycerol phospholipid domain in hDGAT2 was also found to be conserved in Dga1p (45% identity over 80 amino acids).

*Deletion of plh1* and *dga1* Resulted in a Viable Yeast Cell Without Detectable TAG—To determine whether Phlp1 and Dga1p are involved in TAG synthesis in the fission yeast, we generated ∆plh1, ∆dga1 single and ∆plh1∆dga1 double deletion (referred to as the DKO strain thereafter) mutants by homologous recombination. All mutants were viable at 16 °C, 30 °C, and 37 °C on rich or minimal media and on different carbon sources (data not shown). We were also unable to observe any obvious morphological changes in the DKO cells under light microscope. To investigate whether cellular TAG mass was affected in these strains, cells were grown to mid-log phase, and lipids were extracted, separated by TLC, and stained by iodine vapor. Although the TAG mass in each single deletion mutant was virtually indistinguishable from that of the wild type cells, virtually no TAG mass could be seen for DKO cells (data not shown). The sterol ester mass was clearly visible for all mutants, ruling out a lipid extraction error for the DKO strain. To further examine the ability of these strains to synthesize TAG, cells in log phase were pulse-labeled with [3H]oleate, and its incorporation into TAG was measured (Fig. 1A). No significant differences in oleate incorporation into TAG were detected between wild type and the ∆dga1 mutant. However, TAG synthesis decreased by nearly 50% due to the loss of Phlp1. Most notably, the double mutant was almost totally deficient in TAG synthesis. In contrast, sterol ester biosynthesis was normal in all mutants (data not shown). In the budding yeast, it has been confirmed that Are1p and/or Are2p are responsible for the residual TAG synthesis activity (about 5% of the wild type level). In *S. pombe*, there are two proteins...
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(GeneDB systematic names: SPAC13G7.05 and SPCP1E11.05) that share strong homology with Are1p and Are2p. We have determined that these two homologs catalyze sterol esterification in S. pombe (data not shown); however, whether these proteins have a role in TAG synthesis remains to be examined. To confirm that either Plh1p or Dga1p was sufficient for TAG synthesis, we overexpressed plh1Δ and dga1Δ in wild type and DKO strains. Both genes were placed under the control of a modified nmt1 promoter (21), and each gene was able to complement the TAG synthesis defect in the DKO mutant, indicating an overlapping function of these two genes (Fig. 1B). Overexpression of plh1Δ and dga1Δ also caused a significant increase in TAG synthesis in WT and mutant strains, suggesting these genes could be regulated at transcription level. These results imply that TAG synthesis is mediated by two gene products in fission yeast, whereas Plh1p plays a major role at log phase. To further confirm the absence of TAG in the DKO strain, we treated yeast cells with Nile Red, a fluorescent dye with strong and specific affinity for neutral lipids (28). In both of the wild type and DKO cells, cytoplasmic fluorescent droplets could be seen in early stationary phase cultures. However, the number and intensity of the droplets observed in DKO cells was significantly less than those in wild type strains (Fig. 1C).

In Vitro Microsomal Assays of DAG Esterification—The results described above demonstrated the essential roles of Plh1p and Dga1p in TAG synthesis; however, they did not reveal the exact molecular function of these two proteins. Based on sequence homology and experimental data from previous studies (8–11), it is highly likely that both of Plh1p and Dga1p carry out DAG esterification, with Dga1p functioning as an acyl-CoA DAG acyltransferase (DGAT), whereas Plh1p functioning as a phospholipid DAG acyltransferase (PDAT). To confirm this hypothesis, we analyzed DAG esterification using microsomes prepared from the wild type and deletion mutant strains. When 14C-oleoyl-CoA and unlabeled diacylglycerol were added to the microsomes, the DGAT activity in Δplh1 cells was significantly higher than the level in WT cells up to 25 μM oleoyl-CoA, whereas it was nearly undetectable in microsomes from Δdga1 and DKO cells (Fig. 2A). When 14C-diacylglycerol and cold acyl-CoA were used, almost no DAG incorporation into TAG could be detected in the DKO strain. The Δdga1 microsomes showed ~50% DAG esterification activity of the normal microsomes, whereas the Δplh1 microsomes had similar activity as the wild type microsomes (Fig. 2B). When 1-palmitoyl-2-[1-14C]oleoyl phosphatidylethanolamines (PEs) and unlabeled DAG were added, microsomes from wild type cells and Δdga1 cells incorporated radiolabeled fatty acid into TAG at similar rates (Fig. 2C). This activity was absent in Δplh1 microsomes and in microsomes prepared from the DKO strain, indicating that Plh1p mediates esterification of DAG using the sn-2 acyl group of PE as the acyl donor. The substrate specificities of Plh1p and Dga1p were also investigated using the same in vitro assay system. Plh1p showed the best activity with PE, followed by phosphatidylethanolamine and phosphatidylinositol. Dga1p prefers palmitate over oleate (data not shown). Lastly, normal microsomes showed no incorporation of fatty acid from PE into sterol esters (data not shown), indicating that ergosterol is not a substrate for Plh1p under these assay conditions.

Cells Deleted for Both plh1Δ and dga1Δ Underwent Apoptosis upon Entering Stationary Phase—Schaffer and colleagues (12) have recently demonstrated that TAG synthesis protected against fatty acid-induced lipotoxicity in Chinese hamster ovary (CHO) cells. Other recent studies also implicated a critical role for TAG synthesis in cell viability in Drosophila and in the oleaginous yeast (29, 30). Previous reports indicated that the sole growth phenotype in budding yeast cells without neutral lipids was a prolonged lag phase with no significant change of growth in exponential or stationary phases. We examined the growth properties of wild type and DKO S. pombe cells and found that, when stationary phase cells with the same A0.95 value were used to start a growth culture, there was indeed a significant delay in the onset of log phase for DKO cells; however, when log phase cells were used to start the culture, there was no such lag phase (data not shown). These data suggest that, among other possibilities, most of the double mutant cells in the stationary phase could not be revived. To test this hypothesis, a colony forming assay was performed (Fig. 3A), and as expected, most of the DKO cells started losing viability upon entry into the stationary phase. In addition, when the stationary phase cells were stained with DAPI, remarkable nuclear DNA fragmentation was detected in the majority of the DKO cells, whereas no such fragmentation was observed in any WT cells (Fig. 3B). The mechanisms of this stress-induced cell death were further investigated by the TUNEL assay. Cleavage of DNA during apoptosis produces free 3′-OH termini, which can be effectively labeled by fluorescently tagged nucleotides in a process catalyzed by terminal deoxynucleotidyl transferase. It was found that, at early stationary phase, DNA fragmentation occurs in the majority (over 50%) of mutant cells, as seen by the extensive TUNEL reaction (Fig. 3C). This extensive labeling was not observed in any of the wild type cells. Log phase cells in general did not show positive labeling, a result that is consistent with the findings that, in the logarithmic growth phase, the double mutant has a comparable viability with the wild type strain. The TUNEL-positive phenotype suggested to us that the DKO cells could undergo apoptotic cell death when starved; therefore, we look for more apoptotic markers. In mammalian and Saccharomyces cerevisiae cells, exposure of phosphatidylserine (PS) at the outer periphery of the plasma membrane is an early marker for apoptosis (31). To test whether the same process occurs in S. pombe, spheroplasts of WT and DKO cells derived from stationary phase cultures were incubated with FITC-labeled annexin V. As shown in Fig. 3D, strong fluorescence could be seen in the periphery of about 10% of mutant cells, suggesting that phosphatidylserine is indeed exposed to the outer leaflet of plasma membrane. Membrane integrity was intact, because propidium iodine was excluded from most of the annexin V-positive cells. In contrast, no FITC fluorescence was observed for WT cells. Production of reactive oxygen species (ROS) represents another prominent marker for apoptosis in yeast (26). We treated WT and DKO cells with dihydroethidium, which can be oxidized by ROS to fluorescent ethidium. As shown in Fig. 3E, over 50% of the mutant cells at the stationary phase fluoresced strongly, whereas less than 5% of WT cells showed fluorescence (Fig. 3E).

DAG Accumulates in DKO Cells and Triggers Apoptosis—Loss of viability, nuclear DNA fragmentation, exposure of PS, and generation of reactive oxygen species lend strong support to the conclusion that these DKO cells underwent apoptosis upon nutrient starvation. Apoptosis has been shown to exist in the unicellular organism S. cerevisiae, with ROS playing a central regulatory role and the newly identified caspase homolog Yea1p as a possible central executioner (32, 33). However, how internal and environmental cues stimulate the production of ROS is largely unknown and whether there are other factors or pathways that might function independently of ROS or caspase to regulate apoptosis in yeast remains to be explored. We sought to understand the mechanism of this novel form of apoptotic cell death caused by the inability of DKO cells to produce TAG. S. cerevisiae cells begin accumulating neutral lipids upon exiting logarithmic growth phase, probably as a...
result of phospholipids remodeling by altering the activities of phosphatidate phosphotases (34). Due to the loss of DAG esterification capability in DKO cells, DAG and long chain fatty acids, two signaling molecules and major substrates for TAG synthesis, could accumulate upon entry into the stationary phase and induce apoptosis. We therefore examined the quantity of DAG and palmitic and oleic acids in WT and DKO strains. Steady-state labeling experiments showed that there was almost 300% more DAG in DKO cells than in WT cells at early stationary phase (Fig. 4A), whereas there was little difference between the two strains at log phase. Surprisingly, no significant differences in total fatty acid levels between WT and DKO cells were observed as measured by gas chromatography/mass spectrometry (not shown). This result suggests that accumulation of DAG might be the key to the death of DKO cells at stationary phase. We reasoned that if the apoptotic cell death of the double mutant cells was indeed caused by accumulation of DAG upon entry into the stationary phase, we

**Fig. 2.** *In vitro* assays of DAG esterification. Microsomes were extracted from WT and deletion strains grown to log phase. DAG esterification activities were assayed *in vitro* at 23 °C for 25 min with varying substrates. At 25 min, the enzyme activities for each substrate at all concentrations used fall within the linear range (data not shown). A, 0–50 μM [1-14C]oleoyl-CoA and 150 mM 1,2-dioleoyl-sn-glycerol. B, 0–70 μM 1-stearoyl-2-[1-14C]arachidonoyl-sn-glycerol and 50 mM oleoyl-CoA. C, 0–60 μM 1-palmitoyl-2-[1-14C]oleoyl phosphatidylethanolamines and 150 mM 1,2-dioleoyl-sn-glycerol. Assays were performed in triplicates.
should be able to kill exponentially growing cells with exogenously added DAG. We therefore treated log phase cells with a membrane-permeable DAG analog called DiC₈ DAG (1,2-dioctanoyl-sn-glycerol). Early log phase cells were treated with various concentrations of DAG for 0–3 h. As expected, prominent nuclear DNA fragmentation and cell death were observed.
in the DKO strain, but not in the wild type. The percentage of cells showing DNA fragmentation generally increased with time (up to 3 h) and concentration of DAG (up to 0.3 mM). The results of cells treated with 0.2 mM DAG for 2 h are shown in Fig. 4 (B–D).

**Fatty Acids Induce Apoptosis through DAG**—To further investigate the possible role of DAG in yeast apoptosis, we focused our attention on free fatty acids. We reasoned that if DAG is indeed the apoptosis-inducing molecule in the DKO cells, treating the cells with free fatty acids would also trigger apoptosis, because excessive free fatty acids would increase the production of, among other molecules, DAG. We treated WT and DKO cells with palmitate and oleate, and not surprisingly, both of them caused the DKO cells to undergo apoptosis (Fig. 5, A–C). To prove that the fatty acids-induced apoptosis is caused by DAG, we first measured cellular DAG level after fatty acids were added to growth media. Because steady-state labeling would not be a feasible method to estimate DAG in this case, a DAG kinase kit was used instead to estimate cellular DAG after cells were incubated with 0.8 mM palmitate or oleate for 2 h. As expected, the DAG level in DKO cells increased from 1 nmol/mg dry weight before addition of palmitate to 3.5 nmol/mg of dry weight after a 2-h incubation. In wild type cells, the change was mild (from 1 to 1.5 nmol/mg of dry weight). With oleate, there was a less but significant increase in DAG level (from 1 to 2.5 nmol/mg of dry weight) in DKO cells. The different effects of palmitate or oleate could be due to substrate preference of glycerol-3 phosphate acyltransferases in *S. pombe*. In fact, it has been demonstrated in *S. cerevisiae* that 16-carbon fatty acids are preferred substrates of Gat2p, one of the two newly identified enzymes that control the initial steps of glycerolipid synthesis (35). To further prove that fatty acids cause apoptosis through increased *de novo* synthesis of DAG, we tested whether removal of DAG could attenuate or reverse palmitate/oleate-induced apoptosis. A bacterial DAG kinase (DGK) was expressed in the double deletion strain under the control of a modified nmt1 promoter (21, 22). The expression resulted in a DAG kinase activity of 35 pmol/min/mg as determined by [γ-32P]ATP incorporation into phosphatidic acid, more than 5-fold higher than basal activity. As shown in Fig. 6A, nearly 60% of cells survived as a result of DGK expression, whereas only a 5% survival rate was observed in cells containing the empty plasmid. Further evidence was obtained by DAPI staining and the TUNEL assay. Significantly fewer cells with DGK expression showed DNA fragmentation and positive TUNEL reaction (Fig. 6, B and C). Based on these results, we could conclude that the apoptosis-inducing effect of fatty acids is mediated, if not exclusively, by DAG.
Palmitate can also induce de novo synthesis of sphingolipids, some of which are potent pro-apoptotic molecules in other experimental systems. To determine whether dihydrosphingosine (DHS), phytosphingosine (PHS), or ceramide plays a role in palmitate-induced apoptosis, WT and DKO cells were treated with various concentrations of DHS, PHS, or C2-ceramide for different periods of time (0–3 h). These compounds could kill *S. pombe* cells at high concentrations; however, no DNA fragmentation was observed (only the effect of a 2-h treatment by 20 μM C2-ceramide was shown in Fig. 7, A–C). These data are consistent with previous findings that ceramide causes cell cycle arrest, not apoptosis, in *S. cerevisiae* (24). We also examined the effect of fumonisin B1 (inhibitor of ceramide synthase) and myriocin (inhibitor of serine palmitoyl transferase) on the growth of double deletion cells treated with 0.8 mM palmitate. As expected, no rescue of apoptosis was observed, and as a matter of fact, more cells underwent apoptosis in the presence of fumonisin B1 or myriocin (data not shown). These results suggest that sphingolipids are not involved in palmitate-induced apoptosis in *S. pombe*, and when sphingolipid synthesis is blocked, palmitate could be channeled to other pathways, such as the glycerolipid pathway.

**Generation of ROS Is Essential to Lipoapoptosis, whereas Deletion of a Caspase Homolog Has No Effect—**Oxidative stress has been shown to act as a key regulator of apoptosis in *S. cerevisiae* (26) and in other organisms (36, 37). In addition, palmitate-induced lipoapoptosis in CHO cells required in-
increased production of ROS (38). We therefore sought to determine whether generation of ROS is required for the death of DKO cells. We have shown that ROS were generated when DKO cells entered stationary phase (Fig. 3E). Incubation of log phase cells with DAG (not shown), oleate (not shown) or palmitate (Fig. 8C) also induced dramatic ROS production. To determine whether ROS are required for lipoapoptosis in our yeast strain, we examined the effect of the free radical spin trap TMPO on cell viability and DNA fragmentation. Mutant cells were pretreated with TMPO for 2 h before 1 mM palmitate was added for 1 h. As shown in Fig. 8, TMPO could effectively scavenge ROS (Fig. 8C) and prevent DNA fragmentation (Fig. 8B) and cell death (Fig. 8A).

The recent discovery of Yca1p, a caspase homolog in *S. cerevisiae*, has generated much interest and excitement about regulators of apoptosis in yeast. We identified a Yca1 homolog in *S. pombe* (GeneDB systematic name: SPCC1840.04) and named it *pca1* 

Fig. 7. Sphingolipids are not involved in free fatty acids-induced cell death. DKO cells were grown to early log phase and treated with various concentrations of C2-ceramide for 2 h. Cell viability (A), DAPI (B), and TUNEL (C) assays were performed as in Fig. 4. Only the results of selected concentrations of C2-ceramide are shown for each assay.

Fig. 8. Generation of ROS is essential to lipoapoptosis. Log phase WT and DKO cells were pretreated with or without 125 μg/ml of TMPO for 2 h and then incubated with 1 mM palmitate for an additional 2 h. Colony forming (A), DAPI staining (B), and ROS production (C) were performed as described in Fig. 4 and under “Experimental Procedures.”

when log phase cells were treated with DAG or fatty acids (data not shown). Adding caspase inhibitor zVAD-fmk also failed to prevent cell death and DNA fragmentation in our experimental system (data not shown). These results suggest that caspase does not play an essential role in lipoapoptosis in the fission yeast.
invasion, that mediate the last step of TAG synthesis in the fission yeast S. pombe. Deletion of plh1" and dga1" caused cells to undergo apoptosis upon entry into the stationary phase. We demonstrated, for the first time, that apoptotic cell death could result from deletion of endogenous genes in S. pombe. In addition, clear evidence was provided to show that DAG, not sphingolipids, induced apoptosis in S. pombe. Elevation of cellular DAG might represent one of the most primitive pro-apoptotic signals. We further demonstrated that ROS is generated and required for lipid apoptosis in the fission yeast.

Similar to their S. cerevisiae counterparts, Dga1p has DGAT activity, whereas Plh1p is a PDAT. One important difference is that, unlike in S. cerevisiae, a small but significant amount of TAG can be detected in LRO1 and DGA1 double-deletion cells, we were not able to observe any significant TAG synthesis when both plh1" and dga1" were deleted. This could be due to the sensitivity of our assay, however, it is more likely that the two yeasts are different in this respect. The requirement of two mechanistically different reactions to synthesize the same product would be meaningful to a cell if the two enzymes are differentially regulated, alternatively localized, or preferentially recognized by different DAG species. In agreement with Oelkers et al. (10), our results indicate that Plh1p or the PDAT activity is responsible for the majority of TAG synthesis when yeast cells are undergoing exponential growth. Whether the DGAT activity is more important at stationary phase remains to be investigated. In addition, PDAT activity utilizes phospholipids, an essential component of all eukaryotic membranes. It is therefore conceivable that, in addition to its role in TAG synthesis, Plh1p might function to modulate membrane lipid composition and related cellular events such as cross-membrane transport and vesicular trafficking. For instance, the yeast Sec14 pathway clearly demonstrated the dynamic interface between phospholipids metabolism and Golgi function (23). Lastly, although both DGAT and PDAT activities could be detected in the microsomal fractions in the budding yeast, the S. pombe Dga1p appears to localize exclusively to the lipid droplets while PDAT localizes to the endoplasmic reticulum (data not shown). The significance of this pattern of localization remains to be understood.

The most important finding in this study is that S. pombe cells without detectable TAG undergo apoptosis upon entry into the stationary phase. Apoptosis is a form of cell death that plays a critical role in the development and homeostasis of all multicellular organisms. Recent studies have proven that apoptosis also exists in unicellular organisms, such as the yeast S. cerevisiae (33). Various stimuli can cause apoptotic cell death in S. cerevisiae, including oxygen radicals, sexual pheromone, UV, salt stress, and expression of pro-apoptotic mammalian genes, e.g., Bax (reviewed in Ref. 33). Mutations in certain S. cerevisiae genes could also trigger apoptosis (31, 39). All cases of apoptosis documented so far in S. cerevisiae were shown to be associated with increased production of ROS, which is believed to be a key regulator for apoptosis in both uni- and multicellular organisms. A recent landmark study (32) identified a caspase-related protease that appeared to regulate apoptosis in S. cerevisiae, further supporting a common mechanism underlying yeast and mammalian apoptotic processes. Apoptosis in S. pombe is much less well characterized, and the only reported cases were induced by overexpression of mammalian Bak (40) or Caenorhabditis elegans Ced-4 (41). In this report, we found that S. pombe cells unable to synthesize TAG lost viability upon entry into the stationary phase and displayed prominent apoptotic markers, which were not observed when cells were treated with C2-ceramide or sphingoid bases. One surprising result was the lack of effect on the death of DKO cells when the caspase homolog pca1" was deleted. In other experimental systems, caspase-independent cell death pathways do exist, and one of such pathways is controlled by AIF, the apoptosis-inducing factor (42). There is a homolog to mammalian AIF encoded by the fission yeast genome, and whether this homolog is important to the death of DKO cells is under investigation. Interestingly, the Yea1p homolog in Aspergillus nidulans was not required for its apoptotic cell death induced by sphingoid long-chain bases (43). The molecular details of the primitive apoptotic pathways in unicellular organisms therefore require further investigation. Nonetheless, our results added S. pombe to a growing family of unicellular organisms in which apoptotic cell death can be endogenically triggered.

The fact that DKO cells lost viability upon entry into the stationary phase is both intriguing and informative. Normal yeast cells arrest cell growth and enter a resting state called the stationary phase upon nutritional limitation (44). It is known that yeast cells accumulate neutral lipids after diauxic shift, possibly as a result of phospholipid remodeling. Although TAG might be required for yeast cells to survive the stationary phase, possibly as an energy source, it is more likely that TAG serves as an inert storage depot for such bioactive molecules as DAG and fatty acids. Failure to convert DAG and fatty acids into TAG could result in deleterious consequences. In fact, Schaffer and colleagues have recently reported that accumulation of TAG protects against fatty acid induced lipotoxicity in mammalian cells (12). In addition, a mutation in Dro sophila DAG gene led to apoptotic cell death of egg chamber cells, although the exact mechanism was unclear (29). In the current study, we provided several lines of convincing evidence supporting a critical role of DAG in the death of DKO cells: first, mutant cells grown in rich media accumulated DAG upon entry into the stationary phase; second, exogenous DiC8 DAG caused exponentially growing mutant cells to undergo apoptosis; third, addition of palmitate and oleate induced DAG synthesis and triggered apoptosis, which could be rescued by overexpression of a bacterial DAG kinase; fourth, DHS, PHS, or ceramide could kill mutant cells in a manner other than apoptosis. The mechanism by which DAG induces apoptosis is largely unclear. Protein kinases Cs (PKCs) are a classic family of proteins that could be activated by DAG, and the activated isoforms of PKC could be either pro-apoptotic or anti-apoptotic (45). PKC homologs do exist in S. pombe; however, their interaction with DAG and their role in apoptosis are yet to be established (46). It is highly likely that DAG might activate proapoptotic proteins other than PKC. For instance, a mammalian protein Munc13 could bind DAG and induce apoptosis when overexpressed (47). The next obvious challenge is to identify the target of DAG and how it activates the apoptotic pathway in the fission yeast. Lastly, although we have thus far focused on the role of lipids in causing apoptotic cell death, it is of importance to note that nutrient depletion not only changes cellular lipid metabolism but that it itself is a common form of stress. In both yeast and mammalian cells, stress-activated mitogen-activated protein kinase pathways are known to play an important role in the activation of apoptosis (48, 49). It would be interesting to examine how the TAG-deficient cells respond to other forms of stress such as oxidative or osmotic stresses. The eventual apoptotic cell death reported here could probably result from the interplay between accumulation of toxic lipids (DAG) and stress signaling.

The fact that S. cerevisiae cells deficient in TAG or neutral lipid synthesis showed no obvious growth defects is puzzling. Sandager et al. (8) reported a 3.7-fold decrease of DAG in cells.
deficient in neutral lipid synthesis at the stationary phase in S. cerevisiae, which clearly is not the case in S. pombe mutants. This discrepancy highlights the differences between the two yeasts and emphasizes the necessity to conduct parallel studies in both model systems. S. pombe has been extensively used to study the molecular mechanisms of many aspects of cell physiology, including cell cycle and stress signaling; however, little work has been done in the area of lipid cell biology. Therefore, examination of certain aspects of lipid metabolism in the fission yeast might yield valuable information.

Free fatty acids play a key role in the pathogenesis of type II diabetes, and many studies suggested that a high level of free fatty acids in the plasma and excessive accumulation of fatty acids in non-adipose tissues cause insulin resistance and cell death, especially apoptosis of the pancreatic beta cells (50, 51). Using Zucker diabetic fatty fa/fa (ZDF) rats, Unger and colleagues showed that fatty acids and over-accumulation of TAG caused pancreatic beta cells to undergo lipopapoptosis, which was probably mediated by increased production of ceramide and nitric oxide (NO) (reviewed in Ref. 51). Studies by Schaffer and colleagues (38) demonstrated that ROS, rather than ceramide, was critical in the fatty acids-induced apoptosis of CHO cells. The involvement of DAG and protein kinase C (PKC) in palmitate-induced generation of ROS was demonstrated when cultured aortic smooth muscle cells were incubated with high levels of palmitate (52). The S. pombe mutant cells deficient in TAG synthesis may serve as an excellent model system to study the molecular mechanisms of lipotoxicity and lipopapoptosis, because the effect of fatty acids on cell growth are more pronounced and can be easily detected in these mutants. As shown in this study, we provided convincing evidence that DAG and ROS are critical for fatty acids-induced apoptosis in yeast. Powered by the genetic tractability of S. pombe, more components of the lipopapoptotic pathway are expected to be identified and characterized; whether the same components and mechanisms exist in mammalian systems, especially during the course of the development of human type II diabetes, would be highly interesting and worthy of future investigation. Lastly, the fact that the S. pombe strain could offer a novel platform to screen for compounds that might prevent fatty acids-induced lipopapoptosis.

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Schizosaccharomyces pombe Cells Deficient in Triacylglycerols Synthesis Undergo Apoptosis upon Entry into the Stationary Phase
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