Yeast AMID Homologue Ndi1p Displays Respiration-restricted Apoptotic Activity and Is Involved in Chronological Aging

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Apoptosis-inducing factor (AIF) and AIF-homologous mitochondrion-associated inducer of death (AMID) are both mitochondrial flavoproteins that trigger caspase-independent apoptosis. Phylogenetic analysis suggests that these two proteins evolutionarily diverge back from their common prokaryote ancestor. Compared with AIF, the proapoptotic nature of AMID and its mode of action are much less clarified. Here, we show that overexpression of yeast AMID homologue internal NADH dehydrogenase (*NDI1*), but not external NADH dehydrogenase (*NDE1*), can cause apoptosis-like cell death, and this effect can be repressed by increased respiration on glucose-limited media. This result indicates that the regulatory network of energy metabolism, in particular the cross-talk between mitochondria and the rest of the cell, is involved in Ndi1p-induced yeast cell apoptosis. The apoptotic effect of *NDI1* overexpression is associated with increased production of reactive oxygen species (ROS) in mitochondria. In addition, *NDI1* overexpression in *sod2* background causes cell lethality in both fermentable and semi-fermentable media. Interruption of certain components in the electron transport chain can suppress the growth inhibition from Ndi1p overexpression. We finally show that disruption of *NDI1* or *NDE1* decreases ROS production and elongates the chronological life span of yeast, accompanied by the loss of survival fitness. Implication of these findings for Ndi1p-induced apoptosis is discussed.

INTRODUCTION

Apoptosis is a form of programmed cell death essential to the development and tissue homeostasis of multicellular organisms. Although programmed cell death or apoptosis is widely accepted as an essential process in multicellular organisms, until a few years ago it was generally considered to be unnecessary for single cell organisms that do not form multicellular structures. Recent studies demonstrated that apoptosis also occurs in bacteria and yeast (Madeo *et al.*, 1997; Engelberg-Kulka and Glaser, 1999; Wadskog *et al.*, 2004). Like mammalian cells, yeast cells (*Saccharomyces cerevisiae*) undergoing apoptosis show characteristic markers such as DNA cleavage, apoptosis-typical chromatin condensation, externalization of phosphatidylserine, and cytochrome *c* release from mitochondria (Madeo *et al.*, 1997; Manon *et al.*, 1997; Ludovico *et al.*, 2002). Reactive oxygen species (ROS), the central regulator in the metazoan apoptosis, has also been identified as essential in at least some yeast apoptosis, indicating that the similarity between the two processes is not restricted to the appearance (Madeo *et al.*, 1999). The past several years have seen the discovery of several yeast orthologues of crucial apoptotic regulators (Laun *et al.*, 2001; Ligr *et al.*, 2001; Blanchard *et al.*, 2002; Madeo *et al.*, 2002; Chae *et al.*, 2003; Fahrenkrog *et al.*, 2004; Fannjiang *et al.*, 2004; Herker *et al.*, 2004; Sauder and Aebi, 2004; Ahn *et al.*, 2005). These findings firmly established that

yeast and metazoan apoptosis are in essence the same cellular program and lay the foundation of using yeast as a tool for apoptosis research. The observation that old yeast cells as well as dying yeast cells in stationary phase produce oxygen radicals and die apoptotically provides possible clues to a similar sequence of events in mammalian ageing (Laun *et al.*, 2001; Herker *et al.*, 2004).

Mitochondria, both as an essential organelle targeted for destruction and as a perpetrator of the death pathway, play a central role in mammalian apoptosis. Recently, an apoptosis-inducing factor (AIF) orthologue, YNR074C, was identified in yeast that displays sequence similarity to AIF and AIF-homologous mitochondrion-associated inducer of death (AMID) and regulates apoptosis in a similar way to that of AIF in mammalian cells (Wu *et al.*, 2002; Wissing *et al.*, 2004). However, the closest homologue of AMID in yeast is Ndi1p instead of Aif1p. In this report, we describe the role of internal NADH dehydrogenase (*NDI1*) and its homologue external NADH dehydrogenase (*NDE1*) in apoptosis and ageing. We demonstrated that overexpression of *NDI1*, but not *NDE1*, induces apoptosis-like cell death in glucose-rich media, and their deletion can elongate yeast chronological lifespan accompanied with fitness loss.

MATERIALS AND METHODS

Media and Strains

Standard yeast media and growth conditions, except otherwise noted, were used (Sherman, 1991). All strains were in BY4742 (MAT α *his3 Δ 1 leu2 Δ 0 lys2 Δ 0 URA3 Δ 0*) background.

Phylogenetic Tree

Protein sequences of Nde1p and Ndi1p were from *Saccharomyces* Genome Database (www.yeastgenome.org) and sequences of human AIF and AMID

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Table 1. Proteins of AIF family and AMID family used in the phylogenetic tree

Abbreviation	Gene	Protein ID in NCBI	Species
H-AIF	<i>PDCD8</i>	gi:4757732	<i>Homo sapiens</i>
H-AMID	<i>AMID</i>	gi:14318424	<i>H. sapiens</i>
G-AIF	<i>PDCD8</i>	gi:55926141	<i>Gallus gallus</i>
G-AMID	<i>LOC423720</i>	gi:50749348	<i>G. gallus</i>
D-AIF	<i>PDCD8</i>	gi:37682113	<i>Danio rerio</i>
C-wah-1	<i>Wah-1</i>	gi:32564386	<i>Caenorhabditis elegans</i>
P-AIF	<i>PF07_0085</i>	gi:23612574	<i>Plasmodium falciparum</i>
P-AMID	<i>PFI0735c</i>	gi:23613669	<i>P. falciparum</i>
S-NDI1	<i>NDI1</i>	gi:6323515	<i>S. cerevisiae</i>
S-NDE1	<i>NDE1</i>	gi:6323794	<i>S. cerevisiae</i>
S-AIF1	<i>AIF1</i>	gi:6324402	<i>S. cerevisiae</i>
E-AMIDh	<i>nirB</i>	gi:16130618	<i>E. coli</i>
E-NADH	<i>ygbD</i>	gi:26247252	<i>E. coli</i>
E-AIF-h	<i>hcaD</i>	gi:16130467	<i>E. coli</i>
Me-NADH	<i>MJ0649</i>	gi:15668830	<i>Methanococcus jannaschii</i>
A-AIF	<i>At1g63940</i>	gi:30696930	<i>A. thaliana</i>
A-AMID	<i>At1g07180</i>	gi:18390737	<i>A. thaliana</i>
A-AMIDh	<i>At5g22140</i>	gi:18390737	<i>A. thaliana</i>

ID, identification; NCBI, National Center for Biotechnology Information.

were from protein database of the National Center for Biotechnology Information. Other sequences in Table 1 (except E-AMIDh and A-AMIDh) were obtained by BLASTp search from National Center for Biotechnology Information with human AIF, AMID, and yeast Ndi1p as queries. Similar results were obtained with either AMID or AIF sequence as query except that two proteins (E-AMIDh and A-AMIDh), from bacteria (*Escherichia coli*) and plant (*Arabidopsis thaliana*), respectively, could be identified as homologues of human AMID only. To generate a phylogenetic tree, complete alignment of all these sequences (identifications shown in Table 1) was used. Unrooted tree was obtained by ClustalX version 1.81 (Thompson *et al.*, 1997), which was evaluated by Bootstrap statistic method in 1000 times.

Gene Expression and Disruption

Open reading frames (ORFs) of *NDI1* and *NDE1* were PCR amplified from BY4743 genomic DNA with primers flanked by appropriate restriction sites. The PCR products were cloned into expression vector pYES2 or pADH-YES2, which is derived from pYES2 (Invitrogen, Carlsbad, CA) in which the *GAL* promoter was replaced with that of *ADH*. Yeast transformation was performed by standard lithium acetate method (Gietz and Schiestl, 1991). Empty vectors were also introduced into parallel cultures to serve as controls. Transformed cells were selected and grown on uracil dropout media [SD-URA or SGal(galactose)R(raffinose)-URA].

The *NDI1* and *NDE1* ORFs were disrupted with a PCR-mediated method with *URA3* or kanamycin resistance gene (*Kan^r*) as a selection marker (Güldener *et al.*, 1996). PCR primers were designed to amplify the *URA3* or *Kan^r* cassette flanked by 40–50 bases corresponding to immediately downstream and upstream region of *NDI1* or *NDE1* ORFs. Yeast cells were transformed with the PCR product, and integrants were selected on SD-URA or YPD plates containing geneticin (G418; Invitrogen) at 200 mg/l. Gene deletions were verified by PCR.

Growth and Survival Tests

Growth was monitored by plate assays. Yeast were grown overnight, adjusted to identical optical density (OD_{600}) and diluted 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-4} , respectively. Then, 5 μ l of each diluted yeast culture was spotted onto SD-URA or other plates. For ageing experiments, yeast cells were grown until they reached the exponential phase, and aliquots were taken out and continuously incubated in fresh media (with media change every 3 d). The number of surviving colonies was determined by plating a small aliquot on YPD plates.

4,6-Diamidino-2-phenylindole (DAPI) Staining and Microscopy

The basic protocol for DAPI staining of nuclei was used (Streiblova, 1988). Cells were collected, resuspended in 70% (vol/vol) ethanol for brief fixation and permeabilization, and stained with DAPI solution. Cell images were recorded from a fluorescence microscope (model BH-2RFCA; Olympus, Tokyo, Japan) with a digital camera (model C35AD-4; Olympus) and captured on a Lenovo

Tianjiao series computer. Images were processed using Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

ROS Production

For flow cytometric analysis of the production of free intracellular radicals, cells were incubated with dihydrorhodamine 123 (DHR) for 0.5 h and analyzed using an FACSCalibur (BD Biosciences, San Jose, CA) at low flow rate with excitation and emission settings at 488 and 525–550 nm (filter FL1), respectively.

$\Delta\psi_m$ Assay

Cells of overnight culture ($\sim 10^7$ /ml) in SD-URA or SG-URA media were collected and resuspended in 20 mM HEPES buffer, pH 7.4, containing 50 mM glucose. Then, 1 ml of the cell suspension was loaded with 2 μ M rhodamine 123 (Rh123) for 30 min, centrifuged, washed, and resuspended in 100 μ l of phosphate-buffered saline (PBS). $\Delta\psi_m$ was expressed as a fluorescence intensity of Rh123, which was read using an FACSCalibur (BD Biosciences) with excitation at 480 nm and emission at 530 nm.

Annexin V Staining

Existence of exposed phosphatidylserine was detected by staining with FITC-coupled Annexin V (ApoAlert Annexin V apoptosis kit; Clontech, Palo Alto, CA). Yeast cells were washed in sorbitol buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, and 35 mM potassium phosphate, pH 6.8), digested with 5.5% glusulase (Roche Molecular Biochemicals, Mannheim, Germany) and 15 U/ml lyticase (Sigma-Aldrich, St. Louis, MO) in sorbitol buffer for 2 h at 28°C, harvested, washed in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$; Clontech) containing 1.2 M sorbitol, and then harvested again and resuspended in binding buffer/sorbitol. Finally, 2 μ l of Annexin-fluorescein isothiocyanate (FITC) and 2 μ l of propidium iodide (500 μ g/ml) were added to 38 μ l of cell suspension and then incubated for 20 min at room temperature. The cells were finally suspended in binding buffer/sorbitol for examination and analysis.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL)

For the TUNEL test, DNA ends were labeled using the In Situ Cell Death Detection kit (POD, Roche Diagnostics), and cells were prepared as described previously (Madedo *et al.*, 1999). Briefly, yeast cells were fixed with 3.7% formaldehyde, digested with lyticase, and applied to a polylysine-coated slide as described for immunofluorescence (Adams and Pringle, 1984). The slides were then rinsed with PBS and incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature to block endogenous peroxidases. After rinsing with PBS, the slides were incubated in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice, rinsed twice again with PBS, incubated with 10 μ l of TUNEL reaction mixture (200 U/ml terminal deoxynucleotidyl transferase, 10 mM FITC-labeled dUTP, 25 mM Tris-HCl, 200 mM sodium cacodylate, and 5 mM cobalt chloride; Roche Diagnostics) for 60 min at 37°C, and then rinsed three times with PBS. For the detection of peroxidase, cells were incubated with 10 μ l of Converter-POD (anti-FITC Fab fragment from sheep, conjugated with horseradish peroxidase) for 30 min at 37°C, rinsed three times with PBS, and then stained with 3-3'-diaminobenzidine tetrahydrochloride-substrate solution (Roche Diagnostics) for 10 min at room temperature. Coverslips were mounted on with a drop of Kaiser's glycerol gelatin (Merck, Whitehouse Station, NJ). As staining intensity varies, only samples from the same slide were compared.

Electron Microscopy

NDI1- and *NDE1*-overexpressing cells were grown at 30°C in SD-URA to an OD_{600} of 1.0, at which point aliquots were fixed by adding an equal volume of 6% paraformaldehyde and 4% glutaraldehyde in 0.2 M potassium phosphate buffer, pH 6.5, to the growth medium. After 1-h fixation at 20°C, the cells were collected by centrifugation, washed three times in 0.1 M potassium phosphate buffer, pH 6.5, and three times in water, and then treated with 1% $KMnO_4$ for 2 h on ice, followed by three rinses with water. The samples were subsequently dehydrated and then embedded in Spurr's low-viscosity media (EM Scientific, Gibbstown, NJ) as described by the manufacturer. Thin sections (60–80 nm) were cut, stained with lead citrate and uranyl acetate, and examined under a Philips CM10 electron microscope. Images were captured with a Gatan digital camera, viewed with Digital Micrograph software, and processed with Adobe Photoshop 7.0.

RESULTS

Ndi1p Is the Closest Yeast Homologue of AMID

In mammalian cells, AMID induces a novel caspase-independent apoptotic pathway (Wu *et al.*, 2002). Although Wissing *et al.* (2004) claimed that *S. cerevisiae* AIF (S-AIF) has

Figure 1. Ndi1p is the yeast homologue of AMID. Neighbor-joining phylogeny of different organisms' AIF and AMID homologues was created by ClustalX by using complete alignment. The sequences used in the calculation and abbreviations presented are listed in Table 1. The branch lengths are proportional shows the ideal total amino acids substitution, and the scale bar indicates amino acid substitutions per site.

significant similarity to both AIF and AMID, sequence comparison revealed that Ndi1p of *S. cerevisiae* is the closest homologue of AMID, displaying 28% identity and 46% similarity to human AMID. Most importantly, compared with AIF, both AMID and Ndi1p lack a nuclear localization signal. In the phylogenetic tree, AIF and AMID belong to two distinct subgroups, both of which seem to have diverged ever since their prokaryote ancestor (Figure 1). *NDI1* encodes NADH:ubiquinone oxidoreductase, localized on the mitochondrial inner membrane, and is involved in the transfer of electrons from NADH to ubiquinone in the respiratory chain. *NDE1*, a close homologue of *NDI1*, also encodes NADH:ubiquinone oxidoreductase and is localized on the mitochondrial external membrane. In contrast to the multi-subunit respiratory complex I in higher eukaryotic cells, Ndi1p and Nde1p do not pump protons across mitochondrial inner membrane during respiration.

***NDI1*-overexpressing Yeast Cells Display Growth Defect on SD-URA Medium and the Defect Can Be Attenuated by Increased Respiration**

To determine whether overexpression of *NDI1* or its homologue *NDE1* can induce cell death in yeast, we constructed

two kinds of expression plasmids. The first is based on pYES2, in which the cloned *NDI1* or *NDE1* is driven by *GAL1* promoter, and thus the target gene is induced by galactose and repressed by glucose. The second is a modified form of pYES2, in which the original *GAL1* promoter is replaced by a constitutive *ADH* promoter. When driven by *GAL1*, neither *NDE1* nor *NDI1* displayed growth defect on fermentable or nonfermentable media (our unpublished data). However, when *NDI1* is driven by the *ADH* promoter, the host strain exhibits severe growth defect on SD-URA medium, but not on non- or semifermentable (respiratory) media such as glycerol, ethanol, raffinose, or galactose (Figure 2). As a further confirmation that the growth defect of *NDI1*-overexpressing strain is respiratory dependent, we reduced the concentration of glucose to 0.1% and found this alone can significantly improve yeast growth on SD-URA medium (Figure 2).

Overexpression of NDI1 Accelerates Apoptosis-like Cell Death and Ageing When Respiration Is Repressed

To test whether the defect from overexpression of *NDI1* is apoptotic in nature, subcellular markers of apoptosis were examined. Chromatin condensation/fragmentation is a typical marker of apoptosis. Often, fragments are aligned as a ring close to the nuclear envelope (Clifford *et al.*, 1996). Chromatin condensation indicated by DAPI staining and transmission electron microscopy (TEM) was detectable in ~10% of the *NDI1*-overexpressing cells cultured on SD-URA media (Figures 3 and 4), and DNA fragmentation as revealed by TUNEL staining was observed in ~15% of the cells but almost none in wild-type or *NDE1*-overexpressing cells (Figure 5, A–D). DAPI staining of *NDI1*-overexpressing cells that were cultured in SD-URA media showed that in ~10–20% of cells chromatin fragments were arranged in a half-ring or nuclear fragments were distributed. Nucleus of wild-type strain displayed a single round spot without fragmentation in all cells. Electron microscopic investigation of *NDI1*-overexpressing cells also revealed extensive chromatin condensation and aggregation along the inner part of nuclear envelope, a typical sign of apoptosis (Figure 4). In comparison, nuclei of cells cultured in fermentable media are normal and homogeneous in shape and density in both DAPI staining and under electron microscopic examination (Figures 3 and 4).

In multicellular organisms, autophagolysosomes, which are lysosomes with autophagic bodies in them, often occur when the cells undergo starvation or apoptosis (Clark, 1990). Vacuoles in yeast have a similar function to that of lysosomes in multicellular organisms. Autophagic bodies are often seen in the vacuoles of starved yeast cells (Takeshige *et al.*, 1992). TEM revealed discernible autophagic bodies and

Figure 2. *NDI1*-overexpressing cells display growth defect on SD-URA medium. Yeast cells were spotted on corresponding positions of different plates with indicated carbon sources by serial 10-fold dilutions. The percentages in front of SD-URA indicate the amount of glucose used in the media. *NDE1*, *NDE1*-overexpressing cells; *NDI1*, *NDI1*-overexpressing cells.

Figure 3. *NDI1*-overexpressing cells displayed chromatin fragmentation and cell surface wrinkling when cultured in SD-URA medium. Shown here are DAPI stain (A, C, E, G, I, and K) and their phase contrast representation (B, D, F, H, J, and L) of BY4742, with either empty vector, or *NDE1* or *NDI1*. Cells were cultured in SD-URA or SGR-URA (SGalR-URA) media. Bar, 10 μ m.

some other abnormal structures embedded with intact membrane in the vacuoles of *NDI1*-overexpressing cells cultured in SD-URA medium (Figure 4, C and D). These abnormal appearances were not observed in wild-type cells grown under similar conditions. The presence of autophagic bodies in the vacuoles of *NDI1*-overexpressing cells is therefore another indicator of apoptosis-like cell death.

The simple model system, yeast, also shows similarity to higher cells in cellular, and perhaps organismic, ageing in terms of the following changes: old cells are much bigger than young cells (Mortimer and Johnston, 1959; Egilmez *et*

al., 1990; Nestelbacher *et al.*, 1999); the cell cycle as well as protein synthesis slow down during ageing (Mortimer and Johnston, 1959; Motizuki and Tsurugi, 1992); and the cell surface has a loose and wrinkled appearance (Pichova *et al.*, 1997). Surfaces of *NDI1*-overexpressing cells often display loose and wrinkled appearance, and they are bigger than the other cells under the same conditions. Similarly sized controls such as BY4742 and *NDE1*-overexpressing cells do not have these characteristics (Figure 3).

An early morphological marker of apoptosis is the exposure of phosphatidylserine at the outer leaflet of the cytoplasmic membrane, a feature conserved from yeast to mammalian cells (Martin *et al.*, 1995; Madeo *et al.*, 1997). In yeast, phosphatidylserine can be detected by FITC-labeled Annexin V staining upon cell wall digestion. Concomitantly, cells were checked for membrane integrity by incubation with propidium iodide (Herker *et al.*, 2004). About 5.4% of the *NDI1*-overexpressing cells grown in SD-URA medium were both propidium iodide and Annexin V positive. Some cells (4.7%) were stained with Annexin V but did not take up propidium iodide, indicating an intact membrane (Figure 5E).

A strict correlation between the flow cytometric profile of DNA content and the morphological changes typical of apoptosis has been reported, indicating that FACS may be used to measure apoptosis in yeast (Del Carratore *et al.*, 2002). We therefore examined the profile of DNA content in *NDI1*-overexpressing cells that were cultured in fermentable media. Control cells showed two peaks, corresponding, respectively, to G_1 and G_2/M phases of the cell cycle, whereas very few cells were present in the hypodiploid sub- G_1 phase (not more than 0.1% of the total events acquired) (Figure 5F). For *NDI1*-overexpressing cells cultured in SD-URA medium, the number of the hypodiploid population increased to 12.1%. At the same time, these cells displayed a block in the G_1 phase associated with a nearly complete disappear-

Figure 4. *NDI1*-overexpressing cells showed typical apoptotic chromatin condensation and aggregation. Cells after 24 h of growth in SD-URA (about at the middle of exponential growth phase) were harvested and prepared for electron microscopy. (A) Wild-type control BY4742 with empty vector. (B–D) *NDI1*-overexpressing strain. n, nucleus; v, vacuole; chromatin condensation is marked by arrows. Bar, 1 μ m.

Figure 5. Overexpression of *NDI1* results in DNA breakage, exposure of phosphatidylserine, and disruption of membrane integrity. Cells of BY4742 (A), *NDE1* (B), and *NDI1* overexpression strains (C) grown on SD-URA at the end of log phase (36 h after inoculation) were stained for DNA strand breaks with TUNEL. Bar, 5 μ m. (D) Percentage of TUNEL-positive cells. (E) Exposure of phosphatidylserine and membrane integrity of cells when cultured in SD-URA media. Exposure of phosphatidylserine at the outer leaflet of the cytoplasmic membrane, as detected by FITC-labeled Annexin V staining upon cell wall digestion, and membrane integrity, as indicated by propidium iodide staining, were analyzed by flow cytometry. (F) Distribution of DNA content of the indicated strains when cultured on SD-URA media. DNA was stained with propidium iodide and analyzed by flow cytometry.

ance of the G_2 phase. This result is another indication of apoptosis, as reported by Del Carratore *et al.* (2002).

ROS Is Possibly an Important Mediator in *Ndi1p*-induced Apoptosis-like Cell Death

Whether ROS is a regulator of all apoptosis in yeast is controversial (Madeo *et al.*, 1999; Wysocki and Kron, 2004). To determine whether ROS plays an important role in *NDI1*-induced apoptosis, we first measured the ROS production of yeast cells cultured in nonfermentable or fermentable media by flow cytometry. Dihydrorhodamine 123, which can be oxidized by ROS to become fluorescent chromophore rhodamine 123, was mixed with yeast cells. Flow cytometric analysis showed that overexpression of either *NDI1* or *NDE1* caused significant ROS production as revealed by fluorescent rhodamine 123, and the fluorescence intensity in *NDE1*-overexpressing cells was higher than that in *NDI1*-overexpressing cells (Figure 6A). This measured fluorescence difference between *Nde1p*- and *Ndi1p*-overexpressing strains may be because ROS produced by overexpression of *NDI1* is localized mostly in mitochondria, where most dihydrorhodamine 123 is possibly less accessible, whereas ROS produced by overexpression of *NDE1* is

likely more in the cytosol. We further tested the role of ROS scavenging systems such as Sod1p, which is located in both the cytosol and the mitochondrial intermembrane space, and Sod2p, which is located within the mitochondrial matrix (O'Brien *et al.*, 2004) in this process. We reasoned that if ROS plays a key role in the *NDI1*-induced apoptotic-like cell death, then the deletion of any one of those two genes may enhance the effect of *Ndi1p*. When we introduced *NDI1*-overexpressing plasmid into Δ *sod1* cells, transformants can only grow on semi-fermentable media, but not on glucose-rich media. When we used Δ *sod2* as host, despite repeated efforts we could not get any *NDI1*-overexpressing transformants on either glucose-rich or semi-fermentable media. To explicitly prove that Sod2p is required for the survival of *NDI1*-overexpressing yeast, we co-transformed *NDI1*-overexpressing plasmid and a control plasmid (with a different nutrition-selective marker). Whereas both plasmids were able to transform normal 4742 strain, as indicated by the appearance of colonies in both selective semi-fermentable media, only the control plasmid was able to transform into Δ *sod2* strain. The vital roles of ROS scavenging systems, especially Sod2p, in antagonizing the effect of *Ndi1p* indicate that ROS is an important factor in mediating *Ndi1p*-induced apoptosis.

Figure 6. Overexpression of *NDI1* induces both ROS generation and $\Delta\psi_m$ loss. (A) Indicated strains cultured on SD-URA or SG-URA media were analyzed for accumulation of ROS by DHR staining with flow cytometry. (B) Mitochondrial membrane potential of indicated strains cultured on SD-URA or SG-URA media were analyzed with flow cytometry. (C) Some mutants of mitochondrial electron transport chain can alleviate *NDI1*-induced toxicity. Shown here is Δcox12 yeast harboring *NDI* expression plasmid on SD-URA, with Δndi1 strain carrying *NDI* expression plasmid as the control.

Mitochondrial electron transport chain is a major ROS-generating site. We thus explored the involvement of the electron transport chain in *Ndi1p*-induced toxicity by examining corresponding gene's role in alleviating or aggravating the *Ndi1p*-induced growth inhibition. Among ~20 mutants studied that affect various parts of the electron transport chain, most do not seem to influence the *Ndi1p*-induced toxicity in either way. However, several of them, including *cox12*, *qcr8*, *cox8*, and *cor1*, significantly or partially relieve yeast cells from the *Ndi1p*-induced toxicity (Figure 6C). This suggests that mitochondrial electron transport system is intimately involved in the *Ndi1p*-induced toxicity, although it is still unclear why some of the mutants have obvious effect, whereas others do not.

Mitochondrial membrane permeabilization is a rate-limiting step in the sequence of biochemical events leading to cellular demise. By analogy to cell cycle control, $\Delta\psi_m$ loss can be considered as (one of) the checkpoint(s) regulating cell death (Kroemer, 2002). We thus tested whether *NDI1*-induced apoptosis is also accompanied with $\Delta\psi_m$ disruption. The results unambiguously showed that some *NDI1*-overexpressing cells lost $\Delta\psi_m$ when cultured in SD-URA

media, in contrast to very little $\Delta\psi_m$ disruption in *NDE1*- or *NDI1*-overexpressing cells cultured in nonfermentable media or in wild-type cells (Figure 6B).

ndi1 and *nde1* Cells Manifest Elongated Chronological Life Span Accompanied with Fitness Loss

Many ageing theories are based on the hypothesis that ageing is caused by oxidative damage as well as other macromolecular modifications that lead to the accumulation of random intracellular molecular defects (Kirkwood, 1977; Kirkwood and Rose, 1991; Warner, 1994; Holliday, 1995). Recent studies have supported this idea by demonstrating a correlation between increased superoxide dismutase activity; decreased oxidative damage; and increased life span in fruit flies, nematodes, and yeast (Larsen, 1993; Agarwal and Sohal, 1994; Sohal *et al.*, 1995). It has been shown that external NADH dehydrogenases *NDE1* and *NDE2* are involved in the generation of intracellular oxidative stress. Deletion of these two genes can decrease the levels of H_2O_2 production of mitochondria and nuclear mutation frequency (Davidson and Schiestl, 2001). We therefore examined whether the deletion of *NDI1* or *NDE1* can elongate the chronological life

Figure 7. *nde1* Δ and *ndi1* Δ cells display elongated chronological life span accompanied with reduced fitness. (A) Chronological aging of wild-type and $\Delta nde1$ and $\Delta ndi1$ strains. Data represent mean $\pm$ SEM. (B) Growth defect of $\Delta ndi1$ cells when cultured on nonfermentable media. Same amount of indicated yeast cells were spotted on YPD or YPG media by serial 10-fold dilutions. (C) Competition assay between wild-type and $\Delta nde1$ strain. Equal amounts of cells of wild-type and $\Delta nde1$ strains at exponentially growing phase were mixed. After subculturing four times in YPD or YPG media, the percentage of living cells of both strains was monitored. Data represent mean $\pm$ SEM.

span of yeast. As we can see from Figure 7A, at the first 24 d there was no significant difference of mortality among wild type, $\Delta ndi1$, and $\Delta nde1$ strains, but after this time point, $\Delta ndi1$ and $\Delta nde1$ displayed a substantially higher survival rate than the control. This indicates that deletion of *NDE1* or *NDI1* can significantly elongate the chronological life span of yeast cells.

We next analyzed survival fitness of $\Delta ndi1$ and $\Delta nde1$ cells. When $\Delta ndi1$ yeast were grown on nonfermentable media such as YPG, they manifested moderate growth defect compared with wild type, whereas no significant difference of growth was observed when they were grown on fermentable media such as YPD (Figure 7B). However, $\Delta nde1$ displayed no significant difference of growth rate with wild-type strains when cultured on either fermentable or nonfermentable media. To find out whether wild-type or $\Delta nde1$ cells have competitive growth advantage when growing together, we mixed the same amount of cells of both strains during exponential growth, followed by a few passages of subculturing in liquid YPD or YPG media. To discriminate between these two strains, aliquots were plated on YPD and YPD-geneticin plates ($\Delta nde1$ is geneticin resistant and wild type is not). When cultured in YPG media, after subculturing four times, $\Delta nde1$ completely disappeared and only wild-type cells remained (Figure 7C). Unexpectedly, when cultured in liquid YPD medium, the wild-type cells also prevailed over $\Delta nde1$. Hence, $\Delta nde1$ cells are not able to survive efficiently when they have to compete side by side against wild-type cells when cultured in either nonfermentable or fermentable media.

DISCUSSION

Database search and phylogenetic analysis indicated that AMID possesses homologous counterparts in eubacteria but not in archaeobacteria, in contrast to AIF, which possesses significantly homologous genes in both eubacteria and archaeobacteria. In the phylogenetic tree, AMID and AIF belong to two distinct subgroups, suggesting distant evolutionary relationship. Besides its oxidoreductase function, there has not been any previous report about the apoptotic activity of yeast AMID homologue Ndi1p. We now show that Ndi1p exerts an apoptotic function, based on analysis of a number of major landmarks of apoptosis, including DNA fragmentation, apoptosis-typical chromatin condensation, mitochond-

rial membrane permeabilization, exposed phosphatidylserine, and increased ROS production. Furthermore, we showed that the surface of some *NDI1*-overexpressing cells displayed a loose and wrinkled appearance, one of the characteristics of ageing cells (Pichova *et al.*, 1997). Because there is good evidence that normal ageing of yeast is also a process of apoptosis (Laun *et al.*, 2001; Jazwinski, 2003; Herker *et al.*, 2004), the apoptotic phenotype of *NDI1*-overexpressing cells may be because of the accelerated ageing process.

NDI1-overexpressing yeast cells manifest severe growth defect when cultured on SD-URA medium, and this growth defect is dependent on glucose supply. In glucose-rich media, *S. cerevisiae* preferentially uses glucose through fermentation and turns down a large number of genes involved primarily in respiration or the use of other carbon sources (Ronne, 1995). This is thought to be an energy-saving response, a phenomenon named as glucose repression (Schuller, 2003). When glucose becomes limiting, energy generation of cells becomes more dependent on respiration, and the glucose-repressed genes are induced. *NDI1* and *NDE1* are two of the genes that are repressed when cultured on glucose-rich media (Giaever *et al.*, 2002). Overexpression of *NDI1* in glucose-rich media accelerates apoptosis and ageing. In agreement with this result, there was another report that overexpression of a glucose-repressed gene, *SNF1*, can cause a decreased life span in yeast, possibly through phosphorylation of one or more cellular proteins or enzymes that normally affect ageing (Ashrafi *et al.*, 2000; Bakker *et al.*, 2000). Snf1p is required for induction of glucose-repressed genes in response to glucose starvation, and both *NDI1* and *NDE1* are possible targets of *SNF1* (Ashrafi *et al.*, 2000).

The percentage of apoptotic cells was 10–15% in both our TUNEL and PS assays. Is this an underrepresentation of apoptotic death, or are there other cell fates in Ndi1p-expressing cells? Although we cannot exclude certain levels of underdetection, we think a significant portion of cells did not undergo apoptosis; instead, their growth was just inhibited. Leading to this hypothesis was the observation that in glucose-rich media, many Ndi1p-overexpressing yeast cells still grew, albeit at much slower pace and at reduced numbers. When these cells were transferred to semi-fermentable media later, most of them grew up to colonies. As glucose level was reduced and respiration increased, this growth inhibition could be further relieved. Thus, overexpression of

Ndi1p in normal strain only leads to modest apoptosis under our glucose-rich media. The apoptotic rate might be much higher when *SOD2* is impaired, as reflected by the observation that $\Delta sod2$ cells with Ndi1p overexpression could not be obtained in either glucose-rich or semifermentable media.

Both *NDE1* and *NDI1* encode NADH dehydrogenases. Why does overexpression of *NDI1* accelerates apoptosis and ageing, but not *NDE1*? The difference in the two gene products is that Ndi1p catalyzes the intramitochondrial NADH (Bakker *et al.*, 2000), whereas Nde1p oxidizes NADH at the cytosolic side of the inner membrane (Luttik *et al.*, 1998). Because overexpression of either of these products increases ROS production, one explanation for this observed difference is that ROS produced in *NDE1*-overexpressing cells is possibly more in the cytosol and ROS produced from *NDI1*-overexpressing cells is more localized in mitochondria. It is possible that intramitochondrial ROS is a more potent apoptosis inducer. In addition, cytosolic ROS may well be scavenged by Sod1p, but intramitochondrial ROS may not be so well by Sod2p, which is expressed at low levels (5- to 10-fold less) during growth in glucose medium (Maris *et al.*, 2001). Therefore, when cultured in a glucose-rich medium such as normal SD-URA, ROS produced by *NDI1* overexpression cannot be detoxified efficiently, which in turn damages mitochondria (Figure 8). Alternatively, it is possible that the ROS detection method used is more effective in detecting cytosolic ROS instead of mitochondrial ROS, thus the ROS generated by Ndi1p is artificially underestimated compared with that of Nde1p. Another intriguing observation is that overall ROS produced for Ndi1p cells is similar in glucose-rich and semifermentable media, but apoptosis is much less obvious in the latter medium. We consider that semifermentable medium mitochondria may have better tolerance of ROS because of increased *SOD2* expression. When *SOD2* is deleted, apoptosis could happen even in semifermentable medium and in a much more aggravated manner, as evidenced by our inability to obtain corresponding yeast colonies. Thus, ROS may not be the sole factor in determining apoptosis. The antioxidant system that copes with ROS is equally important.

There seems to be much more to be understood concerning how ROS generation and electron transport are related, and the ROS and mitochondria story could be more complicated than it first seemed. Among the 20 mutants that are defective in various parts of the electron transport system, several affect (reduce) the Ndi1p-induced toxicity. One apparent explanation is that some mutants can and others cannot reduce the ROS production in the presence of excessive Ndi1p activity. Another puzzling observation is that Ndi1p overexpression dramatically inhibits the growth of petite cells, obtained by ethidium bromide treatment. Petite cells have seriously defective mitochondria and presumably lack electron transport chains. The inhibitory effect happens in both glucose-rich and semifermentable media (our unpublished data). We think that although petite is normally expected to generate less ROS, it is possible that when Ndi1p, the first component of the electron transport chain, is strongly expressed there might still be significant production of ROS. In addition, Sod2p activity might also be affected in petite background. Combination of the above-mentioned two factors could result in more severe apoptosis in petite cells. It would be interesting to investigate when Ndi1p is overexpressed how petite cells, which have seriously malfunctioned mitochondria, undergo apoptosis (if they really do, instead of just senescence).

Figure 8. A model for how Ndi1p induces yeast apoptosis. Ndi1p overexpression induces mitochondrial ROS production, whereas Nde1p induces mainly cytosol ROS production. Reduced level of Sod2p such as in glucose medium or *sod2* mutant cells leads to ROS-induced yeast apoptosis. See text for details.

It has been shown that the oxidoreductase activity of AIF can be dissociated from its apoptotic function (Miramar *et al.*, 2001). In this regard, the apoptotic effect of *NDI1* overexpression could be independent of its NADH dehydrogenase activity. We have not yet obtained direct evidence in support of this possibility. However, because some mutants that are defective in electron transport chain can suppress the toxicity of Ndi1p-induced toxicity, it seems more likely that the Ndi1p-associated apoptosis is linked to its NADH dehydrogenase activity, an important electron transport component. If this is indeed the case, Ndi1p-associated apoptosis could arise from altered electron transport system because of increased expression of NADH dehydrogenase activity (Ndi1p), which in turn leads to the elevated ROS production. We also tried to investigate whether Ndi1p migrate to nucleus, because AIF (possibly its yeast homologue YNR074C, too) does during apoptosis (Susin *et al.*, 1999; Wissing *et al.*, 2004). Unfortunately, when we used Myc to tag Ndi1p, Ndi1p-Myc significantly lost its apoptotic activity, precluding our further cellular localization analysis.

The question as to whether mitochondria are necessary for all yeast apoptosis is still debated, with some authors suggesting an important role for mitochondria, whereas others report that this process does not necessarily involve mitochondria (Gross *et al.*, 2000; Kissova *et al.*, 2000; Mazzoni *et al.*, 2003). Although we cannot prove that mitochondria are required for apoptosis in other systems, our results are consistent with the argument of mitochondrial involvement because Ndi1p itself is an integral part of mitochondria and the observation that some components of the mitochondrial electron transport chain closely relate to Ndi1p-associated toxicity. In addition, mitochondrial ROS is possibly an important mediator in this apoptotic process. Finally, our results together with those of Ashrafi *et al.* (2000) suggest there was a retrograde regulatory mechanism or glucose repression mechanism between fermentation and mitochondrial respiration during the process of Ndi1p-induced apoptosis.

Why is the apoptotic activity of Ndi1p dependent on the cross-talk between mitochondria and the rest of the cell? We have no clear answer, but we may better understand the process from an evolutionary perspective. Mitochondria are thought to have originated from proteobacteria that were taken up by a nucleated cell. Thus, besides the observed harmony between cell and mitochondria, there might be residual signs of a competition and even conflict between them under certain, special conditions. It is possible that yeast represent an early form of eukaryotic cells in which coordination between mitochondria and the rest of the

cell is still not as smooth as that in some multicellular organisms, evidenced by the fact that yeast without mitochondria can still survive, whereas multicellular organisms cannot. When primitive eukaryotic cells grew on nutrient-rich environment with enough glucose and their own metabolism was low, proteobacteria might have sensed that they could survive better independently without the protection of the host. Therefore, an apoptotic program based on a primitive AMID (Ndi1p) form was developed. The progressive transfer to the nucleus of the essential mitochondrial genes involved in the regulation of programmed cell death may have represented an important step in the emergence of a regulated participation of the mitochondria in self-destruction. The host developed a system to downregulate the expression of the primitive AMID (Ndi1p) when glucose is abundant. The development of these two programs thus reflects an evolutionary arms race between the proteobacteria and the protoeukaryotic cell, leading to a resolution of these conflicts and to enforced cooperation through a process of reciprocal addiction (Ameisen, 1996, 2002). From this perspective, our observed Ndi1p-induced apoptosis might be a revolutionary remnant during the evolution of AMID-induced apoptosis.

It has been reported that disruption of yeast AIF1 (YNR074C) significantly delayed the onset of age-induced cell death, suggesting a proapoptotic role of yeast Aif1p (Wissing *et al.*, 2004). This is consistent with our results in that disruption *NDI1* or *NDE1* can significantly elongate the chronological life span of yeast. In mammals, caloric restriction continues to be the only experimental manipulation that increases maximum longevity (Santos-Pinto *et al.*, 2001). It decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA (Gredilla *et al.*, 2001). In yeast, it has been shown that deletion of *NDE1* decreases H₂O₂ production of mitochondria (Davidson and Schiestl, 2001). So, the chronological life span elongation of $\Delta ndi1$ and $\Delta nde1$ strains also may be because of the decreased ROS production of mitochondria. This suggests that decreasing ROS generation through a decline in the activity of complex I or NADH dehydrogenase is likely a highly conserved mechanism during the evolution of longevity.

A paradox between our results and that reported by Lin *et al.* (2004) is that they showed that overexpression of *NDE1* and *NDE2* can significantly increase the life span of yeast on 2% glucose, but not on 0.5% glucose. This may be because of what they determined is the replicative life span, but in our system, chronological life span. Indeed, no correlation between chronological and replicative life span was apparent, and the deletion of genes reducing replicative longevity does not affect chronological longevity in the same strain, implying that the genetic control of both forms of ageing may be unrelated (Maskell *et al.*, 2003). Chronological life span might be expected to relate to stress resistance and be regarded as a model for investigating the factors that influence ageing of postmitotic tissues in higher organisms (Longo *et al.*, 1996; Fabrizio *et al.*, 2001; Maclean *et al.*, 2001). In our experiments, there was clearly fitness loss accompanied with the elongation of chronological life span of the strains. The key difference between our ageing experiment and those of others such as Wissing *et al.* (2004) and Herker *et al.* (2004) is what they compared the disadvantage during ageing, whereas we investigated the disadvantage of competing growth or surviving fitness. An important theory about why we age is the "disposable soma theory," which is based on optimal allocation of metabolic resources between somatic maintenance and reproduction (Kirkwood, 1977; Kirkwood and Austad, 2000). As for *S. cerevisiae*, the replicative life span may stand for reproduction investments, and

the chronological life span may stand for investments in soma maintenance. *NDI1* may be one of the key elements that regulates the equilibrium between somatic maintenance and reproduction and be used as a balancer to control the life span and fitness to optimally adapt to various environments.

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