Increased Resistance of Complex I Mutants to Phytosphingosine-induced Programmed Cell Death

Ana Castro§†, Catarina Lemos§†, Artur Falcão§†, N. Louise Glass§, and Arnaldo Videira§††
From the †IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal, the ‡UFPP - Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Rua Carlos da Maia 296, 4200-150 Porto, Portugal, the §Department of Plant and Microbial Biology, University of California, Berkeley, California 94720-3102, and the ¶ICBAS - Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Largo Prof. Abel Salazar 2, 4099-003 Porto, Portugal

We have studied the effects of phytosphingosine (PHS) on cells of the filamentous fungus Neurospora crassa. Highly reduced viability, impairment of asexual spore germination, DNA condensation and fragmentation, and production of reactive oxygen species were observed in conidia treated with the drug, suggesting that PHS induces an apoptosis-like death in this fungus. Interestingly, we found that complex I mutants are more resistant to PHS treatment than the wild type strain. This effect appears to be specific because it was not observed in mutants defective in other components of the mitochondrial respiratory chain, pointing to a particular involvement of complex I in cell death. The response of the mutant strains to PHS correlated with their response to hydrogen peroxide. The fact that complex I mutants generate fewer reactive oxygen species than the wild type strain when exposed to PHS likely explains the PHS-resistant phenotype. As compared with the wild type strain, we also found that a strain containing a deletion in the gene encoding an AIF (apoptosis-inducing factor)-like protein is more resistant to PHS and H₂O₂. In contrast, a strain containing a deletion in a gene encoding an AMID (AIF-homologous mitochondrial-associated inducer of death)-like polypeptide is more sensitive to both drugs. These results indicate that N. crassa has the potential to be a model organism to investigate the molecular basis of programmed cell death in eukaryotic species.

The term apoptosis (apo-from, ptosis-falling) means “dropping off” of petals or leaves from plants or trees and was described as a particular mode of cell death that is characterized by rounding up of the cell, reduction of cell volume, condensation of chromatin, fragmentation of the nucleus, and maintenance of the intact plasma membrane until the very late stage of the death process (1). Apoptosis is a form of programmed cell death (PCD) that plays a central role in tissue homeostasis and maintenance in multicellular organisms. Cytological phenotypes associated with apoptosis include chromatin condensation, DNA fragmentation, phosphatidylserine externalization, vacuolization, activation of caspases, and increased production of reactive oxygen species (ROS). Although the importance of apoptosis in stroke, neurodegenerative disorders, and cancer is increasingly evident, many details of its regulation and production of apoptotic phenotypes are poorly understood (for recent reviews, see Refs. 2–4).

In addition to the well established role of mitochondria in ATP production, regulation of cell death has emerged as a second major function of these organelles. This function appears to be related to the role of mitochondria as the major intracellular source of ROS, mainly generated at complexes I and III of the respiratory chain (5). Enhanced ROS production has been associated with mtDNA mutations, aging, and cell death (6) and also with a wide variety of pathologies, including cancer, atherosclerosis, and neurodegenerative diseases (7). ROS overproduction induces an osmotic imbalance across the inner mitochondrial membrane. This leads to the swelling of the mitochondrial matrix and, consequently, the disruption of the outer mitochondrial membrane and release of proteins from the intermembrane space into the cytosol (2). Some of these released proteins, including cytochrome c, Smac/Diablo, and apoptosis-inducing factor (AIF), are proapoptotic regulators that subsequently activate cellular apoptotic programs (8, 9). The mitochondrial or intrinsic pathway is a major apoptotic pathway, where the release of cytochrome c from the mitochondrion and the production of ROS are the two main events proposed as integral control elements in the cell decision to die. In the second major pathway, the extrinsic pathway, caspases are activated directly (10).

Programmed cell death and apoptotic mechanisms are ubiquitous in both prokaryotic and eukaryotic species (11). The complex regulatory network and the sometimes contradictory results obtained with human cell lines make it desirable to investigate cell death mechanisms in simpler model systems.

§This work was supported, in whole or in part, by National Institutes of Health Grant RO1 GM60468 (to N. L. G.). This work was also supported by research grants from Fundação para a Ciência e a Tecnologia from Portugal and the Programa Operacional Ciência, Tecnologia, Inovação program of QCA III (co-participated by Fundo Europeu de Desenvolvimento Regional) and by a sabbatical fellowship from Fundação Luso-Americana. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
†The on-line version of this article (available at http://www.jbc.org) contains two supplemental figures.
‡To whom correspondence should be addressed. Tel.: 35-1-226074900; Fax: 35-1-226099157; E-mail: avideira@ibmc.up.pt.

1 The abbreviations used are: PCD, programmed cell death; PHS, phytosphingosine; ROS, reactive oxygen species; AIF, apoptosis-inducing factor; AMID, AIF-homologous mitochondrial-associated inducer of death; GFP, glucose, fructose, sorbose; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.

2 The on-line version of this article (available at http://www.jbc.org) contains two supplemental figures.

3 To whom correspondence should be addressed. Tel.: 35-1-226074900; Fax: 35-1-226099157; E-mail: avideira@ibmc.up.pt.
For example, apoptotic-like programmed cell death has been observed in the unicellular yeast, *Saccharomyces cerevisiae* (12, 13), and in a number of filamentous fungi, including *Aspergillus spp.*, *Podospora anserina*, and *Candida albicans*, following exposure to some environmental stimuli, such as acetic acid, hydrogen peroxide, sphingoid bases, viral killer toxins, and UV radiation (14–16). The fungal genomes contain a subset of metazoan apoptotic genes, although the genomes of filamentous fungi contain homologues of PCD genes of metazoans that are not present in *S. cerevisiae* (17), thus making them attractive models for dissecting mechanisms of programmed cell death.

The filamentous fungus *Neurospora crassa* is a morphologically complex multicellular organism with many differentiated cell types (18). In addition, it is well studied, its genome was sequenced, and many molecular tools have been developed over the years to work with the organism (19–21). In addition to PCD induced by external factors, *N. crassa* and other filamentous fungi utilize a cell death pathway for non-self recognition that occurs when two genetically different colonies come in contact (22). Thus, it has the potential to be very useful for research on the mechanisms and evolution of programmed cell death in eukaryotic species.

The fact that phytosphingosine (PHS) induces apoptosis in the filamentous fungus *Aspergillus nidulans* (23) prompted us to initiate the characterization of the sphingolipid-induced PCD in wild type and respiratory mutants of *N. crassa*. In this work, we describe the programmed cell death phenotype of the fungal cells exposed to PHS and the finding that respiratory chain complex I is involved in the process.

**MATERIALS AND METHODS**

*Strains and Growth Techniques*—The *N. crassa* wild type strain 74-OR23-1A (74A) and mutants in the respiratory chain complex I genes *nuo9.8, nuo14, nuo21, nuo21.3c, nuo30.4, nuo51*, and *nuo78* have been described previously (reviewed in Ref. 24). Deficient mutants in the mitochondrial complexes III (fes-1; FGSC11184), IV (cyt-12; FGSC4506), and V (oli; FGSC8738) and in cytochrome c (cyc-1; FGSC3558), as well as the AIF (*ΔNClu08580, here called aif-1; FGSC11900*) and AIF-homologous mitochondrion-associated inducer of death (AMID) (*ΔNcu06061; FGSC12090*) deletion strains (20), were obtained from the Fungal Genetics Stock Center (FGSC) (25). General procedures for growth and handling of the fungal strains have been published (26). When indicated, PHS from Avanti Polar Lipids (Alabaster, AL) was added to the media from a stock solution of 4 mg/ml in ethanol. The viability of conidia was determined by plating the cells on GFS agar medium, which induces colonial growth, and counting colony-forming units after incubation at 26 °C. Conidial germination in Vogel’s minimal medium (27) at 26 °C was evaluated by observation under a Nikon optical microscope. These types of experiments, as well as the following methods, were repeated at least three times.

*TUNEL Assay*—DNA strand breaks were analyzed by terminal deoxynucleotidyl transferase biotin–DUTP nick end labeling (TUNEL) using the *in situ* cell death detection kit fluorescein (Roche Applied Science), as described previously (28). *N. crassa* conidia were preincubated in minimal medium for 30 min, washed in water, and then treated with phytosphingosine for 60 min and washed again. Spheroplasts were prepared (29), and fixation was performed 5.5 h after treatment with the drug. Images were collected in a Zeiss Axiovert 200 M microscope using an AxioCam (Carl Zeiss, Germany). Flow cytometry analysis was performed in a FACSCalibur (BD Biosciences). Twenty-thousand cells per sample were analyzed. Data were acquired and analyzed with CELLQuest PRO 3.3 (BD Biosciences).

*Spot Assays*—Conidia from different strains were harvested with distilled water, and the cell suspensions were adjusted to a concentration of 6 × 10⁷ cells/ml. Three-fold serial dilutions of each strain were spotted on plates containing GFS medium containing various concentrations of the appropriate drugs. Images were taken by scanning the plates after about 3 days incubation at 26 °C.

*ROS Detection*—ROS production by mitochondria was monitored by using dihydorhodamine 123 from Sigma. The reduced form of dihydrorhodamine 123 does not fluoresce until encountering an actively respiring cell, where it is oxidized by ROS to a green fluorescent compound that is sequestered in the mitochondria (30). Conidial cells were harvested and suspended in minimal medium at a concentration of 5 × 10⁶ cells/ml. Then, 50 μg/ml dihydorhodamine 123 was added, and the cell suspensions were incubated at 26 °C during 30 min and washed before the addition of PHS. ROS production was evaluated using a FACSCalibur cytometer (BD Biosciences), and data were analyzed by CELLQuest version 3.3 (BD Biosciences) with excitation at 480 nm and emission at 530 nm.

**RESULTS**

*PHS Induces an Apoptosis-like Cell Death in N. crassa*—The sphingolipid PHS has potent antifungal activity toward *A. nidulans*, inducing a caspase-independent apoptosis in this organism (23). In test effects in *N. crassa*, we evaluated the viability of asexual spores (conidia) upon exposure to PHS. Conidial cells were incubated in the presence of 10 μg/ml PHS, samples were withdrawn at different times and plated in GFS medium (which induces colonial growth of *N. crassa*), and the resulting colonies were counted. As shown in Fig. 1A, exposure of *N. crassa* wild type conidia to PHS resulted in cell death. The kinetics of cell viability, estimated by colony-forming unit counts, showed a dramatic decrease in the cell viability (around 60%) after a short period of incubation with the drug (5 min). Cell viability continued to decrease, reaching about 15% cell viability after a 120-min incubation with PHS. This effect was not seen in the control experiment where cell viability was nearly 100% and indicates that PHS possesses a potent activity against *N. crassa*.

We also evaluated whether exposure to PHS impaired the germination of *N. crassa* conidia. Untreated wild type conidia progressively undergo germination when incubated in liquid medium; the majority of cells initiate germination within a couple of hours. In contrast, germination was inhibited in cells treated with PHS with less than 50% of the conidia initiating germination after a 4-h exposure (Fig. 1B).

We observed that *N. crassa* conidia exposed to PHS underwent nuclear condensation (data not shown). Nuclear conden-
sation is a hallmark of apoptosis, as is chromatin fragmentation. To determine whether chromatin fragmentation was also associated with cell death caused by PHS in *N. crassa*, we employed the TUNEL assay. TUNEL detects DNA fragmentation and has been used as a discriminating technique to detect programmed cell death at a single cell level (16). *N. crassa* wild type conidia were treated with PHS, and the TUNEL staining was subsequently performed. The results were visualized by fluorescence microscopy (Fig. 2A) and analyzed by flow cytometry (Fig. 2B). Control conidial cells, either treated with PHS but unlabeled or labeled but untreated, were TUNEL-negative. TUNEL-positive cells, indicative of DNA fragmentation, were only found in the positive control and among cells treated with PHS (Fig. 2A). Fluorescence-activated cell sorter analysis was performed for the quantification of TUNEL-positive conidia from PHS-treated cells by comparing with the labeled but untreated negative control cells. Under the conditions tested, about 12% of the cells exposed to PHS revealed DNA fragmentation (Fig. 2B). Taken together, our results indicate that PHS induces an apoptosis-like cell death in *N. crassa*, similarly to what has been observed in *A. nidulans*.

**FIGURE 1.** Effects of phytosphingosine on the survival (A) and germination (B) of *N. crassa* conidia. A, time course survival. Conidia were incubated in the presence (circles) or absence (squares) of 10 μg/ml PHS during the indicated time periods and plated in GFS medium. The number of colony-forming units was determined relative to time 0. B, conidial germination. After a preincubation for 30 min in minimal medium, conidia were incubated in the presence (light gray) or absence (dark gray) of 10 μg/ml PHS. Samples were taken at the indicated time points to calculate the percentage of germinated cells. The vertical bars represent standard deviations.

**FIGURE 2.** TUNEL assays. A, typical images of TUNEL staining of *N. crassa* conidia. Cells were preincubated in minimal medium for 30 min and then treated with 20 μg/ml PHS in H₂O for 60 min. Fixation was performed 2.5 h thereafter. Unlabeled cells treated with PHS (upper left), labeled untreated cells (upper right), a positive control of DNase-treated cells (lower left), and cells exposed to 20 μg/ml PHS for 60 min (lower right) are shown. Control cells either unlabeled or untreated showed a negative TUNEL staining. DNA fragmentation was only detected in the positive control and in cells that were treated with PHS. B, quantification by fluorescence-activated cell sorter analysis of TUNEL-positive cells from labeled untreated cells (upper panel) and labeled cells treated with PHS (lower panel). FITC, fluorescein isothiocyanate.
Complex I Mutants Are More Resistant to PHS than the Wild Type Strain—Mitochondria are an essential mediator of apoptosis. To evaluate whether mitochondria are also involved in PHS-induced death as suggested (23), we analyzed the behavior of mitochondrial mutants of *N. crassa* when exposed to the sphingolipid. We started with our collection of complex I mutants by testing the viability of conidial cells from *nuo9*, *nuo14*, *nuo21*, *nuo21.3c*, *nuo30.4*, *nuo51*, and *nuo78* mutant strains following PHS treatment. Interestingly, we found that all complex I mutants are more resistant to PHS than the wild type strain. Fig. 3 shows the results obtained with the *nuo51* and *nuo21* mutant strains. It can be seen that the *nuo51* mutant is clearly more resistant to PHS. Roughly, more than half of the *nuo51* cells remain viable after a 90-min exposure to PHS, whereas the viability of wild type cells dropped below 20% under the same conditions. The *nuo51* mutant (which lacks the 51-kDa subunit) is still capable of assembling complex I. However, this complex I is non-functional in electron transfer because the 51-kDa protein harbors the FMN prosthetic group, which is the entry point of electrons into the respiratory chain (31). The resistance of the *nuo21* mutant toward PHS lies between that of the *nuo51* mutant and a wild type strain. This observation is in agreement with the fact that strains lacking the 21-kDa subunit assemble a functional complex but show lower amounts of complex I enzyme than wild type (32). The complex I mutants were also more resistant than the wild type strain to inhibition of conidial germination by PHS (data not shown).

To investigate whether the resistance to PHS is specific for complex I mutants, we tested the response to this sphingolipid in mutants that are defective in other components of the mitochondrial respiratory chain. In these experiments, we employed strain FGSC3558, which has a mutation in the cytochrome c structural gene, strain FGSC4506, which has a different mutation in cytochrome c but is also defective in complex IV, strain FGSC11184, which contains a deletion of the Rieske iron-sulfur subunit of complex III, strain FGSC8738, which contains a mutation in the dicyclohexylcarbodiimide-binding subunit of complex V and is defective in energy transduction (33), plus the previous series of complex I mutants. Serial dilutions of conidial suspensions from each of these strains were spotted on media containing PHS, incubated at 26 °C, and the growth of the strains was qualitatively analyzed. Typical results are shown in Fig. 4. All strains grew similarly under control conditions (minimal medium) with the exception of the complex III defective mutant FGSC11184, which displayed reduced growth under all conditions. When PHS was included in the medium, the complex I mutants were more resistant to the drug than the wild type strain, as expected. In contrast, all of the other respiratory complex mutants were more sensitive to PHS than the wild type strain. These results point to a specific involvement of complex I in PHS-induced cell death.

In addition to PHS, a number of other environmental stimuli induce apoptotic-like death in both filamentous fungi and unicellular yeasts. In particular, exposure to hydrogen peroxide has been reported to induce apoptotic-like death in *Aspergillus fumigatus*, *Colletotrichum trifolii*, *C. albicans*, and *S. cerevisiae* (34–36). We therefore evaluated whether mutations in the different respiratory complexes affected sensitivity of these mutants to hydrogen peroxide. We observed that complex I mutants showed a similar resistance to *H*₂⁰₂ exposure as they showed upon exposure to PHS (Fig. 4, see also below). However, strains containing mutations in other respiratory complexes (FGSC3558, cytochrome c mutant; FGSC4506, defective in complex IV; FGSC11184, defective in complex III; FGSC8738, defective in complex V) showed a similar sensitivity to PHS and *H*₂⁰₂ (Fig. 4). These data suggest that the response of *N. crassa* to PHS and hydrogen peroxide likely share common mechanisms.

AIF and AMID are flavoproteins/oxidoreductases (similar to com-

![Image](image-url)
complex I) that promote caspase-independent apoptosis (37, 38). In S. cerevisiae, mutations in the AIF homolog (AIF1) rescue cells from ROS stress and delays age-induced apoptosis (39). In N. crassa, there is one AIF-like homologue, NCU05850 (see supplemental Figs. S1 and S2), and two AMID-like predicted genes, NCU06016 and NCU11413, although NCU11413 is divergent. We therefore tested the sensitivity of deletion mutants in the AIF-like (FGSC11900) and an AMID-like (FGSC12090) predicted proteins of N. crassa toward both PHS and H₂O₂, using serial dilutions of conidia as outlined above. We observed that the N. crassa aif1 (FGSC11900) mutant was more resistant to exposure to both PHS and H₂O₂, whereas the mutant containing mutation in the predicted AMID homologue (FGSC12090) was more sensitive than the wild type strain to both drugs (Fig. 4).

Complex I Mutants Generate Fewer ROS—As shown above, complex I mutants of N. crassa are more resistant to oxidative stress caused by hydrogen peroxide than the wild type strain (Fig. 4). We hypothesized that this resistance (and resistance to PHS) is related to the production of reactive oxygen species. ROS are involved in programmed cell death in many species, including filamentous fungi (2, 16). Exposure to hydrogen peroxide may elicit a “vicious cycle” of production of more ROS (40, 41). Thus, complex I mutants could produce more ROS than the wild type strain, resulting in up-regulation of defense mechanisms for scavenging ROS from the cell. In this case, they should be more resistant to exogenously applied drugs that induce ROS, such as PHS and H₂O₂. Alternatively, complex I mutants could be more resistant to exogenously added ROS because they produce fewer endogenous ROS than wild type. To discriminate between these two hypotheses, we measured by flow cytometry the production of ROS upon PHS treatment after an 80-min exposure to PHS. The results show that complex I mutants produced between ¼ and ½ of the ROS produced by the wild type strain under identical conditions. Thus, the inability to generate high levels of ROS upon exposure to PHS likely accounts for the increased resistance of complex I mutants toward both PHS and hydrogen peroxide.

DISCUSSION

In this study, we evaluated whether the filamentous fungus N. crassa is a suitable model organism to investigate molecular mechanisms associated with PCD. Our results indicate that phytosphingosine exhibits a potent antifungal activity against Neurospora, as deduced by the dramatic decrease in viability of fungal cells upon treatment with the sphingolipid. We further observed that PHS induced rapid nuclear condensation, as revealed by 4’,6-diamidino-2-phenylindole dihydrochloride staining of DNA. Conidia with condensed nuclei did not take up propidium iodide (data not shown). This mitosis-independent nuclear condensation and propidium iodide exclusion are characteristic features of apoptosis in other eukaryotic cells (42). In addition, DNA fragmentation and ROS production are also hallmarks of apoptotic cells (16). Based on the TUNEL assay, we detected DNA fragmentation exclusively in N. crassa conidia exposed to PHS. Furthermore, we found that the sphingolipid stimulates the production of ROS by these cells. Altogether, these results indicate that PHS induces an apoptosis-like cell death in N. crassa, as shown previously in A. nidulans (23).

Interestingly, we found that respiratory chain complex I mutants are less prone to die upon exposure to PHS than the wild type strain, clearly implicating mitochondria in the death process. The complex I involvement appears to be specific since of wild type N. crassa versus the complex I mutant nuo14, using dihydrorhodamine 123 as a marker. Conidia from wild type and the nuo14 mutant were exposed to PHS, and the production of ROS was determined at different times (50–200 min) after drug treatment. This time course study showed a dramatic increase in ROS production in wild type cells treated with PHS, with a maximum production of ROS around 80 min (Fig. 5). More than half of the cells (about 60%) exhibited detectable production of ROS at this time point. In sharp contrast, ROS production of the complex I mutant nuo14 exposed to PHS under the same conditions was almost negligible. Only about 15% of the nuo14 cells displayed detectable ROS levels.

We performed similar kinetics of ROS production with other complex I mutants. Fig. 6 shows the results obtained with these strains.
mutants defective in other components of the oxidative phosphorylation system, such as complex III, IV, and V mutants, do not show a similar resistance to PHS (or hydrogen peroxide) as the complex I mutants. Although ATP is required for apoptosis (2, 43), all the oxidative phosphorylation system mutants are expected to be deficient in ATP production. Thus, ATP deficiency alone cannot explain why complex I mutants are more resistant to PHS-induced death. Rather, a more direct involvement of the complex I enzyme is anticipated. The participation of complex I in PCD is predictable from the findings that the NUO14 subunit is homologous to the mammalian cell death regulator GRIM-19 (44). Other work has shown that complex I inhibition induces apoptosis in mammalian cells (45, 46) and that caspase cleavage of the 75-kDa subunit of complex I was claimed to be required for apoptosis (47). Furthermore, it appears that complex I is also involved in virus-induced apoptosis (48, 49) and in the interferon-β and retinoic acid-induced cancer cell death (50). Our results are in line with these findings and point to a central role of complex I in mediating programmed cell death in filamentous fungi. The simplicity of N. crassa as compared with higher eukaryotic systems is attractive in unraveling the role of complex I in programmed cell death and apoptosis.

We further investigated the mechanisms connecting complex I to cell death. A central phenomenon in most instances of apoptosis is the accumulation of ROS as a result of mitochondrial dysfunction, although it is not clear whether ROS play a secondary role or may act as both signaling molecules and regulators of PCD (51). Various oxidants stimulate, whereas antioxidants inhibit apoptosis, suggesting a role for ROS as initiators or downstream mediators of apoptosis (52). ROS appear as necessary and sufficient to induce certain forms of apoptosis in plant and yeast cells (53, 54), but its role has been questioned in other cases (23). We found that complex I mutants are also more resistant to exogenously added hydrogen peroxide than wild type N. crassa, in contrast to the other respiratory chain mutants. Interestingly, the response of the different mutant strains to H₂O₂ mirrored their response to PHS in terms of sensitivity. Hydrogen peroxide can cause oxidative stress and cell death since it easily permeates through cellular membranes (55) and it induces the production of more ROS (41). We reasoned that ROS production/removal could be a key factor in PHS-induced cell death and explain why different strains display different responses to the drug. Thus, we carried out a time course analysis of ROS production in N. crassa and compared the results obtained with wild type versus the complex I mutants. Upon PHS treatment, wild type cells initiated a massive production of ROS with a maximum at about 80 min. ROS production preceded DNA fragmentation, which was only detected some hours later, in agreement with evidence that ROS accumulation precedes the onset of apoptosis (56). In sharp contrast, fewer ROS were generated by the complex I mutants under the same conditions. Taken together, these data strongly suggest that ROS production is a crucial mediator of PHS-induced PCD in N. crassa and that decreased ROS production in the complex I mutants is responsible for their higher resistance toward PHS and H₂O₂.

The low levels of induction of ROS production observed in complex I mutants are in accordance with findings that most ROS inside cells are generated by the respiratory chain at the level of complex I (52). We speculate that the PHS-induced ROS generation involves the FMN prosthetic group, as described for ROS production arising from complex I inhibition when the NADH/NAD⁺ ratio is high (57). This suggestion follows from the fact that the nuo51 mutant displays decreased ROS production as compared with the wild type strain. FMN is bound to the 51-kDa subunit of complex I, and only this polypeptide is lacking from the assembled non-functional complex I of the nuo51 mutant (31).

Our results of decreased ROS production in complex I mutants might appear contradictory to findings that inhibition of this enzyme elevates ROS production in mammalian systems (45, 46). However, we believe that these situations are dissimilar. Inhibition of complex I results in ROS formation likely by disturbing "normal" electron transfer of the enzyme, whereas complex I from the (PHS-resistant) complex I mutants does not perform electron transfer (in most cases, it is not even properly
PHS-induced Death in Neurospora

assembled). On the other hand, complex I dysfunction is associated with a large number of human mitochondrial diseases (58–62), and there is evidence for ROS involvement in death resulting from complex I inhibition in a rat model of Parkinson disease (45). It will be of interest to extend these studies of ROS generation in additional models of mitochondrial disease.

We show that a mutant in the N. crassa homologue of the apoptosis-inducing factor (AIF) is more resistant to PHS than the wild type strain, suggesting that this polypeptide is also involved in PHS-induced PCD. This scenario was already suggested in the case of PHS-induced apoptosis in A. nidulans because the process was found to be independent of caspases (23). Caspase-independent apoptosis is regulated by AIF in mammals (37). It was hypothesized that PHS-induced apoptosis due to lack of the AIF protein (23), but a divergent orthologue of AIF was subsequently found in this organism (39). Our results showing the involvement of complex I in PHS-induced death in N. crassa suggest an alternative explanation. Complex I is a ubiquitous enzyme from bacteria to eukaryotic species but is absent from S. cerevisiae. This fact could as well explain the resistance of S. cerevisiae to PHS-induced apoptosis. In mammalian systems, AIF seems to be involved in complex I biogenesis (63). These data suggest that, besides an independent role of AIF and complex I in programmed cell death, AIF can also affect the process indirectly through its influence on complex I assembly.

Another interesting discovery was that an N. crassa deletion mutant in an AMID-like protein (NCU06061) is more sensitive to PHS (and H2O2) than the wild type strain. Like AIF, AMID and its yeast homologue Nd1p are mitochondrial flavoproteins described as apoptotic proteins (30, 38), although their role during apoptosis has not been as well characterized as that of AIF. It would be expected that an N. crassa AMID mutant would better resist killing by death-inducing drugs. Therefore, the N. crassa AMID encoded by NCU06061 either is not involved in PHS-induced death or, alternatively, it has a protective (antiapoptotic?) role. The equivalent of the yeast AMID homologue Nd1p is the N. crassa internal alternative NADH dehydrogenase ND11 (64). It is not known whether this polypeptide is involved in cell death. In addition, the N. crassa genome encodes another smaller protein (NCU11413) with similarity to AMID proteins. It is possible that either or both of these proteins (ND11 and NCU11413) are equivalent of the apoptotic AMID protein of mammals and S. cerevisiae, in contrast to the apparently antiapoptotic role of the AMID-like protein (NCU06061) described in this work. The continued comparison of the role of specific proteins in different organisms is likely to bring new insights on the mechanisms associated with programmed cell death.

Acknowledgments—We thank Simon Monard for help with flow cytometry and Dr. Charles Hall for assistance with the phylogenetic analysis.

REFERENCES
1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
2. Skulachev, V. P. (2006) Apoptosis 11, 473–485
3. Madeo, F., Herker, E., Wissing, S., Jungwirth, H., Eisenberg, T., and Fröhlich, K.-U. (2004) Curr. Opin. Microbiol. 7, 655–660
4. Green, D. R., and Kroemer, G. (2004) Science 305, 626–629
5. Andready, A. Y., Kushnareva, Y. E., and Starkov, A. A. (2005) Biochemistry (Moscow) 70, 200–214
6. Orrenius, S. (2007) Drug Metab. Rev. 39, 443–455
7. Dröge, W. (2002) Physiol. Rev. 82, 47–95
8. Kroemer, G., and Reed, J. C. (2000) Nat. Med. 6, 513–519
9. Wang, X. (2001) Genes Dev. 15, 2922–2933
10. Putcha, G. V., Harris, C. A., Moulder, K. L., Easton, R. M., Thompson, C. B., and Johnson, E. M., Jr. (2002) J. Cell Biol. 157, 441–453
11. Koonin, E. V., and Aravind, L. (2002) Cell Death Differ. 9, 394–404
12. Ludovico, P., Madeo, F., and Silva, M. (2005) IUBMB Life 57, 129–135
13. Eisenberg, T., Büttner, S., Kroemer, G., and Madeo, F. (2007) Apoptosis 12, 1011–1023
14. Semighini, C. P., Hornby, J. M., Dumitru, R., Nickerson, K. W., and Harris, S. D. (2006) Mol. Microbiol. 59, 753–764
15. Hamann, A., Brust, D., and Osiewacz, H. D. (2007) Mol. Microbiol. 65, 948–958
16. Lu, B. C. K. (2006) in Growth, Differentiation and Sexuality, (Kües, U., and Fischer, R., eds) pp. 167–187, Springer Berlin
17. Fedorova, N. D., Badger, J. H., Robson, G. D., Wortman, J. R., and Nierman, W. C. (2005) BMC Genomics 6, 177–191
18. Davis, R. H. (2000) Neurospora: Contributions of a Model Organism, Oxford University Press
19. Galagan, J. E., Calvo, S. E., Borkovich, K. A., Selker, E. U., Read, N. D., Jaffe, D., FitzHugh, W., Ma, L. J., Smirnov, S., Purcell, S., Rehman, B., Elkins, T., Engels, R., Wang, S., Nielsen, C. B., Butler, J., Endrizzi, M., Qui, D., Ianakiev, P., Bell-Pedersen, D., Nelson, M. A., Werner-Washburne, M., Selitrennikoff, C. P., Kinsey, J. A., Braun, E. L., Zelter, A., Schulte, U., Kothe, G. O., Jedd, G., Mewes, W., Staben, C., Marcotte, E., Greenberg, D., Roy, A., Foley, K., Naylor, J., Stange-Thomann, N., Barrett, R., Gnerre, S., Kamal, M., Kamvysselis, M., Maucci, E., Bielke, C., Rudd, S., Frishman, D., Krystofova, S., Rassmann, C., Metzenberg, R. L., Perkins, D. D., Kroken, S., Cogoni, C., Macino, G., Catcheside, D., Li, W., Pratt, R. J., Osmani, S. A., DeSouza, C. P., Glass, L., Orbach, M. J., Berglund, J. A., Voelker, R., Yarden, O., Parnam, M., Seiler, S., Dunlap, J., Radford, A., Agramayo, R., Natvig, D. O., Alex, L. A., Mannhaupt, G., Ebbole, D. J., Freitag, M., Paulsen, I., Sachs, M. S., Lander, E. S., Nusbaum, C., and Birren, B. (2003) Nature 422, 859–868
20. Colot, H. V., Park, G., Turner, G. E., Ringelberg, C., Crew, C. M., Litvinkova, L., Weiss, R. L., Borkovich, K. A., and Dunlap, J. C. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 10532–10537
21. Davis, R. H., and Perkins, D. D. (2002) Nat. Rev. Genet. 3, 397–403
22. Dementhon, K., Iyer, G., and Glass, N. L. (2005) Eukaryot. Cell 5, 2161–2173
23. Cheng, J., Park, T.-S., Chio, L.-C., Fischl, A. S., and Ye, X. S. (2003) Mol. Biol. Cell. 13, 163–177
24. Marques, I., Duarte, M., Assunção, J., Ushakov, A. V., and Videira, A. (2005) Biochim. Biophys. Acta 1707, 211–220
25. McCluskey, K. (2003) Adv. Appl. Microbiol. 52, 245–262
26. Davis, R. H., and de Serres, F. J. (1970) Methods Enzymol. 17A, 79–143
27. Vogel, H. J. (1956) Microb. Genet. Bull. 13, 42–46
28. Madeso, F., Frolich, E., and Frolich, K.-U. (1997) J. Cell Biol. 139, 729–734
29. Duarte, M., Sousa, R., and Videira, A. (1995) Genetics 139, 1211–1221
30. Li, W., Sun, L., Liang, Q., Wang, J., Mo, W., and Zhou, B. (2006) Mol. Cell 17, 1802–1811
31. Fecke, W., Sled, V. D., Ohnishi, T., and Weiss, H. (1994) Eur. J. Biochem. 220, 551–558
32. Ferreirinha, F., Duarte, M., Melo, A. M., and Videira, A. (1999) Biochem. J. 342, 551–554
33. Perkins, D. D., Radford, A., and Sachs, M. S. (2001) The Neurospora Compendium, Academic Press London
34. Phillips, A. J., Sudbery, I., and Ramsdale, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14327–14332
35. Mousavi, S. A. A., and Robson, G. D. (2004) Microbiology (Read.) 150, 1937–1945
36. Chen, C., and Dickman, M. B. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 3459–3464
