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Report

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Apoptosis-inducing factor (AIF), a key regulator of cell death, is essential for normal mammalian development and participates in pathological apoptosis. The proapoptotic nature of AIF and its mode of action are controversial. Here, we show that the yeast AIF homologue Ynr074cp controls yeast apoptosis. Similar to mammalian AIF, Ynr074cp is located in mitochondria and translocates to the nucleus of yeast cells in response to apoptotic stimuli.

Introduction

Apoptosis-inducing factor (AIF) is a flavoprotein with oxidoreductase activity localized in the mitochondrial intermembrane space (Susin et al., 1999; Miramar et al., 2001). Upon apoptosis induction, AIF translocates to the nucleus, where it leads to chromatin condensation and DNA degradation (Susin et al., 1999). AIF has been suggested to control a caspase-independent pathway of apoptosis, important for neurodegeneration and normal development (Susin et al., 1999; Cregan et al., 2002).

Recently, the yeast Saccharomyces cerevisiae has become a useful model organism for the study of apoptosis. Apoptotic markers were observed in association with a mutation in the AAA-ATPase gene CDC48 (Madeo et al., 1997), whose metazoan orthologues were subsequently implicated in the regulation of apoptosis (Shirogane et al., 1999; Wu et al., 1999). Yeast apoptosis is often accompanied by the generation of oxygen radicals (Laun et al., 2001; Mazzoni et al., 2003; Weinberger et al., 2003), and orthologues of core regulators of mammalian apoptosis such as caspases, HtrA2/Omi, and the proteasomal death pathways have been shown to be conserved in yeast (Blanchard et al., 2002; Madeo et al., 2002; Fahrenkrog et al., 2004). In addition, physiological scenarios of yeast apoptosis have been described during aging processes (Laun et al., 2001; Herker et al., 2004).

Here, we describe an orthologue of AIF in yeast cells. Yeast Aif1p shows the same localization and exhibits similar death executing pathways as mammalian AIF. We demonstrate that yeast Aif1p is dependent on cyclophilin A (CypA) and partially on caspase action.

Results and discussion

Ynr074cp (Aif1p) is the yeast homologue of AIF

Sequence comparison revealed that ORF YNR074C of S. cerevisiae encodes a protein of 41.3 kD, showing significant similarity with both AIF as well as AMID (AIF-homologous mitochondrion-associated inducer of death; Wu et al., 2002). Ynr074cp displays 22% identity and 41% similarity with human AIF (Fig. 1). Human AIF is a flavoprotein
Aif1p translocates from mitochondria to the nucleus upon apoptosis induction

To determine the cellular localization of Aif1p in yeast cells, we expressed GFP-tagged Ynr074cp (Aif1p<sup>GFP</sup>). Fluorescence microscopy revealed the colocalization of the Aif1p-GFP construct with a mitochondria marker DsRed Su1-69 (Fig. 2 A).

Low doses of reactive oxygen species are necessary and sufficient to induce yeast apoptosis (Madoe et al., 1999). After treatment with 0.6 mM H<sub>2</sub>O<sub>2</sub> for 5 h, Aif1p<sup>GFP</sup> translocated from mitochondria to the nucleus, as revealed by colocalization of Aif1p<sup>GFP</sup> with the nuclear marker DsRed-NLS (Fig. 2 B). Recently, we have shown that chronological aging is a physiological trigger for apoptosis in yeast (Herker et al., 2004). Consistently, we observe a predominantly nuclear localization of Aif1p<sup>GFP</sup> in aged yeast cells (Fig. 2 C). These data confirm that, like mammalian AIF, <i>S. cerevisiae</i> Aif1p translocates from mitochondria into the nucleus during apoptosis.

Subcellular fractionation of yeast cells with chromosomally GFP-tagged Aif1p confirmed the distribution of Aif1p<sup>GFP</sup> (Fig. 2 D). It should be noted that in untreated cells a nuclear Aif1p<sup>GFP</sup> localization is observable in cell fractionation but not in in vivo fluorescence. This might be due to the conditions during fractionation, in particular the digestion of the cell wall seems to be stressful for the cells, as it leads to a nuclear localization of Aif1p<sup>GFP</sup> (unpublished data).

Aif1p induces apoptosis in yeast

To explore the contribution of Aif1p to yeast cell death, wild-type and AIF1 disruptant (Δaif1) cells were exposed to H<sub>2</sub>O<sub>2</sub> or to acetic acid, which also induces apoptosis in budding yeast (Madoe et al., 1999; Ludovico et al., 2001). Δaif1 cells treated with 0.4 mM H<sub>2</sub>O<sub>2</sub> (Fig. 2 F) or 200 mM acetic acid (not depicted) showed a significantly higher survival rate compared with similarly treated isogenic wild type. Therefore, Aif1p is required for efficient apoptotic cell death in budding yeast.

Next, we investigated whether overexpression of Aif1p might sensitize yeast to apoptotic stimuli. Overexpression of AIF1 for 20 h did not compromise cell survival in the absence of additional apoptotic stimuli (Fig. 2 G). However, exposure of Aif1p overexpressing cells to low doses of H<sub>2</sub>O<sub>2</sub> resulted in massive cell death after 20 h compared with H<sub>2</sub>O<sub>2</sub>-treated isogenic controls (Fig. 2 G). Due to the incubation time required for overexpression (20 h), cell densities are much higher than in Fig. 2 F and hence low doses of H<sub>2</sub>O<sub>2</sub> do not induce massive cell death in the isogenic control strains. To test whether this Aif1p-facilitated cell death is of apoptotic nature, subcellular markers of apoptosis were examined. Chromatin condensation indicated by DAPI staining was detectable in 30% of the Aif1p overexpressing cells treated with H<sub>2</sub>O<sub>2</sub> (Fig. 2 H). Also, overexpression of Aif1p leads to DNA fragmentation in 80% of the cells, as revealed by TUNEL staining (Fig. 2 H). Moreover, plasmid DNA was completely degraded after incubation with cell extracts from strains overexpressing yeast Aif1p but not with control extracts from isogenic strains (Fig. 2 I). Interestingly, the Aif1p overexpressing yeast need peroxide treatment to yield lysates capable of digesting plasmid DNA. Immunoblotting of Aif1p overexpressor reveals a fivefold accumula-
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The localization of Aif1p after treatment with H$_2$O$_2$ for 5 h on SCD is observed, with cells expressing Aif1p$_{EGFP}$ in both the mitochondria and the nucleus (Fig. 2 J). This could be due to the differential localization of Aif1p or different transcription/translation of apoptotic cells.

We next asked whether purified recombinant Aif1p (Fig. 2 J) from E. coli is capable of degrading DNA. Indeed, Aif1p shows a DNase activity on purified yeast nuclei (Fig. 2 K) and plasmid DNA (Fig. 2 L). This function was dose dependent, as well as dependent on divalent cations (Fig. 2 L). DNase activity of Aif1p requires refolding of the protein and is destroyed by heat (100°C; Fig. 2 L). But refolding and the ability of DNA degradation did not require the presence of FAD, indicating that the predicted FAD binding domain and the NADH oxidoreductase activity are not involved, similar to mammalian AIF (Susin et al., 1999). The required concentration of Aif1p for degradation of plasmid DNA is ~1 μg protein per 1 μg plasmid DNA indicating that Aif1p works together with other cofactors for catalyzing nucleolytic attack of DNA in vivo. Purified yeast nuclei are degraded to random fragments and not internucleosomally. This may be caused by the S. cerevisiae chromatin structure with approximately no linker DNA between the nucleosomes (Lowary and Widom, 1989).
The apoptotic function of Aif1p is partially caspase dependent

We next addressed the question of whether the mechanism of Aif1p-induced cell death is conserved between mammals and fungi. Although early reports described human AIF as a mediator of a caspase-independent way of cellular suicide (Suisin et al., 1999), the release of AIF from mitochondria may be subordinated to earlier caspase activation (Arnoult et al., 2003), supporting the notion that caspases and AIF would be engaged in cooperative or redundant pathways. As described above, Aif1p overexpression stimulates apoptotic cell death only in synergy with mild oxygen stress. The yeast caspase Yca1p is activated by the same apoptotic stimulus (Madeo et al., 2002), which leads to physiological-induced cell death functions. Therefore, we investigated whether Aif1p function also depends on cyclophilin action. Aif1p was overexpressed in two yeast strains in which either the CPR1 or CPR2 genes, which encode cyclophilin homologues, had been disrupted. It appears of note, that Aif1p overexpression resulted in a lower expression level in the CPR1 disrupted strain compared to the wild type (unpublished data). Therefore, we adjusted the Aif1p overexpression in the wild type to a comparable level (see Materials and methods). Disruption of CPR1, the yeast homologue of human CypA (Dolinski et al., 1997; Fig. 3 C), but not disruption of the cyclophilin B homologue CPR2 (not depicted) abrogated cell death induced by overexpression of AIF1. In mammals, the cooperative effects of CypA and AIF in apoptosis occur independently of the peptidylprolyl cis-trans-isomerase activity of CypA (Candé et al., 2004). We also observe that cyclosporin A (CsA), which inhibits the peptidylprolyl cis-trans-isomerase activity of CypA did not increase survival of cells overexpressing Aif1p (Fig. 3 D).

CypA is required for Aif1p-induced cell death

AIF function in apoptosis has been shown to critically depend on cyclophilins (Candé et al., 2004), a large family of highly conserved proteins acting as peptidylprolyl cis-trans-isomerase enzymes throughout protein folding. It has been suggested that CypA has a latent nuclease activity (Montague et al., 1997) and Kroemer and colleagues (Candé et al., 2004) recently discovered that human AIF interacts with CypA to induce lysis of chromatin. Therefore, we investigated whether yeast Aif1p function also depends on cyclophilin action. Aif1p was overexpressed in two yeast strains in which either the CPR1 or CPR2 genes, which encode cyclophilin homologues, had been disrupted. It appears of note, that Aif1p overexpression resulted in a lower expression level in the CPR1 disrupted strain compared to the wild type (unpublished data). Therefore, we adjusted the Aif1p overexpression in the wild type to a comparable level (see Materials and methods). Disruption of CPR1, the yeast homologue of human CypA (Dolinski et al., 1997; Fig. 3 C), but not disruption of the cyclophilin B homologue CPR2 (not depicted) abrogated cell death induced by overexpression of AIF1. In mammals, the cooperative effects of CypA and AIF in apoptosis occur independently of the peptidylprolyl cis-trans-isomerase activity of CypA (Candé et al., 2004). We also observe that cyclosporin A (CsA), which inhibits the peptidylprolyl cis-trans-isomerase activity of CypA did not increase survival of cells overexpressing Aif1p (Fig. 3 D).

AIF1-deficient cells survive better during chronological aging

Long-term cultivation causes an aging processes in the whole yeast culture, called chronological aging (Fabrizio and Longo, 2003), which leads to physiological-induced apoptosis in yeast (Herker et al., 2004). Therefore, we investigated whether Aif1p participates in chronological aging. We observed that disruption of AIF1 significantly delayed the onset of age-induced cell death, which is consistent with the proapoptotic role of Aif1p (Fig. 3 E). This
Materials and methods

Strains and plasmids

Experiments were performed in BY4741 (MATa hisΔ1 leu2Δ0 met15Δ0 ura3Δ0), obtained from Euroscarf. Yeast strains BY4741, Δaif1 (YNR074C), Δcpr1, Δcpr2, and Δyca1 were obtained from Euroscarf and were grown on SC medium containing 0.17% yeast nitrogen base (Difco), 0.5% raffinose, 1% galactose for the wild type.

For adaptation of protein levels of Aif1p in wild type BY4741 cells, they were cultured for 1 day in SC (10 mg/l leucine), 30 mg/l adenine, and 320 mg/l uracil with 2% glucose as carbon source (SCD). For adaptation of protein levels of Aif1p in wild type BY4741 and Δcpr1 cells we used SC/DCD media containing 1% glucose, respectively, 1% galactose for the wild type.

Genomic DNA was isolated from BY4741. To construct Aif1p<sup>ΔC</sup>ΔAG, AIF1 was amplified by PCR, cut with EcoR1 and Not1 and ligated into vector pESC-His (Stratagene). The construct codes for a COOH terminally flag-tagged protein and was expressed under the control of a inducible Gal1 promoter. Chromosomal COOH terminally YEGFP-tagged AIF1 was generated using the protocol of Knop et al. (1999). Vector pYM12 was used as template and kanMX6-YEGFP-tag cassette was assembled by PCR with primers containing homologous regions to AIF1. The amplified cassette was transformed into BY4741. To generate an Aif1pΔCΔEGFP fusion under the control of the MET25 promoter, AIF1 was amplified by PCR from plasmid pYCG<sub>YNR074c</sub> (EUROSCARF) cut with BamHI-EcoR1 and ligated into vector pUCG35.

Test for apoptotic markers

Overexpression of Aif1p with and without hydrogen peroxide for survival assays was performed as described previously for yeast strain FMV21 (Madeo et al., 2002), with a modified lysis buffer (150 NaCl, 50 mM Tris-Cl, pH 7.4, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM PMSE). The indicated amount (micrograms) of cell extract 1 μg of plasmid DNA (pESC-His; Stratagene) and 2 mM MgCl<sub>2</sub>/CaCl<sub>2</sub> was added. The reaction mix was incubated for 90 min at 37°C and DNA fragments were separated on a 1% agarose gel.

The indicated amount of purified recombinant Aif1p or BSA was added to 1 μg plasmid DNA (pYES2; Invitrogen) and incubated at 30°C for 30 min in the presence or absence of 2 mM MgCl<sub>2</sub>/CaCl<sub>2</sub> followed by gel electrophoresis. Heat-inactivated Aif1p was incubated for 10 min at 100°C. 16 μg of purified recombinant Aif1p or BSA was added to purified yeast nuclear (200 μg of protein) for the indicated time. After incubation the genomic DNA was isolated by incubation in 1% SDS, 1% Triton X-100, 50 mM NaCl, 50 mM Tris-Cl, pH 6.4, and 0.66 μg μg/ml proteinase K for 2 h, phenol/chloroform extraction, alcohol precipitation, and incubation in 1 mg/ml RNase. Samples were analyzed by gel electrophoresis.

In vitro mitochondrial import

Radiolabeled preproteins were prepared by in vitro transcription and translation reactions using TNT T7 coupled reticulocyte lysate (Promega) in the presence of redissolved L-[35S]methionine (Amersham Biosciences), creating an Aif1p with a NH<sub>2</sub>-terminally c-Myc tag. Import mixtures contained 1–5% reticulocyte lysate (vol/vol) in 1% BSA (wt/vol), 600 mM sorbitol, 50 mM KCl, 2 mM potassium phosphate, 10 mM MgCl<sub>2</sub>, 50 mM Hesper-KOH, pH 7.4, 2 mM NADH, and 2 mM ATP, and were incubated with mitochondria (25–50 mg protein) at 25°C for 1 h. Mitochondria were converted to mitoplasts by 10-fold dilution in ice-cold 20 mM Hepes, pH 7.4. Protease treatment was performed by addition of 50 μg/ml proteinase K to the reaction and incubation for 30 min at 0°C. The protease was inactivated by the addition of 1 mM PMSE. Mitochondria or mitoplast were centrifuged at 14,000 g for 15 min at 4°C washed with ice-cold 600 mM sorbitol, 80 mM KCl, 20 mM Hepes, pH 7.4, resuspended in Laemmli buffer, loaded on a 12% SDS polyacrylamide gel, electrophoresed onto an Immobilon PVDF membrane (Millipore), and detected by autoradiography.

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References

Aris, J.P., and G. Blobel. 1989. Yeast nuclear envelope proteins cross react with an antibody against mammalian pore complex proteins. J. Cell Biol. 108: 2059-2067.

Arnould, B., B. Gaume, M. Karbowski, J.C. Sharpe, F. Cecconi, and R.J. Youle. 2003. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J. 22:4385–4399.
Blanchard, F., M.E. Rusiniak, K. Sharma, X. Sun, I. Todorov, M.M. Castellano, C. Gutierrez, H. Baumann, and W.C. Burhans. 2002. Targeted destruction of DNA replication protein Cdc6 by cell death pathways in mammals and yeast. *Mol. Cell. Biol.* 13:1536–1549.

Candé, C., N. Vahsen, I. Courant, E. Schmitt, E. Daugas, C. Spahr, J. Luban, R.T. Kroemer, F. Giordano, C. Garrido, et al. 2004. AIF and cyclophilin A co-operate in apoptosis-associated chromatinolysis. *Oncoogene.* 23:1514–1521.

Cregan, S.P., A. Fortin, J.G. MacLaurin, S.M. Callaghan, F. Cecconi, S.W. Yu, T.M. Dawson, V.L. Dawson, D.S. Park, G. Kroemer, and R.S. Slack. 2002. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J. Cell Biol.* 158:507–517.

Daum, G., P.C. Bohni, and G. Schatz. 1982. Import of proteins into mitochondria. Energy-dependent, two-step processing of the intermembrane space enzyme cytochrome b2 by isolated yeast mitochondria. *J. Biol. Chem.* 257:13028–13033.

Dolinski, K., S. Muir, M. Cardenas, and J. Heitman. 1997. All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 94:13093–13098.

Fabrizio, P., and V.D. Longo. 2003. The chronological life span of *Saccharomyces cerevisiae*. *Aging Cell.* 2:73–81.

Fahrenkrog, B., U. Sauser, and U. Aebi. 2004. The *Saccharomyces cerevisiae* HtrA-like protein Nma111p is a nuclear serine protease that mediates yeast apoptosis. *J. Cell Sci.* 117:115–126.

Herker, E., H. Jungwirth, K.A. Lehmann, C. Maldener, K.U. Frohlich, S. Wissing, S. Buttnner, M. Fehr, S. Sigrist, and F. Madeo. 2004. Chronological aging leads to apoptosis in yeast. *J. Cell Biol.* 164:501–507.

Jakobs, S., N. Martini, A.C. Schauss, A. Egner, B. Westermann, and S.W. Hell. 2003. Spatial and temporal dynamics of budding yeast mitochondria lacking the division component Fis1p. *J. Cell Sci.* 116:2005–2014.

Jazwinski, S.M. 1990. Preparation of extracts from yeast. In *Methods in Enzymology*. Academic Press, Inc., San Diego, CA. 154–174.

Knop, M., K. Siegers, G. Pereira, W. Zacharias, B. Winsor, K. Nasmyth, and E. Schiebel. 1999. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast.* 15:963–972.

Laur, P., A. Pichova, F. Madoe, A. Ellinger, S.D. Kohlwein, K.U. Frohlich, L. Dawes, and M. Breitenbach. 2001. Aged mother cells of *Saccharomyces cerevisiae* show markers of oxidative stress and apoptosis. *Mol. Microbiol.* 39:1166–1173.

Lowary, P.T., and J. Widom. 1989. Higher-order structure of *Saccharomyces cerevisiae* chromatin. *Proc. Natl. Acad. Sci. USA.* 86:8266–8270.

Ludovico, P., M.J. Sousa, M.T. Silva, C. Leao, and M. Corte-Real. 2001. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology.* 147:2409–2415.

Madoe, F., E. Frohlich, and K.U. Frohlich. 1997. A yeast mutant showing diagnostic markers of early and late apoptosis. *J. Cell Biol.* 139:729–734.

Madoe, F., E. Frohlich, M. Ligt, M. Grey, S.J. Sigrist, D.H. Wolf, and K.U. Frohlich. 1999. Oxygen stress: a regulator of apoptosis in yeast. *J. Cell Biol.* 145:757–767.

Montague, J.W., F.M. Hughes Jr., and J.A. Cidlowski. 1997. Native recombinant cyclophilins A, B, and C degrade DNA independently of peptidylprolyl cis-trans-isomerase activity. Potential roles of cyclophilins in apoptosis. *J. Biol. Chem.* 272:6677–6684.

Rodrigues, F., M. van Hermet, H.Y. Steensma, M. Corte-Real, and C. Leao. 2001. Red fluorescent protein (DiRed) as a reporter in *Saccharomyces cerevisiae*. *J. Bacteriol.* 183:3791–3794.

Sermersh, F., G.R. Fink, and J.B. Hicks. 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 139–142.

Shirogane, T., T. Fukada, J.M. Muller, D.T. Shima, M. Hibi, and T. Hirano. 1999. Synergistic roles for Pim-1 and c-Myc in STAT3-mediated cell cycle progression and antiapoptosis. *Immunity.* 11:709–719.

Susin, S.A., H.K. Lorenzo, N. Zamzami, I. Marzo, B.E. Snow, G.M. Brothers, J. Mangion, J. Jacotot, H. Fyfe, and J.A. Cidlowski. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature.* 397:441–446.

Weinberger, M., L. Ramachandran, and W.C. Burhans. 2003. Apoptosis in yeasts. *JUBMB Life.* 55:467–472.

Wu, D., P.J. Chen, S. Chen, Y. Hu, G. Nunez, and R.E. Ellis. 1999. *C. elegans* MAC-1, an essential member of the AAA family of ATPases, can bind CED-4 and prevent cell death. *Development.* 126:2021–2031.

Wu, M., L.G. Xu, X. Li, Z. Zhai, and H.B. Shu. 2002. AMID, an apoptosis-inducing factor-homologous mitochondrion-associated protein, induces caspase-independent apoptosis. *J. Biol. Chem.* 277:25617–25623.