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Ysp2 mediates death of yeast induced by amiodarone or intracellular acidification

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Abstract

Recently we have found that the drug amiodarone induces apoptosis in yeast, which is mediated by reactive oxygen species (ROS). Here we have used this finding as a tool to screen for genes involved in the death program. We have described a novel mitochondrial protein, Ysp2, acting in the amiodarone-induced death cascade. After amiodarone addition both the control and amiodarone-resistant ysp2-deleted cells formed ROS, but the mutant (unlike the control) did not undergo the mitochondrial thread-to-grain transition. To test whether the action of Ysp2 is amiodarone-specific we tried to induce PCD by other agents. We have found that acetic acid-induced PCD also depends on Ysp2. We also demonstrate that, like acetic acid, propionic acid or nigericin triggered intracellular acidification causing ROS-dependent death. We suggest that intracellular acidification results in the protonation of superoxide anion ($O_2^{-\cdot}$) to form HO_2, one of the most aggressive ROS, which in turn induces Ysp2-mediated PCD.

Keywords: Apoptosis; Programmed cell death; Acetate; ROS; superoxide; pH; Acid stress

1. Introduction

Programmed cell death plays a crucial role during the development, immune response, antitumor defense and maintenance of tissue homeostasis in multicellular organisms. Unicellular organisms also possess suicide programs, which represent a particular case of phenoptosis (programmed death of organism [1–3]). For instance, a number of treatments including hydrogen peroxide, acetic acid, hyperosmotic shock and Bax expression trigger death in *S. cerevisiae* yeast accompanied by appearance of typical apoptotic markers ([4–7]). There are at least two possible physiological inducers of yeast suicide: mating pheromones [8] and chronological aging [9,10]. Consistent with their possible role as natural suicide-promoting factors, both pheromones and chronological aging in stationary cultures are relatively inefficient in triggering PCD. Indeed, pheromone kills no more than 30% of yeast cells present in the cultures, and death in aging cultures begins only after several days of the culture incubation. This complicates their use as laboratory models to study PCD.

Is it possible to increase the efficiency of death induction in any of these models? Recently we have shown that amiodarone, a Ca^{2+} channel-targeted drug, induced PCD via the same pathway as pheromone. Moreover, amiodarone is proved to be much more efficient than pheromone as a death-inducing agent [11]. Here we have used this finding as a basis to screen for PCD-inducing genes in *S. cerevisiae*. We have found one novel gene, (ORF YDR326c), deletion of which confers resistance to

Abbreviations: PCD, programmed cell death; ROS, reactive oxygen species; CFU, colony-forming units, H_2-DCF-DA, dichlorofluorescein diacetate; MES, 2-[N-morpholino] ethanesulfonic acid; DIC, differential interferential contrast

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both amiodarone- and acetic acid-induced PCDs. We also show that the latter type of PCD is likely to be mediated by a drop in intracellular pH.

2. Material and methods

2.1. Strains and growth conditions

S. cerevisiae strains used in this study were derivates of W303-1A [THY40 (background strain), THY40 Δ*ysp2* (*ydr326c*::*TRP1*) and THY40 ysp2-GFP (*ydr326c-GFP*::*HIS3*)] or BY4741 [background strain, Δ*ysp2* (*ydr326c::kanMX4*), Δ*trp1* (*trp1:: kanMX4*, Δ*nma111* (*nma111::kanMX4*)]. Mutants of W303-1A genetic background were obtained by one-step PCR transformation. The strains of BY4741 genetic background were taken from EUROSCARF deletion collection. Typically, yeast cells were inoculated and grown overnight to 5×10^6 cells/ml in media containing 1% yeast extract (Difco), 2% bactopeptone (Difco) and 2% dextrose or glycerol (ICN). Dextrose was used during the screening because using this carbon source provided the highest efficiency of the mutant selection. Glycerol was used in the experiments on low pH-induced death because we aimed to perform these types of experiment on cells with fully functional mitochondria.

2.2. Induction of cell death and assay for cell survival

To induce PCD, yeast cells were treated with 80 μM amiodarone [11] or 80 mM acetic acid [12]. Induction of death with propionic acid, inorganic phosphate or MES (pH of cultivation medium was adjusted with HCl) was performed in the same way as with acetic acid. Yeast survival was measured as described in [11]). Nigericin was used at 13 μM, α-tocopherol at 50 μM concentrations.

2.3. Fluorescence microscopy

Intracellular ROS concentrations were visualized by treating yeast with 50 μM H2DCF-DA (Molecular Probes) as described [11]. Mitochondria were stained with Mitotracker orange (0.1 μg/ml, Molecular probes). To image the mitochondrial network (Fig. 1E), optical Z-sections were collected and collapsed into a single image [11].

3. Results and discussion

3.1. Amiodarone-based screen

Following our observation that amiodarone induces PCD in yeast via raising cytosolic Ca^{2+} concentration [11], we performed a genetic screening for yeast pro-apoptotic genes. To do that, transposon-mutagenized yeast cells [13] were treated with 80 μM amiodarone, and the surviving cells were grown in liquid media overnight. The overnight incubation allowed lowering the proportion of cells with non-functional mitochondria and other mutations which retarded the growth rate. The treatment-growth cycle was repeated four times. We reasoned that the cell survival could be either due to a specific loss of a protein involved in the death cascade or changes in Ca^{2+} homeostasis. As mitochondrial ROS formation is downstream of amiodarone-induced Ca^{2+} rise [11], estimating ROS levels in the treated cells allowed us to eliminate the latter possibility. Therefore, we treated the survived cells with amiodarone in the presence of ROS-sensitive dye H_2-DCF-DA and selected ROS-positive cells using fluorescence microscopy. We identified one gene, a deletion of which conferred amiodarone resistance without lowering the ROS levels. The gene (YDR326c) encodes an ORF, which we named Ysp2 (yeast suicide protein 2, by analogy with Ysp1 [11]), with no clear homologs to genes in higher organisms. Analysis of the amino acid sequence predicts GRAM domain and a transmembrane domain (Fig. 1A). Ysp2-GFP co-localizes with mitochondria (Fig. 1B), suggesting that Ysp2 is a novel mitochondrial membrane protein of unknown function. Importantly, while amiodarone-induced ROS staining seemed to be even higher in the deletion mutant than in the control (Fig. 1C), the survival of the mutant cells was at least 5 times higher (Fig. 1D). The distributions of the ROS dye proved to be different in two cell types. The control cells showed a diffuse ROS staining, whereas *ysp2*Δ displayed ROS in filamentous structures (Fig. 1C). Since the initial amiodarone-induced ROS accumulation occurs in mitochondria [11], data shown in Fig. 1C suggest that amiodarone treatment of the deletion mutant

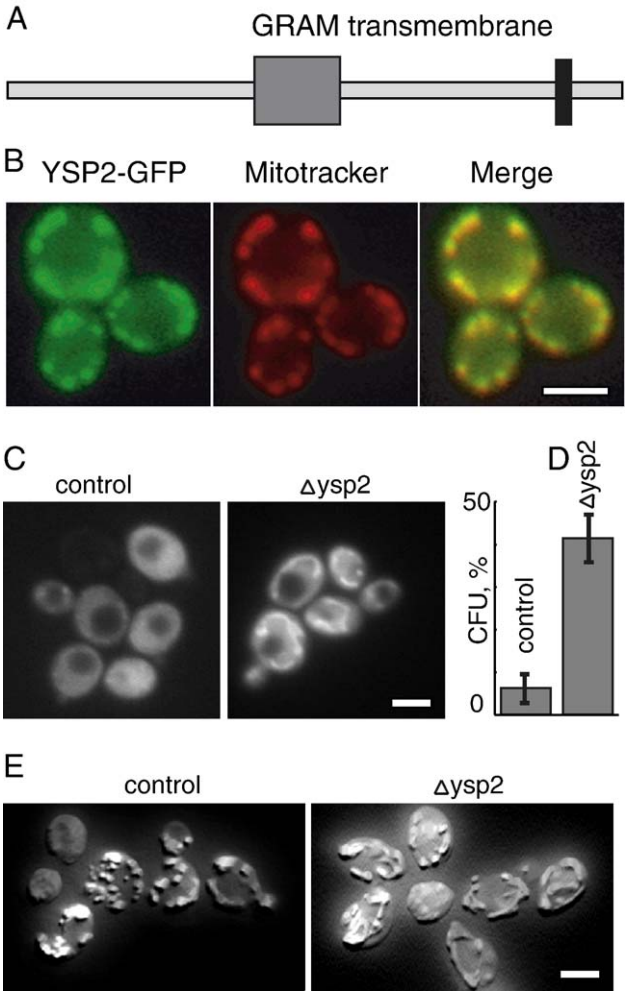

Fig. 1. Ysp2 localization and function. (A) Domain structure of Ysp2 predicted by SMART program includes a single GRAM and transmembrane domains. (B) Colocalization of the Mitotracker orange and Ysp2-GFP inside the yeast cell. Bar, 5 μm. (C) While ROS staining in the control cells is diffuse, ROS in *ysp2*Δ are localized to the filamentous structures. (D) Deletion of *ysp2* increases cell survival after amiodarone treatment. (E) Amiodarone-induced mitochondrial thread-to-grain transition is inhibited in *ysp2*Δ cells.

(unlike the control strain) does not cause thread-to-grain transition of mitochondria. To confirm this hypothesis we treated both the control and the *ysp2*Δ cells with amiodarone in the presence of Mitotracker orange and found that while in the control cells mitochondria fragmented into round particles, mitochondria in the mutant remained mainly filamentous (Fig. 1E).

3.2. PCD induced by acidification

Is the PCD-resistant phenotype of *ysp2*Δ specific to amiodarone or does the deletion also protect yeast from amiodarone-independent PCD induction? To test this, we used acetic acid to activate the PCD cascade. Previously, it was convincingly shown that acetic acid triggers apoptotic cell death in yeast. First, we confirmed the data of Ludovico et al. [12,14] on formation of ROS during acetic acid-induced apoptosis (Fig. 2A). Importantly, like amiodarone, acetic acid at pH equal to 3 induced a similar increase in the levels of ROS in the control and *ysp2*Δ cells (Fig. 2A).

Next, we checked whether *ysp2* deletion conferred resistance to acetic acid. Here it should be mentioned that, as we used the EUROSCARF mutant collection, the deletion mutants contained kanMX4 gene. To test the effect of kanMX4 on acetic acid-induced death we used Δ *trp1* strain with kanMX4. As shown by Fig. 2B, the *trp1*, kanMX4 transformation caused a small increase in the survival, suggesting that the kanMX4 strain should be considered as a control in this type of experiments. Data presented in Fig. 2B show that the survival of *ysp2*Δ was significantly higher than that of the kanMX4 control. Interestingly, the deletion of *nma111* (apoptotic protease described in [15]) had a similar effect on the survival (Fig. 2B).

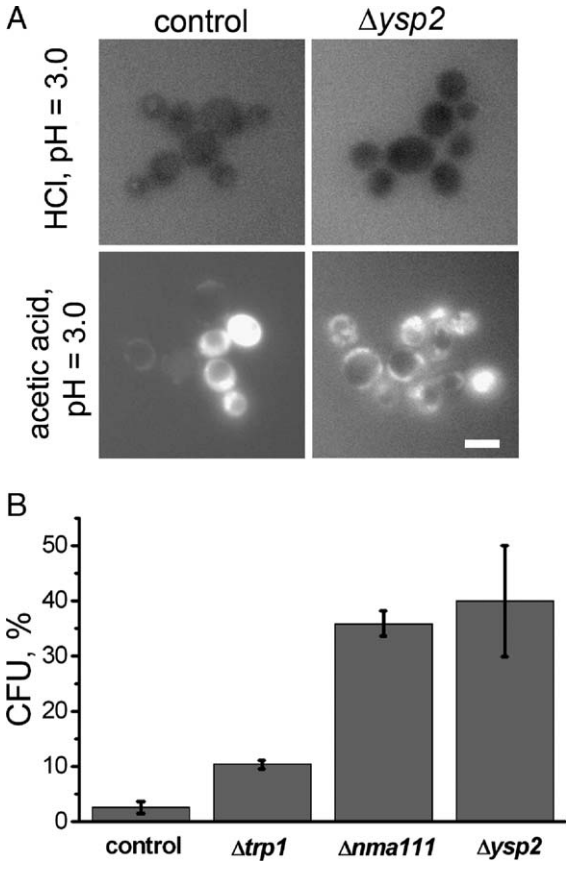

Fig. 2. Survival and ROS of acetic acid-treated yeast. Acetic acid induces ROS formation in both the control and *ysp2*Δ cells (A). (B) Deletions of *ysp2* and *nma111* (apoptotic protease) increase cell resistance to acetic acid-induced PCD. Bar, 5 μm.

Obviously, amiodarone and acetic acid are entirely different in their chemical nature. Nevertheless, the data of Ludovico et al. [12,14] together with ours ([11] and this paper) suggest that both types of PCD proceed via overlapping pathways. Indeed, both pathways share the same features, namely, a requirement for active mitochondria, Ysp2 involvement and ROS production. What is the convergence point of the two pathways? To answer this question let us consider the molecular mechanism of acetic acid-induced PCD. As shown by Ludovico et al. [12], acidification of the culture medium by HCl, unlike acetic acid, does not cause PCD. A possible explanation for this difference is based on the fact that CH_3COOH and HCl are weak and strong acids respectively. As CH_3COOH is a hydrophobic molecule, it is capable of diffusing across the cell membrane. Therefore, acidification of the medium by acetic acid (but not by HCl) is expected to directly induce a strong drop in the intracellular pH. To test whether the difference between the effects of HCl and acetic acid could be explained by the hydrophobicity of the latter, we acidified the yeast cultures to pH equal to 3 by MES, phosphoric (hydrophilic), or propionic (hydrophobic) acids. As shown by Fig. 3A, both hydrophilic acids did not cause cell death, while the effect of propionic acid was similar to that of acetic acid. Addition of an antioxidant (α-tocopherol) was shown to alleviate both propionic acid- and acetic acid-induced cell deaths. To confirm that the triggering of PCD by intracellular acidification is not a unique feature of acetic and propionic acids, we tried to induce the intracellular acidification by adding the K^+/H^+ antiporter nigericin. This ionophore facilitates proton flow across membranes in exchange for potassium ions. Fig. 3B shows that at pH value equal to 3 nigericin induces cell death, which can be inhibited by α-tocopherol addition. Consistent with that, H_2-DCF-DA staining reveals accumulation of ROS in HCl/nigericin treated cells (Fig. 3C). An alternative explanation for the effect of nigericin treatment is that the disturbance of K^+ (not H^+) homeostasis causes the cell death. Although this explanation cannot be ruled out, it seems unlikely: as $[K^+_{in}]$ is at least thousand times higher than $[H^+_{in}]$, a primary effect of K^+/H^+ antiporter is expected to be on ΔpH rather than ΔpK. Thus, our experiments suggest that the action of acetic acid as PCD-inducing agent can be explained by the acidification of an intracellular compartment.

4. Discussion

Why does intracellular acidification cause death of yeast? The simplest explanation is that low intracellular pH directly activates intracellular damage produced by endogenous ROS.

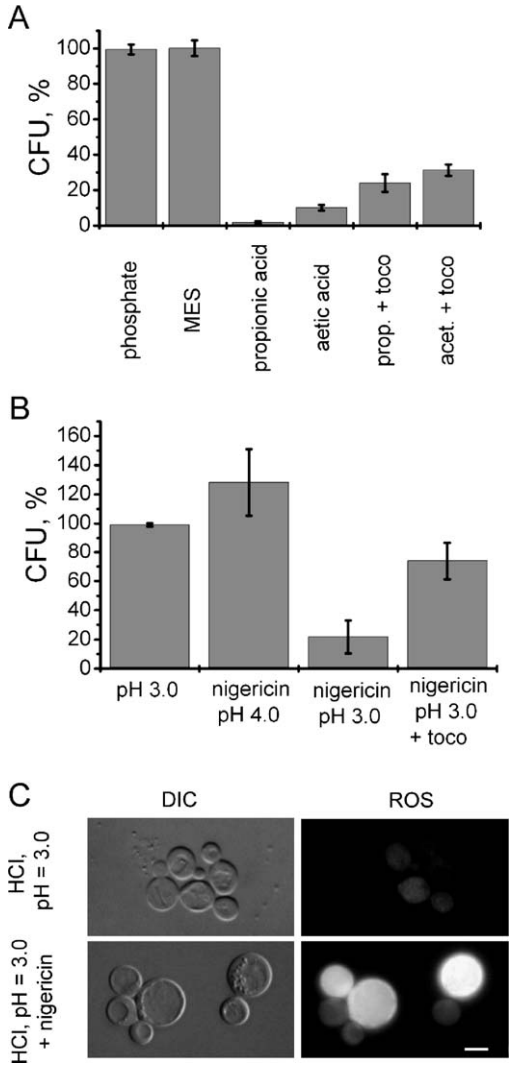

Fig. 3. Intracellular acidification causes ROS-dependent cell death. (A) While acidification of the culture media containing hydrophilic anions of strong acids (MES, phosphate) does not cause cell death, low pH medium containing weak acids (acetic, propionic) trigger α-tocopherol-sensitive cell death. Nigericin combined with low pH_{out} causes ROS-dependent cell death (B) and ROS accumulation (C). Bar, 5 μm.

Indeed, at low pH superoxide anion ($O_2^{-\cdot}$), the primary ROS produced by the mitochondrial respiratory chain, is protonated to $HO^{2\cdot}$. $HO^{2\cdot}$ is one of the most active forms of ROS. Moreover, $HO^{2\cdot}$ (a small uncharged hydrophobic molecule unlike the charged hydrophilic O_2) should readily diffuse through the membranes causing massive oxidative damage in all intracellular compartments (for review, see [16]). Such a damage usually causes decomposition of mitochondrial filaments — a necessary step in the PCD cascade [17].

The above reasoning may explain why acetic acid and nigericin kill yeast at extracellular pH (pH_{out}) equal to 3 but not to 4, the killing being almost completely prevented by antioxidant. Indeed, on one hand, the pK_a of superoxide protonation is 4.8. On the other hand, the data on intracellular pH in yeast show that, under experimental conditions similar to ours, there is approximately one or two units difference between pH_{in} and pH_{out} values upon acetic acid treatment [18,19]. As a result, pH_{in} at pH_{out} equal to 4 is expected to be above the superoxide pK_a value, and at pH_{out} equal to 3 — close to or below it. Thus, it seems likely that protonated superoxide is a key intermediate in the acetic acid-induced PCD. This suggests that both amiodarone- and low pH_{in}-induced death pathways are mediated by and converge at the level of intracellular ROS. This conclusion is consistent with the observations that for both amiodarone-induced [11] and acetic acid-induced [20] PCDs ROS are essential inter mediates of the death cascades rather than their by-products.

Another question arising from the works of Ludovico et al. [12,14] and the current study is whether acetic acid-induced PCD is related to any natural scenarios of yeast cell death. Here it is important to mention that during chronological aging-induced PCD (which is considered to be a natural form of yeast suicide) a significant acidification of the medium takes place [21]. Importantly, inhibition of the acidification by pH-buffering agents completely inhibits chronological aging-induced PCD [21]. As organic acids (including acetic acid) are natural products of yeast metabolism, it is possible that during chronological aging yeast cells die of low pH_{in}-induced apoptosis.

To summarize, we suggest that similar to amiodarone-induced PCD, low pH_{in}-induced apoptosis can be used as a high-efficiency model system to study natural PCD induced by chronological aging. We have also identified a new protein, Ysp2, which plays a role in both amiodarone- and low pH_{in}-induced cell deaths. We also show that, at least in the case of amiodarone-induced PCD, Ysp2 acts downstream of ROS formation. Currently we are studying the role of Ysp2 in pheromone- and chronological aging-induced PCDs. Independently of the outcome of these experiments, intracellular localization of Ysp2 and its effect on the thread-to-grain transition suggest a direct function of this protein in mitochondria-mediated PCD.

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