ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 11, pp. 1254-1259. © Pleiades Publishing, Ltd., 2008.
Published in Russian in Biokhimiya, 2008, Vol. 73, No. 11, pp. 1561-1568.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-057, October 12, 2008.

Mitochondrial Matrix Fragmentation as a Protection Mechanism of Yeast *Saccharomyces cerevisiae*

D. A. Knorre, S. M. Ojovan, V. B. Saprunova, S. S. Sokolov, L. E. Bakeeva, and F. F. Severin*

Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-318; E-mail: severin@belozersky.msu.ru

Received February 18, 2008
Revision received March 31, 2008

Abstract—It was shown that separate fragments of the inner mitochondrial compartment (mitoplasts) can exist under a single non-fragmented outer membrane. Here we asked whether fragmentation of the inner mitochondria could prevent rupturing of the outer membrane and release of pro-apoptotic molecules from the mitochondrial intermembrane space into the cytoplasm during mitochondrial swelling. First, we showed that in *Saccharomyces cerevisiae* yeast addition of amiodarone causes formation of electrically separate compartments within mitochondrial filaments. Moreover, amiodarone treatment of Δ*ysp2* mutant produced a higher proportion of cells with electrically discontinuous mitochondria than in the wild type, which correlated with the survival of cells. We confirmed the existence of separated mitoplasts under a single outer membrane using electron microscopy. Mitochondria with fragmented matrixes were also detected in cells of the stationary phase. Our data suggest that such fragmentation acts as a cellular protective mechanism against stress.

DOI: 10.1134/S0006297908110126

Key words: yeast, mitochondrion, mitochondria fragmentation, *ysp2*, amiodarone, apoptosis

It has been shown that local laser ablation (0.1 μm^2) of an elongated mitochondrion (5-10 μm) in cultured fibroblasts depolarizes the whole mitochondrial filament [1]. It has been concluded that in these cells, mitochondria exist as electrically coupled organelles and their inner membranes are continuous along the entire lengths of mitochondrial filaments. At the same time, under hypoxic conditions one mitochondrion can consist of several mitoplasts surrounded by a single outer membrane [2]. What is the physiological reason for the existence of such structures? It is known that mitochondria play a key role in programmed cell death in all organisms including unicellular fungi (reviewed in [3, 4]). It is also known that an increase of ion flow across the inner membrane (i.e. upon formation of Ca^{2+}-dependent permeability pore) can lead to swelling of the matrix [5]. This in turn can lead to rupturing of the mitochondrial outer membrane and the exit of pro-apoptotic proteins into the cytoplasm, thus activating the programmed cell death cascade (reviewed in [6]). Here we suggest that matrix fragmentation can promote mitochondrial resistance to swelling. Indeed, the maximal volume of the swollen matrix depends on two factors: first, the total surface area of the inner membrane (which can be assumed to stay constant for the duration of the swelling) and, second, on the number of mitoplasts surrounded by the outer membrane. Therefore fission of the inner mitochondria might serve as a mechanism to reduce the maximal total volume (Fig. 1a) of mitochondrial matrix and thus to protect the cell against mitochondrial swelling.

Here we tested whether such structures can be formed in wild type yeast cells and in the mutants resistant to mitochondria-mediated programmed cell death.

MATERIALS AND METHODS

Yeast strains and culture conditions. The yeast *Saccharomyces cerevisiae* strains used in this study are W303 (mat *a*) and isogenic strain Δ*ysp2* (*ysp2::TRP1*). Cells were typically grown in liquid culture up to logarithmic phase (2·10^6 cells/ml) or to stationary phase (~10^8 cells/ml). Cells were grown in YEPD (containing 2% glucose) or in YEP-raffinose (containing 2% raffinose) medium as in [7].

Fluorescent and electron microscopy. Cells of W303 and Δ*ysp2* strains were grown up to stationary phase and then incubated at 5·10^7 cells/ml concentration in 2%

Abbreviations: FCCP) carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; GFP) green fluorescent protein; TMR) tetramethyl rhodamine.

* To whom correspondence should be addressed.

glycerol solution containing 25 mM Mes, pH 5.5, and 1 μM of fluorescent dye JC-1 for 15 min. After that, 60 μM of amiodarone was added. Local ablations with a laser were performed using the experimental setup described by Tolic-Norrelykke et al. [8]. Electron microscopy was done as in Yang et al. [9]; JC-1 was omitted in the electron microscopy samples.

Mitotracker Green/Mitotracker Orange (Invitrogen, USA) staining was performed in the YEPD growth medium. Mitochondrial fragmentation and cell death were induced by 40-μM amiodarone.

For staining of mitochondria with mito-GFP/TMR (tetramethyl rhodamine) combination, W303 cells were transformed with pYX223 plasmid pretreated with Nhe1 restriction enzyme. This plasmid encodes GFP (green fluorescent protein) fused to mitochondrial targeting sequence [10]. Cells were grown up to late stationary phase on solid agarized YEP-raffinose/galactose media (containing 2% raffinose or galactose, respectively) and transferred into phosphate buffered saline containing 2 μM TMR.

RESULTS AND DISCUSSION

Formation of separated mitoplasts under the intact outer membrane can be detected by visualization of mitochondrial depolarization. If the matrix is non-fragmented, a local damage to the inner membrane will lead to depolarization of the entire mitochondrial filament. In case the outer membrane covers several electrically separate compartments, a local damage will depolarize only a part of the filament (Fig. 1b). Thus we tested mitochondrial depolarization after local laser ablations in wild type cells and also in a mutant resistant to mitochondria-mediated death, Δ*ysp*2 [11, 12]. Yeast cells were stained with mitochondrial dye JC-1 and then treated with amiodarone at 30-μM concentration. We showed earlier that amiodarone-induced killing of yeast cells is accompanied by (and depends on) mitochondrial hyperpolarization [11]. As JC-1 accumulation in mitochondria is membrane potential-dependent, amiodarone addition leads to an increase in JC-1 staining.

We also confirmed our earlier observations [12] that after amiodarone addition mitochondria stay filamentous for 10 min and then fragment. At the same time, the *ysp2* deletion inhibits amiodarone-induced fragmentation; in the mutant mitochondria remain in filaments until 30 min following amiodarone treatment ([12] and unpublished data). We subjected mitochondria of both cell types to local laser ablations. After short incubation times with amiodarone (10 min or less) mitochondria in the wild type cells and in the mutant responded in the same way to local laser damages: we detected long-distance loss of JC-1 staining (Fig. 2). Interestingly, longer incubation times (10-30 min) of Δ*ysp*2 cells with amiodarone lead to JC-1 loss which seemed to be limited to the area of laser ablation (Fig. 2). Semi-quantitative analysis of these data is presented in table. As it was technically difficult to measure precisely the lengths of depolarized stretches, we divided all laser ablations into two categories: ones causing long-distance depolarization and ones causing local depolarization of mitochondrial filaments. Figure 2 shows that the mitochondrial matrix of the mutant strain (unlike the wild type) fragments faster than the outer mitochondrial membrane. This supports the idea that delayed fragmentation of the outer membrane (relative to the matrix) in the mutant is responsible for amiodarone resistance.

Next we tried another way of testing our hypothesis. If matrix fragmentation takes place, then the values of mitochondrial membrane potential may vary in different parts of the same mitochondrion (Fig. 1b). Thus the exis-

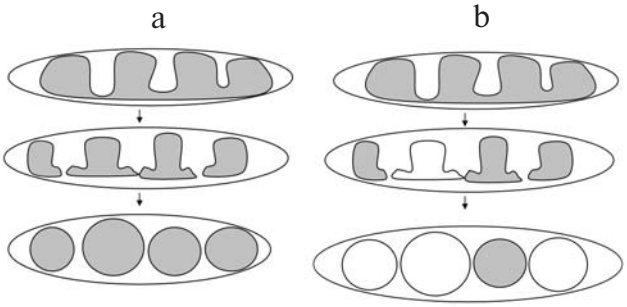

Fig. 1. Model of a mitochondrion containing several mitoplasts surrounded by a single outer membrane. a) Mitoplast fragmentation reduces the maximal volume of the matrix. b) Non-uniform distribution of membrane potential along mitochondrial filament indicates matrix fragmentation.

Number of the events of local depolarization and filament depolarization

Depolarization	Wild type cells	Δ*ysp2*	Wild type cells	Δ*ysp2*
	5 min		10 min	
Filament bleaching	7	7	9	5
Spot bleaching	0	1	0	8

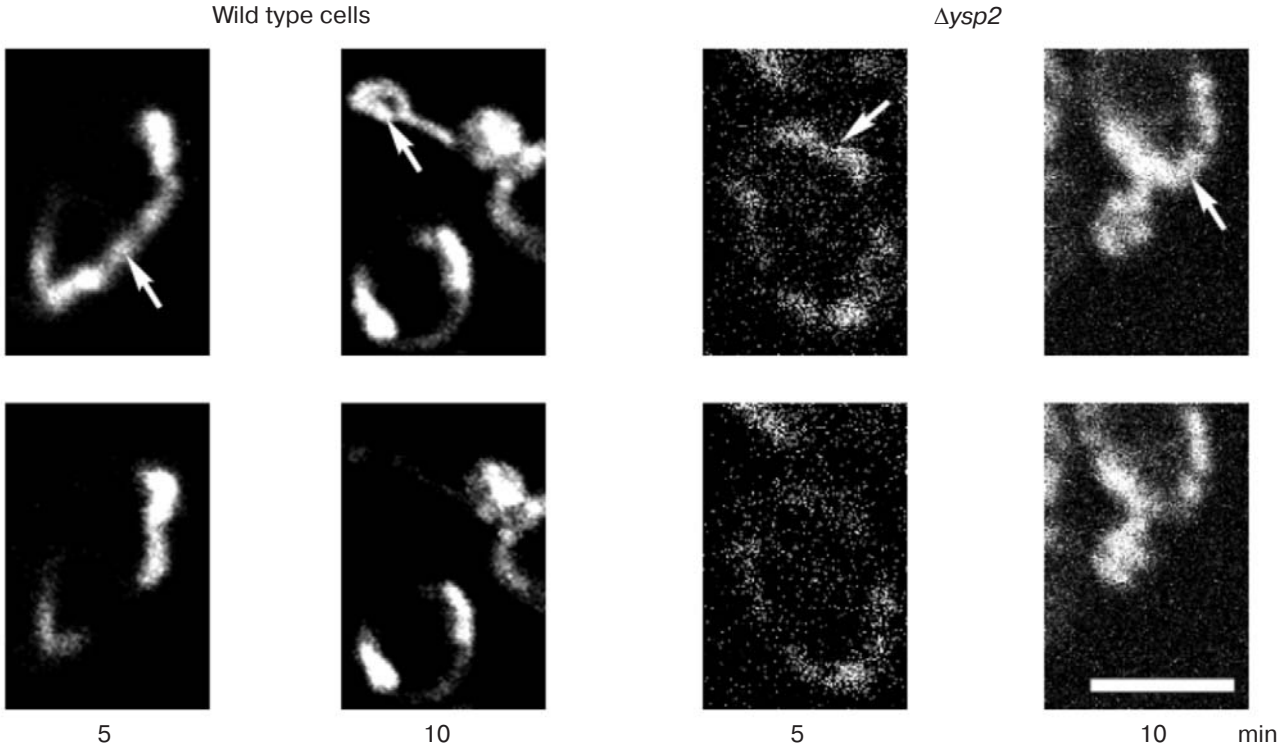

Fig. 2. Local laser damage indicates existence of electrically separated compartments within the mitochondrial filament. JC-1 fluorescence before and after laser damage (the damage spot is marked by an arrow). Bar, 5 μm.

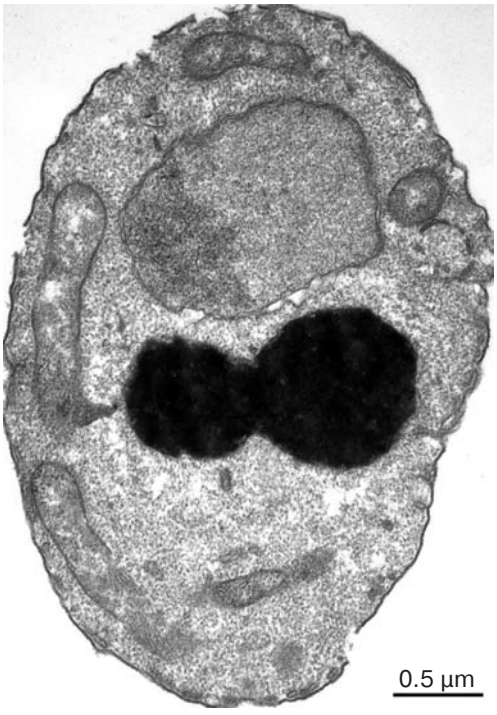

Fig. 4. Mitochondria of *S. cerevisiae* wild type cells visualized by electron microscopy.

tence of mitochondria with non-uniform distribution of membrane potential ("mosaic mitochondria") would indicate the presence of separate mitoplasts surrounded by the outer membrane. To look for such structures we stained mitochondria of amiodarone-treated cells with two dyes—Mitotracker Green, which stains mitochondria independently of membrane potential, and potential-dependent dye Mitotracker Orange. The untreated cells displayed weak mitochondrial accumulation of both dyes (not shown). In the presence of amiodarone approximately 10% of Δ*ysp2* cells displayed "mosaic" mitochondria—filamentous mitochondria (Mitotracker Green staining) containing a limited number of energized compartments (Mitotracker Orange staining) (Fig. 3a; see color insert). This experiment and the laser ablations data support the idea that amiodarone treatment causes formation of mitochondria with fragmented matrixes.

Similar experiments were performed using fluorescent compound JC-1. This dye accumulates in mitochondria depending on the membrane potential. At high concentration, JC-1 forms aggregates with emission spectrum in a long-wavelength range thus allowing estimation of the value of membrane potential. We found that JC-1 staining displayed the most reproducible results when performed on cells grown to stationary phase. Thus we treated stationary phase cultures with amiodarone and

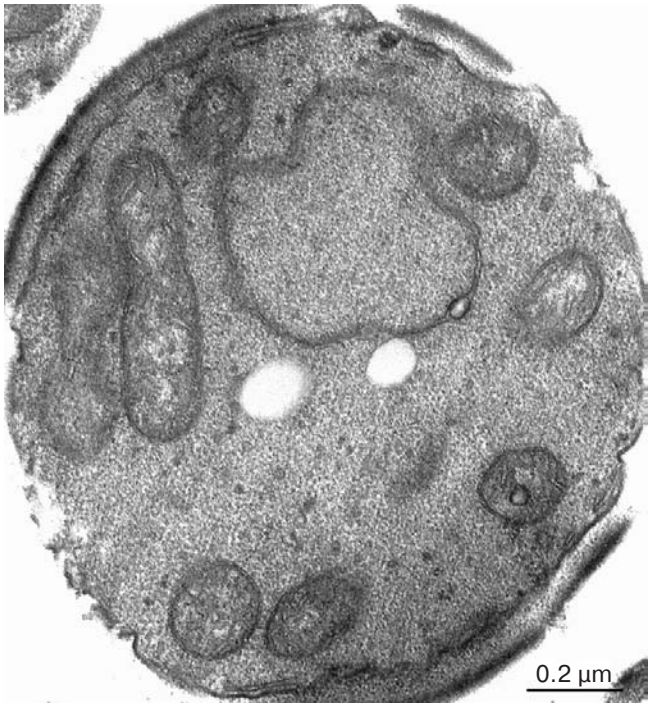

Fig. 5. Mitochondria of *S. cerevisiae* Δ*ysp2* cells visualized by electron microscopy.

counted cells containing mosaic mitochondria. As shown by Fig. 3b, the mutant strain contained a much higher percentage of cells with mosaic mitochondria than the wild type. At the same time, the death rate was higher in the wild type strain. The percentages of dead cells were estimated by propidium iodide accumulation (Fig. 3b) and by counting the colony forming units (not shown).

Interestingly, a minor percentage (~1%; data not presented) of untreated cells in stationary cultures contained mosaic mitochondria (Fig. 3c). Similar data were reported for heat-shocked cells [13]. This indicates that amiodarone is not a unique inducer of mitochondrial matrix fragmentation.

Data obtained using fluorescent microscopy support the existence of mitochondria with fragmented matrixes. At the same time, one can think of alternative explanations of the data. First, the mosaic staining of mitochondria might reflect nonspecific accumulation of dyes in lipid droplets located next to the mitochondria. To test this possibility we repeated JC-1 staining of amiodarone-treated cells and then added uncoupler FCCP (1 μM). It appeared that in this case the mosaic staining was lost. Thus we can rule out membrane potential-independent accumulation of JC-1.

Next we studied the structural details of mitochondria in the wild type and in Δ*ysp2* cells using electron microscopy. Figures 4 and 5 show typical mitochondria of the wild type and the mutant cells from stationary cultures. Mitochondria of both cell types are elongated with sparse cristae oriented at approximately 90° relative to the longitudinal axis of the mitochondrion. A 10-min incubation of both cell types with 50-μM amiodarone caused

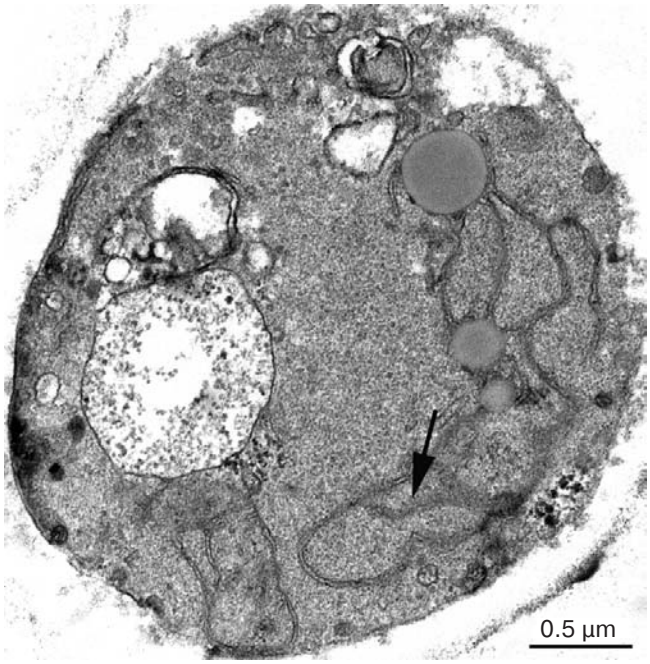

Fig. 6. Electron microscopy photographs of wild type cells incubated for 10 min with 50-μM amiodarone. Arrow shows cristae in the newly formed structures.

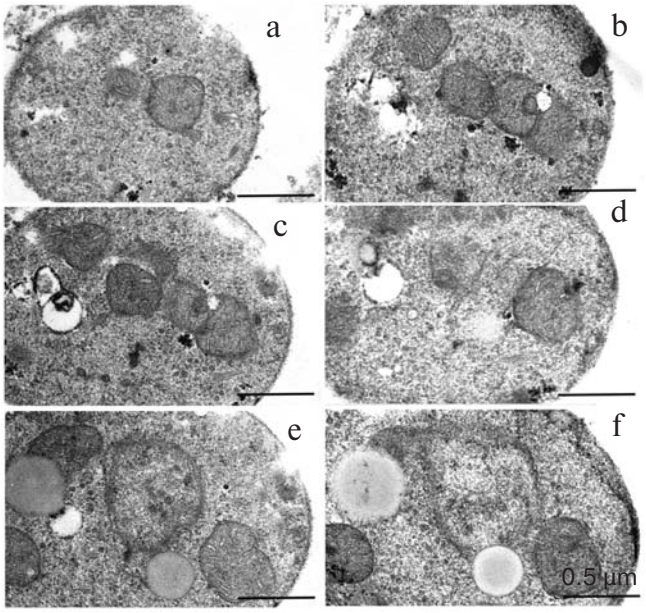

Fig. 7. Electron microscopy photographs of Δ*ysp2* cells incubated for 10 min with 50-μM amiodarone. a-f) Serial sections showing a single mitochondrial cluster.

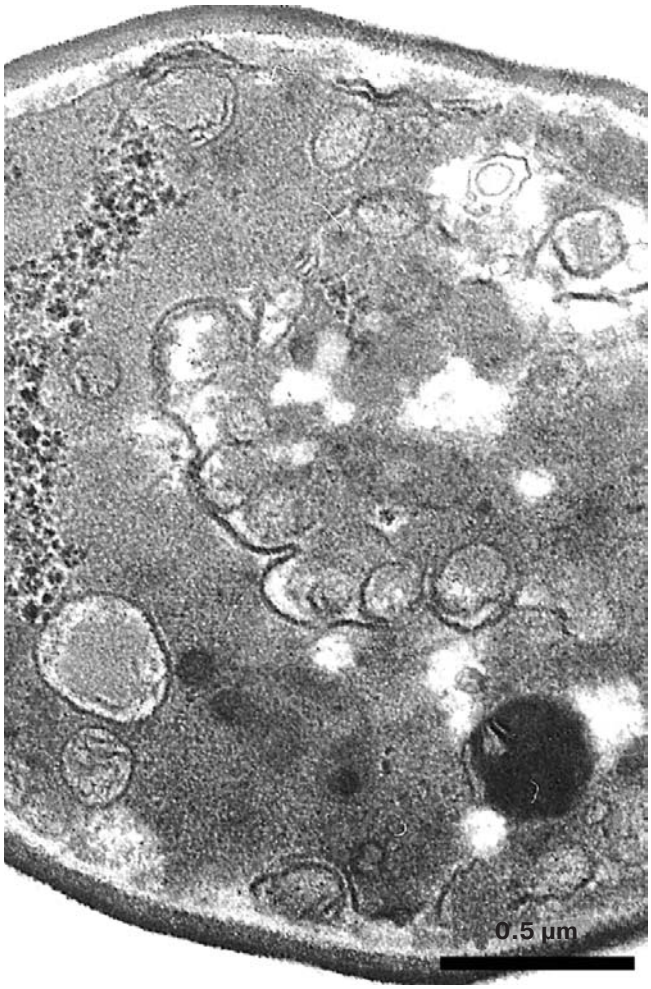

Fig. 8. Electron microscopy photographs of wild type cells incubated for 20 min with 50-μM amiodarone. Mitochondria disintegrate into separated mitoplasts.

dramatic changes in their mitochondrial structure. Amiodarone caused wild type cells to accumulate large double membrane structures consisting of several compartments (Fig. 6). Some of these compartments display typical cristae. It is possible that these structures form as a result of mitochondrial fusion.

Amiodarone triggered similar changes in the mitochondria of the mutant cells (Fig. 7). Serial section reconstruction shows that amiodarone caused formation of large mitochondrial clusters containing several mitoplasts surrounded by a single outer membrane (Fig. 7, a-f).

Longer incubation time with amiodarone (20 min) caused mitochondrial damage in both the wild type and Δ*ysp2* cells (Figs. 8 and 9, respectively). Mitochondrial outer membranes appear to be ruptured, and a large number of single membrane vesicles (fragmented mitochondrial inner membrane) can be seen in the cytoplasm.

It is important to mention that similar changes were observed in mitochondria of in the Δ*ysp2* cells from deep stationary culture (three days on solid medium). Figure 10 shows a cell from this preparation containing a mitochondrion with more than 10 separated mitoplasts. Serial section reconstruction analysis showed that the mitoplasts are indeed topologically separate (data not shown). Presumably in this case matrix fragmentation is a part of the cell conservation mechanism: from laboratory practice it is known that when grown on solid media cells from deep stationary cultures are highly resistant to a wide variety of stresses.

Currently it is accepted that mitochondrial fragmentation is a key step in activation of the cell suicide cascade [2, 14, 15]. For instance, inactivation of Drp1 and Fis1 proteins prevents mitochondrial fission and apoptosis in many cell types [16-18]. At the same time, the reasons for that are still not exactly clear. Our data allow us to put forward a simple hypothesis which links programmed cell death and mitochondrial fragmentation. We suggest that fragmentation of mitochondrial matrixes leads to the formation of mitochondria resistant to swelling. This resistance is due to a decrease in the maximum possible volume of the matrix. The same structure might form as a result of fission of outer membranes in the absence of fission of the inner ones. Our data suggest that both processes take place.

Recently it has been reported that fragmentation of mitochondrial matrixes (vesicularization) also takes place during etoposide-induced apoptosis of HeLa cells [19]. The authors suggest that this is a step in the apoptotic cas-

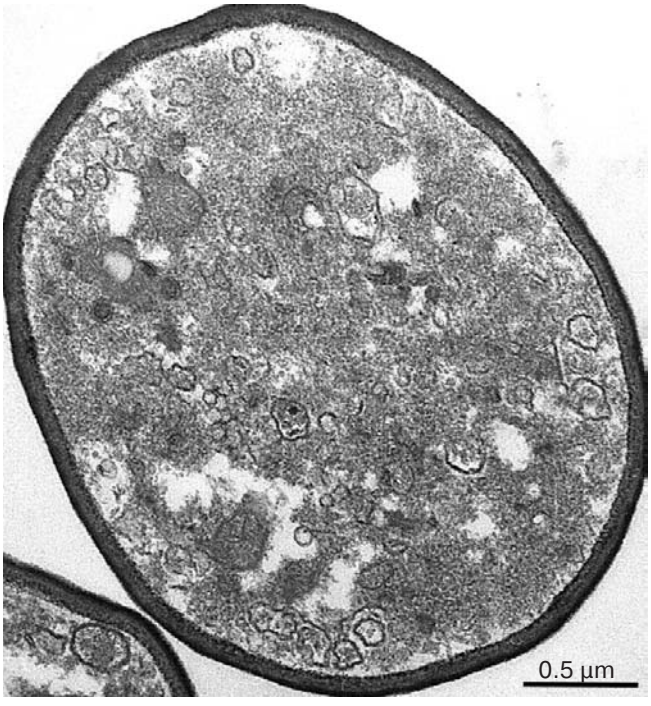

Fig. 9. Electron microscopy photographs of Δ*ysp2* cells incubated for 20 min with 50-μM amiodarone. Intact mitochondria are absent; multiple mitoplasts can be seen.

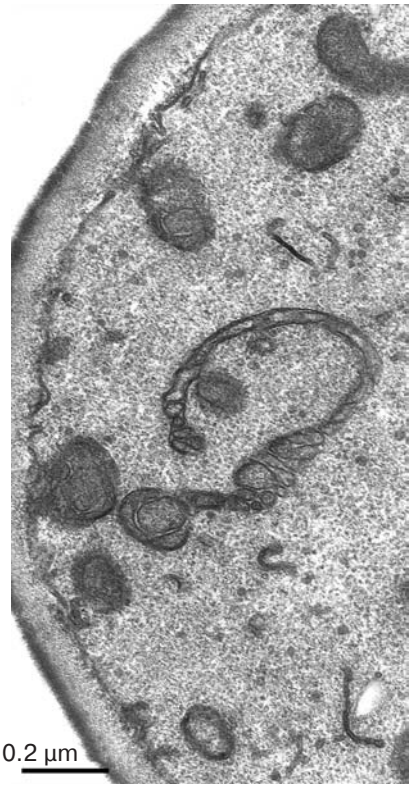

Fig. 10. Electron microscopy photographs of Δ*ysp2* cells grown to deep stationary phase on solid media. The mitochondrion in the center contains at least 10 separated mitoplasts surrounded by a single outer membrane.

cade necessary to accelerate fragmentation of the mitochondrial network. Our data indicate that fragmentation of the inner mitochondrial membranes is not a step in the apoptotic cascade but rather a defensive response acting to protect the cell from mitochondria-mediated death. The above data obtained on HeLa cells together with our observations ([11, 12] and this work) are consistent with this hypothesis, but they are not sufficient to prove it. We have shown that *ysp2* deletion increases the percentage of mosaic mitochondria and cell resistance to mitochondrial stresses. Currently we are testing other known mutations affecting mitochondrial fission and fusion and looking for correlations between the accumulation of mosaic mitochondria and stress resistance.

We thank I. Tolic-Norrelykke, I. Raabe, and R. Chave-Cox for discussions and technical help.

The work was supported by BMBF RUS 04/016, RFBR grants 06-04-49555 and 07-04-00223, and a FEBS Short-Term Fellowship.

REFERENCES

1. Amchenkova, A. A., Bakeeva, L. E., Chentsov, Y. S., Skulachev, V. P., and Zorov, D. B. (1988) *J. Cell Biol.*, **107**, 481-495.
2. Skulachev, V. P., Bakeeva, L. E., Chernyak, B. V., Domnina, L. V., Minin, A. A., Pletjushkina, O. Y., Saprunova, V. B., Skulachev, I. V., Tsyplenkova, V. G., Vasiliev, J. M., Yaguzhinsky, L. S., and Zorov, D. B. (2004) *Mol. Cell Biochem.*, **256/257**, 341-358.
3. Madeo, F., Herker, E., Wissing, S., Jungwirth, H., Eisenberg, T., and Frohlich, K. U. (2004) *Curr. Opin. Microbiol.*, **7**, 655-660.
4. Gordeeva, A. V., Labas, Y. A., and Zvyagilskaya, R. A. (2004) *Biochemistry (Moscow)*, **69**, 1055-1066.
5. Kaasik, A., Safiulina, D., Zharkovsky, A., and Veksler, V. (2007) *Am. J. Physiol. Cell Physiol.*, **292**, C157-C163.
6. Skulachev, V. P. (2002) *Ann. N. Y. Acad. Sci.*, **959**, 214-237.
7. Sherman, F. (2002) *Meth. Enzymol.*, **350**, 3-41.
8. Tolic-Norrelykke, I. M., Sacconi, L., Stringari, C., Raabe, I., and Pavone, F. S. (2005) *Curr. Biol.*, **15**, 1212-1216.
9. Yang, H., Ren, Q., and Zhang, Z. (2006) *FEMS Yeast Res.*, **6**, 1254-1263.
10. Westermann, B., and Neupert, W. (2000) *Yeast*, **16**, 1421-1427.
11. Pozniakovsky, A. I., Knorre, D. A., Markova, O. V., Hyman, A. A., Skulachev, V. P., and Severin, F. F. (2005) *J. Cell Biol.*, **168**, 257-269.
12. Sokolov, S., Knorre, D., Smirnova, E., Markova, O., Pozniakovsky, A., Skulachev, V., and Severin, F. (2006) *Biochim. Biophys. Acta*, **1757**, 1366-1370.
13. Lewandowska, A., Gierszewska, M., Marszalek, J., and Liberek, K. (2006) *Biochim. Biophys. Acta*, **1763**, 141-151.
14. Vieira, H. L., Boya, P., Cohen, I., El Hamel, C., Haouzi, D., Druillenec, S., Belzacq, A. S., Brenner, C., Roques, B., and Kroemer, G. (2002) *Oncogene*, **21**, 1963-1977.
15. Breckenridge, D. G., Germain, M., Mathai, J. P., Nguyen, M., and Shore, G. C. (2003) *Oncogene*, **22**, 8608-8618.
16. Fannjiang, Y., Cheng, W. C., Lee, S. J., Qi, B., Pevsner, J., McCaffery, J. M., Hill, R. B., Basanez, G., and Hardwick, J. M. (2004) *Genes Dev.*, **18**, 2785-2797.
17. Frank, S., Gaume, B., Bergmann-Leitner, E. S., Leitner, W. W., Robert, E. G., Catez, F., Smith, C. L., and Youle, R. J. (2001) *Dev. Cell*, **1**, 515-525.
18. Scheckhuber, C. Q., Erjavec, N., Tinazli, A., Hamann, A., Nystrom, T., and Osiewacz, H. D. (2007) *Nat. Cell Biol.*, **9**, 99-105.
19. Sun, M. G., Williams, J., Munoz-Pinedo, C., Perkins, G. A., Brown, J. M., Ellisman, M. H., Green, D. R., and Frey, T. G. (2007) *Nat. Cell Biol.*, **9**, 1057-1072.