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The yeast cAMP protein kinase Tpk3p is involved in the regulation of mitochondrial enzymatic content during growth

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Abstract

During aerobic cell growth, mitochondria must meet energy demand either by adjusting cellular mitochondrial content or by adjusting ATP production flux, allowing a constant growth yield. On respiratory substrate, the Ras/cAMP pathway has been shown to be involved in this process in the yeast *Saccharomyces cerevisiae*. We show that of the three cAMP protein kinase catalytic subunits, Tpk3p is the one specifically involved in the regulation of cellular mitochondrial content when energy demand decreases. In decreased energy demand, the Δ*tpk3* mitochondrial enzymatic content decreases leading to a subsequent decrease in the cellular growth rate. Moreover, enzymatic content decreases in the Δ*tpk3* isolated mitochondria, suggesting that the amount of cellular mitochondria is not affected, but rather that the mitochondria are modified. Our study points to an important decrease in the cytochrome *c* content in the Δ*tpk3* mitochondria, which leads to a decrease in the slipping process at the level of cytochrome-*c*-oxidase.
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Keywords: Yeast; Mitochondria; Oxidative phosphorylation; cAMP protein kinase; Slipping

1. Introduction

During growth of the eukaryotic cell, mitochondria must meet energy demand, which can vary by significant lengths. One can expect mitochondria to use two means in order to do so: (i) it is now well established that oxidative phosphorylations in the living cell are not functioning to their fullest, i.e., the maximal respiratory capacity is usually higher than the spontaneous respiratory rate; an increase in oxidative phosphorylation rate would thus allow an increase in energy production; (ii) an increase in energy demand could also be met by an increase in the amount of mitochondria per cell, with a constant oxidative phosphorylation steady state. The latter seems to be the way favored by the eukaryotic cell in order to adjust energy production. Indeed, numerous papers now describe modulations in the mitochondrial amount in response to modifications in energy demand (see Refs. [1,2] for review). More specifically, it has been shown in yeast that the respiratory capacity and the mitochondrial enzyme content simultaneously decrease when the growth rate slows down [3–5]. Moreover, the latter phenomenon is correlated with the maintenance of growth yield, i.e., the amount of respiratory substrate assimilated into biomass as compared to that used for respiration [5]. Similar decreases in respiration have been observed for various cultured mammalian cells before reaching confluence [6–8] and when cells were deprived of serum [9] or nitrogen [8]. Therefore, during growth, a modulation of the mitochondrial content seems to be involved in the long-term regulation of oxidative phosphorylation. In this respect, the *ccs1-1* yeast strain, mutated in the *ira2* gene encoding for an activating protein of the Ras-GTPase activity, was characterized in the late exponential phase by a higher cellular respiration associated with a higher cytochrome content [10–12]. Moreover, this mutant exhibits a number of characteristics of cells overactivated for the Ras/cAMP pathway, i.e., a nutrient starvation

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sensitivity and a reduced glycogen accumulation. Altogether, these observations suggest that the Ras/cAMP/PKA pathway is involved at least in the regulation of the cellular mitochondrial content in the transition phase.

Previous work from our laboratory has shown that, upon aerobic growth, the activity of the Ras/cAMP pathway is involved in the regulation of the cellular mitochondrial content upon the transition phase [13,14]. Yeast has three A kinase catalytic subunits, which have greater than 75% identity and are encoded by the *TPK* (*TPK1*, *TPK2* and *TPK3*) genes [15]. Although they are redundant for viability and functions such as glycogen storage regulation, the three A kinases are not redundant for other functions such as pseudohyphal growth, regulation of genes involved in trehalose degradation and water homeostasis as well as iron uptake, which are all regulated by Tpk2p [16–18]. Tpk1p is required for the derepression of branched chain amino acid biosynthesis genes that seem to have a second role in the maintenance of iron levels and DNA stability within mitochondria [19]. These data provide evidence for a specificity of signaling through the three PKA catalytic subunits. In order to elucidate a potential role of one or more of these subunits in the regulation of mitochondrial biogenesis in response to energy demand during growth, we investigated the role of each one of the TPK for this process. We show that the yeast protein kinase Tpk3p is specifically involved in the regulation of mitochondrial enzymatic content at the time of the transition phase. Mitochondria isolated from Δ*tpk3* cells in the transition phase are highly modified, and both enzymatic activities as well as the cytochrome *c* mitochondrial content are reduced. This leads to a decrease in the cytochrome-*c*-oxidase intrinsic uncoupling. Physiological consequences of these modifications are discussed.

2. Materials and methods

2.1. Yeast strains, culture medium and growth condition

The *S. cerevisiae* strains used in this study are the following:

BY4742: *MATα ura3Δ0 lys2Δ0 leu2Δ0 his3Δ0* (euroscarf)
Y11261: *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tpk1::kanMX4* (euroscarf)
Y11089: *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tpk2::kanMX4* (euroscarf)
Y15016: *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tpk3::kanMX4* (euroscarf).

Cells were grown aerobically at 28 °C in the following medium: 1% yeast extract (Difco), 1% bactopeptone, 0.1% potassium phosphate, 0.12% ammonium sulfate, containing 2% of D,L-lactate (w/v) (Aldrich), pH 5.5. Growth was measured at 600 nm in a Safas spectrophotometer (Monaco). Dry weight determinations were performed on samples of cells harvested throughout the growth period and washed twice in distilled water.

2.2. Oxygen consumption assays

The oxygen consumption of cells, spheroplasts permeabilized with nystatin [20] and isolated mitochondria was measured polarographically at 28 °C using a Clark oxygen electrode in a 1-ml thermostatically controlled chamber. Respiratory rates (JO_2) were determined from the slope of a plot of O_2 concentration vs. time. Respiration assays of growing cells were performed in the growth medium, except in the case of uncoupled respiration rate which was performed after cells were harvested in the following buffer: 2 mM magnesium sulfate, 1.7 mM sodium chloride, 10 mM potassium phosphate, 10 mM glucose and 100 mM ethanol, pH 6.8. Respiration assays of permeabilized spheroplasts were performed in the following buffer: Sorbitol 1 M, NaCl 1.7 mM, $MgSO_4$ 2 mM, KH_2PO_4 10 mM, EGTA 0.5 mM, BSA 0.1% m/v, pH 6.8. Respiration assays for isolated mitochondria (isolated by enzymatic method as described in Ref. [21]) were performed in the following buffer: 0.65 M mannitol, 0.36 mM EGTA, 5 mM Pi, 10 mM Tris–maleate pH 6.8. Respiratory substrates were used at 10 mM except for ethanol 100 mM, ADP 1 mM and CCCP 10 μM.

2.3. Obtainment of spheroplast lysates and enzymatic assays

The spheroplasts were obtained by enzymatic digestion of the cell wall with cytohelicase as described in Ref. [20]. Protein concentration was measured using the biuret method. When needed, spheroplasts lysates were obtained by directly adding 0.05% Triton X-100. Citrate synthase (EC 4.1.3.7) activity was measured as described in Ref. [5]. Oligomycin-sensitive F0F1 ATPase activity was measured at 28 °C and pH 8.4 as described in Ref. [22]. Cytochrome-*c*-oxidase activity was determined by measuring oxygen consumption in the presence of 5 mM ascorbate, 1 mM *N*,*N*,*N'*,*N'*-tetramethyl-1,4-phenylendiammoniumdichlorid (TMPD), 0.5 μg mg^{-1} protein antimycin A and 10 μM CCCP as described previously [20].

2.4. Cytochrome content determination

The cellular and mitochondrial content of $c+c_1$, b, and $a+a_3$ hemes was calculated as described in Ref. [5] taking into account the respective molar extinction coefficient values and the reduced minus oxidized spectra recorded using a dual beam spectrophotometer (Aminco DW2000).

2.5. Proteins extraction, electrophoresis and blotting

Isolated mitochondria protein solubilization was achieved in 1:1 (v/v) mixture of 10% SDS and sample

buffer (0.1 M Tris, 2% SDS, 2% β-mercaptoethanol, 25% glycerol, 0.002% bromophenol blue). After quantification with a Biorad kit, proteins were analysed by 12% SDS-PAGE performed according to the method of Laemmli [23]. After electrotransfer onto PVDF membranes (Amersham Pharmacia), blots were probed with the desired antibodies. The proteins were visualized by enhanced chemiluminescence (ECL), according to the manufacturer's (Amersham) instructions.

Antibodies: anti-Cox2p antibody was purchased from Molecular Probes, anti-cytochrome *c* antibody was purchased from Eurogentec and anti-cytochrome b–c_1 antibody was a gift from Dr. U. Brand.

2.6. Protonmotive force measurement on isolated mitochondria

Electrical transmembrane potential was evaluated in isolated mitochondria respiratory buffer by measurement of the fluorescence quenching of rhodamine 123 (1 μM) with an SFM Kontron fluorescence spectrophotometer [24]. Since the quenching varied linearly with the potassium diffusion potential, we were able to establish a straight standard relationship between the fluorescence signal and the membrane potential established at different values of potassium diffusion potential in the presence of $^{86}Rb^+$, allowing us to quantitate the electrical membrane potential on isolated mitochondria with the fluorescent dye rhodamine 123. Moreover, the ΔpH component of the protonmotive force was transformed in $\Delta\Psi$ by adding nigericin (25 ng mg pr^{-1}) and 10 mM KCl.

3. Results

In growing yeast cells, it has previously been shown that when the growth rate slows down, such as during the transition phase, both respiratory capacity and the mitochondrial enzyme content decrease [5]. This adjustment allows the cell to maintain its growth yield and has been shown to be related to the Ras/cAMP pathway [13,14]. Indeed, cells exhibiting an overactivated Ras/cAMP pathway are no longer able to adjust their mitochondrial content in this transition phase.

This suggests a role of the Ras/cAMP pathway in the regulation of mitochondrial biogenesis. In the yeast *Saccharomyces cerevisiae*, the three cAMP-dependent protein kinases, namely, Tpk1p, Tpk2p and Tpk3p, elicit numerous and often redundant functions (see Introduction). Because the Ras/cAMP pathway is involved in the regulation of mitochondrial biogenesis, and since we have shown that a Bcy1p mutant strain is affected in this regulation [14], we investigated the possible role of these kinases in the regulation of mitochondrial content during growth.

3.1. The mutant strain Δ*tpk3* exhibits a decreased growth rate in the transition phase.

Wild-type and *tpk* mutant strains (Δ*tpk*1, Δ*tpk*2 and Δ*tpk3*) were grown aerobically in rich medium with 2% lactate as carbon and energy source. In these conditions, growth rate is dependent on mitochondrial functions. In the exponential phase, all four strains exhibit a similar growth rate: μWT=0.22±0.01 h^{-1}; μΔ*tpk*1=0.22±0.02 h^{-1}; μΔ*tpk*2=0.22±0.01 h^{-1}; μΔ*tpk3*=0.21±0.02 h^{-1}. However, whereas in the transition phase, wild-type, Δ*tpk*1 and Δ*tpk*2 strains have a similar growth rate (not shown), the Δ*tpk3* strain exhibits a highly decreased growth rate (Fig. 1). Since growth in lactate medium is related to ATP synthesis through oxidative phosphorylation, we measured respiratory rates for these four strains in the different growth phases. Two distinct respiratory rates were considered, spontaneous respiratory rate (oxygen uptake during growth conditions) and uncoupled respiratory rate, which is measured in the presence of the protonophoric uncoupler CCCP and is an indication of the maximal respiratory rate achieved by the cells [25]. As previously shown [5], yeast cells exhibit a decrease in both spontaneous and maximal respiratory rates in the transition phase (Table 1). This decrease is enhanced in stationary phase and leads to very low respiratory rates. Table 1 shows that whereas wild-type, Δ*tpk*1 and Δ*tpk*2 cells exhibit comparable respiratory rates in all phases of growth, in the transition phase, the Δ*tpk3* strain has decreased respiratory rates. Indeed, spontaneous and maximal respiratory rates are decreased by 40% and 30% respectively. Cytochrome content was assessed in these strains during this growth phase. Wild-type, Δ*tpk*1 and Δ*tpk*2 cells exhibit comparable cytochrome content (not shown) but in the Δ*tpk3* cells, the amount of c+c_1

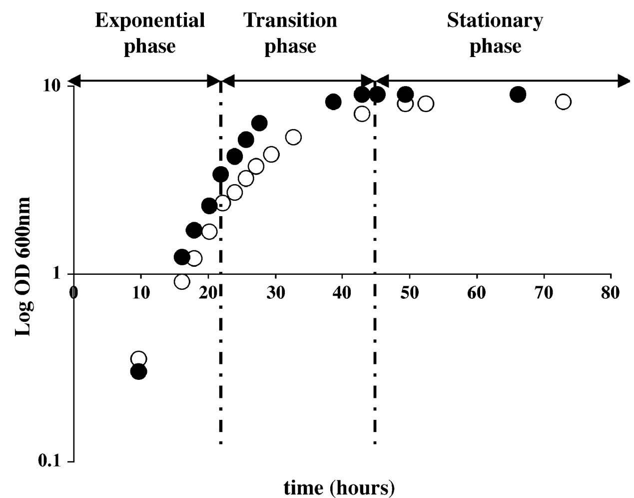

Fig. 1. Transition from exponential to stationary phase during the growth of wild-type (●) and Δ*tpk3* (○) cells in batch culture with lactate as substrate. Cells were grown aerobically in complete medium containing 2% (w/v) D,L-lactate. Growth was measured at 600 nm as described in Materials and methods.

Table 1
Respiratory rates during growth in wild-type and Δ*tpk* mutant strains

Strain	Exponential phase		Transition phase		Stationary phase	
	Spontaneous respiratory rate (nat O/min/mg dry weight)	Uncoupled respiratory rate (nat O/min/mg dry weight)	Spontaneous respiratory rate (nat O/min/mg dry weight)	Uncoupled respiratory rate (nat O/min/mg dry weight)	Spontaneous respiratory rate (nat O/min/mg dry weight)	Uncoupled respiratory rate (nat O/min/mg dry weight)
WT	209±20	313±13	116±17	253±21	24±4	101±11
Δ*tpk1*	200±22	316±3	105±10	246±17	21±4	93±10
Δ*tpk2*	199±8	318±20	105±10	237±20	21±4	92±13
Δ*tpk3*	188±10	311±5	65±12*	183±13*	20±4	95±12

Cells were grown aerobically in rich medium containing 2% lactate. Respiratory rate was determined as described in Materials and methods. Values are means±S.D. of at least three measurements performed on three independent cell cultures.

* Statistically significant difference.

cytochromes was significantly decreased compared to wild type, whereas a+a_3 and b were not significantly modified (Table 2). Since the amount of c_1 vs. the amount of b within the b–c_1 complex is likely to be constant, the decrease in c+c_1 mostly originates from a decrease in cytochrome c content. Interestingly, despite an overall decrease in cellular respiratory rates in the Δ*tpk3* strain during the transition phase, the only cytochrome affected is the cytochrome c.

3.2. Respiratory rates and mitochondrial enzyme contents are decreased in the Δtpk3 strain

To further characterize the modifications induced by the absence of the yeast cAMP-dependent protein kinase Tpk3p on the mitochondrial content in the transition phase, we investigated the functional properties of the mitochondria from both strains in permeabilized isolated spheroplasts. This approach has the advantage of preserving both the structural and functional integrities of the mitochondrial compartment within permeabilized cells. Three distinct respiratory states were considered: non-phosphorylating, phosphorylating and uncoupled. Fig. 2 shows that whichever respiratory state is considered, there is a two fold decrease in respiration in the Δ*tpk3* strain vs. the wild-type strain during the transition phase. Respiratory control ratio (phosphorylating vs. non phosphorylating rates) is about the same in both strains, which suggests that the mitochondrial oxidative phosphorylations in the Δ*tpk3* strain are achieved in a way comparable to that of the wild-type strain. NADH was chosen as a substrate in this study in order to avoid eventual kinetic control due to substrate transport across the mitochondrial membrane. Similar results were obtained with succinate as a substrate (not shown), which indicates that there is an overall decrease in mitochondrial content in the Δ*tpk3* strain.

However, since a decrease in mitochondrial respiratory activities could also be linked to a kinetic regulation at the respiratory chain level, we then assessed enzymatic activities on spheroplasts extracts. Citrate synthase is a matricial enzyme, commonly used as a marker of mitochondrial content within a cell. As shown in Table 3, this enzyme's specific activity is significantly decreased in the Δ*tpk3* strain. Also, in accordance with cytochrome content measurements, cytochrome-*c*-oxidase activity decreased by almost 50%. ATPase activity is decreased in the Δ*tpk3* strain vs. the wild-type strain. Glucose-6-phosphate dehydrogenase activity, used as a cytosolic marker, was unaffected in this strain, which suggests that the *TPK3* deletion specif-

Table 2
Cytochrome content in wild-type and Δ*tpk3* cells during the transition phase

Cytochrome content (pmol mg dry weight)	Wild type	Δ*tpk3*
a+a_3	10±1	9.5±0.5
b	24±5	18±5
c+c_1	37±4	25±4*

Wild-type and Δ*tpk3* cells were grown aerobically in rich medium containing 2% lactate. Cytochrome content was determined as described in Materials and methods. Values are means±S.D. of at least three measurements performed on three independent cell cultures.

* Statistically significant difference.

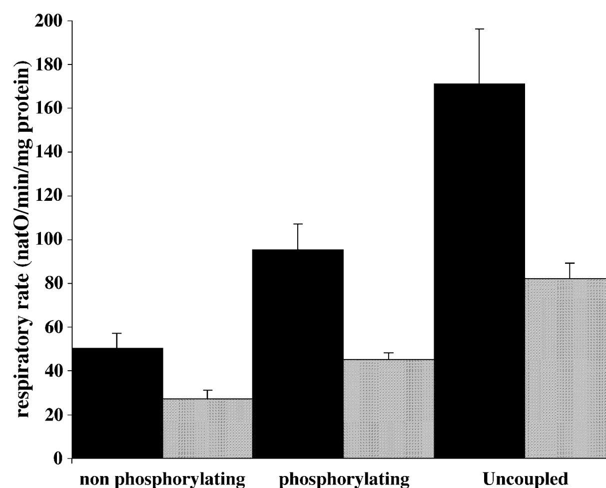

Fig. 2. NADH linked respiratory rate in isolated spheroplasts. Wild-type (black) and Δ*tpk3* (gray bars) cells were grown aerobically with 2% lactate as carbon source. «Transition Phase» spheroplasts were prepared as described in Materials and methods. Respiratory rate was measured in the presence of 10 mM NADH (non-phosphorylating), 10 mM NADH+2 mM ADP (phosphorylating), 10 mM NADH+10 μM CCCP (Uncoupled). Results are means of at least three independent experiments±S.D.

Table 3
Enzyme activities in both wild-type and Δ*tpk3* spheroplasts isolated in the transition phase

Enzyme specific activity (mU/mg cellular proteins)			
	Wild type	Δ*tpk3*	Wild type/ Δ*tpk3*
Citrate synthase	432±38	293±38*	1.5±0.3
Cytochrome-*c*-oxidase	266±21	150±14*	1.79±0.07
ATPase	440±20	315±21*	1.4±0.1
Glucose-6-phosphate dehydrogenase	81±13	90±13	0.92±0.16

Wild-type and Δ*tpk3* cells were grown aerobically in rich medium containing 2% lactate. Citrate synthase, ATPase and glucose-6-phosphate dehydrogenase activities were measured on spheroplasts lysates as described in Materials and methods. Cytochrome-*c*-oxidase activity was measured on permeabilized spheroplasts as described in Ref. [23]. Values are means±S.D. of at least three measurements performed on three independent spheroplasts preparations.

* Statistically significant difference.

ically affects the mitochondrial compartment under our experimental conditions.

3.3. Isolated mitochondria in the Δtpk3 strain exhibit different energetic parameters and protein content compared to wild type

The results presented above point to a decrease in the mitochondrial protein content in the Δ*tpk3* strain vs. the wild type during the transition phase. This could be interpreted in two different ways: either the mutant strain has fewer mitochondria with similar energetic parameters or the mitochondria in the mutant strain are highly modified, leading to a decrease in the considered enzymatic activities. In order to determine which of the above hypotheses was true for Δ*tpk3* cells, we isolated mitochondria from both the wild type and the Δ*tpk3* strains during the transition phase, and characterized their energetic parameters as well as their enzymatic content.

Yeast respiratory chain has no proton pumping complex I but exhibits NADH dehydrogenase activities on the external face of the internal membrane as well as on the internal face. In isolated mitochondria, NADH is thus a substrate of external NADH dehydrogenases, whereas ethanol is a substrate of the matricial alcohol dehydrogenase which produces internal NADH (substrate of internal NADH dehydrogenase). We examined energetic parameters of wild-type and mutant mitochondria using various respiratory substrates as shown in Table 4. Whatever the substrate and the respiratory state considered, isolated mitochondria from the mutant strain exhibit a decreased respiratory rate when compared to the wild type. Moreover, the higher the respiratory flux, the higher the decrease of the flux in the mutant mitochondria, i.e., an average of 24% decrease of the respiratory flux is measured in state 4 (non-phosphorylating) whereas an average of 39% decrease is measured in both state 3 (phosphorylating) and uncoupled state. ADP/O ratio, which represents oxidative phosphorylation yield, is not significantly modified in the Δ*tpk3* mitochondria (Table 4). As shown in Table 5, citrate synthase activity is decreased by one third in the mutant mitochondria. Other enzymatic markers such as oligomycin-sensitive ATPase and cytochrome-*c*-oxidase exhibit a comparable decrease. In order to determine the respiratory chain content of the isolated mitochondria in the Δ*tpk3* strain, we assessed cytochrome content in this strain. As shown in Table 6, there is a heterogeneous decrease in the cytochrome content of the Δ*tpk3* mitochondria when compared to the wild type. The cytochrome a-a_3 are not significantly different in the wild-type and Δ*tpk3* mitochondria, and the cytochrome b exhibits a slight decrease in the mutant mitochondria, whereas the cytochrome c-c_1 are decreased by one third. First, this correlates with the results obtained on whole cells from both strains (see Table 2). Second, if one assumes that

Table 5
Enzyme activities in both wild-type and Δ*tpk3* isolated mitochondria

Enzyme activities			
	wt	Δ*tpk3*	Wt/ Δ*tpk3*
ATPase (nmol min^{-1} mg^{-1})	1975±213	1150±141*	1.72
Citrate synthase (U mg^{-1})	2.5±0.3	1.7±0.1*	1.47
Cytochrome-*c*-oxidase (nat O/min/mg^{-1})	1372±85	960±10*	1.43

Citrate synthase, cytochrome-*c*-oxidase and ATPase activities were measured on isolated mitochondria as described in Materials and methods. Values are means±S.D. of at least three measurements performed on three independent mitochondria preparations.

* Statistically significant difference.

Table 4
Respiratory rates in mitochondria isolated from wild-type and Δ*tpk3* cells

Substrate	Non-phosphorylating		Phosphorylating		Uncoupled		ADP/O ratio	
	wt	Δ*tpk3*	wt	Δ*tpk3*	wt	Δ*tpk3*	wt	Δ*tpk3*
Ethanol	146±17	112±12*	339±31	205±22*	394±11	222±12*	1.7±0.1	1.7±0.1
NADH	184±13	141±14*	506±41	299±37*	838±55	534±25*	1.5±0.1	1.6±0.1
Glycerol-3-phosphate	178±34	142±16	292±50	172±17*	338±23	223±18*	1.5±0.1	1.4±0.1

Cells were grown aerobically with 2% lactate as carbon source. «Transition Phase» isolated mitochondria were prepared as described in Materials and methods. Respiratory rate was measured in the presence of 10 mM respiratory substrates (except for ethanol: 100 mM) (non-phosphorylating), +0.2 mM ADP (phosphorylating), +10 μM CCCP (uncoupled). Results are means of at least three independent experiments on three independent mitochondrial preparations±S.D.

* Statistically significant difference.

Table 6
Cytochrome content in both wild-type and Δ*tpk3* isolated mitochondria

Cytochrome content ($pmol\ mg^{-1}$)			Cytochrome c extraction ($pmol\ mg^{-1}$)			
	Wild type	Δ*tpk3*			Wild type	Δ*tpk3*
b	370±53	307±15	Pellet content	b	342±30	302±14
c-c_1	435±64	281±29*		c_1	158±9	158±28
a-a_3	138±30	127±21		a-a_3	137±15	124±9
			Supernatant	c soluble	279±32	178±6*

Cytochromes were measured on isolated mitochondria as described in Materials and methods. In order to quantitate cytochrome c, it was extracted by hypotonic lysis (0.02 M Tris–HCl pH 7) followed by a salt wash (0.5 M NaCl). After centrifugation (30,000×g, 30 min) only cytochrome c was released from the mitochondria (no other cytochrome could be detected by spectroscopy) and thus was quantitated in the supernatant fraction whereas the other cytochromes were quantitated in the pellet fraction. Values are means±S.D. of at least three measurements performed on three independent mitochondria preparations.

* Statistically significant difference.

the stoichiometry of the b–c_1 complex remains fixed (i.e., 2 cytochrome b hemes for one c_1), which is very likely, this result shows an important decrease in the cytochrome c amount in the mutant mitochondria. The hypothesis of a decrease in the cytochrome c content is correlated by three distinct results: the activity of the cytochrome-c-oxidase (see Table 5), the amount of cytochrome c released from the isolated mitochondria (see Table 6) and the amount of cytochrome c estimated by Western blot. Even though Western blotting is not a highly quantitative measurement, Fig. 3 and Table 6 clearly show that the amount of b–c_1 is comparable in both strains, whereas the amount of cytochrome c is highly decreased in the Δ*tpk3*. Thus, isolated mitochondria from the Δ*tpk3* strain are highly modified and these modifications are not a result of a homogeneous decrease in the oxidative phosphorylation complexes.

3.4. Intrinsic uncoupling is decreased in the Δtpk3 isolated mitochondria compared to wild type

Since the respiratory rates were decreased in the mutant mitochondria, we asked whether these mitochondria were able to maintain the same proton electrochemical potential difference (Δp) across the mitochondrial inner membrane. The Δp measured in both state 4 and state 3 showed no significant difference between the wild type and the Δ*tpk3* isolated mitochondria (140±5 mV, state 4 wild type; 138±6 mV, state 4 Δ*tpk3* and 125±3 mV, state 3 wild type; 123±4 mV, state 3 Δ*tpk3*). These transmembranal electrical potentials were measured fluorimetrically with the dye rhodamine 123 and are comparable since the isolated mitochondria volumes are not significantly different (not shown). This raises the question as to whether the flux–force relationships are similar in both strains. Indeed, under non-phosphorylating conditions, the same protonmotive force is maintained in the mutant strain for a lower respiratory rate. Under these conditions, the respiratory rate is directly related to the compensation of the energy waste. There are two kinds of energy waste: (1) the proton leak which is directly related to the membrane permeability to protons, related to the Δp, and (2) the proton slip, which is due to a modification in the efficiency of proton pumping. Proton slipping is independent of the membrane proton conductance and depends not only on the Δp but also other forces such as the redox potential and all the kinetic properties of the proton pumps involved [26,27]. Indeed, numerous previous studies have shown a non linear relationship between Δp and state 4 respiration at high potentials, which can be explained by an increase in intrinsic uncoupling at the level of respiratory chain proton pumps themselves such that some catalytic cycles of the complexes might be able to occur in the absence of stoichiometric proton translocation across the membrane [26,27]. In order to determine whether the non-phosphorylating flux–force relationships are modified in the Δ*tpk3* strain, we performed a respiratory rate/Δp titration with a b–c_1 complex inhibitor, namely myxothiazol on mitochondria isolated from both strains. As shown on Fig. 4, the relationship between JO_2 and Δp in the ohmic region of the titration is similar in both strains, which shows that the membrane proton leakage properties are not modified in the Δ*tpk3* strain. However, in the non-ohmic region, where one can measure an increase in respiratory rate for a constant protonmotive force, the maximal respiratory rate is lowered in the mutant mitochondria. This

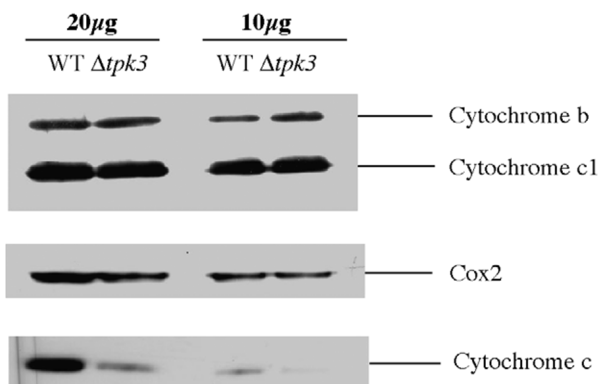

Fig. 3. Protein content in the wild-type and Δ*tpk3* isolated mitochondria. Mitochondria were isolated from the WT and the Δ*tpk3* strain in the transition phase as described in Materials and methods. Proteins were isolated and quantified as described in Materials and methods. This result is representative of three such experiments.

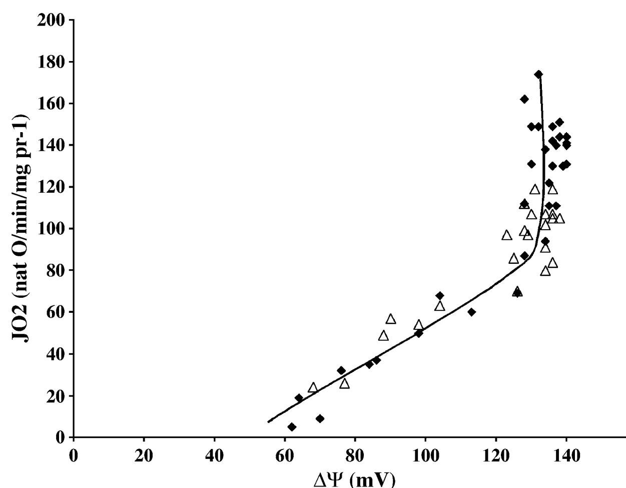

Fig. 4. $JO_2/\Delta p$ relationship in the wild-type and Δ*tpk3* isolated mitochondria. Mitochondria were isolated from the WT and the Δ*tpk3* strains in the transition phase as described in Materials and methods. JO_2 and Δp were titrated using myxothiazol as a respiratory chain inhibitor. Δp was measured with the fluorescent dye rhodamine 123 as described in Materials and methods. Results are representative of three such experiments.

can only be interpreted as a large decrease in proton slipping in these mitochondria. Since one of the potential proton slipping sites is the cytochrome oxidase [28] and since proton slipping depends on the electron flux, the decrease in proton slipping measured in the Δ*tpk3* isolated mitochondria could be due to the decrease in the amount of cytochrome *c*, which decreases electron flux through cytochrome oxidase.

4. Discussion

Previous studies from our laboratory have shown that when reaching the transition phase, cells adjust their mitochondria content in order to maintain a constant growth yield [5]. This process is highly related to the activity of the Ras/cAMP signaling pathway. We have shown that an increase in the activity of this pathway induces an increase in the mitochondrial content, leading to a loss of the adjustment of mitochondria activity to the growth yield [13]. Briefly, in yeast, the Ras/cAMP pathway is as follows: Cdc25p catalyzes the conversion of GDP-Ras1p and 2p into GTP-Ras1p and 2p, which are the activators of Cyr1p, the adenylate cyclase. Cyr1p catalyzes cAMP synthesis. The intracellular concentration of cAMP thus depends on the respective activities of Cyr1p and the phosphodiesterases Pde1p and 2p. High cAMP concentrations promote the dissociation of the regulatory subunit (Bcy1p) from the catalytic subunits (Tpk1,2,3p), activating the catalytic subunits of the protein kinase A, which phosphorylate a variety of substrates. We have shown that, from the top of the pathway down to Bcy1p, any mutation leading to an overactivation of this pathway leads to an increase in the mitochondrial content [14]. This shows that the signaling of this pathway to mitochondrial content regulation goes through the PKA, i.e., Tpk1p, 2p and 3p in yeast. These three catalytic subunits of the PKA have redundant functions. However, specificity in their respective signaling has recently been proposed [16–19]. In this paper, we show that Tpk3p is the PKA catalytic subunit specifically involved in the regulation of mitochondrial content in the transition phase. This further enforces the hypothesis of a specificity in the signaling of the distinct isoforms of the PKA.

Compared to wild type, the Δ*tpk3* strain shows a decrease in the spontaneous respiration rate as well as the growth rate in the transition phase. This shows that in the exponential phase, the Tpk3p is not involved in the regulation of mitochondrial content. Thus, this PKA is involved in the cellular response leading to a decrease in the growth rate when reaching the stationary phase (i.e., transition phase). Energetic characterization of spheroplasts isolated from the wild-type or Δ*tpk3* strain correlates with results obtained on isolated mitochondria, showing that when mitochondria are isolated from either strain, they are representative of the cellular mitochondrial fraction. Indeed, whichever enzymatic activity was measured, a comparable enrichment factor is obtained when isolated mitochondria are compared to permeabilized spheroplasts. Thus, for the sake of simplicity, energetic parameters will be discussed below at the isolated mitochondria level. Phosphorylating and uncoupled respiratory rates and enzymatic activities (i.e., citrate synthase, cytochrome-*c*-oxidase and oligomycin-sensitive ATPase) are decreased (around 40%) in the mutant compared to the wild type. However, when cytochrome content is measured in both strains, there is clearly a modification in the composition of the respiratory chain. Whereas the cytochrome a-a_3, b and c_1 are not significantly affected in the mutant strain, the *c* content is significantly decreased (40%). The decrease in phosphorylating and uncoupled respiratory rates, as well as cytochrome-*c*-oxidase activity, originates in this decrease in cytochrome *c* content.

One surprising result relative to the mutant mitochondria is that the non-phosphorylating respiratory rate is significantly lower than for the wild-type mitochondria. The non-phosphorylating respiratory rate is controlled by energy waste which has two distinct phenomenological origins: the passive membrane permeability to protons (i.e., proton leakage) and modification in the apparent stoichiometry H^+/e^- of the cytochrome-*c*-oxidase (slipping). Proton leakage is dependent on the protonmotive force as well as the inner membrane proton conductance while slipping depends on the forces (redox potential and protonmotive force) as well as kinetic constraints [27,28]. Since in the mutant strain, the protonmotive force measured on isolated mitochondria is comparable to the wild-type one and the membrane lipid composition is likely to be the same, the difference in respiratory rates measured could be linked to a decrease in the amount of cytochrome *c* in the Δ*tpk3* strain leading to a

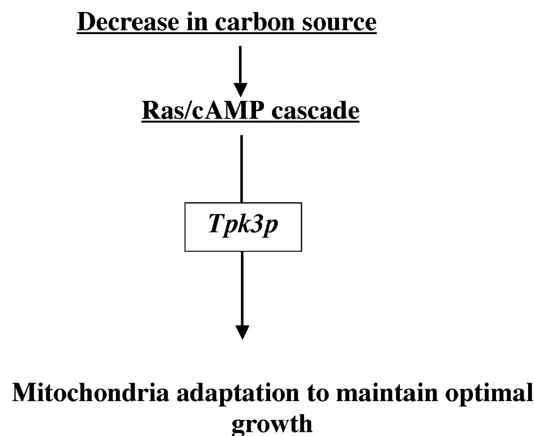

Fig. 5. The role of Tpk3 in the regulation of mitochondrial adaptation.

decrease in the slipping process [27]. This is confirmed by the inhibitor titration of both respiratory rate and protonmotive force under non-phosphorylating conditions (see Fig. 4). Both wild-type and Δ*tpk3* mitochondria have comparable $JO_2/\Delta p$ relationships in the ohmic region of this titration, which is in agreement with the hypothesis formulated above of a comparable leak in both kinds of mitochondria. However, the non-ohmic region almost completely disappears in the Δ*tpk3* strain, which implies an important decrease of the slipping process in the Δ*tpk3* mitochondria. It is worth noting that the consequence of such a decrease is that non-phosphorylating respiratory rate is directly proportional to cytochrome-*c*-oxidase activity. This has previously been described in liver mitochondria isolated from different rat models [29]. This confirms that an important part of the oxygen consumption under non-phosphorylating conditions is due to slipping which, in yeast, is essentially controlled by cytochrome-*c*-oxidase activity.

Under phosphorylating conditions, the oxidative phosphorylation yield is comparable in the wild-type and mutant mitochondria. This is a peculiar condition where ADP is saturating on isolated mitochondria. In situ (growing cells), the steady state of oxidative phosphorylation is situated in between state 3 (saturating ADP) and state 4 (non-phosphorylating). Thus, one could wonder about the growth yield on non-fermentable substrate in the Δ*tpk3* strain (i.e., conditions where ATP is only produced via oxidative phosphorylation) in the transition phase. Our results (Fig. 1 and Table 1) clearly show that the amounts of oxygen consumed to increase the biomass to the same extent in both strains is comparable (57 μat O/mg dry weight for the wild type and 54 μat O/mg dry weight for the Δ*tpk3*). Since previous studies from our laboratory have shown that lactate combustion under these conditions is complete [5], we can consider that growth yield (produced biomass/consumed lactate) is identical in the wild-type and Δ*tpk3* strains during the transition phase.

This work confirms that the mitochondrial content adjustment during growth is related to the activity of the ras/cAMP pathway. More specifically, in the transition phase, the yeast Tpk3p plays a major role in this process. It can thus be hypothesized that when the carbon source in the medium decreases, *tpk3*p plays a key role in mitochondrial adaptation leading to an optimal growth rate (see Fig. 5). This paper also confirms that as long as growth can be modulated, there is a clear homeostasis in growth yield ensured by mitochondrial activity.

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