A cytochrome *c* mutant with high electron transfer and antioxidant activities but devoid of apoptogenic effect

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A cytochrome *c* mutant lacking apoptogenic function but competent in electron transfer and antioxidant activities has been constructed. To this end, mutant species of horse and yeast cytochromes *c* with substitutions in the N-terminal α -helix or position 72 were obtained. It was found that yeast cytochrome *c* was much less effective than the horse protein in activating respiration of rat liver mitochondria deficient in endogenous cytochrome *c* as well as in inhibition of H_2O_2 production by the initial segment of the respiratory chain of intact rat heart mitochondria. The major role in the difference between the horse and yeast proteins was shown to be played by the amino acid residue in position 4 (glutamate in horse, and lysine in yeast; horse protein numbering). A mutant of the yeast cytochrome *c* containing K4E and some other 'horse' modifications in the N-terminal α -helix,

proved to be (i) much more active in electron transfer and antioxidant activity than the wild-type yeast cytochrome *c* and (ii), like the yeast cytochrome *c*, inactive in caspase stimulation, even if added in 400-fold excess compared with the horse protein. Thus this mutant seems to be a good candidate for knock-in studies of the role of cytochrome *c*-mediated apoptosis, in contrast with the horse K72R, K72G, K72L and K72A mutant cytochromes that at low concentrations were less active in apoptosis than the wild-type, but were quite active when the concentrations were increased by a factor of 2–12.

Key words: antioxidant effect, apoptosis, mutagenesis, respiration.

INTRODUCTION

Cytochrome *c* was first described in 1930 by David Keilin [1]. For more than 60 years it was assumed that cytochrome *c* was simply a small, stable, water-soluble protein that operated as a carrier of electrons from the bc_1 complex to cytochrome oxidase in the respiratory chain. However, the recent discovery that cytochrome *c* is also involved in activation of programmed cell death (apoptosis) [2–5] renewed interest in this protein.

Subsequent studies of antioxidant defence mechanisms suggested additional roles for cytochrome *c*, namely: (i) oxidation of superoxide back to O_2 by cytochrome *c* desorbed from the inner mitochondrial membrane; and (ii) specific inhibition by extramitochondrial cytochrome *c* of the generation of reactive oxygen species (ROS) in the initial segment of the respiratory chain [6,7]. Thus it became clear that cytochrome *c* is polyfunctional. Therefore the problem arose: which parts of the cytochrome *c* molecule are responsible for each of these functions?

Moreover, it was found that the cytochrome *c*-mediated apoptotic mechanism is paralleled by cytochrome *c*-independent pathways also leading to programmed cell death (for review see [6]). The contribution of these mechanisms to various apoptotic cases might be elucidated by a knock-in study, namely by replacing the cytochrome *c* gene with a mutated gene encoding cytochrome *c* deprived of apoptogenic activity but still retaining its other functions.

The first indication that the electron-transfer and apoptogenic functions of cytochrome *c* could be separated was obtained when it was found that a cytochrome *c* derivative containing Zn^{2+} instead of Fe^{2+} , which is inactive as an electron carrier, still induces apoptosis [4]. In the same paper, it was shown that the yeast cytochrome *c* lacks all apoptogenic activity [4], although it is known to function (but with different efficiency) in the respiratory chain of mammalian mitochondria [8]. The yeast cytochrome *c* is also less active in inhibiting mitochondrial ROS formation [7].

The inefficiency of yeast cytochrome *c* in the apoptotic cascade was quite recently found to be due to two different reasons [9]. First, the ϵ -amino group of Lys⁷² is trimethylated in yeast but not in animal cytochrome *c*. The expression of horse cytochrome *c* in yeast results in formation of the trimethylated protein, which proved to be ineffective in apoptosis. On the other hand, the yeast cytochrome *c* expressed in *Escherichia coli* is also inactive even though Lys⁷² is not trimethylated. This means that there is something besides trimethylation in the yeast cytochrome *c* structure that prevents it from being operative in apoptosis. This is hardly surprising if we take into account that (i) approx. 45% of the amino acid residues in the yeast and mammalian cytochromes *c* are different [10], and (ii) the affinity of the mammalian cytochrome *c* for apoptosis protease-activating factor-1 (Apaf-1), its partner in the apoptotic mechanism, is extremely high (K_a is approx. $10^{11} M^{-1}$ [11]), a fact pointing to a very high specificity of interaction between these two proteins and,

Abbreviations used: AIF, apoptosis-inducing factor; Apaf-1, apoptosis protease-activating factor-1; DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-p-nitroanilide; ROS, reactive oxygen species.

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hence, strict requirements that their structures must precisely fit each other.

In the present study we characterize the contribution of various amino acid residues to the respiratory, antioxidant and apoptogenic functions of cytochrome *c*. So far, we have mainly dealt with the first α -helix of cytochrome *c*, where the most dramatic differences in amino acid sequence between mammalian species and yeast occur [10].

Until recently, studies on recombinant cytochromes *c* were impeded by lack of a convenient system of expression for obtaining preparative amounts of the protein. In the eukaryotic systems the yield was very low, whereas expression in prokaryotes was useless, since bacterial haem lyases are inefficient in covalent binding of haem with ectopic apocytochromes. For these reasons, we employed a construct allowing co-expression in *E. coli* of native or mutant cytochromes *c* and yeast haem lyase [12,13]. Among the mutants studied, a yeast cytochrome *c* mutant was found that was still inactive in apoptosis but much more active in the respiratory-chain electron transfer as well as in an antioxidant effect than the wild-type yeast cytochrome *c* when tested in mammalian mitochondria. The mutant in question was a 'yeast $\rightarrow$ horse' hybrid having five 'horse' substitutions in the N-terminal α -helix and lacking, like the horse cytochrome *c*, five N-terminal amino acids.

While the present work was in progress, a publication by Yu et al. [14] appeared which claimed that the horse K72A cytochrome *c* mutant, like the K72-trimethylated protein, 'showed no detectable caspase 9-activating activity at all'. The electron transfer and antioxidant activities of the mutant were not studied. On the face of it, the K72A cytochrome *c* mutant looks like a candidate for a knock-in study if it is competent in other (non-apoptotic) functions. Therefore, in addition to the 'yeast $\rightarrow$ horse' hybrid cytochromes *c*, we also studied the horse K72A mutant and three other K72-substituted mutants. We found that these mutant cytochromes *c* are quite active in the non-apoptotic functions. Unfortunately, all of them proved also to be active in apoptosis when added at 2–12-fold higher concentrations than the wild-type protein, and therefore they cannot be used in the suggested knock-in study, in contrast with the yeast $\rightarrow$ horse mutant described above.

EXPERIMENTAL

Production of mutants and purification of proteins

To select mutants that might be interesting for the present study, database searches and alignments of cytochrome *c* sequences were performed, using the GCG package [15]. The WHAT IF program [16] was used to visualize the three-dimensional structures of cytochromes *c* and to assess the environments of amino acid residues [16].

To express corresponding mutant cytochrome *c* genes in *E. coli*, oligonucleotide primers were used taking into account the frequency of the presence of various codons in the *E. coli* genome. The Quick Change Site-Directed Mutagenesis Kit (Stratagene) was used for the experiments. The mutagenesis procedure included synthesis of the full-length single-stranded plasmid DNA with the thermostable DNA polymerase *Pfu*, using two complementary primers that contained the necessary substitutions. Each primer was composed of 30–35 nt. The substitutions were localized in the middle part of the sequence and were flanked by approx. 15 nt. All of the mutant genes were sequenced to confirm their primary structure.

Cytochrome *c* was expressed in *E. coli* JM 109 cells [17] and purified from the supernatant, obtained after disruption of the cells by a French press and centrifugation (50 000 *g* for 20 min at

4 °C), using ammonium sulphate fractionation and ion-exchange chromatography on a Mono-S column. The final purity of the cytochrome *c* was > 98 % according to SDS/PAGE.

Preparation of mitochondria and mitoplasts

Rat liver mitochondria were prepared as described elsewhere [18]. To disrupt the outer mitochondrial membrane, mitochondria (3–4 mg of protein/ml) were incubated in hypotonic medium (5 mM Mops/KOH, 25 mM sucrose and 1 mM EGTA, pH 7.4; 0 °C) for 5 min with gentle stirring. The medium was then supplemented with 125 mM KCl and incubated for 25 min, at 0 °C, to desorb cytochrome *c*. Following this procedure, the suspension was diluted (1.5-fold) with cold medium containing 5 mM Mops/KOH, 150 mM KCl and 1 mM EGTA, pH 7.4, and centrifuged (14 000 *g* for 10 min at 4 °C). Mitoplasts were suspended in a buffer containing 5 mM Mops/KOH, 0.25 M sucrose, 1 mM EGTA and 3 mg/ml BSA, pH 7.4, centrifuged again under the same conditions and resuspended in the same buffer to obtain 150–180 mg of protein/ml.

Rat heart mitochondria were prepared as described previously [19], but treatment with H₂O₂ and aminotriazole was omitted.

Measurement of cytochrome *c* activities

Activity of cytochrome *c* as a respiratory-chain electron carrier was monitored by measuring stimulation of respiration of cytochrome *c*-depleted rat liver mitoplasts by the addition of cytochrome *c*. Oxygen consumption was measured with a Clark-type O₂ electrode. The incubation mixture contained 5 mM Mops/KOH, 0.25 M sucrose, 0.5 mM EGTA, 5 mM succinate, 2 μ M rotenone and mitoplasts (2–3 mg of protein/ml; pH 7.4; 30 °C). The respiration rate was measured at the second minute after the addition of cytochrome *c*.

Superoxide oxidase activity of cytochrome *c* was measured as the rate of the cytochrome *c* reduction by xanthine in the presence of xanthine oxidase (for details see [7]).

The antioxidant effect of cytochrome *c* under conditions of reverse electron transfer via Complex I was studied in rat heart mitochondria as described previously [7]. The rate of H₂O₂ production was measured using scopoletin and horseradish peroxidase. The scopoletin response was calibrated using the glucose oxidase system [20]. The incubation mixture contained 10 mM Mops/KOH, 0.15 M sucrose, 0.5 mM EDTA, 6 mM succinate and rat heart mitochondria (1 mg of protein/ml; pH 7.3; 25 °C).

Apoptogenic activity of various cytochrome *c* species was determined as described by Kluck et al. [9]. Briefly, the cytosolic extracts from *Xenopus* eggs or from mouse T-hybridoma A1.1 cells were preincubated with cytochrome *c*, 2 mM ATP, creatine kinase and phosphocreatine for 10 or 30 min at 22 °C. Activity of caspases was measured in the extracts by monitoring the hydrolysis of a colorimetric substrate, 40 μ M *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA) at 22 °C. The *Xenopus* and A1.1 extracts contained 40 and 20 mg of protein/ml respectively.

The horse cytochrome *c* (0.25 μ M) was found to induce maximal activation of caspases in *Xenopus* extract within 10 min. Increasing the preincubation time to 30 min and the cytochrome *c* concentration to 100 μ M did not cause further activation. In line with an earlier observation [9], maximal caspase stimulation in the A1.1 extract was reached at higher concentrations of cytochrome *c* (5–10 μ M) and longer preincubation (30 min). The maximal DEVD-pNA cleaving activity was 2.2 μ mol/h per ml in the *Xenopus* extract and 0.8 μ mol/h per ml in the A1.1 extract.

RESULTS AND DISCUSSION

Mutant cytochromes *c*

Our preliminary comparative analysis of cytochromes *c* from yeast and approx. 40 other (predominately mammalian) species showed that the first α -helix contains a number of exposed amino acids that are conserved through mammalian species but are different in the yeast cytochromes *c*. In particular, amino acids Asp², Glu⁴, Lys⁷, Lys⁸ and Val¹¹ (horse cytochrome *c* numbering) found in horse and other animal cytochromes *c* are replaced by serine, lysine, alanine, threonine and lysine respectively in yeast. These amino acids form a hydrophilic outer surface on the first α -helix and might play a role in interactions of cytochrome *c* with its partners. Moreover, the yeast iso-1-cytochrome *c* contains five additional amino acid residues at the N-terminus [10]. We constructed a mutant of the yeast iso-1-cytochrome *c* that contained the 'horse' amino acids in the above-mentioned positions and lacked the five extra N-terminal amino acid residues. Similarly, a horse mutant protein was constructed containing some components of the yeast sequence in the same region of the protein.

Figure 1 shows the N-terminal sequences of the horse cytochrome *c*, the yeast iso-1-cytochrome *c*, and three mutant cytochromes *c*, namely (i) the horse cytochrome *c* with E4K, V11K and Q12T substitutions (horse $\rightarrow$ yeast), (ii) the yeast iso-1-cytochrome *c* with five N-terminal amino acid residues removed, containing S2D, K4E, A7K, T8K and K11V substitutions (yeast $\rightarrow$ horse), and (iii) the yeast iso-1-cytochrome *c* containing only one substitution, K4E [yeast $\rightarrow$ horse(K4E)]. Moreover, for the reasons described in the Introduction section, four horse mutants with changes in position 72 were prepared, i.e. K72R, K72A, K72L and K72G.

Efficiency in the respiratory chain

We first compared mutant cytochromes *c* for their ability to supplement the respiratory chain in the cytochrome *c*-depleted rat liver mitoplasts. Figure 2 shows that both the horse and yeast

Figure 2 Stimulation of the respiratory chain of cytochrome *c*-deficient rat liver mitoplasts by various cytochrome *c* species

The degree of stimulation of oxygen consumption by added cytochrome *c* is shown (fold increase). *y(r)* corresponds to recombinant yeast cytochrome *c*. Other abbreviations are defined in the legend to Figure 1. The rate of oxygen consumption without added cytochromes was 3 nmol of O₂/min per mg of protein. Data from a single experiment representative of 5–8 experiments are shown.

cytochromes *c*, when added at high concentrations, stimulated respiration by a factor of 5–6. However, at low concentrations, the horse protein appears to be much more efficient than the yeast protein. It should be mentioned that such a difference between the efficiencies of low amounts of two cytochromes took place only if we employed a gentle procedure of mitoplast formation described in the preceding section. Nevertheless, under any conditions used, the concentration causing half-maximal effect for the horse cytochromes *c* was at least 3-fold lower than that for the yeast cytochrome.

All 'hybrid' cytochromes *c* listed in Figure 1 had activities lower than the horse protein and higher than the yeast protein (Figure 2 and Table 1). The efficiency of the horse $\rightarrow$ yeast

1	2	3	4	5	6	7	8	9	10	11	12	13	14	
G	D	V	E	K	G	K	K	I	F	V	Q	K	C	<i>h</i>
G	D	V	K	K	G	K	K	I	F	K	T	K	C	<i>h</i> $\rightarrow$ <i>y</i>
G	D	A	E	K	G	K	K	L	F	V	T	R	C	<i>y</i> $\rightarrow$ <i>h</i>
TEFKA	G	S	E	K	G	A	T	L	F	K	T	R	C	<i>y</i> $\rightarrow$ <i>h</i> (K4E)
TEFKA	G	S	K	K	G	A	T	L	F	K	T	R	C	<i>y</i>

Figure 1 N-terminal sequences of various cytochrome *c* species studied in the present paper

Black and white squares represent amino acid residues specific for the horse and yeast cytochromes *c* respectively. *h*, horse cytochrome *c*; *y*, yeast cytochrome *c*; *h* $\rightarrow$ *y*, horse cytochrome *c* where three amino acids are replaced by those of the yeast cytochrome *c*; *y* $\rightarrow$ *h*, yeast cytochrome *c* with five amino acids from the horse sequence and without the five N-terminal amino acids; *y* $\rightarrow$ *h*(K4E), yeast cytochrome *c* with the indicated substitution.

Table 1 Efficiency of various cytochrome *c* species in respiratory-chain electron transfer and in the inhibition of H₂O₂ formation by mitochondria

Abbreviations are defined in Figures 1 and 2. Each cytochrome *c* species was studied in 5–8 separate experiments. *P*, probability that the difference observed was occasional.

Cytochrome <i>c</i> species	Concentration causing half-maximal effect (nM)			
	Stimulation of respiration		Inhibition of H ₂ O ₂ formation	
	Mean ± S.E.M.	<i>P</i>	Mean ± S.E.M.	<i>P</i>
<i>h</i>	50 ± 10		190 ± 10	
<i>y</i>	900 ± 210	< 0.01 (compared with <i>h</i>)	400 ± 40	< 0.01 (compared with <i>h</i>)
<i>y</i> → <i>h</i>	220 ± 60	< 0.01 (compared with <i>y</i>)	240 ± 20	< 0.02 (compared with <i>y</i>)
<i>y</i> → <i>h</i> (K4E)	180 ± 40	< 0.01 (compared with <i>y</i>)	210 ± 10	< 0.01 (compared with <i>y</i>)
<i>h</i> → <i>y</i>	480 ± 190	< 0.01 (compared with <i>h</i>)	370 ± 20	< 0.01 (compared with <i>h</i>)
<i>y</i> (<i>r</i>)	1290 ± 180	> 0.10 (compared with <i>y</i>)	420 ± 60	> 0.10 (compared with <i>y</i>)

cytochrome *c* mutant was closer to that of the yeast protein, whereas the efficiency of the yeast → horse mutants was closer to that of the horse cytochrome *c*. The activity of the wild-type yeast cytochrome *c* expressed in *E. coli* was similar to that of the native yeast protein (Figure 2 and Table 1).

The data indicate that glutamate at position 4 in the horse cytochrome *c* (instead of lysine in the yeast protein) strongly contributes to the higher efficiency of the horse cytochrome *c* in mammalian mitoplasts, whereas amino acids at positions 2, 7, 8 and 11, as well as the additional five amino acids at the N-terminus of the yeast cytochrome *c*, are much less important.

The trimethylation of Lys⁷², consistent with previous observations [8,21], is without any effect (compare native yeast cytochrome *c* with recombinant yeast protein expressed in bacteria, where trimethylation did not occur). Substitutions K72A and K72G, as well as K72R and K72L (results not shown), did not influence the electron transfer efficiency of the horse cytochrome *c*.

Antioxidant efficiency

Besides participating in respiratory-chain electron transfer, cytochrome *c* takes part in the antioxidant defence of the cell. Being desorbed from the inner mitochondrial membrane to the inter-membrane space, cytochrome *c* can operate as a superoxide oxidase [6]. We compared the superoxide oxidase activities of the horse and yeast cytochromes *c* in solution using the xanthine–xanthine oxidase system to generate superoxide. The activities were the same in the two cytochromes *c* (results not shown). For this reason, the mutant cytochromes were not tested in this respect.

On the other hand, the cytochrome variants studied had different efficiencies when tested for another antioxidant effect, namely, inhibition of ROS formation in mitochondria carrying out reverse electron transfer via Complex I. As shown in Table 1, the range of activities of various cytochromes *c* as inhibitors of ROS formation resembles that of the same proteins as respiratory-chain electron carriers. However, the Zn²⁺ derivative of horse cytochrome *c*, being completely ineffective as an electron carrier, still showed some antioxidant activity. This activity was recently shown to be due to a specific effect of extramitochondrial cytochrome *c* that did not require disruption of the outer mitochondrial membrane and was not inhibited by superoxide dismutase [7]. It was hypothesized that the antioxidant effect is due to binding of cytochrome *c* to a putative receptor localized on the outer surface of the outer mitochondrial membrane. Such binding was assumed to produce a signal transmitted to Complex I [7].

Apoptogenic efficiency

The cytochrome *c* mutants were tested in the cell-free systems used to study apoptogenic activity of this protein. To this end, cytochrome *c*-induced caspase activation was studied. The horse → yeast mutant containing the 'yeast' amino acids in positions 4, 11 and 12, the remaining residues being identical to the native horse cytochrome *c*, was found to be still fully active in apoptosis, just like the wild-type horse cytochrome *c* expressed in *E. coli* (Figure 3A). On the other hand, the yeast → horse mutant, which is even more active in the respiratory-chain electron transfer and antioxidant effect than the horse → yeast mutant, was inactive (even when added in 400-fold excess) in inducing apoptosis in both the amphibian and mammalian cell-free systems (Figure 3A). The effect of substitutions in position 72 on apoptogenic activity of the horse cytochrome *c* in the amphibian system is shown in Figure 3(B). The K72R substitution decreased the activity only slightly, whereas the K72G and especially the K72A and K72L substitutions resulted in more pronounced inhibition. The decrease in apoptogenic activity was approx. 2-fold for K72R, 3–4-fold for K72G and approx. 12-fold for K72A and K72L. Nevertheless, all the horse cytochromes *c* with changes in position 72 showed the same caspase activation as the wild-type protein if high cytochrome concentrations were used. Similar data were obtained in the mammalian system.

The above results are at variance with the conclusion of Yu et al. [14] that the horse K72A cytochrome *c* mutant has no pro-apoptotic activity at all. Most probably, Yu et al. [14] failed to observe apoptogenic activity of this mutant protein since the concentration range of the added K72A cytochrome *c* was too narrow (the maximal concentration tested was less than 10-fold higher than that of the wild-type cytochrome *c* [14]). Thus the K72A mutant can hardly be a candidate for *in vivo* knock-in studies on the role of cytochrome *c*-linked apoptosis. On the other hand, the yeast → horse mutant, showing no apoptogenic activity even when added in 400-fold excess, looks much more promising.

In this context, a recent publication by Joza et al. [22] should be discussed. They showed that an alternative cytochrome *c*-independent apoptotic pathway, mediated by apoptosis-inducing factor (AIF), is required for the first wave of apoptosis inherent in the development of mouse embryos at the stage of cavitation of embryoid bodies. This conclusion was made based on an AIF knock-out study. Since no function of AIF other than activation of apoptosis is known, the authors concluded that the AIF-mediated apoptosis is involved at this stage of ontogenesis.

As to the cytochrome *c* knock-out embryos, they die a week later as was found by Li et al. [23]. This may be because

Figure 3 Caspase activation by various cytochrome *c* species in cell-free *Xenopus* and mammalian systems (A and B) and the *Xenopus* system (B)

h(Y97L), h(K72R), h(K72L), h(K72A), and h(K72G) correspond to horse cytochromes *c* with the indicated substitutions. Other abbreviations are defined in the legends to Figures 1 and 2. Caspase activity (measured as the rate of DEVD-pNA cleavage) equivalent to 2.2 and 0.8 μmol of DEVD-pNA/h per ml corresponds to 100% in the *Xenopus* extract and in the mammalian extract respectively.

cytochrome *c*-mediated apoptosis is involved at a later stage of prenatal development or, alternatively, this stage cannot be carried out without cytochrome *c* as an electron carrier and/or antioxidant.

On the face of it, the validity of the former possibility seems to be confirmed by the data of Yoshida et al. [24] and Hakem et al. [25] that the Apaf-1 and caspase 9 knock-outs respectively, result in the death of mouse embryos approximately at the same stage as the cytochrome *c* knock-out [23]. However, these observations cannot rule out the latter possibility, since cytochrome *c* is operating upstream in the apoptotic cascade, and the existence of cytochrome *c*-mediated Apaf-1- and caspase 9-independent mechanisms of cell death seem possible. As was recently shown by Sherman and co-workers [26], and M. Sherman, personal communication) energy deprivation of Hgc2 myogenic cells results in a necrotic cell death mediated by stress kinase (c-Jun N-terminal kinase), accompanied by a drop of the mitochondrial membrane potential. This death was found to be caspase-independent. Sherman suggests that cytochrome *c* release under conditions of low [ATP] actuates a cell suicide mechanism of a necrotic type. Golstein and co-workers [27] reported that interdigital cell death in mouse embryo limb buds can occur through necrotic and caspase-independent pathways. This effect was shown in buds incubated *in vitro* when caspase-mediated apoptosis was inhibited by a pan-caspase inhibitor or when buds were obtained from a Apaf-1 knock-out mouse embryo. It is obvious that the possible involvement of cytochrome *c* in such phenomena could be proved using the yeast $\rightarrow$ horse cytochrome *c* mutant.

It should be stressed that some physiologically significant properties of cytochrome *c* were outside the scope of our study. Among them are: (i) the interaction of cytochrome *c* with cytochrome *b*₅, which is activated, for example, on cold exposure of mammals in a thyroid hormone-dependent fashion [28–31]; (ii) the interaction of apocytochrome *c* with component(s) of the outer mitochondrial membrane, required for its translocation from cytosol to the intermembrane space [32]; and (iii) the interaction of apocytochrome *c* with haem lyase. An indication for the existence of one more function of cytochrome *c* was recently reported by Li et al. [23], who showed that a cytochrome *c* knock-out cell line is much more sensitive to apoptosis induced by tumour necrosis factor- α [33], since the anti-apoptotic effect of this factor mediated by nuclear factor κB does not occur.

This is why it is desirable in the future to perform a knock-in experiment using a cytochrome *c* variant that is as close as possible to that of the mammalian cytochrome *c*, but still lacking its apoptogenic activity. For this reason, the yeast $\rightarrow$ horse mutant is a better candidate for this role than the yeast $\rightarrow$ horse (K4E) mutant which contains more 'yeast' amino acids (see above and Figure 1).

A novel observation reported in the present paper is that glutamate in position 4 is essential for high affinity of animal cytochrome *c* to its mitochondrial targets responsible for electron transfer and antioxidant effect (see Figure 2 and Table 1). On the other hand, it does not contribute to pro-apoptotic activity, which is in line with the report by Yu et al. [14].

As for Lys⁷², it can be replaced by leucine, alanine or glycine without any effect on the electron transfer and antioxidant properties (not shown in Figures). Lys⁷² substitutions partially decrease the pro-apoptotic activity, the glycine and especially the leucine and alanine substitutions being more significant than the arginine substitution (see Figure 3B).

In conclusion, a mutant yeast cytochrome *c* has been constructed that has high respiratory and antioxidant efficiencies, but which cannot activate the apoptotic cascade. Such a mutant

is a good candidate for knock-in studies to elucidate the physiological role of cytochrome *c*-mediated cell death.

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