Octameric mitochondrial creatine kinase induces and stabilizes contact sites between the inner and outer membrane

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We have investigated the role of the protein ubiquitous mitochondrial creatine kinase (uMtCK) in the formation and stabilization of inner and outer membrane contact sites. Using liver mitochondria isolated from transgenic mice, which, unlike control animals, express uMtCK in the liver, we found that the enzyme was associated with the mitochondrial membranes and, in addition, was located in membrane-coated matrix inclusions. In mitochondria isolated from uMtCK transgenic mice, the number of contact sites increased 3-fold compared with that observed in control mitochondria. Furthermore, uMtCK-containing mitochondria were more resistant to detergent-induced lysis than wild-type mitochondria. We conclude that octameric uMtCK induces the formation of mitochondrial contact sites, leading to membrane cross-linking and to an increased stability of the mitochondrial membrane architecture.

Key words: adenine nucleotide translocator (ANT), electron microscopy, micro-compartment, mitochondrion, octameric mitochondrial creatine kinase, outer membrane pore.

INTRODUCTION

Cells with high or fluctuating energy demands express cytosolic and mitochondrial creatine kinases (EC 2.7.3.2). Creatine kinases shuttle 'high-energy' phosphates formed in mitochondria, to the cytosol, where they are utilized [1,2]. MtCK (mitochondrial creatine kinase), located in the inter-membrane compartment [3] uses ATP, supplied by the ANT (adenine nucleotide translocase), to form PCr (phosphocreatine) [4], which is delivered via the outer membrane VDAC (voltage-dependent anion channel) to the cytosol [5,6]. In the cytosol, PCr is used by cytosolic creatine kinase to phosphorylate ADP, thereby securing a more stable ATP concentration [1,2,7].

The MIM (mitochondrial inner membrane) and MOM (mitochondrial outer membrane) form MCSs (mitochondrial contact sites), that are enriched in ANT, VDAC, hexokinase and MtCK [6,8–12]. It has been proposed that these MCSs constitute a micro-compartment for PCr synthesis [13,14]. Evidence supporting the micro-compartment model has come from several experiments. Respiration measurements have demonstrated that ADP, formed by MtCK during mitochondrial PCr synthesis, is shuttled directly into the mitochondrial matrix via ANT for re-phosphorylation without reaching the bulk phase [4,15]. Functional studies have shown that MtCK can create permanent contacts between membranes of reconstituted MIM and MOM [16,17]. This property is largely dependent on the octameric state of MtCK [16,17]. The octameric MtCK can be dissociated into dimers by TSAC (transition-state analogue complex). Dimeric MtCK has a significantly reduced capacity for membrane binding and cross-linking as compared with the octameric enzyme [18]. Binding studies of uMtCK (ubiquitous MtCK) have indicated that it interacts both with acidic phospholipids, mainly cardiolipin, and with VDAC [18,19]. Therefore it is thought that octameric

MtCK plays a central role in the formation and maintenance of MIM/MOM contact sites. Anthracyclines, such as doxorubicin, can compete with uMtCK for binding to cardiolipin and thereby release the protein from the membrane [20].

To elucidate a possible structural role of MtCK in MIM/MOM contact site formation and in stabilizing mitochondrial ultra-structure, we used a transgenic mouse line where uMtCK is expressed under the control of a liver-specific promoter [21]. MtCK is not expressed at a detectable level in the liver of WT (wild-type) mice under normal conditions.

In the present paper, we report new data obtained by immuno-electron microscopy and by quantitative analysis of MCSs in WT compared with transgenic, uMtCK-expressing liver. In uMtCK-containing liver mitochondria, the number of MCSs was increased 3-fold. Furthermore, we found that the resistance to detergent-induced lysis was markedly higher in uMtCK-containing mitochondria. The detergent resistance was diminished by reagents that are known to interfere either with the oligomeric state or with the membrane binding of uMtCK. From these results, we conclude that octameric uMtCK induces the formation of MCSs, which, apart from playing an important role in energy metabolism, also stabilize the mitochondrial membrane architecture.

EXPERIMENTAL

Tissue extracts and isolation of mitochondria

Transgenic mice expressing uMtCK in liver were bred as described previously [21]. Mice (3–4 months of age) were anaesthetized by CO_2 and killed by cervical dislocation. Liver, brain and heart were homogenized separately at $4°C$ in a medium containing 250 mM sucrose, 10 mM Hepes/KOH, pH 7.4, 1 mM

EDTA and 0.5 % (w/v) fatty-acid-free BSA, using a Teflon/glass Potter homogenizer. The total tissue homogenate was centrifuged at 700 **g** for 10 min. The resulting supernatant was collected and centrifuged further at 7000 **g** for 10 min. The resulting supernatant was termed cytoplasm and was collected for further analysis. The pellet containing mitochondria was resuspended in 30 ml of a medium containing 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 100 μM EGTA, 25 % (v/v) Percoll (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 100000 **g** for 35 min. The fraction enriched in mitochondria was collected and washed twice by centrifugation at 7000 **g** for 10 min in the same medium without Percoll. As shown previously [4], the yield and the respiratory control ratio were similar for mitochondria of WT and uMtCK-expressing liver.

Immunoblotting

Protein extracts from liver, brain and heart were separated by SDS/PAGE (10–12 % gels) and transferred on to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were incubated for 1 h in TBS(T) (Tris-buffered saline with Tween) medium containing 150 mM NaCl, 25 mM Tris/HCl, pH 7.4, and 0.05 % Tween, supplemented with 5 % (w/v) fat-free milk powder, washed for 30 min with TBS(T) and subsequently incubated for 2 h with rabbit anti-MtCK serum diluted 1:1000 in TBS(T), or with monoclonal anti-human VDAC diluted 1:2000. After washing with TBS(T) for 30 min, the membranes were incubated with goat HRP (horseradish peroxidase)-conjugated anti-rabbit antibody diluted 1:10000 in TBS(T) (Sigma, St. Louis, MO, U.S.A.) or HRP-conjugated anti-mouse antibody diluted 1:10000. Immunoreactive bands were visualized using the Renaissance Western Blot Chemiluminescence Reagent Plus Kit (NEN, Boston, MA, U.S.A.) according to the manufacturer's instructions. All steps were carried out at room temperature (25 °C). The anti-MtCK antibody was prepared and characterized as described previously [22].

Electron microscopy of liver tissue

Liver tissue samples from both WT and transgenic mice were fixed by perfusing with PBS, containing 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, supplemented with 2 % (w/v) para-formaldehyde, 2 % (w/v) glutaraldehyde, 0.1 % (v/v) acrolein and 2 % (v/v) DMSO, at a flow-rate of 3 ml/min for 10 min at room temperature. After fixation, tissue cubes of approx. 1 mm^3 were embedded, sectioned and stained as described in [23]. Sections were viewed in a JEOL s100 transmission electron microscope.

Immunoelectron microscopy samples were prepared according to the method of Griffiths [24]. Liver tissue was fixed by perfusion with PBS supplemented with 4 % (w/v) paraformaldehyde and 0.05 % (w/v) glutaraldehyde, for 10 min at room temperature. The liver was then cut in 2-mm-thick slices, which were subsequently incubated for 2 h in the same medium. For post-fixation, liver slices were incubated in 1 % tannic acid, whereupon they were embedded in sucrose-polyvinylpyrrolidone and were thin-sectioned at −100 °C. The thin sections were incubated in PBS supplemented with 10 % (v/v) foetal calf serum for 1 h whereupon they were immunoreacted with rabbit anti-uMtCK serum or pre-immunization serum diluted 1:200 in PBS for 1 h. After washing the thin sections with PBS three times for 1 h, they were incubated with 10 nm colloidal gold-conjugated protein A for 1 h. Thin sections were subsequently incubated for 1 min with PBS supplemented with 4 % (w/v) formaldehyde and 1 % (w/v) glutaraldehyde, rinsed with PBS and finally stained with a solution of 2 % (w/v) methylcellulose and 0.4 % (w/v) uranyl acetate.

Detergent-resistance measurements

Mitochondria from transgenic and WT liver tissue were suspended at 0.5 mg protein/ml in measurement medium containing 250 mM sucrose, 10 mM Hepes/KOH, pH 7.4, and 100 μM EGTA at 25 °C. Digitonin or Triton X-100 was added at concentrations up to 1 mg/mg of mitochondrial protein. Dissociation of the octameric uMtCK was initiated by the addition of TSAC containing 4 mM ADP, 5 mM MgCl$_2$, 20 mM creatine and 50 mM KNO$_3$ [18]. Mitochondrial intactness was monitored as turbidity at 540 nm using a multi-well plate reader (Molecular Devices, Basel, Switzerland). The turbidity was set to 1 arbitrary unit by dividing the measured turbidity (A) at time t by the initial turbidity (A_0) at $t = 0$ min. The effect of the detergents was quantified as the rate of change in turbidity according to the formula $(A/A_0)/\Delta t$. Where mentioned, samples of the mitochondrial suspensions were taken directly from the spectrometer cuvette for electron microscopy. Negative staining of mitochondria directly adsorbed to the electron microscopy grids was performed with 2 % (w/v) ammonium molybdate as described previously [25].

Chemicals and reagents

Doxorubicin hydrochloride was a gift from Pharmacia (Milan, Italy). All other chemicals were of highest grade and purchased from Sigma.

RESULTS

In order to elucidate the role of uMtCK in MCS formation and stabilization, we compared liver mitochondria from uMtCK transgenic mice with those of WT mice, which lacked uMtCK in their liver. We first verified that uMtCK was expressed in the liver of the transgenic mice. Protein extracts prepared from liver, heart and brain were separated on SDS/PAGE gels and immunoreacted with an anti-MtCK antibody. Confirming previous findings [21], the results indicated that the liver of the transgenic mice contained a high level of uMtCK (Figure 1). In the transgenic animals, the expression level of uMtCK in liver was similar to MtCK levels in the brain and heart (Figure 1A). As expected, uMtCK could not be detected in WT liver. Western blot analysis of isolated mitochondria compared with cytosol confirmed that uMtCK in transgenic liver was correctly targeted to the mitochondria (Figure 1B). For comparison, we determined the expression of the outer membrane protein VDAC, a putative contact-site protein. The results revealed that the amount of VDAC was similar in mitochondria of WT and uMtCK-expressing mice (Figure 1B). These data suggest that the amount of mitochondria in the WT and transgenic liver was similar.

We then examined the ultrastructure of uMtCK-containing and WT liver tissue using electron microscopy. Interestingly, there were distinct differences in the mitochondrial ultrastructure of transgenic and WT liver. Mitochondria from transgenic mice frequently contained electron-dense membrane-coated matrix inclusions (Figures 2A, 2C and 2D). These inclusions did not occur in mitochondria of WT mice (Figure 2B). To visualize uMtCK, thin sections were immunoreacted with an anti-MtCK antibody, which was labelled with colloidal gold particles. The results showed that the matrix inclusions were very strongly labelled with colloidal gold, indicating that high amounts of uMtCK were present in the inclusions (Figure 2C). Gold particles were also associated with the mitochondrial membranes (Figure 2C). These findings show that uMtCK was located both in matrix inclusions and on the MIM/MOM interface.
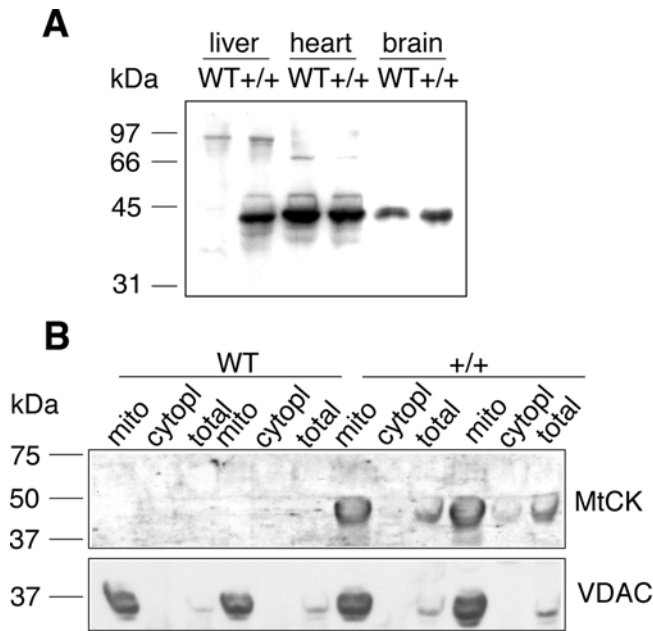
Figure 1   Immunoblot analysis of uMtCK expression in mouse tissues

Protein extracts of WT and uMtCK-expressing (+/+) mice were separated by SDS/PAGE and immunoblotted. (**A**) Protein extracts from liver, heart and brain, blotted with an anti-MtCK-antibody. (**B**) Protein extracts from liver (total), liver cytoplasm (cytopl) and liver mitochondria (mito), blotted first with an anti-MtCK antibody, followed by an anti-VDAC antibody. The results from two WT and two uMtCK-expressing animals are shown.

Figure 2   Ultrastructure of transgenic and WT mouse liver tissue

Liver tissue samples were prepared for transmission electron microscopy as described in the Experimental section: transgenic liver (**A**), WT liver (**B**), transgenic liver immuno-reacted with anti-MtCK antibodies (**C**), transgenic liver immunoreacted with pre-immunization serum (**D**), MIM/MOM contact sites (indicated by arrows) (**E**). Scale bar, 200 nm. (**F**) The number of contact sites was divided by the mitochondrial circumference, calculated from 20 randomly chosen mitochondrial sections in each group. The difference in the number of contact sites between transgenic and WT mice was statistically significant ($P < 0.0005$ as assessed by ANOVA). Results are means $\pm$ S.E.M.

In a quantitative approach, we then proceeded to count the number of MCSs in uMtCK-expressing and WT liver. MCSs were counted, and the total number was divided by the mitochondrial circumference measured in micrometers (Figure 2E). We found that the number of visible MCSs was three times higher in uMtCK-containing mitochondria than in WT mitochondria (Figure 2F).

It has been demonstrated that MtCK is able to bind to mitochondrial membranes and to cross-link the MIM with the MOM *in vitro* [16,17]. As this cross-linking may increase the physical stability of the membranes and mitochondria as such, we then studied in a quantitative fashion the resistance of mitochondria to detergent-induced lysis. Following detergent addition, mitochondrial lysis was measured as turbidity. In parallel, mitochondrial ultrastructure was examined by electron microscopy. The results demonstrated that addition of digitonin (0.5 mg/mg mitochondrial protein) to WT mitochondria lead to a rapid decrease in turbidity indicating that mitochondrial disruption and lysis occurred (Figure 3A, inset trace d). In contrast, the turbidity of uMtCK-containing mitochondria decreased at a significantly lower rate following digitonin addition, suggesting that uMtCK-containing mitochondria were capable of retaining their organization, at least partially, in the presence of detergent (Figure 3A, inset trace c). The relationship between digitonin concentration and the rate of change in turbidity (Figure 3A) indicated that uMtCK-containing mitochondria were more resistant to lysis than WT mitochondria, especially at higher digitonin concentrations. Furthermore, substituting Triton X-100 for digitonin confirmed that uMtCK-containing mitochondria were more resistant to detergent-induced lysis than WT mitochondria (Figure 3B). Electron microscopy of mitochondria that were negatively stained with ammonium molybdate, after detergent treatment for 30 min, supported the results of the turbidity experiments. The organization of the membranes of WT mitochondria was disrupted, and stain had
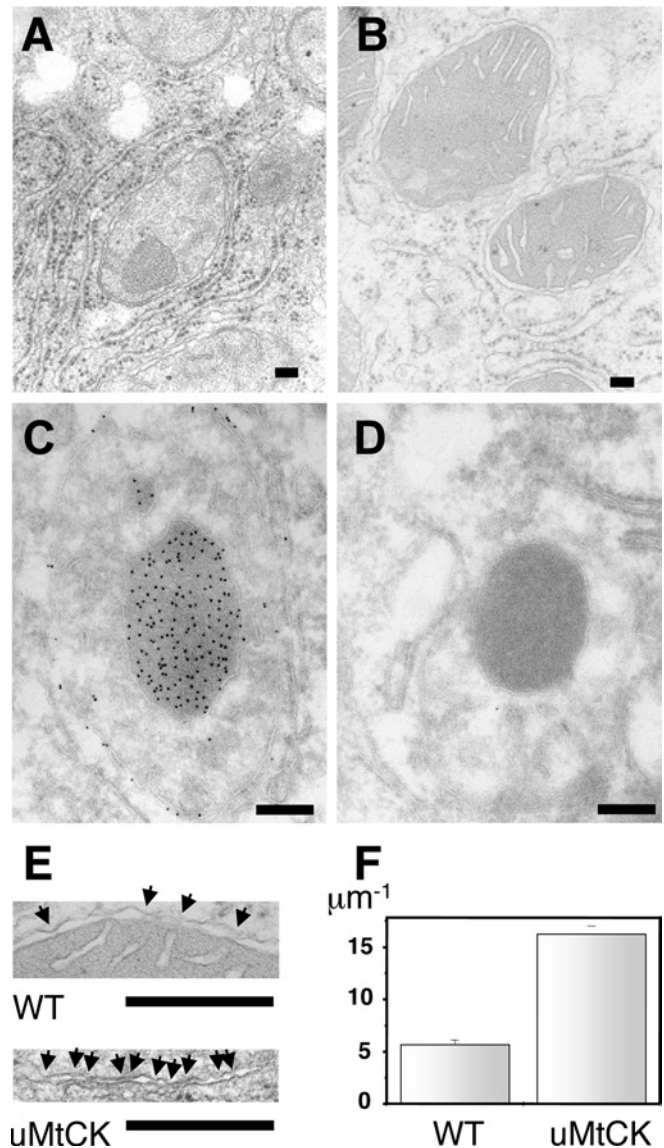
penetrated into the matrix compartment (Figure 3F). In contrast, the ultrastructure of uMtCK-containing mitochondria remained essentially unchanged without penetration of stain into matrix (Figure 3E). For comparison, uMtCK-containing and WT mitochondria without digitonin are shown in Figures 3(C) and 3(D).

We next addressed the question as to whether the greater detergent-resistance of uMtCK-containing mitochondria was due to a direct effect of uMtCK. For this purpose, we studied TSAC, which specifically dissociates uMtCK-octamers [18], and doxorubicin, which reduces the membrane-binding of octameric uMtCK and thus releases the enzyme from mitochondrial membranes [20]. The results indicated that the resistance of
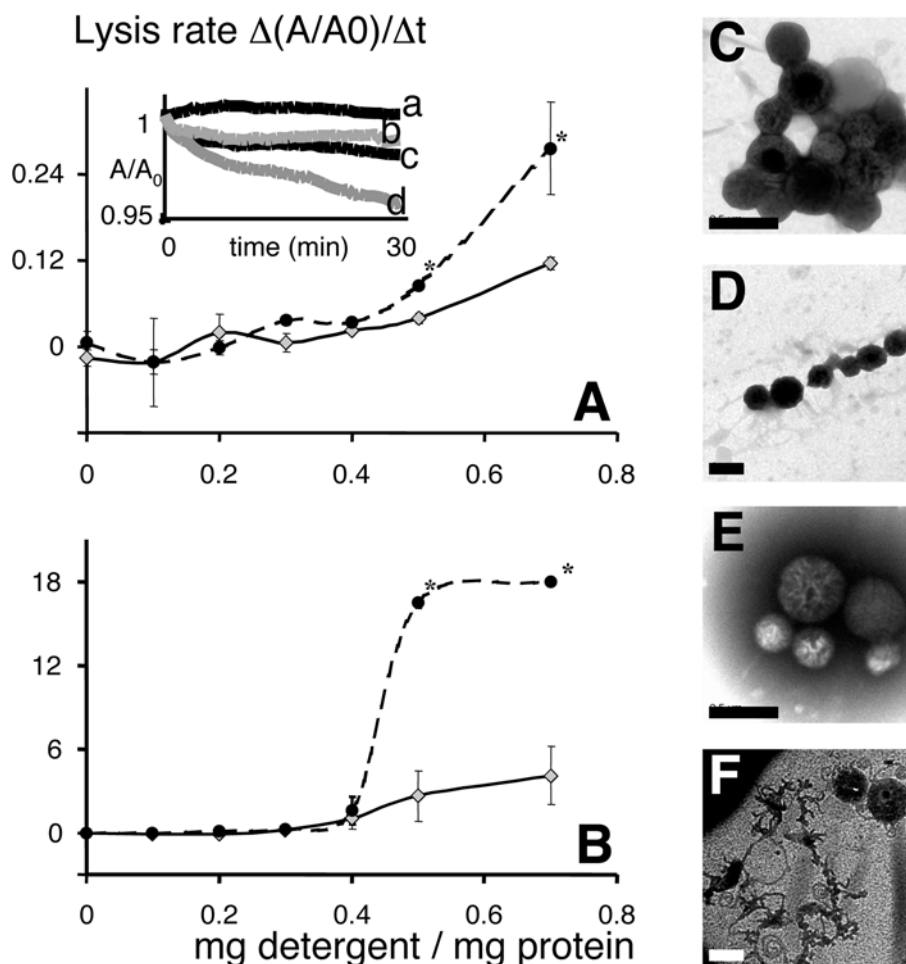
Figure 3   Detergent-resistance of uMtCK-containing mitochondria

Mitochondria were suspended in sucrose-based medium at a concentration of 0.5 mg of protein/ml. Detergent was added and the turbidity of the mitochondrial suspension was monitored at 540 nm. In parallel, aliquots of the mitochondrial suspension were taken from the cuvette after 30 min and stained with 2 % ammonium molybdate for electron microscopy. (**A**) Rate of change in turbidity as a function of digitonin concentration for uMtCK-containing mitochondria (grey diamonds) and WT mitochondria (filled circles). Inset shows turbidity for: WT mitochondria plus digitonin, 0.5 mg/mg mitochondrial protein (trace d); uMtCK-containing mitochondria plus digitonin, 0.5 mg/mg mitochondrial protein (trace c); WT mitochondria without detergent (trace b); uMtCK-containing mitochondria without detergent (trace a). (**B**) Rate of change in turbidity as a function of TX-100 concentration for uMtCK-containing mitochondria (grey diamonds) and WT mitochondria (filled circles). Results in (**A** and **B**) are the means $\pm$ S.E.M. of three different mitochondrial preparations. Points marked with asterisks were statistically different for uMtCK-containing mitochondria compared with the control ($P < 0.001$ by ANOVA). (**C**) uMtCK-containing mitochondria without detergent. (**D**) WT mitochondria without detergent. (**E**) uMtCK-containing mitochondria plus digitonin, 0.5 mg/mg mitochondrial protein. (**F**) WT mitochondria plus digitonin, 0.5 mg/mg mitochondrial protein, in negative staining. Scale bar in (**C**–**D**), 1 μm.

uMtCK-containing mitochondria to digitonin was shifted to that of control mitochondria by both TSAC (Figure 4A) and doxorubicin (Figure 4B). This finding shows that the increased stability of uMtCK-containing mitochondria was a direct effect of octameric uMtCK tethering the MIM and MOM together.

DISCUSSION

We have studied the effect of uMtCK on the formation of MCSs and their role in conferring stability on the mitochondrial membrane architecture. For this purpose, we used a mouse line in which uMtCK is expressed under the control of a liver-specific promoter [21]. Immuno-electron microscopy showed that uMtCK was located near the mitochondrial membranes and in membrane-coated matrix inclusions. We found that the number of MCSs was increased 3-fold in uMtCK-containing mitochondria compared with WT mitochondria lacking uMtCK. We also found that

the resistance to detergent-induced lysis was markedly higher in uMtCK-containing mitochondria. These results indicate that uMtCK induces the formation of MCSs resulting in membrane cross-linking and an increased stability of the mitochondrial membrane architecture.

Previous studies have shown that uMtCK is present in an enzymically active form in the liver of transgenic mice [4,21,26]. In these livers, many mitochondria show unusual morphological features [21], indicating that MtCK can influence mitochondrial membrane topology and ultrastructure. The data of our study showed that uMtCK was associated with the mitochondrial membranes, indicating that all components required for correct targeting and function of uMtCK on the MIM/MOM interface exist in liver mitochondria of transgenic animals. However, we also found that a significant proportion of uMtCK was located in inclusions in the mitochondrial matrix. The inclusions were membrane-coated, suggesting that they were formed by a dilation of mitochondrial cristae, and therefore connected with the
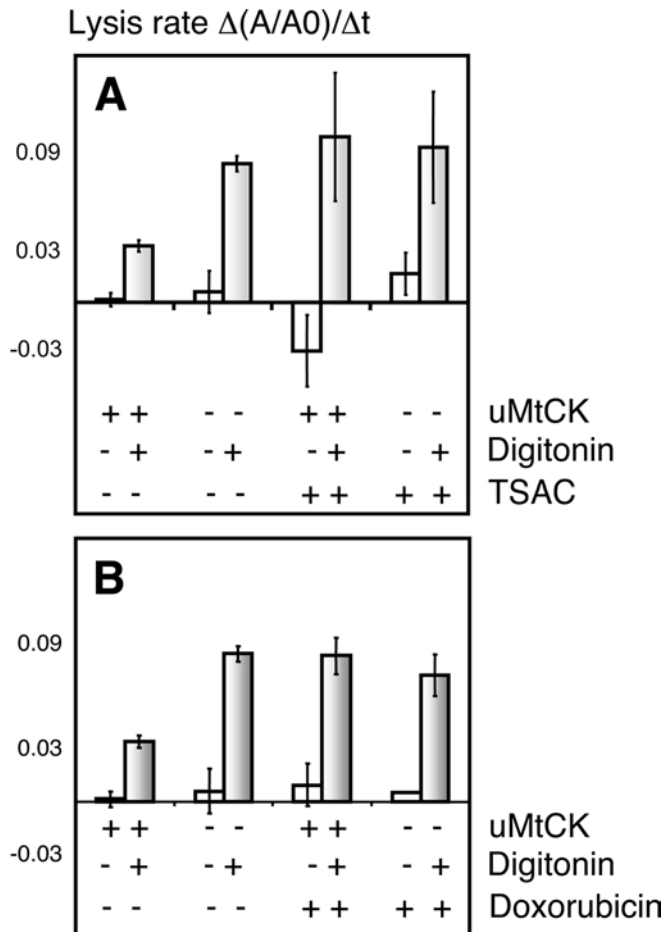
Lysis rate Δ(A/A0)/Δt

A

+ +	− −	+ +	− −	uMtCK
− +	− +	− +	− +	Digitonin
− −	− −	+ +	+ +	TSAC

B

+ +	− −	+ +	− −	uMtCK
− +	− +	− +	− +	Digitonin
− −	− −	+ +	+ +	Doxorubicin

Figure 4   Effects of TSAC and doxorubicin on detergent-resistance of uMtCK-containing mitochondria

Turbidity measurements were performed as described in Figure 3. Mitochondria from WT and uMtCK-expressing mice were incubated in measurement medium supplemented with either TSAC (**A**) or 50 μM doxorubicin (**B**). Digitonin (0.5 mg/mg of mitochondrial protein) was added, and the rate of change in turbidity was calculated as described in the Experimental section. Results are the means $\pm$ S.E.M. for three different mitochondrial preparations.

intermembrane compartment. The uMtCK within these inclusions did not form the characteristic crystalline array structure observed in muscular myopathies [27] or after creatine depletion [28].

Our results demonstrated that the number of MCSs was increased 3-fold in uMtCK-containing mitochondria. As the uMtCK-induced MCS were most likely to be composed of uMtCK, we conclude that the uMtCK-independent MCSs, occurring in WT liver mitochondria, were composed of different molecular assemblages, possibly including complexes of the protein-import machinery [29,30].

If the observed MCSs induce stable cross-links between the MOM and MIM, mitochondria should be more resistant to disruptive forces. To test this hypothesis, we measured the resistance of mitochondria against detergent-induced lysis and analysed the effects of detergents on mitochondrial ultrastructure. The results indicated that uMtCK-containing mitochondria were more resistant to detergent-induced lysis than WT mitochondria. This increase in detergent resistance could be abolished by addition of TSAC, which dissociates uMtCK-octamers, or doxorubicin, which prevents the binding of uMtCK to cardiolipin. These results suggest that TSAC and doxorubicin released uMtCK from its membrane binding sites thereby destabilizing the MIM/MOM

cross-links, leading to a decreased detergent resistance. This interpretation is supported by the finding that octameric uMtCK can cross-link model membranes more effectively than dimeric uMtCK [16,17]. It is likely that octameric uMtCK facilitates membrane cross-linking due to its cubic symmetry having two identical top and bottom faces [31], where the membrane binding sites are located [31,32]. These findings indicate that uMtCK-induced contact sites stabilize the mitochondrial membranes.

During the execution phase of apoptosis, mitochondria undergo structural and functional changes that lead to the release of several proteins from the intermembrane compartment to the cytosol [33]. The results of this and other studies [16,19,18,34] suggest strongly that uMtCK plays a role in maintaining the organization of the MIM/MOM interface [10]. Therefore it is possible that uMtCK plays a role in the control of apoptosis. This hypothesis is gaining support from the finding that MtCK, in the presence of creatine, prevents mitochondrial permeability transition [2,35], a critical apoptotic event leading to the release of intermembrane proteins by rupture of the MOM [33,36]. Furthermore, dissociation of the octamer by TSAC in reconstituted VDAC–MtCK–ANT complexes promotes permeability transition [10]. Since the MOM is tethered to the MIM by uMtCK, the protective effect of creatine on cell death [37,38] may be due to the increased number of MCSs.

We conclude that uMtCK has the dual role of functioning both as a key enzyme in energy metabolism and as a structural protein. uMtCK might confer physical stability on the mitochondrial membranes and thereby contribute to the organization of the entire organelle.

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