Methylation-controlled J-protein MCJ acts in the import of proteins into human mitochondria

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Loss of expression of the methylation-controlled J gene, MCJ (DNAJC15), is observed in cases of several tumors and plays a crucial role in the chemoresistance of ovarian cancer cells. Aside from the pathophysiological effects, almost nothing is known about the cellular function of MCJ. Here, we provide the first evidence that MCJ acts in the biogenesis of mitochondria. Our results demonstrate that MCJ is located in mitochondria. It is anchored in the mitochondrial inner membrane with the C-terminal J domain facing the matrix space. We show that MCJ forms a stable subcomplex with a component of the mitochondrial import motor, MAGMAS, a protein overexpressed in cells treated with granulocyte-macrophage colony-stimulating factor and in prostate carcinomas. In addition, MCJ and MAGMAS interact with the core components of the TIM23 pre-protein translocase. We demonstrate that the recombinant soluble MCJ domain stimulates the ATPase activity of the human mtHsp70 chaperone, mortalin, the central component of the import motor of the TIM23 translocase. This stimulation is counteracted by MAGMAS. Moreover, pre-protein import into mitochondria is impaired in the absence of MCJ. Interestingly, MCJ is able to take over the function of Tim14, the essential J co-chaperone of the mitochondrial protein import motor in yeast. In summary, our results show that MCJ functions as J co-chaperone of the human TIM23 pre-protein translocase, suggesting a link between mitochondrial pre-protein import and tumorigenesis.

INTRODUCTION

Hypomethylation and hypermethylation of tissue genes are well-known modifications involved in cancer. These modifications can lead to silencing of tumor-suppressor genes and activation of growth-promoting genes. The methylation-controlled J gene (MCJ) was first described as a gene differentially expressed in ovarian tumor cell lines and short-term cultures of normal ovarian epithelial cells (1). The inhibition of its expression in primary ovarian tumors and ovarian carcinoma cell lines was linked to methylation of CpG islands within the first exon and the first intron of the MCJ gene (2). In addition, epigenetic inactivation by hypermethylation of MCJ appears to play a role in Wilms’ tumors, in malignant pediatric brain tumors and in melanomas (3–5). The methylation status of the CpG islands also correlates with the expression of MCJ in non-tumorigenic cells (2). In ovarian epithelial cells, methylation and low expression of MCJ were observed, whereas expression from the non-methylated MCJ gene was reported with fibroblasts and lymphocytes (2). Interestingly, the hypermethylation-controlled loss of MCJ expression confers resistance to chemotherapeutic drugs, such as paclitaxel, topotecan and cisplatin, and also correlates with poor survival and therapeutic response of ovarian tumor patients (1,6,7). A parental MCJ-non-expressing ovarian carcinoma cell line was less chemoresistant, when it was stably transfected with MCJ, indicating that the chemoresistance phenotype can be rescued by expression of MCJ (1). MCJ is expressed in drug-sensitive, but not in multidrug-resistant breast cancer cells (6). In the absence of the MCJ protein, the expression levels of the ABCB1 drug transporter were reported to be increased (6). The increased levels of multidrug transporter probably contribute to the observed chemoresistance, since they may prevent intracellular accumulation of drugs.

The MCJ protein is a member of the family of J proteins which was named after the founding member, the DnaJ of...
E. coli, and is characterized by the presence of a typical J domain (1). In humans, there are 41 J proteins reported, which are grouped according to their domain structure into three classes: DNAJA, DNAJB and DNAJC (8,9). MCJ, also named DNAJC15, belongs to the third class, the DNAJC members, as it has a J domain, but lacks the G/F-rich and Cys-repeat regions of DnaJ. Almost all members of the J domain proteins function in conjunction with Hsp70 chaperone proteins in various cellular processes, such as folding of proteins, prevention of protein aggregation, disaggregation of proteins and protein transport (10–12). In mediating these processes, the Hsp70 chaperones bind and release unfolded polypeptide chains by the coordinated action of their ATPase and substrate binding domains. The reaction cycle of Hsp70 is driven by the hydrolysis of ATP and supported by two types of co-chaperones. The nucleotide exchange factors accelerate the release of ADP and the exchange of ADP to ATP and the J-proteins employ the J domain to stimulate the ATPase activity of Hsp70 chaperones.

Based on overexpression studies in human cell lines, the MCJ protein has been suggested to be present in the Golgi apparatus (6). MCJ appears to be a membrane-anchored protein, since it contains a putative transmembrane (TM) segment. The amino acid residues C-terminal to this segment contain the typical J domain, whereas the N-terminal 40 amino acid residues do not harbor any known motif. There are a number of other J proteins known to be anchored in a membrane (8). Within the family of J proteins, the MCJ proteins have the highest sequence similarity to the membrane-anchored TIM14 proteins, which have a crucial function in the TIM23 pre-protein translocase of yeast (13,14). The TIM23 translocon mediates the import of precursor proteins containing amino-terminal mitochondrial targeting signals across and into the inner membrane of mitochondria in a membrane-potential and ATP-dependent manner (15–17). Almost all of the knowledge about this translocon was obtained from studies in S. cerevisiae, in which the translocon contains eight essential subunits. The multiple membrane-spanning subunits Tim23 and Tim17 are connected to the receptor in the intermembrane space (IMS), Tim50, and to the ATP-dependent import motor on the matrix side of the inner membrane (15–20). The import motor represents a specialized Hsp70 chaperone system. Besides its co-chaperone Tim14 and the soluble nucleotide exchange factor Mge1, the import motor contains two additional essential subunits, Tim44 and the J-related protein without J activity, Tim16. Tim16 counteracts the stimulatory activity of Tim14 on the mtHsp70 ATPase activity (21–25). MAGMAS, the recently reported human ortholog of Tim16, is able to rescue the deletion of TIM16 in yeast (26). Recombinant MAGMAS has been shown to form a stable subcomplex with the mtHsp70 stimulating J co-chaperone DNAJC19, a human homolog of yeast Tim14 and a homolog of MCJ (26,27). Interestingly, in contrast to yeast Tim14, DNAJC19 appears not to be essential for cell viability. However, the absence of functional DNAJC19 has been observed to cause the severe disease of dilated cardiomyopathy with ataxia (DCMA) (28,29).

Despite the clinical importance of MCJ, almost nothing is known about the function of MCJ in normal and cancerous cells. In order to obtain more insight into the role of MCJ in cancer, we have analyzed its physiological function. Here we show that MCJ is a mitochondrial protein. It resides in the mitochondrial inner membrane with its N terminus facing the IMS and its C-terminal J domain facing the matrix space. MCJ is a subunit of the TIM23 translocon and its depletion affects pre-protein import into mitochondria. In consistency with its association with the translocon, it stimulates the ATPase activity of human mtHsp70, mortalin. Interestingly, MCJ but not DNAJC19 rescues the deletion of the TIM14 gene in yeast. In summary, we conclude that MCJ acts as a J co-chaperone in the import motor of the TIM23 translocon.

RESULTS
MCJ is located in mitochondria

To analyze the function of MCJ, we first characterized its subcellular location. Subcellular fractions of HeLa cells were obtained by differential centrifugation and then analyzed with antibodies against MCJ and marker proteins of mitochondria (MAGMAS, TIM23, TIM44), ER (Calnexin), Golgi (GM130) and lysosomes (EEA1).

Figure 1. MCJ is a mitochondrial protein. HeLa cells were disrupted and crude mitochondrial and post-mitochondrial fractions were separated by differential centrifugation. Mitochondria were further purified by gradient centrifugation (purified mitochondria). Equal amounts of protein were analyzed by SDS–PAGE and immunodecoration using antibodies against MCJ and marker proteins of mitochondria (MAGMAS, TIM23, TIM44), ER (Calnexin), Golgi (GM130) and lysosomes (EEA1).
MCJ resides in the inner mitochondrial membrane

Next, we addressed the submitochondrial location of MCJ. Analyzing the amino acid sequence of MCJ in silico indicated the absence of a typical N-terminal cleavable mitochondrial targeting sequence (Fig. 2A). Moreover, a hydrophobic TM segment and a J domain in the C terminus of MCJ were predicted. To test whether MCJ is indeed a membrane protein, mitochondria were subjected to alkaline carbonate extraction. MCJ was recovered in the pellet fraction like the integral membrane protein TIM23, and not in the soluble fraction, confirming MCJ to be an integral membrane protein (Fig. 2B).

MCJ is a component of the TIM23 translocase

In order to obtain information about the function of MCJ in mitochondria, we searched for interaction partners. Since MCJ is a J protein anchored to the inner membrane of mitochondria, we analyzed whether MCJ associates with components of the mitochondrial TIM23 pre-protein translocase. To this end, we lysed mitochondria with digitonin-containing buffer and then performed a co-immunoprecipitation with antibodies against TIM17B, an integral component of the pre-protein translocase. These antibodies precipitated TIM17B together with TIM23, but not the TIM17 isoform TIM17A from the mitochondrial extract (Fig. 3A). In addition, a fraction of MCJ and of MAGMAS and TIM50 was co-precipitated, but not the matrix protein Hsp60, used as control for unspecific binding. These results suggest that MCJ is associated with the TIM23 translocase. Moreover, antibodies against MCJ co-precipitated MAGMAS together with MCJ and vice versa (Fig. 3B). A fraction of TIM17A and TIM17B was also isolated together with MCJ and MAGMAS. These results demonstrate that MCJ is indeed a component of the TIM23 translocase. When mitochondria were lysed with Triton X-100-containing buffer, a large fraction of MAGMAS, but not TIM17, was co-precipitated with antibodies against MCJ (Fig. 3C, data not shown). Since the TIM23 translocase is not stable under these conditions of membrane solubilization, this result indicates the presence of a subcomplex of MAGMAS and MCJ. We also co-expressed the soluble domains of MCJ and MAGMAS, hereinafter denoted as MCJ(s) and MAGMAS(s) in E. coli and purified MAGMAS(s) via his-tag by Ni-NTA agarose chromatography from the bacterial extract. Indeed, MCJ(s) was co-isolated with his-tagged MAGMAS(s) (Fig. 3D). The formation of the MCJ(s)/MAGMAS(s) complex in the heterologous E. coli system supports the previous result showing interaction of MCJ and MAGMAS under physiological conditions. Taken together, the results strongly suggest that MCJ is part of the mitochondrial import motor of the TIM23 translocase.

MCJ plays a role in the pre-protein import into mitochondria

Next, we addressed in an in vitro import assay whether import of proteins into human mitochondria is affected by the lack of MCJ. The radiolabeled mitochondrial pre-protein pSu9-DHFRmut was incubated with mitochondria isolated from MCF7 cells and MCF7/siMCJ cells. In contrast to the situation in MCF7 cells, MCJ was virtually absent in MCF7/siMCJ cells.
The pre-protein was imported in a mitochondrial membrane-potential-dependent manner into mitochondria of both cell types (Fig. 4). The import rates of mitochondria lacking MCJ were decreased when compared with the import rates of MCF7 mitochondria, indicating that the absence of MCJ impairs the import of pre-proteins into human mitochondria. The reduced import of pre-protein into mitochondria that was still detected in mitochondria depleted of MCJ was most likely due to the presence of the second human homolog of Tim14, DNAJC19. In summary, these results strongly support that MCJ acts as an import component of the TIM23 translocase.

MCJ stimulates the activity of mitochondrial Hsp70

Since MCJ contains a C-terminal J domain and is present in the TIM23 translocase, we asked whether MCJ acts as a co-chaperone stimulating the ATPase activity of the human mtHsp70 chaperone, mortalin, the main motor component of the TIM23 translocase. To this end, we tested the purified soluble C-terminal domains of MCJ and DNAJC19 (MCJ(s) and DNAJC19(s)), in an enzyme-coupled ATPase activity assay with recombinant mortalin and the human nucleotide exchange factor MGE1. In presence of MGE1, mortalin had a low basal ATPase activity of 0.033 mol ATP per mol mortalin per minute (Fig. 5). The addition of the J co-chaperone, DNAJC19(s), increased the activity of mortalin about 3-fold, consistent with previously reported results (26). Upon addition of MCJ(s), the ATPase activity of mortalin was also stimulated by a factor of 3.5. The stimulatory effects of MCJ(s) and DNAJC19(s) were confirmed using another ATPase activity assay, which measures the hydrolysis rates of radioactive $\gamma^{32}$P-ATP (30) (data not shown). Thus, the results from both assays demonstrate a role of MCJ in the stimulation of the ATPase activity of mortalin.

The stimulatory activity of MCJ is counteracted by MAGMAS

The stimulatory activity of the J protein of the yeast import motor, Tim14, is inhibited by Tim16 (21–25). Therefore, we asked whether the presence of MAGMAS, the Tim16...
homolog in human, affects the stimulation of the mortalin ATPase activity by MCJ. Therefore, we pre-incubated MAGMAS(s) with MCJ(s) prior to their addition into the ATPase assay. In contrast to the stimulation of the ATPase activity of mortalin by MCJ, the ATPase activity was not altered upon addition of the pre-incubated mixture of MCJ(s) and MAGMAS(s) (Fig. 5). A direct effect of MAGMAS on mortalin was excluded, since the basal ATPase activity of mortalin was not decreased in the presence of MAGMAS(s). Upon pre-incubation of his-tagged MAGMAS(s) with MCJ(s), the proteins formed a stable complex, which could be isolated in NiNTA pull-down experiments (data not shown). These results confirm that MAGMAS(s) interacts with MCJ(s) and strongly suggest that MAGMAS(s), by interaction with MCJ(s), abolishes the MCJ-dependent stimulation of the mortalin ATPase activity. These findings were confirmed using the stable MAGMAS(s)/MCJ(s) subcomplex that was purified from E. coli. The subcomplex of MCJ(s) and MAGMAS(s) also did not stimulate the ATPase activity. In conclusion, MAGMAS counteracts the stimulatory effect of MCJ on the ATPase activity of mortalin by binding to MCJ.

Expression of MCJ restores growth of yeast cells lacking Tim14

The deletion of the TIM14 gene that encodes the J protein of the TIM23 translocase in S. cerevisiae is lethal (31). To test whether the human J co-chaperones MCJ and DNAJC19 are able to complement the growth defect of the deletion of TIM14, centromeric plasmids expressing MCJ or DNAJC19. As a positive control, a plasmid expressing yeast Tim14 was used. Growth of cells was tested on plates containing 5-fluoro-orotic acid to select for cells that have lost the URA plasmid.

Figure 4. MCJ acts in the pre-protein import into mitochondria. Mitochondria were isolated from MCF7 and MCF7/siMCJ cells depleted of MCJ. The radiolabeled precursor protein pSu9-DHFRmut was synthesized in reticulocyte lysate and incubated in the presence or absence of mitochondrial membrane potential (ΔΨ) with mitochondria for the indicated time periods at 34°C. The import reaction was stopped and then incubated with protease K to remove non-imported material. Mitochondria were re-isolated and subjected to SDS–PAGE. The imported and processed mature form of the pre-protein (mSu9-DHFRmut) was detected by autoradiography and quantified by densitometry. Import into MCF7 mitochondria at the longest time point was set to 100% (control). The upper panel shows the autoradiogram of a representative import experiment and the lower panel the quantification of four independent experiments with SEM.

Figure 5. The stimulatory effect of MCJ on the ATPase activity of mortalin is inhibited by MAGMAS. The ATPase activity of recombinant mortalin was determined in the absence and presence of a 7.5-fold molar excess of soluble MCJ (MCJ(s)), a 10-fold excess of soluble DNAJC19 (DNAJC19(s)), a 7.5-fold molar excess of the recombinant MCJ(s) pre-incubated with an equimolar amount of MAGMAS(s), of the recombinant MCJ(s)/MAGMAS(s) complex or of MAGMAS(s). The basal activity of mortalin was set to 1 and the fold stimulation compared with the basal activity is indicated. The graph shows mean values ± standard deviation (n ≥ 5).

Figure 6. MCJ is able to rescue the deletion of TIM14 in yeast cells. A haploid deletion strain of TIM14 harboring a wild-type copy of TIM14 on the URA plasmid was transformed with plasmids expressing either MCJ or DNAJC19. As a positive control, a plasmid expressing yeast Tim14 was used. Growth of cells was tested on plates containing 5-fluoro-orotic acid to select for cells that have lost the URA plasmid.
as a J co-chaperone in the mitochondrial import motor of the TIM23 translocase.

**DISCUSSION**

The analysis of the cellular function of MCJ is a prerequisite to elucidate its role in pathological processes. Here we show that MCJ acts in the biogenesis of mitochondria, an essential cellular process. Our results indicate that MCJ is a component of the mitochondrial TIM23 pre-protein translocase in human and that deletion of MCJ affects the import of pre-proteins into mitochondria. MCJ is a membrane-anchored J co-chaperone that stimulates the ATPase activity of mortalin, the central player of the import motor of the translocase. Thus, MCJ resembles in its properties its homolog in yeast, Tim14. Indeed, MCJ was able to restore growth of yeast cells lacking Tim14, clearly demonstrating that MCJ is able to fulfill a J co-chaperone function in the TIM23 translocase. Taken together, we provide the first evidence that MCJ acts under physiological conditions in the import of proteins into mitochondria.

Interestingly, human cells have a second Tim14 homolog, DNAJC19 (13,14,28). Both proteins MCJ and DNAJC19 harbor a conserved TM segment and a J domain with additional, highly conserved residues amino terminal of the J domain. The polypeptide stretches present C-terminally of the TM segments in the proteins share no sequence similarities. However, according to secondary-structure predictions, these stretches have the ability to form an amphipathic helix. The combination of a TM segment followed by an amphipathic helix has the potential to act as a mitochondrial targeting signal, as described in yeast for Bcs1, another mitochondrial inner membrane protein (32). Our demonstration of the location of MCJ in mitochondria is in full agreement with this prediction of the mitochondrial targeting signals. The previously reported localization of MCJ to the Golgi compartment was derived from experiments using protein overexpression (6), a procedure often resulting in mislocalization of proteins. The mitochondrial location described here avoided overexpression and is confirmed by our functional data presented and by the complementation of the deletion of TIM14 by MCJ in yeast.

MCJ and DNAJC19 exhibit a major structural difference: DNAJC19 lacks an N-terminal segment, whereas MCJ contains an N-terminal segment of 39 amino acid residues in front of the TM segment, facing the IMS. MCJ shares the presence of such an N-terminal IMS segment with yeast Tim14. However, these segments are not conserved at the level of the amino acid sequence. What is the function of the N-terminal IMS segment? The first insights into this question were obtained from the analysis of yeast Tim14. A strain harboring Tim14 lacking the IMS segment was still viable indicating that this segment is not essential (33,34). Nevertheless, the deletion of the IMS segment led to a growth defect on lactate medium at higher temperature of 37°C, thereby suggesting an adverse effect on mitochondrial oxidative phosphorylation. Moreover, the IMS segment has been shown by biochemical and genetic experiments to interact with Tim17, suggesting that it stabilizes the interaction of Tim14 with the pre-protein translocase (21,35). Interestingly, the removal of the IMS segment increased the import efficiencies of inner membrane pre-proteins (36). The lateral release of proteins into the inner membrane might be inhibited by the IMS segment. The analysis of MCJ and its N-terminus in future studies might help elucidate the role of the IMS segment in more detail.

The stimulatory activity of MCJ and DNAJC19 on mortalin is counteracted by MAGMAS, the human homolog of yeast Tim16 (Fig. 5) (26). This property of MCJ and DNAJC19 is similar to that of their yeast homolog, Tim14, whose stimulatory effect is counteracted by Tim16 (21–25). Like Tim16, MAGMAS contains an N-terminal hydrophobic stretch and a C-terminal J-like domain which lacks the HPD motif that is present in J domains and crucial for Hsp70 stimulation (22,23,26). The structural and functional interaction of MAGMAS with Tim14 and DNAJC19 has been reported to resemble that of Tim16 with Tim14 (26,27). The X-ray structure of the Tim16–Tim14 complex indicates an interaction of the J-like with the J-domain (34). Moreover, Tim16 has been shown to be crucial for the recruitment of Tim14 to the TIM23 translocase and thus for the import of mitochondrial proteins (23,33,37,38). It may also regulate the activity of Tim14 in the translocase, so that Tim14 is able to stimulate the mtHsp70 activity only when an incoming pre-protein is present in the translocase (22–24,34). We show here that MAGMAS forms a stable subcomplex with MCJ. MCJ and MAGMAS can replace Tim14 and Tim16 in yeast, respectively (Fig. 6) (26). Thus, the interaction of MAGMAS with MCJ is most likely very similar to the interaction of Tim16 with Tim14.

There is also a second homolog of Tim14 present in yeast, Mdj2, that has a similar domain architecture of a TM segment and a C-terminal J domain (39). Like Tim14, Mdj2 has been shown to function as a J co-chaperone of the TIM23 translocase (25). In contrast to Tim14, however, Mdj2 is not the crucial J protein in the translocase. Mdj2 is dispensable for the cell and its physiological function remains unclear. It differs in its biochemical properties from Tim14. Only upon overexpression, Mdj2 was able to rescue the deletion of TIM14 (25). The sequences of MCJ and DNAJC19 are clearly more similar to the sequence of yeast Tim14 than to the one of Mdj2. In contrast to the other proteins, Mdj2 has a wider spacing between the TM segment and the J domain, uncharged residues next to the GGF motif in front of the J domain and an altered helix 2 of the J domain. Thus, MCJ and DNAJC19 do not resemble a Tim14/Mdj2 pair in human, but rather represent two Tim14 proteins.

Why are two Tim14 homologs present in vertebrates? Like MCJ, DNAJC19 interacts with subunits of the TIM23 translocase and stimulates the ATPase activity of mortalin (Fig. 5) (26). Thus, both proteins MCJ and DNAJC19 likely have similar and/or overlapping functions. So far, no tissue specificities of MCJ and DNAJC19 have been observed. Expression analysis of MCJ and DNAJC19 at the RNA level revealed expression in all tissues tested; MCJ was found to have the highest expression levels in testis (1,28). However, there might be cells and/or tissues which express only one of the homologs. Since Tim14 is essential for viability in yeast, a J co-chaperone is most likely also obligatory in human. The presence of two J co-chaperones may explain
why cells survive in the absence of either one of them. Ovarian cancer cells and MCF7 cells survive and are even more resistant against chemotherapeutic agents upon depletion of MCJ (1,2,6). Low amounts of MCJ and/or the presence of DNAJC19 appear to be sufficient for cell viability. The presence of DNAJC19 probably also explains why the import of pre-proteins into mitochondria is not strongly decreased upon depletion of MCJ. On the other hand, DNAJC19 plays a crucial role, even in the presence of MCJ, since a stop mutation in the DNAJC19 gene of patients leads to a severe phenotype of DCMA syndrome (28,29). MCJ may partially fulfill the function of DNAJC19, but it is not sufficient for normal function and development of the cells.

MCJ and DNAJC19 are not the only genes encoding components of the TIM23 translocase that are present in two copies in the human genome. For example, the two genes TIM17A and TIM17B encode TIM17 subunits. It has been shown that they are present in distinct TIM23 complexes (40). However, it still needs to be resolved whether these distinct TIM23 translocases have different substrate specificities or whether they have redundant functions and are able to replace each other. MCJ and DNAJC19 might be part of two distinct TIM23 pre-protein translocases with specific function and substrate specificity, as well. A major challenge will be to address whether distinct translocases with different structural and functional properties exist. The ability to employ and switch between distinct translocases may allow different cell types and/or cells under certain environmental conditions to adapt to varying physiological situations.

The expression level of MCJ was reported to correlate with the chemoresistance of ovarian cancer cells (1,6,7). Thus, the processes of mitochondrial import and of chemoresistance of cancer cells might be linked. At present, it can only be speculated whether and how the regulation of mitochondrial protein import affects the chemosensitivity of cancer cells. Increased chemoresistance can be attributed to several mechanisms, such as enhanced cellular drug efflux, increased metabolism of drugs or decreased expression of drug targets (41,42). Moreover, the inhibition of apoptotic pathways plays a major role in the development of chemoresistance. Notably, chemo-resistant ovarian cancer cells lacking MCJ were shown to be less sensitive to apoptosis (1). Mitochondria are a central player in the intrinsic pathway of apoptosis (43). In the absence of MCJ, an altered biogenesis and import of many and/or specific mitochondrial proteins might trigger changes in the mitochondrial physiology and dynamics and, thereby, might inhibit the function of mitochondria in apoptosis, e.g. by inhibiting the release of pro-apoptotic factors. On the other hand, an altered mitochondrial physiology might trigger signaling from mitochondria to the outside of mitochondria. This might induce changes in other cellular processes, such as gene expression or enzymatic activities, finally leading to reduced concentration of drugs. Interestingly, other components of the mitochondrial import machinery were reported to be involved in diseases and malignancies, such as MAGMAS in prostate carcinomas, DNAJC19 in DCMA and mortalin in carcinogenesis (28,44,45). It will be important to elucidate in future studies the relationships between mitochondrial protein import and diseases.

**MATERIALS AND METHODS**

**Plasmids and strains**

The yeast deletion strain of TIM14 expressing the corresponding wild-type gene from the URA3 carrying plasmid pVT-U was described before (25). For complementation of the deletion of TIM14 in yeast, the open reading frames of human MCJ and human DNAJC19 were amplified by PCR and cloned using the restriction sites EcoRI and XhoI into the centromeric vector pYX142 that express the open reading frames under the TPI promoter. The plasmids were transformed into the strain described above. Viability of the deletion strains of TIM14 harboring human MCJ or human DNAJC19 was tested by selection of cells that had lost the URA plasmid expressing wild-type Tim14 on plates containing 5-fluoroorotic acid. As a control for complementation, a plasmid expressing yeast Tim14 under its own promoter and with its own terminator (pRS314-yTim14) was used (33).

For bacterial co-expression of the soluble domains of MCJ and MAGMAS, the nucleotide sequence coding for the soluble domain of MAGMAS, MAGMAS(s) (amino acid residues 24–125), was cloned in frame with the sequences coding for an N-terminal his-tag and the TEV cleavage site into the pETDuet-1 vector using the restriction enzymes BamHI and NotI. The coding sequence for the soluble domain of MCJ, MCJ(s) (amino acid residues 57–150), was cloned into the second multiple cloning site of the generated plasmid with the restriction enzymes NdeI and Xhol, generating the plasmid pETDuet-HisMAGMAS(s)-MCJ(s).

The nucleotide sequences coding for the soluble domain of MCJ (amino acid residues 57–150) and for the soluble domain of DNAJC19 (amino acid residues 24–116) were cloned into the pGEX6p1 using the restriction sites EcoRI and XhoI to express these domains as GST fusion proteins.

**Isolation of mitochondria and submitochondrial localization**

HeLa cells were collected by centrifugation and resuspended in SEM buffer (70 mM sucrose, 220 mM mannitol, 20 mM HEPES, 5 mM EDTA, pH 7.2) containing complete EDTA-free protease-inhibitor cocktail (Roche). They were disrupted by 20 strokes in a Dounce homogenizer, type A. The homogenate was centrifuged to remove nuclei and cell debris. The supernatant was then separated into the pellet fraction (crude mitochondria) and a post-mitochondrial fraction by centrifugation at 20 000g for 10 min. Crude mitochondria were further purified by sucrose step gradient ultracentrifugation at 134 000g for 1 h at 2°C. The gradient consisted of four sucrose layers (15, 23, 32, 60%). Mitochondria at the interface of the 32 and 60% sucrose layer were harvested, pelleted and resuspended in SEM buffer.

Mitoplasts were generated by disrupting the outer membrane. To this end, mitochondria were diluted 10-fold in 20 mM HEPES-KOH, pH 7.4. Mitoplasts and mitochondria were incubated with or without of 50 μg/ml proteinase K for 20 min on ice. Mitochondria and mitoplasts were re-isolated, washed and precipitated with 12% (w/v) TCA. The samples were analyzed by SDS–PAGE and immunodecoration with
antibodies against mitochondrial marker proteins. Carbonate extraction was performed to distinguish integral membrane proteins from soluble proteins. Isolated mitochondria were incubated in 0.1 M sodium carbonate buffer at pH 11.5 for 20 min on ice. Soluble and membrane proteins were separated by centrifugation at 124,000 g for 30 min at 4°C. The supernatant containing the soluble proteins was precipitated with 12% TCA. Samples were then analyzed by SDS–PAGE and immunodecoration with antibodies against the indicated proteins.

**In vitro import into mitochondria**

For *in vitro* import, the precursor protein pSu9-DHFRmut, an established model precursor protein (46), was used, which consists of the N-terminal 69 amino acid residues of the subunit 9 of the ATP synthase of *Neurospora crassa* fused to a mutant variant of mouse dihydrofolate reductase. Radiolabeled precursor protein was synthesized by coupled transcription-translation (Promega) in reticulocyte lysate in a mutant variant of mouse dihydrofolate reductase. Radiolabeled precursor protein was used, which consists of the N-terminal 69 amino acid residues of the subunit 9 of the ATP synthase of *E. coli* (47). Bacteria were grown, induced, harvested and lysed as described above and mortalin was purified via a Ni-NTA agarose column. Following purification, the protein sample was loaded onto a PD10 column (Amersham) to exchange the buffer for storage buffer (20 mM HEPES-KOH, pH 7.4, 100 mM KCl, 5 mM MgCl2, 5% glycerol). The purity of the samples was examined by SDS–PAGE and Coomassie blue staining.

Mature mortalin (amino acid residues 51–679) with an N-terminal his-tag and a TEV cleavage site was expressed in the presence of yeast Hsp1 in a ΔDnaK *E. coli* strain (47). Bacteria were grown, induced, harvested and lysed as described above and mortalin was purified via a Ni-NTA agarose column. Following purification, the protein sample was loaded onto a PD10 column (Amersham) to exchange the buffer for storage buffer (20 mM HEPES-KOH, pH 7.4, 100 mM KCl, 5 mM MgCl2, 5% glycerol).

The plasmids coding for the soluble domains of MCJ (MCJ(s)) and DNAJC19 (DNAJC19(s)) as GST fusion proteins containing a Prescission Protease cleavage site were transformed into a ΔDnaK *E. coli* strain. Cells were harvested, lysed and sonicated in buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.6 mM KH2PO4, 1 mM PMSF, pH 7.3) as described above. Triton X-100 was added to a final concentration of 0.5% and the samples were incubated at room temperature for 30 min. The supernatant of the clarifying spin was loaded onto a GSH sepharose column. Following washing steps, the fusion protein bound to the beads was incubated with a GST-tagged Prescission Protease. The beads were removed and the supernatant containing the released MCJ(s) or DNAJC19(s) protein was loaded onto a PD10 column to exchange the buffer for storage buffer.

The pET21d vector carrying either the nucleotide sequence coding for his-tagged human MGE1 or for the his-tagged soluble domain of MAGMAS (amino acid residues 24–125) was used for expression of MGE1 and MAGMAS(s). When indicated, the mitochondrial membrane potential was dissipated by addition of 2 μM valinomycin. Following incubation, the samples were treated with 100 μg/ml proteinase K for 15 min on ice. The protease was stopped by addition of 2 mM PMSF. Mitochondria were re-isolated, washed and subjected to SDS–PAGE.

**Co-immunoprecipitation**

Mitochondria isolated from HeLa cells were lysed in buffer A [20 mM TrisHCl pH 7.4, 80 mM KCl, 1 mM PMSF with complete EDTA-free protease-inhibitor cocktail (Roche)] containing either 2.5% digitonin (Merek) or 1% Triton X-100 (Sigma-Aldrich) for 20 min at 4°C. After a clarifying spin (12,000 g, 4°C, 20 min), the lysate was incubated for 90 min at 4°C with antibodies or pre-immune IgGs prebound to protein-A sepharose. The supernatant was removed and beads were washed twice with 1 ml of buffer containing 0.1% digitonin or 0.05% Triton X-100. Bound proteins were eluted with Laemmli buffer. Proteins of total cell lysates, of the supernatant after the immunoprecipitation and of the eluate were analyzed by SDS–PAGE and immunodecoration with antibodies against the indicated proteins.

**Purification of recombinant proteins**

MCJ and MAGMAS were co-purified from bacteria carrying the plasmid pETDuet-HisMAGMAS(s)-MCJ(s). Cells were grown at 37°C in LB media containing 100 μg/ml ampicillin to an OD600 of 0.5. Protein expression was induced by addition of 1 mM IPTG. Cells were harvested after 3 h and resuspended in 20 ml of buffer A (50 mM TrisHCl pH 8.0, 200 mM NaCl, 5% glycerol, 20 mM imidazole) containing 1 mg/ml lysozyme and 1 mM PMSF. Following incubation of the cells for 45 min at 4°C, the samples were sonicated (level 4, duty cycle 80%, 10 times 12 s). Cell debris was removed by centrifugation at 27,500 g for 15 min at 4°C and the supernatant was loaded onto a Ni-NTA agarose column. The column was washed with 20 column volumes of buffer A. Bound proteins were eluted with buffer B (50 mM TrisHCl, pH 8.0, 200 mM NaCl, 5% glycerol, 500 mM imidazole) and stored in storage buffer (20 mM HEPES-KOH, pH 7.4, 100 mM KCl, 5 mM MgCl2, 5% glycerol). The purity of the samples was examined by SDS–PAGE and Coomassie blue staining.

**Measurement of the ATPase activity**

The ATPase activity of mortalin was determined using a coupled photometric enzyme assay. The hydrolysis of ATP is coupled to the oxidation of NADH to NADH*+* that can be photometrically monitored in a spectrophotometer (JASCO) at 360 nm. The assay was carried out at 25°C in buffer containing 20 mM HEPES pH 7.4, 80 mM KCl, 5 mM MgCl2, 2 mM ATP, 3 mM PEP, 0.12 mM NADH, 39 U/ml PK, 37 U/ml LDH and 2 μM of human MGE1. The reaction was started by addition of 1 μM of mortalin. The rates of ATP hydrolysis and standard deviations were calculated from the changes in absorbance measured in time (n ≥ 5). When indicated, 7.5 μM of purified MCJ(s) or MAGMAS(s) or 10 μM of purified DNAJC19(s) was added to the reaction. To test for the inhibition by MAGMAS, the purified MCJ(s) was...
either pre-incubated for 5 min at 25°C in an 1:1 molar ratio with purified MAGMAS(s) and the mixture added to the assay or purified MCJ(s)–MAGMAS(s) complex was assayed.

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