Mutations in the nuclear gene coding for the mitochondrial aspartyl-tRNA synthetase, a key enzyme for mitochondrial translation, are correlated with leukoencephalopathy. A Ser\textsuperscript{45} to Gly\textsuperscript{45} mutation is located in the predicted targeting signal of the protein. We demonstrate in the present study, by \textit{in vivo} and \textit{in vitro} approaches, that this pathology-related mutation impairs the import process across mitochondrial membranes.

\textbf{Key words:} aminoacyl-tRNA synthetase, organelle, pathology-related mutation, protein import, translation machinery, translocation.

\section*{INTRODUCTION}

The mitochondrion has many fundamental functions, e.g. in metabolic pathways, redox processes, energy production or apoptosis. The link between mitochondrial energetic dysfunction and cancer, aging phenomena and a broad range of metabolic and degenerative diseases is becoming more and more recognized [1]. Mitochondrial disorders can be caused by mutations in mtDNA (mitochondrial DNA) genes encoding either a core protein of an oxidative phosphorylation complex, or rRNAs and tRNAs required for mitochondrial translation. Additionally, mutations in nuclear genes encoding mitochondrial proteins are increasingly found to be associated with mitochondrial disorders, with the hypothesis that they would even be more common than mtDNA mutations [2].

An emerging field of neuromuscular and neurodegenerative disorders is linked to mutations in nuclear-encoded factors of the mitochondrial translation machinery [3] and especially mt-aaRS (mitochondrial aminoacyl-tRNA synthetases). LBSL (leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation) is a monogenic disease associated with mitochondrial disorders, with the link between mitochondrial energetic dysfunction and cancer, aging phenomena and a broad range of metabolic and degenerative diseases is becoming more and more recognized [1]. Mitochondrial disorders can be caused by mutations in mtDNA (mitochondrial DNA) genes encoding either a core protein of an oxidative phosphorylation complex, or rRNAs and tRNAs required for mitochondrial translation. Additionally, mutations in nuclear genes encoding mitochondrial proteins are increasingly found to be associated with mitochondrial disorders, with the hypothesis that they would even be more common than mtDNA mutations [2].

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Although mutations in \textit{ARS2} and \textit{YARS2} alter aminoacylation properties of mt-ArgRS and mt-TyrRS, consequences of the mutations affecting mt-AspRS are intriguingly different. Indeed, \textit{in vitro} activities of the subset of mutants tested so far only revealed a limited loss of their aminoacylation properties. Additionally, when respiratory chain complex activities were examined in fibroblast and lymphoblasts, no apparent dysfunctions were observed. Tissue-specific parameters and/or consequences on alternative functions of the aaRS have to be considered [4,5].

In the present paper, we are interested in the molecular consequences of the S45G change in mt-AspRS that was identified in LBSL patients. Ser\textsuperscript{45} resides within the predicted MTS (mitochondrial-targeting sequence) of mt-AspRS [11] (Figure 1). Most nuclear-encoded mitochondrial proteins possess a unique MTS, necessary for their presence in mitochondria. Mitochondrial proteins synthesized in the cytosol are then targeted to the surface of mitochondria via their interaction with chaperones. The MTSs are then specifically recognized by mitochondrial outer membrane receptors before guiding the precursor proteins through translocon complexes of the outer and inner membrane. Finally, MITs are identified and cut-off by mitochondrial-processing peptidases, releasing mature proteins (reviewed in [12–16]). In the present study, we have investigated the consequences of the S45G mutation on targeting, binding, translocation and processing steps of mt-AspRS. We show that this pathogenic mutation strongly affects mt-AspRS translocation through the mitochondrial membranes. This is the first time that an import defect of a mitochondrial translation machinery factor is associated with a severe human brain disorder.

\section*{EXPERIMENTAL}

\section*{Cloning}

Constructs used in the confocal microscopy experiments were cloned into pcDNA3.1/CT-GFP-TOPO® vector (Invitrogen) following the manufacturer’s protocol. PCR fragments corresponding to MTS–GFP (green fluorescent protein),

Abbreviations used: (mt-) aaRS, (mitochondrial) aminoacyl-tRNA synthetase; (mt-) ArgRS, (mitochondrial) arginyl-tRNA synthetase; (mt-) AspRS, (mitochondrial) aspartyl-tRNA synthetase; GFP, green fluorescent protein; (mt-) GluRS, (mitochondrial) glutamyl-tRNA synthetase; (mt-) GlyRS, (mitochondrial) glycyl-tRNA synthetase; HEK, human embryonic kidney; LBSL, leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation; mtDNA, mitochondrial DNA; MTS, mitochondrial-targeting sequence; mt-TyrRS, mitochondrial tyrosyl-tRNA synthetase.

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**MTS-mt-AspRS−GFP and MTS(S45G)-mt-AspRS−GFP** were generated using the pEGFP-N1-DARS2 and pEGFP-N1-(S45G)-DARS2 plasmids. These plasmids contain the full-length mt-AspRS-coding sequence (either wild-type or containing the mutation corresponding to S45G) cloned into pEGFP-N1 (Clontech). The mt-AspRS amplicon was amplified from pQE70-mt-AspRS [11]. Constructs used in the *in vitro* import and processing assays, PCR-generated truncated mt-AspRS versions (from amino acids 1 to 313, with or without the mutation), were cloned into pCR®2.1 vector (Invitrogen) downstream of a T7 promoter. A Kozak consensus sequence (5′-GCCATG-3′) was introduced during the PCR for the translation of the corresponding proteins. The plasmid expressing the first 81 amino acids of *Arabidopsis thaliana* mt-GluRS (mitochondrial glutamyl-tRNA synthetase) upstream of the GFP sequence has been published previously [17].

**Cell culture and visualization of the mt-AspRS−GFP fusion protein variants**

HEK (human embryonic kidney)-293T cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium, Invitrogen) supplemented with 10% FBS (fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C, in a 10% CO₂ humidified incubator. Cells were seeded on to coverslips at 3 × 10⁵ cells per 35 mm dish. At semi-confluence, cells were transiently transfected by the calcium phosphate method with 500 ng of plasmids. After 36 h, MitoTracker Red CM-H₂XRos (Invitrogen) was added to a final concentration of 100 nM for 30 min. Images were obtained using confocal microscopy as described previously [17].

**Human mitochondria purification**

Mitochondria were purified from HEK-293T cell lysates (Waring blender) by differential centrifugation at 1500 g followed by a final step at 20500 g for 25 min in 5 mM Heps/KOH (pH 7.5), 210 mM mannitol, 70 mM sucrose, 2 mM EDTA, 0.5% BSA and 2 mM 2-mercaptoethanol. The pellet of mitochondria was washed twice either in the import buffer [10 mM Heps/KOH (pH 7.5), 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM KH₂PO₄/K₂HPO₄, 0.05 mM EDTA and 5 mM MgCl₂] or in the import buffer without EDTA for the processing assay, and then kept on ice. The concentration of mitochondrial proteins was measured using the Bradford assay.

**In vitro assays**

*In vitro* synthesis of proteins in the presence of [³⁵S]methionine (1000 Ci/mmol, Amersham) was carried out with the TnT® T7 Coupled Reticulocyte Lysate System (Promega) following the manufacturer’s protocol. Import assays were performed as described previously [17]. The incubation time (30 min) is sufficient to cover the full process of import and maturation [18]. *In vitro* processing assays were performed as described previously [19]. Briefly, sonicated HEK-293T mitochondria (3 rounds of sonication for 10 s, medium speed, bioruptor, Diagenode) were diluted 2-fold in a processing buffer containing 40 mM Heps (pH 7.3), 2 mM MnCl₂, 0.2% Tween, 2 mM DTT (dithiothreitol) and 1× EDTA-free protease inhibitor cocktail. Then 5 μl of ³⁵S-labelled fusion proteins were added to 30 μl of mitochondrial suspension. After 3 h incubation at 30 °C, samples were analysed by SDS/PAGE and autoradiography.

**RESULTS AND DISCUSSION**

Mutated mt-AspRS co-localizes with mitochondria

To determine the possible consequences of the mutation on any of the mitochondrial protein import steps, targeting to mitochondria was first explored. HEK-293T cells, transfected with GFP-fusion protein constructs, were visualized by confocal microscopy. Although mt-AspRS lacking its theoretical MTS
Aminoacyl-tRNA synthetase import defect in mitochondrial disorder

Figure 2 Subcellular localization of GFP-fused mt-AspRS derivatives in human HEK-293T cells

Schematic representations of the four GFP-fused protein constructs are shown. Cells were transiently transfected with full-length (amino acids 1−645) or partial (amino acids 1−48 or 48−645) sequences of mt-AspRS and fluorescence signals were visualized by confocal microscopy. The S45G mutation is highlighted with an arrowhead. (A) GFP fluorescence; (B) Mitochondrial marker (MitoTracker Red CM-H2XRos); and (C) merge. The integrity of nuclei of HEK-293T cells following transient expression of MTS-mt-AspRS−GFP or MTS(S45G)-mt-AspRS−GFP was verified by staining with DAPI (4′,6-diamidino-2-phenylindole) (results not shown).

(residues 1−48) is exclusively present within the cytosol, both mt-AspRS and (S45G)mt-AspRS possessing their MTS co-localized with mitochondria (Figure 2). It is worth noting that GFP fused to the mt-AspRS MTS alone also co-localized with the mitochondrial-specific marker, showing that the N-terminal 48 amino acids of mt-AspRS are sufficient to guide this protein to mitochondria in vivo. In addition, S45G impaired neither targeting nor binding of mt-AspRS to mitochondria. Next, import through the mitochondrial membranes was analysed.

Mutated MTS prevents import of mt-AspRS into mitochondria

The impact of the mutation on the import process was tested by in vitro import assays of 35S-radiolabelled proteins into mitochondria purified from HEK-293T cells. mt-GluRS−GFP (N-terminal 81 amino acids of A. thaliana GluRS fused to GFP) was used as a positive control. This protein was previously successfully used for assays studying import and processing into isolated plant mitochondria under well-defined experimental conditions [17]. Figure 3(A) shows that this protein is also imported and processed into purified human mitochondria. Comparable with the A. thaliana mt-GluRS variant, a truncated version of mt-AspRS was used (N-terminal 313 amino acids including the MTS) for good resolution of precursor and processed proteins on SDS/PAGE. Processed forms of mt-AspRS, resistant to proteinase K, were observed, confirming proper import of this protein into mitochondria (Figure 3B). The size difference of ∼6 kDa between the precursor and processed forms is in agreement with the expected removal of the theoretical MTS. Import was restrained as expected in the presence of valinomycin, an uncoupling agent inhibiting protein import [17]. Two observations were made for (S45G)mt-AspRS. First, the mutated precursor protein bound to mitochondria as efficiently as the wild-type form since both proteins were recovered with mitochondria after a sucrose cushion purification step (Figures 3B and 3C, lane 2). This binding is consistent with the confocal microscopy experiments (Figure 2). Secondly, in contrast with mt-AspRS, no processed versions of the protein were visible (Figure 3C, lanes 2 and 3). This absence of mature product strongly suggests that the S45G mutation affects the import process, either at the translocation step through the double mitochondrial membrane or at the MTS-processing step. Actually, it has been shown that a non-processed protein is thermally less stable in the mitochondrial matrix and more rapidly degraded than a mature protein [20]. Therefore the effect of mutation on those two steps has been investigated further. In addition, incomplete degradation of mt-AspRS or (S45G)mt-AspRS by proteinase K (Figures 3B and 3C, lane 3) suggests that in vitro import of these human proteins is slower or less efficient than the mt-GluRS−GFP fusion protein (Figure 3A, lane 3).

Mutated AspRS precursor can be cleaved off by mitochondrial peptidases, but is not translocated through the mitochondrial membranes

To discriminate between the two above-mentioned steps, an in vitro processing experiment was performed. The same radio-labelled proteins [mt-GluRS−GFP, mt-AspRS and (S45G)mt-AspRS] were incubated with a mitochondrial enzymatic extract from HEK-293T cells containing active mitochondrial-processing peptidases [19]. No processing products were visible in the absence of the mitochondrial enzymatic extract for any of the three proteins (Figure 4). Incubation with the mitochondrial extract led to the expected processed products, with identical differences in size between precursor and processed forms with the ones obtained in the in vitro import assay. Interestingly, the processing...
assay shows that the pathogenic mutation S45G did not prevent removal of MTS by mitochondrial peptidases. Therefore, if the mutated (S45G)mt-AspRS is present inside the mitochondria, its MTS could be processed by mitochondrial peptidases. Combining these results indicates that the absence of a processed form of (S45G)mt-AspRS (Figure 3C) appears to be due to non-translocation of the protein and not to some inherent defect in its ability to be processed.

**Mitochondrial import failure of an aaRS in LBSL**

Many mutations in DARS2 have been associated with LBSL. In the present study, we have analysed the sole mutation known to be located in the MTS and nearby the predicted processing site (Figure 1). Interestingly, although the import machineries are well conserved between species of the same kingdom, each mitochondrial protein has its own MTS. Consequently, rules regarding the nature and the position of crucial residues for mitochondrial import cannot be precisely defined. The identity of those amino acids is likely to be studied on a case-by-case protein basis, and molecular consequences of mutations within the MTS cannot be anticipated. For instance, in *A. thaliana*, an alanine-to-serine mutation at MTS position 2 of mt-GluRS and mt-MetRS (mitochondrial methionyl-tRNA synthetase), or a threonine-to-glycine change of mt-PheRS (mitochondrial phenylalanyl-tRNA synthetase) respectively enhances, inhibits or does not affect their mitochondrial import [21]. Thus the consequence of the mutation S45G on the import process of mt-AspRS could not be foreseen before the present study; however, the strong conservation of Ser^45^ within the MTS of mammalian mt-AspRSs (Figure 1) suggested a role in their import. In the present study we show that S45G has a clear effect on the import process. Confocal microscopy imaging reveals that it does not affect the targeting, or the binding to mitochondria. *In vitro* import and processing assays show the import, and more precisely the translocation step, to be impaired by the mutation. This is a novel point of impact for a pathology-related mutation regarding a translation machinery protein. Strikingly, S45G has been described to not have any consequence on mitochondrial activity [4]. This remains puzzling, but an answer may arise from the fact that patients are compound-heterozygous for this mutation, the other mutation leading to only a weak expression of wild-type protein. This low level is apparently sufficient to support mitochondrial translation in the cells that were tested. Whether this is different in brain white matter cells has not been studied. It could also be that the mutation affects another, but still unknown, function of the protein [22]. Remarkably, and for unknown reasons, this compound-heterozygosity phenomenon is common to all of the DARS2 mutations so far reported [5].

**Mitochondrial import failure and diseases**

Two major types of mitochondrial import defects have been reported to be associated with disorders (for a review, see [23]). Either the components of the mitochondrial import and/or processing machinery are directly affected or mutations are present in the MTS, reducing the mitochondrial import of the corresponding protein. For example, deafness dystonia syndrome as well as a form of cardiomyopathy are caused by mutations in the genes encoding small proteins of the inner mitochondrial membrane [24, 25]. Only two cases have been so far reported with mutations affecting the MTS: a pyruvate dehydrogenase deficiency leading to abnormalities in the central nervous system [26], and partial arrest of manganese superoxide dismutase precursor protein within the inner mitochondrial membrane probably modulating susceptibility to various diseases [27]. For LBSL we describe a novel finding: the impairment of the import of a crucial translation machinery protein is correlated with a white
matter disorder. It can be anticipated that new disease-related mutations will be found in the MTS of proteins involved in any aspect of mitochondrial biogenesis.

AUTHOR CONTRIBUTION

Marie Messmer, Hagen Schwenzer, Gert Scheper and Laurence Maréchal-Drouard performed experiments. Marie Messmer, Laurence Maréchal-Drouard and Marie Sissler designed the research. Laurence Maréchal-Drouard and Marie Sissler supervised the present work. Marie Messmer, Catherine Florentz, Laurence Maréchal-Drouard and Marie Sissler analysed the data and wrote the manuscript. Hagen Schwenzer, Gert Scheper and Marjo van der Knaap critically read the manuscript prior to submission.

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REFERENCES


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