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A human pathology-related mutation prevents import of an aminoacyl-tRNA synthetase into mitochondria

Marie Messmer[†], Catherine Florentz[†], Hagen Schwenzer[†], Gert C. Scheper[‡], Marjo S. van der Knaap[‡], Laurence Maréchal-Drouard^{§*} and Marie Sissler^{†*}

[†] Architecture et Réactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 15 rue René Descartes, F-67084 Strasbourg, France.

[‡] Department of Pediatrics and Child Neurology, VU University Medical Center, 1081 HV Amsterdam, The Netherlands.

[§] Institut de Biologie Moléculaire des Plantes, UPR 2357, CNRS, conventionné avec l'Université de Strasbourg, 12 rue du Général Zimmer, F-67084 Strasbourg, France.

***, To whom correspondence should be addressed:

E-mail: m.sissler@ibmc-cnrs.unistra.fr. Phone: +33 (0)3 88 41 70 57 – Fax: +33 (0)3 88 41 60 22 18

E-mail: laurence.drouard@ibmp-cnrs.unistra.fr. Phone: +33 (0)3 88 41 72 40 – Fax: +33 (0)3 88 61 44 42

Short title (page heading): Aminoacyl-tRNA synthetase import defect in mitochondrial disorder

SYNOPSIS

Mutations in the nuclear gene coding for the mitochondrial aspartyl-tRNA synthetase, a key enzyme for mitochondrial translation, are correlated with leukoencephalopathy. Ser₄₅ to Gly₄₅ mutation is located in the predicted targeting signal of the protein. We demonstrate here, by *in vivo* and *in vitro* approaches, that this pathology-related mutation impairs the import process across mitochondrial membranes.

Keywords: Organelle / Pathology-related mutation / Aminoacyl-tRNA synthetase / Protein import / Translation machinery / Translocation

INTRODUCTION

The mitochondrion has many fundamental functions in *e.g.* 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 (mt) disorders can be caused by mutations in mt DNA genes encoding either a core protein of an oxidative phosphorylation complex or rRNAs and tRNAs required for mt translation. Additionally, mutations in nuclear genes encoding mt proteins are increasingly found to be associated with mt disorders, with the hypothesis that they would even be more common than mt DNA mutations [2].

An emerging field of neuromuscular and neurodegenerative disorders is linked to mutations in nuclear encoded factors of the mt translation machinery [3] and especially mt aminoacyl-tRNA synthetases (mt-aaRS). “Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation” (LBSL) is a monogenic disease associated with a large variety of mutations affecting the human nuclear gene *DARS2*, encoding the mt aspartyl-tRNA synthetase (mt-AspRS) [4] and novel mutations in this gene were reported since [5, 6]. Deleterious mutations in *RARS2* (mt-ArgRS) are associated with pontocerebellar hypoplasia [7] and mutations in *YARS2* (mt-TyrRS) with myopathy, lactic acidosis and sideroblastic anemia [8]. Missense mutations in *GARS* (encoding both cytosolic and mt-GlyRS) cause a variant of Charcot-Marie-Tooth disease [9, 10].

While mutations in *RARS2* and *YARS2* alter aminoacylation properties of mt-ArgRS and mt-TyrRS, consequences of the mutations affecting mt-AspRS are intriguingly different. Indeed, *in vitro* activities of the subset of tested mutants 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].

Here, we are interested in the molecular consequences of the p.Ser45Gly (S45G) change in mt-AspRS that was identified in LBSL patients. Ser₄₅ resides within the predicted mitochondrial targeting sequence (MTS) of mt-AspRS [11] (Fig. 1). Most nuclear-encoded mt proteins possess a unique MTS, decisive for their presence in mitochondria. Mt proteins synthesized in the cytosol are first targeted to the surface of mitochondria *via* their interaction with chaperones. The MTSs are then specifically recognized by mt outer membrane receptors before guiding the precursor proteins through translocase complexes of the outer and inner membrane. Finally, MTSs are identified and cut off by mt processing peptidases, releasing mature proteins (reviewed in *e.g.* [12-16]). We have investigated the consequences of 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 mt membranes. This is the first time an import defect of a mt translation machinery actor is associated with a severe human brain disorder.

EXPERIMENTAL

Cloning

Constructs used in the confocal microscopy experiments were cloned into pcDNA3.1/CT-GFP-TOPO® vector (Invitrogen) following the manufacturer's procedures. PCR fragments corresponding to MTS-GFP, 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 aa 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 81 first aa of *A. thaliana* mtGluRS upstream the GFP sequence was published before [17].

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

HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C, in a 10% CO₂ humidified incubator. Cells were seeded onto 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 [17].

Human mitochondria purification

Mitochondria were purified from HEK 293T cell lysates (warring blender) by differential centrifugations at 1,500 *g* followed by final step at 20,500 *g* for 25 min in 5 mM HEPES-KOH pH 7.5, 210 mM mannitol, 70 mM sucrose, 2 mM EDTA, 0.5% BSA, 2 mM β-mercaptoethanol. The pellet of mitochondria was washed twice either in the import buffer (10 mM HEPES-KOH pH 7.5, 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM KH₂PO₄/K₂HPO₄ pH 7.5, 0.05 mM EDTA, 5 mM MgCl₂) or in the import buffer without EDTA for the processing assay and kept on ice. Concentration of mt proteins was measured by a 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 procedures. Import assays were performed as previously described [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 previously described [19]. Briefly, sonicated HEK 293T mitochondria (3 rounds of sonication for 10 sec, medium speed, bioruptor, Diagenode) were diluted 2-fold in a processing buffer containing 40 mM HEPES pH 7.3, 2 mM MnCl₂, 0,2% Tween, 2 mM DTT and 1X protease inhibitor cocktail EDTA free. Five µl of [³⁵S]-labeled fusion proteins were then added to 30 µl of mt suspension. After 3 h incubation at 30 °C samples were analyzed by SDS-PAGE and autoradiography.

RESULTS & DISCUSSION

Mutated mt-AspRS co-localizes with mitochondria

To determine the possible consequences of the mutation on any of the mt protein import steps, targeting to mitochondria was first explored. HEK 293T cells, transfected with GFP fusion protein constructs, were visualized by confocal microscopy. While mt-AspRS lacking its theoretical MTS (residues 1-48) is exclusively present within the cytosol, both mt-AspRS and (S45G)mt-AspRS possessing their MTS co-localize with mitochondria (Fig. 2). Noteworthy, GFP fused to the mt-AspRS MTS alone also co-localizes with the mt specific-marker, showing that the 48 N-terminal amino acids (aa) of mt-AspRS are sufficient to guide

this protein to mitochondria *in vivo*. In addition, S45G neither impaired targeting nor binding of mt-AspRS to mitochondria. Next, import through the mt membranes was analyzed.

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 [S^{35}]-radiolabeled proteins into mitochondria purified from HEK 293T cells. MtGluRS-GFP (first 81 N-term aa of *Arabidopsis 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 3A shows that this protein is also imported and processed into purified human mitochondria.

Comparable to the *A. thaliana* mtGluRS variant, a truncated version of mt-AspRS was used (N-terminal 313 aa including 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 into mitochondria of this protein (Fig. 3B). The size difference of ~6 kDa between the precursor and processed products 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 (Fig. 3B and 3C, lane 2). This binding is consistent with the confocal microscopy experiments (Fig. 2). Second, in contrast to mt-AspRS, no processed versions of the protein were visible (Fig. 3C, lanes 2 and 3). This absence of mature product strongly suggests that mutation S45G affects the import process, either at the translocation step through the double mt-membrane, or at the MTS-processing step. Actually, it has been shown that a non-processed protein is thermally less stable in mt matrix and more rapidly degraded than a mature protein [20]. Therefore, effect of mutation on those two steps has been further investigated. In addition, incomplete degradation of mt-AspRS or (S45G)mt-AspRS by proteinase K (Fig. 3B and 3C, lane 3) suggests that *in vitro* import of these human proteins is slower or less efficient than the mtGluRS-GFP fusion protein (Fig. 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 radiolabeled proteins (mtGluRS-GFP, mt-AspRS, (S45G)mt-AspRS) were incubated with a mt enzymatic extract from HEK 293T cells containing active mt processing peptidases [19]. No processing products were visible in the absence of the mt enzymatic extract for any of the three proteins (Fig. 4). Incubation with the mt extract led to the expected processed products, with identical differences in size between precursor and processed forms than 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 mt peptidases. Therefore, if the mutated (S45G)mt-AspRS would be present inside the mitochondria, its MTS could be processed by mt peptidases. Combining these results indicates that the absence of a processed form of (S45G)mt-AspRS (Fig. 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 aminoacyl-tRNA synthetase in LBSL

Many mutations in *DARS2* have been associated with LBSL. Here, we have analyzed the sole mutation known to be located in the MTS and nearby the predicted processing site

(Fig. 1). Interestingly, while the import machineries are well conserved between species of a same kingdom, each mt protein has its own MTS. Consequently, rules about the nature and the position of crucial residues for mt import cannot be precisely defined. The identity of those aa is likely to be studied on a case-by-case protein basis and molecular consequences of mutations within MTS cannot be anticipated. For instance, in *A. thaliana*, Ala to Ser mutation at MTS position 2 of mt-GluRS and mt-MetRS or Thr to Gly change of mt-PheRS respectively enhances, inhibits or doesn't affect their mt import [21]. Thus, the consequence of mutation S45G on the import process of mt-AspRS could not be foreseen before this study. However, the strong conservation of Ser45 within MTS of mammalian mt-AspRSs (Fig. 1) suggested a role in their import. Here we show that S45G has a clear impact 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 designate 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 mt 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 mt 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 the *DARS2* mutations reported so far [5].

Mitochondrial import failure and diseases

Two major types of mt import defects have been reported to be associated with disorders (for a review, see [23]). Either the components of the mt import and/or processing machinery are directly affected or mutations are present in the MTS, reducing the mt import of the corresponding protein. The Deafness Dystonia Syndrome as well as a form of cardiomyopathy are for example caused by mutations in the genes encoding small proteins of the inner mt membrane [24, 25]. Only two cases were reported so far 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 mt membrane likely 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 MTS of proteins involved in any aspect of mitochondrial biogenesis.

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FIGURE LEGENDS

Figure 1. Multiple sequence alignment of the N-terminal region of human mt-AspRS with a selection of known AspRSs. Accession numbers of sequences are mt *H. sapiens*, NP_060592.2; mt *C. jacchus*, XP_002760400; mt *B. taurus*, NP_001095692; mt *E. caballus*, XP_001493136; mt *M. musculus*, NP_766232; mt *A. thaliana*, NP_195102; mt *S. cerevisiae*, NP_015221; *E. coli*, NP_416380; *B. subtilis*, NP_390633; *S. aureus*, NP_372154. Black and gray boxes highlight identical and similar residues, respectively. Predicted cleavage position of mt-AspRS mitochondrial targeting sequence is indicated by a gray arrowhead. The S residue mutated into G at position 45 in the human mt-AspRS is pinpointed by a black arrowhead.

Figure 2. Subcellular localization of GFP-fused mt-AspRS derivatives in human HEK 293T cells. Schematic representations of the 4 GFP-fused protein constructs are indicated. Cells were transiently transfected with full-length (1-645 aa) or partial (1-48 or 48-645 aa) sequences of mt-AspRS and fluorescence signals visualized by confocal microscopy. S45G mutation is highlighted with an arrowhead. Column **A**: GFP fluorescence; column **B**: mt marker (Mitotracker Red CM-H₂XRos); column **C**: merge. 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 4',6-diamidino-2-phenylindole (DAPI) (not shown).

Figure 3. *In vitro* import experiments with purified human mitochondria. Experiments were performed with three constructs: **A**. As a control, mtGluRS-GFP (81 first aa of *A. thaliana* mtGluRS upstream the GFP sequence) [17], **B**. Human mt-AspRS 313 first amino acids and **C**. (S45G)mt-AspRS. Radioactive full-length precursors obtained by *in vitro* transcription/translation (lane 1), were incubated with mitochondria purified from human HEK 293T cells (lanes 2 to 5) for 30 min, and further purified on sucrose gradient before separation on SDS-PAGE and autoradiography. Proteinase K degrades proteins present at the mt surface but does not affect proteins located inside mitochondria. Preincubation with valinomycin inhibits protein import. Molecular weights were calculated based upon marker proteins. Each assay was independently performed at least three times, with no significant variations.

Figure 4. Processing of precursor proteins by a human mitochondrial peptidase extract. The experiment was performed with the same constructs used in Figure 3. Radioactive full-length precursor proteins of mtGluRS-GFP, MTS-mt-AspRS and (S45G)MTS-mt-AspRS obtained by *in vitro* transcription/translation were incubated in the absence (-) or in the presence (+) of a HEK 293T mt enzymatic extract. Three independent sets of experiments were performed and showed identical patterns. Precursor and processed forms are indicated. Molecular weights have been calculated according to marker proteins. * = non specific products synthesized with the TNT® T7 Coupled Reticulocyte Lysate System.





