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Activation of Human Mitochondrial Lysyl-tRNA Synthetase upon Maturation of Its Premitochondrial Precursor

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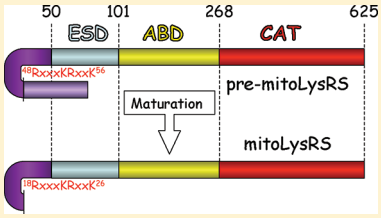
ABSTRACT: The cytoplasmic and mitochondrial species of human lysyl-tRNA synthetase are encoded by a single gene by means of alternative splicing of the KARS1 gene. The cytosolic enzyme possesses a eukaryote-specific N-terminal polypeptide extension that confers on the native enzyme potent tRNA binding properties required for the vectorial transfer of tRNA from the synthetase to elongation factor EF1A within the eukaryotic translation machinery. The mitochondrial enzyme matures from its precursor upon being targeted to that organelle. To understand how the cytosolic and mitochondrial enzymes are adapted to participate in two distinct translation machineries, of eukaryotic or bacterial origin, we characterized the mitochondrial LysRS species. Here we report that cleavage of the precursor of mitochondrial LysRS leads to a mature enzyme with reduced tRNA binding properties compared to those of the cytoplasmic counterpart. This adaptation mechanism may prevent inhibition of translation through sequestration of lysyl-tRNA on the synthetase in a compartment where the bacterial-like elongation factor EF-Tu could not assist in its dissociation from the synthetase. We also observed that the RxxxKRxxK tRNA-binding motif of mitochondrial LysRS is not functional in the precursor form of that enzyme and becomes operational after cleavage of the mitochondrial targeting sequence. The finding that maturation of the precursor is needed to reveal the potent tRNA binding properties of this enzyme has strong implications for the spatiotemporal regulation of its activities and is consistent with previous studies suggesting that the only LysRS species able to promote packaging of $tRNA^{Lys}$ into HIV-1 viral particles is the mature form of the mitochondrial enzyme.



Aminoacyl-tRNA synthetases (aaRS) activate amino acids and transfer them on cognate tRNAs.[1] These enzymes are ubiquitous to all living cells that generally possess the full set of 20 enzymes, one for each amino acid. In eukaryotic cells, in addition to the cytosolic translation machinery, a distinct translation system is found in mitochondria and in plant chloroplasts. Because these two organelles are of endosymbiotic origin, from an α-proteobacteria or from a cyanobacteria, for mitochondria and chloroplasts, respectively, two or three sets of tRNA and aminoacyl-tRNA synthetases are required in these organisms.[2] All aaRS are encoded by the nucleus and routed to the appropriate cellular compartment. In human cells, most mitochondrial aaRS are of prokaryotic origin and are more similar in sequence to the bacterial enzymes than to the cytosolic counterparts. There are two exceptions, where the cytosolic and mitochondrial synthetases are expressed from a single gene. This is the case for glycyl-tRNA synthetase, where the two species are translated from a single mRNA from two in-frame initiation codons,[3] and lysyl-tRNA synthetase (LysRS), which is produced from two mRNAs arising by alternative splicing of the unique KARS gene.[4]

Cytosolic LysRS (cLysRS) is composed of a core enzyme, corresponding to the catalytic and anticodon-binding domains, that is 43% identical in amino acid sequence with bacterial LysRS and possesses a eukaryote-specific, N-terminal polypeptide extension of 73 residues that provides the core synthetase with potent tRNA binding capacities.[5,6] A peptide from this extension may adopt an α-helical conformation, providing a tRNA-anchoring platform[7,8] for the acceptor-TΨC stem−loop portion of tRNA.[6,9] The structural model of the complete *Brugia malayi* asparaginyl-tRNA synthetase,[10] a synthetase closely evolutionarily related to LysRS, recapitulates the properties ascribed to the polypeptide extensions of class IIb eukaryotic aaRS. These polypeptide appendices are believed to mediate processivity of tRNA handling in translation, from the synthetase to elongation factor EF1A.[11]

The cLysRS species is also a component of the multi-aminoacyl-tRNA synthetase complex (MARS) containing eight other aaRS (arginyl-, aspartyl-, isoleucyl-, glutaminyl-, glutamyl-, leucyl-, methionyl-, and prolyl-tRNA synthetases) and three auxiliary proteins (p18, p38, and p43) involved in complex assembly.[12−14] Association of cLysRS with MARS primarily involves interaction of the catalytic domain of the enzyme with the 42 N-terminal amino acid residues of p38, the scaffold protein of the complex.[13−15] The complex is believed to accommodate two LysRS dimers per p38 dimer.[16] The crystal structure of the core enzyme, lacking its eukaryote-specific N-terminal polypeptide extension, has been determined.[17] A three-dimensional model of the LysRS−p38 subcomplex, comprising one p38 dimer and two LysRS dimers, has been proposed.[18]

Even if the main structural state of cLysRS is its associated form within MARS, and its main physiological role is tRNA aminoacylation within the cytosolic translation machinery,

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other alternative functions have been ascribed to cLysRS. For example, it has been shown that cLysRS is one of the most efficient synthetases in synthesizing diadenosine tetraphosphate (Ap_4A), and that this efficiency is increased after its dissociation from MARS.[19] It was recently reported that cLysRS is released form MARS after MAPK-dependent phosphorylation in activated mast cells and is translocated into the nucleus where it activates transcription factor MITF in an Ap_4A-dependent manner.[20] Moreover, the activity of cLysRS has been shown to be regulated by syntenin-1, a PDZ protein involved in various cellular functions, such as transcription, cellular trafficking, and cell adhesion,[21] or has been reported to be secreted in response to TNF-α to trigger a proinflammatory response.[22]

The translation product for the premitochondrial species of LysRS (pmLysRS) possesses a specific N-terminal sequence of 49 amino acid residues that is predicted to be processed between Lys^{16} and Thr^{17} during mitochondrial import to give the mature species (mLysRS).[4] Whereas pmLysRS and mLysRS, which share the catalytic domain of cLysRS, have the intrinsic capacity to bind p38, they are never recovered within MARS.[15] This suggests that pmLysRS is strictly routed *in cellulo*, which prevents its interaction with cytosolic proteins. Mitochondrial LysRS also has additional functions in addition to aminoacylation. A mutation in Cu,Zn-superoxide dismutase (SOD1), which causes amyotrophic lateral sclerosis (ALS), induces an aberrant interaction between SOD1 and mLysRS, resulting in mitochondrial disorders.[23] Early studies reported the presence of LysRS in HIV-1 viral particles,[24] but polyclonal antibodies used in this study did not discriminate between the cytosolic and mitochondrial LysRS species. The same authors suggested that cellular LysRS could be targeted to the virions by interaction with the polyproteins Gag[25] and Pol.[26] More recently, the mitochondrial species of LysRS was shown to be a key element for the biology of the human immunodeficiency virus (HIV-1). Cytosolic $tRNA_3^{Lys}$, the primer for reverse transcription of the HIV-1 genome, is hijacked into viral particles as a complex containing the viral protein GagPol and mLysRS.[15,27]

The involvement of cLysRS and mLysRS in several functional pathways beyond translation suggests that the spatiotemporal organization of the translation machinery is a key element for the regulation of their functions and of cellular homeostasis. To understand how pmLysRS reaches the mitochondrial compartment without interfering with cytosolic translation, we determined the signal sequences of pmLysRS involved in its mitochondrial targeting and processing and characterized the aminoacylation activity and tRNA binding capacity of the precursor (pmLysRS) and mature (mLysRS) mitochondrial LysRS species. Our data show that the activity of mLysRS is enhanced upon pmLysRS processing.

EXPERIMENTAL PROCEDURES

Expression and Purification of LysRS Expressed in *Escherichia coli*, Yeast, or Insect Cells. Human cytosolic and mitochondrial LysRS were expressed in the yeast *Saccharomyces cerevisiae*[27] and purified[6] as described previously.

The premitochondrial form of human LysRS was expressed either in High Five insect cells after its cDNA had been subcloned into pFastBac1 or in BL21(DE3) *E. coli* cells after being subcloned into pET28b. When the compound was expressed in insect cells, a crude extract was prepared in 100 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM KCl, 5 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, 1% Triton X-100, 10 mM benzamidine, and 1 mM Pefabloc and sonicated, and the mitochondrial LysRS that matured in vivo was purified essentially as described previously,[6] by chromatography on S-Sepharose FF, Mono Q HR 5/5, and Mono S HR 5/5 columns (GE Healthcare). This procedure gave 2.5 mg of homogeneous protein per liter of culture. Analysis by nanoESI-Q-TOF indicated that the purified protein was cleaved after Gly^{30}. When expressed in *E. coli*, premitochondrial LysRS was not processed and was purified as described below.

The protein was expressed in *E. coli* BL21(DE3) grown in LB medium supplemented with kanamycin (50 μg/mL). The culture (6 L) was grown at 37 °C to an A_{600} of 0.4 and then transferred at 20 °C. When the A_{600} was increased to 1.0, and expression was induced by addition of 1 mM IPTG for 5 h. Cells were washed twice with ice-cold extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM EDTA, 10 mM $MgCl_2$, 10% glycerol, and 10 mM 2-mercaptoethanol], resuspended in the same buffer (1 mL/g of cell pellet) containing 2 mM diisopropyl-fluorophosphate, 2 mM PMSF, and 2 μg/mL antipain and chymostatin, and lysed in an Eaton press. All subsequent steps were conducted at 4 °C. After a 2-fold dilution with extraction buffer, the extract was sonicated to fragment nucleic acids and diluted by addition of 1 volume of extraction buffer containing 800 mM KCl, and cell debris was removed by centrifugation at 48000*g* for 30 min. Nucleic acids were removed by precipitation with Polymin P at a concentration of 0.5%. The clear supernatant was diluted by addition of 2 volumes of extraction buffer without KCl and applied to a 65 mL S-Sepharose FF column (GE Healthcare). pmLysRS was eluted with a linear gradient (20 column volumes) of NaCl from 150 to 600 mM in 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 10% glycerol, and 10 mM 2-mercaptoethanol. After dialysis against the same buffer containing 75 mM NaCl, fractions were applied to a 4.6 mL SOURCE 15Q column (GE Healthcare), equilibrated in the same buffer, and eluted with a linear gradient (20 column volumes) of NaCl from 75 to 500 mM. Fractions containing pmLysRS were directly applied on a Mono S HR 5/5 column (GE Healthcare), equilibrated in the same buffer containing 200 mM NaCl, and eluted with a linear gradient (50 column volumes) of NaCl from 200 to 800 mM. Fractions containing pmLysRS were concentrated by ultrafiltration, dialyzed against 25 mM potassium phosphate (pH 7.5), 2 mM DTT, and 55% glycerol, and stored at −20 °C. Protein concentrations were determined by using a calculated absorption coefficient of 0.77 A_{280} units mg^{-1} cm^2. Analysis by nanoESI-Q-TOF and by automatic Edman degradation showed that the purified protein corresponded to pmLysRS with the removal of the N-terminal Met residue.

Expression of LysRS in HeLa Cells. The cDNA of human premitochondrial LysRS (pmLysRSHs), cytoplasmic LysRS (cLysRSHs), and the cDNA of a N-terminally truncated version of cLysRS (ΔNLysRSHs), containing 529 residues including the anticodon-binding domain (ABD) and the catalytic domain (CAT) were amplified by polymerase chain reaction (PCR). The sense oligonucleotides used were GGGGAATTCATAATGTTGACGCAAGCTGCTGTAAGG, GGGGAATTCATAATGGCGGCCGTGCAGGCGGCCGA, and GGGGAATTCATAATGGTGGACCCAAATCAATAC-TACA for pmLysRSHs, cLysRSHs, and ΔNLysRSHs respectively. The antisense oligonucleotide was common to the three reactions and was GGGGAATTCCTAGACAGAACTGC-

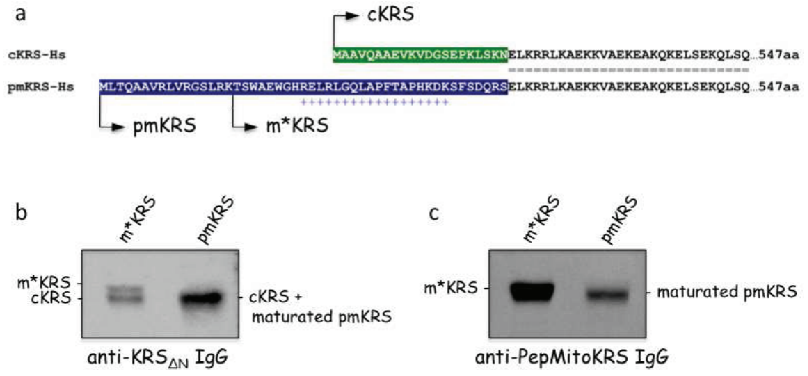

Figure 1. Expression of the cytoplasmic and mitochondrial forms of LysRS in HeLa cells. (a) The 576 C-terminal amino acid residues encoded by exons 3−15 of the human KARS1 gene are common (=) to the cytoplasmic (cKRS) and mitochondrial (pmKRS) species of LysRS. The 21 N-terminal residues of cKRS are encoded by exon 1, and the 49 N-terminal residues of pmKRS are encoded by exon 2. The peptide sequences used to raise antibodies specific for the mitochondrial species of human LysRS are denoted with pluses. The putative maturation site of pmKRS (m*KRS) is indicated. (b and c) The two gene products m*KRS and pmKRS were expressed in HeLa cells. Total extracts were analyzed by Western blotting using antibodies directed to the core domain of LysRS (anti-KRSΔ_N IgG) (b) or the synthetic peptide specific for the mitochondrial form of human LysRS (anti-PepMitoKRS IgG) (c).

CAACTGTTG. The PCR products were digested with *Eco*RI and inserted into the *Eco*RI site of eukaryotic expression vector pSG5 (Agilent). The encoded proteins correspond to full-length pmLysRSHs, cLysRSHs, and the N-terminally truncated version of cLysRSHs (ΔNLysRSHs). The sequence of the recombinant plasmids was verified by DNA sequencing. HeLa cells were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, and a 100 μg/mL penicillin/streptomycin mixture. Cells were transfected with Effectene (QIAGEN). Cell extracts were prepared in sodium dodecyl sulfate (SDS) sample buffer 48 h post-transfection, and expression of proteins was checked by Western blot analysis with antibodies as indicated.

Confocal Imaging. HeLa cells were grown in F12 medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, a 100 μg/mL penicillin/streptomycin mixture. Cells were transfected with Effectene (QIAGEN). For localization experiments, cells were cotransfected with a pEGFP-N1 derivative and with pDsRed2-Mito (BD Biosciences). Cells were grown in eight-well Lab-Tek II chambers (Nalge Nunc International) and observed by confocal laser scanning microscopy using a Leica TCS SP2 confocal microscope equipped with a DD488/543 mirror. GFP was excited using a 488 nm laser line of an Ar laser and detected at 500−535 nm. DsRed was excited at 543 nm with a He−Ne laser and detected at 584−659 nm. Imaging of GFP and DsRed fluorescence was performed in a sequential manner. The expression and the stability in HeLa cells of the fusion proteins containing LysRS sequences fused to GFP were checked by Western blot analysis with anti-GFP antibodies.

Purification of Mitochondrial LysRS Expressed in HeLa Cells. The cDNA of human premitochondrial LysRS (pmLysRS) was amplified via PCR between oligonucleotides GGGGAATTCATAATGTTGACGCAAGCTGCTGTAAGG and GGGGAGATCTACAGAACTGCCAACTGTTG. The PCR product was digested with *Eco*RI and *Bgl*II and inserted into the *Eco*RI−*Bam*HI sites of eukaryotic expression vector pSG5(His). The encoded protein corresponds to full-length pmLysRS bearing an additional C-terminal His^6 sequence and was verified by DNA sequencing.

The six-His-tagged pmLysRS was expressed in HeLa cells. Cells were grown to 50% confluency in 25 Petri dishes (145 mm diameter, approximately 2×10^8 cells) and transfected by the $CaCl_2$ method with pSG5-KRSpm-6His. The cells were harvested 48 h post-transfection, lysed in extraction buffer [20 mM Tris-HCl (pH 7.5), 10 mM imidazole, 500 mM NaCl, 5 mM 2-mercaptoethanol, 0.5% Triton X-100, and 2.5 mM benzamidine], and homogenized by sonication to disrupt mitochondria. After lysis, the supernatant recovered after centrifugation at 10000*g* for 15 min was loaded on a 0.5 mL nickel-nitrilotriacetic acid Superflow matrix (Qiagen) charged with Ni^{2+}. After being washed with buffer A [20 mM Tris-HCl (pH 7.5), 10 mM imidazole, 500 mM NaCl, and 5 mM 2-mercaptoethanol], bound material was eluted by addition of 5 mL of buffer B [20 mM Tris-HCl (pH 7.5), 200 mM imidazole, 500 mM NaCl, and 5 mM 2-mercaptoethanol]. Fractions (1 mL) were collected, and fractions containing mLysRS-His^6 were pooled (~2 mL) and dialyzed against 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). After dialysis, mLysRS-His^6 was applied to a MonoQ HR 5/5 anion exchange column (GE Healthcare) equilibrated in dialysis buffer. Bound material was eluted with a 20 mL linear gradient from 100 to 500 mM NaCl in the same buffer; 500 μL fractions were collected and frozen at −80 °C.

Gel Retardation Assay. Plasmid ptRNA$_3^{Lys}$ encoding human cytosolic tRNA$_3^{Lys}$ was linearized with *Fok*I and subjected to in vitro transcription with T7 RNA polymerase as described previously.[6,28]

Protein−RNA interactions were analyzed using a band shift assay. Homogeneous LysRS proteins were incubated at increasing concentrations with radiolabeled RNA (25000 cpm per point) in a 11 μL volume containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10% glycerol, and 0.1 mg/mL bovine serum albumin (BSA). After incubation at 25 °C for 30 min, the mixture was placed on ice and loaded on a 6% polyacrylamide gel (29:1 mono:bis ratio) containing 5% glycerol in 0.5× TBE (pH 8.0) at 4 °C. After electrophoresis, the gel was fixed, dried, and subjected to autoradiography. Free and bound tRNA was quantified by densitometry measurements.

tRNA Aminoacylation Assay. Initial rates of tRNA aminoacylation were measured at 25 °C in 0.1 mL of 20 mM imidazole-HCl buffer (pH 7.5), 100 mM KCl, 0.5 mM DTT,

12 mM $MgCl_2$, 2 mM ATP, 180 μM ^{14}C-labeled lysine (NEN, 16.66 Ci/mol), and saturating amounts of tRNA.[5] Human $tRNA_3^{Lys}$ expressed in *E. coli* (lysine acceptance of 780 pmol/A_{260}) was used as a tRNA substrate. The incubation mixture contained catalytic amounts (1−5 nM) of enzymes appropriately diluted in 10 mM Tris-HCl (pH 7.5) and 10 mM 2-mercaptoethanol, containing 4 mg/mL BSA. One unit of activity is the amount of enzyme producing 1 nmol of lysine-$tRNA^{Lys}$ per minute at 25 °C. For the determination of K_m values for tRNA, $tRNA^{Lys}$ concentrations of 0.1−50 μM were used. Michaelian parameters were obtained by nonlinear regression of the theoretical Michaelis−Menten equation to the experimental curve using KaleidaGraph version 3.6.5 (Abelbeck Software).

RESULTS

Identification of the N-Terminal Maturation Site of Mitochondrial LysRS. Mitochondrial LysRS is expressed as a precursor protein (pmKRS) containing a specific N-terminal sequence made of 49 amino acid residues, as compared with the cytoplasmic protein encoded by the same gene by a mechanism of alternative splicing (Figure 1a). Mitoprot predicted that maturation of the mitochondrial precursor of LysRS might occur between residues Lys[16] and Thr[17], leading to the putative mature mitochondrial species of LysRS, m*KRS. When m*KRS was expressed in HeLa cells, a polypeptide migrating with an electrophoretic mobility slower than that of the endogenous cytoplasmic form of LysRS (cKRS) was observed with antibodies directed to the sequences common to cytosolic and mitochondrial LysRS (Figure 1b), consistent with its larger size. This polypeptide was not directed to mitochondria because it lacks the targeting sequence (see below). By contrast, when pmKRS was expressed in HeLa cells, no polypeptide displaying an electrophoretic mobility similar to that of m*KRS was observed, but the intensity of the polypeptide corresponding to the migration of cKRS was increased (Figure 1b), suggesting that maturation of pmKRS occurred beyond Lys[16]. This gene product was directed to the mitochondria[27] (see below), and thus, the polypeptide recovered after expression in HeLa cells corresponds to the naturally in vivo processed, mature mitochondrial LysRS. When the same samples were probed with antibodies directed to the Arg[25]−Lys[42] peptide of pmKRS, the intensity of the polypeptide corresponding to the endogenously cleaved pmKRS was much weaker than the intensity of the non-processed m*KRS species. These data suggested that the maturation site of pmKRS could be located between Arg[25] and Lys[42], leading to the loss of one part of the antigenic peptide in the mature protein.

To experimentally determine the maturation site of pmKRS in vivo, pmKRS was overexpressed in HeLa cells with a C-terminal His tag. After being expressed for 48 h, the endogenously processed LysRS species were purified. Three polypeptides were observed in the purified fraction (Figure 2). The N-terminal sequence of the purified products was determined by automated Edman degradation and liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis. One minor product was cleaved between Glu[26] and Leu[27]. It might correspond to an incomplete maturation product, because of overexpression of pmKRS. The two major products were cleaved between Gly[30] and Gln[31] and between Phe[44] and Ser[45]. Because the maturation product identified in the crude extract from HeLa cells comigrates with cKRS and no smaller maturation product could be observed by direct analysis of the crude extract by Western blot analysis (Figure 1), we surmise that the product cleaved between Phe[44] and Ser[45] is the result of uncontrolled proteolysis that occurred during purification of the mitochondrial species of LysRS. When pmKRS was expressed in High Five insect cells and purified to homogeneity, a single maturation product was recovered, corresponding to a cleavage between Gly[30] and Gln[31] (Figure 2). When pmKRS was expressed in *E. coli*, the purified protein corresponded to a nonmature mitochondrial LysRS containing all the presequence required for its targeting into mitochondria (Figure 2), as assessed by automated Edman degradation.

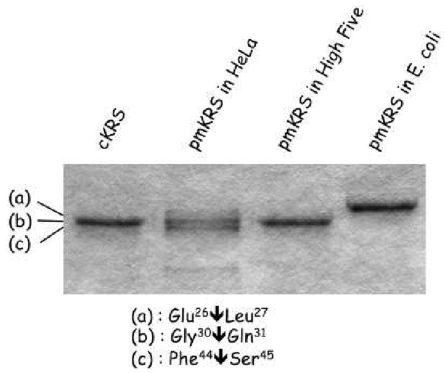

Figure 2. Analysis of pmLysRS expressed in human, insect, or bacterial cells. The pmKRS gene product was expressed in human cells (HeLa), in insect cells (High Five), or in bacteria (*E. coli*), purified to homogeneity, analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis, and visualized by Coomassie staining. The cKRS gene product expressed in yeast is shown at the left. Entries a−c represent the three polypeptides recovered after expression in HeLa cells. Their corresponding N-terminal cleavage sites determined by Edman degradation and LC−MS/MS analyses are indicated.

Determination of the Mitochondrial Targeting Sequence of LysRS. To identify the minimal amino-terminal sequence necessary for mitochondrial targeting of pmKRS in human cells, N-terminal sequences of pmKRS of variable lengths were fused to GFP (Figure 3). The fusion of full-length pmLysRS with GFP led to a classical pattern of complete mitochondrial localization. The GFP fusion proteins containing only 8 or 16 N-terminal residues of pmKRS were not targeted to mitochondria. Addition of 27 or 38 N-terminal residues of pmKRS to GFP led to partial (27 residues) or complete (38 residues) mitochondrial localization of the GFP fusion proteins. Insertion of additional residues of pmKRS did not change the pattern of GFP localization. Thus, the 38 N-terminal residues of pmKRS are sufficient to trigger mitochondrial localization. Most of this mitochondria targeting sequence is removed (cleavage between Gly[30] and Gln[31]) during the import process.

The tRNA Binding Capacity of Mitochondrial LysRS Is Revealed upon Maturation of Its Premitochondrial Precursor. The eukaryote-specific tRNA-binding domain of the cytoplasmic form of human LysRS increases the catalytic efficiency of the enzyme and provides the core synthetase with a potent tRNA binding capacity.[5] The tRNA-binding motif has been mapped to the peptide ^{20}KxxxKRxxK^{28} of cLysRS, with the four basic residues being essential for tRNA binding.[6] From this consensus sequence, the N-terminal K[20] residue belongs to the cytoplasm-specific, N-terminal sequence of 21 residues encoded by exon 1 (Figure 4). In the mitochondrial species of LysRS, this Lys residue is replaced with an Arg residue encoded

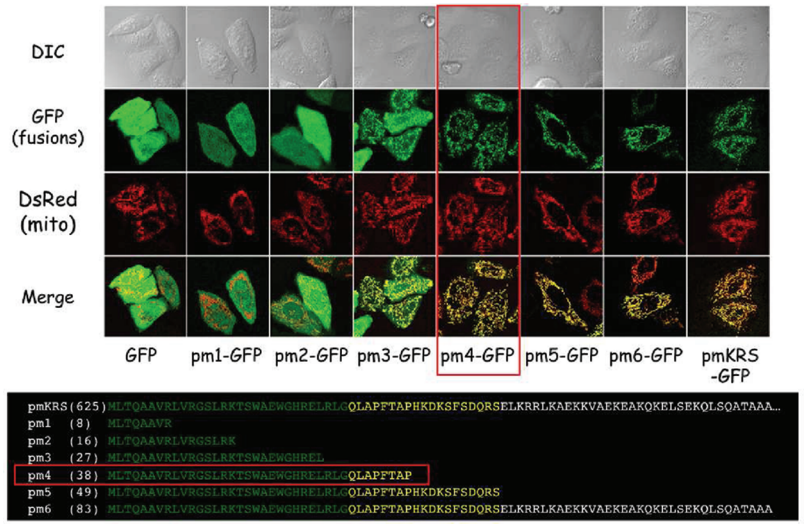

Figure 3. Determination of the minimal signal sequence for targeting pmLysRS to mitochondria in human cells. The subcellular localization of GFP fusion proteins was analyzed by confocal laser scanning microscopy. HeLa cells were cotransfected with plasmids expressing GFP alone, or the LysRS−GFP fusion proteins, and with pDsRed2-Mito, a plasmid that expresses a mitochondrial protein marker. The pmKRS−GFP fusion protein contains the full pmKRS sequence; the pmx−GFP fusion proteins contain the 8, 16, 27, 38, 49, or 83 N-terminal residues of pmKRS, as indicated. Differential interference contrast (DIC), GFP fluorescence, and DsRed fluorescence images are shown. The merge image shows a perfect match between GFP and DsRed imaging when at least 38 residues of pmKRS are fused to GFP. The amino acid sequences from the mitochondria targeting sequence are colored green, those from the pmKRS-specific sequence yellow, and those common to cKRS and pmKRS white.

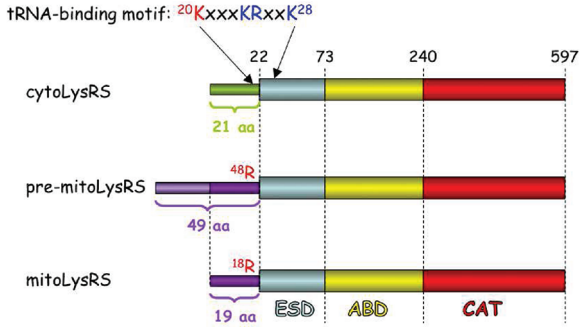

Figure 4. Domain structure of the three LysRS species from human cells. The three major domains of LysRS are indicated: the catalytic domain (CAT, amino acid residues 241−597), the anticodon-binding domain (ABD, amino acid residues 74−240), and the eukaryote-specific domain (ESD, amino acid residues 23−73). The numbering of the cytoplasmic enzyme is given. The N-domains of 21, 49, and 19 amino acid residues, specific for the cytoplasmic (cytoLysRS), premitochondrial (pre-mitoLysRS), and mitochondrial (mitoLysRS) forms of LysRS, respectively, are shown. The tRNA-binding motif identified in cytoLysRS is indicated; the Lys residue at position 20, which is an arginine in the mitochondrial species, is colored red. It corresponds to Arg^{48} or Arg^{18} in pre-mitoLysRS or mitoLysRS, respectively.

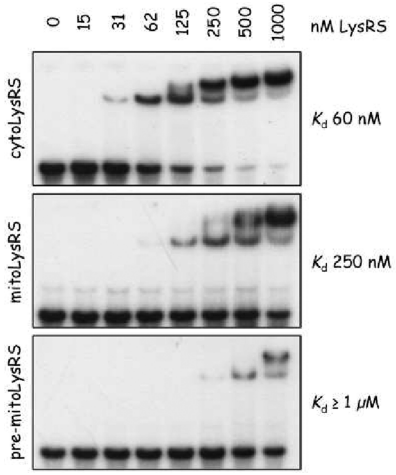

Figure 5. tRNA binding properties of the three LysRS species from human cells. ^{32}P-labeled in vitro transcribed $tRNA_3^{Lys}$ was incubated with cytoLysRS, with mitoLysRS, or with pre-mitoLysRS, at different concentrations (0−1000 nM, expressed as monomer concentrations). After electrophoresis at 4 °C on a 6% native polyacrylamide gel, the mobility shift of tRNA was visualized by autoradiography. In each assay, the bottom band corresponds to the free tRNA species. The deduced dissociation constants (K_d) for tRNA are indicated.

by exon 2. To determine the consequence of this amino acid substitution on the tRNA binding properties of the mitochondrial enzyme, we analyzed the capacity of pmLysRS and mLysRS (Figure 4) to bind $tRNA_3^{Lys}$ in a gel retardation assay (Figure 5). Because mitochondrial $tRNA^{Lys}$ transcribed in vitro does not fold into a proper cloverleaf structure because of the absence of essential modified nucleotides,[29] we chose to use human cytosolic $tRNA_3^{Lys}$ for functional studies with mitochondrial LysRS. Because the anticodon-binding and catalytic domains of mitochondrial and cytosolic LysRS are identical, the capacities of the two enzymes to bind and aminoacylate $tRNA^{Lys}$ of mitochondrial or cytosolic origin should be similar. Indeed, it was shown that the additional tRNA-binding domain appended to human cytosolic LysRS provides a general anchoring platform for tRNAs but does not discriminate between specific and nonspecific tRNAs in vitro.[5] Also, the choice of human cytosolic $tRNA_3^{Lys}$ allowed us to address the capacity of mitochondrial LysRS to target cytosolic $tRNA^{Lys}$ to HIV-1 viral particles.

A derivative of human LysRS with a truncation of its eukaryote-specific domain, ΔN-LysRS, displays a markedly reduced affinity for tRNA ($K_d \sim 6$ μM) compared with that of native, cytoplasmic LysRS [K_d = 60 nM (expressed as monomer concentration)].[5] The mature mitochondrial species

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of LysRS interacted with $tRNA_3^{Lys}$ with an apparent dissociation constant of 250 ± 40 nM (monomers of LysRS). This K_d value was ~4-fold higher than that reported for association of cLysRS with $tRNA_3^{Lys}$, suggesting that the replacement of the lysine residue at position 20 with an arginine significantly weakens the interaction between the tRNA and the synthetase. More surprisingly, the presence of the mitochondrial targeting sequence of 30 amino acid residues in pmLysRS completely concealed the tRNA binding properties of the enzyme, which bound tRNA as weakly as ΔN-LysRS does. Therefore, activation of mLysRS for tRNA binding occurs following maturation and removal of the first 30 amino acid residues of pmLysRS.

Aminoacylation Activity of Premitochondrial and Mitochondrial LysRS. To analyze the consequences of the presence of different N-terminal sequences on the functioning of the enzyme, the catalytic parameters of cLysRS, pmLysRS, and mLysRS in the $tRNA^{Lys}$ aminoacylation reaction were determined and compared with the kinetic constants of LysRS with a deletion of the complete eukaryote-specific domain, ΔN-LysRS (Table 1). The catalytic parameters determined for the mature mitochondrial species, mLysRS, were similar to those determined for the cytoplasmic enzyme. In contrast, the nonmature mitochondrial species, pmLysRS, showed a 4-fold increase in K_M for $tRNA_3^{Lys}$ compared to that for cLysRS, with a value of 6.4 μM being very similar to that determined for ΔN-LysRS (7.7 μM). These data suggest that the mitochondrial targeting sequences of pmLysRS neutralize the tRBD of LysRS, presumably by masking the tRNA-binding motif, as observed above in the tRNA binding assay.

Table 1. Kinetic Constants[a] of Cytoplasmic (cLysRS), Premitochondrial (pmLysRS), Mitochondrial (mLysRS), and N-Terminally Truncated LysRS in $tRNA_3^{Lys}$ Aminoacylation

LysRS	K_M (μM) ($tRNA_3^{Lys}$)[b]	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})
cLysRS	1.5 ± 0.3	4.3 ± 0.8	2.87
pmLysRS	6.4 ± 0.7	6.8 ± 0.9	1.06
mLysRS	2.8 ± 0.5	4.9 ± 0.8	1.75
ΔN-LysRS	7.7 ± 0.9	8.1 ± 0.8	1.05

[a]Standard errors were determined from at least two independent data sets. [b]$tRNA_3^{Lys}$ acceptance of 780 pmol/A_{260}.

DISCUSSION

It is remarkable that, despite the fact that the two LysRS species present in the cytoplasm and in the mitochondria of human cells are encoded by the same gene by means of alternative splicing and share 576 amino acid residues, they show a clear adaptation to the translation machinery of the compartment to which they are directed. Indeed, a major difference between cytosolic and mitochondrial translation results from the endosymbiotic origin of mitochondria. While eukaryotic translation evolved an integrated system where tRNA is vectorially transferred from the synthetase, to elongation factor 1A, to ribosome,[30] such a subcellular organization does not exist in bacteria. To ensure that aminoacyl-tRNAs are not released in the cellular fluid after aminoacylation on the synthetases and before they form a ternary complex with the EF1A–GTP species, aaRS acquired in evolution additional protein domains that sequester aminoacyl-tRNA to ensure processivity of tRNA handling in translation from the synthetase to elongation factor EF1A.[11] The tRNA-binding domain of cLysRS, carrying the ^{20}KxxxKRxxK^{28} tRNA-binding motif, has been extensively studied and characterized.[5,6,8,9] In human mitochondria, most aaRS are bacterial-like enzymes and do not possess these additional tRBDs. Because mLysRS is a eukaryotic-like enzyme, it was interesting to understand how a member of the eukaryotic translation apparatus can participate in mitochondrial translation. Indeed, the presence of a potent tRBD on mLysRS would introduce a limiting step in tRNA release and inhibit mitochondrial translation.

In this context, it is noteworthy that the tRNA-binding motif of mLysRS (K_d = 250 nM) is significantly less potent than the tRNA-binding motif of cLysRS (K_d = 60 nM). The replacement of the first Lys residue in the KxxxKRxxK motif, encoded by exon 1 in cLysRS, with an Arg residue, encoded by exon 2 in mLysRS, could be an adaptation mechanism to prevent sequestration of aa-tRNA by the synthetase in the mitochondrial compartment. Previous studies have shown that this Lys residue is especially important for tRNA binding; its replacement with an Ala residue led to a significant increase in the dissociation constant for tRNA (K_d = 300 nM).[6]

During functional characterization of mLysRS, we have also discovered that its precursor protein, the pmLysRS species produced in the cytosol before proteolytic maturation during mitochondrial import, behaves as the N-terminally truncated derivative of LysRS. This means that pmLysRS does not possess the tRNA binding capacity of cLysRS or even of mLysRS. This implies that its tRBD is neutralized by the very N-terminal signal sequence of pmLysRS involved in mitochondrial targeting. Activation of the tRNA-binding capacity of mLysRS requires removal of the first 30 N-terminal residues occurring during mitochondrial import (Figure 6). This maturation step results in the exposure of the RxxxKRxxK motif of mLysRS. The reason why the cell uses this strategy to preclude that the pmLysRS species could interfere with cytosolic translation is unclear but may be related to a need to efficiently segregate cytosolic and mitochondrial translation, to achieve a strict compartmentalization of the two mechanisms. Because cLysRS and mLysRS are also involved in other cellular functions beyond translation, such as the immune response,[20] signal transduction,[22] and HIV-1 replication,[27] the finding that mLysRS needs to be activated during its maturation process also suggests that this strategy might be a prerequisite for the spatiotemporal regulation of its activities. It is noteworthy that a similar mechanism has been recently observed for another LysRS species, the mitochondrion-specific LysRS from the kinetoplastid *Trypanosoma brucei*.[31] In this organism, cytosolic and mitochondrial LysRS are encoded by distinct genes. The mitochondrial gene product possesses a

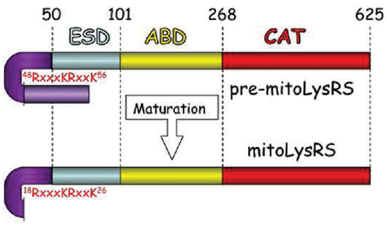

Figure 6. Maturation of premitochondrial LysRS reveals its tRNA-binding domain. The ^{48}RxxxKRxxK^{56} tRBD motif of pmLysRS is masked by the mitochondrial import sequence. Its removal upon proteolytic maturation leads to its functional exposure in mLysRS.

specific C-terminal polypeptide extension and is completely inactive for amino acid activation and tRNA aminoacylation. It becomes active upon removal of this C-terminal extension, probably by a mitochondrial protease.

The finding that pmLysRS is a poor tRNA-binding protein is also of prime interest for the understanding of the role of mitochondrial LysRS in the packaging of $tRNA_3^{Lys}$ into HIV-1 viral particles.[27] We show here that pmLysRS functionally mimics ΔN-LysRS for tRNA binding. However, N-terminally truncated LysRS, which binds poorly to $tRNA^{Lys}$, is not able to promote the packaging of $tRNA^{Lys}$ into viral particles.[32] We previously observed that the electrophoretic mobility of the mitochondrial LysRS species recovered into HIV-1 particles was not consistent with the packaging of the pmLysRS precursor or of the predicted m*LysRS mature species (cleavage between Lys^{16} and Thr^{17}) but was similar to that of cLysRS.[27] The data that we report here provide a rational explanation for these observations. Maturation of pmLysRS upon translocation into mitochondria does not occur after Lys^{16}, but after Gly^{30}, leading to a mLysRS species similar in size to cLysRS (Figures 2 and 4). The very weak tRNA binding capacity of pmLysRS is enhanced after maturation into mLysRS, which could then serve as a carrier of $tRNA^{Lys}$ into viral particles. The mechanism of release of mLysRS from mitochondria after HIV-1 infection is not properly understood, but the viral protein Vpr could be involved in this process.[33] Because tRNA should be readily released in the virions to participate in the initiation of reverse transcription of the viral RNA genome, the weaker tRNA binding properties of mLysRS, as compared to those of cLysRS, should favor its effectiveness in the replication process.

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REFERENCES

(1) Ibba, M., and Söll, D. (2000) Aminoacyl-tRNA synthesis. *Annu. Rev. Biochem. 69*, 617−650.

(2) Duchene, A. M., Pujol, C., and Marechal-Drouard, L. (2009) Import of tRNAs and aminoacyl-tRNA synthetases into mitochondria. *Curr. Genet. 55*, 1−18.

(3) Mudge, S. J., Williams, J. H., Eyre, H. J., Sutherland, G. R., Cowan, P. J., and Power, D. A. (1998) Complex organisation of the 5′-end of the human glycine tRNA synthetase gene. *Gene 209*, 45−50.

(4) Tolkunova, E., Park, H., Xia, J., King, M. P., and Davidson, E. (2000) The human lysyl-tRNA synthetase gene encodes both the cytoplasmic and mitochondrial enzymes by means of an unusual alternative splicing of the primary transcript. *J. Biol. Chem. 275*, 35063−35069.

(5) Francin, M., Kaminska, M., Kerjan, P., and Mirande, M. (2002) The N-terminal domain of mammalian lysyl-tRNA synthetase is a functional tRNA-binding domain. *J. Biol. Chem. 277*, 1762−1769.

(6) Francin, M., and Mirande, M. (2003) Functional dissection of the eukaryotic-specific tRNA-interacting factor of lysyl-tRNA synthetase. *J. Biol. Chem. 278*, 1472−1479.

(7) Agou, F., Yang, Y. S., Gesquiere, J. C., Waller, J. P., and Guittet, E. (1995) Polyanion-induced α-helical structure of a synthetic 23-residue peptide representing the lysine-rich segment of the N-terminal extension of yeast cytoplasmic aspartyl-tRNA synthetase. *Biochemistry 34*, 569−576.

(8) Yiadom, K., Hammamieh, R., Ukpabi, N., Tsang, P., and Yang, D. C. H. (2003) A peptide from the extension of Lys-tRNA synthetase binds to transfer RNA and DNA. *Peptides 24*, 987−998.

(9) Francin, M., and Mirande, M. (2006) Identity elements for specific aminoacylation of a tRNA by mammalian lysyl-tRNA synthetase bearing a nonspecific tRNA-interacting factor. *Biochemistry 45*, 10153−10160.

(10) Crepin, T., Peterson, F., Haertlein, M., Jensen, D., Wang, C., Cusack, S., and Kron, M. (2011) A hybrid structural model of the complete *Brugia malayi* cytoplasmic asparaginyl-tRNA synthetase. *J. Mol. Biol. 405*, 1056−1069.

(11) Mirande, M. (2010) Processivity of translation in the eukaryote cell: Role of aminoacyl-tRNA synthetases. *FEBS Lett. 584*, 443−447.

(12) Kaminska, M., Havrylenko, S., Decottignies, P., Gillet, S., Marechal, P. L., Negrutskii, B., and Mirande, M. (2009) Dissection of the structural organization of the aminoacyl-tRNA synthetase complex. *J. Biol. Chem. 284*, 6053−6060.

(13) Quevillon, S., Robinson, J. C., Berthonneau, E., Siatecka, M., and Mirande, M. (1999) Macromolecular assemblage of aminoacyl-tRNA synthetases: Identification of protein-protein interactions and characterization of a core protein. *J. Mol. Biol. 285*, 183−195.

(14) Robinson, J. C., Kerjan, P., and Mirande, M. (2000) Macromolecular assemblage of aminoacyl-tRNA synthetases: Quantitative analysis of protein-protein interactions and mechanism of complex assembly. *J. Mol. Biol. 304*, 983−994.

(15) Kobbi, L., Octobre, G., Dias, J., Comisso, M., and Mirande, M. (2011) Association of mitochondrial lysyl-tRNA synthetase with HIV-1 GagPol involves catalytic domain of the synthetase and transframe and integrase domains of Pol. *J. Mol. Biol. 410*, 875−886.

(16) Mirande, M. (2005) Multi-Aminoacyl-tRNA Synthetase Complexes. In *The Aminoacyl-tRNA Synthetases* (Ibba, M., Francklyn, C., and Cusack, S., Eds.) pp 298−308, Landes Bioscience, Georgetown, TX.

(17) Guo, M., Ignatov, M., Musier-Forsyth, K., Schimmel, P., and Yang, X. L. (2008) Crystal structure of tetrameric form of human lysyl-tRNA synthetase: Implications for multisynthetase complex formation. *Proc. Natl. Acad. Sci. U.S.A. 105*, 2331−2336.

(18) Fang, P., Zhang, H. M., Shapiro, R., Marshall, A. G., Schimmel, P., Yang, X. L., and Guo, M. (2011) Structural context for mobilization of a human tRNA synthetase from its cytoplasmic complex. *Proc. Natl. Acad. Sci. U.S.A. 108*, 8239−8244.

(19) Wahab, S. Z., and Yang, D. C. (1985) Synthesis of diadenosine 5′,5‴-P1,P4-tetraphosphate by lysyl-tRNA synthetase and a multienzyme complex of aminoacyl-tRNA synthetases from rat liver. *J. Biol. Chem. 260*, 5286−5289.

(20) Yannay-Cohen, N., Carmi-Levy, I., Kay, G., Yang, C. M., Han, J. M., Kemeny, D. M., Kim, S., Nechushtan, H., and Razin, E. (2009) LysRS serves as a key signaling molecule in the immune response by regulating gene expression. *Mol. Cell 34*, 603−611.

(21) Meerschaert, K., Remue, E., De Ganck, A., Staes, A., Boucherie, C., Gevaert, K., Vandekerckhove, J., Kleiman, L., and Gettemans, J. (2008) The tandem PDZ protein Syntenin interacts with the aminoacyl tRNA synthetase complex in a lysyl-tRNA synthetase-dependent manner. *J. Proteome Res. 7*, 4962−4973.
(22) Park, S. G., Kim, H. J., Min, Y. H., Choi, E. C., Shin, Y. K., Park, B. J., Lee, S. W., and Kim, S. (2005) Human lysyl-tRNA synthetase is secreted to trigger proinflammatory response. *Proc. Natl. Acad. Sci. U.S.A. 102*, 6356−6361.
(23) Kawamata, H., Magrane, J., Kunst, C., King, M. P., and Manfredi, G. (2008) Lysyl-tRNA synthetase is a target for mutant SOD1 toxicity in mitochondria. *J. Biol. Chem. 283*, 28321−28328.
(24) Cen, S., Khorchid, A., Javanbakht, H., Gabor, J., Stello, T., Shiba, K., Musier-Forsyth, K., and Kleiman, L. (2001) Incorporation of lysyl-tRNA synthetase into human immunodeficiency virus type 1. *J. Virol. 75*, 5043−5048.
(25) Javanbakht, H., Halwani, R., Cen, S., Saadatmand, J., Musier-Forsyth, K., Gottlinger, H., and Kleiman, L. (2003) The interaction between HIV-1 Gag and human lysyl-tRNA synthetase during viral assembly. *J. Biol. Chem. 278*, 27644−27651.
(26) Saadatmand, J., Guo, F., Cen, S., Niu, M., and Kleiman, L. (2008) Interactions of reverse transcriptase sequences in Pol with Gag and LysRS in the HIV-1 $tRNA_3^{Lys}$ packaging/annealing complex. *Virology 380*, 109−117.
(27) Kaminska, M., Shalak, V., Francin, M., and Mirande, M. (2007) Viral hijacking of mitochondrial lysyl-tRNA synthetase. *J. Virol. 81*, 68−73.
(28) Tisné, C., Rigourd, M., Marquet, R., Ehresmann, C., and Dardel, F. (2000) NMR and biochemical characterization of recombinant human $tRNA_3^{Lys}$ expressed in *Escherichia coli*: Identification of posttranscriptional nucleotide modifications required for efficient initiation of HIV-1 reverse transcription. *RNA 6*, 1403−1412.
(29) Helm, M., Brule, H., Degoul, F., Cepanec, C., Leroux, J. P., Giege, R., and Florentz, C. (1998) The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial tRNA. *Nucleic Acids Res. 26*, 1636−1643.
(30) Negrutskii, B. S., Stapulionis, R., and Deutscher, M. P. (1994) Supramolecular organization of the mammalian translation system. *Proc. Natl. Acad. Sci. U.S.A. 91*, 964−968.
(31) Espanol, Y., Thut, D., Schneider, A., and de Pouplana, L. R. (2009) A mechanism for functional segregation of mitochondrial and cytosolic genetic codes. *Proc. Natl. Acad. Sci. U.S.A. 106*, 19420−19425.
(32) Cen, S., Javanbakht, H., Niu, M. J., and Kleiman, L. (2004) Ability of wild-type and mutant lysyl-tRNA synthetase to facilitate $tRNA^{Lys}$ incorporation into human immunodeficiency virus type 1. *J. Virol. 78*, 1595−1601.
(33) Kaminska, M., Francin, M., Shalak, V., and Mirande, M. (2007) Role of HIV-1 Vpr-induced apoptosis on the release of mitochondrial lysyl-tRNA synthetase. *FEBS Lett. 581*, 3105−3110.