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Research paper

Association of human mitochondrial lysyl-tRNA synthetase with HIV-1 GagPol does not require other viral proteins

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Abstract

In human, the cytoplasmic (cLysRS) and mitochondrial (mLysRS) species of lysyl-tRNA synthetase are encoded by a single gene. Following HIV-1 infection, mLysRS is selectively taken up into viral particles along with the three $tRNA^{Lys}$ isoacceptors. The GagPol polyprotein precursor is involved in this process. With the aim to reconstitute in vitro the HIV-1 $tRNA_3^{Lys}$ packaging complex, we first searched for the putative involvement of another viral protein in the selective viral hijacking of mLysRS only. After screening all the viral proteins, we observed that Vpr and Rev have the potential to interact with mLysRS, but that this association does not take place at the level of the assembly of mLysRS into the packaging complex. We also show that $tRNA_3^{Lys}$ can form a ternary complex with the two purified proteins mLysRS and the Pol domain of GagPol, which mimicks its packaging complex.

Keywords: HIV-1; $tRNA_3^{Lys}$; Lysyl-tRNA synthetase; Packaging complex

1. Introduction

An essential process in the viral life cycle of HIV-1, the human immunodeficiency virus type 1, is the conversion of its viral genomic RNA (vRNA) into proviral DNA. One of the crucial step of this mechanism is viral packaging of $tRNA_3^{Lys}$ from the host cell, a molecule that serves as a primer for initiation of reverse transcription [1]. Even though formation of a specific vRNA/$tRNA_3^{Lys}$ complex primarily involves annealing of the 3′-terminal 18 nucleotides of $tRNA_3^{Lys}$ to the complementary sequence located in the 5′-untranslated region of vRNA corresponding to the primer binding site (PBS), the complete process of initiation and transition to elongation requires a more complex pattern of interactions [2]. Because initiation of reverse transcription starts after maturation of new viral particles before infection of target cells, or before disruption of the capsid following infection, packaging of $tRNA_3^{Lys}$ during viral assembly is essential to produce effective virions [3]. However, the three $tRNA^{Lys}$ isoacceptors are present in the virus [4], including $tRNA_1^{Lys}$ and $tRNA_2^{Lys}$ that cannot anneal to the PBS, which establishes that vRNA/tRNA annealing is not responsible for tRNA packaging.

Lysyl-tRNA synthetase (LysRS) from infected cells, the enzyme that aminoacylates $tRNA_3^{Lys}$ to give the aminoacyl-tRNA used for protein synthesis, is also packaged into HIV-1 particles [5,6]. Human LysRS possesses a 73 amino acid residue N-terminal polypeptide extension that contributes a potent tRNA-binding domain [7,8] required for efficient tRNA packaging [9]. Although it has long been believed that cytosolic LysRS (cLysRS) was involved in this process [5], the actual source of LysRS present in the virions is the mitochondrial enzyme [6]. Maturation of pmLysRS, the precursor of mitochondrial LysRS (mLysRS), after cleavage of its mitochondria-targeting sequence between Gly^{30} and Gln^{31} upon translocation into mitochondria, generates an active enzyme with potent tRNA-binding properties [10].

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The HIV-1 *gag* reading frame encodes the Gag polyprotein precursor containing the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins (Fig. 1A). The GagPol polyprotein precursor containing the transframe (TF, also named p6*), protease (PR), reverse transcriptase (RT) and integrase (IN) proteins in addition to MA, CA and NC, is produced via a −1 ribosomal frameshifting occurring between the NC and p6 coding regions of *gag* (Fig. 1A). It is believed that the TF and IN domains or the Pol region of the GagPol polyprotein precursor form molecular tweezers that interact with the catalytic domain of mLysRS to build the GagPol:mLysRS:$tRNA_3^{Lys}$ packaging complex [11]. The Gag and GagPol polyprotein precursors are maturated into the virions via the viral protease to generate individual proteins, thus releasing $tRNA_3^{Lys}$ into the virus particle, which allows initiation of reverse transcription to occur.

Whereas cLysRS and pmLysRS, which are produced by alternative splicing of exon 2 of the KARS gene [12], share the same catalytic domain, and have the intrinsic capacity to bind Pol [11], only the mLysRS species is recovered within the virions [6,10]. This suggests that the LysRS species are strictly targeted *in cellulo*, which prevents association of mLysRS with p38 in the cytosolic multi-aminoacyl-tRNA synthetase complex, and of cLysRS with GagPol in HIV-1 infected cells [11]. In order to get a precise view of the composition of the tRNA packaging complex, we explored the possibility that another yet unidentified viral protein could be involved in the assembly of this complex. We analyzed the capacity of the Env polyprotein precursor, and of the auxiliary proteins Vif, Vpr, Tat, Rev, Vpu and Nef of HIV-1 to interact with LysRS, and to participate in the assembly of the GagPol:mLysRS:$tRNA_3^{Lys}$ packaging complex. Then, we showed that

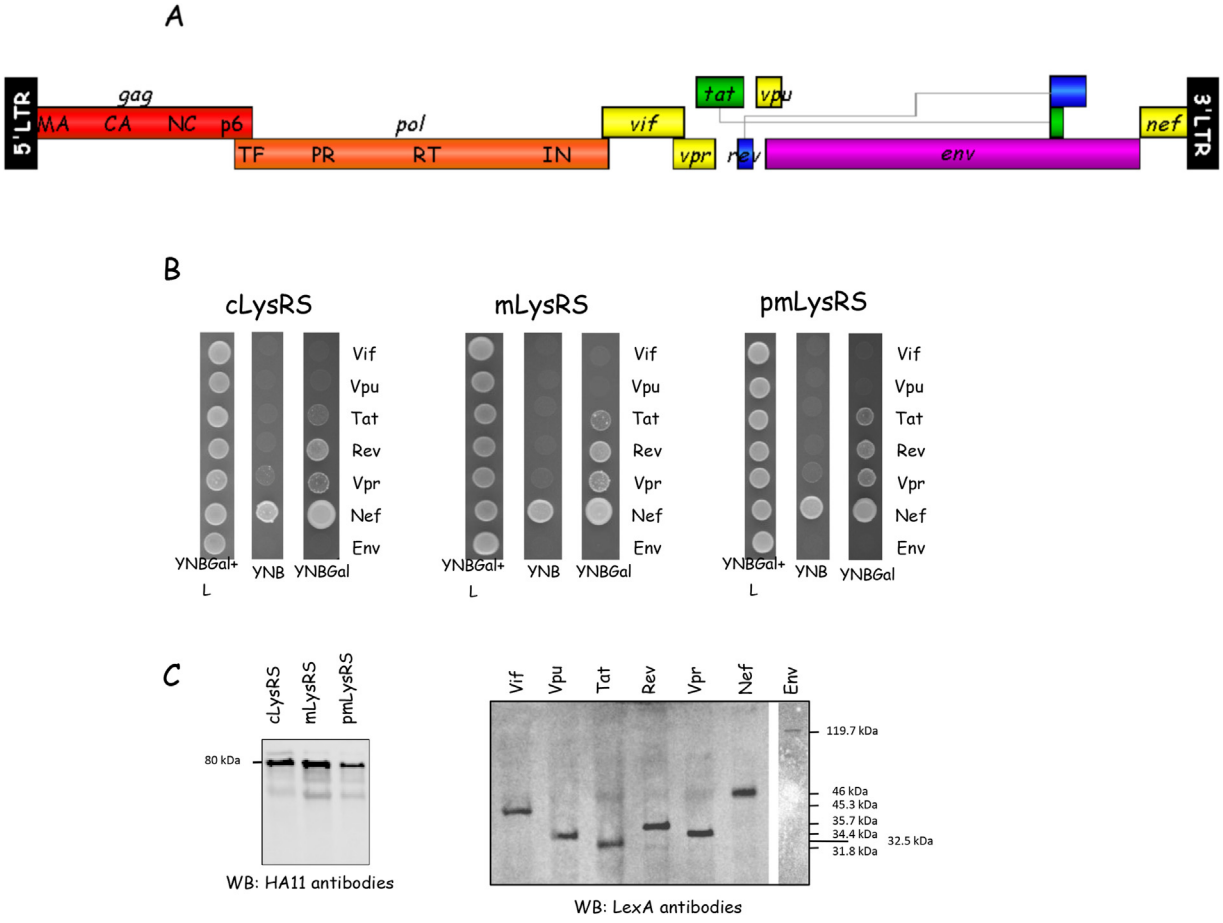

Fig. 1. Two-hybrid analysis of LysRS:pHIV-1 interaction. (A) The complete genome of HIV-1 is shown. The polyproteins Gag, GagPol and Env, and proteins Vif, Vpr, Tat, Rev, Vpu and Nef are encoded on the three reading frames of the viral genome. The viral (pHIV-1) proteins were expressed fused to LexA in pEG202. (B) The two-hybrid protein:protein interaction assay between pHIV-1, and cLysRS, mLysRS, or pmLysRS, was conducted in EGY48 strain. The three LysRS species for the cytoplasmic (cLysRS), mitochondrial (mLysRS), and premitochondrial (pmLysRS) enzymes were expressed fused to the B42 transcription activator, under the control of a galactose-inducible promoter in pJG4-5. As a control, all the strains grew on a galactose medium in the presence of leucine (YNBGal + L). When the strains were plated on a galactose medium in the absence of leucine (YNBGal), a protein:protein interaction resulted in the expression of the LEU2 gene, as observed following coexpression of any of the three LysRS species with Nef, Vpr, Rev or Tat. If the expression of LEU2 requires interaction between LysRS and pHIV-1, then strains should not grow in the absence of leucine on a glucose medium (YNB) where LysRS is not expressed. All the strains grew on YNB in the presence of leucine (not shown). (C) Expression and stability of the fusion proteins produced in yeast. Left: Western blot analysis of the fusion proteins expressed in yeast from the pJG4-5 plasmid. The cLysRS, mLysRS or pmLysRS species (80 kDa) were expressed fused to the HA11 hemagglutinin epitope. Right: Western blot analysis of the fusion proteins expressed in yeast from the pEG202 plasmid. The Vif (45.3 kDa), Vpu (31.8 kDa), Tat (32.5 kDa), Rev (35.7 kDa), Vpr (34.4 kDa), Nef (46 kDa) and Env (119.7 kDa) fusion proteins were expressed fused to the LexA DNA-binding domain.

$tRNA_3^{Lys}$ can be readily accommodated in vitro into the complex made of mLysRS and of the Pol domain of GagPol.

2. Materials and methods

2.1. Yeast two-hybrid analysis

The yeast two-hybrid system developed by Brent et al. was used [13]. The Vif, Tat, Rev, Vpu and Env coding regions from pNL4-3 were introduced between the *Eco*RI and *Xho*I sites of pEG202. The Vpr and Nef coding regions were introduced between the *Bam*HI and *Xho*I sites, or *Eco*RI and *Nco*I sites of pEG202, respectively. The LexA DNA-binding domain-fused proteins in pEG202 are expressed under the control of a constitutive promoter. To test whether the LexA:pVIH-1 fusion proteins are able to enter the nucleus and bind LexA operators, the repression assay with the pJK101 reporter was used [13]. The cDNAs encoding the human cytoplasmic, premitochondrial and mitochondrial forms of LysRS, the catalytic (CAT, from Asp238 to Val597), anticodon-binding (ABD, from Val71 to Asn237), or eukaryotic-specific (cESD, mESD, pmESD, from their respective N-terminus to Ser70) domains of LysRS introduced between the *Eco*RI and *Xho*I sites of pJG4-5 have been described [11]. The B42 activation domain-fused proteins in pJG4-5 also carry a hemagglutinin epitope tag, and are expressed under the control of a galactose-inducible promoter.

The yeast strain EGY48 (*Matα his3 leu2::3LexAop-LEU2 ura3 trp1*) which contains a chromosomal *LEU2* gene placed under the control of LexA operators was transformed to his^+ with pEG202-derivatives and to trp^+ with pJG4-5 derivatives. At least four independent colonies were analyzed for their ability to grow in the absence of leucine (expression of *Lexop-LEU2*). A pair of interactive proteins was scored as positive when transformants did not grow on glucose medium lacking leucine (no expression of B42-fusions) but grew on galactose medium lacking leucine (expression of B42-fusions that interacted with LexA-fusions).

2.2. Immunoprecipitation

The Rev, Vpr, Tat and Nef coding sequences from pNL4-3 were inserted into the *Bgl*II site of pRS315-PGK (*CEN/ARS, LEU2* [14]). FLAG-tagged proteins were expressed with an N-terminal MGDYKDDDDKPW sequence corresponding to the FLAG epitope (underlined). Plasmids pRS315-PGK-FLAG or pRS315-PGK-FLAG-(Rev, Vpr, Tat, or Nef) were used to transform yeast W303-1Bα strain (*leu2*, *his3*, *trp1*, *ura3*, *ade2*). Cells were grown at 28 °C in 1 l of minimal YNB medium (0.7% yeast nitrogen base without amino acid, 2% glucose) supplemented with uracil, adenine, histidine and tryptophan, to an A_{600} of 1. Cells were washed, resuspended in 5 ml of ice-cold extraction buffer (100 mM Tris–HCl pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) and lysed at 2100 bar in a "One Shot" cell disrupter (Constant Systems Ltd) in the presence of 1 mM benzamidine and 1 mM PMSF. Extracts were clarified by centrifugation at 12,000 *g* for 15 min at 4 °C, followed by a 40 min centrifugation step at 160,000 *g*, at 4 °C. The supernatant was incubated 2 h, at 4 °C, after addition of 120 µl of a 1:1 slurry of ANTI-FLAG M2 affinity gel (Sigma). The matrix was extensively washed and resuspended in NETN50 (20 mM Tris–HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.5% NP40). Anti-FLAG agarose incubated with an extract of yeast transformed with pRS315-PGK-FLAG-Pol is denoted FLAG-Pol matrix, or FLAG matrix when incubated with the control extract of yeast transformed with pRS315-PGK-FLAG. Before incubation with purified proteins, a fraction of the matrix was treated with SDS and analyzed by Western blotting with anti-FLAG antibodies to assess that similar amounts of the different FLAG-proteins were bound to the matrix.

Purified mLysRS expressed in insect cells and purified as described [10] was incubated (0.1 ml at 2 µM) with 30 µl of FLAG or FLAG-(Rev, Vpr, Tat, or Nef) matrix in NETN50 containing BSA at 10 µg/ml. The mixture was incubated 1 h at 4 °C with constant shaking. After centrifugation 1 min at 4 °C (13,000 × g), agarose beads were washed twice with 200 µl NETN50, once with 200 µl NETN250 (NETN50 containing 250 mM NaCl), and twice with 200 µl NETN50. Beads were resuspended in 36 µl NETN50 and specific elution from ANTI-FLAG M2 affinity gel was performed after addition of 4 µl of FLAG peptide (Sigma) at 5 mg/ml. After incubation for 30 min at 4 °C, the supernatant was recovered by centrifugation. Fractions were analyzed by Western blotting.

2.3. Reconstitution of protein complexes

The Pol, IN, Rev and Vpr coding regions from pNL4-3 and mLysRS sequences were introduced between the *Eco*RI and *Xho*I sites of pFastBac1 (Life Technologies). The Pol and IN sequences contain a His^6 tag at the C-terminus. Recombinant plasmids were integrated at the Tn7 transposition site of EMBacY (gift from Imre Berger, EMBL, Grenoble) to give the recombinant bacmids [15]. Initial baculoviruses were obtained after transfection of adhesive Sf21 cells grown in Grace medium supplemented with 10% FCS. Baculovirus amplification was performed by infection of Sf21 cells grown in suspension in SF-900 II medium (Life Technologies). To express recombinant proteins, baculoviruses were used to infect High Five cells grown in suspension in Express Five SFM medium (Life Technologies). After 48–56 h of culture at 27 °C, cells were harvested by centrifugation, washed with ice-cold PBS, and the cell pellet was stored at −80 °C. Cells were lysed by addition of 1 vol. of ice-cold extraction buffer (20 mM K-phosphate pH 7.5, 150 mM NaCl, 10 mM imidazole, 5% glycerol, 5 mM 2-mercaptoethanol, 1% Triton X-100) in the presence of 1 mM Pefabloc, 10 mM benzamidine and 10 mM PMSF. Extracts were clarified by centrifugation at 12,000 *g* for 15 min at 4 °C. The cell extracts containing different recombinant proteins were mixed as described, incubated 1 h at 4 °C. After addition of 1 vol. of buffer A-NiNTA (20 mM K-phosphate pH 7.5, 500 mM NaCl, 50 mM imidazole, 5% glycerol, 5 mM 2-mercaptoethanol), the mixture was incubated with 20 µl of a 1:1 slurry of Ni-NTA

Superflow (Qiagen), during 1 h at 4 °C. Beads were washed with 4 × 1 ml of buffer A-NiNTA, and elution was performed by adding 2 × 200 µl of buffer B-NiNTA (20 mM K-phosphate pH 7.5, 500 mM NaCl, 400 mM imidazole, 5% glycerol, 5 mM 2-mercaptoethanol). Eluted proteins were analyzed by western blotting. Where indicated, $tRNA_3^{Lys}$ was added during incubation of cell extracts. Human $tRNA_3^{Lys}$ was produced in *Escherichia coli* JM101Tr transformed with plasmid pBSTK3 [16] and purified as described [17].

2.4. Antibodies and western blot analysis

Monoclonal antibodies directed to the hemagglutinin epitope were from BAbCo. Polyclonal antibodies to LexA were a gift of Patrice Moreau (Laboratoire de Chimie Bactérienne, Marseille). Polyclonal anti-ΔNLysRS antibodies have been described previously [18]. Monoclonal anti-IN and anti-Rev antibodies were from Abcam. Polyclonal anti-Vpr antibodies were a gift from Uwe Tessmer (Heinrich-Pette-Institute, Hamburg, Germany). Western blot analyses were conducted with goat anti-rabbit or goat anti-mouse secondary antibodies conjugated with peroxidase (Chemicon) and the SuperSignal West Pico chemiluminescent substrates (Pierce).

2.5. Northern blot analysis

An oligonucleotide (K3) complementary to the region ranging from nucleotide 27 to nucleotide 52 of $tRNA_3^{Lys}$ was labeled with digoxigenin-ddUTP as described by the supplier (Roche). After electrophoresis on a 15% polyacrylamide gels containing 8 M urea, tRNA was electrotransfered to a nylon membrane (Nytran 0.45 µm, Schleicher & Schuell), hybridized to the labeled probe, and revealed with the DIG luminescent detection kit (Roche), as recommended by the supplier.

3. Results

3.1. Search for viral proteins interacting with lysyl-tRNA synthetase

We previously determined that the Pol domain of GagPol, but not the Gag domain, interacts with the catalytic domain of mLysRS. More specifically, mLysRS interacts with the N-terminal (TF) and C-terminal (IN) domains of the Pol region of GagPol (Fig. 1A) [11]. Despite the fact that only mLysRS is recovered into viral particles [6], cLysRS which shares its catalytic domain with mLysRS has also the intrinsic ability to interact with Pol in vitro. Now, we performed an exhaustive search to investigate the putative role of the other HIV-1 viral proteins in establishing a specific tRNA packaging complex with mLysRS. All the viral proteins, except Gag and GagPol which were used in previous studies [11], were subjected to a protein:protein interaction search with mLysRS. This included the Env polyprotein precursor, gp160, specifying the surface (SU, gp120) and transmembrane (TM, gp41) glycoproteins, as well as the auxiliary proteins Vif, Vpr, Tat, Rev, Vpu and Nef (Fig. 1A). Association of LysRS with these viral proteins was first screened by the yeast two-hybrid method. The viral sequences were expressed fused with the LexA DNA-binding domain, and fusion proteins of the expected sizes were detected by western blot (Fig. 1C). The fusion proteins have to be present in the nucleus to activate transcription in the two-hybrid assay. To ascertain that the encoded proteins were at least in part translocated into the yeast nucleus, we used the repression assay developed by Brent et al. [13]. Briefly, a lacZ reporter gene is placed under the control of a GAL1 promoter that also contains a LexA operator. When LexA or a LexA-fusion protein binds LexA operator, expression of lacZ is repressed, leading to a reduced synthesis of β-galactosidase [13]. With the exception of LexA-Nef, all other fusion proteins repressed lacZ expression to different extents, exemplifying their presence in the nucleus (Table 1). The cytoplasmic, mitochondrial and premitochondrial LysRS species, cLysRS, mLysRS and pmLysRS, were expressed in yeast fused with the B42 transcription activation domain. These constructs also contain a nuclear localization signal that targets them to the nucleus and an HA11 hemagglutinin epitope that allows identification of the fusion proteins expressed in yeast (Fig. 1C).

Expression on galactose medium (YNBG, Fig. 1B) of LexA-Rev, LexA-Vpr or LexA-Tat with B42-cLysRS, B42-mLysRS or B42-pmLysRS allowed yeast EGY48 to grow in the absence of leucine, suggesting that all these protein pairs are able to associate through protein:protein interactions. On a glucose medium (YNB, Fig. 1B), where B42-fusion proteins are not expressed, no growth was observed, also suggesting that interaction between Rev, Vpr or Tat with LysRS is necessary for growth. In the case of LexA-Nef, growth was independent of the expression of LysRS (Fig. 1B, YNB and YNBG), suggesting that LexA-Nef by itself activates transcription, a false positive usually observed with proteins carrying acidic patches [13]. This observation is also consistent with the results of the repression assay (Table 1), showing that the level of β-galactosidase expressed in the presence of

Table 1
β-Galactosidase activity in the repression assay.[a]

Plasmid used	%
pRS313[b]	100
pEG202[c]	17 ± 3
pEG202-Vif	79 ± 5
pEG202-Vpu	67 ± 4
pEG202-Tat	21 ± 2
pEG202-Rev	23 ± 2
pEG202-Vpr	21 ± 3
pEG202-Nef	121 ± 10
pEG202-Env	75 ± 6

[a] Activity is expressed as the percentage of activity in the given strain as compared to the activity determined in the control strain that does not express LexA (pRS313).
[b] Control plasmid that does not express LexA.
[c] Control plasmid that expresses wild-type LexA.

LexA-Nef was higher than the level observed with pRS313, when no repression occurs. Expression of Vif, Vpu or Env with any of the three forms of LysRS did not lead to expression of LEU2.

3.2. Identification of the protein domain of LysRS involved in its interaction with viral proteins

Because Rev, Vpr and Tat were scored positive by the yeast two-hybrid assay with the three forms of LysRS, this suggested that a domain common to all LysRS species was involved in their association.

The structural domains of LysRS (Fig. 2A) were independently expressed fused to B42 (Fig. 2B). This includes the C-terminal catalytic domain of LysRS (CAT), its tRNA anticodon-binding domain (ABD), and its N-terminal eukaryote-specific sequences (ESD) that promote the formation of a stable complex between $tRNA^{Lys}$ and LysRS in humans [7]. The cESD, mESD and pmESD domains also contain the cytoplasmic-, mitochondrial- and premitochondrial-specific sequences, respectively. Expression of CAT, but of none of the other cESD, mESD, pmESD, or ABD domains, with Rev, Vpr or Tat, revealed a protein–-protein interaction (Fig. 2A). No interaction was detected with Vif, Vpu or Env. As observed above, expression of Nef leads to expression of the LEU2 gene in the absence of LysRS expression, consistent with its peculiar ability to activate transcription in yeast.

3.3. Control of protein:protein interactions by immunoprecipitation

To evaluate the validity of the protein:protein interactions detected between Rev, Vpr and Tat with LysRS by the two-hybrid assay, association of mLysRS with these three proteins was assessed by immunoprecipitation of LysRS with FLAG-tagged viral proteins produced in yeast (Fig. 3). The viral proteins were expressed in yeast with a FLAG-tag appended to their N-terminus, and purified by adsorption on anti-FLAG agarose. The resulting FLAG-proteins bound to anti-FLAG agarose, or an aliquot of anti-FLAG agarose saturated with FLAG peptide, were incubated in the presence of purified mLysRS. After washing of agarose beads, specific elution of interacting proteins was accomplished by adding an excess of FLAG peptide that released FLAG-proteins from the agarose matrix by competition. mLysRS was recovered with FLAG-Rev and FLAG-Vpr, but mLysRS was not recovered from FLAG-Tat or from FLAG alone (Fig. 3).

This immunoprecipitation assay was also conducted with FLAG-Nef, because, as described above, the association between Nef and LysRS could not be appraised by the yeast two-hybrid approach. mLysRS did not significantly interact with Nef in this assay (Fig. 3).

Thus, only Rev and Vpr showed a robust association with mLysRS by the two-hybrid and immunoprecipitation approaches and were considered for further experiments. The putative association between Tat and LysRS was only observed by the two-hybrid assay, and was not judged as robust.

3.4. Rev and Vpr do not associate to mLysRS within the Pol:mLysRS complex

To assemble and isolate the $tRNA^{Lys}$ packaging complex, containing GagPol, mLysRS and $tRNA^{Lys}$, the GagPol protein precursor, or the Pol polyprotein only, both expressing an inactive protease domain, and mLysRS were expressed in insect cells. The baculovirus expression system allows synthesis of proteins in a eukaryotic expression system closely mimicking the translation machinery of a human cell, where HIV-1 proteins are naturally expressed. The level of expression of GagPol was low, and the polyprotein was rapidly degraded by cellular proteases into individual components. Thus, we chose to associate mLysRS with Pol, which contains the two domains TF and IN required to establish a potent interaction with mLysRS (Figs. 4 and 5).

Pol was expressed with a C-terminal His-tag allowing its recovery on a NiNTA column. An extract of insect cells expressing Pol was mixed with an extract of insect cells expressing mLysRS in excess as compared to Pol, and incubated for 1 h before recovery of Pol on a NiNTA matrix. As observed Fig. 4C, mLysRS was recovered with Pol, showing that the Pol:mLysRS complex easily forms between the two proteins. It is noticeable that Pol-H6 appears as a doublet in the input fraction (Fig. 4A), but as a single band in the output fraction (Fig. 4B, but also Fig. 5 below). The lower band of the doublet corresponds to a degradation product of Pol, with a deletion of its N-terminal TF domain. Therefore, because the IN:mLysRS interaction is the only one that can form with this truncated derivative of Pol, its affinity for mLysRS was much weaker and it was not recovered in the pull-down fraction. When Pol and mLysRS were coexpressed in insect cells after coinfection with the two baculovirus species, the Pol:mLysRS complex could also be recovered after incubation with NiNTA matrix directly after cell lysis (not shown).

Because Rev and Vpr bind to the free form of mLysRS, we analyzed their ability to bind mLysRS into the Pol:mLysRS complex. Vpr and Rev were expressed in the baculovirus expression system, and the effect of the addition of these two proteins on the assembly of the Pol:mLysRS complex was analyzed as described above (Fig. 4). As a control, the integrase (IN) domain of Pol, corresponding to one of the two domains of Pol interacting with mLysRS [11], expressed in insect cells with a C-terminal His-tag, was also used.

Viral proteins IN, Vpr or Rev were incubated alone, with Pol, with mLysRS, or with Pol and mLysRS (Fig. 4A). The protein samples recovered on NiNTA matrix contained the His-tagged proteins Pol and IN, where applicable, but did not contain Vpr or Rev (Fig. 4B), showing that Vpr and Rev, which associated with free mLysRS (Fig. 3), do not interact with mLysRS into the Pol:mLysRS complex (Fig. 4B). In addition, this experiment also shows that Vpr and Rev do not interact with Pol. The amount of mLysRS recovered with Pol

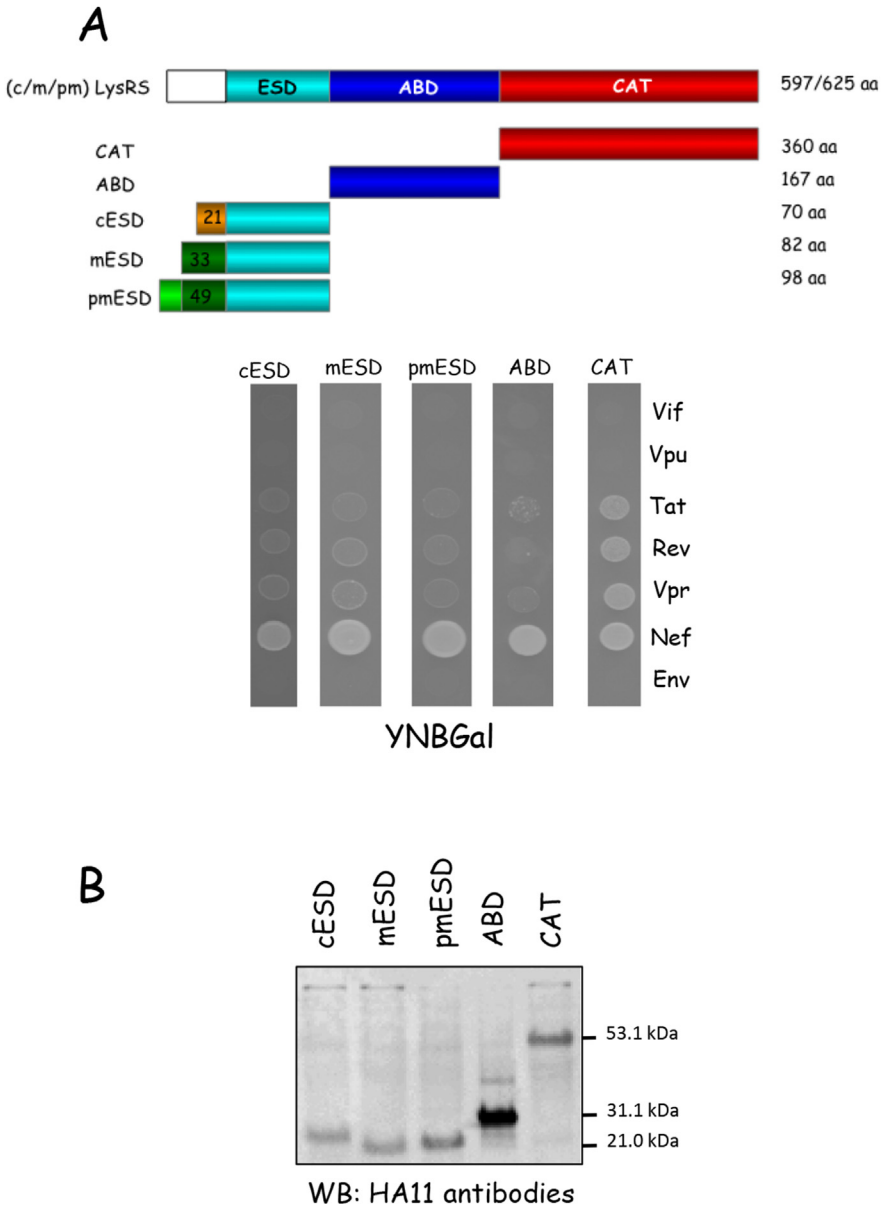

Fig. 2. Two-hybrid analysis of the LysRS domains interacting with pHIV-1. (A) The cytoplasmic- (cESD), mitochondrial- (mESD), or premitochondrial- (pmESD) eukaryote-specific domains, the anticodon-binding domain (ABD), and the catalytic domain (CAT) of LysRS were expressed fused to the B42 transcription activator, under the control of a galactose-inducible promoter in pJG4-5. The two-hybrid protein:protein interaction assay between the LysRS constructs and the pHIV-1 constructs expressed fused to LexA in pEG202, was conducted in EGY48 strain. Expression of the CAT domain alone was as efficient as the expression of the three full-length LysRS species to restore growth in the presence of Vpr, Rev, or Tat. No growth was observed on YNB plates, except for the strain expressing the Nef construct. These data also show that none of the HA-LysRS fusions activate transcription of the reporter gene in the absence of a suitable B42-fusion protein. For clarity, only the YNBGal selection plate is shown. (B) Expression of the fusion proteins in yeast was analyzed by Western blot. The cESD (19.3 kDa), mESD (21.1 kDa), pmESD (22.7 kDa), ABD (31.1 kDa) or CAT (53.1 kDa) domains of LysRS, were expressed fused to the HA11 hemagglutinin epitope.

in the Pol:mLysRS complex was also not affected by the presence of Vpr or Rev (Fig. 4C), showing that their presence did not impair association of mLysRS with Pol. By contrast, when IN was added in excess in the presence of Pol, more mLysRS was recovered from the NiNTA matrix (Fig. 4C), consistent with the observation that IN, carrying a His-tag, is also able to associate with mLysRS, as observed when mLysRS is incubated with IN alone (Fig. 4C). Thus the interaction between mLysRS and Vpr or Rev observed Fig. 3, is abolished in the presence of Pol, suggesting that the sites of interactions of Vrp and Rev on mLysRS are overlapping with the sites of interaction with Pol, and that the later are much more robust. Altogether, these data show that the functional significance of the interaction between mLysRS and Rev or Vpr, if any, is likely not to be involved in the assembly of the $tRNA^{Lys}$ packaging complex.

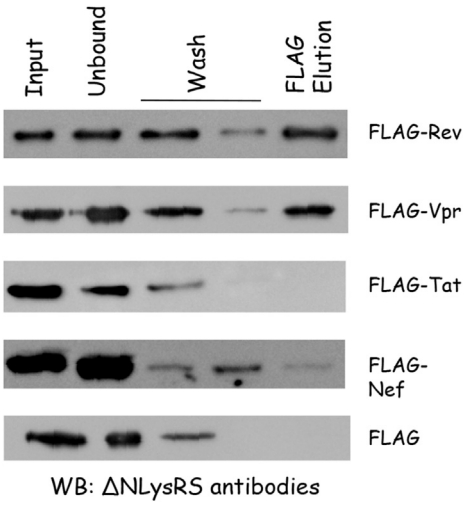

Fig. 3. Co-immunoprecipitation of mLysRS with HIV-1 proteins. Anti-FLAG agarose beads carrying FLAG peptide (FLAG) or the FLAG-Rev, FLAG-Vpr, FLAG-Tat or FLAG-Nef fusion proteins were incubated with the mitochondrial species of LysRS (Input). After removal of the unbound fraction, beads were washed and then incubated with FLAG peptide in excess, and the eluted fraction was recovered (FLAG elution). mLysRS present in these fractions was analyzed by Western blotting with anti-ΔNLysRS antibodies. The 'Input' and 'Unbound' fractions were diluted 10×.

3.5. Assembly of the ternary complex Pol:mLysRS:tRNA$_3^{Lys}$

The functional role of association of mLysRS to GagPol is to carry tRNA^{Lys} to viral particles during the packaging process. To test whether the Pol:mLysRS complex observed in Fig. 4 can accommodate tRNA$_3^{Lys}$ to build the Pol:mLysRS:tRNA$_3^{Lys}$ packaging complex, extracts of insect cells containing Pol-H^6 and mLysRS were also supplemented with tRNA$_3^{Lys}$ in excess (1 μM in the incubation mixture) (Fig. 5). The addition of tRNA$_3^{Lys}$ during incubation of Pol-H^6 with mLysRS did not impair the recovery of mLysRS with Pol in the pull-down fraction (Fig. 5A). The amount of tRNA$_3^{Lys}$ recovered in the pull-down fraction obtained in the presence of the two proteins, Pol and mLysRS, was much above the background level observed with Pol or mLysRS alone (Fig. 5B). This experiment recapitulates the assembly of the three components into the tRNA$_3^{Lys}$ packaging complex.

4. Discussion

We previously showed that association of HIV-1 GagPol with the cellular protein mLysRS to form the GagPol:mLysRS complex involves interaction of the TF and IN domains of the Pol region of GagPol with the catalytic domain of mLysRS [11]. Because a single gene encodes the cytoplasmic and mitochondrial species of LysRS in human by means of alternative splicing [12], the three cellular species of LysRS, cLysRS, pmLysRS, and mLysRS, share 576 amino acid residues, corresponding to the catalytic and anticodon-binding domains. Accordingly, association of GagPol to the catalytic domain of LysRS is not restricted, in vitro, to the mLysRS species [11]. However, cLysRS, a member of the cytosolic multisynthetase complex MARS [19], is never recovered into the virions [6], suggesting that its interaction with the p38 scaffold protein of MARS and its routing to this complex prevents its interaction with GagPol in HIV-1 infected cells [11].

In this study, we explored the possibility that another yet unidentified viral protein could be a component of the tRNA$_3^{Lys}$ packaging complex. We found that mLysRS is also able to interact with the Rev and Vpr proteins, and determined that this association mainly involves the catalytic domain of mLysRS, common to the three LysRS species. Association of cytoplasmic LysRS with Vpr was already described in another study [20], but was suggested to be not highly specific [21] since BSA added in excess could inhibit this interaction. In the present study, interaction of Rev and Vpr with mLysRS is displaced by Pol (Fig. 4), suggesting that these interactions are not involved in the assembly of the GagPol:mLysRS:tRNA$_3^{Lys}$ packaging complex. Indeed, we show that the Pol polyprotein, mimicking GagPol, and mLysRS are sufficient to associate tRNA$_3^{Lys}$ within the packaging complex. Since the complex between Pol and mLysRS can form in the absence or in the presence of tRNA$_3^{Lys}$, this suggests that tRNA binding does not induce large structural rearrangements on either Pol or mLysRS. Structural changes induced on the synthetase following tRNA binding could be tolerated by the flexibility of the Pol polypeptide, as it may be expected for a polyprotein containing flexible hinge peptides at the borders of its individual domains.

The data presented in this paper suggest that cellular compartmentalization is the key mechanism for selective targeting of only mLysRS to HIV-1 virions. It also suggests that mLysRS can fulfill other functions in the HIV-1 life cycle, via its association to Vpr and Rev.

Viral protein R (Vpr), one of the regulatory/accessory proteins encoded by HIV-1 genome, is involved in several aspects of host–virus interactions [22]. One of the main cellular roles of Vpr seems to be induction of cell cycle arrest in G2 following HIV-1 infection. Vpr causes hyperphosphorylation of Cdc2, a cyclin-dependent kinase that regulates the cell cycle, through inhibition of the Cdc25 phosphatase and activation of the Wee1 kinase. A small fraction of Vpr is also phosphorylated on Ser^{79} and is essential for nuclear import of the HIV-1 genomic DNA preintegration complex. An important role of Vpr in induction of apoptosis through permeabilization of the mitochondrial membrane has also been reported. Finally, association of Vpr with numerous cellular proteins was observed. It is therefore conceivable that association of Vpr with LysRS could regulate one or several of these processes. For instance, association of Vpr with mLysRS could be required to induce relocalization of mLysRS in the cytoplasm of HIV-1 infected cells, as previously observed following exposure of HeLa cells to exogenous Vpr [21]. The Vpr-assisted release of mLysRS from mitochondria would be a prerequisite for its association with tRNA$_3^{Lys}$ and GagPol to form the packaging complex. Alternatively, binding of cLysRS to Vpr could be a regulatory

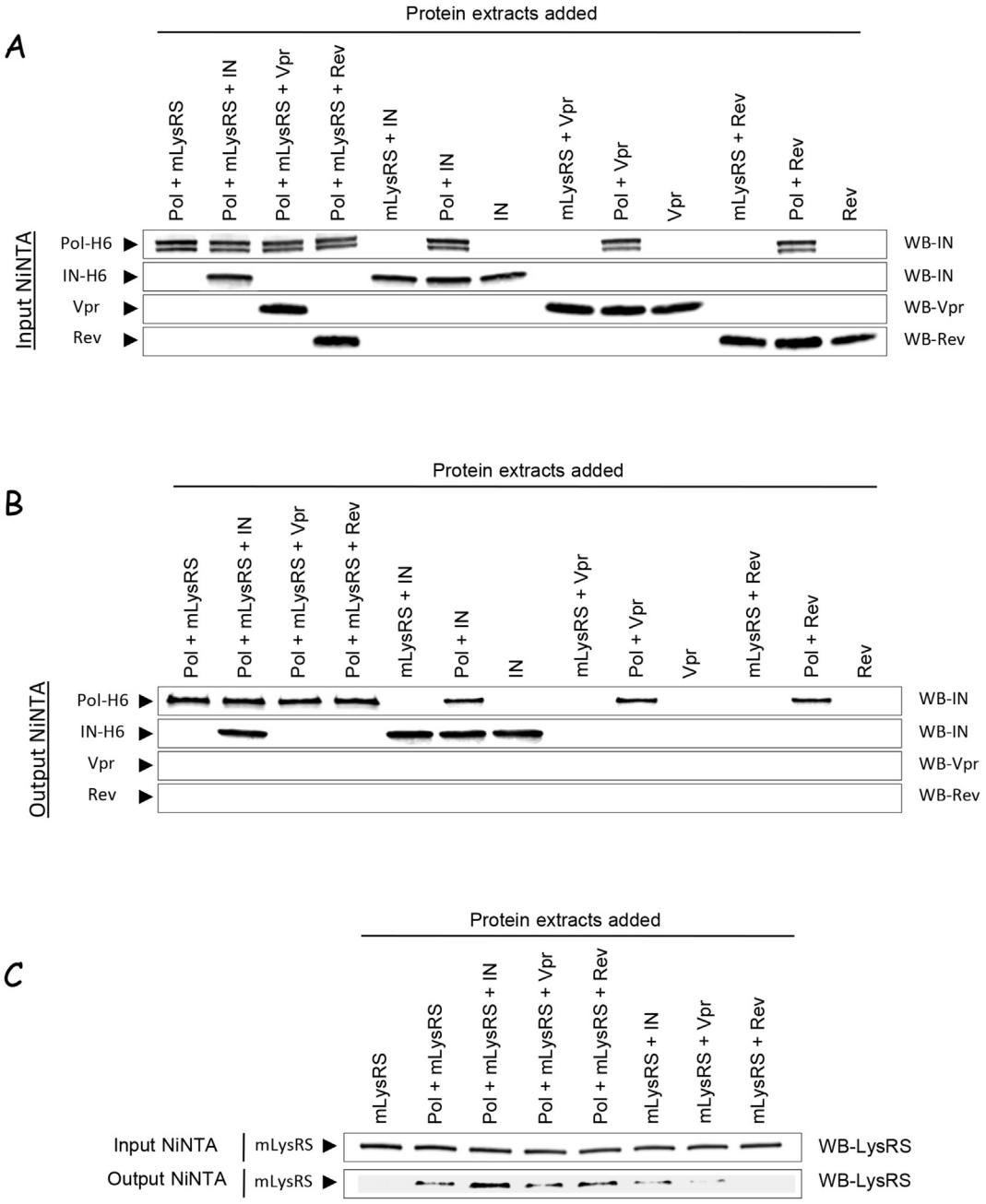

Fig. 4. Rev and Vpr do not copurify with the mLysRS:Pol complex. The mLysRS species, Pol or IN with a C-terminal His-Tag (Pol, IN), Vpr and Rev were expressed independently in insect cells. Extracts were mixed as indicated, and subjected to NiNTA-agarose pull-down. The fractions obtained before (A: Input) or after (B: Output) NiNTA pull-down were analyzed by Western blotting with anti-IN (for Pol and IN), anti-Vpr, anti-Rev, or (C) with anti-LysRS antibodies.

mechanism that controls the other functions of Vpr, in cell cycle arrest or nuclear import.

Rev, the Regulator of Expression of Virion proteins, is an essential component of HIV involved in nuclear export of intron-containing viral RNAs [23]. Rev is a nucleo-cytoplasmic shuffling protein containing four functional domains: a nuclear localization signal and a nuclear export signal for efficient trafficking, an RNA binding domain and an oligomerization domain involved in binding the Rev-responsive elements located on unspliced or partially unspliced mRNAs. In the absence of Rev, unspliced RNAs are not efficiently exported, and GagPol and Env are poorly expressed. Rev functions as a molecular switch for the translation of viral proteins from unspliced transcripts, and several cellular proteins were shown to regulate its activity. For instance, a component of the translation machinery, the eukaryotic initiation factor 5A (eIF-5A) has been shown to be essential for Rev-mediated viral RNA export [24]. The finding that LysRS, its cytosolic or mitochondrial counterpart could also be involved in regulation of Rev functions opens new

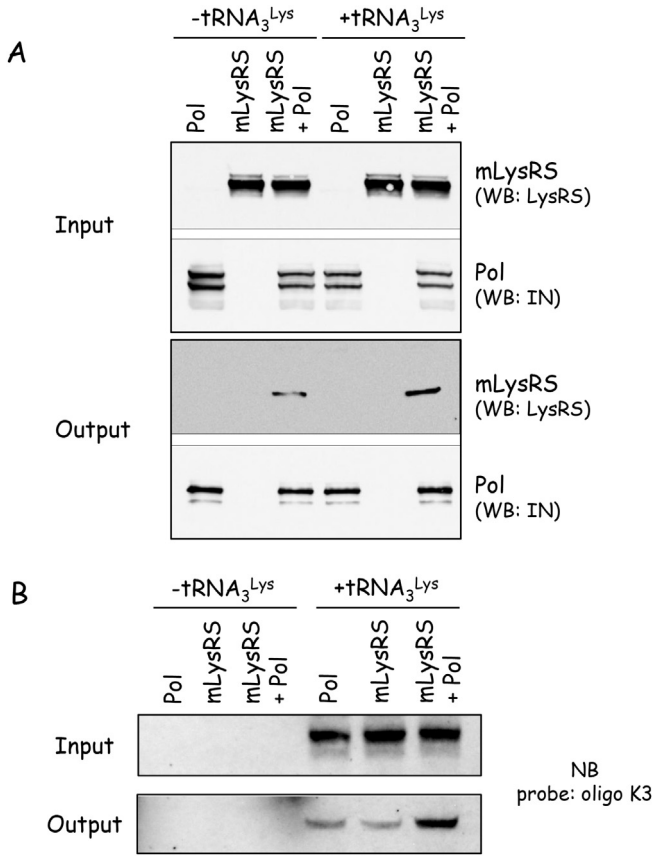

Fig. 5. Association of mLysRS with Pol in the absence or in the presence of $tRNA_3^{Lys}$. Pol with a C-terminal His-Tag and mLysRS were expressed independently in insect cells. Individual extracts or mixed extracts (mLysRS + Pol) were incubated in the absence ($-tRNA_3^{Lys}$) or in the presence ($+tRNA_3^{Lys}$) of $tRNA_3^{Lys}$ and subjected to NiNTA-agarose pull-down. (A) The initial fractions (Input) and the fractions obtained after NiNTA pull-down (Output) were analyzed by Western blotting with anti-LysRS or anti-integrase antibodies. (B) The presence of $tRNA_3^{Lys}$ in the corresponding fractions was determined by Northern blotting with a digoxigenin-labeled oligonucleotide complementary to $tRNA_3^{Lys}$ (K3). With Pol or mLysRS alone, the background level of $tRNA_3^{Lys}$ recovered corresponds either to nonspecific binding of tRNA on NiNTA beads, or to the binding of tRNA on proteins from the total extracts that bind nonspecifically to the beads.

perspectives on the role of this host protein on the biology of HIV-1.

Conflict of interest

The authors declare no competing financial interests.

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