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Noncanonical functions of lysyl-tRNA synthethase in HIV-1 packaging

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ABSTRACT

Lysyl-tRNA synthetase (LysRS) catalyzes the formation of Lys-tRNA^{Lys}. Addition to its role in the translation apparatus, LyRS has other alternative functions which are of great interest. LysRS involves in the production of diadenosine tetraphosphate, acts as a cytokine-like molecule and play a major role in HIV-1 packaging. Interestingly, reverse transcription of the HIV-1 genome is primed by a human tRNA^{Lys3} which is packaged into the virion by the HIV-1 Gag and LysRS. However, the structural basis for simultaneous packaging of tRNA^{Lys3}, LysRS and Gag is still not understood. Better understanding of the interaction among tRNA^{Lys3}, LysRS and Gag might open the way for the design and screening of small peptides and/or other small molecules capable of potentially interfering with the HIV-1 life cycle by blockading interactions between LysRS and the Gag CA-C domain.

Keywords: lysyl-tRNA synthetase, diadenosine polyphosphate, HIV-1 capsid protein, HIV-1 packaging.

INTRODUCTION

Translation is one of the most complex biological processes, involving diverse protein factors and enzymes as well as messenger and transfer RNA. To obtain the correct protein, faithful translation of genetic information is required, which depends on the correct matching of amino acids to their cognate tRNAs. This is achieved in a reaction catalyzed by 20-aminoacyl-tRNA synthetases (aaRS), each of which is specific for one amino acid and one or more isoaccepting tRNAs. Therefore, the two main functions of aminoacyl-tRNA synthetase are to catalyze the aminoacylation reaction, and to discriminate between cognate and noncognate substrates. However, these aaRSs have been found to have several roles in the cell beyond their primary role in protein synthesis. Lysyl-tRNA synthetase (LysRS) was found in recent year to have a various functions. LysRS is found mainly in the cytoplasm as a part of a multisynthetase complex, can produce diadenosine polyphosphate (Ap_nA), acts as a cytokine-like molecule and importantly involves in the formation of the tRNA^{Lys} packaging complex in HIV-1. In this review, the noncanonical functions of LysRS relating to HIV-1 virus are focused. The structures and functions of LysRS are briefly described, a nontranslational function of LysRS as Ap_nA synthetase is also discussed and finally the possible role of LysRS as a novel anti-HIV-1 target is well examined.

Structural and molecular characteristics

LysRS catalyzes the formation of Lys-$tRNA^{Lys}$. It is important to note that this enzyme is found in both unrelated families of aminoacyl-tRNA synthetase. In bacteria and eukaryotes, all known LysRSs are subclass IIb, whereas some archaea have been shown to have characteristics of Class I-type LysRS (Ibba *et al*., 1997a; Ibba *et al*., 1997b; Terada *et al*., 2002). Unlike most prokaryotic aminoacyl-tRNA synthetases, two isoforms of LysRS from *E. coli* is coded by two genes, a constitutive *lysS* and a stress-inducible *lysU* (Kisselev, 1972; Charlier and Sanchez, 1987; Hassani, 1991; 1992). Even though the homology between the amino acid sequences of the two *E. coli* LysRS is of 88%, LysS is twice as active as LysU in ATP-PP_i exchange as well as in the $tRNA^{Lys}$ aminoacylation reaction (Brevet *et al*., 1995; Onesti *et al*., 1995). Furthermore, the dissociation constant (K_d) of LysU-lysine complex is 8 times smaller than that of the LysS-lysine complex. An interesting function of aminoacyl-tRNA synthetase *in vitro* is that the enzyme can synthesize adenylated bis(5′-nucleosidyl) polyphosphates (Ap_nN), with Ap_4A being the major product from LysRS. When human LysRS (hLysRS) and *E. coli* LysRS (eLysRS) are compared (Fig. 1A), the highest similarity exists for the C-terminal aminoacylation domain (50%), while there is still 26% similarity for the N-terminal anticodon-binding domain (Guo *et al.*, 2008). In eukaryotes, LysRS contains a lysine-rich N-terminal appendage which has been shown to enable nonspecific tRNA binding and thus allowing an increase in catalytic efficiency of the enzyme, especially at the low concentration of deacylated tRNA. (Francin *et al.*, 2002; Francin and Mirande, 2003). Recently, the crystal structure of a tetrameric form of hLysRS has been elucidated (Guo *et al.*, 2008). While the crystal structure of eLysRS is shown as dimer, the crystals structure of hLysRS revealed an unusual tetramer (Fig. 1B), a structure not seen with any of the other class II aaRS. This crystal structure has already revealed important insights regarding possible interaction mechanisms between LysRS and proteins such as AIMP2-one of the three nonenzymatic components of a high molecular mass multisynthetase complex (MSC) (Guo *et al.*, 2008). Four molecules of hLysRS with closely similar structures are found in the asymmetric unit. The structure of each molecule harbors the N-terminal OB-fold anticodon-binding domain (S70-P214) that is specific to LysRS, AspRS, and AsnRS, and a C-terminal catalytic domain (L220- K574). The structure of hLysRS closely resembles those of the two similar eLysRSs (LysU and LysS). Interestingly, this tetramer probably functions in aminoacylation because docking of tRNA molecules to the tetrameric LysRS does not cause any steric clash between LysRS and tRNA molecules. Although a symmetric surface of the two dimers is used, the dimer:dimer interaction is not symmetrical. Guo *et al*. (2008) surmised that this tetramer is an intermediate state of a loose tetramer that is captured in the crystal packing. It is possible that dimer–tetramer equilibrium could be shifted under the crystallization conditions of high concentrations of LysRS toward creating a major population of tetrameric species (through α_2–α_2 contacts) that was preferentially packed into the crystal (Guo *et al.*, 2008).

LysRS-catalyzed Ap_4A synthesis

In addition to aminoacylating their cognate tRNAs, most aminoacyl-tRNA synthetase can catalyze the synthesis of dinucleotide derivatives (Brevet *et al*., 1995). In general, Ap_4A synthesis by aminoacyl-tRNA synthetases requires ATP, Mg^{2+}, and the cognate amino acid substrate. In some aminoacyl-tRNA synthetases, Zn^{2+} was found to

stimulate the synthesis of Ap_4A (Blanqet *et al*., 1983). LysU is one of the most effective Ap_4A synthetase - 80% of total Ap_4A synthesis in *E. coli* cell extracts has been ascribed to LysRS catalysis (Charlier and Sanchez, 1987).

A plausible mechanism for this Ap_4A formation by LysU is shown in Fig. 2 (Wright *et al*., 2006). Dinucleoside polyphosphates are small molecules discovered more than 30 years ago though their functions are still unclear. The existence of

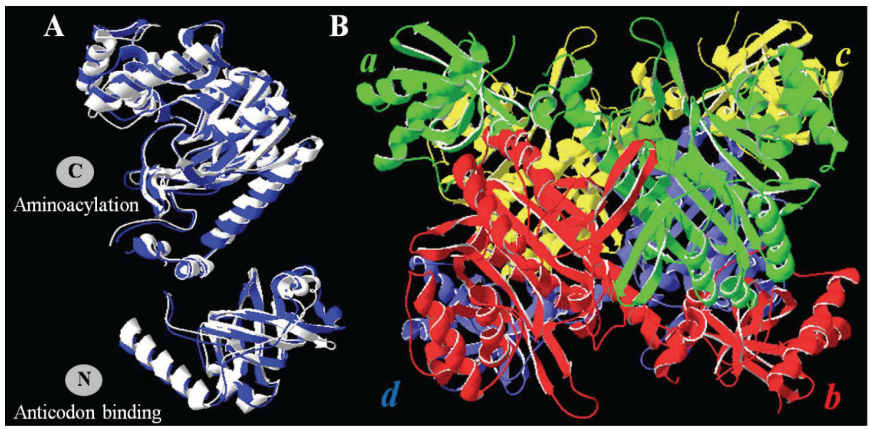

Figure 1 The overall structural features of the human LysRS tetramer. (A) Superimposition of the catalytic domain in human LysRS (white) and with that of *E. coli* LysRS (blue). (*B*) The asymmetric unit containing four LysRS molecules shown in different colors. The four molecules form two canonical dimers of LysRS (LysRS-*ab* and LysRS-*cd*). See color figure on the journal website.

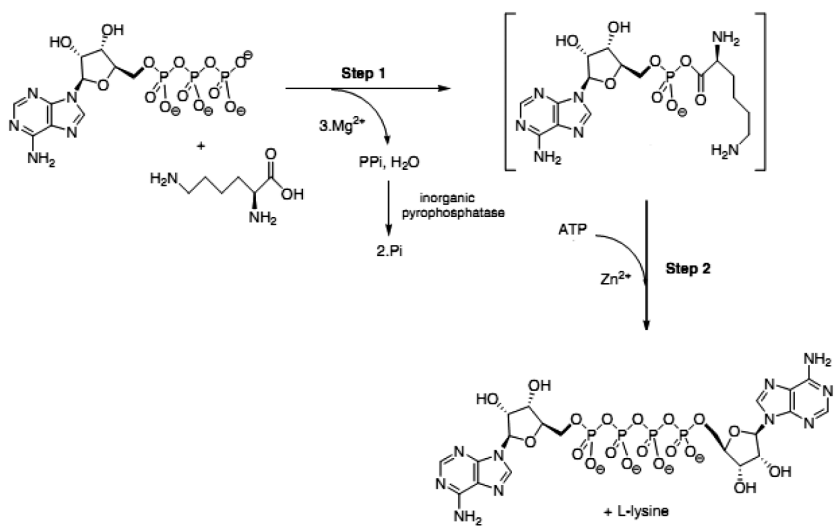

Figure 2 LysU-catalyzed Ap_4A synthesis. The mechanism of Ap_4A synthesis by LysU is catalyzed in the presence of Mg^{2+}, Zn^{2+} and inorganic pyrophosphate. The reaction is a two-step process; step 1: formation of a lysyl-adenylate intermediate; step 2: combination of intermediate with ATP to form Ap_4A with the concurrent release of L-lysine (modified from Wright *et al*., 2006).

these molecules relates to some aspects of cellular metabolism. They function in both intracellular and extracellular signaling. The naturally occurring Np_nN's consists of two nucleoside moieties linked 5′-5‴ by a polyphosphate bridge containing from 2 to 7 phosphate groups. The most abundant and widely studied of these compounds contain either one or two adenosine bases in their structure. Diadenosine polyphosphates (Ap_nA) are the most thoroughly investigated dinucleoside polyphosphates. Ap_nA consist of two adenosine rings joined in 5′-5′ linkage by a chain of phosphodiester linkages, where n is commonly in the range 2-6. Diadenosine 5′-5‴ $-P^1$, P^4 -tetraphosphate (Ap_4A) is the most common dinucleoside polyphosphate. The exact function of Ap_4A is still uncertain. The intracellular concentration of Ap_4A is about 0.05-1 μM during normal growth in mammalian cells (Garrison and Barnes, 1992). The level of this molecule also correlates with the proliferation of the cell or tissue. However the measurements during the cell cycle are unclear. Some reported that the concentration of Ap_4A increases 50- to 100-times at the G1/S boundary after mitogenic stimulation of quiescent cells or it is a 50-fold decrease prior to S-phase in sea urchin embryos but not in mammalian cells (Rapaport and Zamecnik, 1976; Weinmann-Dorsch *et al*., 1984). Various studies also suggest that Ap_4A has a role in the initiation of DNA replication or in the regulation of replication after DNA damage. Indeed, enzymes which are able to synthesize Ap_4A are found in the nucleus, where they bind to a protein that associates with DNA polymerase-α/primase. Their levels are also increased by agents that produce strand breaks in DNA, which can induce apoptosis in certain cells. Therefore, Ap_4A is also called the S-phase component of the DNA damage checkpoint system, which interrupts cell cycle progression when damage to the genome is detected (Paulovich *et al*., 1997). The levels of Ap_4A and several Np_nN's are known to be involved in cellular stress response such as oxidation and heat shock. They were also named as alarmones signaling the onset of cellular and metabolic stress. Ap_4A also plays a different role in nondividing cells. It was suggested that the dinucleotides may act as second messengers to mediate the glucose-induced blockade of K_{ATP} channels (Martin *et al*., 1998). Np_nN's also modulate some enzymes involved in purine and pyrimidine nucleotide metabolism (Günther Sillero and Cameselle, 1992). Since Ap_4A is an ATP analogue, its accumulation would either inhibit or activate adenylate kinases and other protein kinases. In summary, it seems that diadenosine tetraphosphates could be unavoidable by-products of some biological pathways, and their accumulation might be toxic due to their structural similarity to ATP and other important mononucleotides (Mclennan, 2000). These compounds may therefore play some physiological functions and to ascertain their function is one of the challenging projects.

HIV-1 assembly and formation of the $tRNA^{Lys}$ packaging complex

Assembly is the last step of HIV-1 life cycle before the budding of the virus. The viral genomic RNA, Gag, Gag-Pol, $tRNA^{Lys3}$ and LysRS are packed together into a new virion, called the $tRNA^{Lys3}$ packaging complex (Kleiman and Cen, 2004; Kleiman *et al.*, 2010). There are several interactions that involve in the formation of this complex. Gag interacted with Gag-Pol to form the viral like particles (VLPs). The $tRNA^{Lys3}$ interacted with Gag-Pol and LysRS in order to incorporate $tRNA^{Lys3}$ into VLP. The critical region for the protein-protein interaction between LysRS and Gag has been mapped out and the interaction is dependent on Gag sequences within the C-terminal domain of the capsid protein (CA-CTD) (Javanbakht *et al*., 2003).

The interaction between LysRS and CA-CTD can be helpful to maintain the $tRNA^{Lys3}$ into the $tRNA^{Lys3}$ packaging annealing complex. While $tRNA^{Lys}$ is targeted for incorporation into HIV-1 by a specific interaction of HIV-1 Gag with LysRS, RT sequences within Gag- Pol must also be present, or LysRS is packaged without $tRNA^{Lys}$ (Khorchid *et al.*, 2000; Cen *et al.*, 2001). Predicted relationships within the putative $tRNA^{Lys}$ packaging complex are schematically shown in Fig. 3.

The association of Gag-Pol with Gag is most likely driven by an interaction between homologous Gag sequences in both molecules. HIV-1 genomic RNA is packaged into the virus through interactions between nucleocapid (NC) sequences in Gag and specific stem/loop structures at the 50 end of the genomic RNA (Berkowitz *et al.*, 1996; Geigenmuller and Linial, 1996). The primer-binding site (PBS) is located within 100 nucleotides upstream of these sequences. Additional interactions between LysRS and genomic RNA proximal to the PBS may also facilitate tRNA targeting and placement. During viral maturation, the first PR cleavage is between SP1 and NCp7 (Pettit *et al.*, 2004). An interaction between Pol and Gag could insure that Pol is retained in the partially closed budding particle if proteolytic cleavage initiates during assembly. In support of this model, an interaction between LysRS and the connection domain (CD)/RNaseH domains in RT has been reported (Saadatmand *et al.*, 2008). LysRS and mature RT do not interact *in vitro*, and thus this interaction may only occur in the context of Pol or may be indirect, and the function of this interaction is not yet clear. Since C-terminal deletions of Gag-Pol that include the RT CD do not inhibit $tRNA^{Lys3}$ packaging (Cen *et al.*, 2004), the LysRS/ RT interaction is not involved in facilitating $tRNA^{Lys3}$ packaging. In summary, it is estimated that within the $tRNA^{Lys}$ packaging complex, there may be approximately 300 molecules of Gag, 100 molecules of Gag-Pol, 25 molecules of LysRS (Cen *et al.*,

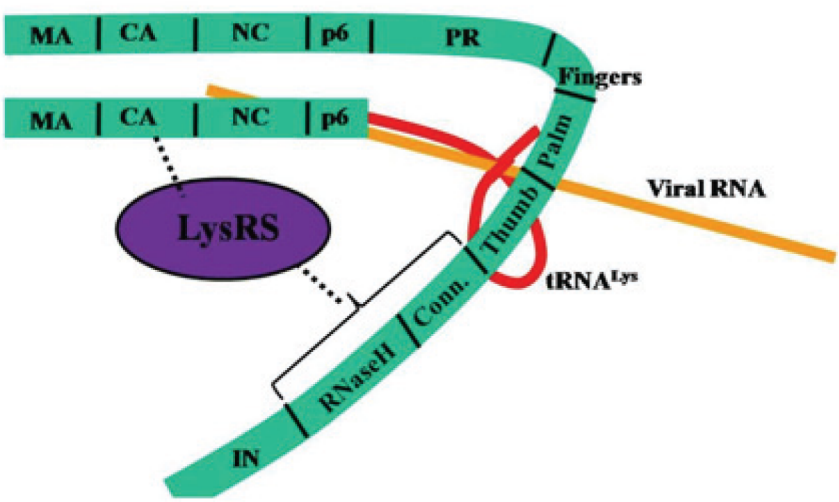

Figure 3 Proposed relationships existing between components of the $tRNA^{Lys3}$ packaging/annealing complex. Both pictures show a previously formed Gag/Gag-Pol/viral RNA complex interacting with a $tRNA^{Lys3}$/LysRS complex, with Gag interacting with both Gag-Pol and LysRS, and Gag-Pol also interacting with $tRNA^{Lys}$.

2002), and 20–25 molecules of $tRNA^{Lys}$ (Huang *et al.*, 1994) in addition to viral genomic RNA. Gag-Pol, LysRS and $tRNA^{Lys3}$ should be proximal to the Gag molecules that are chaperoning tRNA annealing, in order to facilitate transfer of $tRNA^{Lys3}$ from hLysRS to the PBS via RT.

LysRS and $tRNA^{Lys3}$

Human lysyl-tRNA synthetase is packaged into newly formed HIV-1 virions (Cen *et al.*, 2001). The absence of other aminoacyl-tRNA synthetases (aaRSs) suggests that packaging is specific to hLysRS. LysRS directly interacts with Gag *in vitro* and can be packaged into virus-like particles (VLPs) composed only of Gag, independent of $tRNA^{Lys3}$ or Gag- Pol (Cen *et al.*, 2001). Therefore, the current hypothesis for $tRNA^{Lys}$ packaging involves an interaction between a GagD Gag-Pol complex and LysRSD $tRNA^{Lys3}$ complex. Analyses of $tRNA^{Lys3}$ anticodon mutants revealed a direct correlation between their ability to be incorporated into virions and their ability to undergo aminoacylation (Javanbakht *et al.*, 2002). Because the aminoacylation defect of these tRNA variants was primarily in the K_m parameter, it was suggested that binding to LysRS rather than aminoacylation per se is a pre-requisite to the packaging. This conclusion was subsequently verified in a separated study that LysRS mutants lacking aminoacylation activity were still packaged into HIV particles, which also contained wild-type levels of $tRNA^{Lys}$ primer (Cen *et al.*, 2004). The over-expression of exogenous wild-type LysRS in cells results in a two-fold increase in the uptake of both LysRS and $tRNA^{Lys}$ into virions (Gabor *et al.*, 2002). Interestingly, an N-terminally truncated LysRS variant with approximately 100-fold weaker affinity for $tRNA^{Lys}$ showed a slight increase in incorporation into virion compared to wild-type LysRS, possibly as a result of the higher amounts present in the cytoplasm (Cen *et al.*, 2004). However, virion $tRNA^{Lys}$ levels displayed a slight decrease (Cen *et al.*, 2004). Taken together, the binding of $tRNA^{Lys}$ to LysRS is critical for $tRNA^{Lys}$ packaging into HIV, whereas aminoacylation is not. In addition, LysRS packaging is independent of tRNA packaging. Although aaRSs cognate to the primer tRNA are strong candidates for packaging signals, the selective packaging of the aaRS itself differs among retroviruses (Cen *et al.*, 2002). Western blot analysis of viral and cell lysates for the presence of LysRS, TrpRS and ProRS, cognate to primer tRNAs in HIV-1, Rous sarcoma virus and murine leukemia virus (MLV) revealed that LysRS was detected in HIV-1 and TrpRS was seen in Rous sarcoma virus viral lysates, ProRS was not detected in MLV, suggesting that ProRS may not be a packaging signal for $tRNA^{Pro}$ (Cen *et al.*, 2002). Gabor and coworkers (Gabor *et al.*, 2002) showed that overexpression of exogenous $tRNA^{Lys3}$ results in higher incorporation into a virion, an increase tRNA annealing to viral RNA and greater infectivity of the virus. The absence of an accompanying increase in Gag-Pol D Gag levels indicates that LysRS may be the limiting factor for $tRNA^{Lys3}$ packaging (Gabor *et al.*, 2002). Moreover, using small interfering RNA to silence LysRS mRNA causes an 80% decrease in newly synthesized hLysRS in the cellular pool and a corresponding decrease in viral LysRS (Guo *et al.*, 2003). Viral $tRNA^{Lys}$ isoacceptor levels reduce to approximately 40–50% of wild-type levels and a similar decrease in $tRNA^{Lys}$ annealing and viral infectivity is also observed (Guo *et al.*, 2003).

LysRS and Capsid protein C-terminal domain (CA-CTD) of HIV-1

LysRS is one of the nine aaRSs in the high molecular weight multisynthetase complex observed

in higher eukaryotic cells (Nathanson and Deutscher, 2000). A recent solved X-ray crystal structure of a tetrameric form of LysRS provided insight into possible interactions with other proteins that comprise the multi-synthetase complex (Guo *et al.*, 2008). Based on the finding that VLPs composed only of HIV Gag protein package LysRS, it was hypothesized that interactions between Gag and LysRS dictate LysRS packaging. An interaction between the proteins was confirmed by *in vitro* glutathione S-transferase pull-down studies using wild-type LysRS and truncated LysRS mutants, followed by testing their ability to be packaged into Gag VLPs *in vivo* (Javanbakht *et al.*, 2003). Similar experiments with truncated Gag constructs identified the sites of interaction in Gag and LysRS to residues 308–362 at the C-terminal end of CA and 208–259 of the LysRS motif 1 (Javanbakht *et al.*, 2003). Interestingly, both regions are critical for formation of the homodimer interfaces within each protein. Site-directed mutants that disrupted homo-dimerization of either LysRS or Gag have no significant effect on the Gag-hLysRS interaction, possibly as a result of the formation of a heterodimeric Gag-hLysRS complex (Kovaleski *et al.*, 2006). Gel chromatography studies were consistent with heterodimer formation and an equilibrium binding constant of 310 ± 80 nm was determined for the Gag-hLysRS complex using fluorescence anisotropy (Kovaleski *et al.*, 2006). A comparison of X-ray crystal structure data suggested that the interaction domain of CA can adopt different dimerization interfaces by swapping the major homology region (MHR) element between monomers (Ivanov *et al.*, 2005; Worthylake *et al.*, 1999). The MHR, part of helix 1 is a highly conserved domain present in all retroviral CA proteins (Wills and Craven, 1991). Fluorescence anisotropy binding measurements revealed that LysRS missing the N-terminal 219 residues retains a high affinity to CA, and that the CA-CTD is sufficient to bind hLysRS (Kovaleski *et al.*, 2007). Using NMR spectroscopy, chemical shift perturbations of residues in and around helix 4 (211-LEEMMT-216) of CA-CTD were observed upon LysRS binding. Residues T210, M214 and M215, along with a nearby H226, are implicated in this specific interaction as shown by peptide binding studies and alanine scanning mutagenesis (Kovaleski *et al.*, 2007). Computational docking and biochemical data supported a direct interaction between helix 7 of hLysRS and helix 4 (C4) of CA-CTD (Kovaleski *et al.*, 2007).

LysRS-tRNA^{Lys3}-Gag-CA ternary complex

As described previously, the interaction among LysRS, tRNA^{Lys3} and Gag-CA is crucial for HIV-1 packaging and the structural basis for this ternary complex is still not clearly understood. Until recently, Guo (Guo *et al*., 2010) has performed the docking analysis of LysRS-Gag as well as carried out several experiments to show that LysRS-Gag interface overlaps the surface of the α2-LysRS dimer interface. This implies that the dynamic equilibrium of LysRS between monomer and dimer states should direct the protein from aminoacylation to HIV-packing. Fig. 4 shows the model of the human LysRS-tRNA^{Lys3}-Gag-CA ternary complex. It can be seen that Gag-CA-CTD is positioned far from tRNA^{Lys3} in the complex; however, the C-terminal end of CTD points to tRNA^{Lys3} in the modeled complex (see the arrow in Fig. 4). This could allow the C-terminal nucleocapsid part of Gag to access the packaged tRNA^{Lys3} readily. Additionally, H3 and H4 of CA-CTD bind to the interdomain cavity of the LysRS monomer. This area of interaction can be used as a target for a drug. Since H3, H4, and the C-terminal loop of CA-CTD

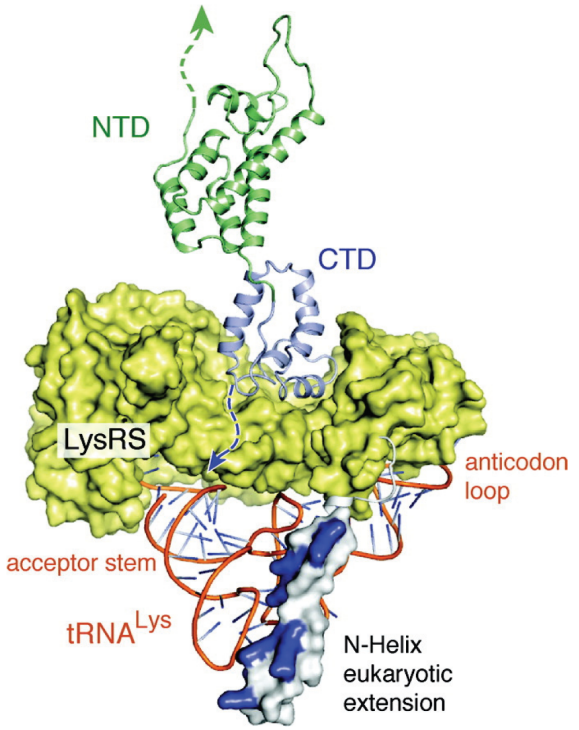

Figure 4 Model of the human LysRS-tRNA^{Lys3}-Gag-CA ternary complex. NTD of Gag-CA is shown in green. The N-terminal helix of human LysRS is shown as a surface representation. All Lys/Arg residues on the N-terminal helix are colored in deep blue, with most of them facing the bound tRNA. The arrows indicate the potential location of N-terminal and C-terminal regions of Gag outside of the CA structure (Guo *et al*., 2010) [permission from American Chemical Society (ACS) publications]. See color figure on the journal website.

are critical for HIV virion assembly, if one can design the inhibitor covering the bottom side of H3 and H4, the inhibitor may not only inhibit LysRS binding and tRNA^{Lys3} incorporation but also would be useful for blocking virion assembly.

LysRS and a peptide derived from CA-CTD

Since tRNA^{Lys3} and LysRS are selectively packaged into the virion through Gag during the viral assembly and tRNA^{Lys3} is used as a primer for the reverse transcription process, LysRS plays a vital role in specifically targeting tRNA^{Lys3} for viral packaging. Therefore, the interaction between LysRS and the HIV-1 packaging machinery can represent a novel target for the development of anti-HIV-1 therapy. Thus understanding the molecular basis for specificity of such peptide inhibitors which are derived from HIV-1 CA-CTD against LysRS involvement in the packaging process of the virus would be crucial. The structural insights of this interaction are expected to help the researcher in optimizing the peptide-based inhibitors for HIV-1 target. Kovaleski and coworker was the only group who reported on the interaction of hLysRS and CA-CTD (Kovaleski *et al.*, 2007). Helix 4 of CA-CTD, containing 17 amino acid residues, was

found to bind strongly to LysRS according to the fluorescence anisotropy binding study. Most of the important residues resided on the helical region of helix 4 of CA-CTD. It has also been confirmed that H3, H4, and the C-terminal loop of CA-CTD are critical for HIV virion assembly (Guo *et al*., 2010). In attempt to narrow down the size of the peptide that can be used as an inhibitor to block LysRS-CA-CTD interaction, Na-nakorn *et al.* (2011) has reported that only 10 amino acid residues are necessary for this interaction and using a shorter peptide would provide new angle on the peptide-LysRS interaction.

Fig. 5A shows how the sh-H4 peptide (a short helix of HIV-1 CA-CTD, LEEMMTACQG) can bind to LysRS. The peptide was laid against helix 7 of LysRS in the perpendicular direction. Five hydrogen bonds are observed in LysRS-sh-H4 interaction in which R247 and R241 play the major role (Fig. 5B). These amino acid residues are conserved in LysRS from several organisms; hence, they appear to be a vital feature of the LysRS-CA-CTD complex and may ultimately lead to the inhibitor design to block the Gag-LysRS interaction.

CONCLUSION

Human LysRS has multiple ex-aminoacylation function besides its function in translation. A critical role of LysRS in HIV viral genesis has drawn much attention as LysRS is packaged into the HIV virion via its interaction with the Gag-protein. Therefore, LysRS seems to have a vital role in the life cycle of the virus. The main interaction between CA-CTD and LysRS has been mapped out. This could clearly open the way for further developments in the design of HIV-1 inhibitor. Moreover, studies of the regulation of LysRS function *in vivo* would provide new insights for the basic mechanism of the enzyme in its various noncanonical roles.

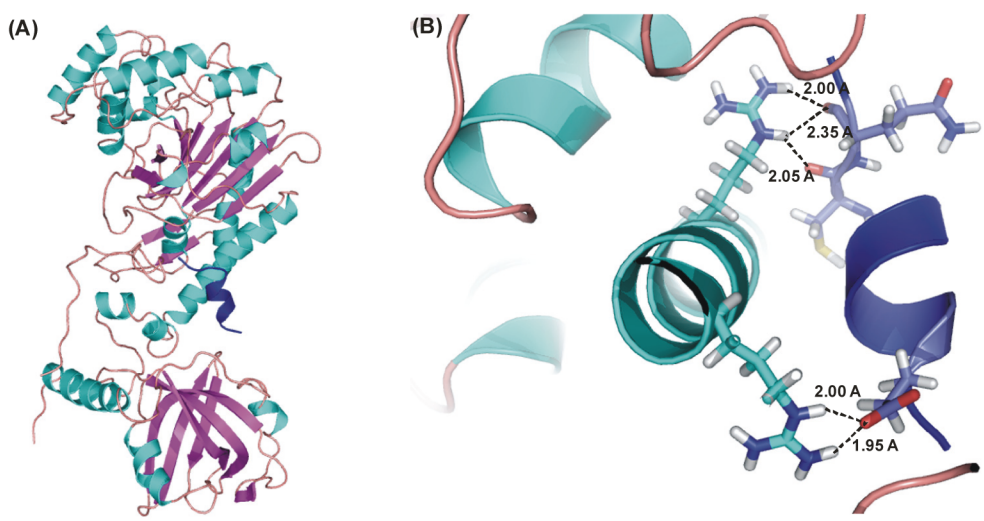

Figure 5 The complex between LysRS and sh-H4 peptide. (A) The ribbon representation of the sh-H4 peptide with monomer of LysRS. (B) The hydrogen bond interactions between helix 7 of LysRS and sh-H4 peptide. Figure reproduced from Na nakorn *et al*. (2011). See color figure on the journal website.

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