Inhibition of Cellular HIV-1 Protease Activity by Lysyl-tRNA Synthetase*

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During early assembly of human immunodeficiency virus type 1 (HIV-1), an assembly complex is formed, the components of which include genomic RNA, Gag, GagPol, tRNA<sup>Lys</sup>, and lysyl tRNA synthetase (LysRS). Directly increasing or decreasing cellular expression of LysRS results in corresponding changes in viral infectivity and in the viral concentrations of LysRS, tRNA<sup>Lys</sup>, and, surprisingly, reverse transcriptase (RT). Since altering the cellular expression of LysRS does not lead to a change in the incorporation of the RT precursor protein, GagPol, in protease-negative HIV-1, we propose that the altered viral content of RT resulting from alterations in cellular LysRS concentration results from the ability of LysRS to inhibit premature activation of GagPol viral protease within the complex. Supporting this hypothesis, we find that increases and decreases in cellular LysRS expression are accompanied by 5–8-fold increases and 5-fold decreases, respectively, in the cytoplasmic proteolysis of Gag and GagPol to mature viral proteins. Using a novel bioluminescence resonance energy transfer assay to directly measure HIV-1 protease activity in vivo also indicates that the overexpression of LysRS in the cell reduces viral protease activity.

During viral assembly, the Gag and GagPol precursors are processed into the final mature viral proteins by the viral protease located within GagPol (1). Proteolysis is believed to occur during or immediately after viral budding (2), and mechanisms that prevent premature cytoplasmic processing of the viral precursors and their loss from the virion are clearly desirable. Since protease dimerization is required for the activity of the enzyme (1), the concentration of GagPol at the plasma membrane could be an important factor favoring dimerization. Another mechanism regulating protease activity could involve a GagPol conformation, rendering it resistant to protease dimerization during its transit to the plasma membrane. Such a GagPol transport complex will include Gag and RNA since interaction with Gag is required for GagPol incorporation into budding virions (3, 4), and this interaction requires the RNA-facilitated multimerization of Gag (5).

This RNA/Gag/GagPol complex will also include tRNA<sup>Lys</sup> and lysyl tRNA synthetase (LysRS), the enzyme that aminoacylates tRNA<sup>Lys</sup><sup>*α</sup>. During HIV-1<sup>1</sup> assembly, both LysRS (6) and the tRNA<sup>Lys</sup> isoacceptors, tRNA<sup>Lys</sup><sup>α1</sup>, tRNA<sup>Lys</sup><sup>α2</sup>, and tRNA<sup>Lys</sup><sup>α3</sup> (the primer tRNA for reverse-transcriptase (7)), are selectively packaged into HIV-1 (8). This incorporation requires the presence of both Gag, which binds specifically with LysRS (9), and GagPol, which interacts with both Gag (3, 4) and tRNA<sup>Lys</sup><sup>*α</sup> (10). LysRS incorporation into Gag viral-like particles occurs independently of tRNA<sup>Lys</sup> incorporation (6), which requires the additional presence of GagPol, suggesting that LysRS itself may act as a signal for targeting tRNA<sup>Lys</sup> for selective packaging into HIV-1. A complex containing Gag, GagPol, and LysRS has been immunoprecipitated with anti-integrase from lysates of 293FT cells infected with protease-negative HIV-1 (9).

GagPol present in an early form of this assembly complex may be more resistant to dimerization and protease activation than at later stages when budding is initiated. In support of this hypothesis, we present data herein that indicate that altering the content of one of the packaging complex components, LysRS, produces corresponding changes in viral protease activity.

**EXPERIMENTAL PROCEDURES**

Plasmids—BH10 is a simian virus 40-based vector that contains full-length wild-type HIV-1 proviral DNA. BH10.P– is similar to BH10 but contains an inactive viral protease (D25G). hGagPol codes for Gag and protease-positive GagPol. hGagPolAF5APR was constructed by deleting 5 thymidines in the frameshift site and codes for GagPol but not Gag. This protein contains an inactive protease due to an R42G mutation in the active site. Both proteins are made from mRNAs that have had their codons optimized for mammalian cell codon usage. The “humanized” proteins have identical amino acid sequences to their viral counterparts. pcDNA1LysRS contains cDNA encoding full-length (1–597 amino acids) human LysRS cloned into pcDNA3.1 (Invitrogen) and was constructed through PCR amplification of the cDNA as described previously (11). It is C-terminally tagged with V5. BH10Lys3 and BH10Lys2 contain both wild-type HIV-1 proviral DNA and a human tRNA<sup>Lys</sup><sup>*α</sup> or tRNA<sup>Lys</sup><sup>*α</sup> gene, respectively. These vectors were constructed as described previously (12). For bioluminescence resonance energy transfer (BRET) analysis, a DNA construct was prepared coding for a fusion protein between humanized sea pansy Renilla reniformis Luciferase (hRLuc2) and a humanized green fluorescent protein (hGFP2). The HIV-1 p2/p7 protease cleavage site (4 amino acids on each side of

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1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; LysRS, lysyl-tRNA synthetase; CA, capsid; RT, reverse transcriptase; BRET, bioluminescence resonance energy transfer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hGFP2 and hRLuc, green fluorescent protein and Renilla luciferase, respectively, made from codon-optimized (humanized) mRNA; siRNA, small interfering RNA; RIPA, radioimmunoprecipitation buffer.
Inhibition of HIV-1 Protease

Overexpression of tRNA<sub>Lys</sub> in the Cell Increases the Viral Concentration of Both LysRS and RT—293T cells were transfected with a plasmid containing HIV-1 proviral DNA alone (BH10) or a plasmid containing HIV-1 proviral DNA and a gene for either tRNA<sub>Lys</sub> (BH10Lys3) or tRNA<sub>2</sub>Lys (BH10Lys2). Previous studies have shown that overexpression of tRNA<sub>Lys</sub> results in an increase in its packaging into the virion, with a corresponding decrease in the viral concentration of the other tRNA<sub>Lys</sub> isoacceptors, tRNA<sub>1</sub>Lys and tRNA<sub>2</sub>Lys, i.e. the total viral tRNA<sub>Lys</sub> virion remains constant (11, 12). Viruses were purified, and Western blots of viral protein were probed with anti-RT, stripped, and reprobed with anti-CA (Fig. 1A). Quantitative analysis using the UN-SCAN-IT gel™ automated digitizing system indicated that there was ~1.7 times more RT/CA in virions produced from BH10Lys3 or BH10Lys2 than in wild-type virus.

This increase of RT in virions as a result of overexpression of tRNA<sub>Lys</sub> is not associated with an increase in the overall viral concentration of tRNA<sub>Lys</sub> (11, 12). This suggests that some other molecule may be responsible for the increased viral RT. Since LysRS is also selectively packaged into HIV-1 during assembly (6), an increase in LysRS concentration in the virus could be associated with the increase in RT. In Fig. 1B, we show that the overexpression of tRNA<sub>Lys</sub> does in fact result in an increased concentration of viral LysRS. 293T cells were transfected with either BH10 or BH10Lys3, and Western blots of viral proteins shown in Fig. 1B were probed first with anti-LysRS and then with anti-CA. It can be seen that the overexpression of tRNA<sub>Lys</sub> not only results in an increase in RT (Fig. 1A) but also results in an increase in the viral concentration of LysRS.

The Viral Concentration of RT Is Directly Proportional to the Amount of LysRS Expressed in the Cell and Packaged into the Virion—The experiments represented in Fig. 2 demonstrate that the change in the concentration of RT in the virus is directly proportional to the change in the concentration of LysRS in the cell and in the virus. Fig. 2A, left panel, shows Western blots of lysates of viruses produced from 293T cells cotransfected with the BH10 plasmid and either a vector plasmid (pcDNA3.1) or this plasmid containing the human LysRS gene (pcDNA-LysRS). We have previously shown that the expression of exogenous LysRS in the cell results in an approximate 2-fold increase in the viral incorporation of both major tRNA<sub>Lys</sub> isoacceptors and of LysRS (11, 20). The Western blots, containing equal amounts of CAp24, were probed with anti-RT and then with anti-CA. The scanned results indicate that the direct overexpression of LysRS results in a 1.8-fold increase in the viral concentration of RT.

We have reported that transfection of 293T cells with small interfering RNA specific for LysRS (siRNA<sub>LysRS</sub>) will reduce the incorporation of LysRS in virions 80%, and this reduction in viral LysRS is correlated with a similar reduction in the synthesis of new LysRS in the cytoplasm (15). Fig. 2B shows Western blots of lysates of viruses produced from 293T cells first transfected with either siRNA<sub>LysRS</sub> (specific for luciferase) or siRNA<sub>LysRS</sub> and transfected 24 h later with the BH10 plasmid. Viruses were harvested 48 h after transfection of the BH10 plasmid, and the Western blots of viral protein were probed consecutively with anti-RT and anti-CA. The results, analyzed by UN-SCAN-IT gel™ automated digitizing system, indicate that the reduction in the cellular expression and viral incorporation of LysRS also results in an ~50% decrease in the viral concentration of RT.
Proteolysis of Viral Protein in the Cytoplasm Is Correlated with the Cytoplasmic Concentration of LysRS—Since LysRS and GagPol are found in the same tRNALys packaging complex, the increased concentration of viral RT due to the increased incorporation of LysRS could reflect an increased recruitment of GagPol into this complex by the excess LysRS. However, this is not likely since we have previously shown that in a protease-negative virion, overexpression of LysRS results in close to a 2-fold increase in both LysRS and tRNALys, without changing the GagPol:Gag ratio (11). An alternative explanation is that LysRS inhibits premature activation of viral protease in this cytoplasmic complex. The data in Figs. 3 and 4 support this premise.

Fig. 3 shows Western blots of lysates of 293T cells that have been cotransfected with a plasmid coding for either protease-positive HIV-1 DNA (A and C, BH10) or protease-negative HIV-1 DNA (B and D, BH10P−) and a second plasmid or small interfering RNA that will alter the expression of cytoplasmic LysRS. Western blots were probed with anti-CA, anti-RT, anti-LysRS, or anti-β-actin. In A and B, the second plasmid is either the vector alone (pcDNA3.1) or this vector containing the gene for LysRS (pcDNALysRS). The results in A, which are quantitated in E, show that there is less proteolysis of viral proteins upon overexpression of LysRS in the cytoplasm, i.e. the cytoplasmic Gag/CA and GagPol/RT ratios are ~4.5- and 8-fold greater, respectively, when the LysRS/β-actin ratio is increased. B is a control showing the cytoplasmic viral protein pattern when no viral protease is present.

The cells represented in C and D have been transfected with either siRNALuc or siRNAtdhLys as well as either BH10 (C) or BH10P− (D). The results in C, which are quantitated in E, show that there is more proteolysis of viral proteins when the concentration of cytoplasmic LysRS is decreased, i.e. when the LysRS/β-actin ratio is reduced ~90%, the cytoplasmic Gag/CA and GagPol/RT ratios are reduced ~80–85%. D is again a control showing the cytoplasmic viral protein pattern when no viral protease is present, indicating that the proteolysis of the viral proteins is not due to the activation of cellular proteases due to the reduced LysRS in the cell.
Effect of LysRS Expression on Viral Protease Activity Using BRET Analysis—We next examined the effect of LysRS concentration upon HIV-1 protease activity using BRET to assay for HIV-1 protease activity in living cells (13). BRET analysis has been used to study Gag binding partners in HIV-1, homodimerization of integral membrane receptors, protein-protein interactions during transcription, and more recently, in an ubiquitination biosensor assay (16–18, 21). The assay takes advantage of the physical distance between a donor and acceptor molecule, and when these are within close proximity (between 10–100 Å), a resonance energy transfer (RET) occurs between the donor and acceptor pair. To initiate this reaction, a membrane soluble coelenterazine, DeepBlueC (PerkinElmer Life Sciences), is added to cells in culture. In the presence of oxygen, humanized sea pansy Renilla reniformis luciferase (hRLuc) catalyzes the DeepBlueC into coelenteramide with concomitant light emission (λ\text{em} = 395 nm). The acceptor of this emission is a codon-optimized (humanized) green fluorescent protein 2 (hGFP2) that is engineered to maximally absorb λ\text{em} and emit green light (λ\text{em} = 510 nm). The broad spectral emission spectra exhibited by these molecules enable a

**FIG. 3. Effect of LysRS expression upon cytoplasmic processing of viral proteins.** 293T cells were transfected or cotransfected with the different plasmids listed along the top of each panel. pcDNA3.1 is a vector plasmid, whereas pcDNA-LysRS is this plasmid containing the cytoplasmic form of human LysRS cDNA. This cDNA is tagged at the C terminus with the V5 epitope and has a slower electrophoretic mobility than endogenous LysRS (20). 48 h post transfection with BH10, cells were lysed in RIPA buffer, and proteins in the cell lysates were resolved by Western blotting and probed with anti-CA, anti-RT, anti-LysRS, and anti-β-actin. Detection of proteins was performed by enhanced chemiluminescence (PerkinElmer Life Sciences) using as secondary antibodies anti-mouse (for anti-capsid, anti-reverse transcriptase, and anti-β-actin) and donkey anti-rabbit (for anti-LysRS), obtained from Amersham Biosciences. A and B, cells were cotransfected with either BH10 or BH10P- and with either pcDNA3.1 or pcDNA-LysRS. C and D, cells were cotransfected with either BH10 or BH10P- and with either siRNA\text{Luc} or siRNA\text{LysRS}. E, quantitation of bands was performed using UN-SCAN-IT gel™ automated digitizing system, and ratios are listed in E.

**Effect of LysRS Expression on Viral Protease Activity Using BRET Analysis**—We next examined the effect of LysRS concentration upon HIV-1 protease activity using BRET to assay for HIV-1 protease activity in living cells (13). BRET analysis has been used to study Gag binding partners in HIV-1, homodimerization of integral membrane receptors, protein-protein interactions during transcription, and more recently, in an ubiquitination biosensor assay (16–18, 21). The assay takes advantage of the physical distance between a donor and acceptor molecule, and when these are within close proximity (between 10–100 Å), a resonance energy transfer (RET) occurs between the donor and acceptor pair. To initiate this reaction, a membrane soluble coelenterazine, DeepBlueC (PerkinElmer Life Sciences), is added to cells in culture. In the presence of oxygen, humanized sea pansy Renilla reniformis luciferase (hRLuc) catalyzes the DeepBlueC into coelenteramide with concomitant light emission (λ\text{em} = 395 nm). The acceptor of this emission is a codon-optimized (humanized) green fluorescent protein 2 (hGFP2) that is engineered to maximally absorb λ\text{em} and emit green light (λ\text{em} = 510 nm). The broad spectral emission spectra exhibited by these molecules enable a
FIG. 4. **Effect of LysRS expression upon cytoplasmic processing of a GFP/p27-hRLuc fusion protein.** A, the HIV-1 p2/p7 protease cleavage site was fused between and in-frame with the BRET donor hRLuc2 and the BRET acceptor hGFP2 to create a hGFP2-p2/p7-hRLuc construct. The site of protease cleavage of Gag p2/p7 is indicated by the exclamation point in the hGFP2-p2/p7-hRLuc. B, Western blots of lysates of 293T cells: mock-transfected (lane 1); cotransfected with hGFP2-p2/p7-hRLuc and hGagPol, which expresses HIV-1 Gag and protease-positive GagPol (lanes 2–6); cotransfected with hGFP2-p2/p7-hRLuc, hGagPol, and increasing concentrations of pcDNA-LysRS (0.5, 1, or 2 μg, lanes 3–5, respectively), a vector that expresses full-length hLysRS C-terminally tagged with V5. Cell extracts were prepared at 40 h after transfection. 40 μg of total protein, containing approximately equal amounts of GAPDH, was loaded in each well, and expression levels of V5-tagged LysRS were detected using a rabbit anti-V5 antibody. Lanes 6–8 represent 293T cells cotransfected with hGFP2-p2/p7-hRLuc and hGagPolDPSFDP, a plasmid coding for protease-negative GagPol (lane 6), hRLuc alone (lane 7), or hGFP2 alone (lane 8). C, BRET analysis of HIV-1 protease activity. The average BRET ratios are the means ± standard deviations of experiments performed three or more times.

To determine whether LysRS expression affects viral protease activity in live cells, the amino acids that encode the p2/p7 protease cleavage site in Pr55Gag were fused in-frame with the donor protein, hRLuc, and the acceptor protein, hGFP2, to create hGFP2-p2/p7-hRLuc (Fig. 4A). 293T cells were cotransfected with this construct, a plasmid coding for codon-optimized protease-positive Gag and GagPol (hGagPol (22)), and increasing amounts of pcDNA-LysRS. 40 h after transfection, cells were exposed to the membrane-diffusible substrate for luciferase, DeepBlueC, and analyzed by BRET. After BRET analysis, cells were lysed, and Western blots of cell lysates were probed with anti-V5 and anti-GAPDH to show the increased ratio of LysRS/GAPDH (Fig. 4B).

BRET analysis is shown in Fig. 4C. A close association between hGFP2 and hRLuc will result in a maximum BRET ratio (lane 6). However, if hGFP2 and hRLuc are separated as a result of cleavage by the viral protease, there will be a decreased BRET ratio (lane 2). Fig. 4C shows that the BRET ratio increases with increasing cellular concentrations of LysRS (lanes 2–5), indicating the inhibition of hGFP2/hRLuc cleavage. Fig. 4C, lanes 6–8, represent control experiments in which cells are either cotransfected with the plasmid coding for hGFP2-p2/p7-hRLuc and a plasmid (hGagPolaΔFSAPR (22)) coding for protease-negative codon-optimized GagPol (lane 6), hRLuc alone (lane 7), or hGFP2 alone (lane 8). The BRET ratio is maximal when no cleavage occurs (lane 6), and in this experiment, the ratio is reduced by 60% when only endogenous levels of LysRS are expressed (lane 2). Thus, these BRET data also indicate that LysRS has an inhibitory effect upon viral protease activity.

**DISCUSSION**

GagPol incorporated into the assembling Gag particle through its interaction with Gag (3, 4, 23, 24). The concentration of GagPol at the cell membrane is believed to facilitate the concentration of GagPol molecules, allowing for GagPol dimerization, a prerequisite to protease activation. Premature dimerization of GagPol at the wrong cellular site could result in the production of processed viral proteins that will not take part in viral assembly. GagPol interacts with multimeric Gag (5), and because pre-plasma membrane complexes of Gag/GagPol may exist in the cytoplasm, means may be necessary for preventing premature protease dimerization and activation in such complexes until they reach the proper cellular compartment.

Our data indicated that the interaction between Gag and GagPol is not sufficient to prevent premature protease activation and that another member of the assembly complex, LysRS, may also help stabilize GagPol protease during the transition to the plasma membrane. In the virion, estimates for Gag are ~1500–1800 molecules/virion (1), and based on estimated differences in translation rates of Gag and GagPol, there may be ~150–200 molecules of GagPol. This is considerably more than the estimated 20–25 molecules LysRS/virion (25), and unsaturated LysRS binding sites may exist in the Gag/GagPol RNA complex. The overexpression of LysRS results in an approximate 2-fold increase in LysRS incorporation into HIV-1 (20) without any increase in GagPol incorporation (11), and the limited increase in LysRS packaging may reflect either limited cellular LysRS overexpression or some other limiting factor. These results suggest that within the experimental conditions used here (such as overproduction of virions), there may exist in the cell multiple assembly complexes of varying stabilities due to limiting amounts of cellular factors such as LysRS.

The mechanism by which LysRS blocks proteolytic activity is not known. It could be inhibiting the viral protease activity generally, or it could act to block access to cleavage sites by the protease. The first cleavage is believed to occur at the p2/Ncp7 site (26), the site examined by BRET. Although it is clear that proteolytic activity at later cleavage sites is also inhibited (such as the ones producing mature CAp24 and RTp66/p51), later cleavages might be prevented as a result of preventing the first cleavage. Nevertheless, it seems unlikely that the relatively small number of LysRS molecules associated with virions could directly protect the much larger number of cleavage sites or protease molecules. We have been unable to detect an interaction between Pol and LysRS using coimmunoprecipitation studies (data not shown), and we suggest that the most likely cause of inhibition of protease activity may be changes induced by LysRS in the GagPol conformation, which might result in either inhibiting protease activity or blocking one or more cleavage sites in GagPol.

The changes observed in the RT incorporation into virions induced by alterations in the cellular concentration of LysRS (Fig. 2) are consistent but not large. Increases and decreases in cellular LysRS cause increases and decreases in the RT/Cap24 ratios of 1.77 and 0.56, respectively. On the other hand, the data in Fig. 3 show that the cellular concentration of LysRS has a much larger influence on the amount of Gag or GagPol processed in the cytoplasm. Thus, a 5-fold increase in the cytoplasmic concentration of LysRS produces a 4.6- and 7.8-fold increase in the Gag/Cap24 and GagPol/p66 ratios, respectively. Similarly, an almost 10-fold decrease in cytoplasmic LysRS shows a 4- and 5-fold decrease in Gag/Cap24 and GagPol/p66 ratios, respectively. Thus, an increase in the amount of premature processing of GagPol may have less effect upon the amount of RT incorporated into virions (i.e. RT/Cap24) because there is also less Gag produced.
Thus, the data obtained from the Western blots shown in Figs. 1–3 indicate inhibition of viral protease activity by LysRS, and as shown in Fig. 4, this conclusion is supported using a very different experimental technique, BRET. In this technique, HIV-1 protease activity is detected by its ability to act upon the cleavage site between Gag p2 and p7 that is present in the hGFP2-p2/p7-hRLuc molecule, thus separating donor hRLuc from acceptor hGFP2. Since there is no evidence that this molecule can be packaged into virions, it is implied that it is the cleaved viral protease, free of the Gag/GagPol complex, that is acting upon hGFP2-p2/p7-hRLuc.

Other work has shown that the infectivity of the HIV-1 population increases 2.5–3-fold when LysRS and tRNALys are overexpressed in the cell. The viral population increases its incorporation of LysRS and tRNA\textsuperscript{Lys}, and there is also an increase in tRNA\textsuperscript{Lys} annealing to viral RNA (11). Although this increase in tRNA\textsuperscript{Lys} annealing had been thought to be the prime factor responsible for the increased infectivity, it is now clear that other factors, such as the decreased premature proteolysis of Gag and GagPol studied here, may also contribute to increased infectivity of the viral population.

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