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The HIV-1 Tat protein: mechanism of action and target for HIV-1 cure strategies

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Abstract

The general mechanism involved in Tat activation of RNA Polymerase II (RNAP II) elongation of the integrated HIV-1 was elucidated over 20 years ago. This mechanism involves Tat binding to the TAR RNA element that forms at the 5′ end of viral transcripts and recruiting a general RNAP II elongation factor termed P-TEFb. This elongation factor consists of CDK9 and Cyclin T1, and when recruited by Tat to TAR RNA, CDK9 was proposed to phosphorylate the carboxyl terminal domain of RNAP II and thereby activate elongation. Research in the past two decades has shown that the mechanism of Tat action is considerably more complicated than this simple model. In metabolically active cells, CDK9 and Cyclin T1 are now known to be largely sequestered in a RNA-protein complex termed the 7SK RNP. CDK9 and Cyclin T1 are released from the 7SK RNP by mechanisms not yet fully elucidated and along with Tat, bind to TAR RNA and orchestrate the assembly of a Super Elongation Complex (SEC) containing several additional proteins. CDK9 in the SEC then phosphorylates multiple substrates in the RNAP II complex to activate elongation. Importantly for therapeutic strategies, CDK9 and Cyclin T1 function are down-regulated in resting $CD4^+$ T cells that harbor latent HIV-1, and their up-regulation is required for reactivation of latent virus. Current strategies for a functional cure of HIV-1 infection therefore are likely to require development of latency reversal agents that up-regulate CDK9 and Cyclin T1 function in resting $CD4^+$ T cells.

Keywords

HIV-1; Tat; P-TEFb; CDK9; Cyclin T1; HIV latency

Following HIV-1 entry into the host cell, the viral genomic RNA is reversed transcribed into cDNA and integrated into a cellular chromosome. RNA polymerase II (RNAP II) is then recruited to the HIV-1 promoter located in the viral 5′ Long Terminal Repeat (LTR) sequences and the viral proviral genome can be transcribed back into genomic RNA. Over 30 years ago, it was observed that expression of an HIV-1 LTR reporter plasmid was nearly 1,000-fold higher in HIV-infected cells than in control cells[1]. This potent stimulatory activity of viral gene expression is due to the activity of the HIV-1 Tat protein. There has been intense research over the past three decades into the mechanism of action of Tat and its

Conflicts of Interest

The author has no conflicts of interest.

importance to HIV-1 replication. The first decade of this research was rather contentious as several distinct mechanisms of Tat action were proposed. After this initial decade, however, a consensus emerged about the general mechanism whereby Tat stimulates viral gene expression. Research conducted in the past two decades has elucidated in increasingly greater detail the mechanisms involved in Tat action. This review will first describe studies that made key contributions to our understanding of Tat. As these mechanistic studies were being conducted, it was also observed that Tat function and therefore HIV-1 replication are regulated in $CD4^+$ T cells and monocytes/macrophages – the two predominant cell types infected by HIV-1 *in vivo*. These studies of the regulation of Tat function will be reviewed, and we will end with a discussion of the significance of this regulation to current efforts to develop a functional cure for infection.

Mechanism of Tat activation of HIV-1 gene expression

The cis-regulatory sequences in the viral LTR that respond to Tat were mapped to sequences downstream from the site of RNAP II transcriptional initiation[2], and the major function of Tat was found to stimulate transcriptional elongation rather than initiation[3]. Using a genetic approach, the cis-regulatory sequences that respond to Tat were shown to function *in vivo* as a stem-loop RNA element encompassing nucleotides +1 to +59, and this RNA element is termed TAR RNA[4]. Biochemical experiments subsequently showed that Tat binds directly to TAR RNA *in vitro*[5], and this binding *in vivo* requires a cellular TAR RNA loop-binding factor[6].

After the step in gene expression that is most strongly activated by Tat was elucidated – RNAP II elongation via Tat binding to TAR RNA – the key issue became the identification of cellular factors that mediate this function of Tat. A key finding was that a recombinant Tat protein specifically bound *in vitro* to a cellular protein kinase activity that was termed TAK for Tat-associated kinase[7]. TAK was subsequently shown to hyperphosphorylate the carboxyl terminal domain (CTD) of RNAP II, a pattern of phosphorylation implicated in activation of RNAP II elongation[8]. An analysis of multiple wild type and mutant Tat proteins demonstrated that Tat binding to TAK *in vitro* is strictly correlated with Tat function *in vivo*[8]. Shortly after the publications describing TAK, it was shown that TAK is identical to a general RNAP II elongation factor known as P-TEFb[9], and the catalytic subunit of TAK/P-TEFb was identified as CDK9 (originally named PITALRE), a member of the cyclin–dependent protein kinase family[9,10]. The cyclin regulatory subunit of CDK9 was then identified as Cyclin T1[11,12], which was shown to be the previously genetically defined factor required for Tat binding to TAR RNA *in vivo*[6]. This cellular kinase composed of Cyclin T1 and CDK9 that mediates Tat function is now termed P-TEFb.

Mechanism of Tat activation found to be complex

The studies described above led to a simple model which proposed that Tat merely recruits CDK9 and Cyclin T1 to TAR RNA at the 5′ end viral transcripts, whereupon CDK9 phosphorylates the CTD of RNAP II and elongation is stimulated. However, work from numerous laboratories over the past two decades has revealed that the mechanism of Tat action is much more complex than this simple model. Research in the transcription field

identified two multiprotein complexes termed NELF and DSIF that associate with the RNAP II complex and function to inhibit elongation[13]. Studies in the HIV-1 system found that when P-TEFb and Tat are recruited to the TAR RNA element, CDK9 phosphorylates subunits of both the NELF and DSIF complexes, as well as the CTD of RNAP II, resulting in a potent activation of transcriptional elongation[14,15].

Biochemical studies further revealed that P-TEFb exists in three distinct complexes. The first complex is "Core P-TEFb" and it is composed of CDK9, CyclinT1, and Brd4; Brd4 contains two bromodomains which bind to histone acetyl-lysine residues in active chromatin. The second P-TEFb complex is the "Super Elongation Complex" (SEC) composed of CDK9, Cyclin T1, ELL1/ELL2, AFF4, and ENL/AF9 [reviewed in[16]]. The third P-TEFb complex is the "7SK RNP" and it is composed of 7SK RNA, CDK9, Cyclin T1, HEXIM1/2, LARP7, and MEPCE; CDK9 is not catalytically active in the 7SK RNP [reviewed in[17]].

Tat can utilize CDK9/Cyclin T1 in each of these three P-TEFb complexes by binding directly to CDK9 and Cyclin T1. Tat captures the Core P-TEFb complex by displacing Brd4 from CDK9/Cyclin T1[18]. Tat participates in assembly of the SEC at TAR RNA[19,20], and Tat can extract active CDK9/Cyclin T1 from the 7SK RNP[21–23]. Recent evidence suggests that the 7SK RNP is recruited to the HIV-1 promoter and following transcription of TAR RNA, Tat extracts Cyclin T1/CDK9 from the RNP through binding to TAR RNA[24,25]. A current model of Tat activation of HIV-1 LTR-directed transcriptional elongation is shown in Figure 1. As shown in this model, cis-regulatory elements in the viral LTR direct binding of the RNAP II complex; these regulatory elements include binding sites for NF-κB, NFAT (not shown), and Sp1.

Regulation of P-TEFb in cells relevant to HIV-1 infection

In primary resting $CD4^+$ T cells, it was observed that Cyclin T1 protein levels are low and cellular activation up-regulates Cyclin T1 by a post-transcriptional mechanism[26–28]. Four miRNAs are expressed at high levels in resting $CD4^+$ T cells and appear to repress translation of Cyclin T1 mRNA in these cells: miR-27b, miR-29b, miR-150, and miR-223[29]. Notably, agents that minimally activate resting $CD4^+$ T cells, such as a combination of cytokines (TNF-α, IL-2, IL-6) or the PKC agonist prostratin, up-regulate Cyclin T1 and CDK9 kinase activity without inducing T cell proliferation or T cell activation markers[26,28]. This point is highly relevant to HIV cure research and is discussed below.

Unlike Cyclin T1, CDK9 is generally expressed at a high basal level in resting $CD4^+$ T cells[26]. However, phosphorylation of threonine 186 in the T-loop of CDK9, a post-translational modification essential for kinase activity, is low to absent in these cells[30]. Upon T cell activation, there is a rapid phosphorylation of the CDK9 T-loop[30] and CDK7 appears to be the kinase responsible for this phosphorylation[31]. The phosphatase PPM1A regulates T-loop dephosphorylation in $CD4^+$ T cells, as siRNA depletion of PPM1A in resting $CD4^+$ T cells leads to elevated T-loop phosphorylation and enhanced HIV-1 gene expression[32]. Three other phosphatases are capable of dephosphorylating the CDK9 T-loop in transformed cell lines: PP2B, PP1α, PPM1G[25,33]. The relative importance of these three CDK9 T-loop phosphatases in primary $CD4^+$ T cells is not known.

In monocytes isolated from healthy blood donors, Cyclin T1 levels are low and are up-regulated as the cells differentiate to macrophages[34,35]. MiR-198 has been identified as a miRNA that can repress Cyclin T1 protein expression in monocytes[36]. Similar to resting $CD4^+$ T cells, CDK9 T-loop phosphorylation is low in monocytes and is up-regulated during macrophage differentiation[35]. Upon extended culture time of primary macrophages, the Cyclin T1 protein that was induced during differentiation is down-regulated by proteasome-mediated proteolysis[34], and this down-regulation can be accelerated by IL-10[37]. After its shut-off in late-differentiated macrophages, Cyclin T1 can be re-induced through post-transcriptional mechanisms by pathogen-associated molecular patterns (PAMPs) or HIV-1 infection[38,39].

The 7SK P-TEFb complex is present at very low levels in resting $CD4^+$ T cells and monocytes, reflecting the low levels Cyclin T1 and CDK9 T-loop phosphorylation in these cells[26,40–42]. Following T cell activation or macrophage differentiation, there is a large increase in the level of the 7SK P-TEFb complex[26,41,42]. Signaling pathways have been identified that when activated cause release of Core P-TEFb from the 7SK RNP in metabolically active cells, with the subsequent activation of HIV-1 proviral transcription. T-cell receptor stimulation of Jurkat $CD4^+$ T cell leads to dissociation of CDK9 and Cyclin T1 from the 7SK RNP and reactivation of a latent HIV-1 provirus[43]. Activation of PKC can also lead to dissociation of Core P-TEFb and activation of proviral transcription[44].

Tat function and a functional HIV cure

Current combination anti-retroviral therapy (cART) is effective in suppressing HIV-1 replication in individuals with access to cART. However, a number of toxicities are associated with cART, including hyperlipidemia, bone loss, cardiovascular complications, and neuropathy. When cART is discontinued, a latent viral reservoir spontaneously reactivates in the vast majority of infected individuals and they must resume cART. Therefore, cART is currently required for the lifetime of infected individuals. As a consequence of these issues with cART, there is a major effort to develop therapeutic strategies to disrupt the latent viral reservoir and enhance the immune system's ability to clear HIV-1. It is hoped that a strategy, termed "shock and kill," can lead to a functional cure of infection. It has been proposed that latency reversal agents (LRAs) can be developed to "shock" latent HIV-1 to become transcriptionally active and therefore begin to express viral antigens. The "kill" involves methods to enhance the immune system's ability to attack HIV-infected cells, perhaps the result of a therapeutic vaccine, which in combination with cART can clear reactivated virus. It is hoped that this strategy will reduce the size of the reservoir, if not eliminate it, and boost the immune system of infected individuals so that viral replication can be contained without the need for continued cART, resulting in a functional cure of infection.

The best understood component of the latent reservoir is that of memory $CD4^+$ T cells that contain a transcriptionally silent but replication-competent provirus. As these latent viruses do not express viral RNA or protein, they are invisible to the immune system. As described above, P-TEFb and therefore Tat function are down-regulated in resting $CD4^+$ T cells. Furthermore, it has been shown in a primary $CD4^+$ T cell model of HIV-1 latency that P-

TEFb must be up-regulated for reactivation of latent virus[45]. Therefore, the “shock” component of a shock and kill strategy will require the induction of P-TEFb.

It is critical that LRAs which induce P-TEFb and other cellular factors required for viral reactivation do not lead to generalized immune activation. Several cellular factors have been shown to function as signal-depedent activators for the initiation of RNAP II transcription of the viral LTR: NF-κB, NFAT, STAT5, and AP-1[46]. LRAs capable of inducing these factors with only minimal effects on immune activation will be valuabe for the “shock,” as an increase in RNAP II initiation will contribute of high levels of viral reactivation if P-TEFb function can also be induce. An induction of P-TEFB without generalized immune activation is likely to be achievable as a combination of cytokines (TNF-α, IL-2, IL-6) or the PKC agonist prostratin have been shown to induce P-TEFb in resting $CD4^+$ T cells without inducing the T cell activation marker CD25 or cellular proliferation[26,28]. A synthetic prostratin derivative has been developed that is potent in reactivating latent HIV-1 in patient cells *ex vivo* and does not induce significant levels of CD25[47]. Additional PKC agonists termed Ingenol esters have been shown to up-regulated P-TEFb and reactivate latent HIV-1 in patient cells *ex vivo*[48–50].

An important component of the LRA “shock” to latent HIV-1 is an activity that can relieve repressive chromatin, such as LRAs that function as histone deacetylase inhibitors (HDACis)[51]. Interestingly, broad spectrum HDACis (vorinostat and panobinostat) that reactivate latent HIV-1 through relief of repressive chromatin also up-regulate P-TEFb through increased levels of T-loop phosphorylation, suggesting that some HDACis have multiple mechanisms of action as LRAs[52,53].

Going forward, high-throughput screens for small molecules that up-regulate P-TEFb in resting $CD4^+$ T cells can be used to develop novel LRAs. Microscopy-based assays with antisera against Cyclin T1 and the CDK9 T-loop have potential as robust assays for such screens. Additionally, release of Core P-TEFb from the 7SK RNP contributes to an increase in RNAP II elongation of the HIV-1 proviruses, and therefore small molecules that stimulate this release have potential as LRAs.

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Abbreviations

cART	combination anti-retroviral therapy
cDNA	complementary DNA
CTD	carboxyl terminal domain
DSIF	DRB sensitivity inducing factor
HDACi	histone deacetylase inhibitor

HIV-1	human immunodeficiency virus type 1
LRA	latency reversing agent
LTR	long terminal repeat
NELF	negative elongation factor
P-TEFb	positive transcriptional elongation factor b
RNAP II	RNA Polymerase II
RNP	ribonucleoprotein
SEC	super elongation complex

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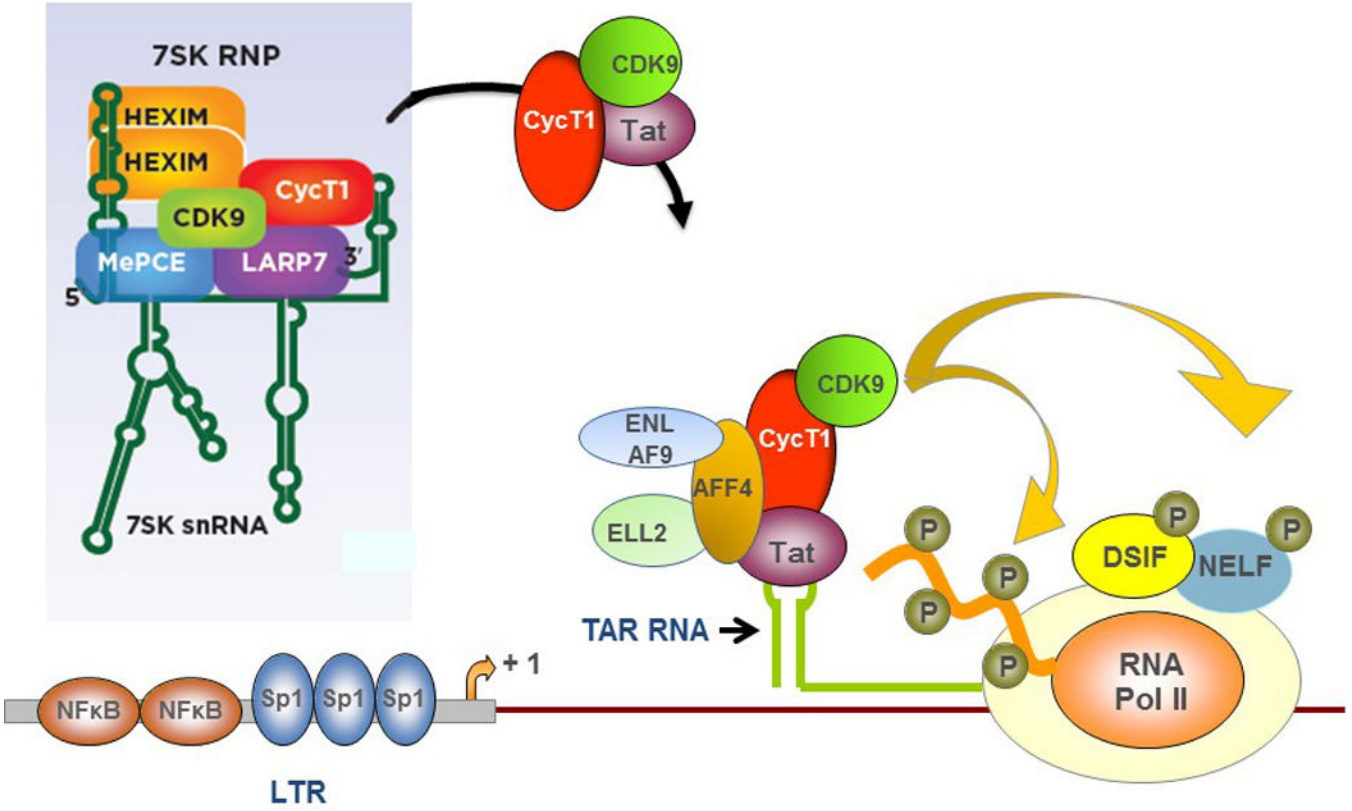

Figure 1. Mechanism of action of Tat

Cis-regulatory elements (e.g., NF-κB, Sp1) in the HIV-1 LTR direct binding of RNAP II to the viral promoter, transcriptional initiation occures, and TAR RNA at the 5′ end of the nascent viral transcript is produced. Tat binds to TAR RNA along with Cyclin T1 and CDK9 and this orchestrates the assembly of the Super Elongation Complex consisting of ENL/AF9/ELL2/AFF4. CDK9 phosphorylates subunits of DSIF and NELF and thereby overcomes their inhibition of RNAP II elongation. The CTD of RNAP II is hyperphosphorylated by CDK9. Cyclin T1 and CDK9 can be extracted from the 7SK P-TEFb RNP by Tat as indicated.