Dominant negative mutant cyclin T1 proteins that inhibit HIV transcription by forming a kinase inactive complex with Tat

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Transcription of the human immunodeficiency virus type 1 (HIV) requires the interaction of the cyclin T1 (CycT1) subunit of a host cellular factor, the positive transcription elongation factor b (P-TEFb), with the viral Tat protein, at the transactivation response element (TAR) of nascent transcripts. Because of this virus-specific interaction, CycT1 may potentially serve as a target for the development of anti-HIV therapies. Here we report the development of a mutant CycT1 protein, containing three threonine-to-alanine substitutions in the linker region between two of the cyclin boxes, which displays a potent dominant negative effect on HIV transcription. Investigation into the inhibitory mechanism revealed that this mutant CycT1 interacted with Tat and the cyclin-dependent kinase 9 (Cdk9) subunit of P-TEFb, but failed to stimulate the Cdk9 kinase activity critical for elongation. This mutant CycT1 protein may represent a novel class of specific inhibitors of HIV transcription which could lead to development of new antiviral therapies.

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The transcription of the human immunodeficiency virus type 1 (HIV) is a highly regulated process in which several host cellular co-factors and the viral transactivator protein, Tat, are involved (Karn, 1999; Taube et al., 1999). Tat stimulates the elongation of transcription with the aid of the positive transcription elongation factor b (P-TEFb). The active form of P-TEFb is a heterodimer comprising cyclin T1 (CycT1) and cyclin-dependent kinase 9 (Cdk9) (Peterlin & Price, 2006). Tat and CycT1 bind to the transactivation response element (TAR), an RNA stem–loop structure located at the 5′ end (+1 to +59) of all HIV transcripts (Bieniasz et al., 1998; Fujinaga et al., 1999; Garber et al., 1998b). This interaction results in the recruitment of Cdk9 and the subsequent stimulation of its kinase activity by Tat (Kim et al., 2002). Among 726 amino acids, the first 272 amino acids of CycT1 that form the cyclin box repeats are sufficient for HIV transcription, since these residues are responsible for the interactions with Tat, TAR and Cdk9 (Bieniasz et al., 1998, 1999; Fujinaga et al., 1999; Garber et al., 1998a, b; Ivanov et al., 1999).

Since P-TEFb is the essential host cellular co-factor for Tat, it serves as a potential target for anti-HIV therapeutics. Several approaches have been taken to block HIV transcription by targeting P-TEFb (Richter & Palu, 2006), which include the use of small compounds or mutant proteins that inhibit Cdk9 kinase activity (Chao et al., 2000; Fujinaga et al., 2002; Heredia et al., 2005; Mancebo et al., 1997) or disrupt the interaction between Tat, TAR and CycT1 (Hwang et al., 2003; Lind et al., 2002; Mischietti et al., 2001; Okamoto et al., 2000), using intrabodies against CycT1 (Bai et al., 2003), or oligomerization chain reaction to inactivate Cdk9 (Napolitano et al., 2003). It is important to note, however, that since P-TEFb is involved in the transcription of many cellular genes (Chao & Price, 2001), it is critical to exclusively block HIV-specific pathway(s) in order to develop safe and effective anti-HIV therapies.

Construction of mutant cyclin proteins that show dominant negative effects against endogenous cyclin–Cdk complexes has been quite challenging. This is presumably because the precise mechanism by which these cyclins activate the kinase activity of their corresponding Cdks is not fully understood. In order to clarify the structure–function relationship of CycT1, which is critical for development of effective dominant negative CycT1 mutants, we have previously determined the crystal structure of the cyclin box region of CycT1 (Anand et al., 2007). The resultant three-dimensional structure indicated that CycT1 shares structural similarities with other cyclins.
(Anand et al., 2007). These results suggest that certain functional motifs are well-conserved among cyclin molecules, although there is wide sequence diversity among them. Previously, Diehl et al. have demonstrated that a threonine residue (T156) at the C-terminal end of the linker region between two of the cyclin boxes of CycD1 is critical for nuclear import and activation of Cdk4 (Diehl & Sherr, 1997). Mutation of this threonine to alanine resulted in a strong dominant negative blockage of nuclear import and phosphorylation of Cdk4 (Diehl & Sherr, 1997). In CycT1, there are three threonines (T143, T149 and T155) that are very well conserved between CycT1 and T2, at the corresponding region (a linker region between two cyclin boxes). We therefore mutated each of these threonines to alamines either singly or in combination in the expression plasmid encoding the N-terminal 280 amino acids of CycT1, which are sufficient for supporting HIV transactivation. The constructs CycT1-280 (T143A), CycT1-280(T149A), CycT1-280 (T155A) and CycT1-280(T143, 149, 155A) were first tested for their ability to support Tat-transactivation (Fig. 1a). The plasmids encoding wild-type (wt) or mutant CycT1 were co-transfected with HIV-LTR-Luciferase (Luc) reporter plasmids and Tat in NIH 3T3 cells. As previously reported, Tat alone exhibited a modest transactivation of HIV-LTR Luc in these cells, due to the inability of the murine endogenous CycT1 to interact with Tat/TAR (Bieniasz et al., 1998; Fujinaga et al., 1999; Garber et al., 1998a) (Fig. 1b, lane 2). CycT1 proteins containing a single mutation showed levels almost equal to the wild-type level of Tat-transactivation (Fig. 1b, lanes 4–6). In sharp contrast, the triple mutant CycT1-280 (T143, 149, 155A) was unable to support Tat-transactivation (Fig. 1b, lane 7). All mutant proteins were expressed at similar levels as the wt CycT1, as assessed by Western blot analysis (Fig. 1d for the expression of CycT1-280 (T143, 149, 155A]).

Next, the dominant negative effect of CycT1-280 (T143, 149, 155A) was examined by transient overexpression in the presence of Tat in HeLa/HR-Luc cells that contain chromosomal HIV-LTR-Luc reporter genes introduced by lentiviral vector (Kim et al., 2006). An increasing amount of CycT1-280 (T143, 149, 155A) proteins inhibited HIV-Luc reporter gene expression (up to ~85%) in the presence of Tat in HeLa cells (Fig. 1c, lanes 3–5). Overexpression of CycT1-280 (T143, 149, 155A) proteins inhibited neither the basal HIV transcription nor the transcription from the CMV promoter, which indicates that the dominant negative effect of CycT1-280 (T143, 149, 155A) is also highly specific for Tat-transactivation (Supplementary Fig. S1, available in JGV Online). HeLa/HR-Luc cells stably expressing CycT1-280 (T143, 149, 155A) were also established using a pHr- lentiviral vector and employed in titration of Tat activity. In these mutant-expressing cells, Tat showed a lower activity on HIV transcription than in HeLa/HR-Luc cells stably carrying the empty lentiviral vector (Fig. S1, available in JGV Online). HeLa/HR-Luc cells at a similar level to wild-type, as determined by Western blot analysis using anti-HA antibodies. (e) Tat has lower activity in cells stably expressing CycT1-280 (T143,149,155A). Upper panel: an increasing amount of Tat-expression plasmid was transiently transfected into HeLa/HR-Luc cells stably carrying a lentiviral vector encoding no protein (empty vector: closed circles) or HA-CycT1-280 (T143, 149, 155A: open circles). Lower panel: HA-CycT1-280 (T143, 149, 155A) proteins are detected in the lysate of the stable cells (‘mutCycT1’), but not in the control cell lysates (‘vector’), as seen by Western blot analysis using anti-HA antibody.

Fig. 1. Mutant CycT1 proteins containing triple T-to-A mutations in the N-terminal region also block HIV transactivation. (a) A schematic representation of the mutant CycT1-280 (T143A), CycT1-280 (T149A), CycT1-280 (T155A) and CycT1-280(T143, 149, 155A) were first tested for their ability to support Tat-transactivation (Fig. 1a). The plasmids encoding wild-type (wt) or mutant CycT1 were co-transfected with HIV-LTR-Luciferase (Luc) reporter plasmids and Tat in NIH 3T3 cells. As previously reported, Tat alone exhibited a modest transactivation of HIV-LTR Luc in these cells, due to the inability of the murine endogenous CycT1 to interact with Tat/TAR (Bieniasz et al., 1998; Fujinaga et al., 1999; Garber et al., 1998a) (Fig. 1b, lane 2). CycT1 proteins containing a single mutation showed levels almost equal to the wild-type level of Tat-transactivation (Fig. 1b, lanes 4–6). In sharp contrast, the triple mutant CycT1-280 (T143, 149, 155A) was unable to support Tat-transactivation (Fig. 1b, lane 7). All mutant proteins were expressed at similar levels as the wt CycT1, as assessed by Western blot analysis (Fig. 1d for the expression of CycT1-280 (T143, 149, 155A)].

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Our previous studies indicated that mutant CycT1 proteins defective in interacting with Tat or Cdk9 showed only a small dominant negative effect on HIV transcription (Fujinaga et al., 2002). Therefore, we next tested whether this CycT1-280 (T143, 149, 155A) protein was able to interact efficiently with Tat or Cdk9. HA-tagged CycT1 proteins were co-expressed with myc-tagged Tat in 293T cells. The wt and mutant CycT1 proteins were immunoprecipitated with anti-HA antibodies. Tat and the
endogenous Cdk9 proteins associated with HA-CycT1 were detected by Western blot analysis using anti-myc and anti-Cdk9 antibodies, respectively. As shown in Fig. 2(a), CycT1-280 (T143, 149, 155A) retained the same ability to interact with Cdk9 and Tat as wt CycT1. Recent work has demonstrated that approximately 50% of P-TEFb molecules in HeLa cells are transcriptionally inactive due to an association with cellular HEXIM1 proteins and 7SK small nuclear RNA (snRNA) (Nguyen et al., 2001; Yang et al., 2001). Therefore, we subsequently examined whether the truncated wt and mutant CycT1 proteins can associate with HEXIM1 by co-immunoprecipitation. As shown in Fig. 2(b), neither wt nor mutant CycT1 (CycT1-280) proteins interacted with HEXIM1, whereas the full-length protein did associate with HEXIM1 in the presence of Tat. These results are despite the fact that it is the N-terminal region which contains the HEXIM1-binding domain (Michels et al., 2003). This is presumably because Tat can compete with HEXIM1 for CycT1 binding, as has recently been demonstrated (Barboric et al., 2007; Schulte et al., 2005). Indeed, the interaction between CycT1-280 and HEXIM1 was observed in the absence of Tat, and co-expression of Tat diminished this interaction (Supplementary Fig. S2). Interestingly, when the mutant CycT1-280 (T143, 149, 155A) proteins were co-expressed, the amount of endogenous CycT1 associated with Tat was significantly decreased as examined by co-immunoprecipitation assays (Fig. 2c, Western blot: α-CycT1 and α-HA, lanes 1 and 2), indicating that the mutant CycT1 can efficiently form a complex with Tat by competing with the endogenous CycT1. These results indicate that use of the truncated CycT1 is advantageous since these proteins are not segregated by 7SK/HEXIM1, and therefore remain as a heterodimer with Cdk9, which can preferentially interact with Tat (Barboric et al., 2007; Dames et al., 2007; Schulte et al., 2005). Since CycT1 (1–280) proteins do not interact with 7SK snRNA and HEXIM1 proteins in the presence of Tat, it can bypass the 7SK/HEXIM-mediated complex regulatory pathway and be exclusively directed towards Tat-dependent transactivation. This makes CycT1(1–280) proteins highly specific to Tat. Indeed, the full-length version of these mutant CycT1 proteins exhibited less potent dominant negative effect on HIV transcription (data not shown), which may potentially provide important insights into the mechanism of 7SK/HEXIM1-mediated P-TEFb regulation. Finally, the Tat-associated kinase assays revealed that the kinase complex associated with the mutant CycT1-280 (T143, 149, 155A) had a lower CTD-kinase activity (Fig. 2c, lower panel, lane 2), although a similar amount of the endogenous Cdk9 associated with Tat (Fig. 2c, Western blot: anti-Cdk9, lanes 1 and 2). These results indicate that the mutant CycT1-280 (T143, 149, 155A) proteins can interact with Tat and Cdk9 just as wt, without HEXIM1-interaction, and block HIV-transcription by forming a kinase-inactive complex. A computer simulation of the predicted conformational change of the cyclin boxes by these mutations was performed using the 3D-Jigsaw program (Fig. 3). A shift of the region between Glu 95 and Glu 102, which is important for the interaction with Cdk9, is observed in the mutant CycT1 (Fig. 3). This prediction indicates that these mutations may have an impact on the conformation of the Cdk9-binding domain of CycT1 necessary for stimulation of Cdk9 kinase activity.

HIV utilizes cellular transcriptional machinery for its own replication. Therefore, it is important to specifically inhibit this step without disturbing cellular functions. Given the interaction of CycT1 with Tat and TAR, it holds promise as an excellent target for the development of safe and effective anti-HIV therapies. Here we present the first example of a dominant negative CycT1 molecule which specifically blocks HIV transcription. Studying the precise mechanism by which certain mutant CycT1 proteins can inhibit HIV transcription may unveil novel regulatory pathway(s) of
Recruitment of a protein complex containing Tat and cyclin T1 to the HIV life cycle and therefore provide valuable insight for designing anti-HIV agents.

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