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FAST TRACK

Distinct Regions of CyclinT1 are Required for Binding to CDK9 and for Recruitment to the HIV-1 Tat/TAR Complex

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Abstract Tat-mediated activation of the HIV-1 promoter activity requires Tat-dependent recruitment of the cyclinT1/CDK9 complex (P-TEFb) to the transacting element (TAR) RNA. Tat interaction with the cyclinT1, the regulatory partner of CDK9, results in a specific recruitment of the heterodimer CycT1/CDK9 complex to TAR, whereby it promotes transcription elongation of the HIV-1 LTR-mediated transcription. Using the yeast two-hybrid protein interaction assay we analyzed the binding between cyclinT1 and CDK9. Moreover, using a modified three-hybrid yeast interaction system, we analyzed the recruitment of CycT1 to the Tat/TAR complex. The data presented here demonstrated that distinct domains of cyclinT1 interact with CDK9 and Tat/TAR in vivo. These findings will be instrumental for the designing of proper dominant-negative P-TEFb components capable to interfere with Tat function. J. Cell. Biochem. Suppl. 36:247–253, 2001. © 2001 Wiley-Liss, Inc.

Key words: CycT1-CDK9 binding; CycT1-Tat/TAR complex; P-TEFb; cyclin box

The virally encoded transactivator Tat is essential for human immunodeficiency virus type 1 (HIV-1) gene expression, and it is required for virus replication. Tat plays a key role in the regulation of viral gene expression by binding to the HIV-1 RNA at the trans-activating response (TAR) region [Jones and Peterlin, 1994]. HIV-1 Tat is a unique activator because it is recruited to the transcription complex by binding to nascent RNA, rather than to promoter DNA, and it has been reported that Tat almost exclusively stimulates chain elongation [Jones and Peterlin, 1994; Jones, 1997; Cullen, 1998]. Although the Tat protein is able to transactivate the HIV-1 promoter in the absence of any viral encoded protein, the enhancement of processivity brought about by Tat requires at least one cellular co-factor. Considerable evidences exhibit that Tat interacts with cellular proteins and that such interactions are involved in its transactivation function [Jones and Peterlin, 1994]. Tat contains two regions that are important for its function: an arginine-rich region that mediates the binding to TAR RNA and an activation domain that mediates the interaction with cellular co-factors. A number of cellular factors that include P-TEFb [Zhu et al., 1997], TIP30 [Xiao et al., 1998], the co-activators p300/CBP, and PCAF [Benkirane et al., 1998; Marzio et al., 1998], have been found to interact with Tat and to potentiate transcription from the HIV-1 LTR. The positively acting elongation factor P-TEFb has been proposed to stimulate transcription elongation of RNAPII transcripts [reviewed in Price, 2000] and is specifically required for Tat transactivation [Mancebo et al., 1997; Zhu et al., 1997; Fujinaga et al., 1998; Zhou et al., 1998; Flores et al., 1999]. The P-TEFb contains a catalytic subunit (CDK9) which in association with a regulatory subunit (cyclinT1) has the ability to phosphorylate the CTD of RNAPII [Price, 2000]. CyclinT1 appears to be a dedicated human-specific co-factor for Tat, as demonstrated by the inability of the mouse

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ehomologue to support Tat-mediated transcription [Bieniasz et al., 1998; Garber et al., 1998; Chen et al., 1999]. The current model for recruitment of P-TEFb to the LTR, predicts the formation of the Tat-P-TEFb complex which efficiently binds TAR, causing hyperphosphorylation of the CTD, which enhances processivity of RNAPII to produce full-length mRNAs [Jones, 1997]. Collectively, these findings demonstrated that cyclinT1 can binds simultaneously to both the catalytic partner CDK9 and Tat/TAR. However, little is known on the crucial regions on cyclinT1 responsible for these interactions in vivo.

Using the yeast protein–protein interaction assay, we analyzed the binding between cyclinT1 and CDK9. In addition, using a modified three-hybrid yeast interaction protocol, here we report an extensive analysis of the in vivo interactions between cyclinT1 and Tat/TAR. The findings presented here highlight the crucial regions of cyclinT1 responsible for binding to CDK9 and Tat/TAR, respectively.

MATERIALS AND METHODS

Plasmids

To construct the pBTM-CycT1 plasmid, the EcoRI fragment encoding the full-length CycT1 cDNA from pCMVHA-CycT1 was inserted into EcoRI site of pBTM116 plasmid [Bartel and Fields, 1995]. The pBTM-CycT1(1-290), pBTM-CycT1(1-188), pBTM-CycT1(1-133) and, pBTM-CycT1(290-726) were constructed by subcloning the corresponding CycT1 regions from pCMVHA-CycT1 using appropriate restriction enzymes [Majello et al., 1999]. The pBTM-CycT1/2xmut was obtained by inserting the EcoRI fragment containing the full-length cDNA from the pCMVHA-CycT1/2xmut into the pBTM116. The specific mutations (K93L and E96K) in CycT1wt cDNA, were generated by PCR-based mutagenesis. To construct pGAD-CDK9 the full-length CDK9 cDNA was cloned into BamHI-EcoRI sites of pGAD10 [Clontech]. The pACTII-CycT1 was constructed by inserting NcoI fragment from pBTM-CycT1 into NcoI sites of pACTII [SenGupta et al., 1996]. The pACTII-CycT1(1-290), pACTII-CycT1(1-188), pACTII-CycT1(133Δ188), pACTII-CycT1(290-726) were constructed by subcloning the corresponding regions of CycT1 from either the pCMVHA-CycT1 or GST-CycT1 in the pACTII vector. The pACTII-CycT1/2xmut was generated by inserting NcoI fragment from pCMVHA-CycT1/2xmut into NcoI site of pACTII. The pACTII-CycT2A(1-285) was constructed by cloning EcoRV-EcoRI fragment encoding the first 285 amino acids of cyclinT2A [Napolitano et al., 1999], into SmaI–EcoRI sites of pACTII. The pRS313-TAT plasmid was obtained by cloning a cDNA encoding the complete Tat protein (86 amino acids) into EcoRI site of pRS313-GAL1 [Stone and Reed, 1990]. All plasmid constructs were verified by DNA sequencing. Full details of plasmid construction are available upon request.

Yeast Strains

L40 yeast strain was used in the two-hybrid assays. The strain is auxotroph for tryptophan, leucine, and histidine and contains HIS3 and LacZ reporter gene under control of LexA binding sites; the genotype is *MATa, ura3-52, leu2-3,112, his3Δ200, trp1Δ1, ade2, LYS2::(LexA op)$_4$-HIS3, ura3::(LexA-op)$_8$-LacZ*. A L40 derived yeast strain, called L40-coat [SenGupta et al., 1996], was used in the modified three-hybrid assays. The strain isn't auxotroph for tryptophan and carries an integrated copy of gene encoding the LexA DNA-binding domain fused to bacteriophage MS2 coat protein; the genotype is *MATa, ura3-52, leu2-3,112, his3Δ200, trp1Δ1, ade2, LYS2::(LexA op)$_4$-HIS3, ura3::(LexA-op)$_8$-LacZ, LexA-MS2 coat (TRP1)*.

Yeast Two- and Three-Hybrid Methods

The yeast two-hybrid assays were performed as follow. L40 yeast cells were co-transformed with pBTM-CycT1 (and mutants thereof), and pGAD-CDK9 plasmids and selected on media lacking tryptophan and leucine. Positive interactions were validated on media selecting for expression of HIS3 reporter gene (SD lacking tryptophan, leucine, and histidine), and by β-galactosidase activity. To analyze the binding between cyclinT1 and Tat/TAR a modified three-hybrid yeast interaction protocol was employed essentially as previously described [SenGupta et al., 1996; Bieniasz et al., 1998]. Briefly, L40-coat yeast cells were co-transformed with pIIIA/TAR-MS2, pRS313-TAT, and pACTII-CycT1 (and mutants thereof) plasmids. Transformants were selected on media lacking uracil, histidine, and leucine and the colonies assayed for β-galactosidase activity either by a filter β-gal assay or by quantitative liquid assay.

RESULTS

The N-Terminus 188 Amino Acids of CycT1 are Necessary and Sufficient to Bind CDK9 In Vivo

The P-TEFb complex is composed by the catalytic unit CDK9, which can be found associated with several regulatory partners: cyclinT1, T2A and T2B and K [Peng et al., 1998; Fu et al., 1999]. However, only the CDK9/CycT1 complex interacts with the viral transactivator Tat protein [Bieniasz et al., 1998; Garber et al., 1998]. Tat interaction with CycT1/CDK9 complex occurs through direct binding between Tat and cyclinT1. Consequently, cyclinT1 is able to interact with both Tat and CDK9. To gain more insights on the crucial regions of cyclinT1 responsible for interaction with both CDK9 and Tat, we performed an extensive analysis of the in vivo interactions using the yeast two and three hybrid systems.

First, using the yeast two-hybrid system we analyzed the binding between cyclinT1 and CDK9. We used the pBTM116 vector [Bartel and Fields, 1995], carrying a *TRP1* marker, for expression of LexA DNA-binding domain, and the pGAD10 vector, carrying the *LEU2* marker, for expression of GAL4 activation domain. The CDK9 full-length cDNA was cloned into pGAD10 to generate plasmid expressing the GAL4 AD-CDK9 fusion protein. The CycT1 full-length cDNA and a series of mutants were cloned into pBTM116 to generate plasmids encoding the relative LexA BD-CycT1 fusion proteins. When L40 yeast cells were co-transformed with pBTM-CycT1 and pGAD-CDK9 plasmids a significant growth on media lacking histidine and a high level of β-gal expression were detected. In contrast, control experiments in which yeast cells were transformed with either empty DNA-binding domain (pBTM116) or activation domain (pGAD10) plasmids, displayed low levels of reporter genes expression (Fig. 1). Thus, according with previous findings [Bieniasz et al., 1998; Peng et al., 1998; Wei et al., 1998] we found that CycT1 interacts efficiently with CDK9 in our two-hybrid interaction system. Next, a series of cyclinT1 mutants were tested for the ability to associate with CDK9. First, we tested the CycT1(1-290) mutant that encodes the first 290 amino acids of the cyclinT1 corresponding to the "cyclin homology box". This region is the most conserved among different members of the cyclin-family. Yeast cells co-transformed with pBTM-CycT1(1–290) and pGAD-CDK9, revealed a strong activation of the reporter genes, indicating the capacity of the "cyclin homology box" to bind CDK9. In contrast CycT1(290–726), encoding the C-terminal region of CycT1, failed to bind CDK9 (Fig. 1). To better define the minimal region within the "cyclin homology box" that can interact with CDK9, two further CycT1 mutants were assayed. When we co-transformed L40 yeast cells with pGAD-CDK9

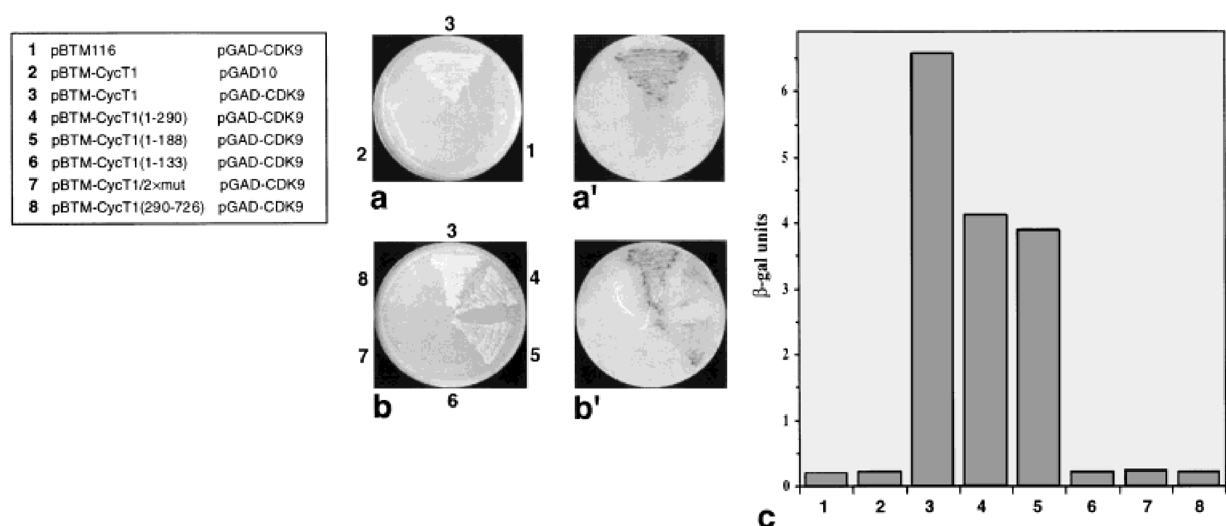

Fig. 1. Interaction of cyclinT1 proteins with CDK9 in a yeast two-hybrid assay. L40 yeast cells were co-transformed with plasmids indicated on the left and growth selection was performed as described in the text (**a** and **b**). Positive transformants were assayed for LacZ activity by filter (a′ and b′) and quantitative β-gal assays (**c**).

and either pBTM-CycT1(1–188) or pBTM-CycT1(1–133), only in the former case a detectable expression of the reporter genes was evidenced (Fig. 1). Thus, it appears that the region of cyclinT1 from amino acid 1 to 188 is necessary and sufficient to interact with CDK9 in vivo.

To further substantiate the critical role of the N-terminus 188 amino acids of cyclinT1 for CDK9 binding we tested CycT1/2xmut in the two-hybrid assay. The CycT1/2xmut contains the Lys-93 and Glu-96 substituted by Leu and Lys, respectively. In analogy with other cyclins, these two residues are predicted to be directly involved in the interaction with the kinase partner [Anderson et al., 1997]. As reported in Figure 1, we found that yeast cells carrying pGAD-CDK9 and pBTM-CycT1/2xmut plasmids displayed no detectable expression of the reporter genes, indicating that CycT1/2xmut has lost the ability to bind CDK9 in vivo. A similar result has been recently documented by others [Bieniasz et al., 1999].

In Vivo Interaction Between CycT1 and Tat/TAR Requires the Integrity of the "Cyclin Homology-Box"

The ability of CycT1 to bind Tat protein has been previously reported [Bieniasz et al., 1998; Wei et al., 1998] and it has been demonstrated that such interaction mediates Tat function by recruitment of P-TEFb complex to TAR. Using a modified yeast three-hybrid system we analyzed the in vivo binding between CycT1 and Tat/TAR complex. This approach represents the experimental system in which the interacting molecules are most likely present in contest near to physiological condition. A schematic representation of the modified three-hybrid system is presented in Figure 2. The pACTII vector [SenGupta et al., 1996] carrying a *LEU2* marker, was used for the expression of the GAL4 activation domain. CycT1 full-length cDNA and a series of CycT1 mutants were inserted in this vector to generate the relative fusion proteins. The pRS313-TAT plasmid carrying a *HIS3* marker was used for the expression of the Tat protein. The pIIIA/TAR-MS2 plasmid [SenGupta et al., 1996] carrying a *URA3* marker, was used for the expression of hybrid MS2-TAR RNA molecules. Yeast L40-coat cells contain an integrated copy of LacZ and HIS3 reporter genes positioned 3′ to LexA binding sites and also constitutively express a fusion protein consisting of the LexA DNA-binding domain fused to bacteriophage MS2 coat protein. Thus, co-transformation of L40-coat cells with plasmids expressing hybrid MS2-TAR RNA and activation domain fusion protein results in reporter gene expression only if the fusion proteins can be recruited on the TAR target (Fig. 2).

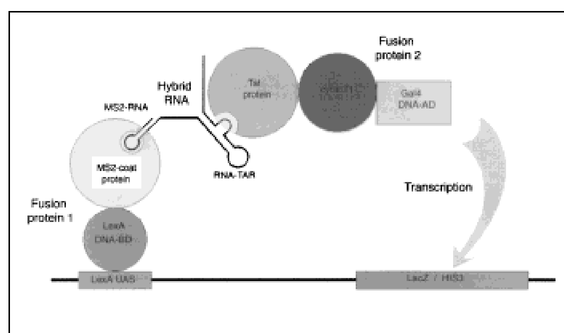

Fig. 2. Diagram of modified yeast three-hybrid system. Fusion protein 1 containing a LexA DNA-binding domain fused to an RNA-binding domain (the coat protein of bacteriophage MS2) localizes to the upstream regulatory sequences of reporter gene (LacZ/HIS3). Fusion protein 2 contains a GAL4 transcription activation domain fused to human cyclinT1. Hybrid RNA molecule consists of two MS2 RNA-binding sites linked to TAR RNA. Tat protein binds both to TAR RNA and cyclinT1; the formation of such TAR-Tat-CycT1 ternary complex brings the GAL4 activation domain in close proximity to the upstream regulatory sequences and results in detectable expression of reporter gene.

As showed in Figure 3, when L40-coat cells were co-transformed with pIIIA/TAR-MS2, pACTII-CycT1, and the empty pRS313 plasmid no activation of LacZ gene was detected. However, when the Tat expression plasmid pRS313-TAT was included in the co-transformations, high levels of β-gal expression were found, indicating the ability of CycT1 to efficiently interact with Tat/TAR element in vivo. To validate the specificity of such interaction, the pACTII-CycT2A(1–285) plasmid expressing a GAL4 AD-CycT2A(1–285) fusion protein was used. Previous results have shown that the cyclinT2A does not interact with Tat in both in vitro and in vivo interaction assays [Napolitano et al., 1999; Wimmer et al., 1999]. Accordingly with these findings we found that yeast cells co-transformed with the pACTII-CycT2A(1–285) plasmid failed to activate the LacZ reporter, thus, demonstrating the specificity of Tat-mediated recruitment of CycT1 to TAR.

To delineate the relevant regions of CycT1 required for in vivo interaction with the Tat/

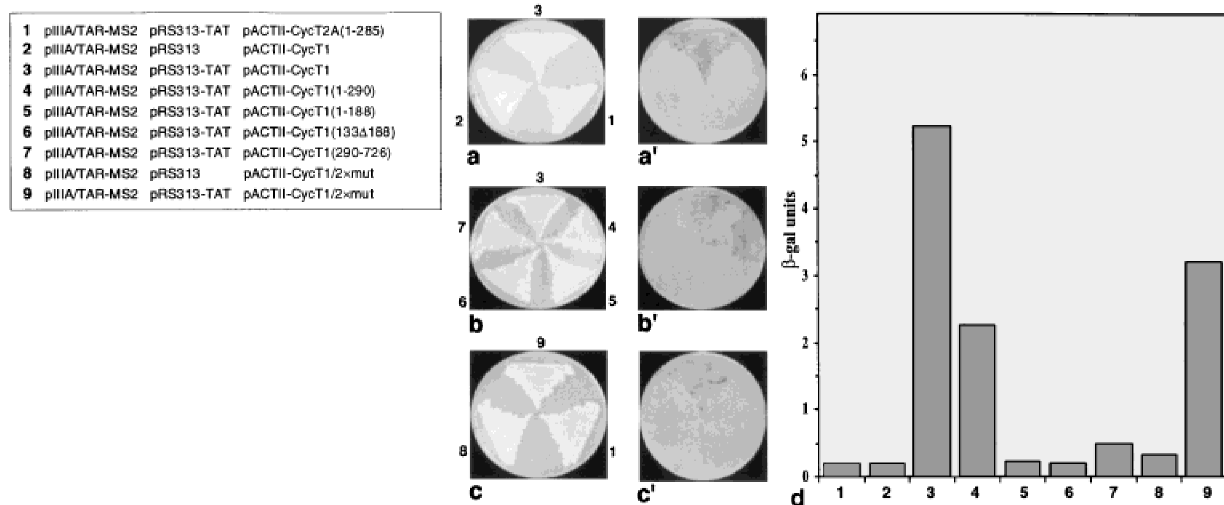

Fig. 3. Interaction of cyclinT1 proteins with Tat/TAR. L40-coat yeast cells were co-transformed with plasmids indicated on the left, and selected for the presence of these plasmids on media lacking leucine, uracil, and histidine (**a**, **b**, and **c**). The yeast triple transformants were assayed for β-galactosidase activity either by a filter β-gal assay (a′, b′, and c′), or by a liquid quantitative β-gal assay (**d**).

TAR complex a series of CycT1 mutants were tested. When L40-coat yeast cells were co-transformed with pIIIA/TAR-MS2, pRS313-TAT, and pACTII-CycT1(1–290) high levels of β-gal activity were detected, demonstrating that the "cyclin homology box" (1–290) suffices for interaction with Tat/TAR. In contrast, yeast transformants carrying pIIIA/TAR-MS2, pRS313-TAT, and pACTII-CycT1(1–188) displayed no detectable β-gal activity, indicating that the further truncation within the "box" until amino acid 188 completely abolished the ability of CycT1 to bind Tat/TAR (Fig. 3). This data is supported by previous studies that identified a Tat/TAR recognition motif (TRM) at the carboxyl-terminal edge of "cyclin homology box" between aa 254 and 272 [Garber et al., 1998]. Moreover, when we tested the ability of CycT1(133Δ188) mutant to bind Tat/TAR no significant activation or LacZ gene was detected (Fig. 3). The CycT1(133Δ188) mutant encodes the first 487 amino acids of CycT1, with a deletion of 55 amino acids between 133 and 188 residues. This mutant encodes the TRM region (aa 254–272), and because it fails to bind Tat/TAR, it appears that the integrity of the entire "cyclin box" is required for binding to Tat/TAR in vivo. Finally, CycT1/2xmut was tested for Tat/TAR binding and as reported in Figure 3, we found that L40-coat yeast cells co-transformed with pIIIA/TAR-MS2, pRS313-TAT, and pACTII-CycT1/2xmut expressed high levels of β-gal expression. Thus, these findings indicate that cyclinT1/2xmut, that is unable to interact with CDK9, retains the wild-type level of binding to Tat/TAR in vivo.

DISCUSSION

Transcription activation of the HIV-1 LTR by the Tat protein involves the recruitment of the P-TEFb complex to the viral TAR element. CyclinT1, the regulatory component of the P-TEFb complex, mediates the interaction of P-TEFb with Tat, and it has been shown that binding of CycT1 to Tat enhances the affinity and specificity of the CycT1-Tat-TAR interaction. Since the active form of P-TEFb for Tat transactivation consists of the CDK9-CycT1heterodimer, the cyclinT1 is capable to bind to both Tat/TAR complex and CDK9 simultaneously. Here we report an extensive analysis of the interactions between CycT1-CDK9 and CycT1/Tat-TAR, respectively. A summary of such interactions is showed in Figure 4.

Accordingly with previous studies we found that the "cyclin homology box" (i.e., amino acids 1–290) is both necessary and sufficient for binding in vivo to both the CDK9 partner and the Tat/TAR complex. In analogy with crystallographic data of cyclinA and cyclinH [Jeffrey et al., 1995; Anderson et al., 1997], the cyclinT1 homology-box presents a globular structure consisting of two characteristic α-helical

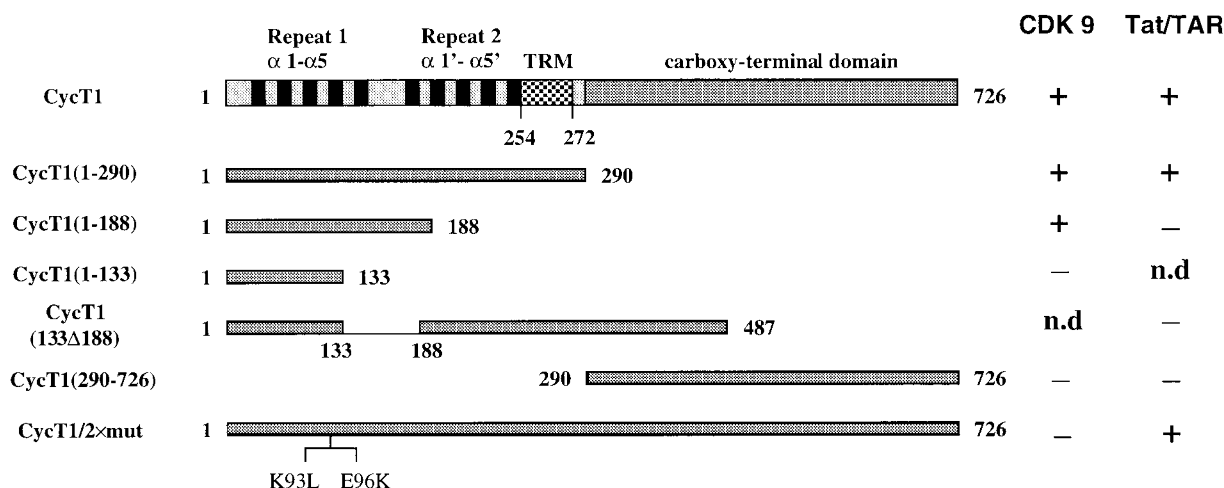

Fig. 4. Summary of the ability of cyclinT1 mutants to bind CDK9 and Tat/TAR. A schematic map of the cyclinT1 protein along with the different mutants is shown. The capability to bind to both the kinase partner CDK9 and the Tat/TAR complex is reported. Relevant regions of cyclinT1 are represented.

domains (each containing five helices) referred to as repeat 1 (α1–α5) and repeat 2 (α1′–α5′). The deletion of 102 amino acids at the carboxy-terminal edge of the "box", mutant CycT1(1–188), did not influence the binding of cyclinT1 to CDK9, but the further truncation to amino acid 133 completely abolished the binding to CDK9. The former deletion removes most of repeat 2 of the "cyclin homology box", while the latter deletion destroys also repeat 1. Taken together these findings demonstrate that the first of the two repeats of the "cyclin-homology-box" contains the crucial sites for CDK9 binding. Accordingly, we found that the Lys-93 and Glu-96 residues are essential for CDK9 recognition; in fact CycT1/2xmut is not capable to interact with CDK9. Similar results have been recently reported [Bieniasz et al., 1999]. Our results demonstrated that the "cyclin homology box" of cyclinT1 is able to bind the Tat/TAR complex, but the deletion within the "box" until amino acid 188 abolished such capacity. In fact, the TRM domain, which is critical for interaction with Tat, extends between amino acids 254 and 272, inside the deleted region. These data suggest that the cyclinT1 recognition site for Tat/TAR lies 3' to CDK9 binding sites within repeat 2, in a location that may overlap with α'5. However, CycT1(133Δ188) encodes the TRM region, but did not rescue the ability to bind Tat/TAR. Since the deletion (i.e., amino acids between 133 and 188) removes most of repeat 2 as well as the last α-helix of repeat 1, the integrity of the entire "homology-box" appears to be required for the interaction between cyclinT1 and Tat/TAR.

Finally, we found that, while CycT1/2xmut has lost the ability to interact with CDK9 it can efficiently bind Tat/TAR complex. The ability of the CycT1/2xmut to be recruited by Tat to TAR RNA target can be exploited for the designing of an efficient dominant-negative inhibitor of Tat function. Because the CycT1mut2x has completely lost the ability to associate with the catalytic partner CDK9, we postulate that enforced expression of the CycT1mut2x protein might inhibit Tat function by forming an efficient CycT1mut2x/Tat/TAR complex which is unable to recruit the CDK9 protein to the HIV-1 LTR. Consequently, over-expression of the CycT1mut2x might competes with endogenous CycT1wt in Tat/TAR association, resulting in a dominant-negative inhibition of the HIV-1 LTR transcription.

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