Recruitment of a protein complex containing Tat and cyclin T1 to TAR governs the species specificity of HIV-1 Tat

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Human cyclin T1 (hCycT1), a major subunit of the essential elongation factor P-TEFb, has been proposed to act as a cofactor for human immunodeficiency virus type 1 (HIV-1) Tat. Here, we show that murine cyclin T1 (mCycT1) binds the activation domain of HIV-1 Tat but, unlike hCycT1, cannot mediate Tat function because it cannot be recruited efficiently to TAR. In fact, overexpression of mCycT1, but not hCycT1, specifically inhibits Tat–TAR function in human cells. This discordant phenotype results from a single amino acid difference between hCycT1 and mCycT1, a tyrosine in place of a cysteine at residue 261. These data indicate that the ability of Tat to recruit CycT1/P-TEFb to TAR determines the species restriction of HIV-1 Tat function in murine cells and therefore demonstrate that this recruitment is a critical function of the Tat protein.

Keywords: cyclin/HIV-1/Tat/transcription

Introduction

The Tat proteins of the lentivirus subfamily of retroviruses are essential gene products that potently activate transcription from the viral long terminal repeat (LTR) promoter (reviewed by Jones, 1997; Cullen, 1998). Uniquely among transcriptional activators, the Tat proteins of the primate lentiviruses and some non-primate lentiviruses, including equine infectious anemia virus (EIAV), act via an RNA target (TAR) located at the 5' end of viral mRNAs. The mechanism of action of Tat has, until recently, remained entirely unclear. However, in contrast to the majority of DNA sequence-specific transcriptional activators, which synergistically enhance both initiation and elongation, Tat appears to act almost exclusively to enhance the elongation competence of initiated transcription complexes (Kao *et al.*, 1987; Feinberg *et al.*, 1991; Marciniak and Sharp, 1991; Blair *et al.*, 1996; Blau *et al.*, 1996).

Mutational analysis has demonstrated that the 86 amino acid human immunodeficiency virus type 1 (HIV-1) Tat protein contains two distinct functional domains. The first is an activation or cofactor-binding domain which comprises a cysteine-rich region, a conserved hydrophobic 'core' sequence and poorly defined residues in the N-terminal portion of the protein. The activation domain is functionally autonomous, i.e. it is an activator when

recruited to the HIV-1 LTR via a heterologous RNA-binding protein and, when expressed alone, is a dominant inhibitor of the full-length Tat protein, presumably by titrating an essential cellular cofactor(s) (Selby and Peterlin, 1990; Southgate *et al.*, 1990; Tiley *et al.*, 1992; Madore and Cullen, 1993). The second functional domain is a highly basic region that is responsible for both the nuclear localization and the TAR RNA-binding activities associated with Tat (Dingwall *et al.*, 1990; Weeks *et al.*, 1990).

HIV-1 TAR forms an RNA secondary structure composed of a single stem containing a U-rich bulge and a terminal hexanucleotide loop (Feng and Holland, 1988). While purified recombinant HIV-1 Tat can bind the TAR bulge *in vitro*, several lines of evidence indicate that the participation of an additional cellular protein(s) is essential for RNA recognition *in vivo*. Thus, although Tat–TAR binding *in vitro* is only sensitive to mutations in the bulge and upper stem of TAR, the ability of TAR to support transactivation *in vivo* requires additional sequences in the TAR loop (Feng and Holland, 1988; Roy *et al.*, 1990). Furthermore, the RNA-binding domain of Tat does not function autonomously *in vivo*; Tat-mediated recruitment of a heterologous protein to TAR in mammalian cells also requires an intact Tat activation domain (Luo *et al.*, 1993). The ability of mutant Tat proteins to inhibit transactivation by wild-type Tat is also dependent on activation domain integrity; activation domain mutants are recessive, i.e. they cannot compete for TAR binding, in contrast to RNA-binding domain mutants, which are dominant negative (Madore and Cullen, 1993). In addition, Tat activity is characterized by a marked species specificity. For example, HIV-1 Tat is a potent activator in primate cells but only poorly functional in rodent cells (Newstein *et al.*, 1990; Alonso *et al.*, 1992). However, activation can be rescued efficiently in mouse cells by recruiting Tat to the viral LTR via a heterologous RNA target (Madore and Cullen, 1993). These genetic data indicate that the HIV-1 Tat activation domain is fully functional in murine cells and suggest that the species specificity of Tat function is due to the lack of a cellular cofactor required for the recruitment of a Tat–coactivator complex to TAR. The observation that murine cells containing human chromosome 12 are partially able to support Tat function via TAR led to the suggestion that a human protein, encoded on chromosome 12, might be required for efficient recruitment of Tat to TAR (Newstein *et al.*, 1990; Alonso *et al.*, 1992).

It has been documented that the RNA polymerase II C-terminal domain (CTD) is hyperphosphorylated in elongating transcription complexes (Dahmus, 1996). This observation is of particular significance to Tat function because Tat-mediated transcriptional activation requires the CTD and is sensitive to the kinase inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) (Marciniak and

Fig. 1. Amino acid sequence comparison of human and murine CycT1. Identical amino acids are boxed and the conserved cyclin box indicated. The single residue here shown to control the ability of hCycT1 to mediate Tat function is indicated by an asterisk. The sequence of murine CycT1 has been deposited in the DDBJ/EMBL/GenBank database (accession No. AF095640).

Sharp, 1991; Okamoto *et al.*, 1996; Parada and Roeder, 1996; Yang *et al.*, 1996). In addition, Tat associates with a CTD kinase activity (TAK) in nuclear extracts (Herrmann and Rice, 1995). The identity of TAK has been reported as CDK7, a component of TFIIF (Cujec *et al.*, 1997; Garcia-Martinez *et al.*, 1997), and by others as CDK9, a component of positive transcription elongation factor-b (P-TEFb) (Mancebo *et al.*, 1997; Yang *et al.*, 1997; Zhu *et al.*, 1997). While depletion of either protein from nuclear extracts abrogates Tat transactivation *in vitro*, the ability of a panel of kinase inhibitors to block Tat transactivation correlates more closely with inhibition of CDK9, rather than of CDK7 (Mancebo *et al.*, 1997). Nevertheless, considerable data suggest that a direct interaction between Tat and TFIIF could represent a critical step in the Tat-mediated activation of the HIV-1 LTR promoter (Blau *et al.*, 1996; Parada and Roeder, 1996; Cujec *et al.*, 1997; Garcia-Martinez *et al.*, 1997).

Recently, a human protein, cyclin T1 (hCycT1), that interacts with Tat and can mediate loop-specific binding of Tat to TAR *in vitro*, was identified (Wei *et al.*, 1998). Importantly, hCycT1, which is one of three known cyclin partners for CDK9 and a component of the transcription elongation factor P-TEFb (Peng *et al.*, 1998), is encoded on human chromosome 12 and can rescue Tat function in murine cells when overexpressed (Wei *et al.*, 1998). While these data demonstrate that CycT1 is a critical cofactor for Tat function, it has remained unclear why, or indeed whether, the murine form of CycT1 (mCycT1) is unable to support Tat function.

In this report, we describe the cloning of the murine homolog of cyclin T1 (mCycT1) and demonstrate that overexpression of mCycT1, which is ~90% identical to hCycT1, fails to rescue Tat function in murine cells and inhibits TAR-dependent Tat function in human cells. Although both human and murine CycT1 can interact

specifically with both human CDK9 and the activation domain of HIV-1 Tat, the Tat–CycT1–TAR ternary complex can only form efficiently when the CycT1 is of human origin. A single amino acid difference between hCycT1 and mCycT1 is shown to control the ability of CycT1 to form this ternary complex and also to govern the ability of CycT1 to rescue Tat function in murine cells and inhibit Tat–TAR function in human cells. Taken together, these data provide compelling genetic evidence that a critical function of Tat is to recruit CycT1 and associated factors to the TAR element present in the HIV-1 LTR promoter.

Results

Molecular cloning of mCycT1

A BLAST search of databases revealed the existence of two murine sequences (EST 519976 and EST 605445) that were closely related to internal regions of the published hCycT1 sequence (Peng *et al.*, 1998; Wei *et al.*, 1998), and were probably derived from a murine CycT1 gene. Therefore, RACE PCR was used to obtain sequences encoding the N- and C-terminal domains of mCycT1, and subsequently, mCycT1-specific primers were used to amplify the complete mCycT1 coding sequence. Comparison of the amino acid sequences of human and murine CycT1 revealed that the two proteins are 90% identical (Figure 1). In particular, the cyclin homology domain in the N-terminal portion of the proteins is almost completely conserved, with scattered and less extensive regions of sequence identity throughout the remainder of the proteins.

HIV-1 Tat function in murine cells is enhanced by hCycT1 but not by hCycT2A, hCycT2B or mCycT1

Although mouse cells support only low levels of Tat activity, a recent study indicated that this defect could be rescued by overexpression of hCycT1 (Wei *et al.*, 1998). To determine whether this is a specific property of the human protein or simply a consequence of generic CycT1 overexpression, murine LmTK[−] cells were transfected with expression plasmids for hCycT1 or mCycT1 along with an HIV-1 LTR CAT reporter plasmid in the presence and absence of Tat. As can be seen in Figure 2A, in the absence of a CycT expression plasmid, Tat only poorly activated (<10-fold) the HIV-1 LTR in these murine cells. Neither hCycT1 nor mCycT1 significantly affected the low basal level of CAT expression when transfected alone (0.9- and 1.4-fold activation, respectively), but CAT expression was increased ~70-fold by Tat when hCycT1 was co-expressed, a value that approaches the level of Tat activity observed in human cells (Madore and Cullen, 1993). Importantly, this enhancement of Tat activity was not observed when mCycT1 was co-expressed; simultaneous transfection of Tat and mCycT1 resulted in only a 5-fold activation of reporter gene expression. Thus, the ability of CycT1 to mediate Tat-dependent transactivation of the HIV-1 LTR is a specific property of the human protein.

CycT1 exists in mammalian cells predominantly as a complex with an associated kinase, CDK9. Other cyclin partners for CDK9 include CycT2, which is expressed in two alternatively spliced forms, CycT2A and T2B (Peng

et al., 1998). To determine whether the human forms of either of these other CDK9-associated cyclins would be able to reproduce the hCycT1 phenotype in murine cells, identical experiments to those described above were performed using hCycT2A and hCycT2B. In contrast to the dramatic effect on Tat function observed following transfection of hCycT1, neither form of hCycT2 had any significant effect on either the basal or the low Tat-activated level of HIV-1 LTR-dependent CAT expression in murine cells (Figure 2A).

A series of chimeric human–murine CycT1 expression plasmids was constructed to determine the sequences that are responsible for the species-specific CycT1 phenotype. These are depicted in Figure 2B and are named according to which amino acids are of human origin. Replacement of mCycT1 amino acids 1–352, 134–726, 134–352 or 246–300 with corresponding hCycT1 sequences resulted in chimeric CycT1 proteins that were able to support Tat activity in murine cells. The smallest region exchanged that resulted in a functional chimera, amino acid residues 246–300, contains nine changes relative to the parental mCycT1 sequence. Therefore, a set of single amino acid mutants of mCycT1 were generated that substituted each of these mCycT1 residues with the corresponding hCycT1 sequence. These mutant mCycT1s were then assayed for their ability to rescue Tat function in murine LmTK[−] cells. A representative experiment, featuring four of these mutants, is presented in Figure 2C. Substitution of Tyr261 for cysteine, but not any other substitution, resulted in a mutant mCycT1 that enhanced Tat function in murine cells as least as effectively as hCycT1. Thus, the differential ability of human and murine CycT1 proteins to support HIV-1 Tat function is governed by a single (Y261C) amino acid change.

To demonstrate that the level of expression of particular CycT1 variants was not a significant determinant of the different *in vivo* phenotypes shown in Figure 2A and C, we used Western blot analysis to measure the steady-state level of expression in transfected cells of hCycT1, of two mCycT1 variants able to rescue Tat function in murine cells [Y261C and M(H246–300)] and of two inactive forms of mCycT1 [wild-type mCycT1 and M(H300–352)]. To allow identification of the introduced CycT1 proteins, all five CycT1 variants were modified by addition of a C-terminal epitope tag derived from the influenza virus hemagglutinin (HA) protein. Addition of this epitope tag did not significantly affect the ability of these five CycT1 variants to rescue Tat function in murine cells (data not shown). The Western analysis shown in Figure 2D reveals that all four of the mCycT1 variants were expressed at comparable levels in transfected cells, thus demonstrating that differential stability cannot explain the efficient rescue of Tat function in murine cells by Y261C and M(H246–300), but not by wild-type mCycT1 or the M(H300–352) chimera. Surprisingly, however, the wild-type hCycT1 protein was found to be expressed at a significantly lower level than the four mCycT1 variants. This lower level of expression may explain the somewhat more efficient rescue of Tat function by certain mCycT1 variants, such as M(H246–300), in transfected murine cells when compared with hCycT1 (Figure 2A).

Fig. 2. Human but not murine CycT1 enhances HIV-1 Tat activity in murine cells. **(A)** Tat activity in murine cells is enhanced by hCycT1 but is unaffected by hCycT2A, hCycT2B or mCycT1. Murine LmTK⁻ cells were transfected with 200 ng of pTAR/CAT, 100 ng of pcTat, 100 ng of pBC12/CMV/lacZ and 500 ng of a pBC12/CMV/CycT expression plasmid. CAT activities, normalized for minor variations in the level of β -gal activity, are given as the mean $\pm$ SD of three transfections. **(B)** Chimeric human-murine CycT1 proteins were constructed as described in Materials and methods, and chimeras designated according to which amino acid sequences are of human origin. For example, M(H134-352) contains sequences of human origin from amino acid 134 to 352 in an otherwise mCycT1 background. **(C)** A single amino acid change in mCycT1 restores its ability to support Tat function. LmTK⁻ cells were transfected as in (A) using the indicated CycT1 chimeras and mutants bearing single amino acid substitutions in the mCycT1 context. **(D)** The indicated wild-type and mutant forms of CycT1 were modified by addition of a C-terminal HA tag and then expressed by transfection of the human cell line 293T. Extracts were prepared ~56 h after transfection, separated by SDS-PAGE, subjected to Western blot analysis using a monoclonal antibody directed against the HA tag and visualized by chemiluminescence.

Both hCycT1 and mCycT1, but not hCycT2A or hCycT2B, bind to the activation domain of HIV-1 Tat

To determine why hCycT1 enhances HIV-1 Tat function in murine cells while other CDK9-associated cyclins fail to exert this phenotype, we first examined which CycTs could bind to Tat using the yeast two-hybrid protein interaction assay (Fields and Song, 1989). As a control, to demonstrate adequate functional expression in yeast, the ability of the CycTs to bind CDK9, which has been demonstrated previously *in vitro* (Peng *et al.*, 1998; Wei *et al.*, 1998), was determined simultaneously. As expected, hCycT1, as well as both hCycT2A and hCycT2B, bound to human CDK9 (Figure 3A). In addition, mCycT1 was also found to be able to interact efficiently with human

CDK9, a finding that is expected given the near identity of the cyclin homology domains found in hCycT1 and mCycT1 (Figure 1). In contrast, there was a clear difference in the ability of the CycTs to bind Tat. While HIV-1 Tat was able to interact with both hCycT1 and mCycT1, no interaction with either hCycT2A or hCycT2B could be detected (Figure 3A).

The specificity of the Tat-hCycT1 and Tat-mCycT1 interactions was investigated next using a panel of truncated or mutated Tat proteins (Fridell *et al.*, 1995). Tat proteins harboring deletions in the C-terminal domain, which is dispensable for Tat activity *in vivo*, retained hCycT1- and mCycT1-binding activity. Deletion of the conserved Tat core sequence, a domain which is essential for Tat activity *in vivo*, resulted in a loss of CycT1-binding

Fig. 3. Interaction of CycT proteins with Tat or hCDK9 in the yeast two-hybrid assay. (A) Yeast were transformed with plasmids expressing the indicated GAL4 DNA-binding domain and VP16 activation domain fusion proteins. β -Gal activities (mean $\pm$ SD of three assays) were determined as described in Materials and methods and are expressed as milli-optical density units/h (mOD/h). The approximate sensitivity of this assay is 10 mOD/h. (B) Interaction between hCycT1 or mCycT1 and HIV-1 Tat mutants, HIV-2 Tat or EIAV Tat. Relative β -gal activities are expressed as percentages of the values obtained with full-length HIV-1 Tat.

activity. A Tat protein lacking amino acids residues 1–11, which retains partial activity *in vivo*, also retained a reduced ability to bind either CycT1. However, further truncation (amino acids 1–21) destroys both Tat activity and CycT1 binding. Finally, single amino acid substitutions in the Tat activation domain that either inactivate (C22S, C37S and K41A) or do not inactivate (C31S) Tat (Fridell *et al.*, 1995) resulted in a commensurate loss or retention of CycT1-binding activity, respectively. Overall, there was an absolute correlation between retention of a functional activation domain, i.e. with the predicted ability of these truncated or mutant Tat proteins to bind an essential cellular cofactor (Fridell *et al.*, 1995), and their actual ability to bind CycT1 *in vivo*. In addition, the Tat sequence

requirements for binding hCycT1 and mCycT1 were indistinguishable. Thus, while the failure of hCycT2A and hCycT2B to mediate Tat function can be explained readily by their failure to bind Tat, mCycT1 must be defective for Tat function for some other reason.

A closely related Tat protein, from HIV-2, which is functional in human cells was also found to interact with either CycT1. EIAV Tat is a more distantly related Tat which is non-functional in human cells when targeted via its cognate TAR element but a fully functional activator when targeted via a heterologous RNA–protein interaction (Madore and Cullen, 1993). Furthermore, overexpression of the EIAV Tat activation domain can inhibit HIV-1 Tat function in human cells, presumably by sequestration of a shared essential cofactor. Consistent with this hypothesis, EIAV Tat interacted with both human and murine CycT1 (Figure 3B).

Tat-dependent binding of hCycT1, but not mCycT1, to TAR

As mCycT1 is functional for binding to both CDK9 and HIV-1 Tat, the failure of mCycT1 to support Tat function must reflect a defect in some other step in the mechanism of action of Tat. To determine whether there is a species-specific difference in the ability of CycT1 proteins to be recruited to TAR, a modified yeast three-hybrid assay was employed (Sengupta *et al.*, 1996). Yeast L40-coat cells contain an integrated *lacZ* gene positioned 3' to LexA-binding sites and also constitutively express a fusion protein consisting of the LexA DNA-binding domain fused to the bacteriophage MS2 coat protein. Thus, transformation of L40-coat cells with plasmids expressing hybrid MS2-containing RNAs and activation domain fusion proteins results in β -galactosidase (β -gal) expression only if the fusion protein can bind the hybrid RNA. When L40-coat cells were transformed with plasmids expressing a hybrid MS2-TAR RNA and either a VP16-hCycT1 or a VP16-mCycT1 fusion protein, only very low levels of β -gal expression were detected (Figure 4A). However, when yeast were also transformed with a wild-type HIV-1 Tat expression plasmid, high levels of β -gal activity were detected in yeast expressing the MS2-TAR RNA and the VP16-hCycT1 protein. This Tat-dependent hCycT1–TAR interaction was blocked by mutations in either the activation domain (K41A) or the RNA-binding domain (Δ ARK) of Tat, and also by mutations in either the bulge (BM) or loop (LM) structures of TAR. Thus, mutations that inactivate Tat or TAR function in mammalian cells also disrupt the formation of a hCycT1–Tat–TAR ternary complex in the yeast cell nucleus.

In contrast to the above, only low levels of β -gal were detected in yeast expressing MS2-TAR RNA, Tat and VP16-mCycT1 (Figure 4B). Since we have demonstrated earlier, using the yeast two-hybrid assay, that a VP16-mCycT1–Tat complex readily forms in the yeast nucleus and can activate β -gal expression potently when recruited to an integrated *lacZ* reporter gene (Figure 3A), these experiments demonstrate that mCycT1–Tat complexes are selectively defective for binding to TAR RNA. When chimeric and mutant CycT1 proteins were examined using this assay, the same sequences that permit the rescue of Tat–TAR activity in murine cells (Figure 2) were also found to govern the differential ability of hCycT1 and

Fig. 4. Tat-dependent interaction between CycT1 and TAR. Yeast L40-coat cells were transformed with plasmids expressing hybrid MS2-TAR RNAs, non-fused Tat proteins and VP16 activation domain-CycT1 fusion proteins. (A) hCycT1 and mCycT1 interaction with wild-type (WT), bulge mutant (BM) and loop mutant (LM) TAR RNAs in the absence (–) or presence of wild-type or mutant (K41A and ΔRK) Tat proteins. (B) Interaction of chimeric and mutant mCycT1 proteins with wild-type TAR RNA in the presence of wild-type HIV-1 Tat. β-Gal activities in pools of >20 yeast transformants were determined in triplicate and are expressed as the mean ± SD.

mCycT1 to bind TAR in a Tat-dependent manner (Figure 4B). Most notably, the single amino acid change that regulates the differential mCycT1 versus hCycT1 phenotype in mammalian cells (Figure 2C) also governs the formation of a Tat–CycT1–TAR ternary complex in a yeast nucleus (Figure 4B). Thus, the failure of murine CycT1 to support Tat-mediated transactivation is due to its failure to be recruited effectively to TAR.

Murine CycT1 inhibits Tat function in human cells in an RNA target-specific manner

If mCycT1 interacts with HIV-1 Tat and human CDK9 effectively, yet cannot mediate recruitment of the resulting

Fig. 5. Experimental strategy and constructs to examine TAR-dependent inhibition of HIV-1 Tat function by murine CycT1. A Tat-Rev fusion protein can transactivate an HIV-1 LTR when recruited via a Tat RNA target (TAR) or a Rev RNA target (SLIIB), but only the Tat–TAR interaction is predicted to be affected by the RNA-binding properties of the bound CycT1 protein. In pSLIIB/TR/CAT and pTAR/TR/CAT, Tat-Rev and CAT are expressed using a bicistronic, IRES-containing RNA. Tat-Rev activates its own expression by a positive feedback mechanism; the sole difference between these two constitutively active expression plasmids is the RNA target for Tat-Rev.

complex to TAR, then overexpression of mCycT1 should inhibit Tat function in human cells by competing for Tat binding with endogenous hCycT1, leading to the formation of an inactive Tat–mCycT1 complex. Importantly, this inhibition is predicted to occur only when Tat acts via the HIV-1 TAR element, and not if Tat is recruited to the HIV-1 LTR via a heterologous RNA–protein interaction.

A fusion protein consisting of HIV-1 Tat fused to the HIV-1 Rev RNA-binding protein has been shown previously to activate transcription from the HIV-1 LTR when targeted via TAR or via a minimal HIV-1 Rev response element RNA target, termed SLIIB (Tiley *et al.*, 1992; Madore and Cullen, 1993). Importantly, Tat-Rev is equivalently active in human cells when recruited via either RNA element, but in murine cells Tat-Rev is only active when recruited by SLIIB (Madore and Cullen, 1993). As noted above, overexpression of mCycT1 in human cells should sequester the co-expressed Tat-Rev into a complex that retains full activation function and is selectively defective for TAR, but not SLIIB, binding. To test this hypothesis, two bicistronic reporter plasmids that express both Tat-Rev and CAT under the control of an HIV-1 LTR were constructed (Figure 5). These two vectors differ only in the RNA target for Tat-Rev (TAR or SLIIB). Since Tat-Rev is able to transactivate its own expression from either plasmid, transfection of either the TAR- or SLIIB-based vector into human 293T cells results in constitutive, approximately equivalent, CAT expression (Table I). However, co-transfection with a mCycT1 expression plasmid resulted in RNA target-dependent inhibition of transactivation, i.e. TAR-mediated CAT expression was reduced ~5-fold, whereas SLIIB-mediated expression was unaffected. Importantly, when the series of chimeric CycT1 expression plasmids were assayed for TAR-specific inhibition of Tat-Rev function, the same hCycT1 sequences that permitted rescue of Tat function in murine cells (Figure 2) and TAR binding in the yeast three-hybrid assay (Figure 4) also alleviated TAR-specific mCycT1 inhibition of Tat-Rev function in human cells (Table I). For example, chimera M(H246–300), which can rescue Tat–TAR function in murine cells and differs in sequence from the

Table I. TAR-specific inhibition of Tat-Rev function by mCycT1

CycT1 protein tested	Relative reporter gene expression (%) ^a			Tat cofactor activity ^b
	TAR/TR/CAT	SLIIB/TR/CAT	CMVlacZ	
None	100 ± 16	130 ± 22	100 ± 28	
hCycT1	237 ± 38	230 ± 18	221 ± 28	+
mCycT1	24 ± 3	110 ± 28	100 ± 15	–
M(H1–352)	226 ± 27	211 ± 39	223 ± 62	+
M(H134–726)	276 ± 34	196 ± 36	189 ± 19	+
M(H134–352)	303 ± 15	239 ± 45	295 ± 72	+
M(H246–300)	89 ± 18	70 ± 8	103 ± 22	+
M(H300–352)	16 ± 4	92 ± 24	128 ± 41	–
mCycT1(Y261C)	93 ± 6	81 ± 18	129 ± 29	+

^aHuman 293T cells were transfected with 100 ng of pTAR/TR/CAT or pSLIIB/TR/CAT, 100 ng of pBC12/CMV/lacZ and 500 ng of a pBC12/CMV/CycT1 expression plasmid. Reporter gene expression is given as a percentage of that observed upon transfection of pTAR/TR/CAT and pBC12/CMV/lacZ in the presence of a negative control plasmid, together with the standard deviation observed, and is reflective of data derived from three separate transfection experiments. The 100% values reflect a CAT activity of 31 450 c.p.m./h, with a background value of ~200 c.p.m./h and a β-gal activity of 3680 mOD/h.

^bThe ability of the tested CycT1 protein to rescue Tat function in mouse cells (Figure 2) and to mediate TAR binding (Figure 4 and data not presented) is indicated.

authentic mCycT1 at only nine amino acid residues, failed to inhibit Tat-Rev activity on either target. In contrast, the chimera M(H300–352), which differs from mCycT1 at 10 positions, yet fails to rescue Tat activity in murine cells, retained the ability to inhibit Tat-Rev activity selectively via a TAR RNA target. In fact, the same single amino acid change that confers upon mCycT1 the ability to rescue Tat function in murine cells (Figure 2A), and bind TAR in the presence of Tat (Figure 4B), also completely blocked its ability to inhibit Tat-Rev–TAR-mediated transactivation (Table I). These data strongly support the hypothesis that hCycT1 and functional CycT1 chimeras and mutants rescue Tat function in murine cells by forming Tat–CycT1 complexes that can be recruited effectively to TAR.

Overexpression of hCycT1 and of chimeric CycT1 proteins retaining certain hCycT1 sequences resulted in enhanced (~2- to 3-fold) CAT expression (Table I). This enhancement was independent of the RNA sequence used to recruit Tat-Rev, and paralleled the documented non-specific hCycT1-induced enhancement of gene expression in human cells from other constitutively active mammalian promoters, for example the CMV IE promoter (Peng *et al.*, 1998).

Discussion

Since the discovery that the HIV-1 Tat protein is a potent transactivator of viral gene expression, a number of cellular proteins that interact directly or indirectly with either Tat or with TAR have been described and proposed to play a role in mediating the transactivation properties of Tat (Shibuya *et al.*, 1992; Kashanchi *et al.*, 1994; Fridell *et al.*, 1995; Blau *et al.*, 1996; Zhou and Sharp, 1996; Cujec *et al.*, 1997; Sune *et al.*, 1997; Xiao *et al.*, 1998). While it remains possible that one or more of these factors could play a critical role in promoting distal steps in the Tat-mediated activation of HIV-1 LTR-dependent gene expression, the *in vivo* evidence presented in support of any of these earlier candidate proteins has not been sufficient to satisfy the stringent criteria for the essential Tat-binding protein and cofactor that have been suggested

by genetic analyses of Tat function (Madore and Cullen, 1993). As documented here, and in the previous work of Wei *et al.* (1998), cyclin T1 fulfills most, if not all, of these expectations.

First, under both stringent *in vitro* conditions (Wei *et al.*, 1998), and in the more physiologically relevant environment of a eukaryotic nucleus, CycT1 binds specifically to the Tat activation domain (Figure 3). Mutated or truncated Tat proteins that retain a functional activation domain bind CycT1, while inactive Tat mutants do not. This interaction does not require TAR, and thus explains why Tat proteins can be made functional activators in normally non-permissive cells when recruited via a heterologous RNA–protein interaction (Madore and Cullen, 1993). Secondly, Tat–hCycT1 complexes bind to TAR in a manner that recapitulates the sequence requirements for TAR function *in vivo*, i.e. an intact bulge and loop are both required (Figure 4A), in contrast to the *in vitro* Tat–TAR interaction, which is only bulge specific (Dingwall *et al.*, 1990; Roy *et al.*, 1990; Weeks *et al.*, 1990). Thirdly, since Tat is not active in rodent cells, it would be predicted that the murine homolog of CycT1 should be non-functional in some way. In fact, mCycT1 is at least as active as its human counterpart in terms of Tat binding (Figure 3). However, unlike the Tat–hCycT1 complex, the Tat–mCycT1 complex is unable to bind efficiently to TAR (Figure 4B) and thus does not support high levels of transactivation. This finding explains the previous observation that Tat, while only poorly functional in rodent cells on a TAR RNA target, is a potent transactivator when fused to an RNA-binding protein whose RNA target is substituted for TAR (Alonso *et al.*, 1992; Madore and Cullen, 1993). Furthermore, overexpression of mCycT1, but not of hCycT1, in human cells suppresses Tat function (Table I). Importantly, this inhibition is critically dependent on the RNA target used in that a Tat-Rev fusion protein is inhibited by mCycT1 when targeted to TAR but remains fully active when targeted via the Rev SLIIB RNA-binding site.

Each of the phenotypic differences between hCycT1 and mCycT1, i.e. (i) TAR binding *in vivo*, (ii) rescue of Tat–TAR activity in murine cells and (iii) TAR-specific

inhibition of Tat-Rev function in human cells, is governed by precisely the same CycT1 sequences. This finding, coupled with the fact that phenotypic differences are manifested only in the presence of TAR, provides compelling evidence for the hypothesis that the differential ability of hCycT1 and mCycT1 to be recruited to TAR is the mechanism by which the different phenotypes of hCycT1 and mCycT1 are manifested. Remarkably, a single amino acid (C261 in hCycT1 versus Y261 in mCycT1) determines the differential TAR-binding abilities (and associated phenotypes) of these two very similar proteins.

A remaining mechanistic question is whether CycT1 directly binds TAR RNA or whether CycT1 instead induces a change in the RNA-binding properties of Tat, as proposed by Wei *et al.* (1998). Clearly, the observations contained herein demonstrate that Tat binding to a CycT1 protein is not in itself sufficient to change the conformation of Tat in such a way as to modify its TAR-binding affinity and specificity. Rather, a single amino acid change in CycT1 modifies the RNA-binding properties of the resultant Tat-CycT1 complex. The most likely explanation for this finding is that CycT1 itself directly binds to the loop of TAR, a hypothesis strengthened by the recent observation that CycT1 displays a significant non-specific affinity for structured RNA (Zhou *et al.*, 1998). Nevertheless, while this single mutation clearly segregates the properties of Tat binding and TAR binding in CycT1, it remains formally possible that mCycT1, while fully competent to bind Tat, fails to modify the Tat structure in such a way as to increase its TAR affinity and simultaneously modify its specificity to include loop residues.

When viewed in conjunction with data presented recently by several other groups (Mancebo *et al.*, 1997; Yang *et al.*, 1997; Zhu *et al.*, 1997; Gold *et al.*, 1998; Wei *et al.*, 1998; Zhou *et al.*, 1998), the data reported here provide compelling evidence that activation of the HIV-1 LTR promoter by Tat requires the recruitment of a protein complex, minimally containing CycT1, CDK9 and Tat itself, to the viral LTR, presumably resulting in hyperphosphorylation of the CTD and processive transcription. It appears likely that the host cell protein complex recruited by Tat to TAR is identical to the CycT1-containing form of the P-TEFb elongation factor, which is known to include not only CycT1 and CDK9 but also four other, as yet unidentified proteins of ~68, ~55, ~49 and ~32 kDa (Zhou *et al.*, 1998). These observations suggest approaches to the development of small animal models of HIV-1 infection and for therapeutic intervention in HIV-1-induced disease. Specifically, the finding that mCycT1 is a dominant inhibitor of Tat-TAR function suggests that strategies for the development of an optimally HIV-1-susceptible mouse by introduction of the hCycT1 gene should either also include the disruption of the endogenous mCycT1 gene or, ideally, the 'repair' of the endogenous murine allele by introduction of the single amino acid change required to support Tat function, thus avoiding any potential perturbation of normal murine P-TEFb function and tissue distribution. In fact, mCycT1 proteins containing only small hCycT1 sequence insertions are modestly, but consistently, more active than full-length hCycT1 in their ability to rescue Tat function in murine cells (Figure 2A). More importantly, the finding that mCycT1 is unable to support the recruitment of the Tat-

CycT1 complex to TAR demonstrates that the sequences in CycT1 that mediate this recruitment are not evolutionarily conserved. This result implies that it may be possible to design reagents that block this essential step in the HIV-1 life cycle without affecting critical cellular processes. Clearly, efforts to understand the functional organization of hCycT1, and the role of other P-TEFb components in both viral and cellular transcriptional regulation (Zhu *et al.*, 1997; Peng *et al.*, 1998; Zhou *et al.*, 1998), will need to be addressed in the near future.

Materials and methods

Molecular cloning of mCycT1

A BLAST search using the published hCycT1 sequence identified two murine expressed sequence tags (ESTs) that are homologous to the published hCycT1 sequence (Peng *et al.*, 1998; Wei *et al.*, 1998). Nested RACE PCR primers derived from these sequences were used to amplify N- and C-terminal coding sequences of mCycT1 using marathon cDNA (Clontech) as template. Thereafter, N- and C-terminal-specific primers were used to amplify full-length mCycT1 from the same source, the resulting PCR products were cloned into pBC12/CMV (Tiley *et al.*, 1992) and the complete sequence of three independent clones determined. hCycT1 was amplified similarly from HeLa marathon cDNA using PCR primers derived from the published sequence.

Plasmid construction

The mammalian expression vectors pcTat, pcRev and pTat-Rev, and pBC12/CMV have been previously described (Tiley *et al.*, 1992). pBC12/CMV/HA is a derivative of pBC12/CMV that contains an *EcoRI* site located 5' to sequences encoding an HA epitope tag followed by an in-frame translation stop codon. Sequences from pcTat-Rev encoding a Tat-Rev fusion protein were inserted into the *HindIII* site of pTAR/CAT and pSLIIB/CAT (Tiley *et al.*, 1992) to generate pTAR/TR/CAT and pSLIIB/TR/CAT, respectively. Thus, constitutively active bicistronic vectors were generated in which both Tat-Rev and CAT are expressed under the control of the HIV LTR, with Tat-Rev targeted to the LTR via either SLIIB or TAR, and with CAT translation dependent on the presence of a poliovirus internal ribosome entry site (IRES). Human and murine CycT1 alleles lacking an identical C-terminal 19 amino acid sequence containing a putative proline-rich PEST sequence were derived by PCR and inserted into pBC12/CMV. Each contained in-frame *EcoRI* sites immediately 5' to both the start and stop codons. Mutant/chimeric CycT1 proteins were derived by recombinant PCR, exchange of restriction fragments or using the Quickchange mutagenesis kit (Stratagene). Wild-type and selected chimeric/mutant CycT1 sequences were also inserted into pBC12/CMV/HA.

The yeast expression vector pVP16 has been described previously (Bogerd *et al.*, 1993). Sequences encoding hCycT1, mCycT1, hCycT2A and hCycT2B, as well as chimeric and mutant CycT1s, were inserted into pVP16 to generate plasmids expressing CycT proteins fused to the HSV-1 VP16 activation domain. pPGK is derived from pVT101UR (Vernet *et al.*, 1987), but the existing *URA3* gene has been replaced with *HIS3* and the existing *ADH* promoter and terminator sequences have been replaced by those of the *PGK* gene flanking a polylinker. The coding sequences of wild-type Tat, as well as the Δ RK and K41A mutants (Fridell *et al.*, 1995), were inserted into pPGK to derive plasmids expressing Tat proteins in yeast and conferring histidine auxotrophy. Tat proteins and CDK9 fused to a GAL4 DNA-binding domain were expressed in yeast using pGAL4 (Bogerd *et al.*, 1993) or pGBT9 (Clontech).

Oligonucleotides containing the wild-type TAR sequence and mutants either entirely lacking the U-rich bulge (TAR BM) or containing a mutation in the hexanucleotide loop (TAR LM) were inserted into pIII/MS2 (Sengupta *et al.*, 1996). Thus plasmids which express hybrid MS2-TAR RNAs in yeast under the control of the RNase P1 promoter while conferring uracil auxotrophy were generated. The LM loop mutation (5'-CTGGGA-3' to 5'-CCAAAA-3') previously has been demonstrated to destroy TAR function *in vivo* without affecting Tat binding *in vitro* (Roy *et al.*, 1990).

Yeast two- and three-hybrid assays

For two-hybrid assays, Y190 yeast cells (Harper *et al.*, 1993) were transformed with pGAL4 HIV-1 Tat (and mutants thereof), and

pVP16-CycT plasmids and transformants selected on media lacking histidine and leucine. Alternatively, yeast were transformed with pGBT9-HIV-2 Tat, pGBT9-EIAV Tat or pGBT9-CDK9 and pVP16-CycT plasmids and transformants selected on media lacking tryptophan and leucine. For three-hybrid assays, L40-coat cells (Sengupta *et al.*, 1996) were transformed with pIII/MS2/TAR, pPGK/Tat and pVP16-CycT1 (and mutants thereof). Transformants were selected on media lacking uracil, histidine and leucine. Pools of >20 transformed yeast colonies were scraped into β -gal assay buffer, normalized according to optical density, and β -gal activities were assayed as previously described (Bogerd *et al.*, 1993).

Cell culture and transfections

Human 293T and murine LmTK⁻ cells were transfected by calcium phosphate co-precipitation and DEAE-dextran respectively, as previously described (Cullen, 1987). To assay rescue of Tat function in murine cells, LmTK⁻ cells were transfected with 200 ng of pTAR/CAT, 100 ng of pBC12/CMV/lacZ, 100 ng of pcTat and 500 ng of pBC12/CMV/CycT. Control transfections lacking either Tat or CycT were performed similarly; in these cases, the total amount of DNA per transfection was maintained by inclusion of the requisite amount of the parental pBC12/CMV plasmid. To assay inhibition of Tat-Rev function, 293T cells were transfected with 100 ng of pBC12/CMV/lacZ, 500 ng of pBC12/CMV/CycT1 and 100 ng of either pTAR/TR/CAT or pSLIIB/TR/CAT. In all transfection experiments, CAT enzyme levels were determined 48 h after transfection as previously described and, except where indicated, normalized to the level of β -gal in cell lysates (Madore and Cullen, 1993).

Western blot analysis

To compare the steady-state expression levels of wild-type and mutant/chimeric CycT1 proteins, 293T cells were transfected (Cullen, 1987) with 1 μ g of each pBC12/CMV/CycT1/HA plasmid. Total cell lysates were prepared 56 h after transfection and separated by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes which were then probed sequentially with the anti-HA 12CA5 monoclonal antibody (Boehringer Mannheim) and peroxidase-conjugated sheep anti-mouse IgG (Amersham). Bound antibodies were visualized using chemiluminescent detection reagents.

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