

Evidence for conformational flexibility in the Tat–TAR recognition motif of cyclin T1

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Received 4 June 2003; returned to author for revision 8 October 2003; accepted 8 October 2003

Abstract

Cyclin T1 (CycT1) is a cellular transcription elongation factor that also participates in Tat-mediated activation of several lentiviral promoters. In human immunodeficiency virus (HIV), CycT1 is required for Tat to bind tightly to TAR and interacts in the ternary complex via its Tat–TAR recognition motif (TRM). In the related bovine immunodeficiency virus (BIV), Tat recognizes its cognate TAR element with high affinity and specificity in the absence of CycT1. At both promoters, CycT1 recruits the Cdk9 kinase, which phosphorylates RNA polymerase II to generate processive transcription complexes. To examine the physical properties of CycT1, we purified a functional domain corresponding to residues 1–272 and found that it possesses a stably folded core, as judged by partial proteolysis and circular dichroism experiments. Interestingly, the C-terminal 20 residues corresponding to the TRM appear conformationally flexible or disordered. The TRM of the bovine CycT1 (bCycT1) is similarly sensitive to proteolysis yet differs in sequence from the human protein. In particular, bCycT1 lacks a cysteine at residue 261 known to be critical for HIV but not BIV ternary complex formation, and mutagenesis data are consistent with a proposed role for this cysteine in metal binding. The apparent flexibility of the TRM suggests that conformational rearrangements may accompany formation of CycT1–Tat–TAR ternary complexes and may contribute to different TAR recognition strategies in different lentiviruses.

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Keywords: Tat–TAR recognition motif; Cyclin T1; Conformational flexibility

Introduction

Like many complex retroviruses, human immunodeficiency virus (HIV) utilizes several virally encoded regulatory proteins during its replication life cycle. One essential regulatory protein, Tat, is required to stimulate RNA polymerase II-mediated transcription initiated from the 5′ long-terminal repeat (LTR) of the integrated proviral genome. Tat binds to an RNA hairpin in the 5′ LTR, known as the transactivating response (TAR) element, and enhances transcription processivity (for review, see [Karn, 1999](#)). One key cellular protein, cyclin T1 (CycT1), was found to interact with Tat and TAR ([Wei et al., 1998](#)) and, along with its associated cyclin-dependent kinase (Cdk9), is part of a

transcription elongation complex known as P-TEFb ([Jones, 1997](#); [Price, 2000](#)). A current working model suggests that Tat recruits P-TEFb to the nascent viral transcripts, allowing Cdk9, and possibly other kinases, to hyperphosphorylate the C-terminal domain (CTD) of polymerase and render it processive ([Majello et al., 1999](#); [Napolitano et al., 2000](#)).

Two functional domains in Tat have been identified: an arginine-rich RNA-binding domain (residues 49–57) that recognizes a bulge region of TAR ([Aboul-ela et al., 1995](#); [Calnan et al., 1991](#); [Long and Crothers, 1999](#); [Puglisi et al., 1992](#); [Roy et al., 1990](#); [Weeks and Crothers, 1991](#)), and an activation domain (residues 1–48) that interacts with CycT1 and increases the affinity and specificity for TAR ([Bieniasz et al., 1998](#); [Chen et al., 1999](#); [Fujinaga et al., 1999](#); [Garber et al., 1998](#); [Ivanov et al., 1999](#); [Kwak et al., 1999](#); [Wei et al., 1998](#)). In the context of this ternary complex, the central loop nucleotides of TAR are crucial for binding, consistent with the known loop requirements for Tat-mediated activation in vivo ([Feng and Holland, 1988](#); [Madore and Cullen,](#)

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1993). Recent RNA crosslinking and footprinting studies suggest that residues between 252 and 261 of CycT1 directly contact nucleotides in the loop and may also help position Tat near the loop (Richter et al., 2002b). It is currently unclear whether CycT1 causes conformational changes in Tat that permit interactions with the loop.

CycT1 is 726 amino acids in length, with residues 1–272 sufficient to form complexes with Tat and TAR and mediate Tat activation (Bieniasz et al., 1998; Chen et al., 1999; Fujinaga et al., 1998; Garber et al., 1998; Ivanov et al., 1999). A region near the C terminus of CycT1 1–272, termed the Tat–TAR recognition motif (TRM), is important for forming Tat–CycT1–TAR ternary complexes (Bieniasz et al., 1998; Fujinaga et al., 1999; Garber et al., 1998). The TRM is one of the least conserved regions of CycT1, which otherwise show >96% sequence identity among species. Indeed, differences in the TRM sequence provided important proof that CycT1 is an essential Tat cofactor. It had long been known that Tat functions poorly in rodent cells, and it was discovered that substituting Tyr261 of mouse cyclin T1 (mCycT1) with a cysteine residue found in the human cyclin (hCycT1) fully rescued Tat activation in mouse cells (Bieniasz et al., 1998; Chen et al., 1999; Fujinaga et al., 1999; Garber et al., 1998; Kwak et al., 1999; Wei et al., 1998). Here we present biochemical evidence that CycT1 1–272 contains a well-folded core with substantial α -helical content, as in other types of cyclins, but that the C-terminal TRM, including Cys261, is conformationally flexible.

Studies of other lentiviral Tat proteins have further demonstrated the functional importance of CycT1 in Tat-mediated activation. The Tat protein from equine infectious anemia virus (EIAV) can activate the EIAV LTR using equine or canine CycT1, but not hCycT1 (Albrecht et al., 2000; Taube et al., 2000). Substituting residues 29, 79, and 80 of hCycT1 with the corresponding residues of equine CycT1 rescued activity (Albrecht et al., 2000; Taube et al., 2000). Interestingly, the equine and canine cyclins contain a cysteine at position 261, like hCycT1, and support activation of the HIV LTR by HIV Tat, further indicating that important species-specific differences exist among different Tat–cyclin–TAR complexes (Albrecht et al., 2000; Taube et al., 2000).

Bovine immunodeficiency virus (BIV) and the closely related bovine Jembrana disease virus (JDV) show yet other differences in the assembly of Tat–TAR complexes and different sequence requirements for the TRM. The arginine-rich RNA-binding domain of BIV Tat adopts a β -hairpin conformation and recognizes BIV TAR with high affinity in the absence of CycT1, utilizing several amino side chains to make sequence-specific RNA contacts (Chen and Frankel, 1994, 1995; Puglisi et al., 1995; Ye et al., 1995). In contrast, the HIV Tat RNA-binding domain binds HIV TAR at least an order of magnitude more weakly in the absence of hCycT1, utilizing one arginine from an extended peptide chain to make a sequence-specific contact (Aboul-ela et al., 1995; Calnan et al., 1991; Puglisi et al., 1992). The loop

nucleotide sequence of BIV TAR is not important for BIV Tat binding or activation (Chen and Frankel, 1994, 1995), and activation through the BIV Tat–TAR interaction is supported in murine, lapine, and human cells, further suggesting a relaxed requirement for CycT1 (Barboric et al., 2000; Bogerd et al., 2000; Chen and Frankel, 1994).

To further examine species-specific differences among CycT1 proteins, we cloned the bovine CycT1 (bCycT1) and compared its biochemical behavior to that of hCycT1. Like mCycT1, bCycT1 lacks a cysteine at position 261 and is unable to support HIV Tat function. Like hCycT1, partial proteolysis of bCycT1 1–272 showed apparent conformational flexibility at its C terminus, consistent with the relatively poor sequence conservation of the TRM. Mutagenesis of residue 261 is consistent with the hypothesis that the C-terminal region binds a metal ion near the Tat-binding interface, perhaps utilizing Cys261 as a metal ligand as previously proposed (Garber et al., 1998). Differences between hCycT1 and bCycT1 in the flexible C-terminal region may affect interactions at the Tat–CycT1 interface and contribute to differences in the HIV and BIV TAR recognition strategies.

Results

Purified recombinant hCycT1 is monomeric

To begin to examine the biochemical properties and structural details of CycT1, we generated a series of bacterial expression vectors in which fragments of hCycT1 corresponding to predicted endpoints of the secondary structure (residues 1–272, 1–317, and 1–420) were fused to GST (in a pGEX2T vector) or expressed directly (in a pET21d vector). The nonfused hCycT1 fragment (residues 1–272) was expressed most highly, with approximately 50% in a soluble form (Fig. 1A), and we refer to this functional fragment as hCycT1 throughout the paper. We developed a two-step purification protocol utilizing hydrophobic affinity and anion exchange chromatography that produced preparations of >95% purity (Fig. 1A). Mass spectrometry indicated a molecular mass of 31535 ± 20 Da, in agreement with the calculated mass of 31533 Da. hCycT1 is predominantly monomeric as judged by size exclusion chromatography (Fig. 1B). RNA-binding gel shift assays showed formation of loop-dependent HIV Tat–CycT1–TAR complexes (C.D. and A.D.F., in preparation) as previously observed (Fujinaga et al., 1999; Ivanov et al., 1999; Richter et al., 2002a; Wei et al., 1998; Zhou et al., 1998).

hCycT1 is helical and moderately stable

To assess the degree of hCycT1 folding, secondary structure, and protein stability, we recorded CD spectra and performed thermal and chemical denaturation experiments. The spectrum of hCycT1 (Fig. 2A) is characteristic of a

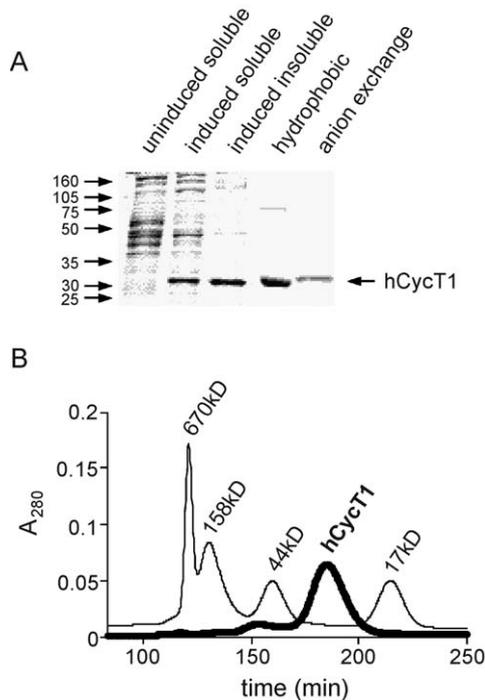


Fig. 1. Expression and purification of hCycT1 1–272. (A) *E. coli* expression levels and solubility of hCycT1 were assessed by running samples of crude lysate and equivalent amounts of the insoluble fraction on SDS-PAGE gels and staining with Coomassie blue. The lysates from uninduced BL21(DE3) cells transformed with the pET21d expression vectors are shown as controls. hCycT1 was purified using hydrophobic interaction (butyl) and anion exchange (Q-Sepharose) FPLC columns, resulting in protein of >95% purity. (B) Elution profile of hCycT1 from a Sephacryl S-100 size exclusion column, with protein standards used for calibration.

folded, highly helical protein with an estimated helix content of 50%. We next examined protein stability by monitoring the CD melting curve at 222 nm (Fig. 2B) and observed a cooperative unfolding transition with an apparent T_m of approximately 45 °C. The melting curve was not reversible, probably due to protein aggregation. To better estimate protein stability, we monitored denaturation induced by guanidinium hydrochloride and again observed a cooperative unfolding transition (Fig. 2C), with a C_m of approximately 1.7 M at 4 °C. Thus, hCycT1 appears well folded and has a helical secondary structure characteristic of other cyclins (Andersen et al., 1996, 1997; Brown et al., 1995; Card et al., 2000; Kim et al., 1996; Kobayashi et al., 1992; Russo et al., 1996), but it appears to be only moderately stable.

The TRM of hCycT1 is sensitive to proteolysis

To examine the folding and stability of hCycT1 in more detail, we performed limited proteolysis using chymotrypsin, trypsin, or subtilisin and examined the digestion products by sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). In all cases, a predominant approximately 29-kDa fragment (approximately 2.5 kDa shorter than hCycT1 1–272) was produced within the first few minutes of cleavage

and was the only product observed after 1 h (Fig. 3A shows cleavage by chymotrypsin). This proteolytically stable fragment from chymotryptic digestion was transferred to a PVDF membrane and sequenced by Edman degradation. The first six residues, MEGERK, corresponded to the intact N terminus of hCycT1, indicating that cleavage had occurred near the C terminus.

We used mass spectrometry to map the cleavage site more precisely. After 15 min of digestion with chymotrypsin, we observed uncleaved hCycT1 along with two digestion products that corresponded to cleavage after Trp256 and Leu252 (Fig. 3B). The amino acid assignments are consistent with the reduction in mass of approximately 2 and 2.5 kDa observed by gel and the known preference of chymotrypsin to cleave after hydrophobic residues. Thus, it appears that the C-terminal 20 amino acids of hCycT1, which includes the TRM and Cys261, may be disordered or near a proteolytically sensitive loop.

We wished to ensure that the C-terminal proteolytic sensitivity was not caused by the somewhat arbitrary truncation of full-length hCycT1 at residue 272, which might have disrupted an important element of secondary or tertiary

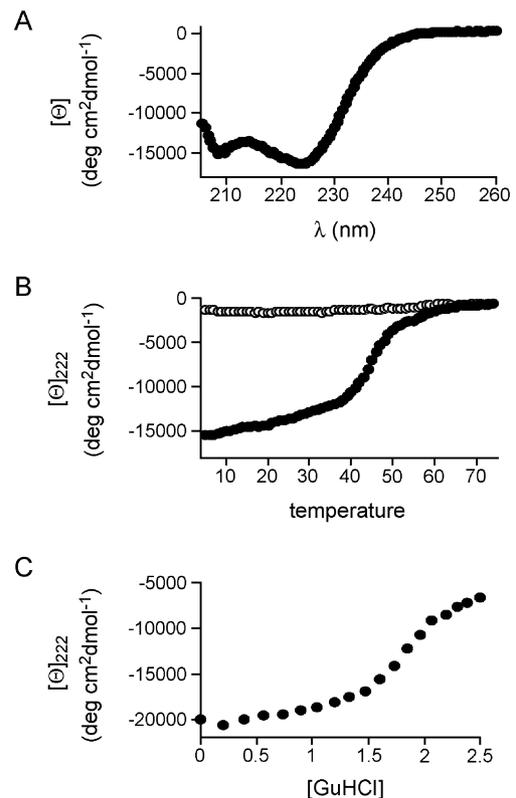


Fig. 2. Circular dichroism spectroscopy of hCycT1. (A) The spectrum of hCycT1 was recorded at 4 °C in 10 mM K_2HPO_4 and 70 mM KF at 75 ± 20 $\mu\text{g/ml}$ protein and mean residue ellipticity was calculated. The minima near 222 and 208 nm are indicative of α helix. (B) The CD signal was recorded at 222 nm as a function of increasing temperature (filled circles), followed immediately by a reverse melt (open circles). (C) The CD signal was recorded at 222 nm as a function of increasing guanidinium hydrochloride concentration, with a C_m observed at approximately 1.8 M.

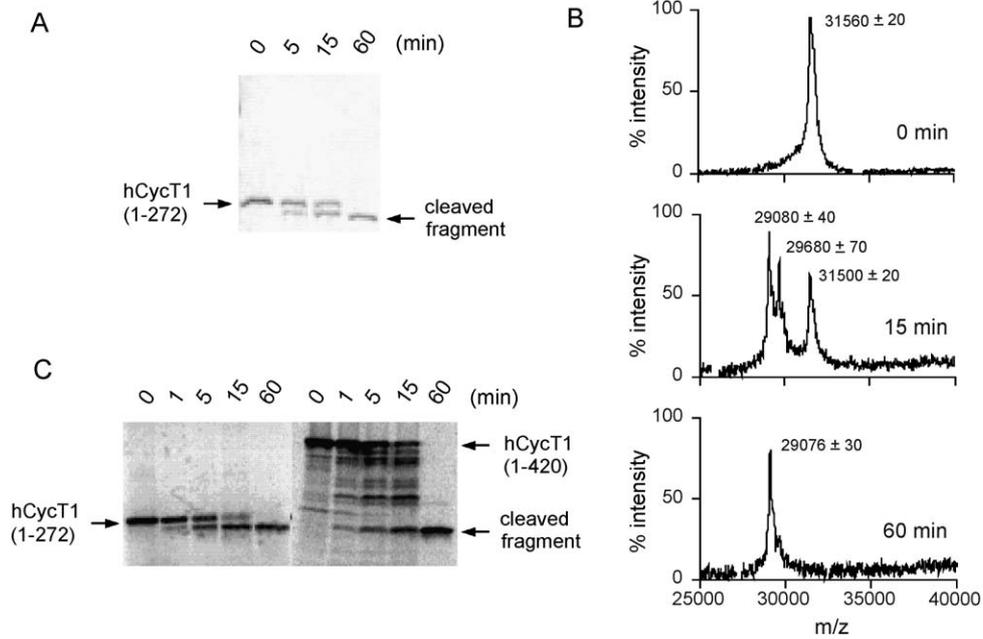


Fig. 3. Partial proteolysis of CycT1 proteins. (A) Time course of chymotrypsin digestion at a 1:60 chymotrypsin/hCycT1 ratio monitored by SDS-PAGE. (B) MALDI-TOF mass spectrometry of the hCycT1 chymotryptic products from A. Before cleavage (0 min), the measured mass to charge ratio was 31560 ± 20 (calculated value = 31533). After 15 min of digestion, two cleavage products were observed with masses of 29680 ± 70 (calculated value for cleavage after Trp256 = 29679) and 29080 ± 40 (calculated value for cleavage after Leu252 = 29096). After 60 min of digestion, only the smaller fragment (29076 ± 30) was observed. (C) Time course of chymotrypsin digestion of in vitro translated hCycT1 1–272 and 1–420 (approximately 1:100 chymotrypsin:hCycT1) monitored by SDS-PAGE.

structure. We were unable to obtain longer soluble versions of hCycT1 by bacterial expression to test whether cleavage would still occur at the same position; however, we could successfully produce hCycT1 residues 1–420 by in vitro translation. As shown in Fig. 3C, chymotryptic digestion of

in vitro translated 1–420 or 1–272 fragments resulted in identical approximately 29-kDa products even after 1 min of digestion (corresponding to cleavage at Leu252), strongly suggesting that the site is truly accessible in its native context.

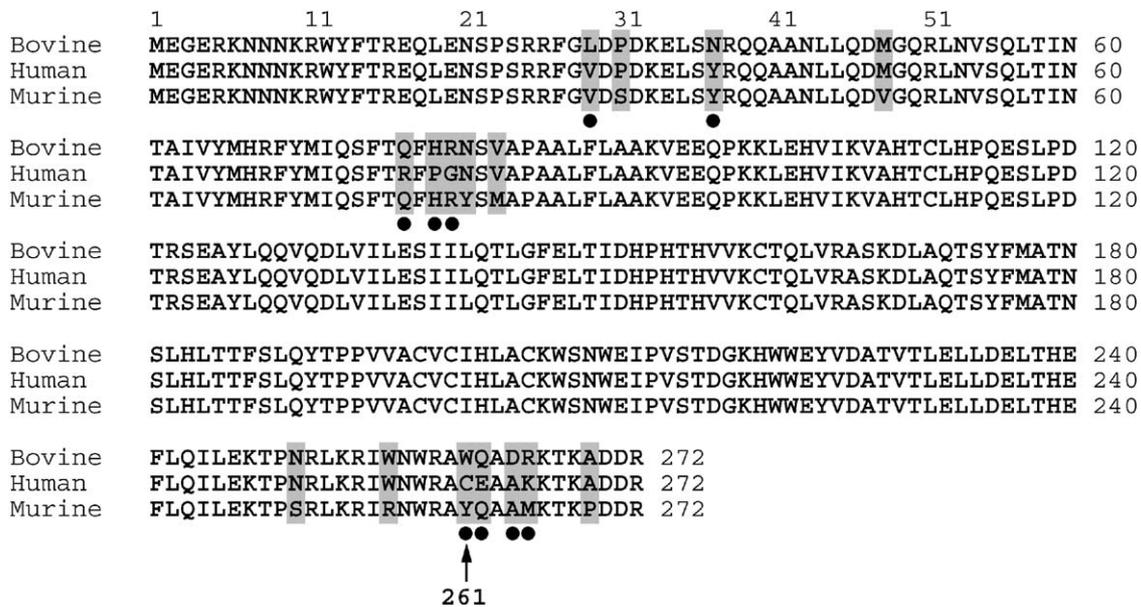


Fig. 4. Multiple sequence alignment of residues 1–272 from bCycT1 (bovine), hCycT1 (human), and mCycT1 (murine) by ClustalW (version 1.81). Boxes indicate positions that differ in any pairwise combination, closed circles indicate the nine positions that differ between the bovine and human proteins, and an arrow marks the important residue at position 261.

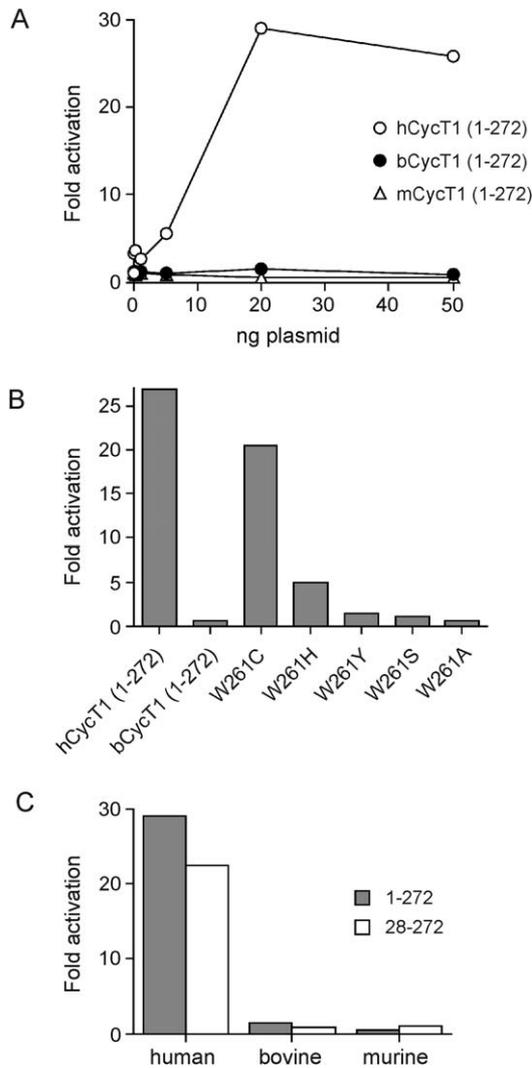


Fig. 5. (A) Activities of bCycT1, hCycT1, and mCycT1 in HIV Tat-mediated activation of the HIV LTR. Mouse NIH 3T3 cells were co-transfected with 20 ng of a pHIV-LTR-CAT reporter, 100 ng of the pSV2tat72 Tat expression plasmid, and the indicated concentrations of pcDNA3 CycT1 expression plasmids. Fold activation was calculated by normalizing to the levels of CAT expression in the absence of cotransfected CycT1. (B) Activities of bCycT1 variants at position 261 in HIV Tat-mediated activation of the HIV LTR. Mouse NIH 3T3 cells were co-transfected with 10 ng of a pHIV-LTR-CAT reporter, 100 ng of the pSV2tat72 Tat expression plasmid, and 20 ng of pcDNA3 CycT1 expression plasmids. Fold activation was calculated as in A. (C) Activities of CycT1 N-terminal deletion mutants in HIV Tat-mediated activation of the HIV LTR. Mouse NIH 3T3 cells were co-transfected with 10 ng of a pHIV-LTR-CAT reporter, 100 ng of the pSV2tat72 Tat expression plasmid, and 50 ng of pcDNA3 CycT1 expression plasmids. Fold activation was calculated as in A.

Bovine CycT1 contains tryptophan at position 261

As described in the Introduction, the Tat–TAR complexes of different lentiviruses show rather distinct modes of binding and dependence on CycT1. For example, the HIV Tat–TAR complex requires hCycT1 and Cys261 in the TRM whereas the BIV Tat–TAR complex forms with high

affinity in the absence of CycT1. To examine the biochemical and functional behavior of a CycT1 TRM that is not essential for TAR recognition, we next cloned bCycT1 from a cDNA library based on nucleotide sequence conservation between hCycT1 and mCycT1. The alignment of amino acids 1–272 (Fig. 4) shows that bCycT1 and hCycT1 are 97% identical in this region and about as similar as hCycT1 and mCycT1 (96% identity). Of the nine amino acid differences between bCycT1 and hCycT1, most notable is tryptophan at position 261 in place of the critical cysteine in hCycT1. In mCycT1, which does not support HIV Tat activation, residue 261 is a tyrosine (Bieniasz et al., 1998; Chen et al., 1999; Fujinaga et al., 1999; Garber et al., 1998; Kwak et al., 1999; Wei et al., 1998). The full-length bCycT1 (728 residues) is 81% identical to hCycT1 but is even more closely related (89% identity) to caprine CycT1, which also has a tryptophan at position 261 (Mata and Vilotte, 2002).

Activity of bCycT1 requires a metal ligand at position 261

Because BIV Tat binds BIV TAR in a CycT1-independent mode, it is able to activate transcription in a variety of cell types, including rodent cells that express CycT1 with Tyr261 and are therefore nonpermissive for HIV Tat activation (Barboric et al., 2000; Bogerd et al., 2000; Chen and Frankel, 1994). To test the activity of our cloned bCycT1, we asked whether it could stimulate BIV Tat-mediated activation of its cognate BIV LTR using CAT reporter assays. No effect of bCycT1 was observed in either HeLa or mouse NIH 3T3 cells (data not shown), as expected because hCycT1 and mCycT1 are both able to support activation in the BIV context (Barboric et al., 2000; Bogerd et al., 2000). To provide a better context for monitoring

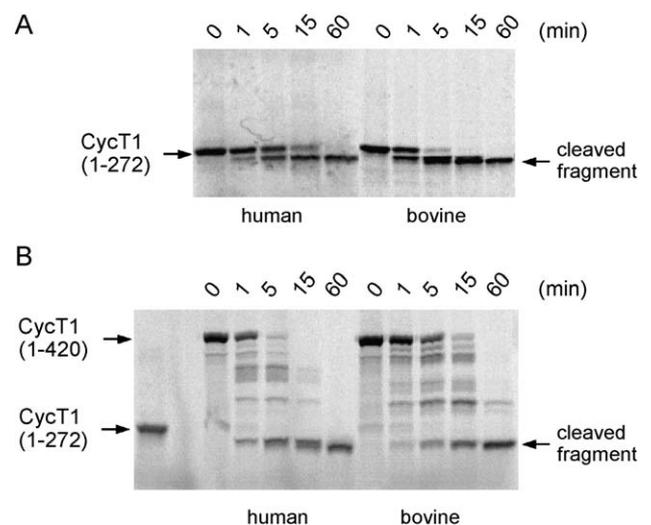


Fig. 6. (A) Time course of chymotrypsin digestion of in vitro translated human and bovine CycT1 1–272 (approximately 1:100 chymotrypsin/CycT1) monitored by SDS-PAGE. (B) Time course of chymotrypsin digestion of in vitro-translated human and bovine CycT1 1–420 (approximately 1:100 chymotrypsin/CycT1).

showed partial activity, consistent with a possible role of this residue in metal binding (Garber et al., 1998) (see below).

The C-terminal region of bCycT1 is sensitive to proteolysis

Given that the TRM of hCycT1 is proteolytically sensitive and probably conformationally flexible or exposed, we wished to determine whether the corresponding C-terminal region of bCycT1 displayed similar behavior despite the amino acid differences. We generated bCycT1 1–272 by *in vitro* translation as we were unable to produce any soluble form of bCycT1 by bacterial expression, and compared its proteolytic sensitivity to that of the corresponding hCycT1 1–272 fragment. The chymotryptic time courses for both were very similar, with a proteolytically stable approximately 29-kDa fragment seen even after 60 min of digestion (Fig. 6). A similar approximately 29-kDa fragment was seen after digestion of *in vitro* translated bCycT1 1–420, as also observed with hCycT1 (Fig. 6), further indicating that the C-terminal protease sensitivity did not result from the truncation at residue 272. Thus, it appears that the functionally active 1–272 fragments of both hCycT1 and bCycT1 are largely folded but contain a disordered or proteolytically susceptible region near their C-termini.

The N-terminal region of CycT1 is not required for Tat-mediated activation

Given that the proteolytically sensitive C-terminal region of hCycT1 also corresponds to the TRM, we attempted to model the position of the C terminus within the cyclin domain to help identify other regions that might influence the structure, and potentially the function, of the TRM. We aligned the sequence of CycT1 with those of three other cyclins (CycA, CycH, and CycM) whose structures are known (Brown et al., 1995; Kim et al., 1996; Schulze-Gahmen et al., 1999) and that are approximately the same length as CycT1 1–272 (Fig. 7). All three contain a conserved cyclin fold (two consecutive domains of five helices each) and an extra N-terminal helix, but have differing C-terminal structures and orientations of the termini (Fig. 7). In CycH, a C-type cyclin like CycT1, the N- and C-termini are in close proximity, and we hypothesized that the putative N-terminal helix of CycT1 might influence the disordered C-terminal region, perhaps even maintaining it in a proteolytically sensitive state. In CycH and CycM, the N-terminal helices also contact their Cdk partners (Andersen et al., 1997; Schulze-Gahmen et al., 1999). To examine the importance of this putative helix, we deleted the N-terminal 27 residues of hCycT1 (generating 28–272), and also of bCycT1 and mCycT1 to test whether deletion might unmask activity, and measured their ability to stimulate HIV Tat-mediated activation in the HIV LTR CAT reporter assay. The deletions neither diminished hCycT1 activity nor enhanced the activity of bCycT1 or mCycT1 (Fig. 5C),

suggesting that the N-terminal region is dispensable for TAR binding and Tat-mediated activation.

Discussion

We have characterized a hCycT1 fragment known to be sufficient for HIV Tat-mediated activation (residues 1–272) and found that the protein is largely helical, characteristic of a cyclin fold. Interestingly, residues 1–252 form a proteolytically resistant core whereas the C-terminal 20 amino acids, which includes the previously defined HIV Tat–TAR recognition motif (TRM) and Cys261 known to be essential for HIV Tat function (Bieniasz et al., 1998; Fujinaga et al., 1999; Garber et al., 1998), appear to be conformationally flexible or disordered. bCycT1, whose sequence we report here, contains Trp at position 261 and consequently does not function in HIV Tat-mediated activation; nevertheless, bCycT1 1–272 also shows a stable core and a flexible C terminus similar to the human protein.

The C-terminal region is the least conserved part of CycT1, consistent with a flexible structure. Of the nine amino acid differences between bCycT1 and hCycT1 within residues 1–272, four are in the C-terminal region between residues 261 and 265. Only the difference at position 261 appears functionally important, as mutation of Trp261 in bCycT1 to Cys generates a protein active for HIV Tat-mediated activation (Fig. 5B). Cys261 is not required for activation of the BIV LTR by BIV Tat or of the HIV LTR when Tat is delivered via the BIV Tat–TAR interaction, which occurs with high affinity in the absence of CycT1 (Barboric et al., 2000; Bogerd et al., 2000; Chen and Frankel, 1994). Thus, it appears that the critical importance of Cys261 is coupled to a role in HIV TAR RNA recognition, presumably mediated by interactions between the C-terminal TRM and HIV Tat. This is supported by cross-linking experiments in which residues within 252–261 of hCycT1 were found to crosslink to the HIV TAR loop in the context of ternary complexes with Tat (Richter et al., 2002b). It is not possible to definitively establish a role for bCycT1 in BIV Tat-mediated activation because BIV Tat functions in all cell types tested (bovine, human, murine, and lapine) and apparently can utilize any of these CycT1s, which share >79% sequence identity, or other cyclins to recruit Cdk9 and function in transcription (Barboric et al., 2000; Bogerd et al., 2000). Because BIV Tat binds with high affinity to BIV TAR in a β -hairpin mode independent of CycT1, it seems reasonable that the requirements for the Tat–CycT1 interface may be more relaxed than in the HIV complex.

There is some evidence that the flexible C-terminal region of CycT1, and Cys261 in particular, may participate in metal binding, at least in the HIV complex. Previous RNA-binding experiments demonstrated Zn-dependent formation of Tat–hCycT1–TAR complexes, leading to a

model in which a metal ion helped bridge the Tat–hCycT1 interface (Garber et al., 1998). Our mutagenesis results with bCycT1 support a role for position 261 in metal binding and formation of ternary complexes on HIV TAR. In particular, substitution of Trp261 with Cys rescued activation of the HIV LTR, as is also the case with mCycT1 (Bieniasz et al., 1998; Chen et al., 1999; Fujinaga et al., 1999; Garber et al., 1998; Kwak et al., 1999; Wei et al., 1998), and substitution with His, another potential Zn-binding ligand, rescued approximately 25% of the activity (Fig. 5B). The rescue of activity with His is interesting in that a similar substitution in mCycT1 did not support formation of HIV Tat–CycT1–TAR ternary complexes in vitro (Garber et al., 1998), perhaps indicating that metal-binding conditions or additional factors in vivo assist in complex formation. Besides Cys261, there are 6 other cysteines and 11 histidines within hCycT1 and bCycT1 1–272 that could serve as potential metal ligands. However, a recent study has shown that mutating any of these residues to alanine had no effect on HIV Tat activation (Koh et al., 2002), suggesting that they are not metal ligands, that individual mutations do not completely disrupt metal binding, or that their metal binding is not critical for Tat function.

The bridging interaction between hCycT1 and HIV Tat has been proposed to involve some of the seven cysteines in the Tat activation domain that are essential for activity and participate in the formation of metal-bridged homodimers in vitro (Frankel et al., 1988; Rice and Carlotti, 1990; Ruben et al., 1989). Interestingly, the activation domain of BIV Tat preserves the seven cysteines (Chen and Frankel, 1994) yet does not require Cys261 for activity, suggesting that interactions between BIV Tat and, presumably, the C-terminal region of bCycT1 are somewhat different than in the HIV complex. Alternatively, it is possible that Cys261 directly contacts HIV TAR and thereby contributes to loop-binding specificity.

Our proteolysis results suggest that the C-terminal regions of both hCycT1 and bCycT1 are conformationally flexible or disordered. Previous studies have shown that the HIV TAR loop in its unbound state is also flexible (Abouela et al., 1995; Huq et al., 1999), suggesting that mutually induced conformational changes may occur in Tat, CycT1, and TAR upon complex formation. It seems likely that any induced structural changes, now observed in many ribonucleoprotein complexes (see Frankel, 2000; Frankel and Smith, 1998; Leulliot and Varani, 2001; Williamson, 2001 for recent reviews), will differ in the HIV and BIV complexes, including possible metal-dependent interactions or conformations, highlighting the need for more detailed structural comparisons.

Conformational flexibility at the C terminus of cyclin domains may be a common theme, and these are often regions that interact with other proteins (Andersen et al., 1997; Kobayashi et al., 1992). Studies of CycA and CycH have reported disorder in the crystal structure at the end of

the helical cyclin domain or proteolytic degradation of 20–35 C-terminal residues (Brown et al., 1995; Kim et al., 1996; Schulze-Gahmen et al., 1999). It seems reasonable that this region of CycT1 is similarly disordered. In general, the cyclins encompass a wide family of proteins (for a review see Noble et al., 1997) defined by a cyclin box motif that displays relatively weak sequence identity but related patterns of hydrophobicity and helical propensity (Gibson et al., 1994). The cyclin fold is roughly defined as two repeats of 5-helix bundles, with the pair often flanked by additional helices (Fig. 7). The first repeat (the cyclin box) contains amino acids required for binding target kinases or maintaining the cyclin fold, and these are often the most, albeit weakly, conserved residues (Andersen et al., 1996, 1997; Brown et al., 1995; Card et al., 2000; Kim et al., 1996; Kobayashi et al., 1992; Russo et al., 1996). The sequences of the second repeat are even more variable, leading to a variety of tertiary arrangements as demonstrated by the structures of the cell cycle regulator CycA (Brown et al., 1995; Russo et al., 1996), transcription factor CycH (Andersen et al., 1996; Kim et al., 1996), and γ -herpesvirus CycM (Card et al., 2000) (Fig. 7). The relative orientation of the repeats, the secondary structure of the regions surrounding the repeats, and the relative orientations of the terminal helices are quite different.

The diversity of cyclin sequence and structure prevents us from developing a good homology-based model of CycT1. hCycT1 was first described as a C-type cyclin based on its sequence similarity to cyclin Pcht1(+) from *S. pombe* (Wei et al., 1998), and thus the structure of CycH (C-type) was previously used to model the possible position of Cys261 (Garber et al., 2000; Taube et al., 2000). Both studies placed Cys261 within the ‘extra’ helix C-terminal to the second repeat, which also located it near the N-terminal helix. This positioning was consistent with a comparison between equine CycT1 and hCycT1 that identified N-terminal residues required for equine infectious anemia virus (EIAV) Tat function (Taube et al., 2000), although another study has reported conflicting results (Albrecht et al., 2000). Nonetheless, it seems difficult to draw strong conclusions about the position of the C-terminal TRM in hCycT1 based on weak cyclin sequence alignments and variable structural arrangements (Fig. 7), and indeed, deletion of the presumed N-terminal helix does not affect hCycT1 activity (Fig. 7). More studies are needed to better position Cys261 and the TRM within the hCycT1 structure.

Recent studies have shown that CycT1 plays a broader role in regulating transcription elongation by binding the small 7SK RNA in addition to CDK9 (Nguyen et al., 2001; Yang et al., 2001). It will be of interest to determine whether 7SK recognition is related to TAR recognition and whether similar regions of CycT1, perhaps including the C-terminal region, are involved in both RNA and protein interactions.

Materials and methods

Plasmid construction

For CycT1 expression in bacteria, PCR fragments were cloned into the *NcoI* and *EcoRI* sites of pET21d (Novagen). Previously described pHIV LTR CAT reporter and pSV2tat72 expression vectors (Frankel and Pabo, 1988; Sodroski et al., 1985) were used for Tat activation assays in mammalian cells. PCR fragments encoding the human, bovine, and mouse CycT1 proteins (residues 1–272 or 28–272) were cloned into the *HindIII* and *EcoRI* sites of pcDNA3 (Invitrogen) and used for CycT1 expression. PCR fragments for the human and mouse clones were amplified from pGEX-2T expression vectors (kindly provided by Dr. K.A. Jones) (Garber et al., 1998), and fragments for the bovine clones were amplified from the cDNA phage stocks described below. Site-directed mutants of bCycT1 were generated using PCR primers containing the appropriate mutations. The sequences of all inserts were confirmed (Biomolecular Resource Center, UCSF).

Protein expression and purification

The pET21d expression vectors for hCycT1 and bCycT1 were transformed into BL21(DE3) cells, single colonies were inoculated into Luria broth (LB) containing 50 µg/ml ampicillin, cultures were grown at 30 °C, and protein expression was induced at OD₆₀₀ = 0.8 by adding 0.1 mM IPTG for 6 h. Cells were lysed by sonication in 80 ml (per l of culture) lysis buffer containing 20 mM Tris–Cl pH 8.0, 30 mM NaCl, 250 mM ammonium sulfate, 10% glycerol, 0.5% NP-40, 5 mM DTT, and 1 mM PMSF, and centrifuged. As described in Results, bCycT1 was insoluble and could not be purified further in its native form. For hCycT1 (residues 1–272), the crude supernatant was applied to a Butyl fast flow hydrophobic column (Pharmacia) equilibrated with lysis buffer, the column was washed with 20 column volumes of lysis buffer, and protein was eluted in buffer A (20 mM Tris–Cl pH 8.0, 30 mM NaCl, 5 mM DTT, 10% glycerol). hCycT1 eluted as a single peak which was dialyzed into buffer A, applied to a Q-Sepharose fast-flow column (Pharmacia), and eluted in a 30–250 mM NaCl gradient in buffer A. hCycT1 eluted at approximately 50 mM NaCl and was >95% pure.

Protein characterization

The purity of hCycT1 1–272 was estimated on Coomassie blue-stained sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), and protein concentrations were measured by Bradford assays (Biorad). N-terminal sequences of hCycT1 or proteolytic fragments were determined by Edman degradation of samples transferred to PVDF membranes (Dr. Christoph Turck, Howard Hughes Medical Institute, UCSF). Oligomeric state was assessed by analytical size exclusion

chromatography on a Sephacryl S-100 column (Pharmacia). hCycT1 (100 µg) was loaded and eluted from the column in buffer A, and the molecular weight was estimated by comparing elution volumes to size exclusion standards (Biorad).

Mass spectra were acquired using a Voyager-DE MALDI/TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser ($\lambda = 337$), single stage reflector, and timed ion selector, at an ion path length of 1.2 m. Samples were collected in positive ion-linear mode with delayed extraction, using an accelerating voltage of 20 K and 140 ns extraction delay time. hCycT1 samples in 20 mM Tris–Cl buffer (pH 8.0), 30 mM NaCl, 0.5 mM DTT, and 10% glycerol were diluted to approximately 1 µM in 10 mg/ml sinapinic acid, 0.3% TFA, and 50% acetonitrile and dried on the sample target. Three spectra were acquired for each sample and the values reported are averages (\pm standard deviation), using bovine α -chymotrypsinogen $[M + H]^+ = [25666]$ (average) and *S. cerevisiae* alcohol-dehydrogenase $[M + H]^+ = [36685]$ (average) as external calibration standards. Theoretical molecular masses for hCycT1 1–272 and proteolytic fragments (see below) were calculated from their amino acid sequence using an internet-based calculator (Swiss Institute of Bioinformatics; http://www.expasy.ch/tools/pi_tool.html).

Circular dichroism (CD) spectra were recorded on an Aviv 62DS spectropolarimeter at 4 °C in 10 mM K₂HPO₄ (pH 7.5) buffer and 70 mM KF (CD buffer). Ellipticity was averaged for 4 s at 1-nm intervals. Helical content was estimated from the mean residue ellipticity at 222 nm (Chen et al., 1974). For thermal denaturation experiments, samples were equilibrated by stirring for 1 min at 1 °C intervals from 4 °C to either 50 or 70 °C using thermoelectric temperature control. For chemical denaturation experiments, aliquots of 8 M guanidinium hydrochloride (ultrapure, Sigma) were added to hCycT1 1–272 initially at 0.1 mg/ml, and the sample was equilibrated after each addition by stirring at 4 °C for >2 min. Ellipticity values at 222 nm were corrected for protein dilution, and protein stability was calculated by linear extrapolation of the melting curve to zero guanidinium (Schmid, 1989).

Partial proteolysis

Proteolysis of purified hCycT1 was performed in CD buffer by incubating 0.79 nmol hCycT1 (24 µg) at 22 °C with 0.4 µg of α -chymotrypsin (TLCK treated, Sigma), trypsin (TPCK treated, Boehringer Mannheim), or subtilisin (Boehringer Mannheim). At each time point, one-tenth of the reaction mixture was removed and quenched by adding SDS-PAGE buffer and heating at 90 °C for 5 min, and analyzed on gels. To prepare proteolyzed samples for mass spectrometry, chymotryptic reactions were carried out in 20 mM Tris–Cl buffer (pH 8.0), 30 mM NaCl, 0.5 mM DTT, and 10% glycerol at approximately 1:100 chymotrypsin/hCycT1, and the reactions were quenched at appropriate

times by adding a sinapinic acid mixture and drying on sample plates. Partial proteolysis was also performed on *in vitro*-translated hCycT1 and bCycT1 proteins. Proteins were synthesized in coupled rabbit reticulocyte transcription–translation reactions by adding 40 μ l of the premixed cocktail (Invitrogen) to 2 μ l of 35 S-labeled methionine and 0.5–1 μ g of template DNA and incubating at 30 °C for 2 h. Partial proteolysis was performed with 5 μ l of protein translation mixture and 0.04 μ g of chymotrypsin, and 0.95- μ l aliquots were removed and quenched as above at appropriate time points.

bCycT1 cloning

Initial PCR primers were designed based on highly conserved nucleotide sequences within hCycT1 and mCycT1 (5'-CCG GAT CCA TAT GGA GGG AGA GAG GAA GAA C-3' and 5'-AAC TGG ATC CTC ACT CAC TAG TCC GAT GAC CCT G-3'). A 1069-nt fragment near the 5' end of the gene was amplified from a bovine cDNA library (Clontech) using TaqPlus (Stratagene) and cloned into pPCRscript SK(+) (Stratagene). This partial cDNA clone was sequenced and found to encode a novel CycT1 homolog. A radioactive probe was prepared from the clone and used to screen a size-selected bovine cDNA phage library (Clontech). Positive plaques were isolated and their inserts amplified using PCR primers based on the vector sequence and internal sequence of bCycT1. A clone containing the largest N-terminal sequence (nts 1–1651) was then used to generate another hybridization probe targeted to the C terminus, the phage library was re-screened, and a clone containing the remaining C-terminal sequence (nts 1051–2181) was isolated. The full-length bCycT1 gene was constructed from these two cDNA clones (Genbank Accession No. AY428555).

Transient transfection and CAT assays

NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml), and grown in 12-well plates to approximately 85% confluence for transfection experiments. Reporter and expressor plasmids (typically 20 ng of the pHIV-LTR-CAT reporter, 100 ng of the pSV2tat72 expressor, and varying amounts of the pcDNA3 CycT1 vectors) were mixed with a carrier Bluescript plasmid (adjusted to 1 μ g total DNA) in 250 μ l Optimem low serum medium (BRL). A mixture of Lipofectamine (Invitrogen) and Optimem [at a 1:62.5 (v/v) ratio in 250 μ l] was incubated at room temperature for 45 min, added to the DNA mixture, and incubated for an additional 15 min. DNA was then added to cells that had been grown in DMEM/10%FBS, washed with PBS, pre-incubated in Optimem at 37 °C for 10 min, and washed again with phosphate-buffered saline (PBS). After incubating at 37 °C for 4 h, the medium was removed and fresh

DMEM/10%FBS was added. Cells were grown for an additional 48 h and harvested for CAT assays as described previously (Chen and Frankel, 1994).

Sequence alignment and molecular graphics

The alignment of multiple cyclin sequences (obtained from the Swiss Prot database; <http://www.ebi.ac.uk/swissprot/>) was performed using ClustalW (1.8) with default parameters (European Bioinformatics Institute; <http://www.ebi.ac.uk/clustalw/>). Atomic coordinates of cyclin A (1FIN), cyclin H (1KXU), and cyclin M (1F5Q) were obtained from the Protein Data Bank (<http://www.rcsb.org/pdb/>), with cyclin A coordinates extracted from a cyclin A-Cdk2 complex. Structures were visualized using the program Molmol (Koradi et al., 1996) and aligned using the “Fit” command with default parameters.

Acknowledgments

We thank Katherine Jones for kindly providing hCycT1 and mCycT1 clones and expression vectors, Maria Gadzinski and Yan Sun for help with experiments, Christoph Turck for N-terminal sequencing, Ami Bhatt, Koh Fujinaga, Baruch Harris, Doug Jeffrey, Muluye Liku, Robert Nakamura, and David Steger for helpful experimental suggestions, and Valerie Calabro, Baruch Harris, Stephen Landt, Wendell Lim, Robert Nakamura, and Brian Yeh for comments on the manuscript. This work was supported by NIH grant AI29135 (A.D.F.), CSU Affirmative Action Faculty Development Award (L.C.), NIH postdoctoral fellowship GM65036 (S.P.E.), National Science Foundation Graduate Research fellowship (C.D.), and an American Foundation for Pharmaceutical Education fellowship (C.D.).

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