Expression, Purification and Characterization of hCyclinT the essential cellular Hostfactor for the highly specific HIV−1 Tat−TAR Interaction

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ABSTRACT

Transcriptional transactivation of the human immunodeficiency virus type 1 (HIV−1) long terminal repeat (LTR) promotor element by interaction of virus encoded transactivator protein (Tat) and the TAR−RNA requires recruitment of the cellular hCyclinT1, an essential component of P−TEFb [1]. Up to now no structural data for CyclinT1 do exist and only predictive models based on *in vitro* and *in vivo* experiments for the ternary complexes of Tat, CyclinT1 and TAR of different lentiviral systems are available [2]. Residues 1−272 in the 726 amino acids comprising hCyclinT1 (hCycT1−272Δ) are sufficient to mediate the Tat−TAR interaction [3,4].

We report here the overexpression in *E. coli* and the purification to apparent homogeneity of recombinant hCycT1−272Δ. Using a purification protocol which requires only two to three days it is possible to purify up to 2.5 mg recombinant hCycT1−272Δ per 1 g wet cells. Recombinant hCycT1−272Δ is biologically active and shows the expected α−helical CD−spectrum as well as a 1D−NMR spectrum of a mainly α−helical protein of more than 30 kDa. Large quantities (even isotopically labeled) hCycT1−272Δ for further structural studies of the protein alone or in its complex with Tat and TAR are now available for experiments that lead to a better understanding of lentiviral replication and build a basis for the construction of new potential viral inhibitors.

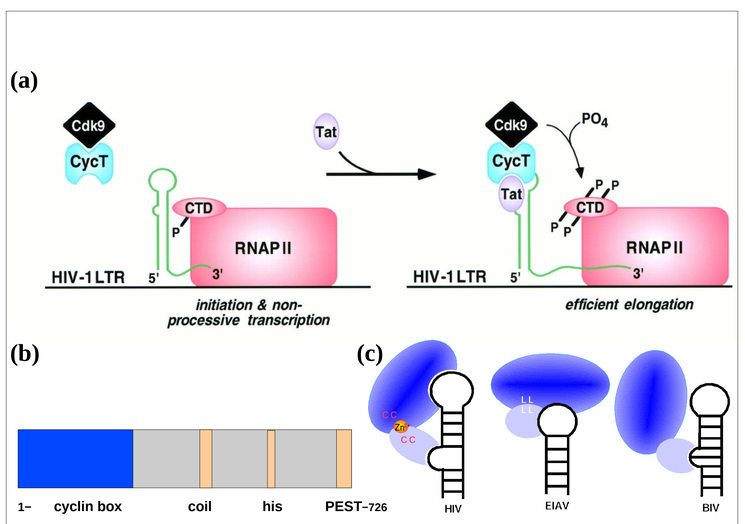

Figure 1: Simplified model [5] of hTat action during HIV replication **(a)**. Sequence domains of hCyclinT 1 **(b)** and predictive models [2, 3] of the ternary complexes between Tat, Cyclin T1 and TAR of different lentivital systems **(c)**.

INTRODUCTION

Cyclins are well known as the positive regulatory subunits of cyclin−dependent kinases (CDK). By binding to the catalytic subunits of CDKs, they play a crucial role in the coordination of the eukaryotic cell cycle [6]. Cyclins share distant sequence homology over a 100 amino acid region called the cyclin box. Up to now 3−dimensional X−ray structures of cyclin A , cyclin H and a viral encoded cyclin from herpesvirus saimiri have been determined uncomplexed, complexed with CDK or in a ternary complex including a CDK inhibitor. Cyclin H was also identified as a subunit of the general transcription factor TFIIH, a multiprotein complex involved in transcription by RNA polymerase II (RNAPII) [7]. Therefore, participation of cyclins and their specific CDKs seems to be not only a common theme in cell cycle control but even on the level of transcriptional regulation. Remarkably, the recruitment of cellular human CyclinT1 is an essential step during the transcriptional transactivation of the human immunodeficiency virus type 1 (HIV−1) long terminal repeat (LTR) promotor element [1]. HIV itself encodes a nuclear transcriptional activator, Tat, which acts together with its cis acting transactivating response element (TAR) to enhance the processivity of RNAPII complexes that would otherwise terminate viral transcription prematurely (Fig. 1a). In the absence of hCyclinT1, however, Tat and TAR do not develop transactivating activity and no efficient copying of the viral genom can be observed. Residues 1−272 in the 726 amino acids comprising hCyclinT1 (hCycT1−272Δ) are sufficient to mediate the above described Tat−TAR interaction (Fig. 1b). Up to now, no structural data for CyclinT1 do exist, and only predictive models based on *in vitro* and *in vivo* experiments for the ternary complexes of Tat, CyclinT1 and TAR of different lentiviral systems are available (Fig. 1c).

A potent protocol for the production and purification of the relevant hCycT1 −272Δ fragment in *E. coli* was established in this work, one of the key requirements for further structural studies.

RESULTS

Structural studies demand high amounts of purified protein. For the characterization of protein structures by NMR techniques additionally large amounts of isotopically labeled forms of the protein are required. To keep the costs reasonable, overexpression is preferably done in *E. coli* cells, which can be grown in isotopically enriched minimal media.

In this work we present a purification protocol for the amino−terminal 272 amino acids of hCyclinT1, which mediate transcriptional transactivation of the HIV−1 LTR. Cyclins are not particulary stable, an observation made for a variety of eukaryotic transcription factors. Therefore we avoided preparative methods to recover hCycT1−272Δ from cytoplasmatic inclusion bodies that have to be solubilized by denaturation, followed by a not yet established refolding protocol. As the use of three different affinity−tags (His−, GST−, Strep−tags) posed a variety of unforeseen problems, we decided to develop a protein purification scheme for native hCycT1−272Δ testing »classical« techniques such as, e.g., hydrophobic interaction chromatography (HIC). A good overproduction of the protein using the expression vector pET11a could be obtained (Fig. 2a, lanes 1−4).

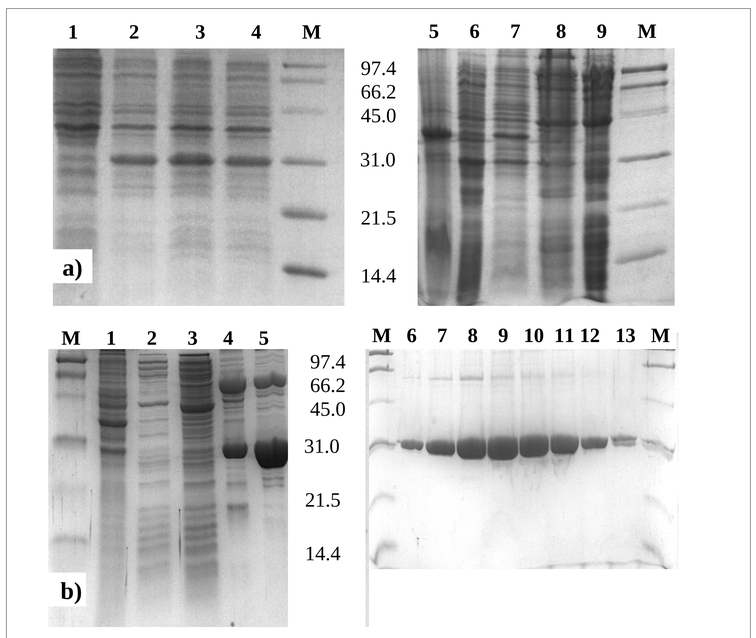

Figure 2: Purification of native recombinant hCycT1−(272Δ

a) hCycT1−272Δ was overexpressed using the pET11a vector system and *E. coli* BL21(DE3) as host strain (lane 1 − 4 are before and 1 h, 2 h and 3 h after induction with 1 mM IPTG, respectively). The cell extract was divided into an insoluble (lane 5) and a soluble fraction (lane 6) which was subsequently used for ammonium sulphate precipitation (lane 7−9, 35 %, 45% and 55 % ammonium sulphate saturation, respectively).

b) Hydrophobic interaction chromatography resulted in hCycT1−272Δ with apparent homogeneity. Proteins which precipitated at 35 % ammonium sulphate were applicated on a butyl−sepharose fast flow column (lane 1) using 0.25 M ammonium sulphate in the starting buffer. hCycT1−272Δ binds tightly to the butyl moieties as no significant amounts of the protein can be detected in the flow through (lane 2) and wash fractions (lane 3). With a decreasing gradient from 0.25 to 0 M ammonium sulphate most of the bound cellular proteins can be eluted, whereas parts of hCycT1−272Δ coelute under these conditions (lane 4 and 5 after 60 and 70 min, respectively). Pure hCycT1−272Δ can be eluted subsequently using an increasing gradient from 0 % to 30 % isopropanol (lanes 6−13 after 110, 120, 125, 130, 135, 145, 160 and 170 min).

In spite of the observed low salt tolerance of hCycT1−272Δ, HIC was tested as a purification step. Our working assumption was, if hCycT1−272Δ shows intrinsic hydrophobic properties perhaps small amounts of ammonium sulphate may be sufficient for mediating binding to certain HIC media. Additionally, a fast ammonium sulphate precipitation was performed prior to HIC in order to remove at least parts of the crude extract proteins (Fig. 2a, lanes 7−9). Best results could be observed with butyl−sepharose as media and the unusual low concentration of 0.25 M ammonium sulphate as start point. Even under this low salt conditions about 15 % isopropanol was neccessary to elute the 31 kDa protein from the column (Fig. 2b, 3). This protein showed good solubility in the eluat fractions after HIC, the isopropanol was removeable via dialysis and the protein could be concentrated without aggregation up to a final concentration of about 0.1 mM/ml.

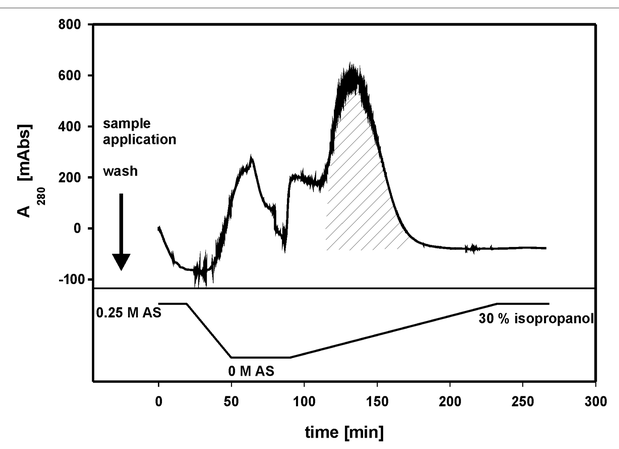

Figure 3: Elution profile of hCycT1−272Δ during HIC on butyl−sepharose fast flow. Fractions containing hCycT1−272Δ with a purity over 95 % (shaded) were pooled.

N−terminal sequencing of the protein resulted in MEGERKNNNKRWY, the N−terminal sequence of hCycT1−272Δ.

To further characterize the obtained hCycT1−272Δ, an aliquot was subjected to a gel filtration column (Fig. 4). hCycT1−272Δ behaves in solution like a monomer of approximately 30 kDa, in agreement with the expected molecular weight.

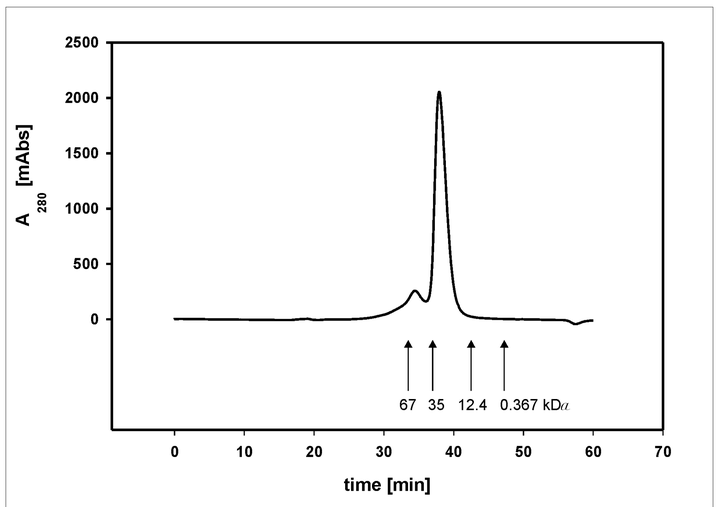

Figure 4: Analysis of hCycT1−272Δ by SEC using a Superdex 200 HR26/60 column. The elution times of calibration proteins (BSA: 67 kDa, β−lactoglobulin: 35 kDa, cytochrom C: 12.4 kDa and CMP: 0.367 kDa) under the same buffer conditions are indicated.

In order to demonstrate the integrity of the recombinant hCycT1−272Δ fragment spectroscopic methods as well as biological test systems were applied. Figure 5a shows the circular dichroism (CD) spectrum of hCycT1−272Δ, which is very similiar to that of Cyclin H. Even the 1D−NMR spectrum is typical for a structured but mainly α−helical protein of more than 30 kDa (Fig. 5 b).

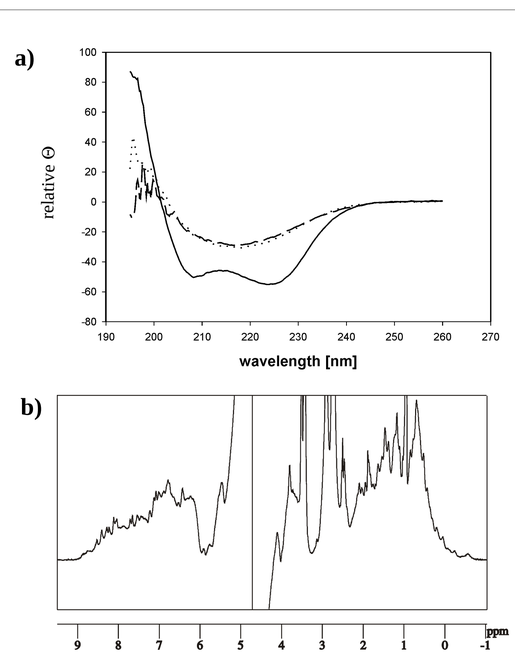

Figure 5: Spectroscopic characterization of hCycT1−272Δ

a) The CD−spectrum of hCycT1−272Δ is typical for an α−helical protein with minima at 208 and 222 nm and an intense positve signal at 195 nm. Heating to 95°C resulted in significant loss of the α–helical character of the protein (dotted line), a process which could not be reversed by cooling to room temperature (broken line). b) 1D−NMR spectrum of hCycT1−272Δ recorded on a Bruker DRX600 spectrometer.

The biological activity of the purified hCycT1−272Δ fragment was tested using a green fluorescence protein (EGFP) reporter system. Murine 3T3 cells which were cotransfected with plasmids pEGFP−HIV−1−LTR and pcDNA−HIV−1−tat alone did not show any fluorescence, whereas addition of plasmid pSV40−CycT1−272 (as positive control) or recombinant hCycT1−272Δ protein (via scrape loading) resulted in a significant number of fluorescent cells.

ACKNOWLEDGEMENT

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