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Leishmania amazonensis: Partial purification and study of the biochemical properties of the telomerase reverse transcriptase activity from promastigote-stage

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ABSTRACT

Telomeres are protein–DNA complexes that protect chromosome ends from degradation and fusion. In *Leishmania* spp., telomeric DNA comprises a conserved TTAGGG repeat and is maintained by telomerase. Telomerase is a multisubunit enzymatic complex that ensures the complete DNA replication by adding new telomeric repeats to the G-rich strand. In this report we aimed to purify and study the biochemical properties of *Leishmani amazonensis* telomerase. In a first trial we used affinity chromatography with antisense 2′-*O*-methyl oligonucleotide without success since the *Leishmania* telomerase, similarly to *Trypanosoma cruzi* enzyme, was not eluted by competition, but instead, it remained bound to the column. Partially purified *L. amazonensis* telomerase activity was achieved by fractionation of extracts on complementary ion exchange and Heparin columns. Further purification of these fractions on a G-rich telomeric DNA affinity chromatography enriched for telomerase activity. The knowledge of telomerase characteristics in *Leishmania* could help to develop new strategies to overcome leishmaniasis.

1. Introduction

Telomeres are the protein–DNA complexes that form the ends of eukaryotic chromosomes. The structure and integrity of telomeres are essential for genome stability since telomere dysregulation can lead to cell death, cell senescence, or abnormal cell proliferation (Blackburn, 2005; Autexier and Lue, 2006).

In most eukaryotes, telomeres are maintained by the action of the ribonucleoprotein enzyme telomerase (Greider, 1998), which adds new telomeric repeats to the 3′ G-rich overhang, thus enabling DNA polymerase to synthesize the complementary strand (Blackburn, 2005). By contrast, some insects rely on retrotransposon-mediated recombination to replace DNA at chromosome termini and 5–10% of human tumors replicate telomeres using a telomerase-independent mechanism known as ALT (alternative lengthening of telomeres) (Pardue and DeBaryshe, 2003; Reddel, 2003). Absence of telomerase activity compromises telomere length maintenance from yeast to man eventually preventing cell divisions (Lundblad and Szostak, 1989; Harley et al., 1990; Singer and Gottschling, 1994; Miller and Collins, 2000; Dreesen et al., 2005).

Telomerase activity was first purified from the ciliate *Euplotes aediculatus* (Lingner and Cech, 1996). In addition, many studies have shown that enzyme activity is present in extracts from *Tetrahymena thermophila* (Greider and Blackburn, 1985), trypanosomatids (Cano et al., 1999) and yeast (Cohn and Blackburn, 1995), and that it is required for indefinite proliferation of the cell population. In trypanosomatid parasitic protozoa, enzyme activity was previously detected in cell extracts of one developmental stage of *Trypanosoma brucei* and of two *Leishmania* species, and from different *Trypanosoma cruzi* and *Plasmodium falciparum* replicative stages (Cano et al., 1999; Muñoz and Collins, 2004; Bottius et al., 1998; Aldous et al., 1998). In multicellular organisms, like humans, telomerase can be detected in extracts of highly proliferative cells from early embryogenesis, germ line and a subset of epithelial and lymphoid progenitors, but enzyme activity declines with age (Forsyth et al., 2002). Telomerase is also upregulated in about 85–90% of human tumors, but it is repressed in most somatic tissues and in some adult stem cells (Kim et al., 1994; Serakinci et al., 2008). Since telomerase is consistently activated in cancer cells and in single-celled organisms, it is believed to be a valid target for the development of new therapeutics (Mergny et al., 2002).

Telomerase is minimally composed of a catalytic subunit (TERT, telomerase reverse transcriptase) and an intrinsic RNA component (TER, telomerase RNA). TERT is a specialized cellular ribonucleoprotein (RNP), which has a reverse transcriptase (RT) homology domain as well as other essential conserved domains (Blackburn,

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2005). TERT elongates telomeres by copying a short template sequence, a region complementary to 1.2–1.9 telomeric repeats, within the RNA component. TER is bound at high affinity to conserved TERT domains that lie outside the RT homology domain. These and the interactions between TERT, TER and other enzyme subunits are critical to telomerase function, activity and processivity (Blackburn, 2001; Lue, 2004).

Despite the taxonomic distance, the composition and structure of telomerase from yeast to man is very similar (Collins, 1999; Cong et al., 2002; Chen and Greider, 2004; Smogorzewska and de Lange, 2004; Harrington, 2005; Sýkorová and Fajkus, 2009). Some of the TERT subunits described in protozoa, which includes *Leishmania amazonensis* TERT (LaTERT), are the largest telomerases described so far and show greater homology with each other than with the proteins of other eukaryotes (Giardini et al., 2006; Sýkorová and Fajkus, 2009).

In the present report, we study the biochemical properties and show the partial purification of enzyme activity from *L. amazonensis* promastigotes. First we tried to purify *L. amazonensis* telomerase activity from an affinity column containing an antisense 2′-*O*-methyl oligonucleotide, complementary to the putative template region of trypanosome telomerase RNA component and similar to the *L. amazonensis* 3′G-rich overhang (Cano et al., 1999; Muñoz and Collins, 2004; Conte and Cano, 2005). But, in resemblance to *T. cruzi* telomerase activity (Muñoz and Collins, 2004), the *L. amazonensis* enzyme showed high affinity for the antisense oligonucleotide and remained bound to the column beads even in the presence of excess of competitor. In contrast, enzyme activity was semi-purified from S100 and nuclear extracts fractionated on complementary chromatography columns. More effective enzyme purification was achieved when these semi-purified fractions were further fractionated on a G-rich telomeric DNA affinity column (G-DNA). The importance of uncovering the enzymatic properties of *L. amazonensis* telomerase from promastigote-stage is discussed.

2. Materials and methods

2.1. L. amazonensis promastigote cultures

Promastigotes of *L. amazonensis* strain MHOM/BR/73/M2269 were cultivated in exponential growth in Schneider's Insect medium (Sigma), supplemented with 10% heat inactivated fetal bovine serum (Cultilab) and 1× antibiotic/antimycotic solution (Invitrogen) at 28 °C.

2.2. Preparation of L. amazonensis S100 and nuclear extracts

Approximately 1.2×10^{11} promastigote cells collected by centrifugation were washed in ice cold phosphate-buffered saline supplemented with 2% glucose (PBS-G), and resuspended in buffer A (20 mM Tris–HCl pH 7.5, 1 mM EGTA pH 8.0, 1 mM EDTA pH 8.0, 1 mM spermidine, 0.3 M spermine and 5 mM 2-mercaptoethanol) containing 1× protease inhibitor cocktail SetIII (Calbiochem) followed by incubation on ice for 30 min. The S100 extracts were prepared as described in Cano et al. (1999). For nuclear extracts, cells resuspended in buffer A were homogenized using a Douncer homogenizer and centrifuged at 12,000 rpm for 20 min. The resulting pellet was incubated on ice in buffer D (50 mM Tris–HCl pH 8.0, 20% glycerol, 10% sucrose, 0.42 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT and 1× protease inhibitor cocktail) for 30 min under mild agitation. The suspension was cleared by centrifugation at 4 °C for 1 h and 30 min in a Beckman 90Ti rotor at 100,000 *g*. The recovered supernatant was dialyzed against 1× TMG buffer (10 mM Tris–HCl pH 8.0, 1.2 mM MgCl2 and 10% glycerol) and referred as nuclear extract. Protein concentration was determined by the Bradford method (Bio–Rad).

2.3. Partial purification of telomerase activity using complementary chromatographic columns

Approximately 20 mg of protein obtained from promastigote S100 or nuclear extracts were equilibrated with 300 mM KCl in 1× TMG and applied onto a DEAE–sepharose column also equilibrated with 300 mM KCl in 1× TMG. Enzyme activity was eluted with step salt gradient at 400–800 mM KCL in 1× TMG. Column fractions were desalted to 50–60 mM KCl in 1× TMG, concentrated in YM-Amicon 30 filter membranes (Millipore) and tested for telomerase activity by modifications of both One-tube and Two-tube TRAP assays (Cano et al., 1999). Fractions containing telomerase activity (~700 μg of proteins) were pooled and loaded onto a 1 ml Heparin HiTrap column (GE Healthcare). Telomerase activity was eluted with 120–450 mM KCl in 1× TMG. These protein fractions (~250 μg of proteins) were pooled, desalted to 50–60 mM KCl in 1× TMG and concentrated in YM-Amicon 30 filter membranes. Fractions obtained from different purification events were used as the input fractions for the 2′-*O*-methyl oligonucleotide and the G-DNA affinity columns.

2.4. Partial purification of telomerase activity using a G-rich telomeric DNA affinity column

The telomerase positive fraction obtained from complementary chromatographies in DEAE and Heparin columns (~250 μg of proteins) was loaded onto an affinity column containing gel-purified 5′-biotinylated G-rich telomeric oligonucleotides (TelG-DNA). This DNA sequence was designed with a linker between the 5′ biotin and the telomeric sequence (Table 1). Telomerase was eluted with 300 mM KCl in 1X TMG supplemented with 0.5 mM DTT and 20U RNaseOUT (Invitrogen). Protein concentration in this affinity purified fraction was about 62 μg total.

2.5. Antisense 2′-O-methyl oligonucleotide-based affinity purification of telomerase activity

Protein fractions with telomerase activity obtained from complementary purification of nuclear extracts in DEAE and Heparin columns (~250 μg of proteins) were loaded on an antisense affinity purification column. The column ligand was a 5′-biotinylated chimerical oligonucleotide containing an antisense 2′-*O*-methyl oligonucleotide sequence complementary to three repetitions of the putative TbTER and TcTER template regions and similar to the *L. amazonensis* 3′G-overhang (Cano et al., 1999; Muñoz and Collins, 2004; Conte and Cano, 2005; Table 1). This oligonucleotide was designed with a DNA linker between the 5′ biotin and the antisense 2′-*O*-methyl oligonucleotide sequence, which does not share homology to any sequence in the *Leishmania* spp. genome (data not shown). Telomerase was eluted by competition using a displacement oligonucleotide (DISPL1, Table 1) complementary to the entire chimerical 5′-biotinylated oligonucleotide as previously described (Schnapp et al., 1998; Muñoz and Collins, 2004). The protein content was determined using the Bradford method. Beads containing non-eluted telomerase bound to the antisense 2′-*O*-methyl oligonucleotide were also tested for telomerase activity.

2.6. Telomerase activity measured by One-tube TRAP assay

Telomerase activity was assayed using the modified PCR-based telomerase assay named One-tube TRAP assay according to Cano et al. (1999). The amount of protein in the DEAE semi-purified extracts used in each reaction was around 0.1–1 μg. Proteins were