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Ribonucleotide reductase is regulated via the R2 subunit during the life cycle of *Trypanosoma brucei*

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Abstract We have examined the occurrence of the R1 and R2 subunits of ribonucleotide reductase during the life cycle of *Trypanosoma brucei*. Whereas the R1 protein is present throughout the life cycle, the R2 protein is not found in cell cycle-arrested short stumpy trypanosomes. RT-PCR/hybridization analysis revealed almost equal amounts of the *R1* and *R2* mRNAs in all life cycle stages of the parasite. The data indicate that ribonucleotide reductase of African trypanosomes is developmentally controlled by post-transcriptional regulation of the R2 subunit.

Key words: Ribonucleotide reductase; Differentiation; Life cycle; Cell cycle; *Trypanosoma brucei*

1. Introduction

Trypanosoma brucei, the causative agent of African sleeping sickness, has a complex life cycle during which it shuttles between stages in the mammalian host and the tsetse fly vector, and between proliferating and non-proliferating forms [1]. In the blood and tissue fluids of the mammalian host the parasite occurs as rapidly multiplying long slender forms which differentiate into cell cycle-arrested short stumpy forms at the peak of parasitemia. The short stumpy trypanosomes are preadapted to the life in the insect vector. After ingestion of a blood meal by the tsetse fly, short stumpy parasites differentiate into dividing procyclic trypanosomes which colonize the insect midgut. The differentiation from bloodstream into insect forms is accompanied by a switch in energy metabolism from glycolysis to oxidative phosphorylation and by a change of surface antigens from the variant surface glycoprotein (VSG) to the procyclic acidic repetitive protein (PARP) (for a review see [1]). In vitro, differentiation into procyclic forms can be easily achieved by culturing short stumpy trypanosomes at 27°C in the presence of the tricarboxylic acid cycle intermediates citrate and *cis*-aconitate [2–4].

Ribonucleotide reductase plays a central role in DNA synthesis by catalyzing the conversion of nucleoside diphosphates into deoxyribonucleotides. Most eukaryotes contain a class I ribonucleotide reductase which is a tetramer composed of each two subunits R1 and R2 [5–7]. The large R1 subunit harbors the active site as well as effector binding sites, the small R2 subunit contains a tyrosyl radical essential for catalysis. The genes encoding the *T. brucei* R1 and R2 proteins have been cloned and overexpressed in *Escherichia coli* [8,9]. The parasite proteins show about 60% identities with ribonucleotide reductases of other eukaryotes [8,9].

In this paper, we studied the life cycle and cell cycle regulation of ribonucleotide reductase in *T. brucei*.

2. Materials and methods

2.1. Trypanosomes

The pleomorphic *T. brucei* variant clone AnTat 1.1 [10] was used throughout this study. Bloodstream form trypanosomes were grown in NMRI mice and purified by DEAE-cellulose chromatography [11]. Long slender and short stumpy trypanosomes were harvested 3 and 5 days after infection, respectively. For differentiation into procyclic trypanosomes, purified short stumpies were seeded at a density of 5×10^6 cells/ml in SDM-79 medium [12] supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 3 mM citrate/*cis*-aconitate following incubation at 27°C [2–4] in closed culture flask. Established procyclic cultures were maintained in SDM-79 medium plus serum but in the absence of citrate/*cis*-aconitate. For immunoblotting, trypanosomes (6×10^7 cells/ml) were suspended and lysed in 50 mM HEPES, 2.5 mM EDTA, 2 mM EGTA, pH 7.0 containing 200 μM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 400 μM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 2 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), 1 μM pepstatin A (lysis buffer [13]) on ice until no intact cells could be observed microscopically. SDS was added to a final concentration of 1% and the protein concentration of the cell lysates was determined using the microwell plate bicinchoninic acid protein assay (Pierce).

2.2. Western blot analysis

SDS–PAGE and Western blotting were performed as previously described [13,14]. The blots were developed using an ECL (enhanced chemiluminescence) system as described by the manufacturer (Amersham Pharmacia Biotech). Polyclonal rabbit antibodies against recombinant R1 and R2 subunits of *T. brucei* ribonucleotide reductase were produced by Eurogentec. Polyclonal antibodies against AnTat 1.1 variant surface glycoprotein (VSG AnTat 1.1) were raised in BALB/c mice. The VSG AnTat 1.1 used for immunization was purified from trypanosomes as previously described [15]. Rabbit antiserum against *T. brucei* aldolase was kindly provided by Prof. Christine Clayton, ZMBH, Heidelberg, Germany. The monoclonal antibodies against *T. brucei* major lysosomal glycoprotein (CB1-gp; clone CB1) [16] and against the procyclic acidic repetitive protein (PARP; clone TBRP1/247) were purchased from Cedarlane.

2.3. RT-PCR/hybridization analysis

Total RNA was purified from long slender, short stumpy, and procyclic trypanosomes by the single-step guanidine method. The mRNAs encoding the R1 and R2 proteins were reverse transcribed and amplified using Ready-To-Go RT-PCR Beads (Pharmacia Biotech). 1.5 μg RNA was incubated with a spliced leader primer (5′TA-

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Abbreviations: CB1-gp, major lysosomal membrane protein; DIG, digoxigenin; PARP, procyclic acidic repetitive protein; R1, large subunit of ribonucleotide reductase; R2, small subunit of ribonucleotide reductase; VSG, variant surface glycoprotein

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GAACCAGTTTCTGTACTATATTG3′) and a gene specific primer (*R1*: 5′TGATGATCGCACACTGTTTGAGT3′; *R2*: 5′CATCGCAAATGAACTCTCGTTCAA3′) at 42°C for 30 min, then at 95°C for 4 min, followed by 32 cycles of 1 min at 95°C, 1 min at 56°C and 1 min at 72°C, and a final extension at 72°C for 5 min. The reaction mixture was applied onto a 1.2% agarose gel, transferred and hybridized with digoxigenin (DIG) labeled probes using standard procedures. The *R1* specific probe covered 963 bp of the open reading frame (codons 1–321), the *R2* specific probe corresponded to the full length coding region of the gene. The DNA fragments were amplified from genomic DNA by PCR using gene specific primers (*R1*: 5′ATGTTGGAAACGGTGAAG3′ and 5′GGAAACCAAAGATATCCG3′; *R2*: 5′ATGCCACCCAAATCTCAC3′ and 5′CTAGAAATCTGCGTCCAG3′) and directly labeled with DIG as described by the manufacturer (Boehringer Mannheim). After hybridization, the blots were incubated with peroxidase-conjugated anti-DIG Fab fragments (Boehringer Mannheim) and developed using the ECL system.

3. Results

The occurrence of the R1 and R2 subunits of ribonucleotide reductase during the life cycle of *T. brucei* was studied by Western blot analyses. As shown in Fig. 1, the R1 protein (94 kDa [8]) is present throughout the life cycle, although at slightly different levels being lowest in short stumpy forms. In contrast, the R2 protein (39 kDa [8,9]) is missing in the cell cycle-arrested trypanosomes. The amounts of R1 and R2 present in the three life cycle stages were determined by scanning densitometry of immunoblots containing recombinant R1 and R2 proteins as standards. The evaluation of the blots revealed that long slender forms contain approximately 46 000 R1 and 15 000 R2 molecules/cell, short stumpy forms 16 000 R1 molecules/cell, and procyclic forms 27 000 R1 and 31 000 R2 molecules/cell, respectively. The homogeneity of the parasites was confirmed using antibodies against stage specific proteins (Fig. 1). The variant surface glycoprotein (VSG) is present only on bloodstream parasites, whereas the procyclic acidic repetitive protein (PARP) is found exclusively on procyclic forms. The major lysosomal membrane protein (CB1-gp) is developmentally regulated showing the highest level in short stumpy trypanosomes [17]. Western blot analysis using antibodies against aldolase confirmed that equal amounts of protein were loaded onto the gels (Fig. 1). Aldolase expression is constant in the various life cycle stages of *T. brucei* [18].

The occurrences of R1 and R2 during the differentiation of short stumpy into procyclic forms are shown in Fig. 2. Short stumpy trypanosomes were cultured at 27°C in the presence of citrate and *cis*-aconitate in order to induce differentiation [2–4]. After 12 h, the level of the R1 protein increased and the R2 protein – which was absent from short stumpy parasites – appeared. Subsequently, the amounts of R1 and R2 increased continuously reaching the level of established procyclic trypanosomes after 48 h. The increased levels of R1 and R2 were accompanied by proliferation of the cells, loss of VSG and the decline of CB1-gp (Fig. 2). PARP was already detectable 3 h after initiating differentiation in agreement with previous observations [4]. The signal was highest between 6 h and 12 h and then decreases again. After 48 h, the level of PARP reached that of established procyclic cells (compare Figs. 1 and 2).

In contrast to most cell systems, *T. brucei* bloodstream forms cannot be synchronized in vitro, e.g. by treatment with hydroxyurea [19]. However, upon differentiation of short stumpy into procyclic parasites the first cell cycle is synchronous [2,4,20]. To study a possible cell cycle dependent regulation of ribonucleotide reductase, the occurrence of R1 and R2 was followed between 10 h and 22 h after induction of differentiation (Fig. 3). The synchronous re-entry of short stumpy trypanosomes into the cell cycle was confirmed by determining the DNA content by flow cytometry. There was no evidence for a regulation of R1 and R2 protein during this first synchronous cell cycle (Fig. 3).

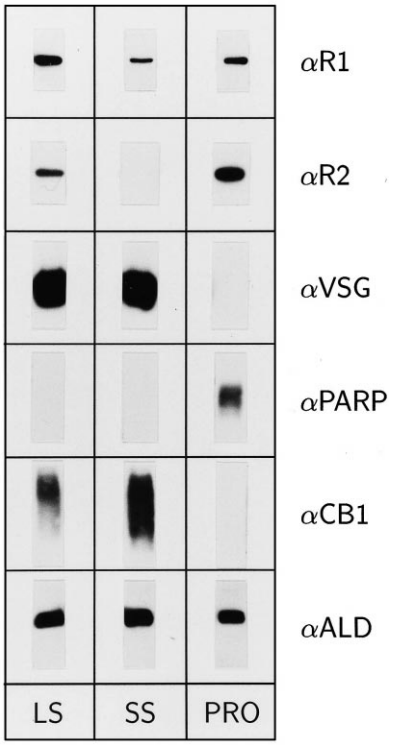

Fig. 1. Occurrence of R1, R2, and marker proteins during the life cycle of *T. brucei*. Long slender (LS), short stumpy (SS), and procyclic (PRO) parasites were lysed and analyzed by immunoblotting using antibodies against R1 (αR1; 1:400), R2 (αR2; 1:5000), VSG AnTat 1.1 (αVSG; 1:2000), PARP (αPARP; 1:2000), CB1-gp (αCB1; 1:3000) and aldolase (αALD; 1:1000) as described in Section 2.

In order to distinguish between transcriptional and post-transcriptional regulation, expression of the *R1* and *R2* genes during the life cycle of *T. brucei* was studied. Internal fragments of the *R1* and *R2* transcripts were amplified by RT-PCR. Subsequent hybridization with *R1* and *R2* specific probes revealed fragments of expected size, 760 bp and 800 bp, in all three developmental stages of the parasite (Fig. 4). These results clearly show that both *R1* and *R2* mRNAs are not down regulated in the non-dividing short stumpy forms.

4. Discussion

The activity of ribonucleotide reductase depends on the presence of both R1 and R2 proteins. As shown here, in African trypanosomes ribonucleotide reductase is developmentally regulated by the occurrence of the R2 protein while the R1 protein is present, although at different levels, throughout the life cycle. The level of R2 is determined by a post-transcriptional mechanism as no change in *R2* mRNA was detectable during the life cycle. Thus, our data confirm previous Northern blot analysis demonstrating that *R2* mRNA is

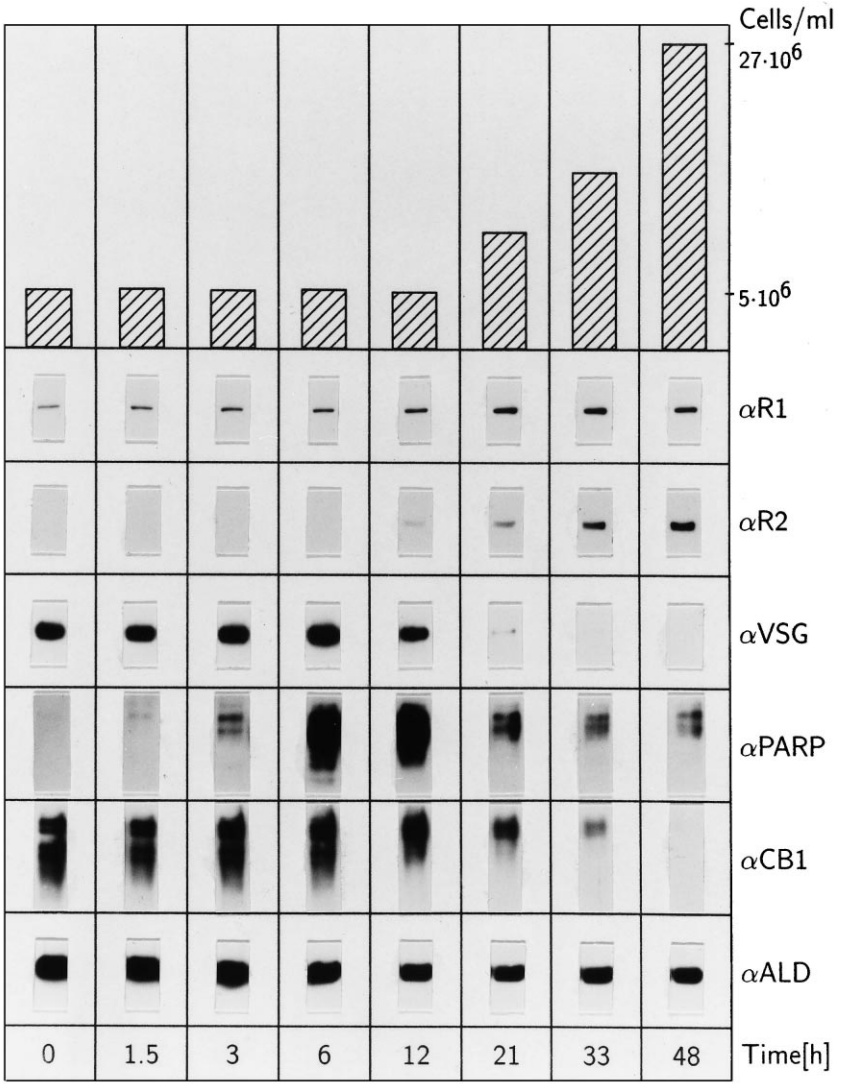

Fig. 2. Cell density and occurrence of R1 and R2 during differentiation from short stumpy into procyclic trypanosomes. Short stumpy *T. brucei* were purified from the blood of infected mice and differentiation was initiated with 3 mM citrate and *cis*-aconitate at 27°C. At the indicated times, the cell density was determined using a hemocytometer and 3×10^7 parasites were harvested by centrifugation. The trypanosomes were lysed and analyzed as described in Fig. 1.

present in almost equal amounts in all three life cycle stages of *T. brucei* [9]. Regulation of ribonucleotide reductase was also demonstrated in mammalian cells during terminal differentiation. In contrast to *T. brucei*, however, it seems that both R1 and R2 proteins are down regulated [21,22].

During the differentiation of short stumpy into procyclic forms we observed reappearance of R2 after 10 h to 12 h. This result is consistent with previous observations that DNA synthesis monitored by thymidine incorporation into the parasites begins between 6 h and 12 h after initiating the differentiation process [23].

In mammalian cells, yeast cells, and malaria parasites, the synthesis of ribonucleotide reductase is regulated at the level of transcription during the cell cycle [24–28]. However, due to the long half-life of the R1 protein, the R1 level remains constant in mammalian cells while synthesis of R2 is linked with the S-phase [24]. In contrast, no regulation of R1 and R2 protein was found during the first cell cycle of differentiating short stumpy trypanosomes. Future studies will reveal if *T. brucei* ribonucleotide reductase is generally not regulated during the cell cycle. At present, it is not possible to synchronize established cultures of bloodstream or procyclic forms of African trypanosomes.

Protein coding genes in trypanosomes are arranged within long polycistronic transcription units [29,30]. This particular organization probably explains why most regulation of gene expression in trypanosomes appears to be post-transcriptional [31–33]. In all cases examined so far, regulatory sequences have been found in the 3′-untranslated region [34–36]. Whether the developmental regulation of the R2 subunit of ribonucleotide reductase in *T. brucei* is also mediated through regulatory sequences in the 3′-untranslated region remains to be elucidated.

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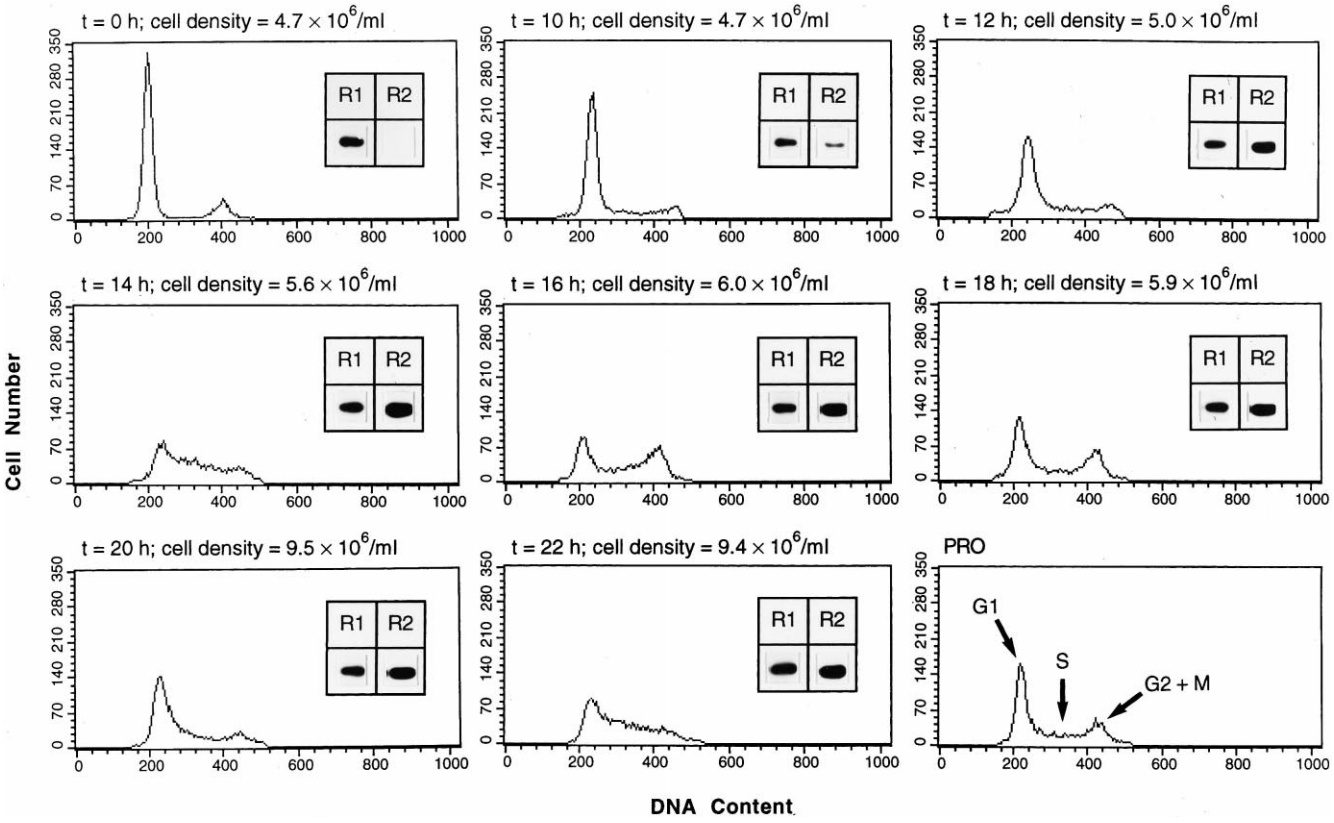

Fig. 3. DNA content and occurrence of R1 and R2 in differentiating trypanosomes during the first synchronous cell cycle. Differentiation of short stumpy trypanosomes was induced as described in Fig. 2. At the indicated times, the cell density was determined and the parasites were harvested. An aliquot of the trypanosomes was lysed and analyzed for R1 and R2 as described in Fig. 1. The residual parasites were fixed in ethanol, stained with propidium iodide, and the DNA content was determined by flow cytometry essentially as described in [19]. The frequency of events per channel which is equivalent to the cell number is plotted against the relative propidium iodide fluorescence which is directly proportional to the DNA content. Each histogram represents data collected from 10 000 events. The positions of the G1, S, and G2+M phases are indicated in the histogram for established asynchronous procyclic trypanosomes (PRO) shown for comparison.

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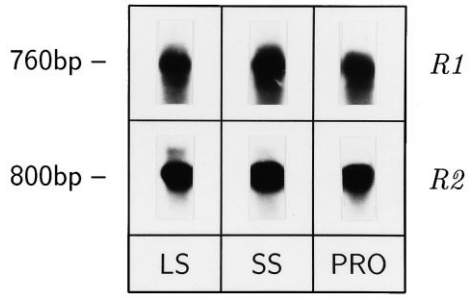

Fig. 4. Transcription of the *R1* and *R2* genes during the life cycle of *T. brucei*. Total RNA was purified from long slender (LS), short stumpy (SS) and procyclic (PRO) trypanosomes and the mRNA reverse transcribed into cDNA. Internal fragments of *R1* and *R2* were amplified from cDNA with a spliced leader and a respective gene specific primer. Equal amounts of the PCR reaction mixtures were separated on a 1.2% agarose gel, transferred to a Nylon membrane and hybridized with DIG labeled *R1* and *R2* probes. Fragments of expected size (*R1* = 760 bp; *R2* = 800 bp) were detected in all life cycle stages.

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