Evidence that J-binding protein 2 is a thymidine hydroxylase catalyzing the first step in the biosynthesis of DNA base J

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Abstract

The genomic DNA of kinetoplastid parasites contains a unique modified base, β-β-glucosyl-hydroxymethyluracil or base J. We recently reported that two proteins, called J-binding protein (JBP) 1 and 2, which regulate the levels of J in the genome, display features of the family of Fe(II)–2-oxoglutarate dependent dioxygenases and are likely to be the enzymes catalyzing the first step in J biosynthesis. In this study, we examine the effects of replacing the four conserved residues critical for the activity of this class of enzymes on the function of Leishmania tarentolae JBP2. The results show that each of these four residues is indispensable for the ability of JBP2 to stimulate J synthesis, while mutating non-conserved residues has no consequences. We conclude that JBP2, like JBP1, is in all probability a thymidine hydroxylase involved in the biosynthesis of base J.

Abbreviations: JBP, J-binding protein; HMU, hydroxymethyluracil; OG, 2-oxoglutarate; TRF, terminal restriction fragment; BrdU, bromodeoxyuridine.

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Fig. 1. Generation of LtJBP2−/− cells. (A) A schematic presentation of the LtJBP2 locus and the inactivation constructs used in this study (not in scale). PstI restriction sites and the position of the LtJBP2 3′-flanking region (flanking region) probe used for Southern blotting are shown. TUB, alpha-tubulin intergenic sequences from L. enriettii; PUR, puromycin N-acetyltransferase; BLA, blasticidin S-deaminase; KO, knock-out. (B) Southern blot to show the genotypes of WT, LtJBP2+/− cells carrying the two different knock-out constructs (BLA and PUR) and LtJBP2−/− (BLA + PUR) cells. Genomic DNA was digested with PstI, run in an agarose gel, blotted and hybridized with the LtJBP2 3′-flanking probe. To generate the knock-out cell lines, 1 × 10⁷ L. tarentolae (TarLi, cultured as in [10]) cells were transfected with 1–5 µg DNA using the Amaxa Nucleofector II electroporator and the Amaxa Human T-cell buffer kit. In our hands, this transfection method improves the transfection efficiency 5- to 10-fold in comparison to conventional methods (data not shown). The cells were allowed to recover for 18–24 h before starting selection with the appropriate antibiotics. (C) Western blot to show the specificity of the rabbit polyclonal anti-LtJBP2 antiserum. The antibody recognizes a single major band of about 120 kD in WT but not in LtJBP2−/− cells. The cells were fixed with 1% formaldehyde, blocked in 1% blocking solution (Roche), and the primary (anti-LtJBP2) and secondary (Alexa 488 (A488)-conjugated goat-anti rabbit (Invitrogen)) antibodies were incubated in 3% BSA/PBS at 37°C for 1 h and 30 min, respectively. After the antibody incubations, the cells were washed for 3× 5 min with PBS. Finally, the samples were mounted in antifade solution (Vectashield; Vector Laboratories) containing TOPro-3 DNA stain (Invitrogen). The images were captured with a Leica TCS SP2 AOBS confocal microscope.

References [9,11]). We originally intended to use T. brucei procyclic cells for this study. These cells would form an optimal system for studying the functionality of the LtJBP2 mutants for several reasons: they completely lack J, do not endogenously express JBP2, and ectopic expression of T. brucei JBP2 results in J synthesis in them [8]. Unexpectedly, however, our repeated attempts to express LtJBP2 in the procyclic T. brucei cells were unsuccessful (data not shown). Therefore, we had to turn to L. tarentolae for further studies.

To generate inactivation constructs for LtJBP2, the 5′- and 3′-flanking sequences of LtJBP2 were subcloned into Leishmania expression vectors coding for puromycin and blasticidin resistance genes (the vectors have been described in [11]) (Fig. 1A). Using a conventional knock-out strategy, we successfully inactivated both of the LtJBP2 alleles (Fig. 1B). To be able to study the expression levels and localization of LtJBP2, we raised a rabbit polyclonal antibody using as immunogen a His-tagged full-length LtJBP2, expressed in and purified from E. coli. The resulting antibody recognizes a single major band of the expected size (120 kD) in WT but not in LtJBP2−/− cells (Fig. 1C). Immunofluorescent staining with the antibody reveals a nuclear staining in WT cells, while only background signal is detected in LtJBP2−/− cells (Fig. 1D).

Thus, unlike JBP1, JBP2 is dispensable for Leishmania. The LtJBP2−/− cells grow normally and lack major morphological abnormalities, but the amount of J in their DNA gradually drops, reaching a four- to eightfold reduction in comparison to WT over a period of approximately 6 months (Fig. 2A). Thereafter, the J levels in the LtJBP2−/− cells remain stable for a period of at least 2.5 years of continuous culturing, the longest observation point that we have. Quantitatively, this reduction resembles the phenotype of the T. brucei JBP2−/− [10], but the kinetics of the decline are different. Interestingly, while our previous data strongly suggest that base J is essential in Leishmania [11], the results with the LtJBP2−/− cells now show that 25% or even less (four- to eightfold reduction) of the WT J levels are sufficient for normal growth and viability.

As the vast majority of base J is found in the telomeres in Leishmania [4], we tested whether some aspects of telomere biology...
are affected by the low amounts of J in the LtJBP2−/− cells. Terminal restriction fragment (TRF) analysis (performed as in [12]) showed no gross alterations in the average telomere length of the LtJBP2−/− cells of any age (data not shown). Probing the TRF-blots with the anti-J antibodies demonstrated that base J is lost universally from all telomeres, with a slight tendency of the shortest ones to be affected first (data not shown). Further, fluorescence in situ hybridization with a telomeric probe revealed no differences in the subnuclear localization of the telomeres in WT and LtJBP2−/− cells (data not shown). To study the telomere length regulation in more detail, we cloned out WT and LtJBP2−/− cells of different age, let the cells grow for 300 generations (approximately 3 months) and took a sample for telomere analysis after every 50 cell divisions. While in WT cells the telomeres slowly grow, as expected [12], telomere shortening took place in theLtJBP2−/− cells (Fig. 2B). This phenotype was present already in the young knock-out cells (Fig. 2B). The significance of this phenomenon, however, remains unclear. As we have observed no net loss of telomeric material in the LtJBP2−/− cells even over prolonged periods of time (Fig. 2A, B and data not shown), the shortening either must be compensated for by periods of telomere growth or there should be a selective pressure favoring the survival of cells with long telomeres. The latter option appears unlikely, though, as even the old LtJBP2−/− grow at rates comparable to WT (see below).

While the telomeric phenotype of the LtJBP2−/− cells is rather mild, we found that these cells display dramatic hypersensitivity to bromodeoxyuridine (BrdU) (Fig. 2C), a thymidine analogue that lowers J levels in kinetoplastids by an unknown mechanism [2]. This sensitivity is concentration dependent and correlates with the age (and thus J levels) of the knock-out cells (Fig. 2C): the growth rate of young (0–5 months in culture) LtJBP2−/− cells in the presence of 0.6 μM BrdU is on average 30% (±10%, n = 8) of the growth rate in the absence of BrdU, while for old (>6 months in culture) LtJBP2−/− cells, this growth rate is only 0.1% (±0.04%, n = 12). This difference, as well as the difference between WT and old LtJBP2−/− cells [the growth rate of WT cells in 0.6 μM BrdU is 90 ± 10% (n = 12) of the growth rate in the absence of BrdU] is highly significant (p < 0.001 and p < 0.0001, respectively). We believe that the BrdU sensitivity is
The WT LtJBP2 (Fig. 3B). Thus, we conclude that the mutations proteins localized to the nucleus in a pattern resembling that of 1 (Fig. 3A). Immunofluorescent staining showed that all the mutant proteins were generated by selecting for paramomycin resistance. We also designed constructs for expression in Leishmania as a template. We replaced the four amino acids (2×His, 1×Arg and 1×Asp) critical for the function of the Fe(II)–2OG dependent dioxygenases by Ala (Table 1; mutants 2–5). We therefore generated LtJBP2 mutants by site-directed mutagenesis, using the WT LtJBP2 construct for expression in Leishmania as a template. We replaced the four amino acids (2×His, 1×Arg and 1×Asp) critical for the function of the Fe(II)–2OG dependent dioxygenases by Ala (Table 1; mutants 2–5). We also designed a control LtJBP2 mutant, in which a non-conserved Val in the vicinity of the critical residues is replaced by Ala. This construct was found to contain a random Leu to Gln mutation at position 548 in addition to the intended one (Table 1; mutant 1). The expression constructs for WT LtJBP2 and the five mutant-LtJBP2s were transfected into L. tarentolae by DNA transfection. We also confirmed that the expression levels of JBP2 were analyzed by Western blotting with anti-LtJBP2 antibodies. All the cell lines expressed a protein of the expected size (Fig. 3A), and the expression levels of JBP2 mutants 2–5 were repeatedly found to be higher than those of WT or mutant 1 (Fig. 3A). Immunofluorescent staining showed that all the mutant proteins localized to the nucleus in a pattern resembling that of the WT LtJBP2 (Fig. 3B). Thus, we conclude that the mutations introduced did not affect the folding or trafficking of LtJBP2 in the cells.

Table 1
The LtJBP2 mutants used in the study.

| Mutant 1 | V462A + L548Q |
| Mutant 2 | R478A |
| Mutant 3 | H465A |
| Mutant 4 | D416A |
| Mutant 5 | H414A |

* The numbers refer to the positions of the amino acids in LtJBP2.

not caused by the lack of LtJBP2 per se, but related to the reduced levels of J in the knock-out cells. The mechanisms of the BrdU-induced growth defect will be subjects of future studies.

As shown in Fig. 2A and C, the major phenotypes of the LtJBP2−/− cells (reduced levels of J, BrdU sensitivity) can be completely reversed by the presence of an ectopic copy of LtJBP2 and thus, the knock-out cells can be used as a system to study the functionality of LtJBP2. We therefore generated LtJBP2 mutants by site-directed mutagenesis, using the WT LtJBP2 construct for expression in Leishmania as a template. We replaced the four amino acids (2×His, 1×Arg and 1×Asp) critical for the function of the Fe(II)–2OG dependent dioxygenases by Ala (Table 1; mutants 2–5). We also designed a control LtJBP2 mutant, in which a non-conserved Val in the vicinity of the critical residues is replaced by Ala. This construct was found to contain a random Leu to Gln mutation at position 548 in addition to the intended one (Table 1; mutant 1). The expression constructs for WT LtJBP2 and the five mutant-LtJBP2s were transfected into L. tarentolae by DNA transfection. The results of this study show that the key residues conserved in the family of Fe(II)–2OG-dependent dioxygenases are critical for the function of LtJBP2, as they are for LtJBP1 [9]. LtJBP2 carrying a mutation in any of these residues is expressed and has a subcellular localization similar to that of the WT protein, but is unable to restore the levels of J in LtJBP2−/− cells. In addition, the sensitivity of the LtJBP2−/− cells to BrdU is unaffected by the expression of these mutant proteins, while WT and a control mutant LtJBP2 completely complement both of these phenotypes. Curiously, the non-functional mutants seem to be expressed at a higher level than WT or control mutant LtJBP2, suggesting that there is a selective pressure against high levels of functional JBP2 in L. tarentolae.

The hallmark amino acid signature of the Fe(II)–2OG-dependent dioxygenases is a unique feature of this class of enzymes, and required for full catalytic activity of all the family members studied thus far [13]. Hence, the results presented here lend further support to our earlier notion that JBP1 and JBP2 belong to the family of Fe(II)–2OG-dependent dioxygenases and could thus be the thymi...
dine hydroxylases responsible for the first step in J biosynthesis [9]. Efforts to directly demonstrate the enzymatic activity of these proteins in vitro have thus far failed, but the accumulating in vivo evidence motivates further studies.

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References