A highly basic sequence at the N-terminal region is essential for targeting the DNA replication protein ORC1 to the nucleus in *Leishmania donovani*

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The conserved eukaryotic DNA replication protein ORC1 is one of the constituents of prereplication complexes that assemble at or very near origins prior to replication initiation. ORC1 has been shown to be constitutively nuclear in *Leishmania major*. This study investigates the sequences involved in nuclear localization of ORC1 in *Leishmania donovani*, the causative agent of visceral leishmaniasis. Nuclear localization signals (NLSs) have been reported in only a few *Leishmania* proteins. Functional analyses have delineated NLSs to regions of ~60 amino acids in length in the tyrosyl DNA phosphodiesterase I and type II DNA topoisomerase of *L. donovani*, and in the *L. major* kinesin KIN13-1. Using a panel of site-directed mutations we have identified a sequence essential for nuclear import of LdORC1. This sequence at the N terminus of the protein comprises residues 2–5 (KRSR), with K2, R3 and R5 being crucial. Independent mutation of the K2 residue causes exclusion of the protein from the nucleus, while mutating the R5 residue leads to diffusion of the protein throughout the cell. This sequence, however, is insufficient for targeting a heterologous protein (β-galactosidase) to the nucleus. Analysis of additional ORC1 mutations and reporter constructs reveals that while the highly basic tetra-amino acid sequence at the N terminus is essential for nuclear localization, the ORC1 NLS in its entirety is more complex, and of a distributive character. Our results suggest that nuclear localization signalling sequences in *Leishmania* nuclear proteins are more complex than what is typically seen in higher eukaryotes.

INTRODUCTION

The nuclear envelope in eukaryotes allows the trafficking of proteins and other macromolecules between the nucleus and the cytoplasm, through nuclear pore complexes (Görlich, 1997). Most eukaryotic nuclear proteins have a nuclear localization signal (NLS) that is recognized by and complexes with a specific receptor molecule in the cytoplasm, and then is transported into the nucleus. The archetypal NLS is typified by that of the SV40 large T antigen, and is a highly basic stretch of seven amino acids (PKKKRKV). A modification of this monopartite NLS sequence is the bipartite NLS, consisting of two highly basic stretches of amino acids that are about 10 residues apart in mammalian proteins, as seen in nucleoplasmin. In addition, an NLS named M9 has also been identified in higher eukaryotes, apart from some atypical NLS sequences (Görlich, 1997; Lufei & Cao, 2009).

While NLSs have been well characterized in other eukaryotes, only a few reports describe NLS sequences in trypanosomatids. A monopartite NLS has been reported to be responsible for nuclear targeting of the La protein in *Trypanosoma brucei* (Marchetti et al., 2000), and bipartite NLS sequences target histone H2B (Marchetti et al., 2000) and p14 splicing factor (Westergaard et al., 2010) to the nucleus in *Trypanosoma cruzi*, with the *T. brucei* ESAG8 protein also having a bipartite NLS (Hoek et al., 2000). The monopartite NLS in the *T. brucei* La protein is a sequence of seven amino acids at the extreme end of the protein’s C terminus, and is rich in basic residues. The bipartite NLS in ESAG8 consists of two clusters of basic residues separated by 16 amino acids, while the NLS sequences in Tcp14 and TbH2B, though bipartite and highly basic, are more complex. Functional dissection of some *Leishmania* proteins has successfully demarcated the domains responsible for nuclear localization (Banerjee et al., 2010; Dubessay et al., 2006; Sengupta et al., 2003). However, to date, no defined sequence has been assigned the role of nuclear localization in *Leishmania* species. The DNA replication protein ORC1, which is responsible for origin recognition,
is conserved across all eukaryotes, including *Leishmania* species. Previous studies in our laboratory have demonstrated ORC1 of *Leishmania major* to be nuclear throughout the cell cycle (Kumar et al., 2008). This report presents the results of our investigation of the sequences regulating nuclear localization of the ORC1 protein of *Leishmania donovani*. We find that while a short stretch of highly basic sequence in the N terminus is essential for targeting LdORC1 to the nucleus, with three of the residues of this sequence being critical for nuclear localization, this sequence is by itself insufficient for nuclear targeting of a heterologous protein (β-galactosidase; β gal), indicating that the signal sequences responsible for nuclear localization of ORC1 are more complex.

**METHODS**

*Leishmania* cultures. *L. donovani* 1S promastigotes were cultured in M199 medium as described previously (Kumar et al., 2008).

Cloning and subcloning of ORC1. *L. donovani* genomic DNA was isolated by phenol/chloroform extraction followed by ethanol precipitation, as described previously (Kumar et al., 2009), and the ORC1 gene was cloned by amplification of the gene using *Leishmania* genomic DNA as template and *Phu* DNA polymerase (Finnzymes). Amplification was carried out using end primers designed based on the *L. major* genome sequence, ORC1-F and ORC1-R (Table 1). The obtained PCR products were cloned into pUC18 for sequencing. To express ORC1 in *L. donovani* promastigotes for immunofluorescence analysis the gene was amplified using primers ORC1-GFP-F and ORC1-GFP-R (Table 1) and the amplicon was cloned into the *Bam*HI and *Sfi* I sites of the vector pXG−/GFP+ (a kind gift from the laboratory of Dr S. M. Beverley, Washington University in St Louis; Ha et al., 1996) to create plasmid pXG/LdORC1-GFP.

Site-directed mutagenesis of ORC1 and creation of ORC–GFP fusion constructs. N-terminal mutants of LdORC1 were made by PCR-based mutagenesis, using mutagenic forward primers (ORC1-K2A-R3A-R5A-R6A-F, ORC1-K2A-F, ORC1-R3A-F, ORC1-S4A-F, ORC1-R5A-F, ORC1-R6A-F; Table 1) and primer ORC1-GFP-R, and cloning the mutagenized genes into pXG−/GFP+ as described above. ORC1-R32A-K33A-R38A and ORC1-K53A-H54A-H55A mutants were made by overlap PCR. To create ORC1-R32A-K33A-R38A, primers ORC1-GFP-F and ORC1-R32A-K33A-R38A-R (Table 1) were used to amplify the N-terminal part, and ORC1-R32A-K33A-R38A-F (Table 1) and ORC1-GFP-R were used to amplify the C-terminal part. ORC1-GFP-F and ORC1-GFP-R primers were then used to amplify the full-length mutated gene. ORC1-K53A-H54A-H55A was likewise created using ORC1-K53A-H54A-H55A-F and ORC1-K53A-H54A-H55A-R (Table 1). The amplicons were cloned into pXG−/GFP+, creating mutated ORC1–GFP fusion constructs.

Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>ORC1-F</td>
<td>5’TACCGGATCCCATATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-R</td>
<td>5’TCCTGCAATGCTCAAAAGTCCGCGCGCG-3’</td>
</tr>
<tr>
<td>ORC1-GFP-F</td>
<td>5’TGGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-GFP-R</td>
<td>5’TGGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-K2A-R5A-R6A-F</td>
<td>5’TACCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-K2A-F</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-R3A-F</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-S4A-F</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-R5A-F</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-R6A-F</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>β gal-F</td>
<td>5’CCGGCGGAATTCGCTTGGCTCTATCTGAG-3’</td>
</tr>
<tr>
<td>β gal-R</td>
<td>5’CCGGCGGATTCGCTTGGCTCTATCTGAG-3’</td>
</tr>
<tr>
<td>Orc1-6-F</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>Orc1-6-R</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>Orc1-13-F</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>Orc1-13-R</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
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<td>Orc1-27-R</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
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<tr>
<td>Orc1-50-R</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
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</tr>
<tr>
<td>ORC1-R32A-K33A-R38A-R</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-K53A-H54A-H55A-F</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
</tr>
<tr>
<td>ORC1-K53A-H54A-H55A-R</td>
<td>5’TACGCGGATCCACATGAGCGGACGCCGCGCGACG-3’</td>
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ORC1-GFP-F and Orc1-27-R (Table 1) and cloning the amplicon so obtained into the Smal site of β gal-GFP. ORC1-50/β gal-GFP was constructed by amplification using ORC1-GFP-F and Orc1-50-R (Table 1) and cloning the amplicon so obtained into the Smal site of β gal-GFP.

Preparation of Leishmania extracts. Leishmania whole-cell extracts were prepared using the M-PER kit (Pierce Biotechnology) as per the manufacturer’s instructions.

Leishmania transfections and expression analysis. Leishmania promastigotes were transfected by a high-voltage protocol as described elsewhere (Robinson & Beverley, 2003). Selection pressure was induced 42 h after transfection by the addition of G418 (100 µg ml⁻¹). Expression of LdORC1–GFP (wild-type and mutants) was analysed ~20 days after incubation in drug-containing medium.

RESULTS

Cloning and sequence analysis of the Leishmania ORC1 gene

The L. donovani ORC1 gene was amplified from L. donovani genomic DNA using end primers designed based on the L. major genome sequence (Ivens et al., 2005), as no sequence from L. donovani was available at the time. The ORC1 amplicon obtained was cloned and sequenced (GenBank accession no. HQ424455). A comparative analysis of the LdORC1 amino acid sequence with those of other eukaryotes revealed that LdORC1 shares between 25 and 31 % identity and between 58 and 61 % similarity with ORC1 from other eukaryotes (data not shown). On comparing the LdORC1 sequence with those of the other trypanosomatids T. brucei and T. cruzi, we found that LdORC1 shared between 56 and 58 % identity and between 79 and 82 % similarity (data not shown). ORC1 was highly conserved among the various Leishmania species (Ivens et al., 2005; Peacock et al., 2007), with LdORC1 showing

![Fig. 1](http://mic.sgmjournals.org)

(b) (c) ORC1–GFP DAPI GFP–DAPI merged Merged

**Fig. 1.** (a) Comparison of the amino acid sequence (N-terminal end) of L. donovani ORC1 with those of ORC1s from other trypanosomatids. CLUSTAL W analysis viewed using Jalview multiple alignment editor (Waterhouse et al., 2009). L. infantum, Leishmania infantum; L. braziliensis, Leishmania braziliensis. The putative NLS is indicated by a box. (b) Western blot analysis of ORC1–GFP expression in Leishmania transfectant promastigotes. Lane 1, control whole-cell lysate from non-transfectant Ld1S promastigotes; lane 2, whole-cell lysate from Leishmania promastigotes harbouring plasmid pXG/LdORC1-GFP. Upper panel, analysis with anti-GFP antibody (Invitrogen, 1:2000 dilution); lower panel, analysis with anti-tubulin (loading control; Zymed, 1:5000 dilution). Arrowhead indicates LdORC1–GFP (~72 kDa). (c) Immunofluorescence analysis of Leishmania promastigotes harbouring plasmid pXG/LdORC1–GFP. DAPI staining indicates a larger nucleus (N) and short rod-like kinetoplast (K). Images were acquired by collecting Z-stacks using a confocal microscope and ×100 objective. Bars, 2 µm.
A stretch of four amino acids at the N terminus of ORC1 is essential for nuclear localization

ORC1 in L. major has already been reported to be nuclear throughout the cell cycle (Kumar et al., 2008). Towards examining the putative NLS in LdORC1, we first expressed LdORC1 in fusion with GFP (GFP fused at C-terminal end of the protein) to analyse the expression and subcellular localization of the ORC1 protein. The ORC1 amplicon was cloned into vector pXG-GFP, and the resultant plasmid pXG/LdORC1-GFP was transfected into Leishmania promastigotes. Western blot analysis of whole-cell extracts made from transfectant promastigotes using anti-GFP antibodies revealed that LdORC1–GFP was robustly expressed (Fig. 1b), and immunofluorescence analysis indicated that, as in L. major, the protein was constitutively nuclear (Fig. 1c).

To determine whether the sequence KRSRR at the N terminus of LdORC1 is indeed an NLS, we mutated the sequence and examined the subcellular localization of ORC1–GFP mutant proteins. A panel of six mutants was created (Fig. 2a), wherein each of the residues of the putative NLS was mutated to alanine. A mutant with all four basic residues mutated to alanine was also created. The plasmids pXG/LdORC1-GFP/ORC1-K2A-R3A-R5A-R6A, pXG/LdORC1-GFP/ORC1-K2A, pXG/LdORC1-GFP/ORC1-R3A, pXG/LdORC1-GFP/ORC1-R6A-GFP/ORC1-R5A and pXG/LdORC1-GFP/ORC1-R6A were transfected into Leishmania promastigotes. Expression of the ORC1–GFP mutant proteins was examined by Western blot analysis of whole-cell lysates made from transfectant cultures, using anti-GFP antibodies (Fig. 2b). All ORC1–GFPs were found to be robustly expressed. The subcellular localization of ORC1–GFP mutants was examined by direct fluorescence of transfected cells (Fig. 3). In the case of all the mutants we found that the pattern of distribution of ORC1 was similar in all cells of a particular mutant type. We found that ORC1-R6A-GFP and ORC1-S4A-GFP behaved like wild-type ORC1–GFP in that the proteins were nuclear in all cells examined. In sharp contrast, ORC1-K2A-R3A-R5A-R6A-GFP, ORC1-K2A-GFP, ORC1-R3A-GFP and ORC1-R5A-GFP were largely cytosolic, underlining the importance of the sequence the KRSRR in mediating nuclear localization. Mutation of the K2 residue (ORC1-K2A-GFP) resulted in the protein being excluded from the nucleus, while mutation of the R5 residue (ORC1-R5A-GFP) caused the protein to be uniformly distributed throughout the cell. Thus, our results indicate the importance of the K2, R3 and R5 residues in directing the protein to the nucleus.

The penta-amino acid sequence is insufficient for nuclear targeting of heterologous protein

To ascertain whether the sequence KRSRR was sufficient for nuclear import of the protein, we took the approach of appending the sequence to β gal and tracking the localization of the fusion protein. Accordingly, the β gal gene was cloned into pXG–/GFP + in order to express it in fusion with GFP at its C-terminal end. β gal–GFP was found to be cytosolic, as expected (data not shown). When we expressed β gal–GFP with the first six amino acids of ORC1 fused at its N terminus (ORC1-6/β gal–GFP; Fig. 4a), we found that the protein remained cytosolic (Fig. 4b), indicating that the penta-amino acid sequence cannot by itself target proteins to the nucleus.

We then expressed β gal–GFP with the first 13, first 27 and first 50 amino acids of ORC1 fused at its N terminus (ORC1-13/β gal–GFP, ORC1-27/β gal–GFP and ORC1-50/β gal–GFP, respectively; Fig. 4a). Immunofluorescence analysis revealed that ORC1-13/β gal–GFP and ORC1-27/β gal–GFP were largely cytosolic, whereas ORC1-50/β gal–GFP was found to be nuclear (Fig. 4a).
gal–GFP were excluded from the nucleus similarly to β gal–GFP ORC1-6/β gal–GFP (Fig. 4b). ORC1-50/β gal–GFP behaved somewhat differently, with the protein localizing to both the nucleus and cytosol (Fig. 4b). We attempted to express β gal–GFP with ORC1-60, ORC1-70 and ORC1-80 fragments fused at the N terminus, but were unable to detect any expression of the protein in these cases, even after weeks of drug-induced selection. The reasons for this are not certain; it is possible that for some reason β gal is unstable when these longer sequences are tagged at the N terminus. These results demonstrated that the ORC1 NLS was more complex than we initially believed it to be.

Upon scanning the amino acid sequence of ORC1 we found basic residues present at multiple positions in the N-terminal region. In addition to the penta-amino acid sequence at the N terminus, two clusters of basic residues were detected in the first 75 amino acids: R32, K33 and R38 in one cluster, and K53, H54 and H55 in another cluster. Two mutant ORC–GFP constructs were created, one in which the basic residues of the first cluster were changed to alanine residues (pXG/ORC1-R32A-K33A-R38A-GFP; Fig. 5a), and one in which the basic residues of the second cluster were changed to alanine residues (pXG/ORC1-K53A-H54A-H55A-GFP; Fig. 5a). Both constructs were

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**Fig. 3.** Subcellular localization of ORC1–GFP and ORC1–GFP mutants. Immunofluorescence analysis of transfected promastigotes expressing ORC1–GFP (wild-type and mutants). Cells were viewed and images acquired using a ×100 objective, utilizing a confocal microscope (Leica TCS SP5) equipped with a high-resolution camera. DAPI staining indicates a larger nucleus and short rod-like kinetoplast (N, nucleus; K, kinetoplast). Bars, 2 μm.
transfected into promastigotes and expression was analysed by direct fluorescence. ORC1-R32A-K33A-R38A–GFP and ORC1-K53A-H54A-H55A–GFP were both found to be nuclear, implying that these two clusters of basic residues (R32, K33 and R38; K53, H54 and H55) are not essential for nuclear localization of ORC1.

**DISCUSSION**

*Leishmania* species cause the group of diseases collectively called leishmaniasis, manifested in three forms: cutaneous, subcutaneous and visceral. Visceral leishmaniasis (VL) can be fatal if not treated in timely fashion. In the Indian subcontinent *L. donovani* is the causative agent of VL. In the light of emerging drug resistance to the first-line drug sodium stibogluconate (which is therefore now being replaced by an oral drug, miltefosine), the development of new and improved means of therapeutic intervention has become one of the primary focuses of *Leishmania* research. A clear understanding of the cellular biology of the organism is highly desirable in order to identify new potential drug targets. Annotation of the whole genome sequence of *Leishmania* species (Ivens et al., 2005; Peacock et al., 2007) reveals that while DNA replication in *Leishmania* and other trypanosomatids broadly resembles that of higher eukaryotes, significant differences must exist, as several proteins of the pre-replication complex (pre-RC) of other eukaryotes are missing in trypanosomatids. This suggests that novel *Leishmania* proteins may be involved in this life process. The pre-RC protein ORC1 is conserved across all eukaryotes studied thus far, and in most eukaryotes is a part of a six-subunit ORC (Orc1–6) that is responsible for origin recognition. Previous work has demonstrated that ORC1 in *L. major*, *T. brucei* and *T. cruzi* is nuclear throughout the cell cycle (Kumar et al., 2008; Godoy et al., 2009).

Nuclear import and export of proteins is a thoroughly investigated area of research in higher eukaryotes. Proteins that are imported into the nucleus generally have an NLS in their sequence. Various proteins involved in nuclear import interact with the NLS to bring about entry into the nucleus (Görlich et al., 1996; Nigg, 1997; Powers & Forbes, 1994). While NLS sequences are conserved across higher eukaryotes, not much has been investigated in *Leishmania*. Analysis of the ORC1 sequence revealed the presence of an identical penta-amino acid sequence that is highly basic at the N terminus of ORC1 (Fig. 1a). This sequence has also been

![Fig. 4. (a) ORC1 sequences appended to β gal–GFP. The first six (ORC1-6), first 13 (ORC1-13), first 27 (ORC1-27) and first 50 (ORC1-50) amino acids of ORC1 were fused to the N terminus of β gal–GFP. (b) Subcellular localization of ORC1/β gal–GFP fusion proteins. Images acquired using a ×100 objective, utilizing a confocal microscope (Leica TCS SP5) equipped with a high-resolution camera. DAPI staining indicates a larger nucleus and short rod-like kinetoplast. Bars, 2 μm.](image-url)
detected in ORC1 of *T. brucei* and *T. cruzi* (Godoy et al., 2009), and is reminiscent of the prototypical monopartite NLS detected in several eukaryotes; therefore, we decided to examine any possible role of this sequence in directing ORC1 to the nucleus in *L. donovani*.

Studies on the characterization of nuclear targeting sequences in *Leishmania* have thus far been limited to narrowing down NLSs to stretches of 40–60 amino acids by deletion analyses (Banerjee et al., 2010; Dubessay et al., 2006; Sengupta et al., 2003). Tyrosyl DNA phosphodiesterase I of *L. donovani* harbours an NLS at its C-terminal end, within the last 60 amino acids; deletion of these residues results in the distribution of the protein in the cytoplasm of the cell (Banerjee et al., 2010). The *L. major* kinesin KIN13-1 has an atypical NLS that is ~60 amino acids in length and lies in the C-terminal domain of the protein. The specific residues responsible for nuclear targeting are scattered through this region (Dubessay et al., 2006). Deletion analysis of *L. donovani* type II DNA topoisomerase has demarcated an ~60 amino acid region in the C-terminal domain to be responsible for nuclear localization (Sengupta et al., 2003). Among *Trypanosoma* species, a monopartite NLS has been reported in the La protein in *T. brucei* (Marchetti et al., 2000), with bipartite NLS sequences being reported in the case of histone H2B and p14 splicing factor (Marchetti et al., 2000; Westergaard et al., 2010) in *T. cruzi*, as well as in the *T. brucei* ESAG8 protein (Hoek et al., 2000).

The present study is believed to be the first report of a defined sequence being essential for nuclear localization of a protein in any *Leishmania* species. It is also the first identification of a discrete sequence playing a role in nuclear targeting of an ORC1 protein, across all the species in which the ORC proteins have been characterized. This sequence resembles the classic NLS exemplified by the SV40 large T antigen, and is a highly basic stretch of amino acids (KRSR) at the N terminus, with the first, second and fourth residues of the motif being functionally the most critical, as mutating any one of these residues results in the protein being dispersed throughout the cell (Fig. 3). However, the sequence does not encompass the entire ORC1 NLS, as it is incapable of localizing β gal to the nucleus. Appending additional ORC1 sequences to β gal and analysing the localization of the fusion proteins did not lead to unambiguous demarcation of the complete NLS (Fig. 4). Mutation of other possible candidate sequences did not shed further light on the enigma either. It is evident, however, from our findings, as well as those of Dubessay et al. (2006), that *Leishmania* NLSs are atypical and significantly different from the conventional NLSs seen in other eukaryotes.

**Fig. 5.** (a) Panel of ORC1 mutants. N-terminal amino acid sequences of ORC1 (wild-type and mutated). Mutated residues are indicated in bold type. WT, wild-type. (b) Subcellular localization of ORC1–GFP mutants. Imaging was done using a confocal microscope (Leica TCS SP6) equipped with a high-resolution camera. DAPI staining indicates a larger nucleus and short rod-like kinetoplast. Bars, 2 µm.
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