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A *Leishmania donovani* gene that confers accelerated recovery from stationary phase growth arrest

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Abstract

We have isolated a gene, LdGF1, from the protozoan parasite *Leishmania donovani*. Overexpression of this gene confers a strong selective advantage in liquid culture after stationary phase growth arrest. We could show that recombinant *L. donovani* or *Leishmania major*, when overexpressing *LdGF1*, recover faster from a stationary phase growth arrest than control parasite strains. While no advantage of *LdGF1* overexpression could be observed in log phase cultures or after a hydroxyurea-induced S-phase growth arrest, recovery from a cell cycle arrest due to serum deprivation was faster in *LdGF1*-overexpressing strains. This was found to be due to an accelerated release from a G_1 cell cycle arrest. By contrast, in a BALB/c mouse infection system, overexpression of *LdGF1* in *L. major* resulted in reduced virulence. We conclude that increased levels of LdGF1 are beneficiary during recovery from G_1 cell cycle arrest, but pose a disadvantage inside a mammalian host. These results are discussed in the context of the observed loss of virulence during in vitro passage of *Leishmania* parasites.

Keywords: *Leishmania*; Growth factor; Cell cycle; Virulence; Attenuation; In vitro passage

1. Introduction

Leishmania spp. are valuable model systems for studying host parasite interactions as well as protozoan cell biology and genetics. The availability of animal infection models (Handman, 1979; Hill, 1986) along with the relatively simple cultivation of the insect form, the promastigote, and the applicability of various reverse and forward genetics strategies (Laban and Wirth, 1989; Cruz and Beverley, 1990; Kapler, et al., 1990; Laban, et al., 1990; Cruz et al., 1991; Ryan, et al., 1993; Beverley and Turco, 1995) are features that, to this degree, are shared by only few other eukaryotic pathogens. In addition, several *Leishmania* species allow the in vitro, axenic cultivation of the amastigote stage that usually resides in the antigen presenting cells of mammalian hosts (Bates et al., 1992; Bates, 1993; Pan, et al., 1993; Hodgkinson, et al., 1996; Balanco, et al., 1998; Saar, et al., 1998; Sereno et al., 1998). Nevertheless, the flagellated, highly proliferative promastigote stage is primarily used in cultivated form and as a model for research.

Leishmania spp. appear in two morphologically distinct stages which are defined by the two hosts required for the life cycle. In the hindgut of phlebotomine sandflies of the genera *Phlebotomus* and *Lutzomyia*, the *Leishmania* appear as flagellated promastigotes. In mammals, the parasites reside in the phagolysosome of macrophages and other antigen-presenting cells as aflagellated amastigote stages. Stage conversion is triggered at least in part through the environmental temperature within poikilothermic insects and homeothermic mammals (Clos and Krobitsch, 1999) and mediated by the 90 kD heat shock proteins (Wiesgigl and Clos, 2001).

When *Leishmania* parasites from infected tissue are cultivated in vitro, the initial growth is often slow. After an adaptive period, however, the parasites begin to proliferate more rapidly. Most laboratory reference strains in use have been adapted to in vitro growth, in some cases for decades.

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In many of these strains, adaptation to in vitro culture correlates with an attenuation of in vivo virulence in susceptible laboratory animals (Neal, 1984; Nolan and Herman, 1985; Segovia et al., 1992; De and Roy, 1999). This suggests that the growth conditions in vitro and in the mammalian host select for mutually exclusive genetic traits.

Usually, virulence in laboratory strains is maintained by regular infection of test animals and subsequent re-isolation of virulent parasites from infected tissues. Aliquots of recovered parasites are stored frozen and used to start fresh liquid cultures at regular intervals. However, the use of double-targeted gene displacement (Cruz et al., 1991) or other reverse genetics approaches (Beverley and Turco, 1998; Beverley et al., 2002) may require prolonged selective cultivation in vitro, raising concerns about a spontaneous loss of virulence.

Attempts have been made to identify genes that, if overexpressed from episomal gene copies, can restore virulence to attenuated laboratory strains and several such genes were identified (Ryan et al., 1993; Beverley and Turco, 1995). To our knowledge, the reverse approach, i.e. the search for genes that support rapid in vitro growth, has not been tried. Genes identified in such screens could then be tested for their effects on parasite virulence and/or survival within experimental hosts.

In the course of a genetic complementation screen designed to identify thermotolerance markers (Hoyer et al., 2001), we isolated a cosmid from an *Leishmania donovani* genomic DNA library that has significant impact on the in vitro growth of recombinant *Leishmania major* strains. Overexpression of one gene from this cosmid, dubbed *L. donovani* growth factor *1* (*LdGF1*), has a significant effect on the recovery from growth arrest and affords a selective advantage to *L. major* promastigotes under in vitro culture conditions. Our data show that overexpression of *LdGF1*, while being of selective advantage in vitro, has a negative impact on virulence in vivo and may thus be viewed in the context of the observed in vitro attenuation of *Leishmania* parasites.

2. Materials and methods

2.1. Parasites

For our analyses, we used *L. major* strain MHOM/-SU/77/5ASKH, originally obtained from D. Evans. The strain was subjected to periodic passage through BALB/c mice to maintain infectivity and virulence. We also used *L. donovani* strain Lo8 (a gift from Dan Zilberstein), both as donor of genomic DNA and for growth experiments. Promastigotes were routinely cultivated at 25 °C in supplemented M199 medium (Krobitsch et al., 1998). Cells were counted using a Schaerfe System CASY Cell Counter.

2.2. DNA constructs

Construction of the *L. donovani* genomic DNA cosmid library in the vector pcosTL (Kelly et al., 1994) has been described (Hoyer et al., 2001). Briefly, a cosmid DNA library of *L. donovani* strain Lo8 was prepared by cleavage of the shuttle cosmid vector pcosTL with *Sma* I and *Bam* HI and ligation with size-selected *Sau* 3AI partial digest products of *L. donovani* strain Lo8 genomic DNA. After packaging using the Gigapack Gold II kit (Stratagene), the complexity of the library was tested, and the library was amplified and stored at −70 °C.

The cosmid pcos 11.2 was sequenced using a combination of shotgun and directed approaches as described previously (Platzer et al., 1997). Open reading frames were selected using the MacMolly Tetra freeware package (http://www.mologen.com/english/Mologen7.html). Minimum requirements were defined as follows: >450 bp and >60% G/C content.

For integration of genes into the rRNA gene cluster of *L. major*, a derivative of plasmid pIRSAT1, a gift from Stephen Beverley, was used. Insertion of a multiple cloning site into the single Bgl II site and the deletion of the single Hind III site created plasmid pIRmcs3 + (Fig. 1).

Inserts for pIRmcs3 + were generated by enzymatic amplification of the respective open reading frames (ORFs) using specific primers that add the desired restriction sites for *Nde* I and *Bam* HI respectively, to the 5′- and 3′ ends. We used primers ORF8-Nde 5′CCCCATATGC TGTTACGCTA TACG-3′ and ORF8-Bam 5′-CCCGGATCCT ACAAGTTGAT GAACGG-CAC-3′ for ORF8, and ORF9-Nde 5′-CCCCATATGG

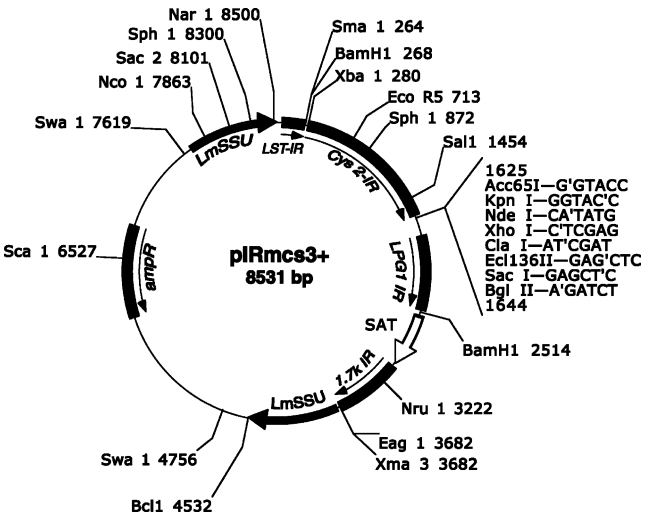

Fig. 1. Plasmid map of pIRmcs3 + showing restriction sites, coding sequences and regulatory flanking sequences. ampR: *β-lactamase*gene; LmSSU: *Leishmania major* small subunit rRNA coding sequence; LST-IR: intergenic region of *L. major LST* gene; Cys2-IR: intergenic region of *Leishmania mexicana cysteine protease 2* gene; LPG1 IR: intergenic region of *L. major LPG1* gene; SAT: nourseothricine resistance gene; 1.7 k IR: intergenic region from the *L. major* 1.7 kb minicircle.

CATACTTGCC TACAAAC-3′ and ORF9-Bam 5′-GGGGGATCCT AGTCGAACAC GGCCATCTG-3′ for ORF9.

2.3. Generation of recombinant leishmania

Electrotransfection of *Leishmania* promastigotes was carried out as described (Krobitsch et al., 1998). Promastigotes were harvested during late log phase of growth, washed twice in ice-cold PBS, once in pre-chilled electroporation buffer and suspended at a density of 1×10^8 ml^{-1} in electroporation buffer. 2 μg of linearised DNA or 50 μg of circular DNA in an electroporation cuvette was mixed on ice with 0.4 ml of the cell suspension. The mixture was immediately subjected to electroporation using a Bio-Rad Gene Pulser apparatus. Electrotransfection of DNA was carried out at 3.750 V/cm and 25 μF in a 4 mm electroporation cuvette. Mock transfection of *Leishmania* was performed in identical fashion, however, without plasmid DNA, to obtain negative control strains for antibiotic selection. Following electroporation, cells were kept on ice for 10 min before they were transferred to 10 ml drug-free medium. The appropriate drug for selection, G418 (50 μg ml^{-1}) or ClonNAT (nourseothricine, 150 μg ml^{-1}), was added after 24 h.

2.4. Recombinant expression, antibody production and immunoblot analysis

The ORF9 was amplified with primers ORF9-Nde and ORF9-Bam which introduce *Nde* I and *Bam* HI sites, respectively, to the 5′ and 3′ ends. After cleavage of the PCR products with *Nde* I and *Bam* HI, the coding sequence was ligated into the plamsid pJC45 (Schlueter et al., 2000), linearised with *Nde* I and *Bam* HI. The ORF9 was then expressed in *E. coli* BL21(DE3)[pAPlacI^{Q}]. The recombinant protein was purified using Ni-NTA agarose (Qiagen) following an established protocol (Clos and Brandau, 1994). Mice were immunised with 50 μg each of purified recombinant protein in complete adjuvant, and boostered twice with the same amount of protein in incomplete adjuvant. Serum from immunised mice was used at 1:250 in immunoblot analysis. Detection was performed using anti-mouse IgG:AP conjugate (Dianova) with nitroblue tetrazolium and X-phosphate.

2.5. Flow cytometry

Leishmania major promastigotes were harvested by mild centrifugation, washed twice in PBS and fixed for 1 h in 70% methanol at 4 °C, followed by treatment with 20 μg/ml RNase A in PBS (20 min, 37 °C). Cells were resuspended in citrate buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS pH7.0, 0.1% TritonX-100) and labeled for 20 min with 1 μM SYTOX Green nucleic acid stain (Molecular Probes). Following staining, cells were washed twice in PBS supplemented with 5% fetal calf serum and 0,01% sodium azide. Samples were stored in the dark at 4 °C until analysis. Fluorescence of 10,000 cells per sample was analysed on a fluorescence activated cell sorter (Becton Dickinson, Heidelberg).

2.6. Experimental infection

Stationary phase promastigotes were collected by centrifugation (1000 × *g*, 10 min, 4 °C), washed twice in ice cold PBS and resuspended at a density of 4×10^8 ml^{-1} or 4×10^7 ml^{-1}. Parasites were inoculated in a volume of 50 μl in the right hind footpad of 6–8 week-old BALB/c mice. Footpad size was measured weekly using an ODITEST calliper rule (Kroeplin, Schlüchtern, FRG).

Mice were sacrificed when lesions were close to ulceration. Footpad tissue was removed, homogenised in supplemented M199 medium (Krobitsch et al., 1998), and the parasites were grown in vitro. As soon as promastigotes were detectable, the parasites were distributed in limiting dilution on microtitre plates for clonal amplification. Genomic DNA from parasite clones was purified using the PureGene Genomic DNA Isolation Kit (Gentra Systems). Using primers CL043 (see Section 2.4) and pIR-P3′ (CGACTCTAAG ATGTCACGGA GG), PCR was performed on the genomic DNA samples to test for the presence of the ORF9 transgene.

3. Results

A population of *L. major* promastigotes that had been transfected with cosmid DNA from a *L. donovani* genomic DNA library was subjected to temperature challenge (Hoyer, et al., 2001). From the survivors, several cosmids were isolated and retransfected into *L. major*. After 12 weeks of in vitro passage the population contained one predominant cosmid, pcos11.2, regardless of whether antibiotic pressure was applied. We hypothesised that pcos11.2 conferred a selectable advantage to *L. major* promastigotes in liquid culture.

We therefore transfected the cosmid pcos11.2 both into *L. major* and *L. donovani*. From either species, wild type and recombinant promastigotes from log phase cultures were seeded at an equal density of 1×10^6 cells ml^{-1} in fresh medium and incubated for 2 days until late logarithmic growth was reached. No significant difference in proliferation could be observed between *L. major* and *L. donovani* transfected with either pcos11.2 or pcosTL (Fig. 2A).

We then seeded the same recombinant parasite strains from cultures that were in stationary phase growth arrest. Outgrowth from stationary phase seed populations was significantly retarded. However, both *L. major* [pcos11.2] and *L. donovani* [pcos11.2] promastigotes resumed growth significantly faster compared with the respective control

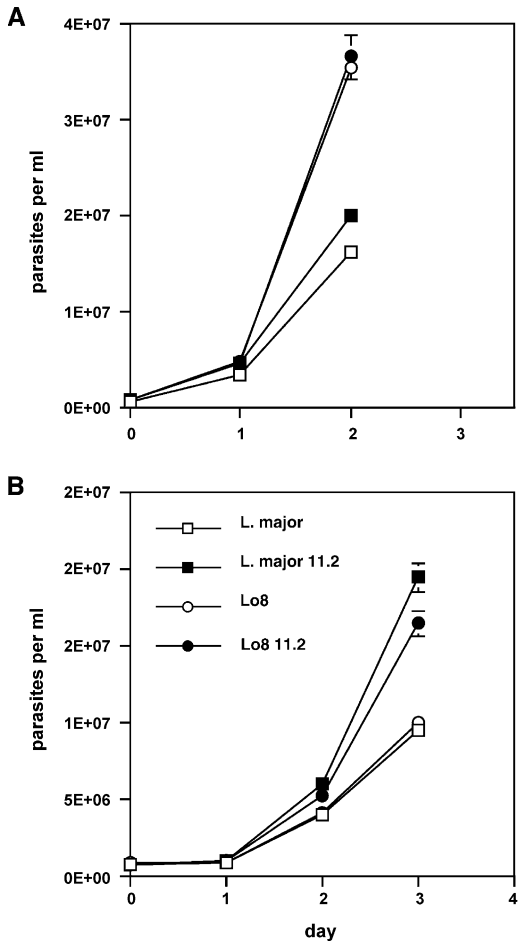

Fig. 2. Proliferation kinetics of wild type and recombinant *Leishmania major* and *Leishmania donovani*. *Leishmania major* and *L. donovani* (open squares and circles, respectively) or recombinants carrying the cosmid pcos11.2 (solid squares and circles, respectively) were taken from a logarithmically growing culture (A) or from a culture that had been in stationary phase for 2 days (B). The promastigotes were seeded at 1×10^6 ml^{-1} in fresh medium 199, and cell density was monitored for 2 and 3 days, respectively. The experiments were performed in quadruplicate. The error bars represent the standard deviation.

strains (Fig. 2B). This effect was somewhat more pronounced in *L. major* than in *L. donovani*. Nevertheless, the result suggested that recovery from stationary phase growth arrest was accelerated by the overexpression of gene(s) in the cosmid pcos11.2. The fact that *L. donovani* was affected, too, by the additional gene copies suggested that the observed effect is, at least in part, due to a gene dosage effect.

In order to identify the gene(s) responsible for the observed growth acceleration, we subjected cosmid pcos11.2 to a partial digest with *Sau*3A1. The fragments were size selected for the 3–6 kb range and subcloned in the pcosTL vector. The parent plasmid was modified to replace the pBluescript SK vector backbone carrying the beta-lactamase resistance marker with pBGS9. pBGS9 has the neomycin phosphotransferase gene in place of the beta-lactamase gene and confers kanamycin resistance rather than ampicillin resistance to *E. coli*. DNA from this sub-library was used to transfect *L. major* promastigotes. The resulting recombinant parasites were selected by periodic stationary phase growth arrest and dilution into fresh medium for 10 weeks. Episomal DNA from the resulting population was extracted and used to transform *E. coli*. A clonal analysis of 50 bacterial clones identified two subclones of pcos11.2 that were dominant in the parasite population (data not shown). The ends of the inserts of both plasmid subclones were sequenced.

In parallel, the cosmid pcos11.2 was completely sequenced. Within the 36,240 base pairs of the *L. donovani* genomic DNA insert (GeneBank Acc. No AC093553), nine putative open reading frames were discovered (Fig. 3). Usually, coding sequences in *Leishmania spp*. show a >60% G/C content owing to the strong preference for G and C in the wobble position of codons (Alvarez et al., 1994; Martinez-Calvillo et al., 2001). Only ORFs 3, 6, and 9 match this criterion of *Leishmania* coding sequences. ORFs 1 and 2 have G/C contents of 56 and 58% respectively, the rest have G/C ratios of 50% only and are not likely gene candidates. ORF1 and ORF9 are similar to hypothetical ORFs identified in the *L. major* genome project (Accession Nos CAC01956, 87% identity, and CAB98657, 86% identity). ORF1 encodes for a protein that has similarities with apolipoprotein A1.

Using the partial sequence information from the two plasmids that had been selected by screening of the plasmid sublibrary, we could establish the endpoints of the plasmid inserts on the pcos11.2 sequence. Only two ORFs, namely ORF8 and ORF9, are in the region present within the subclones' inserts (Fig. 3). To distinguish which of these

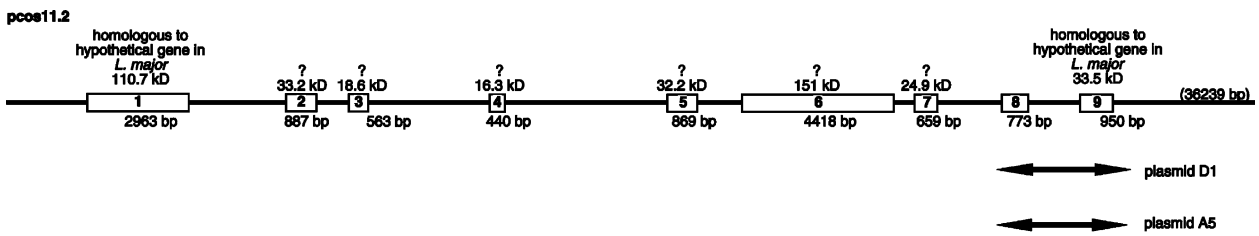

Fig. 3. Schematic representation of the insert sequence of cosmid pcos11.2. The open boxes represent nine putative ORFs. The lengths of the ORFs in bp and the predicted molecular weight of the encoded polypeptides in kDa is given, as well as homologies to hypothetical coding sequences identified in the *Leishmania major* genome project. The double-headed arrows indicate the DNA regions contained within the two plasmids, D1 and A5, that were identified by the selection of the plasmid sublibrary.

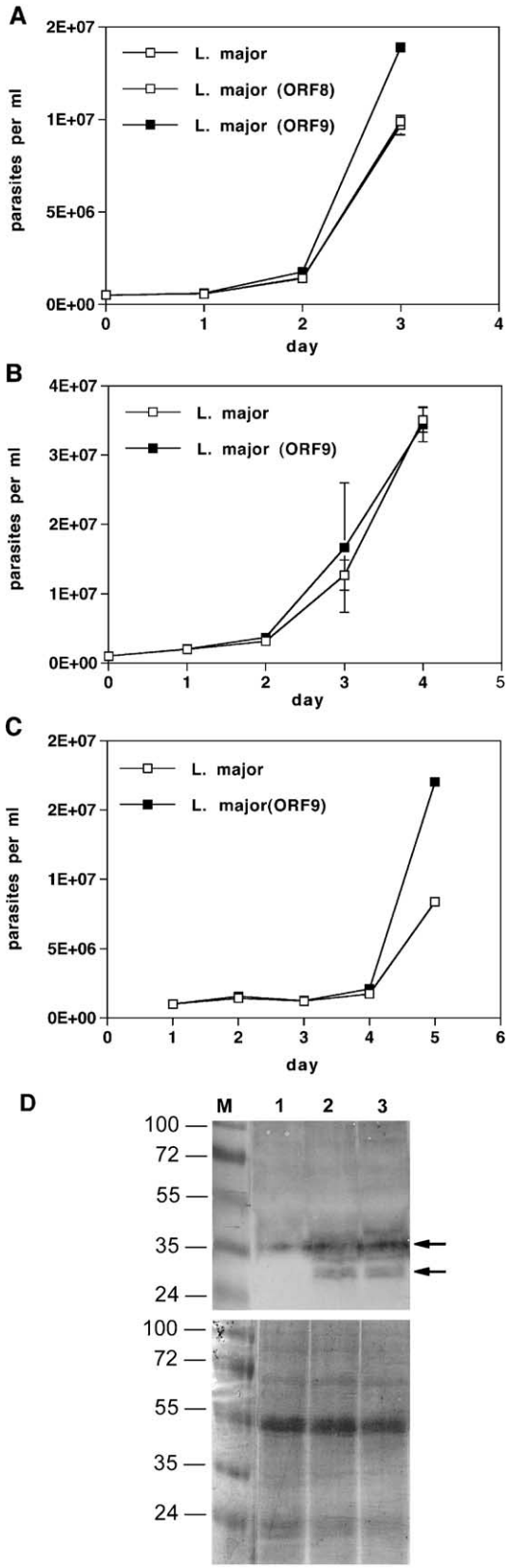

Fig. 4. Proliferation kinetics of recombinant *Leishmania major*. Promastigotes were subjected to a gene replacement, exchanging one copy of the rRNA genes against the *Swa* I fragments from pIRmcs3 + (open squares), pIRORF8 (open triangles), or pIRORF9 (solid squares). Parasites were seeded at 1×10^6 ml^{-1} in fresh medium 199, and cell density was determined daily for 3 to 5 days. Seeds were derived from stationary phase culture (A), hydroxy urea-treated culture arrested in S phase (B), and a culture that was growth arrested by serum deprivation for 4 days (C). The experiments were performed in quadruplicate. The error bars represent the standard deviation. (D) Expression control experiment. Cell lysates equivalent to 2×10^7 promastigotes from *L. major* [pIRmcs3 +], *L. major* [pIRORF9.1], and *L. major* [pIRORF9.4] were separated by SDS-PAGE. Protein patterns were visualised by immunoblot with anti-LdGF1 antiserum (upper panel) or by Coomassie Brilliant Blue (loading control, lower panel). The arrows point at bands that are induced in the overexpressing strains.

two ORFs was responsible for the accelerated outgrowth of *L. major* promastigotes, ORF 8 and ORF 9 were amplified. We used specific primers to create suitable restriction sites for directional cloning into the integration vector pIRmcs3 + (Fig. 1). The amplified ORFs were then ligated into pIRmcs3 + to create plasmids pIRORF8 and pIRORF9, respectively. Two clones from each construct, (pIRORF8.1, pIRORF8.3, pIRORF9.1, and pIRORF9.4), as well as the parent vector pIRmcs3 + were linearised with *Swa* I. The *Swa* I fragments were transfected into *L. major* promastigotes. The transfectants were then selected for stably integrated recombinant DNA by ClonNAT selection. Integration of ORF8 and ORF9, respectively, was verified by PCR analysis of genomic DNA (not shown).

Recombinant parasites were allowed to proliferate until they reached stationary phase growth arrest. Equal numbers were then seeded into fresh medium and the proliferation in these cultures was monitored for 3 days. Fig. 4A shows the result. The *L. major* strain transfected with ORF9 constructs showed faster outgrowth compared with the vector control or the strains transfected with ORF8. We conclude that the gene product of ORF9 is responsible for the observed stimulatory effect on proliferation after growth arrest.

ORF9 encodes a putative polypeptide of 317 amino acids with a calculated mass of 33,485. BLAST searches against the available public databases did not reveal similarities to known proteins or structural motifs. A homologous gene fragment (CHR12_tmp.0490) is found in the database of the *L. major* Genome Project and is classified as a sequence orphan with no known functional motifs. Thus, the function of the ORF9 gene product is unknown. We therefore tentatively assign the name LdGF1, *Leishmania donovani* growth *f*actor *1*, to both the gene and the protein.

To ascertain that LdGF1 is indeed overexpressed in the recombinant parasites, we performed a Western Blot analysis of *L. major* transfected with either pIRmcs3 vector (Fig. 4D, lane 1) or with pIRORF9.1 (lane 2) or pIRORF9.4 (lane3). The equivalent of 2×10^7 promastigotes was lysed in SDS sample buffer and subjected to electrophoresis. After Western transfer, the membrane was probed with an anti-LdGF1 antiserum. The arrows point at protein bands corresponding to molecular masses < 35000 that react with the antibody and are weaker or absent from the pIRmcs control strain. This is in good agreement with the calculated

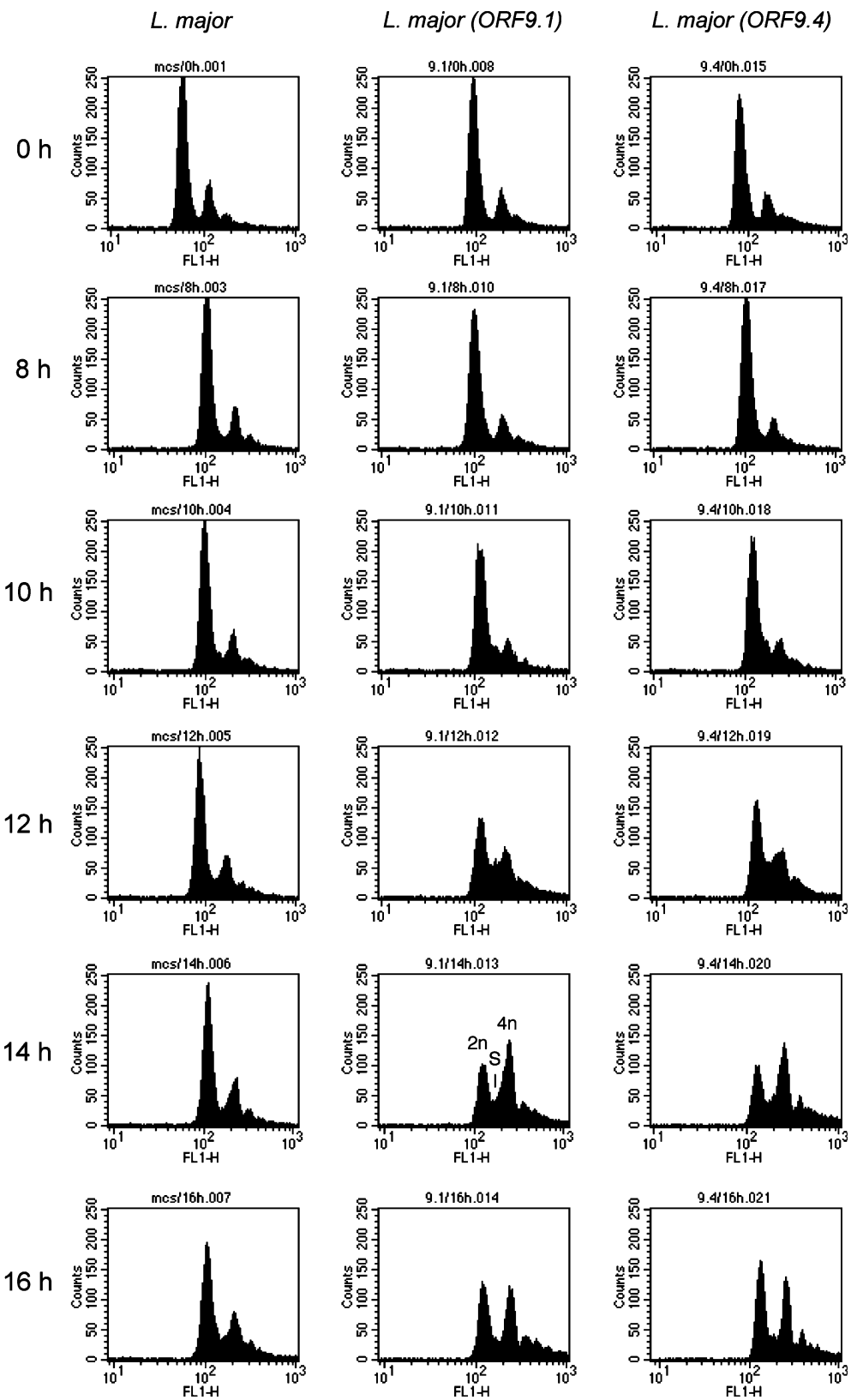

Fig. 5. Flow cytometric analysis. *Leishmania major* transfected with pIRmcs3 + vector or with two independent clones of pIRORF9 were arrested by serum deprivation for 4 days. Growth arrested parasites were seeded in fresh medium containing 20% FCS (0 h), and aliquots were taken. Starting 8 h after the seed, aliquots were taken every 2 h and subjected to SYTOX fluorescent staining. Samples were then analysed for incorporation of fluorescent dye by flow cytometry. The position of the fluorescence peaks that represent the 2 n and 4 n populations (G_1 and G_2 phase of the cell cycle, respectively) are marked in one insert, together with the area that represents the cells in S phase.

mass of 33,485. It also shows that LdGF1 has a very low abundance in wild type *L. major* and is overexpressed substantially in the recombinant strains.

We next tried whether *LdGF1* can confer an advantage after growth arrest induced by other treatments. When we arrested the growth of *L. major* and *L. major* (ORF9) using hydroxy urea which inhibits DNA synthesis and arrests cells at the entry into S phase, the parasites that overexpressed *LdGF1* did not recover faster after drug removal (Fig. 4B). When we arrested recombinant parasites by serum deprivation, however, the positive effect of LdGF1 overexpression was very pronounced (Fig. 4C). We concluded that *LdGF1* accelerates re-entry into the cell cycle after G_1 arrest, but not after an S-phase arrest.

We next established the kinetics of recovery for control parasites and for *LdGF1* overexpressing strains. Proliferation arrest by serum removal is a better-defined parameter than stationary phase growth arrest. Therefore, we arrested the parasite populations by serum withdrawal for 4 days. Serum was added back, and parasite aliquots were taken at time intervals from 0 to 16 h after serum addition. Parasites were stained with SYTOX and subjected to FACS analysis. Fig. 5 shows the result of this study. While the control population remained in G1 arrest for at least 16 h after serum addition, the *LdGF1*-overexpressing strains entered into S phase after 10 h and showed a pronounced G2/M phase peak after 12 h. This result corroborates our finding that overexpression of *LdGF1* accelerates re-entry into the cell cycle after a G_1 growth arrest induced either by high parasite density or by serum withdrawal Fig. 6.

Lastly, we tested the effect of *LdGF1* overexpression on the virulence of *L. major* in mice. *Leishmania major* promastigotes that were transfected with vector pIRmcs3 + or with one of two clones of pIRORF9, pIRORF9.1 and pIRORF9.4, were inoculated into the footpads of two BALB/c mice each. Lesion growth was monitored for up to 16 weeks. All four mice infected with *LdGF1*-overexpressing parasites showed retarded lesion development compared with the mice infected with the control strain. We conclude that overexpression of *LdGF1* is not beneficial during the mammalian stage of the parasites, but rather poses a disadvantage. Nevertheless, when we analysed the re-isolated recombinant parasites from footpad lesions, 52 out of 60 clones had retained the integrated transgene, as evidenced by transgene-specific PCR (data not shown). The result attests to the relative stability of transgenes integrated into the rRNA gene cluster. It also suggests that overexpression of LdGF1 does not abolish virulence alltogether, but rather attenuates *L. major*.

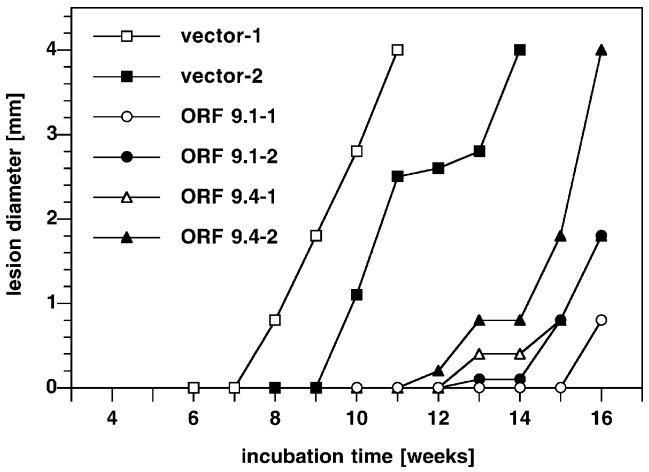

Fig. 6. Time course of *Leishmania major* infections. Two mice each were infected with each *L. major* strain. 5×10^6 promastigotes of *L. major (pIRmcs3 +)* (open and solid squares), *L. major (pIRORF9.1)* (open and solid circles), and *L. major (pIRORF9.4)* (open and solid triangles) were inoculated into the right hind footpads of BALB/c mice. Lesion size, defined as difference of foot thickness of right over left hind foot in mm, was measured weekly.

4. Discussion

The factors that govern the cell cycle and proliferation rate in *Leishmania spp*. are, for the most part, unknown. A search in the *L. major* genome project database revealed only four entries under the heading cell cycle control, all defined by analogies to known proteins. Several proteins were identified that have an impact on the cell division. Among these are the *L. donovani* centrin homologue (Selvapandiyan et al., 2001), *L. donovani* HSP90 (Wiesgigl and Clos, 2001), CRK3 protein kinase (Wang et al., 1998; Hassan et al., 2001), and the proteasome (Robertson, 1999). Of these, CRK3 was shown to rescue a cdc2 yeast knock out mutant. For the other proteins, a negative impact on G2/M transition was demonstrated by mutation analysis or by pharmacological inactivation Fig. 7.

We have, in this study, identified a novel gene from *L. donovani* with significant impact on the cell cycle. *LdGF1* overexpression accelerates the recovery from a G_1 cell cycle arrest induced either by high cell density or by serum withdrawal. The effect of *LdGF1* is dependent on the gene dosage. Its overexpression confers a strong selective advantage to cultivated *L. major* and *L. donovani* promastigotes, provided the cultivation involves frequent cell density-induced growth arrests, something that cannot be avoided entirely in most laboratory settings. Thus, a selective pressure on the increased expression of *LdGF1* can be envisioned during repeated in vitro passage.

Leishmania major parasites that harboured cosmid pcos11.2 could not be recovered from infected tissue of mice after experimental infection (Hoyer et al., 2001). The zero recovery rate of pcos11.2 after mouse passage was lower than the recovery rate of *L. major* transfected with the parent cosmid vector, pcosTL. This indicated that, during the mammalian stage of *L. major*, overexpression of *LdGF1* constitutes a selective disadvantage to *L. major*. This finding was confirmed by the fact that *L. major* (pIRORF9) showed a reduced virulence compared with control strains in BALB/c mice. This may explain why, under natural conditions, there is no selection for an amplification of this gene in wild type

Fig. 7. Sequence alignment of LdGF1 and the putative product of ORF Chr12.18 (GenBank Acc. No CAB98657). The consensus sequence is shown underneath. Sequence similarity is 94.7%.

Leishmania parasites. However, expression of genes such as *LdGF1* will constitute an advantage during in vitro culture. This may be part of the molecular mechanisms responsible for some of the instances where a loss of virulence was observed after prolonged in vitro passage of *Leishmania* parasites (Neal, 1984; Nolan and Herman, 1985; Segovia et al., 1992; De and Roy, 1999).

Interestingly, the parasites that were recovered from the delayed lesions still harbored the transgene. Future experiments should address the question whether the transgenes still allow for overexpression after the mouse passage, i.e. whether they are silenced, and whether the immune response to *L. major* is in any way affected by the overexpressed transgene.

The only homologue found in the public databases is a hypothetical open reading frame, CHR12_tmp.0490, annotated in the *L. major* genome database. The observed identity/similarity between both putative amino acid sequences is 92.4/94.7%.

Since *LdGF1* has no readily identifiable homologues in the database of known proteins, its function may be performed by other, structurally unrelated, proteins in higher eukaryotes. Alternatively, LdGF1 and its unknown function may be unique to *Leishmania spp.* or to kinetoplastid protozoa. In both cases, if its function proves to be essential to *Leishmania*, LdGF1 may be an addition to the ever-growing list of potential target molecules for therapeutic intervention. Especially so, since an amplification of this gene in drug-resistant parasite clones would, in all likelihood, render the parasite less virulent in the mammalian host.

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