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A mitogen-activated protein (MAP) kinase homologue of *Leishmania mexicana* is essential for parasite survival in the infected host

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The parasitic protozoon *Leishmania mexicana* undergoes two major developmental stages in its life cycle exhibiting profound physiological and morphological differences, the promastigotes in the insect vector and the amastigotes in mammalian macrophages. A deletion mutant, Δ*lmsap1/2*, for the secreted acid phosphatase (SAP) gene locus, comprising the two SAP genes separated by an intergenic region of ~11.5 kb, lost its ability to cause a progressive disease in Balb/c mice. While *in vitro* growth of promastigotes, invasion of host cells and differentiation from promastigotes to amastigotes was indistinguishable from the wild-type, the mutant parasites ceased to proliferate when transformed to amastigotes in infected macrophages or in a macrophage-free *in vitro* differentiation system, suggesting a stage-specific growth arrest. This phenotype could be reverted by complementation with 6 kb of the intergenic region of the SAP gene locus. Sequence analysis identified two open reading frames, both encoding single copy genes; one gene product shows high homology to mitogen-activated protein (MAP) kinases. Complementation experiments revealed that the MAP kinase homologue, designated LMPK, is required and is sufficient to restore the infectivity of the Δ*lmsap1/2* mutant. Therefore, LMPK is a kinase that is essential for the survival of *L.mexicana* in the infected host by affecting the cell division of the amastigotes.

Keywords: attenuated strain/cell division/deletion mutant/*Leishmania mexicana*/mitogen-activated protein kinase

Introduction

Infection with *Leishmania*, a parasitic protozoon, represents a major health problem in the tropics and subtropics, with ~380 000 cases annually and 367 million people at risk of infection (Ashford *et al.*, 1996). Depending on the species, *Leishmania* causes a spectrum of diseases ranging from self-healing cutaneous lesions to lethal visceral forms (Alexander and Russell, 1992). During their life cycle, the parasites undergo profound morphological changes. The spindle-shaped, flagellated procyclic promastigotes, which proliferate in the gut of the sandfly, differentiate into non-dividing infective metacyclic cells. When the insect takes a blood-meal, the metacyclics are transmitted into the skin of the mammal. Once in the mammal, the parasites enter macrophages where they reside in phagolysosomes. In this low-pH, hydrolytic environment, they transform into the oval, non-motile amastigotes and proliferate. Morphologically, amastigotes are smaller than promastigotes and have a rudimentary flagellum buried in the flagellar pocket. Ultrastructurally, *Leishmania mexicana* amastigotes have large lysosome-like organelles called megasomes, which are absent in promastigotes. Moreover, they differ in their surface architecture and in their secretory products. The surface of the promastigotes is composed of lipophosphoglycan (LPG), the surface protease gp63 and glycoinositolphospholipids (GIPLs) as dominant surface markers (McConville and Ferguson, 1993). At least in *L.mexicana*, amastigotes lack LPG and gp63, having only GIPLs and host-derived glycosphingolipids on their surface (Bahr *et al.*, 1993; Winter *et al.*, 1994).

It is likely that the changes in the biochemistry and morphology of *Leishmania* during its life cycle are the result of programmed changes in gene expression in response to the changes in the external environment of the parasite. However, the signal transduction pathways involved in promastigote-to-amastigote differentiation and vice versa, and in life stage-specific gene expression are not known. Through analogy with other eukaryotic cells (Treisman, 1996), protein phosphorylation can be expected to be important for differentiation and proliferation of *Leishmania*. Therefore, protein kinases and phosphatases, and their regulation, are likely to be critical for the parasite's development. In fact, changes in phosphoprotein abundance and in the overall phosphorylation pattern in *Leishmania* and other kinetoplastids, such as *Trypanosoma brucei* and *Trypanosoma congolense*, have been documented throughout their life cycles (Mukhopadhyay *et al.*, 1988; Aboagye-Kwarteng *et al.*, 1991; Parsons *et al.*, 1991, 1993, 1995; Dell and Engel, 1994), but their significance for parasite differentiation remains unknown. In a recent review, Boshart and Mottram (1996) summarized the knowledge of protein phosphorylation and protein kinases in trypanosomatids. They presented a collection of cloned genes of various protein phosphatases and kinases: protein phosphatases 1 and 2A from *T.brucei* and 2C from *Leishmania chagasi*; cdc2-related kinases from *L.mexicana*, *T.brucei* and *Crithidia fasciculata*; a NIMA-related, a polo-like and a potential mitogen-activated protein (MAP) kinase from *T.brucei*; protein kinase A from *L.major* and *T.brucei*; MAP kinase kinases from *L.chagasi* and *Leishmania major* (Boshart and Mottram, 1996, and references therein).

Here, I report the identification of a *L.mexicana* gene coding for a protein with strong homology to MAP kinases of yeast and higher eukaryotes that is required for amastigote survival and proliferation in macrophages. Therefore, this gene is essential for the infection of mammals.

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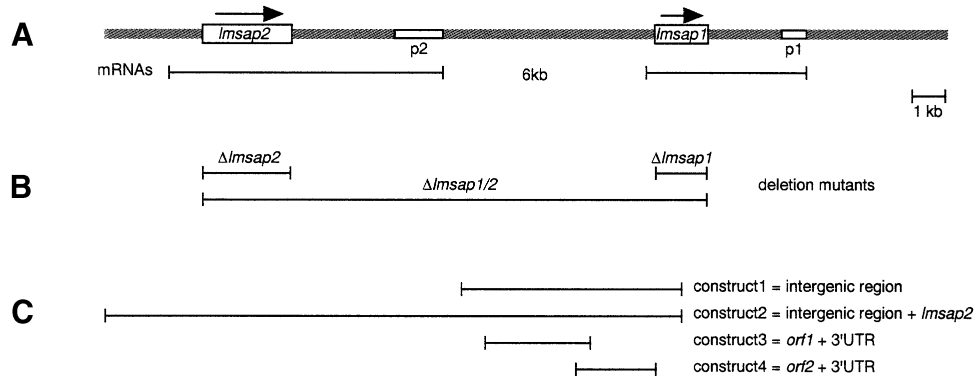

Fig. 1. Genomic organization of the SAP gene locus, *lmsap* deletion mutants and complementation strategy. (**A**) Genomic organization of the SAP genes *lmsap1* and *lmsap2*. The open reading frames are marked by arrows indicating the direction of transcription. Approximate sizes of DNA regions covered by the mRNAs are indicated below the respective genes leaving an intervening region of ~6 kb. p1 and p2 are gene-specific probes used to map the transcribed regions in a Northern-blot analysis. (**B**) DNA regions replaced by resistance markers in the deletion mutants Δ*lmsap1*, Δ*lmsap2* and Δ*lmsap1/2*. (**C**) DNA regions cloned into pX63PHLEO or pX63polPHLEO and used in complementation experiments of the null mutant Δ*lmsap1/2*.

Results

A non-pathogenic deletion mutant of Leishmania mexicana

In a previous publication (Wiese *et al.*, 1995), we reported the construction of gene deletion mutants in *L.mexicana* encompassing parts of the secreted acid phosphatase (SAP) gene locus (Figure 1A and B), in order to analyse the composition and structure of the unique filamentous complex formed by this enzyme (Ilg *et al.*, 1991). Two closely related genes, *lmsap1* and *lmsap2*, were found to be tandemly arranged. The open reading frames are separated by an intergenic region of ~11.5 kb. Three mutants were derived lacking either both alleles of *lmsap1* (Δ*lmsap1*), *lmsap2* (Δ*lmsap2*) or both genes including the intergenic region (Δ*lmsap1/2*; Figure 1). Owing to the speculation that the SAPs may contribute to the pathobiology in Leishmaniasis by eliciting a humoral immune response (Chang *et al.*, 1990), we tested all mutants for their infection potential in Balb/c mice. Both single-gene deletion mutants showed lesion development comparable with control mice infected with wild-type *L.mexicana* (data not shown). In contrast, promastigotes of the third deletion mutant, Δ*lmsap1/2*, did not cause lesion development in mice infected with promastigotes.

In culture, proliferation of Δ*lmsap1/2* promastigotes was indistinguishable from wild-type promastigotes. Mutant promastigotes of late logarithmic growth phase could be transformed in the absence of macrophages into amastigote-like cells by pH and temperature shifts to pH 5.5 and 34°C, respectively (Figure 2A). Starting at cell densities of either 1×10^6 or 4×10^6 promastigotes/ml, the cell counts increased over 4 days to ~5×10^6 and 3×10^7 cells/ml, respectively, and then remained constant. In contrast, the wild-type reached a cell density of 3.5×10^7 cells/ml in either 4 or 6 days, irrespective of the size of the inoculum, indicating growth to stationary phase. After 4 weeks at 34°C and pH 5.5 with intermittent supply of fresh medium, an elevation of the pH to 7.0 and a reduction of the temperature to 26°C, which are the optimal conditions for *L.mexicana* promastigote growth *in vitro*, resulted in some of the cultivated Δ*lmsap1/2* mutant amastigote-like parasites re-transforming into promastigotes, indicating their viability and a reversibility of differentiation independent of the deletion. Infection of peritoneal macrophages derived from Balb/c mice revealed that the Δ*lmsap1/2* mutant retained its ability to enter host cells and to differentiate into the amastigote form. However, in the course of 6 days after the infection, the ratio of infected to uninfected macrophages dropped from 0.9 on the first day to 0.09 on day 6, indicating that the parasites ceased to proliferate and were cleared by the macrophages (Figure 2B). In contrast, the wild-type reached densities up to 25 amastigotes per infected macrophage within 3 days post-infection (results not shown). In summary, the Δ*lmsap1/2* mutant is impaired in the ability to grow axenically as amastigotes, and it cannot proliferate in macrophages or mice.

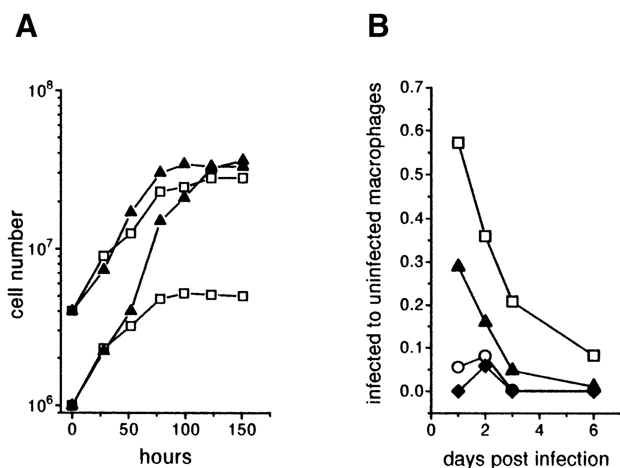

Fig. 2. Growth of wild-type and Δ*lmsap1/2* null mutant amastigotes. (**A**) Growth curves of *in vitro* transformed promastigotes starting at 1×10^6 and 4×10^6 cells/ml, respectively. Transformation was achieved by pH and temperature shifts as described in Materials and methods. (▲) *L.mexicana* wild-type, (□) Δ*lmsap1/2*. (**B**) Infection of peritoneal macrophages with the mutant Δ*lmsap1/2*. Macrophages were grown on glass coverslips overnight, infected with promastigotes at a ratio of 20:1, coverslips were removed at the indicated time points and processed for inspection as described in Materials and methods. Three times, 100 macrophages were randomly inspected, the average taken and the macrophages grouped according to their parasite load. (□) 1–3 amastigotes/macrophage, (▲) 4–6 amastigotes/macrophage, (○) 7–9 amastigotes/macrophage, (◆) >10 amastigotes/macrophage.

The intergenic region is responsible for the Δlmsap1/2 phenotype

The deletion of both SAP genes in the mutant Δ*lmsap1/2* did not impair the parasites with regard to their uptake by macrophages or their differentiation to amastigotes. Therefore, it appeared very likely that in *L.mexicana* the SAP is not required for the initial steps of the infection, but may be necessary to establish a progressive infection later on. However, we have not been able to detect any material reactive with an anti-protein mAb specific for SAP (mAb LT8.2; Ilg *et al.*, 1993) using immunofluorescence in macrophages infected with wild-type promastigotes *in vitro*, or using immunoelectron microscopy of infected macrophages in mouse lesions (M.Wiese, T.Ilg and Y.-D.Stierhof, unpublished results). This could be due to a modification or loss of the mAb LT8.2 epitope located on the exposed C-terminus after the highly *O*-glycosylated repetitive region of SAP (Wiese *et al.*, 1995; Y.-D.Stierhof, M.Wiese, T.Ilg, P.Overath, M.Häuer and U.Aebi, in preparation), preventing the binding of the antibody, or simply to the absence of the protein despite the presence of a *lmsap* mRNA (Wiese *et al.*, 1995). If the SAP is not required to establish an infection, it appears likely that there is at least one additional gene located within the 11.5 kb intergenic region of the SAP gene locus, which has been removed concomitantly with the SAP genes, *lmsap1* and *lmsap2*, in the null mutant, Δ*lmsap1/2*. To clarify this situation, two plasmids were designed and introduced into the deletion background. First, the transcribed regions of the two SAP genes were mapped roughly in a Northern blot experiment using gene-specific probes (p1 and p2; Figure 1A) that hybridized to the 3′-flanking DNA of either *lmsap2* or *lmsap1* (data not shown). This defined a 6 kb portion of the *lmsap* intergenic region that could carry additional genes (Figure 1A) responsible for the mutant phenotype. A suitable DNA fragment carrying most of this portion was subcloned into the leishmanial expression vector pX63PHLEO (Freedman and Beverley, 1993) resulting in construct 1 (Figure 1C). Construct 2 was obtained by the addition of the *lmsap2* gene and its flanking regions to the first construct, thereby forming a plasmid encompassing *lmsap2*, the intergenic region and a truncated *lmsap1* gene (Figure 1C). These two constructs were introduced into the Δ*lmsap1/2* mutant background. Lesion development after injection of 1×10^6 mutant or wild-type promastigotes into hind footpads of Balb/c mice was monitored for 70 weeks (Figure 3). *Leishmania mexicana* wild-type promastigotes, included as a control, gave rise to the most progressive lesion development. Both plasmid constructs conferred the potential to cause the disease, however, with a lag of ~10 weeks, indicating that the prolonged *in vitro* culture used to obtain the mutants may have resulted in a decrease in their infectious potential. The Δ*lmsap1/2* null mutant did not lead to a significant swelling of the inoculated footpads. The overlapping bars for the standard deviations show clearly that there is no significant difference in the course of the infection in the Δ*lmsap1/2* mutant complemented with a construct carrying or lacking *lmsap2*. The slower kinetics of lesion development compared with the wild-type could be due to the episomal location of the essential gene that could either lead to a heterogeneous expression of the corresponding gene product in the amastigotes resulting in protein levels too low to survive in some cells, or the plasmid could be lost in some parasites during division, resulting in the death of the segregants, and thereby slowing down the progression of the disease. This shows that this plasmid-based complementation system can only yield qualitative information when compared with the wild-type. Despite the slower progression of lesion development in the complemented Δ*lmsap1/2* null mutants the final lesion size in infected mice was comparable with wild-type infections resulting in metastasis and finally death of the mice. Therefore, the SAP of *L.mexicana* is not required either for the initial steps of the infection, for the differentiation of promastigotes to amastigotes or for their survival and proliferation in the mammalian host. This result is not unexpected, as a different pathogenic species, *L.major*, does not secrete an acid phosphatase (Lovelace and Gottlieb, 1986).

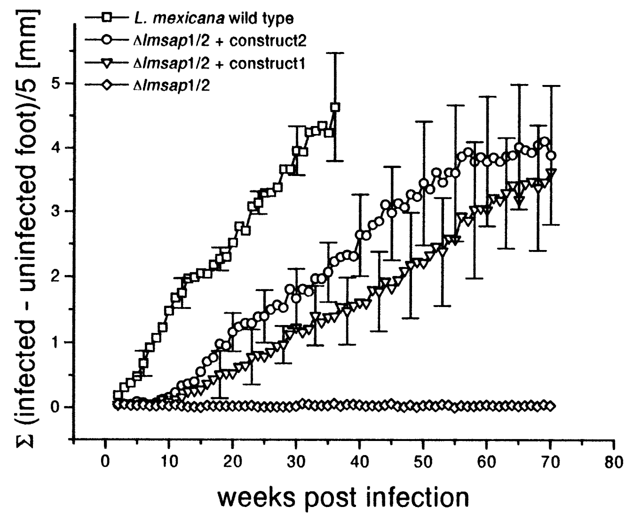

Fig. 3. Footpad infection experiment of SAP null mutant Δ*lmsap1/2* complemented with constructs 1 and 2. (□) *L.mexicana* wild-type, (○) Δ*lmsap1/2* + construct 2, (▽) Δ*lmsap1/2* + construct 1, (◇) Δ*lmsap1/2*; compare Figure 1C. Bars represent standard deviations in each group of five infected mice.

Identification of lmpk and orf2

As the inability of the mutant Δ*lmsap1/2* to develop lesions in infected mice was not due to the loss of the SAP, it was very likely that at least one other gene, which was essential for amastigote survival in the infected host cell, was located within the 6 kb of intergenic region of the SAP gene locus sufficient to complement the null mutant. Sequence analysis of this region led to the identification of two open reading frames, *orf1* and *orf2* (Figure 4A). *Orf1* consists of 1074 bp coding for a protein of 358 amino acids, with a calculated molecular mass of 41 kDa. A homology search in the DDBJ/EMBL/GenBank database revealed significant homology to MAP kinase genes, e.g. 55.6% amino acid identity to the KFR1 MAP kinase homologue of *T.brucei* (Hua and Wang, 1994). Figure 5 shows an alignment of the *L.mexicana* kinase homologue encoded by *orf1* with other protozoan kinases (*T.brucei* KFR1 kinase, *Dictyostelium discoideum* MAP kinase, *Plasmodium falciparum* MAP kinase homologue) and one representative each of yeast (*Saccharomyces cerevisiae* KSS1), plant (*Arabidopsis thaliana* MAP

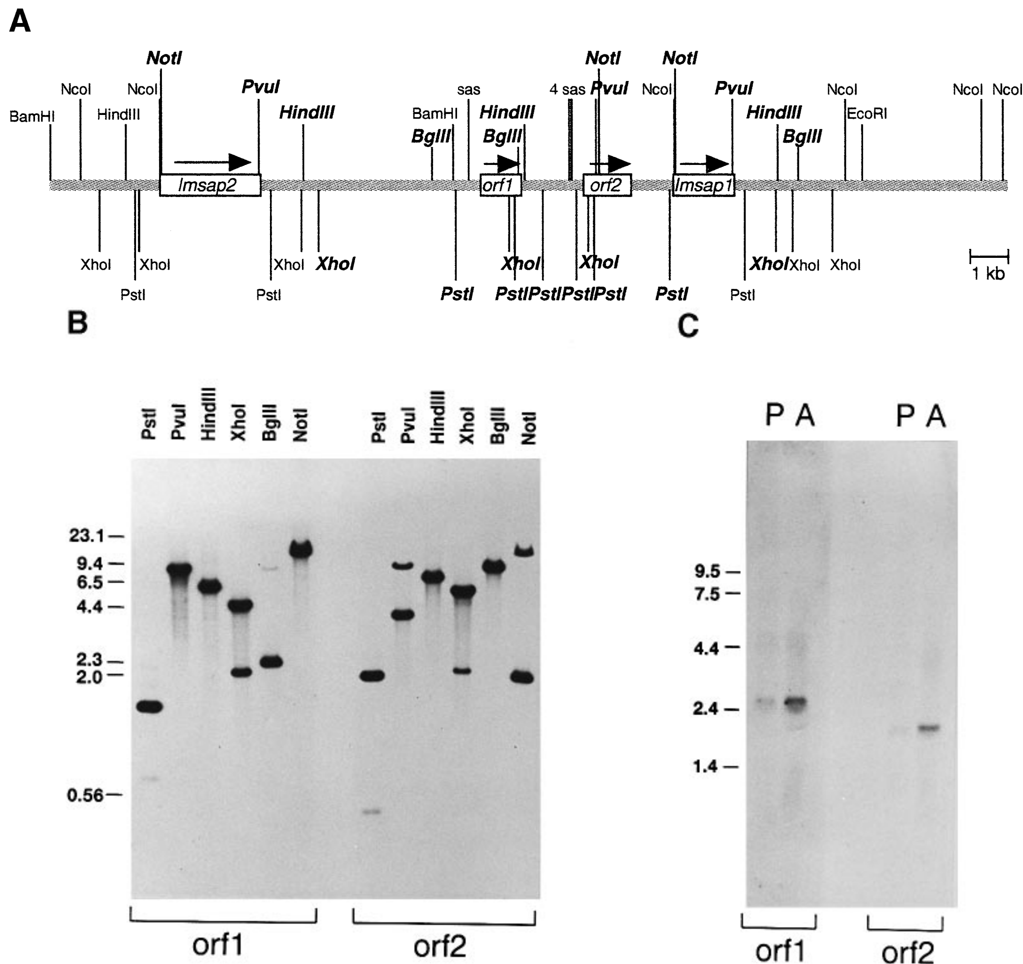

Fig. 4. Southern and Northern analysis of *orf1* (*lmpk*) and *orf2*. (**A**) Genomic organization of *orf1* (*lmpk*) and *orf2* in the SAP gene locus showing all relevant restriction sites and splice addition sites (sas). Restriction sites in bold italics produce hybridizing bands in the Southern blot experiment. The open reading frames are boxed, the arrows indicate the direction of transcription. (**B**) Southern analysis of genomic DNA from *L.mexicana*. DNA (5 μg) was cleaved with the indicated restriction enzymes, separated on a 0.7% agarose gel, blotted onto a nylon membrane and probed with DIG-labelled, gene-specific probes for either *orf1* or *orf2* as indicated. (**C**) Northern analysis of total RNA from *L.mexicana* promastigotes (P) and amastigotes (A). Total RNA (20 μg) was separated on a 1% denaturing formaldehyde agarose gel, blotted onto a positively charged nylon membrane and probed with the respective radiolabelled, gene-specific probes. Numbers indicate the size of DNA and RNA standards (kb).

kinase), and mammalian (*Rattus norvegicus* ERK2 kinase) kinases. The sequence displays the typical 11 major conserved subdomains (I–XI) and the sequence motifs characteristic for proline-directed serine/threonine protein kinases. These motifs are the phosphate anchor ribbon for ATP binding, Gly21-Ser22-Gly23-Ala24-Tyr25-Gly26, at the N-terminus (consensus Gly-X-Gly-X-X-Gly), the potential regulatory phosphorylation sites at Thr176 and Tyr178 in the phosphorylation lip (Leu158-Arg185), the P+1 specificity pocket (Ala180-Thr181-Arg182-Trp183-Tyr184-Arg185) and the catalytic site residues Lys43, Arg57, Arg60, Glu61, Arg136, Asp137, Lys139, Asn142, Asp155 (Mg^{2+} ligand), Arg160 and Thr181 (Zhang *et al.*, 1994; Wang *et al.*, 1997). Because of the high homology and the presence of all the typical MAP kinase features, *orf1* was designated *lmpk* for the *L.mexicana* protein kinase gene. The corresponding protein is termed LMPK.

Orf2 showed no significant homology to the sequences in the database. It codes for a putative protein of 418 amino acids with a calculated molecular mass of 45 kDa. There is no potential signal sequence for import into the endoplasmic reticulum. Interestingly, the protein is rich in Arg (11 mole per cent), has an overall charge of 21 and an isoelectric point of 11.66. Moreover, it contains a number of phosphorylation motifs for cAMP-dependent protein kinase, casein kinase II and protein kinase C as revealed by a motif search using the GCG DNA analysis program (Devereux *et al.*, 1984) and two potential MAP kinase phosphorylation sites matching the consensus Pro-X-Ser/Thr-Pro where X is any amino acid (Ruderman, 1993).

Southern-blot analysis of both genes with a selection of restriction enzymes, cleaving inside and next to the open reading frames using gene-specific probes, resulted in a pattern of hybridizing bands corresponding to the mapped SAP gene locus (Figure 4A and B), indicating that both genes are present in one copy per haploid genome. Moreover, both genes are transcribed in promastigotes and amastigotes with a bias to higher transcript levels in the mammalian amastigote stage (Figure 4C). The sizes of the mRNAs are 2.6 and 2.0 kb for *lmpk* and *orf2*, respectively. Therefore, these mRNAs occupy nearly all of the 6 kb of the, as yet uncharacterized, intergenic region of the SAP gene locus. In Kinetoplastidae, mature mRNAs

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                                                  1                                 31
Leish LMPK   ---------- ---------- --------MT SYGIDGEVEQ RY-RILRHIG SGAYGVVWCA
 Tryp KFR1   ---------- ---------- --------MV .FS...RI.. ..-K..C... ..........
  Dict MPK   ---------- ---------- --------MS .ED..KH.LR K.-EVFHK.. K....I..E.
  Plas MPK   ---------- ---------M PKEDCKTEKS IDS..EN.LK K.-D..KKV. K.......FKG
  Arab MPK   MSAESCFGSS GDQSSSKGVA THGGSYVQYN V..NLF..SR K.VPP..P.. R....I.CA.
  Rat ERK2   ---------- ---------- MAAAAAAGPE MVRGQVFDVG PRYTN.SY.. E....M.CS.
Yeast KSS1   ---------- ---------- ---------M ART.TFDIPS Q.-KLVDL.. E....T.CS.
                                                                        I

             32                                                             88
Leish LMPK   LDRRTGKCVA LKKVYDAFGN VQDAQRTYRE VMLLQRLRHN PFIVGILDVI RA---ANDID
 Tryp KFR1   ...V.KMK.. I......... R......... .....ST.QLD N-..PL.N.. .S---I.GT.
  Dict MPK   I.KKPHHT.. ...IF...Q. AT.....F.. I.F..E.HGH EN.IKL.N.. K.---D..R.
  Plas MPK   RCKKNKNI.. V..IFG..Q. CT.....F.. IIF.YE.NGH DN.IKLM... K.---K..N.
  Arab MPK   TNSE..EE.. I..IGN..D. II..K..L.. IK..KHM-DH ENVIAVK.I. KPPQRE.FN.
  Rat ERK2   Y.NLNKVR.. I..IS-P.EH QTYC...L.. IKI.L.F..- EN.I..N.I. ..PTIEQMK.
Yeast KSS1   IHKPS.IK.. I..I-QP.SK KLFVT..I.. IK..RYFHEH EN.IS...KV .PVSIDKLNA
                  II                   III                   IV

             89
Leish LMPK   LYLVFELIET DLTAIIRKN- ----LLQRDH KRFLTYQLLR TVAQLHAQNI IHRDLKPANV
 Tryp KFR1   .......A.. ..SVVL.H.- ----IMESVQ RQYVA..IVY A..G...RGV ..........
  Dict MPK   I.....HM.. ..H.V..AK- ----I.EEI. .QYTI....K ALKYM.SA.V L...I..S.L
  Plas MPK   I..I.DFM.. ..HEV.KAD- ----..EEI. .KYII..... ALKYI.SGGL L...I..S.I
  Arab MPK   V.I.Y..MD. ..HQ...S.Q P----.TD.. C..FL..... GLKYV.SA.V L......S.L
  Rat ERK2   V.I.QD.M.. ..YKLLKTQH -----.SN.. ICYFL..I.. GLKYI.SA.V L......S.L
Yeast KSS1   V...E..M.. ..QKV.NNQN SGFST.SD.. VQYF...I.. ALKSI.SAQV ....I..S.L
                 V                                                  VI

             144                                                           202
Leish LMPK   FVSSDCSIKL GDFGLARTFR SGYDNEQEFL D-LTDYIATR WYRSPEILVK SRAYSTAMDM
 Tryp KFR1   .LN...N... .......C.N TQGGDN---- .-..E..... ......V... .TC.T.....
  Dict MPK   LLN.E.LV.V A.....SIT .LESIAEANP V-..E.V... ...A....LG .TK.TKGV..
  Plas MPK   L.N.E.H..V A.....SI- .THV..NKVP I-....V... ...A....LG .TH.TEDV..
  Arab MPK   LLNAN.DL.. .......--- -----TKSET .FM.E.VV.. ...A..L.LN CSE.TA.I.I
  Rat ERK2   LLNTT.DL.I C......V-- --A.PDHDHT GF..E.V... ...A...MLN .KG.TKSI.I
Yeast KSS1   LLN.N.DL.V C......CLA .SS.SRETLV GFM.E.V... ...A...MLT FQE.T....I
                  VII                              VIII

             203                                                           262
Leish LMPK   WAIGCVIGEM LLGRPLFEGR NTLDQLRLII EAIGVPSDAD VRSLHSPELE KLINSLPTPL
 Tryp KFR1   ..V..IL..L FT.S...T.N S..H.IG..V A.L.E..AE. LE..K.E.TW P..D...-AI
  Dict MPK   .S...IL..L .GEKAM.P.N S.MN..D... .VT.R..AE. IEAIK..FAG TMLE...-.S
  Plas MPK   .SL..IM..L .C.K...T.N S.MN..EK.. QV..K.NKK. IEDIR..FA. .I.S.FV-D.
  Arab MPK   .SV..IL..T MTRE...P.K DYVH.....T .L..S.D.SS LGF.R.DNAR RYVRQ..-QY
  Rat ERK2   .SV..ILA.. .SN..I.P.K HY....NH.L GIL.S..QE. LNCIINLKAR NYLL...-HK
Yeast KSS1   .SC..ILA.. VS.K...P.. DYHH..W..L .VL.T..FE. FNQIK.KRAK EY.AN..-MR
                IX                          X

             263                                                           320
Leish LMPK   IFSPLVGNKN LKD--SEATD LMMKLIVFNP KRRLSAVEAL QHPYVAPFVQ HGELEKIQGL
 Tryp KFR1   DPD..PERLS KYN--AD.V. .IC.C...D. NK.PT.R... ....F...TS PSDF.ELDKA
  Dict MPK   NPRS.SDMYP SAS--VD.L. .LK..SQ... DK.IT.E... A..F.TQ.HN PA.EPHFDRI
  Plas MPK   KKKN.KDICY KAS--N.SL. .LE..LQ... SK.I..EN.. K.K..EE.HS IIDEPTCRHI
  Arab MPK   PRQNFAARFP NMS--AG.V. .LE.ML..E. S..ITVD... C...L..LHD IN.EPVCVRP
  Rat ERK2   NKV.WNRLFP NA.--.K.L. .LD.MLT... HK.IEVEQ.. A...LEQYYD PSDEPIAEAP
Yeast KSS1   PPL.WETVWS KT.LNPDMI. .LD.MLQ... DK.I..A... R...L.MYHD PSDEPEY-PP
                                       XI

             321                                         358
Leish LMPK   DPLV------ ----LPLVDE KIYTKEEYKA NLYDEIGMRY RYHITDVY*- ----------
 Tryp KFR1   V.IK------ ----..FP.. EERPADQ.RS A...I..N.R KQKLAL*--- ----------
  Dict MPK   --IK------ ----ISID.G QKFPIA..RN R..ND.IKK- ---------- --KKEERKKQ
  Plas MPK   --IT------ ----I.IN.N TK.RVNF.RN VV.FV.MR.N KF.SNVLNQG ESKKEEKKDR
  Arab MPK   FNFD------ -------FEQ PTL.E.NI.E LI.R.TVKFN PQDSV*---- ----------
  Rat ERK2   FKFD------ -------MEL DDLP..KL.E LIFE.TARFQ PGYRS*---- ----------
Yeast KSS1   LN.DDEFWKL DNKIMRPEE. EEVPI.ML.D M....LMKTM E*-------- ----------
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Fig. 5. Alignment of LMPK with various MAP kinase amino acid sequences. Leish LMPK, *L.mexicana* MAP kinase homologue; Tryp KFR1, KFR1 kinase from *T.brucei* (Hua and Wang, 1994; accession No. L10997); Dict MPK, *D.discoideum* MAP kinase (Segall *et al.*, 1995; accession No. L33043); Plas MPK, *P.falciparum* MAP kinase (accession No. X82646); Arab MPK, *A.thaliana* MAP kinase (Mizoguchi *et al.*, 1993; accession No. D21840); Rat ERK2, *R.norvegicus* ERK2 kinase (Boulton *et al.*, 1991; accession No. M64300) and Yeast KSS1, *S.cerevisiae* KSS1 kinase (Courchesne *et al.*, 1989; accession No. M26398). **I–XI** indicate MAP kinase typical domains. Diamonds mark potential regulatory phosphorylation sites at Thr176 and Tyr178, arrows indicate catalytic site residues, brackets depict the phosphate anchor ribbon (Gly21-Gly26) and the P+1 specificity pocket (Ala180-Arg185) and asterisks mark the C-terminus of the protein. Dashes indicate gaps introduced to optimize the alignment; dots represent identical amino acid residues. Numbering corresponds to LMPK.

are formed from a polycistronic precursor by transfer of the so-called mini-exon carrying a cap structure to the 5′-end of protein coding RNAs, in a *trans*-splicing reaction using an AG dinucleotide splice-acceptor site (Laird, 1989). *Trans*-splicing is accompanied by polyadenylation on the 3′-end of the RNA. Whereas the splice-acceptor site is determined by the AG dinucleotide, there is no consensus for poly(A) addition. In contrast, poly(A) site selection of a given gene has been shown to be dependent on the location of the splice-acceptor site of the next gene located further downstream (LeBowitz *et al.*, 1993). Splice-acceptor sites for *lmpk* and *orf2* were determined using total RNA in a reverse transcriptase polymerase chain reaction (RT–PCR) with gene internal and mini-exon primers (see Materials and methods). Amplified fragments were cloned and sequenced. A single AG splice-acceptor site for *lmpk* was located 320 bp upstream of the ATG translation initiation codon. *Orf2* was found to use

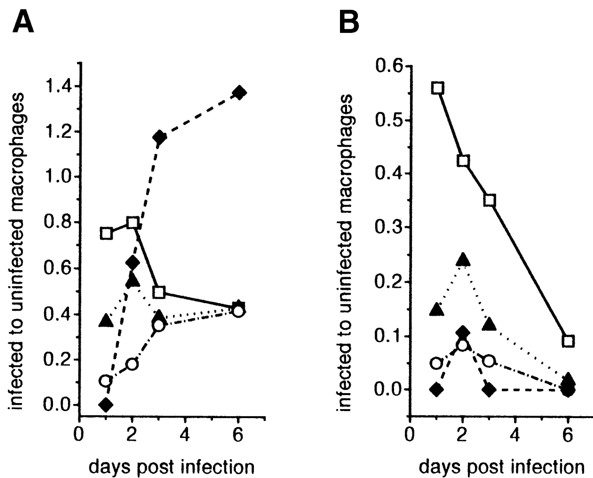

Fig. 6. Infection of peritoneal macrophages with the complemented SAP null mutant, Δ*lmsap1/2* (compare with Figure 2B). (**A**) Δ*lmsap1/2*+*orf1* (*lmpk*), (**B**) Δ*lmsap1/2*+*orf2*, (□) 1–3 amastigotes/macrophage, (▲) 4–6 amastigotes/macrophage, (○) 7–9 amastigotes/macrophage, (◆) >10 amastigotes/macrophage.

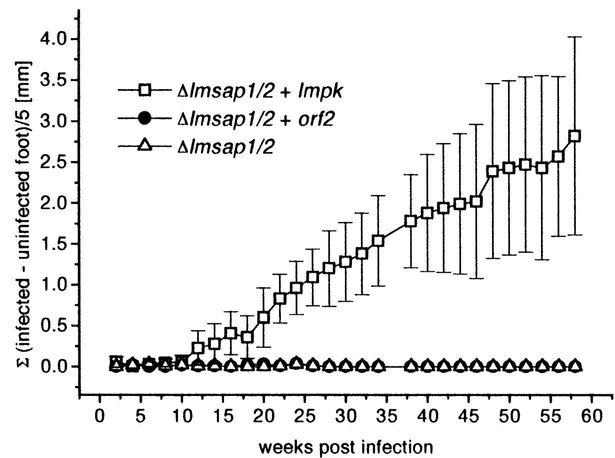

Fig. 7. Footpad infection experiment of SAP null mutant Δ*lmsap1/2* complemented with constructs 3 and 4. (□) Δ*lmsap1/2* + construct 3 (*lmpk*), (●) Δ*lmsap1/2* + construct 4 (*orf2*); (△) Δ*lmsap1/2*; compare with Figure 1C. Bars represent standard deviations in the group of five infected mice.

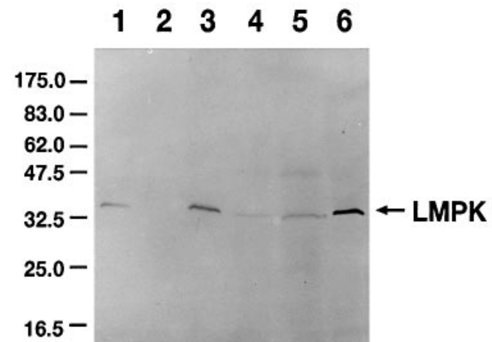

Fig. 8. Immunoblot of LMPK from promastigote and amastigote total cell lysates with a polyclonal antiserum against a C-terminal peptide. Lane 1, wild-type promastigotes; lane 2, Δ*lmsap1/2* promastigotes; lane 3, Δ*lmsap1/2* + *lmpk* promastigotes; lane 4, wild-type amastigotes from mouse lesions; lane 5, Δ*lmsap1/2* + *lmpk* amastigotes from mouse lesions; lane 6, Δ*lmsap1/2* + *lmpk* promastigotes freshly transformed from lesion-derived amastigotes. The molecular mass of standard proteins in kDa is indicated.

at least four adjacent splice-acceptor sites in distances of 383, 379, 335 and 313 bp upstream of the first likely ATG translation initiation codon. The genomic organization of the genes in the SAP gene locus is in accordance with the already described tight packing of genes in the *Leishmania* genome (LeBowitz *et al.*, 1993).

DNA from representatives of every *Leishmania* species was tested in a Southern blot experiment with gene-specific probes for *lmpk* and *orf2*. Specifically hybridizing fragments revealed that both genes are present in at least one copy per haploid genome of *L.amazonensis*, *L.braziliensis*, *L.tropica*, *L.major*, *L.aethiopica* and *L.donovani* (data not shown). Despite ~58% nucleotide identity between *lmpk* and *kfr1*, corresponding to 55.6% amino acid identity (Figure 5), no hybridization was observed with *T.brucei* DNA, showing that hybridization and washing conditions were stringent enough to detect only highly homologous DNA sequences.

lmpk is essential for amastigote proliferation

The first complementation experiment showed clearly that the defect of the mutant Δ*lmsap1/2* is due to the lack of the intergenic region. Therefore, a second set of complementation experiments was performed to discriminate whether one of the two genes of the intergenic region, or both, are essential for the survival of the parasites in macrophages or mice. Either *lmpk* or *orf2*, including the respective 3′-untranslated regions (3′-UTR) (Figure 1C, constructs 3 and 4), was subcloned into pX63polPHLEO and reintroduced into the null mutant, Δ*lmsap1/2*. Peritoneal macrophages from Balb/c mice infected with promastigotes from the resulting mutants Δ*lmsap1/2*+*lmpk* or Δ*lmsap1/2*+*orf2* at a ratio of 20:1 were inspected microscopically for parasite load at various time points after infection (Figure 6). Both mutant parasite lines differentiated into the typical ovoid amastigote form. The initial infection rate reached by both cell types was essentially the same, starting with 1–3 parasites per infected macrophage. However, at later time points, only those parasites complemented with *lmpk* revealed a progressive infection indicated by the increasing number of macrophages harbouring 10 or more amastigotes, while the number of macrophages with a parasite load of 1–3 decreased. Therefore, *lmpk* alone enabled the parasites to proliferate in the macrophages, whereas *orf2* is dispensable. Footpad infection of Balb/c mice with promastigotes from the mutants Δ*lmsap1/2*, Δ*lmsap1/2*+*lmpk* or Δ*lmsap1/2*+*orf2* corroborated the *in vitro* result (Figure 7). Only mice infected with the mutant Δ*lmsap1/2*+*lmpk* began to develop lesions 10 weeks post-infection. Lesion size increased steadily over 60 weeks, indicating a progressive infection in the mice. The mutant containing *orf2* showed no lesion development in Balb/c mice.

Expression of *lmpk*

A polyclonal antiserum against a C-terminal peptide of LMPK (anti-CLMPK) was raised in rabbits, affinity-purified using the peptide and employed to detect LMPK in total cell lysates. On an immunoblot, the antiserum recognized a band with a molecular mass corresponding to the calculated molecular mass of 41 kDa in both *L.mexicana* promastigotes and amastigotes (Figure 8, lanes

1 and 4). As expected, this reaction was absent in a lysate from the mutant Δ*lmsap1/2* (Figure 8, lane 2). The additional band seen in amastigote lysates may be derived from contaminating mouse lesion material or an amastigote protein interacting with secondary antibodies. Freshly prepared Δ*lmsap1/2*+*lmpk* lesion amastigotes were positive for LMPK (Figure 8, lane 5), as well as Δ*lmsap1/2*+*lmpk* promastigotes either freshly transformed from mouse lesion-derived amastigotes or from recombinant parasites that had not been passaged through the mouse (Figure 8, lanes 6 and 3). Both types of promastigotes were kept under selective pressure by the presence of the antibiotic phleomycin in the culture medium, whereas the lesion-derived Δ*lmsap1/2*+*lmpk* amastigotes maintained the *lmpk* gene without selective pressure for >1 year (compare with Figure 7) and expressed the protein in amounts similar to that of wild-type *L.mexicana* amastigotes. The high level of *lmpk* expression in promastigotes freshly transformed from lesion-derived amastigotes, which were then cultivated under antibiotic selection, suggested that *lmpk* is still plasmid encoded and not integrated into the genome by a recombination event. This was confirmed by Southern blot experiments using total DNA from these promastigotes cleaved by a set of restriction enzymes specific for the identification of plasmid-encoded *lmpk*, and by re-isolation of the plasmid by transformation of *Escherichia coli* using total promastigote DNA, followed by restriction analysis of 23 independent plasmid clones (data not shown). All the plasmids showed the same restriction pattern as the original plasmid that was used to transfect the promastigotes of the null mutant Δ*lmsap1/2*, indicating the stability of the plasmid construct as an extrachromosomal element in complemented Δ*lmsap1/2* amastigotes for >14 months without selective pressure.

Discussion

A deletion mutant (Δ*lmsap1/2*) in *L.mexicana* lacking both alleles of the two tandemly arranged *lmsap* genes including the intergenic region, that was originally developed to analyse the structure and function of the filamentous SAP complex, was found to have lost its potential to develop lesions in Balb/c mice and to grow in macrophages. However, mutant promastigotes showed the same initial infection rate of cultured macrophages as wild-type promastigotes, suggesting that the defect of the Δ*lmsap1/2* mutant does not impair their potential to invade macrophages and to differentiate into the amastigote form. This differentiation was independent of the presence of macrophages and could also be induced by temperature and pH shifts from 26°C and pH 7.0 to 34°C and pH 5.5 in the culture medium. In contrast to wild-type cells, the Δ*lmsap1/2* mutant ceased to proliferate after a 4-fold increase in cell number after 4 days without reaching stationary growth phase. This result is interpreted as follows. A late-logarithmic promastigote culture is not synchronized with respect to the differentiation state containing some non-dividing metacyclic cells. After triggering differentiation, the metacyclic cells will be able to differentiate into dividing amastigotes immediately; however, most of the cells will be in the dividing procyclic stage and may undergo one or two more duplications before they transform to metacyclics and then to amastigotes. This implies that the increase in cell number in the Δ*lmsap1/2* mutant may be due to dividing procyclic promastigotes. However, once all cells have differentiated to amastigotes, proliferation of the Δ*lmsap1/2* mutant is arrested suggesting that the loss of the SAP gene locus causes an amastigote stage-specific block in cell division of *L.mexicana*.

To restore the wild-type phenotype parts of the SAP gene locus were subcloned into *Leishmania* expression vectors and reintroduced into the null mutant Δ*lmsap1/2*. The intergenic region without *lmsap* genes was sufficient to restore the wild-type phenotype with regard to survival and proliferation of amastigotes in the infected host cell. Therefore, the SAP is dispensable for establishing an infection. Moreover, this raised the likelihood of at least one additional open reading frame present within the intergenic region of the SAP gene locus. In fact, two open reading frames were identified by sequence analysis, one (*lmpk*) encoding a *L.mexicana* MAP kinase homologue, the other (*orf2*) showing no homology to the sequences in the DDBJ/EMBL/GenBank database. Both genes were tested for their potential to revert the mutant phenotype. Only the reintroduction of *lmpk* into the null mutant restored the ability of the cells to proliferate in the host cell and resulted in fully infective parasites. Therefore, the gene product of *orf2* seems to have no essential function for the parasite or may encode for a functionally redundant protein. However, the gene product of *lmpk* is absolutely necessary for the parasite, but its function is most likely restricted to the amastigote stage.

Because of the high level of homology, the presence of all MAP kinase typical domains carrying the critical amino acids to form the catalytic centre, including the ATP-binding site and the phosphorylation lip, and its necessity for proliferation of the amastigotes, the protein encoded by *lmpk* is likely to be a component of a MAP kinase signal transduction pathway affecting cell division. The protein is present in promastigotes and amastigotes, but exhibits its function only in the amastigote. Therefore, its activity has to be regulated by post-translational modifications. In fact, this mode of functional activation of proteins that are present throughout the cell cycle is an inherent feature of MAP kinases that are part of signal transduction pathways being activated by phosphorylation on threonine and tyrosine residues in the TXY motif of the phosphorylation lip by a specific dual-specificity kinase called MAP kinase kinase, which in turn is activated by another kinase (Waskiewicz and Cooper, 1995). The initial signal for kinase activation could be intracellular, e.g. the onset of replication and division, or extracellular, e.g. osmotic stress, heat shock or the action of mitogens and growth factors. When a MAP kinase is activated by dual phosphorylation, it has been shown to translocate into the nucleus to activate a specific set of transcription factors. However, it has also been shown that ERK1 and 2, which are mammalian MAP kinases, also phosphorylate a number of cytoplasmic proteins, e.g. the pp90 ribosomal S6 kinase, the cytosolic phospholipase A2 and the tail of the EGF receptor (Waskiewicz and Cooper, 1995). Regulated gene expression in trypanosomatids is still not understood. These organisms have no promoter-like sequences to drive the expression of protein coding genes, no transcription

factors have been characterized and transcription initiation seems to occur at random sites on the DNA. Therefore, via the identification of its substrate, the *lmpk* gene product may lead to a factor regulating gene expression in *Leishmania* parasites. It will be interesting to see whether the LMPK substrate is involved in transcription, *trans*-splicing, mRNA stability or translation, which are the major regulatable checkpoints in parasite protein expression.

The null mutant in combination with *lmpk* on a plasmid also has a practical applicability, as it could be used to achieve stable expression of a foreign gene in *L.mexicana* amastigotes without drug selection, e.g. in infected macrophages or in laboratory animals.

Infection of Balb/c and C57/BL6 mice with the null mutant followed by challenge with wild-type parasites after 4 weeks, did not lead to protection of the mice against *L.mexicana*. Therefore, the null mutant cannot be used as an attenuated strain for vaccination. However, LMPK may be a suitable target for drug treatment using specific kinase inhibitors. These should have the same effect on the proliferation of the amastigotes as a gene deletion, thereby preventing a progressive disease. For the mammalian p38 MAP kinase, a specific inhibitor of the pyridinyl-imidazole group, which competes with ATP for binding in the ATP pocket, has been described (Lee *et al.*, 1994), and its specificity for p38 has been confirmed by co-crystallization with the kinase (Tong *et al.*, 1997; Wilson *et al.*, 1997). Because a homologue of *lmpk* is present in representatives of all *Leishmania* species investigated, such a kinase-specific inhibitor may indeed be very useful as a therapeutic agent against Leishmaniasis.

Materials and methods

Parasites

Promastigotes of *L.mexicana* MNYC/BZ/62/M379 (Lainson and Strangeways, 1963) were grown as described (Menz *et al.*, 1991). Amastigotes were isolated from lesions of Balb/c mice. Lesion tissue was cut into pieces, transferred into amastigote stabilization buffer (PSGEMKA; Hart *et al.*, 1981) and passed through a sterile metal grid to disrupt the host macrophages and liberate the amastigotes. Low speed centrifugation at 140 *g* for 10 min at 4°C removed most of the host cells and cellular debris. In a second centrifugation at 1500 *g* for 10 min at 4°C, the amastigotes were pelleted. Cells were either lysed directly by boiling in 1× SDS sample buffer (0.4% SDS, 4% glycerol, 0.0002% bromphenol blue, 50 mM DTT, 12.5 mM Tris–HCl pH 6.8) for 10 min or transferred to semi-defined medium 79 (SDM-79) for transformation to promastigotes at 26°C. Promastigotes were transformed *in vitro* to amastigote-like forms by acidification of the SDM-79 to pH 5.5 or by transfer into Schneider's *Drosophila* medium (Serva, Heidelberg, Germany) supplemented with 20% heat-inactivated fetal calf serum (iFCS; Biospa, Wedel, Germany) and 3.9 g/l 2-(*N*-morpholino)ethane sulfonic acid (Bates *et al.*, 1992) and a temperature shift from 26 to 34°C.

Nucleic acids analysis

Sequence analysis was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using an AutoRead™ Sequencing Kit (Pharmacia-LKB, Freiburg, Germany) and an ALF sequencing device. Both strands were sequenced. The GCG/Wisconsin software (Devereux *et al.*, 1984) was used to analyse the DNA sequences. Nucleic acid isolation and hybridization analyses were performed as described by Ziegelbauer *et al.* (1992), with the exception of using digoxigenin-labelled probes in DNA–DNA hybridizations according to the manufacturer (Boehringer Mannheim, Germany).

Reverse transcriptase polymerase chain reaction (RT–PCR) was performed with 1 μg total RNA from *L.mexicana* promastigotes using the Titan™ One Tube RT–PCR System (Boehringer Mannheim). The RNA was prepared from 4×10^9 cells using the RNA Isolation Kit from Stratagene (La Jolla, CA), yielding 2.2 mg total RNA. To determine the splice addition sites of *lmpk* and *orf2*, the following primer combinations were used in the RT–PCR reactions. For *lmpk*, the mini-exon primer 5′-GCTCTAGAATTCTATATAAGTATCAGTTTCTGTACTTTATTG-3′ (3′-end of mini-exon sequence is underlined) and a gene internal primer 5′-CAACGGGGTCGGAAGGGAGTT-3′ was used; for *orf2* the mini-exon primer was also used, as well as the following gene internal primer: 5′-GAGGAGTGTTGGCCGTGCACGC-3′. RT–PCR was performed as described by the manufacturer with annealing temperatures of 63 and 65°C for *lmpk* and *orf2*, respectively. In a second PCR reaction using the Expand™ High Fidelity PCR System (Boehringer Mannheim) 2.5 μl of the RT–PCR reaction diluted 1:100 was used as the template DNA. In addition to the mini-exon primer, a third primer located in the coding region between the mini-exon and the above gene internal primers was used (*lmpk*: 5′-GCGATGTAGTCGGTCAGGT-3′; *orf2*: 5′-GATGGCC-ATCTGTCTGCGAA-3′). PCR was performed according to the manufacturer with an annealing temperature of 60°C. The resulting PCR products were cloned into pCR2.1 using the TA Cloning Kit (Invitrogen, San Diego, CA) and sequenced.

The sequence data of the intergenic region of the SAP gene locus carrying *lmpk* and *orf2* have been submitted to the DDBJ/EMBL/GenBank databases under accession No. Z95887.

Expression constructs

The SAP gene locus was originally isolated from a genomic DNA library by two overlapping phage clones (Wiese *et al.*, 1995). The insert of the larger clone comprising *lmsap2*, its upstream region, the *lmsap* intergenic region and the 5′ part of *lmsap1* were subcloned into the *Eco*RI site of pBluescriptII SK(+) (Stratagene, La Jolla, CA), using *Eco*RI sites derived from the λ phage so that the *Bam*HI site of the plasmid was placed next to the truncated *lmsap1* gene. From this construct a 6.4 kb *Bam*HI fragment carrying parts of the intergenic region and the partial *lmsap1* gene was isolated and cloned into pX63PHLEO (Freedman and Beverley, 1993) using the *Bam*HI and *Bgl*II restriction sites of the plasmid. The resulting expression plasmid was designated pX-INT. To obtain the second expression construct (pX-INT*lmsap2*), pX-INT was linearized at its single *Bam*HI site and ligated to a 10.8 kb *Bam*HI fragment carrying *lmsap2* and its flanking regions. Therefore, this construct carries the same leishmanial DNA sequences as the original phage.

Leishmania expression constructs for the two open reading frames discovered on the *lmsap* intergenic region were made first by cloning a filled in *Bsp*HI–*Hinc*II fragment containing *lmpk* and its downstream region into the *Eco*RV restriction site of pX63polPHLEO (pX63PHLEO modified by addition of a new multicloning site GGATCCAATTGATAT-CTAGATCT which was ligated into the pre-existing *Bam*HI and *Bgl*II restriction sites). Secondly, an *Nco*I restriction site was introduced at the potential ATG translation initiation codon of *orf2* using oligonucleotides 5′-CAC**GGATCC**ATGGGTTCTGCGCTGCACCCCTCG-3′ and 5′-GCCC**AAGCTT**CACCACGAGGTCGCTCCCGTG-3′ in a PCR, followed by subcloning the obtained fragment into pBluescriptII SK(+) using the added *Bam*HI and *Hin*dIII restriction sites. The *orf2* downstream region was added by replacement of the 3′ terminal 419 nucleotides of *orf2* with an *Aat*II/*Kpn*I fragment carrying these nucleotides, the entire downstream region and parts of *lmsap1*. Finally, an *Nco*I fragment from this construct was isolated, filled in and cloned into the *Eco*RV restriction site of pX63polPHLEO.

The plasmid constructs were introduced into *L.mexicana* Δ*lmsap1/2* promastigotes by electroporation (Wiese *et al.*, 1995), and recombinant cells were selected in liquid culture containing phleomycin (Sigma, Deisenhofen, Germany) up to a final concentration of 5 μg/ml, G418 at 10 μg/ml (Boehringer, Mannheim, Germany) and hygromycin at 20 μg/ml (Sigma).

Immunoblotting

A polyclonal rabbit antiserum (anti-CLMPK) was raised against a 15-residue peptide corresponding to the C-terminus of LMPK. The antibodies were affinity purified on the peptide coupled to SulfoLink™ coupling gel via its N-terminal cysteine residue, according to the manufacturer's instructions (Pierce, Rockford, IL). Lysates of 2×10^7 cells in 1× SDS sample buffer were boiled for 10 min, separated on a 12% SDS–polyacrylamide gel and blotted to poly(vinylidenedifluoride) membranes. The following incubations were carried out at 37°C for 1 h each with shaking. The membranes were blocked in blocking buffer (PBS, 2% BSA, 0.2% Tween-20, 10 mM Tris–HCl pH 7.5), incubated in anti-CLMPK diluted 1:50 in blocking solution, washed five times in PBS, treated with goat-anti-rabbit secondary antibodies coupled to alkaline

phosphatase (Dianova, Hamburg, Germany), washed a further five times in TBS and developed in 1 M diethanolamine, 1 mM $MgCl_2$, 0.5 mM nitrobluetetrazolium chloride, 0.5 mM disodium-5-bromo-4-chloro-3-indolylphosphate pH 9.8.

Infection experiments

Peritoneal macrophages from Balb/c mice were grown in DMEM (Gibco, Eggenstein, Germany) at 34°C overnight on glass coverslips in a Petri dish at a density of 1.25×10^5 cells/ml. Non-adherent cells were removed by washing with fresh medium and the remaining macrophages were infected with promastigotes at a ratio of 20:1. After overnight incubation at 26°C, free promastigotes were removed by extensive washing with fresh medium and the cultures were transferred to 34°C. Coverslips were removed at various time points for DAPI staining and immunofluorescence with a polyclonal antiserum against whole *L.mexicana* amastigotes (Winter *et al.*, 1994), to discriminate between free and intracellular parasites. Coverslips were submerged in 2% formaldehyde in PBS for 15 min, washed twice in PBS, blocked by incubation in 2% BSA in PBS for 15 min and treated with the polyclonal antiserum diluted 1:50 in blocking solution for 20 min at room temperature, followed by four washings in PBS and 20 min incubation in goat anti-rabbit Cy3-conjugated secondary antibodies (Dianova) diluted 1:600 and 10 μg/ml DAPI in blocking solution. After four washings in PBS, the coverslips were mounted with Mowiol/DAPCO. Three times, 100 macrophages were inspected for infection by *Leishmania* parasites by random walk through the sample, the average taken and the macrophages grouped according to their parasite burden. Parasites reacting with the antiserum were omitted because they are not inside a macrophage.

Five Balb/c mice per group were injected in the left hind footpad with 1×10^6 promastigotes resuspended in PBS. Footpad lesion size was measured using a calliper slide and the result corrected by the diameter of the uninfected footpad.

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