SHORT COMMUNICATION

Identification of a Differentially Expressed mRNA in Axenic *Leishmania panamensis* Amastigotes

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Differential display technique was applied in order to identify transcripts which are present in axenic amastigotes but not in promastigotes of the Leishmania panamensis *parasites. One of them was cloned and the sequence reveals an open reading frame of 364 amino acids (aprox. 40 kDa). The deduced protein is homologous to the serine/threonine protein kinases and specially to the mitogen activates protein kinases from eukaryotic species. Southern blot analysis suggest that this transcript, named lpmkh, is present in the genome of the parasite as a single copy gene. These results could imply that lpmkh could be involved in the differentiation process or the preservation of amastigotes in axenic conditions.*

Key words: differential display - axenic amastigotes - *Leishmania panamensis* - mitogen activated protein kinase

Leishmania parasites are the etiologic agents of leishmaniasis, a spectrum of diseases ranging from the self-healing cutaneous to the non-healing visceral form. These parasites are digenetic organisms, which replicate as extracellular flagellated promastigotes in the sand fly's midgut and as intracellular aflagellate amastigotes in the vertebrate host's macrophage (Evans 1993).

The mechanism driving promastigote conversion into amastigote form is not well understood; however the temperature, pH (Zilberstein & Shapira 1994) and host biochemical signals (Gomes et al. 1998) might be important factors involved in this process. Stage-specific or prevalent molecule identification provides valuable information about how the amastigotes survives inside the hostile acidic phagolysosome and contributes to the rational design of drugs and vaccines. Thus, proteins involved in signal transduction pathway, such as the organelle-type Ca2+ ATPase (Lu et al. 1997) and a mitogen activated (MAP) kinase protein homologue (Wiese 1998), have shown to be overexpressed in amastigotes and may be a suitable target for drug treatment using specific inhibitors. In addition, constitutive amastigote proteins, such as P4 (35 kDa) and P8 (30 kDa), together with *Corynebacterium parvum* as adjuvant, provide BALB/c mice with partial to complete protection against the challenge with *L. pifanoi* promastigotes (Soong et al. 1995). Antigen P4, a single strand specific nuclease, selectively elicits a T helper 1 cell (Th1) response (Haberer et al. 1998) which mediates the activation of macrophage to produce activating lymphokines (Overath & Aebischer 1999). With the aim of developing a cell line with amastigote-specific attenuation, the *L. donovani*-inducible A2 gene regulatory system (Charest et al. 1996) was used to differentially express two potential suicide genes (Ghedin et al. 1998). The recent development of methodologies to culture amastigotes of several species, in cell free media conditions (Hodgkinson et al. 1997, Balanco et al. 1998), have facilitated the studies of stage-specific genes.

In *Leishmania* parasites, the differential display technique (DD) (Liang et al. 1993) has been employed to identify virulent versus avirulent transcript variants (Heard et al. 1996) and to correlate different types of leishmaniasis parasites species from separated geographic areas (Noyes et al. 1997). We used differential display as a simple and reproducible methodology to compare two in vitro forms of *L. panamensis* at the transcription level.

L. panamensis stationary phase promastigotes and amastigotes obtained in axenic conditions (F

This work was financed by the presidency of the Republic of Colombia, The Colombian Ministry of Health and Colciencias. Nucleotide sequence reported in this paper is available in the GenBank™ under accession number AF203877.

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Received 14 July 2000
Accepted 18 April 2001

Puentes, unpublished data) were tested. The DD reverse transcriptase-polymerase chain reaction was carried out using an oligo dTCG primer and a set of arbitrary primers, following the manufacturer instructions (Display System biotech.). Bands of interest were excised from the dried gel, hydrated and amplified, cloned and sequenced. A clone, C2 (230 bp), was chosen for molecular characterization. To confirm this particular tag's expression, two primers were designed, C2s (5-GAAACCAAAGA GCAACTC-3) and C2a (5-AATAGCCACAGT GACC-3), for further amplification. The RT-PCR confirmed a 230 bp band in amastigotes but not in promastigotes mRNAs (Fig. 1A). A probe, derived from C2, was labeled with [P^{32}] to obtain a genomic restriction map for this transcript. As can be seen in Fig. 1B, the restriction pattern identified unique fragments with each restriction enzyme used, which suggests a single copy gene per haploid genome. Finally, using a primer specific for the splice leader sequence (5-GAATTCCAGTTTCTGTACTTTA TTG-3) and a C2 internal antisense primer (5-CCCGCCACAAA TCCTCAC-3), the complete transcript was amplified, cloned and sequenced. Fig. 2 shows a complete 1290 bp transcript with a 1092 bp open reading frame coding for a 364 amino acid putative protein (~ 40 kDa), with a 5' UTR and 3' UTR sequence in 33 bp and 134 bp long, respectively. A homology search in the database revealed a 97% sequence identity with a putative mitogen activated protein kinase (MAPK) from *L. donovani* parasite and 66% with a putative *Trypanosoma brucei* MAPK at the amino acid level (GenBank accession numbers AF176312 and Z54341.1 respectively). The sequence display the typical subdomains characteristic for serine/threonine protein kinases from eukaryotic species and the reported *L. mexicana* MAP kinase protein (Hanks et al. 1988, Wiese 1998). It has also some relevant motifs including the catalytic residues Lys49, Arg73, Arg76, Glu77, Arg155, Asp156, Lys158, Asn161; the Mg+2 ligand residues Asp174, Arg179, Thr195; the phosphate anchor ribbon for ATP binding Gly37-Phe38-Gly39-Ala40-Cys41-Gly42; the potential regulatory phosphorylation site Thr190 and Tyr192 and the P+1 specific pocket Thr195-Arg196-Trp197 and Tyr198 (Zhang et al. 1994). Three N-myristoylation sites were also detected using the Generunner software program (Hasting software Co.) (Fig. 2). This sequence was termed *L. panamensis* MAP kinase homologue (*lpmkh*).

Furthermore, protein phosphorylation controls

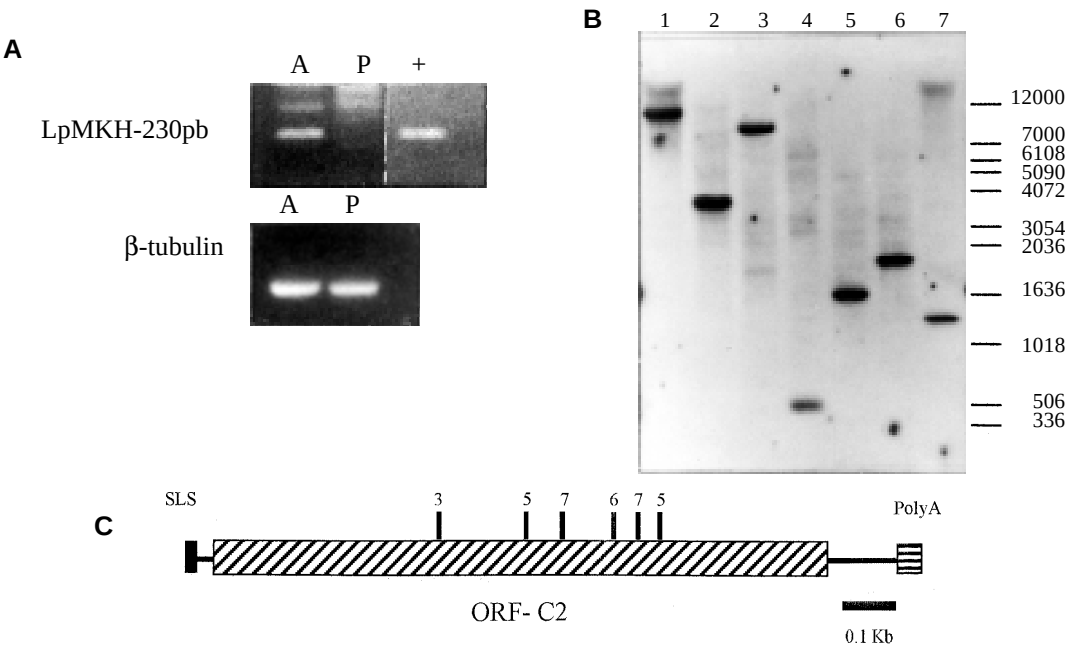

Fig. 1: C2 transcript differential expression, southern analysis and scheme. A: RT-PCR from total RNA from *Leishmania panamensis* stationary phase promastigotes (P) and axenic amastigotes (A). Total RNA (2 µg) was reverse transcribed and PCR was performed with C2s and C2a primers; the products were separated on 2% agarose gel; (+) the original plasmid C2 used as template in PCR. As a control for RNA quality, RT-PCR was performed using primers for the β-tubulin gene. Arrows indicate the expected bands, C2: 230 bp and β-tubulin: 540 bp; B: C2 transcript genomic organization. DNA (5 µg) was cleaved with restriction enzymes and separated on a 0.8% agarose gel, blotted onto a Zeta-probe (Amersham-Pharmacia) membrane and hybridized with a radiolabeled probe derived from C2. 1: EcoRI; 2: SalI; 3: PstI; 4: SacI; 5: HincII; 6: PvuII; 7: BamHI. Numbers on the right are the molecular weight markers 1Kb ladder (Gibco BRL); C: schematic representation of the Gene. SLS: Splice Leader Sequence. Numbers according to the restriction enzyme pattern.

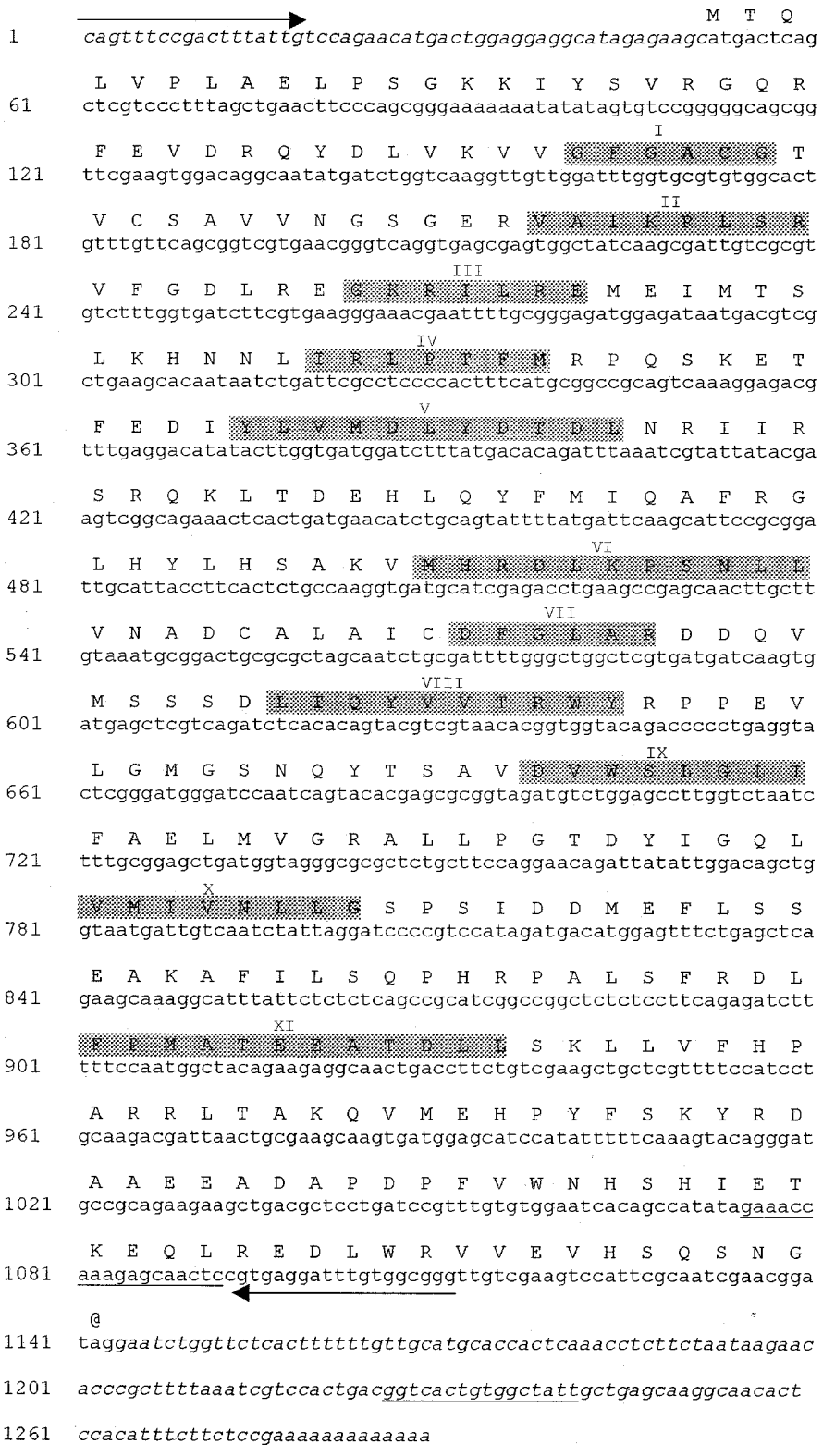

Fig. 2: complete nucleotide and deduced amino acid sequence of the *lpmkh* cDNA. The 5' and 3' untranslated sequences are in italics letters and the open reading frame is in upper case letters. Underlined nucleotides indicate the primers used in the RT-PCR of Fig. 1A. Arrows indicate the positions of the primers used to amplify the entire transcript. Boxed letters show protein kinase domains highly conserved throughout the eukaryotes.

major steps of proliferation and differentiation in eukaryotic organism (Alberts et al. 1995). Several protein kinases have been partially or complete identified in trypanosomatids by biochemical or genetic techniques (Boshart & Mottram 1997). Environmental stimulus, including stress or biochemical factors, can modulate protein kinase activity and could be the case in *Leishmania* differentiation regarding this molecule. Besides, parasites are actively exposed to host molecules such as immunoglobulins and growth factors. It has been shown that insulin-like growth factor, IGF-I, can mediate protein tyrosine phosphorylation in *L. mexicana* promastigotes and amastigotes by a PKC-like pathway (Gomes et al. 1998). Interestingly, a stage-specific change in protein phosphorylation pattern was observed and a 40 kDa phosphoprotein was predominant only in amastigotes. The protein *lmpk* of *L. mexicana* is highly homologous to an eukaryotic MAP kinase and has been shown to be essential for amastigote survival likely as a component of the signal transduction pathway affecting cell division (Wise 1998). Even though, *lpmkh* does not share relevant homology with *lmpk* (17% identity), it is differentially expressed in amastigotes and also has the domains found in eukaryotic kinases. Members of this family can be a suitable target for phosphorylation in the IGF-I assay. Moreover, a sequence from *L. donovani* chromosome 19, almost identical to *lpmkh* , has been reported in the GenBank, coding for a MAP kinase homologue. Significant homology was found with a *T. brucei* ORF encoding a MAP kinase-like protein suggesting a high degree of conservation throughout the trypanosomatidae. Since this transcript seems to be a single copy, it is interesting to create null mutants in *L. panamensis* to asses its function as seen in *L. mexicana* parasites were the protein is essential for the survival in infected host (Wiese 1998). Furthermore, the immunological implications of this protein in the amastigote stage merit further analysis.

ACKNOWLEDGMENTS

To Maryluz Gunturiz and Esperanza Trujillo for automated sequencing assistance, Dr Carlos Parra and Dr Vladimir Corredor for helpful discussion of the work and manuscript.

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