Protein Kinase Involved in Flagellar-Length Control

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During its life cycle, the parasitic protozoon Leishmania mexicana differentiates from a flagellated form, the promastigote, to an amastigote form carrying a rudimentary flagellum. Besides biochemical changes, this process involves a change in overall cell morphology including flagellar shortening. A mitogen-activated protein kinase kinase homologue designated LmxM KK was identified in a homology screening and found to be critically involved in the regulation of flagellar assembly and cell size. LmxM KK is exclusively expressed in the promastigote stage and is likely to be regulated by posttranslational mechanisms such as phosphorylation. A deletion mutant for the single-copy gene revealed motile flagella dramatically reduced in length and lacking the paraflagellar rod, a structure adjacent to the axoneme in kinetoplastid flagella. Moreover, a fraction of the cells showed perturbation of the axonemal structure. Complementation of the deletion mutant with the wild-type gene restored typical promastigote morphology. We propose that LmxM KK influences anterograde intraflagellar transport to maintain flagellar length in Leishmania promastigotes; as such, it is the first protein kinase known to be involved in organelar assembly.

Protein kinases are key regulatory molecules in the proliferation, differentiation, motility, and stress response of all eukaryotic cells. Together with their antagonists, the protein phosphatases, they are organized in complex networks to guarantee proper regulation of cellular processes according to environmental changes and intercellular communication. There is a wealth of information present on protein kinases in higher eukaryotes and Saccharomyces cerevisiae (18, 45). Information on signal transduction processes for other unicellular organisms such as the sporozoan (Plasmodium, Toxoplasma, and Theileria) (22) and the kinetoplastida (Trypanosoma and Leishmania) (40, 50) as causative agents of infectious disease or the green alga Chlamydomonas (48) and the slime mold Dictyostelium discoideum (2) as model organisms for flagellar assembly and differentiation, respectively, is relatively scarce. It is likely that in Leishmania, as in other organisms, signal transduction pathways culminate in altered gene expression. As transcription factors and RNA polymerase II promoters are absent in these parasites, it is generally thought that regulation occurs posttranscriptionally on the level of mRNA maturation and turnover, efficiency of translation, and protein stability (10, 40). However, the regulatory molecules involved in these processes have not been identified. Leishmania parasites undergo a digenetic life cycle, differentiating from the promastigote form in the insect vector, the phlebotomine sand fly, to the amastigote form in the lysosomal compartment of the macrophages of mammals. Promastigotes are spindle-shaped cells, 11 to 20 μm in length and 2 μm in diameter, carrying a single flagellum of at least the length of the cell body at their anterior pole, which pulls the cell forward but also mediates the attachment to the surface of the insect gut (23). On the other side, the amastigotes are significantly smaller, almost spherical cells of 4 to 5 μm in length. Their flagella are almost completely buried in the flagellar pocket, an invagination of the plasma membrane which is the only area of exo- and endocytosis of the cell (38). Differentiation from protoamastigotes and vice versa is induced by changes in temperature and pH (60). However, the mediators that transduce the signals into changes in gene expression are not known. By analogy to higher eukaryotes and yeast, these molecules are likely to be protein kinases and phosphatases. In fact, phosphoprotein abundance and the overall phosphorylation pattern detectable in Leishmania and other kinetoplastids change as they pass through their life cycles (1, 12, 37, 39, 41, 42).

Here, we report the identification of a mitogen-activated protein (MAP) kinase kinase (MKK) homologue from Leishmania mexicana that is required for the maintenance of a full-length flagellum, promastigote shape, and the ability of the cells to swim. This observation makes Leishmania an attractive model for the study of flagellar morphogenesis and function.

MATERIALS AND METHODS

Parasites. Promastigotes of L. mexicana MN1Y/BZ/62/M379 were grown as described previously (34). Amastigotes were isolated from lesions of BALB/c mice as described previously (55).

Gene cloning, sequencing, and nucleic acid analysis. Expand high-fidelity polymerase (Roche, Mannheim, Germany) was used for all PCR applications. LmxM KK was amplified from genomic DNA of L. mexicana by using 15 pmol of two oligomers corresponding to the 5′ end of the open reading frame of the L. chagasi lpk1 gene (28) (5′-GATATCATGAAATCGACCCGCTC-3′) introducing EcoRV and BspHI restriction sites and to the 3′ end (5′-TCTAGAGCCACATCTTATCAAGCTG-3′) introducing a XbaI restriction site, respectively. The reaction was performed with 30 ng of genomic DNA (5 min at 94°C, 10 × [30 s at 94°C, 30 s at 60°C, and 30 s at 72°C], 25 × [30 s at 94°C, 30 s at 60°C, and 1 min at 72°C] plus a cycle elongation of 5 s for each cycle; and 7 min at 72°C). PCR fragments were cloned into pCR2.1 (Invitrogen, San Diego, Calif.), and the resulting construct was designated pCR2.1LmxM KK. The cloned fragment was digoxigenin (DIG) labeled using the oligonucleotides described above and a PCR DIG probe synthesis kit (Roche) and used to screen a genomic DNA library of L. mexicana (56). Positive phage clones were selected and amplified, and their
DNA inserts were subcloned into pBSKII (+) (Stratagene, La Jolla, Calif.). Plasmid isolation, DNA sequencing and analysis, DNA/RNA isolation and blotting, and hybridizations were performed as described before (8).

The splice addition site of LmxxMKK was determined by reverse transcriptase PCR (RT-PCR). It showed that the alternative splicing of promastigote or amastigote total RNA as described in the manufacturer’s protocol (Invitrogen). The reaction was followed by a nested PCR using 2 µl of the cDNA and 15 pmol each of the LmxxMKK-derived oligomer RT1 (5'-CGTATGTTGTCATCACTATA-3') and an oligomer containing part of the L. mexicana minexon sequence MX2 (5'-CTAACGCTATATAAGTACGT-3') in the first primer binding site as follows: 1 min at 94°C, 10 × [50 s at 94°C, 30 s at 55°C, and 30 s at 72°C], 25 × [50 s at 90°C, 30 s at 55°C, and 30 s at 72°C] plus a cycle elongation of 5 s for each cycle; and 7 min at 72°C. A total of 2 µl of the reaction products (diluted 1:100) was subjected to a second PCR using MX2 and RT2 (5'-CCACGCGCCG ACGTTACGT-3') under the same amplification conditions. PCR products were cloned into pCR2.1 and sequenced.

Expression constructs, mutagenesis, and antibody production. The LmxxMKK PCR fragment was cut with EcoRV and XhoI and cloned into pBSKII (+). To generate a constitutively active version of LmxxMKK, all restriction sites between the translational initiation codon of LmxxMKK isolated from pCR2.1LmxxMKK and EcoRV (4 min at 94°C, 30 s at 65°C, and 6 min at 68°C) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The resulting fragment was cut with Acc65I and HindIII and ligated to the annealed oligonucleotides 5'-AGC TTGATGACGACGACGCTGACGACTTCGTTG-3' and 5'-GTC-3' for site-directed mutagenesis as described before (26), lysine 91 was mutated to methionine. Subsequently the gene was released from the resulting constructs LmxxMKK, LmxxMKK(D), LmxxMKK(K91M), and LmxxMKK (K91M(D)) by using BstH and SacI, ligated into pGEX-KG (14), and transformed into Escherichia coli XL1-Blue (Stratagene). Expression of the glutathione-S-transferase (GST) fusion proteins was achieved by induction of a bacterial culture grown to an optical density at 600 nm of 0.8 in Luria-Bertani medium with 10 µM IPTG (isopropyl-ß-D-thiogalactopyranoside) for 2 h at 30°C in a shaking incubator. Bacteria were washed once in cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.4 mM KH2PO4) and resuspended in 50 µl of cold PBS per ml of the original culture volume. The suspension was subjected to sonication on ice with a Branson Sonifier 250 apparatus in pulse mode followed by the addition of Triton X-100 to a concentration of 1%. Solubilization of proteins occurred by end-over-end rotation of the lysate at 4°C for 30 min. Finally, the solution was centrifuged at 4°C and 12,000 × g for 10 min and the supernatant was collected. Purification of the protein was performed on GST-Sepharose 4B by following the instructions of the manufacturer (Amersham-Pharmacia Biotech, Freiburg, Germany). The wild-type GST fusion protein was used for immunization of a rabbit (Charles River, Kisslegg, Germany). Moreover, a rabbit antisera was produced against the peptide CSLENVDVKAQLDKMVL corresponding to the 15 COOH-terminal amino acids of LmxxMKK (Eurogentec, Seraing, Belgium).

Immunoblotting. Lysates of 109 cells ml−1 in 1× lysis buffer (1× PBS, 0.1% sodium dodecyl sulfate [SDS], 50 mM dithiothreitol, 50 µM leupeptin, 25 µM N-α-tosyl-L-lysyl chloromethylketone, 1 mM phenylmethylsulfonyl fluoride, 1,10-phenanthroline [pH 7.2], 1× SDS sample buffer [4% SDS, 4% glycerol, 0.0002% bromophenol blue, 50 mM dithiothreitol, 12.5 mM Tris-HCl, pH 6.8]) were boiled for 10 min, and 20 µl was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and blotted to polyvinylidene difluoride membranes. Immunodetection was carried out as described before (55) with different rabbit antisera and goat-anti-rabbit secondary antibodies coupled to peroxidase (Dianova). Immunodetection was carried out as described before (55) with different rabbit antisera and goat-anti-rabbit secondary antibodies coupled to peroxidase (Dianova, Hamburg, Germany) followed by chemiluminescence using an ECL system (Amersham-Pharmacia Biotech).

Kinase assay. Fusion proteins were bound from bacterial lysates to glutathione-One-Sepharose 4B and washed three times with cold PBS. A total of 10 µl of these beads was resuspended in 100 µl of kinase assay solution (50 mM morpholinepropanesulfonic acid [pH 7.2], 10 mM MgCl2, 2 mM MnCl2, 0.1 M NaCl, 10 µCi of [γ-32P]ATP, 1 mM ATP) and rotated end-over-end at 30°C. Following three washes with PBS, the beads were resuspended in 130 µl of 1× SDS sample buffer with 2× boiled lysate at 95°C for 10 min. After heating, the solution was separated on an SDS–12% PAGE gel, stained with Coomassie blue, destained, dried, and exposed to X-ray films at −70°C.

LmxxMKK deletion constructs. To generate the LmxxMKK null mutant LmxxMKK::Δlmsmkk::HYG (abbreviated LmxxMKK), the flanking regions of LmxxMKK were amplified (using inverted PCR) from genomic DNA of L. mexicana. On the basis of the information from the genomic Southern blot analysis, the PCR products were cloned and sequenced. Two overlapping PCR products (537 and 523 bp, respectively) were used as templates for subsequent two rounds of inverse PCR (from the 5' ends of each PCR product, 3.6 kb of genomic DNA was amplified and ligated to the XhoI restriction enzyme). Antiserum and goat-anti-rabbit secondary antibodies coupled to peroxidase (Dianova) were used to perform immunoblotting as described before (55) with different rabbit antisera and goat-anti-rabbit secondary antibodies coupled to peroxidase (Dianova). The resulting fragment was cut with XhoI and EcoRV and ligated into pBSKII (+). Expression constructs for LmxxMKK complementation. The complementation of LmxxMKK in Δlmsmkk cells under the control of the ribosomal RNA promoter, LmxxMKK and the puromycin resistance gene PAC were integrated into the 18S rRNA locus. The hygromycin B phosphotransferase gene (HYG) of pSSU-int (35) was replaced by LmxxMKK by insertion of the Smal/XhoI fragment from the plasmid and ligation to the EcoRV/XhoI fragment obtained from the initial parental plasmid. The resulting construct was linearized at XhoI and ligated to a PCR fragment obtained at the ends with XhoI and NotI as described previously (8). Finally, the open reading frames of LmxxMKK and PAC were separated at the XhoI site by insertion of the CreB2.8 gene into the 5′ end of LmxxMKK as described previously (6). Both constructs were cut with XhoI and HindIII, gel purified, and used for electroporation in two consecutive rounds, as described previously (56). Transfected were selected on SDM-79 agar plates containing 10 µg of G418 ml−1 and 20 µg of hygromycin B ml−1.

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SEM. For scanning electron microscopy (SEM), the cells were fixed (using final concentrations of 2.5% glutaraldehyde in PBS) for 1.5 h in suspension. Fixed cells were mounted on poly-l-lysine-coated coverslips and postfixed for at least 30 min with 1% osmium tetroxide in PBS. After several PBS washes, cells were dehydrated through a graded ethanol series and critical point dried using CO₂ in a Polaron E 3000 apparatus (Plano, Marburg, Germany). Samples were sputtered (Balt-MEC 010; Balzers, Liechtenstein) with an 8-nm-thick gold palladium coat and examined at 20 kV in a Hitachi S-800 transmission electron microscope (TEM).

For conventional transmission electron microscopy (TEM), logarithmically growing promastigotes of wild-type and ΔLmxMKK L. mexicana were prefixed in suspension for 1 to 1.5 h on ice (after being treated first for 5 min at room temperature) with a final concentration of 2.5% glutaraldehyde in PBS (pH 7.2). After fixation, cells were centrifuged, embedded in 2% low-melting-temperature agarose (Sea Plaques, Rockland, Maine) in PBS, cut into small blocks, washed extensively with double-distilled water, stained with 1% aqueous uranyl acetate (for 1 h in the dark), washed again in double-distilled water, and dehydrated in a graded ethanol series. Afterwards, the blocks were infiltrated stepwise with Epon and the infiltrated samples were polymerized at 60°C for 48 h.

Cryoimmobilization of the cells by high-pressure freezing was performed as described before (17). Briefly, the cells were concentrated by gentle centrifugation in the cultivation medium. The cell suspension was sucked into cellulose microcapillaries, and 2-mm-long capillary segments were transferred to aluminum platelets of 200-μm depth containing 1-hexadecene. The platelets were covered with a second platelet without a cavity and then frozen with a high-pressure freezer (Balt-Tec HPM 010). The frozen capillary tubes were freed from extraneous hexadecane under liquid nitrogen and transferred to 2-ml microtubes with screw caps (no. 72.694; Sarstedt) containing the substitution medium pre-cooled to −90°C. For most structural investigations, the samples were kept in 0.5% osmium tetroxide in anhydrous acetone at −90°C for 34 h, at −60°C for 4 h, and at −40°C for a further 6 h in a freeze substitution unit (Balt-Tec FSU 010). After two washes with acetone, the samples were transferred into an acetone–Epon mixture at −30°C, infiltrated at room temperature in Epon, and polymerized at 60°C for 48 h. Using another approach under the same temperature and time conditions, the samples were replaced with 0.5% uranyl acetate in ethanol. After two washes with ethanol, the samples were infiltrated stepwise in the apolar methacrylate resin Lowicryl HM20 (Polysciences, Eppelheim, Germany) and polymerized by UV irradiation at −35°C. Ultrathin HM20 sections were stained with 1% aqueous uranyl acetate followed by lead citrate. Epon sections need a stronger stain, with a 2% solution of uranyl acetate in 50% ethanol followed by lead citrate. The sections were viewed in a Philips CM10 apparatus and a Philips 201 electron microscope at 60 kV.

Nucleotide sequence accession number. LmxMKK sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AJ243118.

RESULTS

Cloning and molecular characterization of LmxMKK. MKks are key players in signal transduction cascades conferring specificity to a given input signal by phosphorylation of a single specific MAP kinase. One MKK homologue has already been described for L. chagasi (accession number U16029) (28). We used oligonucleotides encoding the NH₂ and COOH termini of this protein to amplify the L. mexicana homologue from genomic DNA. The cloned DNA fragment was used to isolate the gene from a genomic DNA library in λ DASHII BamHI (56), and the gene was designated LmxMKK. Figure 1 shows an alignment of LmxMKK to MKKs from Trypanosoma brucei, Homo sapiens, Arabidopsis thaliana, D. discoideum, and S. cerevisiae. With 64% amino acid identity over the entire amino acid sequence, the T. brucei kinase (accession number AC091483) displayed the highest amino acid similarity to LmxMKK. The Leishmania protein kinase sequence contains the typical 10 most-conserved kinase subdomains (1 to IX) (15). All amino acid residues known to be invariant in MKks and most of the conserved residues are present. LmxMKK contains serine and/or threonine residues at positions in the activation loop between subdomains VII and VIII known to be phosphorylated by MKK kinases in higher eukaryotic cells to activate their substrate MKK.

The protein is encoded by an open reading frame of 1,116 bp corresponding to a length of 372 amino acid residues and with
a calculated molecular mass of 41.5 kDa. Its net charge is -7 and the isoelectric point is 5.38. To determine the splice addition site used for maturation of the mRNA of LmxMKK in trans splicing (27), we performed a reverse transcriptase PCR on total RNA from logarithmic and stationary phase promastigotes and lesion amastigotes. In all cases, a single addition site for the miniexon was found 71 bp upstream of the putative ATG translation initiation codon following an AG dinucleotide. The presence of mature mRNA in both life cycle stages of *L. mexicana* is in accordance with what has been found for the *Leishmania donovani* homologue of LmxMKK (13). Southern blot analysis of genomic DNA from *L. mexicana* was consistent with *LmxMKK* being a single-copy gene in the haploid genome (data not shown).

*LmxMKK* is exclusively expressed in the promastigote stage. Immunoblot analysis using an antisera against the COOH-terminal 15 amino acids of LmxMKK revealed a protein band of approximately 42 kDa in a total promastigote lysate from logarithmically growing cells (Fig. 2, lane 1) but no reaction of the antibodies in total cell lysates of lesion-derived amastigotes (Fig. 2, lane 2). As a control for loading of nondegraded protein, the blot was stripped and reprobed with an antisera against *myo*-inositol-1-phosphate synthase, a protein known to be expressed in pro- and amastigotes (19).

**Recombinant expression, mutagenesis, and autophosphorylation.** Sequence homology to protein kinases and the presence of the typical kinase subdomains indicate that LmxMKK is a functional kinase. To demonstrate its enzymatic activity, the protein was expressed as a GST fusion protein, enriched on glutathione Sepharose beads, and subjected to an autophosphorylation assay (Fig. 3). The protein was readily expressed and found in the soluble fraction of the bacterial cell lysate (lane 2). Lane 2 represents the corresponding autoradiograph, displaying no autophosphorylation activity. It had been shown that replacement of the regulatory phosphorylation sites and their adjacent residues by four aspartate residues in human MKK1 led to constitutive activation (31). Therefore, we replaced the activation loop residues QTLAVSSTY of LmxMKK by the amino acid residues DDDADDH, generating LmxMKK(D). As a control for inherent autophosphorylation activity, a kinase mutant deficient in phosphorylation activity was generated by mutation of the invariant lysine 91 (which is known to be directly involved in the phosphotransfer reaction by positioning of the ATP [15]) to methionine in the wild type and LmxMKK(D), respectively. All of the proteins were expressed and could be bound to glutathione Sepharose (see Fig. 3). The corresponding autoradiograph shows that only LmxMKK(D) revealed autophosphorylation activity (Fig. 3, lane 3) which was abolished completely after additional mutation of K91M (lane 4), confirming that it is not a copurified activity phosphorylating LmxMKK(D). As expected, mutation of lysine 91 to methionine in the wild-type protein also showed no autophosphorylation activity (lane 4).

**Targeted deletion of LmxMKK and complementation.** Both alleles of *LmxMKK* were sequentially replaced by the selective marker genes conferring neomycin (*NEO*) or hygromycin B (*HYG*) resistance. The DNA constructs for deletion were designed to specifically replace the target gene, being composed of approximately 2.4 kb of the 5′ UTR and 2 kb of the 3′ UTR of *LmxMKK* surrounding either of the resistance marker genes. Figure 4B schematically shows some relevant restriction sites in the *LmxMKK* gene locus and the situation after *LmxMKK* had been replaced. Southern blot analysis of the wild type and the null mutant with different probes revealed the bands expected after replacement of both alleles of *LmxMKK* by the resistance marker genes (Fig. 4). Immunoblot analysis of total cell lysates from logarithmically growing wild-type, single-allele deletion mutant, and null mutant promastigotes showed the absence of the protein in the null mutant (Fig. 5, lane 2) and a decrease in its amount in the single-allele deletion mutant (Fig. 5, lane 2). Complementation of the null mutant reintroducing the gene either on a plasmid (Fig. 5, lane 4) or by integration into the rRNA gene locus (Fig. 5, lane 5) led to reexpression.

**The phenotype of the null mutant.** Cell culture of the deletion mutant already showed that the cells had a severe defect in motility but not in growth rate. There were no actively swimming cells; i.e., all cells were found at the bottom of the
culture flask wiggling with their flagella. SEM demonstrated that the null mutant promastigotes have on average a shorter, less voluminous cell body than those of the wild type and that the flagellum is reduced in length to about one-fifth or less of the wild-type flagellum length throughout all cells (Fig. 6). The use of phase-contrast microscopy and Openlab software from Improvision (Heidelberg, Germany) for determination of flagellar lengths of 800 cells from two independent null mutant clones resulted in calculations of an average length of 1.76 μm and a size range from 0 to 5 μm (measured from the cell surface to the tip of the flagellum). TEM of the deletion mutant and the wild type on high-pressure frozen (HPF) and freeze-substituted promastigotes and on chemically fixed cells showed that the mutants also had other defects.

Longitudinal sections through wild-type and mutant cells demonstrated the reduced diameter and length of the flagellum in the mutant (Fig. 7A and B). In chemically fixed cells, vesicular structures were found in both wild-type (data not shown) and mutant (Fig. 7B) flagella. As these were never seen in HPF specimens, they were most likely generated in the course of the preparation. Figures 7C and D show longitudinal sections through HPF cryofixed wild-type and mutant flagella, respectively. The paraflagellar rod (PFR), a crosshatched cytoskeletal structure running along the axoneme in kinetoplastid, euglenoid, and dinoflagellate flagella (4), is seen in the wild type (Fig. 7C) but is missing in most of the mutant (Fig. 7D) promastigotes. In 78.5% of all transverse sections, the flagellum of the mutant displayed an almost circular shape (due to the absence of the PFR) (Fig. 7F and H to J) compared to the oval shape of the wild-type flagellum (Fig. 7E and G). While most of these mutant axonemes had the canonical 9-plus-2 microtubule structure, 11% lacked the central doublet (Fig. 7I and J), which was never observed in the wild type. As some of the 9-plus-0 flagella were found to be separate from all cell bodies (Fig. 7J), the sections were not located in the flagellar pocket and therefore did not represent the transition zone, a region from which the central microtubule doublet is absent (Fig. 7K). The 9-plus-0 structures were also found in flagella attached to the cytoplasmic membrane of the null mutant, displaying tight junction-like structures indicating the proximity to the opening of the flagellar pocket (Fig. 7I). However, most of the transverse sections displaying flagella in their pockets revealed the 9-plus-2 microtubule pattern (Fig. 7M) even in the situation of a dividing cell with the old and new flagella side by side (data not shown). The remaining 21.5% of the flagellar transverse sections revealed a rudimentary PFR in the mutant compared with wild-type PFR (Fig. 7E to H).
Immunoblot analysis using the monoclonal antibody L8C4 against the *T. brucei* PFR protein PFR-A (24) corroborated this result, detecting strongly reduced amounts of PFR2 (the *Leishmania* homologue to the trypanosomatid PFR-A) in total cell lysates (data not shown). In addition to displaying reduced cell size and aberrant flagellar morphology, the mutant promastigotes were found to accumulate membrane fragments in the flagellar pocket (Fig. 7K to M). This observation was not due to a preparational artifact, as these structures are present in both chemically fixed and HPF cells (compare Fig. 7L and M to Fig. 7K).

Null mutant promastigotes were used to infect BALB/c mice. In two independent experiments, lesion development occurred at delayed times after about 30 weeks postinfection (compared to within 5 weeks for the wild type) but then progressed as seen in wild-type infections (data not shown). Parasites were isolated from lesion material and could be transformed back into promastigotes in vitro, again displaying a null mutant phenotype. Lesion material obtained from BALB/c mice infected with the mutant was processed for TEM. Figure 7N shows a representative section through the flagellar pocket region of a mutant amastigote revealing no obvious morphological aberrations.

**DISCUSSION**

We isolated *LmxMKK*, the gene for an MKK homologue from *L. mexicana*. It is interesting that mature mRNA of *LmxMKK* is present in both life cycle stages, the pro- and amastigotes, albeit no protein could be detected on immunoblots of the amastigotes. This suggests that the expression of the protein is regulated on the translational level, affecting translation efficiency or protein stability.

Autophosphorylation has been found to be important for the regulation of the activity of protein kinases, such as (for instance) p42 MAP kinase, MEK kinase 1, MEK1, and WNK1 (11, 49, 57, 58). Wild-type *LmxMKK* expressed as a GST fusion protein in *E. coli* did not show any autophosphorylation. However, the aspartate activation loop mutant had a high level of autophosphorylating activity which was completely abolished by the additional mutation of lysine 91 to methionine. As expected from studies of kinases in other eukaryotes, this lysine is involved in the phosphotransfer reaction that uses ATP as a phosphate donor to the substrate (9). The activation of *LmxMKK* by the introduction of negatively charged residues in the activation loop mimicking negatively charged phosphate groups indicates that at least one of the serine and/or threonine residues is likely to be phosphorylated for the activation of *LmxMKK*. Whether subsequent autophosphorylation occurs in *Leishmania* and whether this will activate the kinase, enhance its activity, or function as a negative feedback control is yet not clear. The identification of the activator(s) and substrate(s) of *LmxMKK* will help to solve this issue.

Deletion of *LmxMKK* resulted in the formation of *L. mexicana* promastigotes with flagella dramatically reduced in length. It is clear from complementation using *LmxMKK* on a plasmid or by integration of the gene into the ribosomal rRNA locus that the shortened flagellum is a consequence of the deletion of the kinase gene. In the *Leishmania* life cycle, flagellum absorption is a normal process during differentiation from promastigotes to amastigotes and flagellar synthesis occurs during differentiation from amastigotes to promastigotes and in cell division, during which a new flagellum is formed adjacent to the old one in the same flagellar pocket. In *Chlamydomonas*, flagellar length is cell cycle regulated (53): flagella are disassembled before cell division, and new basal bodies and flagella are assembled in the daughter cells after division. It has been shown for *Chlamydomonas* that intraflagellar transport (IFT) influences the stability of the axoneme and is required for maintenance of flagellar length (51). Flagellar assembly or elongation requires the transport of subunits from the cytoplasm, where they are synthesized, to the tips of the flagellar microtubules, where they are added to the microtubule ends.
FIG. 7. Ultrastructure of *L. mexicana* wild-type and LmxMKK null mutant (ΔLmxMKK) cells and flagella. (A) Longitudinal section of the flagellar pocket and flagellum of a chemically fixed *L. mexicana* wild-type promastigote (inset shows the cell at reduced magnification). (B) ΔLmxMKK at the same magnification (inset represents the membrane bilayer at the tip of the flagellum). (C to F) Sections of wild-type and ΔLmxMKK flagella after HPF, freeze substitution, and Epon embedding; (C and E) wild-type longitudinal and transverse sections; (D, F, and I) ΔLmxMKK longitudinal and transverse sections. (G, H, and J) Chemically fixed transverse flagellar sections of the wild type (G) and ΔLmxMKK (H and J). (K to M) HPF cryofixed (K) or chemically fixed (L and M) ΔLmxMKK promastigotes, containing large amounts of membrane fragments (mf) in their flagellar pockets. An arrow in panel K indicates a region from which the central microtubule doublet was absent. (N) Amastigote from ΔLmxMKK in lesion material from an infected BALB/c mouse. a, axoneme; f, flagellum; fp, flagellar pocket; g, Golgi; k, kinetoplast; m, mitochondrion; p, PFR; tj, tight junction; tz, transition zone; v, vesicle. Bars, 0.5 μm (A, B, and K to N) and 0.25 μm (C to J).
(21). Kinesin II and cytoplasmic dynein (DHC1b) have been found to be key players in anterograde (base to tip) and retrograde IFT, respectively (25, 43, 44).

It is important that besides appearing to be stable organelles, flagella are dynamic structures that change up to 20% of their polypeptides within 3 h without any changes in their overall length (52). It is not yet clear how flagellar assembly is regulated, but there is some evidence for signal transduction pathways, and over 80 phosphorylated flagellar components have been found in Chlamydomonas (16, 46, 47, 53). It is likely that we identified a protein kinase in L. mexicana which is involved in flagellar length control by regulating the phosphorylation state of proteins involved in flagellar assembly. For kinetochores, flagellar assembly has been studied by looking at the assembly of the PFR, a structure known to be vital for trypanosome motility (6). The two major components PFR-A and PFR-C are incorporated predominantly from the distal tip to the growing PFR, but some protein is also incorporated along its length (3). There is evidence for both anterograde and retrograde IFT coming from PFR mutants in T. brucei and L. mexicana (5, 30). Ablation of the expression of PFR-A (which is required for the assembly of the PFR) resulted in the accumulation of free PFR-C at the tip of the growing flagellum, forming a “blob,” which is resorbed at some point early in the cell cycle prior to the formation of a new flagellum. Likewise, the deletion of PFR1, PFR2, or both also led to a flagellar tip dilation in L. mexicana shown to accumulate detergent-soluble forms of the remaining PFR components (30).

In the LmxM KK null mutant, the overall length of the flagellum is strongly reduced and the PFR is present in electron micrographs as a rudimentary structure of (at most) a fraction of the cells. The shortening of the flagellum during the differentiation from pro- to amastigotes and the lack of expression of LmxM KK in the amastigote stage suggest that LmxM KK is substantially involved in the maintenance of a full-length flagellum in the promastigote. Moreover, secretion of a filamentous acid phosphatase (34) typically found in L. mexicana promastigotes indicates the promastigote nature of the null mutant (results not shown). As balanced IFT is recognized as the major mechanism for the maintenance of flagellar length (32), LmxM KK is likely to be involved in its regulation. After infection of BALB/c mice, the LmxM KK null mutants were able to cause lesions, albeit with a delayed onset at least 30 weeks postinfection. However, the morphology of the lesion amastigotes displayed the typical short flagellum not protruding from the flagellar pocket but sealing it from the surrounding tissues. Therefore, retrograde transport to resorb the flagellum during promastigote-to-amastigote differentiation is still operative in the null mutant, leaving LmxM KK with a potential role in the anterograde transport.

As a kinase, LmxM KK can directly phosphorylate and regulate the activity of components involved in anterograde IFT or influence the expression of these components via specific regulation of gene expression. The latter is likely to occur via a specific MAP kinase in an associated MAP kinase signal transduction cascade. The loss of the central microtubule doublet in some of the cells and the variation in PFR assembly suggests that the degrees of penetrance of the mutation caused by the loss of LmxM KK differ significantly and might reflect the actual age of a cell or its position within the cell cycle. It is not yet clear whether the reduced amount of PFR2 expressed in the null mutant is due to reduced protein synthesis or enhanced degradation as a consequence of the inability to reach its final destination in the PFR. The accumulation of membrane fragments in the flagellar pocket of the null mutant could be due to an overproduction of membrane components such as lipophosphoglycan, glycoinositolphospholipids, and other lipids which would normally be incorporated into the flagellar and cellular membrane (7, 33). On the other hand, the accumulation of the membrane fragments might be an explanation for the smaller size and therefore the reduced surface area and the shortened flagellum in the null mutant. It has been proposed that flagellar movement in its canal might support the continual replacement of flagellar pocket contents (54). The short flagellum of the LmxM KK null mutant moves, although it might not be as active as a full-length wild-type flagellum. Therefore, membrane fragments accumulate in the flagellar pocket. However, the same kinetics of filamentous acid phosphatase secretion into the culture supernatant of wild-type and null mutant promastigotes argues against a defect in the secretion of material from the flagellar pocket (data not shown).

To our knowledge this is the first report of a protein kinase involved in organelle assembly and maintenance. As flagella are widespread in eukaryotic cells from protists to mammals and a number of human disorders are caused by immotile or misassembled flagella (48), study of signal transduction pathways regulating flagellar morphology is of substantial relevance. Here the unflagellated parasite Leishmania could function as a suitable model organism.

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