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Interacting Protein Kinases Involved in the Regulation of Flagellar Length

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A striking difference of the life stages of the protozoan parasite *Leishmania* is a long flagellum in the insect stage promastigotes and a rudimentary organelle in the mammalian amastigotes. LmxMKK, a mitogen-activated protein (MAP) kinase from *Leishmania mexicana*, is required for growth of a full-length flagellum. We identified LmxMPK3, a MAP kinase homologue, with a similar expression pattern as LmxM KK being not detectable in amastigotes, up-regulated during the differentiation to promastigotes, constantly expressed in promastigotes, and shut down during the differentiation to amastigotes. LmxMPK3 null mutants resemble the LmxM KK knockouts with flagella reduced to one-fifth of the wild-type length, stumpy cell bodies, and vesicles and membrane fragments in the flagellar pocket. A constitutively activated recombinant LmxM KK activates LmxMPK3 in vitro. Moreover, LmxM KK is likely to be directly involved in the phosphorylation of LmxMPK3 in vivo. Finally, LmxMPK3 is able to phosphorylate LmxM KK, indicating a possible feedback regulation. This is the first time that two interacting components of a signaling cascade have been described in the genus *Leishmania*. Moreover, we set the stage for the analysis of reversible phosphorylation in flagellar morphogenesis.

INTRODUCTION

Protein kinases are central molecules in differentiation, proliferation, stress response, and apoptosis in all eukaryotic cells. A commonly used signaling pathway is that of the mitogen-activated protein (MAP) kinases. Its core module is comprised of a MAP kinase kinase kinase (MKKK), a MAP kinase kinase (M KK), and a MAP kinase. An external signal is sensed by a cell surface receptor, which subsequently undergoes autophosphorylation and recruits first intracellular signaling molecules. These can activate MKKK, which phosphorylates and activates its substrate MKK. MKKs are dual specificity kinases that activate their substrates, the MAP kinases, by phosphorylation on a threonine and tyrosine residue of the TXY motif in the activation lip region. Finally, MAP kinases can phosphorylate transcription factors, directly influencing the expression of certain genes, or they phosphorylate other soluble kinases or structural proteins of the cell. This is a very simplistic view of the processes occurring in a cell after stimulation, because there are among other proteins, a number of scaffold proteins involved that are responsible for the specificity of the phospha relay. Moreover, protein phosphatases counterbalance the activity of the protein kinases by dephosphorylation.

One aspect of differentiation is the regulation of organelle and overall cell size. Flagellar length regulation is a simple one-dimensional example for maintenance of a defined size of an organelle, the flagellum. A flagellum is a dynamic structure that is built by the assembly of its components at its tip, which is also the site for the disassembly of the structural elements. The building blocks are delivered and removed by a process called intraflagellar transport (IFT). The balance between anterograde (base to tip) and retrograde IFT has been found to determine the length of the flagellum in the green alga *Chlamydomonas reinhardtii* (Marshall and Rosenbaum, 2001). It is likely, however, that protein phosphorylation adds an additional layer to the regulation of flagellar maintenance because >80 flagellar components have been found to be phosphorylated in *Chlamydomonas* (Piperno and Luck, 1976; Piperno et al., 1981; Harper et al., 1993; Tuxhorn et al., 1998). Our studies of the protozoan parasite *Leishmania* have shown that this parasite is a suitable model organism for flagellar length regulation because protein kinases of the MAP kinase signal transduction cascades are critically involved in its regulation (Wiese et al., 2003a; Bengs et al., 2005). Likewise, in *Chlamydomonas* LF4, a protein kinase with homology to MAP kinases and the human male germ cell-associated kinase (MAK), has been found to influence flagellar length as a null mutant displayed elongated flagella (Berman et al., 2003).

*Leishmania* parasites have a digenetic life cycle with the sandfly as their insect vector and mammals as their host. The insect stage promastigotes are spindle-shaped cells, 11–20 μm in length and 2 μm in diameter, with a long flagellum protruding from the flagellar pocket, an invagination of the cytoplasmic membrane at the anterior end of the cell. They are transmitted to a mammalian host during the bloodmeal of the sandfly. In the skin of the host, the promastigotes are taken up by macrophages and end up in the lysosome of the host cell, forming the parasitophorous vacuole. Instead of being killed and degraded, triggered by the elevated temperature and the low pH (Zilberstein and Shapira, 1994), the
Antibody Production and Immunoblotting

A rabbit antisera was produced against the peptide CTACGGSSKNCSCHHH corresponding to the 15-COOH-terminal amino acids of LmxMPK3 (Eurogentec, Seraing, Belgium) and purified on the peptide. Lysates of 1 × 10⁷ cells ml⁻¹ in 1 ℓ lysis buffer (1 ℓ PBS, 0.1% SDS, 50 mM dithiothreitol, 50 µM leupeptin, 25 µM Nα-p-tosyl-L-lysyl-chloromethylketone, 1 mM phenylmethylsulfonyl fluoride, 10 mM 1,10-phenanthroline, and 1 ℓ l SDS sample buffer [0.4% SDS, 4% glycerol, 0.0002% bromphenol blue, 50 mM dithiothreitol, and 12.5 mM Tris-HCl, pH 6.8]) were boiled for 10 min. Then, 20 µl was subjected to 50% polyvinylidene difluoride membranes. Immunodetection was carried out as described previously (Wiese, 1998) with different rabbit or mouse antisera and goat-anti-rabbit or goat-anti-mouse secondary antibodies coupled to horseradish peroxidase (Dianova, Hamburg, Germany) followed by chemiluminescence detection using the Supersignal system (Pierce Chemical, Rockford, IL).

LmxMPK3 Deletion Constructs

To generate the LmxMPK3 null mutants ΔLmxMPK3::HYG/ΔLmxMPK3::NEO and ΔLmxMPK3::PHLEO/ΔLmxMPK3::NEO abbreviated Δ1 and Δ2, respectively, LmxMPK3 and its flanking regions were amplified from a plasmid carrying a DNA-fragment isolated from a genomic DNA library of L. mexicana (Wiese et al., 1995, 2003b). A PCR was performed on this plasmid (5 min at 94°C, 25 × [30 s at 94°C, 30 s at 50°C, 1 min 45 s at 72°C], 7 min at 72°C, 4°C) using the oligomers 5'-CAGACGGGCGACCATCTT-3' and 5'-CTGTGATCCTTCTGTCTGTC-3', the latter introducing an EcoRV restriction site into the 5'-untranslated region (UTR) of LmxMPK3. Because a second EcoRV site is present in the 3'-UTR the amplified fragment was cut using EcoRV and ligated into pBluescript II KS(+) (Stratagene, La Jolla, CA) previously modified to lack a BspLU11I site and linearized at EcoRV resulting in BspLU11I-MspK3ds. To replace LmxMPK3 by different resistance marker genes BspLU11I and NeoI sites were introduced into pBluesupLmxP3ds using the oligomers 5'-CTGTGACTGTTGAGCTCAACCCAG-3' (covering the Scal site in the β-lactamase gene of the plasmid) and 5'-CTGCGCTAGGAGAGAGCTACTGTTGACC-3' containing an AvrII and a BspLU11I site in one PCR to amplify the 5'-flanking region. The resulting DNA-fragments were trimmed using AvrII and Scal and ligated to each other resulting in a plasmid with LmxMPK3 replaced by BspLU11I, AvrII, and NeoI restriction sites. This plasmid was linearized using BspLU11I and NeoI and ligated to DNA-fragments carrying the resistance marker genes for the hygromycin B phosphotransferase HYG, the neomycin phosphotransferase NEO, and the phleomycin binding protein PHLEO, prepared as described previously (Benzel et al., 2000). The constructs were cotransformed into E. coli, gel-purified, and used for electroporation of L. mexicana promastigotes in two consecutive rounds as described previously (Benzel et al., 2005).

Expression of LmxMPK3 and LmxMPK(D) in Leishmania

For episomal expression of LmxMPK3 in L. mexicana, the open reading frame (ORF) coding for the oligonucleotides ACATGTTGGCTACTCTGTGTGC-3' flanking region. The resulting DNA-fragments were trimmed using Scal and ligated to each other resulting in a plasmid with LmxMPK3 replaced by BspLU11I, AvrII, and NeoI restriction sites. This plasmid was linearized using BspLU11I and NeoI and ligated to DNA-fragments carrying the resistance marker genes for the hygromycin B phosphotransferase HYG, the neomycin phosphotransferase NEO, and the phleomycin binding protein PHLEO, prepared as described previously (Benzel et al., 2000). The constructs were cotransformed into E. coli, gel-purified, and used for electroporation of L. mexicana promastigotes in two consecutive rounds as described previously (Benzel et al., 2005).

Materials and Methods

Parasites

Promastigotes of L. mexicana MN6C/BZ/62/M379 were grown as described previously (Menz et al., 1991). Female BALB/c mice were infected into the hind foot pad at the age of 6–8 wk with 1 × 10⁷ promastigote parasites in 30 µl of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 1.4 mM KH₂PO₄). Using a caliper gauge, the course of infection was followed by measuring lesion size relative to the uninfected right hind foot pad at 4- to 5-wk intervals. For axenic amastigotes, cultures were seeded with stationary phase promastigotes at 4 × 10⁶ cells/ml in Schneider's Drosophila medium (Biochrom, Aidhausen, Germany) supplemented with 20% inactivated fetal calf serum and 2 mM glutamine, adjusted to pH 5.5 by the addition of morpholinoethane sulfonic acid, and incubated at 34°C, 5% CO₂. Samples of 1 × 10⁶ cells were taken at defined intervals after initiation of differentiation. For amastigote-to-promastigote differentiation, parasites were isolated from lesions of infected BALB/c mice as described previously (Wiese, 1998). The whole cell suspension was centrifuged for 10 min at 150 × g and 4°C. Then, the supernatant was centrifuged for 10 min at 1500 × g at 4°C. The pellet was resuspended in 10 ml of cold 1 × PBS, and the cells were counted. One hundred milliliters of prewarmed (37°C) SDM-79 medium (Brun and Schoenenberger, 1979) was inoculated with 10⁷ amastigotes. Every 5 h, 5 ml pellets of 1 × 10⁷ cells were collected, washed with cold 1 × PBS, frozen in liquid nitrogen, and stored at −70°C.

Molecular Cloning Methods

Expand High-Fidelity Polymerase (Roche Diagnostics, Mannheim, Germany) was used for all PCR applications. Plasmid isolation, DNA/RNA isolation and blotting, and hybridizations were performed as described previously (Benzel et al., 2000). Both DNA strands of all constructs derived from PCR were verified by DNA sequencing. LmxMPK3 sequence data have been submitted to DDBJ/EMBL/GenBank databases under accession no. AJ292821. Splice-addition sites were determined as described previously (Wiese, 1998) using the aforementioned primers and DNA from L. mexicana ORF) of LmxMPK3 was amplified using the oligomers 5'-CACCAAGTGTTTGATAGG-3' and 5'-CTGTGATCCTTCTGTCTGTC-3', as primers in the reverse transcriptase and polymerase chain reactions.
Philips CM 10 transmission electron microscope at an acceleration voltage of 80 kV. Phase contrast and differential interference contrast (DIC) microscopy and flagellar length determination (the length was measured from the cell surface to the tip of the flagellum using the Openlab software; Improvision, Heidelberg, Germany) were performed on a Zeiss Axioskop 2 Plus and a Leica Leitz DMRB microscope as described previously (Bengs et al., 2005).

**Recombinant Expression**

For recombinant expression of a glutathione S-transferase (GST) fusion protein of LmxMPK3, the ORF was liberated from pCR2.1-23MPK3 (see above) with BpiLU11I and HindIII, and the DNA-fragment was gel-purified and ligated into the Ncol/HindIII-cleaved pEGX-KG (Guan and Dixon, 1991), resulting in pEGX-KG5aBHLmxMPK3. To generate an enzymatically inactive version of LmxMPK3, lysine 62 was mutated to methionine by site-directed mutagenesis, resulting in LmxMPK3KM. A PCR reaction was performed on pEGX-KG5aBHmxMPK3 (5 min at 94°C, 25 × 30 s at 94°C, 30 s at 50°C, 45 s at 72°C), 7 min at 72°C, 4°C) using the oligonucleotides 5’-CTACTCCCAAGATGATAA-3’ and 5’-TCGGCACAATCTCAATGATGACACCTCT-3’. The amplified fragment was cleaved using NruI and XbaI, resulting in pEGX-KG7aBHLmxMPK3 cleaved with NruI and XbaI, resulting in pEGX-KG9BHlmxMPK3KM. Both constructs were transformed into E. coli XL1-Blue (Stratagene). For expression of the fusion proteins, the bacteria were grown in Luria-Bertani medium, induced with 100 µM isopropyl-β-D-thiogalactopyranoside at an optical density at 600 nm (OD 600) of 0.9, further incubated overnight at 18°C, and harvested by centrifugation at 4°C and 4500 × g for 15 min. The cells were washed once in cold PBS, resuspended in 50 mM MOPS, pH 7.0, 10 mM MnCl 2, and heating for 10 min, the recombinant proteins were purified on glutathione-Uniflow resin following the instructions of the manufacturer (BD Biosciences, Heidelberg, Germany). For expression of LmxMKK(D) and LmxMKK(KM) in E. coli XLI-Blue, the constructs described previously (Wiese et al., 2003a) were used under the same conditions as described for LmxMPK3. To cleave the GST-moieity from the fusion protein, 250 µg of protein was incubated with 0.12 U of thrombin (Amersham, Freiburg, Germany) overnight at 20°C.

**Kinase Assay**

To determine the interaction of LmxMKK with LmxMPK3, 2 µg of LmxMKK(D) or LmxMKK(K91M) was incubated at 27°C with 2 µg of GSTLmxMPK3 or GSTLmxMPK3(KM) and 0.1 mM [γ-32P]ATP (1000 cpm.pmol) in a volume of 50 µl containing 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0, 10 mM MnCl 2, and 0.1 M NaCl. Reactions were terminated after 1 h by the addition of 12.5 µl of 5× SDS sample buffer containing 200 mM dithiothreitol and heating for 10 min at 95°C. Then, 25 µl of the solution was separated on a 12% SDS-PAGE, silver-stained, dried, and exposed to X-ray film at –80°C. For quantification, unlabelled ATP replaced [γ-32P]ATP during the phosphorylation of LmxMPK3 by LmxMKK(D) in a reaction of 20 µl, essentially as described previously (Lawler et al., 1997). Then, 350 ng of LmxMKK(D) was incubated with 10 µg of GSTLmxMPK3 at 27°C as described above. After 1 h, 5 µl of this reaction was assayed for GSTLmxMPK3 kinase activity toward MBP at 27°C in a 50 µl reaction containing 50 mM MOPS, pH 7.0, 10 mM MnCl 2, 0.1 M NaCl, 1 mM EGTA, 1 mM Na-orthovanadate, 0.3 µg/µl myelin basic protein (MBP), and 0.1 mM [γ-32P]ATP, resulting in an overall molecular ratio of LmxMKK(D)/GSTLmxMPK3/MBP of 1:17:1000. After incubation for 1 h, 40 µl was spotted on phosphocellulose P81 paper (Whatman, Dassel, Germany) and washed four times with 75 mM H 3 PO 4 and once with acetone. The papers were exposed to x-ray film at 20°C.

**Results**

**Molecular Characterization**

LmxMPK3 is one of nine MAP kinase homologues we identified from L. mexicana in a screen using degenerate oligo-nucleotides encoding highly conserved amino acid regions in subdomains VIIb and VIII of MAP kinases (Wiese et al., 2003b). The gene is comprised of an open reading frame of 1164 base pairs coding for a protein of 388 amino acids with a calculated molecular mass of 43.7 kDa. Homologues are present in other kinetoplastids, such as Leishmania major (98% identical amino acids 382–585; our unpublished data), Leishmania infantum (98% identical amino acids 382–585; our unpublished data), Trypanosoma brucei (70% identical amino acids 262–350), and Trypanosoma cruzi (69% identical amino acids 239–327). Less homologous, but still significant, are the proteins from Chlamydomonas reinhardtii (44% identical amino acids 382–585), from Solanum tuberosum (46% identical amino acids 382–585), and the ERK1 kinase from Dictyostelium discoideum (45% identical amino acids 382–585; Gaskins et al., 1994). The sequence of LmxMPK3 displays the typical 12 kinase subdomains and amino acid residues known to be highly conserved in MAP kinases (Figure 1). There are the subdomain I residues G40–G45 forming the phosphate anchor ribbon for ATP binding with the consensus GxGxxG and the p + 1 specificity pocket in subdomain VIII (I98-R203). In addition, there are the catalytic site residues K62, R76, R79, E80, R160, D161, K163, N166, D167 (Mg 2+ ligand), R184, T194, D195, and Y196, the latter three forming the TXY motif, which is characteristic for MAP kinases and known to be phosphorylated by the activating MAP kinase kinase on threonine and tyrosine to generate the active enzyme. Lysine 62 in subdomain II is a conserved residue found in all protein kinase family members (Hanks et al., 1988) and is essential for catalytic activity (Zoller and Taylor, 1979; Zoller et al., 1981; Kamps and Sefton, 1986; Buechler and Taylor, 1989; Gibbs and Zoller, 1991). The potential common docking (CD)-domain (DEEDE), an accumulation of negatively charged residues that is likely to be involved in protein–protein interactions is depicted in Figure 1 (Tanoue and Nishida, 2003). Splice-acceptor sites for LmxMPK3 were determined using total RNA in a reverse transcriptase-PCR with gene internal and mini-exon primers (see Materials and Methods). Amplified fragments were cloned and sequenced. Three AG splice-acceptor sites were found located 58, 61, and 90 base pairs upstream of the ATG translation initiation codon. Interestingly, all three amplification products were found in RNA obtained from lesion-derived amastigotes despite the fact that the mRNA of LmxMPK3 is down-regulated in this life stage (Wiese et al., 2003b). Southern blot analysis of genomic DNA from L. mexicana proved that LmxMPK3 is a single copy gene in the haploid genome (our unpublished data).

**Immunoblot Analyses**

Using a polyclonal antiserum raised in a rabbit against a carboxy-terminal peptide of LmxMPK3, the protein was readily detectable in a cell lysate of 2× 10 7 promastigotes, whereas it could not be detected in the same number of amastigotes derived from lesions grown in BALB/c mice after infection with L. mexicana wild-type promastigotes (Figure 2). This reflects the above-mentioned down-regulation of LmxMPK3 mRNA in amastigotes (Wiese et al., 2003b). As a control for the presence of comparable amounts of nondegraded protein in each lane, the blot was stripped and treated with a polyclonal antiserum against myo-inositol-1-phosphate synthase, a protein known to be expressed in both life stages (Ilg, 2002). After differentiation of amastigotes to promastigotes using lesion-derived amastigotes to inoculate promastigote growth medium, LmxMPK3 appears within 16–22 h after differentiation initiation (Figure 3A). However, LmxMKK is already detectable in the population...
after 10 h (Figure 3B). At 19–22 h, most of the cells displayed short flagella not allowing the cells to swim (Figure 3C). At 38 h after differentiation initiation, the cells had flagella of normal length and function. After the in vitro differentiation of promastigotes to amastigotes by pH and temperature shift, the analysis of LmxMPK3 levels by immunoblotting revealed that the amount of protein gradually declines to an amount below the level of detection at 48 h after induction of differentiation. Figure 1. Alignment of LmxMPK3 from *L. mexicana* with MAP kinase amino acid sequences from different species. LmxMPK3, *L. mexicana* MAP kinase 3 (accession no. AJ293281); TbMPK3, *Trypanosoma brucei* MAP kinase 3 (accession no. GeneDB Tb927.8.3550); TcMPK3, *Trypanosoma cruzi* MAP kinase 3 (accession no. GeneDB Tb927.8.3550); CrMPK3, *Chlamydomonas reinhardtii* MAP kinase (accession no. AB035141); StMPK2, *Solanum tuberosum* MAP kinase (accession no. AB062138); and DdMPK3, *Dictyostelium discoideum* MAP kinase 3 (accession no. AF050112). Roman numerals I to XI indicate kinase subdomains. The kinase domain is located between the two inverted triangles. Arrows mark the potential regulatory phosphorylation sites in the TXY motif at Thr194 and Tyr196, filled circles indicate conserved amino acid residues, and open circles depict the residues of the phosphate anchor ribbon for ATP binding. The asterisks show the residues of the p+1 specificity pocket. The open triangle marks the invariant lysine residue (Lys62), which is essential for the phosphate transfer reaction. The putative CD-domain is indicated. Dashes indicate gaps introduced to optimize the alignment; dots represent identical residues. Numbering corresponds to LmxMPK3.
differentiation (Figure 3D). During the same time, the cells completely loose their flagella (Figure 3F). For LmxMKK, we observed a slower decline of protein levels with a weak band still present at 72 h (Figure 3E). During this time, the cells adopted the spherical amastigote morphology (Figure 3F).

**Targeted Deletion of LmxMPK3 and Complementation**

The two alleles of LmxMPK3 were sequentially replaced by the selective marker genes conferring hygromycin B (HYG), G418 (NEO), or bleocin (BLE) resistance. Targeted replacement of LmxMPK3 was ensured using 790 base pairs of the 5’/H11032-UTR and 652 base pairs of the 3’/H11032-UTR of LmxMPK3 surrounding either of the resistance marker genes. Southern blot analysis of the wild-type, single-allele deletion, and null mutants with probes detecting the LmxMPK3 wild-type gene locus or the integration of the resistance marker genes revealed the expected bands for accurate replacement (our unpublished data). Immunoblot analysis of total cell lysates from logarithmically growing wild-type, two independent single-allele deletion, and two null mutants derived from these single-allele knockouts could not detect the protein in the null mutants and showed a decrease in its amounts in the single-allele deletion mutant (Figure 4). Complementation of the null mutant reintroducing the gene on a plasmid led to reexpression of the protein, albeit in lower amounts (Figure 4, lanes 6 and 7).

**The Phenotype of the Null Mutant**

The overall appearance of the null mutant promastigotes resembled the appearance of those promastigotes generated by the deletion of LmxMKK, the gene for a MAP kinase homologue from L. mexicana (SEM; Figure 5) (Wiese et al., 2003a). If at all, the cells displayed a flagellum rarely reaching one-half of the length of the wild-type flagellum (Figure 5, A and B). However, as in the LmxMKK null mutant, they were able to wiggle slowly with their flagel-
Figure 6. The \( \Delta \text{LmxMPK3}^-/- \) phenotype. Histograms of flagellar lengths from wild-type and mutant *L. mexicana* promastigotes. The abscissa show groups of flagellar lengths in micrometers; the ordinates indicate the numbers of cells counted. (A) White bars, *L. mexicana* wild type; black bars, \( \Delta \text{LmxMPK3}^-/- \) clone 1 or \( \Delta 1 \); gray bars, \( \Delta \text{LmxMPK3}^-/- \) clone 2 or \( \Delta 2 \). (B) White bars, \( \Delta \text{LmxMPK3}^-/- \) clone 1 + *LmxMPK3* or A1; black bars, \( \Delta \text{LmxMPK3}^-/- \) clone 2 + *LmxMPK3* or A2.

Figure 7. Ultrastructure of *L. mexicana* wild-type and LmxMPK3 mutant (\( \Delta \text{LmxMPK3}^-/- \)) flagella. Cross-sections of flagella of chemically fixed *L. mexicana* wild-type promastigote (A), \( \Delta \text{LmxMPK3}^-/- \) promastigotes (B–F), and episomal complementation (G–I) at the same magnification. a, axoneme; p, paraflagellar rod. Bar, 0.25 \( \mu \text{m} \).

Figure 8. Immunoblot of PFR-2 (mAb L8C4) from *L. mexicana* wild-type and null mutants. Lane 1, \( \Delta \text{LmxMPK3}^-/- \) \( \Delta 1 \) (2 \( \times \) \( 10^7 \) promastigotes); lane 2, \( \Delta \text{LmxMPK3}^-/- \) \( \Delta 2 \) (2 \( \times \) \( 10^7 \) promastigotes); and lanes 3–8, *L. mexicana* wild-type promastigotes. Lane 3, 5 \( \times \) \( 10^4 \); lane 4, 1 \( \times \) \( 10^5 \); lane 5, 5 \( \times \) \( 10^5 \); lane 6, 1 \( \times \) \( 10^6 \); lane 7, 5 \( \times \) \( 10^6 \); and lane 8, 2 \( \times \) \( 10^7 \). The molecular masses of standard proteins are indicated in kilodaltons.
verse sections of the add-backs, the axoneme seemed more condensed (Figure 7, G–I) showing a significantly reduced diameter (160 nm) compared with the wild type (180 nm), but the normal cross-hatched structure of the PFR in the longitudinal section of the flagellum (Figure 7I). Indeed, the null mutant showed the most relaxed structure with an axonomal diameter of 210 nm. As found in the null mutant for LmxMKK, we also observed vesicles and membrane fragments in the flagellar pocket of the LmxMPK3 knockout (Figure 9, A–H). The vesicles revealed single membrane layers (Figure 9, A and H), double layers (Figure 9A), or multiple layers (Figure 9, C–E and G).

L. mexicana wild type, LmxMPK3 single-allele, null mutants, and episomal add-backs were used to infect female BALB/c mice. No significant differences in the progression of lesion development could be observed between the mutant cell lines and the wild-type (our unpublished data), indicating that LmxMPK3 is neither required for the differentiation from promastigotes to amastigotes nor for the proliferation of the amastigotes.

Recombinant Expression and Kinase Assay
LmxMPK3 and its enzymatically inactive version LmxMPK3(K62M) (Carrera et al., 1993) were expressed as glutathione S-transferase fusion proteins and found as 71-kDa proteins on SDS-PAGE (Figure 10A, left, lanes 3 and 4). Both proteins were subjected to kinase assays using MBP as an artificial substrate. As expected, only the wild-type protein displayed auto- and substrate-phosphorylation (Figure 10A, right, lanes 3 and 4). Using recombinant GST-LmxMPK3 and MBP in kinase assays under varying conditions revealed that an increase in temperature from 25 to 40°C at 10 mM Mn2+, pH 6.5, led to an increase in auto- and substrate-phosphorylation. Moreover, manganese is preferred over magnesium at promastigote growth temperature of 27°C and pH 6.5. The optimal pH was found to be a pH range from 6.0 to 7.0 at 2 mM Mn2+, 10 mM Mg2+, and 27°C (our unpublished data).

Activation of LmxMPK3 by LmxMKK
Because the promastigotes of the null mutants for LmxMKK and LmxMPK3 both displayed short flagella, it was likely that the two kinases belong to the same signal transduction pathway. To test whether LmxMKK indeed phosphorylates and activates LmxMPK3, the recombinant proteins and their enzymatically inactive versions (LmxMKK(K91M) and LmxMPK3(K62M)) were used in kinase assays. We have shown previously that recombinant wild-type LmxMKK has no autophosphorylation activity (Wiese et al., 2003a). Using this protein in an assay to phosphorylate MBP also showed no incorporation of radioactive phosphate into the substrate (our unpublished data). Therefore, we used the constitutively active aspartate mutant LmxMKK(D) in which several residues, including the potential phosphorylation sites of the phosphorylation lip, were replaced by aspartate residues in the activation assay (Wiese et al., 2003a). Equal amounts of the two kinases were used with MBP as a substrate, fol-
followed by SDS-PAGE and autoradiography to visualize the incorporation of radioactive phosphate into the different reaction partners (Figure 10A). To separate LmxMKK(D) from LmxMPK3, the GST-moiety was cleaved off after purification of LmxMKK(D), leading to a mobility of the protein at around 42.5 kDa in SDS-PAGE (Figure 10A, left). The gel was exposed for 5 d to prove that LmxMKK(D) and GSTLmxMPK3 are both capable to perform auto- and substrate-phosphorylation (Figure 10A, right, lanes 1 and 3). Furthermore, the KM-versions of LmxMKK(D) and GSTLmxMPK3 showed no phosphorylation at all (Figure 10A, right, lanes 2 and 4). The constitutively active LmxMKK(D) in combination with GSTLmxMPK3 showed stronger phosphorylation of all three components in the kinase assay already after 6 h of exposure (Figure 10A, right, lanes 2 and 4). The constitutively active LmxMKK(D) in combination with GSTLmxMPK3 wild-type reveals the strongest phosphorylation of all three components in the kinase assay already after 6 h of exposure (Figure 10A, middle, lane 5). Especially, MBP phosphorylation is significantly increased. Moreover, LmxMKK(D) and GSTLmxMPK3 show stronger autophosphorylation as in the assay using the proteins on their own. GSTLmxMPK3(K62M) is phosphorylated by LmxMKK(D) (Figure 10A, middle, lane 6) whereas GSTLmxMPK3(K62M) shows no labeling when used on its own (Figure 10A, right, lane 4). MBP is phosphorylated to a higher extent as in the assay using LmxMKK(D) alone (compare Figure 10A, right, lanes 1 and 6), indicating that the interaction with LmxMPK3 increases the activity of LmxMKK. Using LmxMKK(D)(K91M) together with GSTLmxMPK3 also led to a slight increase of the incorporation of labeled phosphate into the MAP kinase and MBP compared with GSTLmxMPK3 alone, but significantly less than in the reaction using additional LmxMKK(D) (compare Figure 10A, right, lane 7 with lanes 3 and 5). Moreover, the GSTLmxMPK3, which has not been activated by LmxMKK(D), not only shows autophosphorylation and phosphorylation of MBP but also phosphorlates LmxMKK(K91M) to some extent, which might indicate feedback regulation (see below). Finally, in the reaction using the two enzymatically inactive kinases residual incorporation of radioactive label into GSTLmxMPK3(KM) could be observed in the long exposure (Figure 10A, right panel, lane 8). Having shown that increased efficiency of MBP phosphorylation depends on the phosphorylation of LmxMPK3 by LmxMKK(D), we quantified the effect caused by the activation of GSTLmxMPK3 with regard to MBP phosphorylation. To preferentially measure MBP phosphorylation by activated GSTLmxMPK3, a method essentially established as described previously was applied (Lawler et al., 1997). In an assay with unlabeled ATP to phosphorylate andactivate GSTLmxMPK3, 17-fold less molecules of LmxMKK(D) were used compared with GSTLmxMPK3. This reaction was diluted 10-fold into an assay containing [γ-32P]ATP to phosphory-
late MBP. Residual incorporation of labeled phosphate into LmxMKK(D) by autophosphorylation could be neglected due to the saturation of the nonradioactive phosphorylation in the activation reaction (our unpublished data). Moreover, the 17-fold lower amount of LmxMKK(D) compared with GSTLmxMPK3 prevents a significant contribution of the MAP kinase to the phosphorylation of MBP. An increase of MBP phosphorylation of up to 35-fold was obtained by preincubation of GSTLmxMPK3 with LmxMKK(D) compared with GSTLmxMPK3 on its own (our unpublished data). To prove the phosphorylation of LmxMPK3 by LmxMKK in vivo, we enriched phosphorylated proteins from promastigotes of the wild type, the LmxMKK null mutant, and cells expressing LmxMKK(D) integrated into the rDNA gene locus in the LmxMKK deletion background. The proteins were subjected to SDS-PAGE and immunoblot analysis using the polyclonal antiserum against the carboxy-terminal peptide of LmxMPK3 (Figure 10B). LmxMPK3 was readily detectable among the phosphorylated proteins of wild-type promastigotes (Figure 10B, lane 2). We could not detect any phosphorylated LmxMPK3 in the LmxMKK null mutant (Figure 10B, lane 1), whereas in the cells expressing the constitutively active LmxMKK(D) significant amounts of phosphorylated LmxMPK3 were present (Figure 10B, lane 3). Therefore, LmxMPK3 is in the same signal transduction cascade as LmxMKK and likely to be directly phosphorylated by LmxMKK in vivo. However, we cannot exclude the albeit unlikely possibility of additional kinases interposed between LmxMKK and LmxMPK3.

Feedback Phosphorylation

Using 1-h preincubation of GSTLmxMPK3 and GSTLmxMKK(D) as single kinases or together in the presence of ATP followed by an incubation with LmxMKK(K91M)(D), an enzymatically inactive version of the aspartate mutant of LmxMKK, led to the phosphorylation of LmxMKK(K91M)(D) by GSTLmxMPK3 (Figures 10C, lanes 5 and 7). Without activation of LmxMPK3 by LmxMKK(D), the phosphorylation of LmxMKK(K91M)(D) is significantly weaker, indicating that activation of LmxMPK3 by LmxMKK(D) is a prerequisite for full feedback phosphorylation. LmxMKK(K91M)(D) shows no autophosphorylation activity (Figure 10C, lane 1). Likewise, the small amounts of GSTLmxMKK(D) used for the preincubation do not incorporate any label in the radioactive assay (Figure 10C, lane 2). It is interesting to note that GSTLmxMPK3 still reveals high autophosphorylating activity after 1 h of preincubation in the presence of unlabeled ATP (Figure 10C, lane 3). GSTLmxMKK(D) is not able to transphosphorylate LmxMKK(K91M)(D) significantly (Figure 10C, lane 4). The interaction of GSTLmxMKK(D) with GSTLmxMPK3 is reflected by the appearance of a smear in the gel, which is phosphorylated (Figures 10C, lanes 6 and 7). Whether the feedback phosphorylation decreases or augments the activity of LmxMKK has not been investigated yet.

**DISCUSSION**

We characterized LmxMPK3, a mitogen-activated protein kinase homologue from *L. mexicana*. The protein is exclusively expressed in promastigotes, the insect stage of the parasite, as is LmxMKK, a MAP kinase kinase homologue (Wiese et al., 2003a). The stage-specific expression is also reflected by a significant decrease of steady-state mRNA for both genes in *Leishmania* amastigotes (Duncan et al., 2001; Wiese et al., 2003b). A null mutant for LmxMKK displayed short flagella and shorter promastigote cell bodies that revealed membrane fragments and vesicles in their flagellar pockets (Wiese et al., 2003a). Indeed, independently generated homoyzous deletion mutants of LmxMPK3 showed a similar phenotype, indicating that the two kinases are components of a common MAP kinase signal transduction pathway involved in the regulation of flagellar length. In wild-type amastigotes, a rudimentary flagellum that lacks the PFR is maintained in the flagellar pocket not protruding from the cell body. As expected PFR-2, a major protein component of the PFR, is not detectable by immunoblot analysis in the amastigote stage of the parasite (Santrich et al., 1997). During differentiation from amastigotes to promastigotes, the flagellum is extended and the PFR is assembled next to the axoneme but is restricted to the part of the flagellum outside the flagellar pocket. Although LmxMKK appears within 10 h after initiation of differentiation, LmxMPK3 is first detectable after 16 h. At this time, short flagella are detectable by light microscopy. In contrast, in cells differentiating from promastigotes to amastigotes LmxMPK3 almost disappears within 24 h. LmxMKK is still detectable after 72 h after initiation of differentiation despite the complete reduction of the flagellum within 24 h, indicating that LmxMKK might have additional functions beside the regulation of LmxMPK3 activity. Most importantly, we found that LmxMKK is involved in the phosphorylation of LmxMPK3 in vivo. No phosphorylated LmxMPK3 could be detected in promastigotes lacking LmxMKK, whereas promastigotes expressing the constitutively active LmxMKK showed phosphorylated LmxMPK3 like the wild type. Autophosphorylation of LmxMPK3 in vivo seems to play a minor role, if at all. Regulatory molecules such as the two kinases obviously have to be present before effector molecules such as PFR-2 and other structural components of the flagellum are expressed and assembled. Indeed, PFR-2 is first detectable after at least 25 h after promastigote differentiation initiation (our unpublished data). At this time, most of the cells already displayed short flagella resembling those of the two kinase null mutants. Interestingly, in the LmxMKK null mutant most of the cells showed no PFR-2 in immunofluorescence analysis, whereas up to 85% of the LmxMPK3 null mutant promastigotes revealed variable amounts of PFR-2 in their flagella (our unpublished data). However, as revealed by electron microscopy PFR-2 was not properly assembled into the lattice-like structure as in the wild type. This indicates that LmxMKK might be a branch point in the regulation of flagellar morphogenesis and has other substrates in addition to LmxMPK3, which are responsible for the expression of PFR-2, whereas LmxMPK3 might affect the assembly of the PFR. MAP kinase kinases exhibit high levels of substrate specificity (Chang and Karin, 2001); however, yeast Ste7 phosphorylates Kss1 and Fus3, MKK7 phosphorylates different isoforms of SAPK1/JNK1 and p38 (Fleming et al., 2000), and mitogen-activated protein kinase kinase 1 phosphorylates extracellular signal-regulated kinase (ERK)1, ERK2, and a potential Golgi-associated FRK (Acharya et al., 1998). A regulatory motif of 10 nucleotides responsible for a 10-fold down-regulation of the amount of mRNA in the amastigote has been identified in the 3′-UTR of all PFR genes in *L. mexicana* (Mishra et al., 2003). This element could not be found in the 3′-UTRs of LmxMKK and LmxMPK3. The proposed additional substrate of LmxMKK might be a MAP kinase that phosphorylates an RNA-binding protein that then stabilizes PFR mRNA, leading to the expression of PFR proteins. LmxMPK3, in contrast, might be involved in the regulation of the IFT, supporting the outgrowth of the flagellum and the proper assembly of the PFR because the null mutants never display its cross-hatched structure. Whether the activity of kinesin II, the molecular motor driving anterograde IFT, is regulated by phosphory-
lation has not been investigated yet; however, potential MAP kinase phosphorylation sites have been identified in both motor proteins forming at least part of the kinesin II complex in *Leishmania* (Wiese, unpublished data).

Using recombinant proteins, we could demonstrate that LmxMPK3 is phosphorylated by a constitutively active LmxMKK, leading to an increase in phosphorylation of the artificial substrate MBP by the activated LmxMPK3. Moreover, the activated LmxMPK3 was able to phosphorylate its activator, revealing a potential feedback mechanism that might either lead to an increase in kinase activity, inactivate the kinase, or mark it for degradation. Higher eukaryotic Cdc2 kinase and its regulating molecules are examples for different roles of phosphorylation. Negative phosphorylation by Wee1 and Myt1 during interphase inactivates Cdc2. At the onset of mitosis Cdc2 is dephosphorylated by Cdc25 phosphatase which in turn is phosphorylated by Cdc2, increasing its phosphatase activity. Active Cdc2 also phosphorylates its inhibitory kinase Wee1, generating a phospho-degron (signal for degradation). This is recognized by an E3 ubiquitin ligase, thereby targeting Wee1 for proteasome-dependent degradation. This is a typical MAP kinase and displays a substrate specificity for serine or threonine followed by a proline residue, there are two potential phosphorylation sites in LmxMKK, serine 117 and threonine 279. It has yet to be tested whether they are indeed phosphorylated to influence the activity or half-life of LmxMKK.

The LmxMKK-LmxMPK3 cascade is not restricted to kinetoplastids because a homologue for LmxMPK3 was found in the flagellated green alga *Chlamydomonas reinhardtii*. Preceding mitosis the two flagella of *Chlamydomonas* are resorbed and grow again once the cell has completed division (Cavalier-Smith, 1974). The LmxMPK3 homologue might be involved in the regulation of the outgrowth of the new flagellum in the daughter cells. Although flagellar resorption is coupled to the differentiation to amastigotes in *Leishmania*, it is likely to be coupled to cytokinesis in *Chlamydomonas*. *Leishmania* might have lost this feature because the promastigotes are attached to the wall of the gut of the sandfly by inserting their flagellum between the microvilli of the gut. Resorption of the flagellum before cytokinesis would detach the cell and bear the risk of being excreted with the feces. Nevertheless, *Leishmania* needs to have a mechanism to initiate the formation of a new flagellum next to the old flagellum before cytokinesis. This cannot be the LmxMKK-LmxMPK3 signaling cascade because amastigotes without expressing the two kinases form a new flagellum before cell division with the difference that it never extends beyond the cell surface. In promastigotes, this mechanism allows for the growth of a short flagellum, lacking an appropriately assembled PFR. A full-length flagellum can only be formed in the presence of both kinases. Because *Chlamydomonas* lacks a PFR, the regulation of PFR assembly in *Leishmania* is likely to be an additional function beside the elongation of the flagellum. Indeed, PFR null mutants display full-length flagella (Maga et al., 1999).

The possibility to activate LmxMPK3 will facilitate the identification of the substrate(s) of this kinase, which could either be a gene regulatory molecule affecting components involved in the elongation and maintenance of the flagellum or components of the IFT itself. Now, we have reached a level very close to the elucidation of the regulation of gene expression in an organism lacking RNA polymerase II promoters and the usual eukaryotic transcription factors.

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