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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 homologue, with a similar expression pattern as LmxMKK being not detectable in amastigotes, is required for growth of a full-length flagellum. We identified LmxMPK3, a MAP kinase homologue, with a similar expression pattern as LmxMKK 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) kinase pathways. Its core module is comprised of a MAP kinase kinase kinase (MKKK), a MAP kinase kinase (MKK), 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 MKKKs, 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 phosphorelay. 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...
parasites differentiate into the spherical amastigote form, which has an overall reduced cellular volume reflected by a length and width of 5–6 μm, a rudimentary flagellum not protruding from the flagellar pocket, and a different cell surface architecture (McConville and Ferguson, 1993). How the signals, a shift in temperature and in pH, are sensed and translated into differentiation is not known yet. However, it is likely that protein kinases and phosphatases play major roles because early investigations on phosphorylation patterns in different life stages of trypanosomatids revealed stage-specific changes in overall protein phosphorylation (Mukhopadhyay et al., 1988; Aboagye-Kwarteng et al., 1991; Parsons et al., 1991, 1993, 1995; Dell and Engel, 1994).

Using deletion analysis, we found that LmxMPK9 is involved in flagellar length regulation (Bengs et al., 2005). A null mutant displayed significantly elongated flagella compared with wild-type promastigotes. Flagellar length was affected in a deletion mutant for the MAP kinase gene LmxMKK (Wiese et al., 2003a). These mutants displayed flagella reduced to one-fifth of the length of the wild-type flagellum, preventing the cells from swimming free in the culture medium.

Here, we analyze LmxMPK3, which is encoded by the only MAP kinase gene investigated so far showing mRNA levels significantly down-regulated in the amastigost stage of Leishmania mexicana (Wiese et al., 2003b). We prove the kinase activity of LmxMPK3 by in vitro kinase assays of the recombinant protein expressed in Escherichia coli. Independently obtained homozygous deletion mutants of LmxMPK3 display flagella significantly reduced in length resembling those of the LmxMKK null mutant (Wiese et al., 2003a). Indeed, the constitutively active LmxMKK phosphorylates and activates LmxMPK3 in vitro and in vivo.

**MATERIALS AND METHODS**

**Parasites**

Promastigotes of L. mexicana MN7C/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 promastigotes 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 isolated from lesions of infected BALB/c mice as described previously (Benzel et al., 1998; Wiese, 1998). For scanning electron microscopy (SEM), parasitized BALB/c mice were cut (Ultra Cut E; Reichert/Leica, Nußloch, Germany) and counterstained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (Zeiss, Jena, Germany).

**Antibody Production and Immunoblotting**

A rabbit antiserum was produced against the peptide CTAGGSSKNCS-GHHH 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 of lysis buffer (1% PBS, 0.1% SDS, 50 mM diithiothreitol, 50 μM leupeptin, 25 μM Nα-p-tosyllysyl-chloromethylketone, 1 mM phenylmethylsulfonyl fluoride, 10 mM 1,10-phenanthroline, and 1× SDS sample buffer [0.4% SDS, 4% glycerol, 0.0002% triton X-100, 50 mM diethiothreitol, and 12.5 mM Tris-HCl, pH 6.8]) were boiled for 10 min. Then, 2 μl was subjected to polyacrylamide gel electrophoresis and transferred to nitrocellulose 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::PHL/O/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 oligonucleotides 5′-GCAAGGAAGGACAC-GGTACAT-3′ and 5′-CTTGATATCT-CTTCTCCGGCTG-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 amplification fragment was cut using EcoRV and ligated into plasmid pBluescriptII SK(+) (Stratagene, La Jolla, CA) previously modified to lack a BspLU11I site and linearized at EcoRV, resulting in pBE5upLmxMPK3ds. To replace LmxMPK3 by different resistance marker genes BspLU11I and NeoI sites were introduced into pBE5upLmxMPK3ds using the oligonucleotides 5′-CTGTCAGCT-GTCAGTACCTAAACAG-3′ (correcting the Scal site in the β-lactamase gene of the plasmid) and 5′-CTGCGCTAGGCACTGGCTCTCTCTCTCTCTGCTG-3′ containing an AvrII and a BspLU11I site in one PCR to amplify the 5′-flanking region. The resulting DNA-fragments carrying the resistance marker genes for the hygromycin B phosphotransferase gene HYG, the neomycin phosphotransferase gene NEO, and the phleomycin binding protein PHL/O were prepared as described previously (Benzel et al., 2003a).

The constructs were ligated into pCR2.1 and electroporated into Escherichia coli, grown to OD₆₀₀ of 0.5, and transformed into Leishmania mexicana promastigotes in two consecutive rounds as described previously (Bengs et al., 2005).

**Expression of LmxMPK3 and LmxMKK(D) in Leishmania**

For episomal expression of LmxMPK3 in L. mexicana, the open reading frame (ORF) encoding the oligonucleotides 5′-CTTCTAC-GAACTGGACAC-GAGG-3′ and 5′-CTGTCAGCT-GTCAGTACCTAAACAG-3′ containing an AvrII and a BspLU11I site in one PCR to amplify the 3′-flanking region. The resulting DNA-fragments carrying the resistance marker genes for the hygromycin B phosphotransferase gene HYG, the neomycin phosphotransferase gene NEO, and the phleomycin binding protein PHL/O were prepared as described previously (Benzel et al., 2003a).

The constructs were ligated into pCR2.1 and electroporated into Escherichia coli, grown to OD₆₀₀ of 0.5, and transformed into Leishmania mexicana promastigotes as described above. Transformants were selected in SDM-79 medium on 96-well tissue culture plates using 5 μg ml⁻¹ bleocin. A 1112-base pair EcoRV/Xbal DNA-fragment was ligated from the pGEX-KG derivative for recombinant expression of LmxMKK(D) generated previously (Wiese et al., 2003a) and used for the construction of a plasmid allowing for integration into the ribosomal DNA gene locus as has been described for LmxMKK (Wiese et al., 2003a). LmxMKK null mutant cells were transformed with 5 μg of a 5.9-kb PstI fragment purified from the final construct and recombinants were selected on SDM-79 agar plates containing 20 μM puroycin.

**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 a specific primer, annealed with the oligonucleotides TAGTATGATTT-3′ and two oligonucleotides derived from LmxMPK3, 5′-CTTCGAAGCCGTGATT3′ and 5′-CTGCACGACACTCTTG-3′, as primers in the reverse transcriptase and polymerase chain reactions.

**Electron and Light Microscopy and Flagellar Length Determination**

For scanning electron microscopy (SEM), Leishmania cells were washed twice in PBS, fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and postfixed with 1% OsO₄. Samples were dehydrated and critical point dried. Samples were stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (Zeiss, Jena, Germany) followed by chemiluminescence detection using the Supersignal system (Pierce Chemical, Rockford, IL).
Phillys 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 5-transferase (GST) fusion protein of LmxMPK3, the ORF was liberated from pCR2.1-LmxMPK3 (see above) with BamHI and HindIII, and the DNA fragment was gel-purified and ligated into the NcoI/HindIII-cleaved pGEX-KG (Guo and Dixon, 1991), resulting in pGEX-KG5aBHLmxMPK3. To cleave the GST-moiety from the protein, 350 ng of LmxMKK(D) was incubated with 10 ng of pGEX-KG5aBHLmxMPK3 cleaved with NruI and XbaI, resulting in pGEX-KG5aBHLmxMPK3 kinase activity toward MBP at 27°C in a 50-μl reaction. After centrifugation at 4°C and 12,000 g for 1 h, 5 μl of this reaction was separated on a 12% SDS-PAGE, silver-stained, dried, and exposed to X-ray film. The lysate was adjusted to 1% Triton X-100 and end-over-end rotated at 4°C overnight at 20°C.

Phosphoprotein Analysis

To determine the interaction of LmxMKK with LmxMPK3, 2 μg of LmxMKK(D) or LmxMKK(KM) was incubated at 27°C with 2 μg of GSTLmxMPK3 of 1:17:1000. After incubation for 1 h, 40 μl of the solution was separated on a 12% SDS-PAGE, silver-stained, dried, and exposed to X-ray film for quantification, unlabeled 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 μM of MBP, 70 μM MnCl2, 0.1 mM NaCl, and 0.1 M NaCl. Reactions were terminated after 1 h by the addition of 12.5 μl of 5X SDS sample buffer containing 200 mM dithiothreitol and heating for 10 min at 95°C. Then, 5 μl of the solution was separated on a 12% SDS-PAGE, silver-stained, dried, and exposed to X-ray film for quantification, unlabeled 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 GSTLmxMKK kinase activity toward MBP at 27°C in a 50-μl reaction containing 50 mM MOPS, pH 7.0, 10 mM MnCl2, and 0.1 M NaCl. Reactions were terminated after 1 h by the addition of 12.5 μl of 5X 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 for quantification, unlabeled 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 GSTLmxMKK kinase activity toward MBP at 27°C in a 50-μl reaction containing 50 mM MOPS, pH 7.0, 10 mM MnCl2, 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 onto phosphocellulose P81 paper (Whatman, Dassel, Germany) and washed four times with 75 mM H2PO4 and once with acetone. The papers were air-dried, and the incorporation of phosphate into MBP quantified by Cerenkov counting in a Beckman LS 5000PC liquid scintillation counter.

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 VIb 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 382/385; our unpublished data), Leishmania infantum (98% identical amino acids 382/385; our unpublished data), Trypanosoma brucei (70% identical amino acids 262/264), and Trypanosoma cruzi (69% identical amino acids 238/240). Less homologous, but still significant, are the proteins from Chlamydomonas reinhardtii (44% identical amino acids 157/160), from Solanum tuberosum (46% identical amino acids 157/160), and the ERK1 kinase from Dictyostelium discoideum (45% identical amino acids 157/160; 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 (I198-R203). In addition, there are the catalytic site residues K62, R76, R79, E80, R160, K163, N166, D179 (Mg2+ 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). Phospho-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 × 107 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

Figure 1. Alignment of LmxMPK3 from \textit{L. mexicana} with MAP kinase amino acid sequences from different species. LmxMPK3, \textit{L. mexicana} MAP kinase 3 (accession no. AJ293281); TbMPK3, \textit{Trypanosoma brucei} MAP kinase 3 (accession no. GeneDB Tb927.8.3550); TcMPK3, \textit{Trypanosoma cruzi} MAP kinase 3 (accession no. GeneDB Tc00.104705300553.60); CrMPK, \textit{Chlamydomonas reinhardtii} MAP kinase (accession no. A0035141); StMPK2, \textit{Solanum tuberosum} MAP kinase (accession no. A086129); and DdMPK3, \textit{Dictyostelium discoideum} MAP kinase 3 (accession no. A56042). 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 lose 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 mutant promastigotes 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 2. Immunoblot of LmxMPK3 from L. mexicana pro- and amastigote total cell lysates. Lanes 1 and 3, 2 × 10^7 promastigotes; lanes 2 and 4, 2 × 10^7 lesion-derived amastigotes. Lanes 1 and 2 were probed with antiserum against a COOH-terminal peptide of LmxMPK3; lanes 3 and 4 were probed with antiserum against recombinant myo-inositol-1-phosphate synthase. The molecular masses of standard proteins are indicated in kilodaltons.

Figure 3. Differentiation of amastigotes to promastigotes (A–C) and promastigotes to amastigotes (D–E). Immunoblots from L. mexicana total cell lysates were probed with LmxMPK3 (A and D) and LmxMKK (B and E) antisera. Hours after differentiation initiation are indicated. The molecular masses of standard proteins are given in kilodaltons. The asterisk depicts the bands corresponding to LmxMPK3; the triangle depicts the bands for LmxMKK. C and F, pictures were taken from fixed cells at the indicated time points using DIC microscopy. For better visualization of flagellar lengths, lines were drawn along the flagella. Bar, 10 μm.

Figure 4. Immunoblot of LmxMPK3 from L. mexicana wild type and various mutants. Lane 1, L. mexicana wild type; lanes 2 and 3, two independent single-allele knockout mutants; lanes 4 and 5, two null mutants derived from the different single-allele knockouts; and lanes 6 and 7, episomal complementation of the two null mutants. The molecular masses of standard proteins are indicated in kilodaltons.

Figure 5. Scanning electron micrograph of L. mexicana. Wild type (A) and LmxMPK3 null mutant (B) promastigotes at the same magnification. Bar, 10 μm.
lum, leading to a tumbling locomotion, but keeping the cells at the bottom of the culture flask. The average flagellar length as determined from 400 individual cells depicted randomly from two independent null mutants by phase-contrast microscopy was 2 μm with a size range from 0 to 6.7 μm, whereas the wild type displayed flagella with an average length of 11.8 ± 2.6 μm (Figure 6A). Indeed, the maximal flagellar length differed in the two mutants being 4.6 μm for mutant Δ1 and 6.7 μm for mutant Δ2. The null mutants were complemented by introducing the wild-type LmxMPK3 cloned into the plasmid pX63polPAC (Bengs et al., 2005) (Figure 6B). These cells generated long flagella again; however, the length varied from 1 to 18 μm (average flagellar length of 8.2 ± 3.7 μm), which is likely due to different expression levels generally observed in Leishmania harboring an episome (Benzel et al., 2000). The ultrastructure of the mutants was analyzed using transmission electron microscopy on chemically fixed cells (Figures 7 and 9). Wild-type flagella showed the typical (9 + 2) pattern of microtubule doublets in the axoneme and the typical lattice-like structure of the paraflagellar rod (PFR) adjacent to the axoneme (Figure 7A). The deletion mutant clones also revealed the typical axoneme; however, the PFR could never be visualized as clear as in the wild type. Instead, different transverse sections could be found either lacking the PFR entirely (Figure 7B), displaying remnants of the PFR (Figure 7C), or various amounts of undefined material around the axoneme (Figures 7, D–F, and 9C), sometimes also displaying vesicles of different size (Figures 7F and 9B). Again, the two null mutants differed from each other. Whereas in the mutant that revealed the longer flagella (mutant Δ2), 17% (34/200) of the transverse sections revealed a PFR at least resembling that of the wild type, no such sections could be observed for the mutant with the shorter flagella (mutant Δ1). Twenty-four percent (48/200) of the sections of mutant Δ2 and 8.5% (17/200) of the mutant Δ1 revealed a rudimentary PFR. No PFR was present in 34% (68/200) of the sections of mutant Δ2 and 29% (58/200) of mutant Δ1. However, mutant Δ1 (61/200; 30.5%) revealed twice as many flagella with material present all around the axoneme as mutant Δ2 (30/200; 15%). Finally, vesicles were present in 10% (20/200) and 32% (64/200) of the flagellar transverse sections for mutant Δ2 and mutant Δ1, respectively. In immunofluorescence analysis, 85% of the mutant promastigotes revealed a fluorescence of varying intensity, 15% showed no reaction with the anti-PFR-2 antibody (our unpublished data). The morphology of the flagella was reflected by a quantification of PFR-2, one of the major protein components of the PFR, by immunoblot analysis (Figure 8). The mutants contain on average roughly 20 times less PFR-2 than the wild type, significantly less than expected, because a mere reduction of flagellar length would only lead to 5 times less PFR-2. In the add-back, small numbers of flagellar sections showed no PFR (15/200), material all around the axoneme (2/200), or vesicles (12/200). A rudimentary PFR was found in 15% (30/200) and a relatively normal architecture of the flagellum in 70.5% (141/200) of the sections, indicating that reexpression of LmxMPK3 is able to complement the null mutant phenotype. It is interesting to note that in flagellar trans-
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 axonemal 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 reveals the strongest 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 phosphorylates 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, 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).

**Figure 10.** Phosphorylation of LmxMPK3 and LmxMKK. (A) LmxMKK activates LmxMPK3 in vitro. Recombinant kinases were subjected to kinase assays with MBP using 10 mM Mn\(^{2+}\), 50 mM MOPS, pH 7.0, at 27°C either in combination or alone as indicated and resolved by SDS-PAGE followed by autoradiography. Left, silver-stained gel. Middle, autoradiograph of the gel exposed for 6 h. Right, autoradiograph exposed for 5 d. MKK(D), constitutively active aspartate mutant of LmxMKK; MKK(KM), enzymatically inactive mutant of LmxMKK; MPK3 WT, GST-fusion protein of LmxMPK3; MPK3(KM), GST-fusion protein of enzymatically inactive mutant of LmxMPK3. (B) In vivo phosphorylation of LmxMPK3 by LmxMKK. Phosphorylated proteins were enriched from total promastigote cell lysates, subjected to SDS-PAGE, blotted, and LmxMPK3 detected using the polyclonal antiserum against a COOH-terminal peptide. Lane 1, null mutant for LmxMKK; lane 2, L. mexicana wild type; lane 3, promastigotes expressing LmxMKK(D) from the ribosomal DNA gene locus in ΔLmxMKK (Wiese et al., 2003a). (C) Feedback phosphorylation of LmxMKK by LmxMPK3. Phosphorylation of an enzymatically inactive version of the aspartate mutant of LmxMKK, designated LmxMKK(KM)(D). Recombinant kinases were subjected to kinase assays either in combination or alone and with or without preincubation with LmxMKK(D) to activate LmxMPK3 as indicated, and resolved by SDS-PAGE. Left, Coomassie-stained gel. Right, autoradiograph of the gel exposed for 6 h. The molecular masses of standard proteins are indicated in kilodaltons.
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 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 late MBP. Residual incorporation of labeled phosphate into wild type, the enriched phosphorylated proteins from promastigotes of the 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 antibody to LmxMKK(D) by autophosphorylation to the phosphorylation of MBP. An increase of the activation reaction (our unpublished data). Moreover, the small amounts of GSTLmxMKK(D) used for the kinase homologue from Leishmania donovani (Duncan et al., 2001; 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 homozygous 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 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 Gogli-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, thus preventing inactivation of Cdc2 (Watanabe et al., 2004). Provided that LmxMPK3 behaves like 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 being excreted with the feces. Nevertheless, this mechanism needs to be elucidated.

REFERENCES


