MPL1, a Novel Phosphatase with Leucine-Rich Repeats, Is Essential for Proper ERK2 Phosphorylation and Cell Motility

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The novel Dictyostelium phosphatase MPL1 contains six leucine-rich repeats at the amino-terminal end and a phosphatase domain at the carboxyl end. Similarly architected phosphatases exist among other protozoa, such as Entamoeba histolytica, Leishmania major, and Trypanosoma cruzi. MPL1 was strongly induced after 5 h of development; ablation by homologous recombination led to defective streaming and aggregation during development. In addition, cyclic AMP (cAMP)-pulsed mpl1− cells showed reduced random and directional motility. At the molecular level, mpl1− cells displayed higher prestimulus and persistent poststimulus ERK2 phosphorylation in response to cAMP stimulation. Consistent with their phenotype of persistent ERK2 phosphorylation, mpl1− cells also displayed an aberrant pattern of cAMP production, resembling that of the regA− cells. Reintroduction of a full-length MPL1 into mpl1− cells restored aggregation, ERK2 regulation, random and directional motility, and cAMP production similar to wild-type cells. We propose that MPL1 is a novel phosphatase essential for proper regulation of ERK2 phosphorylation and optimal motility during development.

Mitogen-activated protein kinases (MAPKs) are central in the regulation of proliferation, differentiation, and cell migration in diverse eukaryotic cells (10, 13, 21, 25, 26, 27). The MAP kinase ERK2 also plays critical roles during Dictyostelium development. ERK2 is essential for initiation and propagation of periodic cyclic AMP (cAMP) pulses during aggregation and differentiation. Chemoattractants, such as cAMP, induce ERK2 activation. Activated ERK2 subsequently inhibits the intracellular cAMP-specific phosphodiesterase RegA, resulting in an increase in the cytosolic cAMP level (15, 19, 22).

erk2− cells, starved for 8 h, exhibited a decrease in motility and a severe chemotaxis defect toward a CAMP gradient. Aberrancy in chemotaxis was aggravated in the presence of a strong CAMP gradient (2 μM) compared to a weak one (0.1 μM) (27). efter2− cells also display defective cytoskeletal remodeling in response to chemoattractant stimulation. A polarized wild-type cell typically displays a single dominant leading edge enriched with F-actin. Myosin II localizes to the lateral side and back of a polarized cell, where it functions to suppress lateral pseudopods and provides tractional force to the back. This single dominant leading edge disintegrates but forms again after 7 min in response to global CAMP stimulation (27). In contrast, efter2− cells, under the same condition, displayed multiple crown-like membranous protrusions, which were enriched not only in F-actin but also in myosin II (27). This aberrant structure, which was proposed to be less stable and unable to provide necessary traction force for cells to move, is believed to be the reason why efter2− cells are less motile than wild-type cells (27).

It is well-established that the dual phosphorylation of threonine176 and tyrosine178 residues of the ERK2 activation loop, often called the TEY motif, mediates activation of ERK2 kinase activity. This dual phosphorylation on the TEY motif of ERK2 peaks around 1 min after CAMP stimulation, but virtually no phosphorylation of the ERK2 remains after 2 to 3 min (14, 27). Adaptation of ERK2 is thus likely mediated by a phosphatase that can dephosphorylate phosphates from both threonine176 and tyrosine178 residues. In Dictyostelium, several tyrosine phosphatases (PTP1, PTP2, and PTP3) have been characterized (4, 6, 7, 8, 9). These phosphatases are involved in cell differentiation or stress response signaling, but it is not known if ERK2 is regulated by any of these tyrosine phosphatases. Currently, the phosphatase responsible for dephosphorylating ERK2 in Dictyostelium is unidentified.

In mammalian systems, several phosphatases are known to decrease MAPK phosphorylation and activity. These MAPK phosphatases belong to the dual specificity phosphatase (DSPase) family, which dephosphorylate both phospho-serine/threonine and phospho-tyrosine residues. Two well-characterized examples of DSPases are Cdc25 and MAP kinase phosphatase (MKP). Cdc25 dephosphorylates and activates cell cycle-dependent kinases to promote cell cycle progression. MKP dephosphorylates and inactivates MAP kinase signaling at the level of MAP kinase (2, 18, 20). A number of mammalian MKPs contain a MAP kinase binding (MKB) domain, but no such domain can be found in the Dictyostelium genome by homology domain search (NCBI, Conserved Domains Search BLAST).

We have isolated and characterized the function of MPL1, a novel Dictyostelium phosphatase with a leucine-rich repeat (LRR) domain. The MPL1 phosphatase domain contains the conserved, functionally critical signature sequence of DSPases: Dx26(V/L)x(V/I)HxAG(I/V)xRSxTx(I/V)xAY(L/I)M (x can be any amino acid). This sequence constitutes a unique structure that enables DSPase to dephosphorylate all three types of
Two other MPL1-like genes exist in the Dictyostelium discoideum genome. A BLAST search for MPL1-like genes resulted in potential DSPase with LRRs in several unicellular protozoans, such as Entamoeba histolytica, Leishmania major, and Trypanosoma cruzi. Serine/threonine protein kinase (PK) and Zn-finger motifs (Zn) are found in several of the genes listed. The combination of potential DSPase and a LRR seems to be unique to protozoans, some of which are well-known parasites that can cause serious illness. Diagrams of these potential DSPases are shown with their amino acid lengths and their gene accession numbers at the right side.

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(C) Northern blot analysis showed that MPL1 expression was low in vegetative-stage cells and highly enhanced during development. Maximum expression was observed around 10 h and declined slightly thereafter. EtBr, ethidium bromide (control).
phospho-amino acids (11, 23). MPL1 contains an LRR domain in its N-terminal region that is composed of six LRR sequences, matching the consensus sequence of LxxLxxLxxN/CxxL (x can be any amino acid, and L can be leucine, isoleucine, or phenylalanine). The LRR domain often forms a horseshoe-shaped domain capable of mediating diverse protein-protein interactions in many eukaryotes (5, 12). The combination of potential DSPases with LRR domains can be found not only in Dictyostelium but also in other parasitic unicellular eukaryotic organisms, such as Entamoeba histolytica, Leishmania major, and Trypanosoma cruzi (16) (Fig. 1A).

Ablation of MPL1 resulted in higher prestimulus and persistent poststimulus ERK2 phosphorylation upon cAMP stimulation. Furthermore, mpl1−− cells displayed strong defects in motility. Similar to regA−− cells, mpl1−− cells displayed more rapid cAMP production during the 2-min window after the stimulation with cAMP compared to wild-type cells. Considering that ERK2 is a negative regulator of RegA, persistent activation of ERK2 would have resulted in a persistent RegA inhibition in mpl1−− cells. Consistently, reintroduction of the full-length MPL1 in mpl1−− cells restored ERK2 regulation, motility, and cAMP production. We propose that MPL1 is a novel phosphatase essential for proper regulation of ERK2 phosphorylation and effective cell movement during Dictyostelium development.

MATERIALS AND METHODS

Dictyostelium culture and development. Dictyostelium cells were grown in axenic medium (7.15 g peptone 3 [Difco], 7.15 g thionate E peptone [Becton Dickinson], 7.15 g yeast extract, 15.4 g glucose, 0.525 g NaHPO₄·7H₂O, 0.48 g KH₂PO₄, in 1 liter of water). Cells were developed on nitrocellulose filters at 1 x 10⁷ cells/cm² in DBF buffer (10 mM sodium phosphate, pH 6.4, 2 mM MgCl₂, and 0.2 mM CaCl₂), or about 100 actively growing Dictyostelium cells were mixed with 200 μl of a saturated overnight culture of Klebsiella aerogenes and plated on a 100 mm SM agar plate (10 g glucose, 10 g Bacto Peptone, 1 g yeast extract, 1 g MgSO₄·7H₂O, 1.9 g KH₂PO₄, 0.6 g K₂HPO₄, 20 g agar in 1 liter of water).

Full-length MPL1 cloning. The full-length MPL1 cDNA (2.505 kb long and contains a single EcoRI site at 2406, 0.65 cistrons and a StyI site at 7174) was generated by PCR using a forward primer 5'-GCTGAATCTTTTC-3' and the reverse primer 5'-AAACTAGTGAAATGATTAATAC-3' using the forward primer 5'-GGAATTCATAGAATTCCATATAG-3' and the reverse primer 5'-GAGCGAAAGCCGAGGAGAG-3'. The PCR product was digested with HindIII and EcoRI and subcloned into the pBluescript II KS(-) (pKS(-)) vector; Stratagen) using BamHI and SpeI sites. The 3' end of the gene (fragment II, 1742 to 2505), 763 bp in length, was generated by PCR using the forward primer 5'-GGAATGCGGCCGCGCCAAGAAGATTCAG and the reverse primer 5'-GTGAATTGATTAATAC-3' using the forward primer 5'-GAATGCGGCCGCGCCAAGAAGATTCAG and the reverse primer 5'-GTGAATTGATTAATAC-3'. One ng of total RNA from either wild-type or mpl1−− cells was used for the RT-PCR template for MPL1 amplification. Ig7 transcripts were amplified using the forward primer 5'-GGGAAACTA GTGAATTGATTAATAC-3', resulting in a product that is 662 bp in length upstream of the Dictyostelium cistronic insertion point. One ng of total RNA from either wild-type or mpl1−− cells was used for RT-PCR template for Ig7 amplification.

Recombinant MPL1 phosphatase and phosphatase assay. The MPL1 phosphatase domain was initially amplified by RT-PCR with the forward primer 5'-CCAAGGAAGGAATGCACAA-3' and the reverse primer 5'-TTATTTGTGTTAATATTTTTCATTAATTG-3' and cloned into the pc2R1-TOPO vector. Positive clones were isolated after screening the whole region. GST-MPL1-PPase was generated by subcloning the MPL1 phosphatase domain into pGEX 4T-2 (Pharmacia Inc.) after EcoRI digestion and filling in with Klenow. GST-MPL1-PPase exhibited a significant increase in absorption by dephosphorylation of pNPP (0.18 ± 0.02) (results are from three independent experiments). Absorption of p-NP at 405 nm was from 1 μg of protein incubated at room temperature for 1 hour.

![Fig. 2. MPL1 encodes an active phosphatase.](image)

- **α-GST**
- **GST**
- **GST-Mpl1-PPase**

**Fig. 2.** MPL1 encodes an active phosphatase. GST and GST-MPL1-PPase were expressed and purified from E. coli. A major ~53-kDa band (GST-MPL1-PPase) and a minor degradation product (*) were detected by Western blotting using anti-GST antibody. Phosphatase activities were compared for purified proteins after normalization by Western blotting. GST alone displayed negligible background (A₄₅₀ 0.02 ± 0.03 [average ± standard deviation]), whereas GST-MPL1-PPase exhibited a significant increase in absorption by dephosphorylation of pNPP (0.18 ± 0.02) (results are from three independent experiments). Absorption of p-NP at 405 nm was from 1 μg of protein incubated at room temperature for 1 hour.

Xhol. All PCR products were confirmed by sequencing both strands after each subcloning step.

Generation of mpl1−− cells and Northern blot analysis of MPL1. The Dictyostelium resistance cassette was subcloned between the LRR and phosphatase (PPase) domain (Gly 583) of MPL1 (Fig. 1B). Transformants were prescreened by PCR (with the forward primer 5'-GACATCTGATAAATCGTGAACGC-3' and the reverse primer 5'-TTCAAGTGTGCTGATTTCTTC-3'). Genomic DNAs from the knockout candidates were isolated, and 5 μg of each sample was digested with EcoRV (see Fig. 3A, below). Knockout cells were analyzed by genomic Southern blotting. The MPL1 expression pattern was determined by Northern blot analysis using a partial MPL1 cDNA encoding the phosphatase domain. The levels of the residual partial MPL1 transcripts in mpl1−− cells were compared to that of wild-type cells by RT-PCR using the forward primer 5'-AGATTATTTTAAAATATTTTTCAAAGG-3' and the reverse primer 5'-GGGAAACTAGTGAATTGATTAATAC-3', resulting in a product that is 662 bp in length upstream of the Dictyostelium cistronic insertion point. One ng of total RNA from either wild-type or mpl1−− cells was used for the RT-PCR template for MPL1 amplification. Ig7 transcripts were amplified using the forward primer 5'-GGTGAGCGAAAGGCCAGAGG-3' and the reverse primer 5'-GACAAATGTACCCGTTCCGGCC-3' as a control. Ten ng of total RNA from either wild-type or mpl1−− cells was used for RT-PCR template for Ig7 amplification.

Chemotaxis and random motility analyses. Log-phase cells were differentiated with 50 nM cAMP pulses at 6-min intervals for 4 h. Pulsed cells were plated at 20°C, cell migration, streaming, and aggregation were monitored using a Leica inverted microscope (DM IRB).
a density of $3 \times 10^4$ cells/cm$^2$ on a 35-mm tissue culture dish cover (Falcon 353001; Becton Dickinson). A Schmazu micromanipulator with a glass capillary needle (Eppendorf Femtotip) filled with either 0.1 mM or 2 mM cAMP solution was used for a chemotaxis assay. For random motility analysis, cells were plated on the same tissue culture plate cover with no cAMP source, and their movements were recorded for 30 min. The responses of the cells were followed by time-lapse video recording with OpenLab software.

Chemotaxing cells were analyzed as described previously (17). An ellipsoid was constructed around the cell to allow the same centroid for both the ellipsoid and the contour. The chemotactic index, defined as the net distance moved to the direction of the pipette divided by the total distance moved, was computed from the centroid positions. The speeds of movement of cells were calculated from positions of the centroids. Statistical significance of differences between wild-type and mutant cells were evaluated by obtaining $P$ values using Student’s $t$ test.

**RESULTS**

**MPL1 is an active phosphatase with leucine-rich repeats.** A group of genes encoding a potential DSPase domain with LRRs can be found in the genome of *Dictyostelium* and several protozoans, such as *Entamoeba histolytica*, *Leishmania major*, and *Trypanosoma cruzi* (Fig. 1A). These hypothetical genes show a potential DSPase (PPase) and LRR domains and sometimes extra domains, such as tandem serine/threonine kinase

FIG. 3. Generation of mpl1$^{-}$ cells and phenotype characterization. (A) The blasticidin resistance cassette was subcloned between LRRs and the PPase domain. Initial screening was performed with PCR, and the selected knockout candidates were confirmed by genomic Southern blot analysis as described in Materials and Methods. Levels of MPL1 messages were compared by RT-PCR, using primers specific for the amino-terminal region before blasticidin cassette insertion. Ig7 transcripts were utilized as an internal control for RT-PCR. (B) Cells were developed on bacterial (*Klebsiella aerogenes*) lawn plates for 7 days. Wild-type cells (Wt) produced numerous fruiting bodies, whereas few multicellular structures were detected from plaques from mpl1$^{-}$ cells. Reintroduction of the full-length MPL1 restored fruiting body formation. Bars, 1 mm. (C) Wild-type and mpl1$^{-}$ cells were developed under DB buffer for 12 h at the indicated densities. mpl1$^{-}$ cells occasionally coalesced together to form loose aggregates at cell densities over $5 \times 10^6$ cells/cm$^2$, yet most of them remained as individual cells at densities where wild-type cells efficiently streamed. Bars, 100 μm.
domains and/or Zn finger domains. No other similarly structured genes were found in the currently available genomes of metazoa or plants.

We have cloned one such Dictyostelium gene, MPL1, which contains a carboxyl-terminal phosphatase domain with conserved residues essential for dual specificity phosphatase function and six potential leucine-rich repeats at the amino-terminus. The protein contains a carboxyl-terminal phosphatase domain with conserved residues essential for dual specificity phosphatase function and six potential leucine-rich repeats at the amino-terminus. The protein was determined by Northern blot analysis using a probe encoding the phosphatase domain, as shown in Fig. 1C. transcripts were analyzed by RT-PCR using a primer set up-stream of the blasticidin cassette insertion point. Compared to wild-type cells, mpl1− cells exhibited a significant decrease in the level of the partial MPL1 transcript, while Ig7 control transcript levels were comparable (Fig. 3A). Although faintly visible, the residual level of the partial MPL1 transcript did not interfere with the rescue of mpl1− phenotypes by the reintroduction of the full-length MPL1 gene into mpl1− cells (see Fig. 4 to 7 and Tables 1 to 3). The aggregation minus (agg-minus) phenotype was evident from 4 to 7 and Tables 1 to 3). The aggregation minus (agg-minus) phenotype was evident from mpl1− cells reintroduced with the full-length MPL1 gene into mpl1− cells (Fig. 3B). Wild-type cells formed a number of fruiting bodies, whereas most of the mpl1− cells failed to display visible structures on the plate. Occasionally, in less than 10% of plaques, mpl1− cells formed various heterogeneous structures, including aggregates and fruiting bodies. This agg-minus phenotype was rescued by the reintroduction of the full-length MPL1 gene (Fig. 3B).

To determine whether mpl1− cells are defective in cell migration, cells were plated to form territorial streams at various cell densities, as described for Fig. 3C. While wild-type cells displayed eminent territorial streaming, even at a density of 1.25 × 10⁴ cell/cm², mpl1− cells, in contrast, consistently failed to form territorial streams, even at a 20 times higher cell density. Ablation of the MPL1 gene was screened by PCR analysis and genomic Southern blotting using an MPL1-specific probe. To determine the level of a partial MPL1 transcript in mpl1− cells, transcripts were analyzed by RT-PCR using a primer set up-stream of the blasticidin cassette insertion point. Compared to wild-type cells, mpl1− cells exhibited a significant decrease in the level of the partial MPL1 transcript, while Ig7 control transcript levels were comparable (Fig. 3A). Although faintly visible, the residual level of the partial MPL1 transcript did not interfere with the rescue of mpl1− phenotypes by the reintroduction of the full-length MPL1 gene into mpl1− cells (see Fig. 4 to 7 and Tables 1 to 3). The aggregation minus (agg-minus) phenotype was evident from mpl1− cells developed on Klebsiella aerogenes plates for seven days (Fig. 3B). Wild-type cells formed a number of fruiting bodies, whereas most of the mpl1− cells failed to display visible structures on the plate. Occasionally, in less than 10% of plaques, mpl1− cells formed various heterogeneous structures, including aggregates and fruiting bodies. This agg-minus phenotype was rescued by the reintroduction of the full-length MPL1 gene (Fig. 3B).

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density. *mpl1−* cells occasionally formed loose aggregates, which might have been formed by nearby cells coalescing together in the absence of directional cell migration.

**mpl1− cells are defective in chemotaxis.** *mpl1−* cells were challenged with weak (0.1 μM) and strong (2 μM) gradients of cAMP for a duration of 60 min to fully examine their behavior. Cells were made responsive to the cAMP gradient by 4 h of cAMP pulses and were challenged for 1 h with a cAMP gradient formed from a micropipette filled with 0.1 μM or 2 μM cAMP. During the initial 20 min, *mpl1−* cells exhibited severely compromised gradient sensing and reduced speed (Fig. 4B and 5B; Tables 1 and 2) compared with wild-type cells (Fig. 4A and 5A and Tables 1 and 2). During the last 20 min, the chemotaxis index of *mpl1−* cells improved ~75% compared to the wild type under either condition, but the degrees of improvement in motility were less (Tables 1 and 2). Although statistically meaningful (*P* = 0.012), the difference of motility under weak and strong gradients was modest. Behaviors of *mpl1−* cells reintroduced with the full-length MPL1 were determined in an equivalent manner. Under 0.1 μM cAMP, these cells displayed a wild-type-like chemotaxis index and motility (Fig. 4C and Table 1). Behaviors of *HS174* cells (27), which lack ERK2, were monitored for comparison with other cell types described in this report. *HS174* cells displayed more severe defects in gradient sensing and directional motility than *mpl1−* cells under both gradients (Tables 1 and 2). These results were obtained from three independent experiments, and the average values are summarized in Tables 1 and 2.

To determine if MPL1 function is necessary for general motility, cells were stimulated with cAMP pulses in the chemotaxis assay, and their movement was monitored in the absence of the cAMP gradient. Wild-type cells, *mpl1−* cells, *mpl1−* cells reintroduced with the full-length MPL1, and *HS174* cells were pulsed as described earlier and were analyzed by tracing their movement for 30 min at 1-min intervals (Fig. 6; Table 3). Compared to wild-type cells, *mpl1−* cells consistently displayed compromised random motility (~60%), whereas *mpl1−* cells reintroduced with the full-length MPL1 showed no significant difference from wild-type cells (Table 3). Cells lacking ERK2 seemed to have more problems than cells suffering from aberrant ERK2 phosphorylation (Table 3; Fig. 7A to E). These results showed that *mpl1−* cells were compromised in both random and directional motility, and reintroduction of MPL1 significantly restored both defects.

**MPL1 regulates poststimulus ERK2 adaptation.** Considering that one of the major dual specific phosphatases are MKPs, we reasoned that *mpl1−* cells may experience aberrant ERK2 phosphorylation. ERK2 phosphorylation in response to cAMP stimulation was tested by Western blot analysis using an anti-phospho-ERK2-specific antibody (Cell Signaling Inc.) (1). Cells

### TABLE 1. Chemotactic indices with 0.1 μM cAMP

| Cell type and analysis period(s) (min) | cAMP concn (μM) | CI   | Speed (μm/min) |
|--------------------------------------|-----------------|------|----------------|
| Wild type                            | 0–20            | 0.1 μM | 0.79 ± 0.13    | 9.5 ± 2.6 |
|                                       | mpl1−           | 0.1 μM | 0.41 ± 0.39    | 2.6 ± 0.8* |
|                                       |                 | 21–40 | 0.55 ± 0.26    | 3.3 ± 2.0 |
|                                       |                 | 41–60 | 0.60 ± 0.33    | 4.2 ± 1.9 |
| flag–MPL1/mpl1−                      | 0–20            | 0.1 μM | 0.63 ± 0.22**  | 8.7 ± 3.3** |
|                                       | erk2−           | 0.1 μM | 0.06 ± 0.36*   | 3.1 ± 1.1* |
|                                       |                 | 21–40 | 0.06 ± 0.38    | 2.9 ± 1.3 |
|                                       |                 | 41–60 | −0.02 ± 0.33   | 2.2 ± 0.9 |

* Average values with standard deviations of chemotaxis indices (CIs) and speeds were obtained from three independent experiments. *, *P* < 0.05 compared with the wild-type control; **, *P* > 0.05 compared with the wild-type control (Student's *t* test).

### TABLE 2. Chemotactic indices with 2.0 μM cAMP

| Cell type and analysis period(s) (min) | cAMP concn (μM) | CI   | Speed (μm/min) |
|--------------------------------------|-----------------|------|----------------|
| Wild type                            | 0–20            | 2.0 μM | 0.82 ± 0.12    | 9.6 ± 2.4 |
|                                       | mpl1−           | 2.0 μM | 0.41 ± 0.34*   | 2.5 ± 1.2* |
|                                       |                 | 21–40 | 0.65 ± 0.25    | 3.3 ± 1.8 |
|                                       |                 | 41–60 | 0.58 ± 0.36    | 2.8 ± 1.3 |
| flag–MPL1/mpl1−                      | 0–20            | 2.0 μM | 0.63 ± 0.17**  | 8.2 ± 3.3** |
|                                       | erk2−           | 2.0 μM | −0.03 – 0.44*  | 2.8 ± 1.3* |
|                                       |                 | 21–40 | −0.05 ± 0.31   | 2.2 ± 1.1 |
|                                       |                 | 41–60 | −0.06 ± 0.53   | 1.7 ± 1.0 |

* Average values with standard deviations of chemotaxis indices (CIs) and speeds were obtained from three independent experiments. *, *P* < 0.001 compared with the wild-type control; **, *P* > 0.05 compared with the wild-type control (Student's *t* test).

### TABLE 3. Movement of cells in the absence of cAMP

| Cell type         | No. of cells analyzed | Speed (μm/min) |
|-------------------|-----------------------|----------------|
| Wild type         | 39                    | 9.0 ± 2.8      |
| mpl1−             | 34                    | 6.2 ± 2.2*     |
| flag–MPL1/mpl1−   | 26                    | 8.7 ± 1.5**    |
| erk2−             | 30                    | 3.3 ± 1.5*     |

* Average values with standard deviations of three independent experiments. *, *P* < 0.001 compared with the wild-type control; **, *P* > 0.05 compared with the wild-type control (Student's *t* test).
were stimulated with pulsatile cAMP for 4 h, treated with 2 mM caffeine for 30 min, and then challenged with 0.1 μM cAMP. Under these conditions, in wild-type cells ERK2 phosphorylation was virtually undetectable before cAMP stimulation and reached its maximum at 1 min after the stimulation. (B) mlp1−/− cells expressing flag-MPL1 were pulsed and stimulated with cAMP and analyzed as described in for panel A. (C) A Western blot assay using anti-flag antibody confirmed the expression of flag-MPL1, which migrated as a ~90-kDa protein as expected. (D) Levels of ERK2 phosphorylation were compared between wild-type cells, mlp1−/− cells, and mlp1−/− cells reintroduced with MPL1. (E) cAMP production was measured from cAMP-pulsed wild-type cells, mlp1−/− cells, and mlp1−/− cells reintroduced with MPL1. mlp1−/− cells overproduced cAMP during the initial 2 min of the poststimulus period, which resembled that of the regA−/− cells. In contrast, mlp1−/− cells reintroduced with MPL1 displayed essentially a wild-type-like pattern of cAMP production. *, P < 0.001 compared with the wild-type control; **, P < 0.05 compared with the wild-type control; †, P > 0.05 compared with the wild-type control (Student’s t test).

FIG. 7. MPL1 regulates ERK2 phosphorylation in Dictyostelium. (A) Both wild-type (Wt) and mlp1−/− cells (10⁶ cells) were stimulated with 50 nM cAMP pulses for 4 h, treated with 2 mM caffeine for 30 min, and further stimulated with 0.1 μM cAMP. Cells were taken at each time point and directly lysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading dye for Western blot analysis using phospho-ERK2-specific antibody and pan-Ras antibody (loading control). Wild-type cells displayed maximal ERK2 activation around 1 min, whereas phospho-ERK2 persisted over 4 min in mlp1−/− cells. Basal phosphorylation of ERK2 was also higher at time zero in mlp1−/− cells than in wild-type cells. (B) mlp1−/− cells expressing flag-MPL1 were pulsed and stimulated with cAMP and analyzed as described in for panel A. (C) A Western blot assay using anti-flag antibody confirmed the expression of flag-MPL1, which migrated as a ~90-kDa protein as expected. (D) Levels of ERK2 phosphorylation were compared between wild-type cells, mlp1−/− cells, and mlp1−/− cells reintroduced with MPL1. (E) cAMP production was measured from cAMP-pulsed wild-type cells, mlp1−/− cells, and mlp1−/− cells reintroduced with MPL1. mlp1−/− cells overproduced cAMP during the initial 2 min of the poststimulus period, which resembled that of the regA−/− cells. In contrast, mlp1−/− cells reintroduced with MPL1 displayed essentially a wild-type-like pattern of cAMP production. *, P < 0.001 compared with the wild-type control; **, P < 0.05 compared with the wild-type control; †, P > 0.05 compared with the wild-type control (Student’s t test).

DISCUSSION

Phosphatases with architectures similar to MPL1 exist in other unicellular eukaryotes. Although multiple MKPs and DSPases exist in mammals, none of the mammalian dual spec-
ficity phosphatases contains LRR domains. The MKPs interact with MAP kinases either directly through the phosphatase domain or indirectly through an MKB domain. No MKB domain was found in the Dictyostelium genome by NCBI homology domain search. There exist, however, several potential DSPases equipped with LRR domains in the genome of certain unicellular eukaryotic organisms, such as Entamoeba histolytica, Leishmania major, and Trypanosoma cruzi as described in Fig. 1A. All three of these organisms are potentially pathogenic, and thus understanding MPL1 function may shed new insight into the biology of these parasitic organisms. It will be interesting to determine if the MPL1-like genes of Entamoeba histolytica, which are highly homologous to MPL1 in domain organization, are also essential for ERK2 regulation and motility.

**MPL1 is an essential regulator of ERK2 function.** In this report, we showed that MPL1 is essential for proper ERK2 regulation. Considering that multiple phosphatases exist in vivo, it is significant to observe aberrantly high levels of phospho-ERK2 in mpl1− cells. In addition, the kinetics of cAMP production of mpl1− cells resembled that of the regA− cells, further supporting the notion that mpl1− cells have higher ERK2 activity (Fig. 7E). Furthermore, reintroduction of MPL1 in mpl1− cells restored ERK2 phosphorylation and cAMP production similar to that of the wild type (Fig. 7B and E). It can be said that MPL1 is essential for proper ERK2 regulation, although it is not clear whether MPL1 directly or indirectly dephosphorylates phospho-ERK2.

In addition to RegA regulation, ERK2 has also been implicated in polarized cytoskeletal reorganization (27). Upon receiving cAMP stimulation during the aggregation process (24), cells lose polarity by disintegrating the leading edge, become rounded up within a minute, and regain polarity by reorganizing a leading edge after 6 to 7 min. Cells lacking ERK2 fail to form the dominant F-actin-filled leading edge. Instead, a leading edge after 6 to 7 min. Cells lacking ERK2 fail to form rounded up within a minute, and regain polarity by reforming cells lose polarity by disintegrating the leading edge, become rounded up within a minute, and regain polarity by reorganizing a leading edge after 6 to 7 min. Cells lacking ERK2 fail to form the dominant F-actin-filled leading edge. Instead, a leading edge after 6 to 7 min.

**mpl1− cells were defective in cell migration.** Quantitative analysis of the chemotaxis of mpl1− cells revealed several defects. Compared to the wild type, mpl1− cells displayed severely compromised chemotaxis index during the initial 20 min, which improved significantly when allowed for an additional 40 min under both weak and strong cAMP gradients. Considering their modest phenotype and the delayed improvement in directionality of mpl1− cells, it is unlikely that MPL1 is the major determinant of gradient sensing.

In contrast, motility was more severely compromised during the first 20 min under both gradients. During the last 20 min, the motility improved modestly under both gradients. The degree of improvement was slightly better under the weak than the strong cAMP gradient. mpl1− cells may experience more problems in ERK2 adaptation under a strong cAMP gradient, but the mechanism behind these observations is currently not clear.

In any case, mpl1− cells displayed a more severe defect in motility than the directionality. Reintroduction of the full-length MPL1 in mpl1− cells significantly restored multiple defects in aggregation, chemotaxis, random motility, ERK2 regulation, and cAMP production, which underscored the significance of MPL1 in these processes.

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