Post-translational Regulation of Mitogen-activated Protein Kinase Phosphatase (MKP)-1 and MKP-2 in Macrophages Following Lipopolysaccharide Stimulation

THE ROLE OF THE C TERMINI OF THE PHOSPHATASES IN DETERMINING THEIR STABILITY*

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Background: MKPs are critical regulators of innate immunity, and yet their regulation remains poorly understood.

Results: Upon LPS stimulation, macrophage MKP-1/2 underwent robust phosphorylation on C-terminal serine residues. Mutating the serine residues to alanine decreased their stabilities while mutating them to aspartate dramatically increased their stabilities.

Conclusion: ERK plays a critical role in MKP-1/2 accumulation.

Significance: MAPKs cross-talk via MKPs to shape inflammatory responses.

MAPK phosphatases (MKPs) are critical modulators of the innate immune response, and yet the mechanisms regulating their accumulation remain poorly understood. In the present studies, we investigated the role of post-translational modification in the accumulation of MKP-1 and MKP-2 in macrophages following LPS stimulation. We found that upon LPS stimulation, MKP-1 and MKP-2 accumulated with different kinetics: MKP-1 level peaked at ~1 h, while MKP-2 levels continued to rise for at least 6 h. Accumulation of both MKP-1 and MKP-2 were attenuated by inhibition of the ERK cascade. Interestingly, p38 inhibition prior to LPS stimulation had little effect on MKP-1 and MKP-2 protein levels, but hindered their detection by an M-18 MKP-1 antibody. Studies of the epitope sequence recognized by the M-18 MKP-1 antibody revealed extensive phosphorylation of two serine residues in the C terminus of both MKP-1 and MKP-2 by the ERK pathway. Remarkably, the stability of both MKP-1 and MKP-2 was markedly decreased in macrophages in the presence of an ERK pathway inhibitor. Mutation of the two C-terminal serine residues in MKP-1 and MKP-2 to alanine decreased their half-lives, while mutating these residues to aspartate dramatically increased their half-lives. Deletion of the C terminus from MKP-1 and MKP-2 also considerably increased their stabilities. Surprisingly, enhanced stabilities of the MKP-1 and MKP-2 mutants were not associated with decreased ubiquitination. Degradation of both MKP-1 and MKP-2 was attenuated by proteasomal inhibitors. Our studies suggest that MKP-1 and MKP-2 stability is regulated by ERK-mediated phosphorylation through a degradation pathway independent of polyubiquitination.

MAPKs are critical regulators of the innate immune response (1), and play a vital role in shaping the adaptive immunity (2–5). As part of the signaling cascades initiated by Toll-like receptors (TLRs), MAPKs participate in the transcriptional induction of a variety of genes that are crucial for the host defense against infectious pathogens (1, 2). Moreover, MAPKs are pivotal modulators in both post-transcriptional regulation and the translation of a myriad of cytokine mRNAs (6–9). MAPK activity is regulated via reversible phosphorylation of a conserved Tyr-Xaa-Thr motif, where Xaa represent amino acid residues characteristic of distinct MAPK subfamilies (10). While phosphorylation of the Tyr and Thr residues of MAPKs by MAPKKs leads to activation (4), dephosphorylation of the Tyr and Thr residues by protein phosphatases leads to deactivation of the MAPK pathways (11). In mammalian cells, deactivation of MAPKs is primarily carried out by a group of dual specificity protein phosphatases often referred to as MAPK phosphatases (MKPs) (11–13). MKP-1 and MKP-2 are two prototypical members of the MKP family. Both are expressed in macrophages, and their levels of expression are elevated in response to TLR ligands (14–17). Although both MKP-1 and MKP-2 have been shown to play important roles in the regulation of the inflammatory response (13, 15–19), these two MKPs have important differences in their biochemical (20) and physiological functions (15, 21–24). MKP-1 prefers p38

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2 The abbreviations used are: TLR, Toll-like receptor; MKP, MAPK phosphatase; BMDM, bone marrow-derived macrophages; His-Ub, hexahistidine-tagged ubiquitin; qPCR, real-time PCR.
The C-terminal Domain in the Regulation of MKP-1 and MKP-2

and JNK as substrates in vivo while MKP-2 appears to favor ERK as its targets. Knock-out studies have shown that MKP-1 functions as a critical negative regulator of the inflammatory response to microbial infections, acting to restrain the host response to infection and restore homeostasis (18, 25, 26). In contrast, MKP-2 appears to potentiate inflammation (15, 27), although an inhibitory role of MKP-2 in the innate immune responses has also been reported (16).

Despite the important biochemical and biological functions in immune defense against microbial pathogens, the regulation of these MKPs is still poorly understood. Previously, it has been shown that MKP-1 stability was regulated by ERK-mediated phosphorylation via an ubiquitin-proteasome pathway (28). We have also demonstrated that MKP-1 stability is attenuated by ERK pathway inhibition (14). To understand the regulation of the inducible MKPs during innate immune response, we investigated the role of MAPK-mediated phosphorylation in the accumulation of MKP-1 and MKP-2 in macrophages following LPS stimulation. We found that both MKP-1 and MKP-2 underwent a robust phosphorylation at their C-terminal domains, which hindered the recognition of these phosphatases by a commercial MKP-1 antibody. Inhibition of the ERK pathway substantially decreased the stability of MKP-2 in LPS-stimulated macrophages. Mutating the two conserved serine residues in the C terminus of both MKP-1 and MKP-2 to alanine markedly decreased the stabilities of these phosphatases, while mutating the serine residues to aspartic acid dramatically enhanced the stabilities of both MKP-1 and MKP-2. Interestingly, deletion of the C-terminal domain also considerably increased the stability of these phosphatases. Surprisingly, we found that the stabilizing mutations of MKP-1 and MKP-2 did not decrease polyubiquitination of MKP-1 and MKP-2. Our studies highlight the critical role of phosphorylation in the accumulation of MKP-1 and MKP-2.

EXPERIMENTAL PROCEDURES

Cell Culture and LPS Stimulation—RAW264.7 and 293T cells were cultured as previously described (14, 29, 30). RAW264.7 cells and their derivatives were stimulated with 100 ng/ml LPS (Escherichia coli 055:B5; EMD Millipore, Billerica, MA) for different lengths of time, as previously described (14, 30, 31). In experiments determining the stability of MKP-1 or MKP-2 proteins, 10 μg/ml cycloheximide (EMD Millipore) was added to the culture medium, and cells were harvested after different periods of time. The MEK inhibitor U0126 (EMD Millipore), the p38 inhibitor SB203580 (EMD Millipore), and the JNK inhibitor 8 (JNK IN 8, MedChem Express, Princeton, NJ) were dissolved in DMSO and added to the medium.

Bone marrow-derived macrophages (BMDM) were generated by culturing murine bone marrow cells in DMEM (Invitrogen, Grand Island, NY) containing 10% FBS (HyClone, Logan, Utah) and 20 ng/ml M-CSF (PeproTech, Rocky Hill, NJ) for 7 days. Cells were then treated with LPS (100 ng/ml) as previously described (14, 30, 31).

Expression Vectors—The mammalian expression vectors pSRα-Flag-MKP-1 and pSRα-Flag-MKP-2 have been previously described (29, 32). The mammalian expression vectors pSRα-Myc-MKP-1 was constructed by cloning a PCR-amplified rat MKP-1 open reading frame into the Srf site of pSRα-Myc-Srf, yielding a MKP-1 protein with three tandem Myc tags at its N terminus. The constructs expressing various MKP-1 or MKP-2 mutants, including pSRα-Flag-MKP-1 S359/364A, pSRα-Flag-MKP-1 S359/364D, pSRα-Flag-MKP-2 S386/391A, and pSRα-Flag-MKP-2 S386/391D, were generated by site-directed mutagenesis, using pSRα-Flag-MKP-1 and pSRα-Flag-MKP-2 as templates. The constructs expressing MKP-1 or MKP-2 lacking their C-terminal domains (designated as pSRα-Flag-MKP-1ΔC, pSRα-Flag-MKP-2ΔC, and pSRα-Myc-MKP-1ΔC, respectively) were generated by introducing a premature stop codon in the open-reading frames through site-directed mutagenesis, using the wild type vectors as templates. The MKP-1ΔC and MKP-2ΔC mutants lack the last 53 and 58 amino acid residues, respectively. The authenticity of all constructs was verified by sequencing reactions. The mammalian expression construct producing a hexahistidine-tagged human ubiquitin (pcDNA-His6-Ub) was kindly provided by Dr. Mark Hochstrasser (33).

Transfection and Establishment of Stable Clones—RAW264.7 and JNK-stimulated wild type MKP-2 together with pcDNA3 (Invitrogen), using FuGENE®6 transfection reagent (Roche) according to the manufacturer’s specifications. Cells were selected in medium containing 250 μg/ml of G418 for 3 weeks, and resistant clones were isolated. Stable clones were maintained in complete medium containing 50 μg/ml of G418.

293T cells were transiently transfected with 5 μg of a mammalian expression vector expressing wild type MKP-1 or MKP-2, or their mutant derivatives, either alone or together with pcDNA3-His6-Ub, using polyethyleneimine as previously described (34).

Antibodies—The antibodies against MKP-1 (V-15, sc-1199; M-18, sc-1102) and MKP-2 (S-18, sc-1200) as well as the M-18 MKP-1 peptide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against total and phosphorylated ERK, JNK, and p38 were purchased from Cell Signaling Technology (Danvers, MA). The mouse monoclonal β-actin and the Flag epitope tag (M2) antibodies were purchased from Sigma-Aldrich. The mouse monoclonal antibody against the Myc epitope tag (9E10) was purchased from Roche (Indianapolis, IN).

Western Blotting and Determination of Protein Half-lives—Western blot analysis was carried out essentially as previously described (14, 35). Endogenous MKP-1 or MKP-2 was detected using a rabbit polyclonal antibody against the respective protein. Phosphorylated ERK, JNK, and p38 were detected using rabbit polyclonal antibodies against the phosphorylated proteins. Flag-tagged MKP-1 or MKP-2 proteins were detected using the mouse monoclonal M2 Flag antibody. Myc-tagged MKP-1 proteins were detected using a mouse monoclonal antibody (9E10).

To quantify the half-lives of MKP-1 or MKP-2 proteins, cells were transiently or stably transfected with a construct that expresses a Flag-tagged version of wild type or mutant MKP-1 or MKP-2. At time 0, cycloheximide (EMD Millipore) was added to the culture medium to a final concentration of 10 μg/ml, and cells were harvested after different periods of time. The decay of
the Flag-tagged MKP protein was monitored by Western blot analyses, using the M2 Flag monoclonal antibody. To assess the effects of proteasomal inhibitors, transfected 293T cells were treated with cycloheximide together with either DMSO or a proteasomal inhibitor for different times. Western blot films were scanned using a Scanmaker 5 (MicroTek) and the images analyzed using ImageQuant TL software (GE Healthcare Life Sciences, Pittsburgh, PA). The half-life of a given protein is estimated using the formula $N_t = N_0(1/2)^{t/\tau}$, where $N_t$ is the quantity that still remains after time $t$, $N_0$ is the initial quantity, and $\tau$ is the half-life.

To assess the effect of U0126 on the stability of endogenous MKP-1 and MKP-2, BMDM were stimulated with 100 ng/ml LPS for 60 min. Cycloheximide was added into the culture together with DMSO or U0126, and cells were harvested at different time-points for Western blot analyses, using the V-15 MKP-1 or S-18 MKP-2 antibody.

Dephosphorylation of Lysate Proteins—To dephosphorylate lysate proteins, including MKP-1 and MKP-2, cells were harvested in phosphate inhibitor-free lysis buffers (10 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 1 mM DTT, 2 $\mu$M leupeptin, 2 $\mu$M aprotinin, and 1 mM PMSF). The soluble lysates (45 $\mu$g protein) were then co-precipitated with 200 units of Lambda protein phosphatase (New England Biolabs, Ipswich, MA) and 5 units of calf intestine alkaline phosphatase (New England Biolabs) at 37 °C for 30 min. Western blot analysis with the dephosphorylated proteins was carried out as described (14, 35).

Analysis of Protein Ubiquitination—Ubiquitination of MKP-1 or MKP-2 proteins was assessed essentially as described by Laney and Hochstrasser (33). Briefly, 293T cells first transfected with a mixture of pcDNA3-His6-Ub (12 $\mu$g) and a control expressing Flag- or Myc-tagged MKP-1 or MKP-2 (12 $\mu$g), or either alone. After 2 days, cells were harvested in lysis buffer (6 $m$ guanidine-HCl, 100 $m$ sodium phosphate buffer-pH 8.0, 5 $m$ imidazole, 5 $m$ N-ethylmaleimide, and protease and phosphatase inhibitor cocktails). The lysates were then sonicated to shear the genomic DNA, and centrifuged at 14,000 × g for 15 min. The soluble lysates were incubated with 75 $\mu$l of Ni-NTA-agarose beads (Qiagen, Hilden, Germany) at 4 °C for 4 h to enrich proteins with a hexahistidine tag. The beads were then extensively washed, and the beads-bound proteins were eluted from the beads in a buffer containing 200 mM imidazole. These eluted proteins were further pelleted after TCA precipitation, and separated using 10% NuPAGE Bis-Tris gels. Western blot analysis was performed on the affinity-purified proteins using the Flag or Myc antibody to detect ubiquitinated MKP-1 or MKP-2 proteins.

Real-time PCR (qPCR)—Total RNA was purified and qPCR was performed essentially as described (21), with the following program: 3 min 95 °C denaturation, 40 thermal cycles composed of a 30 s denaturation step at 95 °C, 10 s annealing step at 60 °C, and 30 s extension step at 72 °C. qPCR results were normalized to the values of the housekeeping gene ribosomal protein L30 (RPL30), which was amplified using primers purchased from Bio-Rad. The following primers were used to quantify the expression levels of MKP-1 and MKP-2 in qPCR reactions: MKP-1, 5′-CTT CCA GTG CAA GAG CAT CCC-3′ and 5′-AAC TCA AAG GCC TCG TCC AG-3′; MKP-2, 5′-TTT CCT CTA CCT CGG CAG TG-3′ and 5′-ATG AAC CAG GAG CTG ATG TC-3′.

Mass Spectrometry—Separation and tandem mass spectrometry analyses of the peptides were performed on a LTQ XL orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an UltiMate™ 3000 HPLC system (Thermo Fisher Scientific). Samples were first desalted on a trap column, followed by separation on a capillary column (0.2 × 150 mm Magic C18AQ 3μ 200A, Michrom Bioresources Inc, Auburn, CA) prior to MS/MS analysis. MS/MS was acquired with a microspray source operated with a spray voltage of 2 kV at a capillary temperature of 175 °C. The resolution of full scan was set at 30,000 for the determination of accurate peptide masses. The collision-induced dissociation fragmentation energy was set to 35%. Based on the accurate masses of the fragments, a theoretical fragmentation list containing b and y fragmentation ions for a peptide was generated using Protein Prospector. Experimental fragmentation ions were then manually compared with the theoretical ones for peptide identification. For relative quantitation, selected ion chromatograph peaks were extracted from total ion chromatograph for the peptide at its $m/z$. When two or more peptides had the same $m/z$, retention time was used to determine the location of the peak of the given peptide. The peak areas were measured, and relative abundance of a given peptide was calculated as (Peak Area of a peptide)/Σ(Peak Area) × 100. Similarly, the relative abundance of a peptide can also be calculated by comparing its peak intensity measured at its $m/z$ with the total peak intensities using the formula: (Peak Intensity of a peptide)/Σ(Peak Intensity) × 100.

RESULTS

The Epitope of the M-18 MKP-1 Antibody—MKP-1 plays an important role during the inflammatory response, and yet its detection is often hindered by the lack of a specific antibody. The M-18 MKP-1 antibody is a commercially available rabbit polyclonal antibody that yielded few nonspecific bands on Western blots. We found that recognition of the antigen(s) by the M-18 antibody was influenced by cell treatment conditions (see the following sections). To understand the mechanisms involved, we made an effort to understand the epitope(s) recognized by the M-18 MKP-1 antibody. Attempts to obtain the amino acid sequence of the immunized antigen from the vendor, Santa Cruz Biotechnology, were unsuccessful, because such information was regarded as “proprietary.” We purchased the M-18 peptide from the vendor, and performed mass-spectrometry analysis. Mass-spectrometry analysis revealed 7 distinct peptides (data not shown), with PTDSALNYLKSPTTSSX and PTDSALDYLKSPTTSSX being the most abundant peptides. These peptides are likely the deamination products of a minor peptide PTNSALNYLKSPTTSSX, which corresponds to the extreme C-terminal region of murine MKP-1 protein. Since the vendor indicated that the M-18 peptide is an 18 amino acid peptide, we reasoned that the M-18 MKP-1 antibody was likely raised against PTNSALNYLKSPTTSSX at the C-terminal end of murine MKP-1. It is worth noting that the last 9 amino acid of both MKP-1 and MKP-2 are identical, and both contain a SPITTSX sequence at the C terminus. Since the C-terminal
8-amino acid sequence of the immunized peptide is shared by both MKP-1 and MKP-2; it is unsurprising that MKP-2 could also be recognized by the M-18 MKP-1 antibody. Since the two serine residues can be phosphorylated by ERK (28), the ERK pathway likely interferes with the recognition of MKP-1 and particularly MKP-2 by the M-18 antibody.

Differential Accumulation of MKP-1 and MKP-2 Proteins in Macrophages Stimulated with LPS—Macrophages were stimulated with LPS for different periods of time. Cells were harvested and Western blot analyses were performed to examine the induction kinetics of MKP-1 and MKP-2 proteins (Fig. 1A). The basal level of MKP-1 proteins was very low, virtually undetectable, as indicated by immunoblotting using a polyclonal V-15 MKP-1 antibody (top panel). LPS stimulation dramatically increased MKP-1 level. MKP-1, a 39 kDa protein, became detectable in 30 min and reached peak levels at 60 min. After 2 h, the MKP-1 level gradually declined. In contrast, MKP-2 protein, which has a predicted molecular mass of 43 kDa, was clearly detectable in unstimulated macrophages by Western blotting using an S-18 polyclonal antibody (3rd panel from the top). While MKP-2 protein level was also increased upon LPS stimulation, the accumulation took place at a slower pace. Unlike MKP-1 that reached the peak and then declined, MKP-2 protein level steadily increased with time for at least 6 h. Curiously, Western blot analyses using the M-18 antibody yielded quite a different picture (2nd panel from the top). First, two bands with a molecular mass of 39 and 43 kDa (marked by asterisks), presumably MKP-1 and MKP-2 respectively, were detected by the M-18 antibody. While the MKP-1 bands detected by the V-15 and M-18 MKP-1 antibodies appeared with somehow similar kinetics, the MKP-2 band detected using the M-18 MKP-1 antibody emerged with kinetics markedly different from that detected using the S-18 MKP-2 antibody. The presence of abundant MKP-2 in LPS-stimulated cells at 4–6 h but lack of recognition by the M-18 antibody strongly suggests that the C terminus of at least MKP-2 was heavily phosphorylated in these cells.

In light of the biochemical function of MKPs in the dephosphorylation and inactivation of MAPKs (11, 13) and the phosphorylation of MKPs by MAPKs, we examined the kinetics of ERK and p38 phosphorylation following LPS stimulation. Unstimulated RAW264.7 cells had an appreciable level of phospho-ERK. LPS stimulation resulted in a marked increase in phospho-ERK levels. The level of phospho-ERK reached its peak at between 30 and 60 min following LPS stimulation, and then gradually declined (Fig. 1A, 4th panel from the top).
contrast, basal phospho-p38 levels were virtually undetectable. In response to LPS stimulation, phospho-p38 levels dramatically increased within 15 min \((5th \text{ panel from the top})\). p38 remained phosphorylated for about 30 min and then returned to nearly basal levels by 60 min. The decline in phospho-p38 levels coincided with the increase in MKP-1 levels. Subsequently, p38 activity gradually increased, which coincided with the decline in MKP-1 levels. The changes in the levels of these proteins were not due to differences in protein loading, since \(\beta\)-actin levels in the various samples were comparable.

qPCR reactions were performed to quantitate the mRNA levels of MKP-1 and MKP-2 in RAW264.7 cells following LPS stimulation (Fig. 1B). MKP-1 mRNA levels were dramatically increased upon LPS stimulation, reaching peak levels at \(~60\) min, and then rapidly declined to close to basal levels within 3 h. In contrast, although MKP-2 mRNA levels were also enhanced by LPS, the increase in MKP-2 mRNA levels were far less profound (notice difference in the scales of \(y\) axis). The induction of MKP-2 mRNA also occurred at a slower pace with peak levels reached at \(~2\) h. Moreover, MKP-2 mRNA levels only modestly declined after 2 h. The discordance between the mRNA and protein levels of MKP-1 and in particular MKP-2 raises an intriguing question regarding the post-transcriptional regulation of these proteins.

**The Effects of MAPK Pathways on MKP-1 and MKP-2**—To understand the roles of p38 and ERK on the induction of MKP-1 and MKP-2, we examined the effects of pharmacological inhibition of these two pathways on the expression of MKP-1 and MKP-2 by Western blot analyses. Three antibodies were used to detect MKP-1 and MKP-2 proteins. With the V-15 MKP-1 antibody, we detected a \(~39\) kDa protein that was not present in unstimulated macrophages but dramatically induced in response to LPS (Fig. 2A, 2nd panel from the top). The p38 inhibitor SB203580 had little effect on MKP-1 protein levels detected by the V-15 antibody in either unstimulated or LPS-stimulated macrophages. In contrast, the ERK pathway inhibitor U0126, either used alone or in combination with SB203580, abolished MKP-1 induction. When the M-18 MKP-1 antibody was used, a different picture emerged (Fig. 2A, 1st panel). As expected, M-18 MKP-1 antibody detected two distinct bands. The lower band of \(~39\) kDa, presumably MKP-1, exhibited a similar pattern of induction and inhibition by U0126 as the band detected by the V-15 antibody. In contrast, the \(~43\) kDa band, presumably MKP-2, exhibited a very different pattern. Although the \(~43\) kDa band was also enhanced upon LPS stimulation, SB203580 pretreatment almost abolished the \(~43\) kDa band. In contrast, pretreatment of cells with U0126 had little effect on the intensity of the \(~43\) kDa band. The combination of SB203580 and U0126 abolished both the \(~39\) kDa and \(~43\) kDa bands. Since the M-18 antibody recognizes both MKP-1 and MKP-2 proteins, we carried out Western blotting using the S-18 MKP-2 antibody, which is specific for MKP-2 (Fig. 2A, 3rd panel). Interestingly, we found that SB203580 in the absence of LPS markedly increased the intensity of the \(~43\) kDa MKP-2

![Image](https://example.com/image.png)
The C-terminal Domain in the Regulation of MKP-1 and MKP-2

Both MKP-1 and MKP-2 Are Heavily Phosphorylated at their C-terminal Serine Residues in LPS-stimulated Macrophages—Since SB203580 and U0126 differentially affected MKP protein detection by M-18 MKP-1 antibody and U0126 caused a marked shift in the MKP-2 band, we postulated that MKP-1 and MKP-2 might undergo phosphorylation, which might interfere with the recognition of MKP-1 and MKP-2 by the M-18 MKP-1 antibody. To test this hypothesis, we pretreated RAW264.7 cells with either vehicle (DMSO), SB203580, U0126, or a combination of both, and then stimulated cells with LPS for 1 h. Cell lysates were prepared using a lysis buffer devoid of any phosphatase inhibitors. Since the M-18 MKP-1 antibody did not detect the 43 kDa band in the SB203580-pretreated sample (Fig. 2A), we treated this cell lysate (40 μg) with a mixture of lambda-phosphatase and calf intestinal alkaline phosphatase for 60 min at 37 °C. Subsequently, the phosphatase-treated sample was compared side-by-side with untreated cell lysates in Western blot analyses. Impressively, treatment of the lysate protein of the SB203580-pretreated, LPS-stimulated cells with phosphatases dramatically enhanced the intensities of both the 39 and 43 kDa bands (Fig. 3 comparing lane 3 with lane 4), indicating that the recognition of MKP-1 and especially MKP-2 by the M-18 MKP-1 antibody is hindered by phosphorylation. Moreover, dephosphorylation of this lysate sample also resulted in a downward shift in both MKP-1 and MKP-2 (Fig. 3A, 2nd and 3rd panels). Since the intensities of the bands detected by the V-15 MKP-1 antibody and the S-18 MKP-2 antibody did not substantially differ after phosphatase treatment (comparing lane 3 with lane 4), the recognition of these proteins by the V-15 and S-18 antibodies is not influenced by phosphorylation. The MKP-2 band in the sample pretreated with a combination of SB203580 and U0126 also ran faster than the band in cells that were pretreated with only U0126 (Fig. 3, 2nd panel), suggesting that p38 plays a role in the phosphorylation, at least, of MKP-2.

Phosphorylation of MKP-1 and MKP-2 by ERK Enhances Their Stability—We have previously shown that phosphorylation of MKP-1 through the ERK pathway enhances the stability of MKP-1 (14). Since MKP-2 also undergoes phosphorylation in LPS-stimulated macrophages, we asked the question whether MKP-2 stability was also affected by MAP kinases. We established RAW264.7 cell clones that stably express a Flag-tagged MKP-2. Cells from two distinct clones (clones 1 and 2) were first stimulated with LPS for 30 min, and then treated with vehicle (DMSO), SB203580, or U0126 for an additional 30 min. Subsequently, protein synthesis in the cells was stopped by adding cycloheximide to the culture medium. As controls, cycloheximide was also added to the cells that were not stimulated with LPS. Cells were harvested at different time points, and the levels of Flag-MKP-2 were assessed in the cell lysates by Western blot analysis using the Flag monoclonal antibody (Fig. 4A). The intensities of the bands were quantified and plotted to calculate the half-lives in the different conditions (Fig. 4A, graphic panel). In the absence of LPS, the half-life of Flag-MKP-2 was ~2.2 h. LPS stimulation dramatically stabilized Flag-MKP-2 protein, increasing the half-life of Flag-MKP-2 to ~16.5 h. Treatment of LPS-stimulated cells with SB203580 had little effect on the stability of Flag-MKP-2. In contrast, treatment of...
The C-terminal Domain in the Regulation of MKP-1 and MKP-2

LPS-stimulated cells with U0126 drastically decreased the stability of Flag-MKP-2, reducing the half-life to ~1.2 h, which is shorter than in unstimulated cells. These results clearly indicate a crucial role of ERK in the post-translational regulation of MKP-2.

To address whether the ERK pathway also enhances the stability of MKP-1 and MKP-2 in primary macrophages, we assessed the effects of the MEK1/2 inhibitor U0126 on the stability of MKP-1 and MKP-2 in LPS-stimulated BMDM (Fig. 4B). BMDM were first stimulated with LPS for 60 min, and then treated with cycloheximide or cycloheximide together with U0126. Cells were harvested at different time-points for Western blotting analyses, using the V-15 MKP-1 antibody or the S-18 MKP-2 antibody (Fig. 4B). In the presence of U0126, the decay of both MKP-1 and MKP-2 protein was markedly accelerated. The half-lives of MKP-1 and MKP-2 were estimated to be 46 and 52 min, respectively. When the ERK pathway was blocked by U0126, the half-lives of MKP-1 and MKP-2 were decreased to ~24 and 27 min, respectively.

Substitution of the Serine Residues of MKP-1 and MKP-2 at their C terminus with Alanine Decreased Stability while Mutation of These Residues to Aspartic Acid Increased Stability—To understand the role of the phosphorylation of the two serine residues at the C terminus of MKP-1 and MKP-2 in the regulation of their stability, we created Ser→Ala and Ser→Asp mutations on wild type Flag-tagged MKP-1 and MKP-2, using site-directed mutagenesis. These mutants and their parental Flag-MKP-1 or Flag-MKP-2 proteins were expressed in 293T cells transiently, and the effects of these mutations on the MKP protein stability were assessed. The steady-state level of wild type Flag-MKP-1 was higher than that of the Ser-359/364→Ala mutant (designated as S359/364A), but substantially lower than that of the Ser-359/364→Asp mutant (designated as S359/364D) (Fig. 5A, lanes 1–4). Similarly, the steady-state level of wild type Flag-MKP-2 was higher than that of the S386/391A mutant, but substantially lower than that of the S86/391D mutant (Fig. 5A, lanes 5–8). Upon treatment with cycloheximide, Flag-MKP-1 protein underwent a rapid decay (Fig. 5B). Compared with wild type MKP-1, the MKP-1 S359/364A mutant was less stable, while the MKP-1 S359/364D mutant was dramatically more stable. The half-life of wild type MKP-1 in transfected 293T cells was ~31 min (Fig. 5, C and D). Mutating the Ser-359/364 residues of MKP-1 to Ala decreased the half-life of MKP-1 to about 15 min. In contrast, mutating Ser-359/364 to Asp increased the half-life of MKP-1 to more than 9 h.

Similarly, we studied the role of the two serine residues in the regulation of MKP-2 stability. The stability of wild type MKP-2 protein was higher than that of the S391/396A mutant, but substantially lower than that of the S386/391D mutant (Fig. 5, C and D). The half-lives of MKP-2, MKP-2 S386/391A, and MKP-2 S386/391D proteins were estimated as 0.94, 0.74, and 15.8 h, respectively.

Deletion of the C Terminal of MKP-1 and MKP-2 Markedly Enhances Their Stability—To understand the function of the C-terminal domain in MKP-1 and MKP-2 in the regulation of their protein stability, we introduced premature stop codons in the open reading frame of MKP-1 and MKP-2 constructs to create C-terminal truncation mutants of their proteins. As a result, the last 53 amino acids in the C terminus of MKP-1 were truncated in the MKP-1ΔC mutant. Similarly, the last 58 amino acids in the C terminus of MKP-2 were truncated in the MKP-2ΔC mutant. The stability of the C-terminal truncation mutants were compared with the wild type and Ser→Asp mutant proteins. In transfection assays, when the same amount of plasmid constructs were used, the constructs of MKP-1ΔC and MKP-2ΔC generated higher levels of protein than did the wild type constructs (Fig. 6A). The decay of the MKP-1ΔC mutant was markedly slower than the wild type MKP-1, although the stability of the MKP-1ΔC mutant was still lower than that of the S359/364D mutant (Fig. 6, B and C). We estimated that truncation of the last 53 amino acid from MKP-1 increased its half-life 3.5-fold, from ~0.67 h to ~2.4 h (Fig. 6D). The MKP-2ΔC mutant was also dramatically more stable than the wild type MKP-2 (Fig. 6, B and C). In fact, both the MKP-
and the MKP-2 S386/391D mutant were so stable that very little decay was observed within the 6-h time-period of the study. We estimated that truncation of the last 58 amino acid from the MKP-2 protein increased its stability by 13.5-fold (Fig. 6D). These experiments clearly indicate that the C-terminal domain of both MKP-1 and MKP-2 serves as a degradation signal.

Previous studies have suggested that MKP-1 stability is regulated by the ubiquitin-proteasome degradation pathway (28). Since mutation of the two serine residues at the C terminus or truncation of the C terminus of MKP-1 and MKP-2 dramatically altered their stability, we assessed whether MKP ubiquitination was attenuated by these MKP-stabilizing mutations and enhanced by MKP-destabilizing mutations. We co-transfected the expression constructs of Flag-tagged wild type or mutant MKP-1 with a construct expressing a hexahistidine-tagged ubiquitin (His-Ub) into 293T cells. After 48 h, cells were lysed in denaturing buffer. His-Ub and proteins conjugated with His-Ub were affinity-purified from the lysates using Ni-NTA-agarose beads. Western blot analyses were performed on the purified proteins using the Flag antibody to detect Flag-tagged MKP-1 proteins (Fig. 7A, upper panel). In the samples purified from cells transfected with His-Ub and wild type, S359/364A, or S359/364D mutant form of MKP-1, a predominant band of ~53 kDa and a minor band of ~44 kDa were detected. Since the minor band was also seen in cells that were transfected with Flag-MKP-1 but not His-Ub, we concluded that the minor ~44 kDa band was residual contamination of the non-ubiquitinated Flag-MKP-1. Presumably, the 53 kDa represents the MKP-1 protein conjugated with one ubiquitin molecule, since it is ~9 kDa bigger than the non-ubiquitinated protein. Additionally, a 62 kDa band and a smear smudge were also seen in the sample purified from the cells transfected with His-Ub and MKP-1 S359/364D mutant, likely representing the MKP-1 mutant conjugated with two or more ubiquitin molecules. However, no band was detected in cells that were either not transfected or only transfected with His-Ub, indicating the specificity of the assay. In the cells co-expressing His-Ub and Flag-MKP-1 C, a major 45 kDa band (likely mono-ubiquitinated MKP-1 C) and a minor 37 kDa band (presumably non-ubiquitinated MKP-1 C) were detected. Western blot of the lysates of the transfected cells revealed a major band of 44 kDa in cells transfected with the wild type, S359/364A, or S359/364D construct (Fig. 7A, lower panel). In addition to the major band, a minor band of ~53 kDa was also seen, particularly in the cells transfected with the S359/364D mutant. A major band of 37 kDa and a minor band of 44 kDa were detected in the lysates isolated from cells transfected with His-Ub and Flag-MKP-1 ΔC.

Since the Flag tag contains arginine residues that can be potential ubiquitination sites, we also examined the ubiquitination of Myc-tagged MKP-1 and MKP-1 ΔC (Fig. 7B). Like what was seen with Flag-tagged MKP-1 proteins, ubiquitination was also seen with Myc-tagged wild type MKP-1 and C terminus-truncated MKP-1. The very similar pattern of ubiquitination of both Flag- and Myc-tagged MKP-1 proteins indicates that ubiquitination occurred on the MKP-1 moiety.
Similarly, ubiquitination was also analyzed for wild type and mutant MKP-2 proteins (Fig. 7C). Both mono- and bi-ubiquitinated MKP-2 proteins were detected in cells transfected with wild type or mutant MKP-2 constructs. Surprisingly, ladders of high molecular weight bands, presumably polyubiquitinated MKP-2 protein, were also seen in the samples expressing the
The C-terminal Domain in the Regulation of MKP-1 and MKP-2

S386/391D and the ΔC mutant, but not in the sample expressing wild type or the S386/391A mutant. Since considerably more polyubiquitination was seen with the Ser→Asp and ΔC mutants of both MKP-1 and MKP-2, we conclude that increased stabilization of these mutants was not caused by decreased protein polyubiquitination.

To examine whether the proteasomes plays a role in the degradation of MKP-1 and MKP-2, we studied the decay of these proteins in the presence or absence of a proteasomal inhibitor (Fig. 7D). The decay of both wild type MKP-1 and MKP-2 was substantially attenuated in the cells treated with 10 μM MG-132, a compound that inhibits the 20 S proteasome, relative to cells treated with the vehicle (DMSO). PS-341, at a concentration of 200 nM, also modestly slowed the degradation of both MKP-1 and MKP-2. Similarly, decay of the Ser→Ala mutants of MKP-1 and MKP-2 was also delayed in the presence of MG-132. Taken together, our results suggest that phosphorylation of MKP-1 and MKP-2 by the ERK pathway increases their stability by inhibiting the proteasome-mediated degradation through a process independent of polyubiquitination.

DISCUSSION

In this report, we showed that MKP-1 and MKP-2 proteins accumulate with very different kinetics, although the mRNAs for the two MKPs were induced with somewhat similar kinetics (Fig. 1). We demonstrated that the accumulation of both MKP-1 and MKP-2 protein following LPS stimulation is primarily controlled by the ERK pathway (Fig. 2). We further defined the epitope of an M-18 MKP-1 antibody, and provided an explanation for the recognition of MKP-2 by the M-18 MKP-1 antibody. We showed that both MKP-1 and MKP-2 are phosphorylated in LPS-stimulated macrophages in a manner largely dependent on the ERK pathway, and that phosphorylation of the C-terminal domains of MKP-1 and MKP-2 interfered with the recognition of these phosphatases by the M-18 antibody (Figs. 2 and 3). We demonstrated that the stability of MKP-1 and MKP-2 protein is positively regulated by the ERK pathway in RAW264.7 macrophages (Fig. 4). Furthermore, stabilization of MKP-1 and MKP-2 by the ERK pathway not only occurs in the immortalized macrophage RAW264.7 cells, but also takes place in macrophages derived from murine bone marrow (Fig. 4B). By site-directed mutagenesis, we unequivocally established that phosphorylation of the two serine residues in the C terminus of both MKP-1 and MKP-2 dramatically increases the stability of these proteins (Fig. 5). Finally, we demonstrated that deletion of the C-terminal domain in MKP-1 and MKP-2 markedly enhances their stability (Fig. 6). Our results suggest that phosphorylation of the C-terminal serine residues renders the degradation signal invisible by the proteasome, thus allowing for the accumulation of MKP-1 and MKP-2, and the resultant dephosphorylation of activated MAPKs. It has been well established that by feedback control of the p38 and JNK pathways MKP-1 plays an important role in the regulation of the inflammatory response (14, 21–23, 26). MKP-2 has also been shown to regulate the inflammatory response (15, 16, 27). The fact that these phosphatases are regulated by the ERK pathway raises the intriguing question of whether growth factors known to activate the ERK pathway could be used to modulate inflammatory responses.

In macrophages, following LPS stimulation MKP-1 and MKP-2 accumulated with very different kinetics (Fig. 1A, comparing the top and 3rd panel). While MKP-1 exhibited a transient increase in protein levels, MKP-2 continued to accumulate for the entire time of the experiment (6 h). Two factors likely contribute to the dramatic differences between the two highly homologous phosphatases. MKP-1 mRNA levels exhibited a remarkable increase after LPS stimulation and then rapidly declined (Fig. 1B). While MKP-2 mRNA levels displayed a modest increase, the increases took place in a delayed fashion. In addition to the difference in mRNA induction, differences in protein stability likely play a dominant role in the dramatic different kinetics of protein accumulation between MKP-1 and MKP-2. In unstimulated macrophages, the half-life of MKP-2 is ~2.2 h (Fig. 5), which is significantly longer than that of MKP-1 (about 50 min)(14). The difference in the stability between MKP-1 and MKP-2 was also seen in the transient transfection assays (0.52 versus 0.94 h). In LPS-stimulated macrophages, the differences in stability between MKP-1 and MKP-2 were even greater, respective half-lives of 3.3 h (14) and 16.5 h. The relatively high ERK activity in RAW264.7 macrophages likely accounts for the greater stability in RAW264.7 cells than in 293T cells. In fact, by 4–6 h after LPS stimulation most MKP-2 protein was phosphorylated, since MKP-2 was not recognized by the M-18 MKP-2 antibody despite the high abundance (Fig. 1A, 2nd panel). Because of the remarkable stability of MKP-2 in LPS-stimulated macrophages, degradation of MKP-2 protein should be minimal. It is likely that following LPS stimulation, the rate of MKP-2 synthesis is significantly increased and rapidly exceeds the rate of MKP-2 degradation, resulting in continued increase in MKP-2 protein level (Fig. 1, 3rd panel). In contrast, the very transient nature of MKP-1 mRNA in combination with the shorter half-life of the MKP-1 protein leads to the more transient response seen following LPS stimulation (Fig. 1, top panel).

A novel finding from this study is the role of the C-terminal domain in the stability of both MKP-1 and MKP-2. Previously, it has been shown that MKP-1 (28) and MKP-2 (38) are phosphorylated by ERK. Brondello et al. observed MKP-1 ubiquitination in their studies and proposed that phosphorylation of MKP-1 prevents MKP-1 degradation through the ubiquitin-proteasomal pathway (28). Our findings that U0126 shortens the half-lives of both MKP-1 (14) and MKP-2 in LPS-stimulated macrophages (Fig. 4) supports the notion that phosphorylation of MKP-1 and MKP-2 enhances their stability. The site-directed mutagenesis experiments indicate that phosphorylation of the two serine residues at the C terminus of MKP-1 and MKP-2 is sufficient for the stabilization of these proteins (Fig. 5).

How does phosphorylation of MKP-1 or MKP-1 at the C-terminal serine residues enhance the stability of these proteins? Our C-terminal deletion experiments provided informative cues. Deletion of the C-terminal domains from MKP-1 and MKP-2 substantially increased the half-lives of the two phosphatases (Fig. 6). A reasonable hypothesis is that the C-terminal domain functions as a part of the degradation signal for the...
recognition by the protein degradation mechanisms. Deletion of the C-terminal domain may compromise their recognition by the protein degradation pathway, thus enhancing their stabilities. Phosphorylation of the two serine residues by ERK or mutation of the two residues to aspartic acid may cause a conformational change, rendering this signal “invisible” to the protein degradation pathway. Since the C-terminal ends of both MKP-1 and MKP-2 share a conserved SPITTSPSC sequence, we speculate that this sequence might be part of the degradation signal.

While our observations support the notion that phosphorylation of MKP-1 and MKP-2 enhances their stability, our results do not support the idea that phosphorylation of these proteins prevents their degradation by the ubiquitin-proteasomal pathway (28). If the ubiquitin-proteasomal pathway were involved, then one would predict that the less stable MKP forms, such as wild type MKP-1, MKP-2, and their Ser→Ala mutants, should more readily become polyubiquitinated, while more stable MKP forms, such as the Ser→Asp and ΔC mutants, should be relatively resistant to polyubiquitination. However, we observed the exact opposite in the transient transfection assays (Fig. 7). In fact, there were far more polyubiquitinated Ser→Asp and ΔC mutant proteins than polyubiquitinated wild type and Ser→Ala mutant proteins in the cell lysates (Fig. 7). Moreover, we found that a small fraction of both MKP-1 and MKP-2 were mono-ubiquitinated or bi-ubiquitinated (Fig. 7). The significance of these types of ubiquitination of MKP-1 and MKP-2 remains to be determined. Since the degradation of both MKP-1 and MKP-2 was attenuated by MG-132 and PS-341 (Fig. 7D), two 20 S proteasomal inhibitors, it is likely that MKP-1 and MKP-2 are degraded by the 20 S proteasomes through a polyubiquitination-independent process. A common feature of the proteins that undergo degradation independent of ubiquitin is that they belong to the intrinsically disordered protein family (37, 38). It has been proposed that the ability of 20 S proteasomes to degrade an intrinsically disordered protein is proportional to the extent of the disorder of the protein (39). Thus, it is plausible that phosphorylation of the two serine residues at the C terminus of MKP-1 or MKP-2 decreases the extent of the disorder, thus inhibiting the degradation by the 20 S proteasomes.

We have previously shown that the ERK pathway can inhibit the p38 pathway via up-regulating the expression of MKP-1 (14). Here we found that p38 can also inhibit the ERK pathway, albeit through a different mechanism. Since SB203580 pretreatment enhanced ERK activity with an associated increase in phospho-MEK (Fig. 2A, 4th and 5th panel), it is likely that the p38 pathway inhibits the upstream signaling process that drives the ERK cascade. This is consistent with a report that p38 can serve as a negative feedback control mechanism limiting TLR signaling (40, 41). Further support for the inhibitory role of p38 on the ERK pathway comes from the observation that SB203580 treatment prior to LPS stimulation hinders the recognition of MKP-2 by the M-18 MKP-1 antibody (Fig. 2A, top panel, comparing lane 5 to lane 6). The M-18 MKP-1 antibody was probably generated by immunizing rabbits with a non-phosphorylated peptide corresponding to the C terminus of MKP-1, which is partially conserved in MKP-2. Phosphorylation of MKP-2 at the two serine residues alters the structure of the C terminus of MKP-2, thus rendering the phosphorylated MKP-2 unrecognizable by this antibody. Dephosphorylation of MKP-2, and to a lesser extent MKP-1, returns these phosphatases to a structure recognizable by the M-18 antibody, thus improving the detection in Western blot analyses (Fig. 3). The fact that SB203580 pretreatment in LPS-stimulated cells makes MKP-2 unrecognizable by the M-18 MKP-1 antibody (Figs. 2 and 3) strongly supports the idea that p38 antagonizes the ERK pathway during the innate immune response.

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The C-terminal Domain in the Regulation of MKP-1 and MKP-2

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