

Identification of MEKK2/3 serine phosphorylation site targeted by the Toll-like receptor and stress pathways

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Members of the mitogen-activated protein kinase kinase (MAP3K) family are crucial for the Toll-like receptor (TLR) signaling and cellular stress responses. However, the molecular mechanisms underlying the TLR- and cellular stress-mediated MAP3K activation remain largely unknown. In this study, we identified a key regulatory phosphorylation site, serine 519 and serine 526, in MAP3K MEKK2 and MEKK3, respectively. Mutation of this serine to an alanine severely impaired MEKK2/3 activation. We generated an anti-p-MEKK2/3 antibody and used this antibody to demonstrate that lipopolysaccharide induced MEKK2 and MEKK3 phosphorylation on their regulatory serine. We found that the serine phosphorylation was crucial for TLR-induced interleukin 6 production and this process is regulated by TRAF6, a key adaptor molecule for the TLR pathway. We further demonstrated that many, but not all, MAPK agonists induced the regulatory serine phosphorylation, suggesting an involvement of different MAP3Ks in activation of the MAPK cascades leading to different cellular responses. In conclusion, this study reveals a novel molecular mechanism for MEKK2/3 activation by the TLR and cellular stress pathways.

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Introduction

The mitogen-activated protein kinase (MAPK) cascades, which include the extracellular signal-regulated kinase (ERK)1/2, the c-Jun N-terminal kinase (JNK), the big MAPK/ERK5, and the p38 MAPK cascades, are intracellular

signaling networks utilized by mammalian cells to respond to a wide spectrum of extracellular stimuli (Seeger and Krebs, 1995; Cobb, 1999). MAPKs are activated through a module that includes an MAPK, an MAPK kinase (MAPKK), and an MAPKK kinase (MAP3K). Located upstream in the MAPK module, an MAP3K phosphorylates and activates its downstream MAPKKs, which in turn phosphorylate and activate the downstream MAPKs. A growing number of MAP3Ks including MEKK1, MEKK2, MEKK3, MEKK4, apoptosis signal-regulating kinase 1 (ASK1), transforming growth factor- β -activated kinase 1 (TAK1), dual leucine-zipper kinase, and tumor progression locus 2 (Tpl2) have been identified that are capable of activating multiple MAPKs (Lange-Carter *et al*, 1993; Yamaguchi *et al*, 1995; Blank *et al*, 1996; Tibbles *et al*, 1996; Gerwins *et al*, 1997; Ichijo *et al*, 1997; Dumitru *et al*, 2000). Biochemical and genetic studies have demonstrated that these MAP3Ks are crucial in relaying distinct cell-surface signals through various downstream MAPK pathways to their cytoplasmic and nuclear effectors. Studies using gene-targeting mice have also revealed that MAP3Ks have both overlapping and distinctive functions. For instance, many MAP3Ks also activate the I κ B kinase (IKK)-nuclear factor κ B (NF- κ B) pathway (Karin and Ben-Neriah, 2000; Yang *et al*, 2001). Despite extensive efforts in the past, precisely how each MAP3K is activated and which upstream molecule is involved in individual MAP3K activation remains unclear.

Toll-like receptors (TLRs) are pattern recognition receptors that are essential for mounting a host defense against infectious pathogens (Takeda *et al*, 2003). The TLR signals are mediated by a conserved Toll/interleukin (IL)-1 receptor (TIR) domain that recruits and assembles intracellular signaling molecules, including MyD88, IL-1R-associated protein kinase, TIR domain-containing adaptor protein/MyD88-adaptor-like, and tumor necrosis factor receptor-associated factor-6 (TRAF6), into complexes (Barton and Medzhitov, 2003; Takeda *et al*, 2003). Members of the MAP3K family including TAK1 (Wang *et al*, 2001), MEKK1 (Lee *et al*, 1997; Kopp *et al*, 1999), NF- κ B-inducing kinase (Malinin *et al*, 1997), Tpl2 (Dumitru *et al*, 2000), MEKK3 (Huang *et al*, 2004), and ASK1 (Matsuzawa *et al*, 2005) have been implicated in mediating the downstream TLR signals that activate the IKK-NF- κ B and MAPK pathways.

MEKK2 and MEKK3 are two closely related MAP3Ks belonging to the MEKK/STE11 subfamily that are widely expressed and are potent activators of the NF- κ B and MAPK pathways (Blank *et al*, 1996; Ellinger-Ziegelbauer *et al*, 1997; Su *et al*, 2001). Extensive *in vitro* studies in the past have shown that they activate the JNK1/2, ERK1/2, p38, and ERK5 MAPKs (Blank *et al*, 1996; Deacon and Blank, 1997; Schaefer *et al*, 1999; Cheng *et al*, 2000; Sun *et al*, 2003). The *in vivo* function of MEKK2 and MEKK3 has also been studied in different physiologic systems, including MEKK2 and MEKK3 knockout mice and cells. These studies have

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shown that MEKK2 regulates T-cell function (Schaefer *et al*, 1999; Su *et al*, 2001; Guo *et al*, 2002), controls cytokine gene expression in mast cells (Sun *et al*, 2003; Kesavan *et al*, 2004), mediates epidermal growth factor (EGF) receptor and fibroblast growth factor-2 (FGF-2) receptor signals (Sun *et al*, 2003; Kesavan *et al*, 2004), and plays a role in rheumatoid arthritis (Hammaker *et al*, 2004). In contrast, MEKK3 is essential for early mouse embryonic development, tumor necrosis factor α (TNF α)-induced IKK-NF- κ B and JNK-p38 activation, and IL-1R-TLR4-induced IL-6 production via the IKK-NF- κ B and JNK-p38 MAPK pathways (Yang *et al*, 2001; Huang *et al*, 2004).

To elucidate the molecular mechanisms of MEKK2 and MEKK3 activation and regulation in response to TLR stimulation and to cellular stresses, we identified a key regulatory serine residue in the activation loop of MEKK2 and MEKK3 and found that this serine phosphorylation in the activation loop of MEKK2 and MEKK3 may serve as a major sensor of the TLR stimulation and cellular stresses.

Results

Identification of the phospho-amino acids in MEKK2 by Maldi-MS

We and others have shown previously that expression of either the full-length MEKK2 or MEKK3, or their respective catalytic domains such as MEKK2CT (an active form of

MEKK2), by transient transfection in cell lines led to their self-activation (Cheng *et al*, 2005). These active MEKK2 and MEKK3 proteins were phosphorylated showing a retarded mobility on an SDS-PAGE gel. In contrast, kinase-inactive MEKK2 or MEKK3 mutants, such as MEKK2CT(KM), were not phosphorylated. Furthermore, dephosphorylation of the activated MEKK2 led to the loss of its catalytic activity, strongly suggesting that phosphorylation plays a critical role in regulating MEKK2 activity.

To identify the potential regulatory phosphorylation residue in MEKK2 and MEKK3, we expressed MEKK2CT and MEKK2CT(KM) in 293T cells and purified them for Maldi-MS analysis (Figure 1A). This experiment revealed one phosphopeptide, 519 SVTGTPTYWMSPEVISGQGYGR 539 , in MEKK2CT but not in MEKK2CT(KM) (Figure 1B). Treatment of MEKK2CT with a protein phosphatase completely abolished this phosphopeptide (Figure 1B). This phosphopeptide was then subjected to further amino-acid sequencing, which revealed that the phospho-residue was Ser 519 in the activation loop of MEKK2 (Figure 1C).

Mutation of Ser 519 but not Thr 521 and 523 into Ala blocked MEKK2CT activation and MEKK2-dependent signaling

To confirm that the Ser 519 phosphorylation is important for MEKK2 activation and signaling, we mutated this residue into an alanine. As shown in Figure 2A, the ability of the

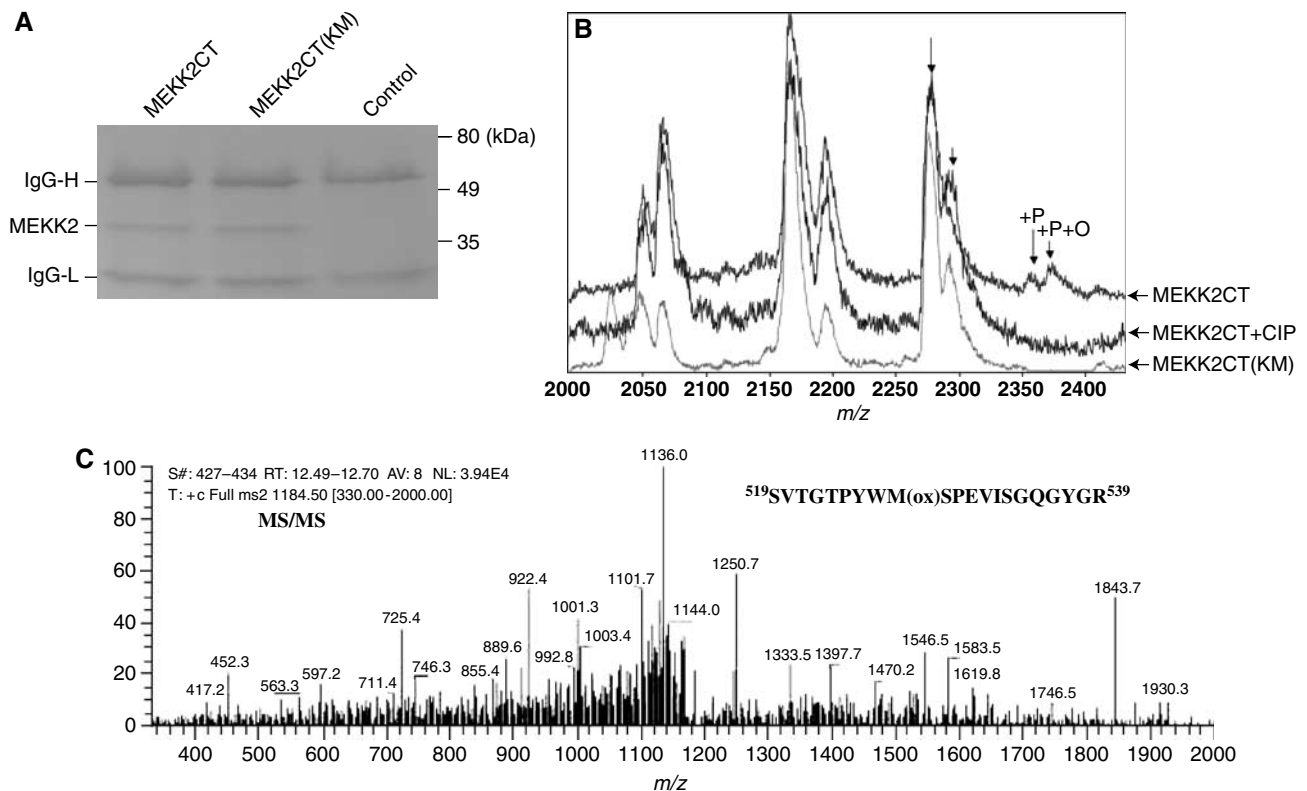


Figure 1 Maldi-MS identification of MEKK2 active phosphorylation site. (A) Purification of the active and inactive MEKK2 catalytic domains transiently expressed in 293T cells. 10 μ g of HA-MEKK2CT or HA-MEKK2CT(KM) expression vectors was transfected into 293T cells. Cell lysates were prepared 36 h later for immunoprecipitation with an anti-HA antibody, and the recombinant proteins were separated by an SDS-PAGE gel and stained with Coomassie blue. (B, C) Maldi-MS identification of MEKK2 Ser 519 phosphorylation. MEKK2CT and CT(KM) proteins shown in panel A were excised from the SDS-PAGE gel for Maldi-MS analysis (B, C). Arrows in panel B indicate the phosphorylated peptide that was further sequenced by MS/MS as shown in panel C.

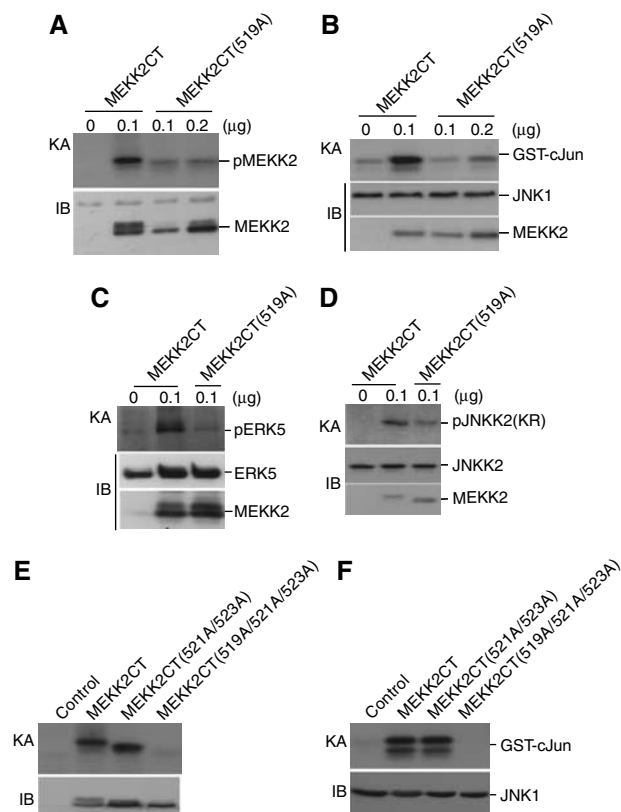


Figure 2 MEKK2 Ser 519 phosphorylation is required for its optimal activation. (A) Mutation of MEKK2 Ser 519 to an alanine impaired its self-phosphorylation. 293T cells were transfected with 0.1 μ g of empty vector or HA-MEKK2CT, or 0.1 or 0.2 μ g HA-MEKK2CT(519A) as indicated. The transfected MEKK2CT proteins were immunoprecipitated 36 h later with an anti-HA antibody for an *in vitro* kinase assay. The expression levels of MEKK2CT and MEKK2CT(519A) were determined by immunoblotting (IB) (bottom panel). (B, C) MEKK2 Ser 519 phosphorylation is required for MEKK2-mediated JNK (B) and ERK5 (C) activation. Flag-JNK1 or Flag-ERK5 expression vector (0.2 μ g) was co-transfected with empty vector, HA-MEKK2CT, or HA-MEKK2CT(519A) into 293 cells. Flag-JNK1 or Flag-ERK5 was immunoprecipitated with an anti-Flag antibody 36 h later for an *in vitro* kinase assay. The expression levels of JNK1, ERK5, and MEKK2 were determined by immunoblotting (IB) (bottom panel). (D) MEKK2 Ser 519 phosphorylation is required for MEKK2-mediated JNKK2 activation. Cell lysates prepared from the COS-1 cells transfected with 0.1 μ g of empty vector, HA-MEKK2CT, or HA-MEKK2CT(519A) were mixed with equal amounts of HA-JNKK2(KR) substrate expressed in COS-1 cells before being subjected to immunoprecipitation with an anti-HA antibody for an *in vitro* kinase assay (top panel). The expression levels of JNKK2(KR), MEKK2CT, and CT (519A) were determined by immunoblotting (IB) (middle and bottom panels). (E, F) MEKK2 Ser 519 but not Thr 521 or Thr 523 is required for MEKK2CT activity. 0.1 μ g of empty vector, or expression vectors for HA-MEKK2CT, HA-MEKK2CT (519A), HA-MEKK2CT (521A/523A), or HAMEKK2CT (519A/521A/523A) were transfected into 293T cells alone (E) or with Flag-JNK1 (0.2 μ g) (F) as indicated. Cell lysates were prepared 36 h later for immunoprecipitation with an anti-HA antibody (E) or an anti-Flag antibody (F) for an *in vitro* kinase assay. The expression levels of MEKK2CT and JNK1 were determined by immunoblotting, as indicated.

Ser 519-to-Ala mutant, MEKK2CT(519A), to self-activate was significantly reduced compared with MEKK2CT, as determined by its autophosphorylation. Consistently, the ability of this mutant to activate the downstream JNK1 and ERK5 MAPKs, two major MAPKs regulated by MEKK2, was significantly reduced (Figure 2B and C). To further show that the

decreased MAPK activation was due to the impaired MEKK2 activation, we measured the ability of MEKK2CT and MEKK2CT(519A) to phosphorylate JNKK2(KR), a preferred substrate for MEKK2 (Cheng *et al*, 2000). Consistently, MEKK2CT(519A) showed significantly decreased activity in phosphorylating JNKK2(KR) (Figure 2D).

Although the Ser 519-to-Ala mutation caused a significant reduction in MEKK2CT activity, this mutant did retain its basal activity and appeared to be phosphorylated, as shown by its mobility retardation (Figure 2A). To determine whether this residual activity was due to a compensatory phosphorylation of the adjacent residues, we also purified this mutant protein for Maldi-MS analysis. We found that the adjacent Thr 521 and Thr 523 on this mutant protein became phosphorylated (data not shown), confirming that the retarded mobility of MEKK2CT(519A) was due to the phosphorylation on these two Thr residues. Interestingly, when these two residues were mutated to an Ala, but the Ser 519 residue remained unchanged, the resultant mutant MEKK2CT(521A/523A) was still active (Figure 2E and F). However, when all three residues, Ser 519, Thr 521, and Thr 523, were mutated to an Ala, the activity of this mutant was almost completely lost, comparable to that of the kinase-inactive mutant MEKK2CT(KM) (Figure 2E and F; data not shown). This suggests that the triple mutation is more severe than Ser 519 mutation in the catalytic domain. However, this may be due to the mutation of the conserved Thr 523 that is normally unphosphorylated (see the following sections). Together, these results confirmed that the MEKK2 Ser 519 is a key regulatory phosphorylation site required for MEKK2 activation.

The Ser 519 and Thr 523 are required for MEKK2 activation

To further investigate the roles of Ser 519, Thr 521, and Thr 523 in full-length MEKK2 activation, we constructed full-length MEKK2 proteins with mutations of these residues and determined the effect of these mutations on MEKK2 self-activation and its ability to activate the downstream targets using the same assays described above. Consistent with our findings for MEKK2CT, we found that the Ser 519-to-Ala mutation also severely impaired activation of the full-length MEKK2 (Figure 3A and B). Interestingly, although mutations of the Thr 521 and Thr 523 into an Ala appeared to have only marginal effect on the activity of MEKK2CT (Figure 2E and F), the mutant MEKK2(521A/523A), like MEKK2(519A) and MEKK2(519A/521A/523A), was also severely defective in JNK1 activation and in inducing JNKK2 phosphorylation (Figure 3A and B). The remaining activity of the MEKK2CT(521A/523A) mutant may be due to its overexpression, because when titrating its expressing level, we found that its autophosphorylation was less than that of its wild-type counterpart MEKK2CT (data not shown). It is also possible that only the MEKK2CT but not the full-length protein was able to have the compensatory phosphorylation on Thr 521 and Thr 523, as we could not detect their phosphorylation by either *in vivo* metabolic labeling experiment or by Maldi-MS analysis (data not shown). Since under normal conditions, neither Thr 521 nor Thr 523 is phosphorylated, the defect in MEKK2(521A/523A) is most likely due to the mutation of these residues rather than their lack of phosphorylation (see results below).

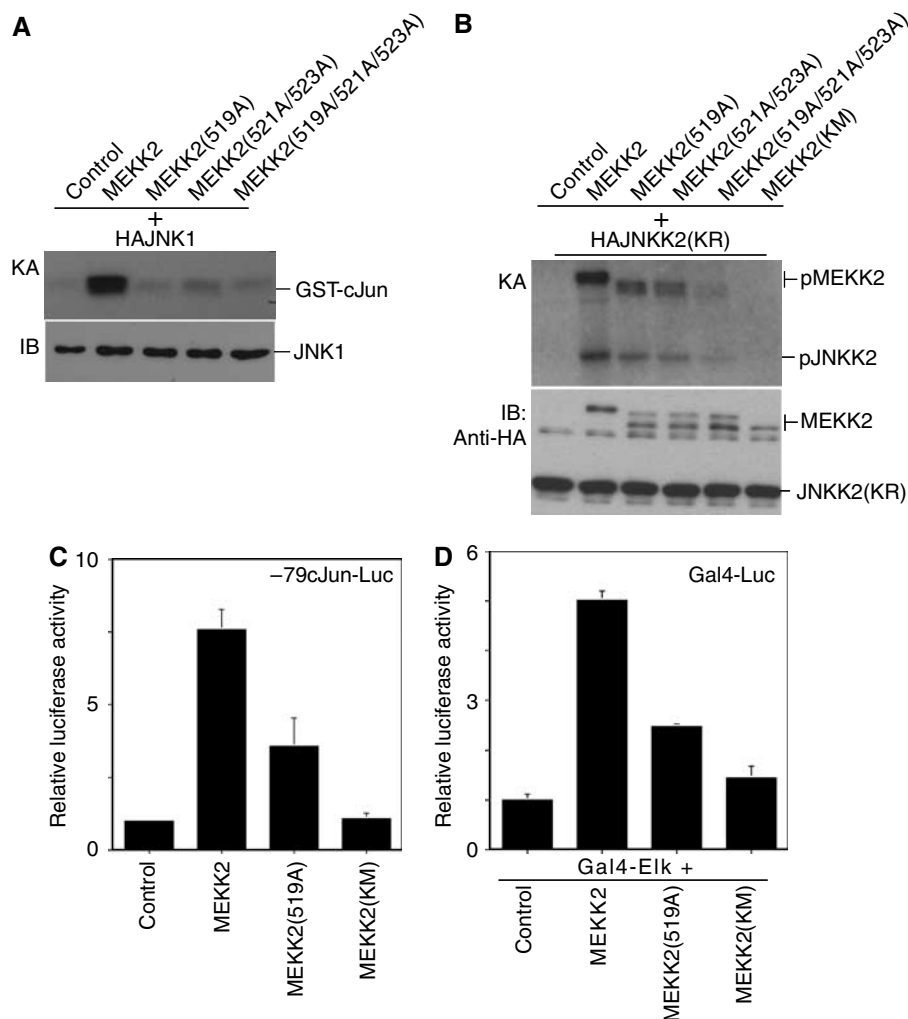


Figure 3 MEKK2 Ser 519 and Thr 521 or Thr 523 are required for the full-length MEKK2 activation. (A) HA-JNK1 (0.2 μ g) was transfected into COS-1 cells with 0.1 μ g of empty vector, or expression vectors for HA-MEKK2 and its mutants as indicated. Cell lysates were prepared 36 h later for immunoprecipitation with an anti-Flag antibody for an *in vitro* kinase assay. JNK1 expression was determined by immunoblotting (IB) (bottom panel). (B) Cell lysates prepared from the COS-1 cells transfected with 0.1 μ g of empty vector, or expression vectors for HA-MEKK2 and its mutants were mixed with equal amounts of HA-JNKK2(KR) substrate expressed in COS-1 cells before being subjected to immunoprecipitation with an anti-HA antibody for an *in vitro* kinase assay (top panel). The expression levels of JNKK2(KR) and MEKK2 were determined by immunoblotting (IB) (bottom panels). (C) MEKK2 Ser 519 phosphorylation is required for MEKK2-dependent reporter gene expression. The AP-1 reporter plasmid -79Jun-Luc (2 μ g/well) was co-transfected with (0.8 μ g) empty vector or HA-MEKK2, HA-MEKK2(519A), and HA-MEKK2(KM) expression vectors into a macrophage line Raw264.7 as indicated. An actin-*Renilla* luciferase reporter (1 ng) was included in all transfection as a control for transfection efficiency. The reporter luciferase activity was determined 36 h later and normalized to the *Renilla* luciferase activity. The results shown are the average of three independent experiments. (D) MEKK2 Ser 519 is required for MEKK2-dependent ELK1 reporter activation. The Gal4-Luc reporter plasmid (2 μ g/well) was co-transfected into Raw264.7 cells with empty vector, or expression vectors (0.8 μ g) for Gal4-ELK1 in the presence of MEKK2, MEKK2(519A), or MEKK2(KM) as indicated. Relative reporter activity was determined as described in panel C.

To determine whether MEKK2 Ser 519 phosphorylation is important for the MEKK2-dependent signaling pathway, we next examined whether MEKK2(519A) is able to activate MEKK2-dependent reporter gene expression. As shown in Figure 3C and D, the ability of MEKK2(519A) to activate the AP-1 reporter and ELK1 reporter gene expression was reduced 60% from that of MEKK2. Interestingly, compared with the kinase-inactive mutant MEKK2(KM), which was almost completely unable to activate the reporter genes, MEKK2(519A) was still able to partially activate reporter gene expression. This result was consistent with our finding that MEKK2CT(519A) and MEKK2(519A) were more active than MEKK2(KM), albeit at a significantly lower level than that of the wild-type MEKK2 (Figures 2 and 3). This result further

supports our conclusion that the Ser 519 is a key regulatory phosphorylation site rather than a structural or catalytic determinant for MEKK2 enzymatic activity.

Generation of an antibody against phospho-Ser 519 in MEKK2

To further characterize the role of Ser 519 phosphorylation in MEKK2 activation, we raised a rabbit polyclonal antibody that specifically detects phosphorylated, but not unphosphorylated, Ser 519 on MEKK2. Because of the high homology between MEKK2 and MEKK3 at the regions that surround this serine residue, our antibody also recognizes phosphorylated, but not unphosphorylated, Ser 526 on MEKK3 (see below for more detailed analysis); thus, we named this

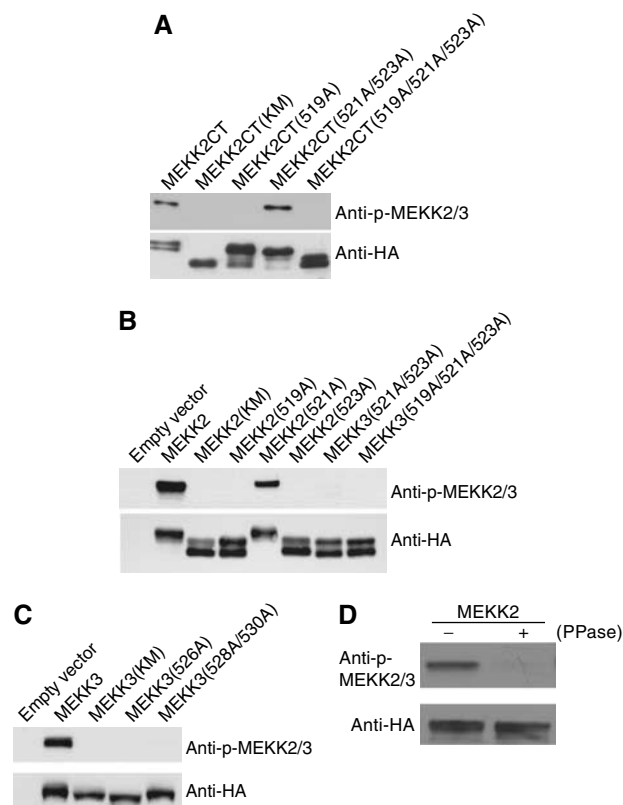


Figure 4 Generation and characterization of MEKK2 Ser 519-specific antibody anti-p-MEKK2/3. (A, C) The anti-p-MEKK2/3 antibody is specific for MEKK2 phospho-Ser 519 and MEKK3 phospho-Ser 526. Cell extracts were prepared from COS-1 cells transfected with expression vectors for the MEKK2 catalytic domain and its derived mutants (A) or for full-length MEKK2 and its derived mutants (B), or MEKK3 and its mutants (C) as indicated, and analyzed by immunoblotting with the anti-p-MEKK2/3 antibody (top panel) and an anti-HA antibody (bottom panel). (D) The anti-p-MEKK2/3 antibody detection of MEKK2 requires phosphorylation. Cell extracts prepared from COS-1 cells transfected with expression vector for HA-MEKK2 were either untreated or treated with calf intestine phosphatase (PPase) for 30 min before being analyzed by immunoblotting with the anti-p-MEKK2/3 antibody (top panel) or an anti-HA antibody (bottom panel).

antibody anti-p-MEKK2/3. As shown in Figure 4A, the anti-p-MEKK2/3 antibody recognized MEKK2CT but not the MEKK2CT(519A) mutant. Mutation of the Thr 521 and Thr 523 to an Ala, however, did not block Ser 519 phosphorylation in MEKK2CT(521A/523A), consistent with this mutant still being an active kinase (Figure 2E and F). As expected, the anti-p-MEKK2/3 antibody did not detect the MEKK2 with mutations of all three potential phospho-acceptor residues. Interestingly, the anti-p-MEKK2/3 antibody also did not detect MEKK2CT(KM), although the protein was expressed at a level similar to that of the active MEKK2CT (Figure 4A). This result suggests that the Ser 519 phosphorylation is mediated by self-activation rather than by another kinase, consistent with our recently reported finding that MEKK2 dimerization is required for its activation (Cheng *et al*, 2005).

The anti-p-MEKK2/3 antibody also detected the full-length MEKK2 and, as expected, it did not detect MEKK2(519A) or MEKK2(519A/521A/523A) (Figure 4B). However, unlike the catalytic domain mutant MEKK2CT(521A/523A), MEKK2(521A/523A) was not detected by the anti-p-MEKK2/3 anti-

body (Figure 4B), consistent with the data shown in Figure 3 that MEKK2(521A/523A) is a defective kinase. To further determine the contribution of Thr 521 and Thr 523 to MEKK2 activation, we constructed the Thr 521-to-Ala and Thr 523-to-Ala mutants MEKK2(521A) and MEKK2(523A), respectively. As shown in Figure 4B, while MEKK2(521A) behaved like the wild-type MEKK2, MEKK2(523A) showed a phenotype similar to that of MEKK2(519A) and MEKK2(521A/523A). This finding suggested that the Thr 523 but not the Thr 521 next to Ser 519 is required for the Ser 519 phosphorylation, and hence MEKK2 activation. Interestingly, the Thr 523 in MEKK2 is conserved in large numbers of Ser/Thr protein kinases that are not members of the MEKK/STE11 subfamily or the MAP3K family (Deak and Templeton, 1997). It is possible that this Thr residue is required for maintaining a functional structure rather than serving as a regulatory phospho-receptor. Indeed, it was reported previously that a conserved Thr residue at this position is not phosphorylated but is rather required to maintain a close contact with the Asp and Lys in the N-terminal lobe (RDI/LKXXN) of the catalytic loop that are absolutely conserved in all kinases (Johnson *et al*, 1996; Szczepanowska *et al*, 1997, 1998). In contrast, the Thr 521 is not conserved in other Ser/Thr kinases except in MEKK3. Given the results from the Maldi-MS analysis that only the Ser 519 is phosphorylated, our mutagenesis study thus confirmed the key regulatory role of Ser 519 phosphorylation in MEKK2 activation.

MEKK2 and MEKK3 are highly related homologs with almost identical catalytic domains (over 95% identity and 98% homology). We therefore reasoned that the Ser 519 phosphorylation should also be conserved in MEKK3. Indeed, the anti-p-MEKK2/3 antibody also detected the full-length MEKK3 (an active form) expressed in COS-1 cells similarly to MEKK2 (Figure 4C). In addition, the anti-p-MEKK2/3 antibody could not detect the kinase-inactive mutant MEKK3(KM), suggesting that similar Ser phosphorylation is required for both MEKK2 and MEKK3 activation. Sequence comparison of MEKK2 and MEKK3 revealed that the Ser 526 in MEKK3 is equivalent to Ser 519 in MEKK2, whereas Thr 528 and Thr 530 are equivalent to Thr 521 and Thr 523. To confirm this homology, we constructed the following MEKK3 mutants: MEKK3(526A) in which the Ser 526 was mutated to an Ala, and MEKK3(528A/530A), in which Thr 528 and Thr 530 were mutated to an Ala. Similar to the findings for the MEKK2 mutants, we found that the anti-p-MEKK2/3 antibody failed to detect either of these mutants (Figure 4C). Finally, we also sequenced the active MEKK3 by Maldi-MS, which confirmed that the Ser 526 is being phosphorylated (data not shown).

To rule out the possibility that the anti-p-MEKK2/3 antibody is detecting something other than phosphorylation, we treated MEKK2 with a protein phosphatase and then performed an immunoblotting analysis. As shown in Figure 4D, the phosphatase treatment completely abolished the ability of anti-p-MEKK2/3 to recognize MEKK2, confirming that the anti-p-MEKK2/3 antibody indeed recognizes the Ser 519 phosphorylation.

MEKK2 Ser 519 and MEKK3 Ser 526 phosphorylation is induced by TLR4 ligand LPS

The above biochemistry study strongly indicated that the Ser 519 and Ser 526 phosphorylation is crucial for inducing

the MEKK2 and MEKK3 signaling pathways, respectively, *in vivo*. If this phosphorylation was physiologically relevant, we would expect that it should be induced by the physiologic stimulation of MEKK2 and MEKK3. To address this question, we stimulated macrophage cell line Raw264.7 with lipopolysaccharide (LPS) and used the anti-p-MEKK2/3 antibody to detect the induction of MEKK2 Ser 519 and MEKK3 Ser 526 phosphorylation in endogenous MEKK2 and MEKK3 immunoprecipitated with MEKK2- and MEKK3-specific antibodies. As shown in Figure 5A and B, stimulation of macrophages by LPS specifically induced Ser 519 and Ser 526 phosphorylation. As a control, no anti-p-MEKK2/3-positive protein band was detected in the immunocomplex using the control preimmune serum.

To further confirm this result, we infected an MEKK2-deficient mouse fibroblast cell line with either an empty retroviral vector or a retroviral vector expressing hemagglutinin (HA)-MEKK2. The infected cells were either untreated or stimulated with LPS for 20 min before being analyzed by immunoprecipitation with an anti-HA antibody and immunoblotting with the anti-p-MEKK2/3 antibody. As shown in Figure 5C, the MEKK2 Ser 519 phosphorylation was clearly induced by LPS in the MEKK2-expressing retroviral vector-infected but not the control vector-infected cells. Together, these results demonstrated that both MEKK2 Ser 519 and MEKK3 Ser 526 are LPS-inducible phosphorylation sites.

Previously, we demonstrated that MEKK3 but not MEKK2 is required for LPS-induced cytokine expression in mouse embryonic fibroblasts (MEFs) (Huang *et al*, 2004). However, it is not clear if MEKK2 is also an LPS downstream signaling molecule in immune cells such as macrophages. Our biochemical studies on MEKK2 Ser 519 phosphorylation strongly indicate that MEKK2 is also required for the LPS signaling. To determine the significance of MEKK2 Ser 519 phosphorylation in LPS signaling, we expressed the MEKK2(519A) mutant in Raw264.7 macrophages together with an MEKK2-dependent reporter plasmid and determined how it may affect the LPS-induced reporter gene expression. Figure 5D shows that overexpression of MEKK2(519A) strongly inhibited the MEKK2-dependent reporter gene expression in a dose-dependent manner, suggesting that MEKK2 is involved in the LPS-TLR4 signal pathway.

LPS-induced Ser 519 and Ser 526 phosphorylation of MEKK2 and MEKK3 is regulated by TRAF6

The above results suggest that LPS activates MEKK2 and MEKK3 by inducing their active Ser phosphorylation. We previously showed that LPS-mediated MEKK3 activation required the adaptor molecule TRAF6 in MEFs (Huang *et al*, 2004). As MEKK3 and TRAF6 physically interact with each other, we reasoned that the TRAF6-associated MEKK3 may be activated and thus Ser 526 phosphorylated. Indeed, using the anti-p-MEKK2/3 antibody, we found that Ser 526 was phosphorylated in the LPS-induced, TRAF6-associated MEKK3 (Figure 5E). Consistent with this result, we found that the MEKK2 and MEKK3 co-precipitated with TRAF6 in the detergent-insoluble fractions were phosphorylated on their respective Ser residues (data not shown).

As TRAF6 has no kinase activity, the MEKK2 and MEKK3 phosphorylation is likely induced by other kinases or by self-activation. In this regard, we recently reported that dimerization of MEKK2 and MEKK3 is crucial for their activation

(Cheng *et al*, 2005). Thus, it is possible that, following LPS stimulation, TRAF6 associates with either MEKK2 or MEKK3 to induce their dimerization or oligomerization resulting in their activation. If this is true, we would expect that the Ser 519 and Ser 526 phosphorylation should also depend on MEKK2 or MEKK3 dimerization, respectively. To test this possibility, we transfected a dominant-negative MEKK2 mutant to disrupt MEKK2 dimer formation (Cheng *et al*, 2005). We found that expression of this mutant led to a significant reduction in MEKK2 Ser 519 phosphorylation (80% reduction) (Figure 5F). This finding further supported a crucial role of Ser 519 and Ser 526 phosphorylation in MEKK2 and MEKK3 activation.

MEKK3 but not MEKK3(526A) mutant synergizes with LPS and IL-1 to induce cytokine production

As the Ser 519 and Ser 526 phosphorylation in MEKK2 and MEKK3 is regulated by LPS-TLR4 signaling, we expected that their phosphorylation should be crucial for LPS-induced proinflammatory cytokine production. To test this possibility, we established cell lines that were infected with control retroviral vector, or with retroviral vectors expressing wild-type MEKK3 or MEKK3(526A) mutant. We then measured the IL-6 production induced by LPS in these cell lines. As shown in Figure 6A, although the MEKK3(526A) mutant expression was similar to that of the wild-type MEKK3 (both are expressed at a low level comparable to that of the endogenous MEKK3), only the wild-type MEKK3 was able to augment the LPS-induced IL-6 production. To further confirm this finding, we infected an MEKK3-deficient MEF line that was unable to produce IL-6 in response to LPS stimulation with either an empty vector, or an expressing vector for wild-type MEKK3 or for the MEKK3(526A) mutant. As shown in Figure 6B, while the wild-type MEKK3 was able to restore the mutant MEFs to produce IL-6, neither the control vector nor MEKK3(526A) mutant-expressing vector was able to restore the mutant MEFs to produce IL-6.

Ser 519 is a general sensor of a spectrum of stress-, cytokine-, and growth factor-mediated signals

MEKK2 and MEKK3 have been demonstrated to be the major MAP3Ks upstream of the MAPK and the IKK-NF- κ B pathways. It is thus likely that they relay multiple extracellular signals to activate these pathways. As the Ser 519 and Ser 526 phosphorylation is essential for MEKK2 or MEKK3 activation, respectively, we reasoned that if MEKK2 or MEKK3 is required for transducing signals to the downstream MAPK and IKK pathways, the agonists upstream of the MAPK and IKK pathways would also induce MEKK2 or MEKK3 Ser phosphorylation. To identify which extracellular stimuli that are known to activate MAPKs and IKKs may also activate MEKK2, we stimulated the stable MEF cell line (Figure 5C) that expressed an HA-MEKK2 at a level about two times higher than the endogenous MEKK2 with EGF, IL-1 β , or TNF α (Figure 7A); with peptidoglycan (PGN), teichoic acid (LTA), and CpG (ligands for TLR1/2, TLR2/6, and TLR9 respectively) (Figure 7B); or with cellular stresses such as ultraviolet light (UV), anisomycin, nocodazole, or sorbitol (Figure 7C). The MEKK2 Ser 519 phosphorylation was then determined in the stimulated cells using the anti-p-MEKK2/3 antibody. We found that almost all the stimuli were able to induce MEKK2 Ser 519 phosphorylation, except anisomycin

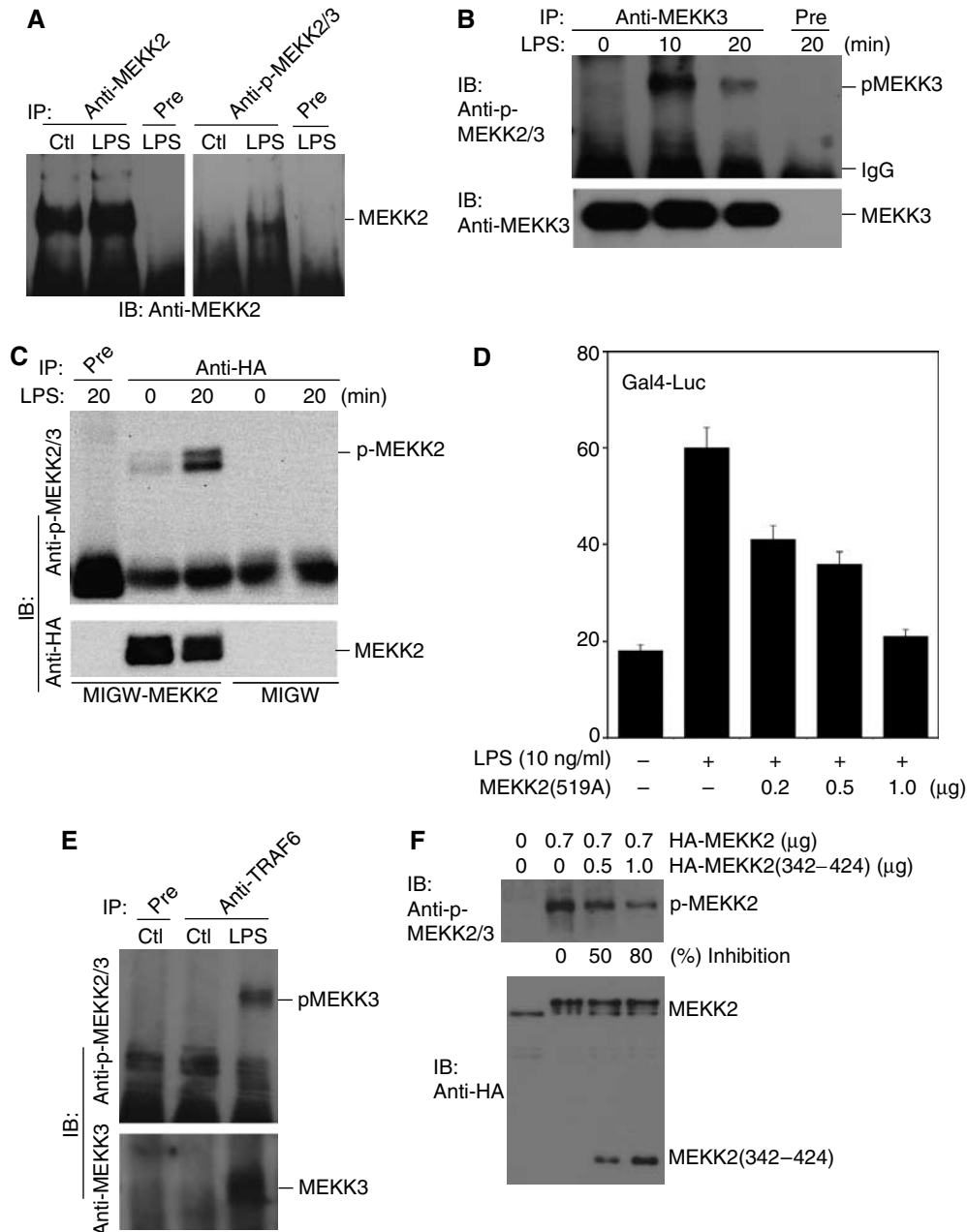


Figure 5 MEKK2 Ser 519 and MEKK3 Ser 526 phosphorylation is induced by LPS. **(A)** Ten million Raw264.7 cells were either untreated or stimulated with LPS for 20 min before being harvested for immunoprecipitation (IP)–immunoblotting (IB) analysis with an anti-MEKK2 antibody and the anti-p-MEKK2/3 antibody. Preimmune serum (Pre) was used as a negative control, as indicated. **(B)** Ten million Raw264.7 cells were either untreated or stimulated with LPS for 10 or 20 min before being harvested for IP–IB analysis with an anti-MEKK3 antibody and the anti-p-MEKK2/3 antibody. Preimmune serum (Pre) was used as a negative control as indicated. **(C)** The anti-p-MEKK2/3 antibody detects LPS-induced HA-MEKK2 in MEKK2-deficient MEFs stably reconstituted with HA-MEKK2. MEKK2-deficient MEFs were infected with an empty MIGW retroviral vector or an HA-MEKK2 expression vector. The viral infected cells (GFP positive owing to an IRES-GFP expression cassette in the MIGW vector) were sorted by a FACS sorter and further analyzed for Ser 519 phosphorylation induction by LPS with an anti-HA antibody and the anti-p-MEKK2/3 antibody as described in panel A. **(D)** Expression of the MEKK2(519A) mutant inhibits LPS-induced ELK1 reporter gene expression. Gal4-Luc reporter plasmid (2 μg) was co-transfected into Raw264.7 cells with expression vectors for Gal4-ELK1 alone or with increasing amounts of MEKK2(519A) as indicated. Cells were stimulated 24 h later with LPS for 12 h. The relative reporter activity was determined as described in Figure 3G. The results shown are the average of three independent experiments. **(E)** TRAF6-associated MEKK3 is phosphorylated on Ser 526. Control untreated or LPS-stimulated MEFs were lysed for IP with preimmune (Pre) or anti-TRAF6 antibody as indicated. The immunocomplex was separated by an SDS–PAGE gel and further analyzed by immunoblotting with antibodies specific to anti-p-MEKK2/3 (top panel) and anti-MEKK3 (bottom panel), as indicated. **(F)** MEKK2 Ser 519 phosphorylation requires MEKK2 dimerization. The HA-MEKK2 expression vector (0.7 μg) was co-transfected with 0, 0.5, or 1 μg of the expression vector for the MEKK2 dimerization motif mutant HA-MEKK2(342–424) into 293T cells. HA-MEKK2 Ser 519 phosphorylation was assayed as described in Figure 4B. Expression levels for HA-MEKK2 and HA-MEKK2(342–424) were determined by IB with an anti-HA antibody.

and nocodazole, which only marginally activated MEKK2. Anisomycin is a potent JNK and p38 activator, and, in fact, was a stronger activator than the other JNK/p38 agonists

such as TLR ligands, growth factors, or cytokines in these MEFs (data not shown). Nocodazole was also able to induce JNK and ERK5 in these MEFs (data not shown). However,

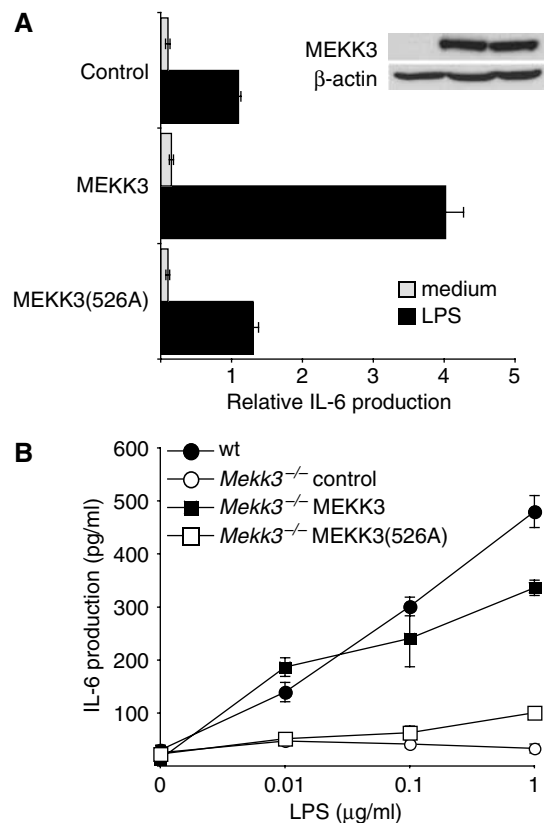


Figure 6 MEKK3 Ser 526 phosphorylation is required for IL-6 production induced by LPS. (A, B) Wild-type (A) and MEKK3-deficient (B) MEFs were infected with a control retroviral vector or vectors for HA-MEKK3 or HA-MEKK3(526A) as indicated. The expression levels of HA-MEKK3 and MEKK3(526A) were determined by immunoblotting with an anti-HA antibody (inset gel) (A). To determine IL-6 induction by LPS, MEFs were either untreated (medium) or stimulated with LPS for 24 h. The IL-6 production in the supernatant was determined by ELISA. The data shown are the average of three independent experiments.

their inability to induce strong MEKK2 phosphorylation and activation suggests that other MAP3Ks, but not MEKK2, are required for anisomycin- and nocodazole-induced downstream MAPK and IKK-NF- κ B activation.

Discussion

One of the key questions regarding the specific activation of each MAPK module is how its activating MAP3K is activated and regulated. Although genetic studies in yeast have provided strong evidence for the existence of yeast MAP3K kinases (also called MAP4K such as Ste20) that act upstream of the yeast MAP3Ks (Elion, 2000), such evidence remains elusive in the mammalian system. One difficulty in studying MAP3Ks in the mammalian system is that, unlike MAP2Ks or MAPKs, the endogenous levels of MAP3K are usually very low, and most MAP3Ks become activated once they are slightly overexpressed by means of transient transfection.

In this study, we identified the Ser 519 and Ser 526 in the activation loop of MEKK2 and MEKK3, respectively, as the key regulatory residues whose phosphorylation is essential for MEKK2 and MEKK3 activation. When MEKK2 Ser 519 was mutated to an Ala, it dramatically decreased MEKK2 self-activation and hence MEKK2-mediated JNK1, ERK5, and

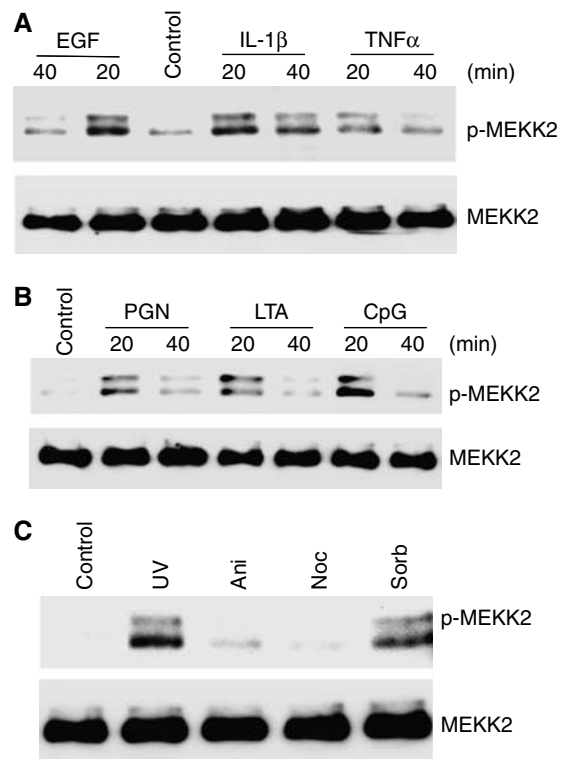


Figure 7 MEKK2 Ser 519 is a general sensor of a wide spectrum of MAPK active stimuli. MEKK2-deficient MEFs (5 million cells at each point) stably expressing HA-MEKK2 were either untreated or stimulated with the cytokines EGF (5 ng/ml), IL-1 β (1 ng/ml), or TNF α (10 ng/ml) for 20 or 40 min (A); with PGN (10 μ g/ml), LTA (1 μ g/ml), or CpG-1668 (5 μ M) for 20 or 40 min (B); or with nocodazole (Noc) (2 μ M), anisomycin (Ani) (50 ng/ml), UV (40 J/M²), or sorbitol (Sorb) (0.2 M) (C) as indicated. MEKK2 Ser 519 phosphorylation was determined as described in Figure 5C. HA-MEKK2 expression was determined by immunoblotting with an anti-HA antibody (A-C, bottom panels).

JNK2 activation. The Ser 519-to-Ala mutation also impaired MEKK2-dependent reporter gene expression. Although mutation of Thr 521 and Thr 523 to an Ala did not significantly affect the activity of MEKK2CT, the catalytic domain of MEKK2, mutation of both Thr 521 and Thr 523 to an Ala or Thr 523 but not Thr 521 to an Ala severely impaired the full-length MEKK2 activation (Figure 3). The Thr 523 mutation alone or together with Thr 521 also blocked Ser 519 phosphorylation in the full-length MEKK2 (Figure 4). This finding suggests that Thr 523 but not Thr 521 is required for Ser 519 phosphorylation induction. Whereas the Thr 521 is not conserved in the MEKK/STE11 family or other Ser/Thr kinases, the Thr 523 is conserved not only in the MAP3K family but also in almost all the Ser/Thr kinases (Johnson *et al*, 1996). MIHCK is a member of the STE20 family that is closely related to MEKK2 and MEKK3. The Thr 631 in MIHCK corresponding to MEKK2 Thr 523 and MEKK3 Thr 530 has been extensively analyzed. The Thr631 in MIHCK was shown to be unphosphorylated. This Thr residue is required to interact with the Asp-(Ile/Leu)-Lys in the catalytic center. A phosphorylation mimic mutation of this residue actually decreased MIHCK enzymatic activity (Szczepanowska *et al*, 1998). As the Asp and Lys residues in the catalytic center are conserved in all kinases, and the Thr residue that interacts with the catalytic center is also conserved in almost all Ser/Thr kinases, the Thr

residue in this position is unlikely to be the regulatory phosphorylation site for Ser/Thr kinases such as MEKK2 and MEKK3. Nonetheless, the fact that this residue is conserved suggests that it is required for maintaining a functional structure by interacting with the catalytic center. On the other hand, the Thr 521 and Thr 528 in MEKK2 and MEKK3, respectively, may not be required for such a regulatory or structural function.

Unlike the highly conserved Thr 523, Ser 519 in MEKK2 is conserved only in MEKK2 and MEKK3 (corresponding to Ser 526) but not among other kinases. This suggests that the Ser 519 and Ser 526 may be the specific regulatory residues for MEKK2 and MEKK3. It is important to note that many Ser/Thr kinases use a Ser or Thr residue located between the VII and VIII subdomains as their regulatory phosphorylation sites. For instance, the Thr-X-Tyr motif in the MAPK family and the Ser-X-X-Ser/Thr motif in the MAP2K family are all located between the VII and VIII subdomains (Davis, 2000). In the MAP3K family, regulatory Ser residues in Raf1 and TAK1 were also identified between the kinase VII and VIII subdomains (Kishimoto *et al*, 2000; Chong and Guan, 2003). Interestingly, these residues are not conserved among other MAP3Ks, suggesting that their phosphorylation may confer the specificity for their regulation. Notably, the Ser 519 mutation seems to block only the inducible but not the basal activity of MEKK2. In contrast, the ATP binding site mutation of MEKK2 (a Lys-to-Met mutation in the subdomain III) completely abolished the MEKK2 activity.

Using the specific anti-p-MEKK2/3 antibody, we found that the kinase-inactive MEKK2 and MEKK3 mutants, MEKK2(KM) and MEKK3(KM), were not phosphorylated on their regulatory serine residues. These results indicated that the basal MEKK2 and MEKK3 activity is required for the regulatory serine residue phosphorylation. These findings also suggest that MEKK2 and MEKK3 are activated through self-phosphorylation. Consistent with having no roles in MEKK2 and MEKK3 activity, we found that Thr 521 in MEKK2 and Thr 528 in MEKK3 had no effect on the regulatory Ser phosphorylation. In contrast, the conserved Thr 523 in MEKK2 and Thr 530 in MEKK3 are required for the regulatory Ser phosphorylation.

It remains unclear whether another kinase (e.g. the putative MAP4Ks) upstream of MEKK2 or MEKK3 is needed to phosphorylate the active Ser residues in MEKK2 and MEKK3. Our current study, however, suggests that both MEKK2 and MEKK3 are capable of self-phosphorylation and activation in the absence of upstream activating kinases. In this regard, we recently found that MEKK2 and MEKK3 form dimers through their catalytic domains and that this dimer formation is required for their activation (Cheng *et al*, 2005). Thus, it is possible that the Ser 519 and Ser 526 phosphorylation can be induced through stimulating MEKK2 and MEKK3 dimer formation via their catalytic domains. Indeed, we found that disruption of MEKK2 dimer formation significantly inhibited Ser 519 phosphorylation.

Previously, we demonstrated that MEKK3 plays a key role in IL-1R-TLR4 signaling in MEFs (Huang *et al*, 2004). However, these studies did not reveal the role of MEKK2 in TLR4 signaling, as the MEKK2-deficient MEFs were still able to respond to LPS. One likely explanation for this finding is that MEKK3 compensated for MEKK2's function. Alternatively, MEKK2 may have cell type-specific function. Indeed, using anti-p-MEKK2/3 antibody, we demonstrated

that MEKK2 and MEKK3 were activated by LPS, a ligand for TLR4 in the innate immune system. In addition, overexpression of MEKK2(519A) mutant also blocked LPS-induced reporter gene expression. Although the dominant MEKK2 mutant could potentially affect both the MEKK2 and MEKK3 signaling, these results together strongly suggest that both MEKK2 and MEKK3 are involved in LPS-TLR4 signaling.

Although it is conceivable that dimer formation may induce Ser 519 and Ser 526 phosphorylation of MEKK2 and MEKK3, respectively, how this dimerization is induced and regulated *in vivo* is not known. Previously, we found that MEKK3 interacts with TRAF6, a ring domain-containing adaptor molecule with E3 ubiquitin ligase activity in the IL-1R-TLR signaling pathways. We also found that TRAF6 interacts with MEKK2 (data not shown). It is possible that TRAF6 may induce MEKK2 and MEKK3 dimer formation, as we found that interaction with TRAF6 also caused the regulatory Ser phosphorylation in MEKK2 and MEKK3. These findings also indicate that the regulatory Ser phosphorylation in MEKK2 and MEKK3 is crucial for LPS-TLR4-mediated innate immune responses. This conclusion was strongly supported by the results showing that only the wild-type MEKK3, but not the MEKK3(526A) mutant, was able to restore LPS-induced proinflammatory cytokine IL-6 production in MEKK3-deficient MEF cells.

Finally, if MEKK2 Ser 519 is indeed the key regulatory phosphorylation site, we would expect it to be induced by many different agonists that activate the MAPK and IKK-NF- κ B pathways. Using the anti-p-MEKK2/3 antibody, we showed that MEKK2 was induced by different types of stimuli, including cellular stress, cytokines, growth factors, and TLR ligands. Importantly, even though all these agonists are known to be potent inducers of the MAPK and IKK-NF- κ B pathways, not all of them induced MEKK2. For instance, nocodazole and anisomycin are two potent MAPK activators (e.g. for p38 or JNK) but they induced only marginal MEKK2 activation. This suggests that although MEKK2 may participate in many cellular responses requiring MAPK and IKK-NF- κ B activation, other MAP3Ks, such as MEKK1, are also crucial and important. Thus, the identification of the key regulatory phosphorylation residues and the generation of antibodies that specifically recognize these residues are critical to understanding the differential involvement of different MAP3Ks in various cellular responses.

In conclusion, we identified a key regulatory serine residue in the activation loop of MEKK2 and MEKK3 whose phosphorylation is crucial for their induction by many agonists, including TLR ligands, cellular stresses, and cytokines.

Materials and methods

Plasmids, proteins, antibodies, and reagents

HA-tagged MEKK2CT, MEKK2CT(KM), MEKK2, MEKK2(KM), MEKK2(342-424), MEKK3, Flag-tagged JNK1, -79Jun-Luc, Gal4-Luc, and Gal4-Elk have been previously described (Cheng *et al*, 2000, 2005). MEKK2 and MEKK3 mutants were constructed by using a polymerase chain reaction-directed mutagenesis method (Stratagen, La Jolla, CA) and confirmed by DNA sequencing. Flag-ERK5 was a gift from Dr Z-G Xia (The University of Washington, Seattle). The retroviral expression vectors were constructed by subcloning the HA-MEKK2- and HA-MEKK3-expressing cassette into the MIGW vector (a gift from Dr X-F Qin, UT MDACC, Houston, TX) at the *Hpa*I site. The expression and purification of GST-cJun were described previously (Su *et al*, 1994).

Anti-HA antibody was purified from 12CA5 hybridoma, anti-Flag antibody M2 was purchased from IBI-K (Rochester, NY), and anti-MEKK2 (8384), anti-MEKK3 (1415), and anti-phospho-MEKK2/3(Ser) antibodies (anti-p-MEKK2/3) were produced by immunizing rabbits with MEKK2 peptide (CRPALSLQETRKAKSSSPKKKQN), MEKK3 peptide (Huang *et al*, 2004), and phospho-MEKK2 peptide (CSGTGMK^(P)SVTGTPYW), respectively. Anti-TRAF6 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LPS, LTA, PGN, sorbitol, anisomycin, and nocodazole were purchased from Sigma Chemical Company (St Louis, MO). CpG (1668) was synthesized by Qiagen (Valencia, CA). TNF α , IL-1 β , and EGF were purchased from PeproTech (Rocky Hill, NJ).

Cell culture and transfection

293T and COS-1 cell culture and transfection with lipofectamine (Life Technologies, Gaithersburg, MD) were described previously (Cheng *et al* 2000). Raw264.7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml), amino acids (0.1 mM), vitamin, glutamine (2 mM), and sodium pyruvate (1 mM), and transfection was carried out with Fugene 6 (Roche Scientific, Indianapolis, IN) according to the manufacturer's protocol. MEFs were cultured as described elsewhere (Yang *et al*, 2001).

Purification of recombinant MEKK2CT and MEKK2CT(KM) and MALDI-MS analysis

HA-MEKK2CT and HA-MEKK2CT(KM) were expressed in 293T cells, purified by immunoprecipitation with an anti-HA antibody, and separated on a 4–20% gradient SDS-PAGE gel. The phosphorylated residue and the amino-acid sequence of the phosphopeptide of interest were identified by mass spectrometric analysis of proteolytic digests of proteins excised from the SDS-PAGE gel, as described elsewhere (Szczezanowska *et al*, 1997).

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Immunoblotting, immunoprecipitation, and in vitro kinase assays

Cell lysate preparation, immunoprecipitation, immunoblotting analysis, and *in vitro* kinase assay were performed as described previously (Su *et al*, 1994; Cheng *et al*, 2000).

Luciferase reporter assay

Luciferase reporter gene assay was performed using dual luciferase reporter assay system (Promega) and a TD-20/20 luminometer (Turner Designs, Promega, Madison, WI) as described previously (Cheng *et al*, 2005).

Preparation of retrovirus and infection of MEF cells

Retroviruses expressing MEKK2, MEKK3, or their derived mutants were prepared in 293T cells as described (Qin *et al*, 2003). MEF cells were incubated with virus-containing medium in the presence of 8 μ g/ml polybrene (hexadimethrine bromide; Sigma Chemical Company, St Louis, MO) during centrifugation at 300 g for 2 h at 32°C. Stable cell lines were established by FACS cell sorting.

Cytokine production

MEFs were cultured with or without LPS for 24 h, as indicated. IL-6 production was determined using an ELISA kit (R&D System Inc., Minneapolis, MN) according to the manufacturer's instructions.

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