Subdomain VIII Is a Specificity-determining Region in MEKK1*

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MAPK/ERK kinase kinase 1 (MEKK1) is a mitogen-activated protein kinase kinase kinase (MAP3K) of the stress-induced JNK pathway. Once activated, MEKK1 phosphorylates the MAP2K MKK4, which in turn phosphorylates JNK. MEKK1 also has the capacity to activate IKK, the central protein kinase of the NF-κB pathway. The molecular determinants responsible for the ability of MEKK1 to recognize specific substrates are poorly understood. We report here that select point mutations in subdomain VIII of the protein kinase domain of MEKK1 (MEKK1Δ) differentially affect its ability to activate MKK4 and IKK, and consequently AP1 and NF-κB reporter genes. Moreover, binding of MKK4 to MEKK1Δ protects the latter from cleavage at an engineered protease target site in subdomain VIII. Collectively these results provide evidence that subdomain VIII of MEKK1 is involved not only in binding to, but also in discrimination of, protein substrates.

A notable feature of MAP3Ks is that they participate in the activation of a distinct signal-induced pathway, the NF-κB pathway. In this pathway, MAP3Ks directly activate IkB kinase (IKK), the central kinase of the NF-κB pathway. IKK, in turn, phosphorylates the NF-κB inhibitor IkB, targeting it for degradation and thereby allowing the nuclear translocation of NF-κB (5). IKK also phosphorylates the p100 subunit of NF-κB, thereby promoting its processing to p52 (6). MAP3Ks of the NF-κB pathway that have been implicated by genetic or RNA studies include MEKK3 (7), NIK (8–11), and TGF-β-activated kinase 1 (12).

MEKK1 is among the best studied of the MAP3Ks and is a 196-kDa protein with a large N-terminal regulatory domain and a C-terminal kinase domain (13). The preferential substate of MEKK1 is MKK4, a MAP2K of the JNK pathway (14). MKK4, in turn, activates JNK (15), which then phosphorylates c-Jun, a component of the AP1 transcription complex (16, 17). The latter phosphorylation event potentiates c-Jun transcriptional activity. MEKK1-deficient cells display defects in JNK activation in response to stimuli such as microtubule disruption, hyperosmolarity, double-stranded RNA, and lipopolysaccharide (18–20). The role of MEKK1 in tumor necrosis factor α- and interleukin-1β-induced JNK activation is more controversial (18–20). Although gene-targeting experiments revealed either more modest or no defects in activation of the ERK or NF-κB pathways, respectively (18–20), MEKK1 can activate MEK1 and therefore the ERK pathway (21), and also has the capacity to activate IKK and therefore the NF-κB pathway (22, 23). Thus, MEKK1 provides an opportunity to study mechanisms by which specificity in these pathways may be achieved.

Direct protein/protein contacts between kinases of a cascade are one important means by which this specificity is maintained. An example of this is a docking interaction that occurs outside of the active site. In the case of the binding of the MAP2K MKK3b to the MAPK p38, recent x-ray crystallographic studies have revealed that this interaction occurs between the N terminus of the former and a docking groove in the latter contributed by subdomains V and VI (24). Mutagenesis studies have also identified charged residues in subdomain VI (the ED domain) and in a C-terminal extension distal to subdomain XI (the CD domain) of MAPK as being critical to this docking interaction (25, 26). Although these charged residues were not directly implicated in the structural studies, which employed an MKK3b N-terminal peptide, they are in the vicinity of the docking groove (24).

Deletion of the N terminus (residues 1–77) of MKK4 abolishes its activation by tumor necrosis factor α (14), raising the possibility that a homologous docking interaction might also mediate the interaction of MKK4 and the upstream kinase MEKK1. However, although MKK4 residues 1–87 alone are sufficient for binding to JNK, it is not for binding to MEKK1 (14). Thus, even if this docking interaction were to play a role in the MEKK1/MKK4 interaction, additional contacts must...
Plasmids—pcDNA-His6-MEKK1 (encoding residues 1174–1493 of mouse MEKK1), pcDNA3-His6-MEKK1 (F1443A), pcDNA3-HA-rMEKK1 (encoding full-length MEKK1), and pCMV2-FLAG-MKK4 were described previously (27). Subdomain VIII mutations of MEKK1 were made from pcDNA3-His6-MEKK1 by QuikChange mutagenesis (Stratagene). Primers used are listed below (only sense primers are shown for brevity): T1381A: GTTGGCATCAAAAGGAGGCGGTCCAGAGGAGGTTCGGCTGAC
T1383A: GGACAGTCTGCGGAAATCTGATCACTCGGCC
I1394A: GTATCTGGGACAGCTGCTCAACCTGGCTGAGGTTCC
G1404A: CATGGCGCCTGAGGTCCTAAGAGCTCAGCAGTATGGTT-
C3197A: GTTACTGGGACAGCTGCTCAACCTGGCTGAGGTTCC
V410A: CATGGCGCCTGAGGACAGCTGCTCAACCTGGCTGAGGTTAGG
L410A: CATGGCGCCTGAGGACAGCTGCTCAACCTGGCTGAGGTTAGG
R4103A: GCCGCGCCTGAGGACAGCTGCTCAACCTGGCTGAGGTTAGG
Q1405R/Q1406R (hereafter referred to as QQ/RR): CCTGAG-
M1397A: GTTACTGGGACAGCTGCATTCGCGGCGCCTGAGG-
L1402A: CATGGCGCCTGAGGTCGCAAGAGGTCAGCAGTATGGT-
G1404A: CATGGCGCCTGAGGACAGCTGCTCAACCTGGCTGAGGTTAGG
L1403A: CATGGCGCCTGAGGACAGCTGCTCAACCTGGCTGAGGTTAGG
QQ/RR) were prepared from pBluescript-MEKK1 by QuikChange mutagenesis (Stratagene). His6-FLAG-MKK4 and His6-FLAG-MEKK1 were obtained from Cell Signaling Technology. Recombinant His6-FLAG-MKK4 and His6-FLAG-MEKK1 were purified from baculovirus-infected insect cells, and GST-MEKK1 (K131A), GST-JNK1 (K55R), and GST-Ikkβ (S223A) were purified from Escherichia coli as described previously (22, 27). His6-ERK2 was obtained from Santa Cruz Biotechnology.

Binding Assays—Lysates of COS-1 cells transiently expressing wild type or mutant MEKK1 were prepared as above and mixed with either corresponding lysates containing transiently expressed FLAG-MKK4, or 4 μg of recombinant His6-FLAG-MKK3. The mixtures were incubated for 2 h at 4 °C with rocking and then immunoprecipitated with anti-FLAG (MA-12, Sigma). The immunoprecipitates were then washed, subjected to SDS-PAGE, and the presence or absence of MEKK1 was examined by Western blotting using anti-MEKK1 antibodies.

Furin Limited Proteolysis—In vitro-translated MEKK1 and MEKK3 (QQ/RR) were prepared from pBluescript-MEKK1 and pBluescript-MEKK3 (QQ/RR), respectively, using Tnt/T7 coupled reticulocyte lysates (Promega) and both [35S]methionine and [35S]cysteine (each at 100 Ci/mmol) as the labeling reagents. In vitro-translated MEKK1 and MEKK3 (QQ/RR) were incubated, with rocking, for 2 h at 4 °C with either 2 μg of anti-MEKK1 antibodies (sc-252, Santa Cruz Biotechnology) and 10 μl of protein G-agarose (Invitrogen), or 2 μg of His6-FLAG-MK4 and 10 μl of anti-FLAG (M2) agarose (Sigma). The incubation mixtures were then washed twice with buffer B (20 mM Hepes, pH 7.0, 0.1 M KCl, 0.2% Nonidet P-40, 10% glycerol, 0.1% bovine serum albumin, and 5 mM 2-mercaptoethanol) and once with furin reaction buffer (100 mM Hepes, pH 7.5, 1% Triton X-100, 1 mM CaCl2, 5 mM 2-mercaptoethanol). The immunoprecipitates were incubated at 30 °C in a total of 20 μl of reaction buffer with four units of furin (New England Biolabs), and after the addition of 2× SDS loading buffer followed by heating at 100 °C for 3 min. The eluates were then subjected to 16% SDS-PAGE and autoradiography.

RESULTS

Mutations in Subdomain VIII of MEKK1 Diminish Its AP1- and NF-κB-inducing Activity—In a previous study (27), we observed that Phe-1443 of subdomain X of MEKK1 might be a contact residue for MKK4. This, in turn, raises the possibility that residues in areas adjacent to subdomain X in the tertiary structure of MEKK1 could be contact sites for MKK4. In JNK2, the sequence between subdomain IX and X (residues 208–230) largely accounts for its higher affinity to c-Jun (30), because replacement of the corresponding region of JNK1 with that from JNK2 increases its binding to c-Jun 30-fold. Thus, we first examined residues N-terminal to F1443. Three charged residues, Glu-1433, Lys-1434, and His-1435, in this region were mutated using a Dual Luciferase Reporter assay system (Promega) and a Wallac LB9507 Luminometer.

Western Blotting—Cell processing, SDS-PAGE, and membrane transfer have been described previously (27). The membranes were then blotted with anti-HA polyclonal (sc-805, Santa Cruz Biotechnology), anti-MEKK1 monoclonal (sc-252, Santa Cruz Biotechnology), anti-IKKα/β polyclonal (sc-7607, Santa Cruz Biotechnology), anti-myc monoclonal (9E10, University of Pennsylvania Cell Center), anti-FLAG monoclonal (M2, Sigma), or anti-phospho-MKK4 (Thr-261) polyclonal (catalog number 9151, Cell Signaling Technology) antibodies.
and there is evidence that subdomain VIII is involved in protein/protein interactions with other protein kinases. For example, p21-activated kinase 1 is present in a latent form due to an interaction with an inhibitory switch domain, and residues in subdomain VIII (Val-436, Arg-438, and Lys-439) form critical interaction with an inhibitory switch domain, and residues in subdomain VIII are involved in promoting binding of this kinase to its peptide substrates (32).

Thus, we examined the potential involvement of subdomain VIII, which is variably conserved among MAP3Ks (Fig. 1A), by performing alanine scanning mutagenesis of residues 1394–1404 in the context of the catalytic domain of MEKK1 (MEKK1Δ) (Fig. 1B). In addition, we included in these studies T1381A and T1393A mutations, because these two threonines have been reported to be autophosphorylation sites critical for MEKK1 activation (33, 34). Excluded from these studies were the residues of the Ala-Pro-Glu motif (residues 1398–1400), because this motif is strongly conserved among protein kinases (35).

We first examined the effects of the MEKK1Δ mutants on the activation of AP1 and NF-κB reporter genes. HeLa cells were cotransfected with expression constructs for wild-type MEKK1 and either AP1 or NF-κB luciferase reporter genes, and subsequently the luciferase activities were measured. As shown in Fig. 2, A and B, overexpression of wild-type MEKK1Δ induced robust activation of both AP1 and NF-κB reporter genes (compare columns 1 and 2). The phospho-acceptor mutants T1381A and T1393A both failed to induce activity from either (columns 3 and 4), consistent with previous studies on these residues (33, 34). Most mutations, including the F1396A, M1397A, V1401A, and G1404A mutations, substantially diminished or abolished both AP1 and NF-κB reporter gene activity (Fig. 2, A and B, columns 6–8 and 11). An exception to this was the R1403A mutation, which diminished AP1 reporter gene activity without diminishing the NF-κB reporter gene activity (Fig. 2, A and B, column 10).

Even more interesting in this regard was the L1402A mutant. This mutant was actually more active (172%) than wild-type MEKK1Δ in activating the NF-κB reporter gene, but was deficient in inducing AP1 activity (39% of wild type; Fig. 2, A and B, column 9). In contrast, the I1394A mutant induced substantial AP1 reporter gene activity (64% of wild type), but almost completely lacked NF-κB-inducing activity (Fig. 2, A and B, column 5). Titration experiments with these two mutants confirmed that the I1394A mutant is more selectively active toward the AP1 reporter gene than the NF-κB reporter gene, whereas the L1402A displays the opposite preference (Fig. 2, C and D).

Mutations in subdomain VIII of MEKK1Δ Impair Its Activation of MAP2Ks and IKK—The principal substrate of MEKK1 is MKK4, so we examined the effects of these mutations on MKK4 phosphorylation and activity. Because MEKK1Δ can also activate MKK7 and MEK1 (13), we examined activation of these MAP2Ks as well. COS-1 cells were cotransfected with expression constructs for wild type or mutant MEKK1Δ, and ones for epitope-tagged MKK4, MKK7, or MEK1. The MAP2K was subsequently immunoprecipitated, and its capacity to phosphorylate recombinant MAP2Ks (GST-JNK1 (K55R) in the case of MKK4 and MKK7, His6-ERK2 in the case of MKK1) then measured in the presence of [γ-32P]ATP. We also performed parallel assays in which endogenous IKK was immunoprecipitated and then assayed for phosphorylation of GST-IκBα (5–55). Endogenous, as opposed to overexpressed, IKK was examined in these experiments due to high basal activity of overexpressed IKK (data not shown) (36).

Wild-type MEKK1Δ, as expected, induced easily detectable MAP2K or IKK activity (Fig. 3, A–D, upper panels, lane 2), whereas the phospho-acceptor mutants T1381A and T1393A were deficient in this regard (Fig. 3, A–D, upper panels, lanes 3 and 4). An additional experiment examining the capacity of MEKK1Δ to phosphorylate Thr-261 in the activation loop of MKK4 demonstrates a close correlation between this activity and the ability of MEKK1Δ to induce MKK4 activity (compare Fig. 3, E and A). With many mutations, the results with all assays were similar. Thus, and consistent with the AP1 and NF-κB reporter gene assays, the F1396A, M1397A, and V1401A mutants showed diminished MKK4 activation (Fig. 3A, upper panel, lanes 6–8), with similar results observed with MKK7, MEK1, and IKK activation (Fig. 3C, D, and B, respectively, upper panels, lanes 6–8).

With the I1394A and L1402A mutants, there were marked differences between activation of the MAP2Ks on the one hand, and IKK on the other. Thus, and again consistent with reporter gene assays, the I1394A mutant induced substantial activation of MKK4 (as well as MKK7 and MEK1) but almost no activation of IKK (Fig. 3, A–D, upper panels, lane 5). In contrast, the L1402A mutant was compromised in its activation of MKK4 (as well as MKK7 and MEK1) but not IKK (Fig. 3, A–D, upper panels, lane 9); indeed, the level of IKK activation was consistently higher than that seen with wild type. In subsequent experiments we focused on these two mutations.

Mutations of Ile-1394 and Leu-1402 Affect the Activity of Full-length MEKK1—We next extended our studies to full-length MEKK1. As shown in Fig. 4, A and B, the full-length MEKK1 I1394A mutant induced wild-type activity toward an AP1 reporter gene, but diminished activity toward an NF-κB reporter gene. Conversely, the L1402A mutant displayed diminished activity toward the AP1 reporter gene but slightly enhanced activity toward the NF-κB reporter gene. Thus, the trends observed with these mutations in the context of MEKK1Δ are also seen in the context of the full-length protein.

![Figure 1](image1.png)

**Fig. 1.** Subdomain VIII sequences in protein kinases. A, sequence comparison of subdomain VIII of select protein kinases. Names of protein kinases are provided on the left. Numbers at top denote MEKK1 residues. Residues conserved in at least four of the nine protein kinases are shaded. Underlined residues indicate amino acids implicated in protein/protein contacts (see “Results”). B, subdomain VIII point mutations of MEKK1 examined in this study. QQ/RR denotes a Q1405R/K1406R mutation. Closed triangle indicates predicted site for furin cleavage.
In conjunction with our previous studies on subdomain X of MEKK1\(\Delta\) (27), the present studies raise the possibility that subdomains VIII and X might both be involved in binding to substrates. As one test of this, we prepared additional mutants of MEKK1\(\Delta\) that consisted of either the I1394A or L1402A mutation in combination with the subdomain X F1443A mutation; the latter mutation was previously found to weaken its interaction with MKK4 (27). As shown in Fig. 4, C and D, the F1443A mutation alone decreased the activity of both AP1 and NF-\(\kappa\)B reporter genes (compare columns 2 and 5), consistent with previous studies (27), and this activity was further diminished by the introduction of either the I1394A or L1402A mutation (columns 3 and 4). Thus, both mutations, including the L1402A mutant that enhanced NF-\(\kappa\)B-inducing activity on its own, accentuate the loss of activity due to the F1443A mutation.

**Fig. 2.** Reporter gene assays with MEKK1\(\Delta\) mutants. HeLa cells grown in 24-well plates were cotransfected with wild type or mutant MEKK1\(\Delta\) expression constructs or pcDNA3, as indicated, 0.2 \(\mu\)g of pAP1-Luc or pNF-\(\kappa\)B-Luc, and 0.05 \(\mu\)g of pRL-TK. Luciferase activities were measured and normalized to that of the renilla luciferase (pRL-TK) internal transfection control. Shown is a representative result, performed in duplicate with standard deviations, from three independent experiments, with activities in relative light units. Activities of MEKK1\(\Delta\) mutants toward AP1 (A) and NF-\(\kappa\)B (B) reporter genes. Titration of I1394A and L1402A mutants toward AP1 (C) and NF-\(\kappa\)B (D) reporter genes. Aliquots of cell lysates were also subjected to Western blotting (WB) using anti-MEKK1 polyclonal antibodies. Position of His\(_6\)-MEKK1\(\Delta\) is indicated.
no significant binding to IKKβ (top panel, lane 5), whereas the L1402A mutant, which induced robust NF-κB activity, bound to IKKβ with an equal or even slightly higher affinity than wild type MEKK1Δ (top panel, lane 6). Collectively, the binding affinities of these mutants correlate with their activities in the reporter gene and immunocomplex kinase assays.

In additional experiments, we sought to examine the role of these residues in other MAP3Ks by substituting sets of residues corresponding to MEKK1 Leu-1402 and Arg-1403 (Val-568 and Leu-569, respectively), MEKK4 residues corresponding to MEKK1 Ile-1394 and Phe-1396 (Ala-1495 and Tyr-1497, respectively), and MLK3 residues corresponding to MEKK1 Leu-1402 and Arg-1403 (Val-294 and Lys-295, respectively). Thus, for example, the NIK mutant consisting of a double V568L/L569R mutation. These mutations variably diminished the native activity of those MAP3Ks, but failed, however, to convert NIK, an NF-κB activator, into a JNK activator; and either MEKK4 or MLK3, both JNK activators, to NF-κB activators, as assessed by reporter gene assays (data not shown).

The Binding of MKK4 to MEKK1Δ Protects the Latter from Limited Proteolysis—The mutagenesis experiments in this report collectively highlight a possible function of subdomain VIII in mediating in MEKK1 substrate specificity. The residues examined could comprise part of the contact site with MKK4; alternatively, their mutations might allosterically alter this contact site. To help distinguish between these possibilities, we performed limited proteolysis experiments. We first introduced a mutation (Q1405R/Q1406R, denoted QQ/RR) just C-terminal to the subdomain VIII residues examined in this study (Fig. 5B), thereby creating an optimal recognition site for the protease furin. It should be noted that this optimal sequence, RXRR X (where X denotes any amino acid and \( \downarrow \) denotes the cleavage site), is not present elsewhere in MEKK1Δ, nor in MKK4. Reporter gene and in vitro binding assays revealed the mutant to display activity, albeit somewhat diminished compared with wild type MEKK1Δ (Fig. 5, A and B). Introduction of a furin recognition sequence at a different site (N-terminal to subdomain VIII) completely abolished activity (data not shown).

We then incubated in vitro-translated, \(^{35}S\)S-labeled MEKK1Δ in the absence or presence of MKK4. In the former case, MEKK1Δ was then immunoprecipitated using antibodies against the C terminus of MEKK1. In the latter case, MEKK1Δ

**Fig. 3.** Immunocomplex kinase assays with MEKK1Δ mutants. COS-1 cells grown in 6-well plates were cotransfected with the indicated wild type or mutant MEKK1Δ constructs or pcDNA3, and 1 μg of either pCMV2-FLAG-MKK4 (A), pCMV-MKK7 (C), or pCMV-HA-MEK1 (D). FLAG-MKK4 (A), endogenous IKKβ (B), myc-MKK7 (C), or HA-MEK1 (D) was then immunoprecipitated and incubated in the presence of 0.5 μg of GST-JNK1 (K55R) (A and C), GST-IκBα (5-55) (B), or His<sub>6</sub>-ERK2 (D) in the presence of \(^{32}P\)ATP at 30 °C for 1 h to assay for kinase activity (KA). Reaction products were then subjected to SDS-PAGE and autoradiography. The relative kinase activities (Rel Act) are shown. E, 5 μl of cell lysates containing transiently expressed wild type or mutant MEKK1Δ were incubated with 0.5 μg of GST-MKK4(K131A) in presence of 100 μM ATP. Reaction mixtures were subjected to SDS-PAGE and Western blotting using antibodies against phospho-MKK4 (Thr-261). Aliquots of cell lysates were subjected to Western blotting using antibodies against the C terminus of MEKK1. In the presence of 0.5 μg of GST-JNK1 (K55R) (A and C), GST-IκBα (5-55) (B), or His<sub>6</sub>-ERK2 (D) in the presence of \(^{32}P\)ATP at 30 °C for 1 h to assay for kinase activity (KA). Reaction products were then subjected to SDS-PAGE and autoradiography. The relative kinase activities (Rel Act) are shown. The mutagenesis experiments in this report collectively highlight a possible function of subdomain VIII in mediating in MEKK1 substrate specificity. The residues examined could comprise part of the contact site with MKK4; alternatively, their mutations might allosterically alter this contact site. To help distinguish between these possibilities, we performed limited proteolysis experiments. We first introduced a mutation (Q1405R/Q1406R, denoted QQ/RR) just C-terminal to the subdomain VIII residues examined in this study (Fig. 5B), thereby creating an optimal recognition site for the protease furin. It should be noted that this optimal sequence, RXRR X (where X denotes any amino acid and \( \downarrow \) denotes the cleavage site), is not present elsewhere in MEKK1Δ, nor in MKK4. Reporter gene and in vitro binding assays revealed the mutant to display activity, albeit somewhat diminished compared with wild type MEKK1Δ (Fig. 5, A and B). Introduction of a furin recognition sequence at a different site (N-terminal to subdomain VIII) completely abolished activity (data not shown).

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complexed with His6-FLAG-MKK4 was immunoprecipitated with anti-FLAG antibodies. The immunoprecipitates were then incubated with furin. Wild type MEKK1Δ, which has a predicted size of 35 kDa, was not cleaved regardless of how it was immunoprecipitated (Fig. 5C, upper panel, lanes 1–3 and 7–9), consistent with its lack of furin recognition sites. In contrast, the QQ/RR mutant immunoprecipitated with the anti-MEKK1 antibody was cleaved by furin in a time-dependent manner, yielding two smaller bands of 26 kDa (band a, upper panel, lanes 5 and 6) and 9 kDa (band b), which are the sizes expected based on the predicted furin cleavage event.

Most importantly, the QQ/RR mutant complexed with MKK4 was resistant to cleavage (Fig. 5C, upper panel, lanes 10–12). No digestion products were observed even upon longer exposures (Fig. 5C, lower panel), nor upon additional repetitions of this experiment in which comparable levels of proteins were immunoprecipitated in all groups (data not shown). It might be noted that for both wild type and the QQ/RR mutant, there was a minor species at ~24 kDa (denoted MEKK1Δ). This species presumably possess an intact C terminus (because the immunoprecipitations in lanes 1–6 were performed using an antibody against the MEKK1 C terminus) and likely generated the 15 kDa band (labeled with *) that appears in the longer exposure. Significantly, neither this nor the other cleavage products were seen with the MEKK1Δ QQ/RR mutant complexed with MKK4 (Fig. 5C, lower panel). Although we cannot rule out the possibility that the anti-FLAG antibody might itself sterically interfere with furin cleavage of MEKK1Δ, in light of the other experiments presented in this report, the most likely explanation is that MKK4 blocks the access of furin to subdomain VIII, thus providing evidence that subdomain VIII is indeed a contact site for MKK4.
DISCUSSION

A fundamental mechanism by which specificity in signal transduction pathways is maintained is by protein/protein contacts. We provide evidence here that subdomain VIII plays a critical role in determining the specificity of the MAP3K MEKK1 for its substrates. One of the notable results of these studies is the identification of two mutations, I1394A and L1402A, that differentially affect the capacity of MEKK1Δ to induce AP1 versus NF-κB reporter genes, and activate MKK4 versus IKK. The results suggest that these residues, and by implication subdomain VIII, is critically involved in discriminating between and in fact contacting substrates, a notion supported by the limited proteolysis studies. One possibility is that Leu-1402 makes a critical contact with MKK4 whereas Ile-1394 makes one with IKK. MKK4 and IKKβ are both phosphorylated in a SXXX(S/T) motif located in subdomain VIII of these protein kinases; hence, it is conceivable that the subdomain VIII of these two substrates might be contact sites. It is also worth noting that mutation of these subdomain VIII residues in MEKK1Δ may also have effects on neighboring subdomain VIII residues, evaluation of which is limited in the present studies by their

**Fig. 5. Limited proteolysis studies with MEKK1Δ.** A, in vitro translated, 35S-labeled, wild type (WT) or Q1405R/ Q1406R (QQ/RR) MEKK1Δ was incubated with His6-FLAG-MKK4. The latter was then immunoprecipitated by anti-FLAG (M2) agarose. Eluates were subjected to SDS-PAGE and autoradiography. B, AP1 reporter gene assays were performed as described in legend to Fig. 2, with activities in relative light units. C, in vitro translated, 35S-labeled, wild type (WT) or QQ/RR MEKK1Δ was incubated in the absence (lanes 1–6) or presence (lanes 7–12) of His6-FLAG-MKK4 and immunoprecipitated with either anti-MEKK1 or anti-FLAG antibodies, respectively. The immunoprecipitates were then incubated with furin at 30 °C for the indicated times. Reactions were terminated and products subjected to 16% SDS-PAGE and autoradiography. Upper panel, short exposure; lower panel, long exposure. Positions of protein molecular mass standards (in kDa) are indicated on the left. The bands that appeared after furin incubation are denoted with a, b, and *, and MEKK1Δ and MEKK1'Δ are indicated on the right (see “Results”).
largely parallel inactivation of both the JNK and NF-κB pathways.

Our experiments cannot distinguish between M KK4 binding to the phosphorylated or the unphosphorylated form of MEKK1. The former is perhaps more plausible, because binding of M KK4 to subdomain VIII of MEKK1 might be predicted to preclude subsequent subdomain VIII phosphorylation of MEKK1. Consistent with this, we find that both T1381A and T1393A mutations of MEKK1Δ substantially diminish its binding to M KK4 (data not shown).

Moreover, a central role for Ile-1394 of MEKK1 in determining specificity comes from the fact that it corresponds to Pro-202 of CAMP-dependent protein kinase α and Arg-189 of ERK2, both of which have been implicated as being part of a pocket that binds the P + 1 site of substrate (35, 37). Along the same lines, a central role for Leu-1402 of MEKK1 is supported by the fact that it corresponds to Val-436 of p21-activated kinase 1, a contact residue for the inhibitory switch domain of the latter (31). The differential effect of different subdomain VIII mutations raises the possibility that individual residues in subdomain VIII may form different contacts with different substrates. In this regard, it should be noted that the docking groove of p38 binds only MKK3b and MEF2A, but that certain residues in the binding groove contact the two differently, such as Glu-160, which forms a hydrogen bond with the latter but not with the former (24).

In conjunction with our previous reports (27, 28), the present study provides evidence that the binding site in MEKK1 for MKK4 consists minimally of residues from subdomain VIII and subdomain X. It is interesting to note that mutagenesis studies on MAP2K/MAPK interactions have implicated both subdomain VIII and subdomain X of ERK as being critical to its interaction with MEK (38, 39). Whether the MAP3K/MAP2K interaction constitutes a homologous interaction that may even include docking groove interactions remains to be determined, and may eventually require structural studies on the MEKK1/ MKK4 complex.

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