Interdependent Domains Controlling the Enzymatic Activity of Mitogen-Activated Protein Kinase Kinase 1[†]

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ABSTRACT: The activation of human mitogen-activated protein kinase kinase 1 (MKK1) is achieved by phosphorylation at Ser218 and Ser222 within a regulatory loop. Partial activation was achieved by replacing these residues with aspartic/glutamic acid. Higher activity was obtained by introducing four acidic residue substitutions in the regulatory loop, indicating that acidic residues in the loop stabilize an active configuration by the introduction of negative charge. Activation of MKK1 is also achieved by deleting residues 44−51, N-terminal to the consensus catalytic core. Although substitution of residues within this segment by alanine does not affect activity, introduction of proline residues elevates kinase activity, indicating that activation results from perturbation of secondary structure within residues 44−51. Pseudosubstrate inhibition, a commonly observed mechanism of kinase regulation, is not operative in this process. Both the acidic substitutions and the N-terminal deletion increase V_{max}, $V/K_{m,ERK2}$, and $V/K_{m,ATP}$, as is also observed following phosphorylation of wild-type MKK1. A synergistic enhancement of these steady-state rate parameters occurs upon combining the mutations, suggesting that conformational changes induced by mutagenesis together mimic those seen upon phosphorylation.

Mitogen-activated protein kinase kinases (MKKs, or MEKs)[1] function in several MAP kinase signaling pathways that mediate cellular growth, differentiation, and stress responses [reviewed by Ahn (1993), Waskiewicz and Cooper, (1995), and Marshall (1995)]. This class of enzymes selectively activates various forms of the mitogen-activated protein kinase (MAPK) family by phosphorylation at conserved Thr and Tyr residues within a regulatory loop between subdomains VII and VIII of the MAPK core (Payne et al., 1991; Nakielny et al., 1992; Hanks & Hunter, 1995); thus the MKKs represent a family of dual-specificity protein kinases (Lindberg, 1992). MKK1 is highly specific for two isoforms of the MAPK family, ERK1 (p44 MAPK) and ERK2 (p42 MAPK) (Ahn et al., 1991; Gomez & Cohen, 1991; Matsuda et al, 1992; Crews & Erikson, 1992). Once activated, the ERKs target cytosolic and nuclear proteins for phosphorylation on serine and threonine residues within the consensus motif Pro-X-(Ser/Thr)*-Pro (Davis, 1993), thereby playing an essential role in regulating their function.

MKK1 is activated by phosphorylation at Ser218 and Ser222 in a regulatory loop between subdomains VII and VIII (Alessi et al., 1994; Zheng & Guan, 1994; Resing et al., 1995). A number of upstream protein kinases are capable of phosphorylating both of these sites *in vitro*, including Mos (Posada et al., 1993; Nebreda & Hunt, 1993), MEKK (Lange-Carter et al., 1993; Xu et al., 1995), and members of the Raf family (Kyriakis, 1992; Dent et al., 1992; Howe et al., 1992). Mos and Raf-1 are protooncogene products which are respectively activated during M-phase in maturing oocytes or during transition through early G_1 in proliferating cells (Yew et al., 1993). Oncogenic variants of these kinases elevate the activity of endogenous MKK1 and promote mammalian cell transformation [reviewed by Yew et al. (1993) and Daum et al. (1994)]. Transformation is also observed upon expression of constitutively active mutants of MKK1 (Mansour et al., 1994a; Cowley et al., 1994; Brunet et al., 1994; Catling et al., 1995), which confirms that sustained signaling by MKK1 deregulates normal programs of cell growth and cell cycle progression and further highlights the essential role played by inhibitory mechanisms that restrict the activation of MKK1 to early G_1 in proliferating cells. The high specificity of MKKs for various MAPKs identifies the MKKs as useful targets for drug intervention of proliferative and inflammatory diseases (Lee et al., 1994; Dudley et al., 1995); therefore, understanding the parameters that contribute to MKK regulation is of significant practical importance.

Activation of MKK1 can be achieved by substituting Ser218 and Ser222 with acidic residues (Mansour et al., 1994a; Cowley et al., 1994; Brunet et al., 1994; Huang et al., 1995) or by deleting residues 32−51 (Mansour et al., 1994). The latter observation is intriguing because it indicates that perturbations outside the catalytic core influence catalytic rates. To further define the mechanism by which these mutations induce enzymatic activation, point mutants within the regulatory loop and deletion and point

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[1] Abbreviations: MKK1, mitogen-activated protein kinase kinase 1; MAPK, mitogen-activated protein kinase, cAK, cyclic AMP-dependent protein kinase; NTA, nitrilo triacetic acid. Deletion mutant nomenclature: ΔN1 deletes residues 1−32; ΔN2 deletes 1−52; ΔN3 deletes 32−51; ΔN4 deletes 44−51; ΔN5 deletes 38−43; ΔN6 deletes 32−37; ΔN7 deletes 47−49; ΔN8 deletes 44−46; ΔN9 deletes 44−49; ΔN10 deletes 36−43.

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mutants in regions flanking the kinase core were constructed and analyzed. The results reveal a unique mechanism for protein kinase activation involving conformational rearrangements driven by electrostatic interactions within the regulatory loop as well as rearrangements in secondary structure at the N-terminus.

MATERIALS AND METHODS

Bacterial Expression Constructs and Protein Purification. Constructs are based on the pRSET plasmids (InVitrogen), which drive gene expression from the T7 promoter in the bacterial strain BL21(DE3)pLysS (Novagen). The genes encoding ΔN1 or ΔN2 MKK1 were expressed in pRSETb, while all other variants were expressed in pRSETa. The two pRSET plasmids contain restriction sites in different reading frames downstream of a hexahistidine tag-encoding sequence. Single-stranded DNA was derived from construct pKH1 (pRSETa + human wild-type MKK1) (Mansour et al., 1994b), and site-directed mutagenesis was carried out using the Muta-gene phagemid in vitro mutagenesis kit (Bio-Rad). Deletions affecting the amino terminus between residues 32 and 51 were generated by changing the sequence of the third *Stu*I site in the open reading frame of MKK1 without affecting the encoded amino acids, followed by substituting the 60-bp fragment between the remaining *Stu*I sites with a double-stranded fragment encoding the desired sequence. Mutagenesis was confirmed by sequencing the targeted DNA region.

Recombinant MKK1 contained a hexahistidine tag at the N-terminus and was purified in soluble form as previously outlined (Mansour et al., 1994b) following elution from a Ni^{2+}−NTA−agarose (Qiagen) affinity column and further purification by DEAE-Sephacel (Pharmacia) chromatography. Protein concentration was determined after resolving MKK by SDS−PAGE and quantifying the Coomassie-stained band by laser densitometry relative to bovine serum albumin standards.

ERK2 expression plasmids encoding Lys52Arg or Thr183Ala mutants were a generous gift of Melanie Cobb. Proteins were purified by Ni^{2+}−NTA−agarose chromatography as described (Robbins et al., 1993).

Kinase Assays and Activity Measurements. Basal activities of wild-type and mutant MKK1 were measured at 30 °C under standard reaction conditions of 20 mM HEPES (pH 7.4), 2 mM dithiothreitol, 0.01% Triton X-100, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (∼2000 cpm/pmol), and 1 μM ERK2 (Lys52Arg or Thr183Ala mutants), at concentrations of MKK ranging from 0.5 to 10 nM. Time points were sampled at 2, 4, and 6 min by quenching assay mixtures with Laemmli sample buffer. Phosphorylation of ERK2 was quantified by SDS−PAGE/PhosphorImager analysis, and rates were determined from linear least squares regression of each time course measured in duplicate (total of six data points). Under the conditions described, time courses of ERK2 phosphorylation by MKK were linear.

Measurements of steady-state rate parameters were carried out under conditions described above, except that ATP concentrations (2.5, 6.4, 16, 40, 100, and 250 μM) were varied against ERK2(Thr183Ala) concentrations (0.1, 0.25, 0.64, 1.6, 4, and 10 μM). Measurements were performed using ERK2(Thr183Ala) as substrate because ERK2(Lys52Arg) showed substrate inhibition at concentrations greater than 3 μM (data not shown). However, the rates of ERK(Thr183Ala) phosphorylation are similar to those of ERK(Lys52Arg) since tyrosine phosphorylation of ERK is more rapid than threonine phosphorylation.[2] Reactions were quenched after 5 min, and phosphorylation of ERK2 was quantified by SDS−PAGE/PhosphorImager analysis. Kinetic parameters were obtained by nonlinear least-squares fit of the data to the steady-state rate expression for two substrate enzymes (Segel, 1975): $v_{obs} = V_{max}/(1 + K_{m,ATP}/[ATP] + K_{m,ERK2}/[ERK2] + K_{i,ATP}K_{m,ERK2}/[ATP][ERK2])$, using the SigmaPlot program (Jandel Corp.). Each set of parameters and their standard errors was obtained from 36 substrate combinations measured in duplicate (total of 72 data points).

Recombinant MKK1 was activated in vitro by v-Mos immunoprecipitated from Swiss 3T3 (Tx-7) cells or by Raf-1 immunoprecipitated from CHO-Raf cells as described (Resing et al., 1995; Mansour et al., 1994a,b). MKK activation reactions were carried out for 2 h at 30 °C and included 240 nM MKK, 0.1 mM ATP, 10 mM $MgCl_2$, 20 mM HEPES (pH 7.4), 2 mM dithiothreitol, and 0.01% Triton X-100, plus immunoprecipitated v-Mos or Raf-1 respectively bound to protein A−or protein G−Sepharose CL-4B beads. Under these conditions, phosphorylation stoichiometries range from 0.5 to 1 mol/mol, although a significant percentage represents phosphate incorporated into several autophosphorylation sites (Resing et al., 1995). The beads were removed by centrifugation, and activated MKK was subsequently diluted to 2 nM with 20 mM HEPES, 2 mM dithiothreitol, and 0.01% Triton X-100 (total volume, 90 μL) and incubated under standard reaction conditions described above at 30 °C in the presence of 1 μM ERK2. Time points were sampled at 2, 4, and 6 min by quenching the assay mixture with Laemmli sample buffer. ERK2 was resolved by SDS−PAGE and the incorporated radiolabel was quantified by PhosphorImager analysis. Control reactions were carried out using nonactivated MKK at a final concentration of 10 nM.

Circular Dichroism Measurements. The ΔN3 peptide ALQKKLEELELDEQQRKRLE, based on the MKK1 sequence between residues 32 and 51, was synthesized by the HHMI Synthesis Facility (University of Texas Southwestern Medical Center at Dallas) and desalted on a SepPak C18 cartridge. Peptide purity and sequence were confirmed by electrospray mass spectrometry. Circular dichroism (CD) measurements were performed on an AVIV 62DS spectrophotometer equipped with a thermoelectric temperature control unit. Samples were placed in 1-mm strain-free quartz cuvettes and equilibrated at 25 °C prior to measurement. CD spectra were scanned using 0.5-nm band steps, 1-nm bandwidth, and 3-s averaging time. The peptide was analyzed at 25 °C, at a concentration of 0.1 mg/mL (w/v) in 40 mM sodium phosphate (pH 7.4) or 20 mM sodium phosphate (pH 7.4)/50% trifluoroethanol (Pierce). Evaluation of α-helical content was determined using SELCON, a self-consistent field numerical method (Sreerama & Woody, 1993).

[2] The $V_{max,app}$ and $K_{m,ERK}$ values for phosphorylation of ERK mutants by MKK(ΔN3/S218E/S222D) at 0.1 mM ATP were: ERK(Lys52Arg) 1.5 min^{-1}, 1.3 μM; ERK(Thr183Ala) 1.7 min^{-1}, 2.0 μM; ERK(Tyr185Phe) 0.5 min^{-1}, 0.6 μM.

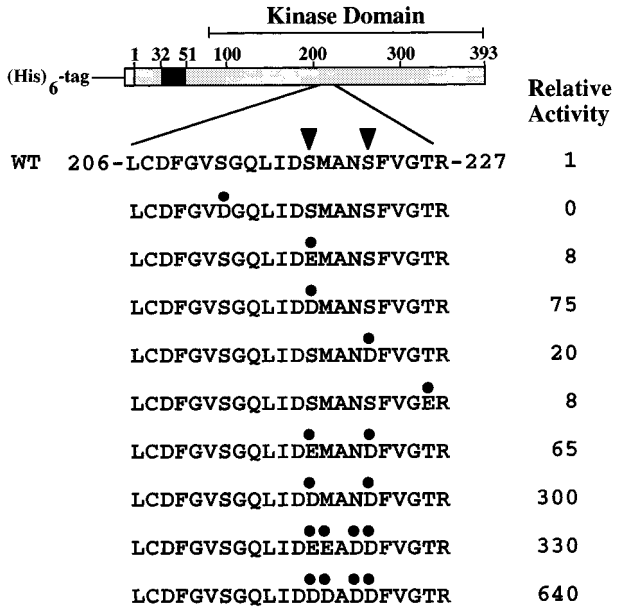

FIGURE 1: Acidic amino acids substitute for phosphoserine in the regulatory loop. The schematic diagram depicts hexahistidine-tagged MKK1 protein. A portion of the polypeptide sequence in the regulatory loop mapping between subdomains VII and VIII is shown, with the arrowheads pointing at the two phosphorylated sites, Ser218 and Ser222. Mutated amino acids are indicated by a black dot on top of the residue. Activities were measured against ERK2(Lys52Arg) as described in Materials and Methods and were normalized to the basal activity of wild-type MKK1 (0.11 nmol min^{-1} mg^{-1} or 0.006 min^{-1}).

RESULTS

Activation of MKK1 by Mutations in the Regulatory Loop. In order to examine the effect of introducing negatively charged residues into the regulatory loop between subdomains VII and VIII of the kinase core, a series of mutants was constructed replacing the activating phosphorylation sites Ser218 and Ser222 with glutamic or aspartic acid. Specific activities were measured toward the substrate ERK2-(Lys52Arg). In general, aspartic acid substitutions yielded higher basal activities than glutamic acid substitutions. Higher basal activities were observed upon substitution of Ser218 compared to Ser222; synergistic enhancement of enzymatic activity was observed when mutations were made in combination (Figure 1). The specific activity of the S218D/S222D mutant was ~35 nmol min^{-1} mg^{-1} (1.8 min^{-1}), 300-fold greater than the basal activity of the wild-type enzyme.

Acidic substitutions were incorporated at other sites within the regulatory loop in order to compare the effect of introducing negative charge near the Ser218/Ser222 sites. Glutamic or aspartic acid was substituted in place of Met219 and Asn221 in addition to Ser218 and Ser222, generating a cluster of negatively charged residues between residues 217 and 222. The substitutions at positions 219 and 221 further elevated the activity of MKK1 by 2−5-fold compared to the Ser218/Ser222 mutants (Figure 1). Again, larger increases in rate were observed when these serine residues were replaced with aspartic acid instead of glutamic acid. Modification of residues outside of this cluster resulted in less substantial effects which were opposite in effect. Replacement of Thr226 with glutamic acid yielded a modest increase in basal activity (Figure 1), which did not synergize with acidic substitutions at either Ser218 or Ser222 (data not shown). Replacement of Ser212 with aspartic acid completely abolished basal kinase activity toward exogenous substrates (Figure 1), although no effect was observed on the ability of this mutant to autophosphorylate (data not shown).

Table 1: Enhancement of MKK1 Mutant Activities after Treatment with v-Mos or Raf-1[a]

	specific activity (nmol min^{-1} mg^{-1})		
	basal	+ v-Mos	+ Raf-1
wild type	0.08	53	12
ΔN1[b]	0.06	13	3.3
ΔN2[c]	0.06	18	2.7
ΔN3[d]	0.65	140	30

[a] ERK2(Thr183Ala) was used as the substrate. [b] ΔN1 lacks residues 1−32. [c] ΔN2 lacks residues 1−52. [d] ΔN3 lacks residues 32−51.

Activation of MKK1 by Deletion of Residues 32−51. The deletion of residues 32−51 increases the basal activity of MKK1 (Mansour et al., 1994a), suggesting that this region of the protein may play a restraining or inhibitory role. We first performed experiments to test whether these residues regulate MKK1 by pseudosubstrate inhibition, a commonly observed regulatory mechanisms for protein kinases, where activity is maintained in a low basal state by intrasteric interactions between pseudosubstrate domains and the catalytic cleft (Kemp et al., 1994).

Deletion of residues 1−32 (ΔN1) or 1−52 (ΔN2) yielded mutants that maintained the low basal activity of wild-type MKK, but were nevertheless activatable by Raf-1 or v-Mos (Table 1). This indicates that the mere elimination of residues 32−51 is insufficient to activate the enzyme; indeed, the elevated basal activity of ΔN3 requires the presence of residues 1−32. A further test for pseudosubstrate inhibition is inhibition of enzymatic activity in trans by synthetic peptides that are exogenously added. The activities of the constitutively active mutants, MKK1(ΔN3) (10 nM) or MKK1(ΔN3/Ser218Glu/Ser222Asp) (0.5 nM), were measured in the presence of excess (1.6 mM) ΔN3 peptide, a synthetic peptide containing MKK1 residues 32−51. Activities changed by less than 15% under standard assay conditions (data not shown), demonstrating lack of trans inhibition. Together, these results indicate that activation by the ΔN3 internal deletion is not caused by removal of a pseudosubstrate sequence.

Perturbations in Secondary Structure at the N-Terminus. In order to determine which amino acids contributed most to stabilization of the inactive conformation, residues between 32 and 51 were all replaced with alanine and the resulting mutant (Ala_{32-51}) was examined against wild-type MKK1. Unexpectedly, the basal specific activity of Ala_{32-51} was comparable to that of wild-type kinase (Figure 2), indicating that substitution of all 20 residues by alanine maintains the enzyme in its inactive state. Both wild-type and Ala_{32-51} were activated by v-Mos or by acidic amino acid substitutions Ser218Glu/Ser222Asp (Figure 2), confirming that the low basal activity of Ala_{32-51} was not due to perturbations in protein folding. The results indicate that specific contacts between side chains in this region and the rest of the molecule have minimal influence on activity and suggest that secondary structure of the N-terminus stabilizes the inactive conformation of MKK1.

Secondary structure predictive parameters, such as the conformational preferences of Chou and Fasman (1978) or

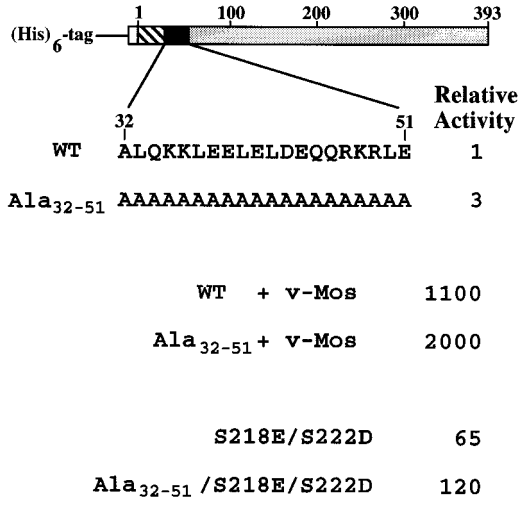

FIGURE 2: Mutant Ala_{32-51} and wild-type MKK1 share similar properties. Mutant Ala_{32-51} replaces MKK1 residues 32−51 with polyalanine. Activities of wild-type and Ala_{32-51} MKK1 before and after phosphorylation by immunoprecipitated v-Mos were measured against 1 μM ERK2(Thr183Ala) and normalized to the basal activity of wild-type MKK1 (0.08 nmol min^{-1} mg^{-1} or 0.004 min^{-1}). Activities of the two mutants, Ser218Glu/Ser222Asp and Ala_{32-51}/Ser218Glu/Ser222Asp, were measured against 1 μM ERK2-(Lys52Arg) and normalized to the activity of wild-type MKK1 (0.11 nmol min^{-1} mg^{-1}).

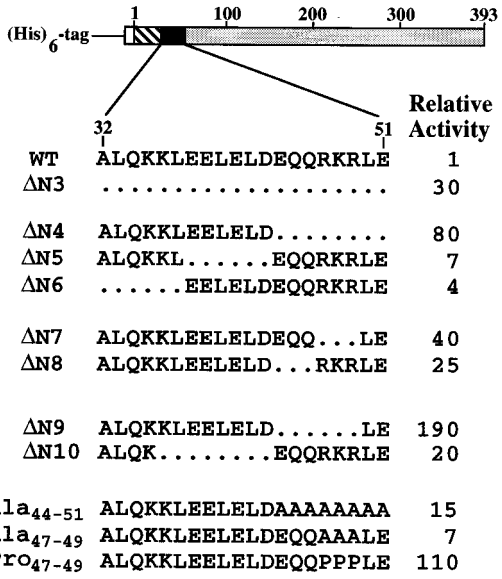

FIGURE 3: Identification of the residues critical for enhancement of enzymatic activity. Mutants containing various internal deletions and substitutions within residues 32−51 were constructed and activities were measured against 1 μM ERK2(Lys52Arg) and normalized to the activity of wild-type enzyme as in Figure 1.

O'Neil and Degrado (1990), as well as the [n; $n + 3$, $n + 4$] spacing between oppositely charged residues within this sequence, suggested that amino acids 32−51 might adopt an α-helical character. Secondary structure predictions using the procedure of Rost and Sander (1993) delineated residues 44−55 as a region of highest α-helical probability (data not shown). Furthermore, alignment of MKK1 against cAMP-dependent protein kinase (Mansour et al., 1994a) shows residues 44−51 overlapping with the A-helix of cAMP-dependent protein kinase (residues 15−31 of cAK) (Veron et al., 1993; Zheng et al., 1993). Several mutants were therefore generated to test whether activation by the ΔN3 deletion involves disruption of α-helical secondary structure.

Residues 32−51 were subdivided into three parts in order to identify the residues critical for enhancing basal activity (Figure 3). Deletion of residues 44−51 (ΔN4) yielded a mutant displaying about 80-fold higher activity, whereas deletions spanning residues 32−37 (ΔN6) or 38−43 (ΔN5) resulted in mutants with minor changes in activity (Figure 3). Four additional mutants were constructed to test whether activation by ΔN4 depends upon the number of amino acids removed vs the removal of specific residues. While deletion of eight amino acids (residues 36-43, ΔN10) from the center of this region resulted in a mutant with relatively low activity, deletion of six amino acids (residues 44−49, ΔN9) from the C-terminus of this region resulted in a mutant with activity 190-fold higher than basal activity (Figure 3), supporting the conclusion that the activation is mainly determined by loss of residues 44−49. Removal of residues 44−46 (ΔN8) or 47−49 (ΔN7) within this region yielded mutants with basal activities significantly lower than ΔN9, further supporting the functional importance of residues 44−49.

Because our approach relied on fusing residues upstream and downstream of a deleted region, the resulting activation might be explained by complex changes in side-chain interactions due to relocalization of residues. To control for this possibility, residues between 44 and 51 were replaced rather than deleted. Replacement of residues 44−51 with polyalanine (Ala_{44-51}) moderately enhanced the catalytic rate but failed to elevate the activity to a level matching the ΔN4 deletion (Figure 3). Polyalanine was chosen in this substitution with the expectation that it might introduce a short helical domain, thus conserving a potential α-helical domain. Other mutants were generated that targeted three amino acids at residues 47−49 by introducing polyalanine (Ala_{47-49}) or polyproline (Pro_{47-49}) substitutions. While the Ala_{47-49} substitution again led to minimal activation, insertion of polyproline yielded an activity 110-fold greater than that of wild-type MKK1 (Figure 3). Again, the behavior of the substitution mutants suggested that secondary structure rather than side-chain interactions stabilize the enzyme in its inactive state. The behavior of the polyproline substitution mutant is consistent with a mechanism of activation involving α-helical disruption.

In order to test whether MKK1 residues 32−51 could at least theoretically adopt an α-helical secondary structure, the far-UV circular dichroism spectrum of a synthetic peptide with this sequence was examined. A helical content of 40% was observed for this peptide in 50% trifluoroethanol (data not shown). Although the spectrum for this peptide in water demonstated a nascent helix with only 13% helical content (data not shown), small peptide fragments derived from α-helical domains of proteins often show only nascent helical content in water (Waltho et al., 1993; Maciejewski & Zehfus, 1995). In less polar solvents like trifluoroethanol, such peptides can sometimes exhibit helical contents similar to those found in intact native proteins (Dyson et al., 1992; Vonderviszt et al., 1992; Maciejewski & Zehfus, 1995).

Two hydrophobic residues, Phe53 and Leu54, located near the C-terminal region of the deleted domain, align with Phe17 and Leu18 of cAK (Mansour et al., 1994a). In the X-ray structure of cAK, Phe17 and Leu18 appear to stabilize the A-helix through hydrophobic contacts with the N-terminal lobe (Veron et al., 1993; Zheng et al., 1993), suggesting that Phe53 and Leu54 might play a similar structural role in MKK1. Substitution of these residues with aspartic acid

Table 2: Activation by the ΔN4 Deletion Does Not Depend on Phosphorylation of S218 and S222[a]

ΔN4[b]	218	222	relative activity
	S	S	1
+	S	S	80
	A	A	1
+	A	A	40

[a] Activities were measured using ERK2(Lys52Arg) as the substrate. [b] + indicates that the deletion is present.

resulted in 10-fold enhancement in enzymatic activity (data not shown). Substitution of the next residue, Thr55, with alanine or aspartic acid had no effect on activity (data not shown).

Activation by ΔN4 Does Not Require Phosphorylation of Ser218 and Ser222. Wild-type MKK1 autophosphorylates at Ser218, Ser298, Ser299, Tyr300, and Thr23 by an intramolecular mechanism, but these reactions are very slow (Resing et al., 1995). In contrast, the constitutively active N-terminal deletion mutants autophosphorylate at a faster rate on serine, threonine, and tyrosine residues (data not shown), raising the possibility that the mechanism of activation by the N-terminal deletions are in part due to enhanced autophosphorylation at Ser218 or Ser222. To test this possibility, a mutant combining ΔN4 with Ser218Ala and Ser222Ala mutations was produced and compared to mutants containing either ΔN4 or Ser218Ala/Ser222Ala alone (Table 2). The ΔN4/Ser218Ala/Ser222Ala mutant was about 40 times more active than wild-type MKK1, indicating that the ΔN4 deletion increases catalytic efficiency by means other than autophosphorylation at these serine residues.

To address whether activation may be caused by autophosphorylation at other sites, we tested the sensitivity of Ser218Glu/Ser222Asp and ΔN3/Ser218Glu/Ser222Asp activity to dephosphorylation by phosphatase 2A. Both mutants retained their elevated basal activity relative to that of wild-type enzyme (data not shown), also suggesting that these mutants are not activated by mechanisms involving autophosphorylation.

Interactions between Regulatory Loop and N-Terminal Mutations. Previous estimates indicated that phosphorylation of MKK1 by Raf-1 results in ~5000-fold increase in kinase activity (190 nmol min^{-1} mg^{-1}, after correction for basal) (Alessi et al., 1994; Dent et al., 1994). In contrast, mutagenesis by regulatory loop substitutions or N-terminal deletions led to activities that were at most 640-fold greater than that of wild type (70 nmol min^{-1} mg^{-1} or 3.5 min^{-1}). Therefore, hybrid enzymes combining these mutations were constructed to see whether activities approaching that of phosphorylated MKK1 could be achieved. Combinations of regulatory loop substitutions with N-terminal deletions led to enhanced activities that were greater than additive, suggesting that the two classes of mutations might cooperate to activate the enzyme (Table 3). In general, activities of the hybrid mutants followed trends observed with regulatory loop substitutions. For example, aspartic acid substitutions within the regulatory loop of hybrid mutants resulted in higher activities than glutamic acid substitutions. However, variations in activities of individual N-terminal deletions were not reflected in hybrid mutant activities; e.g., little difference was observed between hybrid mutants containing ΔN3 vs ΔN4. A hybrid between the activating deletion, ΔN4, and the strongest activating acidic substitutions, Ser218Asp/Met219Asp/Asn221Asp/Ser222Asp, yielded the most active mutant, which had an activity 2300-fold greater than that of wild type (250 nmol min^{-1} mg^{-1} or 13 min^{-1}).

Table 3: Effects of Combining N-Terminal Deletions with Acidic Substitutions[a]

ΔN3	ΔN4	ΔN5	ΔN6	S218	M219	N221	S222	relative activity
				S	M	N	S	1
+[b]				E				100
+							D	270
+				E			D	490
+				D			D	720
	+			E			D	530
	+			D			D	640
		+		E			D	270
			+	E			D	55
	+			E	E	D	D	710
	+			D	D	D	D	2300

[a] Activities were measured using ERK2(Lys52Arg) as the substrate. [b] + indicates that the deletion is present.

Comparison of Phosphorylation vs Mutagenesis on Kinetic Rate Parameters. To extend our kinetic characterization, we compared the effects of phosphorylation vs mutational activation of MKK1 on steady-state rate parameters. K_m for ATP decreased by almost 2 orders of magnitude upon phosphorylation by v-Mos and by 1 order of magnitude upon incorporation of the ΔN4 deletion, acidic substitutions at Ser218 and Ser222, or the combination of these mutations (Table 4). K_m for ERK2 decreased by 55-fold upon phosphorylation and by 8- and 30-fold due to the ΔN4 and acidic substitutions, respectively, with the hybrid enzyme showing a K_m comparable to that of phosphorylated MKK1. V_{max} increased by 4-fold for each mutation alone and increased synergistically by 310-fold upon combining the ΔN4 deletion and the acidic substitutions. This synergy was also revealed by increased $V_{max}/K_{m,ATP}$ and $V_{max}/K_{m,ERK2}$ for the hybrid enzyme, again indicating that the conformational changes induced by the two separate classes of mutations interact with each other to mimic the effects of phosphorylation.

Carboxyl-Terminal Truncations Inactivate MKK1. The carboxyl terminus of MKK1 extends by about 40 amino acids beyond a conserved arginine (Arg349) in subdomain XI, which marks the end of the kinase core (Hanks & Hunter, 1995). The functional importance of the sequence beyond subdomain XI was explored by constructing mutants truncated at Gly373, Glu367, Arg363, His358, or Ile343. The basal activities of these proteins were comparable to that of the wild-type enzyme (data not shown), indicating that the catalytic apparatus was intact in these mutants. However, phosphorylation by immunoprecipitates of v-Mos did not activate these mutants significantly, and the combination of the Gly373 truncation with MKK1(ΔN4/Ser218Glu/Ser222Asp) yielded a hybrid protein with only 10% the activity of ΔN4/Ser218Glu/Ser222Asp (data not shown). Thus the carboxyl-terminal residues appear to play an essential role in enzyme activity but do not play a negative regulatory role.

DISCUSSION

In this study, mutagenesis was employed in order to probe the conformational changes controlling the activation of

Table 4: Steady-State Rate Parameters for Basal and v-Mos-Activated Wild-Type MKK1 and for Constitutively Activated MKK1 Mutants[a]

	V_m (min^{-1})	$K_{m,ATP}$[b] (μM)	$V_m/K_{m,ATP}$ (μM^{-1} min^{-1})	$K_{m,ERK2}$[c] (μM)	$V_m/K_{m,ERK2}$ (μM^{-1} min^{-1})	v_{obs}[d] (min^{-1})
WT MKK (basal)	0.06 ± 0.006	308 ± 39	0.0002	19 ± 2	0.003	0.003
WT MKK (v-Mos)	1.45 ± 0.05 (24)[e]	3.5 ± 0.8	0.42 (2100)	0.34 ± 0.06	4.2 (1400)	1.1 (370)
ΔN4 MKK	0.26 ± 0.01 (4)	32 ± 3	0.008 (40)	2.5 ± 0.2	0.10 (33)	0.072 (24)
S218E/S222D MKK	0.27 ± 0.005 (4)	21 ± 2	0.013 (65)	0.68 ± 0.05	0.40 (130)	0.16 (53)
ΔN4/S218E/S222D	18.8 ± 0.7 (310)	23 ± 3	0.83 (4200)	0.36 ± 0.05	52 (17 000)	13 (4400)

[a] ERK2 (Thr183Ala) was used as substrate; therefore, activity measurements are for tyrosine phosphorylation of ERK2. [b] $K_{m,ATP}$ in the limit of infinite [ERK2]. [c] $K_{m,ERK2}$ in the limit of infinite [ATP]. [d] Initial rates calculated at physiological substrate concentrations (ERK2 $\sim$ 1 μM, ATP $\sim$ 1 mM). [e] Numbers in parentheses represent *x*-fold enhancement compared to wild-type MKK (basal).

MKK1. These mutations centered around three regions of the molecule, the active-site loop that is normally phosphorylated during activation, the N-terminus (residues 1−51) and the C-terminus (residues 343-393). Replacement of several residues in the active-site loop demonstrated the importance of a cluster of five amino acids in which replacement by negatively charged amino acids induced activation. We had previously shown that deletion of 20 residues near the N-terminus (residues 32−51) led to enzyme activation. This effect was narrowed to residues 44−51 and was shown to be caused by perturbations to the secondary structure. Residues 1−31 were required for this effect on activity, presumably through contacts formed with the protein surface. Finally, the C-terminus was shown to be necessary for enzyme activation. The stability of the inactive conformation does not involve intrasteric competition by an autoinhibitory or pseudosubstrate domain. Instead, it appears that structural rearrangements within different regions of the molecule cooperate to switch the enzyme to an active state.

For MKK1, replacement of phosphorylatable serines by acidic groups achieves 300-fold activation, and introduction of additional negatively charged residues further increases this activity by 2−5-fold. This effect suggests that electrostatic interactions between negatively charged residues in the regulatory loop and nearby residues account for part of the mechanism of activation by mutagenesis. Such interactions include salt bridges or formation of polarized hydrogen bonds with other residues. Similarly, electrostatic interactions induced by phosphorylation of the regulatory loop may contribute to conformational changes that lead to activation. Ultimate proof of this model will require more extensive analysis by site-directed mutagenesis.

Different protein kinases that are regulated by phosphorylation show variable effects upon substitution of phosphorylation sites by negatively charged residues. While the strategy has proven useful for constructing constitutively active mutants of MKK 1 and 2 (Mansour et al., 1994a, 1996; Cowley et al., 1994; Brunet et al., 1994; Huang et al., 1995), it does not work for the closely related MKK5 (English et al., 1996). We speculate that kinases may show variable dependences on electrostatic forces in stabilizing their high-activity states. For example, the X-ray crystallographic structure of cAK shows a salt bridge linking a stably phosphorylated residue in the regulatory loop, Thr197, with residue His87 located in the C-helix of the N-terminal lobe (Zheng et al., 1993; Knighton et al., 1991). Mutation of Thr197 to aspartic acid or alanine resulted in 9-fold enhancement of the intrinsic k_{cat} of cAK by the Thr197Asp over the Thr197Ala mutant, indicating that the salt bridge stabilizes the high-activity form by −1.3 kcal/mol (Adams et al., 1995). On the other hand, substitution of Thr183 or Tyr185 phosphorylation sites with glutamic or aspartic acid caused little change in ERK2 activity (Zhang et al., 1995), as did increasing negative charge in this region by substituting neighboring residues with acidic amino acids.[3] Several basic residues are located near Thr183 and Tyr185 in the X-ray structure of ERK2 (Zhang et al., 1994, 1995). While these residues might interact electrostatically, the results from mutagenesis suggest that such interactions would contribute minimally to the free energy of enzyme activation. In agreement, the crystallographic structure of a Thr183Glu/Tyr185Glu ERK2 mutant shows increased disorder in the regulatory loop, suggesting that the consequence of increased negative charge is to enhance motion within this region (Zhang et al., 1995), not to stabilize an active conformation.

Other perturbations of the regulatory loop are known to result in MKK activation. In the yeast MKK homologs, STE7 and MKK1, mutation of Ser368 to proline resulted in gain-of-function phenotypes, and STE7(Ser368Pro) showed 6-fold higher activity in immune complex assays compared to wild-type enzyme (Yashar et al., 1995). Ser368 aligns with Arg227 in human MKK1 (Figure 1) and is adjacent to Thr226 on human MKK1, where substitution by glutamic acid elevates the basal activity by 8-fold (Figure 1). Thus, mutations that perturb the regulatory loop C-terminal to the Ser218/Ser222 cluster elevate MKK activity, suggesting that larger movements may occur on the C-terminal side of the acidic cluster during phosphorylation, while N-terminal residues are more constrained.

A key component in the mechanism of MKK regulation is revealed by the activation seen upon deletion of residues 32−51, in which residues 44−51 play the most important role in maintaining the enzyme inactive state. Mutations in a homologous region also enhanced the specific activity of MKK2 (Mansour et al., 1996). These residues align with the A-helix at the amino terminus of cAK [Veron et al., 1993; Zheng et al., 1993; see Mansour et al. (1994a) for sequence alignment]. The circular dichroism spectrum of a synthetic peptide based on residues 32−51 is consistent with an α-helical conformation, and substitution of residues in this region by polyalanine, which would likely retain α-helical structure, stabilized the inactive conformation of MKK. In contrast, substitution of residues within this region by proline, which would disrupt α-helical structure, led to marked enzymatic activation. These results support our hypothesis that an α-helical conformation in this region stabilizes the inactive conformation. Overall, the data indicate that specific contacts between residues 44−51 and the rest of the molecule are less important than secondary structure in controlling the conformational switch between inactive and active enzyme.

[3] S. J. Mansour, J. M. Candia, and N. G. Ahn, unpublished results.

We hypothesize that residues C-terminal to position 52 in MKK1 are also important for stabilizing the inactive conformation. The behavior of MKK1(ΔN3) compared to ΔN1 or ΔN2 mutants indicates that residues 1−31 are required for enhancement of activity upon deletion of residues 32−51 but not for enhancement of activity upon phosphorylation (Table 1). In the ΔN3 mutant, the placement of residues 1−31 next to residue 52 could augment the disruption of such stabilizing contacts. On the other hand, phosphorylation of the ΔN2 mutant could disrupt these contacts via conformational changes in the regulatory loop without the participation of residues 1−31. This model is consistent with our finding that substitutions at Phe53 and Leu54 with aspartic acid enhance basal activity, suggesting that inhibitory constraints on the enzyme are relieved to some extent by disrupting contacts involving these hydrophobic side chains.

Two mutations of MKK that have been identified in genetic screens might be explained by conformational rearrangements occurring at the N-terminus, similar to those we have observed. An activated MKK1 mutant with a Gln56Pro substitution was identified in a screen for suppressors of transformation-defective v-Ha-Ras in Rat2 cells (Bottorff et al., 1995). This residue maps within the putative N-terminal α-helix, suggesting that proline substitution at this site elevates enzymatic activity by a mechanism similar to that of mutant Pro_{47-49}. A gain-of-function allele of *Drosophila* D-MEK contains an N-terminal mutation, Asp87Val, which aligns with Asp67 of human MKK1 (Lu et al., 1994) and is C-terminal to the putative α-helix. When we incorporated an Asp67Val mutation into human MKK1, a 7-fold enhancement of basal activity was observed.[3]

Comparison of steady-state rate parameters for regulatory loop vs N-terminal MKK1 mutants shows that both mutations reproduce the effects of MKK phosphorylation. Phosphorylation of MKK1 by v-Mos increased V_{max} by 24-fold and decreased K_m for ATP and ERK2 by nearly 2 orders of magnitude. Although the effect in V_{max} was underestimated due to limitations in stoichiometry of phosphorylation by v-Mos, the K_m values accurately reflect changes due to phosphorylation because rate contribution from nonphosphorylated MKK1 is insignificant. [Thus, at physiological concentrations of ATP (1 mM) and ERK2 (1 μM)[4] , the increase in the rate of reaction would primarily be due to the decrease in $K_{m,ERK}$ and increase in V_{max}.) Detailed kinetic studies of cAK, v-Fps, and Csk show that V_{max} can be limited by product dissociation and that $K_{m,ATP}$ and $K_{m,peptide}$ are complex functions of substrate binding/dissociation, intrinsic catalysis, and product dissociation (Adams & Taylor, 1992; Cole et al., 1994; Wang et al., 1996), so we cannot assume that V_{max} for MKK1 reflects the intrinsic k_{cat} or that K_m approximates K_d. However, mutagenesis at the regulatory loop vs the N-terminus led to changes in V_{max}, $K_{m,ATP}$, and $K_{m,ERK2}$ that followed the same trends as those seen upon phosphorylation of wild-type MKK1. Furthermore, whereas either mutation failed to completely mimic the effect of phosphorylation, combining the mutations raised the specific activity to levels previously reported for phosphorylated MKK1. Literature estimates report a specific activity of 190 nmol min^{-1} mg^{-1} after coexpression of MKK1 in Sf9 insect cells with c-Ha-Ras, $pp60^{Src}$ (Y527F), and Δ7-Raf-1 (Dent et al., 1994), and our most active mutant (ΔN4/S218D/M219D/N221D/S222D) exhibited a specific activity of 250 nmol min^{-1} mg^{-1} after expression in *Escherichia coli*.. Together, this is reasonable evidence favoring a model in which activation by N-terminal deletions or phosphorylation-site substitutions mimic conformational changes normally induced by phosphorylation.

Our results are consistent with a model in which disruption of an α-helix at the N-terminus creates new intramolecular interactions which augment structural rearrangements within the regulatory loop, favoring an active conformation. The effects of combining the Ser218Glu/Ser222Asp and ΔN4 mutations on the magnitudes of V_{max}, $V/K_{m,ERK2}$, and $V/K_{m,ATP}$ clearly show a synergistic relationship between these mutations. Therefore, conformational changes induced by the N-terminal deletion enhance the effects of the serine-to-glutamic/aspartic acid substitutions and vice versa. In this respect, it is noteworthy that the C-helix of cAK forms hydrophobic contacts with Trp30 in the A-helix as well as salt bridge interactions with phospho-Thr197 in the regulatory loop (Zheng et al., 1993). It is feasible that phosphorylation of the regulatory loop perturbs the secondary structure at the N-terminus via the C-helix of MKK1, thus explaining how mutagenesis in both regions might contribute to enzymatic activation.[5]

In summary, our findings provide support for the importance of both the N-terminus and the regulatory loop in modulating the activity of MKK. Enzymatic activation can be achieved by at least two mechanisms, one involving enhanced negative charge within the regulatory loop where two residues normally become phosphorylated, and the other involving perturbations in secondary structure N-terminal to the catalytic core. Our study lays the groundwork for a mechanistic understanding of these key modes of protein kinase regulation.

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