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Identification of Two Distinct Regions of p38 MAPK Required for Substrate Binding and Phosphorylation

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The mechanism by which different mitogen activated protein kinases (MAPKs) distinguish between different substrates is poorly understood. For example, p38 and SAPK4 are two closely related p38 MAPKs that both phosphorylate ATF2 and MBP. However, p38 phosphorylates MAPKAPK-2 and -3, whereas SAPK4 does not. In this study, we have used mutagenesis to determine the regions of p38 required for substrate selection. Alanine scanning mutagenesis identified one region of p38 that was required for its ability to phosphorylate MAPKAPK-2 and -3, but that did not significantly affect its binding to these substrates. Chimeras of p38 and SAPK4 identified a second region of p38 that affected the ability of p38 to both bind and phosphorylate MAPKAPK-2 and -3. Hence, we show for the first time that MAPKs contain two distinct regions for recognizing and phosphorylating protein substrates.

Mitogen-activated protein kinases (MAPKs) are now known to be key components of both mitogenic and stress-induced signal transduction cascades regulating a variety of cellular processes ranging from cell growth to cell death (1). At least three families of MAPKs exist in mammalian cells—the ERKs (2), the JNKs (also known as SAPK1) (3) and the p38 MAPKs (also known as CSBP2, RK, SAPK2a and in yeast, HOG1) (4, 5). The ERK family of MAPK is activated primarily by mitogenic stimuli, growth factors and tumor promoters (2) while members of the other two families are activated primarily by environmental stress and inflammatory agents (4–6). These extracellular stimuli activate a cascade of kinases including the MAPKK which activate the MAPKs through phosphorylation of a Thr-Xaa-Tyr motif in an activation loop near the ATP binding site (2, 7).

Once activated, MAPKs phosphorylate their substrates on serine and threonine residues. *In vitro* substrates include myelin basic protein (MBP), ribosomal protein S6 kinases (RSKs), MAPK interacting proteins (MNKs) and c-Myc for ERKs (8–13); c-Jun and ATF2 for JNKs (3, 14), and MBP, ATF2, MNK1, MAPKAPK-2 and MAPKAPK-3 for p38 (5, 6, 11, 12, 15, 16).

Three homologues of p38 have now been identified—p38β/β2 (17, 18) (also called SAPK2b), SAPK3 (19) (also called p38γ (20), and ERK6 (21)), and SAPK4 (also called p38δ) (18, 22, 23). These homologues are between 60 and 74% identical to p38 but show some differences in expression patterns, activation stimuli, sensitivity to the p38 inhibitor SB 203580, and substrate specificities (18, 23). In particular, while p38 and p38β/β2 can phosphorylate MAPKAPK-2 and -3 *in vitro* or *in vivo*, neither SAPK3 nor SAPK4 can phosphorylate MAPKAPK-2, and they show little or no phosphorylation of MAPKAPK-3. By contrast, all four kinases can phosphorylate ATF2 and MBP (18).

In the present study, we have used mutation analysis and p38/SAPK4 chimeras to determine the contribution of various surface regions of p38 to MAPKAPK binding and phosphorylation. We find that the region required for substrate phosphorylation is distinct from the region required for substrate binding/specificity.

EXPERIMENTAL

Plasmid construction and mutagenesis. Cloning and construction of mammalian expression constructs for FLAG-tagged p38 and SAPK4 were described previously (16, 18). Mutants of p38 were generated using a QuikChange site directed mutagenesis kit as directed by the manufacturer (Stratagene) using 40 base pair oligonucleotides containing the indicated amino acid changes in the middle. Nomenclature of the mutants indicates the residue and number of the point mutation of p38 present in that mutant. Numbering of residues is relative to p38 MAPK as shown previously (18). Chimeras of p38 and SAPK4 were generated using PCR amplification of SAPK4 regions inserted into p38 at either *Bst*E II/*Dra* III sites for the more 5′ mutant, or *Dra* III/*Sal* I for the more 3′ mutant. The

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p38/SAPK4 chimeras are named after the restriction fragments used: B stands for *Bst*E II, D for *Dra* III and S for *Sal* I. All mutations were confirmed using a DNA sequencer (Applied Biosystems, Inc.).

Mammalian transfections and preparation of lysates. HeLa cells were maintained in DMEM containing 10% FBS in a humidified 5% CO_2 incubator at 37°C. Transient transfections were performed using Lipofectamine according to the manufacturer's instructions (Life Technologies, Inc.). 24 h after transfection, cells were split into four plates. 48 h later, two plates were treated with 0.4 M sorbitol (Sigma) in DMEM with 10% FBS for 20 minutes, and the other two plates were treated with DMEM with 10% FBS for 20 minutes then harvested as described (16).

Immunoprecipitations, kinase assays and Western blotting. Epitope-tagged proteins were immunoprecipitated from HeLa cell extracts as described previously (16) using anti-FLAG M2 antibody conjugated to agarose (Sigma) then divided into 5 equal portions. Immune complex kinase assays were performed on 4 of the 5 immunoprecipitated aliquots of extract in 30 μl kinase buffer containing 50 μM cold ATP, 2 μCi [γ-^{32}P]ATP (specific activity 4500 Ci/mmol) and one of four substrates—10 μg of MBP, 3 μg GST-MAPKAPK-3, 1 μg GST-MAPKAPK-2 (Upstate Biotech.) or 3 μg GST-ATF2 (amino acids 1–109)—for 30 minutes at 30°C. The reactions were stopped by the addition of SDS sample buffer and the phosphorylated products were analyzed by SDS-PAGE and autoradiography. The radioactive bands were quantitated on a Betagen Betascope. For Western blotting, the other one fifth of the immunoprecipitated extract was subjected to SDS-PAGE, transferred to nitrocellulose, incubated with anti-phospho-Thr180/Tyr182 specific p38MAPK (New England Biolabs, Inc.) antibody and detected by ECL (Amersham) as described by the manufacturer. The blots were then stripped as described by the manufacturer (Amersham), reprobed with anti-FLAG M2 antibody and again detected by ECL. All experiments were repeated at least three times with the results shown being representative of the three experiments.

Binding experiments. Unstimulated HeLa cell extracts were mixed with either 2 μg of GST-MAPKAPK-2 (Upstate Biotech.) for one and a half h at 4°C followed by incubation with Glutathione Sepharose 4B agarose (Pharmacia) for two h at 4°C or with 10 μg of GST-MAPKAPK-3 prebound to Glutathione Sepharose 4B agarose for two h at 4°C. GST-MAPKAPK-3 was prepared in *E. coli* as previously described (16). The beads were washed two times with lysis buffer then resuspended in Laemmli buffer and subjected to SDS-PAGE and Western blotting with the anti-FLAG M2 antibody as described above. Experiments were performed two or three times. In preliminary experiments, the amount of MAPKAP kinase-2 used for precipitation was varied over several μg to determine the minimal amount (2 μg) required to quantitatively precipitate all of the p38 in the lysate sample. An excess (6 μg) of MAPKAPK-2 did not precipitate SAPK4.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed essentially as described (24) with the following modifications. Extracts from transfected HeLa cells treated with or without 0.4 M sorbitol were immunoprecipitated with anti-FLAG M2 antibody. Kinase assays were performed on part of the extract in the absence of exogenously added substrate. Proteins were subjected to SDS PAGE and transferred to Immobilon-P membrane (Millipore). The bands corresponding to phosphorylated protein were excised and analyzed as described (24).

Protein structures. 3D structures of p38 were generated from pdb files using RasMol (RasWin Molecular Graphics Windows Version 2.4, copyright 1993, 1994, by R. Sayle). The Brookhaven reference number for p38 is 1p38.pdb (25).

RESULTS

In order to try to identify residues of p38 involved in substrate binding and phosphorylation, 49 surface residues in the carboxy terminal domain of p38 were mutated, mostly to alanine. After transient transfection into HeLa cells, the mutant proteins were immunoprecipitated using an anti-FLAG antibody and assayed for kinase activity with four substrates—MBP, MAPKAPK-2, MAPKAPK-3, and ATF2 (some examples are shown in Fig. 1A). These immune-complexes also resulted in the phosphorylation of a co-immunoprecipitated molecule (IM) that was also p38 dependent, since it was absent in cells transfected with inactive mutants of p38 (D168A and the double mutant Thr180E, Tyr182E (TY-E)) (Fig. 2B) (26). IM was shown to be p38 itself, since it was immunoprecipitated by both anti-FLAG and anti-p38 antibodies (Figs. 2A and 2B). In the latter case it runs with slightly slower mobility than endogenous p38 due to the amino terminal FLAG tag. Furthermore, phosphoamino acid analysis of IM shows that it is phosphorylated on threonine and tyrosine (Fig. 2B), and mutation of Thr180 and Tyr182 of p38 (TY-E) results in loss of this phosphorylation (Figs. 2B and 2D). Hence IM represents a p38 dependent phosphorylation of p38.

From this analysis, several mutants were identified with significant loss of ability to phosphorylate MAPKAPK 2, 3 and ATF2, of which 6 are illustrated in Fig. 1A. Four of these mutants (R149A, K152A, R189A, Y200A) retained or enhanced the phosphorylation of p38 (IM), two (K152A, L195A) retained the ability to inducibly phosphorylate MBP, and one (D150A) lost all inducible kinase activity (Fig. 1A, panels 1–4). All were expressed and phosphorylated on Thr and Tyr (Fig. 1A, panels 5 and 6). The ability of the mutants to retain kinase activity and/or act as substrates for phosphorylation suggests that none of these mutants had a large change in tertiary structure.

One possible explanation for why these mutants showed a decrease in phosphorylation of specific substrates might be their inability to bind them. To address this question, GST-MAPKAPK-2 and 3 coated beads were used to pull down transfected p38 mutants from transfected HeLa cell nuclear extracts (Fig. 3A). The amount of MAPKAP kinase-2 used was the minimum required to quantitatively precipitate all of the p38 in the extract so as to detect even small reductions in affinity. Despite this, all of the mutants that had shown a decrease or loss of kinase activity against substrate could still bind substrate similarly, suggesting limited changes in affinity. GST coated beads did not pull down p38 (data not shown and (16)). These mutants therefore define a region of p38 required for substrate phosphorylation.

Two recently identified homologues of p38, SAPK3 and SAPK4, are incapable of phosphorylating MAPKAPK-2 and only weakly phosphorylate MAPKAPK-3 (18). This suggested that chimeric p38/SAPK4 proteins could be used to further explore the regions of p38 required for recognition and phosphorylation of MAPKAPK-2 and

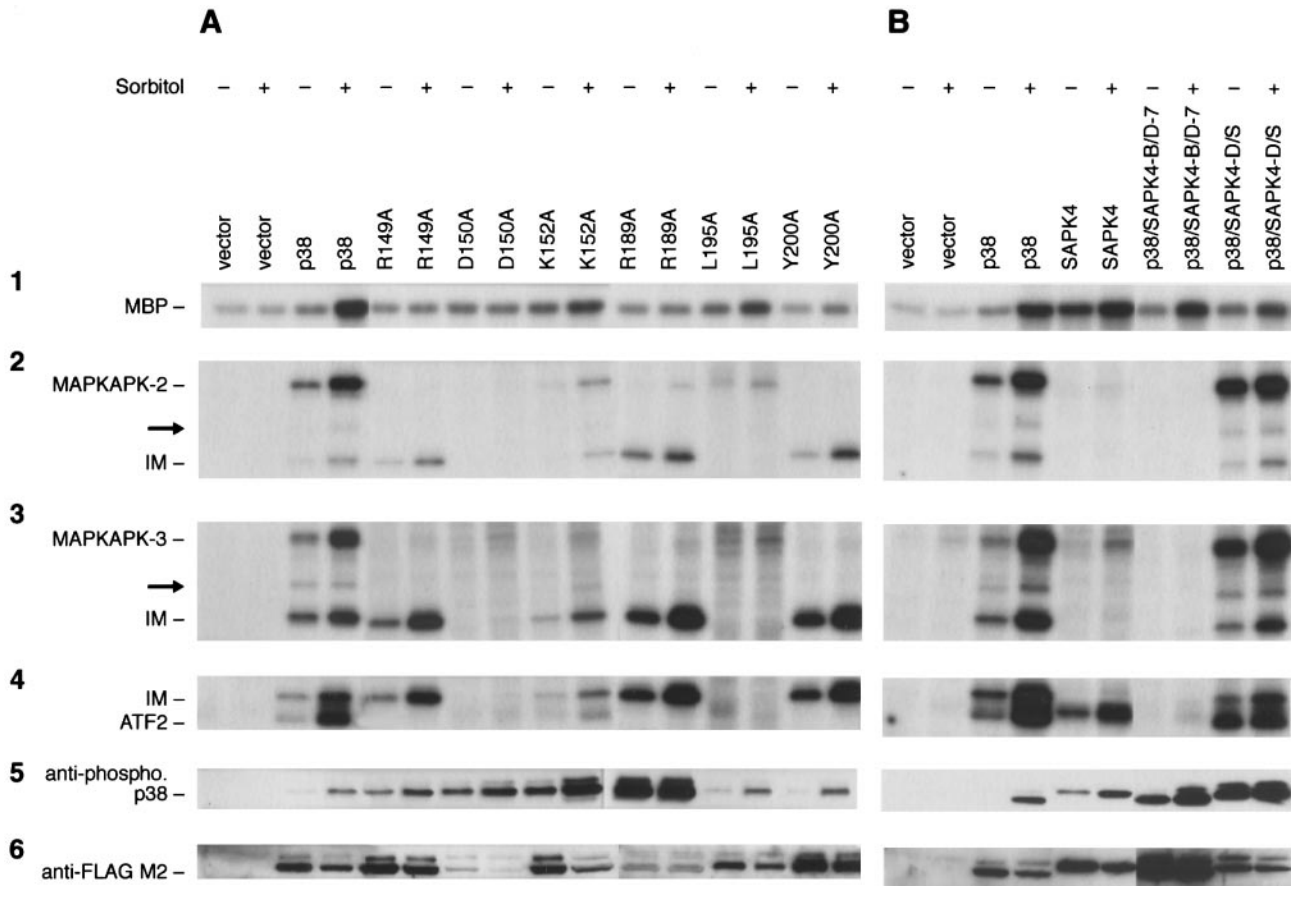


FIG. 1. Kinase activity of alanine scanning and chimeric mutants of p38. (A and B) Extracts from HeLa cells transfected with the indicated constructs and treated with or without 0.4 M sorbitol were immunoprecipitated with anti-FLAG antibody and equal portions tested for kinase activity with MBP (panel 1), GST-MAPKAPK-2 (panel 2), GST-MAPKAPK-3 (panel 3) or GST-ATF2 (panel 4) as substrates or probed by Western blot with anti-phosphospecific-p38 antibody (panel 5) and anti-FLAG M2 antibody (panel 6). The coimmunoprecipitated band IM is p38 and the small arrows in panels 2 and 3 indicate an unidentified endogenous substrate coimmunoprecipitated with anti-FLAG antibody. Nomenclature of mutants indicates the residue and number of the point mutation of p38. The experiments were performed three or four times with similar results.

-3. Chimeras were created in which small regions of SAPK, differing from p38 in 12 or 13 residues, replaced the equivalent regions of p38. As before, the chimeras were transiently transfected into HeLa cells, immunoprecipitated with anti-FLAG antibody and analyzed for kinase activity using four different substrates: MBP, GST-MAPKAPK-2, GST-MAPKAPK-3, and GST-ATF2 (Fig. 1B).

A p38/SAPK4 chimera (p38/SAPK4-B/D-7), in which residues 110 through 184 of p38 were replaced by residues 111 through 184 of SAPK4, results in a change of 12 amino acids from the sequence of p38. Like SAPK4, this chimera was unable to phosphorylate MAPKAPK-2 or -3 but could phosphorylate MBP (Fig. 1B). This chimera only weakly phosphorylated ATF2 even though both p38 and SAPK4 can utilize ATF2 as a substrate, but like SAPK4 did not lead to p38 (IM) phosphorylation. A second chimera (p38/SAPK4-D/S-2), in which residues 193 through 281 of p38 were replaced by residues 193 through 281 of SAPK4 resulting in a change of 13 amino acids, showed no change in phosphorylation of any of the substrates relative to p38.

Chimeras were examined for binding to MAPKAPK2 and 3 in the pull down assay described above (Fig. 3B). Neither SAPK4 nor the chimera p38/SAPK4-B/D could bind to MAPKAPK-2 or -3, directly correlating with the inability of these kinases to phosphorylate these substrates. This lack of binding was shown to be independent of the amount of substrate used, since p38 could bind MAPKAPK-2 at concentrations ranging from 1 to 6 μg of MAPKAPK-2 while SAPK4 showed no binding at any of these concentrations (data not shown). In contrast, a second chimera, p38/SAPK4-D/S, was identical in binding and kinase assays to p38. These results indicate that the several of the 12 p38 amino acids altered in the chimera, p38/SAPK4-B/D, are required for binding of the substrates MAPKAPK-2 and -3 to p38 MAPK. Mutation of individual amino acids in

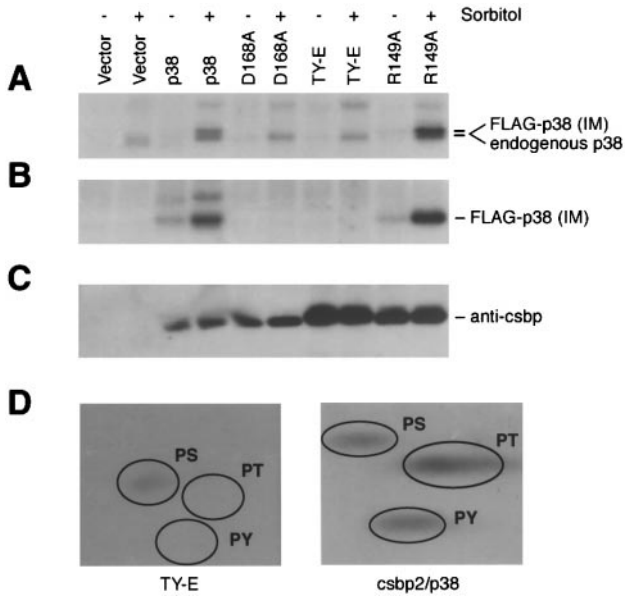


FIG. 2. Identification of IM as p38. Extracts from HeLa cells transfected with the indicated constructs and treated with or without 0.4 M sorbitol were divided into several equal portions. In panels A and B, equal portions of HeLa cell extracts were immunoprecipitated with either anti-p38 or anti-FLAG antibody, respectively, and kinase assays performed in the absence of exogenously added substrate. A second anti-FLAG immunoprecipitated portion was subjected to SDS PAGE, and immunoblotted with anti-p38 antibody (panel C). In panel D, a portion of p38 and the Thr180Glu, Tyr182Glu (TY-E) p38 transfected extract were treated as in panel B, transferred to an Immobilon-P membrane, and the phosphorylated band marked IM excised and subjected to phosphoamino acid analysis.

this region did not affect MAPKAPK-2 binding to p38, suggesting that multiple residues may be important for this interaction (data not shown).

DISCUSSION

We have shown that substrate phosphorylation by p38 is determined by two distinct surface regions. One region is involved predominantly in substrate binding while the second region is important for substrate phosphorylation but does not significantly affect binding to protein substrate. The region which affects substrate phosphorylation (residues 149, 150, 152, 189, 195, 196, 199, 200, 201) appears as a shaded patch in the right hand panel of Fig. 4A. Mutation of any residue in this region to alanine results in a loss or decrease in substrate phosphorylation, but little or no change in binding to substrate (some data not shown). Six of these residues (R149, D150, K152, R189, L195 and Y200) are completely conserved among MAPK in species ranging from *H. sapiens* to *S. cerevisiae* (27). Three of these residues (R149, D150, and K152) are also conserved in other kinases such as cAPK, PKC, CDC28, and Raf (7). The conserved nature of many of these residues indicates that they may be involved in substrate phosphorylation by kinases in general and not specifically by p38. In cAPK and many other kinases the equivalent residues are involved in forming the P + 1 binding pocket or catalytic site (28, 29). It is likely that upon activation the conformation of p38 changes and allows these residues to form this P + 1 pocket for phosphorylation of the substrate. Indeed the equivalent residues of the activated form of ERK2 have been shown to play a role in stabilization of the phosphorylated Thr183 and Tyr185 and in forming the P + 1 pocket for substrate phosphorylation (30).

A second distinct region of p38 (residues 114–126) is required for binding and phosphorylation of MAPKAPK-2 and -3 (shaded in the left-hand panel of Fig. 4A). This is most readily exemplified by the chi-

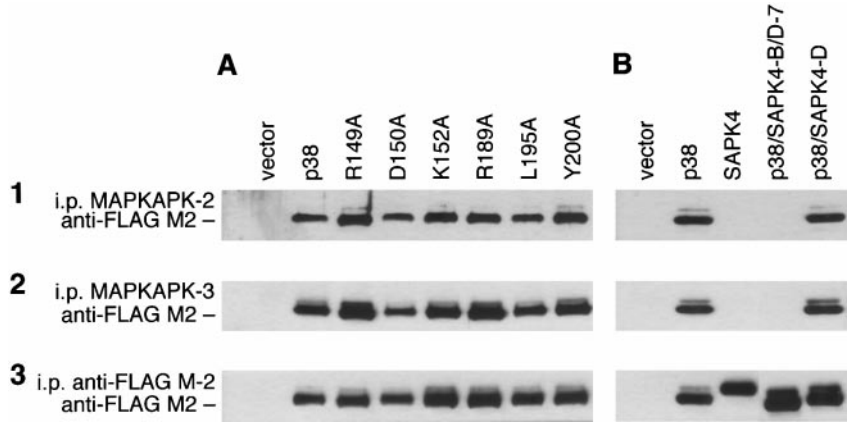
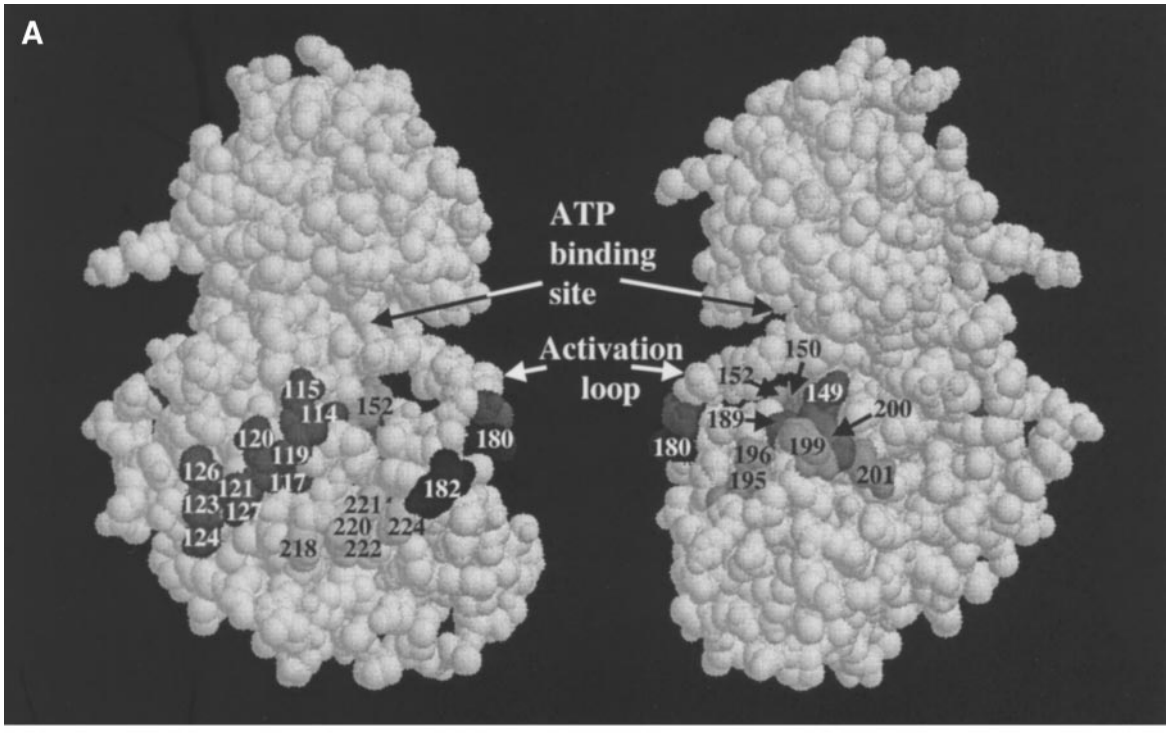


FIG. 3. Binding of selected alanine mutants of p38 to MAPKAPKs. (A and B) Equal portions of unstimulated extracts from HeLa cells transfected with the indicated constructs were mixed with *E. coli* expressed GST-MAPKAPK-2 (panel 1), GST-MAPKAPK-3 (panel 2) and purified with Glutathione Sepharose 4B agarose. A third portion was immunoprecipitated with anti-FLAG M2 antibody (panel 3). After SDS PAGE, p38 was detected by Western blotting with an anti-FLAG antibody. Nomenclature of the mutants is as in Fig. 1. The experiments were performed two or three times with similar results.



B

MAPKAPK-2 binding region c-Jun binding region (JNK2)

	114	127	214	228
p38	-----NNI V KCQKLT DDH V---------AELLTGRTLFPGTDH-----			
p38β	-----. . . . . .A . S . E . .---------. . . . Q . KA . . . . S . Y-----			
SAPK3	-----G KLM . HE . . GE . R I---------. . MI . . K . . . K . S . . -----			
SAPK4	-----Q K . MGME - F SE E KI---------. . M . . . K . . . K . K . Y-----			
JNK1	-----CQV I QME - . DH ERM---------G . MVCHKI . . . . R . Y-----			
JNK2	-----CQV I QME - . DH ERM---------G . . VK . CV I . Q . . . . -----			

FIG. 4. Regions of p38 involved in substrate binding and phosphorylation. (A) Two views of the tertiary structure of p38, 180° apart, are shown. Residues for substrate binding and phosphorylation are identified in the left and right views respectively, as described in the text. Thr180 and Tyr 182 are the residues phosphorylated upon activation. (B) Alignment of sequences of p38 and JNK kinases in the regions which bind substrate.

mera p38/SAPK4-B/D-7, which showed substrate specificity and binding very similar to SAPK4 despite differing from p38 by only 12 amino acids. This region is distinct from the region of JNK2 required for binding to c-Jun (corresponding to the residues 218–224 of p38) (31) and the region of cAPK required for binding to its inhibitor peptide (32). The chimera in which this region of p38 was replaced by SAPK4, (p38/SAPK4-D/S-2) showed no difference in substrate preferences compared to p38, confirming that this region plays no role in determining the substrate preference for MAPKAPK-2 and -3. An alignment of several kinases showing the region of p38 important for substrate binding for MAPKAPK-2 and -3 and the region homologous to the region of JNK2 required for binding c-Jun is shown in Fig. 4B. This shows significant sequence differences between the different isoforms, but similarities between p38 and p38β, which both phosphorylate MAPKAPK-2 and -3 (18). These results support the idea that different regions on this face of MAPKs may contribute to substrate selection.

The chimera p38/SAPK4-B/D, which showed SAPK4 substrate preferences, also had decreased activity against ATF2, despite the fact that p38 and SAPK4 both phosphorylate ATF2. This effect could be due to the substituted region of SAPK4 containing one amino acid less than the equivalent p38 region (residue 121 in Fig. 4B), which might alter the orientation of this region and hence affect ATF2 binding. However, this spacing difference cannot explain the preference of p38 for MAPKAPK-2 and -3, since SAPK3 is not missing an amino acid but is also incapable of phosphorylating MAPKAPK-2 and -3.

We have shown here for the first time that substrate specificity is determined by the both binding interaction and through interaction with the P + 1 site. This finding along with previous work (16, 33) suggests the following mechanism of substrate phosphorylation. First, unphosphorylated p38 binds the substrate. Upon activation of p38 by phosphorylation of the activation loop, ATP binds and the P + 1 site is formed. The P + 1 site then engages the bound substrate so that it can be phosphorylated. It will be of interest to determine

the detailed molecular interactions in these two regions that contribute to substrate selectivity. The discovery of a p38MAPK homologue with altered substrate specificity opens up the possibility of conducting studies *in vivo* that determine the role of its different substrates in p38 induced cellular responses.

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