Mitogen-activated protein (MAP) kinases are a family of serine/threonine kinases that play a role in many signal transduction pathways and affect various cellular phenotypes. Despite the abundance of available data, the exact role of each MAP kinase is not completely defined, in part because of the inability to activate MAP kinase molecules individually and specifically. Based on activating mutations found in the yeast MAP kinase p38/Hog1 (Bell, M., Capone, R., Pashtan, I., Levitzki, A., and Engelberg, D. (2001) J. Biol. Chem. 276, 25351–25358), we have designed and constructed single and multiple mutants of human MAP kinase p38\(\alpha\). Single (p38D176A, p38F327L, and p38F327S) and subsequent double (p38D176A/F327L and p38D176A/F327S) mutants acquired high intrinsic activity independent of any upstream regulation and function in a range of 10 and 25%, respectively, in reference to the dually phosphorylated wild type p38\(\alpha\). The active p38 mutants have retained high specificity toward p38 substrates and were inhibited by the specific p38 inhibitors SB-203580 and PD-169316. We also show that similar mutations can render p38\(\gamma\) active as well. Based on the available structures of p38 and ERK2, we have analyzed the p38 mutants and identified a hydrophobic core stabilized by three aromatic residues, Tyr-69, Phe-327, and Trp-337, in the vicinity of the L16 loop region. Upon activation, a segment of the L16 loop, including Phe-327, becomes disordered. Structural analysis suggests that the active p38 mutants emulate the conformational changes imposed naturally by dual phosphorylation, namely, destabilization of the hydrophobic core. Essentially, the hydrophobic core is an inherent stabilizer that maintains low basal activity level in unphosphorylated p38.

Mitogen-activated protein (MAP) kinases consist of a large family of enzymes that are involved in many critical cellular processes including proliferation, apoptosis, and differentiation. They are also essential for diverse multicellular activities such as development, embryogenesis, and even learning and memory (reviewed in Refs. 1–7). MAP kinases are divided into subgroups based on their biological activities and sequence similarities. They are expressed in each eukaryotic cell and function in distinct pathways. Mammalian subfamilies include the extracellular signal-regulated kinases (ERKs), p38\(\alpha\), c-Jun N-terminal kinases (JNKs), and big MAP kinases (BMK-Erk5 and Erk7) (2, 8, 9). MAP kinases are proline-directed proteins that phosphorylate threonine or serine residues positioned upstream to a proline residue (10, 11). They are characterized by a unique activation mechanism that requires dual phosphorylation of a Thr-Xaa-Tyr sequence located on a flexible loop, also known as the phosphorylation lip. Dual specificity kinases termed MAP kinase kinases (MAPKKs or MKKs) catalyze the threonine and tyrosine dual phosphorylation. The basal activity of the unphosphorylated MAP kinase proteins is very low (2, 12, 13).

The p38 subfamily consists of four isoforms (i.e. p38\(\alpha\), p38\(\beta\), p38\(\gamma\), and p38\(\delta\)) (14–16) that are encoded by different genes. The p38 isoforms share ~60% sequence identity and are differentially expressed in the organism. The p38 MAP kinases become dually phosphorylated and active in response to stresses such as ultraviolet radiation, heat shock, oxidative stress, and osmotic stress (4, 17, 18). They are also activated in response to many intercellular factors including growth factors (19, 20), differentiation factors, and inflammatory signals (21, 22). The p38 subfamily members phosphorylate a variety of substrates including other kinases, tumor suppressors, and important transcription and translation factors (4).

p38 MAP kinases are assumed to mediate protective responses, stress-dependent apoptosis, and inflammation. In some systems, however, p38 may also play a role in differentiation and development (23–27). Despite the abundance of available data, the exact role of each individual MAP kinase in cellular responses is not completely defined. The main limitation in revealing the specific role of a given MAP kinase is the inability to activate any MAP kinase individually. Constitutively active MAP kinases could become powerful tools for studying the individual role of each enzyme. Various approaches for activating particular MAP kinases in vivo are currently being used with limited efficiency and specificity (26, 28–31). Previous attempts to construct constitutively active mammalian MAP kinases independent of MKK activation were essentially unsuccessful (32). Replacing the threonine on the unique Thr-Xaa-Tyr activation motif by glutamate or aspartate failed to produce active enzymes (13, 32). Several genetic screens in Saccharomyces cerevisiae and Drosophila melanogaster resulted in partially active MAP kinases (33–35). Some of the mutations identified in these genetic screens were introduced in conserved residues in the mammalian homologue ERK2 and imposed a negligible increase in activity (36, 37).
Most of the mutants became active only after MKK-mediated activation (33, 37), whereas others manifested very low activity as compared with dual phosphorylated, fully activated kinase (36).

Recently, we have described the isolation of hyperactive mutants of the p38 yeast homologue Hog1 (38, 39). The hyperactive mutants were isolated via a genetic screen that searched for HOG1 alleles that were biologically functional independently of their upstream MKK, Pbs2. Genetic and biochemical analysis of the mutants revealed that they were not only biologically active but also catalytically active in the absence of stimulation. Furthermore, some of the mutants did not require phosphorylation for their activity (40). Combining two activating mutations in a single Hog1 molecule resulted in increased catalytic and biological activity. Cells expressing the Hog1 double mutants showed severe growth retardation (39).

The success in obtaining hyperactive variants of the yeast Hog1 could now be conveyed into mammalian MAP kinases. In fact, we have previously reported the initial attempt to test this approach with the human p38α MAP kinase (note that any indication of p38 refers to p38α unless specified otherwise) and described preliminary results showing that p38Y69H and p38F327S (equivalent to Hog1F322L) are catalytically active in vitro (38).

Here we describe a systematic effort to apply the previously attained data based on the Hog1 hyperactive mutants to develop similar variants of the human p38α MAP kinase. We identify variants that rendered p38α intrinsically active and characterize the biochemical properties of this activity. We also report that combining two single activating mutations in p38α resulted in a substantially increased activity. Furthermore, we describe preliminary results that p38y becomes active using similar mutations as well. In addition, we present a structural analysis of the activating mutants based on the currently available three-dimensional models of p38 and ERK2 (12, 41–44) that provides a plausible mechanism that underlies the activation of the p38 mutants.

**MATERIALS AND METHODS**

**Site-directed Mutagenesis of p38 and the Construction of Double Mutants**—Site-directed mutagenesis was carried out using the QuikChange kit (Stratagene). The reactions were performed on the human p38α gene subcloned into either pET15b (Novagen) downstream and in-frame to the hexahistidine coding sequence or Bluescript (Stratagene). The procedure included PCR using specific complementary primers (Table I) followed by a DpnI digestion. The elongation step was set to 10.5 min at the temperature of 68 °C. All mutated cDNAs were verified by using DNA sequencing of the entire p38 gene. After mutagenesis, all single mutants of human p38α made on Bluescript vectors were subcloned into pET15b (Novagen). To construct double mutants of Y69H combined with A320T, F327S, F327L, or W373R, all pET15b plasmids harboring one of those mutations were digested with AatII and DraIII endonucleases. The 4455-bp digestion product of the plasmid carrying the Y69H mutant was ligated, in separate reactions, with each one of the 934-bp products of the A320T, F327S, and F327L digestions, respectively. These ligations provided hexahistidine-tagged Y69H/A320T, Y69H/F327S, and Y69H/F327L double mutants. To construct plasmids combining the D176A mutation with the A320T, F327L, or F327S mutations, the pET15b plasmids containing each individual mutation were digested with BseRI and BspMI endonucleases. The 450-bp product of the plasmid carrying the D176A mutation was ligated with the 4969-bp digestion products of plasmids carrying the A320T, F327S, or F327L mutants. These ligations provided the D176A/A320T, D176A/F327L, and D176A/F327S double mutants. The construction of the double mutants containing D177A and either A320T, F327L, or F327S was similar to that of the double mutants containing D176A as described above.

**Subcloning and Site-directed Mutagenesis of p38**—The human p38γ coding sequence was amplified using PCR reaction and pcDNA p38γ as a template. Neo and EcoRI restriction sites were added to the PCR product (see specific primers in Table I). The PCR product was cloned into the pHis-Parallel vector using Neo/EcoRI double digestion. Site-directed mutagenesis was carried out similarly to p38α mutagenesis procedures with a specific set of primers (Table I).

**Protein Expression and Purification**—Wild type (WT) and mutant forms of p38 were expressed in Escherichia coli cells as described earlier (43). Each expression plasmid was transformed into BL21(DE3)-pLysS cells (Invitrogen). Cell cultures were grown in volumes of 0.5 liters at 30 °C until they reached an A600 of 0.3–0.4. Protein expression was induced using 0.3 mM isopropyl-β-D-thiogalactopyranoside. The cells were pelleted by centrifugation after 5 h of induction. Cells were washed in a buffer containing 50 mM Tris-HCl, pH 8.5, 0.5 mM imidazole, and 0.3 M NaCl, centrifuged again, flash-frozen in liquid nitrogen, and stored at −80 °C. The frozen pellet was gently thawed on ice and suspended in a buffer containing 50 mM Tris-HCl, pH 8, 5 mM imidazole, 0.3 M NaCl, and a protease inhibitor mixture (Sigma p88849). After mechanical disruption of the cells using a Dounce homogenizer and pH 7.5 disruption of cells using a French press at 11000 psi and 100 °C, all extracts were centrifuged at 40000 × g for 20 min at 4 °C. The supernatant containing the soluble protein was loaded on a 1.5-ml nickel-nitriolactic acid agarose bead (Qiagen) gravity flow open column. The p38 protein was eluted from the column using 50 mM Tris-HCl, pH 8, 500 mM imidazole, 0.3 M NaCl, and 250 mM imidazole. The protein solution was then dialyzed overnight against 12.5 mM Hepes pH 7.3, 100 mM KCl, 6.25% glycerol, and 0.5% dihtiothreitol. After dialysis, protein concentration was determined using a Bradford assay, and the purified protein was then divided into aliquots, flash-frozen in liquid nitrogen, and stored in −80 °C.

**Kinase Assay**—All reactions were conducted in 1.5-mL test tubes. To initialize the reaction, 45 μL of the reaction mixture were added to 5 μL of p38 enzyme (0.2 μg of purified, recombinant hexahistidine tag-p38γ). Final reaction conditions were 100 mM NaCl, 25 mM Hepes pH 7.5, 20 mM MgCl₂, 20 mM 2-glycerolphosphate, 0.1 mM Na₂VO₃, 1 mM diithiothreitol, 20–40 μM of substrate, 50 μM ATP, and 10 μM of ATPγS. The kinase reactions were stopped after 10 or 20 min (to test MKK6-treated or untreated kinases, respectively) by placing the tubes on ice, applying Laemmli sample buffer and boiling at 100 °C for 5 min. In experiments where p38 inhibitors were assayed, the selected inhibitor
TABLE II
Mutations identified in the HOG1 genetic screen and their equivalents in p38α and p38γ

| HOG1 | p38α | p38γ |
|------|------|------|
| Y68H | Y68H | D179A |
| D170A | D176A | D177A |
| A214T | A320T | A320T |
| F318S | F327L | F327S |
| W320R | F327L | F327S |
| F322L | F327L | F327S |
| W332R | W337R | F330S |
| N391D | | |

was added to the test tube prior to the reaction. To activate p38 variants we used recombinant active MKK6 (Upstate Biotechnology). Reaction conditions were similar to those recommended by the manufacturer, except for the use of non-radioactive ATP. Paper-spotted Kinase Assays—Reactions were carried out in 96-well plates with conical bottoms. 0.2 μg of purified recombinant hexahistidine-tag-p38 mutant and WT proteins were used in a final volume of 50 μl/well (i.e. 100 nM kinase). The kinase assays were initialized by the addition of 45 μl of reaction mixture to 5 μl of p38 enzyme. Final reaction conditions were 25 mM Hepes, pH 7.5, 20 mM MgCl₂, and 20 mM 2-glycerophosphate, 5 mM p-nitrophenyl phosphate, 0.1 mM Na₃VO₄, 1 mM dithiothreitol, 64 μg (35.1 μM final) of GST-rat MKK6 ATP2, 250 μM ATP, and 10 μCi of [γ³²P]ATP. The kinase reactions proceeded for 10 or 20 min (to test MKK6-treated or untreated kinases, respectively) and were terminated by the addition of 50 μl of 0.5 M EDTA, pH 8.5 (250 mM final) and placement on ice. Following reaction termination, aliquots of 85 μl from each well were spotted onto 3 × 3-cm Whatman 3MM paper squares and briefly air-dried. Each square was rinsed three times with 10% trichloroacetic acid and 3% sodium pyrophosphate (10 ml/square) overnight was given without shaking. The following day, the squares were rinsed twice with 100% ethanol (4 ml/square) for 20 min each time and air-dried. The radioactivity of each square was counted using a scintillation counter running a ³²P Cherenkov program. Experimental points were usually triplicates.

RESULTS

Design, Expression, and Purification of p38 Active Mutants—The mutations that were found to render Hog1 active are described in Table II (38). Sequence alignment indicated that five of the eight mutated residues identified in Hog1 as active are, in fact, conserved in p38α (Fig. 1). To test whether mutations at these sites would also render p38 active, we constructed the mutants p38Y69H, p38D176A, p38A320T, p38F327L, and p38F327S. Furthermore, we produced two additional mutants, p38Y69H and p38F327S, which were not directly derived from the HOG1 genetic screen. The D177A mutation was constructed because there are two adjacent aspartate residues in the phosphorylation lip of p38 that could be homologous to the Hog1 Asp-170 (Fig. 1 and Table II). The F327S mutation was based on the notion of diversifying the possibilities of mutations in this region. Such diversity was also observed for the p38 family.

The seven mutants of p38 were expressed in E. coli as hexahistidine-tagged proteins and purified utilizing affinity chromatography. The proteins were ~90–95% pure as determined by SDS-PAGE using a Coomassie staining (data not shown). All mutants except p38W337R were expressed at high levels and readily purified. Purified p38 variants were assayed for their capability to phosphorylate GST-ATF2. As expected, WT p38 did not show any activity in this assay. In contrast, the p38D176A, p38F327L, and p38F327S mutants exhibited significant activity (Fig. 2A, left panel). Because the activity was assayed with purified recombinant proteins expressed in E. coli, it is apparent that the activity is intrinsic and does not depend on upstream activation. Having identified Asp-176 and Phe-327 as sites for mutagenesis in p38α, we questioned whether substituting homologous sites in other p38 isoforms would render them hyperactive (Fig. 1 and Table II). We tested this assumption with p38γ and found indeed that mutating Asp-179, which corresponds to Asp-176 in p38α, to alanine rendered p38γ intrinsically active (Fig. 2C). Changing Phe-330 of p38γ to serine however, did not activate the enzyme (Fig. 2C). Thus, some of the activating mutations are of importance in other members of the p38 family.

To further analyze the p38α mutants, we compared their activities to that of the dually phosphorylated, fully activated WT p38. In addition, we also examined whether the p38 mutants could be further activated by MKK6 dual phosphorylation. We thus incubated each of the mutants as well as WT p38 with an activated MKK6 and non-radioactive ATP (see “Materials and Methods”). A Western blot analysis using antiphospho-p38 antibodies verified that MKK6 treatment increased significantly the phosphorylation levels of all p38 variants (Fig. 7, bottom). Subsequent to MKK6 treatment, the WT p38 and the mutants were subjected to a standard kinase assay with GST-ATF2 as a substrate (Fig. 2A, right panel). The results clearly show that all mutants could be further activated by MKK6-mediated phosphorylation. We established and validated (data not shown) a paper-spotted kinase assay (see “Materials and Methods”) that allowed the performance of accurate and quantitative large numbers of kinase assays. Using this approach, we quantified the activities of the WT p38 and mutants (Fig. 2B) and found that dually phosphorylated WT p38 exhibited approximately three orders of magnitude (~800-fold) increased activity relative to non-activated WT p38. The activity of the p38 mutants, in their unphosphorylated forms, reached values of ~10% relative to the fully activated WT p38 reference.

Activity Assay of p38α Double Mutants—In an attempt to obtain p38 mutants with increased activity relative to the p38D176A, p38F327L, and p38F327S mutants, we designed, expressed, and assayed a set of double mutants (Table III). We assumed that combining two active point mutations in p38, each of which may activate the protein in different mechanisms, might result in double mutants with elevated activities. A similar approach in HOG1 resulted in hyperactive mutants (39). The p38 double mutants were assayed for their intrinsic basal activity with reference to the dually phosphorylated WT p38 and the active single mutants. Two of the double mutants, p38D176A/F327L and p38D176A/F327S, exhibited ~192-fold activity compared with the WT p38. This level of activity reached ~25% in reference to the fully activated dually phosphorylated WT p38 (Fig. 3). In contrast, combining the Y69H mutation with other single mutations (A320T, F327L, and F327S) resulted in complete abolition of the intrinsic activity obtained by the single mutants (Table III). Similar results were also obtained by combining D177A with the active single mutants. In an attempt to analyze the contribution of each single mutation and also to try and further increase the activity, we constructed a new set of single, double, and triple mutants with alanine substitutions at selected sites (Table III). Unexpectedly, all of these mutants were completely inactive, although they could become fully activated by MKK6 phosphorylation, indicating that they retained a correct kinase fold (data not shown).

The Active p38 Mutants Preserve an Authentic Substrate Specificity Profile and Sensitivity to Inhibitors—As hitherto there has been no report of any mutation that renders p38 active, the active mutants described above represent a novel type of catalytic activity. We were concerned that perhaps the
active mutants acquired some undesired properties such as loss of substrate specificity, resistance to specific p38 inhibitors, or avoiding upstream activation. To address these matters we first characterized the basic properties of the novel activity and found it to be linear as a function of time (at least 40 min.) (Fig. 4A) and as a function of enzyme concentration (Fig. 4B), indicating that the biochemical results were obtained within the linear range of the activities of the mutants (see "Materials and Methods"). Temperature profile was somewhat surprising, as the mutants were still active at 50 °C although the peak was at 37 °C. The kinetic profile followed the Michaelis-Menten model with $V_{max}$ and $K_m$ values of 37 (picomoles of phosphate incorporated/min/milligrams of enzyme) and 25.3 M, respectively (for p38D176A/F327S) (Fig. 4C). Dually phosphorylated active WT p38 also manifested a Michaelis-Menten kinetic with $V_{max}$ and $K_m$ values of 87.9 (picomoles of phosphate incorporated/min/milligrams of enzyme) and 15.2 M, respectively (Fig. 5A).

By acquiring intrinsic activity, the mutants may have lost their specificity toward downstream p38 substrates, sensitivity to specific inhibitors, and/or recognition by upstream elements. To test substrate specificity of the mutants, we performed kinase assays using either GST-ATF2, myelin basic protein (another known substrate of p38), or GST-c-Jun, a known exclusive substrate of JNKs (45). The results clearly show that the hyperactive double mutants p38D176A/F327L and p38D176A/F327S strongly phosphorylated both ATF2 and myelin basic protein, indicating that they acquired an intrinsic activity that is not substrate-dependent (Fig. 5A). In addition, these double mutants were inert toward c-Jun, indicating that they retained their specificity. Similar results were obtained with the less active single mutants p38D176A and p38F327S (Fig. 5A). Our GST-c-Jun substrate was strongly phosphorylated by JNK (Fig. 5B). Subsequently, we examined whether the specific p38 inhibitors SB-203580 (22, 46) and PD-169316 (47) can inhibit the activity of the two potent double mutants (Fig. 6A). The two compounds efficiently inhibited the active double mutants (Fig. 6A), implying that the mutants share similar structural features with the active WT p38 that enable recognition and binding of the inhibitors. We further quantified the inhibition of WT p38 and p38D176A/F327S by SB-203580 (Fig. 6B) using ATF2 as a substrate. It was apparent that both dually phosphorylated WT p38 and non-phosphorylated p38D176A/F327S proteins exhibited a similar inhibition pattern when using either substrate. We thus assume that the mechanism of action of the mutants emulates the MKK6-dependent active state of the WT p38. To examine whether this active state involves phosphorylation on the Thr-Gly-Tyr motif, we performed a specific Western blot analysis using an antiphospho-p38 antibody (Fig. 7). Although purified as recombinant proteins from E. coli, all of the mutants as well as WT p38 have shown some degree of phosphorylation in the absence of MKK6 treatment. Some of the active mutants (e.g. p38Y69H, p38D176A, and p38D176A/F327S) exhibited significant levels of phosphorylation, whereas others (e.g. p38F327S and p38D176A/F327L) indicated very low phosphorylation levels. We assume that phosphorylation...
Equal exposures were applied for the detection of radioactivity (lower images). The p38D176A, p38D176A/F327L and p38D176A/F327S variants are active independently of MKK6 phosphorylation (Fig. 2A). Using the paper-spotted technique, we quantified and normalized the activities of the mutants to that of the fully activated WT p38 that was defined as 100%. The graph shows averages of results from several independent experiments. S.E. values are <4%.

**p38 Activation May Occur through Destabilization of a Hydrophobic Core in the Vicinity of the L16 Region**—The development of p38 hyperactive mutants was based on activating mutations identified in the yeast Hog1 MAP kinase (38). Because these mutants were discovered via a random, unbiased genetic screen rather than a rational approach, the mechanism of activation of the mutants is unknown. We conducted a comprehensive structural analysis using the available three-dimensional structural data on MAP kinases in order to propose a mechanism that promotes activation of the mutants. p38 (42–44) and ERK2 (12, 41) share a similar topology consisting of two kinase lobes (N-terminal and C-terminal domains) that create the catalytic groove in their interface. The MAP kinases are also characterized by an insertion in the C domain and an extension in the C terminus (located in the N domain). The C terminus extension consists of a loop (the L16 loop) that stretches from the C to the N domains and extends in an α-helix (L16-helix) (Fig. 8). The activation mechanisms of ERK2 and p38 have similar characteristics. In both ERK2 and p38, phosphothreonine is assumed to reorient the phosphorylation lip and thus promote a domain rotation and closure to form the active site, whereas the phosphotyrosine participates in forming the substrate p + 1 recognition site (12, 44).

Throughout the analysis of the unphosphorylated p38 struc-
FIG. 4. Active mutants display linear activity as a function of both time and enzyme concentrations and follow Michaelis-Menten kinetics. A, two active mutants, p38D176A (D176A) and p38D176A/F327S (D176A/F327S), as well as non-activated WT protein were subjected to kinase assay using increasing time intervals and found to be linear at the entire measured time frame (up to 40 min). B, the same mutants as in panel A were subjected to kinase assay using increasing enzyme concentrations (up to 1 µg per reaction well) and found to be linear. C, Lineweaver-Burk plots of the kinetic assay performed on the double mutant p38D176A/F327S (D176A/F327S) and the dually phosphorylated WT p38 (Activated WT). All kinetics profiles followed the Michaelis-Menten model. Using extrapolation, the kinetic parameters were determined to be $V_{\text{max}}$ and $K_m$ values of 37 (picomoles of phosphate incorporated $\times \text{min}^{-1} \times \text{milligrams of enzyme}^{-1}$) and 25.3 µM, respectively, for p38D176A/F327S and $V_{\text{max}}$ and $K_m$ values of 87.9 (picomoles of phosphate incorporated $\times \text{min}^{-1} \times \text{milligrams of enzyme}^{-1}$) and 15.2 µM, respectively, for phosphorylated WT p38.
The hydrophobic core may play a crucial role in stabilizing the L16 loop region, which, in part adopts a 3_10 helical conformation (residues 326–329) (Fig. 5B). Introducing a smaller or charged residue at each of these positions may alter or even disrupt the stability of the hydrophobic core. As reported above, not all substitutions in the vicinity would directly render the kinase active. It should be appreciated that p38F327L showed significantly higher activity than p38Y69H (Fig. 2). In this context, p38F327L did not display any intrinsic activity. It seems, therefore, that disrupting the hydrophobic core is important for activation but that not all substitution in this vicinity would directly render the kinase active. It should be appreciated that p38γ was not examined rigorously and thoroughly in this study, and it is plausible that other mutations in the Phe-330 position would be examined rigorously and thoroughly in this study, and it is plausible that other mutations in the Phe-330 position would not all substitution in this vicinity would directly render the kinase active.

Trp-337 and Tyr-69 are located on L16-helix and C-helix respectively (Fig. 8B). The C- and L16-helices are fundamental segments of the kinase N-terminal domain, which are well stabilized (Fig. 9). Conversely, the L16 loop is stabilized by a limited number of interactions. Thus, the activation level of the hydrophobic core may play a crucial role in stabilizing the L16 loop region, which, in part adopts a 3_10 helical conformation (residues 326–329) (Fig. 5B). Introducing a smaller or charged residue at each of these positions may alter or even disrupt the stability of the hydrophobic core. As reported above, not all substitutions in the vicinity would directly render the kinase active. It should be appreciated that p38F327L showed significantly higher activity than p38Y69H (Fig. 2). In this context, p38F327L did not display any intrinsic activity. It seems, therefore, that disrupting the hydrophobic core is important for activation but that not all substitution in this vicinity would directly render the kinase active. It should be appreciated that p38γ was not examined rigorously and thoroughly in this study, and it is plausible that other mutations in the Phe-330 position would activate p38γ.

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mational changes of domain closure and rotation. Moreover, the L16 loop region undergoes a substantial conformational change upon activation. Consequently, the 310 helical segment observed in the unphosphorylated p38α is becoming completely disordered in residues 330–334 in p38α/H9253(Fig. 8B). One may conclude that the disruption of the hydrophobic core, especially by p38F327L and p38F327S, may result in the disorder of the L16 region and subsequent p38 activation (Fig. 9). In this context, the conformational changes in L16, followed by MKK phosphorylation, could also promote p38 activation once induced by selected mutagenesis.

The p38D176A variant exhibited comparable activity values to p38F327S and p38F327L (∼10% of the fully activated WT p38α) (Fig. 2). Unlike the three residues of the hydrophobic core that play a role in stabilizing L16, Asp-176 is located on the phosphorylation lip. It is solvent-exposed and forms no apparent interactions in the unphosphorylated p38α (Fig. 8A). The phosphorylation lip goes through a substantial conformational change upon kinase activation via MKK phosphorylation (44). The equivalent residue, Asp-179, in the activated p38α/H9253 is shifted 1.0 Å (Cα distance between the corresponding aspartates) toward the L16 vicinity, yet is still extended to the solvent without apparent interactions. It is thus difficult to deduce the direct structural implications of the D176A mutation in p38α (or D179A in p38γ). However, the activated conformation of the phosphorylation lip contains a bulge (residues 177–182) that includes at its tip Asp-179 (equivalent to Asp-176 in p38α). The conformational flexibility at L16 is directly correlated with the activity level, displayed in Fig. 2, attained by the various mutants.

**Fig. 8. p38 structure.** A, ribbon representation of p38α (Protein Data Bank code 1P38) using PyMol (49). Residues that were targeted by mutagenesis are labeled, colored, and represented as transparent space-filling spheres. The L16 loop/helix extension is shown in dark gray. Three residues identified by the HOG1 genetic screen (Tyr-69, Phe-327, and Trp-337) are in close structural proximity to form the hydrophobic core. The Asp-176 side chain is located on the phosphorylation lip and extends to the solvent without any apparent interactions with the protein. B, tube representation of the hydrophobic core using MIDAS (50). In the unphosphorylated p38α (left), both Phe-327 and Trp-337 are contributed by the L16 segment (loop and helix), whereas Tyr-69 is donated by the C-helix. Phe-327 is part of a short 310 helical segment that is assumed to be stabilized by the hydrophobic core, thus inducing low basal activity. Upon activation as seen in dually phosphorylated p38γ (right), the short helical segment, including the phenylalanine residue, is becoming disordered. The corresponding Tyr-72 and Trp-340 (corresponding to Tyr-69 and Trp-337 in p38α) maintain their structural positions because the C- and L16-helices are well stabilized by other intramolecular interactions.

**Fig. 9. Stabilization model of the hydrophobic core in p38.** Other than the hydrophobic core interactions, the two C- and L16-helices are mutually stabilized. The L16 loop at this region is mainly stabilized by the interactions formed among Tyr-69, Phe-327, and Trp-337 (left). Substituting Tyr-69 is partially disrupting the L16 loop stability (middle). Conversely, by mutating Phe-327 the hydrophobic core cannot maintain the L16 conformational stability. Thus, the L16 segment, including the mutated residue, is highly flexible (right). The conformational flexibility at L16 is directly correlated with the activity level, displayed in Fig. 2, attained by the various mutants.
By activation, the phosphorylated loop is in closer proximity to the hydrophobic core residues and L16 in comparison to the inactivated form (Fig. 10). We thus assume that the activation mechanism by p38D176A may have similar structural features as that proposed for p38F327L and p38F327S by altering the L16 conformation. Notably, mutating an adjacent residue Asp-177 to alanine in p38α does not induce any activity. These data may imply that p38D176A activity is not simply a result of lowering the local negative charge in the corresponding segment of the phosphorylation lip.

**DISCUSSION**

In this study we identified two distinct sites, Asp-176 and Phe-327 in p38α and Asp-179 in p38γ, which, upon mutagenesis, independently induce similar activation levels (Fig. 2). Combining two active single mutants resulted in a synergistic effect with a double mutant having relatively high intrinsic activity (Fig. 3). These unique p38 molecules are active independently of upstream regulation in vitro and in mammalian cell cultures. Interestingly, the levels of activity measured for each individual mutant in vitro (this study) correspondingly to its activity in mammalian cells.

We have demonstrated, by using antiphospho-p38 antibodies that all mutants exhibit different levels of phosphorylation prior to MKK6 treatment (Fig. 7) and could be further phosphorylated by MKK6. We assume that the initial phosphorylation of the p38 mutants is a result of autophosphorylation, because these mutants were extracted from a heterologous expression system that totally lacks any components of the MAP kinase signaling pathway. However, the levels of autophosphorylation are not correlated with the activities of the mutants. p38D176A/P327L, one of the most active mutants, manifests only traces of autophosphorylation without MKK6 treatment (Fig. 7). Moreover, p38F327L acquired a similar level of activity as p38D176A yet displayed lower phosphorylation levels prior to MKK activation, indicating that the intrinsic activities of the mutants does not depend on autophosphorylation.

The mutants exhibit specificity toward p38 natural substrates and can be efficiently inhibited by specific p38 inhibitors, indicating that their fold is similar to that of the activated WT protein. The activation of the mutants is proposed to induce conformational changes in the L16 loop that are also observed in activated MAP kinase structures. In contrast to the success with combinations of hyperactivating point mutations, combining a relatively active mutation from the phosphorylation lip with an inactive mutation positioned in the hydrophobic core (e.g. p38G69A/D176A and p38D176A/W337A) resulted in a non-active enzyme. Similarly, combining one of the active mutants from the hydrophobic core with a non-active mutant from the phosphorylation lip (e.g. p38D177A/F327L and p38D176A/F327L) also resulted in complete abolition of the activities of the mutants (Table III). These results may imply that there is a mutual relationship between the two p38 regions (L16 and the phosphorylation lip). This notion is supported by the p38γ model (Fig. 10), where the L16 and the phosphorylation lip are in relatively close proximity in the activated form. We postulate that mutating either or both Asp-176 and Phe-327 disrupts an inherent molecular stabilizer that maintains the low basal activity of p38. The single mutants, regardless of their intrinsic activity, were activated to full extent or even higher by MKK in reference to WT activated p38 (Fig. 2).

Given the successful utilization of HOG1 mutations for producing p38α active mutants and our preliminary results in obtaining a p38γ hyperactive mutant, we assume that it should be possible to apply similar approaches for activating other MAP kinases. Two of the HOG1 and p38α mutation sites that form the hydrophobic core are conserved in the JNK subfamily (Fig. 1). Preliminary results suggest that JNK1 is also activated by mutating those sites. Based on sequence homology (Fig. 1), we postulate that a similar approach with other p38 isotypes (p38δ and p38δ) will most probably result in constitutively active proteins. In ERK1 and ERK2 however, residues that form the hydrophobic core are not aromatic but of similar hydrophobic nature (Fig. 1). The inactive and activated ERK2 models (12, 41) (Protein Data Bank codes 1ERK and 2ERK) indicate that the L16 loop, as in p38, goes through a substantial conformational change. Upon activation, these changes were correlated with the exposure of leucine residues on the L16 and were suggested as promoting ERK2 dimerization (48). One may
assume that the L16 conformational changes are not exclusively aimed to promote dimerization but have a critical role in the kinase activation per se. The structural factors accountable for activation of the p38 mutants have been analyzed and are also supported by biochemical data. Yet, three-dimensional structure elucidation of p38 active mutants would provide insights into the activation mechanism. In principle, the active mutants could be directly emulated by other members of the MAP kinase family. This approach could become significant in identifying their specific role in signaling pathways of various systems. In addition, by applying a similar approach, intrinsically active mutants could be produced for other kinase systems and become a powerful tool in drug design strategies.

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Active Mutants of the Human p38α Mitogen-activated Protein Kinase
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