# p38α Is Active in Vitro and in Vivo When Monophosphorylated at Threonine 180[†]

Nadav Askari,[‡,§] Jonah Beenstock,[‡,§] Oded Livnah,[‡,||] and David Engelberg*[,‡]

*Department of Biological Chemistry and The Wolfson Centre for Applied Structural Biology, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel 91904*

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ABSTRACT: A common feature of the regulation of many protein kinases is their phosphorylation on a conserved Thr residue in the activation loop. In the family of mitogen-activated protein kinases (MAPKs), another phosphorylation event, on a Tyr residue neighboring this Thr (in a TXY motif), is required for activity. Many studies suggested that this dual phosphorylation is an absolute requirement for MAPK activation, assigning an equal role for the Thr and Tyr of the phosphorylation motif. Here we tested this notion by producing p38α variants carrying a T180A or Y182F mutation or both and assessing their activity in vitro and in vivo. These mutations were inserted into the p38α$^{WT}$ molecule or into constitutively active variants of p38α. We found that p38α molecules carrying the T180A mutations lost their activity altogether. On the other hand, p38α$^{WT}$ and intrinsically active mutants carrying the Y182F mutation are activated by MKK6 in vitro and in vivo, although to low levels, mainly due to reduced affinity for the substrate. However, the intrinsically active variants carrying the Y182F mutation lost most of their autophosphorylation and intrinsic activities. Thus, Thr180 is essential for catalysis, whereas Tyr182 is required for autoactivation and substrate recognition. The p38α$^{Y182F}$ mutants are capable of activating reporter genes, suggesting that they are not only catalytically active to some degree but also capable of inducing the relevant downstream pathway. We suggest that p38s are active when only the Thr residue of the phosphorylation lip is phosphorylated, similar to many other kinases in nature.

Protein kinases make up a large family of enzymes that comprise ~518 genes in the human genome (1.7% of all human genes) (*1, 2*). The large number of kinases that evolved in evolution reflects the fundamental role of phosphorylation in regulating all biochemical processes of life. Because of their central role in determining the fate of cells and organisms, the activity of most protein kinases is tightly controlled.

Although each kinase is regulated via a specific combination of regulatory modes, a common regulation of a large number of protein kinases is phosphorylation of a conserved threonine located at the activation loop (*3*−*5*). This phosphorylation, which in many cases is a result of autophosphorylation (*3, 6*−*8*), imposes conformational changes that relieve steric blocking and stabilizes the activation loop in an open and extended conformation that permits substrate binding (*3, 5*).

In some families of kinases, such as the mitogen-activated protein kinase kinases (MAPKKs,[1] also known as MEKs or MKKs) and the mitogen-activated protein kinases (MAPKs), phosphorylation of this conserved residue is not sufficient to evoke catalytic activity. In these kinases, activation is achieved only if yet another residue is simultaneously phosphorylated. In the case of MAPKs, the dual phosphorylation is usually catalyzed by MAPKKs, although MAPKs were reported to possess autophosphorylation capability as well (*9, 10*). MAPKs are further unusual because the additional phosphoacceptor is a tyrosine residue, part of a Thr-X-Tyr motif that is unique to this family of enzymes (*11*−*13*). Structural studies, mainly with MAPK ERK2, suggested that tyrosine phosphorylation contributes mainly to the establishment of substrate binding site and less to catalysis per se (*14*). However, a large number of studies with various MAPKs supported the dogma that phosphorylation on both residues is an indispensable requirement for a MAPK to become activated. Some of these studies clearly showed that altering either of these phosphoacceptors totally abolishes the enzyme's activity. For example, Anderson et al. were able to completely deactivate ERK2 by treating it with either phosphatase 2A, a phosphatase specific for phosphothreonine or phosphoserine, or CD45, a phosphatase specific for phosphotyrosine (*11*). Ahn et al. obtained similar results (*9*). Robbins et al. mutated either Thr183 (to Glu) or Tyr185 (to Phe) in ERK2 and showed that each mutation reduced activity dramatically. Similar mutations in ERK1 abolished activity completely (*16*). Just like in the ERK MAPKs, mutating either the Thr or the Tyr of the phosphorylation motif in JNK1 abolished the capability of this kinase

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* To whom correspondence should be addressed: Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel 91904. Telephone: 972-2-658-4718. Fax: 972-2-658-4910. E-mail: engelber@cc.huji.ac.il.

[‡] Department of Biological Chemistry.

[§] These authors contributed equally to this work.

[||] The Wolfson Centre for Applied Structural Biology.

[1] Abbreviations: MAPKs, mitogen-activated protein kinases; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MKK6, MAPK kinase 6; ERK, extracellular signal-regulated kinases; JNK, c-Jun $NH_2$-terminal kinase; Hog1, high-osmotic glycerol 1; ATF2, activating transcription factor 2; HA, hemagglutinin.

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to become active (*17*). The yeast MAPKs Fus3 and Kss1 were also shown to lose biological and catalytic activity when lacking either phosphoacceptor (*12*). Enslen et al. showed that the mammalian MAPKs p38α and p38*β* could not be activated when either the Thr or the Tyr in the phosphorylation lip was mutated to Ala or Phe, respectively (*18*). These studies combined suggest that the Thr and Tyr of the MAPKs' phosphorylation motif are similarly important for catalysis and biological activity.

Several other studies raised the possibility, however, that phosphorylation of the two phosphoacceptors of MAPKs may not be equally important (*19*−*21*). For example, detailed analysis of reaction kinetics in vitro revealed that monophosphorylated ERK2 molecules are highly active, 2−3 orders of magnitude more than the unphosphorylated kinase, yet they are still 1−2 orders of magnitude less active than fully active bisphosphorylated ERK2 (*20*). It is not known if monophosphorylated ERKs are catalytically and biologically functional in vivo, although such molecules seem to exist in cells (*22, 23*). Some interesting biological data are available for yeast MAPKs. In yeast MAPK Mpk1, mutating the Thr residue abolished biological activity altogether, but mutating the relevant Tyr residue reduced activity only partially. $Mpk1^{Y192F}$ could in fact complement *mpk1*Δ cells (*24*). The catalytic activity of the Mpk1 mutants was not assayed (*24*). Similarly, yeast Hog1, mutated at the relevant Tyr residue (Tyr176), was shown to lose catalytic activity almost completely but was still capable of rescuing *hog1*Δ cells when overexpressed (*19*). On the other hand, Hog1 mutated at Thr174 completely lost biological and catalytic activity (*19*). These studies, together with the existence of phosphatases that hydrolyze the phosphate group from either the Thr or the Tyr residues of MAPKs (*25*), suggest that monophosphorylated MAPKs may have a role in the cell and underscore the need to study them.

Because of the unusual nature of MAPKs, which are inactive unless dually phosphorylated, studying the role of monophosphorylated molecules was performed in two ways. (i) Mutating the relevant residues. This approach addressed in fact the combined effect of the mutations on both activation and activity and does not allow us to distinguish between the two. (ii) Applying specific phosphateses on MAPKK-activated MAPK, an approach that is valid only in vitro. We have been applying yet another approach by using the intrinsically active MAPK variants we have recently developed (*19, 26*). Such variants were obtained so far for yeast MAPK Hog1 (*19, 26, 28*) and for all isoforms of human p38 (*15, 27, 29*). Each variant carries one or two point mutations that rendered it active independent of MAPKK, probably because it acquired a spontaneous autophosphorylation capability (*30*). Although they are spontaneously active, the level of catalysis of all active variants was further elevated following MKK6 treatment (*29*). As these active variants are spontaneously active, they offer an excellent system for testing the effect of each phosphoacceptor on the active state of the enzymes. We previously showed that in the active variants of yeast MAPK Hog1 mutating the Tyr176 residue did not affect the intrinsic catalytic activity but did affect Pbs2-mediated elevation of the activity. Elimination of Thr174 abolished activity altogether (*19*). Accordingly, active Hog1 variants in which Tyr176 was converted to Phe were still independent of any upstream regulation and were capable of rescuing *pbs2*Δ cells. Active mutants lacking Thr174 could not rescue *pbs2*Δ or even *hog1*Δ cells. Thus, in Hog1, Thr174 seems to be essential not just for activation but also for catalysis per se, whereas Tyr176 is required for amplifying the activity.

In this study, we report on the role of the Thr and Tyr phosphoacceptors of human p38α. This MAPK is essential for embryogenesis and development (*31, 32*) and plays an important role in inflammation and cancer (*33*−*36*). Previous studies showed that elimination of either Thr180 or Tyr182 totally abolished catalytic activity as measured in immunoprecipitated molecules expressed in COS cells (*18, 37*). On the other hand, recent in vitro studies, in which specific phosphatases were applied on MKK6-activated p38α, found that p38α, monophosphorylated at Thr180, is catalytically active at a high level (just 1 order of magnitude lower than dually phosphorylated p38α) (*38*). To further test this issue, particularly to assess whether monophosphorylated p38α molecules may function in vivo, we mutated Thr180 and Tyr182 singly and doubly in both $p38\alpha^{WT}$ and constitutively active p38α variants. We report that p38α molecules lacking Tyr182 are catalytically active at low levels in vivo and in vitro, following MKK6 phosphorylation, and are even capable of inducing downstream target promoters. Thus, p38α molecules monophosphorylated on Thr180 could be functional in vivo. We further show, however, that Tyr182 is critical for autophosphorylation activity of p38α, and therefore, basal intrinsic activity is lost when Tyr182 is mutated. p38α molecules lacking Thr180 lost catalysis capabilities altogether.

## MATERIALS AND METHODS

*Antibodies and Other Reagents.* Anti-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p38 and anti-phosphothreonine antibodies were purchased from Cell Signaling. Anti-flag antibodies were obtained from Sigma (Rehovot, Israel). Anti-HA antibody was obtained from 12CA5 hybridomas, and anti-phosphotyrosine antibody was obtained from 4G10 hybridomas. Unless otherwise stated, all other reagents were obtained from Sigma.

*Plasmids.* For bacterial expression, the pET15b vectors were used, with the ORFs hexahistidine-tagged N-terminally, as described previously (*29*). For mammalian expression, the pcDNA3 vector was used (Startagene), with the ORFs tagged N-terminally with 3X-hemagglutinin (3XHA).

*Site-Directed Mutagenesis.* Site-directed mutagenesis, using the Stratagene QuickChange kit, was performed according to the manufacturer's instructions, using pBluescriptIISK+ human p38α as a template, and the specified primers.

*Bacterial and Mammalian Cells.* For bacterial expression, the BL21 strain of *Escherichia coli* was used. For mammalian expression, HEK293T cells were grown in Dulbeco's MEM supplemented with 10% fetal bovine serum and antibiotics. Cells were grown in 37 °C and 5% $CO_2$. Cells were transfected with the calcium phosphate method.

*Protein Purification.* Protein purification from bacterial cells was performed using Ni-NTA beads (Hadar Biotech, Rehovot, Israel) as previously described (*29*).

*In Vitro Kinase Assay.* The kinase assay was performed as described previously (*29*), with GST-ATF2 as a substrate.

Reactions were carried out for 10 min at 30 °C. For activation of p38 with MKK6, two forms of active MKK6 were used: (i) an active MKK6 (purchased from Millipore) which was activated by its upstream MAPKKK and (ii) an active mutant MKK6, in which Ser207 and Thr211 were both mutated to Glu (termed $MKK6^{EE}$), a mutation which renders the protein active (*39*). For kinetic experiments, particularly when the kinetic parameters toward ATP are measured, it was necessary to remove the ATP used for activation of the p38 molecules as well as the activator (MKK6). This was achieved by Ni-NTA bead batch purification. Thirty micrograms of MKK6-activated p38α variants was loaded and bound on 25 μL of Ni-NTA beads using mild agitation at 4 °C for 2 h. The batch was washed twice before the proteins were eluted. The protein buffer was then changed by dialysis. These stages were preformed with the same buffers used for the protein purification from bacterial cells (*29*). Protein concentrations were determined by the Bradford method and verified as p38α by Western blotting.

*Autophosphorylation Assay.* The kinase assay was performed in kinase buffer as described previously (*29*), with each reaction tube containing 1 μg of purified protein in a final volume of 21 μL. Reactions were carried out for indicated time points at 30 °C. Reactions were terminated by transferring reaction tubes to ice and adding 7 μL of 4× Laemmli's buffer.

*Immunoprecipitation and Immune Complex Kinase Assay.* Forty-eight hours post-transfection, cells were lysed in the following way. Cells were washed twice with cold PBS, followed by an addition of $^1/_2$ mL of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μg/mL leupeptin, 10 μg/mL trypsin inhibitor, 10 μg/mL pepstatin A, 313 μg/mL benzamidine, 1 mM $Na_3VO_4$, 1 mM *p*-nitrophenyl phosphate, and 10 mM β-glycerol phosphate] and incubation for 30 min under shaking. Cells were scraped, frozen in liquid nitrogen, and thawed on ice. After a 10 min centrifugation at 20000*g*, supernatant was collected; 300 μg of lysate was incubated (in lysis buffer; see above) with 20 μL of protein-G Sepharose beads (Pharmacia) bound to 1 μg of 3F10 anti-HA antibody for 16 h at 4 °C in a rotating wheel. Samples were then washed twice with 1 mL of lysis buffer and twice with 1 mL of kinase buffer [25 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 1 mM DTT, 20 mM β-glycerol phosphate, 5 mM *p*-nitrophenyl phosphate, and 0.1 mM $NA_3VO_4$]. Supernatants were removed, and 30 μL of kinase buffer containing 20 μg of GST−ATF2 protein, 20 μM ATP, and 10 μCi of [γ-$^{32}$P]ATP were added. Kinase reactions were performed in a 30 °C shaker for 30 min. We terminated reactions by placing the tubes on ice and adding 10 μL of 4× Laemmli's buffer. Samples were boiled for 3 min, and 30 μL of each sample was separated via 10% SDS−PAGE followed by transfer to a nitrocellulose membrane. The membrane was exposed to an X-ray film. To measure the levels of p38 molecules that were immunoprecipitated in each reaction, the same membrane was incubated in a blocking solution (TBS, 1% Tween 20, and 5% low-fat milk), and a Western blot was performed using the specified antibodies.

*Luciferase Assay.* HEK293 cells were plated on 12-well plates ($1 \times 10^5$ cells/well). Cells were transfected with 0.1 μg of either 6×AP-1-luc, c-fos-luc, or c-jun-luc constructs, along with either pcDNA3 empty vector or pcDNA3 containing the specified type of p38α variant (wild type or mutant). In some experiments, pBabe-$MKK6^{EE}$ (0.2 μg) was included. A plasmid (0.1 μg) encoding *Renilla* luciferase (pRL-TK) was also added to each transfection mixture as a control for transfection efficiency. The total amount of DNA was adjusted to 1 μg; 48 h post-transfection, cells were harvested and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

## RESULTS AND DISCUSSION

*$p38\alpha^{TGF}$, but Not $p38\alpha^{AGY}$, Could Be Activated in Vitro by MKK6.* To test whether intact Thr180 and Tyr182 is an absolute requirement for the catalytic activity of p38α, we constructed a series of p38α mutants in which either Thr180, Tyr182, or both were mutated. Thr180 was changed to Ala (yielding an AGY motif), and Tyr182 was changed to Phe (yielding a TGF motif), both singly or in combination (yielding an AGF motif). These substitutions were carried out on the background of wild-type p38α or on the background of the constitutively active variants of p38α ($p38\alpha^{D176A}$, $p38\alpha^{F327L}$, $p38\alpha^{F327S}$, $p38\alpha^{D176A/F327L}$, and $p38\alpha^{D176A/F327S}$) (*29*). All newly mutated proteins were expressed in *E. coli*, purified to near homogeneity, and monitored in an in vitro kinase assay, using GST−ATF2 protein as a substrate. As shown in Figure 1A, recombinant $p38\alpha^{TGY}$ (wild-type protein) did not manifest any basal activity but was strongly induced following incubation with ATP and either $MKK6^{EE}$ or MAPKKK-phosphorylated MKK6. As expected (*18, 37*), $p38\alpha^{AGY}$ had no detectable activity in vitro, with or without preincubation with MKK6 ($MKK6^{EE}$ or phosphorylated MKK6). This result verifies that Thr180 is a mandatory residue in the mechanism of activation of p38α. Unexpectedly, $p38\alpha^{TGF}$ was rendered active by both types of active MKK6, although to relatively low levels, reaching 5% activity compared to that of MKK6-treated wild-type $p38\alpha^{TGY}$ (Figure 1A). Thus, Tyr182 is not essential for catalysis, while Thr180 is. Yet, activity of $p38\alpha^{TGF}$ is very low. In an attempt to explain the basis for this low activity, we first revealed its kinetic parameters. We found that the $K_m$ of $p38\alpha^{TGF}$ toward ATF2 increased significantly compared to that of $p38\alpha^{TGY}$ (250 μM compared to 42 μM). The Y182F mutation also affected the catalytic constant of the reaction, but less severely. The $k_{cat}$ of $p38\alpha^{TGY}$ (WT) was 4.16 $s^{-1}$, while that of $p38\alpha^{TGF}$ was 1.33 $s^{-1}$ (Table 1).

Interestingly, while the Y182F mutation led to a significant decrease in the $k_{cat}$ toward ATP of MKK6-activated $p38\alpha^{TGF}$ compared to that of $p38\alpha^{TGY}$ (0.27 and 11.8 $s^{-1}$, respectively), it had only a small effect on the $K_m$ [6 and 14 μM, respectively (Table 1)]. These results imply that pT180 is sufficient for stabilizing the binding pocket for ATP. pY182 has a minor effect on the enzyme's affinity for ATP. It is rather necessary for optimal binding of other substrates and for achieving the maximal catalytic ability of this enzyme.

The essentiality of Thr180 for p38α catalysis is further emphasized by changing it in the active mutants. An AGY phosphorylation motif on the background of $p38\alpha^{D176A/F327S}$, for example, totally abolished any activity of this mutant, either the intrinsic basal activity or MKK6-induced (in Figure 1B, compare bars 2, 7, and 12 to bars 3, 8, and 13, respectively). Mutating Tyr182 in the background of the active mutant [yielding a $p38\alpha^{(D176A/F327S)TGF}$ protein] reduced

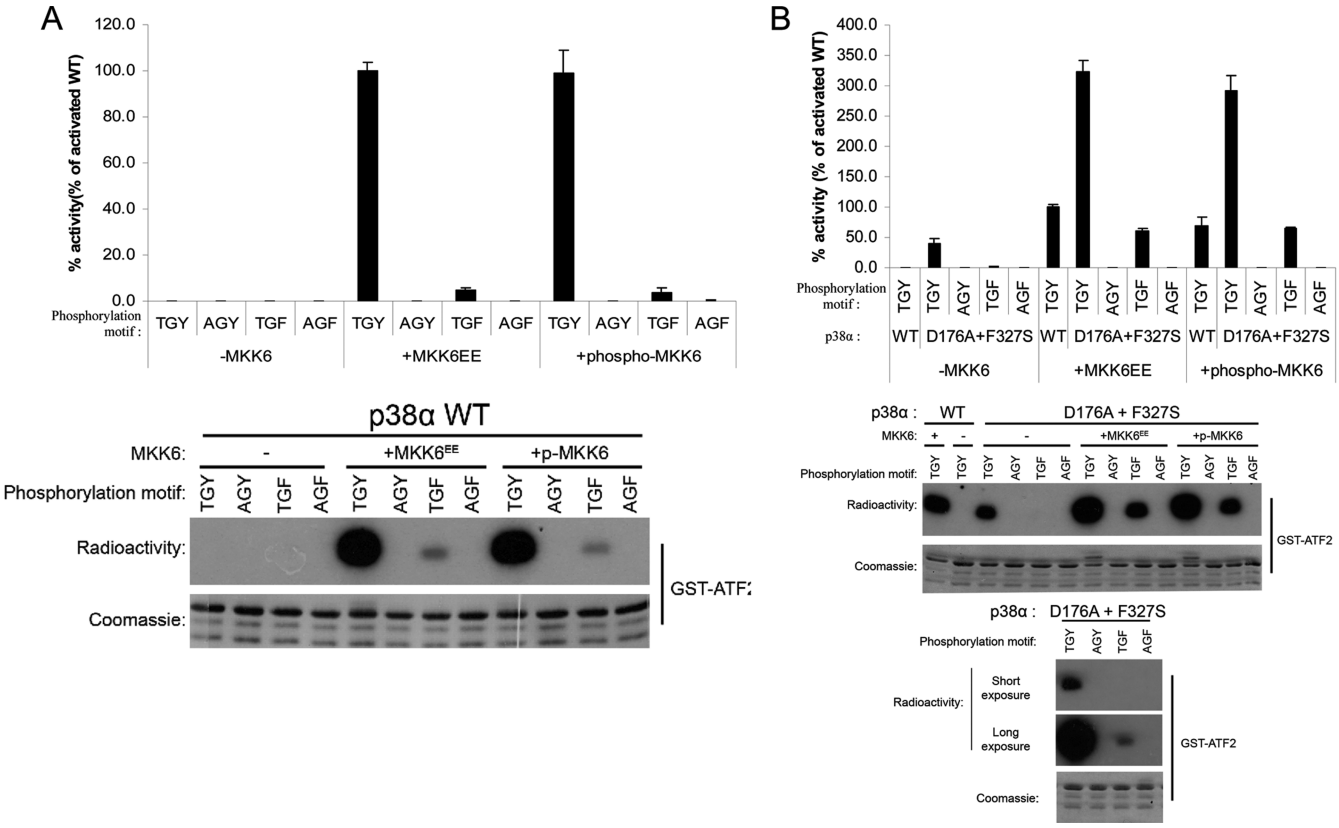


FIGURE 1: p38α molecules lacking Tyr182 are catalytically active. In vitro kinase assays were performed on recombinant proteins of $p38\alpha^{WT}$ (A) or $p38\alpha^{D176A/F327S}$ (B), mutated in the phosphorylation lip as indicated. GST−ATF2 protein was used as a substrate. The graphs in each panel represent the results of a quantitative paper-spot kinase assay. The radioactivity, measured in a scintillation $\beta$ counter, is expressed as a percentage of the activity of MKK6-activated wild-type p38α (100%). In addition, samples from each kinase reaction mixture were subjected to SDS−PAGE. Gels were stained with Coomassie, dried, and exposed to X-ray film. Shown are the autoradiograms and the Coomassie blue staining (which also serve as a loading control). For lanes 3−6 in panel B, a longer exposure of the gel is also shown.

Table 1: Kinetic Parameters of MKK6-Activated p38α

| | $K_m$ ($\mu$M) | | $k_{cat}$ ($s^{-1}$) | |
|---|---|---|---|---|
| p38α | ATP | GST−ATF2 | ATP | GST−ATF2 |
| TGY | 14 | 42 | 4.16 | 11.8 |
| TGF | 6 | 250 | 1.33 | 0.27 |

the intrinsic activity most dramatically, leaving only barely measurable traces of intrinsic activity (Figure 1B, long exposure), yet although Tyr182 seems important for the high intrinsic activity of the active mutants, it is not critical for its MKK6-mediated activity. Namely, $p38\alpha^{(D176A/F327S)TGF}$ could become active following MKK6 phosphorylation (Figure 1B), reaching an activity level that is 61% of that of MKK6-treated wild-type $p38\alpha^{TGY}$, verifying that Tyr182 is not essential for p38α catalysis. Notably, however, the activity of MKK6-treated $p38\alpha^{(D176A/F327S)TGF}$ was only ~19% of the activity of MKK6-treated $p38\alpha^{(D176A/F327S)TGY}$ (Figure 1B), further emphasizing the role of Tyr182 in amplifying the activity. Thus, Tyr182 is not essential for catalysis per se but is important for producing maximal activity after MKK6 phosphorylation, both in the wild-type molecule and in molecules carrying an activating mutation. In the latter, Tyr182 is very important for the MKK6-mediated activity as well. Essentially similar results were obtained with the intrinsically active variants $p38\alpha^{D176A}$, $p38\alpha^{F327L}$, and $p38\alpha^{F327S}$ carrying AGY, TGF, and AGF motifs, respectively (data not shown).

*The Phosphorylation Motif Mutants Are Impaired in Their Ability To Autophosphorylate.* Mechanistic and structural studies suggested that the mechanism of action of the p38α active mutants involves autophosphorylation on the phosphorylation motif, which in turn leads to the activation of the protein (*30*). It could be that both Thr180 and Tyr182 of p38α are essential for autophosphorylation. This notion may explain the fact that active mutants with either an AGY or TGF motif lose intrinsic activity altogether, but their TGF variants are strongly active if phosphorylated by MKK6. To test the effect of mutations of the TGY motif on autophosphorylation, the various mutants were tested in an autophosphorylation assay (Figure 2). $p38\alpha^{TGY}$ had some ability to autophosphorylate, but with very slow kinetics. We were able to monitor a signal only 30 minutes after the start of the reaction (Figure 2A). $p38\alpha^{AGY}$ and $p38\alpha^{TGF}$ possessed a marginal capability of autophosphorylation, and $p38\alpha^{AGF}$ exhibited no autophosphorylation activity (Figure 2A). A strong and rapid autophosphorylation activity was manifested by $p38\alpha^{D176A/F327S}$ (Figure 2B). A T180A mutation reduced this activity to barely detectable levels (Figure 2B, AGY). The Y182F mutation also had a dramatic effect on autophophorylation but did not abolish the activity. The $p38\alpha^{(D176A/F327S)TGF}$ protein could autophosphorylate, at levels that were ~5% of that of $p38\alpha^{D176A/F327S}$. The reduced capability of $p38\alpha^{(D176A/F327S)TGF}$ to autophosphorylate explains its reduced (but not abolished) intrinsic activity (see Figure 1B, long exposure). Thus, Tyr182, although not critical for catalysis, is very important for autophosphorylation.

*Phosphorylation Levels of p38α Mutants in Vitro.* Next, we tested the phosphorylation status of the various phosphorylation motif-mutated p38α variants. As expected, recombinant

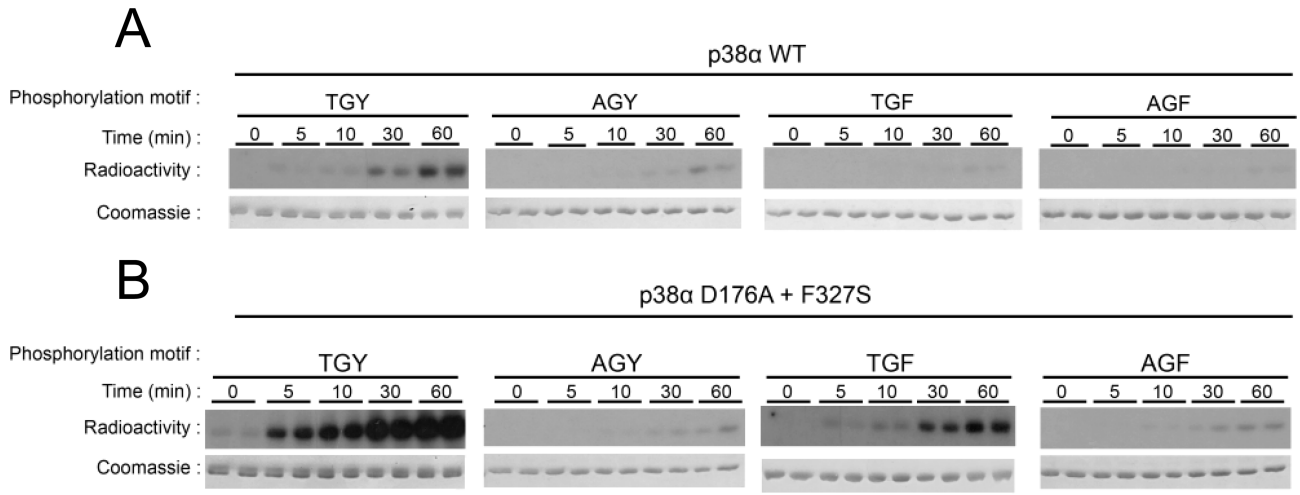


FIGURE 2: Autophosphorylation levels of the phosphorylation motif mutants. In vitro kinase assays were performed as described in the legend of Figure 1, except that GST−ATF2 protein was not included in the reaction mixture. Samples were loaded via SDS−PAGE at the indicated time points (in duplicate): (A) p38α$^{WT}$ and (B) p38α$^{D176A/F327S}$.

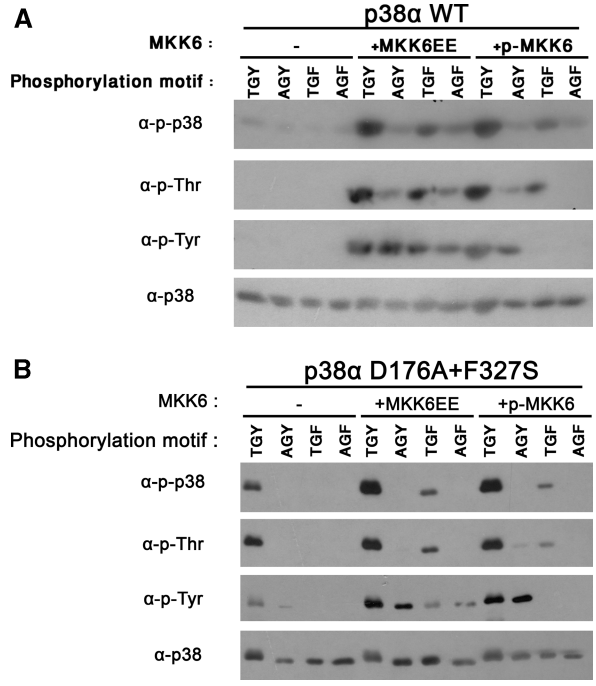


FIGURE 3: Phosphorylation status of the purified recombinant p38α mutants. The indicated mutants were treated or not (as indicated) with two forms of active MKK6: a constitutively active mutant of MKK6 (+MKK6$^{EE}$) or a commercially available MAPKKK-phosphorylated MKK6 (+phospho-MKK6). Samples were assessed via SDS−PAGE, transferred to nitrocellulose membranes, and incubated with the indicated phospho antibodies. Anti-p38 antibodies served as a control: (A) p38α$^{WT}$ proteins carrying the indicated mutations and (B) p38α$^{D176A/F327S}$ proteins carrying the indicated mutations.

p38α$^{WT}$ was not phosphorylated on either Thr or Tyr in the absence of MKK6 phosphorylation (Figure 3A). This protein became phosphorylated by the two forms of active MKK6, confirming its normal activation. In accordance with our previous report (*15*), p38α$^{D176A/F327S}$ was found to be spontaneously phosphorylated (Figure 3B). This phosphorylation was lost in p38α$^{(D176A/F327S)AGY}$, p38α$^{(D176A/F327S)TGF}$, and p38α$^{(D176A/F327S)AGF}$ (Figure 3B), in correlation with the low autophosphorylation activity of these proteins (Figure 2B). MKK6-treated p38α$^{AGY}$ did not react with anti-phospho-Thr antibodies, verifying that the only Thr residue on p38α$^{WT}$ which is phosphorylated by MKK6 is Thr180. However, following preincubation with phosphorylated MKK6, the AGY variants (on the background of either wild-type or intrinsically active p38α) were phosphorylated at Tyr. Similarly, TGF variants that were treated with MKK6 were phosphorylated at Thr (although to lower levels). Thus, MKK6 can phosphorylate Tyr182 or Thr180 regardless of the presence of the other phosphoacceptor. Surprisingly, when using MKK6$^{EE}$ for phosphorylating p38α (either the wild type or the active mutant), p38α$^{TGF}$ and p38α$^{AGF}$ reacted with anti-phospho-Tyr antibodies, suggesting that the MKK6$^{EE}$ mutant phosphorylates another Tyr residue on p38α besides Tyr182. Notably, phospho-MKK6-treated p38α$^{TGF}$ or p38α$^{AGF}$ did not react with anti-phospho-Tyr antibodies. This unexpected MKK6$^{EE}$-mediated phosphorylation probably represents a nonspecific artificial activity of MKK6$^{EE}$. A candidate residue for this nonspecific activity is Tyr323 of p38α, which has been shown to be phosphorylated by ZAP70 in T cells (*10*). However, p38α$^{AGF+Y323F}$ was still phosphorylated at the tyrosine residue by MKK6$^{EE}$ as detected by anti-phosphotyrosine antibodies (data not shown). Thus, the identity of the extra target of MKK6$^{EE}$ (i.e., in addition to Thr180 and Tyr182) is currently unknown to us.

*p38α$^{WT}$ or p38α Active Mutants Could Be Activated in Vivo When Mutated at Tyr182, but Not at Thr180.* The observation that TGF variants are active in vitro raises the possibility that such monophosphoylated p38α molecules are also active in vivo. We tested, therefore, the activity of the phosphorylation motif mutants in mammalian cells. The cDNAs encoding the mutants were subcloned into a mammalian vector (pcDNA3) as 3XHA-tagged proteins. All mutants were transiently expressed in 293 cells and assayed for catalytic activity (following immunoprecipitation) and for phosphorylation status (Figure 4A). In agreement with the results of Raingeaud et al. (*37*), p38α$^{AGY}$ was not activated in mammalian cells by either coexpression of MKK6$^{EE}$ or UV irradiation (Figure 4A, top panel, and data not shown). However, p38α$^{TGF}$ could phosphorylate GST−ATF2 protein when immunoprecipitated from cells coexpressing MKK6$^{EE}$, although the phosphorylation level was significantly lower than that obtained with p38α$^{WT}$. The p38α$^{AGF}$ mutant manifested no activity, like the p38α$^{AGY}$ mutant. Thus, it seems that in the context of the p38α$^{WT}$

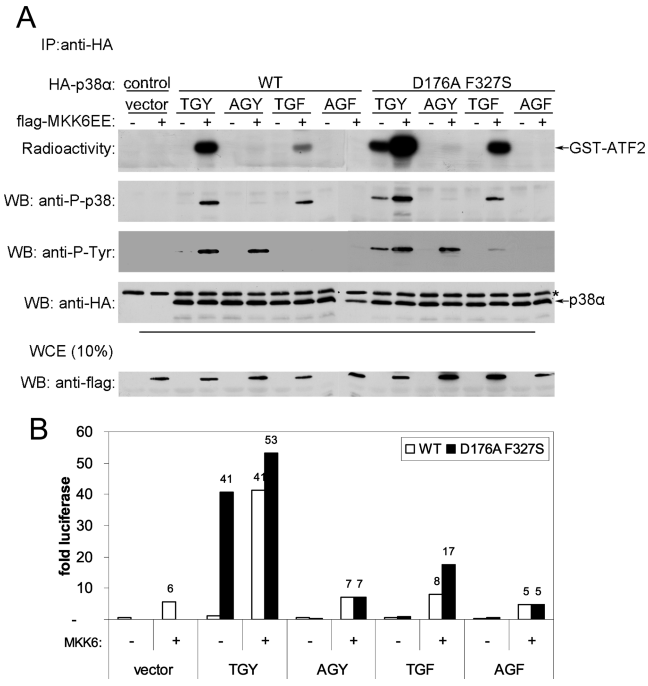


FIGURE 4: Activity and phosphorylation status of the phosphorylation motif mutants expressed in mammalian cells. (A) HEK293T cells were cotransfected with constructs expressing the indicated mutants, either with MKK6$^{EE}$ or with an empty vector (−), as indicated. Forty-eight hours post-transfection, cell lysate were prepared. p38α molecules were immunoprecipitated using anti-HA antibodies, and the immune complex was used in a kinase assay using GST−ATF2 protein as a substrate. Reaction mixtures were subjected to SDS−PAGE, and membranes were exposed to X-ray film. Membranes were then subjected to Western blotting using the indicated antibodies (membranes were stripped and reprobed). The upper bands in the lower-most panel represent the heavy chain of the anti-HA antibody, which was used for the immunoprecipitation (marked with an asterisk). WB, Western blotting; WCE, whole cell extract (10% input). (B) Luciferase assay in cells expressing the mutants. HEK293T cells were cotransfected with a construct containing six AP1 elements fused to the luciferase gene (6XAP1-luciferase), along with an empty vector or MKK6$^{EE}$, and the indicated p38α constructs. Forty-eight hours post-transfection, cells were harvested and lysates were subjected to the luciferase assay as described in Materials and Methods.

protein, Thr180 is vital for the ability of the protein to become active. Tyr182 on the other hand seems dispensable for activity per se but is required to produce maximal levels of activity. These results correlate with the results obtained with the same proteins in vitro (see Figure 1).

When the activity of p38α$^{(D176A/F327S)AGY}$, p38α$^{(D176A/F327S)TGF}$, and p38α$^{(D176A/F327S)AGF}$ was tested in 293 cells, we found that mutating either Thr180 or Tyr182 totally abolished the spontaneous activity of the otherwise hyperactive mutant (in Figure 4A, compare lane 11 to lanes 13 and 15), suggesting that both phosphoacceptors are required for this intrinsic, MKK6-independent activity. However, when these mutants were coexpressed with active MAPKK MKK6$^{EE}$ (or in cells exposed to UV radiation), p38α$^{(D176A/F327S)TGF}$ mutant was activated to high levels (Figure 4A, lane 16, and data not shown). These results indicate that Thr180 is a critical component of p38α catalysis capability, whereas Tyr182 acts as an "amplifier" that increases the enzyme's catalytic activity. However, in the context of the intrinsically active variant, Tyr182 is critical for the intrinsic activity, most probably by contributing to its autophosphorylation activity (Figure 2). Indeed, neither p38α$^{(D176A/F327S)AGY}$ nor p38α$^{(D176A/F327S)TGF}$ is spontaneously phosphorylated when expressed in 293 cells (Figure 4A; see below).

Intrinsically active variants of p38α are spontaneously phosphorylated when expressed in mammalian cells (*15*). This phosphorylation was eliminated by mutating either Thr180 or Tyr182 (Figure 4A), supporting the notion that spontaneous phosphorylation is a result of autophosphorylation. Indeed, both p38α$^{(D176A/F327S)AGY}$ and p38α$^{(D176A/F327S)TGF}$ were phosphorylated on the nonmutated phosphoacceptor in cells coexpressing MKK6$^{EE}$ (Figure 4A). It should be noted that the anti-phospho-p38 antibody, which should recognize only the dually phosphorylated form of p38, could also detect the TGF mutants [which, obviously, do not have any phospho-Tyr182 (Figures 3 and 4A)], in agreement with the work of Bell et al. (*19*). The results in 293 mammalian cells are reminiscent of the autophosphorylation results obtained in vitro (Figure 2).

The finding that p38α (WT or active mutant) is still catalytically active to some degree when mutated in the TGY motif raises the question of whether these mutants also activate cellular downstream targets. To test this question, we measured the ability of the phosphorylation lip-mutated variants to induce the expression of an AP-1-luciferase reporter gene. Components of the AP-1 transcriptional activating complex (such as ATF2 and CREB) are known targets of the p38α pathway (*40*). As shown in Figure 4B, there is a clear correlation between the levels of kinase activity of these variants and their ability to induce AP-1-luciferase. Namely, the p38α$^{D176A/F327S}$ mutant, which was able to spontaneously induce the reporter gene 41-fold, lost this ability when carrying an AGY or TGF motif. However, the p38α$^{(D176A/F327S)TGF}$ mutant was capable of inducing the gene 17-fold in cells coexpressing MKK6$^{EE}$.

In summary, in human 293 cells, p38α$^{TGF}$ and p38α$^{(D176A/F327S)TGF}$ could be catalytically active to low levels. p38α$^{(D176A/F327S)TGF}$ could even induce the expression of the AP-1-luciferase reporter, showing that it activates the downstream pathway all the way to the AP-1 transcriptional activators.

*Thr180 Is Conserved in Many Protein Kinases and Is Critical for Stabilizing the Active Site.* Why are p38α molecules still active to some degree when lacking Tyr182 but are absolutely inactive when lacking Thr180? Structural studies with active ERK2 and active p38γ suggest different roles for the two phosphoacceptors. In dually phosphorylated ERK2, phospho-Thr183 makes eight hydrogen bonds or ionic contacts with the N-terminal domain and is further the focus of two extended networks of bonds that influence the orientation of helix C toward the phosphorylaion lip (*14*). Phospho-Tyr185, on the other hand, forms only four interactions and is largely exposed to the solvent. In dually phosphorylated p38γ, phospho-Thr183 interacts with at least five residues and seems to be responsible, more than phospho-Tyr185, for domain closure and establishment of the active conformation. Furthermore, the threonine residue of the TXY motif of MAPKs is in fact conserved in a large number of kinases; most of them are regulated via phosphorylation of this site (*3, 5*). In contrast, the tyrosine residue is unique to MAPKs. In most kinases studied so far (e.g., cAMP-dependent PKA; CDK2), phosphorylation of this threonine is critical for their catalysis. Just like in ERK2 and p38γ, the analogous phosphothreonine of cPKA (phospho-

Thr197) forms interactions with critical N-terminal domain residues, stabilizing the catalytic site and allowing ATP binding. Importantly, mutating this threonine of cPKA to an aspartate or alanine reduced the level of catalysis significantly, mostly by reducing the affinity for ATP (*3, 5, 41*). Thus, the phosphothreonine of MAPK, just as in many other kinases, is a critical element required for formation and stabilization of catalytic sites. Although the phosphotyrosine of MAPKs also contributes to domain closure and establishment of the active conformation (*14, 42*), its main role seems to be in substrate recognition, particularly in determining the specificity of the kinase toward substrates with a proline at P + 1 (*14*). Thus, it could be that in the absence of Tyr182, the many interactions formed by the phosphothreonine of p38α are sufficient to stabilize the kinase in a conformation close to the optimal active conformation, allowing some level of activity. Substrate recognition, however, could be defective, explaining the reduced activity manifested by the $p38^{TGF}$ molecules. The fact that most other kinases in nature are fully active following phosphorylation of the threonine alone supports the more critical role of the threonine for catalysis per se.

The discussion above suggests that the tyrosine residue of MAPKs may have appeared in evolution mainly as a tool to assist substrate specificity. It could also have an important regulatory role. Phosphorylation of the TXY motif does not occur concomitantly, but rather orderly, with Tyr182 being more favored catalytically (*14*). It could be that efficient phosphorylation of the threonine is dependent on preliminary phosphorylation of the tyrosine. This notion, assigning a role for the phosphotyrosine in regulating threonine phosphorylation and fully activating the kinase, not only provides an explanation for the appearance of the tyrosine later in the evolution of kinases but can also explain the low activity and reduced level of phosphorylation of our $p38^{TGF}$ molecules.

The low activity of TGF mutants could be further explained by the idea of Wilson et al., who suggested that Tyr182 occupies a binding site (*43*), and that the phosphorylated Tyr182 moves by ~15 Å, causing a refolding of the activation lip and potentially exposing the active site (*43*). It could be that altering the Tyr182 phosphoacceptor to a nonphosphorylatable amino acid (Phe) prevents this movement, and therefore, full exposure of the active site is not obtained.

Studying monophosphorylated MAPKs seems relevant because they most likely exist in vivo (*23, 24*). Particularly with regard to p38, Hale et al. showed that p38δ is found in a monophosphorylated form in macrophages after LPS treatment (*44*). Frantz et al., by the use of mass spectroscopic analysis, showed that when expressed in a heterologous system p38α can be found in different phosphorylation states (*45*). The nature of the enzymes regulating MAPKs also supports the notion that monophosphorylated MAPKs do exist in vivo. First, MAPKK-mediated MAPK phosphorylation involves a bi-bi reaction. Therefore, during activation of MAPKs, there is a monophosphorylated intermediate (*46*).

The second reason is the existence of MAPK-specific phosphatases which dephosphorylate only one of the two phosphoacceptors (e.g., PTPN5 and PTPN7 for Tyr dephosphorylation and PP2A and PP2C for Thr dephosphorylation) (*25, 47*).

MAPKs are involved in the cell responses to various stimuli. Their activity must be tightly tuned, reaching the exact level required by the type and intensity of each stimulus. Monophosphorylated MAPKs may serve as another mode taken by the cell to control the intensity of the signal. Monophosphorylated forms of MAPKs may serve, for example, as transmitters of low levels of signals or as tools that preserve some levels of activity after downregulation of the initial spike of activation.

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