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p38β Mitogen-Activated Protein Kinase Modulates Its Own Basal Activity by Autophosphorylation of the Activating Residue Thr180 and the Inhibitory Residues Thr241 and Ser261

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Many enzymes are self-regulated and can either inhibit or enhance their own catalytic activity. Enzymes that do both are extremely rare. Many protein kinases autoactivate by autophosphorylating specific sites at their activation loop and are inactivated by phosphatases. Although mitogen-activated protein kinases (MAPKs) are usually activated by dual phosphorylation catalyzed by MAPK kinases (MAPKKs), the MAPK p38β is exceptional and is capable of self-activation by *cis* autophosphorylation of its activation loop residue T180. We discovered that p38β also autophosphorylates in *trans* two previously unknown sites residing within a MAPK-specific structural element known as the MAPK insert: T241 and S261. Whereas phosphorylation of T180 evokes catalytic activity, phosphorylation of S261 reduces the activity of T180-phosphorylated p38β, and phosphorylation of T241 reduces its autophosphorylation in *trans*. Both phosphorylations do not affect the activity of dually phosphorylated p38β. T241 of p38β is found phosphorylated *in vivo* in bone and muscle tissues. In myogenic cell lines, phosphorylation of p38β residue T241 is correlated with differentiation to myotubes. T241 and S261 are also autophosphorylated in intrinsically active variants of p38α, but in this protein, they probably play a different role. We conclude that p38β is an unusual enzyme that automodulates its basal, MAPKK-independent activity by several autophosphorylation events, which enhance and suppress its catalytic activity.

Enzymes can be divided into nonregulated (substrate-dependent) and regulated (enzyme-dependent) groups (1). The activity of regulated enzymes is controlled in various ways, including allostery, posttranslational modifications, and alteration of subcellular localization. Some regulated enzymes can self-convert their activation state by using their own catalytic activity. In many GTPases, for example, autoregulation via the catalysis of GTP hydrolysis serves as a self-termination mechanism. Some other enzymes are activated by their own catalytic activity. For example, in most eukaryotic protein kinases (EPKs), phosphorylation of a conserved threonine residue, located in a region termed the activation loop, is a requirement for catalytic activity. Because activation loop phosphorylation in the majority of EPKs is achieved via autophosphorylation, these enzymes can be considered self-activators. This phosphorylation imposes dramatic conformational changes that convert kinases from an inactive to an active form (2, 3). In addition to activation loop phosphorylation, a large number of kinases are further phosphorylated at other sites, found either within the putative kinase domain (a domain shared by all EPKs) or in structural motifs specific to subfamilies of kinases. These phosphorylation events serve to regulate the kinase in various manners, including priming for activation, modifying subcellular localization, or determining half-life or interaction with other proteins (for example, see references 4–7). In some rare cases, phosphorylation events might be inhibitory (for example, see reference 8). In most cases, EPKs are inactivated by dephosphorylation, a reaction which is not the reverse reaction of phosphorylation, and is catalyzed by a different group of enzymes, called phosphatases. Therefore, although EPKs can be considered self-activators, they usually do not harbor the capacity to reverse their active conformation. Here we describe an unusual case of a protein kinase, the mitogen-activated protein kinase (MAPK) p38β, which controls its own basal activity by a series of activating as well as inhibitory autophosphorylation events.

The mammalian p38 MAPK family is composed of four isoforms: p38α, p38β, p38γ, and p38δ (9). p38 MAPKs are vital for a plethora of cellular processes (9). Loss of their regulation is associated with various pathologies such as chronic inflammation (9, 10) and cancer (11), illustrating the importance of tight regulation of their activity. Like most EPKs, p38 MAPKs are regulated by activation loop phosphorylation. However, in addition to their activation loop Thr residue, MAPKs possess an adjacent Tyr phosphorylation site, generating a T-X-Y motif (TGY in p38 proteins). Phosphorylation of the TGY motif in p38 MAPKs is commonly catalyzed by the MAPK kinases (MAPKKs) MKK3 and MKK6 (12). Thus, unlike many EPKs, the p38 proteins do not spontaneously autophosphorylate (with the exception of p38β [see below]). As unregulated MAPK activity is associated with various

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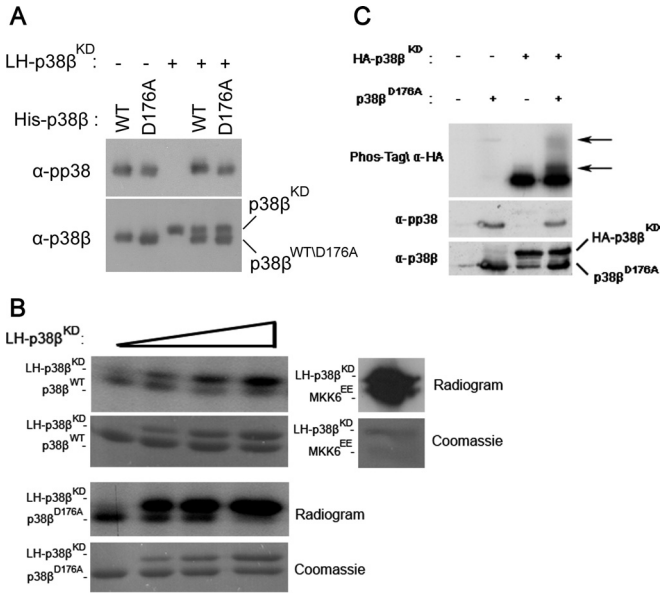

FIG 1 p38β possesses *trans*-autophosphorylation activity that does not target the TGY motif. Recombinant purified His-tagged $p38\beta^{WT}$ or $p38\beta^{D176A}$ was coincubated in an autophosphorylation assay mixture for 1 h with a kinase-dead p38β variant that contains a longer tag (long-His-tagged $p38\beta^{KD}$ [LH-$p38\beta^{KD}$]). (A and B) Samples were separated by SDS-PAGE and analyzed by Western blotting (A) and by exposing gels to an X-ray film (radiogram) (B). (A) Following an autophosphorylation reaction, 100 ng of the indicated proteins was separated and analyzed with the indicated antibodies by Western blotting. (B) Reaction mixtures contained 500 ng $p38\beta^{WT}$ or $p38\beta^{D176A}$ with 0.25 to 1 μg long-His-tagged $p38\beta^{KD}$ and radiolabeled ATP. Five hundred nanograms of MKK6-phosphorylated long-His-tagged $p38\beta^{KD}$ was used as a positive control (right). (C) HEK293 cells were transiently transfected with plasmids expressing the indicated p38 proteins. Cell lysates were prepared 48 h after transfection, and 30 μg protein from each sample was separated by SDS-PAGE, blotted, and probed with the indicated antibodies. Two micrograms of each sample was separated by SDS-PAGE using gels supplemented with the Phos-tag reagent, blotted, and probed with the indicated antibodies. Arrows indicate slower-migrating bands that result from phosphorylation.

diseases (10), it seems likely that suppression of spontaneous activation in MAPKs was selected during the course of evolution to tighten their regulation. Notably, under some conditions, p38 MAPKs were shown to be activated via induced autophosphorylation of the activation loop Thr site (13–17). Monophosphorylation of the Thr residue of the TGY motif is sufficient for catalytic activity and stabilizes an active conformation with properties distinct from those of dually phosphorylated p38 proteins (18–20). This shows that like many EPKs, p38 MAPKs also possess a self-activating capability, but it is tightly controlled and manifested only under specific conditions. It is not known how the autophosphorylation of MAPKs is suppressed. However, because purified MAPKs do not self-activate, there is most probably an inherent structural motif responsible for this suppression (19, 21).

Among MAPKs, p38β is unique and manifests spontaneous activation loop autophosphorylation activity *in vitro* (19). This activity is regulated and does not occur spontaneously in mammalian cell culture (19). The activating autophosphorylation of p38β seems to occur primarily in *cis*, as indicated by its failure to occur on kinase-dead (KD) p38β ($p38\beta^{KD}$) molecules when these molecules are mixed with intact p38β proteins (19) (Fig. 1A). The unique autophosphorylation activity of p38β is generated by a short 13-amino-acid region (R234 to V246) that comprises the G helix and the N-terminal part of the MAPK insert. Grafting this short fragment into p38α is sufficient to induce spontaneous autophosphorylation in p38α *in vitro* and in mammalian cell culture (19). The structural motifs in p38β responsible for suppressing its autoactivation in mammalian cells were also identified. One motif is the C-terminal part of the MAPK insert, and another is the interaction between the C-terminal extension and the N-terminal lobe (19). Notably, the MAPK insert and C-terminal extension are two structural elements unique to MAPKs (22, 23).

In this study, we report two novel autophosphorylation sites that occur on conserved sites in p38β and p38α: T241 and S261. Both sites reside within the MAPK insert. Phosphorylation of T241 and S261 occurs in *trans* and affects the activity of T180-monophosphorylated p38β but not that of dually T180/Y182-phosphorylated p38β. T241-phosphorylated p38β manifests reduced autophosphorylation activity toward the non-activation loop sites. S261-phosphorylated p38β shows reduced activity toward the substrate ATF2. Thus, p38β uses its own enzymatic activity to both activate and downregulate its basal catalytic activity. This is a very rare regulatory mode in enzymes. Using antibodies specifically raised to detect T241-phosphorylated p38β, we found that this site is phosphorylated in muscle and bone tissues in adult mice, suggesting physiological relevance for these phosphorylation events. Furthermore, we show that phosphorylation of T241 is strongly correlated with differentiation of myoblasts to myotubes in culture. T241 and S261 of p38β are highly conserved in p38 molecules from yeast to human and in some other MAPKs as well. Indeed, we further show that the *trans*-phosphorylation capability of p38β is not restricted to other p38β molecules, but it can actually target all p38 isoforms. This pan-p38 phosphorylation activity of p38β targets the same sites in the other p38 proteins. Finally, p38α also manifests such *trans*-isoform activity, but in p38α, these phosphorylated sites seem to have an opposite role, and they are rather required for the activity of T180-monophosphorylated p38α. Thus, p38 proteins are capable of controlling their own basal, MAPKK-independent activity via a variety of autophosphorylation events.

MATERIALS AND METHODS

Mammalian cell culture. HEK293, mouse embryonic fibroblast (MEF) MKK3/6$^{-/-}$ L8, and C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, Na-pyruvate, and antibiotics. MCF10A cells were grown in DMEM–F-12 medium (1:1) with 5% horse serum, 0.02 μg/ml epidermal growth factor (EGF), 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.5 μg/ml cholera toxin, and antibiotics. Cells were grown at 37°C and 5% CO_2. HEK293 cells were transfected by using the calcium phosphate method. MEF cells and MCF10A cells were transfected by using the TransitX2 transfection reagent (Myrus). Cell lines that appear in Fig. 8C were grown under the conditions required for each cell line.

C2C12 and L8 cell differentiation. To induce differentiation in C2C12 and L8 myoblasts, growth medium was replaced with differentiation medium (DMEM supplemented with 2% horse serum, 0.1 μg/ml insulin, Na-pyruvate, and antibiotics) when cultures were subconfluent.

Immune depletion. A total of 1×10^6 MCF10A cells were plated onto a 10-cm plate for 24 h. Growth medium was removed, and the plate was washed with cold phosphate-buffered saline (PBS) twice. Cell lysis was performed with 0.5 ml lysis buffer at 4°C with rotation for 30 min (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 10 μg/ml pepstatin, 313 μg/ml benzamidine, and phos-

phatase inhibitor cocktails 2 and 3 [catalogue numbers P0044 and P5726; Sigma], according to the manufacturer's instructions). Lysates were then scraped with a rubber policeman into a tube, flash-frozen in liquid nitrogen, and thawed on ice. Once thawed, lysates were centrifuged for 10 min at 12,000 relative centrifugal force (RCF) at 4°C. The supernatant was transferred to a new tube, and the protein concentration was determined by using the Bradford method (24). Five hundred micrograms of protein at a final volume of 500 μl was transferred into three tubes and incubated with rotation at 4°C overnight with or without 2 μl of anti-pT241 antibody (rabbit) or anti-p38β antibody (rabbit, catalogue number 9212; Cell Signaling). On the following morning, 25 μl of agarose-protein A (catalogue number IP02; Calbiochem) was added to each tube for 2 h with rotation at 4°C. Beads were pelleted by centrifugation at 1,000 RCF for 2 min. The supernatant was mixed with X4 Laemmli buffer, and 30 μl of each sample was subjected to Western blot analysis with anti-p38β (catalogue number 6187; Santa Cruz), antiactin, or anti-p38α.

siRNA knockdown. A total of 3×10^5 MCF10A cells were plated into a 6-well plate. Twenty-four hours later, cells were transfected with 3.4, 6.8, or 13.6 μl of 10 μM p38β small interfering RNA (siRNA) (catalogue number sc-39116; Santa Cruz) mixed with 10 μl of the Transit X2 transfection reagent (Myrus), according to the manufacturer's instructions. As a control, cells were transfected with 2 μg of pcDNA eGFP. Cell lysates were prepared at 48 h posttransfection and subjected to Western blot analysis.

Protein expression and plasmid construction. For bacterial expression, the pET15b, pET28b, and pGST para vectors were used, with the open reading frames (ORFs) encoding products tagged N terminally with hexahistidine (His) or glutathione *S*-transferase (GST). Protein expression was induced in the *Escherichia coli* Rosetta strain (Novagen). For mammalian expression, the pcDNA3 (Stratagene) and pCEFL (Addgene) vectors were used, with the ORFs encoding products tagged N terminally with a 3× hemagglutinin (3×HA) or a Flag tag. Site-directed mutagenesis, using the *Pfu* Ultra II kit (Agilent), was performed according to the manufacturer's instructions.

Protein purification. Protein purification from *E. coli* cells was performed by using Ni-nitrilotriacetic acid (Ni-NTA) beads (Hadar Biotech) as previously described (25). GST-tagged proteins were purified by using glutathione-Sepharose high-performance beads (GE Healthcare Life Sciences) according to the manufacturer's protocol. Protein concentrations were determined by the Bradford method.

***In vitro* kinase assay.** All reactions were conducted in 96-well plates in triplicates. To initialize the reaction, 45 μl of the reaction mixture was added to 5 μl of p38 enzyme (0.2 μg [100 nM] of purified His-tagged p38). Final reaction mixtures contained 25 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 20 mM 2-glycerolphosphate, 0.1 mM Na_3VO_4, 1 mM dithiothreitol (DTT), 40 μg GST-ATF2, 50 μM ATP, and 0.5 μCi of [γ-^{32}P]ATP. Reactions proceeded for 10 min at 30°C with agitation and were terminated with 50 μl of 0.5 M EDTA (pH 8) (250 mM final). Following reaction termination, aliquots of 85 μl from each well were spotted onto 3-cm by 3-cm Whatman 3-mm paper squares and air dried. Each square was rinsed three times with 10% trichloroacetic acid and 3% sodium pyrophosphate (10 ml/square) for 1.5 h (each time) with gentle agitation, and a fourth wash was performed overnight without shaking. On 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 measured by using a scintillation counter, running a ^{32}P Cherenkov program. For activation of p38 with MKK6, an active mutant of MKK6 in which Ser207 and Thr211 were both mutated to Glu (termed MKK6^{EE}) was used under similar conditions. Autophosphorylation reactions were conducted under similar conditions, with 0.35 μg of His-tagged p38 protein in the same buffer as that described above for the kinase assay (excluding GST-ATF2). To test for *trans* autophosphorylation, 1 μg of the GST-p38 fusion protein (commonly carrying the KD mutation) was included in the reaction mixture. The final volume was 30 μl. Reactions were terminated, unless stated otherwise, after 1 h by adding X4 Laemmli buffer to the mixture, and the mixture was boiled at 100°C for 4 min, separated by SDS-PAGE, stained with Coomassie, dried, and exposed to film.

In-solution tryptic protein digest. Five micrograms of p38α$^{D176A+F327S}$, wild-type (WT) p38β (p38β^{WT}), p38β^{D176A}, and p38β^{T241A} proteins was used to detect autophosphorylation via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples to be analyzed were diluted in 8 M urea and 100 mM ammonium bicarbonate. Before tryptic digestion, cysteine residues were reduced with 2.8 mM DTT (catalogue number D9163-5G; Sigma) for 30 min at 60°C and carboxymethylated with 8.8 mM iodoacetamide (catalogue number I6125-100G; Sigma) for 30 min in the dark at room temperature. Trypsin (sequencing-grade modified, catalogue number V511A; Promega) was added, after the samples were diluted in 2 M urea, at a ratio of 1:50, and the mixture was incubated overnight at 37°C. A second dose of trypsin was added on the following morning for 4 h at 37°C. The samples were then acidified with 1% trifluoroacetic acid (TFA) and desalted with Micro TipColumn C_{18} columns (catalogue number 74-5201; Harvard Apparatus).

In-gel tryptic protein digest. For *trans* phosphorylation, 2.5 μg of GST-p38α^{KD}, GST-p38γ^{KD}, and GST-p38δ^{KD} was first subjected to a kinase reaction in the presence of p38α$^{D176A+F327S}$ or p38β^{D176A}, separated by SDS-PAGE, excised from the gel, and washed at room temperature for 30 min with 100 mM ammonium bicarbonate. The slices were incubated with 150 μl of 100 mM ammonium bicarbonate and 10 μl of 45 mM DTT for another 30 min at 60°C with shaking. Samples were then incubated with 10 μl of 150 mM iodoacetamide at room temperature in the dark for 30 min. Each slice was washed with a solution containing 500 μl of 50% acetonitrile and 100 mM ammonium bicarbonate for 30 min. The top fluid was removed, and 50 μl of 100% acetonitrile was administered to cover the gel for 10 min. The remaining fluids were evaporated by using a SpeedVac for 10 min. The samples were then incubated overnight with trypsin (1 μg trypsin in 25 μl of 10% acetonitrile and 10 mM ammonium bicarbonate) at 37°C. The following day, formic acid was added to a final concentration of 1%, and samples were incubated with shaking for 30 min. The top fluid and gel slices were separated. The top fluid was dried in the SpeedVac and resuspended in 0.1% TFA for desalting on a C_{18} stage tip.

Analysis by liquid chromatography-MS/MS and interpretation of MS data. The tryptic peptides of p38 proteins were resolved by reversed-phase chromatography on pulled 0.075-mm-internal-diameter (ID), ~20-cm-long fused silica capillaries (catalogue number 160-2644-10; Agilent) packed with Reprosil-Aqua C_{18} reversed-phase material (Dr. Maisch GmbH, Germany). The peptides were eluted with linear 30-min gradients of 5% to 28% solvent B (100% acetonitrile), followed by 15-min gradients of solvent B that were increased to 95%. This was followed by a 15-min wash with 95% solvent B. The flow rate was 0.15 μl/min, using an Easy-nLC-1000 capillary high-performance liquid chromatography (HPLC) system. MS was performed with a Q-Exactive-Plus mass spectrometer (Thermo Fisher), using the positive-ion mode and, repetitively, full MS scan, followed by high-collision dissociation (HCD) with multistage activation of the 10 most dominant ions selected from the full MS scan. The mass spectrometry data were analyzed by using MaxQuant versions 1.3.0.5 and 1.5.0.25 (26) with Andromeda (27) search engines, searching against the *E. coli* section of the UniProt database from September 2012, including the human p38 proteins and their mutated variants. Identifications of the proteins and phosphorylation sites were filtered to a 1% false discovery rate (FDR). The resulting identified phospho-site table was filtered to include only phospho-sites on p38 proteins and their mutated variants.

Western blot analysis. For recombinant proteins, 100 ng was mixed with X4 Laemmli buffer and boiled at 100°C for 4 min. Mammalian cell protein lysates were prepared by decanting the growth medium, washing cells with PBS, and adding Laemmli buffer directly to the plate. Lysates were then collected and boiled for 10 min. The different samples were separated via 10% SDS-PAGE and transferred onto nitrocellulose paper. Phos-tag gels were prepared according to the manufacturer's protocol

(Wako). Briefly, gels were supplemented with 25 μM Phos-tag reagent and 50 μM $MnCl_2$, and diammonium peroxysulfate was used instead of ammonium persulfate. Gels were rinsed with 100 mM EDTA-containing transfer buffer for 30 min before transfer onto a polyvinylidene difluoride (PVDF) membrane. Antibodies used in this study were anti-phospho-p38 (catalogue number 9211; Cell Signaling), anti-HA antibodies (obtained from the 12CA5 hybridoma cell line), anti-MAPKAPK2 (catalogue number 3042; Cell Signaling) anti-phospho-MAPKAPK2 (catalogue number 3007; Cell Signaling), anti-Flag (catalogue number A2220; Sigma), anti-p38β (catalogue numbers 9212 [Cell Signaling] and 6187 [Santa Cruz]), antimyogenin (catalogue number 12732; Santa Cruz), anti-p38α (catalogue number 535; Santa Cruz), and antiactin (catalogue number 691001; MP). The anti-phospho-T241 and -S261 antibodies were custom made by Sigma by injecting keyhole limpet hemocyanin (KLH)-conjugated peptides MEVVG(p)TPSPEV (pT241) and RTYIQ(p)SLPPMP (pS261) into rabbits and obtaining reactive serum 3 months after the initial injection.

Sample preparation from mouse tissues. Eight-week-old male C57BL/6N mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation. Tissues were extracted, washed twice in PBS, and diced, and 100 mg was homogenized in 0.5 ml radioimmunoprecipitation assay (RIPA) buffer (containing protease inhibitors, NaV, NaF, and sodium pyrophosphate) in Dounce homogenizers. For bone samples, the femoral bones were separated, cleaned of soft tissues, and snap-frozen in liquid nitrogen. Frozen bones were then pulverized with a mortar and pestle, followed by resuspension in RIPA buffer and homogenization. Extracts were then left on ice for 30 min, followed by 10 min of centrifugation at 10,000 RCF. The supernatant was separated from the pellet and fat and centrifuged again. This was repeated until the supernatant was cleared, at which point it was resuspended in sample loading buffer. Total protein in each sample was quantified by using the Bradford assay.

RESULTS

p38β possesses *trans*-autophosphorylation activity that does not target the TGY motif. p38β is the only p38 isoform that manifests significant intrinsic catalytic activity as a recombinant purified protein (19). This intrinsic activity is a consequence of autoactivation achieved via autophosphorylation of the activation loop Thr residue (T180), part of the TGY motif. We have previously shown that this autophosphorylation occurs in *cis* (19). Accordingly, coincubation of $p38\beta^{WT}$ or $p38\beta^{D176A}$, an active p38β variant, with a K53R-mutated p38β protein ($p38\beta^{KD}$) did not lead to phosphorylation of the kinase-dead protein on the TGY motif (19) (Fig. 1A). Unexpectedly, when $p38\beta^{KD}$ was incubated with $p38\beta^{WT}$ or $p38\beta^{D176A}$ in the presence of [γ-^{32}P]ATP, it was phosphorylated in a dose-dependent manner (Fig. 1B). Furthermore, we found that an increase in the $p38\beta^{KD}$ concentration reduced the phosphorylation level of $p38\beta^{WT}$ or $p38\beta^{D176A}$, indicating that $p38\beta^{KD}$ competes with these proteins for autophosphorylation. Thus, p38β is *trans* autophosphorylated *in vitro* on a site that does not reside within the TGY motif.

To test if this *trans* autophosphorylation occurs in living cells, we coexpressed $p38\beta^{KD}$ and $p38\beta^{D176A}$ in HEK293 cells. $p38\beta^{D176A}$ is spontaneously phosphorylated at the activation loop TGY motif when expressed in mammalian cells (Fig. 1C) (13, 18, 19). As expected, coexpression of $p38\beta^{D176A}$ with $p38\beta^{KD}$ in HEK293 cells did not lead to the phosphorylation of $p38\beta^{KD}$ at the activation loop sites (Fig. 1C). However, the expression of $p38\beta^{D176A}$ seemed to lead to the phosphorylation of HA-$p38\beta^{KD}$ on other residues, as demonstrated by the retarded migration of some HA-$p38\beta^{KD}$ molecules on Phos-tag-supplemented SDS-PAGE gels (28) (Fig. 1C). This is not apparent when HA-$p38\beta^{KD}$ is coexpressed with an empty plasmid, indicating that HA-$p38\beta^{KD}$ phosphorylation is mediated by $p38\beta^{D176A}$. The results of the Western blot assays using the anti-dually phosphorylated p38 (p-p38) (T180/Y182) antibody (Fig. 1A and C), combined with the results of the radioactive kinase assay and the Phos-tag gel analysis (Fig. 1B and C), suggested that the *trans*-autophosphorylation reaction of p38β occurs both *in vitro* and in cell culture.

T241 is a phosphoacceptor of the *trans*-autophosphorylation reaction. Which sites on p38β are autophosphorylated in *trans*? Because p38 proteins, like all MAPKs, are proline-directed kinases (29), we first tested whether any of the serine/threonine-proline (S/T-P) sites within p38β may serve as a potential phosphoacceptor for the *trans*-autophosphorylation activity. p38β harbors three potential sites: T91, T241, and S243 (highlighted in Fig. 2A). T241 and S243, which are located in the MAPK insert, are exposed to the solvent, according to the three-dimensional (3D) structure. T91, which is located in the β4-β5 loop, seems, however, to be buried in the interface with the L16 helix. To examine whether p38β can phosphorylate these residues, we prepared 3 p38β-derived peptides that contain these sites. When incubated with $p38\beta^{WT}$ or $p38\beta^{D176A}$ (Fig. 2A, right), the peptide that contained T241 and S243 was efficiently phosphorylated (peptide 2) (Fig. 2A), whereas the peptide containing T91 was phosphorylated very poorly (peptide 1) (Fig. 2A). Notably, a peptide that includes T180 and Y182 (peptide 3) (Fig. 2A) was only slightly phosphorylated in this assay, further strengthening the conclusion that autophosphorylation of the TGY motif occurs mostly in *cis*.

Substitution of the threonine residue for alanine in peptide 2 reduced the ability of $p38\beta^{WT}$ and $p38\beta^{D176A}$ to phosphorylate this peptide, whereas substitution of the serine residue for alanine did not (Fig. 2B). Thus, T241 seems to be the only potential proline-directed site that is efficiently autophosphorylated. In the context of the whole protein, a kinase-dead GST-tagged p38β protein with a T241A mutation (GST-$p38\beta^{KD+T241A}$) was indeed *trans* phosphorylated by $p38\beta^{D176A}$ at a reduced level compared to that of GST-$p38\beta^{KD}$ (Fig. 2C). However, GST-$p38\beta^{KD+T241A}$ was still *trans* phosphorylated, suggesting that additional phosphoacceptors serve as the substrates for the autophosphorylation activity (see below). Thus, T241 is a p38β *trans*-autophosphorylation site, but another autophosphorylation site(s) apparently exists as well.

MS analysis identifies p38β autophosphorylation on five different sites. To identify all the sites that are autophosphorylated, we performed a capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis on recombinant $p38\beta^{WT}$ and $p38\beta^{D176A}$ proteins purified from *E. coli*. To verify that the phosphorylation of sites identified in this analysis is indeed a consequence of autophosphorylation, we subjected purified recombinant $p38\beta^{WT}$ and $p38\beta^{KD}$ to SDS-PAGE with and without Phos-tag, the phosphoprotein binding reagent (Fig. 3A). In standard SDS-PAGE gels, both proteins migrated as a single band; in gels supplemented with the Phos-tag reagent, only the migration of $p38\beta^{WT}$ was retarded (Fig. 3A). This indicates that $p38\beta^{KD}$ is purified as a nonphosphorylated protein and that all phosphorylation events identified by LC-MS/MS can be attributed to autophosphorylation activity. The LC-MS/MS analysis confirmed the phosphorylation of T180 and T241 in $p38\beta^{WT}$ and $p38\beta^{D176A}$ and further disclosed three additional, previously unidentified, phosphorylation sites: S143, S261, and S359 (Fig. 3B). Interestingly, of all sites, only T241 is proline directed (see Discussion). S143 is located at the C-terminal part of the αE-helix, close to the interface with the common docking domain (Fig. 3C). S359 is very

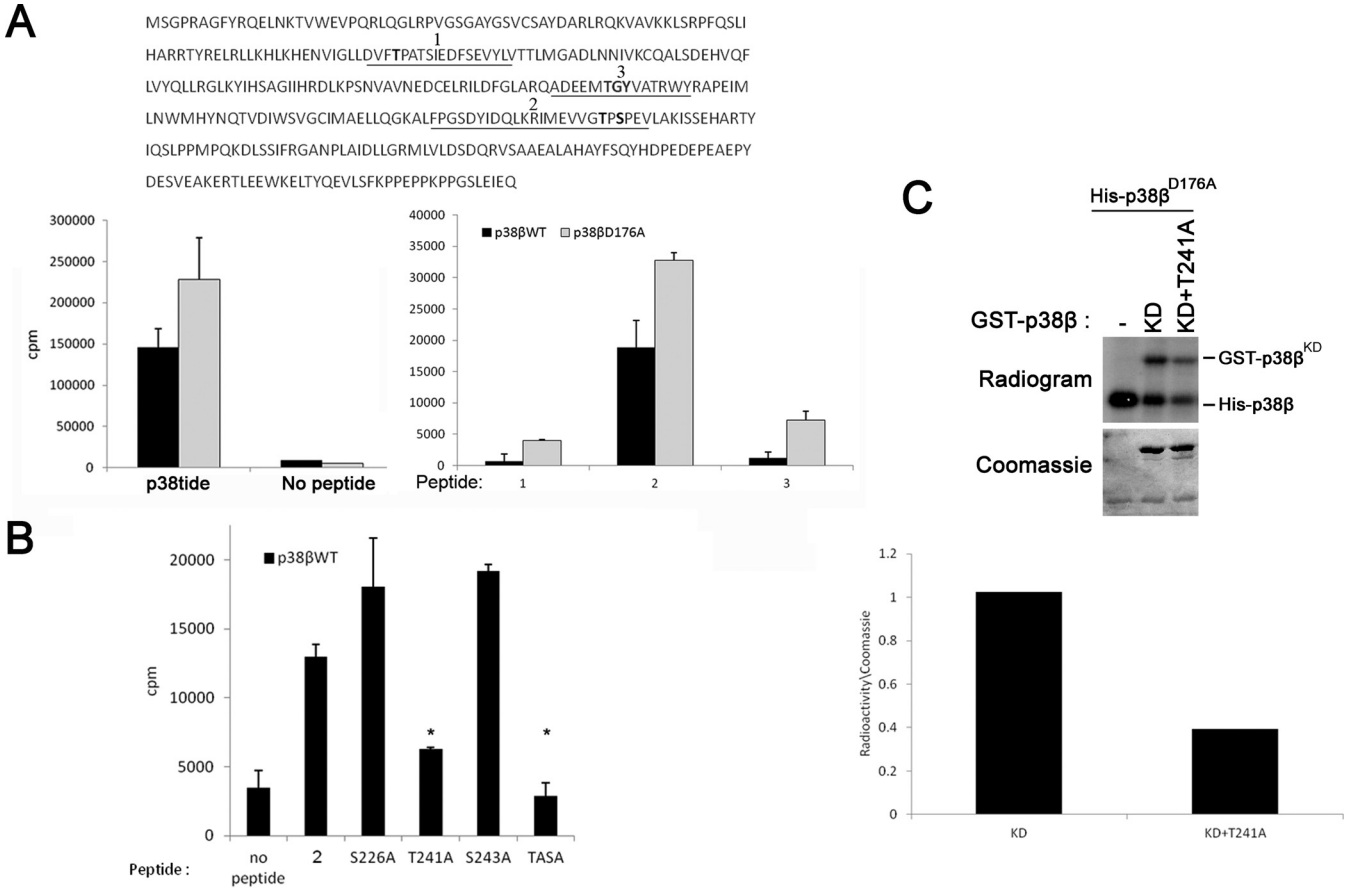

FIG 2 T241 is a *trans*-autophosphorylation site of p38β. (A and B) Recombinant purified p38β^{WT} or p38β^{D176A} was subjected to an *in vitro* kinase assay with radiolabeled ATP and with peptides derived from the p38β sequence. The phosphorylation level of the peptides was monitored with a scintillation counter and is presented as radioactive counts per minute. (A, right) Activity toward peptides 1 to 3, which are underlined in the p38β sequence (top). (Left) activity of p38β^{WT} or p38β^{D176A} toward an optimized p38 peptide substrate, p38tide (49), as a positive control. (B) Activity of p38β^{WT} toward modified peptide 2 variants, which contain single substitutions to alanine in the sites corresponding to the indicated amino acids. *, statistically significant difference (P value < 0.05) compared to unmodified peptide 2 as determined by the Student t test. (C) Recombinant purified p38β^{D176A} was incubated in an autophosphorylation assay mixture for 1 h with the indicated KD GST-tagged p38β variants. Samples were separated by SDS-PAGE, stained with Coomassie, and analyzed by exposure to X-ray film. Quantification was done with ImageJ software. The y axis indicates the ratio of the intensity of the band in the radiogram to the signal shown by Coomassie staining.

close to Q364, the most C-terminal amino acid of p38β. This region, which is not observed in the crystal structure, is part of a flexible region close to the N lobe in the 3D structure. Notably, T241 and S261 are part of the MAPK insert and, according to the 3D structure, reside in close proximity to each other (Fig. 3C). Strikingly, T241 resides within the 13-amino-acid fragment responsible for the unique autophosphorylation activity of p38β, while S261 resides within the neighboring fragment that suppresses this activity (19) (Fig. 3C). Importantly, the activating mutation D176A did not affect the specificity of the autophosphorylation activity, as the LC-MS/MS analysis identified the same sites to be phosphorylated in both the p38β^{WT} and p38β^{D176A} proteins.

p38β^{S261A} possesses increased activity toward ATF2. To assess if the newly identified autophosphorylation events have any effect on the catalytic activity of p38β, we prepared p38β^{S143A}, p38β^{T241A}, p38β^{S261A}, and p38β^{S359A} proteins and subjected them to an *in vitro* kinase assay with ATF2 as a substrate and to an autophosphorylation assay. p38β^{WT}, which is the only p38 isoform with intrinsic catalytic activity (19), manifested MKK6-independent activity toward ATF2 that was measured as ~35% of the activity of MKK6-activated p38β^{WT} (Fig. 4A). The MKK6-independent activities of p38β^{S143A}, p38β^{T241A}, and p38β^{S359A} were similar to that of p38β^{WT}. The MKK6-independent activity of p38β^{S261A}, however, was significantly higher and reached a level of ~75% of that of MKK6-activated p38β^{WT} (Fig. 4A), roughly twice as high as the spontaneous activity of p38β^{WT}. Furthermore, replacing S261 with an acidic glutamic acid, thereby generating the phosphomimetic mutant p38β^{S261E}, reduced the MKK6-independent activity by almost two-thirds (Fig. 4B). The difference in the activities of p38β S261 mutants can be ascribed to their effect on the autophosphorylation of T180 because Western blot analysis showed that p38β^{S261E} phosphorylation on the TGY motif is much lower than that of p38β^{WT}, while that of p38β^{S261A} seems higher (Fig. 4B). Importantly, all p38β variants tested in this assay manifested similar activity after preincubation with MKK6 (Fig. 4A and B), suggesting that phosphorylation of S261 specifically attenuates the MKK6-independent activity of p38β. Notably, elimination of the phosphorylation of S261 in p38β^{D176A} did not further increase the high spontaneous activity of this active protein (Fig. 4C).

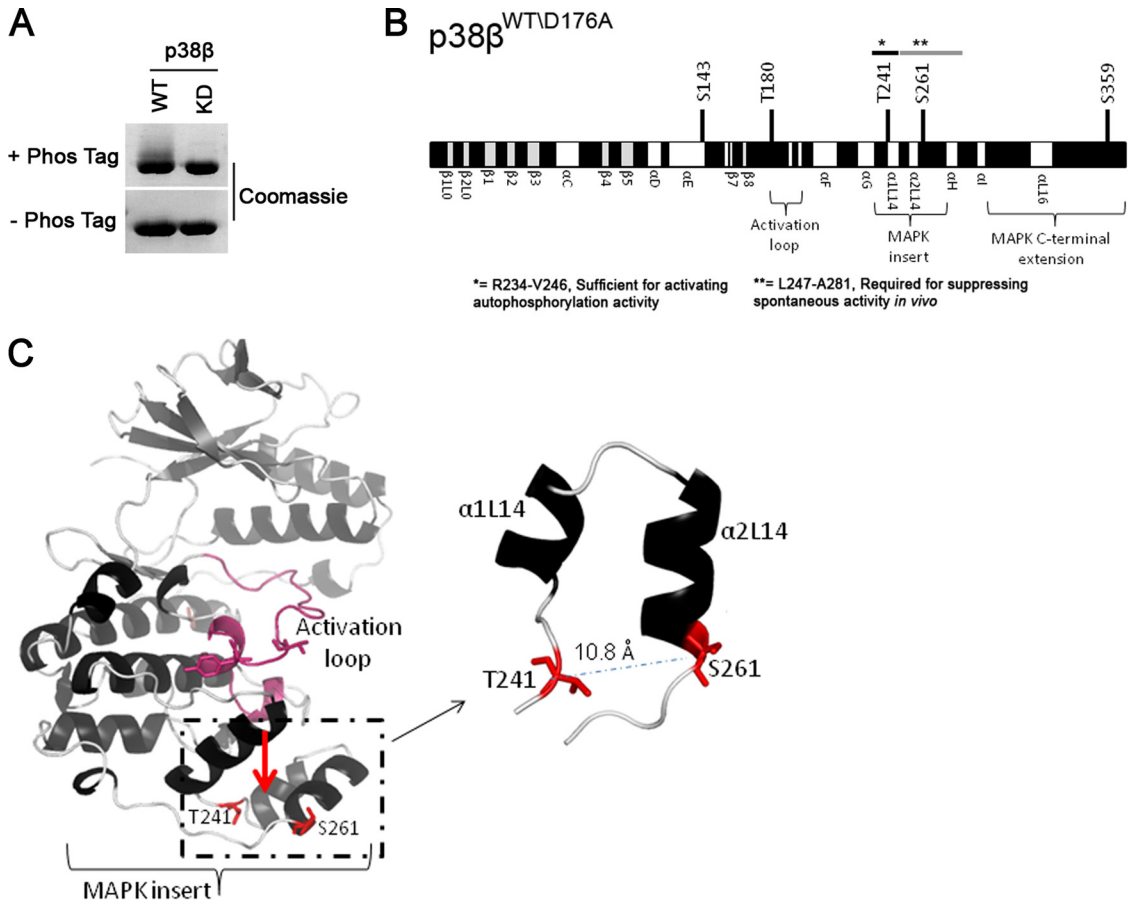

FIG 3 Novel autophosphorylation sites in p38β identified by capillary LC-MS/MS analysis. (A) Two micrograms of the indicated p38β proteins was separated on standard SDS-PAGE gels or SDS-PAGE gels supplemented with the Phos-tag reagent and subjected to Coomassie staining. (B) Schematic representation of the p38β protein. On the scheme are the secondary structures presented according to data reported previously by Sours et al. (50). White rectangles, α-helices; gray rectangles, β-sheets; black, loops and unstructured regions. Below the scheme are the locations of the activation loop, MAPK insert, and C-terminal extension subdomains. Above the scheme are the locations of the phosphorylated sites in $p38\beta^{WT}$ and $p38\beta^{D176A}$ as identified by MS/MS analysis. Also indicated are the regions identified to control autophosphorylation in p38β (19). (C) Locations of T241 and S261 on the crystal structure of p38α (PDB accession number 1P38). The region of the MAPK insert that contains these sites (marked by the dashed rectangle) is enlarged and shown from the angle indicated by the red arrow.

$p38\beta^{T241A}$ manifests increased autophosphorylation activity. We next examined the autophosphorylation activity of p38β molecules mutated at the newly identified phosphorylation sites. We found that the level of autophosphorylation activity manifested by $p38\beta^{T241A}$ was higher than that of $p38\beta^{WT}$ (Fig. 4D). This activity also seemed to be increased toward GST-$p38\beta^{KD}$ (Fig. 4E). Of note, it is unlikely that the observed increase in the autophosphorylation activity of $p38\beta^{T241A}$ occurs on T180, because the activity of $p38\beta^{T241A}$ toward ATF2 and its reactivity with the anti-p-p38 antibody are similar to those of $p38\beta^{WT}$ (Fig. 4A). Indeed, LC-MS/MS analysis revealed that $p38\beta^{T241A}$ molecules are phosphorylated at S261 and S143 at higher levels than $p38\beta^{WT}$ (~2.8- and ~3.5-fold higher than that of $p38\beta^{WT}$) (Fig. 4F). T180 was phosphorylated at similar levels in $p38\beta^{T241A}$ and $p38\beta^{WT}$ (Fig. 4F). Notably, elimination of T241 phosphorylation in $p38\beta^{D176A}$ did not increase the autophosphorylation activity of this protein (Fig. 4G). Finally, combining mutations at T241 and S261 eliminated p38β's spontaneous activity almost completely (Fig. 4H). Because these two residues are located close to each other in the 3D structure (Fig. 3C), mutation of both sites perhaps leads to a disruption of the ternary structure of the MAPK insert. In summary, it seems that the MAPK insert of p38β plays a critical role in this protein's unique autophosphorylation ability. First, the MAPK insert contains part of the 13-residue-long fragment that bestows autophosphorylation activity (19). Second, this subdomain possesses two regulatory phosphorylation sites, T241 and S261. Phosphorylation of T241 suppresses spontaneous autophosphorylation of S143 and S261, whereas phosphorylation of S261 reduces this protein's basal MKK6-independent activity.

Custom-made antibodies detect phosphorylation of p38β at T241 and S261. To be able to monitor the phosphorylation of T241 and S261, we raised antibodies that specifically react with phospho-T241 or phospho-S261 p38β (Fig. 5A and B). Western blot analysis verified that both antibodies reacted with $p38\beta^{WT}$ and $p38\beta^{D176A}$ but not with the respective alanine point-mutated p38β proteins $p38\beta^{T241A}$ (Fig. 5A) and $p38\beta^{S261A}$ (Fig. 5B).

An active p38α variant autophosphorylates T241 and S261. As p38 isoforms are very similar in structure and function, we wondered whether the ability to *trans* autophosphorylate is restricted to p38β. We constructed GST-tagged $p38\alpha^{KD}$, $p38\gamma^{KD}$, and $p38\delta^{KD}$ variants by mutating in each case the invariant Lys residue essential for ATP binding (2). These kinase-dead variants

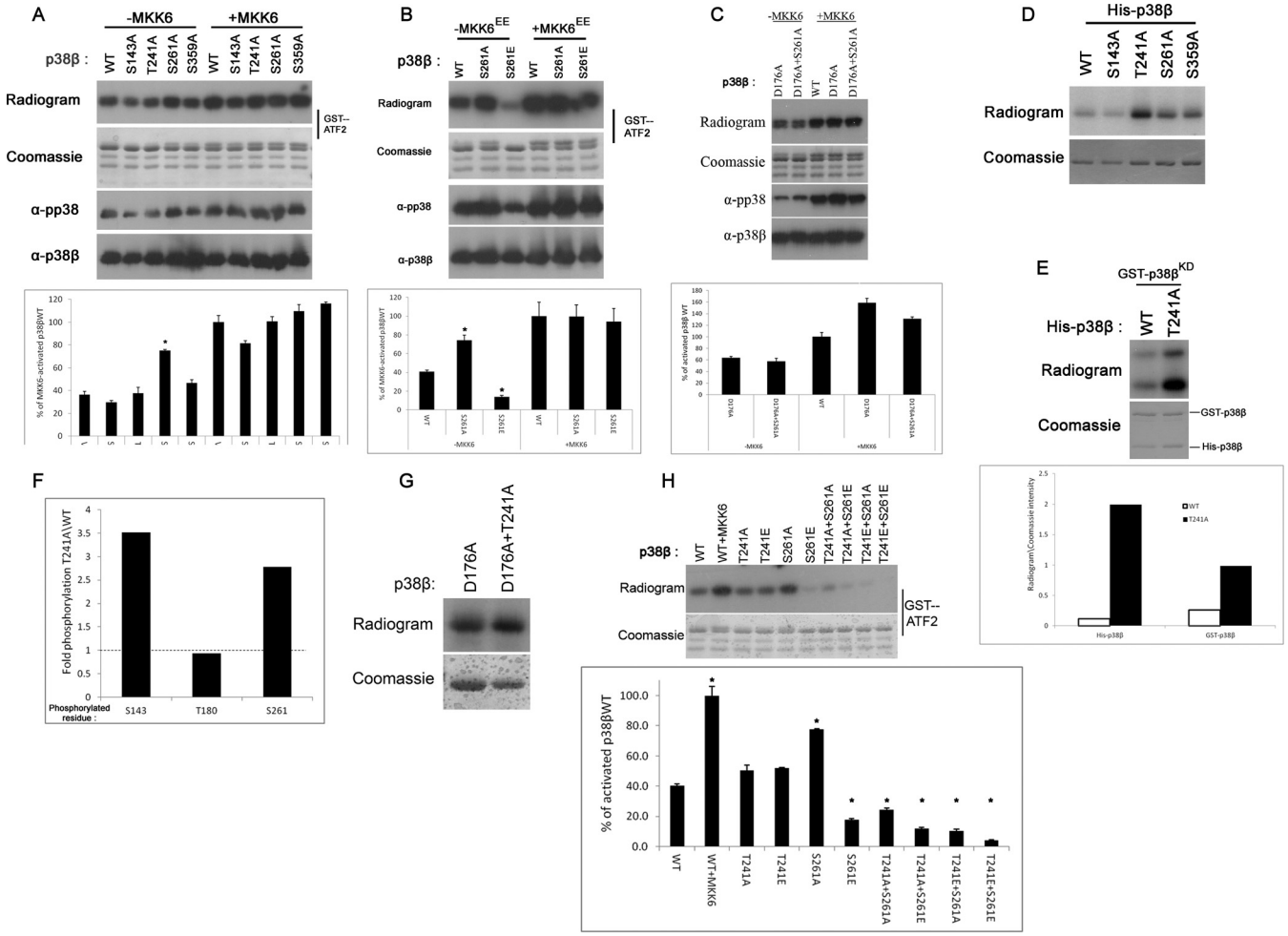

FIG 4 Phosphorylation of T241 and S261 suppresses the activity of T180-monophosphorylated p38β. (A to C and H) An *in vitro* kinase assay was performed with the indicated p38β proteins and with GST-ATF2 as a substrate. Activity is shown in graphs, expressed as a percentage of the activity of MKK6-activated $p38\beta^{WT}$ (100%), and by autoradiograms. *, statistically significant difference (P value < 0.05) between the activity of the indicated mutant and that of $p38\beta^{WT}$ as determined by the Student t test. One hundred nanograms of each protein was also analyzed by Western blotting with the indicated antibodies. (D, E, and G) The indicated recombinant purified His-tagged and GST-tagged p38β proteins were incubated in an autophosphorylation assay mixture for 1 h. Samples were separated by SDS-PAGE, stained with Coomassie, and exposed to X-ray film. (F) Five micrograms of $p38\beta^{WT}$ and $p38\beta^{T241A}$ was subjected to an autophosphorylation reaction. The phosphorylation level of the indicated sites in each protein was determined by LC-MS/MS. The graph presents the ratio between the phosphorylation of modified and unmodified peptides found in $p38\beta^{T241A}$ compared to the same ratio found in $p38\beta^{WT}$.

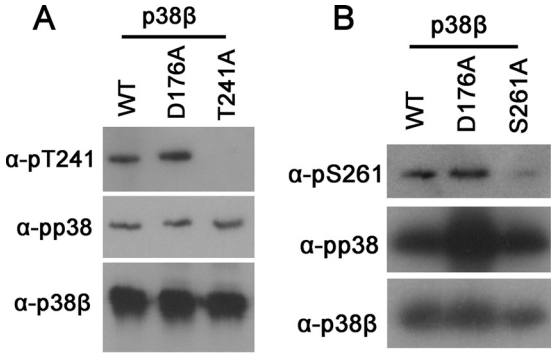

FIG 5 Western blot analysis with custom-made antibodies raised to detect phosphorylation at T241 and S261 verifies phosphorylation of these sites in purified $p38\beta^{WT}$ and $p38\beta^{D176A}$. One hundred nanograms of the indicated proteins was separated by SDS-PAGE and analyzed with the indicated antibodies by Western blot analysis.

did not show any autophosphorylation activity but could still be phosphorylated by $MKK6^{EE}$ (Fig. 6A). Because the basal autophosphorylation activity of $p38\alpha^{WT}$, $p38\gamma^{WT}$, and $p38\delta^{WT}$ is very low, for this experiment, we used intrinsically active variants of these isoforms (13). These variants had acquired increased autophosphorylation activity (Fig. 6B) (13) sufficient to elevate their catalytic activities (15, 18, 30, 31). We thus coincubated the His-tagged WT or active variants of each p38 isoform with increasing amounts of their GST-tagged KD counterpart (Fig. 6B). As expected, $p38\alpha^{WT}$, $p38\gamma^{WT}$, and $p38\delta^{WT}$ manifested very low autophosphorylation activity (Fig. 6B). $p38\alpha^{D176A+F327S}$ (an active p38α variant) successfully *trans* autophosphorylated GST-$p38\alpha^{KD}$ (Fig. 6B). However, $p38\gamma^{D179A}$ and $p38\delta^{F324S}$ (intrinsically active variants of p38γ and p38δ) showed a clear preference for *cis*-autophosphorylation over *trans* phosphorylation of their GST-tagged KD counterparts (Fig. 6B). Notably, $p38\gamma^{D179A}$ displayed the weakest autophosphorylation activity of all of the active p38 variants.

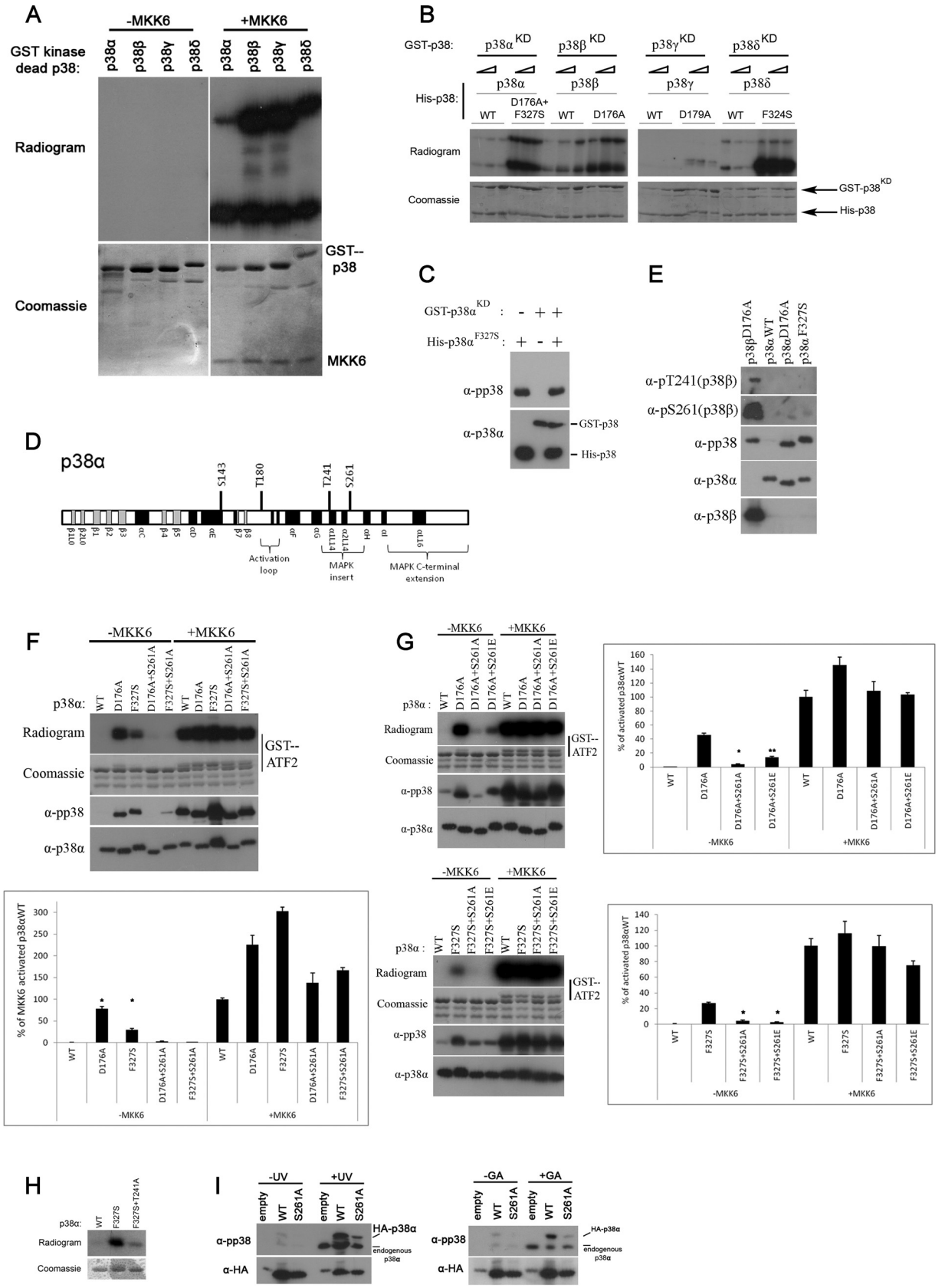

FIG 6 Active variants of p38α also autophosphorylate in *trans* on S261 and T241. (A) The indicated purified GST-tagged kinase-dead proteins were subjected to an *in vitro* phosphorylation assay in the presence of radiolabeled ATP with or without MKK6^{EE}. (B) The same GST-tagged kinase-dead proteins were subjected

Western blot analysis showed that as in p38β, T180 autophosphorylation in p38α$^{D176A+F327S}$ occurs in *cis* (Fig. 6C). This indicates that other sites are *trans* autophosphorylated in p38α. LC-MS/MS analysis showed that p38α$^{D176A+F327S}$ autophosphorylates not only T180 but also S143, T241, and S261 (Fig. 6D), corresponding to the same sites described above to be autophosphorylated in p38β. Importantly, the antibodies that we raised to detect the phosphorylation of T241 and S261 in p38β were not reactive against active p38α mutants (Fig. 6E), most probably because of the differences in the residues surrounding these sites in the two isoforms (19) (see below). Thus, these antibodies are specific reagents for p38β.

We examined the potential effect of phosphorylation of the T241 and S261 sites on the autophosphorylation-dependent activity of p38α. Unlike p38α^{WT}, recombinant p38α^{D176A} and p38α^{F327S} are purified phosphorylated on the TGY motif and possess spontaneous activity (15), measured to be ~70% and ~30%, respectively, of that of MKK6-activated p38α^{WT} (Fig. 6F). Surprisingly, an S261A mutation not only did not augment the activity of p38α^{D176A} and p38α^{F327S} but also almost eliminated TGY phosphorylation and activity completely (Fig. 6F). Most importantly, p38α$^{D176A+S261A}$ and p38α$^{F327S+S261A}$ were both fully active after MKK6 activation, suggesting that intact S261 is essential only for the basal activity. We next tested whether replacing S261 with an acidic amino acid would allow or even enhance MKK6-independent activity. Although p38α$^{D176A+S261E}$ manifested some spontaneous activity, it was significantly lower than that of p38α^{D176A} (Fig. 6G). p38α$^{F327S+S261E}$ was found to be active only after MKK6 activation (Fig. 6G). Similarly, although p38α^{F327S} manifested increased autophosphorylation activity compared to that of p38α^{WT}, the autophosphorylation activity of p38α$^{F327S+T241A}$ was very low (Fig. 6H). Thus, *trans* autophosphorylation at the MAPK insert occurs in more than one p38 isoform and may be physiologically important for activation mechanisms *in vivo*. However, the role of these autophosphorylations in p38α seems opposite to their role in p38β.

We also tested how mutations at S261 affect the autophosphorylation of p38α in mammalian cells. For this, we used MKK3 and MKK6 double-knockout MEF (MKK3/6$^{-/-}$) cells in which p38α phosphorylation can be induced in response to UV irradiation (12) or to the Hsp90 inhibitor geldanamycin (GA) (32) (Fig. 6I). We tested whether p38α^{S261A} could also be induced to autophosphorylate under these conditions and found that, unlike p38α^{WT}, p38α^{S261A} was almost not phosphorylated in response to UV or GA (Fig. 6I). Thus, in p38α, the autophosphorylation sites T241 and S261 seem to be required for autophosphorylation activity on T180. It is not clear if this requirement involves the phosphorylation of these residues.

p38β *trans* phosphorylates all p38 isoforms. The results presented above indicate that p38β and p38α possess *trans*-autophosphorylation activities. p38 isoforms share a high degree of similarity, raising the intriguing possibility that other p38 isoforms could also serve as the substrates for p38α and p38β. Such presumed *trans* phosphorylations between p38 isoforms could potentially serve as a mode of direct cross talk between them. Although the possibility of direct cross talk between p38 isoforms was proposed previously (18), it has not been tested biochemically.

We first examined if the GST-tagged p38 kinase-dead variants could be *trans* phosphorylated by p38β^{WT} and p38β^{D176A}. It was found that not only GST-p38β^{KD} but also all GST-tagged p38 isoforms are indeed *trans* phosphorylated by p38β^{WT} and p38β^{D176A} (Fig. 7A). Notably, p38β *trans*-isoform phosphorylation activity seems to be stronger toward p38α and p38γ than toward p38δ. Thus, both p38β^{WT} and p38β^{D176A} possess *trans*-phosphorylation activity toward all p38 isoforms.

To examine if the *trans*-isoform phosphorylation activity is unique to p38β, we checked whether the active variants of p38α, p38γ, and p38δ are also able to phosphorylate GST-tagged KD versions of the four isoforms (Fig. 7B). We found that, like p38β, p38α$^{D176A+F327S}$ *trans* phosphorylated the other isoforms (Fig. 7B). p38δ^{F324S} manifested some *trans*-phosphorylation activity in this assay; however, this was negligible compared to its total autophosphorylation activity (Fig. 7B). p38γ^{D179A} did not manifest any detectable *trans*-isoform phosphorylation.

To determine the sites on which these *trans* phosphorylations occur, we performed an LC-MS/MS analysis on recombinant purified GST-p38α^{KD}, GST-p38γ^{KD}, and GST-p38δ^{KD} after they were incubated in an *in vitro* kinase reaction mixture with the intrinsically active p38α and p38β mutants. Thus, all phosphorylations identified by this analysis can be attributed to the *in vitro* reaction. Remarkably, all the phosphorylation sites that were identified via this analysis were found to be equivalent to the sites identified above as autophosphorylation sites in p38β (Fig. 3B) and p38α (Fig. 6D). GST-p38α^{KD} was found to be *trans* phosphorylated by p38α$^{D176A+F327S}$ and by p38β^{D176A} on S143. GST-p38γ^{KD} was found to be phosphorylated by p38α$^{D176A+F327S}$ and by p38β^{D176A} on T245, the site corresponding to T241 in p38α and p38β (Fig. 7C). GST-p38δ^{KD} was found to be phosphorylated by p38α$^{D176A+F327S}$ and by p38β^{D176A} on T265 (Fig. 7C). This site is

to a phosphorylation assay with the indicated His-tagged p38 proteins. Following reaction termination, reaction mixtures were separated by SDS-PAGE, stained with Coomassie, and exposed to X-ray film. (C) GST-p38α^{KD} was subjected to an *in vitro* kinase assay with p38α$^{D176A+F327S}$, separated by SDS-PAGE, blotted, and analyzed via Western blotting with the indicated antibodies. (D) Schematic representation of phosphorylation sites identified by LC-MS/MS analysis. Five micrograms of p38α$^{D176A+F327S}$ was subjected to an autophosphorylation reaction. Phosphorylation of the indicated sites was discovered via LC-MS/MS. Results are displayed on a schematic representation of p38α as described in the legend to Fig. 3B. (E) The indicated recombinant purified p38 proteins were subjected to Western blot analysis with the indicated antibodies. (F and G) An *in vitro* kinase assay was performed with the indicated p38 proteins, with GST-ATF2 as a substrate. Activity is shown in graphs, expressed as percentage of the activity of MKK6-activated p38α^{WT} (100%), and by autoradiograms. One hundred nanograms of each protein was also analyzed by Western blotting with the indicated antibodies. *, statistically significant difference (P value < 0.05) between the activity of the indicated mutant and that of p38α^{WT} as determined by the Student t test; **, statistically significant difference (P value < 0.05) between the activity of p38α$^{D176A+S261E}$ and that of p38α$^{D176A+S261A}$ as determined by the Student t test. (H) The indicated recombinant purified His-tagged p38α proteins were incubated in an autophosphorylation assay mixture for 1 h. Samples were separated by SDS-PAGE, stained with Coomassie, and exposed to X-ray film. (I) MEF MKK3/6$^{-/-}$ cells were transfected transiently with plasmids expressing the indicated p38 proteins. Forty-eight hours after transfection, cells were either exposed to UV radiation (120 J/m^2) or treated with 1.8 μM geldanamycin (GA). After 1 h, cell lysates were prepared, and 30 μg protein from each sample was separated by SDS-PAGE, blotted, and probed with the indicated antibodies.

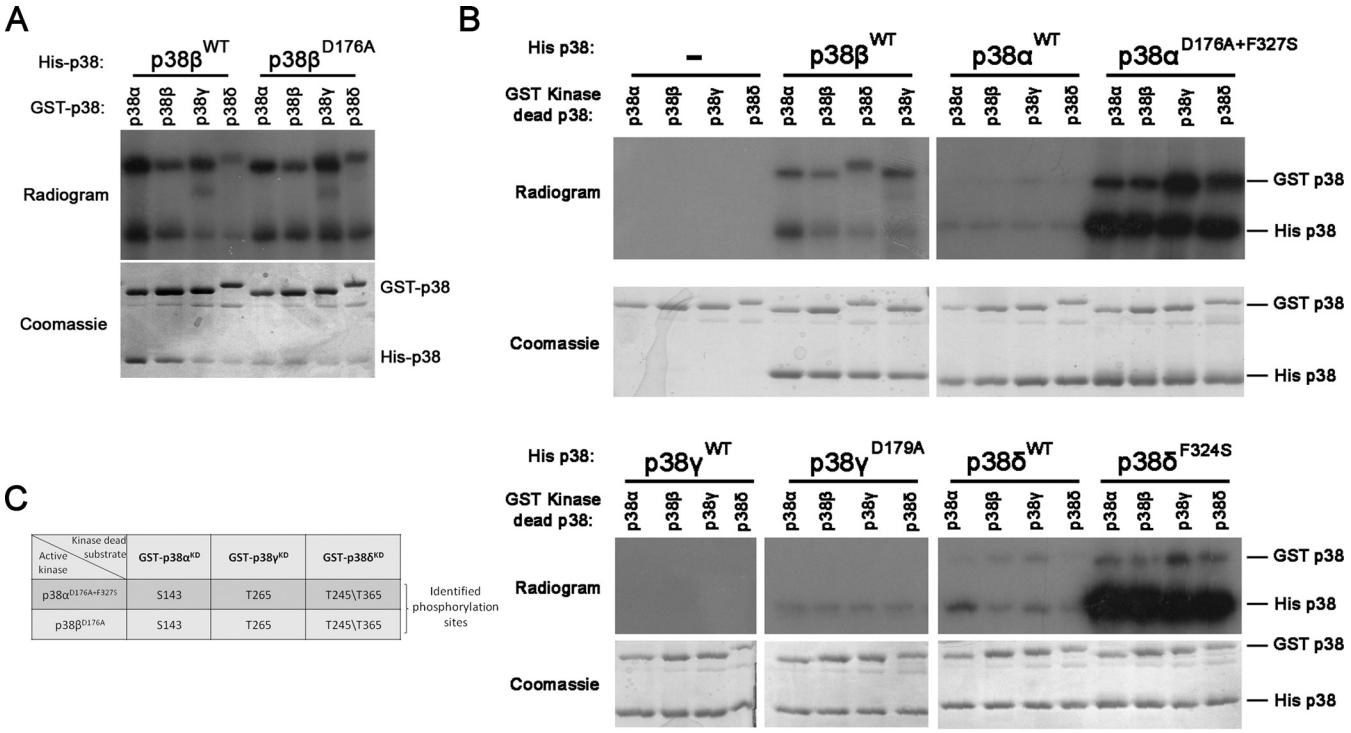

FIG 7 p38α and p38β, but not p38γ or p38δ, possess a general *trans*-isoform phosphorylation activity. The indicated recombinant purified GST- and His-tagged p38 proteins were coincubated in an autophosphorylation assay mixture for 1 h. All GST-tagged p38 proteins harbor the kinase-dead mutation. (A and B) Samples were separated by SDS-PAGE, Coomassie stained, and exposed to X-ray film. (C) Phosphorylation sites in the GST-p38 kinase-dead proteins in each sample, discovered via LC-MS/MS.

located 4 residues C terminal to the S261 residue in p38δ, the site that corresponds to S261 in p38α and p38β.

p38β autophosphorylates T241 in cell culture and *in vivo*. As described above, the novel autophosphorylation sites in p38β were identified *in vitro*. To determine if these phosphorylations occur in mammalian cells, we first expressed p38β proteins in HEK293 cells and subjected the relevant cell lysates to Western blot analysis using anti-p-p38 antibodies and the custom-made anti-phospho-T241 antibodies. Although p38β purified from *E. coli* is spontaneously active, when expressed in mammalian cells, it does not manifest this activity (19). Indeed, p38β^{D176A} was found to be phosphorylated at the activation loop at a much higher level than p38β^{WT} (Fig. 8A). In agreement with the data from the *in vitro* analysis, p38β^{S261A} was found to be slightly hyperphosphorylated at its activation loop compared to p38β^{WT}, while p38β^{S261E} was found to be phosphorylated at lower levels (Fig. 8A). However, p38β^{S261A}'s increased phosphorylation was lower than that of p38β^{D176A} and did not lead to a significant increase in MK2 phosphorylation (Fig. 8A). The most probable explanation is that in these cells, p38β autophosphorylation is not induced (see below). Corresponding to the strong spontaneous phosphorylation of p38β^{D176A} at the activation loop, this variant was found to be strongly phosphorylated at T241, showing that this phosphorylation also occurs in mammalian cell culture (Fig. 8A). Notably, p38β^{T241E} was also found to be hyperphosphorylated and to lead to an increase in MK2 phosphorylation, further emphasizing the importance of these sites in regulating p38β's MAPKK-independent activity (Fig. 8A). Furthermore, coexpression of p38β^{KD} with p38β^{D176A} led to the phosphorylation of p38β^{KD} at T241, indicating that this reaction occurs in *trans* not only *in vitro* but also in mammalian cells in culture and that, under these conditions, the T241 site is not a target of another kinase (Fig. 8B).

We also attempted to measure the status of phosphorylation of the S261 residue in living cells. Unfortunately, however, the antibodies that reacted specifically with S261-phosphorylated recombinant p38β were not specific when tested with samples from cell lysates.

The assays described above verify that ectopically expressed p38β is phosphorylated at T241. We wished to determine if endogenous p38β is also phosphorylated at T241. This task is somewhat problematic because it is impossible to predict the specific conditions and particular cell types in which these phosphorylations may occur. We therefore used the anti-phospho-T241-p38β antibodies to probe samples prepared from an array of mammalian cell lines (Fig. 8C). It was found that the anti-pT241 antibody reacted specifically with lysates prepared from human mammary gland-derived MCF10A cells (Fig. 8C, left) and the human multiple myeloma cell line CAG (Fig. 8C, right). Importantly, in both cell lines, the bands that reacted with the anti-pT241 antibodies corresponded to bands of the same molecular mass that reacted with the anti-p38β antibody. The other proteins that also reacted with the anti-pT241 antibodies in other cell lines, however, might not be p38β. The signal detected with the anti-pT241 in these cell lines does not clearly overlap with that detected with the anti-p38β antibody (Fig. 8C, asterisks). It should be noted that all the p38β proteins identified in this array seemed to migrate slower than the recombinant purified p38β that served as a positive control. For example, p38β in MCF10A cells migrated as an ~50-kDa

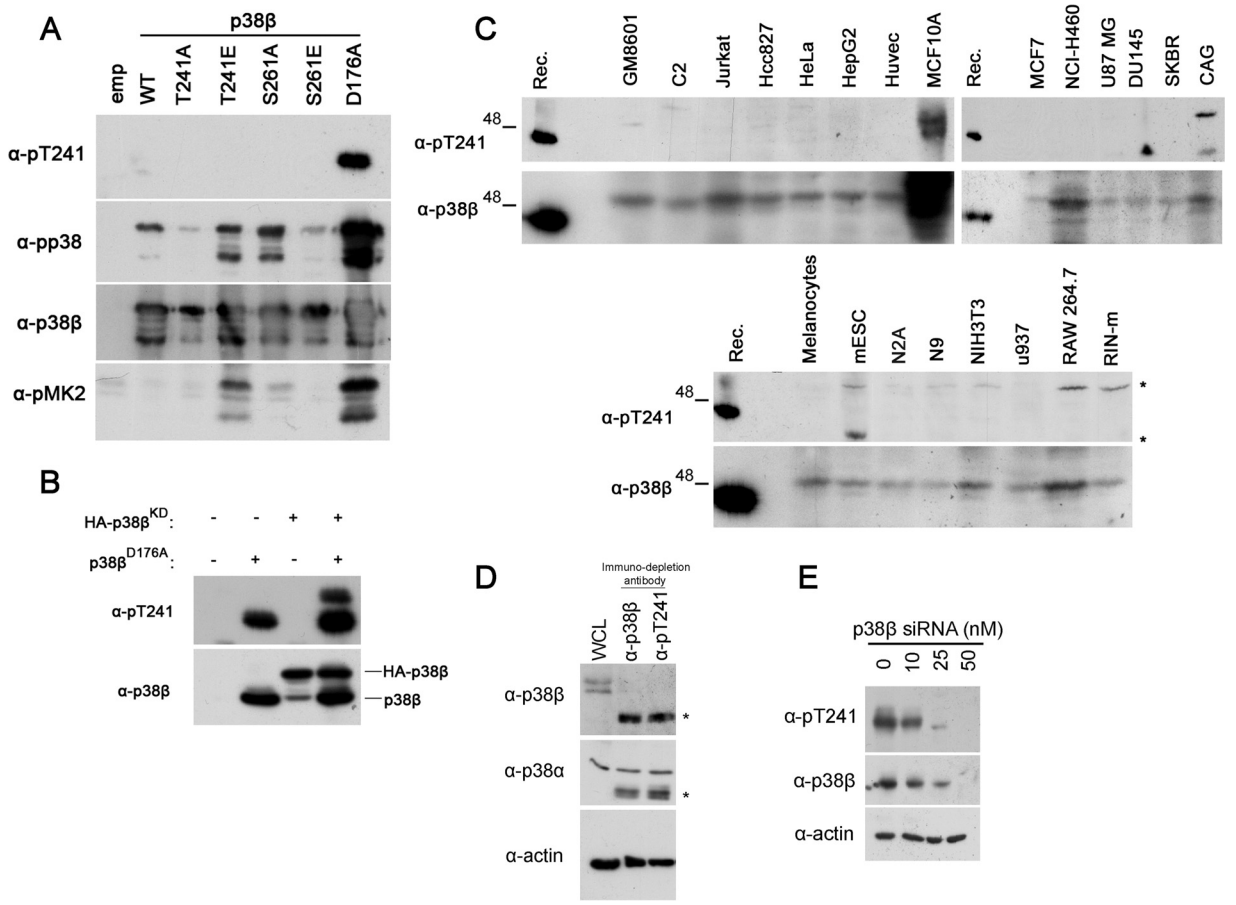

FIG 8 T241 of p38β is phosphorylated in mammalian cells grown in culture. (A and B) HEK293 cells were transfected transiently with plasmids expressing the indicated p38β proteins. (A) Cell lysates were prepared 48 h after transfection, and 30 μg protein from each sample was separated by SDS-PAGE, blotted, and probed with the indicated antibodies. emp., empty. (B) Cells were cotransfected with HA-p38β^{KD} and nontagged p38β^{D176A}. (C) Cell lysates prepared from the indicated mammalian cell lines were analyzed via Western blotting with the indicated antibodies. Forty nanograms of recombinant p38β^{WT} (Rec.) was used as a positive control in each blot. *, bands that appear to be nonspecific (see the text). (D) A native lysate was prepared from MCF10A cells and subjected to immunodepletion with anti-p38β or a anti-pT241 antibodies. Depleted lysates were subjected to Western blot analysis with the indicated antibodies. WCL, whole-cell lysate. (E) MCF10A cells were transfected with siRNA that targets p38β at the indicated final concentrations or with a control plasmid. Lysates were prepared at 48 h posttransfection and subjected to Western blot analysis with the indicated antibodies.

protein and not at ~42 kDa as would be expected on the basis of its calculated molecular mass. The reason for this is not clear to us and may reflect a covalent modification of the p38β molecules. To verify that the anti-pT241 antibody is specific and indeed identifies p38β, we performed an immunodepletion assay (Fig. 8D) and an siRNA knockdown experiment (Fig. 8E). Mixing a native cell lysate prepared from MCF10A cells with the anti-p38β antibody completely eliminated the signal detected by the anti-pT241 antibody. Strikingly, incubation of a lysate prepared from the same cells with an anti-pT241 antibody completely eliminated the signal detected with an anti-p38β antibody as well (Fig. 8D). This indicates that T241 phosphorylation is an abundant modification of p38β in MCF10A cells. Furthermore, siRNA knockdown of p38β eliminated in a dose-dependent manner the phosphorylation signal detected with the anti-pT241 antibody (Fig. 8E). The results combined indicate that p38β possesses an autophosphorylation activity that targets T241 in mammalian cells. Both endogenous proteins and ectopically expressed proteins were phosphorylated.

To determine if p38β is also phosphorylated at T241 *in vivo*, samples from three adult mouse tissues were probed with the anti-phospho-T241 antibody (Fig. 9A). It was found that p38β was phosphorylated on T241 specifically in muscle and bone tissues (Fig. 9A). Thus, the phosphorylation events discovered via the *in vitro* analysis of the *trans* autophosphorylation of p38β occur *in vivo*.

The p38 pathway has a key function in myogenesis (33, 34). Thus, in an attempt to further characterize T241 phosphorylation of p38β in muscle tissue and to determine its potential role, we used the C2C12 (mouse) and L8 (rat) myogenic cell lines (35, 36). These myoblasts proliferate when supplemented with high-serum growth medium. When subconfluent and exposed to differentiation medium, they begin to differentiate and eventually fuse into myotubes (Fig. 9B). We could not detect T241 phosphorylation in proliferating L8 or C2C12 cells. However, in differentiated myotubes, we detected a strong increase in this site's phosphorylation (Fig. 9B). Notably, in differentiated myotubes, there seems to be an increase in the level of total p38β protein, and T241-phosphorylated p38β migrates slightly slower than p38β that is not phosphorylated at this site. This is reminiscent of T241-phosphory-

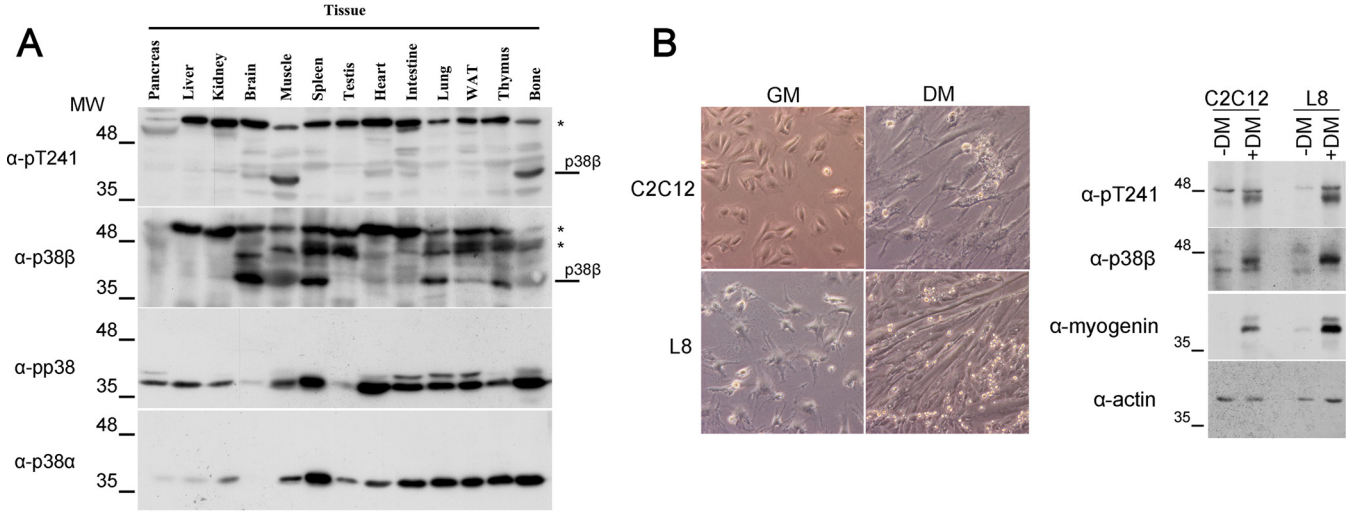

FIG 9 T241 is phosphorylated in muscle and bone tissues of adult mice and is correlated with muscle differentiation in cell lines. (A) Western blot analysis was performed on samples prepared from tissues extracted from three male C57BL/6N mice and homogenized with the indicated antibodies (WAT, white adipose tissue). Shown are representative results from one mouse. Bands that correspond to the molecular weight (MW) (in thousands) of p38β are indicated. *, bands that appear to be nonspecific. (B) C2C12 and L8 cells were cultured in regular growth medium (GM) until they reached subconfluence. The medium was then changed to differentiation medium (DM). Cells were photographed 5 days later, and cell lysates were prepared. Twenty micrograms of protein of the cell lysate was separated by SDS-PAGE, blotted, and probed with the indicated antibodies.

lated p38β in MCF10A cells (Fig. 8C). Thus, p38β is found to be phosphorylated at T241 *in vitro*, in mammalian cell cultures, and *in vivo*. The pattern of this phosphorylation suggests a potential role in muscle tissue differentiation.

DISCUSSION

In this paper, we describe a novel mechanism of autophosphorylation-dependent regulation of the MAPK p38β. This kinase was previously reported to self-activate by *cis* autophosphorylation of T180 in the activation loop, a phosphorylation known to stabilize the active conformation (3, 19, 37). We showed here that p38β possesses another autophosphorylation activity, which does not target the activation loop sites but rather targets S143 and two residues of the MAPK insert: T241 and S261. Here we analyzed the latter sites because of their apparent role in regulating the catalytic activity of p38β. Mutation analysis suggested that phosphorylation of these sites controls the activity output of T180-phosphorylated p38β, which is itself generated by autophosphorylation activity. Thus, what we have described here is a novel autophosphorylation-dependent regulatory mechanism. Identification of the new regulatory sites indicates that the basal activity manifested by p38β is a resultant vector of several phosphorylation events: activating phosphorylation at T180, inhibitory phosphorylation at S261, and phosphorylation at T241 that is inhibitory toward *trans* phosphorylation of S143 and S261. T241 phosphorylation could therefore also have an activating effect, as it would prevent S261 phosphorylation. Thus, p38β evidently possesses inherent regulatory cycles executed by its own catalytic activity. Negative and positive feedbacks are important traits of signaling networks (38) and can serve as mechanisms to modulate the catalytic output, according to the ratio between the positive and negative inputs (38). This type of feedback regulation takes place between components of pathways and networks. Enzymes in which both positive and negative inputs are inherent are extremely rare. We are aware of only one more example of an enzyme that can intrinsically automodulate its activity positively and negatively (8).

Interestingly, T241 is followed in the protein sequence by proline, but S261 is not. Although all MAPKs have a clear preference for proline-directed phosphorylation, p38 proteins have been shown to phosphorylate substrates on sites that are not proline directed (39, 40), including the activation loop T180 residue (14, 16, 17, 19, 37). The selectivity toward the proline-directed site is a result of a specific $P + 1$ accommodating pocket in MAPKs. This $P + 1$ pocket is completely abolished in the structure of nonphosphorylated p38α (25). The appearance of the pocket depends on the presence of phospho-Tyr182, which interacts with a key Arg residue that otherwise accommodates this pocket (41). Similar observations have been made for the MAPK extracellular signal-regulated kinase 2 (ERK2) (21, 23). p38β that is activated via autophosphorylation is found monophosphorylated at T180 and not at Y182 (19). Therefore, in the absence of Y182 phosphorylation, the $P + 1$ pocket will probably acquire a different conformation and will therefore permit phosphorylation of non-proline-directed sites.

Also discussed in this study are the catalytic properties of p38 molecules monophosphorylated at the activation loop (p-p38). Activation of the p38 pathway ultimately leads to MAPKK-dependent dual phosphorylation at the activation loop of p38 proteins (pp-p38), rendering them fully active. However, we and others have shown that autophosphorylation can generate p-p38 (Thr only) molecules (19, 20, 37, 42). Such proteins could also theoretically be generated by the activity of phospho-tyrosine phosphatases on pp-p38 proteins, although this has not yet been shown for p38 proteins *in vivo*. Generation of p-p38α and p-p38β is sufficient for catalytic activity (19, 20, 37). Nonetheless, it seems that this catalytic activity differs from that imposed by dual phosphorylation. For example, p-p38α has been shown to have different kinetic properties (37) and substrate specificity (20) than those of pp-p38α. p-p38α has been shown to be physiologically important,

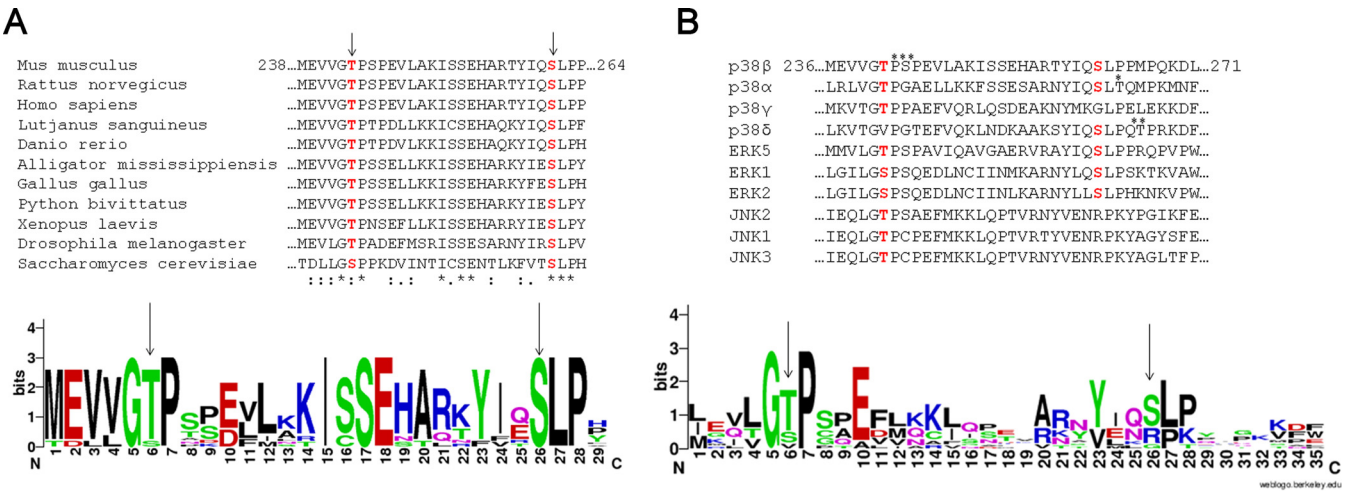

FIG 10 Sequence alignments between MAPKs from different organisms show that T241 and S261 are highly conserved. The linear sequences of p38β proteins from the indicated organisms (A) and the sequences of all *Homo sapiens* MAPKs (B) (NCBI gene database) were aligned by using Clustal Omega software. T241 and S261 are marked in red and indicated by arrows. The conservation of these sites is also shown by using WEBLOGO software. *, T263 phosphorylation in p38α documented previously (47); **, site of *trans* phosphorylation of p38δ by active p38α and p38β variants (Fig. 7); ***, S243 phosphorylation documented previously (48).

for example, in activated T cells (17) and in response to ischemia (43). Therefore, the physiological roles of p-p38s are probably different from those of pp-p38s. An attractive idea is that although the p38 pathway can be fully activated, resulting in the activation of all isoforms, specific isoforms might be individually activated by induced autophosphorylation. This would generate a different signaling output, not only because it would lead to the activation of a specific isoform but also because it would establish a different activation state. In the case of p38β and p38α, an interesting aspect of this particular active conformation could be *trans* phosphorylation of other p38 isoforms.

Here we showed that p-p38β has different properties than pp-p38β. Perhaps the most striking feature is that the activity of p-p38β is regulated by the phosphorylation of T241 and S261, while that of pp-p38β is not. Also, it seems that the phosphorylation of these sites is a distinct property of p-p38β. p38β, the only MAPK that manifests an intrinsic autoactivation capability, seems to execute an intrinsic signaling "subroutine," independent of the canonical MAPKK-dependent p38β activation mode. A model for processing this subroutine may suggest that once a cellular repressing moiety (yet to be identified) is removed, p38β autophosphorylates and activates itself. This may lead to the phosphorylation of a subset of cellular substrates, specific to p-p38β, and to the autophosphorylation in *trans* of S143, T241, and S261 to obtain a lower catalytic steady-state level.

Importantly, phosphorylations of T241 occur *in vivo*, indicating a possible physiological function in muscle tissue differentiation. Little is known about the relative physiological contribution of autophosphorylated p38 proteins compared to that of MAPKK-activated p38 proteins. Owing to the nature of the reagents used in studies of the p38 pathway, mainly the anti-phospho-p38 antibody that recognizes both dually phosphorylated and monophosphorylated p38 proteins (37), phosphorylated p38 detected by this antibody is automatically attributed to MAPKK-dependent activation. In fact, only a few studies have managed to show physiological conditions under which p38 proteins are activated by autophosphorylation (16, 17). Perhaps, the antibodies that we raised against phosphorylated T241, which recognize a site that is probably generated only via autophosphorylation, will prove to be important tools for disclosing more physiological conditions under which autophosphorylation occurs.

Although activation loop phosphorylation is most critical for catalysis, many kinases are phosphorylated, frequently by autophosphorylation, on other sites outside the activation loop. In MAPKs, these phosphorylations can occur either on sites that are part of the canonical kinase domain (44) or on sites that are considered unique for MAPKs (45). For example, ERK proteins have been shown to be phosphorylated at S246 and S248 (ERK2 numeration) (45), located in α1L14, part of the MAPK insert (23, 45). Phosphorylation of these sites is involved in ERK1/2 translocation to the nucleus (4). Phosphorylation of S246 is thought to be mediated by autophosphorylation (45). S246 of ERK2 is the homologous residue of T241 of p38β and is generally very well conserved in all MAPKs (see below). However, in p38β, T241 phosphorylation has no role in nuclear translocation (46) but rather has a role in the regulation of autophosphorylation activity. In addition to T241, we have identified two more previously unnoticed sites, S143 and S261. T241 resides within the 13-amino-acid region that is sufficient, when grafted into p38α, to induce spontaneous autophosphorylation (see above) (19). S261 resides within a region that has been shown to mediate the suppression of the spontaneous activity of p38β in mammalian cells (see above) (19). The MAPK insert in p38 proteins seems to have a major role in regulating autophosphorylation activity. Our results imply that the mechanism through which these regions control autophosphorylation is, at least partially, via the phosphorylation of these sites. The role of S143 phosphorylation is not known but does not seem to affect catalysis. Because this site is close to the CD domain in p38β, it could be that S143-phosphorylated p38β has a different binding repertoire than p38β that is not phosphorylated at S143.

Sequence alignment of p38β proteins from various mammalian species and p38 orthologues from reptiles, chicken, fish, and

yeast shows that T241 and S261 sites are highly conserved among p38β proteins (Fig. 10A). A similar analysis of mammalian MAPKs shows that T241 (p38β numeration) is completely conserved as an S/T-P site in all canonical MAPKs, with the exception of p38δ (Fig. 10B). This indicates that this site might have a functional role not only in p38β (Fig. 4), p38α (Fig. 6), and ERK1/2 (4, 45) but also in other MAPKs. S261, on the other hand, is less well conserved and is not found in p38γ and in Jun N-terminal protein kinase (JNK) proteins (Fig. 10B). The C-terminal part of the MAPK insert, which includes S261, seems to be a "hot spot" for phosphorylation in the p38 family. We have shown that T265 in p38δ, two residues C terminal to S261, is a phosphoacceptor for p38α and p38β *trans*-isoform activity. Furthermore, T263 was found to be phosphorylated in p38α in cells exposed to DNA damage in a global phosphoproteomic study (47), and S243 in p38β was found to be phosphorylated in a similar study performed in human embryonic stem cells (48). Notably, phosphorylation of T241 and S261 has not been detected in global MS-based phosphoproteomic studies. The low abundance of signaling proteins and the frequent low stoichiometry of their phosphorylated forms combined with the cell type-restricted characteristic of the phosphorylation events provide a potential explanation for why these phosphorylations have not yet been observed.

Thus, we conclude that p38β has the ability to self-activate by T180 autophosphorylation and to modulate its activity by autophosphorylation of T241 and S261. Therefore, p38β can be considered a bona fide self-regulatory enzyme that is independent of the canonical MAPKK pathway for activation and of other enzymes for inhibition. Although there are a number of enzymes, including kinases, that can self-activate or self-inhibit their catalytic potency, this is a rare example of an enzyme that does both.

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