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Phosphorylation by p38 MAPK as an Alternative Pathway for GSK3 β Inactivation

Tina M. Thornton¹, Gustavo Pedraza-Alva^{1,*}, Bin Deng^{2,3}, C. David Wood¹, Alexander Aronshtam¹, James L. Clements⁴, Guadalupe Sabio⁵, Roger J. Davis⁵, Dwight E. Matthews^{1,3}, Bradley Doble⁶, and Mercedes Rincon^{1,†}

¹Department of Medicine/Immunobiology Program, University of Vermont, Burlington, VT 05405-0068, USA

²Department of Biology, University of Vermont, Burlington, VT 05405-0068, USA

³Department of Chemistry, University of Vermont, Burlington, VT 05405-0068, USA

⁴Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

⁵Program in Molecular Medicine, University of Massachusetts, Worcester, MA 01605, USA

⁶McMaster Stem Cell and Cancer Research Institute, McMaster University, Hamilton, Ontario, L8N 3Z5, Canada

Abstract

Glycogen synthase kinase 3 β (GSK3 β) is involved in metabolism, neurodegeneration, and cancer. Inhibition of GSK3 β activity is the primary mechanism that regulates this widely expressed active kinase. Although the protein kinase Akt inhibits GSK3 β by phosphorylation at the N terminus, preventing Akt-mediated phosphorylation does not affect the cell-survival pathway activated through the GSK3 β substrate β -catenin. Here, we show that p38 mitogen-activated protein kinase (MAPK) also inactivates GSK3 β by direct phosphorylation at its C terminus, and this inactivation can lead to an accumulation of β -catenin. p38 MAPK-mediated phosphorylation of GSK3 β occurs primarily in the brain and thymocytes. Activation of β -catenin-mediated signaling through GSK3 β inhibition provides a potential mechanism for p38 MAPK-mediated survival in specific tissues.

The p38 mitogen-activated protein kinase (MAPK) is activated through phosphorylation primarily by MAPK kinase 3 (MKK3) and MKK6 in response to cellular stress and cytokines. The p38 MAPK pathway functions in the control of differentiation, the blockade of proliferation, and in the induction of apoptosis (1). It is also activated in response to DNA double-stranded breaks (DSBs) induced by ionizing irradiation or chemotherapeutic drugs, and it participates in the induction of a G₂/M cell-cycle checkpoint (2,3). p38 MAPK can also promote survival (4-6) by unknown mechanisms. During T cell receptor β (TCR β) rearrangement, V(D)J recombination-mediated DSBs also activate p38 MAPK in immature thymocytes at the double negative 3 (DN3) stage of development (7,8). The expression of a constitutively active mutant of MKK6 [MKK6(Glu)] in thymocytes of transgenic mice (MKK6 transgenic mice) activates a p53-mediated G₂/M phase cell-cycle checkpoint (8). Like recombination-activating gene (*Rag*) deficiency, persistent activation of p38 MAPK interferes with the differentiation of thymocytes beyond the DN3 stage. However, MKK6 transgenic thymocytes (but not *Rag*^{-/-} thymocytes) survive and accumulate in vivo (8), suggesting that

*Present address: Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, 62210, México.

†To whom correspondence should be addressed. E-mail: mrincon@uvm.edu

p38 MAPK may also provide a survival signal. A gene expression profile analysis comparing Rag^{-/-} and MKK6 DN3 thymocytes revealed that the MKK6 DN3 thymocytes expressed more *c-myc* and *lef* (fig. S1) [two transcription factors associated with cell survival (9-11)] than did the Rag^{-/-} thymocytes. The increased abundance of c-Myc and Lef proteins in the MKK6 transgenic thymocytes, compared with Rag^{-/-} thymocytes, was confirmed by Western blot analysis (Fig. 1A) (12). Thymocytes from Rag^{-/-} mice crossed with MKK6 transgenic (Rag^{-/-} MKK6) mice contained higher amounts of c-Myc and Lef proteins than did Rag^{-/-} thymocytes, indicating that the activation of p38 MAPK, but not the pre-TCR signals, contributes to the enhanced expression of these transcription factors (Fig. 1B). The *c-myc* and *lef* genes are targets of the β -catenin signaling pathway in certain contexts (13,14). Nuclear accumulation of β -catenin was detected in MKK6 thymocytes, but not in Rag^{-/-} thymocytes (Fig. 1C). Expression of constitutively active MKK6 in 293T cells was also sufficient to increase the amount of β -catenin protein (Fig. 1D), but this had no effect on β -catenin mRNA (fig. S2).

Phosphorylation of β -catenin by glycogen synthase kinase 3 β (GSK3 β) targets β -catenin for ubiquitination and subsequent degradation (15,16). The best-characterized mechanism for the inactivation of GSK3 β is through phosphorylation of its N terminus at Ser⁹ by Akt (17). No increase was observed in the amount of phospho-Ser⁹ GSK3 β in MKK6 thymocytes compared with that in Rag^{-/-} thymocytes (Fig. 2A). Similarly, no increase in phospho-Ser⁹ was observed in 293T cells transfected with constitutively active MKK6 (Fig. 2B). Phosphorylation of Ser⁹ was impaired by Wortmanin, an inhibitor of the PI3K-Akt pathway, but it was not affected by the pharmacological inhibitor of p38 MAPK SB203580 (fig. S3). Thus, p38 MAPK does not appear to regulate the Akt-mediated phosphorylation of GSK3 β on Ser⁹. p38 MAPK immunoprecipitated from MKK6 thymocytes or MKK6-transfected 293T cells phosphorylated recombinant catalytically inactive GSK3 β in vitro, and this phosphorylation was blocked by the p38 MAPK inhibitor (Fig. 2C). No Akt was detected in p38 MAPK immunoprecipitates, and no p38 MAPK was detected in Akt immunoprecipitates (fig. S4), ruling out the presence of residual AKT associated with p38 MAPK. The depletion of Akt before immunoprecipitating p38 MAPK did not affect the phosphorylation of GSK3 β (Fig. 2D). A purified recombinant activated p38 MAPK also phosphorylated GSK3 β in vitro, and this phosphorylation was blocked by SB203580 (Fig. 2E). Coimmunoprecipitation analysis showed that p38 MAPK was present in GSK3 β immunoprecipitates from MKK6 thymocytes (Fig. 2F) and 293T cells (fig. S5). Thus, p38 MAPK physically associates with and phosphorylates GSK3 β at a Ser⁹-independent residue. Although GSK3 α and GSK3 β are thought to be similarly regulated and can compensate for each other for some functions (18,19), GSK3 α was not phosphorylated by recombinant p38 MAPK in vitro (Fig. 2G).

The MAPK extracellular signal-regulated protein kinase (ERK) phosphorylates Thr⁴³ of GSK3 β (20) but does not affect GSK3 β activity. Although SerPro or ThrPro motifs recognized by ERK are also recognized by other MAPK groups, p38 MAPK was still able to partially phosphorylate a GSK3 β -T⁴³A mutant (Fig. 3A), suggesting the existence of additional phosphorylation sites in GSK3 β . Mass spectrometric analysis of recombinant GSK3 β phosphorylated in vitro by p38 MAPK revealed two GSK3 β phospho-peptides containing phosphorylation within a consensus SerPro or ThrPro motif, a phospho-peptide containing Thr⁴³, and a C-terminal peptide (384 to 403) containing the ThrPro motif at Thr³⁹⁰ (corresponding to Ser³⁸⁹ in mouse GSK3 β) (figs. S6 and S7). To confirm Thr³⁹⁰ as a target of p38 MAPK in GSK3 β , catalytically inactive GSK3 β -T³⁹⁰A and GSK3 β -T⁴³A/T³⁹⁰A mutants were used as substrates for p38 MAPK in vitro. Phosphorylation of the GSK3 β -T³⁹⁰A mutant by p38 MAPK was partially reduced but not abrogated (Fig. 3B), but phosphorylation of the GSK3 β -T⁴³A/T³⁹⁰A mutant was abrogated, indicating that these two residues are probably the targets for p38 MAPK in GSK3 β . The T⁴³A mutation (but not the T³⁹⁰A mutation) abrogated

phosphorylation of GSK3 β by ERK (Fig. 3C). Thus, Thr³⁹⁰ of GSK3 β appears to be specifically phosphorylated by p38 MAPK.

We examined the activity of wild-type (WT) GSK3 β and GSK3 β -T⁴³A and GSK3 β -T³⁹⁰A mutants before or after incubation with p38 MAPK or Akt. p38 MAPK inhibited both WT GSK3 β and the GSK3 β -T⁴³A mutant (Fig. 3D), but not the GSK3 β -T³⁹⁰A mutant (Fig. 3D). Akt inhibited WT GSK3 β and the two mutants (Fig. 3D). p38 MAPK did not affect the activity of GSK3 α (fig. S8), in which the Thr³⁹⁰ residue from GSK3 β is not conserved. Together, these results demonstrate that p38 MAPK-mediated phosphorylation of GSK3 β at Thr³⁹⁰ (but not Thr⁴³) is sufficient to inhibit GSK3 β activity. A peptide derived from the N terminus of GSK3 β containing phospho-Ser⁹ specifically inhibits GSK3 β in vitro (21,22). A phospho-Thr³⁹⁰ peptide also inhibited GSK3 β activity, whereas the unphosphorylated-Thr³⁹⁰ peptide did not (Fig. 3E). The phospho-Thr³⁹⁰ peptide inhibited GSK3 β activity as efficiently as the phospho-Ser⁹ peptide (Fig. 3F). Thus, phosphorylation at Thr³⁹⁰ by p38 MAPK may cause an inhibition of GSK3 β comparable to the phosphorylation of Ser⁹ by Akt.

To demonstrate the phosphorylation of this residue in intact cells and in vivo, we generated a specific antibody (Ab) to a mouse phospho-Ser³⁸⁹ GSK3 β peptide. A band corresponding to GSK3 β was detected with this Ab in WT and GSK3 $\alpha^{-/-}$ embryonic stem (ES) cells, but not in the GSK3 $\beta^{-/-}$ ES cells by Western blot analysis (Fig. 4A). This specific band was also present in GSK3 $\beta^{-/-}$ ES cells transfected with a WT GSK3 β , but not with a GSK3 β -S³⁸⁹A mutant (Fig. 4B). Phospho-Ser³⁸⁹ GSK3 β was detected in mouse GSK3 β -transfected 293T cells, but only if active MKK6 was present (Fig. 4C). The presence of the phospho-Ser³⁸⁹ GSK3 β in these cells correlated with an increased amount of β -catenin (Fig. 4C), which is indicative of an inhibition of GSK3 β activity. Ser³⁸⁹-phosphorylation was also detected in WT GSK3 β , but not the GSK3 β -S³⁸⁹A mutant after in vitro incubation with activated p38 MAPK (fig. S9). Phosphatase treatment of GSK3 β previously incubated with activated p38 MAPK abrogated its recognition by the phospho-Ser³⁸⁹ Ab (fig. S9). Together, these results show the specificity of this Ab for phospho-S³⁸⁹ GSK3 β and the phosphorylation of GSK3 β at S³⁸⁹ by p38 MAPK in vitro.

To determine whether activation of p38 MAPK was required for phosphorylation of GSK3 β at Ser³⁸⁹ in intact cells, we treated mouse GSK3 β -transfected 293T cells with SB203580. The inhibition of p38 MAPK abrogated the phosphorylation of Ser³⁸⁹ (Fig. 4D). Similarly, treatment with SB203580 inhibited phosphorylation of endogenous GSK3 β at Ser³⁸⁹ in WT mouse embryonic fibroblasts (MEFs) and ES cells (Fig. 4E). We also examined phospho-Ser³⁸⁹ abundance in MEFs deficient for the major upstream activators of p38 MAPK, MKK3, and MKK6 (23). Phospho-Ser³⁸⁹ was barely detectable in MKK3^{-/-}MKK6^{-/-} MEFs (Fig. 4F). In contrast, the amounts of phospho-Ser⁹ were comparable in WT and MKK3^{-/-}MKK6^{-/-} MEFs (Fig. 4F). Thus, activation of p38 MAPK appears to be required for the phosphorylation of GSK3 β at Ser³⁸⁹. Inhibition of p38 MAPK by either SB203580 (Fig. 4D) or the absence of MKK3 and MKK6 (Fig. 4F) also decreased the amount of β -catenin, consistent with the possibility that p38 MAPK activation is required for repressing GSK3 β activity.

We also examined phospho-Ser³⁸⁹ in different mouse tissues. A high amount of phospho-S³⁸⁹ was detected in the brain, and lesser amounts were detected in thymocytes and spleen cells (Fig. 4G). Phospho-Ser³⁸⁹ was not detected in the kidney (Fig. 4G), liver, or heart (fig. S10). Phosphorylation of GSK3 β at Ser⁹ was detected in practically all of the examined tissues (Fig. 4G). Analysis of the relative abundance of phospho-S³⁸⁹ and phospho-S⁹ showed a predominance of the former in the brain and thymocytes (Fig. 4G), which correlated with the selective high activation of p38 MAPK in these tissues (fig. S11). Inhibition of p38 MAPK by treating animals with SB203580 reduced the levels of phospho-Ser³⁸⁹ GSK3 β in both thymocytes and the brain (Fig. 4H). Analysis of phospho-Ser³⁸⁹ in MKK6 and Rag^{-/-}

thymocytes showed that phospho-S³⁸⁹ GSK3 β was present selectively in MKK6 thymocytes (Fig. 4I). Together, these results support our proposal that GSK3 β is phosphorylated at S³⁸⁹ in vivo by p38 MAPK and that this alternative regulatory mechanism of GSK3 β is tissue-specific.

To date, phosphorylation at Ser⁹ by Akt is the best-characterized mechanism for the inhibition of GSK3 β activity. However, knockin mice in which Ser⁹ was replaced by Ala have only a subtle defect related to insulin regulation of glycogen synthase in their skeletal muscle tissue (24), indicating that alternative mechanisms may be involved in the negative regulation of GSK3 β for certain functions. We propose that the phosphorylation of GSK3 β at S³⁸⁹ by p38 MAPK may be one such mechanism. Conditions that promote the activation of p38 MAPK promote the accumulation of β -catenin in certain scenarios; thus, the activation of the p38 MAPK pathway could be an alternative mechanism to regulate β -catenin/T cell factor signaling (and, potentially, cell survival) through the inactivation of GSK3 β .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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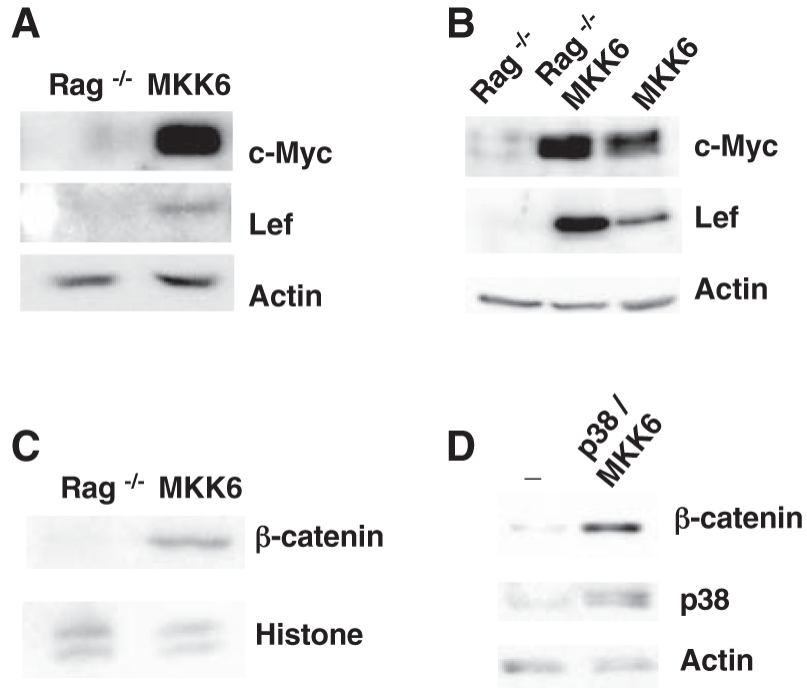


Fig. 1. Regulation of the β -catenin pathway by p38 MAPK. (A) Western blot showing c-Myc and Lef in whole-cell extracts from Rag^{-/-} thymocytes (Rag^{-/-}) and MKK6 thymocytes (MKK6). Actin was examined as a control. (B) Western blot showing c-Myc and Lef in thymocytes from Rag^{-/-}, MKK6, and Rag^{-/-}/MKK6 mice. (C) Western blot showing β -catenin in nuclear extracts from Rag^{-/-} and MKK6 thymocytes. (D) Western blot showing β -catenin and p38 MAPK in whole-cell extracts from 293T cells transfected with GSK3 β (-) or GSK3 β with p38 MAPK and MKK6 (p38/MKK6).

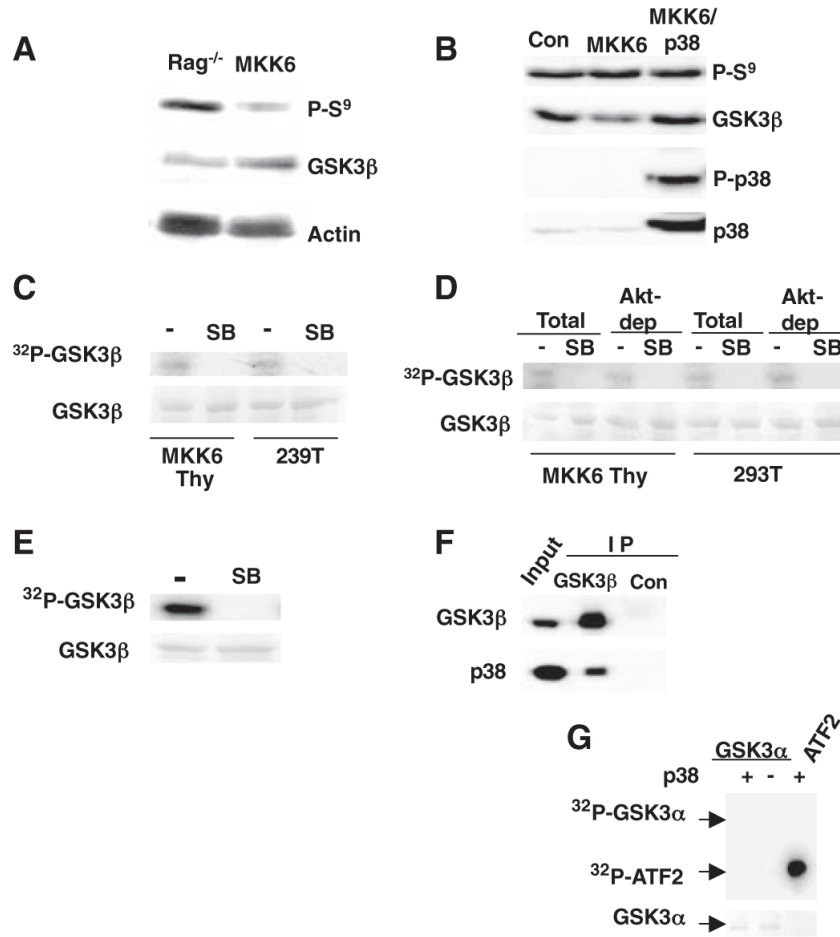


Fig. 2. Direct phosphorylation of GSK3 β by p38 MAPK. (A) Western blot showing phospho-Ser⁹ GSK3 β (P-S⁹) and total GSK3 β in Rag^{-/-} and MKK6 thymocytes. (B) Western blot showing P-Ser⁹ GSK3 β , GSK3 β , phospho-p38 MAPK (P-p38), and p38 MAPK in 293T cells transfected with an empty vector (Con), MKK6 alone, or MKK6 and p38 MAPK (MKK6/p38). (C) In vitro p38 MAPK assay with inactive recombinant GSK3 β as the substrate and p38 MAPK immunoprecipitated from MKK6-thymocytes (MKK6 Thy) or MKK6-transfected 293T cells (293T). In vitro reactions were incubated in the presence (SB) or absence (-) of the specific p38 MAPK inhibitor SB203580. Total GSK3 β was visualized by PonceauS staining, and phosphorylated GSK3 β was detected by autoradiography. (D) In vitro p38 MAPK kinase assay as described in (C), using total or Akt-depleted extracts (Akt-dep) from MKK6 thymocytes (MKK6 Thy) or MKK6-transfected 293T cells (293T). (E) In vitro kinase assay as described in (C), with recombinant active p38 MAPK kinase. (F) Western blot showing GSK3 β and p38 MAPK in the GSK3 β and p21 (Con) immunoprecipitates (IP) and whole-cell extracts from MKK6 thymocytes (Input). (G) In vitro kinase assay for recombinant active p38 MAPK kinase using catalytically inactive GSK3 α as a substrate. Phosphorylation of activating transcription factor 2 (ATF2) was examined as a positive control.

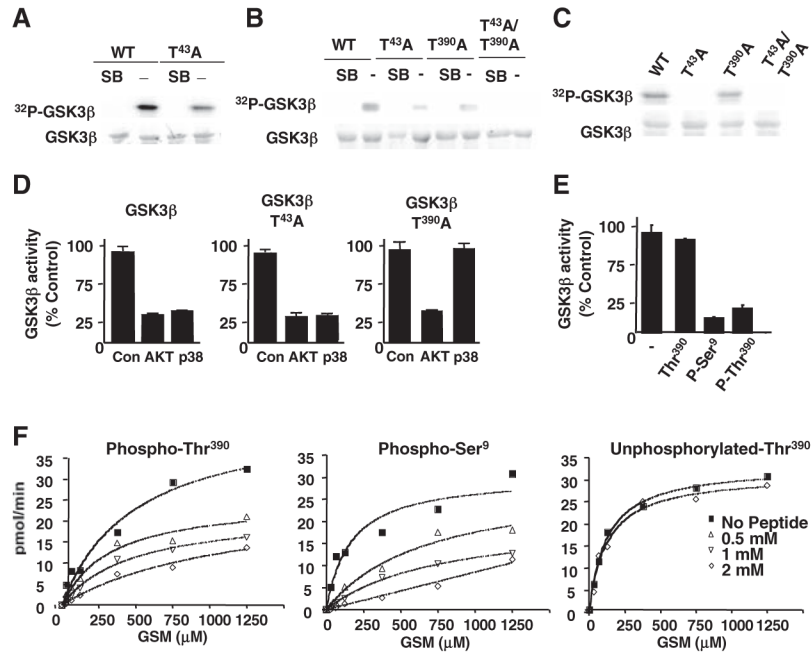
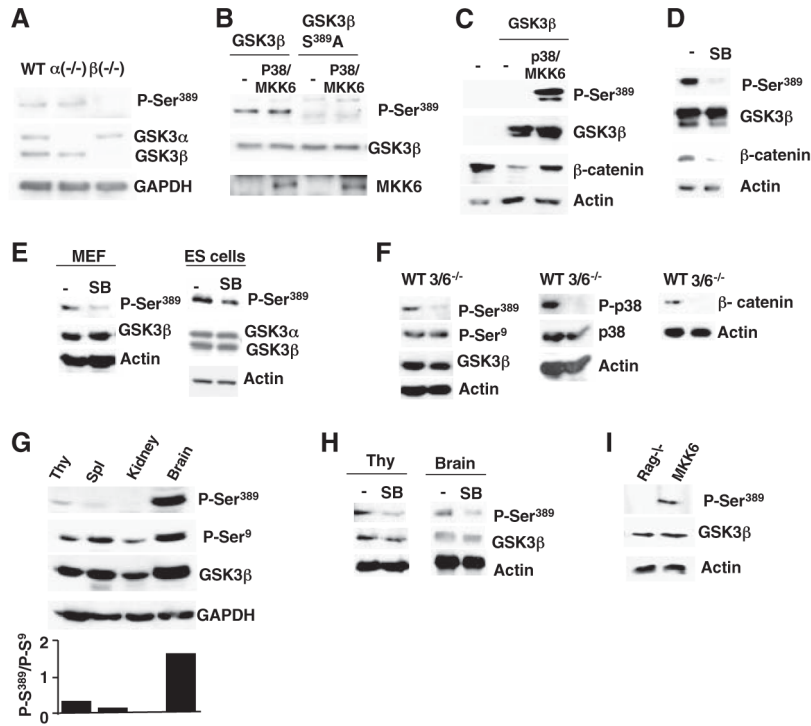


Fig. 3. Inhibition of GSK3 β by p38 MAPK is mediated by phosphorylation at Thr³⁹⁰. (A) In vitro kinase assays for recombinant p38 MAPK with catalytically inactive GSK3 β and GSK3 β -T⁴³A mutant substrates. (B) In vitro kinase assay for recombinant p38 MAPK with kinase-inactive GSK3 β (WT), GSK3 β -T⁴³A, GSK3 β -T³⁹⁰A, and GSK3 β -T⁴³A/T³⁹⁰A mutants as substrates. (C) In vitro kinase assay for recombinant active ERK with catalytically inactive GSK3 β , GSK3 β -T⁴³A, GSK3 β -T³⁹⁰A, and GSK3 β -T⁴³A/T³⁹⁰A mutants as substrates. (D) In vitro kinase assay for active GSK3 β , GSK3 β -T⁴³A, and GSK3 β -T³⁹⁰A mutants before (Con) or after incubation with activated Akt or activated p38 MAPK. GSK3 β activity relative to the activity without Akt or p38 MAPK (Con) is shown. Error bars represent SD (n = 3 replicates). (E) In vitro GSK3 β kinase reactions alone (-) or in the presence of unphosphorylated-Thr³⁹⁰ (Thr³⁹⁰), phospho-Ser⁹ (P-Ser⁹), or phospho-Thr³⁹⁰ (P-Thr³⁹⁰) peptides, as described in (D). Error bars represent SD. (F) GSK3 β in vitro kinase assays as in (D), using various concentrations of phospho-Thr³⁹⁰, phospho-Ser⁹, and unphosphorylated-Thr³⁹⁰ peptides. Each point is the average of two measurements. GSM, modified glycogen synthase peptide.

**Fig. 4.**

Phosphorylation of endogenous GSK3 β by p38 MAPK. (A) Western blot showing the presence of endogenous phospho-Ser³⁸⁹ GSK3 β (P-Ser³⁸⁹) in WT, GSK3 α ^{-/-}, and GSK3 β ^{-/-} ES cells. Total GSK3 α , GSK3 β , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined as controls. (B) Western blot showing P-Ser³⁸⁹ in GSK3 β ^{-/-} ES cells transfected with WT GSK3 β or GSK3 β -S³⁸⁹A mutant alone or with p38 MAPK and MKK6 (p38/MKK6). (C) Western blot showing P-Ser³⁸⁹, Flag-tagged mouse GSK3 β , and β -catenin in nontransfected 293T cells (-) or cells transfected with mouse GSK3 β alone or in combination with p38 MAPK and MKK6 (p38/MKK6). (D) P-Ser³⁸⁹, total GSK3 β , and β -catenin in 293T cells transfected with GSK3 β , p38, and MKK6 in the absence (-) or presence of SB203580. (E) P-Ser³⁸⁹ and total GSK3 β in MEF or total GSK3 α and GSK3 β in ES cells nontreated (-) or treated with SB203580. (F) P-Ser³⁸⁹, P-Ser⁹, total GSK3 β , P-p38, total p38, and β -catenin in WT and MKK3^{-/-}MKK6^{-/-} (3/6^{-/-}) MEF. (G) The tissue distribution of P-Ser³⁸⁹, P-Ser⁹, and total GSK3 β . Quantification of the levels of P-Ser³⁸⁹ relative to P-Ser⁹ in each tissue is also shown (lower panel). Thy, thymocytes; Spl, spleen cells. (H) P-Ser³⁸⁹ and total GSK3 β in thymocytes and brain from WT mice treated in vivo with vehicle (-) or SB203580 (SB). (I) P-S³⁸⁹ and total GSK3 β in thymocytes from Rag^{-/-} and MKK6 transgenic mice.