GSK-3β acts downstream of PP2A and the PI 3-kinase-Akt pathway, and upstream of caspase-2 in ceramide-induced mitochondrial apoptosis

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Summary

The signaling of glycogen synthase kinase-3β (GSK-3β) has been implicated in stress-induced apoptosis. However, the pro-apoptotic role of GSK-3β is still unclear. Here, we show the involvement of GSK-3β in ceramide-induced mitochondrial apoptosis. Ceramide induced GSK-3β activation via protein dephosphorylation at serine 9. We previously reported that ceramide induced caspase-2 and caspase-8 activation, Bid cleavage, mitochondrial damage, and apoptosis. In this study, we found that caspase-2 activation and the subsequent apoptotic events were abolished by the GSK-3β inhibitors lithium chloride and SB216763, and by GSK-3β knockdown using short interfering RNA. We also found that ceramide-activated protein phosphatase 2A (PP2A) indirectly caused GSK-3β activation, and that the PP2A-regulated PI 3-kinase-Akt pathway was involved in GSK-3β activation. These results indicate a role for GSK-3β in ceramide-induced apoptosis, in which GSK-3β acts downstream of PP2A and the PI 3-kinase-Akt pathway, and upstream of caspase-2 and caspase-8.

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Key words: Ceramide, GSK-3β, Apoptosis

Introduction

Glycogen synthase kinase-3β (GSK-3β), originally identified as a regulator of glycogen metabolism, is now known to be important in a variety of signaling pathways that control protein synthesis, cell proliferation, differentiation, motility, and apoptosis (Cohen and Frame, 2001; Frame and Cohen, 2001; Jope and Johnson, 2004). Overexpression of GSK-3β induces cell apoptosis (Pap and Cooper, 1998; Pap and Cooper, 2002; Bijur et al., 2000). The involvement of GSK-3β in apoptotic signaling induced by staurosporine, heat shock, growth factor withdrawal, hypoxia, endoplasmic reticulum stress and mitochondrial complex I inhibitors has also been shown (Bijur et al., 2000; Hetman et al., 2000; King et al., 2001; Somervaille et al., 2001; Bhat et al., 2002; Loberg et al., 2002; Song et al., 2002; Hongisto et al., 2003). Interaction of GSK-3β with various downstream substrates, such as translation initiation factor 2B, β-catenin, $p21^{Cip1}$ and p53, leads to the regulation of cell fate (Cohen and Frame, 2001; Frame and Cohen, 2001; Pap and Cooper, 2002; Rossig et al., 2002; Watcharasit et al., 2003; Jope and Johnson, 2004).

The pro-apoptotic role of GSK-3β has previously been shown to be negatively regulated through the phosphoinositol 3-kinase (PI 3-kinase)-Akt survival pathway (Pap and Cooper, 1998; Cross et al., 1995). Inactivation of GSK-3β through the PI 3-kinase-Akt pathway is mainly due to protein phosphorylation at serine 9 (Cohen and Frame, 2001; Frame and Cohen, 2001; Jope and Johnson, 2004). Blockage of PI 3-kinase-Akt signaling by the selective PI 3-kinase inhibitor LY294002 resulted in GSK-3β activation and cell apoptosis, but this was prevented by dominant-negative GSK-3β (Pap and Cooper, 1998; Pap and Cooper, 2002; Hetman et al., 2002). In addition to the PI 3-kinase-Akt pathway, extracellular signal-regulated kinase (ERK), PKA, PKC, MAP kinase-activated protein (MAPKAP) kinase-1 (also known as p90rsk), p70 ribosomal S6 kinase (p70S6K), and Wnt signaling are also involved in GSK-3β inactivation (Cohen and Frame, 2001; Frame and Cohen, 2001; Hetman et al., 2002; Jope and Johnson, 2004). Furthermore, protein phosphatase 2A (PP2A) activation may concomitantly dephosphorylate and activate GSK-3β directly or indirectly by dephosphorylating Akt (Seeling et al., 1999; Ivaska et al., 2002).

Ceramide is involved in multiple biological functions, including cell survival and apoptosis (Hannun and Obeid, 1995; Dbaibo and Hannun, 1998; Mathias et al., 1998; Ruvolo, 2001; Wagenknecht et al., 2001; Caricchio et al., 2002; Kimura et al., 2003; Ogretmen and Hannun, 2004). A number of apoptotic stimuli – tumor necrosis factor α (TNFα), Fas ligation, serum withdrawal, chemotherapeutic agents and irradiation – may induce generation of cellular ceramide. However, the molecular mechanisms by which ceramide regulates apoptotic events are not fully understood. Mitochondrial damage acts as an apoptotic signaling target of

ceramide (Hearps et al., 2002; Stoica et al., 2003). Activation of caspase-8 and the cleavage of Bid are involved in ceramide-induced neuronal cell death via mitochondrial dysfunction (Darios et al., 2003). Caspase-2 can cleave cytosolic Bid and trigger the release of cytochrome *c* from mitochondria (Guo et al., 2002; Kumar and Vaux, 2002; Lassus et al., 2002; Paroni et al., 2002; Robertson et al., 2002; Schweizer et al., 2003; Troy and Shelanski, 2003; Lin et al., 2004; Wagner et al., 2004). We recently showed sequential activation of caspase-2 and caspase-8 upstream of the mitochondrial apoptotic pathway in ceramide-induced apoptosis (Lin et al., 2004). However, the mechanisms that activate initiator caspases leading to the mitochondrial apoptotic pathways remain unresolved.

Inhibition of the PI 3-kinase-Akt pathway by protein phosphatase has been demonstrated in the apoptotic mechanisms of ceramide (Dobrowsky and Hannun, 1992; Dobrowsky et al., 1993; Wolff et al., 1994; Schubert et al., 2000; Mora et al., 2002; Ruvolo, 2003). Meanwhile, dephosphorylation and activation of GSK-3β induced by ceramide has also been observed (Mora et al., 2002). Lithium chloride (LiCl), a traditional GSK-3β inhibitor, confers neuroprotection against apoptosis induced by ceramide (Centeno et al., 1998; Mora et al., 2002). In the present study, we showed that GSK-3β knockdown using short interfering RNA (siRNA) or specific inhibitors rendered the cells resistant to ceramide-induced apoptosis. GSK-3β inhibition prevented activation of caspase-2 and caspase-8 upstream of the mitochondrial apoptotic pathway. Our study indicates that PP2A and the PI 3-kinase-Akt pathway regulate GSK-3β activation, and that GSK-3β in turn regulates caspase-2 and caspase-8 in ceramide-induced apoptosis.

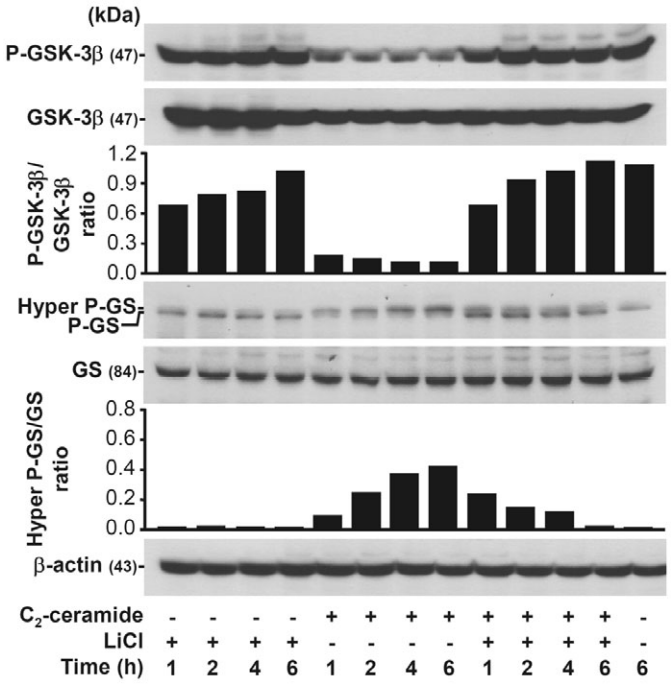

Fig. 1. Ceramide activates GSK-3β. 10I cells were treated with 25 μM of C_2-ceramide for 1, 2, 4 and 6 hours with or without 10 mM LiCl. We used western blotting to determine GSK-3β phosphorylation (P-GSK-3β) at serine 9 and glycogen synthase phosphorylation (P-GS) at serine 641. Total GSK-3β and GS proteins were the control. The relative ratio of P-GSK-3β:total GSK-3β and of hyperphosphorylated GS (hyper P-GS):total GS are shown. β-actin levels were determined as an internal control.

Results

Ceramide activates GSK-3

In an attempt to study the role of GSK-3β involved in the ceramide-induced apoptotic signaling pathway, we treated mouse T hybridoma 10I cells with the ceramide analogue C_2-ceramide and assessed the activation of GSK-3β by its dephosphorylation at serine 9. Western blot analysis showed a decrease in the phosphorylation of GSK-3β compared with the untreated control after a 1-hour ceramide treatment (Fig. 1); this decrease was sustained for 6 hours. Interestingly, LiCl abolished ceramide-induced GSK-3β dephosphorylation. Similar results were shown in other cell types, including human neuroblastoma SK-N-SH and human lung epithelial carcinoma A549 cells (supplementary material Fig. S1). To further confirm the ceramide-induced GSK-3β activation, the phosphorylation of its downstream substrate glycogen synthase (GS) at serine 641 was determined. Our results show that ceramide treatment caused an increase in GS phosphorylation, as evidenced by an increase in its hyperphosphorylated form, but LiCl inhibited this effect (Fig. 1).

GSK-3β activation is required for ceramide-induced apoptosis

We further investigated whether the activation of GSK-3β was required for ceramide to induce apoptosis. We analyzed ceramide-induced 10I cell apoptosis with and without the GSK-3β inhibitors LiCl and SB216763. Cell apoptosis is characterized by nuclear and DNA fragmentation, and was determined using 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) staining, respectively. We then used microscopic and flow-cytometric analysis to determine apoptotic cells. Results showed that ceramide-induced apoptosis was reduced in cells pretreated with LiCl or SB216763 (Fig. 2A), and that this inhibitory effect was dose-dependent (Fig. 2B). Similar findings were obtained using annexin V and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (Fig. 2B and supplementary material Fig. S2A). Furthermore, pretreatment with LiCl or SB216763 also conferred protection against ceramide-induced apoptosis in SK-N-SH and A549 cells (supplementary material Fig. S2B). Treatment with LiCl or SB216763 alone showed a pattern similar to that of the untreated control (Fig. 2B). To further confirm the requirement of GSK-3β in ceramide-induced apoptosis, GSK-3β was silenced using siRNA. First, the specific knockdown of GSK-3β expression and GSK-3β inactivation were confirmed using western blot analysis. Cells with GSK-3β knockdown, which did not change the expression of GSK-3α, showed an inhibition on ceramide-induced GS hyperphosphorylation. Cells with GSK-3α knockdown did not change the expression of GSK-3β. Ceramide-induced GS hyperphosphorylation could still be observed after GSK-3α silencing (Fig. 2C). These results indicated that ceramide predominantly affected GSK-3β rather than GSK-3α, which was demonstrated by the influence of GSK-3β knockdown on GS hyperphosphorylation. After

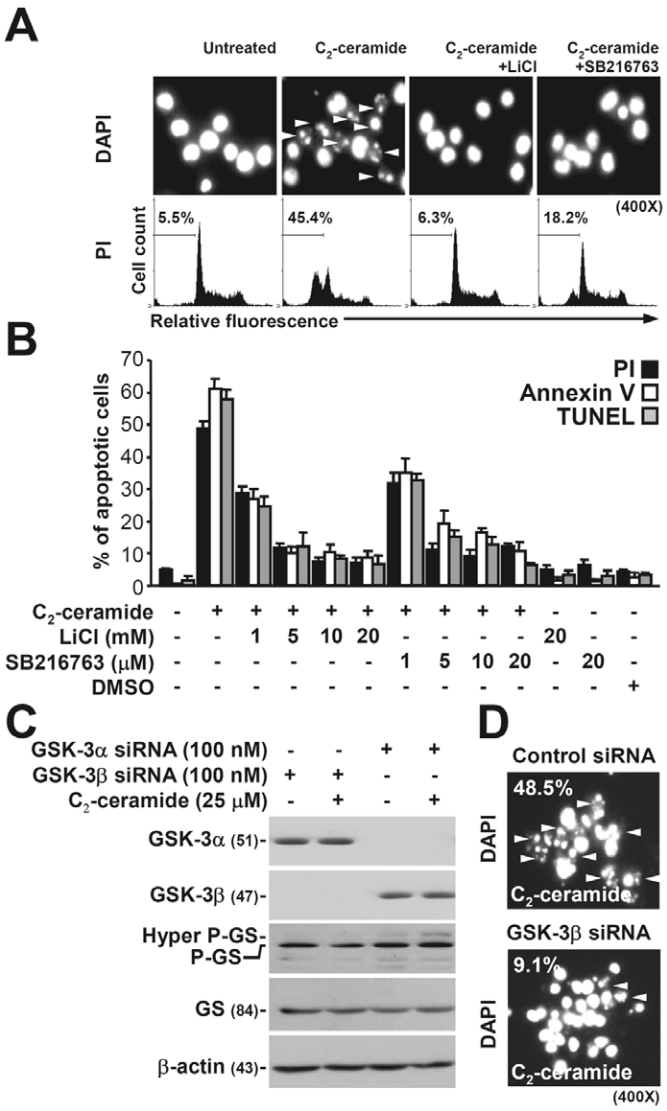

Fig. 2. Inhibition of GSK-3β blocks ceramide-induced apoptosis. (A) 10I cells were treated with 25 μM of C_2-ceramide for 6 hours with or without the GSK-3β inhibitors LiCl (10 mM) and SB216763 (10 μM). Cell apoptosis was determined using DAPI staining and microscopy (to analyze nuclear fragmentation), and propidium iodide (PI) staining and flow cytometric analysis (to analyze DNA fragmentation). Arrowheads indicate apoptotic cells; percentages of apoptotic cells in the subG_0 phase are marked in the histograms. (B) 10I cells were treated with 25 μM of C_2-ceramide for 6 hours with or without various doses of LiCl and SB216763 as indicated. Cell apoptosis was detected using PI, annexin V and TUNEL staining, followed by flow cytometric analysis. The percentage of apoptotic cells is shown (mean ± s.d. of five replicates for PI staining and of triplicates for annexin V and TUNEL staining). Cells treated with DMSO were used as the reagent control. (C) 10I cells were transfected with 100 nM of GSK-3α siRNA (95.8% transfection efficiency) or 100 nM of GSK-3β siRNA (96.1% transfection efficiency) for 24 hours as described in Materials and Methods. After further treatment with 25 μM of C_2-ceramide for 6 hours, the GSK-3α, GSK-3β, GS phosphorylated at serine 641 (P-GS), and levels of total GS (GS) were detected using western blotting. β-actin levels were determined as an internal control. (D) Cells pre-transfected for 24 hours with control siRNA or GSK-3β siRNA (100 nM) were treated with 25 μM C_2-ceramide for 6 hours. We used DAPI staining plus microscopy to determine cell apoptosis characterized by nuclear fragmentation, and PI staining plus flow cytometry to determine cell apoptosis characterized by DNA fragmentation. Arrowheads indicate the apoptotic cells; percentages of apoptotic cells in the subG_0 phase are given in each micrograph.

ceramide treatment, GSK-3β siRNA-transfected cells, in contrast to control siRNA-transfected cells, were resistant to induction of apoptosis (Fig. 2D).

Ceramide-induced apoptosis has been reported to be mitochondria-dependent (Hearps et al., 2002; Darios et al., 2003; Stoica et al., 2003; Lin et al., 2004). Therefore, we next investigated the regulatory role of GSK-3β in ceramide-induced mitochondrial damage. Using the lipophilic cationic fluorochrome Rhodamine 123, we found that the GSK-3β inhibitors LiCl and SB216763 as well as GSK-3β siRNA pretreatment diminished the ceramide-induced loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) (Fig. 3A). To further analyze mitochondrial damage, we examined the release of cytochrome *c* from mitochondria to the cytoplasm. Western blot analysis showed that ceramide caused the release of cytochrome *c*, but that inhibition of GSK-3β blocked this release (Fig. 3B). Inhibition of GSK-3β also blocked ceramide-induced activation of caspase-9 and caspase-3 downstream of the mitochondrial apoptotic pathway (Fig. 3C). These results verified the dependence of GSK-3β signaling in ceramide-induced mitochondrial apoptosis.

GSK-3β regulates activation of caspase-2 and caspase-8, and cleavage of Bid

We have reported previously that caspase-2 and caspase-8 were sequentially activated upstream of the mitochondrial apoptotic pathway during ceramide-induced apoptosis (Lin et al., 2004). However, the mechanisms of ceramide-induced activation of caspase-2 remained unclear. We, therefore, investigated whether ceramide-activated GSK-3β acts upstream of caspase-2 to induce apoptosis. Intriguingly, the GSK-3β inhibitors LiCl and SB216763 and also GSK-3β siRNA blocked ceramide from activating caspase-2 and caspase-8 (Fig. 4A). Because activation of both caspases resulted in the cleavage of Bid (Lin et al., 2004), we used western blotting to analyze the generation of truncated Bid (tBid). Pretreating cells with LiCl, SB216763 or GSK-3β siRNA also inhibited ceramide-induced Bid cleavage (Fig. 4B). As expected, when cells were pretreated with LiCl, tBid did not translocate to mitochondria (data not shown). These results revealed that GSK-3β is necessary for ceramide-induced initiator caspase activation and expression of tBid before mitochondrial damage.

Ceramide induces GSK-3β activation through PP2A

PP2A dephosphorylates and activates GSK-3β (Seeling et al., 1999; Ivaska et al., 2002). Furthermore, ceramide might induce activation of PP2A (Dobrowsky et al., 1993; Ruvolo, 2001). We, therefore, investigated the regulatory role of ceramide-activated PP2A on GSK-3β activation. Okadaic acid (OA), a PP2A inhibitor, blocked ceramide-induced dephosphorylation of GSK-3β. LiCl treatment was used as a control for GSK-3β inactivation (Fig. 5A). To further confirm that PP2A activates GSK-3β, we transfected purified PP2A into cells. We detected GSK-3β dephosphorylation that was inhibited by OA

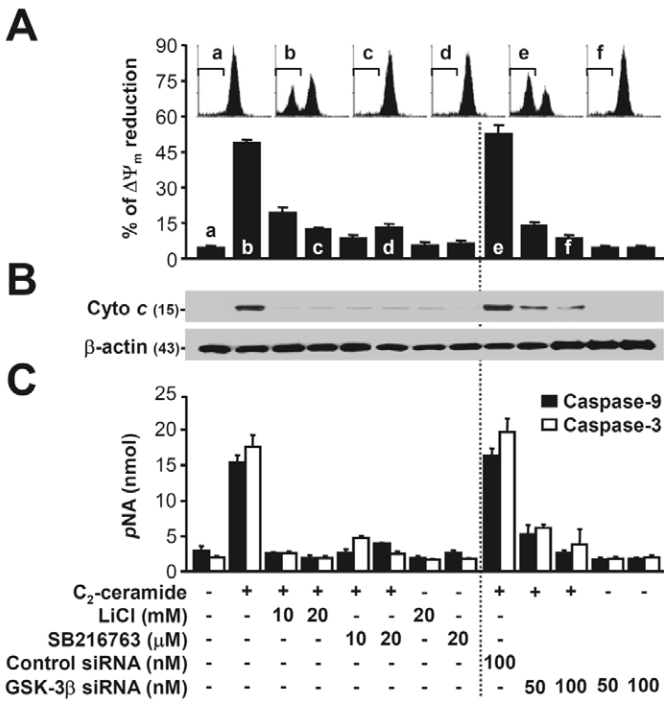

Fig. 3. Inhibition of GSK-3β blocked ceramide-induced mitochondrial apoptosis. 10I cells were treated with 25 μM of C_2-ceramide for 6 hours with or without the GSK-3β inhibitors LiCl (10 and 20 mM) and SB216763 (10 and 20 μM), or pre-transfected for 24 hours with control siRNA (100 nM) or GSK-3β siRNA (50 and 100 nM). (A) We used Rhodamine 123 staining plus flow cytometry to detect the $\Delta\Psi_m$ reduction. Partial histograms are shown. The percentages of cells with $\Delta\Psi_m$ reduction are given as the average of triplicate cultures (mean ± s.d.). (B) We used western blotting to determine the cytosolic levels of cytochrome *c*. β-actin levels were determined as an internal control. (C) We used enzymatic cleavage of the specific substrates benzyloxycarbonyl-Leu-Glu(-OMe)-His-Asp(-OMe)-*p*NA and benzyloxycarbonyl-Asp(-OMe)-Glu(-OMe)-Val-Asp(-OMe)-*p*NA to determine the activities of caspase-9 and caspase-3. Data are given as the average of triplicate cultures (mean ± s.d.). *p*NA, *p*-nitroanilide.

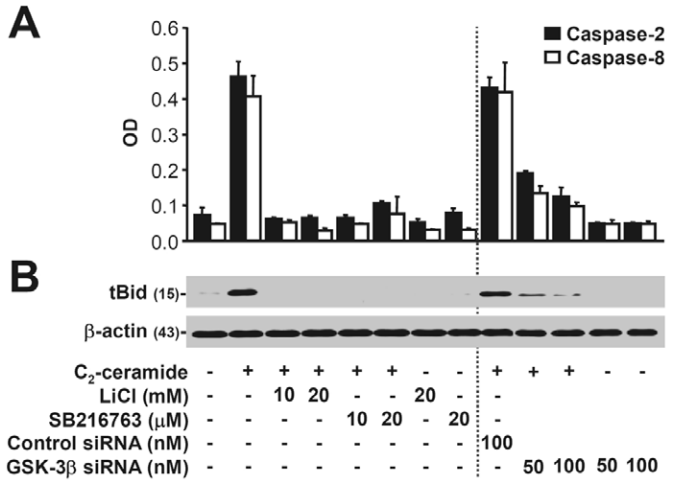

Fig. 4. Inhibition of GSK-3β blocks ceramide-induced activation of caspase-2 and caspase-8 and expression of tBid. 10I cells were treated with 25 μM C_2-ceramide for 6 hours with or without LiCl (10 and 20 mM) and SB216763 (10 and 20 μM), or were pre-transfected for 24 hours with control siRNA (100 nM) or GSK-3β siRNA (50 and 100 nM). (A) We used enzymatic cleavage of the specific substrates benzyloxycarbonyl-Val-Asp(-OMe)-Val-Ala-Asp(-OMe)-*p*NA and benzyloxycarbonyl-Ile-Glu(-OMe)-Thr-Asp(-OMe)-*p*NA to determine the activities of caspase-2 and caspase-8. OD, optical density. Data are given as the average of triplicate cultures (mean ± s.d.). (B) We used western blotting with a tBid-specific antibody to detect tBid expression. β-actin levels were determined as an internal control.

(Fig. 5B). Using immunostaining and confocal microscopy, we observed that ceramide treatment or PP2A transfection caused dephosphorylation of GSK-3β, and that OA reversed this effect (Fig. 5C). In addition to the in vivo experiments described above, we performed cell-free experiments to further examine whether PP2A directly acts on GSK-3β. OA inhibited the elevated activity of immunoprecipitated PP2A obtained from ceramide-treated cells (Fig. 5D, bottom). Co-incubating immunoprecipitated PP2A from ceramide-treated cells with GSK-3β from untreated cells did not affect the levels of GSK-3β phosphorylation (Fig. 5D, top). Furthermore, purified PP2A did not dephosphorylate GSK-3β in vitro (data not shown). Taken together, these results demonstrate that ceramide-activated PP2A (Fig. 5A) or transfected PP2A (Fig. 5B,C) indirectly dephosphorylates and activates GSK-3β (Fig. 5D).

PP2A activates GSK-3β via PI 3-kinase-Akt signaling

Because PI 3-kinase-Akt and MEK/ERK have been implicated in GSK-3β phosphorylation (Cross et al., 1995; Pap and Cooper, 1998; Pap and Cooper, 2002; Hetman et al., 2002), we investigated the effect of PP2A on these signaling pathways. First, ceramide inhibited PI 3-kinase activity, as evidenced by the reduced membrane-associated PI 3-kinase expression in 10I cells (supplementary material Fig. S3). An inhibitory effect of ceramide on PI 3-kinase activity has also been reported previously (Zundel and Giaccia, 1998). In cells not treated with ceramide, the PI 3-kinase inhibitor wortmannin (Fig. 6A, lane 5 versus lane 1) but not MEK inhibitor PD98059 (lane 9 versus lane 1), resulted in dephosphorylation of GSK-3β. In cells treated with ceramide, wortmannin abolished GSK-3β phosphorylation in cells pretreated with LiCl (lane 8 versus lane 4), but pretreatment with PD98059 had no effect (lane 12 versus lane 4). Treatment with wortmannin or PD98059 without LiCl did not reverse ceramide-induced GSK-3β dephosphorylation (lanes 6 and 10 versus lane 3). After using siRNA to knock down the expression of Akt, we found GSK-3β dephosphorylation in Akt-silenced cells. LiCl increased GSK-3β phosphorylation, which was abolished by Akt silencing in ceramide-treated cells (Fig. 6B). Therefore, LiCl might increase GSK-3β phosphorylation by regulating the PI 3-kinase-Akt but not the MEK-ERK pathway. We next clarified the relation between ceramide-induced PP2A, and PI 3-kinase and Akt, which act upstream of GSK-3β. Ceramide-induced dephosphorylation of Akt was abolished in cells pretreated with OA (Fig. 6C). Furthermore, in vitro studies showed that Akt was dephosphorylased by immunoprecipitated PP2A from ceramide-treated cells (Fig. 6D). Also, purified PP2A directly

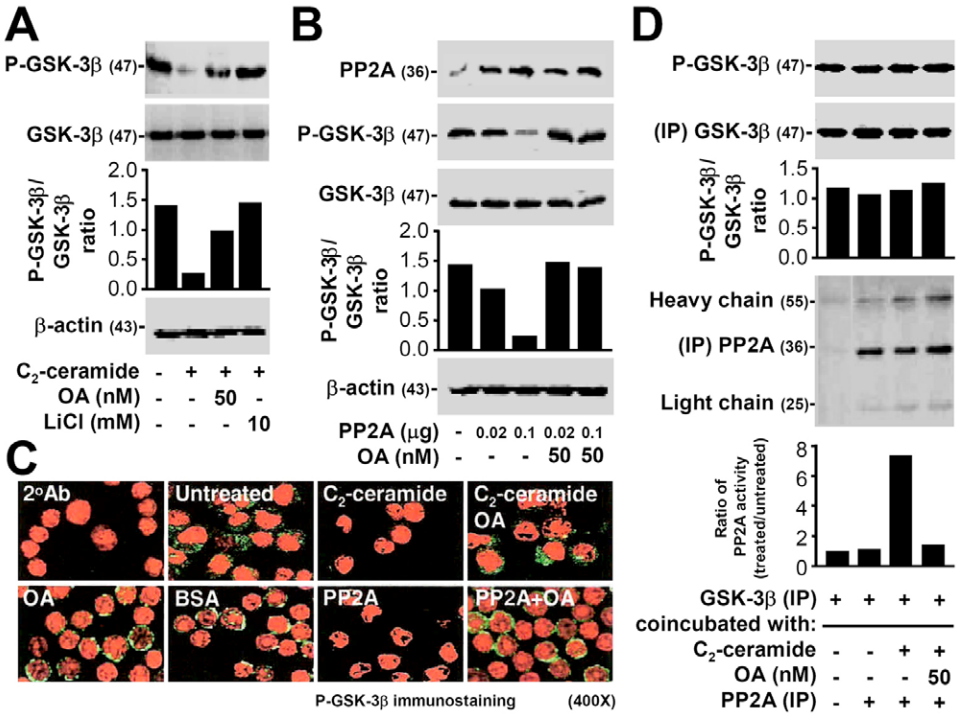

Fig. 5. Ceramide activates PP2A, which then indirectly activates GSK-3β. (A) 10I cells were treated with 25 μM C_2-ceramide for 6 hours with or without LiCl or OA. We used western blotting to determine phosphorylated GSK-3β (P-GSK-3β). Total GSK-3β protein was the control. The relative ratios of P-GSK-3β:total GSK-3β protein are shown. β-actin levels were determined as an internal control. (B) 10I cells were transfected with PP2A (0.02 and 0.1 μg) for 6 hours and incubated without or with OA for an additional 6 hours. The transfection efficiency determined using FITC-labeled immunoglobulin was 92.7% for the cells transfected with 0.02 μg PP2A and 95.8% for those transfected with 0.1 μg PP2A. We used western blotting to determine phosphorylated GSK-3β; the ratio of P-GSK-3β:total GSK-3β protein level is shown. β-actin levels were determined as an internal control. (C) We used immunostaining plus confocal microscopy to determine phosphorylated GSK-3β (green), and PI staining (red) for nuclear staining. (D) To determine whether PP2A directly affects GSK-3β dephosphorylation, GSK-3β was immunoprecipitated (IP) from untreated cells and then incubated together with PP2A that had been immunoprecipitated from 25 μM ceramide-treated cells for 30 minutes at 30°C with or without 50 nM OA. After the cells had been washed, we used western blotting to determine P-GSK-3β; the relative P-GSK-3β:IP GSK-3β protein level is shown. The IP PP2A level was determined using western blotting. PP2A activity was determined using substrate dephosphorylation; relative PP2A activity is shown.

dephosphorylated Akt, an effect that was inhibited by OA (data not shown). These results suggest that ceramide-induced PP2A inactivates Akt resulting in activation of GSK-3β.

Discussion

In the present study, using GSK-3β inhibitors and siRNA, we verified that ceramide-induced mitochondrial apoptosis is GSK-3β-dependent. Also, GSK-3β functions upstream of caspase-2 and leads to mitochondrial damage. The regulatory mechanisms of pro-apoptotic GSK-3β on caspase-2 activation remain still unclear, however. Ceramide-activated protein phosphatases, such as PP2A, might regulate GSK-3β activation. We demonstrated that PP2A indirectly regulates GSK-3β signaling by inactivating the PI 3-kinase-Akt pathway. Fig. 7 presents a summary of how GSK-3β might be involved in ceramide-induced mitochondrial apoptosis. Our previous study demonstrated the sequential activation of caspase-2 and caspase-8 upstream of the mitochondrial apoptotic pathway during ceramide-induced apoptosis (Lin et al., 2004). Moreover, we also showed that PP2A-mediated Bcl-2 dephosphorylation contributed to caspase-2 activation (Lin et al., 2005). However, the relation between PP2A, GSK-3β, Bcl-2 and caspase-2 need to be further clarified.

A number of studies have reported possible mechanisms through which ceramide activates GSK-3β. Cells treated with the ceramide-synthase inhibitor fumonisin B1 showed increased activation of Akt, inhibition of GSK-3β and cell survival (Ramljak et al., 2000). Ceramide significantly inhibited insulin-stimulated phosphorylation of Akt and GSK-3β (Chavez et al., 2003). In general, GSK-3β is inhibited by serine phosphorylation in response to insulin or other growth factors. Several kinases, such as p90rsk, p70S6K, PKA, Akt, PKC and ERK, can inactivate GSK-3β by its phosphorylation at serine 9 (Cross et al., 1995; Pap and Cooper, 1998; Cohen and Frame, 2001; Frame and Cohen, 2001; Hetman et al., 2002; Jope and Johnson, 2004). Here, we have shown that in 10I T cells, GSK-3β phosphorylation was regulated primarily through a PI 3-kinase-Akt-mediated but not an ERK-mediated pathway. This is consistent with a previous report (Stoica et al., 2003), showing that the dephosphorylation of Akt and GSK-3β is associated with ceramide-induced neuronal apoptosis. Ceramide reduced Akt activity by using at least two mechanisms: by interrupting PI 3-kinase signaling and by inducing phosphatase activation. Ceramide-induced PP2A might directly cause Akt dephosphorylation. Ceramide-activated PP2A has also been implicated in GSK-3β activation and cell apoptosis (Ruvolo, 2001; Mora et al., 2002). The PP2A inhibitor OA rescued ceramide-induced activation of GSK-3β. Transfection of purified PP2A into cells caused GSK-3β dephosphorylation. However, an in vitro cell-free experiment indicated that ceramide-activated PP2A does not directly induce GSK-3β dephosphorylation at serine 9. Therefore, ceramide-activated PP2A indirectly induces GSK-3β dephosphorylation and activation. Because other protein phosphatases, such as PP1, might also be affected by OA, and because PP1 is involved in GSK-3β dephosphorylation (Zhang et al., 2003), the involvement of PP1 or other OA-sensitive protein phosphatases cannot be excluded.

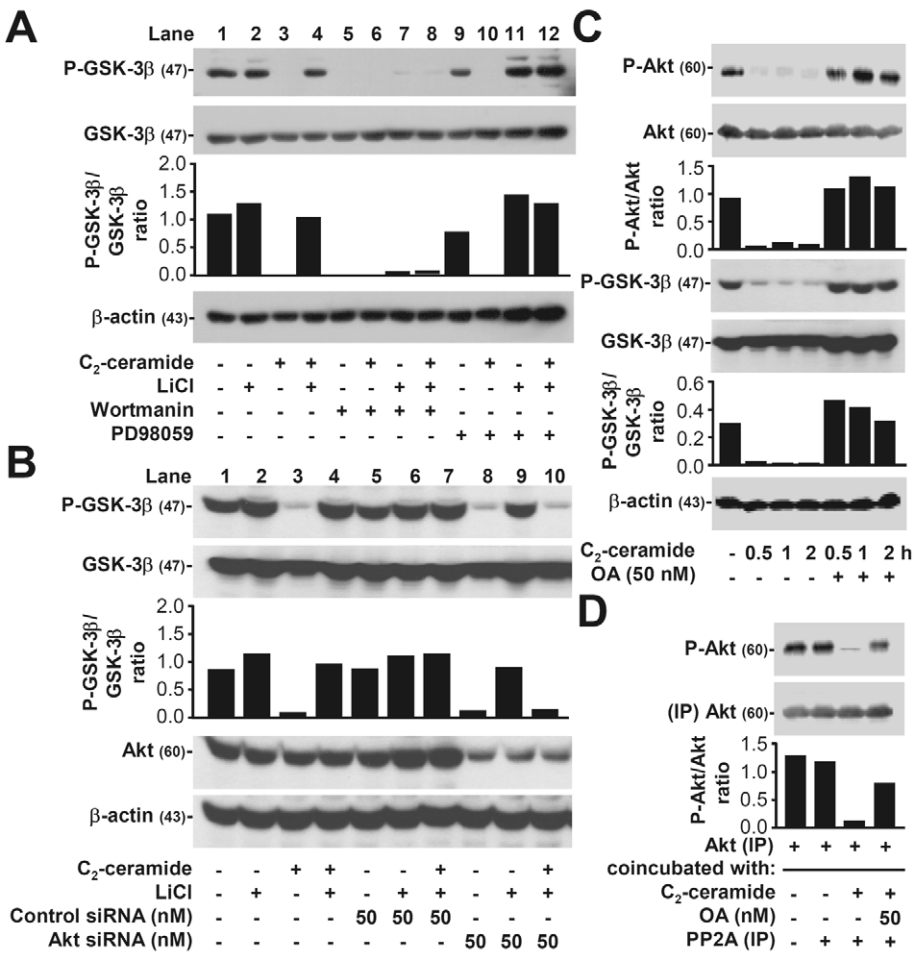

Fig. 6. GSK-3β activation is mediated through PP2A-regulated inactivation of the PI 3-kinase-Akt pathway. (A) 10I cells were treated with 25 μM of C_2-ceramide for 4 hours with or without LiCl, wortmannin (10 nM) or PD98059 (100 μM). We used western blotting to determine GSK-3β phosphorylation (P-GSK-3β) at serine 9; the relative ratio of P-GSK-3β: total GSK-3β protein level is shown. β-actin expression was an internal control. (B) 10I cells were transfected with 50 nM (88.7% transfection efficiency) of Akt siRNA as described in Materials and Methods. After ceramide treatment, levels of P-GSK-3β, GSK-3β and Akt were detected using western blotting. The relative ratio of P-GSK-3β:GSK-3β protein level is shown. β-actin levels were determined as an internal control. (C) 10I cells were treated with 25 μM of C_2-ceramide with or without the PP2A inhibitor OA for different time periods as indicated. We used western blotting to determine phosphorylated GSK-3β (P-GSK-3) and Akt phosphorylated at serine 473 (P-Akt); the relative ratios of P-Akt:total Akt and P-GSK-3β:GSK-3β are shown, respectively. β-actin expression was an internal control. (D) To determine whether PP2A has a direct effect on Akt dephosphorylation, Akt was immunoprecipitated (IP) from untreated cells and then incubated together with PP2A that had been immunoprecipitated from 25 μM of ceramide-treated cells for 30 minutes at 30°C with or without 50 nM of OA. After washing, we used western blotting to determine P-Akt; the relative ratio of P-Akt:IP Akt protein levels is shown.

LiCl inhibits various enzymes including GSK-3β, which may have multiple cellular outcomes (Jope, 2003; Quiroz et al., 2004). LiCl reduces GSK-3β activity in at least two ways: (1) directly, by acting as a competitive inhibitor of Mg^{2+} (Jope, 2003) and, (2) indirectly, by reducing protein phosphatase activity, which leaves GSK-3β phosphorylated and inactive (Jope, 2003; Zhang et al., 2003). We have shown here that LiCl increases GSK-3β phosphorylation through a PI 3-kinase-Akt-mediated pathway. In addition to LiCl, we used siRNA targeting GSK-3β and specific GSK-3β inhibitors to clarify the dependence of the apoptotic signaling of ceramide on GSK-3β. Experiments in this present study focused on the role of GSK-3β and the inolvement of GSK-3α is not clear. Nevertheless, we found that the effect of ceramide on GSK-3β is more dominant than on GSK-3α, a finding that was evidenced by the influence of the GSK-3β knockdown on GS hyperphosphorylation.

The pro-apoptotic role of GSK-3β is controversial. Despite the fact that cell apoptosis can be caused by overexpression of GSK-3β (Pap and Cooper, 1998; Pap and Cooper, 2002; Bijur et al., 2000), GSK-3β-deficient mouse embryos died from severe liver degeneration caused, most probably, primarily by apoptosis of hepatocytes (Hoeflich et al., 2000). Cell survival requires GSK-3β to regulate the activation of NF-κB (Hoeflich et al., 2000; Schwabe and Brenner, 2002). Indeed, GSK-3β also controls cell growth and differentiation (Cohen and Frame, 2001; Frame and Cohen, 2001; Jope, 2003; Jope and Johnson, 2004). GSK-3β is involved in diverse cellular responses, probably because of its enzymatic activities on a broad range of substrates. Cells in which GSK-3β is inhibitied showed resistance to various apoptotic stimuli (Pap and Cooper, 1998; Bijur et al., 2000; Hetman et al., 2000; King et al., 2001; Somervaille et al., 2001; Bhat et al., 2002; Loberg et al., 2002; Pap and Cooper, 2002; Song et al., 2002; Hongisto et al., 2003). In stress-induced apoptosis of the endoplasmic reticulum, GSK-3β is crucial for caspase-3 activation (Song et al., 2002). Our study shows that GSK-3β regulates activation of caspase-2 and caspase-8.

Apoptotic stimuli, such as the Fas ligand, TNF-α, chemotherapeutic agents, irradiation and serum deprivation, are associated with production of ceramide (Hannun and Obeid, 1995; Dbaibo and Hannun, 1998; Mathias et al., 1998; Hannun and Luberto, 2000; Ruvolo, 2001); and ceramide might induce mitochondrial apoptotic pathways (Hearps et al., 2002; von Haefen et al., 2002; Darios et al., 2003; Stoica et al., 2003). We have previously showed that ceramide-induced apoptosis before the damage of mitochondria is caspase-2-dependent (Lin et al., 2004). However, the control of caspase-2 activation remains unclear, although some reports have shown that caspase-2 is activated in a complex containing a variety of

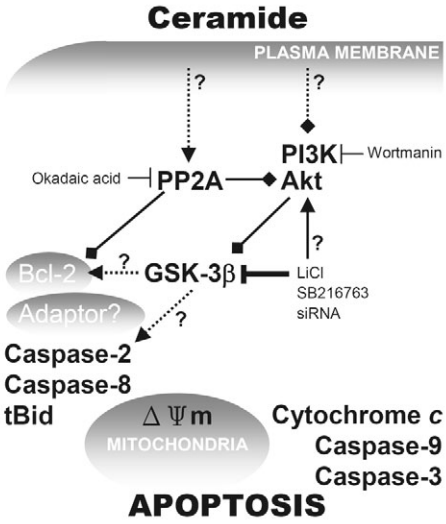

Fig. 7. Schematic diagram of the role of GSK-3β in the signaling pathways of ceramide-induced mitochondrial apoptosis. During ceramide-induced apoptosis, activated PP2A induces GSK-3β dephosphorylation and activation indirectly through a PI 3-kinase-Akt-regulated pathway. How ceramide activates PP2A remains unknown. Also, how GSK-3β activates caspase-2 and caspase-8, which causes mitochondrial damage and apoptosis, needs to be deciphered. It is of particular interest to know whether there is cross-talk between GSK-3β and Bcl-2.

proteins (Read et al., 2002; Tinel and Tschopp, 2004). It has also been shown that ceramide-induced cell apoptosis can be caused by PP2A-mediated Bcl-2 dephosphorylation and rescued by overexpression of Bcl-2 (Ruvolo et al., 1999; Zhang et al., 1996). Bcl-2 rescued ceramide-induced mitochondrial apoptosis by blocking activation of caspase-2 (Lin et al., 2005). In other words, Bcl-2 appeared to downregulate caspase-2. Here, we have shown that GSK-3β is required for ceramide-induced activation of caspase-2. Although PP2A regulates both GSK-3β and Bcl-2, the possible protein-protein interactions remain unclear. A previous report by von Haefen et al. has shown that ceramide can induce mitochondrial apoptosis through a Bax-dependent pathway (von Haefen et al., 2002). In addition, increased GSK-3 activity, caused by growth-factor withdrawal, might regulate apoptosis by triggering conformational change(s) in Bax (Somervaille et al., 2001). It is worth mentioning that GSK-3β is highly activated in mitochondria (Bijur and Jope, 2003). Interestingly, a reduction in GSK-3β activity mediates cell-protective signaling to inhibit the $\Delta\Psi_m$ (Juhaszova et al., 2004). Recent studies (Letai, 2006; Maurer et al., 2006) have shown that GSK-3β-regulated destabilization of Mcl-1, an anti-apoptotic Bcl-2 family member, was involved in the permeabilization of the mitochondrium outer membrane and in apoptosis. The possible relations between GSK-3β, Bcl-2 and Bax in mitochondria are of great interest for future studies.

Materials and Methods

Cell cultures

The mouse T hybridoma cell line 10I (Lai et al., 1987) was kindly provided by M. Z. Lai (Institute of Molecular Biology, Academia Sinica, Taiwan). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics as previously described (Lin et al., 2004). Before the experiments, the cells were washed with serum-free RPMI 1640 and resuspended in hybridoma serum-free medium (Gibco). The human neuroblastoma SK-N-SH and human epithelial carcinoma A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 50 U/ml of penicillin, and 0.05 mg/ml of streptomycin. Cells were maintained at 37°C in 5% CO_2.

Reagents

The ceramide analogue C_2-ceramide (BioMol) was dissolved in dimethyl sulfoxide (DMSO). Lithium chloride (LiCl), which inhibits GSK-3β and okadaic acid (OA) which inhibits PP2A (both from Sigma) were dissolved in cultured medium and DMSO, respectively. SB216763 (inhibiting GSK-3β), wortmannin (inhibiting PI 3-kinase) and PD98059 (inhibiting MEK) (all Tocris Bioscience) were dissolved in DMSO. Purified PP2A ($PP2A_1$ composed of the A, B, and C subunits) was purchased from Sigma.

Analysis of apoptosis

Cell apoptosis, characterized by DNA fragmentation, was detected using propidium iodide (PI; Sigma) staining or terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) reaction by using the ApoAlert DNA fragmentation assay kit (Clontech) according to the manufacturer's instructions. Cells were then analyzed using flow cytometry (FACSCalibur; BD Biosciences). For PI staining, after fixation with 70% ethanol in phosphate-buffered saline (PBS), cells were stained with PI/RNase working solution in PBS containing 40 μg/ml PI and 100 μg/ml RNase A (Sigma) for 30 minutes at room temperature, and then analyzed using flow cytometry. 4′,6-diamidino-2-phenylindole (DAPI; Sigma) was also used (5 μg/ml) for staining of apoptotic cells (room temperature, 30 minutes) that were detected using fluorescent microscopy. Membrane disruption of apoptotic cells, characterized by the presence of phosphatidylserine, was detected using the annexin V-fluorescein isothiocyanate (FITC) detection kit (BioVision).

Detection of caspase activation

Cellular caspase activation was determined using the ApoAlert caspase colorimetric assay kits for caspase-3 and caspase-8, and an ApoAlert caspase fluorescent assay kit for caspase-9 (Clontech) according to the manufacturer's instructions. Caspase-2 activity was detected using a caspase-2 assay kit (Calbiochem). Optical density (OD) measurements were done using a microplate reader. Substrate activities are shown as *p*-nitroanilide (nmol), and were calculated for caspase-3 and caspase-9. For caspase-2 and caspase-8, the relative substrate activity was shown by their OD values.

Western blot analysis

For cytosolic cytochrome *c* detection, cell extract without the mitochondrial fraction was separated using an ApoAlert cell fractionation kit (Clontech) according to the manufacturer's instructions. To detect other proteins, total cell lysates and immunoprecipitated proteins were used followed by western blotting. Briefly, cell extracts or precipitated proteins were separated using SDS-PAGE and then transferred to a PVDF membrane (Millipore). After blocking, blots were developed with a series of antibodies as indicated. Rabbit antibodies against mouse GSK-3α, GSK-3β, phospho-GSK-3α, phospho-GSK-3β, Bcl-xL, and cytochrome *c* (Santa Cruz Biotechnology), PI 3-kinase and PP2A (Upstate Biotechnology), GS and phospho-GS, Akt and phospho-Akt (Cell Signaling Technology), and tBid (Oncogene) were used. Monoclonal antibody against β-actin (Sigma) was used. Finally, blots were hybridized with horseradish peroxidase (HPR)-conjugated goat anti-rabbit IgG or anti-mouse IgG (Calbiochem) and developed using an AEC substrate kit (Zymed Laboratories Inc.) and enhanced chemiluminescence reagent (Pierce).

Mitochondrial functional assay

The mitochondrial transmembrane potential ($\Delta\Psi_m$) was determined using Rhodamine 123 (Sigma). Cells were incubated with 5 μM of Rhodamine 123 for 30 minutes, washed with PBS and analyzed using flow cytometry (FACSCalibur).

GSK-3 and Akt silencing

Expression of GSK-3α, GSK-3β and Akt was silenced using GSK-3 siRNA and Akt siRNA kits, respectively, according to the manufacturer's instructions (Cell Signaling Technology and Upstate Biotechnology). Briefly, before transfection of siRNA, 10^6 cells were washed with serum-free RPMI and then cultured with 2 μl of lipofectamine 2000 (Invitrogen) and various amounts of siRNA in 6-well plates. An FITC-labeled non-targeted negative control siRNA was used to monitor the efficiency of siRNA transfection. After 6 hours of incubation, cells were washed with RPMI containing 10% FCS and maintained for an additional 24 hours before the experiments.

PP2A transfection

PP2A transfection was done using an in vivo protein transfer system (Invitrogen) according to the manufacturer's instructions. Briefly, 10^6 cells were washed with serum-free RPMI and cultured with 2 μl of lipofectamine 2000 and various amounts of purified $PP2A_1$ composed of the A, B, and C subunits (Sigma) in 6-well plates.

After 2 hours of incubation, cells were washed with serum-free RPMI for experiments. An FITC-labeled immunoglobulin was used to monitor the efficiency of protein transfection.

Immunostaining

Cells were fixed with 1% formaldehyde in PBS. For confocal microscopy, rabbit anti-phospho-GSK-3β (Santa Cruz Biotechnology) was used. The cells were then stained with FITC-conjugated goat anti-rabbit IgG (Calbiochem). PI was used for nuclear staining.

Immunoprecipitation

For immunoprecipitation, 100 μg of cell lysate was incubated together with 5 μg of protein G (Amersham Biosciences) and 2 μg of antibodies, including anti-GSK-3β (Santa Cruz Biotechnology), anti-Akt (Cell Signaling Technology), and anti-PP2A/C (Upstate Biotechnology) overnight at 4°C.

PP2A activity assay

Proteins were immunoprecipitated overnight with anti-PP2A antibodies and protein G-agarose beads. The activity of PP2A was analyzed using a nonradioactive PP2A immunoprecipitation phosphatase assay kit (Upstate Biotechnology) according to the manufacturer's instructions. The substrate phosphopeptide KR(*P*)TIRR was detected for its dephosphorylation using PP2A.

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