Inactivation of glycogen synthase kinase-3β, a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells

Muthusamy Kunnimalaiyaan, Abram M. Vaccaro, Mary A. Ndiaye, and Herbert Chen

Endocrine Surgery Research Laboratories, Department of Surgery, University of Wisconsin and the University of Wisconsin Paul P. Carbone Comprehensive Cancer Center, Madison, Wisconsin

Abstract

Glycogen synthase kinase-3β (GSK-3β) is an important regulator of cell proliferation and survival. Conflicting observations have been reported regarding the regulation of GSK-3β and extracellular signal–regulated kinase (ERK1/2) in cancer cells. In this study, we found that raf-1 activation in human medullary thyroid cancer cells, TT cells, resulted in phosphorylation of GSK-3β. Inactivation of GSK-3β in TT cells with well-known GSK-3β inhibitors such as lithium chloride (LiCl) and SB216763 is associated with both growth suppression and a significant decrease in neuroendocrine markers such as human achaete-scute complex-like 1 and chromogranin A. Growth inhibition by GSK-3β inactivation was found to be associated with cell cycle arrest due to an increase in the levels of cyclin-dependent kinase inhibitors such as p21, p27, and p15. Additionally, LiCl-treated TT xenograft mice had a significant reduction in tumor volume compared with those treated with control. For the first time, we show that GSK-3β is a key downstream target of the raf-1 pathway in TT cells. Also, our results show that inactivation of GSK-3β alone is sufficient to inhibit the growth of TT cells both *in vitro* and *in vivo*. [Mol Cancer Ther 2007;6(3):1151–8]

Introduction

Many fundamental processes that regulate cell growth pathways and/or apoptosis are controlled by protein phosphorylation. Glycogen synthase kinase-3β (GSK-3β) is a multifunctional, serine/threonine protein kinase that regulates numerous cellular processes, such as metabolism, cell fate determination, proliferation, and survival (1–4). The activity of GSK-3β is inhibited by phosphorylation of a single serine residue (Ser^9). However, phosphorylated GSK-3β is able to regulate various proteins such as β-catenin, c-myc, c-Jun, Mdm2, and heat shock factor by phosphorylation (5–12). Therefore, GSK-3β may be considered as an important protein kinase, which modulates diverse intracellular signaling pathways. Neuroendocrine tumors such as medullary thyroid cancer (MTC), gastrointestinal and pulmonary carcinoids, and pancreatic islet cell tumors secrete various bioactive hormones that cause many debilitating symptoms in patients (13, 14). Surgery is the only potentially curative therapy for patients with neuroendocrine tumors, but unfortunately, complete surgical resection is often impossible due to widespread metastases (15). This emphasizes the need for the development of new treatment strategies. Several signaling pathways such as the phosphoinositide-3-kinase (PI3K)/Akt pathway and the raf-1/mitogen-regulated extracellular kinase (MEK)/extracellular regulated kinase (ERK) pathway have been shown to play an important role in regulating tumor cell growth (16). The raf-1/MEK/ERK pathway has long been recognized for its importance in cancer biology. Although activation of this pathway is mainly considered to be growth promoting, in certain cell-specific contexts, this pathway acts as a growth suppressor. For example, activation of raf-1 in small cell lung cancer (SCLC; refs. 17, 18) and MTC-TT cells led to both significant phenotypic differentiation and growth reduction (19). We and others have shown that raf-1 activation in TT cells led to growth suppression and a reduction in neuroendocrine tumor markers such as achaete-scute complex-like 1 (ASCL1), chromogranin A (CgA), and calcitonin (16, 19–21). Furthermore, we showed that the reduction in neuroendocrine tumor markers was dependent on MEK1/2 activity in TT cells (21). We have recently shown that *in vivo* raf-1 activation in a mouse xenograft TT model resulted in the inhibition of both tumor development and progression (22). However, the mechanism by which raf-1 pathway activation inhibits MTC cells growth is unclear. Therefore, understanding the mechanism of this pathway could not only provide insight into the process of neuroendocrine tumorigenesis but also lead to the development of new treatments for patients with neuroendocrine tumors. Thus, to understand the regulation of the downstream targets of raf-1 pathway activation, we examined other signaling pathways in TT cells after raf-1 activation.

In the present study, we show that raf-1 activation in TT cells induces GSK-3β phosphorylation, suggesting that GSK-3β might be a downstream mediator. Given the

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Requests for reprints: Herbert Chen, University of Wisconsin, H4/750 Clinical Science Center, 600 Highland Avenue, Madison, WI 53792. Phone: 608-263-1387; Fax: 608-263-7652. E-mail: chen@surgery.wisc.edu

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important role of GSK-3β in other cancer types, we hypothesized that inactivation of GSK-3β might be a mechanism of raf-1–induced growth inhibition. Treatment of TT cells with lithium chloride (LiCl) and SB216763, inhibitors of GSK-3β, suppressed growth and reduced neuroendocrine tumor marker production. Mechanistically, we show that the growth inhibition is mediated by cell cycle arrest at the G_2-M stage. Importantly, treatment with LiCl inhibited growth of TT cells both *in vitro* and *in vivo*.

Materials and Methods

Cell Culture

MTC (TT and TT-raf) cells were kindly provided by Dr. B. Nelkin (Johns Hopkins University, Baltimore, MD) and maintained in RPMI 1640 as described (23). The construction of TT-raf cell line containing the activatable Δraf-1:ER construct, the catalytic domain of raf-1 fused to the hormone binding domain of the human estrogen receptor, has been described previously (24). To induce raf-1 activity in TT-raf cells, 1 μmol/L β-estradiol was added to the media where as an equivalent dilution of ethanol, the carrier for the β-estradiol was used to treat control cells. Mouse embryonic fibroblast cells (NIH-3T3; a kind gift of Dr. Steven Clark, University of Wisconsin, Madison, WI) were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum. All cells were maintained in 5% CO_2 in air at 37°C in a humidified incubator.

Immunoblot Analysis

Cells were harvested after 2 days of treatment and lysed, and the cell lysates were prepared as described previously (25, 26). Total protein concentration was quantified with a bicinchoninic acid assay kit (Pierce, Rockford, IL). Denatured cellular extracts (20–50 μg) were resolved by 10% SDS-PAGE, transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH), blocked in milk and incubated with appropriate antibodies. Antibodies were diluted as follows: GSK-3β, pGSK-3β, pERK1/2, p21, p27, p15, cyclin D1, and phospho-cdc2^{Tyr15} (1:1,000, Cell Signaling Technology, Beverly, MA); mammalian achaete scute homologue-1 (MASH1) for ASCL1, 1:1,000 (BD PharMingen, San Diego, CA); CgA, 1:1,000 (Zymed Laboratories, San Francisco, CA); β-catenin (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA); and glyceraldehyde-3-phospho-dehydrogenase (G3PDH; 1:10,000, Trevigen, Gaithersburg, MD). Horseradish peroxidase–conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Pierce) were used depending on the source of the primary antibody. For protein visualization of the signal, Immunstar (Bio-Rad Laboratories, Hercules, CA) was used except for MASH1, p21, p27, and p15 blots, the membranes were visualized with SuperSignal West Femto kit (Pierce).

Cell Proliferation Assay

Cell proliferation was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (27, 28). Briefly, cells were seeded in quadruplicate on 24-well plates and incubated 24 h under standard conditions to allow cell attachment. The cells were then treated with LiCl or GSK-3β–specific inhibitors at various concentrations and incubated at different time points as indicated in the figure. At each time point, the medium was removed and replaced with 250-μL medium containing MTT (0.5 mg/mL) and incubated at 37°C for 3 h. Then, 750 μL of DMSO was added and mixed thoroughly. Then the plates were measured at 540 nm using a μQuant spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT).

Flow Cytometry Analysis

To determine the stage at which cells were arrested, DNA synthesis was monitored by 5-bromo-2′-deoxyuridine (Sigma, St. Louis, MO) incorporation using FITC-labeled anti–5-bromo-2′-deoxyuridine antibody and processed for cell cycle analysis. Briefly, cells were treated with varying concentrations of LiCl. 5-Bromo-2′-deoxyuridine was added to the cells and continued to incubate for 1 h. Cells were then scraped and collected, and the nuclei were incubated with anti–5-bromo-2′-deoxyuridine antibody overnight at 4°C. Next day, 3 mL of PBS buffer was added, and then the pellet was collected after centrifugation. Then, the pellet was dissolved in PBS buffer containing anti-mouse FITC antibody and incubated for 1 h at room temperature. Finally, the pellets were suspended in propidium iodide staining solution [1 mg/mL RNase A, 33 μg/mL propidium iodide, 0.2% (v/v) Triton ×100 in PBS] and incubated overnight at 4°C in the dark. The following day, the mixture was filtered, and the filtrate was analyzed by flow cytometry for cell cycle distribution using the FACS analysis core facility of the University of Wisconsin Comprehensive Cancer Center. The data were analyzed using FlowJo software version 6.4.1.

Animal Studies

All *in vivo* experiments were done in accordance with our animal care protocol approved by the University of Wisconsin–Madison Animal Care and Use Committee. Athymic immunocompromised *nu/nu* mice, 4 to 5 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA) and maintained under aseptic conditions. Animals were anesthetized via inhalation of isoflourane (Baxter, Deerfield, IL) before all surgical procedures and observed until fully recovered. MTC (TT; 1×10^6) cells were suspended in Hanks' balanced saline solution (HBSS) and injected s.c. into the right flank of each mouse as described (22). Once mice developed palpable tumors, the mice were then divided into two groups of 14 mice in each group. One group (control) received an equal volume of saline every 2 days. The experimental group received 340 mg/kg body weight of LiCl via i.p. injection every 2 days. Length (l) and width (w) of tumors were measured with a vernier caliper every 4 days, and the tumor volumes were calculated using the formula $w^2 \times l \times 0.52$.

Statistical Analysis

ANOVA with Bonferroni post hoc testing (SPSS software version 10.0, SPSS; Chicago, IL) was used for statistical

comparisons. A P value of <0.05 was considered significant. Unless noted, dated are represented as means ± SE.

Results

Raf-1 Activation in TT Cells Decreases Growth, Reduces Neuroendocrine Tumor Markers, and Increases the Level of Phospho-GSK-3β Protein

Previously, we and others have shown that TT and TT-raf cells have high levels of regular extracellular signal–regulated kinase (ERK1/2) and no detectable amount of phospho-ERK1/2 (19, 21, 29). TT-raf cells were created with stable expression of the raf-1 gene fused with the estradiol binding region of the estrogen receptor (19, 21, 29). However, activation of raf-1 in TT-raf cells by estradiol treatment increased the levels of phospho-ERK1/2 in these cells, whereas the TT cells with estradiol treatment did not show any phosphorylated ERK1/2 protein by Western blot analysis, indicating that treatment with estradiol activates raf-1 only in TT-raf cells (19, 21, 29). In the present study, to show raf-1 mediated activation of other pathways, we carried out Western blot analysis for TT and TT-raf cells treated with control and estradiol. As expected, the absence of phosphorylated ERK1/2 and the presence of high levels of ASCL1 are seen in TT cells treated with control and estradiol and TT-raf cells treated with control (Fig. 1A). However, raf-1 activation in TT-raf cells by estradiol treatment resulted in the phosphorylation of ERK1/2, which associated with a reduction in CgA and ASCL1 (21).

As seen in Fig. 1A, raf-1 activation resulted in an increase in the level of phosphorylated GSK-3β protein. Phosphorylation at the Ser^9 residue of GSK-3β has been shown to inactivate GSK-3β. We hypothesized that the mechanism of raf-1–mediated TT-raf growth inhibition reported earlier could be due to GSK-3β inhibition. As expected, the level of regular, total GSK-3β protein remained unchanged. This result suggested that raf-1 pathway activation regulates the phosphorylation of GSK-3β and further indicated that GSK-3β could be a downstream target of the raf-1 pathway. Therefore, we were interested in determining if inactivation of GSK-3β alone was sufficient to inhibit TT growth and hormone production.

Inactivation of GSK-3β by LiCl Leads to a Reduction in Neuroendocrine Tumor Markers

We have previously reported that activation of the raf-1 pathway by either ectopic expression of an activated raf-1 gene or activation of the endogenous raf-1 pathway with a pharmacologic agent led to a reduction in neuroendocrine tumor markers, such as ASCL1, CgA, serotonin, and calcitonin (21, 28, 29). We show in the present study that raf-1 activation induces phosphorylation of GSK-3β, suggesting that raf-1–mediated effects might be due to the

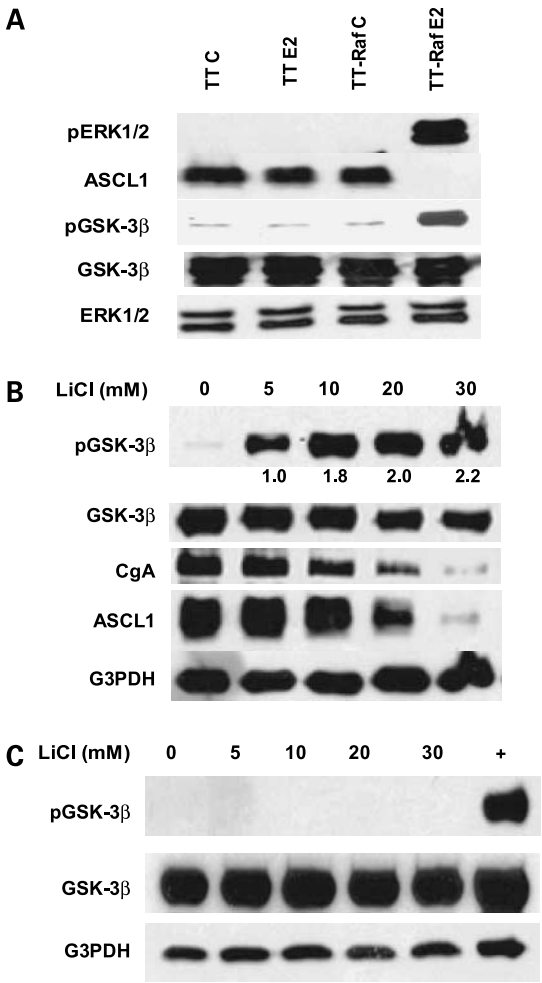

Figure 1. **A,** raf-1 activation induces phosphorylation of GSK-3β in MTC cells. TT-raf cells were previously created by transfecting TT cells with a retroviral construct containing the raf-1 catalytic domain fused to the hormone-binding domain of the human estrogen receptor (Δraf-1: ER; ref. 41). TT and TT-raf cells were treated with ethanol (*C*) and estradiol (*E2*), and the total cellular extracts were isolated and analyzed by Western blot for downstream targets of the raf-1 pathway. Phosphorylation of ERK1/2 was present only in the raf-1 – activated cells, whereas TT cells treated with ethanol and estradiol and TT-raf cells treated with ethanol did not show any detectable amount of phospho-ERK1/2, indicating that the raf-1 pathway is active only in TT-raf treated with estradiol. Protein ASCL1, a neuroendocrine tumor marker, was reduced only in raf-1 – activated cells consistent with our earlier reports. Importantly, high levels of phosphorylated GSK-3β proteins were observed in raf-1 – activated cells. Note that the level of regular GSK-3β was unchanged. Immunoblotting with anti-ERK1/2 antibody confirmed equal protein loading. **B,** inactivation of GSK-3β by LiCl reduces neuroendocrine tumor markers in TT cells. TT cells were treated with various concentrations of LiCl for 2 d. Total cellular extracts were isolated and analyzed by Western blot for the levels of phosphorylated GSK-3β and neuroendocrine tumor markers. LiCl treatment led to an increase in phosphorylated GSK-3β protein. Note that in control cells, there was no detectable level of phosphorylated GSK-3β. In contrast to this, regular active GSK-3β was present at high levels in both control and treated cells. However, there is a slight decrease in the level of total GSK-3β protein. Importantly, the increase in the levels of phospho-GSK-3β led to a significant reduction in neuroendocrine tumor markers, such as CgA and ASCL1. G3PDH was used to confirm equal protein loading. **C,** treatment of NIH3T3 cells with LiCl did not induce phosphorylation of GSK-3β. Various concentrations of LiCl were used to treat NIH3T3, mouse embryonic fibroblast cells as indicated for 2 d. Western blot analysis showed that there was no induction of phosphorylated GSK-3β protein even at higher concentrations of LiCl. Interestingly, high levels of regular total GSK-3β protein present in these cells. To confirm the Western blot worked, we used lysate from TT cells treated with 20 mmol/L LiCl as a positive control. G3PDH was used to confirm equal protein loading.

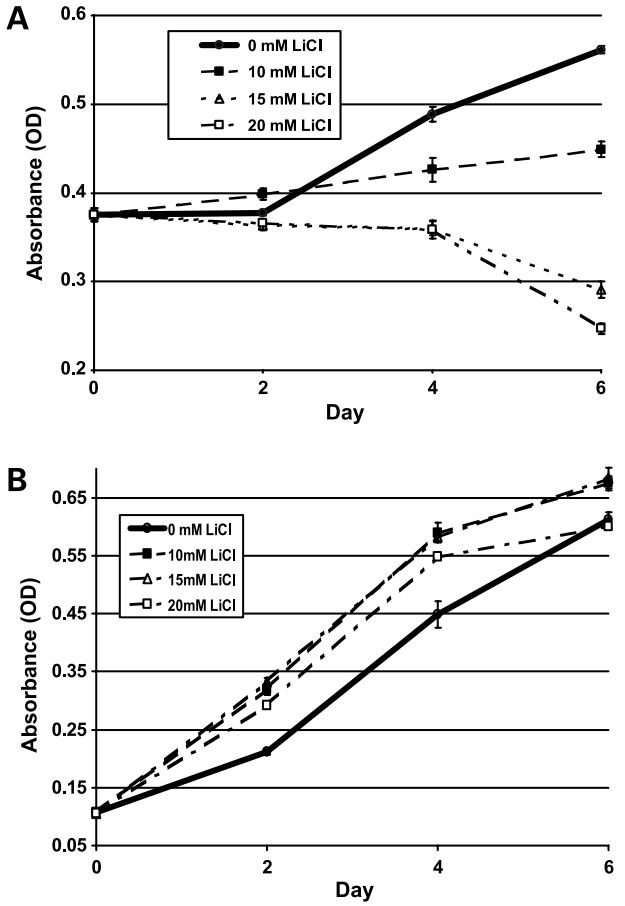

Figure 2. Inactivation of GSK-3β is associated with suppression of TT cells proliferation. Various concentrations of LiCl were used to treat TT (**A**) and mouse embryonic fibroblast (**B**) cells as indicated. Growth assay by MTT was carried out as described in Materials and Methods. Increased concentrations of LiCl treatment significantly and dose-dependently reduced growth in TT cells, whereas in fibroblast cells, there was no reduction even at a higher concentration of LiCl (20 mmol/L). Conversely, there was a growth increase in mouse fibroblast cells treated with LiCl (**B**). At all time points, the growth reductions in TT cells were significant as compared with control treatment ($P = 0.0001$). These experiments were repeated in quadruplicates at least twice.

inhibition of GSK-3β. To determine if the reduction of neuroendocrine tumor markers is also mediated by GSK-3β inactivation, we treated TT cells with various concentrations of LiCl, a well-known inhibitor of GSK-3β. Treatment of TT cells with LiCl led to a progressive increase in the levels of phosphorylated GSK-3β protein (Fig. 1B). However, there was a slight reduction in the level of regular GSK-3β protein with increasing concentrations of LiCl. The intensity of the bands of phosphorylated GSK-3β and regular GSK-3β was measured using ImageQuant software. The ratio showed progressive increase in phosphorylated GSK-3β after being normalized with regular GSK-3β. Interestingly, the levels of neuroendocrine tumor markers such as CgA and ASCL1 were significantly reduced with increasing concentrations of LiCl (Fig. 1B). At the concentration of 20 mmol/L, LiCl, CgA, and ASCL1 were significantly reduced, whereas at 30 mmol/L LiCl treatment, these markers were completely reduced. As a control, NIH3T3 fibroblasts were treated with similar concentrations of LiCl, and the cell lysates were analyzed for the presence of phosphorylated GSK-3β. As shown in Fig. 1C, treatment of NIH3T3 cells with LiCl did not phosphorylate GSK-3β even at the higher concentrations. Furthermore, these cells showed higher levels of the presence of total GSK-3β protein, suggesting that some unknown factor(s) or perhaps other signaling pathway prevented the phosphorylation of GSK-3β after LiCl treatment. At this point in the study, it is difficult to speculate the mechanism preventing the phosphorylation of GSK-3β. Further studies are needed to explore the reasons for the absence of phosphorylated GSK-3β after LiCl treatment in these cells.

LiCl Treatment Inhibits the Growth of TT Cells

Next, we evaluated the effects of LiCl on TT cell growth by MTT assay. As shown in Fig. 2A, treatment of TT cells with LiCl (0–20 mmol/L) resulted in a dose-dependent reduction of cell growth up to 6 days. Interestingly, at 10 mmol/L concentration, there was a modest growth reduction, whereas higher concentrations (15 and 20 mmol/L) of LiCl treatment resulted in significant cell growth reduction (Fig. 2A). The reduction in cellular proliferation in LiCl-treated cells was statistically significant at all treatment points ($P = 0.0001$) compared with control treatment. However, NIH3T3 fibroblast cells, which did not show phosphorylation of GSK-3β after LiCl treatment (Fig. 1C), were not affected by LiCl (Fig. 2B). Interestingly, LiCl treatment seemed to increase the growth of NIH3T3 cells. Taken together, these results suggested that the observed growth reduction in TT cells might be due to the inactivation of GSK-3β.

Inhibition of GSK-3β Is Associated with a Decrease in the Levels of Neuroendocrine Tumor Markers and Growth

Lithium is often used as noncompetitive inhibitor of GSK-3β and displays a number of other activities. To determine if the effect of NE markers reduction and growth is due to GSK-3β inactivation, we treated the cells with SB216763, another pharmacologic GSK-3β inhibitor. SB216763 selectively inhibits GSK-3β in a competitive manner with ATP. Therefore, unlike LiCl, SB216763 does not phosphorylate GSK-3β but inactivates GSK-3β by binding to the active site (ATP binding site) of the protein. Treatment of TT cells with SB216763 resulted in a modest increase and stability of β-catenin protein levels, suggesting that inhibition of GSK-3β kinase activity occurred. Similar results were shown in cultured rat cerebral cortical neurons after treatment with SB216763 (30). As expected, treatment of TT cells with the SB216763 did not increase the phosphorylation of GSK-3β protein, and there was no change in the level of total GSK-3β protein. However, there was a reduction in the expression of neuroendocrine tumor markers such as CgA and ASCL1 (Fig. 3A) with increasing concentrations of SB216763. These results were consistent with the effects of LiCl on these cells in which

phosphorylated, inactivated GSK-3β led to a similar reduction in the expression of these markers.

LiCl treatment of TT cells inhibited growth and reduced neuroendocrine tumor markers. Similarly, treatment by SB216763 also reduced neuroendocrine tumor marker production in TT cells. Therefore, we were interested to determine the effect of SB216763 on cell growth. We carried out the cellular proliferation experiment by MTT assay on TT cells and NIH3T3 fibroblast cells. As shown in Fig. 3B, TT cells treated with indicated concentrations of SB216763 showed significant growth inhibition at or above 10 μmol/L concentrations compared with untreated cells at day 2 and maintained the growth reduction at day 6. However, NIH3T3 cells treated with similar concentrations showed very little or no growth reduction even at higher concentrations (30 μmol/L; Fig. 3C). These results suggested not only that TT cells responded very well to GSK-3β inhibitors compared with NIH3T3 cells, but also showed that inactivation of GSK-3β is associated with growth inhibition and neuroendocrine tumor marker reductions.

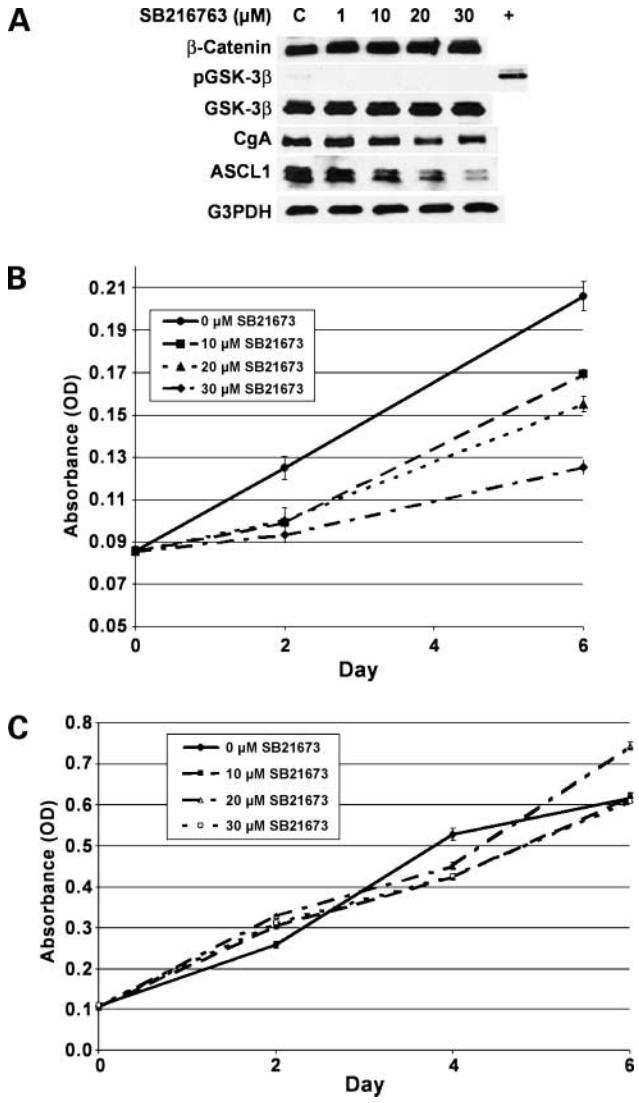

Figure 3. SB216763, a GSK-3β–specific inhibitor, reduces neuroendocrine tumor markers and suppresses growth. **A,** TT cells were treated with varying concentrations of SB216763 for 2 d, and cellular extracts were isolated and analyzed by Western blot for the levels of neuroendocrine tumor markers. Treatment with the GSK-3β inhibitor stabilizes the levels of β-catenin protein and, importantly, does not induce the phosphorylation of GSK-3β protein. The presence of phosphorylated protein in TT cells treated with LiCl was used as a positive control. Importantly, this GSK-3β inhibitor does not alter the levels of GSK-3β protein. Increasing treatment with SB216763 led to a significant reduction in neuroendocrine tumor markers, such as CgA and ASCL1. G3PDH was used to confirm equal protein loading. A reduction in cellular proliferation of TT cells (**B**) with increasing concentrations of SB216763 was seen by MTT assay. The control mouse embryonic fibroblast cells showed no reduction even at higher concentrations (30 μmol/L) of treatment (**C**). The MTT assays were done in quadruplicates at least thrice.

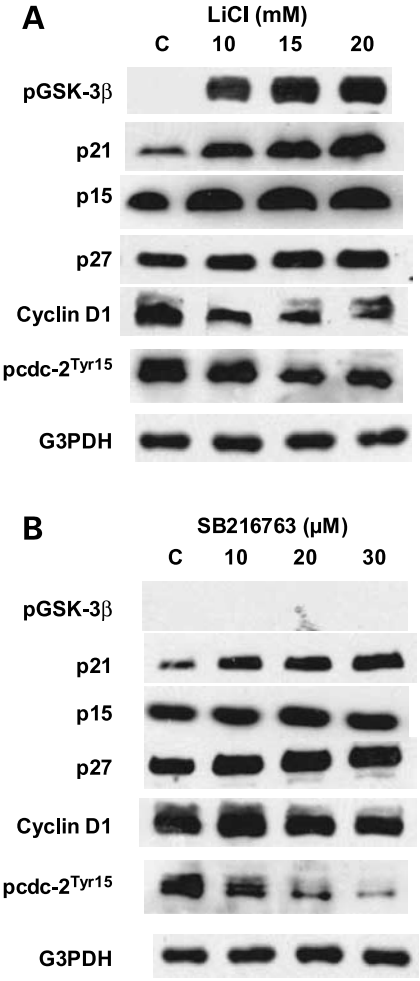

Figure 4. Growth inhibition by inactivation of GSK-3β is mediated by cell cycle arrest at G_1 and G_2-M phase. TT cells were treated with LiCl at 0 (*C*), 10, 15, and 20 mmol/L (**A**) or SB216763 (0, 10,20 and 30 μmol/L) for 2 d (**B**). Total cellular extracts were isolated and analyzed by Western blot using antibodies against phospho-GSK-3β, p21, p27, p15, cyclin D1, and phospho-cdc2^{Tyr15} proteins. The tumor suppressor protein p21 and other cell cycle regulator proteins p15 and p27 were increased with LiCl treatment. The levels of cyclin D1 and phospho-cdc2^{Tyr15} were reduced compared with control.

Growth Inhibition by GSK-3β Inhibitors Is Mediated through Cell Cycle Arrest

To determine the mechanism by which growth inhibition occurs, we treated TT cells with either LiCl or SB216763 at the indicated concentrations for 2 days. Phosphorylation of GSK-3β was maintained only in LiCl-treated cells compared with the nontreated cells (Fig. 4A and B). As shown in Fig. 4A and B, the level of tumor suppressor protein p21, a well-known cyclin-dependent kinase (CDK) inhibitor, was increased with increasing concentrations of LiCl and SB216763. Similar results were previously observed with the inhibition of GSK-3β by LiCl in umbilical vein endothelial cells (31, 32). This suggested that growth inhibition might be due to cell cycle arrest. In addition to an increase in the level of p21, LiCl and SB216763 treatment also increased the levels of p15 and p27, two other well-known CDK inhibitors. Increases in the levels of INK4 (p15) and Cip/Kip (p21 and p27) family members are involved in the destabilization of cyclin D complexes that might lead to a reduction in cyclin D1. As expected, the cyclin D1 level was reduced significantly with GSK-3β inhibitors. Therefore, increased levels of p21 and decreased levels of cyclin D1 suggested that the growth inhibition by GSK-3β inhibitors was associated with cell cycle arrest. To determine the stage at which cell cycle arrest was occurring, we measured the level of phospho-cdc-2^{Tyr15} in treated cells because cdc2 kinase regulates the entry of cells into the M phase. As shown in Fig. 4A and B, the level of phosphorylated cdc2^{Tyr15} was decreased with increasing concentrations of GSK-3β inhibitors. This indicated that cell cycle arrest was taking place at the G_2-M stage. To further confirm this, we carried out flow cytometry analysis of the LiCl-treated cells, and the level of cells in G_2-M phase was increased with LiCl treatment (data not shown). Taken together, the results of the Western blot analysis for cell cycle regulatory proteins clearly indicated that the growth inhibition was due to cell cycle arrest, and that the arrest occurred possibly at the G_2-M phase.

Effects of LiCl on Tumor Growth *In vivo*

Recently, we have reported that activation of raf-1 in a TT xenograft model reduced tumor development and progression (22). Therefore, we were interested to determine similar *in vivo* effects of growth inhibition by inactivation of GSK-3β in a TT xenograft model. Tumors generated by s.c. injection of TT cells were used to assess the effect of LiCl on growth. Eight weeks after TT cells were injected s.c. into nude mice, mice were divided into two groups of 14 mice each. One group received i.p. LiCl injections (340 mg/kg body weight) every 2 days for a total of 10 treatments. The other group as a control received an equal volume of saline. The serum levels of lithium in the mice were between 0.2 and 1.0 mmol/L. These levels have been shown to be safe in human. Furthermore, these levels of lithium can be easily achieved in patients by p.o. dosing with lithium carbonate. We also observed for the weight loss and any behavior changes in mice receiving LiCl. There were no apparent side effects of lithium *in vivo*.

As shown in Fig. 5A, tumor growth was significantly reduced in the treatment group as measured by calculated tumor volume. Mice in the control group reached an average tumor volume of 2,000 mm^3 at 11 weeks after tumor cell injection. However, at that time point, mice in the LiCl-treated group had an average tumor volume of only 1250 mm^3. The differences between the control and LiCl-treated tumor volumes at all time points were statistically significant. To determine the effect of LiCl treatment on the protein levels in the tumors, we carried out Western blot analysis of the lysates extracted from mouse tumors. A representative Western blot analysis of two mice from each groups were shown in Fig. 5B. There is an increase in phosphorylated GSK-3β proteins in the tumor lysates from the mice received LiCl compared with the control mice tumors. This suggested that the i.p. LiCl injections induced the phosphorylation of GSK-3β in TT tumors. Importantly, the increase in phosphorylated GSK-3β is associated with a reduction of CgA consistent with our *in vitro* studies.

Discussion

MTC accounts for 3% to 5% of all thyroid cancers and originates from the C cells of the thyroid (16, 20, 33). MTC

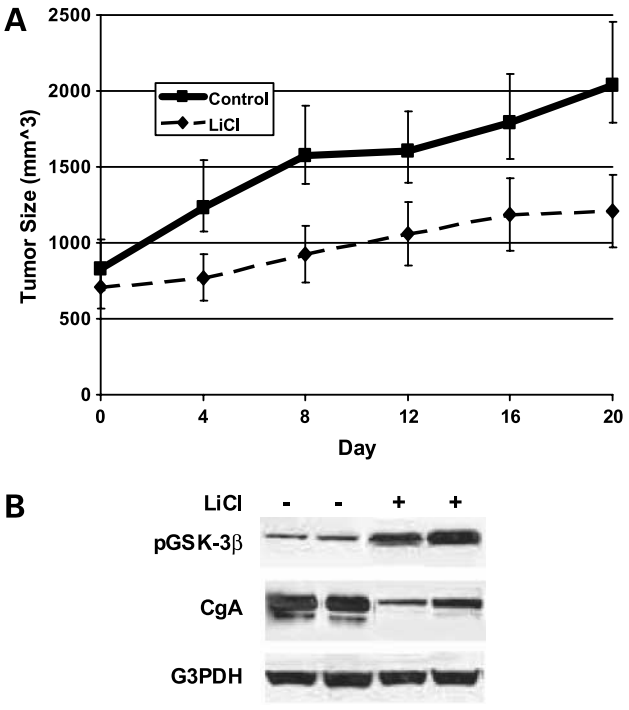

Figure 5. *In vivo* validation of tumor growth inhibition by LiCl treatment. **A,** a s.c. xenograft mouse model was developed for TT cells. After palpable tumors developed, i.p. injections of LiCl (340 mg/kg body weight) were done for up to 20 d, the tumors were measured, and the tumor volume was calculated as described in Materials and Methods every 4 d. As a control, an equal volume of saline was injected into control mice. The growth of the LiCl-treated tumors was reduced compared with the saline-injected mice bearing TT tumors. **B,** representative tumors from control and LiCl treated mice were analyzed by Western blot for the inactivation of GSK-3β by phosphorylation at Ser^9 and its associated effect.

tends to be a slow-growing tumor, but the primary therapy for patients with MTC is surgical resection. However, MTC also frequently metastasizes, especially to the liver and regional lymph nodes, precluding patients from a curative resection. Therefore, there is a great need for the development of new treatment strategies. Various studies by our group and others have shown that ectopic expression of either *Notch1* or *raf-1* leads to growth suppression and significant reduction in neuroendocrine tumor marker expression in neuroendocrine tumor cell lines, such as MTC, SCLC, and pancreatic carcinoid cells (17, 18, 21, 25, 27, 29, 34). A growing number of studies have identified GSK-3β as an important regulator of cell proliferation and survival mediated through modulation of cytoskeletal proteins and transcription factors (1, 2, 6, 8, 9, 12, 35, 36). Inhibition of GSK-3β by specific inhibitors has been shown to reduce tumor growth in several cancers, including pancreas, prostate, and colorectal adenocarcinomas, suggesting that GSK-3β is highly active in cancer cells. In addition, these cancer cell types have active raf-1 pathway, as evidenced by the presence of phosphorylated ERK1/2, and inhibition of the raf-1 pathway in these cancers has been shown to inhibit growth. In contrast to this, the raf-1 pathway is not activated in MTC cells, and indeed, activation of the raf-1 pathway results in growth reduction in MTC as well as carcinoid cells (19, 28). However, the downstream target by which the raf-1 activation inhibits the tumor growth and the regulatory role of GSK-3β is not known in these cells. In the present report, we, for the first time, identify GSK-3β as a key downstream target of the raf-1 pathway in TT cells. We also present in this study that inactivation of GSK-3β by LiCl and SB216763 results in a dose-dependent growth inhibition and a reduction of neuroendocrine tumor markers in TT cells, indicating that inactivation of GSK-3β alone is sufficient to replicate the effects of raf-1 pathway activation observed in TT cells. Mechanistically, we show that cell growth inhibition by GSK-3β inactivation is associated with cell cycle arrest due to an increase in the levels of CDK inhibitors such as p21, a tumor suppressor protein, p27, and p15, and the reduction in phospho-cdc2^{Tyr15} proteins. The CDK inhibitors are important for cell cycle progression and subsequent growth inhibition. Flow cytometry analysis of the LiCl-treated cells further confirmed that the growth inhibition is indeed due to cell cycle arrest possibly at the G_2-M phase (data not shown).

We and others have reported that raf-1 activation in TT and SCLC cells suppressed growth and caused a reduction in neuroendocrine tumor markers (19, 21, 37). Recently, we have shown that *in vivo* activation of raf-1 in a TT xenograft mouse model resulted in the significant reduction in tumor development and also in tumor progression (22). Current studies in our laboratory focusing on the mechanism of tumor growth inhibition by raf-1 pathway activation in TT cells resulted in the identification of GSK-3β inactivation as an important downstream target of the raf-1 pathway. Phosphorylation of GSK-3β by raf-1 activation in TT cells was evidenced by Western blot analysis. However, conflicting recent reports have shown that GSK-3β inhibition induces phosphorylation of ERK1/2 in HT29 cells (38), whereas deletion of GSK-3β abolished ERK1/2 activation in fibroblast cells derived from GSK-3β deleted mice (39, 40). Furthermore, the observation of cell growth inhibition in the present study due to cell cycle arrest by inactivation of GSK-3β in these cells is in contrast to the apoptotic process seen in pancreatic cancer cells. It seems that inactivation of GSK-3β affects cellular proliferation in many cell types. However, the effect by which GSK-3β mediates growth inhibition may not be general. The differences in the regulation of GSK-3β and ERK1/2 and the different mechanisms of growth inhibition seem to be cell type specific.

Furthermore, to validate the *in vitro* findings of growth inhibition in an *in vivo* situation, athymic nude mice were implanted with TT cells. Treatment of LiCl in mice bearing TT cells significantly reduced the tumor volume compared with the control mice treated with saline. Furthermore, we showed that the i.p. LiCl injections inactivated the GSK-3β by phosphorylation of GSK-3β at Ser^9 in TT tumors. The increase in phosphorylated GSK-3β is also associated with a reduction in the neuroendocrine tumor hormone CgA. This result perhaps suggests that the phosphorylated GSK-3β could be used as a marker in a clinical trial as a potential response predictor to therapy. Thus, the possibility that LiCl could be a viable treatment for MTC is exciting. Lithium ions have been used for more than 50 years in humans as mood stabilizers with proven safety.

In summary, this study extends our understanding and appreciation of the importance of the GSK-3β signaling pathway, particularly its role in MTC. Lithium has been given safely, with minimal side effects, for more than five decades as a treatment for mental disorders. This unique situation allows LiCl, as well as other GSK-3β inhibitors, to potentially see clinical trials rather promptly as an effective therapy for patients with MTC.

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