Glycogen Synthase Kinase-3β Participates in Nuclear Factor κB–Mediated Gene Transcription and Cell Survival in Pancreatic Cancer Cells

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Abstract

Recent studies using glycogen synthase kinase-3β (GSK-3β)–deficient mouse embryonic fibroblasts suggest that GSK-3β positively regulates nuclear factor κB (NFκB)–mediated gene transcription. Because NFκB is suggested to participate in cell proliferation and survival pathways in pancreatic cancer, we investigated the role of GSK-3β in regulating these cellular processes. Herein, we show that pancreatic cancer cells contain a pool of active GSK-3β and that pharmacologic inhibition of GSK-3 kinase activity using small molecule inhibitors or genetic depletion of GSK-3β by RNA interference leads to decreased cancer cell proliferation and survival. Mechanistically, we show that GSK-3β influences NFκB-mediated gene transcription at a point distal to the IκB kinase complex, as only ectopic expression of the NFκB subunits p65/p50, but not an IκB kinase β constitutively active mutant, could rescue the decreased cellular proliferation and survival associated with GSK-3β inhibition. Taken together, our results simultaneously identify a previously unrecognized role for GSK-3β in cancer cell survival and proliferation and suggest GSK-3β as a potential therapeutic target in the treatment of pancreatic cancer. (Cancer Res 2005; 65(6): 2076-81)

Introduction

Glycogen synthase kinase-3 (GSK-3) is a Serine/Threonine kinase that was identified as a regulator of glycogen synthesis (1). There are two homologous mammalian isoforms encoded by different genes (GSK-3α and GSK-3β; ref. 1). The classic paradigm places GSK-3β in a complex with the adenomatous polyposis coli, Axin, and β-catenin (1–3). In this model, GSK-3β directly phosphorylates β-catenin and targets it for degradation (1–3). Thus, based on this paradigm, GSK-3β is part of a tumor suppressor complex that controls the levels of the oncoprotein, β-catenin.

Although several reports have suggested a proapoptotic role for GSK-3β in HIV-tat-, PAF-, and staurosporine-induced cell death (1), disruption of the murine gsk-3β gene results in embryonic lethality; and mouse embryonic fibroblast (MEF) derived from these animals are more sensitive to apoptosis (4). Consistent with a model in which GSK-3β contributes to cell survival, two reports show that GSK-3β–deficient MEFs possess an intrinsic defect in the activation of nuclear factor κB (NFκB; refs. 4, 5), although different mechanisms were proposed to explain this effect. Interestingly, some mechanisms of chemoresistance in pancreatic cancer are due to hyperactive NFκB signaling pathways (6). Thus, understanding the mechanisms controlling NFκB-mediated cell survival pathways in pancreatic cancer may aid in the identification of novel anticancer targets.

The observations in the preceding paragraphs lead to conflicting predictions regarding the role of GSK-3β in cancer. On one hand, GSK-3β inactivation would result in sustained β-catenin protein levels and ultimately increased cellular proliferation. This model is difficult to reconcile with the observation that decreased β-catenin expression correlates with more aggressive pancreatic cancer and poor survival (7). On the other hand, recent suggestions that GSK-3β plays a key role in the regulation of NFκB would predict that GSK-3β inactivation would decrease cell proliferation and survival. These conflicting predictions prompted us to investigate the role of GSK-3β in pancreatic cancer. Our results identify GSK-3β as a regulator of pancreatic tumor cell proliferation and survival through the activation of NFκB.

Materials and Methods

Reagents, Cells, and Antibodies. All chemicals were obtained from Sigma (St. Louis, MO). The normal human HMEC and WI38 cell lines were a gift from Dr. Fergus Couch (Mayo Clinic). The GSK-3 inhibitors were obtained from Calbiochem (La Jolla, CA; AR-A014418) and Sigma (SB-216763; refs. 8, 9). The NFκB luciferase reporter has been previously described (10). The E2F1 and p53 luciferase reporter constructs were obtained from Panomics (Redwood City, CA). The constitutively active IκB kinase β (IκKB), and NFκB (p65 and p50) expression vectors were a kind gift from Dr. Carlos V. Paya and Gary Bren (Mayo Clinic). Antibodies to GSK-3β, β-catenin, cyclin D1, Bcl-2, Bcl-xL, and XIAP were from BD PharMingen (San Diego, CA) and GSK-3α and caspase-3 were obtained from Cell Signaling Technologies (Beverly, MA). The PARP antibody was a generous gift from Dr. Scott Kaufmann (Mayo Clinic).

Immunoblot Analysis. For immunoblots, cells were lysed as previously described (11). Whole cell extracts (100 μg) were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed as indicated. Bound antibodies were detected as previously described (11).

Glycogen Synthase Kinase-3β Kinase Assay. The GSK-3β in vitro kinase assay was carried out as previously described using a glutathione S-transferase fusion protein containing amino acids 499-503 of eIF2β as a substrate (10).

RNA Interference. A GSK-3β-specific targeting short hairpin RNA vector was generated as previously described (12) using the target sequence (5'-GATTATACCTCTAGATAGG-3').

Reverse Transcription-PCR. Oligonucleotide sequence and reverse transcription-PCR are described in Supplementary Table 1.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium, Bromodeoxyuridine, and Apoptosis Assays. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and bromodeoxyuridine incorporation assays were carried out over the indicated time course in the presence or absence...
of the GSK-3β inhibitors or suppression of GSK-3β as previously described (13). In some experiments, pancreatic cancer cell lines were transfected with a constitutively active mutant of IKKB, or NFκB p65/p50 expression vectors, and treated as indicated. For apoptosis assays, the indicated cell lines were treated as described in the text, harvested, and nuclei scored for apoptosis as previously described (14).

**Luciferase Assay.** The indicated luciferase reporter constructs were transfected into pancreatic cancer cell lines and luciferase activity was measured using the Dual luciferase assay system (Promega, Madison WI) as previously described (12).

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**Results**

**Pancreatic Cancer Cell Lines Have an Active Pool of Glycogen Synthase Kinase-3β.** As shown in Fig. 1A, all of the pancreatic cancer cell lines examined express GSK-3β. In addition, an *in vitro* kinase assay showed that at least some fraction of GSK-3β is active in the pancreatic tumor cell lines (Fig. 1B). Interestingly, we communoprecipitated β-catenin with GSK-3β from the pancreatic cancer cell lines, and consistent with the *in vitro* kinase assay, we observed phosphorylation of β-catenin in the GSK-3β immunoprecipitates (Fig. 1C). Taken together, these data indicate that pancreatic cancer cell lines contain a pool of active GSK-3β.

**Glycogen Synthase Kinase-3β Regulates Nuclear Factor κB–Mediated Gene Transcription.** Previous studies have shown that NFκB is constitutively activated in most human pancreatic cancer cell lines (15). Recent studies suggest a defect in the activation of NFκB in *gsk-3β−/−* MEFs (4, 5). Consistent with the idea that GSK-3β regulates NFκB-mediated gene transcription, treatment of BXPC-3 cells with a GSK-3β inhibitor, AR-A014418, results in a pronounced decrease in basal NFκB-mediated gene transcription compared with DMSO-treated control cells (Fig. 2A). In fact, by 48 hours after addition of drug, there is a dramatic loss of basal NFκB activity that is further decreased by 72 hours. Importantly, AR-A014418-treated cells did not show a defect in the regulation of P53- or E2F1-mediated gene transcription (Fig. 2B). These data suggest that GSK-3β regulates NFκB-dependent gene transcription in pancreatic cancer cells.

Two different models place GSK-3β either proximal (5) or distal (4) to the IKK complex in the regulation of NFκB activation. We find, that although cells expressing a constitutively active mutant of IKKB show enhanced NFκB-mediated gene transcription, this increase in NFκB activity was blocked by either pharmacologic inhibition or genetic depletion of GSK-3β (Fig. 2C and Supplementary Fig. 1). In contrast, NFκB activity in cells overexpressing the NFκB subunits p65 and p50 were unaffected by GSK-3β inhibition (Fig. 2D). Thus, our data indicate that in pancreatic cancer cell lines, GSK-3β regulates NFκB activity at a point distal to the IKK complex.

**Inhibition of Glycogen Synthase Kinase-3β Results in Decreased Expression of Nuclear Factor κB Target Genes Involved in Cellular Proliferation and Survival.** Treatment of BXPC-3 cells with the GSK-3 inhibitor AR-A014418 led to a reduction in the expression of several NFκB-target genes, including Bcl-2, Bcl-xL, xIAP, and cyclin D1, and XIAP (Fig. 2E). Similar results were observed in several pancreatic cancer cell lines, including MIA-PaCa2, CAPAN2, and PANC1.† To determine if the effect on these NFκB target genes by pharmacologic inhibition was specific to GSK-3β, we depleted GSK-3β expression in the MIA-PaCa2 cell line using RNA interference (12). Consistent with the pharmacologic inhibition of GSK-3β in the BXPC-3 cell line, MIA-PaCa2 tumor cells in which GSK-3β was specifically depleted by short hairpin RNA showed a similar decrease in the expression of NFκB target genes (Fig. 2E). Taken together, these results suggest that GSK-3β is a selective key regulator of NFκB-mediated gene transcription in pancreatic cancer cell lines.

**Glycogen Synthase Kinase-3 Activity Is Required for Pancreatic Tumor Cell Proliferation.** To determine whether GSK-3β activity is required for pancreatic tumor cell proliferation, cells were treated with two structurally distinct inhibitors of GSK-3β (AR-A014418 and SB-216763; refs. 8, 9, 16). BXPC-3 cells show a similar decrease in the expression of NFκB target genes (Fig. 2E). Taken together, these results suggest that GSK-3β is a selective key regulator of NFκB-mediated gene transcription in pancreatic cancer cell lines.

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Figure 1. Expression and activity of GSK-3β in pancreatic cancer cell lines. A, protein lysates from the indicated pancreatic cancer cell lines were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with the antibodies against GSK-3β and β-catenin. B, GSK-3β was immunoprecipitated from the indicated pancreatic cancer cell lines and subjected to an *in vitro* kinase assay using GST-elf2Bα as a substrate. The autoradiography (top), anti-GSK-3β (middle), and GST-elf2Bα (bottom, Coomassie blue stain). C, GSK-3β was immunoprecipitated from the indicated cell lines and the phosphorylation of associated β-catenin was determined using an *in vitro* kinase assay. Top, amount of 32P incorporation into immunoprecipitated β-catenin. Middle and bottom, levels of β-catenin and GSK-3β immunoprecipitated, respectively. Mouse IgG (mIgG) was used as a control immunoprecipitation from the MIA-PaCa2 cell line.

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† A.V. Ougoloff and D.D. Billaudeau, unpublished observation.
effect between 25 and 50 μmol/L for both inhibitors (Fig. 3A). The inhibitory effect on cell viability was not unique to BXPC-3, as several pancreatic cancer cell lines, including MIA-PaCa2, PANC1, ASPC1, and CFPAC, were similarly affected by the addition of GSK-3β inhibitors at a concentration of 25 μmol/L (Fig. 3B and data not shown). Significantly, neither agent affected the proliferation of HMEC or WI38 cell lines (Fig. 3B). Moreover, pharmacologic inhibition or genetic depletion of GSK-3β lead to a significant decrease in cellular proliferation as measured by bromodeoxyuridine incorporation (Fig. 3C). Importantly, and consistent with the data presented in Fig. 2C and D, overexpression of NF-κB subunits p65/p50, but not constitutively active IKKβ, could rescue the decrease in cellular proliferation upon inhibition of GSK-3β (Supplementary Fig. 1). Taken together, these data suggest that GSK-3β contributes to the proliferation of pancreatic cancer cells.

Inhibition of Glycogen Synthase Kinase-3β Leads to Increased Apoptosis in Pancreatic Cancer Cell Lines. Because gsk-3β−/− MEFS are more sensitive to apoptosis (4, 5), we next investigated whether the decrease in cellular proliferation of GSK-3β-inhibited pancreatic tumor cells was accompanied by an increase in apoptosis. To examine this, MIA-PaCa2 cells were genetically depleted of GSK-3β and examined for apoptotic changes by analysis of PARP cleavage and nuclear morphologic changes. Consistent with a role for GSK-3β in regulating cell survival, depletion of GSK-3β lead to a significant increase in apoptosis, as compared with the control transfected cell population (Fig. 4A).

**Figure 2.** Inhibition of GSK-3β decreases NF-κB transcriptional activity. A. BXPC-3 cells were transfected with a 3XNF-κB-luciferase reporter construct and subsequently treated with diluent or AR-A014418 (25 μmol/L) in triplicate. Luciferase activity was measured at the indicated times post-treatment as % luciferase activity in the control-treated population. B. BXPC-3 cells were transfected with either an E2F1-, p53-, or NF-κB-luciferase reporter, treated with diluent or AR-A014418 (25 μmol/L) in triplicate and luciferase activity was measured 72 hours later as described in A. C. BXPC-3 cells were cotransfected with the NF-κB reporter and either a vector control or constitutively active mutant of IKKβ. Transfected cells were treated with diluent or AR-A014418 (25 μmol/L) in triplicate and luciferase activity was measured 48 hours later as described in A. D. BXPC-3 cells were cotransfected with the NF-κB reporter and either a vector control or the NF-κB subunits p65 and p50 expression vectors. Transfected cells were treated with diluent or AR-A014418 (25 μmol/L) in triplicate and luciferase activity was measured 72 hours later as described in A. E. left, BXPC-3 cells were treated with DMSO or AR-A014418; 48 hours post-treatment, the cell pellet was collected, RNA and protein were obtained. Cell lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies to the indicated proteins. RT-PCR analysis was performed as described in supplementary data. E. right, MIA-PaCa2 tumor cells were transfected with a control shVector or the shGSK-3β silencing vector; 72 hours post-transfection, the cell pellet was collected, RNA and protein were obtained. Cell lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies to the indicated proteins. Reverse transcription-PCR (RT-PCR) analysis was performed as described in supplemental data.

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Importantly, the shGSK-3β targeting vector did not affect the levels of GSK-3α within the cells (Fig. 4A), suggesting that the apoptosis effects are due to the down regulation of GSK-3β. In contrast to diluent-treated cells, BXPC-3 cells treated with either AR-A014418 or SB-216763 show nearly 50% apoptosis by 72 hours (Fig. 4B and data not shown). In addition, AR-A014418-treated cells show a time-dependent increase in PARP and caspase-3 cleavage (Fig. 4B). Lastly, consistent with the idea that GSK-3β regulates NFκB activity at a point distal to the IKK complex, overexpression of NFκB subunits p65 and p50 could rescue the apoptotic and transcriptional effects of GSK-3β inhibition (Fig. 4C). Taken together, our data identify GSK-3β as a key participant in the regulation of pancreatic tumor cell survival.

Discussion

We are unaware of any previous report that assessed the role of GSK-3β in the regulation of NFκB-mediated gene transcription and cell proliferation and survival in human cancer. Collectively, the results described above identify a novel role for GSK-3β in regulating pancreatic tumor cell proliferation and cell survival via activation of NFκB-dependent gene transcription. Our data are in agreement with recently published results for MEFs regarding the regulation of cell survival by GSK-3β through an NFκB-dependent pathway (4, 5). However, whereas Takada et al., showed that tumor necrosis factor α stimulation of gsk-3β−/− MEFs fails to induce activation of the IKK complex and degradation of IkB (5), Hoeflich et al., place the defect in NFκB activation downstream of IkB phosphorylation in gsk-3β−/− MEFs (4). Our results, shown in Figs. 2C and D and 3C and Supplementary Figs. 1 and 2 are consistent with the latter model because overexpression of NFκB subunits p65/p50, but not a constitutively active mutant of IKKβ could rescue cells from the decreased cellular proliferation and cell survival effects of GSK-3β inhibition. It is of interest that GSK-3β has been suggested to participate in tumor necrosis factor –mediated regulation of NFκB-dependent gene transcription in hepatocytes through the phosphorylation of p65 between residues 354 and 551 (17). Whether or not this is the mechanism by which inhibition of GSK-3β is affecting basal NFκB-mediated gene transcription in pancreatic cancer cells remains to be determined.
In pancreatic cancer cells, NFκB activity is high and can be further induced by genotoxic stress, thus leading to chemoresistance (6, 18). The inhibition of NFκB activity in pancreatic cancer cells can make them more sensitive to gemcitabine and other chemotherapeutic agents whose mechanism of resistance is due to increased NFκB activity. Indeed, the combination of NFκB inhibitors with other chemotherapeutic agents may be more effective in cancer treatment and clinical trials using such treatment strategies are currently being employed in several human cancers (19). Thus, inhibition of GSK-3β may sensitize gemcitabine-resistant pancreatic cancer cells to gemcitabine, but this remains to be addressed.

Previous studies have shown that pancreatic cancers with low β-catenin levels have a poorer prognosis than those with higher levels (7, 20). This was puzzling as β-catenin is an oncogene that regulates the expression of target genes involved in cell proliferation, including cyclin D1 (1) and might be expected to participate in the proliferation of pancreatic cancer cells, as has been shown in colon cancer cells. The data presented in Fig. 1B provide a potential explanation. These results indicate that there is an active pool of GSK-3β in pancreatic cancer cell lines. This active GSK-3β simultaneously increases β-catenin phosphorylation and degradation at the same time it activates NFκB (Fig. 2), thereby contributing to the proliferation and survival of pancreatic cancer cells (Figs. 3 and 4). Although it remains unclear what role, if any, β-catenin plays in pancreatic cancer, our data suggest, that GSK-3β is uniquely involved in pancreatic cancer cell proliferation and survival, in part through its regulation of NFκB activity.

Further studies are required to determine whether GSK-3β regulates additional molecular pathways that contribute to pancreatic cancer. The observation that GSK-3β participates in the regulation of several proteins involved in controlling cell cycle, protein translation, metabolism, cell survival, and proliferation, suggests that its role in cancer may be broader than previously expected. In summary, the present data identify a novel role for GSK-3β in pancreatic cancer cell lines through the regulation of NFκB-dependent cell proliferation and survival pathways. Whether GSK-3β participates in a similar manner in other human malignancies remains to be determined.

Figure 4. Inhibition of GSK-3β increases apoptosis in pancreatic cancer cells. A, Mia-PaCa2 cells were transfected with either a control shVector or GSK-3β targeting vector. Cells were collected at the indicated times and stained with Hoechst as described previously (14). Three hundred nuclei were counted per field and scored for apoptotic changes (fragmentation of the nucleus into multiple discrete fragments) and are graphically represented. Cell lysates from the transfected cells were probed with the indicated antibodies to demonstrate the specificity and knockdown of GSK-3β and PARP cleavage (right). B, BXPC-3 cells were treated with DMSO or the AR-A014418 (25 μmol/L) for the time indicated. % Apoptotic cells determined by staining with Hoechst as described in A. Cell lysates from the treated cells were probed with the indicated antibodies (right). C, BXPC-3 cells were transfected with a control vector or the NFκB subunits p65 and p50 expression vectors and % apoptotic cells at 48 hours post-transfection determined by staining with Hoechst as described in A. Cell lysates from the treated cells were probed with the indicated antibodies (right).
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References