Genetic Deletion of Glycogen Synthase Kinase-3β Abrogates Activation of IκBα Kinase, JNK, Akt, and p44/p42 MAPK but Potentiates Apoptosis Induced by Tumor Necrosis Factor*

Yasunari Takada‡, Xianjun Fang§, Md. Saha Jamaluddin†, Douglas D. Boyd†, and Bharat B. Aggarwal‡

From the Cytokine Research Laboratory, Departments of Bioimmunotherapy and Cancer Biology, the University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 and the Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

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Glycogen synthase kinase (GSK-3) is a constitutively active, proline-directed serine/threonine kinase that controls growth modulation and tumorigenesis through multiple intracellular signaling pathways. How GSK-3β regulates signaling pathways induced by cytokines such as tumor necrosis factor (TNF) is poorly understood. In this study, we used fibroblasts derived from GSK-3β gene-deleted mice to understand the role of this kinase in TNF signaling. TNF induced NF-κB activation as measured by DNA binding in wild-type mouse embryonic fibroblasts, but deletion of GSK-3β abolished this activation. This inhibition was due to suppression of IκBα kinase activation and IκBα phosphorylation, ubiquitination, and degradation. TNF-induced NF-κB reporter gene transcription was also suppressed in GSK-3β gene-deleted cells. NF-κB activation induced by lipopolysaccharide, interleukin-1β, or cigarette smoke condensate was completely suppressed in GSK-3β−/− cells. Deletion of GSK-3β also abolished TNF-induced c-Jun N-terminal kinase and p44/p42 mitogen-activated kinase activation. Most surprisingly, TNF-induced Akt activation also required the presence of GSK-3β. TNF induced expression of the NF-κB-regulated gene products cyclin D1, COX-2, MMP-9, survivin, IAP 1, IAP 2, Bcl-2, Bcl-xL, Bfl-1/A1, TRAF1, and FLIP in wild-type mouse embryonic fibroblasts but not in GSK-3β−/− cells, and this correlated with potentiation of TNF-induced apoptosis as indicated by cell viability, annexin V staining, and caspase activation. Overall, our results indicate that GSK-3β plays a critical role in TNF signaling and in the signaling of other inflammatory stimuli and that its suppression can be exploited as a potential target to inhibit angiogenesis, proliferation, and survival of tumor cells.

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‡ Ransom Horne, Jr., Distinguished Professor of Cancer Research. To whom correspondence should be addressed: Cytokine Research Laboratory, Dept. of Bioimmunotherapy, the University of Texas M. D. Anderson Cancer Center, Box 145, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-3503/8458; Fax: 713-794-1613; E-mail: aggarwal@mdanderson.org.

† The abbreviations used are: GSK, glycogen synthase kinase; NF-κB, nuclear factor-κB; IκB, inhibitory subunit of NF-κB; IKK, IκB kinase; SEAP, secretory alkaline phosphatase; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; TUNEL, terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling; IAP, inhibitor of apoptosis protein; IL, interleukin; FADD, Fas-associated death domain protein; FLICE, FADD-like IL-1β-converting enzyme; FLIP, FLICE-inhibitory protein; COX, cyclooxygenase; MMP, matrix metalloproteinase; LPS, lipopolysaccharide; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ChIP, chromatin immunoprecipitation; MEF, mouse embryonic fibroblast; EMSA, electrophoretic mobility shift assay; CSC, cigarette smoke condensate; DMEM, Dulbecco’s modified Eagle’s medium; PARP, poly(ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate.

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and degradation and resulting in nuclear translocation of the p50 and p65 subunit complexes of NF-κB. How GSK-3β affects TNF-induced signaling is not well understood. In the study presented here, we used GSK-3β gene-deleted cells to delineate the role of this kinase in TNF signaling. We demonstrate that GSK-3β is required for TNF-induced activation of IKK, JNK, p44/p42 MAPK, and Akt. NF-κB-regulated gene products such as cyclooxygenase (COX)-2, cyclin D1, matrix metalloproteinase (MMP)-9, survivin, inhibitor-of-apoptosis protein 1 (IAP 1), IAP 2, β-catenin, and tumor necrosis factor receptor-associated factor (TRAF)1, and Fas/mouse emed death domain protein (FADD)-like interleukin-1 β-converting enzyme (FLICE)-inhibitory protein (FLIP) were down-regulated by deletion of GSK-3β, which is likely to potentiation of apoptosis induced by TNF.

MATERIALS AND METHODS

Reagents—Bacteria-derived recombinant TNF, purified to homogeneity with a specific activity of 5 × 10⁶ units/mg, was kindly provided by Genentech (South San Francisco, CA). Cigarette smoke condensate (CSC) was kindly provided by Dr. C. Gary Gariola (University of Kentucky, Lexington). Penicillin, streptomycin, RPMI 1640 medium, DMEM, and fetal bovine serum were obtained from Invitrogen. Lipopolysaccharide (LPS) and anti-β-actin antibody were obtained from Sigma. The antibodies anti-p65, anti-p50, anti-IκBα, anti-cyclin D1, anti-MMP-9, anti-poly(ADP-ribose) polymerase (PARP), anti-IAP 1, anti-IAP 2, anti-Bcl-xL, anti-Bfl-1/A1, and anti-FLIP were down-regulated by deletion of GSK-3β, which is likely to potentiation of apoptosis induced by TNF.

Cell Transfection—To introduce IκBα or anti-IKK-β antibody, protein A/agarose slurry (Upstate Biotechnology, Inc.) was used for real time PCR: forward (5′-TGTCCCTTTACTGCCCTGA-3′), reverse (5′-ACTCCAGGCTCTGTCCTCCTC-3′) for Cox-2. These primers correspond to Cox-2 promoter, respectively. For conventional PCR cycle parameters: denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s, with a final incubation for 40 cycles. For cDNA cloning, PCR products were checked in a 10% polyacrylamide gel.

Real time PCR—Real time PCR was performed essentially as described previously (38). Briefly, TNF-treated and untreated cells were fixed with 1% formaldehyde for 8 min to cross-link proteins to DNA. The cells were collected, washed twice in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, and then washed sequentially for 10 min in ice-cold solution 1 (0.25% Triton X-100, 0.5 mM EDTA, 75 mM NaCl, pH 7.5) and thereafter in solution 2 (0.2 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 7.5). The pellet was resuspended in lysis buffer (150 mM NaCl, 25 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors) and sonicated eight times for 15-s bursts. The lysate was diluted to 1 ml in lysis buffer, and an aliquot (5%) was saved as input. Lysate was preclarified sequentially with rabbit IgG and then protein A-agarose. Anti-p65 antibody, 2.5 μg, was added to the lysate and incubated overnight at 4 °C. Immunoprecipitated complexes were collected by adding salmon sperm/protein A-agarose slurry (Upstate Biotechnology, Inc.) for 1 h at 4 °C. Immunoprecipitated were washed once sequentially with preimmune serum and then twice with NF-κB promoter assay buffer (1× PBS, 0.5 M Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40), high salt wash (500 mM NaCl, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris-Cl, pH 8.0), and LiCl wash (250 mM LiCl, 1%, Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 50 mM Tris-Cl, pH 8.0) and then twice with TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). The beads were then treated with RNase (50 μg/ml) at 37 °C for 30 min and then washed, exposed to 1 nM TNF for 24 h, harvested from the cell culture medium, and then analyzed for NF-κB reporter activity according to the protocol essentially as described by the manufacturer (Clontech) using a 96-well fluorescence plate reader (Fluoroscan II; Labsystems, Chicago) with excitation set at 380 nm and emission at 480 nm.

Chromatin Immunoprecipitation (ChIP) Assay—These assays were performed essentially as described previously (38). Briefly, TNF-treated and untreated cells were fixed with 1% formaldehyde for 8 min to cross-link proteins to DNA. The cells were collected, washed twice in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, and then washed sequentially for 10 min in ice-cold solution 1 (0.25% Triton X-100, 0.5 mM EDTA, 75 mM NaCl, pH 7.5) and thereafter in solution 2 (0.2 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 7.5). The pellet was resuspended in lysis buffer (150 mM NaCl, 25 mM Tris-Cl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors) and sonicated eight times for 15-s bursts. The lysate was diluted to 1 ml in lysis buffer, and an aliquot (5%) was saved as input. Lysate was preclarified sequentially with rabbit IgG and then protein A-agarose. Anti-p65 antibody, 2.5 μg, was added to the lysate and incubated overnight at 4 °C. Immunoprecipitated complexes were collected by adding salmon sperm/protein A-agarose slurry (Upstate Biotechnology, Inc.) for 1 h at 4 °C. Immunoprecipitated were washed once sequentially with preimmune serum and then twice with NF-κB promoter assay buffer (1× PBS, 0.5 M Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40), high salt wash (500 mM NaCl, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris-Cl, pH 8.0), and LiCl wash (250 mM LiCl, 1%, Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 50 mM Tris-Cl, pH 8.0) and then twice with TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). The beads were then treated with RNase (50 μg/ml) at 37 °C for 30 min and then washed, exposed to 1 nM TNF for 24 h, harvested from the cell culture medium, and then analyzed for NF-κB reporter activity according to the protocol essentially as described by the manufacturer (Clontech) using a 96-well fluorescence plate reader (Fluoroscan II; Labsystems, Chicago) with excitation set at 380 nm and emission at 480 nm.

Cell Transfection—To introduce IκBα-DN or p65 plasmids, 3 × 10⁵ cells were seeded, and 2 μg of plasmid was diluted in 200 μl of DMEM (without serum and antibiotics) and then mixed with 200 μl of DMEM containing 4 μl of LipofectAMINE 2000. This mixture was added to the cells and incubated for 12 h. The cells were collected and maintained for an additional 48 h before using for experiments.

Immunocytochemistry for NF-κB p65 Localization—The effect of TNF on the nuclear translocation of p65 was examined by an immunocytochemical method as described (39). Briefly, treated cells were plated

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on a poly-L-lysine-coated glass slide by centrifugation using a cytosin
4 (Thermoshenden, Pittsburgh, PA), air-dried, and fixed with 4% paraformaldehyde following permeabilization with 0.2% Triton-X-100.
After being washed in phosphate-buffered saline, slides were blocked
with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 antibody at a 1:100 dilution. After overnight
incubation at 4 °C, the slides were washed, incubated with goat anti-
rabbit IgG-Alexa 594 at a 1:100 dilution for 1 h, and counter-stained for
nuclei with Hoechst 33342 (50 ng/ml). Stained slides were used as control. As shown in Fig. 2A, LiCl suppressed TNF-induced NF-κB activation, but KCl and NaCl did not. These results suggest that the activity of GSK-3β is required for suppression of NF-κB.
GSK-3β Is Required for NF-κB Activation Induced by LPS, IL-1β, or Cigarette Smoke Condensate—NF-κB is activated by a wide variety of carcinogens and inflammatory stimuli other than TNF, through a mechanism that may differ from that of TNF (43–46). Therefore, we investigated the role of GSK-3β in NF-κB activation induced by LPS, IL-1β, or CSC. As shown in
Fig. 2B, all these agents activated NF-κB, and GSK-3β deletion suppressed this activation in every case. These results suggest that GSK-3β must act at a step in the NF-κB activation pathway that is common to all these agents.
Because various combinations of Rel/NF-κB protein can constitute a active NF-κB heterodimer that binds to a specific sequence in the DNA (47), we next showed that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB. We incubated nuclear extracts from TNF-stimulated cells with antibodies to either the p50 (NF-κB1) or the p65 (RelA) subunit of NF-κB. Both shifted the band to a higher molecular mass (Fig. 2C), suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor irrelevant antibody (anti-cyclin D1) had any effect. Excess unlabelled NF-κB (100-fold) caused complete disappearance
of the band, but the mutant oligonucleotide of NF-κB did not affect NF-κB binding activity.
GSK-3β Is Required for TNF-dependent IκBα Degradation—
Translocation of NF-κB to the nucleus is preceded by proteo-
lytic degradation of IκBα (47). To determine whether inhibition of
TNF-induced NF-κB activation in GSK-3β−/− cells was due to inhibition of IκBα degradation, we exposed cells to TNF for the indicated intervals and assayed degradation of IκBα by Western blot analysis. TNF induced IκBα degradation, which precedes NF-κB translocation in control cells as early as 15 min in wild-type fibroblasts. In GSK-3β−/− fibroblasts, however, TNF had no effect on IκBα degradation (Fig. 3A, upper panel). Thus GSK-3β is required for degradation of IκBα.
GSK-3β Is Required for TNF-dependent IκBα Phosphoryla-
tion—The proteolytic degradation of IκBα is known to require phosphorylation at serine residues 32 and 36 (47). To determine
whether GSK-3β deletion affects TNF-induced IκBα phosphorylation, we assayed the TNF-induced phosphorylated form of IκBα by Western blot analysis, using antibody that recognizes the serine-phosphorylated form of IκBα. TNF induced IκBα phosphorylation in wild-type fibroblasts, but in

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GSK-3β−/− fibroblasts, the IκBα phosphorylation induced by TNF was almost completely suppressed (Fig. 3A, middle panel).

**GSK-3β Deletion Inhibits TNF-induced IKK Activation**—Because IKK is required for TNF-induced phosphorylation of IκBα (47), we next determined the effect of GSK-3β deletion on TNF-induced IKK activation. As shown in Fig. 3B, GSK-3β deletion completely suppressed TNF-induced activation of IKK without any effect on the expression of IKK-α or IKK-β. These results suggest that GSK-3β is required for TNF-induced IKK activation.

**GSK-3β Is Required for TNF-induced Phosphorylation and Nuclear Translocation of p65**—Degradation of IκBα leads to nuclear translocation of the p65 subunit of NF-κB. Therefore, we also analyzed the effect of the GSK-3β deletion on TNF-induced nuclear translocation of p65 by Western blot analysis. As shown in Fig. 3C, TNF induced nuclear translocation of p65 in a time-dependent manner; as early as 5 min after TNF stimulation, nuclear p65 was noted in wild-type MEF cells. In GSK-3β−/− fibroblasts, TNF failed to induce nuclear translocation of p65.

TNF induced the phosphorylation of p65, which is required for its transcriptional activity (48). Therefore, we also analyzed the effect of GSK-3β deletion on TNF-induced phosphorylation of p65 by Western blot analysis. As shown in Fig. 3C, TNF induced phosphorylation of p65 in a time-dependent manner; as early as 5 min after TNF-stimulation, p65 was phosphorylated in wild-type fibroblasts. In GSK-3β−/− fibroblasts, TNF failed to induce phosphorylation of p65.

To confirm further the effect of GSK-3β deletion on the suppression of nuclear translocation of p65, we performed an immunocytochemical assay. The results showed that p65 is localized in the cytoplasm, that TNF induced nuclear translocation of p65 in wild-type fibroblasts, and that TNF failed to induce p65 translocation to the nucleus in GSK-3β−/− fibroblasts (Fig. 3D).
GSK-3β Is Required for TNF-induced NF-κB-dependent Reporter Gene Expression—DNA binding does not always correlate with NF-κB-dependent gene transcription (49–51). To determine the role of GSK-3β in TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected cells with the NF-κB-regulated SEAP reporter construct and then stimulated the cells with different concentrations of TNF. NF-κB-regulated reporter gene expression was activated by TNF in a dose-dependent manner in wild-type MEF, but minimal activation was seen in GSK-3β-deleted cells (Fig. 4). These results suggest that GSK-3β is required not only for activation of IKK, nuclear translocation of p65, or p65 binding to the DNA but also for NF-κB-regulated reporter gene expression.

GSK-3β Is Required for TNF-induced Activation of JNK—We investigated the effect of GSK-3β on other signals transduced by TNF. Activation of JNK is one of the earliest events induced by TNF (52). To explore the specific role of GSK-3β in TNF-induced JNK activation, we treated cells with TNF for the indicated intervals, prepared whole-cell extracts, and analyzed them for JNK activity by immune complex kinase assay. TNF induced time-dependent activation of JNK in wild-type fibroblasts but not in GSK-3β−/− fibroblasts (Fig. 5A). These results suggest that GSK-3β is required for TNF-induced JNK activation.
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**Fig. 3.** A, effects of GSK-3β deletion on TNF-induced phosphorylation and degradation of IκBα. One million cells were treated with 0.1 nM TNF for the indicated times. Cytoplasmic extract was prepared, resolved by SDS-PAGE, and electrotransferred onto a nitrocellulose membrane, and then Western blot analysis using anti-IκBα and anti-phospho-specific IκBα antibodies was performed. B, effects of GSK-3β deletion on TNF-induced activation of IKK. One million cells were pretreated with 50 μM proteosome inhibitor ALLN for 1 h and then stimulated with 1 nM TNF for the indicated times. Whole-cell extracts were incubated with anti-IKK-α antibody for 2 h, immunoprecipitated with protein A/G-Sepharose beads, and then analyzed by immunocomplex kinase assay. To examine the level of expression of IKK proteins, the same whole-cell extracts were resolved by SDS-PAGE and performed Western blot analysis using anti-IKK-α and anti-IKK-β antibodies. C, effects of GSK-3β deletion on TNF-induced nuclear translocation of the p65 subunit of NF-κB. One million cells were treated with 0.1 nM TNF for the indicated times; nuclear extract was prepared, resolved by SDS-PAGE, and electrotransferred to a nitrocellulose membrane, and then Western blot analysis using anti-p65 and anti-phospho-specific p65 antibodies was performed. D, immunocytochemical analysis of p65 localization. Cells were treated with 1 nM TNF for 20 min and then cells were subjected to immunocytochemical analysis as described under "Materials and Methods."
PAGE, and performed Western blot analysis by using phospho-specific anti-p44/p42 MAPK antibody. Time-dependent phosphorylation of p44/p42 MAPK occurred in wild-type cells, but p44/p42 MAPK activation was abolished in GSK-3β−/− fibroblasts (Fig. 5B). These results suggest that GSK-3β is required for TNF-induced p44/p42 MAPK activation also.
GSK-3β Is Required for TNF-induced Activation of Akt—Activation of Akt is also the earliest event induced by TNF (53). Activated Akt has been shown to inactivate GSK-3β through phosphorylation (54–56). To explore the specific role of GSK-3β in TNF-induced Akt activation, we treated cells with TNF for the indicated intervals, prepared whole-cell extracts, resolved them by SDS-PAGE, and performed Western blot analysis by using phospho-specific anti-Akt antibody. Time-dependent activation of Akt occurred in wild-type fibroblasts, but not in GSK-3β−/− fibroblasts (Fig. 5C). Thus although Akt can inactivate GSK-3β, GSK-3β is needed for TNF-induced Akt activation.

GSK-3β Is Required for Expression of TNF-induced NF-κB-dependent Cyclin D1, COX-2, and MMP-9 Proteins—TNF has been shown to induce cyclin D1, COX-2, and MMP-9 (39, 57–60); therefore, we determined whether GSK-3β is required for this induction. Cells were treated with TNF for different intervals, prepared whole-cell extracts, and analyzed them by Western blot analysis for the expression of cyclin D1, COX-2, and MMP-9 (Fig. 6A). Cyclin D1, COX-2, and MMP-9 expressions were induced by TNF in a time-dependent manner in wild-type fibroblasts but not in GSK-3β−/− fibroblasts.

GSK-3β Is Required for Expression of TNF-induced NF-κB-dependent Antiapoptotic Proteins—Because NF-κB also regulates the expression of several antiapoptotic proteins including survivin (61), IAP 1/2 (62, 63), Bcl-xL (64), Bfl-1/A1 (65, 66), TRAF1 (67), and cFLIP (68), we tested the effect of GSK-3β on the TNF-induced expression of these antiapoptotic gene products. As shown in Fig. 6B, TNF induced survivin, IAP 1, IAP 2, Bcl-xL, Bfl-1/A1, TRAF1, and cFLIP expression in a time-dependent manner in wild-type fibroblasts but not in GSK-3β−/− fibroblasts. These results further strengthen our evidence that GSK-3β is required for TNF induction of NF-κB-regulated gene products.

GSK-3β and NF-κB Are Required for TNF-induced COX-2 Promoter Activity—It has been shown that NF-κB activation is required for COX-2 expression. Whether the COX-2 promoter is regulated by GSK-3β through modulation of NF-κB was examined. As shown in Fig. 7A, TNF activated COX-2 promoter activity in wild-type MEF but not in GSK-3β-deleted cells, thus suggesting that GSK-3β is needed for COX-2 expression. Whether COX-2 promoter is regulated by NF-κB was examined by using p65 NF-κB-deleted cells. As shown in Fig. 7A, TNF activated COX-2 promoter activity in wild-type MEF but not in p65-deleted cells, thus suggesting that NF-κB is needed for COX-2 expression.

IkBα-DN Transfection Down-regulates and p65 NF-κB Up-regulates Expression of COX-2 and Cyclin D1—Whether suppression of NF-κB by IkBα-DN mimics the loss of GSK-3β in NF-κB-regulated gene expression was also examined. To determine this, wild-type MEF cells were transfected with IkBα-DN plasmid and then treated with 1 nM TNF and analyzed COX-2 and cyclin D1 protein expressions (Fig. 7B). The exogenously transfected IkBα-DN down-regulated TNF-induced expression of COX-2 and cyclin D1 in wild-type MEF cells. TNF-induced COX-2 expression was also abolished in MEFP65−/− cells.

Whether overexpression of p65 reverses the loss of GSK-3β was also examined. To determine this, MEFGSK−3β−/− cells were transfected with p65 plasmid, and then after 48 h cells were treated with 1 nM TNF and analyzed expression of COX-2
and cyclin D1 proteins (Fig. 7C). Ectopic expression of p65 reversed the TNF-induced COX-2 and cyclin D1 expression in GSK-3β-deleted cells. Similarly, COX-2 and cyclin D1 expressions were induced by TNF in MEF/p65+/− cells but not in MEF/p65−/− cells. Taken together, these results provide the evidence that GSK-3β mediates its effect on TNF-induced gene...

**Fig. 7.** A, GSK-3β and NF-κB are required for TNF-induced COX-2 promoter activity. MEF cells (wild-type, GSK-3β-deleted, and p65-deleted) were transfected with the COX-2 promoter linked to luciferase reporter gene plasmid and treated with the indicated concentrations of TNF. After 24 h, cells were lysed and subjected to luciferase assay. B, ectopic expression of IκBα-DN down-regulates COX-2 and cyclin D1 expressions. MEF cells (wild-type, GSK-3β-deleted, and p65-deleted) were transfected with mock or IκBα-DN plasmid for 48 h and then treated with 1 nM TNF. After 24 h, cells were analyzed for COX-2 and cyclin D1 by Western blot analysis. C, ectopic expression of p65 NF-κB subunit up-regulates COX-2 and cyclin D1 expressions. MEF cells (wild-type, GSK-3β-deleted, and p65-deleted) were transfected with mock or p65 plasmid for 48 h and then treated with 1 nM TNF. After 24 h, cells were analyzed for COX-2 and cyclin D1 by Western blot analysis.
FIG. 8. **GSK-3β deletion inhibits binding of NF-κB to the MMP-9 and COX-2 promoters.** Cells (wild-type and GSK-3β-deleted) were treated with 1 nM TNF for the indicated times; the proteins were cross-linked with DNA by using formaldehyde and subjected to ChIP assay by using an anti-p65 antibody with the indicated primers. Reaction products were resolved by electrophoresis (A1 and A2) or quantified by real time PCR (B1 and B2). After analysis, a melting curve was performed to confirm the amplification of a single product (C1 and C2). IP, immunoprecipitation; Ab, antibody.
expression through activation of NF-κB.

**GSK-3β-mediated NF-κB Activation Regulates the Expression of MMP-9 and COX-2 in Vivo**—TNF has been shown to induce MMP-9 and COX-2 (39, 57), both of which have NF-κB-binding sites in their promoters (58–60). Whether the lack of TNF-induced gene expression in GSK-3β-deleted cells is due to suppression of NF-κB activation in vivo, we performed chromatin immunoprecipitation (ChIP) assay. NF-κB-binding sites in the promoters of MMP-9 and COX-2 were examined. Cells were treated with 1 nM TNF, cross-linked in situ DNA-protein complexes, and isolated chromatin and sheared. Subsequently the chromatin was immunoprecipitated with the anti-p65 antibody, purified the DNA, and subjected to real time PCR for quantification using MMP-9 or COX-2 promoter-specific primers. The predicted size of the DNA was found in the wild-type MEF cells (Fig. 8, A1 and A2). By using quantitative PCR, we found that the TNF induced NF-κB binding to MMP-9 and COX-2 promoters (Fig. 8, B1 and B2) isolated from wild-type MEF but not from GSK-3β-deleted cells. These results suggest that NF-κB bound in vivo to the regulatory portion of the MMP-9 and COX-2 promoter in wild-type MEF. A single amplification product was evident in the melting curve following the real time PCR, again confirming specificity of the assay for the MMP-9 and COX-2 promoters (Fig. 8, C1 and C2). Overall, these results also confirm the requirement of GSK-3β for the activation of NF-κB leading to expression of COX-2 and MMP-9.

**Deletion of GSK-3β Potentiates TNF-induced Apoptosis**—Activation of NF-κB has been shown to inhibit TNF-induced apoptosis (69–73). Our results suggest that deletion of GSK-3β might enhance apoptosis induced by TNF through suppression of NF-κB-regulated antiapoptotic gene products. Whether sup-
pression of NF-κB by GSK-3β deletion affects TNF-induced apoptosis was therefore investigated. MTT assay showed that TNF was cytotoxic to cells and that GSK-3β deletion enhanced that cytotoxicity (Fig. 9A). A Live and Dead assay indicated that GSK-3β deletion up-regulated TNF-induced apoptosis from 32.4 to 86.9% (Fig. 9B). Whether enhanced cytotoxicity was due to apoptosis was further investigated. Annexin V staining results showed that TNF-induced apoptosis was enhanced from 23.5% in the wild-type MEF to 87.2% in GSK-3β/− MEF (Fig. 9C). The PARP-cleavage assay showed that GSK-3β deletion potentiated TNF-induced caspase activity (Fig. 9D). All these assay results together suggest that GSK-3β deletion also potentiates TNF-induced apoptosis.

**DISCUSSION**

The goal of the study presented here was to investigate the role of GSK-3β in TNF signaling. Our findings show that TNF-induced NF-κB activation, IKK activation, IκBα phosphorylation, IκBα ubiquitination, IκBα degradation, and NF-κB reporter gene transcription were all suppressed in GSK-3β gene-deleted fibroblasts. NF-κB activation induced by LPS, IL-1β, or CSC was abrogated in GSK-3β/− fibroblasts. Deletion of GSK-3β also abolished TNF-induced JNK, p44/p42 MAPK, and Akt activation. TNF induced the expression of NF-κB-regulated gene products cyclin D1, COX-2, MMP-9, survivin, IAP 1, IAP 2, Bcl-xL, Bfl-1/A1, TRAF1, and FLIP and were all down-
modulated in the GSK-3β–/– fibroblasts, and this correlated with the potentiation of TNF-induced apoptosis.

Our results clearly show that deletion of the GSK-3β gene abolishes TNF-induced NF-κB activation. These results are in agreement with a previous report (18). Our results, however, appear to differ from those of Hoeflich et al. (18) in the mechanism of how GSK-3β suppresses TNF-induced NF-κB activation. They found that deletion of GSK-3β had no effect on TNF-induced IκBα degradation or on the nuclear translocation of p65, but they placed the defect downstream of IκBα phosphorylation and the nuclear translocation of NF-κB. Hoeflich et al. (18) also showed that IκBα resynthesis, which is under the control of NF-κB, is unchanged by deletion of GSK-3β, and suggested that partial inhibition of NF-κB DNA binding is not sufficient for down-regulation of the IκBα gene. In contrast, we found that GSK-3β is needed for activation of IκK, IκBα phosphorylation, IκBα degradation, and NF-κB-dependent gene expression. Similar to our results, the recent findings of Sanchez et al. (31) showed that TNF-induced NF-κB-dependent reporter activity, NF-κB-dependent COX-2 expression, and DNA binding of NF-κB are completely blocked in the GSK-3β mutant S9A in primary astrocytes. Also similar to our results were the findings of Sanchez et al. (31) showing that TNF-induced IKK activation and IκBα degradation are suppressed by the GSK-3β mutant S9A. Moreover, Sanchez et al. (31) showed that the N-terminal region of NEMO (also called IKK-γ), a subunit of IKK, physically interacts with GSK-3β S9A and competes for binding to IKK-α and IKK-β.

We found that deletion of GSK-3β abolishes the TNF-induced phosphorylation of p65. These results are in agreement with a report by Schwabe et al. (74) that recombiant GSK-3β phosphorlates p65 between residues 354 and 551.

We found that the TNF-induced JNK and p44/p42 MAPK activations are abolished on deletion of GSK-3β. Our results are also in agreement with those of Sanchez et al. (31) showing that GSK-3β-mediated apoptosis in primary astrocytes requires inhibition of NF-κB expression. Inhibition of GSK-3β inhibited Wnt-1-dependent NF-κB activation leading to inhibition of growth of PC-12 cells (86). All of these results indicate that GSK-3β has a prosurvival role. Our results demonstrate that this role is mediated through the expression of cyclin D1, survivin, IAP 1, IAP 2, Bcl-2, Bcl-1/A1, TRAF1, and cFLIP, and apoptosis (Fig. 10). Because of the critical role of TNF and NF-κB in cancer, inflammation, diabetes, and neurodegenerative diseases, inhibitors of GSK-3β have therapeutic potential in these diseases (87).

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Yasunari Takada, Xianjun Fang, Md. Saha Jamaluddin, Douglas D. Boyd and Bharat B. Aggarwal

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