A Pathway for Tumor Necrosis Factor-\(\alpha\)-induced Bcl10 Nuclear Translocation

Bcl10 is up-regulated by NF-\(\kappa\)B and phosphorylated by Akt1 and then complexes with Bcl3 to enter the nucleus*‡

Received for publication, October 11, 2005 Published, JBC Papers in Press, November 8, 2005, DOI 10.1074/jbc.M511014200

Pei Yen Yeh‡‡, Sung-Hsin Kuo‡§, Kun-Huei Yeh‡‡, Shuang-En Chuang‡, Chih-Hung Hsu‡‡‡, Wen Ching Chang‡, Hsin-I Lin‡‡, Ming Gao‡‡ and Ann-Lii Cheng‡‡‡‡,† and Kun-Huei Yeh‡‡, Chih-Hung Hsu‡‡‡, Wen Ching Chang‡, Hsin-I Lin‡‡, Ming Gao‡‡ and Ann-Lii Cheng‡‡‡‡,† From the ‡Cancer Research Center and the †Institute of Toxicology, College of Medicine, National Taiwan University, and the ‡§Internal Medicine, National Taiwan University Hospital, and the ‡‡Division of Cancer Research, National Health Research Institutes, Taipei 10016, Taiwan.

Bcl10 overexpression and nuclear translocation were originally identified in mucosa-associated lymphoid tissue lymphoma with t(11;18)(p15;q21) chromosome translocation. DNA amplification of Bcl10 was also found in other solid tumors. We have recently shown that nuclear translocation of Bcl10 is a specific molecular determinant of Helicobacter pylori-independent mucosa-associated lymphoid tissue lymphoma (Kuo, S.-H., Chen, L. T., Yeh, K.-H., Wu, M. S., Hsu, H. C., Yeh, P. Y., Mao, T. L., Chen, C. L., Doong, S. L., Lin, J. T., and Cheng, A.-L. (2004) J. Clin. Oncol. 22, 3491–3497). However, the molecular mechanism of Bcl10 nuclear translocation remains unknown. In this study, we observed that tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) up-regulates the expression of Bcl10 and induces a fraction of Bcl10 nuclear translocation in human breast carcinoma MCF7 cells. Chromatin immunoprecipitation assays and electrophoretic mobility shift assays indicated that an NF-\(\kappa\)B-binding site resides in the Bcl10 5’-untranslated region. This study also demonstrates that Akt1, activated by TNF\(\alpha\), phosphorylates Bcl10 at Ser218 and Ser231 and that phosphorylated Bcl10 subsequently complexes with Bcl3 to enter the nucleus. Either inhibition of Akt1 or depletion of Bcl3 blocks Bcl10 nuclear translocation. In summary, these findings characterize a molecular linkage that directs Bcl10 nuclear translocation in response to TNF\(\alpha\) treatment.

Bcl10 was cloned from the t(1;14)(p22;q32) breakpoint of mucosa-associated lymphoid tissue (MALT) lymphoma and is thought to be linked with progression of this disease (1, 2). Although most studies of Bcl10 have focused on lymphomas, DNA amplification of Bcl10 was also recently found in pancreatic cancer by genomic DNA chip hybridization, indicating that Bcl10 may have a more universal function in different types of tumors (3). Bcl10 normally resides in the cytoplasm to relay antigen receptor-mediated signals to activate NF-\(\kappa\)B of T and B cells (4). The pathway by which Bcl10 activates NF-\(\kappa\)B is not fully characterized; however, several factors have been shown to participate in this biological process. For example, Bcl10 forms a complex with MALT1 (another gene cloned from t(11;18)(q21;q21) of MALT lymphoma (5, 6)) to induce 1\(\kappa\)B kinase activation via TRAF6 ubiquitin ligase and TAK1 kinase (7). Bimp1 and Rip2 have also been reported to play a role in Bcl10-mediated NF-\(\kappa\)B activation (8, 9).

NF-\(\kappa\)B is involved in various cellular responses to extracellular stimuli via the regulation of numerous downstream gene expressions (10–13). Abnormal activation of NF-\(\kappa\)B has been found in different types of disease, including immune disorders and tumors. Although it is well established that NF-\(\kappa\)B is primarily regulated by its subcellular distribution, compelling data indicate that NF-\(\kappa\)B is subjected to multilevel regulation. For example, modification of NF-\(\kappa\)B (particularly phosphorylation and acetylation) may serve as another important mechanism to modulate the strength and duration of transactivating activity by altering the interaction between NF-\(\kappa\)B and transcriptional coactivators (14–16). In addition, rapidly accumulating data indicate that many distinct factors may indirectly regulate NF-\(\kappa\)B activity by acting on components of the NF-\(\kappa\)B signaling pathway.

Akt (also named protein kinase B) is a Ser/Thr kinase believed to transduce a survival signal via targeting various proteins, including NF-\(\kappa\)B (reviewed in Ref. 17). The pathway by which Akt activates the NF-\(\kappa\)B upstream 1\(\kappa\)B kinase (via phosphorylation of 1\(\kappa\)B kinase at Thr23) has been identified (18). Up-regulation of Akt activity is associated with different types of tumors such as ovarian, prostate, pancreatic, gastric, and breast cancers (reviewed in Refs. 19 and 20). Recently, sustained Akt and NF-\(\kappa\)B activity was also found to play a critical role in B cell survival and proliferation (21).

Bcl3 structurally belongs to the 1\(\kappa\)B family, but exerts functions distinct from other 1\(\kappa\)B proteins. Bcl3 is a nuclear protein and is not degraded in the cellular response to extracellular signals. Unlike other members of the 1\(\kappa\)B family, Bcl3 was found to enhance NF-\(\kappa\)B activity, primarily via forming a complex with the NF-\(\kappa\)B p50 homodimer to block the latter’s mediated suppression of target gene expression (22, 23).

We have recently demonstrated that nuclear expression of Bcl10 and NF-\(\kappa\)B is a highly reliable predictor of Helicobacter pylori independence of gastric MALT lymphoma (24), indicating that a close association exists between Bcl10 and NF-\(\kappa\)B and that nuclear translocation of Bcl10 is an important molecular event of this tumor. However, the molecular mechanism underlying this scenario remains unclear. In this study, we demonstrate that tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) increases Bcl10...
expression via the NF-κB signal transduction pathway. We also show that Akt1 activated by TNFα subsequently interacts with and phosphorylates Bcl10, leading to the formation of a Bcl10-Bcl3 complex that translocates into the nucleus.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Chemicals—Human breast carcinoma MCF7 cells were cultured in Dulbecco’s modified Eagles’ medium supplemented with 10% fetal calf serum and incubated in a humidified incubator with 5% CO₂ at 37 °C. The antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. Anti-Akt1 antibody was obtained from Pharmingen. TNFα was purchased from Calbiochem, and other chemicals were purchased from Calbiochem or Sigma.

Plasmid Construction, Site-directed Mutagenesis, and Gene Transfection—The full-length Bcl10 gene was amplified from MCF7 random-primed cDNA according to the sequence information deposited in the GenBank™ Data Bank (accession number NM_003921). The purified PCR product (either sense or antisense direction) was cloned into the bicistronic expression vector pIRES2-EGFP (Clontech) or was cloned in-frame into the pRSET vector (a His6-tagged Escherichia coli expression vector; Invitrogen). The antisense Bcl3 plasmid was constructed by amplifying a 524-bp fragment (positions 273–797) of the Bcl3 gene and cloning into the pCMV vector (Stratagene) in the reverse direction as described previously (25). The Bcl10 C-terminal 27-amino acid deletion mutant was constructed by digesting the Bcl10 gene with BglII and cloning into the pRSET vector. Substitution of Ser218 and/or Ser231 with Ala or Asp in Bcl10 by site-directed mutagenesis was performed by a two-round PCR method as described previously (26), and the mutant gene was cloned in-frame into the pRSET vector. All of the constructed genes were verified by DNA sequencing. Dominant-negative IkBα with an N-terminal 72-amino acid deletion and the NF-κB-driven luciferase reporter plasmid have been described previously (27). Gene transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were selected with 500 µg/ml G418 for 20 days. The surviving colonies were pooled and cultured in medium containing 500 µg/ml G418.

Immunoblotting, Immunoprecipitation, and Kinase Assay—Whole cell lysates and cytoplasmic and nuclear fractions were prepared as described previously (27). Aliquots (15 µg) of cell lysates and cytoplasmic and nuclear fractions were prepared as described previously. Following renaturing of agarose were selected with 500 ng/ml TNFα for the indicated times. The NF-κB DNA-binding activity was determined by EMSA. Supershift experiments were performed by adding anti-p65 or anti-actin antibody to confirm the presence of the NF-κB/DNA complex. The image was visualized after an overnight exposure.

kinase buffer containing 5 µCi of [32P]ATP at 30 °C for 30 min. The images were visualized by exposure to x-ray film.

NF-κB p65 Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed according to a protocol available at www.upstate.com with slight modification. Following TNFα treatment, the protein-DNA complex was cross-linked with 1% formaldehyde for 10 min at 37 °C. The cells were lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-Cl (pH 8.0)). The bulk DNA was sonicated to generate an average fragment size of ~1 kb. The lysates were at least 10-fold diluted with radiolabeled precipitation assay buffer containing protease inhibitors. After one round of protein A/G-agarose preclearing, the NF-κB p65-DNA complex was precipitated with 2 µg of anti-NF-κB p65 antibody and protein A/G-agarose. The complex was eluted with 1% SDS and 0.1 M NaHCO₃, and the cross-link was reversed by adjusting NaCl to 200 mM and incubating at 65 °C for 4 h. DNA was purified and used for PCR. The primer set used to amplify the putative NF-κB p65-binding sites in the Bcl10 or IkBα gene is shown in supplemental Fig. 1. PCR amplification of 18 S rDNA was used to determine the amount of DNA input.

Electrophoretic Mobility Shift Assay (EMSA) and NF-κB-driven Luciferase Activity Assay—The activation of NF-κB was determined by EMSA as described previously (27). The oligodeoxynucleotide probe derived from immunoglobulin (5'-GGATTGGGACTTTCCCCTCC), Bcl10 (5'-GCCCAAGGGCTCTCACTGAG), or IkBα (5'-GGCTGA-CAGGGAAGTACCGGG) genomic sequence was 32P-labeled and incubated with 10 µg of nuclear extract at room temperature for 30 min, and the electrophoretic mobility of the probe was then analyzed on 6% native polyacrylamide gel. The NF-κB/DNA complex was visualized by autoradiography. The identity and specificity of the NF-κB-DNA complex were determined by adding a selective antibody and an unlabeled mutated oligodeoxynucleotide probe (5'-GCCCAAGGGCTCTCACTGAG). NF-κB-driven luciferase activity assay was performed using a luciferase reporter gene assay kit (LucLite, PerkinElmer Life Sciences).

RESULTS

Up-regulation of Bcl10 by TNFα-induced NF-κB Activation—TNFα (a strong NF-κB inducer in various types of cells) was used to activate
NF-κB in this study. As evidenced by EMSA, TNFα activated NF-κB in MCF7 cells (Fig. 1). The NF-κB DNA-binding activity was quickly induced within 15 min of TNFα treatment and then returned to the basal level after 60 min of treatment. In a parallel experiment, the protein levels of Bcl10 and IκBα induced by TNFα in MCF7 cells were determined by Western blotting (Fig. 2). TNFα constitutively increased Bcl10 protein; in contrast, IκBα was degraded at 15 min after treatment and gradually returned to the basal level thereafter.

Because NF-κB is a transcription factor involved in the expression of numerous genes, it is reasonable to consider whether Bcl10 is a downstream gene of NF-κB. To explore this possibility, NF-κB ChIP was performed. The binding of NF-κB to the IκBα gene upstream region was characterized in parallel and served as a control. According to the NF-κB consensus binding sequence, GGGRNYYC (28), and the sequence information deposited in the GenBank™ Data Bank (accession number NM_003921 for Bcl10, accession number AL133163 for IκBα), NF-κB binds to Bcl-10 and IκBα genomic fragments. MCF7 cells were treated as indicated. ChIP was performed by immunoprecipitation of the NF-κB p65-DNA complex with anti-NF-κB p65 antibody. The fragments were PCR-amplified by a primer set specific for Bcl-10 or IκBα. EMSA was used to verify that the core sequence responsible for NF-κB binding resides in the Bcl10 or IκBα gene. NS, nonspecific binding. C, the specificity of the putative κB site of Bcl10 was confirmed by competition assay. A 10- or 50-fold concentration of unlabeled (Cold) wild-type (WT) or mutant (Mut) oligodeoxynucleotide probe was used in the competition assay. The mutated deoxynucleotides are underlined. D, the DNA fragments amplified in ChIP experiments (from the Bcl10 or IκBα gene) were cloned into a pLuc reporter plasmid (pBcl10-Luc or pIκBα-Luc, respectively). The plasmids (0.5 μg) were transfected into MCF7 cells alone or with a dominant-negative IκBα expression vector (1 μg; pBcl10-Luc/DN and pIκB-Luc/DN). At 36 h after transfection, the cells were treated with 10 ng/ml TNFα for 6 h, and then luciferase activity was determined. The reading of luciferase activity was normalized to protein concentration and the reading of the untreated control. The data were from two independent experiments and each has three wells; the S.D. values are shown. E, TNFα induces time course-dependent Bcl10 and IκBα expression. The expression of Bcl10 and IκBα induced by TNFα was determined by reverse transcription (RT)-PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of total RNA input. F, dominant-negative IκBα inhibits TNFα-induced Bcl10 expression. To confirm that NF-κB transactivates Bcl10 expression, MCF7 cells were transfected with dominant-negative IκBα (MCF/DN cells). Following TNFα treatment, the protein level of Bcl10 was determined by immunoblotting (IB). The membrane was reprobed with anti-actin antibody as a loading control.
Activation of NF-κB via Akt1/Bcl10/Bcl3 by TNFα

IκBα genomic sequence, and accession number BC004983 for IκBα mRNA sequence), a Bcl10 5′-untranslated region was expected to be an NF-κB-binding site. The region in the IκBα genome for NF-κB targeting has been mapped previously (29). The relevant sequence information regarding the genomic regions of Bcl10 and IκBα for NF-κB binding is shown in supplemental Fig. 1. The results of p65 ChIP assay indicated that TNFα induced a strong but shorter binding of p65 to the Bcl10 genomic fragment; in contrast, p65 bound to the IκBα genomic fragment for a longer period (Fig. 3A). EMSA was used to demonstrate that the core sequence (gccccggcttaccttag) residing in the 5′-upstream region (positions -270 to -250) of the Bcl10 gene was responsible for NF-κB binding. The binding of NF-κB to the κB site of IκBα was also verified (Fig. 3B). The specificity of NF-κB binding to this κB site of Bcl10 was further confirmed by competition assays with excess unlabeled wild-type or mutated oligodeoxynucleotide probe. As shown in Fig. 3C, excess wild-type probe decreased both the NF-κB-DNA complex and a nonspecific binding complex, whereas the mutated probe decreased the nonspecific binding complex only and had no effect on the NF-κB-DNA complex. To characterize whether this putative κB site of Bcl10 functions as a cis-acting element, the DNA fragments amplified from ChIP experiments (from the Bcl10 or IκBα gene) were cloned into a pLUC reporter plasmid (Stratagene), yielding pBcl10-Luc and pIKB-Luc, respectively. The reporter plasmid pBcl10-Luc was transfected alone or with a dominant-negative IκBα expression vector into MCF7 cells, and then TNFα-induced luciferase activity was assayed in these cells. As shown in Fig. 3D, although TNFα induced luciferase activity in pBcl10-Luc-transfected cells, dominant-negative IκBα suppressed basal as well as TNFα-induced luciferase activities. Similar results were obtained with plκB-Luc-transfected cells. Furthermore, reverse transcription-PCR showed that TNFα induced a marked increase in the RNA level of Bcl10 at 15 min after treatment. In contrast, TNFα induced a later but more persistent increase in IκBα RNA (Fig. 3E). The function of NF-κB in Bcl10 expression was further characterized in dominant-negative IκBα-transfected MCF7 cells (referred to as MCF/DN cells), in which TNFα-induced NF-κB activity was markedly reduced (data not shown). As expected, up-regulation of Bcl10 by TNFα was suppressed in MCF/DN cells (Fig. 3F).

FIGURE 4. TNFα induces Bcl10 nuclear translocation. MCF7 cells were treated with 10 ng/ml TNFα for the indicated times and then subjected to cell fractionation. Nuclear extracts (15 μg) were immunoblotted (IB) with anti-Bcl10 antibody. As shown in Fig. 4, dominant-negative IκBα suppressed the TNFα-induced nuclear translocation of Bcl10.

FIGURE 5. A, TNFα induces Akt1 activation. MCF7 cells were treated as indicated. Whole cell lysates (15 μg) were subjected to immunoblotting (IB) using anti-phospho-Akt1 (pAkt1) antibody to demonstrate Akt1 activation. The membrane was reprobed with anti-Akt1 antibody to show the total amount of protein. B, Bcl10 directly interacts with Akt1. Akt1 or Bcl10 was immunoprecipitated (IP) from whole cell lysates (150 μg) of TNFα-treated MCF7 cells. The interaction between Bcl10 and Akt1 was characterized by immunoblotting of the reciprocal immunoprecipitated complex.

FIGURE 6. A, the Bcl10 C-terminal 27 amino acids are responsible for Akt1 binding. To identify the region of Bcl10 involved in Akt1 targeting, a Bcl10 C-terminal 27-amino acid deletion mutant was constructed. Both wild-type and mutant Bcl10 genes were cloned in-frame into a His6-tagged expression vector. E. coli-expressed Bcl10 protein was adsorbed to nickel-agarose beads and was used for pull-down experiments. Whole cell lysates (150 μg) were prepared from control cells or 30-min 10 ng/ml TNFα-treated MCF7 cells. The pull-down complex was subjected to immunoblotting (IB) with antibodies against Akt1, the Bcl10 N terminus (Bcl10-N), and the Bcl10 C terminus (Bcl10-C). B, Akt1 phosphorylates Bcl10 at Ser218 and Ser231. To identify the phosphorylation site of Bcl10 for Akt1, Ser218 and Ser231 were mutated to Ala, either alone or together. The mutant gene was cloned in-frame into a His6-tagged expression vector. Akt1 was immunoprecipitated from 30-min 10 ng/ml TNFα-treated MCF7 cells. Immunoprecipitated complex kinase assay (Akt1 IKK) was performed using purified E. coli-expressed wild-type (WT) or mutant proteins as exogenous substrates. Aliquots of purified proteins on SDS-polyacrylamide gel were stained with Coomassie Blue to show the amount of protein input. C, TNFα induces multiple phosphorylation of Bcl10. Whole cell lysates were prepared from 30-min 10 ng/ml TNFα-treated MCF7 cells. Wild-type or mutant Bcl10 protein pull-down experiments were performed. The pull-down complex was subjected to the kinase reaction. D, in-gel kinase analysis of the Bcl10 pull-down complex. The wild-type or mutant Bcl10 pull-down complex from 30-min 10 ng/ml TNFα-treated MCF7 whole cell lysates was subjected to in-gel kinase assay with or without wild-type Bcl10 protein embedded in the gel.
TNFα Induces Bcl10 Nuclear Translocation—We next investigated whether TNFα induces Bcl10 nuclear translocation in MCF7 cells. As shown in Fig. 4, a sustained increase in nuclear Bcl10 was observed along the time course of TNFα treatment. The typically dynamic change in nuclear NF-κB p65 induced by TNFα was characterized in parallel and served as a control.

Akt1 Phosphorylates the Bcl10 C-terminal Ser218 and Ser231 Residues—To explore the possible mechanism of Bcl10 nuclear translocation, we first investigated whether TNFα induces Bcl10 phosphorylation. Using Bcl10-co-immunoprecipitated complex kinase assay, we found that Bcl10 was phosphorylated after TNFα treatment and also observed that several phosphorylated proteins coprecipitated with Bcl10. The molecular mass of one of them was close to that of Akt1 kinase (data not shown). Further studies indicated that TNFα induced Akt1 activation in MCF7 cells (Fig. 5A). Whether Akt1 was responsible for Bcl10 phosphorylation after TNFα treatment was therefore explored. The possible interaction between Bcl10 and NF-κB p65 was also determined. E, phosphorylation of Bcl10 at Ser218 and Ser231 alters its affinity for Bcl3. To explore the biological significance of Bcl10 Ser218 and Ser231 phosphorylation, mutations of these two sites, either alone or together, to aspartic acid was performed by site-directed mutagenesis. Wild-type (WT) or mutant Bcl10 was cloned into a His6-tagged expression vector. Wild-type or mutant Bcl10 or the C-terminal 27-amino acid deletion mutant (Bcl10ΔC) was pulled down from 1-h 10 ng/ml TNFα-treated MCF7 cells. The existence of Bcl3 protein was identified by immunoblotting. Bcl10 was probed to show the total protein input.

FIGURE 7. A, Akt1 activity is involved in TNFα-induced Bcl10 nuclear translocation. MCF7 cells were treated with 10 ng/ml TNFα for 0–1 h with or without a 1-h pretreatment with 10 μM LY294002 as indicated. The nuclear lysates (15 μg) were subjected to immunoblotting (IB) with anti-Bcl10 antibody. Western blotting of proliferating cell nuclear antigen (PCNA) served as a loading control. B, Akt1 activity is involved in TNFα-induced Bcl3 nuclear translocation. MCF7 cells were treated as indicated. The nuclear lysates (15 μg) were subjected to immunoblotting with anti-Bcl3 antibody. C, Akt1 triggers Bcl10-Bcl3 complex formation. The Bcl3-co-immunoprecipitated complex was isolated from whole lysates prepared from MCF7 cells with the indicated treatments. Immunoblotting (IB) of Bcl3 and Bcl10 in the complex was performed. D, the Bcl10-Bcl3 complex exists in the nucleus. MCF7 cells were treated with 10 ng/ml TNFα for 0–1 h, and nuclear lysates were prepared. Bcl10 and Bcl3 reciprocal immunoprecipitation, followed by immunoblotting, was performed. The possible interaction between Bcl10 and NF-κB p65 was also determined. E, phosphorylation of Bcl10 at Ser218 and Ser231 alters its affinity for Bcl3. To explore the biological significance of Bcl10 Ser218 and Ser231 phosphorylation, mutations of these two sites, either alone or together, to aspartic acid was performed by site-directed mutagenesis. Wild-type (WT) or mutant Bcl10 was cloned into a His6-tagged expression vector. Wild-type or mutant Bcl10 or the C-terminal 27-amino acid deletion mutant (Bcl10ΔC) was pulled down from 1-h 10 ng/ml TNFα-treated MCF7 cells. The existence of Bcl3 protein was identified by immunoblotting. Bcl10 was probed to show the total protein input.
mutant could not bind to Akt1. To identify the phosphorylation sites of Bcl10 for Akt1, phosphorylation site analysis of this 27-amino acid peptide was performed using NetPhos Version 2.0 (developed by the Technical University of Denmark, available at www.cbs.dtu.dk). Two Ser residues (Ser²¹⁸ and Ser²³¹) were predicted to be the most probable sites for phosphorylation (data not shown). The mutant Bcl10 gene with these two Ser residues substituted with Ala, either alone or together, was therefore constructed in the His₉-tagged pRSET vector. E. coli-expressed wild-type or mutant proteins were used as exogenous substrates in Akt1-immunoprecipitated complex kinase assay. The results indicate that Akt1 could not phosphorylate Bcl10 with the Ser²¹⁸/Ser²³¹ double mutation (Fig. 6B). On the other hand, nickel-agarose-Bcl10 pull-down kinase assay showed a similar level of Bcl10 phosphorylation regardless of whether wild-type or mutant proteins were used (Fig. 6C), suggesting that Bcl10 is subjected to multiple phosphorylation and that Akt1 is not the only kinase targeting Bcl10. Autophosphorylation of Akt1 has been reported previously (31) and further confirmed by in-gel kinase assay in this study. As shown in Fig. 6D, the wild-type or mutant Bcl10 pull-down complex was subjected to in-gel kinase assay with or without wild-type Bcl10 embedded in the gel, and an autophosphorylated protein with a molecular mass corresponding to that of Akt1 was observed in all pull-down complexes.

Ser²¹⁸ and Ser²³¹-phosphorylated Bcl10 Complexes with Bcl3 to Enter the Nucleus—The function of Akt1 in Bcl10 nuclear translocation was further characterized by applying the Akt1 inhibitor LY294002 to suppress TNFα-induced Akt1 activity. As shown in Fig. 7A, LY294002 pretreatment blocked TNFα-induced Bcl10 nuclear translocation, suggesting that Akt1 activity is necessary for Bcl10 nuclear translocation. Close examination of the molecular features of Bcl10 suggested that it does not have a nuclear localization signal (data not shown). Thus, the association of Bcl10 with an uncertain nuclear protein might be necessary for its nuclear translocation.

To search for the possible nuclear protein involved in Bcl10 nuclear translocation, Bcl3 (a unique IkB family protein) was selected for further studies. This was based on several previous findings, including the up-regulation of Bcl3 by NF-κB and the involvement of Bcl3 in NF-κB downstream gene expression (32, 33). In particular, expression of Bcl3 is associated with cytokine-independent growth of lymphocytes (34) and hormone independence of breast carcinoma (35). These findings for Bcl3 are very similar to our previous observations for Bcl10. We first characterized whether TNFα induces Bcl3 nuclear translocation. As shown in Fig. 7B, nuclear translocation of Bcl3 was induced by TNFα and was suppressed by pretreatment with LY294002. Whether Bcl3 is involved in Bcl10 nuclear translocation was first investigated by Western blotting of the Bcl3-co-immunoprecipitated complex from whole cell lysates. As shown in Fig. 7C, increased Bcl10 was associated with Bcl3 after TNFα treatment, and this interaction was interrupted by LY294002 pretreatment. Next, reciprocal co-immunoprecipitation, followed by Western blotting, demonstrated that the Bcl10-Bcl3 complex existed in the nucleus after TNFα treatment. TNFα induces marked NF-κB p65 nuclear translocation. Therefore, the possible interaction between Bcl10 and NF-κB p65 after TNFα treatment was also examined. No Bcl10-NF-κB p65 complex could be detected by reciprocal immunoprecipitation and Western blotting (Fig. 7D), suggesting that Bcl10 specifically interacts with Bcl3. In addition, Bcl10 and Bcl3 were found to colocalize in the TNFα-treated cells by double immunofluorescence staining (data not shown).

To further confirm that phosphorylation of Bcl10 at Ser²¹⁸ and Ser²³¹ is necessary for the formation of the Bcl10-Bcl3 complex, these two residues were mutated to aspartic acid, either alone or together, to mimic their phosphorylation. Wild-type and mutant proteins were used in pull-down experiments. As shown in Fig. 7E, Bcl10 with the Ser²¹⁸/Ser²³¹ double mutation or with the C-terminal deletion exhibited high affinity for Bcl3. The latter finding might support the previous observation that C-terminally truncated Bcl10 exhibits nuclear distribution (30).

The role of Bcl3 in Bcl10 nuclear translocation was further demonstrated using the antisense Bcl3 gene. Transfection of antisense Bcl3 effectively inhibited Bcl3 expression of MCF7 cells (Fig. 8A) and blocked TNFα-induced Bcl10 nuclear translocation (Fig. 8B).

Bcl10 Is Involved in TNFα-induced NF-κB Transcriptional Activity—We previously reported that nuclear expression of Bcl10 and NF-κB is a tightly associated phenomenon in H. pylori-independent MALT lymphoma (24). We also found that myc, an NF-κB downstream gene, is overexpressed in these MALT lymphomas.3 To explore the possibility that Bcl10 might be involved in the NF-κB downstream signaling pathway, MCF/AsBcl10 cells were established by transfection of the antisense Bcl10 gene into MCF7 cells, in which expression of Bcl10 is blocked (Fig. 9A). TNFα-induced myc expression in cells with different treatments was determined by Western blotting. As shown in Fig. 9B, LY294002 pretreatment inhibited TNFα-induced myc expression. The Myc protein level in antisense Bcl3-, antisense Bcl10-, or dominant-negative IkBα-transfected cells was also markedly decreased (Fig. 9C). The involvement of Bcl10 in TNFα-induced NF-κB activity was further confirmed by NF-κB-driven luciferase assay. As shown in Fig. 10, TNFα-induced luciferase activity was suppressed in MCF/AsBcl3, MCF/AsBcl10, and MCF/DN cells.

A recent study suggested that Bcl10 might function as a transcription factor (36). We therefore explored whether Bcl10 or Bcl3 can act as a coactivator of NF-κB by EMSA. As shown in Fig. 11A, depletion of Bcl3 or Bcl10 did not affect TNFα-induced NF-κB DNA-binding activity. In addition, neither Bcl3 nor Bcl10 was found in the NF-κB-DNA complex in supershift experiments (Fig. 11B), indicating that Bcl10 is not a component of the transcription factor for the κB site.

3 S.-H. Kuo and A.-L. Cheng, unpublished data.
DISCUSSION

Bcl10 nuclear translocation has been observed in MALT lymphoma with a t(1;14)(p22;q32) or t(11;18)(q21;q21) chromosome aberration. Expression of nuclear Bcl10 is associated with the progression of MALT lymphoma, particularly with the development of H. pylori independence (2). However, our recent study indicates that Bcl10 nuclear translocation exists in a fraction of MALT lymphoma without these chromosome translocations and can still serve as a reliable predictor of H. pylori-independent status (24), suggesting the existence of an unknown molecular mechanism and biological function of Bcl10 nuclear translocation. In this study, we have characterized a molecular linkage that directs Bcl10 nuclear translocation in response to TNFα treatment. We have provided evidence that TNFα activates NF-κB and Akt1 in MCF7 cells; the former up-regulates Bcl10, and the latter phosphorylates Bcl10, leading to the formation of a Bcl10-Bcl3 complex, which translocates into the nucleus. Our results also suggest that nuclear Bcl10 might be involved in the TNFα-activated NF-κB signaling transduction pathway.

Autoregulation of a signal transduction molecule (either positive or negative) is an important mechanism to enhance or terminate its signaling pathway. This study has provided evidence that, in TNFα-treated MCF7 cells, NF-κB up-regulates Bcl10 expression via a κB site residing in the Bcl10 5′-untranslated region. The increased Bcl10 might affect NF-κB downstream gene expression, as indicated by the finding that depletion of Bcl10 reduces TNFα-induced myc gene expression and NF-κB-driven reporter activity. On the other hand, we have also shown that TNFα induces IκBα expression, which is well documented to provide negative feedback regulation of NF-κB (13). How these two opposite pathways could be precisely regulated remains unknown, but malfunction of either one of them may result in abnormal NF-κB activation.

In this study, we found that phosphorylation of Bcl10 at Ser218 and Ser231 or deletion of the C-terminal 27 amino acids may change the conformation of Bcl10 and expose its Bcl3-binding motif, although this binding region remains to be identified. Accumulating data suggest that protein phosphorylation is an important mechanism in the regulation of protein-protein interaction. For example, Zhong et al. (16) reported that phosphorylation of NF-κB may determine its association with CBP (GAMP-responsive element-binding protein-binding protein)/p300 or histone deacetylase-1. Yui et al. (37) found that differential interaction of Bcl10 with TRAF2 or cIAP is regulated by the phosphorylation status of Bcl10. The interaction of Bcl3 with NF-κB p50 was also reported to be determined by the phosphorylation of Bcl3 (38).

Bcl10 has been shown to interact with many proteins, such as Carm1 (39), MALT1 (40), Bimp1 (8), and Rip2 (9). In this study, we shown that, in response to TNFα treatment of MCF7 cells, Bcl10 is phosphorylated at C-terminal residues Ser218 and Ser231 by Akt1 and subsequently forms a complex with Bcl3 to enter the nucleus. On the other hand, the results from Bcl10 pull-down kinase assay suggest that Akt1 is not the only kinase targeting Bcl10 and that TNFα induces multiple phosphorylation of Bcl10. Furthermore, Bcl10 pull-down in-gel kinase assay

FIGURE 9. A, TNFα-induced Bcl10 expression was suppressed by the antisense Bcl10 gene. MCF7 cells were transfected with the antisense Bcl10 gene (MCF/AsBcl10 cells). The inhibitory effect of the antisense Bcl10 gene on the expression of Bcl10 induced by TNFα was determined by Western blotting. The membrane was reprobed with anti-actin antibody as a loading control. Bcl10 determined by immunoblotting (IB). The membrane was reprobed with anti-actin antibody to show the total protein load.

FIGURE 10. NF-κB-driven luciferase activity assay. Plasmid pxB-Luc was transfected into MCF7, MCF/AsBcl3, MCF/AsBcl10, and MCF/DN cells. At 36 h after transfection, the cells were treated with 10 ng/ml TNFα for 6 h, and NF-κB-driven luciferase activity was determined. The reading of luciferase activity was normalized to protein concentration and the reading of the untreated control. The data are from two independent experiments and each has three wells; the S.D. values are shown.

Activation of NF-κB via Akt1/Bcl10/Bcl3 by TNFα
The results of this study suggest that Bcl10 is involved in the NF-κB tumor development. The mechanism by which the concentration of nuclear Bcl10 is controlled remains unclear. A previous study revealed that the concentration of nuclear Bcl10 and Bcl3 might enhance NF-κB activity. Given that various cytokines, including TNFα, are the downstream genes of NF-κB and that NF-κB is a mediator linking inflammation and tumor formation (43), the elevation of NF-κB activity may be responsible for inflammation-associated tumor development and extracellular stimulus-independent tumor progression.

In summary, we have provided evidence to elucidate a TNFα-induced pathway by which Bcl10 is up-regulated and translocated into the nucleus. Whether our findings in MCF7 cells may be applied to other types of cells, such as MALT lymphoma and inflammation-associated tumors, should be further investigated.

REFERENCES

1. Willis, T. G., Jadayel, D. M., Du, M. Q., Peng, H., Perry, A. R., Abdul-Rauf, M., Price, H., Karran, L., Majekodunmi, O., Wlodarska, I., Pan, L., Crook, T., Hamoudi, R., Isaacson, P. G., and Dyer, M. J. (1999) Cell 96, 35–45

2. Issacson, P. G., and Du, M. Q. (2004) Nat. Rev. Cancer 4, 644–653

3. Hostmann, K., Kohlbammer, H., Schwaneck, C., Wessendorf, S., Kestler, H. A., Schwoerer, A., Rau, B., Radlwimmer, B., Dohner, H., Lichter, P., Gress, T., and Bentz, M. (2004) Cancer Res. 64, 4428–4433

4. Ruland, J., Duncan, E. G., Elia, A., del Barco Barrantes, I., Nguyen, L., Phyte, S., Millar, D. G., Bouchard, D., Watcham, A., Oghashi, P. S., and Mak, T. W. (2001) Cell 104, 33–42

5. Akagi, T., Motegi, M., Tamura, A., Suzuki, R., Hosokawa, Y., Suzuki, H., Otta, H., Nakamura, S., Morishima, Y., Taniwaki, M., and Seito, M. (1999) Oncogene 18, 5785–5794

6. Dierlam, J., Bars, M., Wlodarska, I., Stefanova-Ouzounova, M., Hernandez, M., Hossfeld, D. K., De Wolf-Peeters, C., Hagemeijer, A., Van den Bergh, H., and Maryn, P. (1999) Blood 93, 3601–3609

7. Sun, L., Deng, L., Xu, C. K., Xie, Z. P., and Chen, Z. J. (2004) J. Biol. Chem. 279, 22,607–22,614

8. Ellefson, M., and Woodgett, J. R. (2001) J. Biol. Chem. 276, 30589–30597

9. Ruefl-Brasse, A. A., Lee, W. P., Hurst, S., and Dixit, V. M. (2004) J. Biol. Chem. 279, 1570–1574

10. Dixit, V., and Mak, T. W. (2002) Cell 111, 615–619

11. Ghosh, S., and Karin, M. (2002) Cell 109, 581–596

12. Aggarwal, B. B. (2004) Cancer Cell 6, 203–208

13. Chen, L. F., and Greene, W. C. (2004) Nat. Rev. Mol. Cell. Biol. 5, 392–401

14. Webster, G. A., and Perkins, N. D. (1999) Mol. Cell. Biol. 19, 3485–3495

15. Zhong, H., Voll, R. E., and Ghosh, S. (1999) Mol. Cell. Biol. 19, 661–671

16. Zhong, H., Hoy, M., and Ghosh, S. (2002) Mol. Cell. Biol. 9, 625–636

17. Nicholson, K. M., and Anderson, N. G. (2002) Cell. Signal. 14, 381–395

18. Romankova, J. A., and Makarov, S. V. (1999) Nature 401, 86–90

19. Brazil, D. P., and Hemmings, B. A. (2001) Trends Biochem. Sci. 26, 657–664

20. Scheid, M. P., and Woodgett, J. R. (2001) Nat. Rev. Mol. Cell. Biol. 2, 760–768

21. Sun, S., Perez-Aceijo, P., Perez-Chacon, V., Vargas, J. A., Sanchez, A., Martin-Saavedra, F. M., Ballester, S., Garcia-Marcos, J., Jordá, J., and Duráñez, A. (2004) Leukemia (Basingstoke) 18, 1391–1400

22. Mitchell, T. C., Thompson, B. S., Trent, J. O., and Casella, C. R. (2002) Ann. N. Y. Acad. Sci. 975, 132–147

23. Thornburg, N. J., Pashnathanon, R., and Raab-Traub, N. (2003) Cancer Res. 63, 8293–8301

24. Kuo, S.-H., Chen, L. T., Yeh, K.-H., Wu, M. S., Hsu, H. C., Yeh, P. Y., Mao, T. L., Chen, C. L., Doong, S. L., Lin, J. T., and Cheng, A.-L. (2004) J. Clin. Oncol. 22, 3491–3497

25. Brasier, A. R., Lu, M., Hai, T., Lu, Y., and Boldogh, I. (2001) J. Biol. Chem. 276.
Activation of NF-κB via Akt1/Bcl10/Bcl3 by TNFα
A Pathway for Tumor Necrosis Factor-α-induced Bcl10 Nuclear Translocation: Bcl10 IS UP-REGULATED BY NF-κB AND PHOSPHORYLATED BY Akt1 AND THEN COMPLEXES WITH Bcl3 TO ENTER THE NUCLEUS

Pei Yen Yeh, Sung-Hsin Kuo, Kun-Huei Yeh, Shuang-En Chuang, Chih-Hung Hsu, Wen Ching Chang, Hsin-I Lin, Ming Gao and Ann-Lii Cheng

J. Biol. Chem. 2006, 281:167-175.
doi: 10.1074/jbc.M511014200 originally published online November 8, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M511014200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2005/11/17/M511014200.DC1

This article cites 43 references, 17 of which can be accessed free at
http://www.jbc.org/content/281/1/167.full.html#ref-list-1