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c-Myc Sensitizes Cells to Tumor Necrosis Factor-mediated Apoptosis by Inhibiting Nuclear Factor κB Transactivation*

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Nuclear factor κB (NF-κB) plays a key role in suppression of tumor necrosis factor (TNF)-mediated apoptosis by inducing a variety of anti-apoptotic genes. Expression of c-Myc has been shown to sensitize cells to TNF-mediated apoptosis by inhibiting NF-κB activation. However, the precise step in the NF-κB signaling pathway and apoptosis modified by c-Myc has not been identified. Using the inducible c-MycER system and c-Myc null fibroblasts, we found that expression of c-Myc inhibited NF-κB activation by interfering with RelA/p65 transactivation but not nuclear translocation of NF-κB. Activation of c-Myc promoted TNF-induced release of cytochrome *c* from mitochondria to the cytosol because of the inhibition of NF-κB. Furthermore, we found that NF-κB-inducible gene A1 was attenuated by expression of c-Myc and that the restoration of A1 expression suppressed c-Myc-induced TNF sensitization. Our results elucidate the molecular mechanisms by which c-Myc increases cell susceptibility to TNF-mediated apoptosis, indicating that c-Myc may exhibit its pro-apoptotic activities by repression of cell survival genes.

Tumor necrosis factor (TNF)[1] is a pleiotropic cytokine that plays an important role in apoptosis, inflammation, and cell growth (1). The engagement of TNF receptor 1 by TNF recruits a TNF receptor-associated death domain containing protein (TRADD). TRADD plays a key role in two important signaling pathways: the caspase cascade and nuclear factor κB (NF-κB) activation (2). Through interaction with RIP, TRAF2, TRADD activates NF-κB to promote cell growth and cell survival (2, 3). Conversely, TRADD recruits both Fas-associated death domain containing protein and caspase-8 to induce apoptotic caspase cascade under inhibition of the NF-κB signaling pathway (1, 2).

NF-κB is initially identified and named for its role in the control of immunoglobin κ-chain gene expression in B lymphocytes (4–6). The primary form of NF-κB is a heterodimer consisting of a DNA-binding subunit (p50) and a transactivation subunit (RelA/p65). In most unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB family proteins. Upon stimulation by TNF, IκB kinase complex (IKK) is activated, resulting in the phosphorylation of IκB on two conserved N-terminal serine residues (7, 8). The phosphorylated IκB is ubiquitinated and subsequently degraded by the 26 S proteasome pathway, liberating NF-κB to the nucleus where it activates gene expression (6). Although the induction of nuclear translocation of NF-κB is considered to be an important step for NF-κB-mediated gene transcription, recently, growing evidence has shown that NF-κB activity is also regulated by other mechanisms (9–14). Zhong *et al.* (13) found that lipopolysaccharide stimulated protein kinase A-dependent phosphorylation of p65 and subsequently recruited the transcription co-activators CREB-binding protein/p300 to potentiate NF-κB transcriptional activation. Wang *et al.* (11) demonstrated that TNF induced the phosphorylation of p65 transactivation domain at serine 529 by casein kinase II. This phosphorylation significantly increased the NF-κB transactivation potential. Recently, we and others found that cell survival kinase Akt stimulated NF-κB transcription activities by modulation of the NF-κB transactivation domain, indicating that the NF-κB pathway played an important role in Akt-mediated cell survival (10). In contrast, pro-apoptotic p53 protein had been found to inhibit RelA/p65-dependent transactivation without altering RelA expression or inducible κB-DNA binding. p53-mediated repression of RelA activity was caused by competition for a limiting pool of the transcriptional co-activator protein p300 and cAMP-response element-binding protein-binding protein *in vivo* (12). Additionally, very recently, RelA/p65 activities had been found to be regulated by acetylation and deacetylation (9, 15).

Cell susceptibility to TNF-mediated apoptosis has been found to be regulated by cytokines, growth factors, viral infection, and oncogenes. Because of its potential usage as a cancer therapy agent, cell sensitivity to TNF has been an important issue under intense investigation. We and others found that NF-κB activation played a critical role in the modification of TNF sensitivity (16–19). Several important anti-apoptotic genes were found to be dependent on NF-κB transcription. These molecules included inhibitors of apoptosis family proteins (IAP), TNF receptor-associated factor family proteins, IEX-1L, c-FLIP, NF-κB-inducible death effector domain-containing protein, and the Bcl-2 family members A1 and Bcl-X_L (20–27). TNF sensitivity can be induced in most types of cells by blocking *de novo* protein synthesis or inhibition of NF-κB activation (19). Also, several lines of evidence have demonstrated that cell sensitivity to TNF killing is also regulated by oncogenes and viral infection (28–31). For example, the adeno-

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[1] The abbreviations used are: TNF, tumor necrosis factor; EGFP, enhanced green fluorescence protein; IKK, IκB kinase; NF-κB, nuclear factor κB; SR-IκBα, super-repressor of IκBα; TRADD, TNF receptor-associated death domain-containing protein; OHT, 4-hydroxytamoxifen; IAP, inhibitor of apoptosis protein; CREB, cAMP-response element-binding protein.

virus E1B 19K protein had been found to inhibit TNF-mediated apoptosis by inhibiting the Bax conformation change and interrupted caspase activation downstream of caspase-8 and upstream of caspase-9 (30). On the contrary, tumor cells expressing the adenovirus E1A protein were sensitive to TNF killing in absence of a protein synthesis inhibitor. Studies by Shao *et al.* (31) found that inhibition of IKK activity by E1A was an important mechanism for E1A-mediated sensitization.

c-Myc is another molecule that modifies cell sensitivity to TNF-mediated apoptosis (28, 29, 32). c-Myc is highly expressed in over 30% of human tumors and paradoxically plays an important role in both cell proliferation and apoptosis (33–36). Following serum deprivation or growth factor withdrawal, c-Myc can induce or sensitize cells to apoptosis by inducing cytochrome *c* release that is independent of caspase activation (37). Interestingly, several studies also reveal that there is a regulatory connection between c-Myc and cell death receptor-mediated apoptosis (28, 29, 38). Hueber *et al.* (38) demonstrated that the CD95/Fas signaling pathway was required for c-Myc-induced apoptosis under serum deprivation. On the other hand, c-Myc expression rendered cells sensitive to TNF-mediated apoptosis in the absence of protein synthesis inhibitor, suggesting that c-Myc inhibits the expression of TNF-inducible anti-apoptotic genes (28, 29, 32). Studies by Klefstrom *et al.* (29) suggested that induction of TNF sensitivity by c-Myc was involved in NF-κB activation. However, the precise step in the NF-κB signaling pathway and apoptosis regulated by c-Myc has not been identified.

In the current study, utilizing the inducible c-MycER system and c-Myc null fibroblasts, we found that the activation of c-Myc inhibited NF-κB transcription by predominantly interfering with the NF-κB transactivation potential but not the nuclear translocation of NF-κB. We found that activation of c-Myc potentiated TNF-induced cytochrome *c* release because of inhibition of NF-κB. The results provide new insight into the molecular mechanisms of c-Myc-mediated TNF sensitization.

EXPERIMENTAL PROCEDURES

Cell Culture and Retroviral Infection—Human squamous cell carcinoma cell line KB and Rat-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml). HT1080 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and hygromycin B (200 μg/ml). To establish cell lines stably expressing c-MycER, a retrovirus expression system was utilized, allowing whole populations of cells to be examined with minimal expansion in culture. The retroviruses were generated by transfecting the retroviral construct encoding c-MycER into 293T cells by the calcium phosphate method. Retrovirus-containing supernatant was collected 48 h later and stored in −70 °C. The cells were infected with retroviruses in the presence of 6 μg/ml polybrene. Forty-eight h after infection, the cells were selected with puromycin (1.5 μg/ml) for 1 week. The resistant clones were pooled and confirmed by Western blot analysis.

Western Blot Analysis—Cells were collected, washed with ice-cold phosphate-buffered saline, and pelleted. Whole cell lysates were extracted with RIPA buffer containing 1% Nonidet P-40, 5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, and 1:100 protease inhibitors mixture (Sigma-Aldrich). The protein concentrations were measured using the Bradford protein assay (Bio-Rad). The protein extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane by electroblotting (Bio-Rad). The membranes were blocked with 5% nonfat dry milk and 1× TBST (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20) overnight and probed with primary antibodies for 1 h and then horseradish peroxidase-conjungated secondary antibodies for 1 h. The results were visualized using the ECL kit (Amersham Biosciences) according to the manufacturer's instructions. For internal control, the blots were stripped with Tris buffer (62.5 mM, pH 8) containing 100 mM 2-mercaptoethanol and 2% SDS at 60 °C for 1 h and reprobed with α-tubulin. Primary antibodies were purchased from the following commercial sources: monoclonal antibodies against human caspase 8 (1:1000) and cytochrome *c* (1:1000) from Pharmingen; monoclonal antibodies against α-tubulin (1:7500) from Sigma; monoclonal antibodies against c-Myc (1:500) from Santa Cruz; and secondary antibodies against rabbit or mouse IgG (1:7500) from Promega.

Cell Viability and DNA Fragmentation—The cells were pretreated with OHT (100 nM) and then treated with TNF for 48 h. The detached and attached cells were collected, and cell viability was determined by trypan blue exclusion. To examine DNA laddering, the attached and detached cells were collected at the indicated time points following OHT and TNF treatment. DNA was isolated and separated on a 1.2% agarose gel.

Transfection and Luciferase Assay—0.5×10^5 cells were plated in 6-well plates in triplicate. The cells were transfected using LipofectAMINE according to the manufacturer's protocol (Invitrogen). Briefly, the plasmids were mixed with LipofectAMINE (1:4 ratio) in OPTI medium (Invitrogen), and complexes were incubated for 30 min at room temperature. For internal control, pRL-TK *Renilla* luciferase reporter was co-transfected to normalize for transfection efficiency. The DNA-LipofectAMINE mixtures were added to the cells and incubated for 6 h at 37 °C. After incubation, the cells were replenished with fresh medium. Twenty-four h after transfection, the cells were washed with 1× ice-cold phosphate-buffered saline and lysed in lysis buffer (Promega). The luciferase assays were performed using a dual luciferase system (Promega).

Electrophoretic Mobility Shift Assays—The cells were treated with TNF for the different time periods, were washed with ice-cold phosphate-buffered saline, and then collected. The nuclear extracts were prepared as described previously (25). Five-μg aliquots of cell nuclear extracts were preincubated with 1 μg of poly(dI-dC) in binding buffer (10 mM Tris, pH 7.7, 50 mM NaCl, 20% glycerol, 1 mM dithiothreitol, 0.5 mM EDTA) for 10 min at room temperature. Approximately 1.5×10^4 cpm of ^{32}P-labeled DNA probe containing the class I major histocompatibility complex NF-κB site (underlined) (5′-CAG GGC TGG GGA TTC CCC ATC TCC ACA GTT TCA CTT-3′) was then added, and the reaction proceeded for 15 min. The complexes were resolved on a 5% polyacrylamide gel in Tris-glycine buffer consisting of 25 mM Tris, 190 mM glycine, and 1 mM EDTA at room temperature. The gel was dried at 80 °C for 60 min and exposed for autoradiography.

Immunofluorescence Staining—The cells were seeded in 12-well plates the day before stimulation and then treated with TNF for 16 h following OHT induction for 6 h. The cells were fixed with 4% paraformaldehyde and washed with phosphate-buffered saline. After blocking with normal goat serum for 1 h, the cells were added with primary monoclonal antibodies against cytochrome *c* for 1 h, and the immunocomplexes were detected with a fluorescein isothiocyanate-conjugated secondary antibody against mouse IgG. The results were photographed by a fluorescence microscope using a filter set for fluorescein isothiocyanate.

RESULTS

Inhibition of NF-κB Transactivation by c-Myc—Earlier studies reported that tumor cells that constitutively expressed c-Myc were sensitive to TNF-mediated cytotoxicity (28, 29, 32). To rule out secondary effects caused by chronic transformation induced by c-Myc, we utilized an inducible c-MycER expression system. In this system, c-Myc is fused with a portion of an estrogen receptor and retained in the cytoplasm by chaperone protein. After cells are stimulated with the synthetic steroid OHT, c-Myc is translocated into nucleus where it induces/represses gene expression (37, 38). Human fibrosarcoma cell line HT1080 cells that lacked detectable c-Myc were transduced with retroviruses expressing c-MycER or control empty vector. HT1080 cells stably expressing c-MycER fusion protein (HT1080/MycER) or control HT1080C cells were obtained after antibiotic selection (Fig. 1*A*). As expected, OHT stimulation potently enhanced TNF-induced cell death in HT1080/MycER cells but not in HT1080C cells, indicating that activation of c-Myc sensitized cells to TNF killing (Fig. 1*B*). As shown in Fig. 1C, DNA fragmentation assay demonstrated that cell death induced by TNF in the presence of c-Myc occurred via activation of an apoptotic program. Similarly, activation of c-Myc also enhanced TNF-mediated killing in squamous cell carcinoma cell line (KB cells) (Fig. 1, *D* and *E*) and Rat-1 cells as previously reported (data not shown).

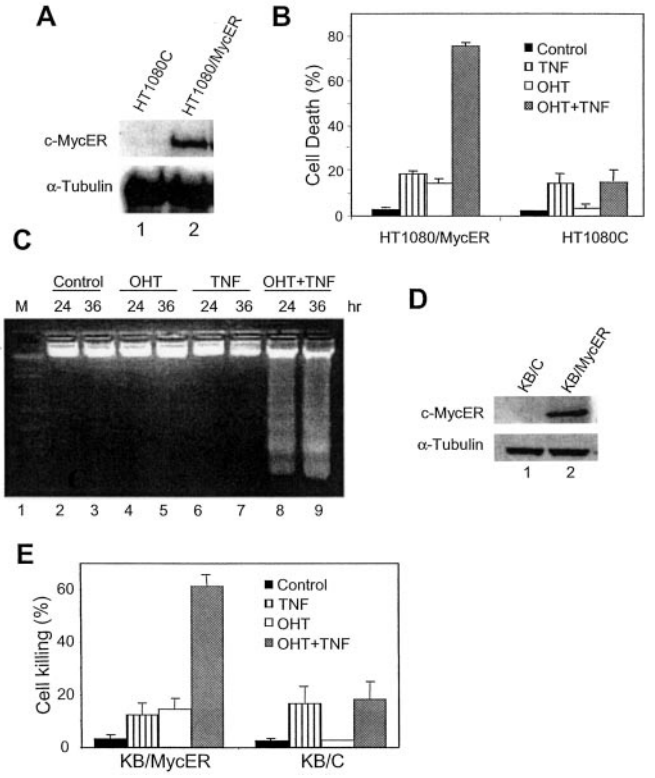

FIG. 1. **Activation of c-Myc sensitizes cells to TNF-mediated apoptosis.** *A*, establishment of HT1080 cells expressing c-MycER. HT1080 cells were transduced with retroviruses expressing c-MycER or control empty vector and selected with puromycin (1.5 μg/ml) for 1 week, and stable clones were pooled. Ectopic expression of c-MycER in HT1080 cells was examined by Western blot analysis. *B*, activation of c-Myc rendered cells sensitive to TNF killing. HT1080/MycER and HT1080C cells were pretreated with OHT (100 nM) for 6 h and then treated with TNF (100 ng/ml) for 48 h. The detached and attached cells were collected, and cell viability was determined with 0.1% trypan blue. The assay was performed in triplicate, and the results represent three independent experiments. *C*, c-Myc potentiated TNF-induced DNA fragmentation. The cells were treated for the indicated times, and the attached and detached cells were collected. DNA was isolated and resolved on a 1.2% agarose gel. *M*, 1-kB DNA ladder marker. *D*, establishment of human squamous cell carcinoma cell line KB expressing c-MycER. The stable transduction was performed as described for *A*. *E*, activation of c-Myc sensitizes KB cells to TNF killing. Cell killing was performed as described for *B*.

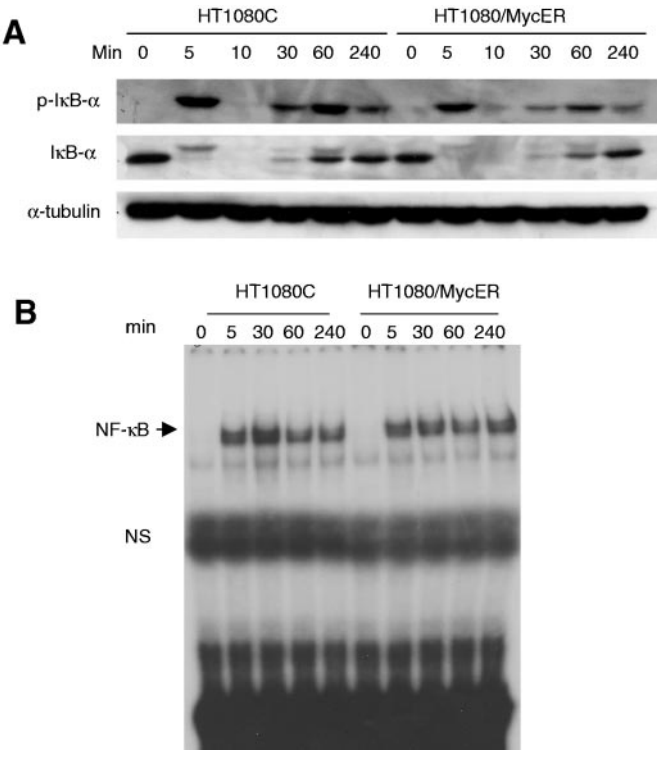

FIG. 2. **Activation of c-Myc did not block the nuclear translocation of NF-κB induced by TNF.** *A*, activation of c-Myc had minimal effects on the phosphorylation and degradation of IκBα. HT1080/MycER and HT1080C cells were pretreated with OHT for 6 h and then treated with TNF for the indicated times, respectively. The whole cell extracts were prepared, and 50-μg aliquots of proteins were used for Western blot analysis. For the loading control, the blots were stripped and reprobed with α-tubulin. *B*, activation of c-Myc did not block the nuclear translocation of NF-κB induced by TNF. HT1080/MycER and HT1080C cells were pretreated with OHT for 6 h and then treated with TNF for the indicated time. The nuclear extracts were prepared, and electrophoretic mobility shift assays were performed as described under "Experimental Procedures."

Although c-Myc has been shown to sensitize cells to TNF-mediated apoptosis by inhibiting NF-κB transcription (29), the precise step of NF-κB activation impaired by c-Myc has not been explored. Because the phosphorylation of IκBα by TNF-stimulated IKK is a primary step to activate NF-κB, we first examined whether c-Myc activation had any affects on IKK activity *in vivo*. The cells were pretreated with OHT for 6 h to activate c-Myc and then treated with TNF for the indicated time. As shown in Fig. 2*A*, TNF rapidly induced IκBα phosphorylation in both HT1080/c-MycER and HT1080C cells, indicating that c-Myc did not inhibit TNF-induced IKK activity *in vivo*. In addition, IκBα protein was also degraded following TNF treatment with a similar kinetics in both cells. Subsequent to IκBα degradation, NF-κB is translocated to the nucleus (4). Thus, we performed electrophoretic mobility shift assays to determine whether activation of c-Myc inhibited the nuclear translocation of NF-κB and/or NF-κB binding activity. As shown in Fig. 2*B*, c-Myc did not significantly inhibit NF-κB nuclear binding activities induced by TNF. Similar results were also obtained in Rat-1 cells (data not shown).

Next, we examined whether expression of c-Myc had effects on NF-κB transcription activity because activation of c-Myc did not interfere with the nuclear translocation of NF-κB. The cells were transfected with 2× κB-responsive luciferase reporter and treated with TNF for 6 h. This time period was before cell death occurred because of activation of c-Myc. As shown in Fig. 3*A*, c-Myc activation inhibited the NF-κB-dependent luciferase reporter assay, confirming a previous study by Klefstrom *et al.* (29). Recently, emerging evidence suggests that the regulation of RelA/p65 transactivation function by oncogenic proteins such as Ras or Akt plays an important role in cell survival. Stimulation of the transactivation potential of NF-κB is independent of its nuclear translocation. Because our results suggested that c-Myc activation did not affect the nuclear translocation of NF-κB, we further examined whether c-Myc had any effects on RelA/p65 transactivation using the Gal4 luciferase reporter system. In this system, the C-terminal transactivation domain of p65 was fused with the DNA-binding domain of *Saccharomyces cerevisiae* Gal4. When co-transfected with a Gal4-responsive luciferase reporter (Gal4-luc), this system allowed us to determine whether cellular signals regulate gene expression by specifically targeting the transactivation domain of p65 independent of nuclear translocation of NF-κB (10). As shown in Fig. 3*C*, c-Myc expression significantly inhibited p65-mediated transactivation (Fig. 3*B*). Previously, c-Myc transformation has been found to be associated with TNF sensitivity. Expression of the c-MycΔ104–136 mutant, in which the residues 104–136 of c-Myc, which are critical for transformation, are deleted, was unable to induce TNF sensitivity (29). Importantly, we found that expression of c-MycΔ104–136 could not

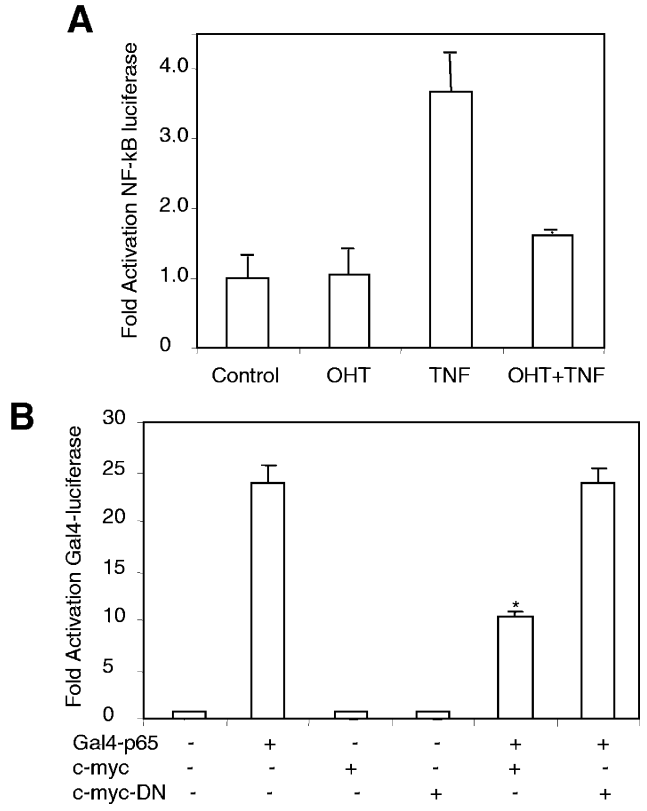

FIG. 3. **Expression of c-Myc attenuates NF-κB transcription by inhibiting RelA/p65 transactivation.** *A*, inhibition of NF-κB transcription by c-Myc. HT1080/c-MycER cells were transfected with 2× κB-dependent luciferase reporter with LipofectAMINE. To normalize transfection efficiency, the cells were co-transfected with the pRK *Renilla* expression vector. Twenty-four h after transfection, the cells were pretreated with OHT (100 nM) for 6 h and then treated with TNF for 6 h. Luciferase activity was measured with a dual luciferase system. The assays were performed in triplicate, and the results represent the average values from three independent experiments. *B*, expression of c-Myc inhibited NF-κB transactivation. HT1080 cells were co-transfected with Gal4 luciferase reporter, Gal4-p65, pCMV-c-Myc, pCMV-c-MycΔ104–136 (*c-myc-DN*), or control vector as indicated. Twenty-four h after transfection, luciferase activity was measured. *, $p < 0.001$

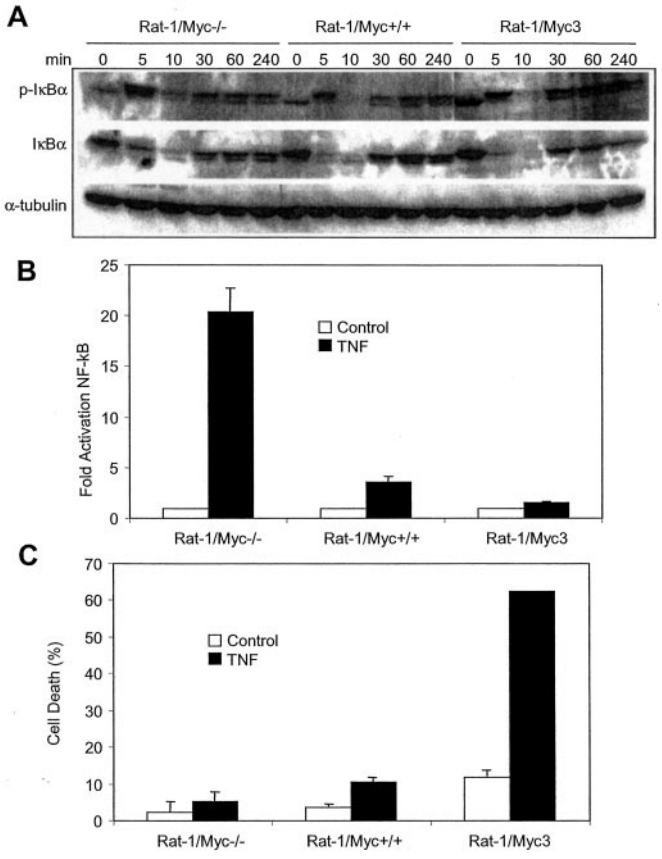

FIG. 4. **Regulation of NF-κB activation by endogenous c-Myc.** *A*, expression of c-Myc did not affect degradation of IκBα. Rat-1/c-*myc*$^{-/-}$, Rat-1/c-*myc*$^{+/+}$, and Rat-1/c-*myc3* cells were treated with TNF for indicated times. Western blot was performed as described in the legend to Fig. 2. *B*, endogenous expression of c-Myc inhibited TNF-induced NF-κB activation in Rat-1 cells. The cells were transfected with 2× κB luciferase reporter with LipofectAMINE. Twenty-four h after transfection, the cells were treated with TNF for 6 h, and luciferase activities were determined as described in the legend to Fig. 4. *C*, c-Myc sensitized cells to TNF killing in Rat-1 cells. The cells were treated with TNF (100 ng/ml) for 24 h, and cell viability was determined with a trypan blue exclusion assay.

inhibit the transactivation potential of p65 (Fig. 3*B*).

Previous studies, as well as works presented above, utilized an inducible c-MycER system in the presence of endogenous c-Myc protein. In this regard, the functional role of endogenous c-Myc on NF-κB activation induced by TNF has not been explored. Additionally, to rule out side effects by OHT treatment, we utilized c-*myc*-deficient Rat-1 cell lines (Rat-1/c-*myc*$^{-/-}$) as described previously (33, 39). As shown in Fig. 4*A*, phosphorylation and degradation of IκBα were induced by TNF with similar kinetics in Rat-1/c-*myc*$^{-/-}$ and in parental wild type cells (Rat-1/c-*myc*$^{+/+}$), demonstrating that c-Myc did not affect IKK activities. In contrast, NF-κB activity in Rat-1/c-*myc*−/− cells was 4-fold higher in comparison with in Rat-1/c-*myc*$^{+/+}$ cells following TNF stimulation, indicating that endogenous c-Myc played an essential role in the regulation of TNF-induced NF-κB activation. Moreover, we also examined whether the restoration of c-Myc expression in Rat-1/c-*myc*$^{-/-}$ cells by retroviral transduction had any affects on NF-κB activation. In this regard, we had previously established a Rat-1/c-*myc3* cell line, a derivative of Rat-1/c-*myc*$^{-/-}$, in which the level of c-Myc expression was 3-fold higher than in Rat-1/c-*myc*$^{+/+}$. As shown in Fig. 4*B*, TNF-mediated NF-κB activation was significantly reduced in Rat-1/c-*myc3* cells compared with in Rat-1/c-Myc$^{+/+}$ cells or Rat-1/c-*myc*$^{-/-}$ cells. Also, overexpression of c-Myc had minimal effects on the phosphorylation and degradation of IκBα (Fig. 4*A*). Furthermore, we determined whether expression of c-Myc modified cellular susceptibility to TNF killing in these cells. Although TNF could not efficiently kill Rat-1/c-*myc*$^{+/+}$ cell, probably because of activation of NF-κB, we consistently observed that Rat-1/c-*myc*$^{-/-}$ null cells had a lower basal level of cell death than Rat-1/c-*myc*$^{+/+}$ cells following TNF treatment (Fig. 4*C*). Rat-1/c-*myc3* cells were very sensitive to TNF killing compared with both Rat-1/c-*myc*$^{+/+}$ and Rat-1/c-*myc*$^{-/-}$ cells (Fig. 4*C*). Taken together, these results confirmed that c-*myc* regulated TNF sensitivity by attenuating NF-κB activation.

c-Myc Potentiated TNF-induced Cytochrome c Release—Biochemical and genetic studies have demonstrated that caspase-8 is an initiator in TNF-mediated apoptosis (1, 40). Active caspase-8 cleaves a pro-apoptotic family member of Bcl-2, called Bid; subsequently the cleaved Bid moves to mitochondria to induce the release of cytochrome *c* to the cytosol, which amplifies apoptosis by activating caspase-9 (41). It was previously demonstrated that activation of NF-κB inhibited TNF-mediated apoptosis by inhibiting caspase-8 activation and cytochrome *c* release (24, 25). Thus, we examined in which step activation of c-Myc might act to potentiate TNF-mediated apoptosis. As shown in Fig. 5*A*, the processing of caspase-8 was not dramatically induced following TNF stimulation. It was likely that the total amount of caspase-8 cleavage was unde-

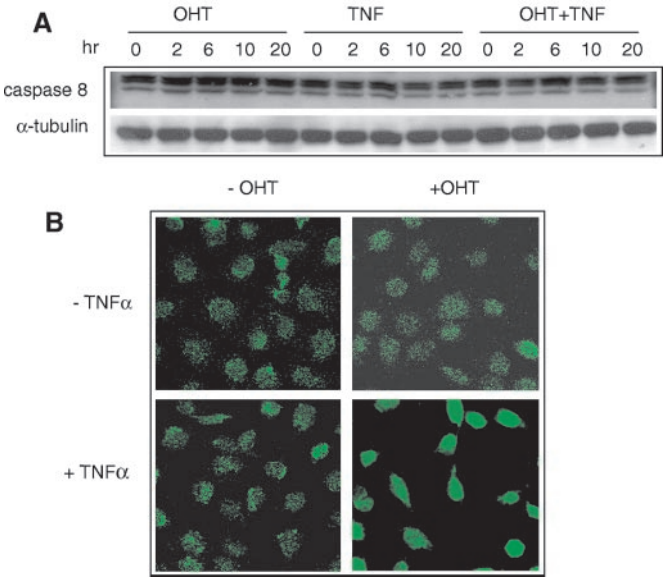

FIG. 5. **Activation of c-Myc potentiates TNF-induced cytochrome *c* release.** *A*, activation of c-Myc had undetectable effects on caspase-8 processing by TNF. The cells were pretreated with OHT for 6 h and then treated with TNF (100 ng/ml) for the indicated times. The protein level of caspase-8 was determined by Western blot. *B*, activation of c-Myc potentiated TNF-induced cytochrome *c* release. HT1080/MycER cells were treated with TNF for 16 h after OHT addition. The cells were fixed and stained with monoclonal antibodies against cytochrome *c*. The results were analyzed by fluorescent microscopy.

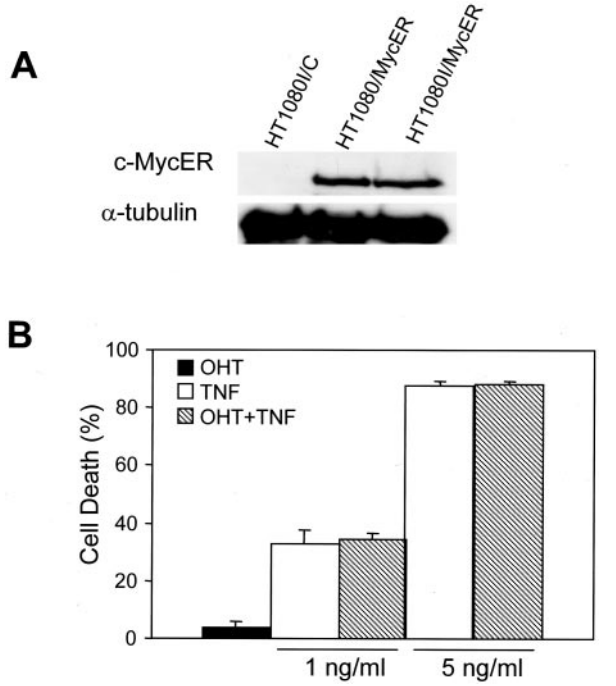

FIG. 6. **c-Myc-mediated sensitization to TNF killing is predominantly caused by the inhibition of NF-κB.** *A*, establishment of HT1080I cells expressing c-MycER (*HT1080I/MycER*). HT1080I cells were transduced with retroviruses expressing c-MycER, and the stable clones were selected with puromycin for 1 week (1.5 μg/ml). The cells expressing c-MycER proteins were determined by Western blot. *B*, c-Myc-mediated sensitization was dependent on inhibition of NF-κB. HT1080I/MycER cells were treated with OHT and/or TNF for 16 h. Cell viability was determined with a trypan blue exclusion assay.

tectable because of the low sensitivity of the Western blot. Nevertheless, elegant studies by Scaffidi *et al.* (42) have demonstrated there are two types of cell death receptor-mediated apoptosis. In type I cell death, caspase-8 can directly activate other caspases to induce apoptosis independent of mitochondria. In type II cell death, active caspase-8 utilizes the mitochondria/cytochrome *c* pathway to amplify caspase cascade, and this is due to the weak processing of caspase-8 after stimulation. Because we observed that TNF killing was much slower in c-Myc expressing cell than in cells expressing the super-repressor of IκBα (SR-IκBα), in which NF-κB activation was completely blocked, we performed the immunofluorescence staining as previously reported by us (43). This was important to determine whether expression of c-Myc promoted TNF-induced cytochrome *c* release. As shown in Fig. 5*B*, cells treated by TNF or OHT only displayed a punctate staining pattern for cytochrome *c* typical of mitochondrial localization. In contrast, cells that were treated by combination of OHT and TNF exhibited a diffuse staining pattern for cytochrome *c*, indicating that c-Myc enhanced TNF-induced cytochrome *c* release.

c-Myc-induced TNF Sensitization Was Dependent on the Inhibition of NF-κB—Although our results suggested that c-Myc potentiated TNF-mediated apoptosis by inhibition of NF-κB, it remains possible that c-Myc might sensitize cells to TNF-mediated apoptosis by NF-κB-independent pathways. To address this question, we utilized a HT1080I cell line expressing SR-IκBα to determine whether c-Myc could enhance TNF-mediated apoptosis in a NF-κB-independent manner. We had previously demonstrated that HT1080I cells were sensitive to TNF-mediated apoptosis because SR-IκBα completely abolished NF-κB activity (19). HT1080I cells were transduced with retroviruses expressing c-MycER, and the stable cell lines expressing c-MycER (HT1080I/MycER) were isolated as described above. Western blot analysis demonstrated that the level of c-MycER expression in HT1080I/MycER cells was comparable with that in HT1080/MycER cells (Fig. 6*A*). As shown in Fig. 6*B*, TNF alone induced apoptosis in HT1080I/MycER cells in a dose-dependent manner as expected. However, activation of c-Myc upon OHT addition did not significantly enhance TNF killing. These results, when compared with HT1080 parental cells, demonstrate that c-Myc sensitizes cells to TNF-mediated killing predominantly by inhibition of NF-κB (compared Fig. 6*B* with Fig. 1).

c-Myc Inhibited the Expression of NF-κB-inducible Gene A1—To gain insight into how c-Myc sensitized cells to TNF-mediated apoptosis by inhibition of NF-κB, we further examined whether the expression of NF-κB-regulated anti-apoptotic genes was regulated by c-Myc. Recently, it had been found that the activation of c-Myc inhibited the expression of Bcl-X_L in primary myeloid and lymphoid progenitors and the expression of Bcl-X_L and Bcl-2 in precancerous B cells from Eμ-myc transgenic mice (44). Because expression of c-Myc potentiated TNF-induced cytochrome *c* release, we first examined whether c-Myc and TNF modified the expression of Bcl-X_L and Bcl-2. As shown in Fig. 7*A*, expression of c-Myc had no effects on the expression of Bcl-2 and Bcl-X_L with or without TNF treatment. Additionally, Bax was also unmodified by TNF and c-Myc expression (Fig. 7*A*). Previously, we and others found that A1, a Bcl-2 homologue, was induced by TNF in the NF-κB-dependent manner and that A1 inhibited TNF-mediated apoptosis (25). Because there was not a specific antibody for detection of A1 protein expression, Northern blot analysis was performed to examine whether c-Myc inhibited A1 expression induced by TNF. As shown in Fig. 7*B*, A1 mRNA was rapidly induced by TNF treatment. However, ~70% of A1 expression induced by TNF was inhibited following expression of c-Myc, as determined by the NIH image analysis. Finally, we also examined whether other NF-κB-inducible genes including TRAF1 and c-IAP2 were inhibited by c-Myc. Interestingly, TNF-induced c-IAP2 was slightly inhibited (Fig. 7*C*, *top panel*), whereas

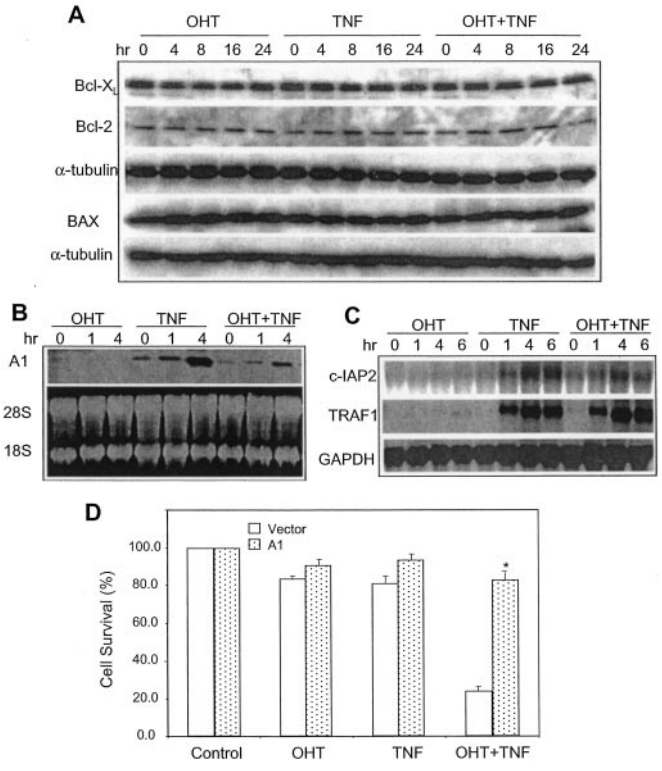

FIG. 7. **Expression of c-Myc inhibits NF-κB-regulated A1 expression induced by TNF.** *A*, c-Myc and/or TNF did not modify the expression of Bcl-2, Bcl-X_L, and Bax. The cells were treated with OHT and/or TNF for the indicated times, and Western blots were performed as described in the legend to Fig. 2. *B*, c-Myc inhibited the expression of NF-κB-inducible gene A1. The cells were pretreated with OHT or vehicle control and then treated with TNF for the indicated times. The cells were harvested, and the total RNA was extracted with Trizol. Northern blot analysis was performed as described under "Experimental Procedures." Fifteen-μg aliquots of RNA from each sample were probed with full-length human A1 cDNA. For loading control, the gels were stained with ethidium bromide and photographed under UV light. *C*, c-Myc had minimal effects on the expression of NF-κB-inducible genes c-IAP2 and TRAF-1. The cells were treated with OHT and/or TNF for the indicated times. Fifteen-μg aliquots of RNA from each sample were probed with full-length c-IAP2 cDNA (*top panel*). The blots were then stripped and reprobed with full-length TRAF1 cDNA (*middle panel*). For the internal control, the blots were stripped again and examined with glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) cDNA probe. *D*, the constitutive expression of A1 inhibited c-Myc-induced TNF sensitization. The cells were transfected with pCMV-EGFP and A1 or control expression vector by the LipofectAMINE method. Twenty-four h after transfection, the cells were pretreated with OHT for 6 h and then treated with TNF for 48 h. The surviving cells were counted from three random fields in each well. The results represent average values from two independent experiments.

TRAF1 expression was totally not effected by activation of c-Myc (Fig. 7*C*, *middle panel*). These results suggested that c-Myc preferentially regulated NF-κB-inducible A1 expression induced by TNF.

Because TNF-induced A1 expression was suppressed by activation of c-Myc, we next determined whether the restoration of A1 expression could rescue c-Myc-induced cellular susceptibility to TNF-mediated apoptosis. The cell survival assays were performed using enhanced green fluorescence protein (EGFP) as a reporter as described previously in our studies (43). HT1080/c-MycER cells were transiently co-transfected with pCMV-EGFP and pcDNA3-A1 expression vector or control empty vector. Twenty-four h after transfection, the cells were treated with OHT and then TNF for 36 h. As shown in Fig. 7*D*, over 70% of cells transfected with control vector were killed by TNF following activation of c-Myc. In contrast, only about 20% of cells transfected with A1 were killed by TNF following activation of c-Myc.

DISCUSSION

The pro-apoptotic activities of c-Myc were initially observed under the condition of serum deprivation and growth factor withdrawal (45, 46). Several target genes regulated by c-Myc, including $p19^{ARF}$ and p53, have been implicated in c-*myc*-induced apoptosis in response to serum deprivation (36). Consistent with its pro-apoptotic functions, c-*myc* was also capable of sensitizing cells to TNF-mediated apoptosis. Until now, only one study by Klefstrom *et al.* (29) showed that c-*myc*-mediated sensitization was caused by impairing NF-κB activation, and the mechanism of this inhibition was unclear. In this study, we found that c-Myc inhibited NF-κB activation by interfering with NF-κB transactivational function but not the nuclear translocation of NF-κB. Activation of c-Myc potentiated TNF-induced release of cytochrome *c* from mitochondria to the cytosol. In this regard, we identified that the NF-κB-regulated anti-apoptotic genes A1 were suppressed by expression of c-Myc. The restoration of A1 expression inhibited c-Myc-mediated TNF sensitization. To the best of our knowledge, the results are the first to elicit the molecular mechanisms by which c-Myc sensitized cells to TNF-mediated apoptosis.

Under serum deprivation, activation of c-Myc has been found to promote apoptosis by induction of the release of cytochrome *c*, which occurs before caspase activation (37). Although both p53 and CD95/Fas signaling was found to play a role in c-Myc-induced apoptosis, neither was required for c-Myc-induced cytochrome *c* release (37, 38). Thus, induction of cytochrome *c* release appears to be a primary event induced by c-Myc when cells are deprived of growth factors. Although the exact mechanisms by which c-Myc induces cytochrome *c* release are not clear, recent studies suggest that Bax is required for promoting cytochrome *c* release by c-Myc under serum or growth factor deprivation (47). In contrast, under normal growth conditions, expression of c-Myc promotes cell proliferation and is unable to induce the release of cytochrome *c* and apoptosis (38). Our studies suggest that c-Myc attenuated NF-κB transcription by impairing p65/RelA transactivation and subsequently sensitized cells to TNF-mediated apoptosis. Thus, it is unlikely that c-*myc* sensitizes cells to TNF-mediated apoptosis by direct induction of cytochrome *c* release. The release of cytochrome *c* observed in TNF-induced apoptosis induced by expression of c-Myc was a secondary effect. Our previous studies have demonstrated that inhibition of NF-κB by SR-IκBα potentiated TNF-induced caspase-8 activation (24). Interestingly, although c-Myc suppressed NF-κB activation, TNF-induced caspase-8 processing was not enhanced by c-Myc. These results suggest that c-Myc may specifically inhibit NF-κB-regulated genes that play an important role in maintaining the integrity of mitochondria.

Recently, c-Myc was found to suppress expression of Bcl-X_L in primary myeloid and lymphoid progenitors and expression of Bcl-X_L and Bcl-2 in precancerous B cells from Eμ-myc transgenic mice. The suppression of Bcl-X_L RNA levels by Myc required *de novo* protein synthesis and was independent of ARF-Mdm2-p53 pathway (44). In contrast, we found that expression of Bcl-2 and Bcl-X_L was not inhibited by c-Myc. These discrepancies might be due to cell type specificities. Previously, we and others found that induction of Bcl-2 homologue A1 expression by TNF was dependent on NF-κB activation and that ectopic expression of A1 blocked TNF-induced cytochrome *c* release (25). In this study, we found that c-Myc inhibited NF-κB activation and thereby attenuated A1 expression, indicating that A1 was a critical target by c-Myc. Nevertheless, our results presented here and the findings of Eischen *et al.* (44) provide new insight into mechanisms of the pro-apoptotic function of c-Myc and suggest that c-Myc promotes apoptosis not

only by induction of pro-apoptotic genes but also through repression of pro-survival genes. Bcl-2 protein levels have been found to be elevated for compensating c-Myc disregulation in lymphomas (44). Along these lines, it would be very interesting to examine whether the expression of A1 is disregulated in c-Myc-associated tumors. NF-κB has also been found to regulate other anti-apoptotic genes such as TRAF-1, TRAF-2, Bcl-X_L, XIAP, c-IAP1, c-IAP2, and NF-κB-inducible death effector domain-containing protein (20–27, 43). Interestingly, we found that disregulation of c-Myc only affects a subset of these genes. For example, we found that c-IAP2 expression was moderately reduced by c-Myc, which might lower the threshold of apoptosis, whereas TRAF-1 expression was not inhibited by c-Myc. The underlying mechanisms were unknown. The results may be related to the regulation of the promoter regions of these genes following c-Myc activation. However, it remains possible that c-Myc may inhibit unidentified NF-κB-regulated anti-apoptotic genes.

The results presented here significantly extended previous studies and demonstrated that disregulation of c-Myc inhibited TNF-induced NF-κB activation through attenuating p65/RelA transactivation potential. In contrast, during the revision of this manuscript, Tanaka *et al.* (49) reported that c-Myc blocked TNF-induced NF-κB binding activities. Currently, we cannot provide an explanation for this difference. Because of the technical difficulty, we could not determine whether the inhibition of NF-κB by c-Myc was required for *de novo* protein synthesis because the luciferase reporter was generally down-regulated in the presence of protein synthesis inhibitors. There are several potential mechanisms by which c-Myc could inhibit p65/RelA transactivation. One is that c-Myc may interact with p65 to inhibit NF-κB transactivation. The c-Myc-binding partner Max has been found to bind to Mad family proteins, which are associated with Sin3, the transcriptional corepressor N-Cor, and histone deacetylases (48). However, our immunoprecipitation experiments found that c-Myc did not interact with p65/RelA or p50. In addition, our supershift assays found that c-Myc did not exist in the NF-κB-binding complex.[2] Thus, it is unlikely that c-Myc inhibits NF-κB transactivation by recruiting co-repressors. However, we could not rule out the possibility that the interaction between p65 and c-Myc was transient and potentially unstable. It is therefore likely that c-Myc suppresses NF-κB transactivation by inducing c-Myc-regulated gene products. In the future, it would be very interesting to determine whether c-Myc-induced genes inhibit NF-κB transactivation using the Gal4-p65 reporter assay. These results will provide new insight into the regulation of NF-κB activation by c-Myc.

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[2] Z. You and C.-Y. Wang, unpublished observation.