Transcription Factor NF-κB Differentially Regulates Death Receptor 5 Expression Involving Histone Deacetylase 1

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The transcription factor nuclear factor κB (NF-κB) regulates the expression of both antiapoptotic and proapoptotic genes. Death receptor 5 (DR5, TRAIL-R2) is a proapoptotic protein considered to be a potential target for cancer therapy, and its expression is mediated by NF-κB. The mechanism of NF-κB-induced DR5 expression is, however, unknown. Herein, we determined that etoposide-induced DR5 expression requires the first intronic region of the DR5 gene. Mutation of a putative NF-κB binding site in this intron eliminates DR5 promoter activity, as do mutations in the p53 binding site in this region. Reduction in p53 expression also blocks p65 binding to the intronic region of the DR5 gene, indicating cooperation between p53 and p65 in DR5 expression. In contrast, the antiapoptotic stimulus, epidermal growth factor (EGF), fails to increase DR5 expression but effectively activates NF-κB and induces p65 binding to the DR5 gene. EGF, however, induces the association of histone deacetylase 1 (HDAC1) with the DR5 gene, whereas etoposide treatment fails to induce this association. Indeed, HDAC inhibitors activate NF-κB and p53 and upregulate DR5 expression. Blockage of DR5 activation decreased HDAC inhibitor-induced apoptosis, and a combination of HDAC inhibitors and TRAIL increased apoptosis. This provides a mechanism for regulating NF-κB-mediated DR5 expression and could explain the differential roles NF-κB plays in regulating apoptosis.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF ligand family. It is capable of inducing apoptosis in transformed cancer cells but not in normal cells (14, 53). TRAIL binds to two receptors, death receptor 4 (DR4, TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2) (33, 40, 41, 52, 57). Upon binding, an adaptor protein called Fas-associated death domain (FADD) is recruited to the death receptors, forming a death-inducing signaling complex (46). Fas-associated death domain constitutively associates with procaspase 8 and, upon recruitment to death receptors, caspase 8 is activated. This leads to further caspase activation and release of mitochondrial proteins and, ultimately, to apoptosis (3, 38).

Chemotherapeutic drugs, including etoposide and doxorubicin in combination with TRAIL, give a synergistic apoptotic response in cancer cells (11, 21, 43). This synergy is due partially to the ability of chemotherapeutic drugs and TRAIL to induce DR4 and DR5 expression. Up-regulation of DR5 in lung cancer cell lines is p53 dependent, and the p53-responsive element responsible for DR5 expression has been identified in the first intron region of the DR5 gene (47). Indeed, primary chronic lymphocytic leukemia cells lacking functional p53 fail to up-regulate DR5 following genotoxic treatment (20). Besides p53 involvement, blockage of the transcription factor NF-κB effectively inhibits etoposide and TRAIL-induced DR5 expression and synergistic apoptotic response (11, 42). The mechanism for NF-κB up-regulation of DR5 is currently unknown, but a putative NF-κB binding site has been identified in the first intronic region of the DR5 gene near the p53 response element (59).

NF-κB is a ubiquitously expressed family of proteins that includes p65 (RelA), p50, c-Rel, and RelB. These proteins form heterodimers or homodimers, with the predominant form of NF-κB being a p65/p50 heterodimer. NF-κB regulates both survival and apoptotic signaling pathways (5, 10, 15, 18, 31, 32, 37, 39). In particular, NF-κB is involved in the up-regulation of antiapoptotic genes, such as Bcl-2, Bcl-xL, Mcl-1, and inhibitor-of-apoptosis protein 1/2 (IAP1/2) that effectively promote cell survival (9, 16, 23, 54, 58). We have previously shown that epidermal growth factor (EGF)-induced NF-κB up-regulates Mcl-1 expression, blocking TRAIL-induced apoptosis (16). In contrast, NF-κB has been implicated in up-regulation of other proapoptotic genes besides DR5, such as the Fas and FasL genes (16). NF-κB activation also cooperates with p53 to induce apoptosis (36). The regulation of NF-κB transcriptional activity that leads to up-regulation of proapoptotic genes, however, is unclear.

Histone acetylation regulates transcription by remodeling chromatin structure (45). Acetylation of the core histones disrupts the nucleosome structure, allowing DNA to unfold and providing access for transcription factors to bind to their targeted promoters. The turnover of acetylated histones is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs), where HATs allow transcription and HDACs repress transcription (12, 13). With cancer, dysregulation of HAT or HDAC activity often occurs (24, 49, 50). HDAC inhibitors have been shown to effectively induce apoptosis in cancer cells, as opposed to normal cells, and are currently in clinical trials for a variety of cancers (22, 25, 26). These inhibitors were also found to give a

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synergistic apoptotic response in combination with TRAIL and to directly activate the TRAIL apoptotic pathway (22, 25, 26).

Herein, we have determined that etoposide-induced DR5 expression is regulated by NF-κB binding to the first intronic region of the DR5 gene, which is similar to p53. Stimulation with the antiapoptotic growth factor EGF also induces NF-κB binding to the DR5 gene but, unlike etoposide, EGF induces HDAC1 association with the DR5 gene mediated by NF-κB. Using HDAC inhibitors, DR5 expression is increased, and this up-regulation plays a role in HDAC inhibitor-induced apoptosis. This provides a mechanism for NF-κB regulation of DR5 expression.

MATERIALS AND METHODS

Reagents. Human DR5 promoter (2.5 kb; bp 1 to 2.5 kb) construct and the 4-kb promoter containing the first intron cloned into a luciferase reporter construct (gift from T. Sakai, Japan). p65 in CMV4 and p50 were purchased from L. Kirshenbaum, St. Boniface, Winnipeg, Manitoba, Canada, and W. C. Greene, University of California, San Francisco, respectively. Etoposide, trichostatin (TSA), and EGFR were purchased from Sigma. Etoposide was dissolved in dimethyl sulfoxide, trichostatin was dissolved in 100% ethanol, and EGFR was prepared in 0.1% bovine serum albumin and 10 mM trichloroacetic acid. Valproic acid (VPA) and lactacystin were purchased from ALEXIS Biochemicals. Proteins were purchased from Affinity BioReagents, Inc. Mouse monoclonal anti-HDAC1 was purchased from Affinity BioReagents. Anti-acetyl H3 (Ac-H3) was purchased from Upstate Biotechnology, and soluble TRAIL was purchased from R&D Systems. Soluble p53 was purchased from Chiron (St. Augustine, FL). Anti-p53 and anti-p50 (Santa Cruz) antibodies were purchased with extracts prior to probe addition where indicated. After preincubation for 5 min at room temperature, probes were added to the binding reactions and the incubation was continued for an additional 30 min. The reaction mixtures were then run onto a 5% nondenaturing polyacrylamide gel. Gels were vacuum-dried and autoradiographed at ~80°C for 18 to 24 h.

Chromatin immunoprecipitation assay (ChiP). ChiP assay was performed as outlined by Spencer (43a). HEK293 cells were fixed to cross-link protein bound DNA by adding formaldehyde at a final concentration of 1% of 30% formaldehyde in normal cells. The percentage of apoptotic cells was determined from 30% output on ice for four 20-s intervals and precleared with protein A Sepharose 4B (Amersham Pharmacia). The lysate was precipitated with the primary antibodies p65 (3 µg), p53 (3 µg), acetylated histone 3 (3 µg), and HDAC1 (3.5 µg) overnight at 4°C. Immunoprecipitates were tested to ensure that the targeted proteins were completely immunoprecipitated (data not shown). Organic extractions were performed to isolate the ChiP DNA and protein using glycogen carrier (Invitrogen) and absolute ethanol. The ChiP DNA pellet was resuspended in 10 µl of double-distilled H2O and analyzed using PCR (94°C, 56°C, 72°C, for each for 1 min for a total of 35 cycles) using primers specific to exon-intron 1 of DR5 (5′ACCACTT GTGGTGGTTGTC3′ and 5′TTCCCCAGCTTACCTG3′). As a negative control, primers specific for 1353 to 1832 of the DR5 gene (5′GATGCCCGTGAGAGCGCGTG3′ and 5′GAAAGGAGAACGGACTCTCG3′) were used. For each treatment, 1% of the chromatin was assayed for equal loading (data not shown). Conditions were set such that equal amounts of nuclear extract were used.

Immunoprecipitation. Cells were treated with etoposide or EGFR and lysed in lysis buffer (50 mM HEPES [pH 7.25], 150 mM NaCl, 50 mM ZnCl2, 50 mM NaF, 2 mM EDTA, 1 mM sodium vanadate, 1% NP-40, and 2 mM phenylmethylsulfonyl fluoride). One mg of protein of each cell lysate was incubated with 2 µg of anti-p65 antibody for 1 h at 4°C, and 50 µl of protein A Sepharose beads were added for 1 h. The immunoprecipitation complex was washed four times with lysis buffer with 5% NP-40. The pellet was boiled in loading dye for 2 min, and the rest of the steps mentioned in the above immunoblots section were followed. Anti-HDAC1 was used as the primary antibody. All blots were stripped and reprobed for p65 NF-κB expression for equal loading (data not shown).

Apoptosis assay. Cells (1 × 106 to 2 × 106) were resuspended in 100 µl of medium by gentle vortexing, and 2 µl of acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) in phosphate-buffered saline was added. Eight µl was removed and placed on a microscope slide. The slide was observed under fluorescent microscope using a fluorescein filter set for the detection of condensed DNA in apoptotic cells. The condensed DNA was determined by intense local staining of DNA in the nucleus compared to the diffuse staining of the DNA in normal cells. The percentage of apoptotic cells was determined from cells containing normal DNA staining compared to cells condensed DNA in blinded samples.

Transfection of HEK293 cells with siRNA. HEK293 cells were plated in six- well plates and grown to 60% confluency. Small interfering RNA (siRNA; 3.5 µg) against p53 (Upstate Biotechnology) or DR5 (as described by Wang et al. [51]) was diluted into 100 µl of medium. Six µl of RNAiFect transfection reagent (QIAGEN) was added to the siRNA and incubated at room temperature for 10 min. The complexes formed between the reagent and siRNA were added dropwise to the cells. The cells were then incubated with the transfection complexes

activity was normalized to β-Gal activity measured at 414 nm and presented as luciferase units.

Immunobots. Cells were lysed using NP-40 lysis buffer (50 mM HEPES [pH 7.25], 150 mM NaCl, 50 mM ZnCl2, 50 mM NaF, 2 mM EDTA, 1 mM sodium vanadate, 1% NP-40, and 2 mM phenylmethylsulfonyl fluoride). Cell debris was removed by centrifugation at 1,200 rpm for 5 min, and protein concentration was determined by the Bradford assay. Ten to 100 µg of cell lysate protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline–Tween 20 solution containing 5% milk. Blots were incubated with either DR5, p53, or HDAC1 antibodies overnight at 4°C. Washing was continued for 3 times with Tris-buffered saline, and incubated for 1 h with appropriate secondary antibody conjugated with alkaline phosphatase. Blots were visualized on X-ray film using enhanced chemiluminescence reagents (Amer sham).

Electromobility shift assay (EMSA). Oligonucleotide probes (Invitrogen) for the intronic region were generated by 5′-end labeling with [γ-32P]ATP (Perkin–Elmer) and T4 polynucleotide kinase (Invitrogen). The DNA was used as the primary antibody. All blots were stripped and reprobed for p65 NF-κB expression for equal loading (data not shown).

Luciferase activity was normalized to β-Gal activity measured at 414 nm and presented as luciferase units.
under normal tissue culture conditions overnight. As controls, scrambled siRNA was used as described above.

RESULTS

First intronic region in the DR5 gene regulates its expression. Genotoxins increase DR5 expression involving p53 binding to the first intronic region in the DR5 gene. To confirm whether this intronic region was required for the promoter activity of DR5 following genotoxin treatment in epithelium-derived cells, the DR5 gene (430 to 411) was cloned in frame with the luciferase gene and compared with a reporter plasmid containing the DR5 promoter alone (gift from T. Sakai, Japan) (Fig. 1A, i). The reporter plasmid containing the promoter alone, when transfected into epithelium-derived MCF-7 cells, showed an increase in transcriptional activity (1.8-fold) only after 4 h following etoposide treatment and returned to basal levels after 6 h (Fig. 1B). Similar results were found for HEK293 cells (data not shown). In contrast, the luciferase reporter plasmid containing the DR5 promoter and first intronic region showed increased promoter activity at both 4 and 6 h following etoposide treatment, peaking at a 3.5-fold increase in transcriptional activity after 6 h. As a negative control
for DR5 promoter activity, growth factor EGF failed to increase the transcriptional activity of both DR5 promoter constructs over this 6-h time course (Fig. 1C). This suggests that the first intronic region of the DR5 gene is required for genotoxin up-regulation of DR5.

**DR5 transcriptional activity requires NF-κB activation.** We have previously determined that blockage of NF-κB activation with ΔIkB, which prevents etoposide-induced DR5 expression (11). There is a putative NF-κB binding site in the first intronic region of the human DR5 gene (Fig. 1A). This is similar to other NF-κB binding sites found in the first intronic region of the mouse and rat DR5 gene (Fig. 1A, ii). To investigate whether NF-κB activation regulates DR5 transcriptional activity through this binding site, we expressed the DR5 luciferase reporter gene containing the promoter and first intron region in HEK293 cells stably expressing ΔIkB (which blocks NF-κB activation) or in HEK293 cells expressing vector alone. In the ΔIkB-expressing cells, transcriptional activity of the DR5 promoter was repressed compared to that of cells expressing vector alone and, following etoposide treatment, transcriptional activity of the DR5 promoter failed to be induced (Fig. 2A, i). In addition, HEK293 cells were transiently transfected with the DR5 promoter and either vector alone or ΔIkB (Fig. 2A, ii). The cells were then treated with etoposide for 4 h. In cells transfected with vector alone, the DR5 promoter activity increased 2.5-fold, but the promoter activity failed to significantly increase in cells expressing ΔIkB (1.3-fold increase) (Fig. 2A, ii). Expression of ΔIkB failed to block increased transcriptional activity of a p53-responsive BAX promoter, indicating the specificity of ΔIkB in blocking NF-κB transcriptional activation (Fig. 2A, iii). To determine whether this NF-κB binding site is responsible for NF-κB-dependent transactivation of DR5 gene, we introduced point mutations within the NF-κB consensus sequence (GGGAGTATCGCTTG) in the first intron of the DR5 gene, we introduced point mutations within the NF-κB consensus sequence (GGGAGTATCGCTTG) in the first intron of the DR5 gene. The mutated DR5 promoter construct was transiently transfected into MCF-7 and HEK293 cells. This resulted in a dramatic reduction in DR5 transcriptional activity compared to the wild-type promoter (Fig. 2B). A promoter construct with the putative NF-κB binding site deleted gave similar results (data not shown). In addition, treatment of HEK293 cells with etoposide over a 16-h time course showed that the mutated
DR5 construct failed to increase luciferase activity compared to the wild-type construct (Fig. 2C). This suggests that the NF-κB putative binding site in the first intronic region is involved in the transactivation of the DR5 gene.

To further confirm the role of NF-κB in DR5 transcriptional activity, p65 or p50 subunits of NF-κB cDNA in an expression vector were transiently coexpressed with the DR5 luciferase reporter gene containing the first intron region in both MCF-7 and HEK293 (Fig. 3A, i) cell lines. We found a threefold increase in DR5 transcriptional activity in the presence of the p65 subunit, and the activity was lowered when the p50 subunit was overexpressed compared to vector alone (Fig. 3A, ii). Furthermore, cotransfection of p65 with the NF-κB binding site mutant DR5 promoter failed to significantly increase transcriptional activity of the DR5 promoter (Fig. 3A, iii). In addition, overexpression of p65 increased transcriptional activity of the DR5 promoter with a mutation in the p53 binding site but failed to reach levels observed with the...
wild-type promoter (Fig. 3A, iii). In p65-overexpressing HEK293 cells, the level of DR5 protein was significantly increased compared to cells expressing vector alone or cells overexpressing p50 (Fig. 3B).

Since mutation of the putative NF-κB binding site in the first intronic region of the DR5 gene reduces its transcriptional activity, we determined whether NF-κB binds to this region. Synthetic oligonucleotides corresponding to the NF-κB consensus site in the first intron region of the DR5 gene were used in an electrophoretic mobility shift assay with nuclear lysates untreated or treated with etoposide. Our results revealed a shift in migration of these labeled oligonucleotides from nuclear extracts of etoposide-treated HEK293 cells over a 6-h time course (Fig. 3C). Etoposide-treated nuclear lysate in the presence of p65 antibodies showed a supershift. These results were similar to that for MCF-7 cells treated with etoposide (data not shown) and indicate the ability of the NF-κB subunit p65 to bind to the NF-κB binding site in the first intronic region of DR5.

To establish whether NF-κB binds to the intronic region of the DR5 gene, we performed ChIP assays on HEK293 cells where the first intronic region of the DR5 gene was amplified. The binding of the p65 subunit of NF-κB to the first intronic region of the DR5 gene increased over the 8-h time course of etoposide treatment and peaked at 4 h. Similar results were found for MCF-7 cells (data not shown). Amplification of the input DNA from the intronic region of the DR5 gene was used as a loading control, whereas lysate with no antibody was used as a negative control under each condition tested (Fig. 3D, i).
Isotype-matched antibody was also used as a negative control and gave similar results (data not shown). To ensure that p65 was binding to the intronic region, we performed a ChIP assay using primers further upstream in the DR5 gene. This revealed no binding of p65 in the DR5 gene (Fig. 3D, ii). Finally, we transfected the DR5 promoter into 3T3 murine fibroblast cells that lacked p65 expression. The transcriptional activity was significantly reduced in the fibroblasts lacking p65 compared to wild-type controls following etoposide treatment (Fig. 3E). These results indicate that endogenous p65 protein binds to the DR5 gene following etoposide treatment and controls DR5 transcriptional activity in epithelium-derived cells.

The tumor suppressor p53 is also required for DR5 promoter activity. Since NF-κB may cooperate with p53 in inducing expression of proapoptotic genes and the presence of a p53-like consensus site in the first intronic region of DR5 (27, 47), we determined whether p53 is important for DR5 transcriptional activity following etoposide treatment. We created point mutations within the p53 consensus binding site (GGG AATATCCGGGCAAGACG) in the first intronic region of the DR5 gene. This construct or the wild-type DR5 promoter construct was transiently expressed in both MCF-7 and HEK293 cells, and the amount of DR5 transcriptional activity was determined. This mutation in the p53 binding site resulted in a significant reduction in DR5 promoter activity compared to the wild-type DR5 gene (Fig. 4A). In addition, a promoter construct with the p53 binding site deleted gave similar results (data not shown). To establish whether p53 binding occurs in the first intronic region of the DR5 gene, we performed ChIP assays. Our results indicate that the binding of p53 to the DR5 gene was detectable in untreated cells and increased following 4 h of etoposide treatment in HEK293 cells (Fig. 4B). In MCF-7 cells, p53 binding to the DR5 gene increased after 2 and 4 h of etoposide treatment (Fig. 4B). To determine...
whether p53 binding to the DR5 gene affects p65 binding, siRNA directed against p53 was transfected into HEK293 cells to reduce p53 expression, and the ability of p65 to bind to the intronic region of the DR5 gene was determined by ChIP assay. Following 4 h of etoposide treatment, p53 and p65 were associated with the DR5 gene in the presence of scrambled siRNA (control). In contrast, etoposide treatment failed to induce p53 and p65 binding to the intronic region of DR5 in the presence of siRNA against p53 (Fig. 4C). Taken together, these results suggest that p53 is required for DR5 expression and cooperates with NF-κB.

**EGF fails to increase DR5 expression but induces NF-κB activation and binding to the DR5 gene.** We have previously demonstrated that the genotoxic etoposide increases DR5 expression in epithelium-derived cells (11). An RNase protection assay was performed to monitor the mRNA expression levels of DR5 in MCF-7 cells in response to etoposide or EGF over a 24-h time course (Fig. 5A and B). MCF-7 cells, following 8 h of etoposide treatment, showed a 2.5-fold increase in DR5 mRNA expression. At 24 h, the level of DR5 mRNA was still at a twofold increase compared to untreated cells. In contrast, EGF treatment showed no induction of DR5 mRNA expression over the same time course. Similarly, DR5 protein levels were increased following etoposide treatment but failed to increase following EGF treatment (Fig. 5A and B).

EGF treatment also activates NF-κB transcriptional activity. We determined whether EGF could induce NF-κB binding to the consensus binding site in the intronic region of DR5. Using the same oligonucleotide probes of the NF-κB binding site in the DR5 gene, EGF treatment of nuclear lysate induced a shift in the probes, indicating binding of NF-κB. Supershift of the complexes was observed in the presence of antibody against p65 and p50 after EGF treatment (Fig. 5C), as with etoposide treatment. These results were also found in MCF-7 cells treated with EGF (data not shown). To determine whether this binding occurs in the DR5 gene, a ChIP assay was performed. As with etoposide, EGF treatment induced binding of p65 to the first intronic region of the DR5 gene, but peaked at 1 h and was reduced to basal levels after 4 h (Fig. 5D). This corresponds to the time course of NF-κB activation following EGF treatment (Fig. 5C). In contrast, p53 was associated with the intronic region of the DR5 gene but failed to increase following EGF treatment (Fig. 5E). This difference in the ability of p53 to bind to the DR5 gene is associated with its level of expression. Upon etoposide treatment, the p53 protein levels increased, whereas upon EGF treatment, p53 protein levels remain unchanged (Fig. 5F). Similarly, etoposide induced serine 15 phosphorylation of p53, indicating p53 activation, whereas EGF failed to phosphorylate this residue (data not shown). This suggests that EGF-induced NF-κB activation causes binding of NF-κB to the DR5 gene but fails to induce its expression.

**HDAC1 recruitment to the DR5 gene following EGF but not etoposide treatment is mediated by NF-κB.** Histone acetylation allows for transcription of genes (45). The ability of etoposide or EGF to increase histone acetylation in the DR5 gene is unknown. To determine if histone acetylation within the DR5 gene occurred under etoposide or EGF treatment, a ChIP assay was performed on untreated and treated HEK293 cells using an anti-acetyl histone H3 antibody. Treatment with etoposide resulted in increased histone acetylation after 16 h of etoposide treatment (Fig. 6A) that corresponds with DR5 expression (Fig. 5A). In contrast, EGF treatment resulted in no increase of histone acetylation within the time course of NF-κB binding to the DR5 gene (Fig. 6B). Histone acetylation of the constitutively expressing pS2 gene was used as a positive control (39a). This indicates that etoposide but not EGF treatment induces histone acetylation required for transcription.

**HDACs repress transcription of genes (12).** We determined whether HDAC1 associates with the DR5 gene following etoposide or EGF treatment. A ChIP assay using HDAC1 antibodies resulted in the association of HDAC1 with the DR5 gene after 2 and 4 h of EGF treatment. Etoposide treatment, however, failed to recruit HDAC1 to the DR5 gene (Fig. 7A). This indicates that following EGF treatment, the DR5 gene is actively repressed by HDAC1. NF-κB has been shown to associate with HDAC1 and has been implicated in transcriptional repression of target genes. To determine whether NF-κB interacts with HDAC1 following EGF or etoposide treatment, we immunoprecipitated NF-κB with anti-p65 antibody over a time course in HEK293 cells and performed Western blotting for HDAC1. We found that the interaction between p65 and HDAC1 increased following EGF treatment, whereas etoposide treatment produced no change compared to the unstimulated control (Fig. 7B). To demonstrate that HDAC1 recruitment to the DR5 gene requires NF-κB, we treated HEK293 cells expressing ΔIkB (which effectively blocks NF-κB activation) or expressing vector alone with EGF (Fig. 7C). The ability of HDAC1 to associate with the DR5 gene following EGF treatment was blocked in ΔIkB-expressing cells.
HEK293 cells. Furthermore, immunodepletion of p65 in HEK293 cell lysates also blocked HDAC1 association with the DR5 gene following EGF treatment (Fig. 7D). This suggests that NF-κB is involved in the recruitment of HDAC1 to the DR5 gene following EGF treatment.

**Inhibition of HDAC activity causes increased DR5 expression.** The association of HDAC1 with the DR5 gene suggests that HDACs repress DR5 expression. In order to determine if inhibition of HDAC proteins had an effect on DR5 gene expression, we performed an RNase protection assay using HEK293 cells treated with HDAC inhibitor TSA or VPA. We found that treatment with TSA or VPA increased DR5 mRNA levels by 2.0- or 2.5-fold, respectively. This is comparable to results with etoposide-treated cells. L32 (housekeeping gene product) was used as the loading control (Fig. 8A). We also treated HEK293 cells with TSA alone or in combination with EGF and determined the expression levels for DR5 and DR4. Upon TSA treatment, DR5 protein levels increased, but the combination of TSA and EGF failed to further increase DR5 expression. DR4 expression remained unchanged following TSA and EGF treatment (Fig. 8B). TSA has been previously demonstrated to activate both p53 and NF-κB through acetylation of these proteins. Following TSA treatment in HEK293 cells, we confirmed that both p53 and NF-κB are activated by using a luciferase reporter assay (Fig. 8C). To determine whether p53 and p65 are bound to the DR5 intronic regions in a manner similar to that seen with etoposide treatment, ChIP experiments were performed on HEK293 cells treated with TSA for 16 h. Following TSA treatment, both p53 and p65 were bound to the intronic regions of DR5 gene, whereas in contrast to results with EGF treatment, HDAC1 failed to bind to this region (Fig. 8D). These results suggest that HDAC inhibitors induce DR5 expression mediated by p53 and NF-κB activation.

**TRAIL apoptotic pathway is involved in HDAC inhibitor-induced apoptosis.** The up-regulation of DR5 could lead to activation of the TRAIL apoptotic pathway. To determine whether HDAC inhibitors activate the TRAIL apoptotic pathway, we incubated MCF-7 cells with DR4:Fc protein that sequesters TRAIL away from its endogenous receptors for 36 h. The amount of HDAC inhibitor-induced apoptosis was determined as described in Materials and Methods. VPA and TSA induced 24% and 34% apoptosis, respectively, in MCF-7 cells, but in the presence of DR4:Fc, the amount of VPA- and TSA-induced apoptosis was reduced to 11% and 16%, respectively (Fig. 9A). In addition, HEK293 cells were transfected with siRNA against DR5 to reduce its expression. The amount of TSA-induced apoptosis was determined over a dose range

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**FIG. 7.** HDAC1 is recruited to the DR5 gene following EGF but not etoposide treatment mediated by NF-κB. (A) HEK293 cells that were untreated, treated with etoposide (100 μM), or treated with EGF (1 μg/ml) over an 8-h time course were used in a ChIP assay with anti-HDAC1 antibody. Sample with no antibody added was used as the negative control. Genomic DNA was used as the input control. (B) HEK293 cells were treated with etoposide (100 μM) or EGF (1 μg/ml) over the indicated time course. The cells were lysed, immunoprecipitated with anti-p65 antibodies, and Western blotted for HDAC1. The blots were stripped and reprobed for p65 for equal loading. (C) HEK293 cells with vector alone or expressing ΔIκB were untreated or treated with EGF (1 μg/ml) over a 4-h time course. These cells were used in a ChIP assay with anti-HDAC1 antibodies as previously described. (D) HEK293 cells were treated with EGF (1 μg/ml) over a 2-h time course. The cells were then lysed and immunoprecipitated with anti-HDAC1 or anti-p65 antibodies as described in Materials and Methods. The immunoprecipitated DNA was isolated, and PCR was performed to detect the first intron region of the DR5 gene. Input represents the input DNA from the cell lysates, and the negative control (-ve Control) represents sample with no antibody control.
In the presence of the siRNA against DR5, the amount of TSA-induced apoptosis was consistently lower than untransfected or transfected with scrambled siRNA (scRNA) as a control (Fig. 9B). DR5 expression was decreased only with siRNA, as determined by Western blotting and flow cytometry (data not shown). Up-regulation of DR5 expression mediated by HDAC inhibitors could lead to increased TRAIL-induced cell death. We treated MCF-7 cells with VPA or TSA for 36 h in the presence or absence of TRAIL. Combined treatment of these HDAC inhibitors with TRAIL induced a synergistic apoptotic response (Fig. 9C). This result was similar to that for HEK293 cells (data not shown). This indicates that DR5 activation may contribute to HDAC inhibitor-induced apoptosis.

**DISCUSSION**

Apoptotic stimuli, including genotoxins, up-regulate DR5 levels, sensitizing cells to undergo programmed cell death. This increased expression makes DR5 a potential target for molecular-based cancer therapy (11, 44). To this end, TRAIL (the ligand for DR5) and antibodies directed against DR5 have been developed for anti-cancer therapy (6, 14, 19, 52). The regulation of DR5 expression may be crucial to making these therapies effective.

We and others have determined that genotoxins increase DR5 expression through activation of transcription factors p53 and NF-κB (11, 42, 47). Herein, we have described a mechanism for NF-κB transactivation of the DR5 gene through binding to the intronic region of the DR5 gene. This binding, along with p53 binding, induces DR5 expression following etoposide treatment. It is well established that there is transcriptional cross talk between NF-κB and p53 (56). These transcription factors could mutually repress each other’s transcription activity by competing for a limited pool of histone acetyltransferase p300/CBP or directly associate with each other (48, 51). In contrast, NF-κB activation could increase p53 levels and p53 could activate NF-κB transcriptional activity, contributing to apoptosis (36). We found that both endogenous p53 and NF-κB bind to the DR5 gene, leading to up-regulation of DR5 and apoptosis following genotoxin treatment. Eliminating p53 binding to the intronic region also prevented p65 binding. This suggests that p53 and NF-κB cooperate to induce DR5 expression. Besides these transcription factors, other proteins could be sufficient to induce DR5 expression. For example, bile acid-
induced apoptosis increases DR5 expression mediated by the c-Jun N-terminal kinase, and interferon-γ-induced DR5 expression involves the transcription factor STAT1 (17, 28). Our results indicate that both p53 and NF-κB in epithelium-derived cells are required for etoposide-induced DR5 expression, and the cross talk between NF-κB and p53 in regulating DR5 expression will be the focus of future investigations.

NF-κB is involved in both cell survival and apoptosis (4, 9, 15). It has been reported that this contradictory function of NF-κB could be due to differentially regulated subunits of NF-κB (8, 34). The p65 subunit was shown to be required for the expression of the BclxL antia apoptotic gene, and the c-Rel subunit is required for up-regulation of DR5 (8, 34). In contrast, we found that the p65 subunit of NF-κB was capable of increasing DR5 expression through binding to the first intronic region of the DR5 gene following etoposide treatment. Furthermore, the overexpression of p65 was sufficient to induce DR5 expression. This difference could be due to the specific cell type used. Mouse embryonic fibroblasts lacking c-Rel expression failed to induce DR5 expression (8), whereas human epithelium-derived cell lines were able to induce DR5 expression in a p65-dependent manner. The c-Rel subunit was ob-
served to be a minor component of the NF-κB activity induced upon apoptosis in HEK293 cells (8). In 3T3 murine fibroblasts lacking p65, the ability of etoposide to induce DR5 transcriptional activity was attenuated, indicating a role of p65 in DR5 expression. Thus, it is unlikely that the subunits of NF-κB are responsible for differential regulation of gene expression leading to cell survival or apoptosis.

It has been demonstrated that in the nucleus of resting cells, NF-κB is complexed with the histone deacetylase HDAC1 and represses NF-κB transcriptional activity (2). Upon NF-κB activation, HDAC1 is released from the complex, freeing NF-κB to target genes for expression (60). Under apoptotic conditions, p65 has been demonstrated to bind to Bel-1 and X-linked IAP promoters, decreasing their expression. This was accomplished through recruitment of HDACs, mediated by p65, to these promoters (7). We have demonstrated that upon EGF treatment, HDAC1 recruitment to the DR5 gene is mediated by NF-κB. The mechanism by which HDAC proteins repress the transcriptional activity of NF-κB is through their deacetylase activity, since treatment with HDAC inhibitor TSA or VPA activates NF-κB and induces DR5 expression. However, the mechanisms regulating the interactions between NF-κB and HDAC proteins are unknown. It is possible that in the case of EGF stimulation, NF-κB is activated early, in contrast to etoposide treatment, which is a late activator of NF-κB. Another determinant for NF-κB involvement in apoptosis could be how long NF-κB is activated. Growth factors activate NF-κB transcriptional activity transiently, whereas genotoxic-induced NF-κB activation is prolonged. This could influence the binding of HDACs to NF-κB, defining whether a gene is expressed or repressed. Furthermore, the lack of p53 recruitment to the DR5 gene following EGF treatment could influence HDAC binding to this gene. Nevertheless, NF-κB activation under survival stimulation (EGF) mediates HDAC1 recruitment to the DR5 gene, likely preventing DR5 expression, whereas both NF-κB and p53 are recruited to the DR5 gene under apoptotic stimuli (etoposide), inducing DR5 expression. This could be a mechanism controlling NF-κB-mediated expression of proapoptotic genes under apoptotic conditions.

HDAC inhibitors have been shown to have antitumor activity (22, 25, 26). Death receptor apoptotic signaling pathways have been shown to be activated following treatment with HDAC inhibitors (1). Using HDAC inhibitors TSA and VPA, we have shown that DR5 expression increased and that treatment with HDAC inhibitors and TRAIL gives a synergistic apoptotic response. Blockade of TRAIL binding to DR5 effectively reduced HDAC inhibitor-induced apoptosis. In leukemia cells, HDAC inhibitors increase DR5 expression, and activation of the TRAIL apoptotic pathway occurs (30). This activation contributed to HDAC inhibitor-induced apoptosis in these cells. Indeed, several groups have shown that activation of the TRAIL apoptotic signaling pathway occurs following treatment with HDAC inhibitors in various cell types (29, 35). Taken together, these results provide a mechanism for HDAC inhibitor-induced up-regulation of DR5 involving the contributions of NF-κB and p53 to the cytotoxicity of these inhibitors. Thus, DR5-induced expression may be an effective target for synergistic chemotherapeutic treatment for cancer.

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