Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)
Decoy Receptor TRAIL-R3 Is Up-regulated by p53 in Breast Tumor Cells through a Mechanism Involving an Intrinsic p53-binding Site

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Tumor necrosis factor-related apoptosis-inducing ligand receptor 3 (TRAIL-R3) is a decoy receptor for TRAIL, a member of the tumor necrosis factor family. In several cell types decoy receptors inhibit TRAIL-induced apoptosis by binding TRAIL and thus preventing its binding to proapoptotic TRAIL receptors. We studied the regulation of TRAIL-R3 gene expression in breast tumor cells treated with the genotoxic drug doxorubicin (DXR). The breast tumor cell line MCF-7 (p53 wild type) responded to DXR with a marked elevation of TRAIL-R3 expression at the mRNA, total protein, and cell surface levels. In contrast, in EVSA-T cells (p53 mutant) DXR did not induce increased expression of TRAIL-R3. In MCF-7 cells overexpressing the human papillomavirus protein E6, which causes p53 degradation, DXR-induced TRAIL-R3 expression was notably reduced. Furthermore, in MCF-7 cells overexpressing a temperature-sensitive p53 mutant (Val135), shifting the cultures to the permissive temperature was sufficient to induce the expression of TRAIL-R3. We also cloned and characterized a p53 consensus element located within the first intron of the human TRAIL-R3 gene. This element binds p53 and confers responsiveness to genotoxic damage to constructs of the TRAIL-R3 promoter in transient transfection experiments. Our results indicate that genotoxic treatments such as DXR, frequently used in cancer therapy, may also induce genes such as TRAIL-R3 that potentially have antiapoptotic actions and thus interfere with the TRAIL signaling system. This is particularly important in view of the proposed use of TRAIL in antitumor therapy.

Tumor necrosis factor (TNF)1-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein that belongs to the TNF family (1). TRAIL can bind specifically to four membrane-bound receptors of the TNF receptor superfamily. Two of these, TRAIL-R1 (DR4) (2) and TRAIL-R2 (DR5, KILLER, or TRICK2) (3, 4), are also known as proapoptotic receptors and contain an intracellular death domain essential for the induction of apoptosis. In contrast, TRAIL-R3 (TRID, LIT, or DcR1) (5–8) and TRAIL-R4 (TRUNDD or DcR2) (9, 10) are antiapoptotic or decoy receptors because although they can bind TRAIL, they are not capable of engaging the cell suicide apparatus and may inhibit TRAIL-induced apoptosis.

TRAIL-R3 lacks the cytoplasmic and transmembrane domains and is bound to the cell surface through a glycosylphosphatidylinositol lipid anchor. Ectopic overexpression of TRAIL-R3 in TRAIL-sensitive cells confers resistance to TRAIL-induced apoptosis (5). Furthermore removal of TRAIL-R3 from the cell surface by phosphatidylinositol-specific phospholipase C has been shown to turn TRAIL-resistant cells into sensitive cells (5, 11, 12). However, studies in other systems showed no correlation between TRAIL-R3 expression and sensitivity to TRAIL-induced apoptosis (13). A definitive demonstration of the antiapoptotic action of TRAIL-R3 in apoptosis induced by TRAIL thus requires further studies.

The TRAIL-R3 gene maps to human chromosome 8p22-21, clustered with the genes encoding TRAIL-R1, -R2 (8), and -R4 (9). It consists of five exons interrupted by four introns and spans ~14.5 kb. The first exon encodes the 5’ untranslated region and the first 60 amino acid residues of the open reading frame. Exon II codes for the next 35 amino acids, from position 61 to 95, and includes the predicted signal peptide cleavage site (8). Exons III and IV encode the two cysteine-rich domains characteristic of the TNF receptor family. The rest of the protein is encoded by exon V. This region contains the extreme carboxyl-terminal hydrophobic region, the 88-amino acid linker sequence, and the 3’ untranslated region.

Tissue and cellular distribution of TRAIL-R3 transcripts seems to be more restricted compared with the other TRAIL receptors (8). Its transcripts have been detected in some normal tissues, and they can also be observed in most transformed cell lines but in substantially lower amounts. However, very little is known about the regulation of TRAIL-R3 expression. Overexpression of c-Rel or activation of endogenous Rel/NF-κB factors by TNF-α have been reported to up-regulate TRAIL-R3 expression and confer resistance to TRAIL-induced apoptosis (11). TRAIL-R3 is also induced by genotoxic agents in a p53-dependent manner and is overexpressed in primary tumors of
TRAIL and TRAIL receptor mRNAs were analyzed by reverse transcriptase PCR.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—RPMI 1640 medium and fetal bovine serum were obtained from Invitrogen. DXR was from Sigma Immunochemicals. Anti-human TRAIL receptor antibodies for Western blots were purchased from Alexis Corp. (San Diego, CA). Anti-human p53 and p21 antibodies were purchased from Calbiochem and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All restriction enzymes were from Roche Applied Sciences. T4 DNA ligase was purchased from New England Biolabs (Beverly, MA).

Cell Lines—The human tumor cell lines MCF-7 and EVSA-T were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 40 μg/ml gentamycin at 37 °C in a humidified 5%CO2, 95% air incubator. MCF-7 cells stably expressing the human papillomavirus type 16 E6 protein or the temperature-sensitive p53 mutant p53Val135 were previously generated in our laboratory and maintained in culture as described previously (17).

Reverse Transcriptase and PCR Assays—Total RNA was isolated from MCF-7 cells with the TRIzol RNA isolation system (Invitrogen) as recommended by the supplier. cDNAs were synthesized from 2 μg of total RNA using a reverse transcriptase PCR kit (PerkinElmer Life Sciences) with the supplied oligo(dT) primer under conditions described by the manufacturer. PCRs were performed using the following primers: hTRAIL sense, 5′-CAACCCGTCGACTGTTAGAAAG-3′, and hTRAIL antisense, 5′-CTGAACACCGAGACACTGCTGTCCAC-3′; hTRA-IL-R1 sense, 5′-CTTGGAGAACGGCAGGCTGTGCAAT-3′; hTRA-IL-R2 sense, 5′-GCTCTTGGACTTGGGAGATGTG-3′; hTRA-IL-R3 antisense, 5′-CTCTTGGACTTGGGAGATGTG-3′; and hTRA-IL antisense, 5′-CTTTGGACACCCGACACTGCTGTGCAAT-3′; hTRA-IL-R1 sense, 5′-CTTGGAGAACGGCAGGCTGTGCAAT-3′; hTRA-IL-R2 sense, 5′-GCTCTTGGACTTGGGAGATGTG-3′; hTRA-IL-R3 antisense, 5′-CTCTTGGACTTGGGAGATGTG-3′; and hTRA-IL antisense, 5′-CTTTGGACACCCGACACTGCTGTGCAAT-3′; human β-actin sense, 5′-TGACGGGGTCAACCACATGGGAGAATCC-3′, and human β-actin antisense, 5′-CTAGAAGCACTGGGTGAAGATGG-3′. The number of cycles varied depending on the TRAIL receptor analyzed. The products were resolved on 1% agarose gel and visualized with ethidium bromide.

**Fig. 1.** Up-regulation of TRAIL receptors in MCF-7 cells treated with DXR. MCF-7 breast tumor cells were treated with or without DXR (500 ng/ml) for 15 h. A, total RNA was isolated, and TRAIL and TRAIL receptor mRNAs were analyzed by reverse transcriptase PCR. Reverse transcriptase PCR products of β-actin and CD95 were used as controls of RNA input and a genotoxic damage-induced gene, respectively. B, cell surface expression of TRAIL receptors was determined by flow cytometry with primary antibodies to TRAIL receptors (solid line) or an irrelevant antibody (dotted line) as described under “Experimental Procedures.” Cell surface expression of CD95 was included as a control of a DXR-induced gene.
Flow Cytometry—To detect TRAIL receptors at the cell surface, control cells or cells treated with DXR (500 ng/ml, 15 h) were detached with RPMI 1640 medium, 3 mM EDTA, washed with PBS, and suspended in 200 μl of PBS. Cells were incubated with primary antibodies (5 μg/ml) at 4 °C for 30 min. After washing with PBS to remove unfixed primary antibody, cells were incubated with rabbit anti-mouse fluorescein isothiocyanate-conjugated antibody (Dako) (dilution 1:20) for 30 min at 4 °C. Cells were then washed again with PBS, suspended in 200 μl of PBS, and analyzed in a FACSscan flow cytometer using the Cell Quest software (BD Biosciences).

Immunoblot Detection of Proteins—After detachment with trypsin, 0.25% EDTA or PBS, 3 mM EDTA, cells (3 × 10⁷) were washed with PBS, and protein content was measured before lysing the cells in Laemmli sample buffer under reducing conditions. Cell lysates were sonicated, and proteins were resolved on SDS-polyacrylamide gels and detected as described previously (17).

Plasmid Constructs—Wild-type and mutant versions of the TRAIL-R3 intronic p53-binding site were generated by annealing the corresponding synthetic complementary oligonucleotides. The double-stranded oligonucleotides had overhang ends to direct their cloning between BamIII (5'-end) and HindIII (3'-end) restriction sites (italic residues in oligonucleotide sequences indicate the restriction enzyme sites). The sequences of the oligonucleotides used were: 5'-GATCCGGCCGGATGACGAGCTCCGTCACGTGGACGACGG-3' for wtp53-BS and 5'-GATCCGGCCGGATGACGAGCTCCGTCACGTGGACGACGG-3' for mutp53-BS.

For the annealing reaction each pair of complementary primers was mixed at a final concentration of 25 μM, each in an annealing buffer containing 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol. The annealing mixture was incubated at 90 °C for 2 min, and the reaction mixture was allowed to cool down to room temperature.

pIBS-506Luc and pmIBS-506Luc constructs, which respectively contain the wtp53-BS and mutp53-BS upstream of a 506-bp portion of the TRAIL promoter, were generated from the TRAIL-R3 promoter reporter plasmid (p-1641luc) described previously (15). p-1641luc was digested with BamHI and HindIII to release a 1135-bp fragment of the TRAIL promoter, were generated from the TRAIL-R3 promoter reporter plasmid. The mutation (underlined nucleotides) was generated with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The DXR concentration in A and B was 500 ng/ml. C, MCF-7 cells stably transfected with empty vector (pCMV-Neo) or a vector encoding E6 protein (pCMV-E6) and treated for 24 h with DXR (A) and MCF-7 cells stably transfected with empty vector (pCMV-Neo) or a vector encoding E6 protein (pCMV-E6) and treated for 24 h with DXR (B). The DXR concentration in A and B was 500 ng/ml. C, MCF-7 cells stably transfected with a temperature-sensitive mutant p53 (MCF-7p53ts) or with empty vector (MCF-7p53ex) and incubated at the permissive (32 °C) or non-permissive (37 °C) temperature for 48 h. Protein levels of p53 and p21 were determined as positive controls of p53 responsiveness. Protein levels of α-tubulin were used as protein loading controls.

Extractions. MCF-7 cells were treated with DXR (500 ng/ml) for 6 h. Cells were lysed in 400 μl of hypotonic buffer (10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM Na₂MoO₄, protease inhibitors, 0.75 mM spermidine, and 0.15 mM spermine) containing 0.6% Nonidet P-40. Nuclei were then centrifuged (13,000 rpm in a microcentrifuge for 10 min, and supernatants containing the nuclear extracts were immediately stored at −80 °C. The protein concentration was quantified by the Bradford procedure.

Electrophoretic Mobility shift assays (EMSAs)—For nuclear protein

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Fig. 3. A p53-binding element (p53-BS) located within intron 1 of TRAIL-R3 gene acts as an enhancer of doxorubicin-induced activation of the TRAIL-R3 gene promoter. A, genomic structure of the human TRAIL-R3 gene. Exons are represented as black boxes and numbered from I to V. p53-BS is located within intron 1 and is highly homologous to the p53 consensus DNA-binding sequence. The percentage
and agcttGAGTTCGTCCTGCCCGGACATGCCCGGCCGg (antisense).

follows: gatccCGGCCGGGCATGTCCGGGCAGGACGAACTCa (sense)

located within the first intron of the human TRAIL-R3 gene) were as
cleotides used in these studies (corresponding to the p53 sequence
indicated reporter plasmid and 0.25
/H9262

To increase the sensitivity of cancer cells to
metabolite drug methotrexate as the genotoxic agent (not
TRAIL, different DNA-damaging treatments have been used
results in MCF-7 cells. DXR failed to elevate the expression of
TRAIL-R1, -R2, or -R3 in EVSA-T cells. These results suggest
that p53 may be required for the up-regulation of TRAIL-
receptors in response to genotoxic stress. To confirm this we used
MCF-7 cells that express the human papillomavirus type 16 E6
protein (17). Overexpression of E6 protein causes the degrada-
tion of p53 via the proteasome pathway and thus prevents the
transcriptional activation of p53 target genes (27). We have
previously reported that overexpression of E6 protein in MCF-7
cells prevents the up-regulation of CD95/Fas receptor that
takes place in response to genotoxic stress (28) As shown in Fig.
2B, MCF-7p53c cells failed to accumulate p53 in response to DXR
treatment and showed a complete absence of TRAIL-R3 up-
regulation. Likewise up-regulation of TRAIL-R1 and -R2 by
DXR was abrogated, and we also observed a markedly lower
induction of WAF1/p21 in MCF-7p53c cells when compared with
MCF-7neo (control) cells.

The data presented so far indicated that p53 accumulation
is a necessary event for the up-regulation of TRAIL-R1, -R2, and
-R3 expression in breast tumor cells treated with DXR. This
prompted us to examine whether accumulation of active p53
was sufficient to elevate TRAIL receptor expression in MCF-7
cells. For this we used MCF-7 cells stably expressing a tem-
perature-sensitive mutant of mouse p53 (Val135) (17). Shifting the
wild-type p53) cells before and after treatment with DXR. We
breast tumor MCF-7 (wild-type p53) and EVSA-T (inactive
expression levels did not change after incubation with DXR.
Contrast, TRAIL-R3 protein could not be detected by flow cy-
tometry at the cell surface of untreated MCF-7 cells, but its
expression was markedly increased upon treatment (Fig. 1B).

Elevated TRAIL-R3 Expression Induced by DXR in Breast
Tumor Cells Is Dependent on p53—It has been reported that
the tumor suppressor protein p53, which participates in the
cellular response to DNA damage, is involved in the up-regu-
lation of the expression of several apoptosis-related genes, in-
cluding death receptors (25). To address the role of p53 in
DXR-induced TRAIL receptor up-regulation in breast tumor
cells, we compared the expression levels of TRAIL receptors in
breast tumor MCF-7 (wild-type p53) and EVSA-T (inactive
mutant p53) cells before and after treatment with DXR. We
also analyzed the effect of DXR in these cell lines on the
accumulation of p53 and the expression of WAF1/p21, a protein
whose gene is regulated by p53 (26). Fig. 2A shows that in
MCF-7 cells, the p53 level was low in untreated cultures and
was markedly elevated after drug treatment. By contrast, un-
treated EVSA-T cells showed a high level of p53 expression,
and there was only a small increase after treatment. Consist-
ent with this, the expression of WAF1/p21 was up-regulated by
DXR treatment in only MCF-7 cells, confirming that p53
expression in EVSA-T cells is transcriptionally inactive. The
results shown in Fig. 2A demonstrate that contrary to the find-
ings in MCF-7 cells, DXR failed to elevate the expression of
TRAIL-R1, R2, or R3 in EVSA-T cells. These results suggest
that p53 may be required for the up-regulation of TRAIL-
receptors in response to genotoxic stress. To confirm this we used
MCF-7 cells that express the human papillomavirus type 16 E6
protein (17). Overexpression of E6 protein causes the degrada-
tion of p53 via the proteasome pathway and thus prevents the
transcriptional activation of p53 target genes (27).

p53 Consensus Sequence Located within the First Intron of
the TRAIL-R3 Gene Mediates DXR-induced Activation of
TRAIL-R3 Gene Promoter Activity—In our recent description
of the minimal promoter region of the TRAIL-R3 gene, we did not
observe p53-mediated regulation of TRAIL-R3 promoter in
MCF-7 cells (15). However, in that study we did not exclude the
existence of p53-responsive elements located in other regions
of the gene as occurs in other members of the TNF family of death
receptors (18, 19). The TRAIL-R3 gene contains five exons
separated by four introns of 8539, 2832, 738, and 1025 bp,
respectively (Fig. 3A). The recent publication in the human
genome data base of the genomic sequence of human TRAIL-R3
(accession number NT_023666) has made it possible to search

homology is indicated, and capital letters indicate nucleotides that match the consensus binding sequence. B, generation of luciferase reporter
plasmids of the human TRAIL-R3 promoter modified to include the p53-BS from intron 1. Wild-type or mutant p53-binding sites from intron 1 of
the human TRAIL-R3 gene were cloned 5’ upstream of a 506-bp fragment of the TRAIL-R3 promoter in the luciferase reporter vector pX2 to
generate the pBS-506Luc and pmBS-506Luc plasmids used in transfection experiments. BHI and HIII are, respectively, the BamHI and HindIII
restriction enzyme sites used for the cloning. The nucleotides mutated to create the mutant p53-binding site are underlined. C, transcriptional
activity, expressed as relative luciferase units (RLU), of TRAIL-R3 reporter plasmids in MCF-7 cells. Cells transfected for 10 h with 0.75 µg of
the indicated reporter plasmid and 0.25 µg of a β-galactosidase plasmid were treated for a further 15 h with 500 ng/ml DXR. Control cultures were
transfected with empty vector (pXP2). The bar chart shows the luciferase activities determined in a representative experiment of three performed.
p506Luc denotes the luciferase reporter vector, which contains the unmodified 506-bp fragment of the human TRAIL-R3 promoter.
for p53-binding elements in this gene. As p53-regulatory sites are frequently found within intron 1 of death receptor genes (18, 19), we were particularly interested in examining the first intron of the TRAIL-R3 gene. Computer-based analysis of intron 1 of TRAIL-R3 with the TRANSFAC program (30) identified a 20-nucleotide sequence (Fig. 3A, p53-BS), sharing 85% homology with the p53 consensus DNA-binding sequence (31).

To examine the potential involvement of this site in the up-regulation of TRAIL-R3 by DXR we performed transient transfection experiments with a luciferase reporter plasmid driven by a modified version of a 506-bp fragment of the TRAIL-R3 promoter (15). In these experiments, the 20-nucleotide putative p53-binding sequence from intron 1 (p53-BS) was cloned upstream of the 506-bp promoter fragment to yield pIBS-506Luc (Fig. 3B). Likewise we generated another luciferase plasmid (pmIBS-506Luc) containing a mutated form of the p53-BS upstream of the promoter sequence (Fig. 3B). Fig. 3C shows that DXR induced a strong transcriptional response only when MCF-7 cells were transfected with the reporter plasmid harboring the intronic p53 consensus sequence. This response was completely canceled by mutation of the p53-BS (Fig. 3C). The inclusion of the p53-BS in pIBS-506Luc increased the transcriptional activity of the luciferase reporter plasmid even in the absence of DXR (Fig. 3C, Control). This result was expected because MCF-7 cells basally express wild-type p53 (Fig. 2, A and B), which may account for the increased transcriptional activity of luciferase constructs that contain strong p53 enhancer elements (28).

To substantiate the role of p53 in DXR-induced activation of the pIBS-506Luc construct, we performed transient transfection experiments in the MCF-7E6 cells, which show a markedly reduced p53 response (Fig. 2B). As shown in Fig. 4A, treatment of these cells with DXR failed to induce the activation of the reporter plasmid containing the p53 enhancer element of the TRAIL-R3 gene. In contrast, the transcriptional response to DXR of MCF-7neo cells transfected with pIBS-506Luc (Fig. 4A) was similar to that observed in control MCF-7 cells (Fig. 3C). Moreover, co-transfection of MCF-7 cells with the pIBS-506Luc reporter plasmid and a p53 expression vector demonstrated that wild-type p53 expression was sufficient to stimulate markedly the transcriptional activity of the luciferase reporter when the intronic fragment was present (Fig. 4B). In these experiments, the inactive mutant p53 (p53 Ala143) did not stimulate luciferase transcription from the pIBS-506Luc construct. Furthermore mutation of the p53-BS element in pIBS-506Luc plasmid completely inhibited luciferase activity induced by wild-type p53 (Fig. 4B).

To further analyze the role of the intronic p53-responsive enhancer in TRAIL-R3 expression in MCF-7 breast tumor cells, we performed EMSAs. EMSAs with nuclear extracts from

**Fig. 4.** p53 is required for the regulation of TRAIL-R3 gene promoter activation by p53-BS upon DXR treatment. A, transcriptional activity of TRAIL-R3 reporter plasmids in MCF-7neo (p53-positive) or MCF-7 neo (p53-negative) breast tumor cells. Cells transfected for 10 h with 0.75 μg of luciferase reporter plasmids containing either the native TRAIL-R3 promoter (p506Luc) or the modified promoter with the wild-type intronic p53-binding site inserted upstream (pIBS-506Luc) were treated with DXR (500 ng/ml) for a further 15 h. Extracts from cells transfected with pXP2 vector were used as control for background luciferase activity. B, transcriptional activity of MCF-7 cells co-transfected for 36 h with p506Luc (empty vector), pIBS-506Luc (wild-type p53), or pmIBS-506Luc (mutated p53) TRAIL-R3 promoter constructs and pCMV plasmids encoding wild-type or mutant p53. C, EMSA of nuclear extracts from untreated and DXR-treated (500 ng/ml, 6 h) MCF-7 cells with a 32P-labeled sequence containing the intronic p53-binding site of the human TRAIL-R3 gene as probe. EMSAs were performed in the presence of an anti-p53 monoclonal antibody (pAb421) or an irrelevant isotype control (IgG). A 100-fold excess of unlabeled competitor p53 oligonucleotide was added to the binding reaction to determine specificity of binding. The specific DNA-p53 complex induced by DXR is indicated by the arrow. In all cases a representative result of three independent experiments is presented. RLU, relative luciferase units.
MCF-7 cells and a labeled oligonucleotide probe containing the p53-BS of TRAIL-R3 intron 1 showed that p53 DNA binding activity in the presence of p53-activating antibody pAb421 (32) was induced by treatment with DXR for 6 h (Fig. 4C). This complex was not observed in the presence of an irrelevant isotype antibody and was completely competed by a 100-fold excess of unlabeled p53-BS oligonucleotide.

**The Intronic p53-binding Sequence Drives Promoter Activation in Constructs of the TRAIL-R3 Genomic Locus**—The above data demonstrate that the intronic p53-BS confers p53 responsiveness to luciferase constructs of TRAIL-R3 gene promoter when located upstream of the promoter sequence. We next investigated whether this p53-binding element could also stimulate the transcriptional activation of TRAIL-R3 gene promoter when located at a position relative to the promoter similar to that found in the genomic locus. For this, we generated a luciferase-reporter plasmid (pTR3Luc) comprising the 506-bp promoter region, exon I, and a 181-bp fragment of the first intron, which harbors the p53-binding site (Fig. 5A). We then performed transient transfections with this reporter plasmid in MCF-7 cells and treated them with DXR. The results depicted in Fig. 5B demonstrate that, whereas the reporter construct of the promoter region (p506Luc) shows only a weak stimulation in response to DXR as previously described (15), the pTR3Luc reporter plasmid was clearly activated by genotoxic treatment. We next analyzed the role of p53 and the intronic p53-binding site in this observed transcriptional activation. We first examined whether activation of the pTR3Luc reporter construct by DXR could still be observed in cells expressing E6 protein. The results shown in Fig. 6A indicate that DXR-mediated activation of pTR3Luc in MCF-7neo cells was almost completely lost as compared with control MCF-7wt cells, suggesting that endogenous p53 is required for genotoxic drug-induced transcriptional activity. Further evidence for a regulatory action of p53 in pTR3Luc transcriptional activation was obtained in co-transfection experiments with expression plasmids for wt p53 or mutant p53 (p53Ala143), wt p53, but not p53Ala143, was able to stimulate potently the transcriptional activity of the pTR3Luc construct (Fig. 6B). Neither p53 variant was able to enhance the activity of a reporter plasmid containing only the 506-bp promoter fragment (Fig. 6B) as described previously (15). Finally, we determined whether the p53-BS located in the intronic fragment of the pTR3Luc construct was responsible for the observed p53-dependent activation of transcription. Site-directed mutagenesis of the p53-BS in the pTR3Luc plasmid was performed to generate the pmTR3Luc construct. Analysis of transcriptional activation of pTR3Luc constructs by DXR demonstrated that mutation of the p53-BS completely abolished the inducing effect of genotoxic stress (Fig. 6C), further indicating the important role of the intronic p53-binding element in the regulation of TRAIL-R3 expression in the context of its genomic locus.

**DISCUSSION**

The regulation of the expression of TRAIL decoy receptors is poorly understood. Whereas in human normal tissues TRAIL proapoptotic and decoy receptors are co-expressed, many cancer cell lines preferentially express TRAIL-R1 and TRAIL-R2, suggesting a differential regulation of the death and decoy receptors. A full analysis of the promoter and regulatory regions of decoy receptor genes should help to increase our understanding of the cellular expression of these receptors. It has been suggested that hypermethylation of the promoters both of TRAIL-R3 and -R4 decoy receptors is involved in the down-regulation of expression of these genes in neuroblastoma and other tumor types (33). Although this could render tumor cells more susceptible to TRAIL-induced apoptosis, down-regulation in the same cells of proapoptotic proteins such as procaspase-8.

![Diagram](http://example.com/diagram.png)
may permit these cells to escape from death receptor-mediated apoptosis (34). By contrast, it has been reported that TRAIL-R3 is overexpressed in primary tumors of the gastrointestinal tract, which may thus gain a selective growth advantage by escaping from TRAIL-induced apoptosis (14). However, the mechanism underlying the observed up-regulation of TRAIL-R3 in these tumors and whether or not p53 is involved are not known. Regulation of TRAIL-R3 function may also take place at the level of cellular localization, suggesting the existence of more complex levels of regulation (35).

TRAIL-R3 (DcR1/TRID) is an antagonist decoy receptor that when overexpressed can protect cells from TRAIL-induced apoptosis (5, 11, 12). TRAIL-R3 mRNA is highly expressed in a number of normal cells but not in many tumor cells (5, 6). Although up-regulation of TRAIL-R3 by DNA damage has been reported in various tumor cells (14), the mechanism involved has remained completely undefined. In the present report, we provide several lines of evidence indicating that TRAIL-R3, like TRAIL-R1 and -R2, is a p53-regulated gene in breast tumor cells. TRAIL-R3 is up-regulated by genotoxic damage in MCF-7 (wild-type p53) but not in EVSA-T cells (mutant and inactive p53). We have previously established the use of these two cell lines as prototypes for the study of p53-mediated cellular effects of DNA-damaging agents (17). Our current results showing the effect of the antitumor drug doxorubicin on MCF-7 cells transfected with human papillomavirus type 16 E6 cDNA further support a role for DNA damage-mediated p53 elevation in regulating TRAIL-R3 expression in breast tumor cells. Finally, a direct demonstration of the importance of p53 in the regulation of TRAIL-R3 expression is provided by our experiments with MCF-7 cells expressing a temperature-sensitive mutant p53. In these studies, overexpressing active p53 at the permissive temperature is sufficient to up-regulate the expression of decoy TRAIL-R3 as well as proapoptotic TRAIL-R1 and -R2. These results indicate that merely increasing the cellular content of active p53 protein leads to an increased expression of TRAIL-R3 in the absence of the DNA-damaging drug doxorubicin. This finding also suggests that although doxorubicin may trigger signaling independently of p53 (36, 37), most of its effects on TRAIL-R3 expression in breast tumor cells are likely to be p53-mediated.

Up-regulation of proapoptotic TRAIL receptors in response to genotoxic stress is thought to contribute to p53-mediated apoptosis in vivo (23). However, we have now established that p53 also regulates the expression of decoy receptor TRAIL-R3, confirming previous suggestions (14). A similar regulation has also been reported for TRAIL-R4 (38). Although the significance of decoy receptor regulation by p53 remains unclear, there is evidence to suggest that by controlling the levels of TRAIL decoy receptors, p53 may reduce its TRAIL-R1/R2-dependent apoptotic effects in p53-mediated apoptosis (14, 38).

In this report, we have identified a previously unknown p53-binding sequence located in the first intron of the TRAIL-R3 gene. This sequence is a potent enhancer element of the genotoxic stress-induced transcriptional activation of TRAIL-R3 in MCF-7 breast tumor cells. The TRAIL-R3 intronic p53-binding site greatly stimulates the p53-dependent activity of pTR3Luc, p506Luc, and pXP2 in MCF-7 cells co-transfected with pCMV (empty vector) or pCMV encoding wild-type or mutant p53 as described under “Experimental Procedures.” Transfection cultures were incubated for 36 h. C, transcriptional activity of pmTR3Luc, pTR3Luc, p506Luc, and pXP2 in MCF-7 cells treated with DXR (500 ng/ml, 15 h) 10 h after transfection. In all experiments shown, cells were harvested, and luciferase activity was determined as described under “Experimental Procedures.” A representative experiment of three independently performed experiments is presented.
TRAIL-R3 Expression Regulation by Intrinsic p53-binding Site

transcriptional activity of luciferase-reporter constructs containing the minimal promoter region of the TRAIL-R3 gene. This effect is observed both when the intrinsic element is located upstream of the TRAIL-R3 promoter and in the context of the endogenous genomic locus.

Genes for all membrane-bound TRAIL receptors (TRAIL-R1, -R2, -R3, and -R4) map to a cluster on human chromosome 8p22-21, which suggests that these genes have evolved from a common precursor through duplication events (8). The coding region of TRAIL-R3 gene is 58, 54, and 70% identical to that of the TRAIL-R1, -R2, and -R4 genes, respectively (39). Moreover the first introns of the human TRAIL-R2 and TRAIL-R3 genes share an important region of sequence similarity that includes the putative p53-binding element (19). This intronic p53-binding sequence is required for DNA damage-induced up-regulation of TRAIL-R2 in various tumor cells (19). It is striking that the p53-binding site in intron 1 of TRAIL-R3 matches 19 bp of the 20-bp sequence in TRAIL-R2. This provides further strong evidence for the importance of this site in the p53-mediated regulation of TRAIL-R3 gene expression. However, we cannot exclude the possibility that in the chromosomal context, p53-binding sites located in other regions of TRAIL-R3 gene may have a role in the transcriptional regulation of the TRAIL-R3 gene. Indeed, in the case of TRAIL-R2 gene, other p53-binding elements have been found in the promoter and second intron that may contribute to its regulation by p53 (19).

The observation that wild-type p53 can regulate both pro-apoptotic and decoy TRAIL receptors in breast tumor cells raises another important issue: the impact of mutant forms of p53 on the regulation of these receptors. Mutations of p53 are found in about 20% of breast cancers (40) and are associated with poor prognosis and resistance to chemotherapeutic drugs (41). Despite this, the impact of these mutations on TRAIL receptor expression has not been evaluated in detail. Differences in the regulation of proapoptotic versus decoy receptors by the mutant p53 proteins frequently found in breast tumor cells may result in different levels of these receptors on the surface of tumor cells and have important consequences for the response to TRAIL. Further studies examining the effect of p53 mutations on the regulation of the various TRAIL receptors in breast tumor cells will help in the design of combination strategies involving TRAIL and genotoxic treatment regimes.

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Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Decoy Receptor TRAIL-R3 Is Up-regulated by p53 in Breast Tumor Cells through a Mechanism Involving an Intronic p53-binding Site

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