Transcriptional Activity of the ΔNp63 Promoter Is Regulated by STAT3*

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The N terminus-truncated splicing variant of TAp63 is known as ΔNp63. ΔNp63 lacks transactivation function and is thought to antagonize the transcriptional regulation of the p53 and TAp63 target genes. Overexpression of ΔNp63 has been observed in a number of human cancers, suggesting a role in carcinogenesis. In the present study we present data showing that the ΔNp63 gene promoter activity is positively regulated by ΔNp63α, and such positive autoregulation is mediated via activation of STAT3 activity. We show that expression of ΔNp63α in Hep3B cells induces Stat3 phosphorylation on Tyr-705 and Ser-727. A putative STAT3-responsive element (STAT3-RE) is identified in the ΔNp63 promoter region. Electrophoretic mobility shift and avidin biotin-Conjugated DNA assays show direct binding of STAT3 to STAT3-RE of the ΔNp63 promoter, and such binding is stimulated by ΔNp63α. Binding of the endogenous STAT3 to the ΔNp63 promoter in Hep3B cells was demonstrated by a chromatin immunoprecipitation assay. The stimulation of the ΔNp63 transcriptional activity by ΔNp63α is abolished by Janus kinase 2 (JAK2)/STAT3 inhibitor AG490, dominant-negative STAT3, STAT3 small interfering RNA, and deletion of the STAT3-RE sequence from ΔNp63 promoter. Taken together these observations clearly indicated that auto-regulation of ΔNp63 gene transcription is mediated through activation of STAT3 and its subsequent binding to the STAT3-RE. Because the activation of STAT3 by interleukin-6 also leads to ΔNp63 up-regulation and the blockade of ΔNp63 or STAT3 expression by siRNA leads to repression of the cell growth, the identified regulatory pathway is presumably of cell physiological significance.

The discovery of the transcription factor p63 was soon followed by back-to-back reports showing that p63 is essential for the development of a number of epithelial structures, including skin, breast, prostate, urothelia, and others (1). High levels of p63 expression were found in the basal cells of many stratified epithelia and is overexpressed in some human cancers including bladder carcinoma, non-small cell lung cancers, nasopharyngeal carcinoma, and liver cancers. These observations suggest that overexpression of ΔNp63 may have important implications in carcinogenesis (5–8). Recently, Harmes et al. (9) reported the presence of a p53 binding site located at −495 to −473 relative to the transcriptional start site of exon-3′ in the ΔNp63 promoter region, suggesting that p53 may regulate the promoter activity of the ΔNp63 gene. In addition, they showed that ΔNp63 is also recruited to this element and may contribute to the down-regulation of ΔNp63 promoter activity. It is likely that ΔNp63 promoter is a positive and negative transcriptional target of p53 and ΔNp63, respectively.

The STAT32 is constitutively activated in many different cancer cell lines and primary tumors, including prostate, breast, and head and neck cancers. Recently, STAT3 was found to negatively regulate the induction of the innate and adaptive immunity by tumor cells, suggesting further implications of the STAT3 pathway in human tumors (10). Leukemia-inhibiting factor signals through a heteromeric receptor to activate STAT3; this signal has been shown to play essential roles in maintaining self-renewal of the stem cells (11). Like other STAT proteins, STAT3 is activated by tyrosine phosphorylation at a single site close to the C terminus (Tyr-705) as well as by serine phosphorylation at a site within the transactivation domain (Ser-727). Tyrosine phosphorylation in response to cytokine stimulation is mediated by a Janus kinase, most often the JAK1. Tyr-705 phosphorylation is required for STAT3 dimerization, nuclear translocation, and DNA binding, resulting in enhanced transcription of STAT-responsive genes, such as the c-fos, junB, vascular endothelial growth factor, and vasoactive intestinal peptide genes (12–14). Several observations suggest a positive role for Ser-727 phosphorylation in STAT3 transcriptional activity, presumably through enhanced recruitment of some necessary transcriptional cofactors (15). To date, a linkage between STAT3 activity and ΔNp63 expression has not been reported.

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In the present study we show that ΔNp63α exerts a positive regulatory effect (autoregulation) on the activity of ΔNp63 promoter in Hep3B human liver carcinoma cells, and activation of STAT3 is critical for the ΔNp63 promoter activity. The ΔNp63α-induced autoregulation was repressed when STAT3 activity was blocked by AG490, transfection of dominant-negative STAT3 (Y705F), and STAT3 siRNA. To elucidate the mechanisms underlying the autoregulation of the ΔNp63 promoter, we identified a putative STAT3-responsive element (STAT3-RE) in the ΔNp63 promoter region. We show that this putative STAT3-RE is involved in the up-regulation of the ΔNp63 promoter activity by ΔNp63.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human liver cancer cell line Hep3B was obtained from Food Industry Research and Development Institute, Taiwan. The cells were maintained in basal medium (Dulbecco’s modified Eagle’s medium/F-12 at 3:1, v/v; Invitrogen) supplemented with 10% fetal bovine serum in a humidified incubator at 37 °C under 5% CO2, 95% air.

**Construction of ΔNp63 Promoter-Luciferase Constructs and Expressing Vectors**—The PCR reactions were carried out with the sequence-specific primer pairs; these primers were all designed to contain a Xhol site and a HindIII site for the subsequent cloning reactions. Desired DNA fragments were PCR-amplified and inserted into luciferase reporter vector pGL3-Basic, a promoter- and enhancer-less vector (Promega, Madison, WI). The inserts were positioned in sense orientation relative to the luciferase coding sequence between Xhol and HindIII sites. Proper insertion was verified by direct DNA sequencing. The nomenclature of pGL3-823-Luc was based on the length of the insert upstream to the transcriptional start site of the ΔNp63 promoter. The 494-bp fragment containing the human GAPDH promoter (16) was amplified from human DNA using primers 5'-CCCCCTCAGGTGAGAAGTTCACCAAAGCTTTC-3' and 5'-CCCCAAAGCTTCGACGCGGA-GGCTGGGCGGTCA-3'. The fragment was inserted into pGL3-Basic via Xhol and HindIII site to obtain vector pGL3-GAPDH-Luc. The STAT3 luciferase reporter, pGL3-STAT3RE-Luc, was constructed with five tandem copies of the consensus STAT3 binding site, −557 to −539 relative to the transcriptional start site of ΔNp63, was deleted. Site-directed mutagenesis of pGL3-823-Luc vector was carried out using PCR methods. Briefly, the PCR mixture contained 1 μg of pGL3-823-Luc vector, 12.5 μl of 2× Extensor Hi-fidelity PCR Master Mix (ABgene) (consisting 350 μM dNTP, 2.25 μM MgCl2, and 2.5 units of extensor PCR enzyme), and 0.1 μg of each primer. The synthetic oligonucleotide primers were 5’-GGACACATT-TATCAGATTGAGTGTGGTTTGGTGTGTTTTTG-3' (sense) and 5’-CAAAAAAGAAAAACACATCCATACTCTGAAAA-TGTTGCC-3' (antisense). pCMV-STAT3-DN (Y705F) and pCMV-STAT3-C (constitutive) were obtained by site-directed mutagenesis. The primers used for these mutations were as described previously (17, 18). PCR was performed by three-temperature cycles; 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 8 min for a total of 12 cycles. After PCR, the methylated parental DNA template was digested with 20 units of DpnI (NEB, Schwabach, Germany) at 37 °C overnight. The DNA fragment containing the desired mutation was transformed into competent Escherichia coli JM109 cells, which are capable of repairing the nicked DNA. Colonies were selected on LB agar plates containing 50 μg/ml ampicillin. The presence of the desired mutation was verified by direct DNA sequencing.

**Luciferase Assay**—Transient transfection of luciferase reporter plasmids was performed using Lipofectamine 2000 (Invitrogen) according to the protocol recommended by the supplier. The cells were seeded in 12-well tissue culture plates at 2 × 105/well in 1 ml of Opti-MEM (Invitrogen) 24 h before transfection. On the day of transfection, the cells were exposed to DNA-Lipofectamine 2000 mixtures containing 0.5 μg of luciferase reporter plasmids and 0.2 μg of pSV-β-galactosidase control vector (Promega). To analyze the ΔNp63 promoter activity in response to appropriate expressing vector, various amounts of pCMV-expressing vector and pCMV empty vectors (Invitrogen) were added to the DNA-Lipofectamine mixtures. The same amounts of pCMV-empty vectors were used as controls. After incubation for 24 h, the cells were rinsed with phosphate-buffered saline and lysed in 150 μl of 1× reporter lysis buffer (Promega). Lysates were used directly for luciferase activity assay (Promega) performed according to the manufacturer’s protocols. β-Galactosidase enzyme assay (Promega) was also performed with the same lysates to standardize the transcription efficiency. All experiments were performed at least three times, and the mean relative luciferase activity was obtained.

**siRNA Transfection**—The sense siRNA sequences were purchased from MWG-Biotech (AG), including STAT3 siRNA, 5’-AGUCAGGGUUGCUUGUCAA-3’ (19), ΔNp63 siRNA, 5’-UCAUGCCGCGACUAAUU-3’, p63-DBD siRNA, 5’-CCAUGACUGACGCGUG-3’ (20), and control non-silencing siRNA, 5’-UUCUCGAAACUGGUGACGU-3’. The Hep3B Cells were grown in Opti-MEM till 60% confluent. The Cells were cotransfected with the desired siRNA (40 nm), pSV-β-galactosidase vector (0.2 μg/ml) and pGL3-823-Luc (0.5 μg/ml) using Lipofectamine 2000 (Invitrogen). Forty-eight hours after

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**Site-directed Mutagenesis of pGL3-823-Luc Vector**—To construct pGL3-823-Luc vector containing a deletion mutation in the STAT3-responsive element (pGL3-823pSTAT3-Luc), the consensus STAT3 binding site, −557 to −539 relative to the transcriptional start site of ΔNp63, was deleted. Site-directed mutagenesis of pGL3-823-Luc vector was carried out using PCR methods. Briefly, the PCR mixture contained 1 μg of pGL3-823-Luc vector, 12.5 μl of 2× Extensor Hi-fidelity PCR Master Mix (ABgene) (consisting 350 μM dNTP, 2.25 μM MgCl2, and 2.5 units of extensor PCR enzyme), and 0.1 μg of each primer. The synthetic oligonucleotide primers were 5’-GGACACATT-TATCAGATTGAGTGTGGTTTGGTGTGTTTTTG-3' (sense) and 5’-CAAAAAAGAAAAACACATCCATACTCTGAAAA-TGTTGCC-3' (antisense). pCMV-STAT3-DN (Y705F) and pCMV-STAT3-C (constitutive) were obtained by site-directed mutagenesis. The primers used for these mutations were as described previously (17, 18). PCR was performed by three-temperature cycles; 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 8 min for a total of 12 cycles. After PCR, the methylated parental DNA template was digested with 20 units of DpnI (NEB, Schwabach, Germany) at 37 °C overnight. The DNA fragment containing the desired mutation was transformed into competent Escherichia coli JM109 cells, which are capable of repairing the nicked DNA. Colonies were selected on LB agar plates containing 50 μg/ml ampicillin. The presence of the desired mutation was verified by direct DNA sequencing.

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**Regulation of the ΔNp63 Gene Transcription**
transfection, the cells were harvested, and luciferase activity and β-galactosidase activity were measured.

Preparation of Cell Lysate and Western Blot Analysis—The cells (1 × 10^6) were seeded into 100-mm tissue culture plates 1 day before transfection and were transfected with an appropriate amount of the expression plasmid or pCMV-empty vector. For the preparation of total cell lysate, the cells were washed with ice-cold phosphate-buffered saline and lysed in Mammalian Protein Extraction Reagent (M-PER; Pierce). Nuclear and cytoplasmic extract were prepared using nuclear and cytoplasmic extraction Kits (NE-PER™ Nuclear and Cytoplasmic Extraction Reagents; Pierce), respectively. All the lysis buffers contained 10 mM NaF, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1× mixture protease inhibitor (Sigma). Protein concentration was determined by the Bio-Rad protein assay kit. Protein samples were fractionated on 10% SDS-polyacrylamide gel and blotted onto Immobilon(TM)-P membranes (Millipore, Bedford, MA), blocked in Tris-buffered saline-Tween, and probed with primary antibodies (1:1000 for phospho-STAT3 (Ser-727 and Tyr-705), phospho-p44/42 MAP kinase (Thr-202/Tyr-204), phospho-JNK (Thr-183/Tyr-185), phospho-p38 MAP kinase (Thr-180/Tyr-182), 1:2000 for p63, STAT3,V5, and 1:10000 for GAPDH) overnight at 4 °C. The membrane was then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000 in Tris-buffered saline-Tween) and were visualized by Enhanced Chemiluminescence (ECL) (Amersham Biosciences). Primary antibodies used were p63 (4A4) monoclonal antibody, glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (Chemicon, Temecula, CA), V5 polyclonal antibody (Novus, Littleton, CO), STAT3 polyclonal antibody, phospho-STAT3 (Ser-727 and Tyr-705) antibody, phospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibody, phospho-JNK (Thr-183/Tyr-185) antibody, and phospho-p38 MAP kinase (Thr-180/Tyr-182) antibody (Cell Signaling, Beverly, MA).

Electrophoretic Mobility Shift Assay—Cells were transfected with various amounts of pCMV-empty or pCMV-ΔNp63α for 24 h. STAT3-specific DNA binding assay was performed using a LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce) according to the manufacturer’s instructions. Nuclear extract prepared from cells transfected with ΔNp63α was used for analysis. Briefly, an aliquot of nuclear extract containing 3 μg of protein was incubated with 2 μl of 10× binding buffer (100 mM Tris-HCl, pH 7.5, 500 mM KCl; 10 mM dithiothreitol) containing 2.5% glycerol, 20 fmo1 of biotin-labeled STAT3-RE probe, and 1 μg of poly(dI-dC)) in a total volume of 20 μl for 20 min at room temperature. The STAT3-RE probe used for the reaction contains the putative STAT3 binding site of the ΔNp63 promoter with a sequence of 5’-GGATTCCTAGTTCCCGGTACATAATATGGAT-3’ and STAT3-REmut, 5’-GGATTCCTATCGTACATAATATGGAT-3’. The reaction mixtures were fractionated on a 6% native polyacrylamide gel and transferred to Immobilon-NY+ membrane (Millipore). After the membrane was incubated with LightShift stabilized streptavidin-horseradish peroxidase conjugate, the luminol/enhancer and stable peroxide solution, the STAT3 and DNA complexes were visualized by exposure of the membrane to x-ray film. The specificity of the identified STAT3 and DNA binding activity was confirmed by using a 200-fold excess of unlabeled hSIE oligonucleotides. hSIE is a high-affinity serum-inducible consensus STAT3 binding element derived from the c-fos promoter.

Avidin Biotin-conjugated DNA Binding Assay—Five hundred μg of pCMV-empty or pCMV-ΔNp63α-transfected whole-cell extracts were precleared with 30 μl of a 50% slurry of streptavidin-conjugated agarose beads (Pierce) in avidin biotin-conjugated DNA binding buffer (20 mM Heps, pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 10 mM NaF, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1× mixture protease inhibitor; Sigma) for 30 min at 4 °C. The beads were pelleted by centrifugation at 5000 × g for 1 min at 4 °C. The suspensions were incubated with 10 μg of poly(dl-dC) and 100 pmol of biotinylated annealed probes at room temp for 20 min. The biotinylated annealed probes were used were STAT3-RE, 5’-GGATTCCTATTCCCGGTACATAATATGGAT-3’, and STAT3-REmut, 5’-GGATTCCTATCGTACATAATATGGAT-3’. The DNA-protein complex was precipitated with 30 μl of streptavidin-agarose for 30 min, washed 3 times in binding buffer, eluted by boiling in 2× SDS loading buffer, analyzed by SDS-PAGE, and visualized by Western blotting using anti-STAT3 (Cell Signaling) antibody for detection.

Chromatin Immunoprecipitation—Chromatin Immunoprecipitation assay was performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer’s instructions with some modifications. Briefly, Hep3B cells were cross-linked with 1% formaldehyde (Sigma) at room temperature for 10 min and stopped by adding glycine to a final concentration of 125 mM for 5 min. The cells were rinsed twice with ice-cold phosphate-buffered saline, pH 7.4, and collected in 1 ml of ice-cold phosphate-buffered saline and centrifuged for 4 min at 2000 rpm. The cell pellet was resuspended in SDS lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS) containing protease inhibitor mixture (Sigma) for 10 min on ice. Cell lysate was then centrifuged for 10 min at 13,000 rpm, and the supernatant was sonicated to shear the DNA to obtain a size of ~200–1000 bp. Sonicated lysate was then diluted to 1 ml with chromatin Immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 167 mM NaCl) followed by preclearcing with 80 μl of salmon sperm DNA/protein A-agarose beads for 30 min at 4 °C with rotation. The precleared lysate was next immunoprecipitated with 1 μg of anti-STAT3 (Cell Signaling) or 1 μg of nonimmunized goat IgG at 4 °C overnight with rotation. Chromatin-antibody complexes were collected with 60 μl of salmon sperm DNA/protein A-agarose beads and washed once with 1 ml each of low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl), and LiCl immune complex wash buffer (250 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1) and twice with TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Immune complexes were then eluted using freshly prepared elution buffer (1% SDS and 0.1 M NaHCO₃). Cross-links
were reversed by heating at 65 °C for 4 h in the presence of NaCl followed by RNase A treatment for 30 min at 37 °C. Samples were treated with proteinase K and incubated at 45 °C for 1 h. The DNA was recovered by a QIAquick PCR purification kit (Qiagen, Valencia, CA). After purification, PCR was used to analyze immunoprecipitated DNA with the following primers: \( \Delta Np63\text{-}719, 5'\text{-CATAGATGTCATACGTGCA-3'} \) (sense); \( \Delta Np63\text{-}464, 5'\text{-GCAATTACAAAATAAGCTACCTG-3'} \) (antisense). One microliter of the purified DNA was subjected to 30 cycles of PCR amplification in a volume of 25 \( \mu l \) (denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 3 min) by means of Go Taq\textsuperscript{R} Green Master Mix (Promega). The PCR reactions was separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

\[ 3-[4,5\text{-Dimethylthiazol}-2-yl]-2,5\text{-diphenyl Tetrazolium Bromide (MTT) Cell Survival Assay} \]—Hep3B cells (5 \( \times 10^5 \)) were seeded in 96-well plate were treated with various concentrations of AG490. After 24 h, 10 \( \mu l \) of MTT labeling reagent was added to each well. Four hours later, the cells were lysed, and absorbance at 550 nm was measured. The assays were performed in triplicate, and the result was presented as means ± S.D.

**Colony Formation Assay**—The Hep3B cells (2 \( \times 10^5 \) cells) were seeded in 35-mm dishes. The cells were transfected with either non-silencing siRNA, \( \Delta Np63 \) siRNA, STAT3 siRNA, or p63-DBD siRNA (40 nM). Twenty-four hours after siRNA transfection, the cells were harvested and seeded in 60-mm dishes at 800 cells per dish. The medium was changed every 3 days, and cell colonies were fixed with 4% formaldehyde and stained with 0.25% crystal violet on day 10.

**RESULTS**

\( \Delta Np63 \) Exerts a Positive Effect on the \( \Delta Np63 \) Promoter Activity—\( \Delta Np63 \) has been suggested to repress \( \Delta Np63 \) gene expression through suppression of the p53-induced \( \Delta Np63 \) transcriptional activity (9). In the present study we reexamined the possible role of \( \Delta Np63 \) on the \( \Delta Np63 \) promoter activity. We cotransfected \( \Delta Np63 \) expressing vector pCMV-\( \Delta Np63 \) with pGL3-823-Luc into Hep3B cells and examined the reporter activity. As shown in Fig. 1, pGL3-823-Luc reporter activity was dose dependently stimulated by an increasing transfection dose of pCMV-\( \Delta Np63 \); an increase of the reporter activity from 1.7- to 4.4-fold was observed when pCMV-\( \Delta Np63 \) was increased by 0.05 to 0.2 \( \mu g/ml \) compared with that of the pCMV-\( \Delta Np63 \)-untransfected control cells. To examine the promoter specificity, we used reporter vector containing GAPDH promoter in the luciferase assay as a parallel control. We showed that an increased transfection dose of pCMV-\( \Delta Np63 \) does not significantly affect the promoter activity of pGL3-GAPDH-Luc (Fig. 1).

**STAT3 Transactivates \( \Delta Np63 \) Gene**—The role of STAT3 in the regulation of the \( \Delta Np63 \) gene expression was further examined. It has been reported (18) that substitution of two cysteine residues in the C-terminal SH2 domain of STAT3 resulted in constitutive activation. The constitutive STAT3 is designated as STAT3-C in this report. The cells were transfected with STAT3-DN- or STAT3-C-expressing vector (both at 0.2 \( \mu g/ml \)) for 24 h and were then treated with IL-6 (50 ng/ml), a STAT3-activating cytokine (21), for another 24 h to induced expression of \( \Delta Np63 \alpha \). \( \Delta Np63 \alpha \) protein level in Hep3B cells was analyzed by Western blotting. As shown in Fig. 2A, in empty vector-transfected cells, the \( \Delta Np63 \alpha \) protein level was increased by IL-6 stimulation compared with that of the unstimulated control cells, and the increase was abolished by STAT3-DN. Transfection with STAT3-C exhibited no further effect on a IL-6-induced increase in \( \Delta Np63 \alpha \) protein level.

To demonstrate STAT3 alone is sufficient to transactivation of \( \Delta Np63 \) gene, STAT3 expressing vector, pCMV-STAT3-C, or pCMV-STAT3-DN (Y705F) was cotransfected with pGL3-823-Luc into Hep3B cells. Fig. 2B shows that pGL3-823-Luc reporter activity (24 h after transfection) was increased by 4.0-fold or was unchanged in cells transfected with STAT3-C or STAT3-DN vector, respectively. These results suggest that activated STAT3 can transactivate \( \Delta Np63 \).
Regulation of the ΔNp63 Gene Transcription

A. IL-6

ΔNp63α

Transfected STAT3

Endogenous STAT3

V5

GAPDH

B. pGL3-823-Luc reporter activity

(Fold induction)

| Condition       | pGL3-823-Luc reporter activity |
|-----------------|--------------------------------|
| Empty           | 1.0 (-fold induction)          |
| STAT3-DN        | 2.5 (+ fold induction)         |
| STAT3-C         | 3.5 (+ fold induction)         |

C. pGL3-823-Luc reporter activity

(Fold induction)

| Expressing Vector | pGL3-823-Luc reporter activity |
|------------------|--------------------------------|
| IL-6 (50 ng/ml)  | 1.0 (-fold induction)          |
| Empty            | 2.5 (+ fold induction)         |
| ΔNp63            | 3.5 (+ fold induction)         |
| ΔNp63α           | 4.5 (+ fold induction)         |

FIGURE 2. STAT3 transactivates the ΔNp63 gene promoter and enhances ΔNp63 expression. A, Hep3B cells were cotransfected with STAT3-DN- or STAT3-C-expressing vector (both at 0.2 μg/ml) for 24 h and then treated with IL-6 (50 ng/ml) for another 24 h. Equal amounts of protein lysate were subjected to Western blotting analysis using antibody against p63, STAT3, V5, and GAPDH. B, Hep3B cells were cotransfected with pGL3-823-Luc (0.5 μg/ml), pSV-β-galactosidase vector (0.2 μg/ml), and STAT3-DN- or STAT3-C-expressing vector (both at 0.2 μg/ml) for 24 h. Data are presented as fold induction relative to pCMV-empty-transfected cells and are expressed as the means ± S.D. from triplicate analysis. An asterisk denotes a significant difference with a p value < 0.05 (t test). C, the Hep3B cells were transfected with pCMV-empty or pCMV-ΔNp63α (0.2 μg/ml) for 24 h and then treated with or without IL-6 (50 ng/ml) for an additional 24 h to activate STAT3, and the luciferase activity was then analyzed. Data are presented as fold induction relative to pCMV-empty-transfected and IL-6-untreated cells. Data are the means ± S.D. from triplicate analysis. We suggested that if ΔNp63α and STAT3 act through the same pathway, then exposure of the ΔNp63α-transfected cells to STAT3 activator (IL-6) would have no further stimulatory effect on the ΔNp63 promoter activity. On the other hand, if STAT3 and ΔNp63α act on separate signaling pathways, the transactivation would be additive. As shown in Fig. 2C, in empty vector-transfected cells, the luciferase activity was increased by 3.5-fold upon IL-6 stimulation compared with that of the IL-6-untreated cells. The IL-6-induced promoter activity was further confirmed by ΔNp63 Western blot analysis as parallel. We suggested that if ΔNp63α and STAT3 act through the same pathway, then exposure of the ΔNp63α-transfected cells to STAT3 activator (IL-6) would have no further stimulatory effect on the ΔNp63 promoter activity. On the other hand, if STAT3 and ΔNp63α act on separate signaling pathways, the transactivation would be additive. As shown in Fig. 2C, in empty vector-transfected cells, the luciferase activity was increased by 3.5-fold upon IL-6 stimulation compared with that of the IL-6-untreated cells. The IL-6-induced promoter activity was further confirmed by ΔNp63 Western blot analysis as shown in Fig. 2A. Clearly, IL-6-induced ΔNp63 promoter activity was not further increased by pCMV-ΔNp63α transfection, suggesting ΔNp63α and STAT3 probably act through the same signaling pathway.

A STAT3-responsive Element Is Present in the ΔNp63 Promoter—For STAT3 to transactivate ΔNp63 gene transcription, it is assumed that at least a STAT3 responsive element (RE) has to be present in the promoter region. We used TRANSFAC data base and MatInspector program to do the search. A putative STAT3 binding site was identified in the ΔNp63 gene promoter flanking region at −557 to −539 (Fig. 3A). To examine if the putative STAT3-RE responds to STAT3 for transcriptional regulation, Hep3B cells were cotransfected with pGL3-STAT3RE-Luc (0.5 μg/ml), pSV-β-galactosidase vector (0.2 μg/ml), and STAT3-DN- or STAT3-C-expressing vector (both at 0.2 μg/ml) for 24 h. Data are presented as fold induction relative to pCMV-empty-transfected cells. Data are the means ± S.D. from triplicate analysis. The double asterisk denotes p < 0.01 (t test) compared with the control.

FIGURE 3. Identification of the STAT3 responsive element in the ΔNp63 promoter. A, nucleotide sequence of the human ΔNp63 gene 5′-flanking region. The sequence extends from −823 to +262 relative to the transcription start site of ΔNp63 gene. Sequences corresponding to potential responsive elements are underlined. B, Hep3B cells were cotransfected with pGL3-STAT3RE-Luc (0.5 μg/ml), and STAT3-DN- or STAT3-C-expressing vector (both at 0.2 μg/ml) for 24 h. Data are presented as fold induction relative to pCMV-empty-transfected cells. Data are the means ± S.D. from triplicate analysis. The double asterisk denotes p < 0.01 (t test) compared with the control.
Regulation of the ΔNp63 Gene Transcription

Hep3B cells were transfected with empty vectors or pCMV-ΔNp63α, and the whole-cell extracts were prepared. The STAT3 were captured by biotinylated annealed probes (STAT3-RE and STAT3-REmut) and detected by Western blot analysis using anti-STAT3 antibody. Fig. 5C shows that anti-STAT3 antibody recognized a band with an apparent molecular size of 92 kDa by Western blot analysis. The STAT3 was efficiently recovered from STAT3-RE but not STAT3-REmut. In addition, STAT3 recruitment to the ΔNp63 promoter was significantly increased, whereas pCMV-ΔNp63α was transfected. These data show that endogenous STAT3 can bind to the ΔNp63 promoter. To demonstrate that endogenous STAT3 is present at the ΔNp63 promoter in living cells, a chromatin immunoprecipitation assay was performed to evaluate the association of STAT3 with the putative STAT3-RE at ΔNp63 promoter. DNA fragments brought down by STAT3 antibody were PCR-amplified using primers that amplify a 255-bp region (−719 to −464) spanning the putative STAT3-RE (−557 to −539) within the ΔNp6 promoter. As shown in Fig. 5D, in vivo association of STAT3 with the ΔNp63 promoter was detected. In contrast, immune complexes with nonimmunized goat IgG yielded no 255-bp band, demonstrating the specificity of the interaction between STAT3 and the ΔNp63 promoter.

To further ascertain the functional significance of the putative STAT3-RE in ΔNp63 autoregulation, the putative STAT3-RE of the ΔNp63 promoter was deleted (Fig. 5E), and the reporter activities of pGL3-823-Luc and pGL3–823ΔSTAT3-Luc were compared in cells with and without pCMV-ΔNp63α cotransfection. As shown in Fig. 5E, deletion of the STAT3-RE attenuated ΔNp63 autostimulation on transcription by about 65% compared with that of the wild type (pGL3-823-Luc) control.

ΔNp63 Autoregulation Is STAT3-dependent—To ascertain that STAT3 is involved in ΔNp63 autoregulation, we examined the possible inhibitory effect of the JAK2/STAT3 inhibitor AG490. Other kinase inhibitors, including the MAP kinase inhibitor (MEK1/2) PD98059, the p38 inhibitor SB203580, the JNK inhibitor DMAP, the phosphoinositide-3-kinase inhibitor (MEK1/2) PD98059, the p38 inhibitor SB203580, the JNK inhibitor DMAP, the phosphoinositide-3-kinase inhibitor (MEK1/2) PD98059, the p38 inhibitor SB203580, the JNK inhibitor DMAP, the phosphoinositide-3-kinase inhibitor (MEK1/2) PD98059, the p38 inhibitor SB203580, the JNK inhibitor DMAP, the phosphoinositide-3-kinase inhibitor (MEK1/2) PD98059. Pretreatment with AG490, the JAK2/STAT3 inhibitor, at 50 μM completely abolished luciferase activity in pGL3–823-Luc–transfected and pGL3–823-Luc/pCMV-ΔNp63α cotransfected cells (Fig. 6A). Pretreatment with PD98059 (50 μM), DMAP (50 μM), or chelerythrine (10 μM) had no obvious effect on the basal and ΔNp63α-induced luciferase activity. Consistent with prior observations (22), PY294002 pretreatment reduced basal luciferase activity by 33% and the ΔNp63α-induced luciferase activity by 53% compared with that of the respective control (Fig. 6A). Moderate inhibition of the luciferase activity was also observed by SB203580 pretreatment.

The inhibitory effect of AG490 was further confirmed by a dose-dependent assay. Fig. 6B shows that the transcriptional activity of pGL3–823-Luc was dose-dependently repressed by...
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FIGURE 5. STAT3-responsive element is involved in the ΔNp63 autoregulation. A, Hep3B cells were cotransfected with pGL3-STAT3-RE-Luc (0.5 μg/ml) and various concentrations of pCMV-ΔNp63α for 24 h. The pCMV-empty vector was added when necessary to keep the total plasmid concentration constant. Data are the mean ± S.D. from three independent analyses. Luciferase activity is presented as -fold induction relative to pcMV-empty vector-transfected cells. Cell lysates were subjected to Western blot analysis with anti-V5 and anti-GAPDH antibodies. B, gel shift analysis with nuclear extracts prepared from cells transfected with increasing concentrations of pCMV-ΔNp63α. Biotin-labeled duplex oligonucleotides containing putative STAT3-responsive elements of the ΔNp63 promoter was incubated with nuclear extracts (3 μg) for 20 min. Excess (200×) unlabeled HSIE competitor was included to indicate specificity. C, putative STAT3-responsive element (STAT3-RE) in the ΔNp63 promoter region was analyzed in vitro for STAT3 binding by avidin biotin-conjugated DNA assay. Biotinylated annealed probes (STAT3-RE) were incubated with pCMV-empty or pCMV-ΔNp63α-transfected Hep3B whole-cell extract, which serves as a source for STAT3. Ten micrograms of pCMV-empty or pCMV-ΔNp63α-transfected Hep3B whole-cell extract was fractionated on separate lane as an input; STAT3-RE with four bases mutated (STAT3-REmut) was used as the negative control. The sequences substituted are underlined. D, chromatin immunoprecipitation was performed using antibodies specific for STAT3 or nonimmunized goat IgG. The precipitated chromatin was analyzed using PCR. PCR primers were designed to yield a 255-bp product (−719 to +464) spanning the Stat3-RE (−557 to −539) of the ΔNp63 promoter. The Input lane represents 0.01% of total chromatin used in chromatin immunoprecipitation assays. Ab, antibody. E, schematic presentations of pGL3-823-Luc and pGL3-823ΔSTAT3-Luc. The deletion was introduced into the STAT3-RE in pGL3-823-Luc luciferase reporter. The sequence of the STAT3-RE is indicated. Hep3B cells were transfected with 0.5 μg of each reporter along with pCMV-ΔNp63α (0.2 μg) for 24 h. Data are the mean ± S.D. from three independent analysis. Luciferase activity is presented as -fold induction relative to pcMV-empty vector-transfected cells.

AG490; an 80% inhibition was observed at 50 μM. The AG490 effect was specific, because the β-galactosidase activity was not inhibited compared with that of the untreated cells (data not shown). Cell viability of the AG490-treated culture was examined by MTT assay. As shown in Fig. 6C, AG490 did not significantly affect the cell viability. The involvement of JAK2/STAT3 pathway in ΔNp63 autoregulation was further confirmed by transfection with wild type or mutated STAT3 construct. Hep3B cells were transfected with pCMV-ΔNp63α and pGL3-823-Luc with or without cotransfection with pCMV-STAT3-W or pCMV-STAT3-DN, and the luciferase activity was then analyzed. As shown in Fig. 6D, cotransfection of the dominant negative STAT3 resulted in the inhibition of the ΔNp63α-induced pGL3-823-Luc luciferase activity. In contrast, cotransfection with STAT3-wild type exhibited no inhibitory effect. Similar results were observed in STAT3 siRNA-transfected cells. Inhibition of STAT3 expression by STAT3 siRNA inhibits pCMV-ΔNp63α-induced pGL3-823-Luc luciferase activity (Fig. 6E). The results clearly suggested that STAT3 is required for the ΔNp63 transcriptional autoregulation.

Knockdown of STAT3/ΔNp63 Pathway Inhibits Cell Proliferation—We have thus far shown that STAT3 activator (cytokine IL-6) stimulated ΔNp63 expression in Hep3B cells. However, the physiological role of STAT3/ΔNp63 pathway activation is still unclear. To examine whether ΔNp63 or STAT3 knockdown has any effect on the proliferation of Hep3B cell, we examined the cell colony size by colony formation assay. The Hep3B cells were transfected with siRNA against ΔNp63, STAT3, or p63-DBD (this siRNA was designed against the DNA binding domain present in all p6 isoforms) to suppress the respective cellular genes.

Colony size was markedly reduced by STAT3 siRNA transfection compared with that by non-silencing siRNA transfection (Fig. 7). In addition, ΔNp63 or p63-DBD knockdown also significantly inhibited the colony growth.

DISCUSSION

The p63 gene encodes multiple isoforms with structural and functional similarities. In contrast to p53, p63 is not frequently mutated in human tumors; instead, altered expressions of ΔNp63 are frequently found (5–8). These observations strongly suggest that aberrant ΔNp63 expression may play important roles in carcinogenesis tissues. As a dominant-negative inhibitor of p63 and p53, ΔNp63 may bind to the promoter of the p53 target genes without transactivation of the transcription, thus blocking the p53-driven cell cycle arrest and apoptosis (4, 23, 24). On the other hand, lacked expression of TAp63 has also been reported in human tumors (7, 8, 25). Because genomic deletion and mutation of the ΔNp63
Gene are uncommon but altered expression is frequent in human cancers, the regulation of the transcription of \( \Delta Np63 \) gene is likely to be an important event in tumorigenesis. Noteworthy, the mechanisms underlying the regulation of \( \Delta Np63 \) expression have begun to unravel. Recently, a p53 binding element at position -495 to -473 relative to the start of transcription in the \( \Delta Np63 \) promotor region has been identified by Harmes et al. (9). They showed that binding of p53 to this element elicits a positive signal for \( \Delta Np63 \) transcription, whereas binding of \( \Delta Np63 \) to this element exerts a negative signal. This observation is consistent with several studies, suggesting that \( \Delta Np63 \) acts as a dominant negative inhibitor of the p53 function (4, 6). It also suggests that \( \Delta Np63 \) is a negative autoregulator of \( \Delta Np63 \) transcription. We found that at lower \( \Delta Np63 \alpha \) levels, a negative regulation is observed acting through binding to the p53 binding element. In contrast, at higher \( \Delta Np63 \alpha \) levels, a positive regulation is observed, and it does not involve a direct interaction of \( \Delta Np63 \alpha \) with its promoter, suggesting that an indirect mechanism is involved.

In a recent report, Barbieri et al. (22) showed that \( \Delta Np63 \alpha \) is a target of the phosphoinositol 3-kinase pathway downstream of the epidermal growth factor receptor in keratinocytes. Treatment of keratinocytes with epidermal growth factor (EGF) resulted in an increase in \( \Delta Np63 \alpha \) expression at the mRNA level, and inhibition of phosphoinositol 3-kinase 3-kinase abrogated the EGF stimulation. In a previous study we identified another regulatory signaling pathway for \( \Delta Np63 \) expression at both mRNA and protein levels, the STAT3 pathway. We found that higher levels of \( \Delta Np63 \) promotes nuclear translocation of the STAT3, and such translocation is correlated with an increased \( \Delta Np63 \) transcription (26). In the present study we clearly show that the binding of STAT3 to the putative STAT3-RE of \( \Delta Np63 \) promoter leads to increased \( \Delta Np63 \) expression (Fig. 8).

STAT3 has also been identified as a potential oncogene for transfection of constitutively activated STAT3, leading to tumor formation in nude mice (17). Constitutive activation of STAT3 correlates with cell proliferation in breast cancer (27) and non-small-cell lung cancer (28) and inhibits apoptosis (29). In contrast, inhibition of STAT3 signaling results in suppression of cancer cell growth and induces apoptosis in various cancers (27, 30). Moreover, it has been shown that abnormalities of the JAK/STAT3 is associated with oncogenic transformation (31, 32).
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A. siRNA

| Non-silencing | ΔNp63 | STAT3 | p63-DBD |
|---------------|-------|-------|---------|
| Colonies (%)  |       |       |         |

FIGURE 7. Knockdown of STAT3 or ΔNp63 pathway suppresses cell proliferation. The Hep3B cells were transfected with siRNA (40 nm) against ΔNp63, STAT3, p63-DBD, or with non-silencing control. The transfected cells were plated in a 60-mm dish at 800 cells/dish and cultured for 10 days. Colonies were fixed and stained with crystal violet. Dishes were photographed as shown in panel A. The area covered by each colony was measured using Image J software (National Institutes of Health). Colonies with an area equal or greater than 1 mm² were scored and presented in panel B. Data computed from non-silencing control-transfected cells were set as 100%.

B. Relative area of colonies (%)

ΔNp63 promoter

Phosphorylation

Dimerization

Cytokine(s)

STAT3-RE

ΔNp63α

FIGURE 8. Proposed model for ΔNp63 autoregulation. Expression of ΔNp63α causes STAT3 activation where it is phosphorylated on Tyr-705 and Ser-727. Phosphorylation of STAT3 leads to dimerization and nuclear translocation and binding to STAT3-RE in ΔNp63 promoter.

In the present study we show that ΔNp63α activates STAT3 through phosphorylation at Tyr-705 and Ser-727, leading to transcriptional activation of the ΔNp63 gene through binding to a putative STAT3-RE located at positions −557 to −539 relative to the start of transcription. Expression of dominant negative STAT3 or treatment with AG490 inhibited the ΔNp63α-induced autoregulation. Our results clearly demonstrate that STAT3 is involved in the mediation of the positive autoregulation of ΔNp63 transcription. The activation of STAT3 by ΔNp63α through phosphorylation is of possible cell biological significance. The tumor suppressor p53 has been shown to inhibit STAT3-dependent transcriptional activity and induces apoptosis in breast cancer cells expressing constitutively active STAT3 (10). It is, therefore, possible that through interactions at the STAT3 pathway, ΔNp63α may act as a dominant-negative effector to antagonize the tumor suppressor function of p53. ΔNp63α is a transcription factor and presumably possesses no kinase activity. How it causes phosphorylation and activation of STAT3 is currently unclear. One possible mechanism is that ΔNp63α might induce the expression of the IL-6 family cytokines, and these cytokines act in an autocrine manner to activate STAT3. IL-6 family cytokines includes IL-6, IL-10, IL-11, and leukemia-inhibiting factor and are the known mediators that stimulate the JAK/STAT3 pathway. In conclusion, our findings identify a possible link between two diverse signal transduction pathways acting together to inhibit apoptosis and, perhaps, promote tumorigenesis.

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