Tumor Necrosis Factor α Induces Spermidine/Spermine $N^1$-Acetyltransferase through Nuclear Factor κB in Non-small Cell Lung Cancer Cells*

Received for publication, February 27, 2006, and in revised form, May 16, 2006. Published, JBC Papers in Press, June 6, 2006, DOI 10.1074/jbc.M601871200

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Tumor necrosis factor α (TNFα) is a potent pleiotropic cytokine produced by many cells in response to inflammatory stress. The molecular mechanisms responsible for the multiple biological activities of TNFα are due to its ability to activate multiple signal transduction pathways, including nuclear factor κB (NFκB), which plays critical roles in cell proliferation and survival. TNFα displays both apoptotic and antiapoptotic properties, depending on the nature of the stimulus and the activation status of certain signaling pathways. Here we show that TNFα can lead to the induction of NFκB signaling with a concomitant increase in spermidine/spermine $N^1$-acetyltransferase (SSAT) expression in A549 and H157 non-small cell lung cancer cells. Induction of SSAT, a stress-inducible gene that encodes a rate-limiting polyamine catabolic enzyme, leads to lower intracellular polyamine contents and has been associated with decreased cell growth and increased apoptosis. Stable overexpression of a mutant, dominant negative IκBα protein led to the suppression of SSAT induction by TNFα in these cells, thereby substantiating a role of NFκB in the induction of SSAT by TNFα. SSAT promoter deletion constructs led to the identification of three potential NFκB response elements in the SSAT gene. Electromobility shift assays, chromatin immunoprecipitation experiments, and mutational studies confirmed that two of the three NFκB response elements play an important role in the regulation of SSAT in response to TNFα. The results of these studies indicate that a common mediator of inflammation can lead to the induction of SSAT expression by activating the NFκB signaling pathway in non-small cell lung cancer cells.

Polyamines are aliphatic cations present in all cells, whose levels are intricately controlled by their transport and metabolic enzymes. Spermidine/spermine $N^1$-acetyltransferase (SSAT) 2 is a rate-limiting step in polyamine catabolism, which catalyzes the transfer of the acetyl group from acetyl-CoA to the $N^1$ position of spermidine or spermine and has a predominant role in the regulation of intracellular polyamine concentrations in mammalian cells (1, 2). Decreases in polyamines have been shown to promote decreased growth or apoptosis (3–6), depending on the cell type and the particular stimulus, suggesting a complex interaction between polyamines, cell growth, and cell death. Therefore, although polyamines are required for cell growth and differentiation, SSAT is thought to prevent overaccumulation of the higher polyamines from becoming toxic to the cell and may play a role in reducing the growth rate by decreasing intracellular polyamines.

Recently, considerable attention has been paid to SSAT as a target for cancer chemotherapy. SSAT activity is highly regulated and is induced rapidly in response to a number of stimuli, including polyamines, polyamine analogues, hormones, physiological stimuli, drugs, and toxic agents (1). It has been shown that the regulation of SSAT by the natural polyamines and the anti-tumor polyamine analogues is through the polyamine response element (7). Further, the superinduction of SSAT by polyamine analogues has been implicated in the cell type-specific cytotoxic response of several important human tumors (8–13). Various nonsteroidal anti-inflammatory drugs like aspirin, sulindac, and indomethacin and chemotherapy drugs like 5-fluorouracil and oxaliplatin have been shown to induce SSAT expression in various cancer cell types (14–17). Sulindac induces SSAT by inducing peroxisomal proliferator-activated receptors, which, once activated, can bind to the peroxisomal proliferator-activated receptor response elements in the SSAT 5′ promoter region (18). Recently, we have shown that aspirin can also induce SSAT expression in colon cancer cells, partly by activating the NFκB signaling pathway, which leads to the binding of NFκB complexes to the NFκB response elements in the SSAT 5′ promoter region (19).

Tumor necrosis factor α (TNFα) is a potent pleiotropic cytokine and is a major mediator of inflammation with multiple biological functions (20). One of the molecular mechanisms responsible for the biological activities of TNFα is the ability to activate nuclear factor κB (NFκB), which plays critical roles in cell proliferation and survival (21, 22). In mammalian cells, five members of the NFκB/Rel family are known: NFκB1 (p50 or its precursor p105), NFκB2 (p52 or its precursor p100), c-Rel, RelA (p65), and RelB (23, 24). Heterodimers composed of p65-p50 are the most abundant active form of NFκB in most cell types. However, NFκB can consist of other homo- and heterodimers

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*The research was supported by National Institutes of Health Grants CA 51085 and CA 98454. The costs of publication of this article were defrayed solely to indicate this fact.

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2 The abbreviations used are: SSAT, spermidine/spermine $N^1$-acetyltransferase; TNFα, tumor necrosis factor α; NSCLC, non-small cell lung cancer; NFκB, nuclear factor κB; IκBα, inhibitor κBα; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation.
that have different abilities to activate target genes (25, 26). In quiescent cells, NFκB resides in the cytosol in a latent form bound to IkBα. Stimulation of the cell with TNFα triggers a series of signaling events that ultimately leads to the phosphorylation and the proteolytic degradation of IkBα and activation of NFκB. The phosphorylation of IkBα is elicited by an IkBα kinase (IKK), which can be activated by mitogen-activated protein kinase (27, 28), whereas the proteolysis of IkBα is mediated by the ubiquitin-proteasome pathway of protein degradation. The degradation of IkBα triggers the translocation of NFκB from the cytoplasm to the nucleus, where it regulates the expression of multiple genes.

In this study, we examined the effects of TNFα treatment on SSAT expression in representative human non-small cell lung cancer cells and identified the specific NFκB response elements responsible for the regulation of SSAT by TNFα.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents, DNA-modifying enzymes, TRIzol® reagent (total RNA isolation reagent), and Lipofectamine reagent were purchased from Invitrogen (Carlsbad, CA). All primers and oligonucleotides were custom made by Invitrogen. Recombinant human TNFα was obtained from R&D Systems, Inc. (Minneapolis, MN). IkBα antibody is from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and p65 antibody is from Upstate (Waltham, MA). Paclitaxel (Taxol®) was obtained from Sigma-Aldrich.

Cell Culture—The non-small cell cancer lines NCI-A549 (adenocarcinoma) and NCI-H157 (squamous) were purchased from American Type Culture Collection (Manassas, VA) and grown overnight. 15 h later, the medium was removed, and cells were refed complete medium, and cells were allowed to recover for 24 h prior to selection in 0.4 mg/ml G418. Cells were maintained in selection medium until a stable population was achieved. Individual colonies were then isolated, expanded, and used for screening and experiments.

Plasmids—Full-SSAT-luc, having a 3.493-kb-long 5’-flanking sequence of the human SSAT gene, was cloned into a promoterless pGL2-basic (Promega, Madison, WI) as previously reported (29). A series of smaller SSAT promoter constructs were made from Full-SSAT-luc, using PCR and subcloned into pGL2-basic vector. 197-SSAT-luc, having 283 nucleotides of the 5’-flanking region of the SSAT promoter; 283-SSAT-luc, having 441 nucleotides of the 5’-flanking region of the SSAT promoter; and 659-SSAT-luc, having 740 nucleotides of the 5’-flanking region of the SSAT promoter, were made from Full-SSAT-luc using PCR and subcloned into pGL2-basic vector. NFκB1-Luc reporter, having two NFκB response elements; dNFκB2-Luc reporter, in which both the NFκB response elements have been deleted; and dominant negative-IκBα (DN/IκBα) and its control (Ctrl/IκBα) plasmids were obtained from Dr. Nancy Davidson (Johns Hopkins University, Baltimore, MD). The DN/IκBα plasmid has a deletion of 36 NH2-terminal amino acids containing Ser32 and Ser36 phosphorylation sites, which was cloned into the pcDNA3.1 mammalian expression plasmid vector (Invitrogen). mNFκB1-SSAT-luc, mNFκB3-SSAT-luc, and dmNFκB1/3-SSAT-luc were made by mutating the NFκB-1, NFκB-3, and both the NFκB-1 and -3 sites, respectively, in the Full-SSAT-luc using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were done by placing four base substitutions in the respective NFκB sites to achieve the same mutated sequences as the probes used for the electromobility shift assays.

Stable Transfection—Exponentially growing cells were plated at 1 x 10⁶ cells/100-mm plate and cultured in normal medium for 24 h and then transfected using 50 μl of Lipofectamine reagent with 10 μg of the DNA plasmid. After 6 h of incubation, with Lipofectamine-DNA complex, cells were supplemented with complete medium having 20% bovine calf serum and 2% penicillin and streptomycin and grown overnight, after which the medium was removed, cells were refed complete medium, and cells were allowed to recover for 24 h prior to selection in 0.4 mg/ml G418. Cells were maintained in selection medium until a stable population was achieved. Individual colonies were then isolated, expanded, and used for screening and experiments.

Transient Transfections—Transient transfections were performed using Lipofectamine reagent according to the supplied protocol. Briefly, 5 x 10⁵ cells were seeded in a 6-well plate and cultured in normal medium for 24 h. Cells in each well were transfected with 1 μg of firefly luciferase reporter construct along with 0.2 μg of pCMV-β-galactosidase expression plasmid, used as a control for transfectional efficiency. After 6 h, cells were supplemented with complete medium having 20% bovine calf serum and 2% penicillin and streptomycin and grown overnight. 15 h later, the medium was removed, and cells were refed with the medium containing 10 ng/ml TNFα for the indicated amount of time. All transfections were performed in triplicates unless otherwise indicated. Transfected cells were washed once with phosphate-buffered saline and lysed, and luciferase activities were measured using 10 μl of cell extract and 50 μl of luciferase reagent (Promega). β-Galactosidase activity was measured using the β-galactosidase assay kit (Invitrogen) according to the supplied protocol.

Quantitative Real Time PCR—Cells were seeded, grown overnight, and then treated as indicated. Total cellular RNA was extracted using TRIzol® reagent and RNA isolation according to the method developed by Chomczynski and Sacchi (30). 3 μg of total RNA was treated with RNase-free DNase I (Roche Applied Science) for 30 min at 37 °C before reverse transcription with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) as described by the manufacturer. The forward and reverse primers were used at 10 μM. The resulting cDNA was subjected to quantitative real time PCR. The DNA-intercalating SyBr green reagent (Quantitect; Qiagen) and melting temperature profiles were used for detection of the PCR product. Agarose gel electrophoresis confirmed the presence of a single PCR product. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The experimental and GAPDH PCRs were done in separate tubes in triplicates in the MyiQ single color real-time PCR machine (Bio-Rad), and the average threshold cycle (C_t) for the triplicate was used in subsequent calculations. SSAT cDNA (180 base pairs) was amplified using the following primers (Invitrogen): forward, 5’-GGATCAAAATTCTGAGAAT-3’; reverse, 5’-ACCCCTTTCCTGACAGATC-3’. As a loading control, GAPDH cDNA (188 base pairs) was also amplified using

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the following primers: forward, 5′-GAAGGTGAAGGTCA-3′; reverse, 5′-GAAGTGGTACGGAGGTGATC-3′.

Immunoblotting—Total cell extracts were obtained by lysing cells on ice in radioimmunoprecipitation buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μg/ml aprotinin, 100 μM sodium orthovanadate, 10 μg/ml phenylmethylsulfonyl fluoride) and centrifuging for 20 min at 4 °C. 30 μg of total protein was loaded per lane and separated on a 10% SDS-polyacrylamide gels for IκBα immunoblot. The proteins were transferred electrophoretically to immunoblot polyvinylidene difluoride membranes for 1 h. Blots were blocked in Blotto A (5% w/v nonfat dry milk in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and probed for IκBα and p65 proteins. Blots were stripped and redetected with β-actin (Santa Cruz Biotechnology) antibody as a loading control. Western blot analyses were repeated at least three times, and a representative blot was chosen for presentation.

In Taxol experiments, Western blot results were quantified using the LICOR immunofluorescence system (LI-COR Biosciences, Lincoln, NE). Briefly, membranes were blocked for 1 h in Odyssey blocking buffer, per the manufacturer’s instructions. Rabbit anti-IκBα and mouse anti-actin (loading control) (Santa Cruz Biotechnology) primary antibodies were then added together at dilutions of 1:1000 and 1:1500, respectively, with 0.1% Tween 20 in blocking buffer for 1 h at room temperature. Following washes with PBS–TWEEN, blots were incubated with appropriate fluorescent dye-conjugated secondary antibodies (1:4000 each, 0.1% Tween 20, in blocking buffer, pro-

Gel Electromobility Shift Assays—Nuclear extracts were prepared from A549 cells as described previously (31). To study the binding of NFκB complexes to the putative NFκB sites, double-stranded oligonucleotides for each of the three putative NFκB sites in the SSAT 5′ sequence were 32P-labeled with polynucleotide kinase (Promega, Madison, WI). The oligonucleotide containing the first putative NFκB site spans from −304 to −280 of the SSAT 5′ sequence and had the sequence NFκB-wild-1 (5′-GCTGCAGAGCTTGCTCCAAAA-3′), whereas the corresponding mutant NFκB-mut-1 (m) had the sequence 5′-GCTGCAGATGACCTTCTTT-3′. The oligonucleotide spanning the third putative NFκB site spanned from −1751 to −1728 of the SSAT 5′ sequence and had the sequence NFκB-wild-3 (3′-GCTAGGGTACCTCAGGATCGGTCT-3′), whereas the corresponding mutant NFκB-mut-3 (m) had the sequence 5′-GCTAGGTTACCTCAGGATCGGTCT-3′. The putative NFκB sequence is underlined, and the mutated bases are shown in lowercase letters. A 10-μl reaction containing 10 μg of nuclear extract was incubated for 15 min at room temperature in a buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2.5 mM EDTA, 250 mM NaCl, 2.5 mM dithiothreitol, 20% glycerol, and 1 μg of poly(dl-dC). Following this, 30,000 cpm of the labeled probe was added, and the reaction mixture was incubated for 30 min at room temperature. The DNA-protein complexes were resolved from the free probe by electrophoresis at 4 °C on a 5% polyacrylamide gel in 0.75 × TBE buffer, pH 8. Band density was quantified using the Typhoon 8600 PhosphorImager and ImageQuant software (Amersham Biosciences).

Chromatin Immunoprecipitation (ChIP)—Chromatin immunoprecipitation assays were performed using the commercially available ChIP kit (Upstate Cell Signaling) with some modifications. A549 cells were seeded at 2 × 10⁶/culture dish and grown overnight. On the following day, they were treated with 10 ng/ml TNFα for increasing times. After treatment, the cells were treated with 1% formaldehyde by adding 0.4 ml of 37% formaldehyde directly to 15 ml of culture medium for 15 min at 37 °C. The cells were washed twice with cold phosphate-buffered saline containing protease inhibitors (leupeptin, phenylmethylsulfonyl fluoride, and aprotinin) and then suspended in 0.7 ml of SDS-lysis buffer (50 mM Tris-Cl, pH 8.1, containing 1% SDS and 10 mM EDTA) plus protease inhibitors. Twenty 10-s sonication pulses with 30-s intervals were done using the Branson Sonifier 250 (cycle 40%, output 1.5/2) to shear chromatin to <1000-bp fragments. The effectiveness of shearing was confirmed by incubating a 100-μl aliquot of the extract with 10 μl of 5 mM NaCl at 65 °C for 4 h (to reverse cross-links) and subsequently subjecting it to electrophoresis on a 1% agarose gel. The sonicated sample was centrifuged at 13,000 rpm for 10 min. 0.2 ml of the cell supernatant was diluted by adding 1.8 ml of ChIP dilution buffer (16.7 mM Tris-Cl, pH 8.1, containing 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, and 1.2 mM EDTA) plus protease inhibitors. 50 μl of this sample was stored for later PCR analysis as the input sample control. To reduce nonspecific background, the remaining amount (1950 μl) was precleared with 75 μl of salmon sperm DNA/protein-agarose 50% slurry (Upstate Cell Signaling, NY) for 1 h at 4 °C with agitation. The cleared supernatant was divided into 0.4-ml aliquots and incubated with either no antibody, a nonspecific antibody (rabbit IgG), 4 μl of anti-p50, or 4 μl of anti-p65 and incubated overnight at 4 °C with rotation, followed by the addition of 30 μl of salmon sperm DNA/protein-agarose 50% slurry and incubated for an additional 3 h.

The agarose was pelleted by centrifugation, and the pellets were washed consecutively with 0.5 ml of low salt buffer (20 mM Tris-Cl, pH 8.1, containing 0.1% SDS, 1% Triton X-100, 150 mM NaCl, and 2 mM EDTA), 0.5 ml of high salt buffer (20 mM Tris-Cl, pH 8.1, containing 0.1% SDS, 1% Triton X-100, 500 mM NaCl, and 2 mM EDTA), 0.5 ml of LiCl wash buffer (10 mM Tris-Cl, pH 8.1, containing 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, and 1 mM EDTA), and 0.5 ml of 1× Tris/EDTA (10 mM Tris-Cl, pH 8.1 containing 1 mM EDTA). DNA and protein were eluted from the pellets by incubating the pellets twice in 0.25 ml of elution buffer (0.1 M NaHCO₃ with 1% SDS), and protein-DNA cross-links were reversed by incubating with 20 μl of 5 mM NaCl at 65 °C for 4 h. Proteins were then degraded by incubating the eluant with 10 μl of 0.5 M EDTA, 20 μl of 1 M Tris-Cl, pH 6.5, and 2 μl of 10 mg/ml Proteinase K for 1 h at 45 °C. DNA was recovered by phenol/chloroform extraction and ethanol-precipitated overnight at −20 °C. Samples were pelleted, washed with 70% ethanol, and dissolved in 100 μl of
sterile water. 2-μl aliquots were used for each PCR to determine binding of either p65 or p50 to the co-immunoprecipitated promoter fragments. The primers used to probe for the NFκB-1 site are forward (5’-TCTTGAGTGGCTCCCACT-3’) and reverse (5’-GGTGTGTCCTCCAGTAAAC-3’), primers for the NFκB-2 site are forward (5’-GGGCAGAAGGAAGTGTC-3’) and reverse (5’-CCAAAGGAATGTKGTCCTG-3’), and primers for the NFκB-3 site are forward (5’-GACCACCCCTCACATTCAAC-3’) and reverse (5’-TGCCAGGAGATGCTAGG-3’).

SSAT Enzyme Activity Determination—For enzyme activities, cells were grown overnight and then treated with TNFα for different times. Cells were harvested after treatment and washed in cold phosphate-buffered saline. The radiochemical assay of the N⁶-acetylated spermidine synthesized from [¹⁴C]acetyl-coenzyme A and unlabeled spermidine, as previously described (8). Fold change was calculated by dividing the enzyme activity for the sample by the vehicle. The enzyme assays were performed in triplicate.

Polyamine Analysis—Cell extracts were prepared in 0.1 N HCl (4 × 10⁶ cells/900 μl). After sonication, the preparation was adjusted to 0.2 N HClO₄, and the supernatant was analyzed by reverse-phase high performance liquid chromatography with 1,7-diaminoheptane as an internal standard (32). Protein was determined by the BCA assay (33).

Statistical Analysis—All transient transfection experiments were performed in triplicates and were repeated at least three times. Quantitative real time PCR and ChIP assays were done at least three times. Representative experiments or mean values ± S.D. are shown. Statistical differences were determined by Student’s t test. A p value of <0.05 was considered significant.

RESULTS

TNFα Induces SSAT mRNA and SSAT Enzyme Activity and Decreases Intracellular Polyamine Contents in Non-small Cell Lung Cancer (NSCLC) Cells—We have previously demonstrated the presence of functional NFκB response elements in the SSAT gene (34). Since SSAT has been implicated as a stress enzyme activity in these NSCLC cells. TNFα, at 10 ng/ml, induces SSAT mRNA expression in both A549 and H157 NSCLC cells within 30 min of treatment (Fig. 1A). SSAT is an enzyme that acetylates both spermidine and spermine. The acetylated products can then be either exported or degraded by the action of polyamine oxidase, thereby leading to a decrease in intracellular polyamine content. Therefore, we next determined whether the increase in SSAT mRNA by TNFα treatment was accompanied by an increase in SSAT enzyme activity and a decrease in the polyamine content. TNFα treatment led to an increase in SSAT enzyme activity in these NSCLC cells (Fig. 1B) and a modest reduction in the intracellular polyamine content (Fig. 2). The decrease in polyamine content is consistent with the -fold induction in the SSAT enzyme activity by TNFα in the NSCLC cells.

TNFα Activates NFκB Signaling in NSCLC Cells—One of the major signaling pathways by which TNFα is known to work is by activating NFκB signaling in the cell. In unstimulated cells, NFκB resides in the cytosol in a latent form bound to IκBα. Stimulation of the cell with TNFα triggers a series of signaling events that ultimately leads to the phosphorylation of IκBα by IKK, which then becomes a target of ubiquitin-proteosomal degradation. The phosphorylation and subsequent degradation of IκBα triggers the translocation of NFκB complex (p65-p50) protein from the cytoplasm to the nucleus, where it regulates the expression of multiple genes. The finding that TNFα induces SSAT expression and the fact that TNFα functions predominantly via NFκB signaling led us to determine the functionality of the NFκB signaling in these NSCLC cells. Treatment of A549 cells with TNFα produced a rapid increase in the IκBα protein (Fig. 3A), with a concomitant rapid increase in nuclear translocation of the p65 protein in the A549 cells (Fig. 3B). Similar effects on IκBα and p65 protein were observed in

FIGURE 1. TNFα induces SSAT expression in NSCLC cells. A, A549 (black bars) and H157 (gray bars) cells were treated with TNFα or its vehicle (V, open bar) for 15, 30, 60, and 180 min and harvested, and total RNA was extracted and used for quantitative reverse transcription-PCR. SSAT expression was normalized to the G3PDH expression in these treatments. -Fold induction of A549 and H157 cells were grown overnight and then treated with either vehicle (V, open bar) or 10 ng/ml TNFα (black and gray bars) for 1 or 3 h. Cells were then harvested, and SSAT enzyme activity was measured as described under “Experimental Procedures.” SSAT enzyme activity values are given as pmol/min/mg of protein. The results represent the mean of three different experiments ± S.D. *, a significance with p < 0.05.
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FIGURE 2. TNFα decreases intracellular polyamines in NSCLC cells. A549 and H157 cells were seeded and grown for 24 h, after which they were treated with either vehicle (white bars) or TNFα (black and gray bars) for 1 h. Putrescine (A), spermidine (B), and spermine (C) were detected by high pressure liquid chromatography. The polyamine levels were normalized to the protein in the samples and plotted. The result is the mean of two different experiments performed in duplicate ± S.D.

the H157 cells (data not shown). Transient transfection experiments were performed with an NFκB reporter construct to determine whether translocation of p65 into the cell nucleus was accompanied by a functional increase in the NFκB-responsive genes. As shown in Fig. 3C, TNFα treatment led to an increase in the luciferase activity from the NFκB2-Luc plasmid in both A549 and H157 cells but had no effect on the control plasmid lacking NFκB response elements, suggesting a functional NFκB signaling mechanism in these NSCLC cells.

Attenuation of TNFα-induced SSAT mRNA by Dominant Negative IκBα—To verify that TNFα induction of SSAT expression was indeed mediated by the nuclear translocation of the NFκB complex, both A549 and H157 cells were stably transfected with the dominant negative IκBα (DN/IκB-A549 and DN/IκB-H157)-overexpressing plasmid. DN/IκB plasmid lacks the NH2-terminal 36 amino acids, thereby removing the Ser32 and Ser36 residues that are required for its phosphorylation by IKK and subsequent proteosomal degradation, but maintains the ability to bind NFκB complex in the cytosol. Two A549 clones with the highest expression of DN/IκBα protein (Fig. 4A) were used to study the effects of TNFα treatment on NFκB and SSAT activation. Two clones from the vector-transfected A549 cells (Ctrl/IκB-A549) were used as the control. Treatment with TNFα resulted in translocation of p65 into the nucleus in the Ctrl/IκB clones, but the dominant negative IκBα overexpression completely blocked the nuclear translocation of the p65 protein in either vehicle- or TNFα-treated cells, suggesting the inability of TNFα to activate the NFκB signaling in the DN/IκB-A549 cells (Fig. 4B). Further, DN/IκB overexpression either abolished (as in clone 1) or attenuated (as in clone 2) SSAT mRNA induction by TNFα in the A549 cells (Fig. 4C). Treatment with TNFα also resulted in p65 translocation and induced SSAT mRNA in the Ctrl/IκB-H157 clones, but the dominant negative IκBα blocked both the nuclear translocation of p65 and the induction of SSAT in the DN/IκB-overexpressing H157 clones (Fig. 4D).

Taxol Induces SSAT by an NFκB-dependent Pathway in A549 Cells—To determine the effects of activating NFκB by an agent other than TNFα on the expression of SSAT in the A549 cells, the effects of Taxol exposure were examined. Taxol has been previously demonstrated to induce NFκB activation through
phosphorylation of IκBα, thereby leading to its degradation, in various cancer cells (35–39). Further, Taxol has been shown to activate NFκB signaling in the A549 cells (40). At both 10 and 50 nM, Taxol reduced IκBα protein to 0.52 ± 0.11 and 0.54 ± 0.08, respectively, of control. Further, these concentrations of Taxol led to the induction of SSAT mRNA in both A549 parental and Ctrl/IκB-A549 cells, but not in the DN/IκB-A549 cells (Fig. 5). These results are consistent with the hypothesis that NFκB plays a role in the regulation of SSAT, whether the source of signaling is from TNFα or the cytotoxic agent, Taxol.

**Mapping of TNFα-responsive Elements in the SSAT Promoter**—The inability of TNFα to induce SSAT expression in DN/IκB-overexpressing cells is consistent with the hypothesis that NFκB plays an important role in the regulation of SSAT by TNFα in the NSCLC cells. Three putative NFκB response elements, NFκB-1 (w) at −286, NFκB-2 (w2) at −594, and NFκB-3 (w3) at −1735 relative to the transcription start site, have been identified in the SSAT 5′ flanking region (19). In order to determine whether these NFκB response elements play a role in the induction of SSAT by TNFα, the 5′-flanking sequences of SSAT were tested for their ability to mediate TNFα-induced transcription of a reporter gene. Four luciferase constructs were used, each containing portions of the SSAT 5′-flanking region linked to a promoterless firefly luciferase gene. Full-SSAT-luc, 659-SSAT-luc, 358-SSAT-luc, and 197-SSAT-luc contained 3.53, 0.74, 0.44, and 0.28 kb, respectively, of the SSAT 5′-flanking sequence (Fig. 6A). Full-SSAT-luc contains all three previously identified NFκB sites, 659-SSAT-luc contains two of the three sites (NFκB-2 and NFκB-3), 358-SSAT-luc contained only one of the three NFκB sites (NFκB-1), and 197-SSAT-luc contained none of the NFκB sites. TNFα treatment of the A549 cells, which have been transiently transfected with either of these SSAT-luc reporter constructs, activated transcription of the Full-SSAT-luc and 358-SSAT-luc reporter construct by 2–3-
fold but did not induce the 197-SSAT-luc and the control pGL2-basic vector (Fig. 6B). TNFα did not induce the 659-SSAT-luc reporter construct, which could be due to the presence of inhibitory cis-acting sequences in the stretch of the SSAT promoter, which are not present in the 358-SSAT-luc promoter construct. These data suggest a role for NFκB-1 and NFκB-3 in the induction of SSAT by TNFα in the NSCLC cells.

**TNFα Induces NFκB Binding to Specific NFκB Response Elements in the SSAT Promoter**—Electrophoretic mobility shift assays were performed to determine whether TNFα exposure induces the binding of NFκB complexes to the NFκB response elements in the SSAT promoter and produce the observed increase in SSAT expression in the NSCLC cells. Electrophoretic mobility shift assays were performed using the nuclear extract from the A549 cells, which had been treated with TNFα for increasing times, and double-stranded oligonucleotides containing the putative wild-type NFκB sequences. As shown in Fig. 7A, we observed binding of nuclear proteins to the NFκB-1 (w) probe. The presence of two bands suggested two different protein complexes that are bound to the (w) probe. TNFα treatment led to a slight increase in the binding of the complexes to the (w) probe as compared with the vehicle-treated nuclear extract, suggested by an increase in the upper band intensity. This binding was specific, since binding to the NFκB-1 mutant (m) sequence was not observed, and the binding to the labeled wild type probe could be competed by cold wild probe (w). Incubation of nuclear extracts with the labeled probe and an antibody to the p50 subunit of the NFκB complex led to the generation of a supershift band, suggesting the presence...
of p50 protein in the complexes bound to the NFκB-1 sequences. This supershift was observed in the absence of the TNFα treatment, indicating the presence of a p50-containing complex at the NFκB-1 sequence. Using other putative NFκB sequences, we found no significant binding of the TNFα-treated A549 nuclear extract with the NFκB-2 (w2) sequence (data not shown) but found that there was binding of NFκB complexes to the NFκB-3 (w3) sequence (Fig. 7B). This binding was increased by TNFα treatment and was specific, since it could be competed by cold (w3) probe and supershifted in the presence of a p50 antibody. No shift was observed when the NFκB-3 mutant (m3) sequence was used as a labeled probe.

To confirm the binding of NFκB protein complexes on the SSAT promoter in response to TNFα in situ, ChIP assays were performed for all three NFκB response elements in the SSAT promoter. A549 cells were treated with TNFα for increasing times, and then the nuclear protein-DNA complexes were cross-linked and then precipitated with either p50 or p65 antibodies. PCR amplification of the DNA present in the anti-p50 and anti-p65 chromatin immunoprecipitate demonstrated that p50 protein is bound to the NFκB-1 sequence in the unstimulated cells, and TNFα stimulation leads to the binding of the p65 protein to the NFκB-1 sequence (Fig. 8A). ChIP assays done with the NFκB-2 sequence in the SSAT promoter showed that p50 protein, but not p65, is bound to this site with or without TNFα treatment (Fig. 8B). ChIP assays done with the NFκB-3 sequence in the SSAT promoter showed that neither p50 nor p65 proteins are bound to this site in the unstimulated state, and TNFα stimulation leads to the binding of both p50 and p65 proteins to the NFκB-3 sequence (Fig. 8C).

To further evaluate the roles of NFκB-1 and NFκB-3 in the regulation of SSAT by TNFα, we introduced mutations in either or both of the NFκB-1 and NFκB-3 sites in the Full-SSAT-luc reporter constructs. The mutated plasmids were then transfected into the A549 cells and then treated with TNFα for 1 h. Expression of constructs containing the wild type NFκB-3 but mutant NFκB-1 sequences (mNFκB1-SSAT-luc) were induced to a greater level than the constructs containing a mutant NFκB-3 and wild type NFκB-1 sequences (mNFκB3-SSAT-luc) (Fig. 9). This suggests a greater role for NFκB-3 in the TNFα-induced SSAT expression. Expression of constructs containing both mutant NFκB-1 and NFκB-3 sequences but wild type NFκB-2 (dmNFκB1/3-SSAT-luc) was not induced by TNFα exposure, indicating that both NFκB-1 and NFκB-3 play a role in the maximal response to TNFα exposure in the NSCLC cells. These results also confirm that NFκB-2 is not capable of mediating the TNFα response, consistent with ChIP results indicating that p65 is not bound to NFκB-2 in TNFα-stimulated cells (Fig. 8C).

**DISCUSSION**

TNFα is a potent pleiotropic proinflammatory cytokine produced by many different cells in response to infection and inflammatory stress. TNFα has been shown to have paradoxical roles in the evolution and treatment of malignant disease (41, 42). Depending on the source, cell type, and level of TNFα produced, it can lead to either cell death or cell survival. Activation of NFκB has been associated with inflammation, increased cel-
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ular proliferation, and decreased programmed cell death, but there is also evidence for the role of NFκB activation in leading to cell death and apoptosis (43, 44). The exact action of the NFκB activation in a cell depends on the cell type, the type of activating stimuli, and the ratio of different genes induced. Aspirin, a nonsteroidal anti-inflammatory drug, can lead to the activation of NFκB in Caco-2 cells, which leads to SSAT induction in these cells (34).

It is demonstrated here that SSAT is one of the genes regulated by TNFα in non-small cell lung cancer cells. Treatment of two non-small cell lung cancer cell lines, A549 and H157, with TNFα leads to a rapid increase in the SSAT mRNA expression and enzyme activity and to a decrease in the polyamine content. SSAT activity leads to increased acetylation of spermidine and spermine, which can be then be back-converted via the polyamine oxidase pathway or exported from the cells. SSAT has been implicated as a stress response gene whose expression can be induced to a substantial level by endogenous and environmental factors, including a variety of growth factors, toxic agents, and the polyamines themselves (1). SSAT overexpression has also been implicated in adaptive responses to environmental stress (9), hypoxia and nutrient depletion (13), polyamine analogues (8, 10–12, 46), and exposure to non-steroidal anti-inflammatory drugs (16, 48). Our data suggest that induction of SSAT by TNFα may provide a mechanism by which cells respond to inflammatory stress by reducing intracellular polyamines and slowing growth.

Superinduction of SSAT by specific agents has been shown to be associated with anti-tumor activity in several important human solid tumor models (12, 49), with the NSCLCs typically responding to the polyamine analogue treatment with a significant induction of SSAT and subsequent cell death (8, 11, 50, 51). In specific instances, the superinduction of SSAT results in production of H2O2 from the constitutive activity of the acetyl-polyamine oxidase, polyamine oxidase. The H2O2 thus produced can lead to DNA damage and ultimately cell death (52), suggesting another mechanism by which TNFα can lead to apoptosis in lung cancer cells.

Interestingly, it has been proposed that polyamines can directly modulate NFκB binding in various cell types. Currently, the data are conflicting as to the precise role of polyamines in regulating NFκB activation and DNA binding (53–57). Our results suggest that a stress stimulus, like TNFα, leads to a rapid induction in SSAT expression via NFκB activation, leading to a reduction in polyamine content. It is possible that this rapid polyamine depletion inhibits NFκB DNA binding activity, thereby directly providing feedback regulation of SSAT expression. More experiments will be necessary to determine whether such a feedback mechanism exists in NSCLC cells.

The molecular mechanisms responsible for the multiple biological activities of TNFα are due to their ability to activate multiple signal transduction pathways, including those involving extracellular signal-regulated kinase, other mitogen-activated protein kinases, and NFκB (21, 22). In the systems reported here, the data are most consistent with TNFα acting through NFκB. Exposure of the NSCLC lines to TNFα reduces IκBα protein, increases nuclear p65 protein, and induces the expression of reporter constructs containing specific NFκB response elements. Additional evidence that TNFα is acting through NFκB is provided by the observed significant reduction in the ability of TNFα to induce SSAT in the DN/IκB-A549 and DN/IκB-H157 cells. It should be noted, however, that the expression of the dominant negative IκB does not completely block SSAT induction by TNFα in DN/IκB-A549 cells, suggesting that TNFα may also function through NFκB-independent signaling pathways (58–62).

Taxol has been shown to activate NFκB signaling by increasing the phosphorylation of IκBα in various cell types. This activation of NFκB has also been associated with resistance to Taxol in these cells (35, 38). Taxol was used here as another NFκB activator to study the role of NFκB activation in the induction of SSAT in these cells, since Taxol has been specifically demonstrated to activate NFκB in the A549 cells (40). We found that Taxol treatment produced an approximately 50% decrease in the IκBα protein, thereby leading to the activation of NFκB and induced SSAT expression in A549 cells. Overexpression of DN/IκB significantly attenuated the induction of SSAT observed in the Ctrl/IκB-A549 cells. Although Taxol treatment does not result in complete degradation of IκBα, the suppression of SSAT induction in the presence of DN/IκB indicates a central role for NFκB activation in the induction of SSAT in response to Taxol.

NFκB complexes are present as homo- or heterodimers of p50 and p65 proteins, which can then bind to specific NFκB response elements in NFκB-regulated genes. Three NFκB response elements in the SSAT promoter have previously been identified (34). The results presented here from electrophoretic mobility shift assays, reporter constructs, and ChIP experiments indicate that two sites, NFκB-1 at −286 and NFκB-3 at −1735, but not NFκB-2 at −594, are responsible for the TNFα response. Mutating either the NFκB-1 or NFκB-3 response elements decreased the induction of SSAT reporter constructs by TNFα, and mutations in both of the NFκB response elements were required to completely abolish the induction of SSAT reporter constructs by TNFα in A549 cells. Further, constructs having mutant NFκB-1 but wild type NFκB-3 showed higher SSAT promoter activity after TNFα treatment than the constructs having mutant NFκB-3 but wild type NFκB-1 sequences. These data suggest that NFκB-3 may play a greater role than NFκB-1 in regulation of SSAT by TNFα in A549 cells. This preference for NFκB-1 over the NFκB-1 could be due to the 5-base difference between the two response elements or due to the different contacts made by the transcriptional activation complex because of the ~1.5-kb distance between them. Further experiments will be required to characterize the exact role of NFκB-3 and NFκB-1 in the actions of TNFα on the SSAT promoter.

It is important to note that the chromatin immunoprecipitation experiments were particularly informative, demonstrating that p50 was bound to the NFκB-1 and NFκB-2 sequences with or without TNFα treatment. Of the NFκB/Rel proteins, only p65 and c-Rel have potent transcriptional activation domains, whereas p50 protein lacks these domains. Hence it is thought that the binding of p50 homodimers functions as a transcriptional repressor (47, 63, 64), as compared with the p50-p65
heterodimers, which can function as transcriptional activators. The data presented here suggest that in the basal uninduced state, NFκB-1 and NFκB-2 are bound by p50 homodimers, thereby keeping the SSAT expression level low, but after TNFα treatment (or stress stimuli), there is a change in the promotor-bound NFκB complexes such that the p50 homodimers are replaced by p50-p65 heterodimers at the NFκB-1 site and a recruitment of p50-p65 heterodimers at the NFκB-3 site, which together lead to the induction of SSAT expression.

The discovery that the expression of SSAT is regulated by a common mediator of inflammation, TNFα, is highly significant both in the context of understanding the regulation of polyamine homeostasis and in understanding tumour cell responsiveness to various anti-tumor strategies. Depending on the extent, the induction of SSAT has been linked to both growth inhibition and cell death (10, 11, 46). Thus, TNFα stimulation of SSAT may, in some cases, allow cells to reduce their growth rate when exposed to a stressful environment, and the observed cytotoxic drug responses may be an exaggerated extension of this normal response. Although more studies will be necessary to test this possibility, it is intriguing to consider the potential cross-talk that may occur between the polyamine response element (7, 45), which is thought to mediate SSAT induction produced by cytokine polyamine analogues, and NFκB and how such cross-talk might be exploited for therapeutic advantage.

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