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Spermidine/Spermine N¹-Acetyltransferase 2 (SSAT2) functions as a coactivator for NF- κ B and cooperates with CBP and P/CAF to enhance NF- κ B-dependent transcription

Nancy L. Vogel^{*}, Marta Boeke^{*}, and Brian P. Ashburner

Department of Biological Sciences, The University of Toledo Toledo, OH 43606

Abstract

Activation of transcription by NF- κ B requires association with coactivator proteins, including CBP/p300 and P/CAF. To identify new coregulatory proteins, a cytoplasmic two-hybrid screen was performed using the C-terminus of the p65 subunit as bait. Through this screen, the spermidine/spermine N¹-acetyltransferase 2 (SSAT2) protein was identified as a potential modulator of NF- κ B activity. SSAT2 was originally identified based on homology to SSAT1, a protein involved in polyamine catabolism. However both proteins contain an acetyltransferase domain that has similarity to the acetyltransferase domains of the GNAT superfamily of coactivators. Although SSAT2 is 46% identical to SSAT1, based on a recent report, SSAT2 does not appear to function in polyamine catabolism. Because of the similarity of SSAT2 to coactivators, we wanted to determine if SSAT2 could function as a coactivator for NF- κ B. Coimmunoprecipitations confirmed the interaction between p65 and SSAT2. In transient transfection reporter gene assays, SSAT2 functions as a transcriptional coactivator for NF- κ B and cooperates with CBP and P/CAF to enhance TNF α -induced NF- κ B activity. Moreover, SSAT2 transiently associates with the promoters of the NF- κ B-regulated cIAP2 and IL-8 genes in response to TNF α . Although the overall function of SSAT2 is not known, it appears that can function as a transcriptional coactivator.

Keywords

coactivator; acetyltransferase; NF- κ B

1.Introduction

NF- κ B is a transcription factor that plays a role in a number of important cellular processes by regulating expression of genes involved in immune and inflammatory responses, cell adhesion, cell cycle regulation, angiogenesis, and apoptosis [1,2]. The NF- κ B family of transcription factors includes five mammalian members: RelA (referred to as p65 from here on), c-Rel, and RelB, as well as p105 and p100 which are processed into the p50 and p52 subunits respectively [1,2]. Each subunit contains a highly conserved Rel homology domain (RHD) that contains the DNA binding and protein dimerization domains, as well as the nuclear localization sequence. The p65, c-Rel, and RelB subunits also contain an extended carboxyl-terminal transactivation domain (TAD) that is capable of inducing high levels of transcription. The NF-

Address correspondence to: Brian P. Ashburner, Department of Biological Sciences, MS 601, The University of Toledo, 2801 W. Bancroft St., Toledo, OH 43606. Tel: 419-530-1542; Fax: 419-530-7737; E-mail: brian.ashburner@utoledo.edu. .

^{*}The first two authors contributed equally to this work.

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κB subunits can homo- or heterodimerize through the RHD forming transcription factor complexes with a wide range of DNA-binding and activation potentials. The most common and well studied form of NF-κB is the p50 and p65 heterodimer [3]. In unstimulated cells NF-κB resides primarily in the cytoplasm bound by a member of the IκB family of inhibitory proteins [1,2]. Upon stimulation, IκB is phosphorylated by the IκB Kinase (IKK) complex that then targets IκB for polyubiquitination and degradation by the 26S proteasome, allowing NF-κB to translocate to the nucleus to activate transcription of its target genes [1,2].

Upon entry into the nucleus, NF-κB binds to *cis*-acting elements within the promoters of its target genes to induce high levels of transcription. Activation of transcription by NF-κB requires transcriptional coactivator proteins, such as those that possess histone acetyltransferase (HAT) activity [1]. These proteins primarily function to alter chromatin structure, allowing recruitment of the basal transcription factors and RNA polymerase II to initiate transcription. NF-κB interacts with several different HATs including the CREB-binding protein (CBP) and its homolog, p300 [4-6], the p300/CBP-associated factor (P/CAF) [6-8], and members of the SRC/p160 family [6-8]. These HATs acetylate the amino-terminal tails of the core histones, as well as the p50 and p65 subunits of NF-κB [7,9,10] to stimulate high levels of NF-κB-dependent gene expression.

In addition to interaction with coactivator proteins, NF-κB also interacts with transcriptional corepressor proteins, including the Class I histone deacetylase proteins HDAC1, HDAC2, and HDAC3 [9,11,12], and the Class III HDAC, SIRT1 [13]. These proteins negatively regulate NF-κB transcriptional activity by deacetylating the aminoterminal tails of the core histones, as well as by deacetylating the p50 and p65 subunits of NF-κB. In support of this, treatment of cells with specific HDAC inhibitors potentiates TNF-induced NF-κB activity [11,12,14,15].

To identify new coregulatory proteins that can interact with the p65 subunit of NF-κB, we carried out a cytoplasmic yeast two-hybrid screen using C-terminus (aa 313-551) of the p65 subunit, which includes the p65 transactivation domains (TAD), as the bait. One of the proteins identified in this screen is the product of the spermidine/spermine N¹-acetyltransferase 2 (SSAT2) gene. SSAT2 contains a predicted acetyltransferase domain similar to the GCN5-related N¹-acetyltransferase (GNAT) family [16]. Mammalian members of the GNAT superfamily include GCN5 and P/CAF [17] and more broadly share structural homology with other HAT proteins including CBP/p300 and the p160/SRC family [17]. Interestingly, P/CAF, as well as CPB/p300 and p160/SRC function as coactivators for NF-κB [6].

SSAT2 is a 170 amino acid protein with a predicted molecular mass of 20 kDa that was originally identified based on its homology to SSAT1 [18,19]. SSAT2 is 64% similar and 46% identical to SSAT1 [16]. SSAT1 functions in polyamine catabolism and contributes to regulation of intracellular pools of the polyamines; putrescine, spermidine, and spermine [20, 21] and over expression of SSAT1 results in an increased level of acetylated polyamines [16]. SSAT2 however, appears to function differently than SSAT1. For example, although over expression of SSAT1 caused a decrease in spermidine and spermine levels, over expression of SSAT2 had no effect on the levels of these polyamines [16]. In addition, SSAT2 has a much lower level of acetyltransferase activity towards various polyamines than SSAT1 [16] and the preferred substrate for SSAT2 appears to be thialysine, not polyamines, suggesting that SSAT2 is not involved in polyamine metabolism [19].

Since we identified SSAT2 as a potential p65 interacting protein by a yeast two-hybrid screen and since SSAT2 encodes a protein with an acetyltransferase domain similar to that of several transcriptional coactivators that are important for NF-κB transcriptional activity, we wished to determine if SSAT2 plays a role in regulation of NF-κB-dependent transcription. Our results confirm the interaction between SSAT2 and p65 by coimmunoprecipitations and also show

that SSAT2 can function as a transcriptional coactivator to enhance the NF- κ B transactivation function. In addition, SSAT2 also cooperates with both the CBP and P/CAF coactivators to further enhance TNF α -induced NF- κ B activity. Interestingly, although SSAT2 has not previously been linked to regulation of transcription, the similarity of the SSAT2 acetyltransferase domain to the acetyltransferase domain found in GNAT family further suggests a possible function for SSAT2 in transcriptional regulation.

2. Materials and methods

2.1. Cells, plasmids, and other reagents

HeLa and HEK293T cells were obtained from ATCC and maintained in DMEM-H (Invitrogen, Carlsbad, CA) supplemented with 10% bovine growth serum (HyClone Laboratories, Logan, UT) and penicillin/streptomycin. CMV-p65, 3X κ B-luc, GAL4-p65, and 5X GAL4-luc have been described previously [11]. The GAL4-AR(N terminus) was obtained from L. Shemshedini (University of Toledo) and is described in [22] and the GAL4-p53 plasmid was obtained from A. Baldwin (University of North Carolina). pGL3-Control (Promega) contains the firefly luciferase reporter gene driven by the constitutive SV40 promoter. pcDNA-CBP was provided by R. Kwok (Univ. of Michigan). CMV-PCAF was provided by P. Nakatani (Dana-Farber Cancer Institute, Boston, MA). The SSAT2 cDNA was isolated by RT-PCR from HeLa total cellular RNA and ligated into the pcDNA-Myc/His vector to produce the pcDNA-SSAT2-Myc/His plasmid. This plasmid expresses SSAT2 with a C-terminal Myc/His epitope tag. TNF α was from Biosource (Camarillo, CA) and was used at a final concentration of 10 ng/ml. Antibodies used in this study include: p65, RNA Pol II, Myc (9E10) (Santa Cruz Biotechnology), and Flag-M2 (Sigma-Aldrich, St. Louis, MO). The SSAT2 antibody was kindly provided by C. Coleman (Penn St. University College of Medicine, Hershey, PA).

2.2. Transient transfection/reporter gene assays

Transient transfection/reporter gene assays were performed in 24-well plates using Fugene 6 (Roche Biochemicals, Indianapolis, IN) according to the manufacturers recommendations. Cells were harvested 48 hours after transfection, lysed with M-PER cell lysis buffer (Pierce, Rockford, IL) and assayed for luciferase activity using a LMax luminometer (Molecular Devices). Data was normalized to total protein assayed.

2.3. Coimmunoprecipitation and Western blot analysis

For coimmunoprecipitations, cells were lysed in cell lysis buffer (50 mM TRIS, pH 7.5; 200 mM NaCl; 1.0% Triton X-100; 0.5% NP-40; 10 mM EDTA with protease inhibitors) for 10 min on ice followed by centrifugation at $16,000 \times g$ for 15 min and protein concentration determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Immunoprecipitations were performed using equal amounts of whole cell extracts with the indicated antibodies and Protein A/G-agarose (Santa Cruz Biotechnology). The immunoprecipitated complexes were washed 4 times with 500 μ l of cell lysis buffer and resuspended in 15 μ l of 2X SDS-PAGE sample buffer. The samples were boiled for 3 min and the proteins separated on a SDS-10% polyacrylamide gel and transferred to PVDF membrane (Immobilion-P, Millipore). Membranes were blocked in 1X TBST with 5% milk and probed with the indicated antibodies. Proteins were visualized using either anti-rabbit IgG-HRP or anti-mouse IgG-HRP (Promega) and PicoWest Chemiluminescent reagent (Pierce, Rockford, IL) followed by exposure to film.

2.4. Chromatin Immunoprecipitation (ChIP) Assays

ChIP analysis was performed essentially as described [23]. Cellular proteins and DNA were cross-linked by adding formaldehyde to the growth medium to a final concentration of 0.1%.

Cells were harvested in cold phosphate-buffered saline and lysed in cell lysis buffer (50 mM TRIS pH 8.0, 85 mM KCl, 0.5% NP-40 with protease inhibitors) on ice for 10 min. Nuclei were recovered by centrifugation at $3,300 \times g$ for 5 min at 4°C. Nuclei were lysed in nuclear lysis buffer (50 mM TRIS, pH 8.0, 10 mM EDTA, 1% SDS with protease inhibitors). Lysates were sonicated using a Sonic Dismembrator, Model 500 (Fisher Scientific) and pre-cleared with protein A-agarose or protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit or mouse IgG (Sigma, St. Louis, MO). Lysates were then rocked overnight at 4°C with the immunoprecipitation antibody. Protein A-agarose or protein A/G-agarose were added and incubated at 4°C for one hour with rocking. Complexes were washed one time in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl), one time in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.0, 500 mM NaCl), and one time in LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.0) followed by two washes in TE buffer (20 mM Tris and 2 mM EDTA). Washed complexes were eluted with freshly prepared elution buffer (1% SDS and 100 mM NaHCO₃) and the Na⁺ concentration was adjusted to 200 mM by adding NaCl followed by incubation at 65°C overnight to reverse cross-links. DNA was purified utilizing the Wizard SV Gel and PCR purification system (Promega, Madison, WI) and one tenth of the eluate was used to amplify the indicated promoter regions. The primers used for PCR were: IL-8 Forward 5'-GGGCCATCAGTTGCAAATC-3', Reverse 5'-TTCCTTCCGGTGGTTTCTTC-3'; cIAP2 Forward 5'-GTGTGTGTGGTTATTA CCGC-3' Reverse 5'-AGCAAGGACAAGCCCAGTCT-3'; GAPDH Forward 5'-AGCGCAGGCCTCAAGACCTT-3', Reverse 5'-AAGAAGATGCGGCTGACTGT-3'. Amplified DNA was visualized by agarose gel electrophoresis using SYBR Gold (Invitrogen/Molecular Probes). Images were captured using a Kodak EAS290 Digital Camera and images were inverted using the software program provided by the manufacturer (Kodak).

3. Results

3.1. SSAT2 interacts with the p65 subunit of NF-κB

We identified SSAT2 in a cytoplasmic yeast two-hybrid screen using the C-terminus of p65 (aa 313-551) as the bait (data not shown). To confirm the interaction between SSAT2 and p65 observed by the yeast two-hybrid screen, a transient transfection/coimmunoprecipitation experiment was performed (Fig. 1A). HEK293T cells transiently expressing FLAG-p65 and/or Myc-SSAT2 were immunoprecipitated with Flag antibody and the immunoprecipitates analyzed by immunoblotting. After transfer of proteins to membrane, the membrane was cut and the upper portion probed with Flag antibody (to detect the immunoprecipitated Flag-p65) while the lower portion was probed with Myc antibody to detect the faster migrating Myc-SSAT2. Immunoblots (Input, Fig 1A) also were performed on whole cell extracts (25 μg) to confirm the expression of Flag-p65 or Myc-SSAT2. As shown in Figure 1A (lower IP panel, Lane 2), Myc-SSAT2 coimmunoprecipitated with Flag-p65 from cells transfected with both the SSAT2 (pcDNA-SSAT2-Myc/His) and p65 (CMV-Flag-p65) expression plasmids. No nonspecific interactions were observed from control transfected cells (Figure 1A, Lanes 1, 3, and 4), supporting the specificity of the SSAT2-p65 interaction.

To further confirm this interaction, coimmunoprecipitations were performed to demonstrate an interaction between endogenous SSAT2 and endogenous p65. Whole cell extracts from HEK293T cells that were either untreated or treated with TNFα for 20 or 40 min to activate NF-κB, were immunoprecipitated with a p65 antibody or with non-specific rabbit IgG and the immunoprecipitates analyzed by immunoblotting. In untreated cells there was no detectable interaction between p65 and SSAT2 (Fig. 1B, lower IP panel) even though both proteins are expressed at high levels (Fig. 1B, Input panels). In contrast, after a 20 min TNFα treatment, a significant level of SSAT2 coprecipitated with p65 (Fig. 1B, lower IP panel). However, this interaction was transient as only a small amount of SSAT2 coprecipitated with p65 after

treatment with TNF α for 40 min (Fig. 1B, lower IP panel). Neither p65 nor SSAT2 were precipitated with the non-specific rabbit IgG antibody (Fig. 1B, IP panel) confirming the specificity of the p65-SSAT2 coimmunoprecipitation. Taken together, the results in Fig. 1 confirm the interaction of SSAT2 and p65 identified in the yeast two-hybrid screen and demonstrate that p65 and SSAT2 can interact in vivo.

3.2. SSAT2 enhances p65-mediated transactivation through the C-terminal TAD of p65

Since SSAT2 has a HAT domain similar to the HAT domains found in the GNAT family of transcriptional coactivators we next determined if expression of SSAT2 had an effect on the ability of p65 to activate transcription. To test this, HeLa cells were transiently transfected with the κ B-luc reporter plasmid and either p65 alone, SSAT2 alone, or with p65 and SSAT2 together. Expression of p65 by itself results in about a 10-fold induction of reporter gene activity compared to the vector control transfected cells, whereas expression of SSAT2 alone had no effect on reporter gene activity (Fig. 2). In contrast, when p65 and SSAT2 were coexpressed, a 35-fold increase in reporter gene activity (a 3.5-fold increase relative to p65 alone) was observed (Fig. 2). These results suggest that SSAT2 can function as a coactivator to enhance the ability of p65 to activate transcription.

SSAT2 interacted with the C-terminus (aa 313-551) of p65 in the yeast two-hybrid screen, suggesting that SSAT2 might exert its coactivator function by enhancing the activity of the C-terminal p65 TAD. To test this hypothesis, we utilized a fusion protein between the GAL4 DNA binding domain (GAL4-DBD) and the p65 C-terminus (aa 280-551). HeLa cells were transiently transfected with the GAL4-dependent luciferase reporter plasmid (5X G4-luc) and either a plasmid expressing just the GAL4-DBD or a plasmid expressing the GAL4-p65 (280-551) fusion in the presence or absence of SSAT2. Expression of the GAL4-DBD alone or coexpression with SSAT2 resulted in only a low, basal level of reporter gene activity (Fig. 3A). In contrast, expression of the GAL4-p65 fusion resulted in about a 60-fold increase in reporter gene activity compared to the basal level of expression induced by the GAL4-DBD (Fig. 3A). When SSAT2 was coexpressed with the GAL4-p65 fusion, an additional 13.4-fold increase in reporter gene activity compared to the GAL4-p65 alone was detected (about 800-fold greater than the basal level of expression) (Fig. 3A). We also tested the effect of SSAT2 expression on the ability of a GAL4-p53 (Fig. 3B) and GAL4-AR (N-terminus) (N-terminal transactivation domain of the human androgen receptor) (Fig. 3C) to activate transcription. In both cases, coexpression of SSAT2 results in about a 2-fold increase in reporter gene activity compared to expression of the GAL4 fusions alone (Fig. 3B and C). Thus, although the effect of SSAT2 expression is not as strong with GAL4-p53 or GAL4-AR compared with the GAL4-p65 fusion, it appears that SSAT2 can function as a general coactivator protein to enhance the activity of multiple transcription factors.

3.4. SSAT2 enhances TNF α -induced NF- κ B activation

To demonstrate that SSAT2 could also enhance the ability of endogenous NF- κ B to activate transcription, HeLa cells were transiently transfected with the κ B-luc reporter plasmid and either a vector control plasmid or increasing amounts of the plasmid expressing SSAT2 (pcDNA-SSAT2-Myc/His). About 40 hr after the transfection, the cells were either left untreated or treated for 6 hr with TNF α (10 ng/ml) and extracts assayed for luciferase activity. In the absence of SSAT2 (vector control), TNF treatment increased reporter gene activity about 25-fold compared to the untreated cells (Fig. 4A). Cotransfection of 10 ng or 25 ng of the SSAT2 expression plasmid resulted in a further increase of 2.8-fold and 7.5-fold respectively over the TNF α treated vector control cells whereas transfection of 50 ng of the SSAT2 expression plasmid resulted in only a 5.7-fold increase, possibly due to a squelching effect (Fig. 4A). In contrast, expression of SSAT2 at any concentration had no effect on the basal level of reporter gene activity indicating that the effect is specific to TNF-induced NF- κ B. In

addition, expression of SSAT2 had no effect on the expression of a constitutive reporter gene (pGL-3 Control vector containing the luciferase reporter gene driven by the constitutive SV40 promoter) (Fig. 4B) suggesting that the SSAT2-dependent increase in the NF- κ B-dependent reporter gene activity was not caused by a general increase in transcription, but was specific to TNF α -induced NF- κ B activity. It is likely however that SSAT2, like most transcriptional coactivators, is able to function as a coactivator for other transcription factors in addition to NF- κ B. This is supported by the enhancement of the GAL4 fusion proteins shown in Fig. 3B & C.

3.5. SSAT2 associates with the promoters of the NF- κ B-regulated cIAP2 and IL-8 genes in a TNF α -inducible manner

To determine if SSAT2 can associate with the promoters of NF- κ B-regulated genes, Chromatin Immunoprecipitation (ChIP) experiments were performed. HeLa cells were untreated or treated with TNF α for the indicated times and protein:DNA complexes crosslinked and DNA sheared. Nuclear extracts were then used in immunoprecipitations with the indicated antibodies and the purified, immunoprecipitated DNA used as the template for PCR using primers specific for the promoters of the cIAP2, IL-8, or GAPDH genes. SSAT2 associated with both the cIAP2 and IL-8 promoters in response to TNF α stimulation (Fig. 5). The association of SSAT2 with both of these promoters was transient with very little or no SSAT2 bound to the promoters in the absence of TNF α stimulation (Fig. 5, Lanes 1 and 6). In response to TNF α treatment, SSAT2 began to associate with the cIAP2 promoter by 15 min, with peak levels of binding at 30 min. Only low levels of SSAT2 binding were detected at 60 and 120 min (Fig. 5, Lanes 1-5). For the IL-8 promoter, a low but reproducible level of SSAT2 binding was detected in the absence of TNF α stimulation (Fig. 5, Lane 6). Similar to the results obtained with the cIAP2 promoter, peak levels of SSAT2 binding were detected at 30 min with lower levels at 60 and 120 min (Fig. 5, Lanes 7-10). The recruitment of SSAT2 to both the cIAP2 and IL-8 promoters occurred with approximately the same kinetics as the recruitment of the p65 subunit of NF- κ B (Fig. 5, Lanes 1-10) indicating that SSAT2 may be recruited to these promoters through its association with p65. However, in contrast to SSAT2, p65 remained associated with both the cIAP2 and IL-8 promoters during the entire course of TNF α treatment (Fig. 5, Lanes 2-5 and 7-10). The recruitment of the RNA Pol II large subunit, in response to TNF α stimulation, to the cIAP2 and IL-8 promoters was used as a positive control (Fig. 5). As expected, no binding of p65 or SSAT2 to the promoter of the constitutively expressed GAPDH gene was detected (Fig. 5). The transient association of SSAT2 may indicate that SSAT2 plays an important role in the initial activation of expression of these genes and that other coactivators, such as CBP and P/CAF, may function to maintain and/or further enhance activation. Based on these data, it appears that SSAT2 is a coactivator that can be transiently recruited to the promoters of NF- κ B regulated genes in response to TNF α stimulation.

3.6. SSAT2 cooperates with CBP and P/CAF to enhance NF- κ B activity

Activation of transcription mediated by NF- κ B requires the recruitment of several transcriptional coactivator proteins including CBP/p300 and p300/CBP Associated Factor (P/CAF) to the promoters of NF- κ B regulated genes [4-6,24]. Since multiple coactivators are required for maximal NF- κ B-mediated transcriptional activation, we wished to determine if SSAT2 could cooperate with both CBP and P/CAF to increase the transactivation potential of TNF α -induced NF- κ B activity. To test this, HeLa cells were transiently transfected with the κ B-luc reporter plasmid and either CBP (Fig. 6A) or P/CAF (Fig. 6B) in the presence or absence of SSAT2. In the vector control transfected cells, TNF α treatment increased reporter gene expression about 25-fold compared to the untreated cells (Fig. 6A & B). Expression of CBP alone resulted in about a 4.8-fold further increase in TNF α -induced reporter gene activity compared to the vector control whereas expression of SSAT2 alone yielded about a 7.6-fold increase (Fig. 6A). In contrast, coexpression of SSAT2 and CBP resulted in a greater than 13.4-

fold increase in TNF α -induced reporter gene activity relative to the vector control (Fig. 6A). Similarly, expression of P/CAF alone resulted in only a 1.4-fold increase in TNF α -induced reporter gene activity, however, coexpression of P/CAF with SSAT2 resulted in a greater than 16-fold increase in activity (Fig. 6B). Therefore, from these results and the ChIP assays demonstrating TNF α -induced recruitment of SSAT2 to the promoters of the cIAP2 and IL-8 genes, it appears that SSAT2 functions as a coactivator that can cooperate with both the CBP and P/CAF coactivators to enhance NF- κ B-dependent transcriptional activation.

4. Discussion

Activation of transcription mediated by all transcription factors, including NF- κ B requires the activity of many different accessory proteins including transcriptional coactivator and corepressors. In order to gain a better understanding of how NF- κ B transcriptional activity is regulated, we carried out a cytoplasmic yeast two-hybrid screen to identify proteins that interact with the C-terminal transactivation domain (TAD) of the p65 subunit. Our goal in carrying out this screen was to identify new proteins that are capable of either positively or negatively regulating the ability of NF- κ B to activate transcription. We identified several proteins through this screen, one of which was the product of the spermidine/spermine N¹-acetyltransferase 2 (SSAT2) gene. This gene was first identified based on its homology to the SSAT1 gene [16]. The SSAT1 protein functions in regulation of the polyamine catabolic pathway that regulates intracellular pools of the polyamines putrescine, spermidine, and spermine [20,21]. Although SSAT2 shares a high degree of similarity to SSAT1, SSAT2 does not appear to function in the polyamine catabolic pathway and was recently described as being a thialysine acetyltransferase [19]. Interestingly, SSAT2 shares homology in its acetyltransferase domain with members of the GCN5-related N¹-acetyltransferase (GNAT) superfamily. Mammalian members of the GNAT superfamily include GCN5 and P/CAF [17] and also share structural homology with other HAT proteins including CBP/p300 and the p160/SRC family [17]. Recently a yeast polyamine acetyltransferase was identified, (Paa1), that shows a genetic interaction with the yeast SAGA coactivator complex [25]. The SAGA complex in yeast is a large (~1.8 MDa) complex that includes the Gcn5 HAT [26]. Homologous complexes exist in mammalian cells that include either the human GCN5 or the P/CAF HAT proteins [26]. Therefore, since P/CAF, CBP/p300, and p160/SRC all function as coactivators for NF- κ B [6] we hypothesized that SSAT2 may also perform a similar function.

To address this question, we first confirmed the interaction between SSAT2 and p65 identified in the two-hybrid screen using coimmunoprecipitation experiments. The interaction between endogenous SSAT2 and p65 is TNF α -inducible and transient with a fairly strong interaction occurring with a 20 min TNF α treatment but no detectable interaction after a 40 min treatment. In transient transfection reporter gene assays, coexpression of SSAT2 with p65 resulted in a dramatic increase in p65-dependent reporter gene activity whereas SSAT2 alone had no effect on reporter gene expression, strongly indicating a potential function of SSAT2 as a transcriptional coactivator protein. In further support of this, expression of SSAT2 enhanced TNF α -induced expression of the NF- κ B-dependent reporter gene. Finally, since the C-terminus of p65 (aa 313-551) was used as the bait in the two-hybrid screen, we wanted to confirm that SSAT2 functions to enhance p65 activity through the C-terminal TAD. To do this, a fusion of the p65 C-terminus (aa 280-551) to the GAL4 DNA binding domain (DBD) was used. Our data show that SSAT2 can specifically enhance the activity of the GAL4-p65 fusion protein but has no effect on the GAL4 DBD alone. Taken together, our data strongly indicate that SSAT2 can function as a transcriptional coactivator protein to enhance NF- κ B-dependent transcriptional activation through the p65 TAD.

To determine if SSAT2 was able to associate with the promoters of NF- κ B-regulated genes Chromatin Immunoprecipitation (ChIP) assays were performed on the promoters of the cIAP2

and IL-8 genes, both of which are activated by NF- κ B in response to TNF α stimulation. Over a TNF α time course a typical pattern of recruitment of the p65 subunit of NF- κ B was observed on both the cIAP2 and IL-8 promoters. SSAT2 was also recruited to both promoters in a TNF α -inducible manner, further supporting our data that SSAT2 functions as a coactivator for NF- κ B. Interestingly, SSAT2 was recruited to both promoters shortly after TNF α stimulation (15 and 30 min) but the association of SSAT2 was very transient with most SSAT2 dissociated from the promoters by 60 min. This result is in agreement with the endogenous coimmunoprecipitation results that also show a very transient interaction between SSAT2 and p65. This may indicate that SSAT2 plays an important function in the early steps of NF- κ B-mediated transcriptional activation and subsequently dissociates from the promoters (and p65) in response to some signal such as phosphorylation and/or acetylation of the p65 subunit.

Activation of transcription mediated by NF- κ B requires multiple coactivator proteins including CBP/p300, P/CAF, and members of the p160/SRC family [6]. It is likely that multiple HAT-containing proteins are required due to substrate specificity of each acetyltransferase protein and that these proteins cooperate with each other to stimulate maximal NF- κ B-dependent gene expression. Our studies have identified another acetyltransferase protein, SSAT2 that displays coactivator function to enhance NF- κ B-dependent transcription. Moreover, we were able to show that SSAT2 can cooperate with both CBP and P/CAF to further enhance TNF α -induced NF- κ B activity. In both cases (coexpression of SSAT2 with either CBP or P/CAF), the level of TNF α -induced NF- κ B-dependent reporter gene expression was greater than the level seen with SSAT2, CBP, or P/CAF alone. Although it is not yet known if SSAT2 can acetylate protein substrates, such as histones or any of the NF- κ B subunits, the homology of SSAT2 to members of the GNAT family of acetyltransferase coactivator proteins coupled with our data showing SSAT2 is able to function as a coactivator suggest that SSAT2 may function by acetylating one or more of these proteins. Future experiments will be aimed at determining if the acetyltransferase domain of SSAT2 is required for its coactivator function and if SSAT2 is capable of acetylating protein substrates.

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Fig. 1.

Coimmunoprecipitation of SSAT2 with p65. (A) Coimmunoprecipitations were performed on whole cell extracts from HEK293T cells transfected with the indicated plasmids. IP panels show the coimmunoprecipitation of Myc-SSAT2 with Flag-p65. Input panels show expression of the epitope tagged proteins in the appropriate lanes. (B) Coimmunoprecipitation between endogenous p65 and endogenous SSAT2. Whole cell extracts from HEK293T cells were immunoprecipitated with a p65 specific antibody and immunoprecipated proteins were subjected to Western blot analysis probing for p65 (as a control for the IP) or SSAT2. Input blots show equal expression levels of SSAT2 and p65 in response to all treatments. UT, untreated cells; 20 and 40, cells treated with TNFα (10 ng/ml) for either 20 or 40 min.

Fig. 2.

SSAT2 enhances p65-mediated transcriptional activation. Hela cells were transiently transfected with the indicated plasmids and the κ B-luc reporter gene plasmid. Cells were harvested 48 hr after transfection and extracts assayed for luciferase activity. Luciferase values were normalized to total protein assayed and expressed as average fold activation $\pm$ st. dev. relative to the vector control transfected cells. Experiment was performed in triplicate and repeated three times with similar results.

Fig. 3.
SSAT2 enhances p65 activity through the C-terminal transactivation domain. (A) HeLa cells were transiently transfected with the indicated plasmids and the 5X Gal4-luc reporter gene plasmid. Extracts were assayed for luciferase activity about 48 hr after the transfection. In addition, HeLa cells were transfected with either the GAL4-p53 (B) or GAL4-AR(N-terminus) (C) fusion proteins as well as the 5X Gal4-luc plasmid and the indicated plasmids and extracts assayed for luciferase activity. Luciferase values were normalized to total protein assayed and expressed as average fold activation $\pm$ st. dev. relative to the GAL4-DBD control transfected cells. Experiments were performed in triplicate and repeated three times with similar results.

Fig. 4.

SSAT2 enhances TNF α -induced activation of endogenous NF- κ B. (A) HeLa cells were transfected with the κ B-luc reporter gene plasmid and the indicated amounts of the Myc-SSAT2 expression plasmid (or the pcDNA-Myc/His vector). About 40 hr after the transfection the cells were either left untreated or were treated with TNF α (10 ng/ml) for 6 hr. (B) HeLa cells were transfected with the constitutive SV40-luc (pGL3-Control, Promega) reporter gene plasmid in the presence or absence of SSAT2. For both (A) and (B), extracts were assayed for luciferase activity and luciferase values normalized to total protein assayed. Values are expressed as average fold activation relative to the untreated vector control transfected cells. All experiments were performed in triplicate and repeated three times.

Fig. 5.
SSAT2 associates with the promoters of the cIAP2 and IL-8 genes. ChIP assays were performed on nuclear extracts from HeLa cells according to the protocol described in Methods and Materials section. Immunoprecipitated DNA was subjected to PCR using primers specific to the indicated promoters and amplified DNA visualized by agarose gel electrophoresis.

Fig. 6.
SSAT2 cooperates with CBP and P/CAF to enhance NF- κ B-dependent gene expression.
 HeLa cells were transiently transfected with the κ B-luc reporter gene plasmid, CBP (A) or P/CAF (B) alone and SSAT2 either alone or in combination with CBP (A) or P/CAF (B). About 40 hr after the transfection the cells were either left untreated or were treated with TNF α (10 ng/ml) for 6 hr. Extracts were assayed for luciferase activity and luciferase values normalized to total protein assayed. Values are expressed as average fold activation relative to the untreated vector control transfected cells. All experiments were performed in triplicate and repeated three times.