The STAT3 NH2-Terminal Domain Stabilizes Enhanceosome Assembly by Interacting with the p300 Bromodomain

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Signal Transducer and Activator of Transcription 3 (STAT3) is a latent transcription factor mainly activated by the Interleukin-6 (IL-6) cytokine family. Previous studies have shown that activated STAT3 recruits p300, a coactivator whose intrinsic histone acetyltransferase activity is essential for transcription. Here we investigated the function of the STAT3 NH2-terminal domain and how its interaction with p300 regulates STAT3 signal transduction. In STAT3−/− MEFs, a stably expressed NH2-terminal-deficient STAT3 mutant (STAT3ΔN) was unable to efficiently induce either STAT3-mediated reporter activity or endogenous mRNA expression. Chromatin Immunoprecipitation (ChIP) assays were performed to determine whether the NH2-terminal domain regulates p300 recruitment or stabilizes enhanceosome assembly. Despite equivalent levels of STAT3 binding, cells expressing the STAT3ΔN mutant were unable to recruit p300 and RNA pol II to the native socs3 promoter as efficiently as those expressing STAT3-full length (FL). We previously reported that the STAT3 NH2-terminal domain is acetylated by p300 at K49 and -87. By introducing K49R/K87R mutations, here we found that the acetylation status of the STAT3 NH2-terminal domain regulates its interaction with p300. In addition, the STAT3 NH2-terminal binding site maps to the p300 bromodomain, a region spanning from aa 995 to 1255. Finally, a p300 mutant lacking the bromodomain (p300-ΔB) exhibited a weaker binding to STAT3 and the enhancement formation on the socs3 promoter was inhibited when p300-ΔB was overexpressed. Taken together, our data suggest that the STAT3 NH2-terminal domain plays an important role in IL-6 signaling pathway by interacting with the p300 bromodomain, thereby stabilizing enhanceosome assembly.
remodeling and transcription activation has been long recognized (7). Several proteins with intrinsic HAT activity have been identified, including GCN5 (8), p300/CREB-binding protein (CBP) homologs (9), p300/CBP-associated factor (P/CAF) (10), and TAFII250 (11). HATs activate transcription by one or more of the following ways: 1. they are able to relax core nucleosome structure by acetylating the NH2-terminal histone tails (12); 2. they can directly acetylate transcription factors and alter their transcription activities (13-17); 3. they function as scaffold proteins to recruit other coactivators to the transcriptional apparatus (18,19); and 4. they serve as bridging factors to physically connect sequence-specific transcription factors with multiple components in the basal transcription machinery (20,21). p300 and its homolog CBP are potent transcriptional coactivators that are actively involved in all the four processes mentioned above. They have been shown interact with several transcription factors, such as MyoD (17), p53 (22) and E2F1 (23), and regulate their activity by reversible acetylation.

There is strong evidence demonstrating that the HAT activity of p300 is required for STAT3 target gene activation (24-26). Our previous studies have shown that overexpression of the p300 inhibitor, adenovirus 12S E1A, significantly inhibits the IL-6-induced activation of human angiotensinogen (hAGT), a vasoactive peptide and acute phase protein controlled by STAT3 (24). The ectopic expression of p300 enhances the induction of hAGT reporter gene stimulated by IL-6 (24). Conversely, expression of a p300 mutant deficient in HAT activity functions as a dominant-negative inhibitor and strongly inhibits STAT3-dependent transcription (24). Moreover, IL-6-inducible p300-STAT3 association causes an increase in histone H4 acetylation on the hAGT promoter (24), indicating that p300 recruitment augments STAT3 transactivation by acetylating histone tails, thereby relaxing chromatin structure.

p300 interacts with STAT3 within both its COOH-terminal TAD and NH2-terminal domain (25,26), and this phenomenon is also confirmed for STAT1 and STAT2 (27,28). The STAT family share a highly conserved modular structure that includes an NH2-terminal domain, a coiled-coil domain, a DNA-binding domain, a linker domain, a SH2 domain and a COOH-terminal TAD (29). The coiled-coil domain is actively involved in protein-protein interaction (30) and the SH2 domain mediates the STAT3 dimerization via intermolecular pTyr-SH2 interactions (31). The COOH-terminal TAD contains a conserved single tyrosine residue that is phosphorylated in STAT activation and facilitates transcriptional activation (32). The function of the NH2-terminal domain in STAT3, however, is poorly understood. In this study we investigated the STAT3 NH2-terminal function by stably expressing a NH2-terminal-deleted mutant (STAT3-ΔN) in STAT3-/- MEFs. Both OSM-inducible γ-FBG reporter gene and endogenous mRNA expression including socs3, c-fos and p21, are significantly reduced in response to STAT3-ΔN expression. Because the STAT3 NH2-terminal domain is involved in p300 binding, the defective activity observed in STAT3-ΔN is probably caused by the reduced cooperation between STAT3 and p300. This hypothesis was then tested in native chromatin by ChIP assays which reveal a reduction in OSM-inducible p300 recruitment to the socs3 promoter in MEFs stably expressing STAT3-ΔN. At the same time, there is a decrease in RNA pol II binding to the socs3 promoter, indicating the STAT3 NH2-terminal domain not only stabilizes coactivator association but also facilitates the assembly of transcription preinitiation complex.

Recent studies identified STAT3 not only as a binding partner of p300 but also as a substrate for its acetylation, where p300 modifies STAT3 at multiple sites. A single acetylation on Lys residue K685 localized in the COOH-terminal TAD is required for STAT3 dimerization and the subsequent DNA binding activity (26). Our lab independently identified two other Lys residues, K49 and -87, in the STAT3 NH2-terminal domain that are also inducibly acetylated by p300 in response to IL-6 and OSM (25). Although these NH2-terminal acetylations
have no effect on STAT3 DNA-binding activity, they are essential for STAT3 dependent transcription because K49R/K87R substitutions significantly inhibit STAT3 target gene expression (25). We also noticed that the K49R/K87R mutations decrease the association between p300 and STAT3, indicating that the inducible NH2-terminal acetylations may augment STAT3-p300 interaction. In this study, we further investigated the interaction between the STAT3 NH2-terminal domain and p300 and found that the acetyl-Lys mimic substitutions (K49Q/K87Q) increase the STAT3 NH2-terminal binding to p300, confirming the hypothesis that the NH2-terminal acetylations stabilize the STAT3-p300 interaction. We also discovered that the STAT3 NH2-terminal binding site maps to the p300 bromodomain. The deletion of the bromodomain in p300 molecule decreases its ability to cooperate with STAT3. In addition, the bromodomain-deficient p300 mutant (p300-ΔB) exhibits weaker chromatin binding.

Taken together, we propose a model in which the IL-6 or OSM-inducible acetylations of STAT3 on K residues 49 and -87 triggers the recognition of NH2-terminal domain by the p300 bromodomain, resulting in a strengthened recruitment of p300 to the promoter of STAT3 target gene, thereby facilitating subsequent enhanceosome assembly.

**EXPERIMENTAL PROCEDURES**

**Materials**-Polyclonal antibodies of p300 (N-15), STAT3 (c-20) and RNA pol II (N-20) were purchased from Santa Cruz Biotechnology. Antibodies against V5 and FLAG were obtained, respectively from Invitrogen and Sigma. Recombinant mouse OSM was provided by R&D Systems.

**Cell Cultures**-The human HepG2 and HEK 293 cell lines (ATCC, Manassas, VA) were cultured as described before (25,33). STAT3 +/- and STAT3 -/- MEFs were generous gifts from Dr. Stephanie Watowich at University of Texas MD Anderson Cancer Center. MEFs were cultured in DMEM (GIBCO, Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine and penicillin (100 U/ml)/streptomycin (100 µg/ml), and maintained at 37°C in a humidified atmosphere of 5% CO2. Cells were serum starved for at least 16 h before treated with OSM (20 ng/ml) or IL-6 (10 ng/ml).

**Plasmid Construction**-The γ−FBG-LUC reporter and pEFF6-V5-STAT3 expression vectors including FL, STAT3-ΔN (aa 133-770) and V5-STAT3-130 (aa 1-130) were constructed as described (33). FLAG-tagged WT-124, KR-124 and KQ-124 were produced by PCR using pEFF6-V5-STAT3-WT, STAT3-K48R/K87R and STAT3-K48Q/K87Q (25) as templates respectively. The primers used for this purpose were: sense primer 5’ CATCGATGGATCCATGGACTACAAAGACGATGACGATAAGGCCCAATGGAATCAGCTACAG 3’ and antisense primer 5’ CGTACCTCTAGACTACTGGGCCGCAGTGGCTGCAGTCTG 3’. The PCR products were digested with Bam HI and Xba I endonucleases, gel purified and cloned into pECFP-Nuc (Clonetech) restricted with the same endonucleases.

The expression vector used for PXFS-FLAG-p300 constructs was described (33). The full length p300 cDNA was amplified from pCMVβ p300 (34) using the following primers: sense: 5’-CAGTCTAGACGTAAGCTTGCCGAGAATGTGGTGGAAACGGG-3’ and antisense: 5’-CTCGTAGATATCCTAGTGTATGTCTAGTGTACTC-3’. The PCR product was restricted with HindIII and EcoRV and cloned into the PXFS-FLAG vector. The four NH2-terminal-deleted truncations (p300-ΔN1, p300-ΔN2, p300-ΔN3 and p300-ΔN4) were produced from p300-FL by PCR using the same antisense primer: 5’-CATGAACACCTACAACATGACC-3’ and different sense primers as following: sense 5’-GGTTCGAGCAAAGCGCTTGATCCAGAAGCGCAAGCTCATCC-3’ for p300-ΔN1; sense 5’-CCATCCACTAAGCTTCGAAACAGTG GCACGAAGATATTAC-3’ for p300-ΔN2; sense
5’-GTCAAAGAAAAAGCTTTTCAAACCCAGAAGAACTACGACAG-3’ for p300-ΔN3; sense 5’-GCGGAAGAAAGATGAAGCTTATCTGTCCTTCACCATGAGATC-3’ for p300-ΔN4. All the PCR fragments were restricted by HindIII and XbaI and cloned into the PXFS-FLAG plasmid restricted with the same endonucleases. The two COOH-terminal-deleted truncates (p300-ΔC1 & p300-ΔC2) were amplified from p300-FL using the same sense primer as for p300-FL, 5’-ACGTCTAGACGTAAGCTTGCCGAGAATGTGGTGGAACCGG-3’, and antisense primer: 5’-GATATCCTAGCTAGTCTGGTAGCTGCTG-3' for p300-ΔC1, or antisense primer: 5’-GATATCCTACGGAGATGACTGGGTAGCT-3’ for p300-ΔC2. The PCR products were digested with HindIII and EcoRV and ligated into the PXFS-Straw-FLAG plasmid restricted with the same endonucleases. p300-ΔB mutant was generated by two steps. First the fragment containing the sequences encoding aa 1 to aa 994 was produced by using primer set: sense 5’-CAGTCTAGACGTAAGCTTGCCGAGAATGTGGTGGAACCGG-3’ and antisense: 5’-CTGAAATAAGCTTCGGCTGAGTATCTGCTG-3’ from p300-FL, producing a PCR product overhang with HindIII site on both 5’ and 3’. This PCR product was then restricted with HindIII and fused into pXFS-FLAG-ΔN4 upstream of the sequences encoding aa 1255 of p300, finally producing a plasmid encoding p300 mutant with the internal region spanning from aa 995 to aa 1255 was deleted (p300-ΔB).

Transient Transfection and Luciferase Activity Assay—Transient transfection using Lipofectamine PLUS reagent (Life Technologies, Inc.) was performed in triplicate plates according to the manufacturer’s instruction. For reporter assay, cells were plated into 6-well plates and cotransfected with the γ-FBG-LUC reporter gene, the indicated expression vectors and the transfection efficiency control plasmid pSV2PAP. Twenty-four hours later, cells were stimulated with OSM or IL-6. Both luciferase and alkaline phosphatase activities were measured 48 h after transfection. For co-immunoprecipitation assay, indicated expression plasmids were co-transfected into 10-cm² dishes using the same protocol and cells were harvested for protein extraction 24 h after transfection.

Preparation of Subcellular Extracts—Sucrose cushion-purified nuclear extract (NE) was prepared as described before (25). In brief, cells were harvested in PBS and centrifuged. Pellets were resuspended in double cell volume of solution A and centrifuged to obtain the supernatants as cytoplasmic fraction. The nuclear pellets were purified in solution B and lysed in solution C. After centrifugation at 12,000×g at 4 °C for 20 m, the supernatants were saved as NE. The protein concentrations were measured by coomassie dye binding (Protein Reagent, BioRad).

Western Immunoblot—Proteins were fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Mass). Membranes were blocked in 5% milk for 0.5-1 h and incubated with indicated primary Ab at 4 °C overnight. Membranes were washed in TBS-0.1% Tween 20 and incubated with secondary Ab at 20 °C for 1 h. Signals were detected by the enhanced chemiluminescence assay (ECL; Amersham) or visualized by the Odyssey Infrared Imaging system. ß-actin is used as a loading control.

Co-Immunoprecipitation Assay—One to two mg of protein was precleared with 40 µl protein A-Sepharose beads (Sigma) for 1 h at 4°C. Immunoprecipitation was performed in the presence of 5 µg of the indicated primary Ab at 4°C overnight. Immune complexes were captured by adding 50 µl protein A-Sepharose beads (50% slurry) and rotated at 4°C for 2 h. After the supernatant was discarded, protein A-Sepharose beads were washed with cold PBS for 4-5 times and immunoprecipitates were fractionated by SDS-PAGE.

Two-Step Chip Assay—Two-step ChIP was performed as described (35). In brief, 4-6 x10⁶ MEFs cells per 100 mm dish were washed twice with PBS after stimulation. Protein-protein crosslinking was first
performed with disuccinimidyl glutarate (DSG, Pierce) followed by protein-DNA cross-linking with formaldehyde. After cells were washed and collected in 1ml PBS, pellets were lysed by SDS lysis buffer and sonicated 4 times, 15 sec each at setting 4 with 10 sec break on ice until DNA fragment lengths were between 200 - 1000 bp. Equal amounts of DNA were immunoprecipitated overnight at 4°C with 4 µg indicated Abs in ChIP dilution buffer. Immunoprecipitates were collected with 40 µl protein-A magnetic beads (Dynal Inc., Brown Deer, WI), and washed sequentially with ChIP dilution buffer, high-salt buffer, LiCl wash buffer, and finally in 1x TE buffer. DNA was eluted in 250 µl elution buffer for 15 min at room temperature. Samples were de-cross-linked in de-cross-linking mixture at 65°C for 2 h. DNA was phenol/chloroform extracted, precipitated by 100% ethanol and used for RT-PCR.

**Quantitative Real-Time PCR (Q-RT-PCR)**-Total cellular RNA was extracted by Tri Reagent (Sigma). 2 µg of RNA was used for reverse transcription using SuperScript III First-Strand Synthesis System from Invitrogen. 2 µl of cDNA products was amplified in 20 µl reaction system containing 10 µl iQ SYBR Green Supermix (BioRad) and 400 nM primer mix. The primers were designed by PrimerExpress v2.0 and shown in Table I. All the reactions were processed in MyiQ Single Color Real-Time PCR thermocycler using two-step plus melting curve program and the results were analyzed by iQ5 program (BioRad). The fold change of mRNA expression was calculated by normalizing the absolute amount to internal control GAPDH and comparing to untreated cells.

For quantitative real-time genomic PCR (Q-gPCR), the primer set used for ChIP assay was first tested by performing QPCR on serial dilutions of mouse genomic DNA (from 40 ng – 25 µg) isolated from MEFs. A standard curve was generated by plotting the logarithm of genomic DNA concentration versus the threshold cycle (Ct value). This standard curve confirmed that ChIP-QPCR results were within the linear range of amplification (not shown). The fold change of DNA in each immunoprecipitate was determined by the comparative Ct method. First the Ct value of each sample was normalized to the corresponding input DNA reference, producing the ΔCt value (ΔCt=Ct_sample-Ct_input). Next, the signals in OSM-treated samples were compared with those of untreated samples by calculating the ΔΔCt value (ΔΔCt=ΔCt_treated- ΔCt_untreated). Here, ΔCt_treated was the Ct value of OSM-treated samples normalized to input DNA and ΔCt_untreated represented the Ct value of OSM-untreated samples normalized to input DNA. Finally, we calculated the fold change relative to that amount in unstimulated cells as 2 -ΔΔCt. The primer set used for Q-gPCR to amplify the STAT3 binding sites on the socs3 promoter included sense primer 5’-CGCGCACAGCCTTTCAGT-3’ and antisense primer 5’-CCCCCGATTCTCTGGAACT-3’.

**RESULTS**

The STAT3 NH2-terminal domain is required for OSM-inducible transcription- Based on our findings that the STAT3 NH2-terminal domain may play an important role in STAT3 function, we generated a V5-epitope tagged STAT3 mutant (V5-STAT3-ΔN), in which the entire NH2-terminal region (aa 1-133) was deleted, and tested its transcriptional activity in luciferase reporter assays. For this purpose, STAT3-/-- MEFs were used to avoid the interference from endogenous STAT3. First, the loss of STAT3 in STAT3-/-- MEFs was confirmed by Western Immunoblot shown (Figure 1A, left panel). STAT3-/-- MEFs were then transiently transfected with V5-STAT3-FL or V5-STAT3-ΔN and ectopically expressed STAT3 measured by Western Immunoblot with anti-V5 Ab. As seen in the right panel of Figure 1A, the expression of V5-STAT3-ΔN was not affected by the removal of NH2-terminal domain.

Different doses of V5-STAT3-FL or V5-STAT3-ΔN were then cotransfected with the reporter gene, γ-FBG-LUC, which contains the three native STAT3-binding sites from the γ-FBG gene. Because MEFs express low levels of IL-6Rα and OSM has
been shown to induce STAT3-p300 cooperation in STAT3-/− MEFs (36), OSM was used in these experiments to activate STAT3 signal transduction. Reporter activity was measured in presence or absence of OSM. As little as 0.25 μg of V5-STAT3-FL was able to increase γ-FBG-LUC activity by about 3-fold and a further increase to 5-fold was observed when the amount of V5-STAT3-FL expression vector was increased (Figure 1B). In contrast to the behavior of V5-STAT3-FL, V5-STAT3-ΔN was able to induce only a 1.5-fold increase in reporter activity and a dose-dependent response was not observed (Figure 1B). Our findings that the STAT3 NH2-terminal domain is sufficient to associate with p300 raised the possibility that this domain regulates STAT3 signaling by mediating p300 recruitment. Therefore, we next tested whether the STAT3 NH2-terminal domain was required for the functional cooperation between STAT3 and p300. To this end, different doses of p300 expression vectors were cotransfected with V5-STAT3-FL or the V5-STAT3-ΔN mutant into STAT3-/− MEFs. p300 further enhanced γ-FBG-LUC activity induced by V5-STAT3-FL; however, in presence of V5-STAT3-ΔN, p300 was unable to rescue the lower levels of reporter activity (Figure 1C).

The STAT3 NH2-terminal domain is required for the OSM-inducible mRNA expression—To further explore the role of the STAT3 NH2 terminus, we analyzed the expression of endogenous STAT3 target genes in MEFs by Quantitative Real-Time PCR (Q-RT-PCR). Socs3, c-fos and p21, well-established STAT3 dependent genes (37-40), were all significantly and rapidly induced by OSM in STAT3+/− MEFs, with a 100-fold induction of socs3 expression, for example, was observed within 30 min of stimulation. This highly inducible upregulation was dramatically decreased in STAT3-/− MEFs, where less than a 5-fold induction of socs3 being observed, indicating that STAT3 was a crucial transactivator for the induction of these genes (Figure 2A-C). To investigate how the NH2-terminal deletion affects STAT3 target gene expression, we stimulated a population of STAT3-/− MEFs stably expressing V5-tagged STAT3-FL or STAT3-ΔN mutant with OSM. In cells expressing STAT3-FL, all three STAT3 target genes were strongly activated by OSM. Conversely, cells expressing V5-STAT3-ΔN, despite its equivalent expression as V5-STAT3-FL (insert panel in Figure 2D), were defective in gene expression (Figure 2D-F). Collectively, these results indicate the necessity of NH2-terminal domain for transcription activation by STAT3 in response to OSM signaling.

The NH2 terminal deletion affects enhanceosome assembly on the socs3 promoter—To clarify whether NH2 terminal of STAT3 regulates its transcription activity by mediating p300 recruitment, two-step Chromain Immunoprecipitation (ChIP) assays were performed to analyze p300 binding to the socs3 promoter. Two STAT3 consensus binding regions have been identified on the socs3 promoter upstream of the transcription initiation site (37,38). A primer pair amplifying both binding sites was designed and optimized by quantitative real time genomic PCR (Q-gPCR) to show a linear dynamic range from 40 ng to 25 μg DNA (Methods). Next, we examined the OSM inducible STAT3 binding to the socs3 promoter in STAT3-/− MEFs complemented with V5-STAT3-FL or V5-STAT3-ΔN mutant in ChIP experiments by using an anti-V5 as the primary Ab. This experiment revealed that both V5-STAT3-FL and V5-STAT3-ΔN inducibly associate with the socs3 promoter within 30 m after OSM stimulation (Figure 3A). This finding was consistent with previous studies showing that NH2-terminal deletion did not affect the nuclear translocation or DNA binding activity of STAT3 (25,41). The ChIP assays were extended further to analyze the effect of OSM on inducible p300 and RNA pol II binding to the socs3 promoter. As a result, we observed a 2.5-fold increase of p300 binding in response to OSM when STAT3-/− MEFs were complemented with V5-STAT3-FL or V5-STAT3-ΔN mutant in ChIP experiments by using an anti-V5 as the primary Ab. This experiment revealed that both V5-STAT3-FL and V5-STAT3-ΔN inducibly associate with the socs3 promoter within 30 m after OSM stimulation (Figure 3A). This finding was consistent with previous studies showing that NH2-terminal deletion did not affect the nuclear translocation or DNA binding activity of STAT3 (25,41). The ChIP assays were extended further to analyze the effect of OSM on inducible p300 and RNA pol II binding to the socs3 promoter. As a result, we observed a 2.5-fold increase of p300 binding in response to OSM when STAT3-/− MEFs were complemented with V5-STAT3-FL, but no significant p300 recruitment was detected when V5-STAT3-ΔN mutant was stably expressed (Figure 3B). In addition, the inducible loading of RNA pol II on the socs3 gene was also inhibited in MEFs stably expressing STAT3-ΔN (Figure 3C). Our explanation for these results is that the reduced p300 binding
caused by the NH2-terminal deletion affects the association RNA pol II due to its direct interaction with p300 (42,43).

**Association between the STAT3 NH2-terminal domain and p300 is regulated by NH2-terminal acetylation**- To study the interaction between the STAT3 NH2-terminal domain and p300, we first sought to determine whether the STAT3 NH2-terminus is sufficient for p300 interaction. For this purpose, cells were cotransfected with pCMVβ p300 and a plasmid expressing V5-tagged NH2-terminal domain of STAT3 (aa 1-130). Nuclear protein was recovered and immunoprecipitated by anti-p300 Ab followed by Western Immunoblot using anti-V5 Ab. As seen in Figure 4A, V5-STAT3 (1-130) was specifically captured by p300 Ab but not by IgG. To determine the effect of STAT3 K49/K87 acetylations on p300 interaction, we generated FLAG-tagged acetylation-deficient (K49R/K87R) and the pseudo-acetylated (K49Q/K87Q) NH2-terminal mutants (aa 1-124), and examined their association with p300. In contrast to the FLAG-STAT3-WT (1-124), the acetylation-deficient FLAG-STAT3 (1-124)-K49R/K87R mutant was barely detectable in p300 immunoprecipitates (Figure 4B). Instead, the pseudo-acetylated FLAG-STAT3 (1-124)-K49Q/K87Q mutant exhibited stronger p300 binding despite the fact that its expression level was comparable to FLAG-STAT3-WT (1-124) (Figure 4C). These results indicate that the STAT3 NH2-terminal domain is sufficient for p300 interaction and the K49/K87 acetylations increase this association.

The binding site of STAT3 NH2-terminal domain maps to the p300 bromodomain-To determine the domain of p300 responsible for the STAT3 NH2-terminal binding, we generated a panel of FLAG epitope-tagged p300 deletions. The related functional domains included in each truncation are shown in Figure 5A. Each of the four NH2-terminal-deleted mutants (p300-ΔN1, -ΔN2, -ΔN3, & -ΔN4) or two COOH-terminal-deleted mutants (p300-ΔC1 & -ΔC2) was individually coexpressed with V5-STAT3 (1-130) and p300-bound-NH2 terminal domain was examined by nondenaturing co-immunoprecipitaion assays. STAT3 NH2-terminal binding was lost when the region between aa 1047 (p300-AN3) and aa 1255 (p300-ΔN4) was deleted (Figure 5B, right panel). Importantly, this region, from aa 1047 to aa 1255, contains the p300 bromodomain, spanning from aa 1053 to aa 1156. Consistent with this finding, the lack of bromodomain in ΔC2 (aa 1-1043) abrogated its interaction with the STAT3 NH2-terminal domain, however the bromodomain-containing mutant ΔC1 (aa 1-1264) still complexed with the STAT3 NH2 terminus (Figure 5C, right panel). To further confirm that the p300 bromodomain mediates its interaction with the STAT3 NH2 terminal region, an expression vector encoding p300 with an internal deletion of the bromodomain (p300-ΔB) was generated and tested. As we expected, no interaction was detected between p300-ΔB and the STAT3 NH2-terminal domain (Figure 5C, right panel). Taken together, these data suggest that the p300 bromodomain binds the STAT3 NH2-terminal domain and the IL-6 or OSM-inducible NH2-terminal acetylations further stabilize this interaction.

The p300 bromodomain facilitates STAT3-dependent transcription-Because the p300 bromodomain was responsible for STAT3 NH2-terminal binding (Figure 5), we propose that the p300-STAT3 interaction will be affected when the p300 bromodomain is deleted. To test this, 293 cells were transfected with expression vectors encoding V5-STAT3 together with FLAG-tagged p300-FL or p300-ΔB mutant. The interaction between these two proteins was then measured by nondenaturing co-immunoprecipitation with anti-FLAG Ab followed by Western Immunoblot with anti-V5 Ab. As we expected, the p300-ΔB mutant showed decreased interaction with STAT3 compared to that of p300-FL (Figure 6A, left panel), despite the similar expression level of these two proteins (Figure 6A, right panel). The remaining association observed between p300-ΔB and STAT3 indicates multiple domains of p300 are involved in
STAT3-p300 interaction. An earlier study using in vitro transcription reactions found that p300 forms a stable complex with chromatin templates which is mediated, at least in part, by the bromodomain (44). To confirm this finding in cellulo, ChIP experiments were performed to capture the binding of p300 to the endogenous socs3 promoter after p300-FL or p300-ΔB mutant was transiently transfected into STAT3+/− MEFs. As seen in Figure 6B, the p300-AB mutant exhibited weaker association with the socs3 promoter than p300-FL. At the same time, reductions in STAT3 and RNA pol II binding were observed in p300-ΔB-transfected cells (Figure 6C,D).

To further test the functional role of bromodomain, different doses of p300-FL or p300-ΔB mutant was cotransfected with γ-FBG-LUC reporter gene into HepG2 cells followed by IL-6 treatment for 24h. As a result, the p300-FL expression further enhanced the IL-6-inducible reporter activity in a dose-dependent manner. The p300-ΔB mutant, however, failed to function as a transcriptional coactivator in this assay (Figure 6E). Taken together, our results suggested that the bromodomain plays a critical role in p300 function because it mediates p300 interaction with STAT3 and facilitates enhanceosome formation.

DISCUSSION

STAT3 is the major transcription factor activated by the IL-6 family of cytokines. Although the STAT3 signaling from the cell membrane to the nucleus is well understood, the molecular events regulating gene transcription needs more elucidation. Like many other transcription factors, the activated STAT3 recruits the p300 coactivator after nuclear translocation (24). The p300-induced acetylation on histone tails is coupled with chromatin remodeling, thereby enhancing target gene expression (24-26). The interaction between p300 and STAT3 is regulated via both the STAT3 NH2-terminal domain and the COOH-terminal TAD (26). In this study, we reported that the NH2-terminal domain plays a critical role in STAT3-mediated signal transduction by regulating enhanceosome assembly at the promoter loci. This conclusion is supported by 1. the NH2-terminal deletion inhibited OSM-induced reporter activity and abolished the cooperation between STAT3 and p300 (Figure 1); 2. the expression of endogenous STAT3 target genes, including socs3, c-fos and p21, was significantly reduced in STAT3-ΔN-complemented STAT3/−/− MEFs (Figure 2); and 3. significantly reduced loading of p300 and RNA pol II to the socs3 promoter were observed when STAT3-ΔN mutant was stably expressed in STAT3/−/− MEFs (Figure 3). These results suggest that the NH2-terminal deletion disrupts enhanceosome formation on the socs3 promoter, resulting into an inhibition in mRNA transcription.

The NH2-terminal domain of STATs comprises approximately the first 130 residues that assembled into an all-helical hook-like structure (45). Although it is highly conserved in the STAT family, the NH2-terminal domain is implicated in diverse functions, including STAT dimeration or tetramization (46,47), phosphorylation/dephosphorylation (48-50), and STAT’s interaction with other transcription factors or coactivators (33,51). One of the identified functions of NH2-terminal region is regulating the cooperative binding of STAT dimer-dimer complexes on two tandemly arranged binding sites. This function is conserved in STAT1 (52), STAT4 (53) and STAT5 (54), and recently is also confirmed in STAT3 when it activates the transcription of α2-macroglobulin (α2-M) gene (47). A tetrameric STAT3 complex is formed on the α2-M enhancer sequence, which is required for the maximum transcriptional activation (47). Two point mutations in the NH2-terminal region (W37A, Q66A) disrupts the tetramer binding (47). The murine socs3 promoter also contains two putative STAT3 binding sites which are highly conserved in the human socs3 promoter (37,55). Both sites are required for the complete activation of the human socs3 promoter stimulated by LIF (55). As for the murine socs3 promoter, the proximal site localized from nucleotide -72 to -64 is essential for LIF-induced transactivation (37). The distal site from nucleotide -95 to -87, however, has not been directly tested.
Although two STAT3 motifs on the murine socs3 promoter are tandemly linked, STAT3 binding is only detected on the proximal site and no obvious dimer-dimer interaction is observed (41). This excludes the possibility that the defective activity of STAT3-ΔN mutant in activating the socs3 expression (Figure 2) is caused by the disruption of STAT3 tetramerization.

Here we describe a new level of STAT3 NH2-terminal function achieved through its interaction with the p300 bromodomain. p300 is known to function as a bridging factor connecting sequence-specific transcription factor with the basal transcription machinery (56). The direct association between p300 and RNA pol II in mammary cells has long been known (42,43). In this interaction, p300 specifically interacts with the unphosphorylated form of RNA pol II which is able to form transcription preinitiation complex (42,57). Our study show that the STAT3 NH2-terminal deletion significantly decreases p300 recruitment to the socs3 promoter (Figure 3B), resulting in an inhibition in RNA pol II binding (Figure 3C). These results reveal the function of NH2-terminal domain to integrate the enhancer binding proteins and facilitate the assembly of transcription preinitiation complex.

The recent finding that STAT3 is also a direct target of p300 for acetylation draws attention to the study of p300-STAT3 interaction. STAT3 is acetylated by p300 at multiple lysine residues. The reversible acetylation on K 685 within TAD is essential for STAT3 dimeration and DNA-binding ability (26). Our earlier studies identified another two acetylation sites (K residues 49 and -87) localized in the NH2-terminal domain of STAT3, sites whose acetylations are also dispensable for STAT3-mediated transactivation (25). Interestingly, this NH2-terminal acetylations, however, have no effect on the inducible STAT3 binding to DNA (25).

In this study, we demonstrate that the STAT3 NH2-terminal domain is sufficient for p300 interaction (Figure 4A) and show that the NH2-terminal acetylations increase the p300-NH2-terminal interaction (Figure 4B & 4C). Here we also report the discovery that the p300 bromodomain is the site for NH2-terminal association (Figure 5). The bromodomain represents a highly conserved protein module that is commonly found in many chromatin-associated proteins and nearly all HATs (58). The function of bromodomain as an acetyl-Lys binding domain was discovered soon after its three-dimensional structure was elucidated. The solution structure of the bromodomain from P/CAF revealed an unusual left-handed four-helix bundle (59), a unique structural fold that was conserved in other chromatin-associated proteins, such as human TAFII250 (60) and the Saccharomyces cerevisiae Gcn5p (61). Although acetyl-Lys is the direct recognition site of the bromodomain, residues flanking both sides of acetyl-Lys are also important for the interaction and contribute to the ligand selectivity of bromodomains (62). The acetylation-enhanced interaction (Figure 4B & 4C) indicates that the acetylations on K residues 49 and -87 provide a binding motif for the bromodomain of p300, leading to a stable complex formation and efficient HAT recruitment to the promoter. There is also a possibility that the NH2-terminal acetylations induce conformational changes in residues flanking the acetyl-Lys that positively affect p300-STAT3 interaction. Interestingly, the acetylation-dependent interaction is also observed between MyoD and the bromodomain of p300/CBP (17). p300/CBP selectively recognizes the acetylated form of MyoD in cell and mutations of acetylation sites in MyoD decrease its ability to cooperate with p300/CBP (17). This suggests that acetylation is probably a common strategy that p300/CBP utilizes to facilitate their interaction with non-histone proteins.

The bromodomain is also reported to mediate p300 binding to chromatin. p300 forms a stable complex with in vitro transcription template and this association is regulated at least partially by the bromodomain (44). Our ChIP result in Figure 6B confirmed this finding in cellulo by showing that the bromodomain deletion decreased p300 occupancy on
the endogenous socs3 promoter. We also noticed that the bromodomain-deficient mutant (p300-ΔB) was still bound to STAT3 (Figure 6A) although the interaction was significantly weaker than that of p300-FL and STAT3. This data indicates that more than one domain of p300 are involved in mediating p300-STAT3 interaction. It has been shown that TAD of STAT3 also associates with p300 although the binding site on p300 has not been identified yet (24,26). Similarly, the interaction between STAT1 and p300/CBP is also mediated by two contact regions. The STAT1 NH2-terminal region is recognized by the CREB-binding domain of p300/CBP and the binding site of TAD maps to the p300/CBP domain that recruits adenovirus E1A protein (28).

In summary, we have provided evidence that the STAT3 NH2-terminal domain interacts with the p300 bromodomain in an acetylation-dependent manner. IL-6 or OSM-induced acetylations on K49 and K87 trigger the recognition of substrate by the p300 bromodomain, resulting in a strengthened association between p300 and STAT3. This interaction further stabilizes the recruitment of other transcription components like RNA pol II to efficiently initiate the transcription of STAT3 target genes.

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5616-5626

FOOTNOTES

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The abbreviations used are: IL-6, interleukin-6; OSM, oncostatin M; STAT, signal transducer and activator of transcription; IL-6Rα, IL-6 receptor α chain; HATs, histone acetyltransferase; RNA pol II, RNA polymerase II; FBG, fibrinogen; TAD, transactivation domain; ChIP, chromatin immunoprecipitation assay; NE, nuclear extract; Q-RT-gPCR, quantitative real time genomic PCR; FL, full length; WT, wild type.

FIGURE LEGENDS

Fig.1. The STAT3 NH2-terminal domain is required for OSM-inducible transcription. A, The left panel is Western Immunoblot showing endogenous STAT3 level in STAT3+/+ MEFs (C4+/-) and the loss of STAT3 expression in STAT3-/- MEFs (L1-/-). In the right panel, the expression of transiently transfected V5-STAT3-FL and V5-STAT3-ΔN in STAT3-/- MEFs was measured by Western Immunoblot with anti-V5 Ab. β-actin was used as a loading control. B, STAT3-/- MEFs were transfected with the γ-FBG-LUC reporter gene together with different doses of V5-STAT3-FL or V5-STAT3-ΔN expression vectors and treated with OSM for 24 h prior to reporter gene assay. Shown is normalized luciferase reporter activity. C, Different doses of pCMVβ p300 were cotransfected with V5-STAT3-FL or V5-STAT3-ΔN together with γ-FBG-LUC reporter followed by OSM stimulation for 24 h. Data shown were means ± SD from three independent transfections. The data was analyzed by Student’s t test. The luciferase reporter activities in V5-STAT3-FL-transfected MEFs were compared with those in V5-STAT3-ΔN-transfected MEFs using a Student’s t-test. *, p value < 0.05, **, p value < 0.01.

Fig.2. The STAT3 NH2-terminal domain is required for OSM-inducible mRNA expression. A-C, STAT3+/+ and STAT3-/- MEFs were treated with OSM (20 ng/ml) for 15- or 30 min. Whole cellular mRNA was isolated and the expression of socs3, c-fos and p21 were measured by Q-RT-PCR. The fold change in OSM-treated cells over OSM-unstimulated control was obtained after correction for the amount of internal control,
GAPDH. The mRNA induction in STAT3 +/- MEFs was compared with that in STAT3-/- MEFs. D-F, STAT3-/- MEFs were transfected with V5-STAT3-FL or V5-STAT3-ΔN expression vectors. Positively transfected cells were selected by puromycin at 48 h and pooled. The insert panel in 4D is Western Immunoblot showing the stably expressed V5-STAT3-FL and V5-STAT3-ΔN after antibiotic selection. The V5-STAT3-FL- or V5-STAT3-ΔN-complemented STAT3-/- MEFs were stimulated by OSM (20 ng/ml) for 15 m or 30 m. Shown is the result of Q-RT-PCR assays plotting the change of mRNA abundance in OSM treated cells normalized to GAPDH expressed as a fold change relative to unstimulated controls. The mRNA induction in V5-STAT3-FL-complemented STAT3-/- MEFs was compared with that in V5-STAT3-ΔN complemented STAT3-/- MEFs. The data was analyzed by Student’s t test. *, p value < 0.05, **, p value < 0.01.

Fig.3. The STAT3 NH2-terminal domain regulates enhanceosome assembly on the socs3 promoter. STAT3-/- MEFs stably expressing V5-STAT3-FL or V5-STAT3-ΔN were treated with OSM (20 ng/ml) for 30 m, and two-step ChIP assay was performed by using Abs specifically recognizing V5 tag (Fig.3A), p300 (Fig.3B) or RNA pol II (Fig.3C). The sequence of the socs3 promoter in the immunoprecipitates was amplified by Q-RT-gPCR using specific primers. Shown is the fold change of signals in OSM-treated samples compared to those of untreated samples. A, Both V5-STAT3-FL and V5-STAT3-AN are induced by OSM to bind to the socs3 promoter. B, The OSM-inducible p300 recruitment to the socs3 promoter is inhibited in cells stably expressing STAT3-ΔN. C, The OSM-inducible RNA pol II association to the socs3 promoter is reduced in STAT3-ΔN-complemented MEFs. The results were expressed as means ± SD from triplicates. The data was analyzed by Student’s t test. *, p value < 0.05, **, p value < 0.01.

Fig.4. Interaction between the STAT3 NH2-terminal domain and p300. A, HepG2 cells were cotransfected with pEF6-V5-STAT3 (aa 1-130) with pCMVβ p300. Cells were treated with IL-6 (10 ng/ml) for 30 m before NE was prepared. 2 mg of NE was immunoprecipitated by anti-p300 Ab. p300-bound V5-STAT3 (1-130) was detected by anti-V5 Ab in Western Immunoblot. The first lane is lysate showing the expression of V5-STAT3 (1-130). The second lane is protein standard (indicated by “M” in Fig. 4A). B, HepG2 cells were cotransfected with either pECFP-FLAG-STAT3-WT (aa 1-124) or pECFP-FLAG-STAT3-K49R/K87R mutant (aa 1-124) together with pCMVβ p300. NE was immunoprecipitated with anti-p300 Ab followed by Western Immunoblot with anti-FLAG Ab. The first two lanes show FLAG-STAT3-WT (1-124) and FLAG-STAT3-ΔK49R/K87R (1-124) expression in nuclear lysate. Lane 3 and lane 4 are immunoprecipitates of FLAG-STAT3-WT (aa 1-124) with anti-p300 Ab (lane 3) or IgG (lane 4). Lane 5 and lane 6 are immunoprecipitates of FLAG-STAT3-ΔK49R/K87R mutant (aa 1-124) with anti-p300 Ab (lane 5) or IgG (lane 6). C, HepG2 cells were cotransfected with either pECFP-FLAG-STAT3-WT (1-124) or pECFP-FLAG-STAT3-K49Q/K87Q mutant (1-124) together with pCMVβ p300. Immunoprecipitation was performed as described in Fig 1B. The first two lanes show FLAG-STAT3-WT (1-124) and FLAG-STAT3-ΔK49Q/K87Q (1-124) expression in nuclear lysate. Lane 3 and lane 4 are immunoprecipitates of FLAG-STAT3-WT (aa 1-124) with anti-p300 Ab (lane 3) or IgG (lane 4). Lane 5 and lane 6 are immunoprecipitates of FLAG-STAT3-ΔK49Q/K87Q mutant (aa 1-124) with anti-p300 Ab (lane 5) or IgG (lane 6).

Fig.5. The STAT3 NH2-terminal domain is associated with the p300 bromodomain. A, A schematic diagram showing different functional domains of p300 and all p300 mutants used in Fig 5B & SC. B&c: PXFS-FLAG-p300-FL, p300 NH2-terminal deletions (p300-ΔN1, -ΔN2, -ΔN3 and -ΔN4), COOH-terminal deletions (p300-ΔC1 & -ΔC2), or p300 mutant without the bromodomain (p300-ΔB) were individually cotransfected with pEF6-V5-STAT3 (1-130) into HEK 293 cells. Immunoprecipitation was performed by
anti-FLAG Ab and the p300-associated STAT3 NH2-terminus was detected by anti-V5 Ab. The left panels are Western Immunoblot showing the expression of each mutant in the cell lysate. The right panels are p300-associated STAT3-NH2 terminus. EV: the empty vector (PXFS-FLAG) was included as a negative control.

Fig.6. The p300 bromodomain facilitates STAT3-dependent transcriptional activation. A, HEK 293 cells were cotransfected with PXFS-FLAG-p300-FL or p300-ΔB mutant together with pEF6-V5-STAT3-FL. 48 h after transfection, 1 mg of whole cell extract was collected and immunoprecipitated by anti-FLAG Ab followed by Western Immunoblot with anti-V5 Ab. The right panel is Western Immunoblot showing that p300-FL and p300-ΔB mutant had comparable expression level.

B, C&D, STAT3+/- MEFs were transiently transfected with PXFS-FLAG-p300-FL or p300-ΔB mutant. 24 h after transfection, cells were treated with OSM (20 ng/ml) for 30 m or left untreated. Two-step ChIP assays were performed by using antibodies specifically recognizing p300 (Fig.6B), STAT3 (Fig.6C) and RNA pol II (Fig.6D). The signals in OSM-treated cells were compared with those in non-treated MEFs. 

E, HepG2 cells were transfected with different doses of PXFS-FLAG-p300-FL or p300-ΔB mutant together with γ-FBG-LUC reporter gene. 24 h later, cells were treated with IL-6 (10 ng/ml) for another 24 h and then luciferase activity was measured. Data shown were means ± SD from three independent transfections. The data was analyzed by Student’s t test. *, p value < 0.05, **, p value < 0.01.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Sense</th>
<th>Anti-sense</th>
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<tr>
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<td>5’ TTGACGCTCAACGTGAAGAAGT 3’</td>
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<tr>
<td>c-fos</td>
<td>5’ CCTGCCCTTCTCAACGA 3’</td>
<td>5’ TCCACGTTGCTGATGCTTCTT 3’</td>
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<tr>
<td>p21</td>
<td>5’ TTCCGCACAGGAGCAAAGT 3’</td>
<td>5’ CGGCGCACACTGCTCCTCTC 3’</td>
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</table>
Fig. 2A

SOCS3

Fold of mRNA expression

Fig. 2B

c-fos

Fold of mRNA expression

Fig. 2C

p21

Fold of mRNA expression
Fig. 5A

![Diagram of a protein domain with labeled sections.]

Fig. 5B

![Western blot images with antibody labeling.]

Fig. 5C

![Western blot images with antibody labeling.]

[^1] The figure illustrates the domain structure and binding sites of a protein, with specific regions labeled and antibody labeling results shown.
Fig. 6A

Fig. 6B

Fig. 6C
The STAT3 NH2-terminal domain stabilizes enhanceosome assembly by interacting with the p300 bromodomain
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