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Regulation of Inducible Nitric Oxide Synthase Expression by p300 and p50 Acetylation[1]

Wu-Guo Deng and Kenneth K. Wu[2]

To determine whether p300 is involved in inducible NO synthase (iNOS) transcriptional regulation, we evaluated the effect of p300 overexpression on iNOS expression and characterized p300 binding to iNOS promoter in RAW 264.7 cells. p300 overexpression increased iNOS expression which was abrogated by deletion of the histone acetyltransferase (HAT) domain (Δ1472–1522). DNA-binding and chromatin immunoprecipitation assays revealed binding of p300 to several DNA-bound transactivators at basal state. Following stimulation with LPS plus IFN-γ, binding of p300, p50/p65 NF-κB, and IFN-regulatory factor-1 was increased by ~2-fold. Nuclear p50 was complexed with and acetylated by p300 at the basal binding state which was increased by LPS and IFN-γ stimulation. p300 overexpression resulted in increased p50 acetylation which was reduced by HAT mutation. p50 acetylation correlated with increased NF-κB binding and enhanced p300 recruitment. Co-overexpression of E1A abolished the augmentation of p50 acetylation and p50 binding induced by p300 overexpression, and a correlative suppression of p300 recruitment to the complex. We conclude that p300 is essential for iNOS transcription. Our results suggest that p300 HAT acetylates the p50 subunit of NF-κB, thereby increasing NF-κB binding and NF-κB mediated transactivation. *The Journal of Immunology,* **2003, 171: 6581–6588.**

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Inducible NO synthase (iNOS)[3] is a calcium-independent member of the NOS gene family that catalyzes the synthesis of a large quantity of NO (1). It plays diverse pathophysiological roles including microbial killing, inflammation, septic shock, and ischemic tissue injury (2). iNOS levels in resting cells are absent or very low but are highly inducible by LPS, IFN-γ, and other cytokines (3). Many studies in murine and human cells have shown that LPS and cytokines induce iNOS expression at the transcriptional level. iNOS promoter activation by combined LPS and IFN-γ (LPS/IFN-γ) has been extensively characterized in murine RAW 264.7 cells. The 5′-flanking region of murine iNOS gene contains two clusters of *cis*-acting regulatory elements that are essential for iNOS transcription of which the proximal cluster is required for LPS-induced and the distal cluster is essential for IFN-γ-induced activation (4, 5). LPS/IFN-γ synergistically activates iNOS promoter by stimulating binding of several transcription activators, notably NF-κB, IFN-regulatory factor-1 (IRF-1), and C/EBPβ to their respective cognitive sites in these two clusters of regulatory elements (6–8). Transmission of the transactivator message to the transcription machinery requires recruitment of interacting proteins such as coactivators (9–11). Coactivators that are involved in iNOS promoter activation have not been reported. In this study, we determined binding of p300 coactivator to iNOS promoter region and evaluated the role of p300 in regulating iNOS promoter activity. Our results indicate that LPS/IFN-γ increased p300 interaction with promoter-bound transactivators. p300 overexpression augmented LPS/IFN-γ-induced p300 binding and iNOS promoter activity. Adenoviral E1A abrogated the stimulating effects of LPS/IFN-γ and p300 overexpression. We provided evidence that p300 acetylated the p50 subunit of NF-κB thereby increasing NF-κB binding.

Materials and Methods

Cell culture

The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS and 1:100 dilution of an antibiotic-antimycotic solution (Invitrogen, Grand Island, NY). For all experiments, 80–90% confluent cells were cultured in serum-free medium for 24 h, washed with PBS and incubated in fresh medium in the presence or absence of 2 μg/ml LPS (*E. coli* 026:B6; Sigma-Aldrich, St. Louis, MO) plus 400 U/ml IFN-γ (Sigma-Aldrich) at 37°C for 8 h. After washing with chilled PBS three times, the cells were harvested. All the tissue culture regents were obtained from Life Technologies (Grand Island, NY).

Plasmids

A fragment of murine 1.63 kb iNOS promoter/enhancer region (−1486 to +145) was constructed into a luciferase reporter vector pGL3 as previously described (8). A full-length p300 expression construct (pCL.p300) and its histone acetyltransferase (HAT) deletion mutant (pCL.p300ΔHAT, Δ1472–1522) (12) were provided by Dr. J. Boyes (Institute of Cancer Research, London, U.K.). p300 C- and N-terminal deletion mutants were constructed by cloning the deletion mutants amplified by PCR into *Hind*III/*Xho*I sites of a mammalian expression vector pCMV-Tag2 (Stratagene, La Jolla, CA). Expression vectors for 12S E1A and its deletion mutant (Δ2–36) (13) were provided by Dr. P. Raychaudhuri (University of Illinois, Chicago, IL).

Transfection and luciferase assay

The transfection procedure was performed as previously described (14). In brief, 10 μl of Lipofectamine 2000 reagent (Invitrogen) and 4 μg of DNA constructs were mixed, and the mixture was slowly added to cells in a 6-well plate and incubated for 24 h. The cells were washed, incubated in serum-free medium for 24 h, and treated with or without LPS/IFN-γ for 8 h. Luciferase activity was measured using a kit from Promega (Madison, WI) and the emitted light was determined in a luminometer (TD-20/20). To

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[3] Abbreviations used in this paper: iNOS, inducible NO synthase; HAT, histone acetyltransferase; IRF-1, IFN-regulatory factor-1; ChIP, chromatin immunoprecipitation; WT, wild type; Ac-p50, acetylated p50.

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evaluate the effect of p300 overexpression, 10 μg of p300 constructs were mixed with 25 μl of Lipofectamine 2000 reagent and the mixture was added to cells cultured in a 10-cm dish. In all experiments, transfections were performed in duplicate.

Western blot analysis

Western blot analysis was performed as previously described (15). In brief, proteins were separated by electrophoresis in a 4–15% SDS-polyacrylamide gradient minigel (Bio-Rad, Hercules, CA) and electrophoretically transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Western blots were probed with affinity purified rabbit polyclonal IgG against iNOS, p300, p50, p65, C/EBPβ, c-Jun, or IRF-1 at 1 μg/ml each (Santa Cruz Biotechnology, Santa Cruz, CA). The protein bands were detected by ECL (Amersham Pharmacia Biotech). A number of p50 Abs were available from commercial sources. We evaluated several Abs purchased from Santa Cruz Biotechnology and Upstate Biotechnology (Boston, MA) for their sensitivity in detecting p50 proteins in RAW264.7 cell lysates. The Santa Cruz catalogue no. SC-7178, SC-114 and Upstate catalogue no. 06-886 rabbit polyclonal Abs exhibited a similar sensitivity. The Santa Cruz catalogue no. SC-8414 rabbit polyclonal Ab and catalogue no. 1192 goat polyclonal Ab had low sensitivity. We used the SC-7178 or SC-114 Ab (1 μg/ml each) in all experiments. For p65 (Rel A) immunoblots, we used a Santa Cruz rabbit polyclonal Ab catalogue no. SC-109 (1 μg/ml dilution). Another Santa Cruz Ab catalogue no. SC-372 and a polyclonal Ab from Oncogene (Boston, MA) had a similar sensitivity as SC-372.

Coimmunoprecipitation

Coimmunoprecipitation was performed as previously described (16). Nuclear extracts were prepared from 80–90% confluent RAW 264.7 treated with or without LPS/IFN-γ for 8 h and their protein concentrations were determined. Specific Abs against p300 or transactivators were added. The samples were incubated at 4°C overnight. Protein A/G-agarose beads (Santa Cruz Biotechnology) were added, incubated for 2 h, and centrifuged. The beads were washed three times with wash buffer (50 mM Tris, pH 8, NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). The immunoprecipitates were mixed with SDS loading buffer and analyzed by 4–15% SDS-PAGE (Bio-Rad) followed by Western blotting using a specific Ab. A rabbit nonimmune normal IgG was included as a negative control.

In vitro DNA-protein binding assay

Binding of p300 protein or transactivators to iNOS promoter DNA was assayed as described previously (17). Biotin-labeled double-stranded oligonucleotide probes corresponding to iNOS promoter sequence (−1168 to −1) were synthesized by Integrated DNA Technologies (Coralville, IA). The binding assay was performed by mixing 500 μg of nuclear extract proteins, 5 μg of biotin-labeled DNA, and 50 μl of streptavidin agarose beads with 70% slurry. The mixture was incubated at room temperature for 1 h with shaking. Beads were then pelleted and washed with cold PBS for three times. The binding proteins were separated by 4–15% PAGE, followed by Western blot analysis probed with Abs against p300 or transactivators.

Chromatin immunoprecipitation (ChIP)

The ChIP assay was done as described with minor modifications (18). A total of 80–90% confluent RAW 264.7 cells were serum starved for 24 h and treated with or without LPS/IFN-γ for 8 h. Formaldehyde (1%) was added to the culture medium and after incubation for 20 min at 37°C, cells were washed twice in PBS, scraped, and lysed in lysis buffer (1% SDS, 10 mM Tris-HCl, pH 8.0, with 1 mM PMSF, pepstatin A, and aprotinin) for 10 min at 4°C. Lysates were sonicated five times for 10 s each and the debris was removed by centrifugation. One third of the lysate was used as DNA input control. The remaining two-thirds of the lysate were diluted 10-fold with a dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) followed by incubation with an anti-p300 Ab or a nonimmune rabbit IgG (Santa Cruz Biotechnology) overnight at 4°C. Immunoprecipitated complexes were collected by using protein A/G-agarose beads (Santa Cruz Biotechnology). The precipitates were extensively washed and incubated in the elution buffer (1% SDS and 0.1 M $NaHCO_3$) at room temperature for 20 min. Cross-linking of protein-DNA complexes was reversed at 65°C for 5 h, followed by treatment with 100 μg/ml proteinase K for 3 h at 50°C. DNA was extracted 3 times with phenol/chloroform and precipitated with ethanol. Pellets were resuspended in TE buffer and subjected to PCR amplification using specific iNOS promoter primers (5′ primer: −498 CTGCCCAAGCTGACTTACTAC −478, 3′ primer −1 GACCCTGGCAGCAGCCATCAG −21). The resulting product of 498 bp for iNOS was separated by 1% agarose gel electrophoresis.

Acetylation of transactivators

p50, p65, IRF-1, C/EBPβ, or c-Jun in nuclear extracts was immunoprecipitated with a specific Ab (1 μg/ml) described above and the immunoprecipitates were collected by using protein A/G-agarose beads. After extensive washing proteins were separated by 4–15% SDS-PAGE and acetylated transactivators were detected by Western blots using a mAb against acetylated lysine (0.2 μg/ml) (Cell Signaling Technology, Beverly, MA).

Results

Regulation of iNOS expression by p300

p300 proteins were detected in resting RAW 264.7 cells which were not increased by treatment with LPS/IFN-γ for 8 h (Fig. 1*a*). To determine whether p300 is involved in iNOS expression, we cotransfected RAW 264.7 cells with a wild-type (WT) or a deletion mutant (ΔE1A) of adenoviral E1A construct and an iNOS-luciferase expression vector. The WT E1A inhibited the basal and LPS/IFN-γ-stimulated iNOS promoter activity whereas the ΔE1A mutant had no inhibitory effect (Fig. 1*b*). Neither E1A nor ΔE1A overexpression alone influenced the luciferase activity. Overexpression of p300 by transient transfection (Fig. 2*a*) resulted in increased basal iNOS protein levels and augmented iNOS protein levels stimulated by LPS/IFN-γ (Fig. 2*b*). Furthermore, p300 transfection dose-dependently increased basal and LPS/IFN-γ-stimulated iNOS promoter activity, suggesting that iNOS expression is regulated by p300 levels (Fig. 3). As the p300 protein contains multiple binding domains at C- and N-terminal regions,

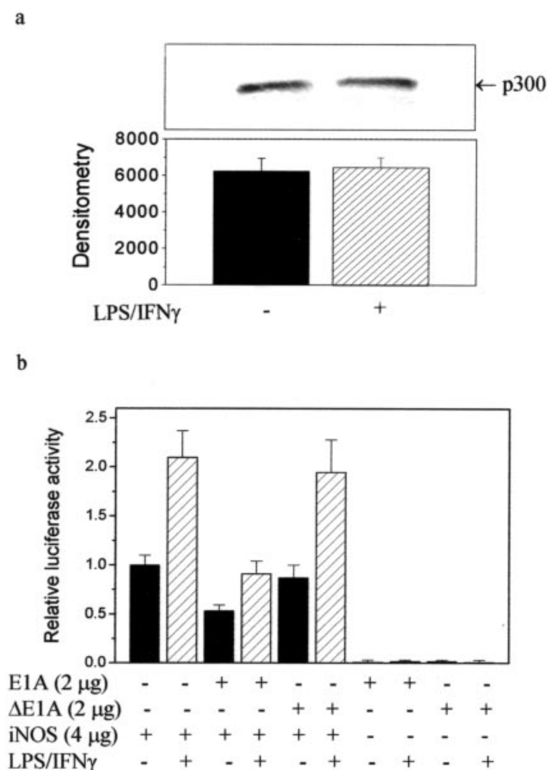

FIGURE 1. Role of p300 in iNOS promoter activation. *a,* p300 protein levels in RAW 264.7 cells treated with or without LPS/IFN-γ for 8 h. The densitometric analysis shows mean ± SD of three experiments. *b,* iNOS promoter activity determined by transfecting an iNOS-luciferase construct. Cotransfection with E1A or E1A deletion mutant (ΔE1A) construct was performed as described in Methods section. Each bar is mean ± SD of three independent experiments.

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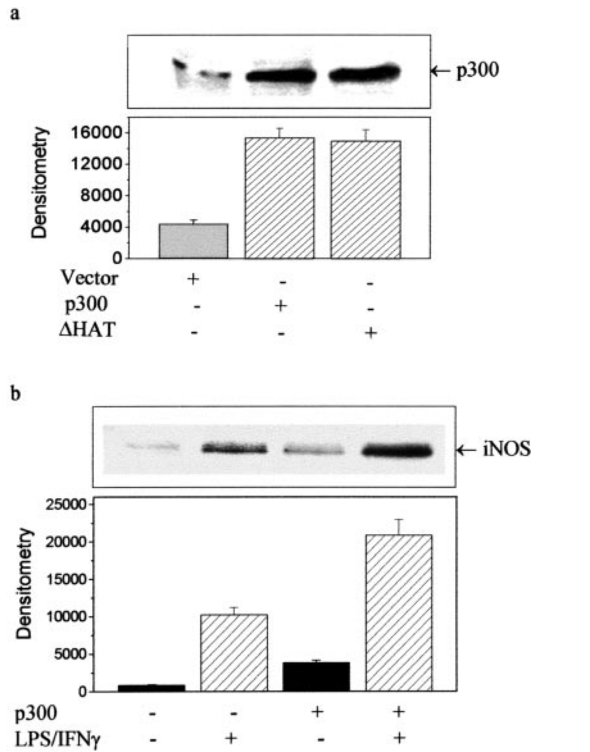

FIGURE 2. Effect of p300 overexpression on iNOS protein levels in RAW 264.7 cells treated with and without LPS/IFN-γ. *a,* p300 protein levels in cells transfected with a plasmid control (vector), WT p300 or p300 HAT deletion mutant (ΔHAT). The same amount of DNA construct (1 μg) was used in transfection experiments. Densitometry shows mean ± SD of three experiments. *b,* iNOS protein levels determined by Western blot analysis. p300 denotes p300 transfection. Densitometry shows mean ± SD of three experiments.

we evaluated the influence of C- and N-terminal deletion mutations on iNOS promoter activity. Deletion of 714 and 1220 aa from the C terminus or 671 aa from the N terminus resulted in a complete abrogation of the stimulatory activity of p300 (Fig. 4). Mutation of p300 HAT (ΔHAT) also abrogated the p300 stimulatory activity, consistent with the involvement of p300 HAT in iNOS transactivation. Roscovitine, an indirect inhibitor of p300 HAT (19), inhibited basal and LPS/IFN-γ-stimulated iNOS promoter activity in a concentration dependent manner, confirming the importance of p300 HAT in iNOS promoter activation (Fig. 5).

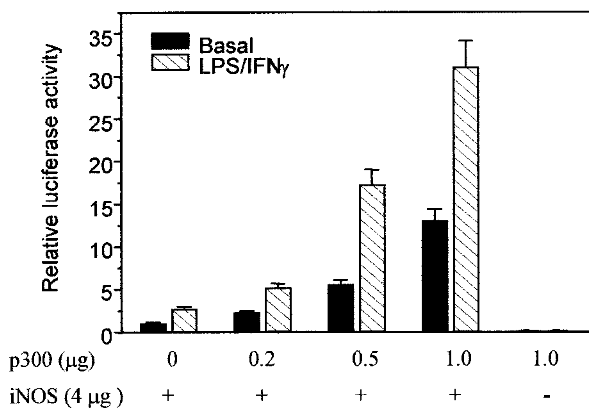

FIGURE 3. Concentration-dependent augmentation of iNOS promoter activity by p300 overexpression. p300 and iNOS denote cotransfection of these two constructs with or without LPS/IFN-γ stimulation. Each bar is mean ± SD of three experiments.

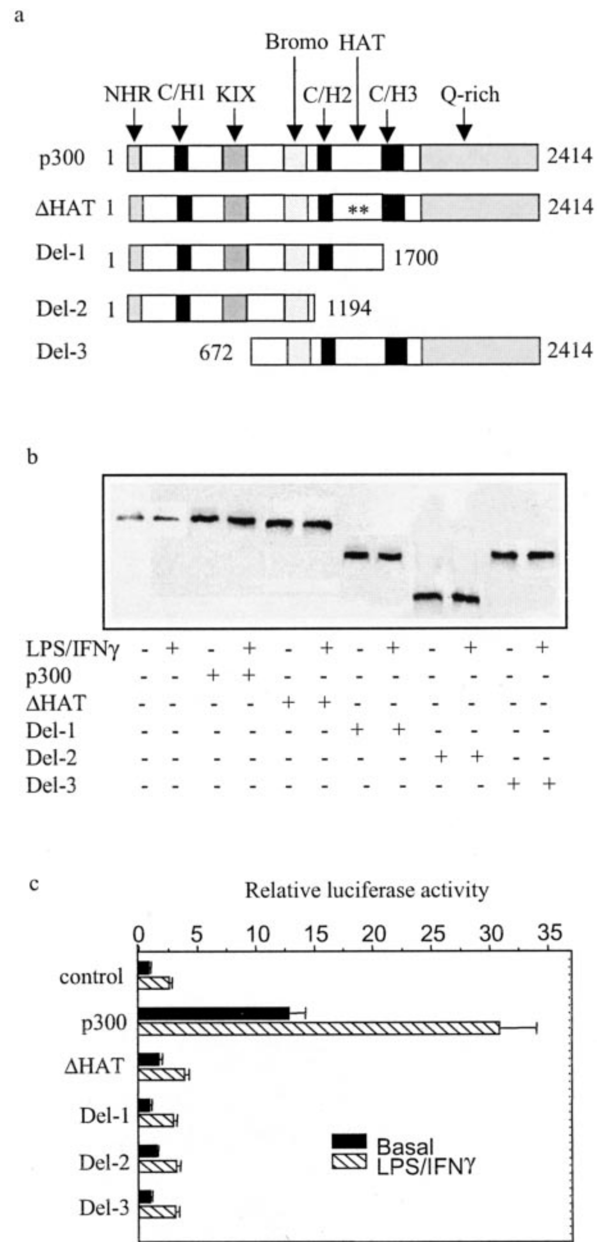

FIGURE 4. p300 domain requirement for augmenting iNOS promoter activity. *a,* A linear p300 structure illustrating multiple domains. NHR, nuclear hormone receptor domain; C/H 1, 2, and 3, cysteine-histidine rich domains 1, 2 and 3; KIX, CRE binding protein domain; bromo, bromo domain; HAT, HAT domain; and Q rich, glutamine-rich domain. ΔHAT denotes HAT deletion mutant (residues 1472 to 1522 are deleted). Del-1, -2 and -3 are mutants with deletion of C-terminal residues 1701–2414, 1195–2414 and N-terminal residues 1–671, respectively. *b,* iNOS promoter activity in cells transfected with WT p300 or various deletion mutants in the presence or absence of LPS/IFN-γ stimulation. Each bar shows mean ± SD of three experiments.

Regulation of p300 binding to iNOS promoter

Several transactivators, notably NF-κB and IRF-1, have been shown to be required for iNOS transcriptional activation. By using a streptavidin-agarose pull-down assay, we detected p50 and p65 NF-κB isoforms, IRF-1, C/EBPβ, and c-Jun in the ternary complex in resting cells (Fig. 6). There was a ∼2-fold increase in

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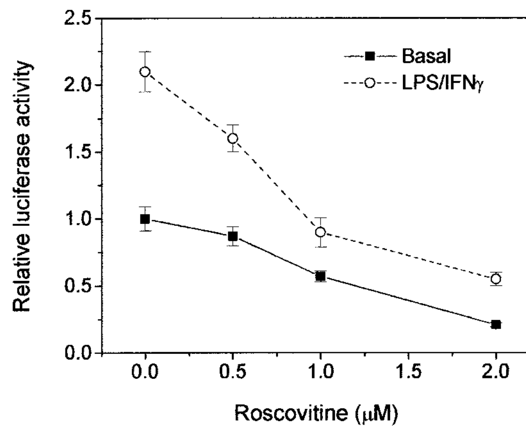

FIGURE 5. Effect of roscovitine on iNOS promoter activity. Each concentration point represents mean ± SD of triplicates.

NF-κB and IRF-1 binding in cells stimulated with LPS/IFN-γ for 8 h (Fig. 6). Neither C/EBPβ nor c-Jun binding was influenced by LPS/IFN-γ treatment (Fig. 6). p300 was also detected in the complex at the basal state which was increased by ~2-fold after stimulation with LPS/IFN-γ for 8 h (Fig. 6). Results from the ChIP assay also show binding of p50/p65, IRF-1 and p300 to chromatin

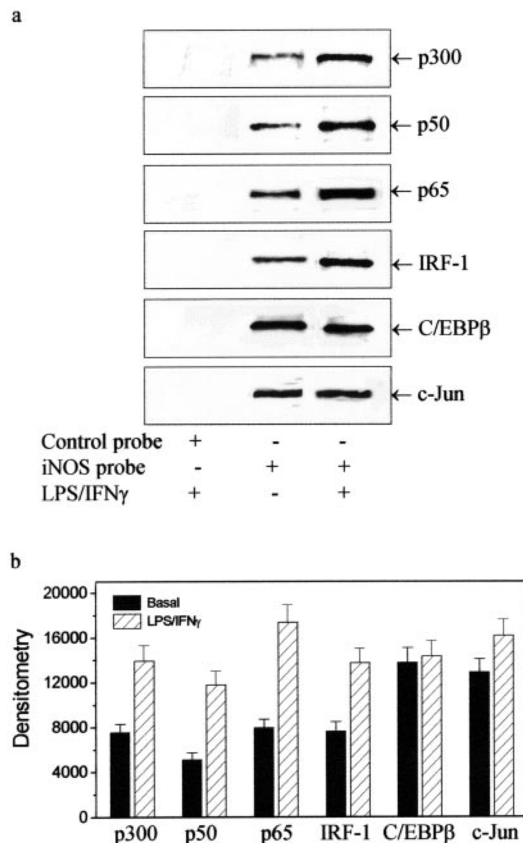

FIGURE 6. Binding of transcriptional activators and p300 coactivator to a biotinylated iNOS probe. Nuclear extracts from cells treated with and without LPS/IFN-γ were incubated with the biotinylated probe and the complex was pulled down with streptavidin-agarose beads. After extensive washing, proteins in the complex were analyzed by Western blotting using Abs to p300 or the indicated transactivators. Control probe is a biotinylated 22-bp nonrelevant DNA sequence. *a,* A representative binding profile. *b,* Densitometric analysis of three separate experiments. Each bar is mean ± SD of three experiments.

iNOS promoter region in resting cells, which was significantly increased by LPS/IFN-γ treatment (Fig. 7).

Complex of p300 with transactivators in nuclear extracts

To provide evidence for a direct interaction between p300 and NF-κB or IRF-1, we prepared nuclear extracts from resting and LPS/IFN-γ-treated cells and performed immunoprecipitation with Abs specific for p50/p65 or IRF-1. p300 in the immunoprecipitate was resolved by Western blots. p300 complexed with p50/p65 NF-κB and IRF-1 was significantly increased by LPS/IFN-γ treatment (Fig. 8*a*). A control IgG did not precipitate a p300-containing complex. We next immunoprecipitated the nuclear extracts with anti-p300 Abs and detected transactivators in the precipitate by Western blots using Abs specific for p50, p65 and IRF-1. The levels of transactivators in the precipitate were significantly increased by LPS/IFN-γ (Fig. 8*b*). Thus, p300 binds p50/p65 and IRF-1 in nucleus at basal cellular state and the binding is upregulated by LPS/IFN-γ.

Augmentation of NF-κB binding and p300 recruitment by p50 acetylation

We observed that p50 binding to the iNOS promoter probe was augmented by WT p300 but not ΔHAT mutant overexpression in resting and LPS/IFN-γ-treated cells (Fig. 9). Like p50, p65 binding was also augmented by WT p300, but not ΔHAT. By contrast, IRF-1 binding was not affected by WT or ΔHAT p300 (Fig. 9). To determine whether p300-mediated p50 binding might be regulated by p50 acetylation, we prepared nuclear extracts from resting and LPS/IFN-γ-treated cells, immunoprecipitated the nuclear extract proteins with anti-p50, anti-p65 or other transactivator Abs. Transactivator acetylation was detected with an anti-acetyl-lysine Ab. Acetylated p50 (Ac-p50) was detectable at basal cell state which

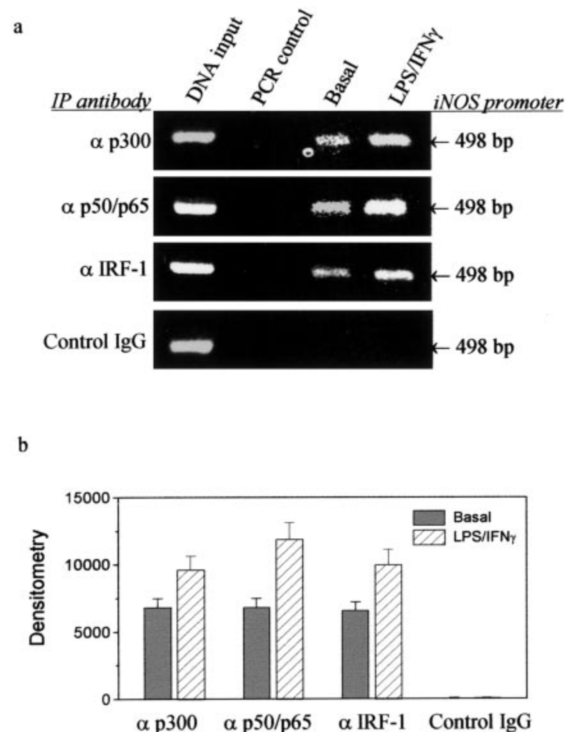

FIGURE 7. Binding of p300, p50/p65, and IRF-1 to chromatin iNOS promoter region determined by ChIP. Chromatin was immunoprecipitated with Abs to p300 (α p300), p50/p65 (α p50/p65), IRF-1 (α IRF-1) or a normal rabbit IgG. *a,* A representative ChIP gel. *b,* Densitometry of three experiments showing mean ± SD.

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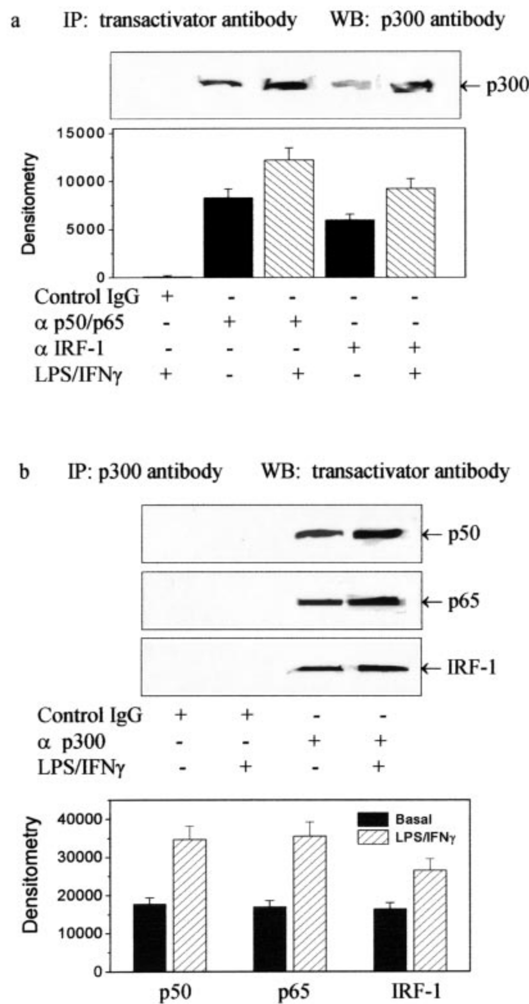

FIGURE 8. Interaction of p300 with transactivators. *a,* Nuclear extracts were immunoprecipitated with a rabbit polyclonal p50/p65 (α p50/p65) or IRF-1 (α IRF-1) Ab and p300 levels in the precipitate were detected with a p300 Ab. Control IgG denotes IP with a normal rabbit IgG. *b,* A reciprocal procedure in which nuclear extracts were immunoprecipitated with a rabbit polyclonal p300 Ab (α p300) and p50, p65, or IRF-1 in complex was identified by a specific Ab. Control IgG denotes IP with a normal rabbit IgG in place of p300 Ab. Each error bar represents mean ± SD of three experiments.

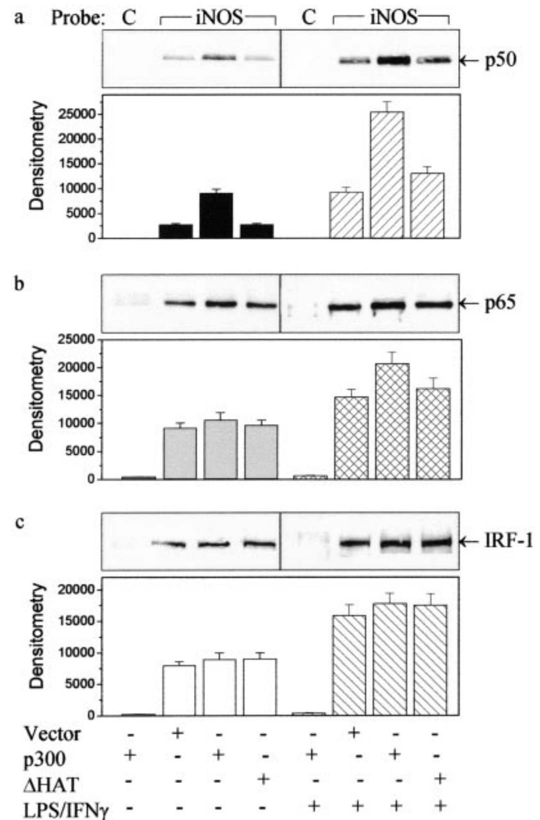

FIGURE 9. Influence of p300 or ΔHAT overexpression on p50 (*a*), p65 (*b*), and IRF-1(*c*) binding to biotinylated iNOS promoter. C denotes the use of a nonrelevant probe as a negative control. Vector denotes transfection with an empty plasmid. Each bar is mean ± SD of three experiments.

was increased by ~2-fold in LPS/IFN-γ treated cells (Fig. 10). Traces of Ac-p65 and Ac-c-Jun were detected and there was no detectable Ac-IRF-1 or Ac-C/EBPβ (Fig. 10). The level of Ac-p50 increase correlated with that of p50 binding (Fig. 9). We next evaluated the effects of WT and ΔHAT p300 overexpression on p50 acetylation. WT p300 overexpression resulted in an almost 2-fold increase in Ac-p50, whereas ΔHAT overexpression did not induce an increase in Ac-p50 (Fig. 11*a*). Total p50 protein levels were unaltered by p300 or ΔHAT overexpression (Fig. 11*b*). It is interesting to note that p300 binding was enhanced by ~2-fold by WT p300 transfection but not the ΔHAT mutant (Fig. 12). These results suggest a relationship between p50 acetylation and p50/p300 binding to iNOS promoter.

Inhibition of p50 acetylation and binding by E1A

To ascertain the role of p300 in p50 acetylation, we cotransfected cells with p300 and E1A and determined Ac-p50 in the presence or absence of LPS/IFN-γ stimulation. E1A overexpression resulted in a reduced level of Ac-p50 at basal state and in cells stimulated with LPS/IFN-γ (Fig. 13*a*) without an effect on p50 levels (Fig. 13*b*). We next determined the effect of E1A overexpression on p50 binding by the streptavidin pull-down assay. p50 binding at the basal state and in the presence of LPS/IFN-γ was severely suppressed by E1A (Fig. 14*a*). Reduced p50 binding was correlated with a diminished p300 level in the binding complex (Fig. 14*b*).

Discussion

Results from this study provide new insight into the role of p300 in iNOS transcriptional activation. Data from E1A overexpression experiments indicate that p300 is essential for iNOS promoter activity induced by LPS/IFN-γ. The action of E1A is specific for p300 as WT E1A abrogates the iNOS promoter activity stimulated by LPS/IFN-γ whereas a N-terminal deletion mutant of E1A has no effect on LPS/IFN-γ-stimulated iNOS promoter activity. Results from p300 binding experiments provide additional support for the p300 requirement. p300 binding to iNOS promoter-bound transactivators is enhanced by LPS/IFN-γ stimulation which is abrogated by E1A overexpression. Our findings further indicate that iNOS transcriptional activation is regulated by p300 protein levels. Overexpression of p300 by transient transfection of RAW 264.7 cells with various concentrations of p300 constructs elicits a concentration-dependent increase in iNOS promoter activity stimulated by LPS/IFN-γ. These data are in agreement with a general concept that p300 proteins in most cells are expressed in low abundance and are a limiting factor in gene transcription. Another potential factor that regulates p300 transactivation is its posttranslational modification by phosphorylation. p300 and/or

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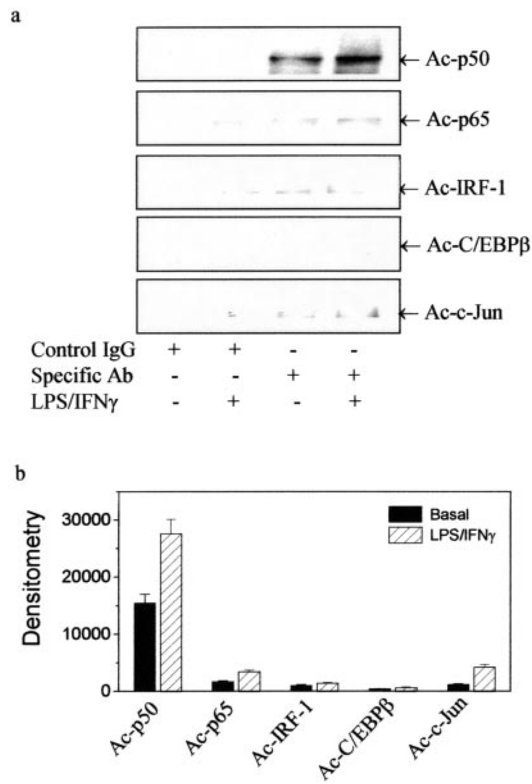

FIGURE 10. Effect of LPS/IFN-γ on levels of Ac-p50 and other acetylated transactivators by Western blots using a specific monoclonal acetyl-lysine Ab. A mouse IgG was included as control. *a,* A representative blot. *b,* Densitometric analysis of blots from three experiments. The error bar is mean ± SD.

CBPphosphorylation has been demonstrated during cell differentiation, cell cycle progression and cell signaling via the protein kinase C pathway (19–22). It has been shown that CBP is phos-

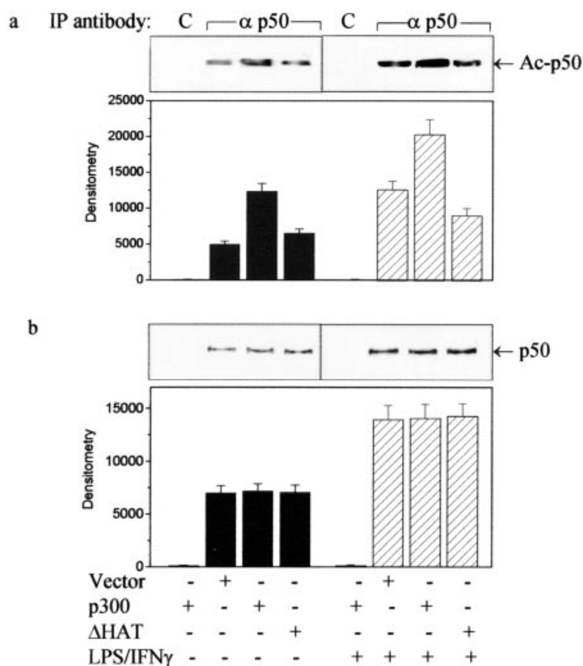

FIGURE 11. Influence of p300 or ΔHAT overexpression on Ac-p50 (*a*) and total p50 levels (*b*). p300 but not ΔHAT enhanced Ac-p50 levels without altering the total p50 levels. C denotes control IgG and α p50, specific Ab. The data presented are mean ± SD from three experiments.

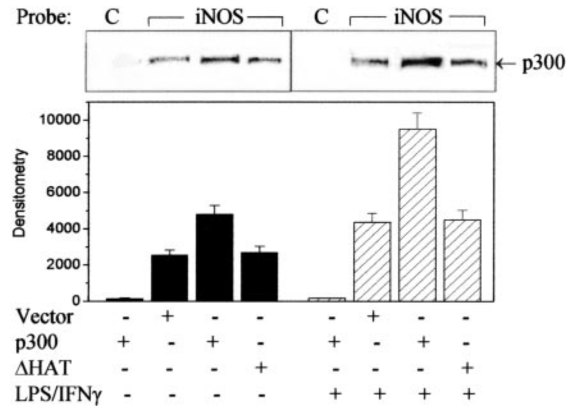

FIGURE 12. Influence of p300 or ΔHAT overexpression on p300 levels in biotinylated iNOS probe complex. p300 but not ΔHAT overexpression was correlated with an increased p300 level in the promoter complex. Each bar is mean ± SD of three experiments.

phorylated by cyclin E-CdK2 during cell cycle progression and the phosphorylated CBP exhibits an enhanced HAT activity (19). Roscovitine, an inhibitor of cyclin E-CdK2, abrogates the increase in HAT activity. We, therefore, chose to evaluate the effect of roscovitine on iNOS promoter activity. Our data indicate that roscovitine dose-dependently blocks not only the iNOS promoter activity induced by LPS/IFN-γ but also the basal promoter activity. These results suggest that p300 phosphorylation by cyclin E-CdK2 may play a critical role in regulating iNOS transcriptional activation. Further studies are needed to provide direct evidence for this.

p300 contains multiple domains at the N- and C-terminal regions that interact with myriad DNA-bound transcription activators including p65 subunit of NF-κB (9–11). Results from our p300 deletion mutant transfection experiments suggest that multiple domains are required for stable binding of p300 to iNOS promoter-bound transactivators. As expected, the core HAT domain is also required for p300-mediated iNOS promoter activity. p300

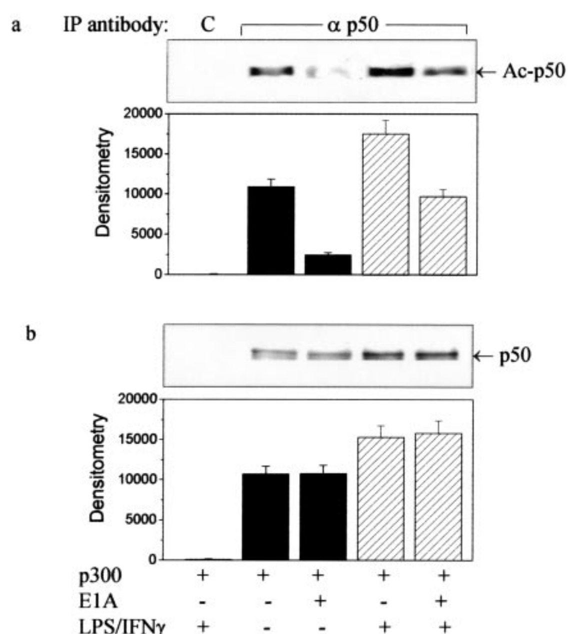

FIGURE 13. Effect of E1A overexpression on Ac-p50 (*a*) and total p50 (*b*) levels in p300 over-expressed cells treated with or without LPS/IFN-γ. Each bar is mean ± SD of three experiments.

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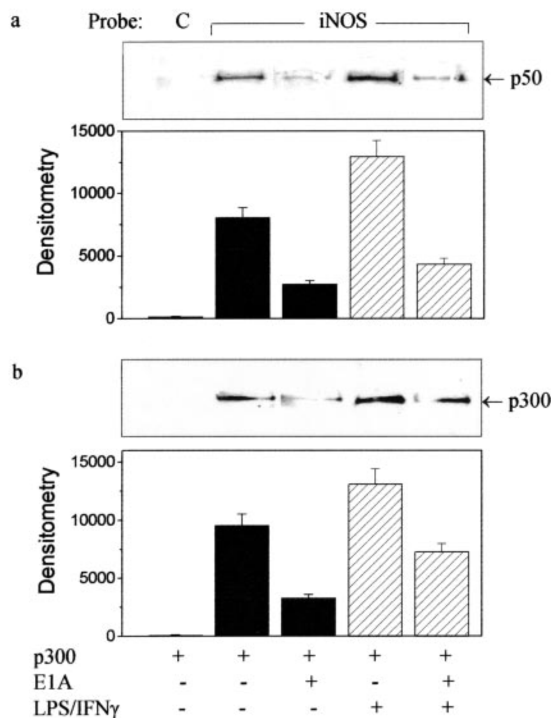

FIGURE 14. Effect of E1A overexpression on p50 (*a*) or p300 (*b*) binding to biotinylated iNOS promoter probe. A biotinylated nonrelevant sequence was used as a control. There was a concordant reduction in binding of p50 and p300 by E1A. Each bar is mean ± SD of three experiments.

HAT plays a major role in chromatin modification by acetylation of lysine residues at the N-terminal tail of core histones (23), whereby it opens up the chromatin structure at the iNOS promoter region and makes *cis*-acting regulatory elements accessible to transactivators such as NF-κB and IRF-1. Our results suggest that p300 HAT may also regulate interaction of p300 with promoter-bound transactivators. Direct assessment of p300 binding to DNA-transactivator complex by electrophoresis mobility shift assay is impossible because of very large molecular masses of the complex. By using a streptavidin-agarose pulldown assay, we were able to analyze the candidate proteins in the complex. Our data indicate that transfection of WT p300 results in an increased p300 binding whereas transfection of ΔHAT fails to increase p300 binding despite a similar level of p300 protein overexpression as WT p300. The mechanism by which p300 regulates its own recruitment to iNOS promoter-transactivator complex is unclear. Our results suggest that p50 acetylation by p300 may represent a possible mechanism. We found a good correlation between p50 acetylation, p50 binding and p300 recruitment in RAW264.7 cells treated with LPS and IFN-γ. Furthermore, WT p300 transfection induces a concordant increase in p50 acetylation, p50 binding, and p300 recruitment whereas transfection of the HAT deletion mutant increases neither p50 acetylation nor the binding of p50 or p300. Immunoprecipitation experiments reveal that p300 formed complex with p50 in nucleus of resting cells which was enhanced by LPS and IFN-γ. Interestingly, Ac-p50 in p50-p300 complex was also enhanced by LPS and IFN-γ treatment. p300 also complexes with p65, C/EBPβ, IRF-1, and c-Jun in nucleus. Only traces of Ac-p65 and Ac-c-Jun were detected which were not regulated by LPS and IFN-γ. These results suggest that LPS/IFN-γ enhances nuclear p50/p300 complex formation wherein p50 acetylation is up-regulated. p50 acetylation may represent one mechanism by which p50/p300 binding to iNOS promoter is facilitated.

The p50 NF-κB isoform joins several transcriptional activators including p53, GATA-1 whose DNA binding activities have been shown to be enhanced by p300-catalyzed acetylation (12, 24). p50 has been reported to be acetylated in lymphocytes infected by HIV-1 (25). The viral Tat protein appears to be a requisite cofactor for p50 acetylation by p300. Several lysine residues of p50 were proposed to be potential acetylation sites (25). It is unclear whether p50 acetylation by p300 in RAW264.7 cells also requires a cofactor. Rel A (p65) binding to iNOS promoter is also up-regulated by LPS and IFN-γ. However, we detected only very low level of Ac-p65 in resting cells and there was no apparent induction of Ac-p65 by LPS and IFN-γ or p300. Thus, binding of p65 to iNOS promoter may not be directly regulated by acetylation in RAW264.7 cells in response to stimulation with LPS and IFN-γ. However, it is possible that its binding is enhanced via Ac-p50 by forming heterodimers with p50.

It has been reported that Rel A (p65) is acetylated by p300 at lysine 218, 221 and 310 (26). Acetylation at K221 in Rel A enhances DNA binding and inhibits IkBα interaction, thereby retaining Rel A in the nucleus (27). Acetylation of K310 is required for transcription without a direct effect on DNA binding or IkBα interaction. In those studies, Rel A acetylation in 293T or COS-7 cells was enhanced by treatment with TNF-α. It is unclear why we detected only a very low level of Ac-p65 in RAW264.7 cells which was not induced by LPS and IFN-γ. One potential shortcoming of our study is that we had not determined p65 acetylation with [^{3}H]acetate which may be more specific than acetyl-lysine Ab. We were also concerned whether our results might be influenced by cross-reactivity of NF-κB Abs. We, therefore, evaluated several p50 and p65 Abs and found variations in sensitivities in detecting p50 on Western blot analysis. Three p50 and p65 Abs each exhibited a similar degree of specificity and sensitivity in recognizing p50 and p65 proteins, respectively and consistently show induced p50 but not p65 acetylation by LPS and IFN-γ. Thus, it is unlikely that our results are due to cross-reactivity of Abs. We cannot exclude the possibility that differences in p50 and p65 acetylation between the present study and the previous reports is due to different cell types under stimulation by different agonists. It should be emphasized that several lysine residues in p50 have been identified as potential sites for p300-catalyzed acetylation (25). Thus, both p50 and p65 comprise lysine residues for acetylation by p300. Their acetylation and the functional consequences may depend on the signaling from external stimuli and the cellular program.

In summary, p300 plays a major role in iNOS transcriptional activation. Our results suggest that it interacts with p50, and acetylates p50 in nucleus, thereby increasing NF-κB binding to its corporate sites. It also complexes with IRF-1 but does not acetylate IRF-1 or C/EBPβ. IRF-1 and C/EBPβ binding may thus depend on other posttranslational modifications. As NF-κB promotes the expression of myriad proinflammatory genes (28), it will be important to determine whether p300-mediated p50 acetylation and binding is a common mechanism for gene regulation.

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References

1. Griffith, O. W., and D. J. Stuehr. 1995. Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol. 57:707.*
2. Nathan, C. 1997. Inducible nitric oxide synthase: what difference does it make? *J. Clin. Invest. 100:2417.*
3. Wu, K. K. 1995. Inducible cyclooxygenase and nitric oxide synthase. *Adv. Pharmacol. 33:179.*

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4. Xie, Q. W., R. Whisnant, and C. Nathan. 1993. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *J. Exp. Med. 177:1779*.
5. Lowenstein, C. J., E. W. Alley, P. Raval, A. M. Snowman, S. H. Snyder, S. W. Russell, and W. J. Murphy. 1993. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon γ and lipopolysaccharide. *Proc. Natl. Acad. Sci. USA 90:9730*.
6. Xie, Q. W., Y. Kashiwabara, and C. Nathan. 1994. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem. 269:4705*.
7. Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, J. Gerecitano, D. Shapiro, J. Le, S. I. Koh, T. Kimura, S. J. Green, et al. 1994. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science 263:1612*.
8. Cieslik, K., Y. Zhu, and K. K. Wu. 2002. Salicylate suppresses macrophage nitric-oxide synthase-2 and cyclooxygenase-2 expression by inhibiting CCAAT/enhancer-binding protein-beta binding via a common signaling pathway. *J. Biol. Chem. 277:49304*.
9. Eckner, R., M. E. Ewen, D. Newsome, M. Gerdee, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingstone. 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300 KD protein (p300) reveals a protein with properties of a transcriptional adapter. *Genes Develop. 8:869*.
10. Giles, R. H., D. J. Peters, and M. H. Breuning. 1998. Conjunction dysfunction: CBP/p300 in human disease. *Trends Genet. 14:178*.
11. Janknecht, R., and T. Hunter. 1996. Transcription: a growing coactivator network. *Nature 383:22*.
12. Boyes, J., P. Byfield, Y. Nakatani, and V. Ogryzko. 1998. Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature 396:594*.
13. Raychaudhuri, P., S. Bagchi, S. D. Neill, and J. R. Nevins. 1990. Activation of the E2F transcription factor in adenovirus-infected cells involves E1A-dependent stimulation of DNA-binding activity and induction of cooperative binding mediated by an E4 gene product. *J. Virol. 64:2702*.
14. Deng, W. G., M. A. Saunders, D. W. Gilroy, X. Z. He, H. Yeh, Y. Zhu, M. I. Shtivelband, K. H. Ruan, and K. K. Wu. 2002. Purification and characterization of a cyclooxygenase-2 and angiogenesis suppressing factor produced by human fibroblasts. *FASEB J. 16:1286*.
15. Xu, X. M., L. Sansores-Garcia, X. M. Chen, N. Matijevic-Aleksic, M. Du, and K. K. Wu. 1999. Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc. Natl. Acad. Sci. USA 96:5292*.
16. Liou, J. Y., W. G. Deng, D. W. Gilroy, S. K. Shyue, and K. K. Wu. 2001. Colocalization and interaction of cyclooxygenase-2 with caveolin-1 in human fibroblasts. *J. Biol. Chem. 276:34975*.
17. Zhu, Y., M. A. Saunders, H. Yeh, W. G. Deng, and K. K. Wu. 2002. Dynamic regulation of cyclooxygenase-2 promoter activity by isoforms of CCAAT/enhancer-binding proteins. *J. Biol. Chem. 277:6923*.
18. Luo, R. X., A. A. Postigo, and D. C. Dean. 1998. Rb interacts with histone deacetylase to repress transcription. *Cell 92:463*.
19. Ait-Si-Ali, S., S. Ramirez, F. X. Barre, F. Dkhissi, L. Magnaghi-Jaulin, J. A. Girault, P. Robin, M. Knibiehler, L. L. Pritchard, B. Ducommun, et al. 1998. Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A. *Nature 396:184*.
20. Yaciuk, P., and E. Moran. 1991. Analysis with specific polyclonal antiserum indicates that the E1A-associated 300-kDa product is a stable nuclear phosphoprotein that undergoes cell cycle phase-specific modification. *Mol. Cell Biol. 11:5389*.
21. Kitabayashi, I., R. Eckner, Z. Arany, R. Chiu, G. Gachelin, D. M. Livingston, and K. K. Yokoyama. 1995. Phosphorylation of the adenovirus E1A-associated 300 kDa protein in response to retinoic acid and E1A during the differentiation of F9 cells. *EMBO J. 14:3496*.
22. Yuan, L. W., and J. E. Gambee. 2000. Phosphorylation of p300 at serine 89 by protein kinase C. *J. Biol. Chem. 275:40946*.
23. Ogryzko, V. V., R. L. Schiltz, V. Russanova, B. H. Howard, and Y. Nakatani. 1996. The transcriptional co-activators p300 and CBP are histone acetyltransferases. *Cell 87:953*.
24. Gu, W., and R. G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell 90:595*.
25. Furia, B., L. Deng, K. Wu, S. Baylor, K. Kehn, H. Li, R. Donnelly, T. Coleman, F. Kashanchi. 2002. Enhancement of nuclear factor-κB acetylation by coactivator p300 and HIV-1 Tat proteins. *J. Biol. Chem. 277:4973*.
26. Chen, L-f., Y. Mu and W. C. Green. 2002. Acetylation of Rel A at discrete sites regulates distinct nuclear functions of NF-κB. *EMBO J. 21:6539*.
27. Chen, L., W. Fischle, E. Verdin, and W. C. Greene. 2001. Duration of nuclear NF-κB action regulated by reversible acetylation. *Science 293:1653*.
28. Li, X., and G. R. Stark. 2002. NFκB-dependent signaling pathways. *Exp. Hematol. 30:285*.

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