THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 51, Issue of December 19, pp. 51150–51158, 2003
Printed in U.S.A.

Differential Activation of a C/EBPβ Isoform by a Novel Redox Switch May Confer the Lipopolysaccharide-inducible Expression of Interleukin-6 Gene*

Received for publication, May 27, 2003, and in revised form, October 3, 2003
Published, JBC Papers in Press, October 6, 2003, DOI 10.1074/jbc.M305501200

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C/EBPβ, a member of the CCAAT/enhancer binding protein (C/EBP) family, is one of the key transcription factors responsible for the induction of a wide array of genes, some of which play important roles in innate immunity, inflammatory response, adipocyte and myeloid cell differentiation, and the acute phase response. Three C/EBPβ isoforms (*i.e.* LAP*, LAP, and LIP) were known to arise from differential translation initiation and display different functions in gene regulation. C/EBPβ is known to induce interleukin (IL)-6 gene when P388D1 cells are treated with lipopolysaccharide (LPS). Exactly how the transcriptional activities of C/EBPβ isoforms are involved in the regulation of the IL-6 gene remains unclear. Here we report that LPS-induced expression of IL-6 gene in P388D1 cells is mediated by a redox switch-activated LAP*. The intramolecular disulfide bonds of LAP* and LAP have been determined. Among the cysteine residues, amino acid 11 (Cys^{11}) of LAP* plays key roles for determining the overall intramolecular disulfide bonds that form the basis for redox switch regulation. The DNA binding activity and transcriptional activity of LAP* are enhanced under reducing condition. LAP and LIP, lacking 21 and 151 amino acids, respectively, in the N-terminal region, are not regulated in a similar redox-responsive manner. Our results indicate that LAP* is the primary isoform of C/EBPβ that regulates, through a redox switch, the LPS-induced expression of the IL-6 gene.

Members of the C/EBP[1] family of proteins have been implicated in the differentiation of myelomonocytic cells and the regulation of gene expression during activation of macrophages (1, 2). Regulation of macrophage gene expression by lipopolysaccharide (LPS) is of potential importance in the pathogenesis of infection, inflammation, and septic shock. A detailed understanding of the molecular basis of such disease requires a thorough analysis of the mechanisms by which the actions of LPS are transduced to the nucleus to alter gene expression. The expression of C/EBPβ is inducible in monocytes and macrophages by LPS. C/EBPβ has been implicated in the regulation of proinflammatory cytokines as well as other genes associated with macrophage activation and the acute phase response (3, 4). The promoter regions of the genes for IL-6, TNFα, IL-1, IL-8, and granulocyte colony-stimulating factor contain C/EBPβ-binding sites (3, 5–7). The stable expression of C/EBPβ in a murine P388 lymphoblast cell line is sufficient to confer LPS-inducible IL-6 expression (8, 9). Conversely, inhibition of endogenous C/EBPβ expression by antisense RNA interference blocks LPS induction of IL-6 expression in the murine P388D1(IL1) macrophage-like cells (8). C/EBPβ can also activate the proximal promoter of the human TNF-α gene in RAW264.7 monocytic cells, whereas overexpression of a dominant negative C/EBPβ inhibits LPS-induced activation (10). In the murine P388D1(IL1) cell line, C/EBPβ is constitutively expressed, and IL-6 and TNF-α can readily be induced by agents such as LPS. The exact molecular mechanisms of LPS stimulation of IL-6 and TNF-α gene expression and how this relates to C/EBPβ-mediated activation of these genes remain unclear.

Structurally, members of C/EBP family are bZIP proteins with sequence-specific DNA binding activity when homo- or heterodimerized. The DNA-binding and transactivation domains are located in the C- and N-terminal regions, respectively (11). The intronless *c/ebpβ* gene (also known as *lap* in rat, *agp/ebp* in mouse, and NF-IL6 in human) encodes three isoforms, including two activators (termed LAP* and LAP, respectively) and a repressor (*i.e.* LIP). These isoforms of C/EBPβ are differentially translated from the same mRNA (12). The expression of these isoforms is developmentally and hormonally regulated (12–14).

The functional differences between the two activator isoforms, LAP* and LAP, have been addressed in two examples (15, 16). The 21 amino acids of the N terminus region of LAP* are responsible for its functional interaction with NF-κB and for recruiting to the SWI-SNF complex involved in chromatin remodeling. Both NF-κB and C/EBPβ can activate the inflammatory cytokine genes, and conversely, these cytokines can induce NF-κB and C/EBPβ (17). These results suggest that the mechanisms for activating C/EBPβ and NF-κB may converge on a post-translational level. There is a cysteine residue, Cys^{11}, located in the 21-amino acid stretch of the N-terminal region of LAP*. The possibility of Cys^{11} involved in the tertiary structure

* This work was supported by National Science Council Grants NSC90-2321-B002-003 and NSC92-2320-B002-188 and by funds from the Institute of Biological Chemistry, Academia Sinica, Taiwan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: C/EBP, CCAAT/enhancer binding protein; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; PDTC, pyrrolidine dithiocarbamate; NAC, *N*-acetylcysteine; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; ChIP, chromatin immunoprecipitation; IGF, insulin-like growth factor; IGFBP, IGF-binding protein.

or redox sensing that results in the differential activation of LAP* and LAP deserves to be investigated.

The activities of transcription factors may be regulated in a redox-sensitive manner. LPS has been shown to induce changes in cellular redox states. The cellular levels of thioredoxin, thioredoxin reductase, and NADPH, as well as the reactive oxygen species, may be elevated by LPS treatment (18–20). Redox-based transcriptional regulation has been studied extensively in prokaryotes (21–24) and demonstrated in eukaryotic cells (25–31). Among the redox-sensing proteins, thioredoxin, thioredoxin reductase, and other related oxidoreductases are involved in the regulation of activities of many key proteins in species ranging from bacteria to eukaryotic cells. REF-1 has been characterized as a factor that imparts strong DNA binding activity to the AP-1 complex by maintaining the cysteines as reduced sulfhydryl groups (32). The DNA binding activity of transcription factors can be affected by redox modifications (25, 27, 29, 33). Cysteine residues of several transcription factors, although not directly involved in DNA binding, modulate DNA binding activity in response to the cellular redox states (25–27, 33–37).

There are six, five, and two cysteines in LAP*, LAP, and LIP, respectively. To address the potential regulation of LAP* and LAP activity by LPS, we performed detailed biochemical and functional studies on the differential regulation of LAP* and LAP. Here we report that only LAP* is activated by LPS-induced redox switch. Upon reduction, LAP* is activated to up-regulate the transcription of IL-6 gene in P388D1(IL1) cells. We have determined that disulfide bonds formed in LAP* and LAP. A cysteine residue located in the N-terminal 21-amino acid region plays key roles in determining the tertiary structure and the redox-regulated activation of LAP*. The importance and the biological significance of this discovery are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection—293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone). P388D1(IL1) cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal calf serum and 2 mM L-glutamine (Invitrogen). Both cultures were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. All nuclear extracts from 293T or P388D1(IL1) cells were fresh prepared with nitrogen gas. Transient transfection of 293T cells was performed with a calcium phosphate precipitation method. The calcium phosphate-plasmid DNA precipitate contained, in each well of a 6-well plate, 1 μg of AGP-CAT, 0.3 μg of pSV-β-galactosidase, and various constructs of pCMV-C/EBPβ (detailed in the legend of figures). pCMV plasmid DNA was used to give a final amount of 2 μg of DNA for each transfection. 20 h post-transfection, the cultures were replaced with fresh medium and treated with either 10 μM pyrrolidine dithiocarbamate (PDTC) or 1 mM *N*-acetylcysteine (NAC) for 16 h. The cells were harvested, and CAT activity was determined and normalized with β-galactosidase activity. At least two independent, duplicate experiments were performed to each assay.

Plasmids—The AGP-CAT and pCMV-C/EBPβ plasmids were obtained as described elsewhere (38). FLAG-tagged LAP*, LAP, and LIP constructs were obtained by cloning the full-length and the respective N-terminal deletion constructs into pCMV-tag2 expression vector (Stratagene). Mutants of each of the six cysteine residues (*i.e.* Cys → Ser) were generated with the M13mp18 site-directed mutagenesis system (Promega) using the following synthetic primers: C11S, 5′-ACGCAGCAAGCCTCCCG-3′; C33S, 5′-AGCCCGACAGCCTGGAA-3′; C123S, 5′-CCGCCCGCAAGCTTTCCG-3′; C143S, 5′-CCGCGGACAGCAAGCGC-3′; C201S, 5′-CCCGCCGCAAGCTTCGCG-3′; and C296S, 5′-CGGGCCACAGCTAGCGCGGCGCG-3′. The mutated nucleotides are underlined. The mutated cDNAs were then cloned into pCMV-tag vector.

Antibodies—18F8 monoclonal antibody was produced from hybridomas generated by fusing splenocytes from BALB/c mouse immunized with MHRLLAWDAACLPPPAAFPKLH with myelomas. 1H7 monoclonal antibody was obtained by immunizing BALB/c mice with recombinant LIP. A16 and other antibodies were described previously (15).

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays were modified from previously described methods (39). Briefly, P388D1(IL-1) cells were cross-linked with 1% formaldehyde for 10 min at 37 °C. The nuclei were isolated and sonicated into oligonucleosomes of ~600 bp in length. The sheared chromatin was immunoprecipitated with 18F8 and 1H7 antibodies overnight, followed by 1 h of incubation with protein G-agarose, cross-linking reversal, and deproteination. The presence of immunoprecipitated DNA was detected by PCR with the following primers: IL-6, sense, 5′-GCTTCTTAGGGCTAGCCTCA-3′, and antisense, 5′-AGCTACAGACATCCCCAGTC-3′; TNF-α, sense, 5′-TTCCGAGGGTTGAATGAGAGCT-3′, and antisense, 5′-TTTCTGTTCTCCCTCCTGGCTA-3′; and C/EBPβ, sense, 5′-GTAGCTGGAGGAACGATC3′, and antisense, 5′-TCGGGAACACGGAGGAGC-3′. PCR was performed for 25 cycles. The products were resolved by 2% agarose gels and visualized with ethidium bromide staining. UV-illuminated images were photographed and analyzed by AlphaImager 1220 (Alpha Innotech Corp.)

Northern Blot Analysis—P388D1(IL1) cells were cultured to 80% confluent and stimulated with 1 μg/ml LPS (*Escherichia coli* 0111:B4; Sigma). The total RNA was extracted with TRIZOL reagent (Invitrogen). Approximately 10 μg of total cellular RNA of each sample was separated by electrophoresis in a 1% agarose gel and analyzed by Northern blotting according to standard protocols. IL-6 probe was generated by reverse transcription-PCR from P388D1(IL1) RNA with the following primers: IL-6 forward primer, 5′-ATGAAGTTCCTCTCTGCAAGAG-3′, and IL-6 reverse primer, 5′-CTAGGTTTGCCGAGTAGATCTCA-3′. Full-length cDNA was used to probe C/EBPβ RNA as described previously (4).

Alkylation of Free Thiol Groups with Iodoacetamide in Vivo—The alkylation method was performed as described previously (40). P388D1(IL1) cells were stimulated with 1 μg/ml LPS in the presence of 0.1 mM iodoacetamide (Sigma) to prevent free thiol groups from oxidizing to disulfide. Direct lysates were prepared at the indicated times with SDS sample dye containing 200 mM iodoacetamide at room temperature. The lysates were then resolved by nonreducing SDS-PAGE followed by Western blot analysis.

Electrophoretic Mobility Shift Assay—The specific probe for C/EBPβ was prepared by annealing the oligonucleotides 5′-GATCATTTTGTGTAAGAC-3′ and 5′-GATCGTCTTACACAAAAT-3′. The probe was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Nuclear extract (5–10 μg) from P388D1(IL1) cells or transfected 293T cells was incubated with the probe (1 ng) and 300 ng poly(dI-dC) in either the absence or presence of DTT, NADPH (Sigma), thioredoxin (Sigma), or the combination of NADPH, thioredoxin, and thioredoxin reductase (Sigma) at 37 °C for 20 min. For supershift assay, after the reaction mixture was incubated, 1–2 μl of anti-C/EBPβ antibody was added, and the incubation continued at room temperature for additional 20 min. The reaction mixtures were separated by 5% PAGE in Tris/glycine/EDTA buffer.

Disulfide Bond Determination by Liquid Chromatography Electrospray Ionization Mass Spectrometry —FLAG-C/EBPβ isoforms were expressed in 293T cells, immunoprecipitated by M2 beads, and resolved in SDS-PAGE under nonreducing conditions. The in-gel tryptic digest of C/EBPβ was dissolved in 0.1% formic acid, separated with an ABI 140D HPLC (Perkin-Elmer, 150 × 0.5 mm Brownlee reversed phase C18 column packed with 5-μm particles with a 300-Å pore), and on-line detected with a Finnigan Mat LCQ ion trap mass spectrometer. The mobile phase of HPLC consisted of various mixing ratios of 0.1% aqueous formic acid (solution A) and 0.1% formic acid in acetonitrile (solution B). The HPLC analysis was run using a gradient: 5% B for the first 25 min, 5–15% B in 25–30 min, 15–50% B in 30–100 min, and 50–65% B in 100–105 min; this final isocratic solvent was used until all of the peptides were eluted. The eluted peptides were analyzed under positive ion mass spectrometry scan mode over an *m/z* range of 395–1605. A table of detected masses was generated from the acquired mass spectra by the SEQUEST Browser software (Finnigan). The acquired ion masses were then manually examined for possible combinations of peptides containing cysteine residues.

RESULTS

LAP Is Recruited to IL-6 Gene Promoter after LPS Treatment*—Schematic representation of the C/EBPβ isoforms is shown in Fig. 1*A*. There are six cysteine residues in the mouse LAP* (*i.e.* Cys^{11}, Cys^{33}, Cys^{123}, Cys^{143}, Cys^{201}, and Cys^{296}), five in LAP (*i.e.* Cys^{33}, Cys^{123}, Cys^{143}, Cys^{201}, and Cys^{296}), and two

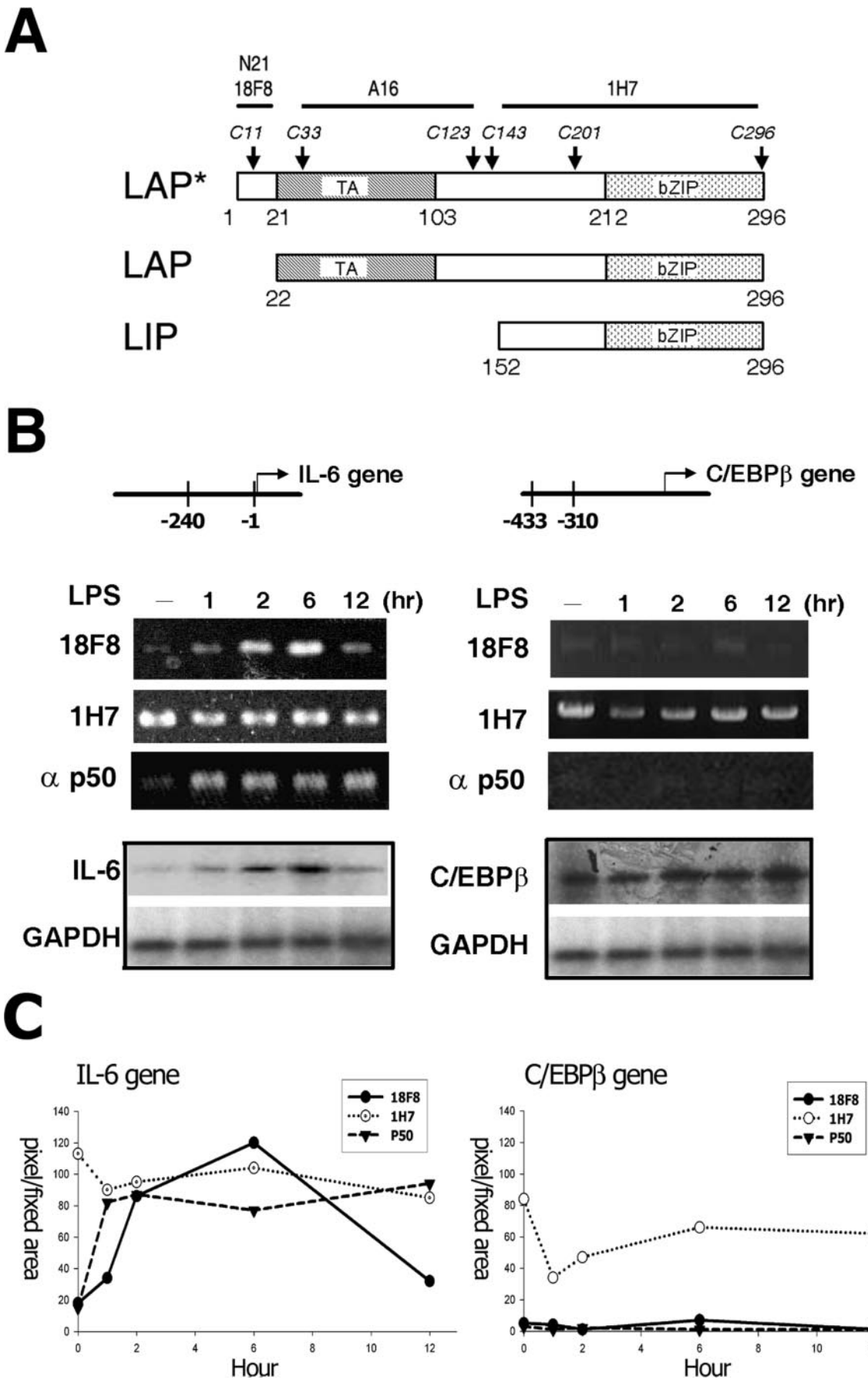


FIG. 1. **LAP* is specifically recruited to IL-6 promoter upon LPS stimulation.** *A*, schematic representation of the C/EBPβ isoforms. The associated *bars* indicate the antigen used to produce the three monoclonal antibodies (18F8, A16, and 1H7) and polyclonal antibody N21. *Arrows* mark the six highly conserved cysteine residues. *TA*, transactivation domain; *bZIP*, basic leucine zipper domain. *B*, the kinetics of promoter binding

in LIP (*i.e.* Cys^{201} and Cys^{296}). Monoclonal and polyclonal antibodies that recognize only LAP* (*i.e.* 18F8 and N21), both LAP* and LAP (*i.e.* A16), as well as all three isoforms (*i.e.* 1H7) were generated (Fig. 1*A*, the regions recognized by these monoclonal antibodies are indicated at the *top* of this figure). To investigate the differential recruitment of C/EBPβ isoforms to target gene promoters, 18F8 and 1H7 were employed for chromatin immunoprecipitation (ChIP) experiments of IL-6 and C/EBPβ genes from LPS-treated P388D1(IL1) cells. Schematic representations of these target genes are shown in the *top panels* of Fig. 1*B*. Semi-quantitative analysis of the amounts of DNA immunoprecipitated by 18F8, 1H7, and anti-p50 antibody is shown in Fig. 1*C*. LAP* is selectively recruited to the IL-6 gene promoter only after LPS stimulation, whereas the binding of the sum of all three C/EBPβ isoforms as visualized by ChIP using 1H7 remains constant, independent of the time course of LPS treatment (Fig. 1*B*, *18F8* and *1H7* of *left panels*). The binding of LAP* to the promoter region of C/EBPβ is undetectable as demonstrated by ChIP assay with 18F8, but the time course of 1H7 binding to the C/EBPβ promoter remains unchanged upon LPS treatment (Fig. 1*B*, *18F8* and *1H7* of *right panels*).

NF-κB, known as the predominant factor responsible for the induction of inflammatory cytokine genes (41), was also tested in ChIP experiment with anti-p50 antibody. Indeed, NF-κB was also recruited to the IL-6 promoter, albeit with different kinetic pattern from that of LAP* (Fig. 1*B*, *α p50* of *left panels*). The kinetics of LAP* recruitment to the IL-6 promoter correlates well with the expression of IL-6 mRNA (Fig. 1*B*, *18F8* and *IL-6* of *left panels*). C/EBPβ is constitutively expressed in P388D1(IL1) cells (Fig. 1*B*, *C/EBPβ* of *right panels* and data not shown). Neither LAP* nor NF-κB was recruited to the C/EBPβ promoter upon LPS treatment of P388D1(IL1) cells (Fig. 1*B*, *18F8* and *α p50* of *right panels*). Taken together, these results suggest that LAP* may play key roles for LPS-induced IL-6 expression but not in the constitutive expression of C/EBPβ gene. In addition to, or in conjunction with LAP*, NF-κB could also play important roles in LPS-mediated IL-6 gene expression.

LAP Activation Correlates with the Reduction of Intramolecular Disulfide Bonds*—We next aimed to examine the potential mechanisms that govern the LPS-induced recruitment of LAP* to the IL-6 gene promoter. A number of different pathways have been described to activate C/EBPβ, including transcriptional up-regulation, post-translational modifications, and nuclear translocation (42). The Northern data in Fig. 1*B* (*C/EBPβ panel*) exclude the possibility of transcriptional up-regulation in the present assay system. Immunological analyses demonstrated that both protein level and subcellular localization of C/EBPβ isoforms remain unchanged regardless of whether the P388D1(IL1) cells were treated with LPS or not (data not shown). Because LPS treatment is associated with alterations of the redox state, we hypothesized that LAP* could be regulated by a redox switch. Interestingly, amino acid alignment shows that the cysteine residues in C/EBPβ are highly conserved among species (Fig. 2*A*). Moreover, an extra cysteine residue is located in the N-terminal 21-amino acid stretch that is unique to LAP* (Fig. 2*A*). To assess whether these cysteine residues were involved in disulfide bond rearrangements during LPS treatment, we performed Western blot analysis to LPS-treated P388D1(IL1) under nonreducing conditions. Iodoacetamide was used to trap the free thiol groups during LPS treatment. At least two species of LAP* displaying slower mobility were observed 1 h after LPS treatment (Fig. 2*B*), suggesting the differential reduction of intramolecular disulfide bonds. The kinetics of these mobility changes in LAP* also correlates with the ChIP pattern of 18F8 antibody and the induction of IL-6 gene depicted in Fig. 1*B*. However, when the same protein preparations were subjected to a treatment with the reducing agent DTT, no mobility change could be observed (Fig. 2*B*, *upper panel*). On the contrary, LAP does not show a similar change of mobility under nonreducing conditions (Fig. 2*B*, *bottom panel*). These observations suggest that endogenous LAP* exists in a more compact conformation, which is relaxed by disulfide bond reduction upon LPS stimulation. To further confirm this finding, we treated the nuclear extract of P388D1(IL1) cells with various concentrations of DTT and analyzed by Western blot under nonreducing conditions. As shown in Fig. 2*C*, the N21 antibody detected three different migration forms of LAP* (*upper panel*). A slightly slower migration form was also observed for LAP from the same extracts (Fig. 2*C*, *lower panel*), whereas no mobility change was detected in LIP (data not shown). The same migration behavior was also observed for FLAG-tagged recombinant C/EBPβs expressed in 293T cells (Fig. 2*D*).

Identification of the Intramolecular Disulfide Bonds in LAP and LAP*—To determine which cysteine residues are involved in the intramolecular disulfide bond formation in LAP* and LAP, we performed mass spectrometric analysis. FLAG-LAP* was digested with trypsin, and the resulting peptides were analyzed by mass spectrometer. Disulfide bonds between Cys^{11} and Cys^{33}, between Cys^{123} and Cys^{143}, and between Cys^{201} and Cys^{296} of LAP* were identified (Fig. 3*A*). When FLAG-LAP was subjected to a similar analysis, only Cys^{123} and Cys^{201} were found to form intramolecular disulfide bond. This result is consistent with the barely detectable mobility shift of endogenous LAP shown in Fig. 2*B*. The major determinant in disulfide bond formation between LAP* and LAP hinges on the Cys^{11} residue of LAP*. To assess the importance of this residue in the overall disulfide bond formation, we performed site-directed mutagenesis that substituted the cysteine with a serine residue. Analysis of this mutant FLAG-LAP* (C11S) reveals that only Cys^{123} and Cys^{201}, and not Cys^{11} and Cys^{33} nor Cys^{201} and Cys^{296}, were linked by disulfide bond formation. When Cys^{11} is substituted, the tertiary structure of FLAG-LAP*(C11S) is apparently similar to that of LAP. To correlate these results, we expressed each mutant of the cysteine residues of LAP* and examined their migration pattern under nonreducing SDS-PAGE. As shown in Fig. 3*B*, all of the mutants migrate more slowly than the wild type protein. The mobility of C123S, C143S, C201S, and C296S was somewhat diffused as compared with that of C11S and C33S. The mobility of C11S and C33S was similar, in accordance to their partnership in disulfide bond formation. Taken together, these data provide evidence that all of the cysteine residues in LAP* are linked by disulfide bond formation. Cys^{11} plays a central role in determining the tertiary structure of LAP*. If the disulfide bond between Cys^{11} and Cys^{33} is disrupted by point mutation, LAP* assumes a conformation that is similar to that of LAP.

Transcriptional Activity of LAP Is Greatly Enhanced under Reducing Conditions*—Having demonstrated that LAP* under-

and the expression profiles of C/EBPβ target genes. Transcription initiation site on IL-6 and C/EBPβ gene promoters (*arrows*) and the PCR fragments amplified (*line drawings*) are indicated. ChIP experiments were performed with 18F8, 1H7, and anti-NFκB p50 antibodies from P388D1(IL1) cells at the indicated times after LPS stimulation (1 μg/ml). Steady-state RNA levels are monitored by Northern blotting, GAPDH serves as a loading control (*boxed panels*). *C*, semi-quantitative analysis of the ChIP results are represented as the mean intensity over the fixed area normalized against background values.

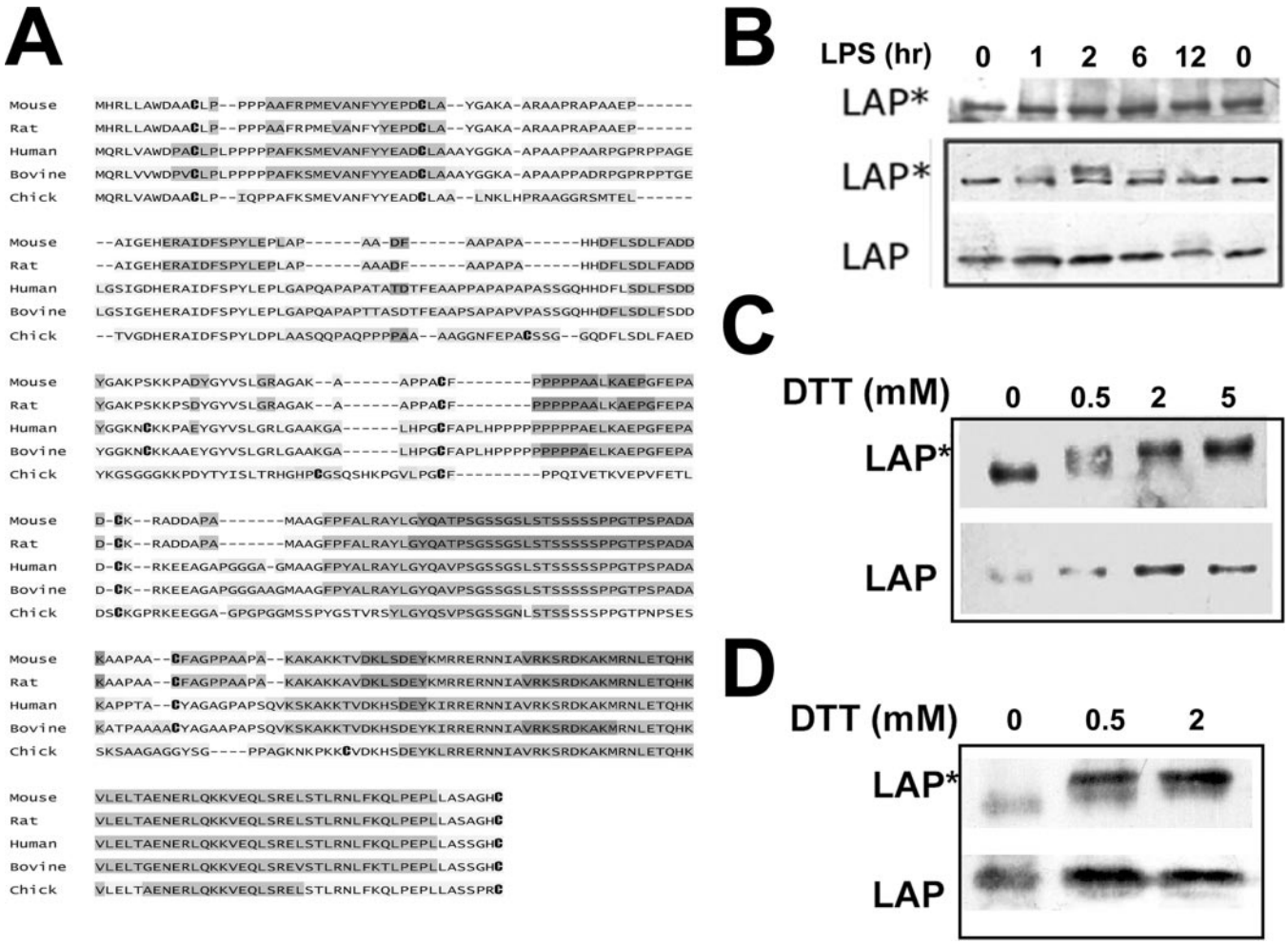
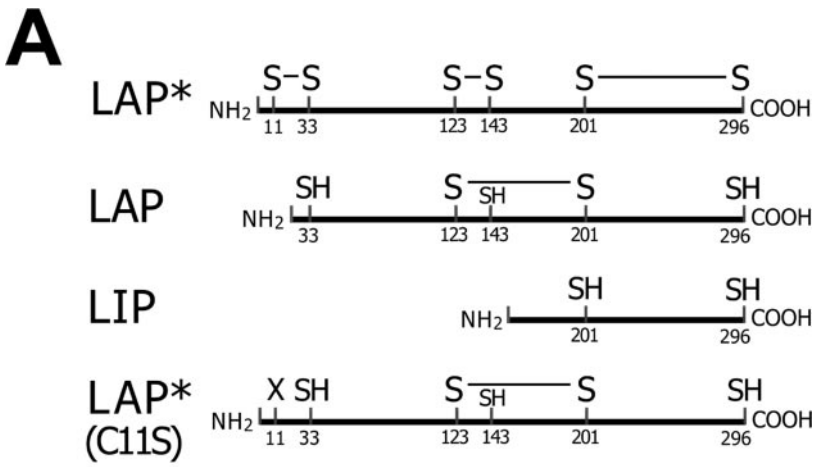


FIG. 2. **LPS induces reduction of disulfide bonds in LAP*.** *A*, amino acid sequence alignment of C/EBPβ from various species. The conserved cysteines are shown in *bold type*. *B*, LAP* displays redox-sensitive mobility changes during LPS stimulation. P388D1(IL1) cells were treated with 1 μg/ml LPS in the presence of 0.1 mM iodoacetamide. The cell lysates harvested at the indicated time were subjected to reducing and nonreducing (*boxed panels*) SDS-PAGE and visualized by Western blot with the N21 (LAP*) and A16 (LAP) antibodies. *C* and *D*, presence of intramolecular disulfide bonds in endogenous and recombinant LAP*. Nuclear extracts from P388D1(IL1) cells (*C*) and C/EBPβ-transfected 293T cells (*D*) were prepared with buffers devoid of DTT. Nuclear extract was incubated with various concentrations of DTT at 37 °C for 20 min. The reaction mixtures were subjected to nonreducing SDS-PAGE and visualized as in *B*.

goes reduction of disulfide linkages and activation upon LPS stimulation, we then evaluated the effect of reducing power on the transcription activity of LAP*. The expression vectors of LAP* and LAP were transfected into 293T cells together with AGP-CAT reporter. Consistent with our previous results published elsewhere, LAP is more active than LAP* when tested by transient transfection experiments (15). When LAP* transfectants were treated with the reducing agents PDTC or NAC, the activation of reporter gene was enhanced in a PDTC and NAC concentration-dependent manner. These stimulatory effects were not observed for LAP transfectants (Fig. 4, *A*, *PDTC*, and *B*, *NAC*). These reducing agents may exert their effect by directly reducing the disulfide bonds in LAP*. To explore this possibility, we assayed the transcription activity of the cysteine mutants, which mimic the reduction of their respective disulfide bonds. For C11S and C33S, the transactivation of AGP-CAT was greatly enhanced as compared with that of wild type LAP* and other cysteine mutants (Fig. 4*C*). Because the results shown in Fig. 3 implicate that the C11S mutant (and possibly C33S as well) adopt the same disulfide linkage as in LAP, the most reasonable explanation for these observations is that PDTC or NAC also promotes the disengagement of disulfide linkage between Cys^{11} and Cys^{33}, rendering LAP* to acquire the disulfide pairing as in LAP, which is the most transcriptionally active isoform.

*Reducing Conditions Enhance the DNA Binding Activity of LAP**—To determine the mechanism by which change in redox state influences the transcription activity of LAP*, we performed electrophoretic mobility shift assays. The DNA binding activity of FLAG-LAP*, but not FLAG-LAP and FLAG-LIP, was enhanced by the addition of DTT (Fig. 5*A*, compare *lanes 3* and *6* for LAP*, *lanes 4* and *7* for LAP, and *lanes 5* and *8* for LIP). Similarly, the DNA binding activity of endogenous LAP* was detected only in the DTT-treated extracts, as demonstrated by the antibody-specific supershifts (Fig. 5*B*, compare *lanes 3*, *4*, *9*, and *10*; *asterisks* mark the supershifted complexes). The DNA binding activity of LAP did not appear to be influenced by the presence of DTT (Fig. 5*B*, compare *lanes 5*, *6*, *11*, and *12*; supershifted complex detected by A16). One could argue that DTT may not reflect *in vivo* conditions. To explore the impact of endogenous redox-sensing systems on LAP*, we tested the effects of NADPH, thioredoxin, and the combination of NADPH, thioredoxin, and thioredoxin reductase in electrophoretic mobility shift assays. FLAG-LAP* was purified with M2 beads to avoid the consumption of reducing agents from other proteins. As shown in Fig. 5*C*, DTT (*lanes 2* and *3*) and



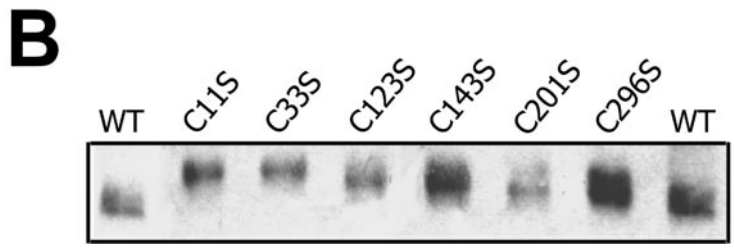


FIG. 3. **Mapping of intramolecular disulfide bond(s) in C/EBPβ.** *A*, schematic representation of the identified intramolecular disulfide bonds of C/EBPβ isoforms. FLAG-tagged C/EBPβs were expressed in 293T cells, immunoaffinity-purified with M2 beads, and recovered from SDS-PAGE. The peptides generated by trypsin digestion were analyzed with LCQ (Finnigan) and MALDI-TOF. *B*, wild type (*WT*) and mutant LAP*s migrate differently in nonreducing SDS-PAGE. FLAG-LAP* and its cysteine mutants (Cys → Ser) were expressed in 293T cells. Nuclear extract prepared with buffer devoid of DTT was subjected to SDS-PAGE and visualized by Western blot with M2 antibody.

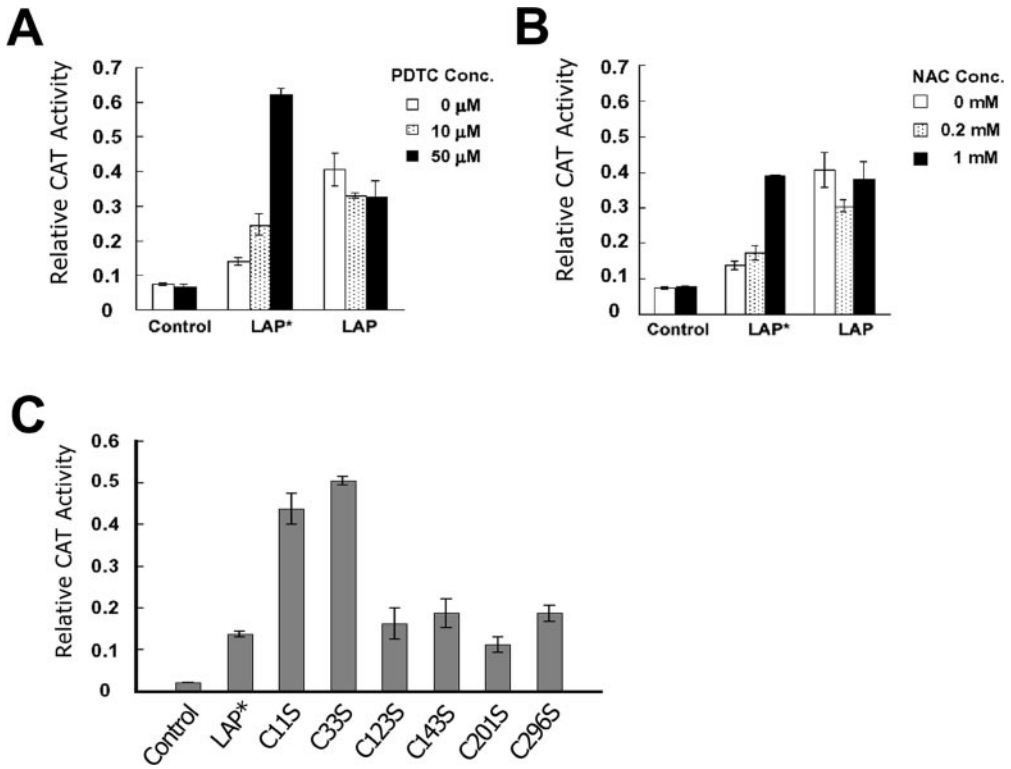


FIG. 4. **The transcriptional activity of LAP*, but not LAP, is enhanced under reducing conditions.** *A* and *B*, treatment with antioxidants stimulates the transactivity of LAP*. 293 T cells were transfected with 1 μg of AGP-CAT, 0.5 μg of pSV-β-galactosidase, and 50 ng of pCMV-tag-LAP* or pCMV-tag-LAP. The transfected cells were treated with various concentrations of PDTC (*A*) and NAC (*B*) for 16 h before harvesting for CAT assays. The CAT activity was normalized with β-galactosidase. *C*, transcriptional activity of LAP* and each cysteine mutants were measured by CAT assay. 293T cells were co-transfected with 0.75 μg of AGP-CAT, 0.25 μg of pSV-β-galactosidase, and 0.02 μg of each corresponding LAP* construct. The CAT assays were performed 36 h post-transfection. Each normalized CAT value represents the means of four independent experiments.

thioredoxin (*lanes 6* and *7*) were effective in enhancing the DNA binding activity of LAP*. The reducing effect of thioredoxin alone seemed less effective than that of DTT or the combination of thioredoxin, thioredoxin reductase, and NADPH. This may be attributed to the decreased reducing power by partially oxidized form of thioredoxin. NADPH has an almost undetectable effect (Fig. 5*C*, *lanes 4* and *5*). When LAP* was treated with thioredoxin, NADPH, and thioredoxin reduc-

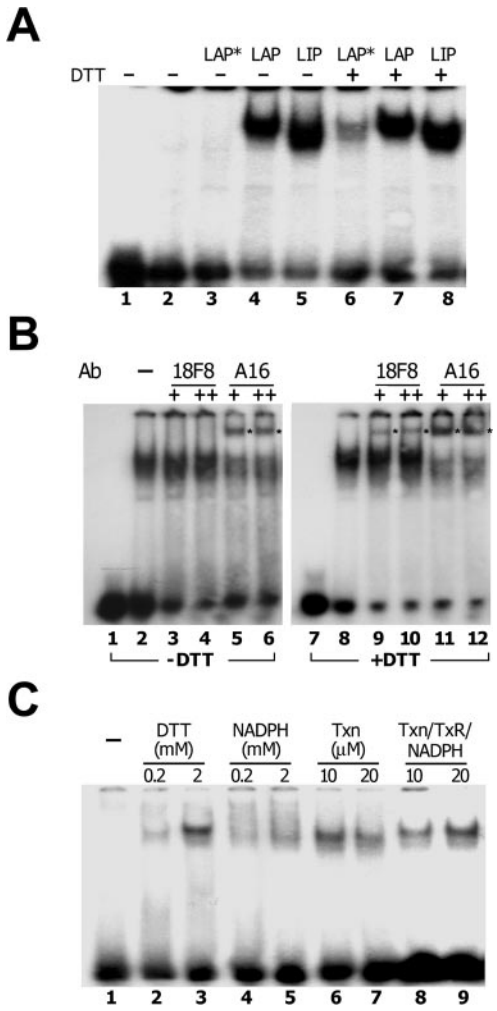


FIG. 5. **The DNA binding activity of LAP* is enhanced under reducing conditions.** *A*, nuclear extracts (5 μg) from 293T cells expressing various recombinant C/EBPβ isoforms were incubated in the absence (*lanes 1–5*) or presence (*lanes 6–8*) of DTT, reacted with [α-^{32}P]ATP-labeled probe, and then separated by 5% native PAGE. *Lane 1*, probe only; *lane 2*, nuclear extract from mock transfected 293T cells; *lanes 3* and *6*, LAP*; *lanes 4* and *7*, LAP; *lanes 5* and *8*, LIP. *B*, 10 μg of nuclear extract from P388D1(IL1) cells were treated in the absence or presence of DTT. The reaction mixtures were then incubated with labeled probe in the absence or presence of antibodies specific to C/EBPβ isoforms. The *asterisks* indicate the supershifted complexes. Two different dosages of antibodies were used: 1 μl (+) and 2 μl (++). *C*, recombinant FLAG-LAP* was purified from 293T transfected cells by immunoprecipitation and eluted with FLAG peptide. The purified FLAG-LAP* was incubated with various reducing agents and further reacted with probe. *Lane 1*, FLAG-LAP* only; *lanes 2* and *3*, 0.2 and 2 mM of DTT; *lanes 4* and *5*, 0.2 and 2 mM NADPH; *lanes 6* and *7*, 10 and 20 μM thioredoxin (*Txn*); *lanes 8* and *9*, thioredoxin reductase (*TxR*) and NADPH and 10 and 20 μM Txn were added to the reaction mixtures.

tase, its DNA binding activity was comparable with that of DTT-treated samples (Fig 5*C*, *lanes 8* and *9*). Taken together, our findings provide strong evidence that the DNA binding activity of LAP* may be regulated in a redox-sensitive manner. The thioredoxin and thioredoxin reductase are among the candidate players in the activation of LAP*. In contrast, LAP and LIP are insensitive to the change in redox condition.

DISCUSSION

C/EBPβ is one of the key players in the regulation of growth, differentiation, metabolism, and inflammation. Understanding the mechanism of differential regulation of C/EBPβ is an important issue for basic and applied biological research. Although functional differences have been described between LAP* and LAP (15, 16), how they are separately regulated remains completely unknown. In the present study, we report for the first time that upon LPS stimulation, the full-length isoform LAP* is differentially activated by a redox switch mechanism, and consequently, LAP* gains DNA binding activity and is recruited to the IL-6 promoter for its transcriptional induction.

Because numerous studies revealed that C/EBPβ regulation is extremely complicated and cell type-specific (42), we focused our studies in the P388D1(L1) cells, in which both inducible (IL-6) as well as constitutive regulation (C/EBPβ itself) by C/EBPβ are well documented. The observation that although a bulk of C/EBPβ is permanently associated with both target promoters, the induction of IL-6 does not occur until the recruitment of LAP* strongly suggests that LAP* is the determinant isoform in LPS-inducible IL-6 expression. On the other hand, LAP* does not seem to be involved in the regulation of constitutively expressed genes, as exemplified by C/EBPβ itself. This is in pretty good agreement with our previous report that LAP* preferentially interacts with NF-κB in a synergistic manner, whereas LAP does not. NF-κB is known to be a crucial factor in the LPS-inducible expression of inflammatory cytokine genes (41). Our ChIP data confirmed its initial recruitment to the IL-6 promoter simultaneously with LAP*; nevertheless, NF-κB binding persisted long after decreased IL-6 mRNA production. This difference in promoter binding kinetics prompted us to examine another LPS-inducible gene, TNF-α, for which NF-κB is considered as the determinant factor of its expression. We found that both C/EBPβ and NF-κB were recruited to the TNF-α promoter with the same kinetics (data not shown). A possible implication of these results is that although the activation of C/EBPβ and NF-κB are both necessary for the induction of LPS-up-regulated genes, they may differ in playing the triggering roles for specific genes. In light of the perfect correlation between IL-6 transcription and LAP* recruitment to its promoter, our data strongly suggest that LAP* may play the switch roles for IL-6 transcription, whereas NF-κB plays the key role for the induction of TNF-α gene.

A previous report described the association of LAP* with the SWI-SNF complex through the 21-amino acid stretch in the N terminus (16). It is then speculated that LAP* may be involved in the activation of those target genes requiring chromatin remodeling activity provided by the SWI-SNF complex. The roles of LAP* on chromatin remodeling of the IL-6 and C/EBPβ genes would be crucial for further investigation. Moreover, our biochemical studies demonstrated a novel function for the extra amino acids at the N terminus of LAP*. Specifically, Cys^{11} is clearly involved or affected in the intramolecular disulfide bond formation of LAP*. When cDNA of C/EBPβ is transfected into cells, proteins of all three isoforms are produced. To obtain any single species of C/EBPβ isoform, FLAG-tagged cDNA is used for generating recombinant protein by transient transfection into host cells. When we performed SDS-PAGE and Western blot analysis under nonreducing and reducing conditions using P388D1(IL1)-derived and FLAG-tagged proteins, the mobility of endogenous-derived and recombinant proteins are similar. This result suggests that the intramolecular disulfide bond formation for endogenous and recombinant proteins is likely to be the same. Mass spectra analysis indicates that Cys^{11} and Cys^{33}, Cys^{123} and Cys^{143}, and Cys^{201} and Cys^{296} of LAP* form three intramolecular disulfide bonds. LAP, lacking the N-terminal 21 amino acids and thus Cys^{11}, is different from LAP* in tertiary structure. The intramolecular disulfide bond of LAP is between Cys^{123} and Cys^{201}. The intramolecular disulfide bond of LAP*(C11S) is the same as that of LAP. These results indicate that Cys^{11} of LAP* is key to the tertiary structure of LAP*. The Cys^{11} and Cys^{33} disulfide bond could affect the function of

the transactivation domain of LAP*. Maybe when Cys^{11} gets reduced under redox switch, the conformation of LAP* changes to facilitate its association with the SWI-SNF complex and hence turning on target gene expression (16). Therefore, the activities of LAP* and LAP are likely to be different, and LAP* may be the primary factor among C/EBPβ isoforms that confers the redox-sensitive transactivation of target genes.

Closely related family member proteins were known to display different activities because of the difference in their intramolecular disulfide bonds (43). For example, insulin-like growth factor (IGF)-binding proteins (IGFBPs 1–6) are responsible for modulating the action of IGFs. IGFBPs 1–5 contain 18 conserved cysteines, but IGFBP-6 lacks two of the 12 N-terminal cysteines. The N-terminal intramolecular disulfide linkages of IGFBP-6 differ significantly from those of IGFBP-1 as determined by mass spectrometry. This difference could contribute to the distinctive IGF binding properties of IGFBP-6 (which has the highest affinity for IGF-II). Our present results on the difference of intramolecular disulfide bonds between LAP* and LAP are analogous to those of IGFBPs.

How does LAP* get reduced in LPS-treated P388D1(IL1) cells? The more likely physiological scenario is through oxidoreductases (*e.g.* thioredoxin and thioredoxin reductase). There is a report on the elevation of thioredoxin and thioredoxin reductase levels in placenta of pregnant mice exposed to LPS (18). The LPS-treated P388D1(IL1) cells may generate high levels of thioredoxin, thioredoxin reductase, and NADPH and thus facilitate the activation of LAP*. Alternatively, oxidoreductase such as protein disulfide isomerase may be activated under LPS-stimulated conditions and may then activate LAP*. Our results demonstrated that LAP* gets reduced when P388D1(IL1) cells are treated with LPS (Fig. 2*B*). These results suggest that post-translational events, such as the reduction of disulfide bonds are responsible for the activation of LAP*. Consistent with the ChIP results, transient transfection experiments using wild type LAP* also demonstrated that LAP*, not LAP, is specifically activated by PDTC or NAC treatment. When treated with 50 μM PDTC, the transcription activity of LAP* is higher than that of LAP. The transactivation activity of LAP*(C11S) or LAP*(C33S) is much higher than that of wild type LAP*, suggesting that the reduction of intramolecular disulfide bond between Cys^{11} and Cys^{33} of LAP* may be important for its activation. The intramolecular disulfide bond between Cys^{201} and Cys^{296} may pose a constraint for dimerization of LAP*, which must be reduced or disrupted preceding DNA binding. Alternatively, Cys^{11} or Cys^{33} may influence the overall tertiary structure and conformation during protein translation. That is why when Cys^{11} is mutated, the intramolecular disulfide bond of LAP*(C11S) is the same as LAP. The disulfide bond between Cys^{123} and Cys^{201} may be buried in the molecule based on the results of our molecular modeling (data not shown). The DNA binding activity of LAP* is activated by reducing agents, whereas LAP is constitutively active. In addition to playing roles of direct disulfide bond formation, Cys^{11} could influence the folding of LAP* during translation. Mutation of cysteine residues other than Cys^{11} and Cys^{33} could have very different effects on intramolecular disulfide bond formation and folding of LAP*. The results from transient transfection experiments performed with various cysteine mutants clearly define that Cys^{11} and Cys^{33} are functionally different from other cysteines (Fig. 5*C*). In addition to dimerization and DNA binding, domain(s) located near the N-terminal region is important for the transactivation of LAP*. Cys^{11} and Cys^{33} could influence the disulfide bonds formation mediated by the other four cysteines; however, the other mutants of cysteines (*i.e.* Cys^{123}, Cys^{143}, Cys^{201}, and Cys^{296}) may or may not influence the disulfide bond formation between Cys^{11} and Cys^{33}. These possibilities are to be addressed in future experiments. Any results derived from the mutation of cysteine must be interpreted with caution. It should also be noted that, because resident genes are embedded in chromatin but the ectopically transfected plasmids are not, the activities of LAP* defined by transient transfection and those derived from the ChIP for are likely to be different.

The results from this study suggest that LAP* may function as a cellular sensor for the sudden surge of reducing potential that is required for switching the key regulatory genes as exemplified by the induction of IL-6 gene. LAP, on the other hand, does not respond to the reducing power but may regulate the transcription of genes with house keeping functions. Taken together, these results demonstrate that redox switch is responsible for the activation of LAP*, but not LAP, for targeting genes encoding key regulatory proteins. This novel type of molecular switch for differential regulation of two transcriptional activators resulted from alternative translation of the same mRNA, may be found in other systems, and should have general interest in studying gene regulation.

Acknowledgments—We thank Li-Ping Tu, Ruey Chao, and Han Ting Chou for excellent technical assistance.

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