Distinct Functions of CCAAT Enhancer-binding Protein Isoforms in the Regulation of Manganese Superoxide Dismutase during Interleukin-1β Stimulation*

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The mitochondrial antioxidant enzyme manganese superoxide dismutase (Mn-SOD) is crucial in maintaining cellular and organismal homeostasis. Mn-SOD expression is tightly regulated in a manner that synchronizes its cytoprotective functions during inflammatory challenges. Induction of Mn-SOD gene expression by the proinflammatory cytokine IL-1β is mediated through a complex intronic enhancer element. To identify and characterize the transcription factors required for Mn-SOD enhancer function, a yeast one-hybrid assay was utilized, and two CCAAT enhancer-binding protein (C/EBP) members, C/EBP β and C/EBP δ, were identified. These two transcription factors responded to IL-1β treatment with distinct expression profiles, different temporal yet inducible interactions with the endogenous Mn-SOD enhancer, and also opposite effects on Mn-SOD transcription. C/EBP β is expressed as three isoforms, LAP* (liver-activating protein), LAP, and LIP (liver-inhibitory protein). Our functional analysis demonstrated that only the full-length C/EBP β/LAP* served as a true activator for Mn-SOD, whereas LAP, LIP, and C/EBP δ functioned as potential repressors. Finally, our systematic mutagenesis of the unique N-terminal 21 amino acids further solidified the importance of LAP* in the induction of Mn-SOD and emphasized the crucial role of this isoform. Our data demonstrating the physiological relevance of the N-terminal peptide also provide a rationale for revisiting the role of LAP* in the regulation of other genes and in pathways such as lipogenesis and development.

The superoxide dismutases (SODs)2 are the first line of cellular defense against damage caused by superoxide anion radicals produced as byproducts during normal cellular respiration as well as being a consequence of the inflammatory response (1–3). Three types of SODs have been identified in eukaryotic cells, the cytoplasmic copper/zinc SOD (CuZn-SOD) (4), the extracellular CuZn-SOD (5), and the mitochondrially localized manganese SOD (Mn-SOD) (6). All family members efficiently catalyze the conversion of superoxide radicals (O2) to H2O2 and molecular oxygen. Mn-SOD is the most highly regulated via induction by proinflammatory mediators such as lipopolysaccharide, tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and IL-6, and interferon γ to confer potent cytoprotective functions (7–13). The physiological importance of Mn-SOD has been clearly demonstrated by two independent groups who found that Mn-SOD null mice die within 10–21 days after birth with dilated cardiomyopathy and steatosis in liver and skeletal muscle, as well as metabolic acidosis (14, 15).

Previous investigations (8) and other laboratories (16) have demonstrated that the stimulus-dependent elevation in Mn-SOD mRNA level is because of increased de novo transcription and that maximum induction of the gene requires an enhancer region, located in intron 2 in both rodent and human Mn-SOD genes (17–19). This enhancer is highly conserved over a 200–500-bp region, depending on the species, even though it resides in an intron in all the species studied. Sequence analysis, together with electrophoretic mobility shift assay, has indicated the potential involvement of various transcription factors with the intronic enhancer during the stimulus-dependent up-regulation of Mn-SOD transcription (19–22). However, these mechanisms have not been studied in detail.

By employing a yeast one-hybrid strategy to identify cognate regulatory factors that bind to conserved enhancer sequences, we have identified CCAAT enhancer-binding protein (C/EBP) β and δ as candidate factors involved in Mn-SOD gene induction. The C/EBPs are a subfamily of the larger basic region/leucine zipper (bZIP) transcription factor family (23). To date six C/EBP family members, C/EBP α, β, γ, δ, ε, and ζ, have been identified and cloned. C/EBP α, β, γ, and δ genes contain no intron, whereas C/EBP ε and ζ contain two and four exons, respectively (reviewed in Refs. 24 and 25). All of the six members share noteworthy sequence identity in the C-terminal bZIP domain. All members are able to form homodimers as well as heterodimers in all intrafamilial combinations (24, 25) and, with the exception of C/EBP ζ (26), at least in vitro, bind to an identical canonical R↓T↓G↓C↓G↓Y↓A↓Y sequence (27), where R is A or G, and Y is C or T. In addition, members of the C/EBP family can form heterodimers with other bZIP subfamily members, such as CREB/ATF and AP-1, with alterations in the DNA binding sequence (28, 29). Moreover, C/EBP proteins were also

* This work was supported, in whole or in part, by National Institutes of Health Grants HL35953 and HL67456 (to H. S. N.). 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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2 The abbreviations used are: SOD, superoxide dismutase; TNF, tumor necrosis factor; IL, interleukin; C/EBP, CCAAT enhancer-binding protein; bZIP, basic region/leucine zipper; LAP, liver-activating protein; MEF, mouse embryonic fibroblast; LIP, liver-inhibitory protein; HA, hemagglutinin; siRNA, small interfering RNA; hGH, human growth hormone; ChIP, chromatin immunoprecipitation; TFIIIB, transcription factor II B.
shown to form protein-protein interactions with other non-bZIP factors, such as the subunits of NFkB (30, 31) and the glucocorticoid receptor (32). Different C/EBP members function specifically through collaboration, but some level of redundancy is also believed to exist (reviewed in Ref. 33). The C/EBPs perform critical roles in a variety of physiological and pathophysiological processes, such as cell differentiation, metabolic regulation, cell proliferation, and inflammation (24, 33, 34).

Both C/EBP \( \beta \) and C/EBP \( \delta \) have been shown to be involved in the inflammatory response (35–37). C/EBP \( \beta \) mRNA is intron-less and gives rise to three different protein isoforms through the alternative use of three inherent translation start codons (38). These isoforms include full-length LAP* (liver-activating protein), medium-length LAP, and short-form LIP (liver-inhibitory protein). Both LAP* and LAP are usually considered to be transcriptional activators, whereas LIP is usually considered a dominant negative isoform, as it lacks all activation domains. Since the identification of the isoforms by Dr. Ueli Schibler’s group (38, 39), the functional relevance of the unique N-terminal amino acids in full-length LAP* has been addressed only in a limited number of studies. For example, Kowenz-Leutz and Leutz (40) reported that the first \( \sim 20 \) amino acids are essential for recruiting the SWI/SNF complex in neuronal synapses, which can also regulate the transcriptional activation of LAP* (43).

The results of the present study have demonstrated that the knockdown of C/EBP \( \beta \) by siRNA or its absence in C/EBP \( \beta^{-/-} \) mouse embryonic fibroblast (MEF) cells leads to the inhibition of IL-1\( \beta \)-dependent induction of Mn-SOD gene expression. We have also shown that the different isoforms of C/EBP \( \beta \) as well as C/EBP \( \delta \) have unique and specific functions related to Mn-SOD gene expression. Full-length LAP* serves the role of a transcriptional activator, whereas medium-length LAP, LIP, and C/EBP \( \delta \) are able to efficiently block IL-1\( \beta \)-dependent induction. We also confirmed the specific in vivo binding of LAP* and C/EBP \( \delta \) to the Mn-SOD enhancer. Most importantly, we have systematically demonstrated the importance of specific N-terminal amino acids unique to LAP*, solidifying the importance of this domain and LAP* in the transcriptional activation of Mn-SOD by IL-1\( \beta \). Overall, our data also strongly suggest that the full-length C/EBP \( \beta \)/LAP* may have a much more important physiological role than previously realized.

**MATERIALS AND METHODS**

Reagents and Antibodies—Normal rabbit and mouse IgG (sc-2027, sc-2025), antibodies against the C/EBP \( \beta \) C terminus (sc-150, recognize LAP*, LAP, and LIP), C/EBP \( \delta \) (sc-151), HA tag (sc-7392), and TFIIB (sc-225) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The LAP* antibody (ab18327), specific to the first 21 amino acids, was purchased from Abcam Inc. Protein A-Sepharose CL-4B, protein G-Sepharose, an ECL kit, and secondary antibodies were purchased from Amersham Biosciences/GE Healthcare.

**Yeast One-hybrid Screening**—The top and bottom strands of the Mn-SOD intronic enhancer sequence (5‘-GTAGG-GAAAAAGCTAGTTGGAAATCCTTT-3’) were synthesized by Invitrogen as three tandem repeats with either EcoRI/Sall or EcoRI/Xbal restriction sites on each end. The oligonucleotides were annealed and then phosphorylated at the 5‘-ends by T4 polynucleotide kinase (New England Biolabs). After purification, the double-stranded EcoRI/Xbal oligos were ligated into the pPHI vector, and the EcoRI/Sall oligos were ligated into the pLacZi vector obtained from Clontech. Ligation products were transformed, and positive clones were verified by sequencing. Each clone was linearized with either Xho (pPHI) or Ncol (pLacZi) and used for transformation/integration into the yeast YM4271 strain. Integration was confirmed through plating on auxotrophic media, and subsequent transformation of the library was confirmed similarly with drop-out media, and increasing concentrations of 3-amino-1,2,4-triazole. One-hybrid screening was done using the manufacturer’s recommendations for the MATCHMAKER protocol (Clontech). 5 \( \times \) 10\(^4\) colony-forming units were screened from a rat lung cDNA library obtained from Clontech. Positive clones were identified by sequencing.

**Cell Culture**—L2 cells, a rat pulmonary epithelial-like cell line (ATCC CCL 149), and MEFs, both wild type and C/EBP \( \beta \)-deficient, were provided by Dr. P. Johnson (National Institutes of Health) via Dr. M. Kilberg (University of Florida). L2 and MEFs were grown in Ham’s modified F12K medium (Sigma) and Dulbecco’s modified Eagle’s medium (Sigma), respectively, with 10% fetal bovine serum (Invitrogen), ABAM (penicillin G (100 units/ml), streptomycin (0.1 mg/ml), amphotericin B (0.25 \( \mu \)g/ml; Invitrogen), and 4 mM glucose at 37 °C in incubators supplied with 5% CO\(_2\) 2 ng/ml IL-1\( \beta \) (R&D Systems) was used to treat cells for the indicated times.

**Recombinant Plasmid Construction and Site-directed Mutagenesis**—Coding sequences for each of the C/EBP \( \beta \) isoforms were cloned into pcDNA3.1 using the EcoRV and HindIII sites. The C/EBP \( \delta \) cDNA was originally obtained from a yeast one-hybrid library screening of the Clontech rat lung cDNA library and subcloned from the pACT2 vector (Clontech) into the HindIII and Xbal sites of pcDNA3.1. Mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene). An HA tag was subcloned into the pLAPS C terminus by a QuikChange site-directed mutagenesis kit using primers 5‘-CTCGGGGCCTGCTACTCCATCCATCACAATTCG-ACGGTACAGCACTAGCTAGTTGAAAGCTTAAGGTTAACCGC-3 and 5‘-GGGGTTTAATAGCTTACAAGGCATGTTAGGTTCACCGG-3’. The reporter construct (Mn-SOD promoter/enhancer-human growth hormone (hGH)) (17) contains a 919-bp Mn-SOD enhancer fragment (+1278 to +2196) and a 2.5-kb Mn-SOD promoter fragment (−2489 to +42) in the otherwise promoterless pUC12-based hGH expression vector (44).
RNA Isolation, Northern Analysis, and Statistical Analysis—Total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform extraction method described by Chomczynski and Sacchi (45) with modifications (10) or with the Qiagen RNeasy kit (Qiagen). 15 μg of total RNA was size-fractionated on a 1% agarose-formaldehyde gel and electrotransferred to a charged nylon membrane (Zetabind, Cuno Inc., Meriden, CT) followed by UV cross-linking. Membranes were then incubated overnight at 61 °C with a 32P radiolabeled gene-specific probe generated by random primer extension, washed with a high stringency buffer at 66 °C, and then analyzed by autoradiography. Densitometry was performed by direct scanning of the original autoradiograph and analyzed using Scion Image analysis from the National Institutes of Health. Statistical analysis was derived from a minimum of three independent experiments. The errors are presented as the standard error of the means, and comparisons were performed using Student’s t test.

First-strand DNA Synthesis and Real-time PCR—1 μg of total RNA isolated with the Qiagen RNeasy kit (Qiagen) was used to generate first-strand DNA for real-time PCR analysis using the SuperScript™ first-strand synthesis kit (Invitrogen). The final product was diluted to 100 μl, and 2 μl was used as the template for each real-time PCR. To this, each primer at 0.3 μM, 12.5 μl of iTaq™ SYBR® Green Supermix with ROX (Bio-Rad) and water were added to a final volume of 25 μl. The primers used for amplification to measure Mn-SOD mRNA levels were: sense, 5’-CCGCTGCTCTACATCAGGA-3’ and antisense, 5’-TCAATAATGGCTTCTCAGATAGTCA-3’. The ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA) was used with the following parameters: cycle 1, 95 °C for 10 min; cycle 2, 95 °C for 15 s, 60 °C for 1 min, for 40 cycles. After PCR, melting curves were acquired by a stepwise increase of the temperature from 55 to 95 °C to ensure that a single product was amplified in the reaction. The cyclophilin A mRNA level was also measured at the same time as the internal control. The primers for amplification were: sense, 5’-GGTG-GCAAGTCATCATACGG-3’ and antisense, 5’-TCACCTTCCCAAGACACACT-3’. Real-time PCR was done in triplicate with samples from at least three independent experiments. The errors are presented as the standard error of the means, and comparisons were performed using Student’s t test.

Transient Transfection—L2 cells were cultured as described previously and transfected at ~60% confluency in 10 cm dishes. Transfection was done using FuGENE 6 transfection reagent (Roche Applied Science). For protein overexpression analysis, 5 μg of the indicated plasmid was transfected, and total cell extract was collected 48 h after transfection and subjected to immunoblot analysis. For reporter assay analysis, 0.2 μg of reporter plasmid and 0.5 μg of the indicated transcription factor constructs were used, and empty vector pcDNA 3.1 was used to bring the total DNA amount to 4 μg. After 24 h, the cells were split 1:2 and incubated for another 16 h. IL-1β (R&D Systems) was added to one set of plates to a final concentration of 2 ng/ml. After 8 h, total RNA was isolated for Northern analysis.

Immunoprecipitation—Total cell extracts were prepared in radioimmune precipitation assay lysis buffer (150 mM NaCl, 1% Nonidet P-40, 1.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 7.5) at the time points indicated and incubated at 4 °C overnight with antibody against C/EBP β. Complex capture was completed by incubating with protein A-Sepharose beads at 4 °C for 2 h. Complexes were washed four times with radioimmune precipitation assay buffer followed by immunoblot analysis with the indicated antibody.

Immunoblot Analysis—Immunoprecipitates, 10 μg of total cell lysate, or 40 μg of nuclear extracts prepared at the indicated time points were separated on a 10.5–14% Tris-HCl polyacrylamide gel (Bio-Rad) and then electrotransferred to a nitrocellulose membrane (Bio-Rad). The membranes were then blocked for 1 h with 8% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5, 0.1% (v/v) Tween 20, 200 mM NaCl) at room temperature. The membranes were incubated at 4 °C with primary antibody overnight, washed three times with TBST, incubated with a peroxidase-conjugated secondary antibody for 1 h, washed again three times, and subjected to enhanced chemiluminescence (ECL, Amersham Biosciences).

Chromatin Immunoprecipitation Analysis—Chromatin immunoprecipitation (ChIP) analysis was performed according to a modified protocol from Upstate Biotechnology, Inc. (Charlottesville, VA) as described. Protein-DNA cross-linking was performed at the indicated time points by adding formaldehyde directly to the culture medium to a final concentration of 1% and then stopped 10 min later by adding glycine to a final concentration of 0.125 M. Cross-linked chromatin was sonicated with a Branson model 500 ultrasonic dismembrator (Fisher Scientific). Cell extract was incubated with 2 μg of the indicated antibodies overnight, and a normal rabbit or mouse IgG was used as the nonspecific antibody control. The antibody-bound complex was precipitated by protein A/G-Sepharose beads (Amersham Biosciences). The DNA fragments in the immunoprecipitated complex were released by reversing the cross-linking at 65 °C for 5 h and purified using a QiAquick PCR purification kit (Qiagen). Real-time PCR analysis was performed to determine the ratio of precipitated DNA to the input controls. Serial dilutions of input chromatin were used to generate a standard curve for determining the relative amount of product. Forward primer 5’-CTGAGGGTGAGCATAGCCA-3’ and reverse primer 5’-CCGCTGCTCTCCTCAGAACA-3’ were used to amplify the promoter region, −249 to +66; forward primer 5’-AAGTGTGCTTTATTTAGCATTAGTGTGA-3’ and reverse primer 5’-AGAGAAAAGTTGCGATGTGCACC-3’ were used to amplify the intronic Mn-SOD enhancer region, +1716 to +1940; forward primer 5’-CGAGTTCCATACATCAGGA-3’ and reverse primer 5’-TGAATAGCAGCGGAGCTG-3’ were used to amplify an intergenic region as negative controls. Data were plotted as the fraction of immunoprecipitated to the total input DNA sample.

RESULTS AND DISCUSSION

Identification of Transcription Factors Regulating Mn-SOD Enhancer Function by Yeast One-hybrid Analysis—The intronic enhancer regions of the Mn-SOD gene are highly conserved among rat, mouse, chimpanzee, and human (17–19, 22). An enhancer fragment that is highly conserved among mammalian species (5’-TGAGTGGGGAAAGCCCAGTTG-
against cyclophilin B or C/EBP from untransfected or mock-transfected (Dharmafect alone) L2 cells or L2 cells transfected with siRNA specifically for the transcription factors C/EBP β (also referred to as LAP, IL6-DBP, and AGP/EBP) (47–52) and C/EBP δ (also referred to as NF-IL6, CRP3, CELF, and C/EBP2) (50, 53–56). Both proteins belong to the bZIP family and are derived from intronless genes. In contrast to C/EBP δ, which has only one protein form, C/EBP β can utilize three different start codons (AUG) within the unique C/EBP β mRNA to produce three isoforms (38, 39, 57, 58), which we will refer to as full-length C/EBP β or LAP*, starting at Met-1; medium-length LAP, starting at Met-2; and the short form, LIP, starting at Met-153.

Effect of IL-1β on C/EBP β and C/EBP δ—The potent antioxidant enzyme Mn-SOD is highly inducible by a number of proinflammatory stimuli including IL-1β (10, 59), TNF-α (11, 60), lipopolysaccharide (59, 61), and IL-6 (9). We (17) and others (18, 19, 22) have shown that the induction of Mn-SOD by IL-1β, TNFα, and lipopolysaccharide is in fact mediated through the conserved intronic enhancer element. To investigate the effect of IL-1β treatment on C/EBP β or C/EBP δ cellular protein levels or localization, we isolated cytosolic and nuclear fractions from L2 cells at the indicated times after exposure to 2 ng/ml IL-1β, and both fractions were subjected to immunoblot analysis. Neither C/EBP β nor C/EBP δ was detectable in the cytosolic fraction (data not shown). In the nuclear fraction, C/EBP δ protein induction was first detectable at 1 h, peaking at ~2–3 h, with a gradual decline at 24 h to a level still significantly higher than the untreated control. Immunoblot analysis of TFIIB was used as the loading control (Fig. 1A, top panel). As mentioned previously, the translation of the C/EBP β mRNA can lead to three protein isoforms, designated LAP*, LAP, and LIP. In the nucleus, when utilizing an antibody which recognizes all three protein isoforms (α-C/EBP β), we observed the induction by IL-1β of each of these isoforms, with a time course unique to each protein (Fig. 1A, bottom panel). These data, therefore, demonstrated that LAP* and LAP were both detectable in untreated cells, showing a gradual increase in protein levels with a plateau after about
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3 h. LIP, on the other hand, was extremely low in control cells, showing an induction at ~1 h reaching a plateau at about 5 h. Unfortunately, our attempts to utilize a commercially available monoclonal antibody (ab18327, Abcam Inc.) specific to the N-terminal 21 amino acids of LAP* in conventional immunoblot analysis were unsuccessful on whole cell or nuclear extracts (data not shown). To demonstrate that LAP* was indeed present in these cells and inducible, and to verify that the top band in Fig. IA was indeed LAP*, we collected total cell extracts at increasing time points after IL-1β treatment and performed an immunoprecipitation with the antibody that recognizes all three C/EBP β isoforms (α-C/EBP β). The precipitant was fractionated by SDS-PAGE, and immunoblot analysis was performed with the antibody specific to LAP* (ab18327, Abcam Inc.) (Fig. 1B), confirming the presence of endogenous LAP* and the induction of this isoform by IL-1β.

Effect of C/EBP β Knock-out and Knockdown on IL-1β-dependent Induction of Mn-SOD—To demonstrate the functional role of C/EBP β in the IL-1β-dependent induction of Mn-SOD, we evaluated the cytokine response in C/EBP β−/− MEF cells (62). RNA was isolated from both wild type (β+/+) and C/EBP β knock-out (β−/−) MEF cells with or without 8 h of IL-1β treatment followed by Northern analysis (Fig. 2A). IL-1β exposure caused the expected induction of Mn-SOD mRNA levels in wild type MEF cells; however, the levels of Mn-SOD mRNA were unchanged in the C/EBP β knock-out (β−/−) MEF cells, implicating the relevance of C/EBP β in IL-1β induction of Mn-SOD, which is consistent with published data (20). To further verify the importance of C/EBP β in the IL-1β induction of Mn-SOD, we also utilized siRNA specific to C/EBP β to knock down endogenous C/EBP β protein levels. Immunoblot analysis of cells exposed to the transfection reagent alone or an unrelated siRNA showed no effect on the levels of the endogenous C/EBP β isoforms, whereas the siRNA specific to C/EBP β knocked down all C/EBP β isoform levels by ~80% (Fig. 2B). The C/EBP β-specific siRNA-treated cells were subjected to IL-1β treatment, and real-time RT-PCR data from three independent experiments were analyzed for Mn-SOD mRNA levels (Fig. 2C). Untreated levels of Mn-SOD mRNA were unaffected by any of the conditions, whereas specific knockdown of C/EBPβ blocked IL-1β induction of Mn-SOD by more than 60%.

Interaction of C/EBP β and C/EBP δ with the Mn-SOD Enhancer Element—To investigate the interactions of C/EBP β and C/EBP δ with the Mn-SOD gene in intact cells following IL-1β exposure, ChIP analysis was performed with L2 cells collected at specific time points after IL-1β treatment. A nonspecific IgG antibody gave minimal background signals (data not shown). Antibodies against C/EBP β (Fig. 3A) or C/EBP δ (Fig. 3B) showed significant inducible interactions with the Mn-SOD intronic enhancer following IL-1β treatment, with minimal binding observed at the promoter as well as at an intergenic control region. The differences between these two transcription factors were more obvious when considering the time dependence of the binding. C/EBP β showed a ~7.4-fold increase in the occupancy of the intronic enhancer after 15 min of IL-1β exposure with sustained binding up to ~11.4-fold at 5 h (Fig. 3A). This is consistent with the initial induction of LAP* and LAP protein levels (Fig. 1). The binding of C/EBP δ, on the other hand, showed only a ~2.6-fold increase in occupancy at 15 min but a ~10.3-fold increase at 2 h, which coincided with the significant increase in protein levels at 2 h (Fig. 1A). The differences in the timing of occupancy may reflect a difference in function associated with these two transcription factors in the regulation of Mn-SOD expression following IL-1β treatment, as eluded to below.

Functional Importance of C/EBP β and C/EBP δ in Mn-SOD Transcriptional Regulation—Classically speaking, the LAP* and LAP isoforms have been identified as activators (63–65), whereas LIP has been considered as a repressor (39, 66). However, our data suggested a more distinct role for these isoforms. Given the existence and inducibility of all three C/EBP β isoforms in L2 cells, it was logical to assume that the C/EBP β protein isoforms functioned differently to coordinate the regulation of Mn-SOD expression during the induction by IL-1β. To test this hypothesis, we cloned the cDNAs that would initiate at each of the three methionine residues into the mammalian expression vector, pcDNA3.1, with the designations pLAP*/LAP/LIP indicating the production of all three proteins, LAP*, LAP, and LIP, pLAP/LIP refers to the synthesis of only LAP and LIP, and pLIP is the designation for the production of LIP alone (Fig. 4A, top panel). We tested the functional importance of these three constructs on Mn-SOD gene regulation following transient co-transfection of L2 cells with an hGH reporter driven by the Mn-SOD promoter coupled to its cognate intronic enhancer (Mn-SOD promoter/enhancer-hGH). We
found that co-transfection of the expression vector pLAP*/LAP*/LIP increased hGH expression (Fig. 4A, bottom panel, lane 2 versus 1), whereas co-transfection with pLAP/LIP or pLIP had no effect on the basal expression of the Mn-SOD promoter/enhancer (lanes 3 and 4 versus 1). As reported previously (17), when cells were co-transfected with Mn-SOD promoter/enhancer-hGH and the empty expression vector, pcDNA3.1, exposure to 2 ng/ml IL-1β for 8 h induced hGH expression (Fig. 4A, lane 5 versus 1), demonstrating the responsiveness of the intronic enhancer to IL-1β. Co-transfection with pLAP/LIP or pLIP blocked the IL-1β-dependent induction (Fig. 4A, lanes 7 and 8 versus 5), whereas co-transfection with pLAP*/LAP*/LIP in IL-1β-treated cells showed a somewhat additive response compared with IL-1β alone (lane 6 versus 5).

To systematically study the role of each protein isoform separately and with all possible combinations, we selectively mutated methionine 22 and/or methionine 153 to alanine, generating four possible combinations, pLAP*/LIP, pLAP*/LAP, pLAP*, and pLIP (Fig. 4B, top panel). As described for the constructs in Fig. 4A, the designations for each construct reflect the protein isoforms expressed from each construct. The anticipated results were verified by isolating total protein extracts from L2 cells transfected with the respective mutant expression vectors followed by immunoblot analysis with antibodies specifically against LAP* or C/EBP β (recognizes all three isoforms). The bottom right panel in Fig. 4B shows a darker exposure of a similar experiment performed with antibody against C/EBP β to illustrate the presence of LIP in the appropriate lanes. C, L2 cells were co-transfected with the Mn-SOD promoter/enhancer-hGH reporter plasmid together with an empty pcDNA3.1 plasmid or the indicated expression vectors. At 48 h post-transfection, total RNA was collected, with or without 8 h of IL-1β treatment, and subjected to Northern analysis. As described for A and B, the name of each expression vector denotes the proteins produced from the respective construct.

To determine whether methionine to alanine mutations would affect the transcriptional activity of the respective isoforms, the indicated expression vectors were co-transfected with the Mn-SOD promoter/enhancer-hGH reporter con-
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**FIGURE 5. Transcriptional regulation of Mn-SOD by LAP*, LAP, LIP, and C/EBP δ.** A, L2 cells were co-transfected with Mn-SOD promoter/enhancer-hGH reporter plasmid along with an empty pcDNA 3.1 vector or the expression vectors for LAP*, LAP, LIP, or C/EBP δ. 40 h post-transfection, cells were either untreated or stimulated by exposure to 2 ng/ml IL-1β for 8 h. Total RNA was then collected and subjected to Northern analysis with Cath B as the loading control. B, densitometry was derived from five independent experiments, and data are presented as means ± S.E. * indicates statistical significance of transcriptional activities in unstimulated conditions compared with the empty vector, pcDNA 3.1, with p < 0.05. A cross indicates statistical significance in enhancing or impeding IL-1β induction of Mn-SOD promoter/enhancer-hGH compared with empty vector pcDNA 3.1 (p < 0.05).

struct. Total RNA collected with or without 8 h of IL-1β treatment was subjected to Northern analysis (Fig. 4C). Those constructs that express LAP* (Fig. 4C, lanes 2–5), significantly enhanced hGH expression (compare with lane 1) in the absence of IL-1β. Most relevantly, the induction observed for the pLAP* construct (Fig. 4C, lane 5), expressing only LAP*, was very efficient at inducing Mn-SOD promoter/enhancer function. However, when transfected cells were exposed to IL-1β, a slight additive response was observed (Fig. 4C, lanes 9–12 versus 8). Additionally, lanes 6 and 7 (Fig. 4C) confirm that in the absence of LAP*, hGH expression is not increased. Furthermore, lanes 13 and 14 (Fig. 4C) demonstrate that pLAP and pLAP/LIP strongly repress hGH expression when exposed to IL-1β.

To further demonstrate the roles of LAP*, LAP, LIP, and C/EBP δ during IL-1β-induced Mn-SOD expression, empty pcDNA3.1 or vectors expressing only LAP*, LAP, LIP, or C/EBP δ were co-transfected with the Mn-SOD promoter/enhancer-hGH reporter plasmid, and hGH mRNA levels were examined by Northern analysis (Fig. 5A). A densitometry analysis of three independent experiments is shown in Fig. 5B. As demonstrated in Fig. 5, LAP* caused a ~2.5-fold induction of hGH expression in the absence of IL-1β, whereas overexpression of either LAP, LIP, or C/EBP δ had no effect on basal expression. Overexpression of LAP* in conjunction with IL-1β treatment further added to the IL-1β-enhanced hGH expression, whereas overexpression of LAP, LIP, or C/EBP δ significantly repressed Mn-SOD promoter/enhancer-driven hGH expression (Fig. 5B).

**FIGURE 6. Association of LAP* with the endogenous Mn-SOD gene.** A, top panel, L2 cells were transfected with 5 μg of empty vector pcDNA 3.1 or expression vectors for HA-tagged LAP*, and total cell extracts were investigated by immunoblot analysis with antibodies against HA or C/EBP β. Bottom panel, L2 cells were co-transfected with Mn-SOD promoter/enhancer-hGH reporter plasmid together with either an empty pcDNA 3.1 plasmid or vectors expressing non-tagged or HA-tagged LAP*. 48 h post-transfection total RNA was collected and subjected to Northern analysis. L2 cells transfected with Mn-SOD promoter/enhancer-hGH and pcDNA 3.1 were also exposed to 2 ng/ml IL-1β for 8 h to illustrate normal induction by IL-1β. Three independent plates were transfected for each construct. B, L2 cells were transfected with 10 μg of expression vector for HA-tagged LAP* in 150-mm plates, and a ChIP assay was conducted as described under "Materials and Methods" at 48 h post-transfection using a nonspecific mouse IgG or an antibody against HA. Data were plotted as the fraction of immunoprecipitated to the total input DNA sample.

Binding of LAP* to the Mn-SOD Enhancer—With the unique role that LAP* played as a potent transcriptional activator of Mn-SOD, we felt it necessary to demonstrate that LAP* could specifically interact with the endogenous Mn-SOD enhancer element. Given that the antibody against C/EBP β used in the ChIP analysis in Fig. 3A recognizes all three C/EBP β protein isoforms and that the antibody specifically against LAP* did not possess adequate affinity for immunoprecipitation in our hands, we generated a vector expressing C-terminal HA-tagged LAP*. Immunoblot analysis of protein from cells transfected with HA-LAP* vector with antibodies either to the HA tag or to C/EBP β demonstrated that the construct produced the expected protein (Fig. 6A, top panel). Furthermore, co-trans-
Infection of cells with pHA-LAP* and the Mn-SOD promoter/enhancer-hGH reporter plasmid demonstrated that the HA-tagged protein behaved identically to the untagged LAP* in its ability to induce hGH expression (Fig. 6A, bottom panel). With these results, we then transfected L2 cells with the vector expressing HA-LAP* and evaluated the binding of LAP* to MnSOD by ChIP analysis with the HA-specific antibody. The data shown in Fig. 6B clearly demonstrates that HA-LAP* is specifically bound to the Mn-SOD enhancer, whereas no actual binding was observed with the Mn-SOD promoter, an intergenic region, or samples using nonspecific mouse IgG.

Importance of the First 21 Amino Acids of LAP*—Our results thus far have demonstrated that LAP* is the only isoform of C/EBP family proteins that can function as a transcriptional activator of Mn-SOD gene expression. The only difference between the isoforms, LAP* and LAP, is the N-terminal 21 amino acids. With only crystal structures of the bZIP domain (67, 68) and no available crystal structure for either LAP or LAP* protein in the literature, our best prediction of the inherent relevance of this sequence could be derived only from an alignment of the N-terminal peptide from a variety of mammalian species (Fig. 7A). This alignment illustrates that the N-terminal domain of LAP* is highly conserved among human, chimpanzee, bovine, mouse, and rat, among which 13 amino acids were identical.

To evaluate the functional importance of the conserved amino acids to the transcriptional activity of LAP*, we mutated each conserved residue to alanine with the exception of Ala-18, where valine was the substitution. Bracketed triangles Pro-13–Pro-16 (designated as 4P) were mutated to alanines within a single vector. B, L2 cells were co-transfected with Mn-SOD promoter/enhancer-hGH reporter plasmid together with the indicated expression vectors. Top panel, whole cell extracts were collected and subjected to immunoblot analysis with an antibody against C/EBP β. Bottom two panels, total RNA was collected with or without 8 h of IL-1β treatment and then subjected to Northern analysis. The quadruple mutant P13A, P14A, P15A, P16A is referred to as 4P-A. C, densitometry analysis of Northern data for each individual site-directed mutant in untreated and IL-1β-exposed cells. The top graph depicts data from three independent experiments for mutants that showed no obvious change compared with the pLAP* construct. The bottom panel summarizes data from six independent experiments showing those mutants that displayed an effect either in untreated or IL-1β-exposed cells. Data are presented as the ratio of hGH to Cath B. Asterisk, indicates statistical significance of changes in transcriptional activities in unstimulated conditions compared with LAP* (p < 0.05); double asterisks, p < 0.01; cross, indicates statistical significance in enhancing or impeding IL-1β induction of Mn-SOD promoter/enhancer-hGH compared with empty vector pcDNA 3.1 (p < 0.05).
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Whole cell extracts were collected from L2 cells transfected with the indicated expression vectors, and immunoblot analysis was performed with antibody against C/EBP β (Fig. 7B, top panel). Most mutants had similar overexpression levels compared with pLAP*, with only the mutants R3A, W7A, and D8A exhibiting lower expression levels. Northern analysis of mRNA levels from all of the overexpression vectors, however, showed no differences in the level of transcription (data not shown), indicating that R3A, W7A, and D8A mutations could result in reduced protein translation or protein stability.

All mutant LAP* vectors were then co-transfected with the Mn-SOD promoter/enhancer-hGH reporter to evaluate the transcriptional activity. A representative Northern analysis is shown for untreated and IL-1β-treated cells (Fig. 7B, middle and bottom panels). Based on densitometric analyses of three independent experiments (Fig. 7C), we separated the mutations with no effects, mutants L4A, C11A, L12A, A18V, and F19A (Fig. 7C, top panel) from the mutants R3A, W7A, D8A, and the quadruple mutant P13A, P14A, P15A, P16A, which resulted in a significant reduction in LAP*-dependent transcriptional activation (Fig. 7C, bottom panel). These mutants also lost the ability to act additively with IL-1β, and of particular note, LAP*D8A and LAP* P13A, P14A, P15A, P16A also caused a statistically significant inhibition of IL-1β-dependent induction, demonstrating a repressive affect.

To further evaluate the chemical and/or structural importance of amino acid residue Asp-8, we substituted the aspartate with either asparagine, to eliminate the charge, or glutamate, to maintain the charge. Overexpression levels of these mutants were similar to pLAP* (Fig. 8A, top panel). Northern analysis of hGH (Fig. 8A, bottom panel) showed substitution of Asp-8 with alanine or asparagine caused a significant reduction in LAP*-dependent transcriptional activation in untreated cells, whereas substitution with glutamate maintained high transcriptional activation analogous to LAP*. When treated with IL-1β for 8 h, the D8A mutant possessed statistically significant repressive activity as confirmed by densitometric analysis based on three independent experiments (Fig. 8B). However, the D8N mutant lacked the repressive affect, indicating that the stereo structure of Asp-8 may also be important although not as crucial as the negative charge. The D8E substitution, on the other hand, displayed characteristics identical to LAP* in its transcriptional activation and its ability to cause an additive induction in IL-1β-treated cells.

To further examine the importance of Arg-3, we generated two additional mutant constructs, pLAP*R3Q and pLAP*R3K, which either abolished the charge while partially maintaining the stereo structure or replaced the arginine with lysine to maintain the charge, respectively. Unexpectedly, all three substitutions caused a significant decrease in the expression level as well as transcriptional activity (data not shown). To eliminate the possibility that reduced transcriptional activation was caused by decreased protein levels, a titration of the three mutants was performed (Fig. 9A, top panel). The shaded co-transfections in Fig. 9A, top panel, were chosen for hGH expression analysis (Fig. 9A, bottom panels). Densitometric analysis (Fig. 9B) also showed that all three mutations, R3A, R3Q, and R3K, resulted in a dramatic reduction in LAP* transcriptional activity. When exposed to IL-1β, R3A and R3Q mutants appear to inhibit the Mn-SOD induction by this proinflammatory cytokine; however, the statistics yielded a p value slightly greater than 0.05. These results would imply that the arginine residue at position 3 is important for LAP* function because of both its inherent structure and its positive charge.

To test the functional relevance of the aromatic nature of Trp-7, we constructed another mutant, W7F. Similar to the approach used for the Arg-3 mutants, we transfected cells with increasing concentrations of pLAP*W7A plasmid to equalize its expression level with that of wild type LAP* (Fig. 9C, top panel). Guided by these data, we studied the hGH expression levels with the co-transfections of 1.0 μg of pLAP*W7A and 0.5 μg of pLAP*W7F, with or without 8 h of IL-1β treatment, as compared with 0.5 μg of LAP*. As illustrated by the results of a Northern analysis (Fig. 9C, bottom panel) and the corresponding densitometry analysis (Fig. 9D), the W7A mutant was unable to illicit an induction of the Mn-SOD enhancer/promoter-driven hGH expression in untreated cells, and moreover it definitively lost the ability to act additively with IL-1β. Substitution of Trp-7 with phenylalanine displayed functional characteristics essentially identical to tryptophan at this position. These results clearly delineate the critical importance of an aro-
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FIGURE 9. Functional examination of mutants at sites R3 and W7. A, L2 cells were co-transfected with the Mn-SOD promoter/enhancer-hGH reporter construct and the indicated amounts of LAP* or LAP*R3 mutants. pcDNA 3.1 was used to bring the total amount of DNA transfected to 4 µg for each transfection. At 48 h post-transfection total cell extracts were collected followed by immunoblot analysis with antibodies against C/EBP β and actin (top panel). Shaded concentrations (top panel) denote the transfection conditions used in Northern analysis (bottom panel). RNA was collected from cells either left untreated or exposed to IL-1β for 8 h followed by Northern analysis with Cath B serving as the loading control (bottom panel). B, densitometry was performed on three independent experiments. Data are presented as the -fold induction derived from the hGH to Cath B ratios relative to the control (pcDNA). Asterisk, indicates statistical significance of changes in transcriptional activities in unstimulated conditions compared with LAP* (p ≤ 0.05); double asterisks, p ≤ 0.01; cross, indicates statistical significance in enhancing IL-1β induction of Mn-SOD promoter/enhancer-hGH compared with empty vector pcDNA 3.1 (p ≤ 0.05). C, analysis of Trp-7 mutants. L2 cells were co-transfected with the Mn-SOD promoter/enhancer-hGH reporter construct and the indicated amounts of LAP* or LAP*W7 mutants. pcDNA 3.1 was used to bring the total amount of DNA transfected to 4 µg for each transfection. At 48 h post-transfection total cell extracts were collected followed by immunoblot analysis with antibodies against C/EBP β and actin (top panel). Shaded concentrations (top panel) denote the transfection conditions used in Northern analysis (bottom panel). RNA was collected from cells either left untreated or exposed to IL-1β for 8 h followed by Northern analysis with Cath B serving as the loading control (bottom panel). D, densitometry was performed on three independent experiments. Data are presented as the -fold induction derived from the hGH to Cath B ratios relative to the control (pcDNA). Asterisk, indicates statistical significance of changes in transcriptional activities in unstimulated conditions compared with LAP* (p ≤ 0.05); cross, indicates statistical significance in enhancing IL-1β induction of Mn-SOD promoter/enhancer-hGH compared with empty vector pcDNA 3.1 (p ≤ 0.05).

mastic residue at this position. It is thus increasingly clear from our mutagenesis studies that the N-terminal amino acid extension of full-length C/EBP β/LAP* plays a central role in the activation of Mn-SOD.

Summary—The regulation of Mn-SOD gene expression can be considered of central importance to cellular and organismal homeostasis with postnatal death observed in gene ablation mouse models at 10 days to 3 weeks (14, 15). For the past 30 years, extensive studies have convinced the research community that Mn-SOD expression levels are lower in primary tumors and in cancer cell lines (69–71) and that overexpression of manganese superoxide dismutase and its catalytic mutants can inhibit cancer cell growth (72–76) and tumors in vivo (72, 76–78). Furthermore, numerous studies have demonstrated that elevated levels of Mn-SOD are able to protect against radiation damage (reviewed in Ref. 79), N-methyl-D-aspartate-initiated neurotoxicity (80), and TNF-α-mediated apoptosis (81). Other studies have also demonstrated that cardiac overexpression of Mn-SOD can confer protection of cardiac mitochondria, thereby reducing diabetic cardiomyopathy (82). Therefore, it is clear that an understanding of the underlying mechanisms controlling the cellular regulation of Mn-SOD could be of central importance to a variety of disease pathologies as well as to the aging process.

The present studies have focused on the regulation of Mn-SOD gene expression by proinflammatory cytokines, specifically IL-1β. Our results have thus demonstrated the central importance of specific C/EBP β isoforms and another family member, C/EBP δ, in the IL-1β-dependent regulation of Mn-SOD. We have shown that full-length C/EBP β/LAP*, LAP, and LIP, as well as C/EBP δ protein levels, are rapidly induced by treatment with IL-1β.

Most relevantly, our data have elucidated the specific role of LAP* as the only isoform of C/EBP β that serves as a specific transcriptional activator of Mn-SOD gene expression. This conclusion was based on the ability of LAP* overexpression to act specifically through the Mn-SOD enhancer as well as additively with IL-1β. In addition, our ChIP analysis demonstrated the specific contact of LAP* with the endogenous intronic Mn-
SOD enhancer element. In contrast, we found that medium-length C/EBP β/LAP when overexpressed could inhibit IL-1β-dependent induction of Mn-SOD, as was true for the established dominant negative isoform LIP and the other family member identified by one-hybrid analysis, C/EBP δ. A review of the literature has established these results as novel observations in the regulation of gene expression by C/EBP. We also believe that these data provide convincing evidence that could implicate LAP* as having an enhanced role in the regulation of other genes and pathways previously linked to C/EBP β or more specifically LAP. In fact, in many studies in which C/EBP β is linked to gene expression, no clear distinction is made as to which isoform of the protein is responsible for the documented regulatory signals (65, 83, 84).

We have further solidified the importance of LAP* in the induction of Mn-SOD by systematically demonstrating the importance of the N-terminal 21 amino acids unique to LAP*. Our efforts have identified three individual conserved amino acids, Trp-7, Asp-8, and Arg-3, along with a set of four consecutive proline residues, that when mutated are either unable to induce Mn-SOD promoter/enhancer expression or can behave as a repressor, inhibiting IL-1β induction. Our mutagenesis studies also addressed the relevance of the entire N-terminal peptide to LAP* as the only transcription activator among C/EBP β isoforms and C/EBP δ. We are aware of only one other previous mutagenesis study that addresses the importance of the cysteine at position 11 (41). This study implicated Cys-11 as a potential site for disulfide bond formation with another internal cysteine. However, our results indicate that this position is not required for LAP* transcriptional activation in our system.

Furthermore, our mutagenesis studies establish these N-terminal amino acids as an important transcriptional activation domain. For example, these data illustrate that the aromatic nature of Trp-7 is necessary for LAP* function, because the protein substituted with another aromatic amino acid (W7F) retained wild type function as a transcriptional activator. Similarly, the conserved substitution at position Asp-8 with another negatively charged amino acid (D8E) maintained the transcriptional activity, illustrating the importance of the positive charge at this position. However, for the Arg-3 site, any substitution led to the loss of LAP* transcriptional activity. This occurred even when Arg-3 was replaced with a similarly charged amino acid (R3K), indicating that the size/volume of the residue, and/or possibly the positive charge, is important. In addition, it may also be that the R3K substitution affects the modification status of the protein, as both residues can be phosphorylated, although through the action of different enzymes.

Of particular note is the importance of the stretch of 4–5 proline residues given that substitution of these 4 prolines to alanines abolished the transcriptional activity without affecting the protein expression level. The proline-rich sequence tends to form a polyproline II (PPII) helix, which is rigid and of great advantage in rapidly recruiting interchangeable protein partners. Proline-rich regions are often involved in signal transduction and are also found in some transcription factors. A number of studies have shown that proline-rich motifs (reviewed in Ref. 85) are potential binding partners for proteins containing the Src homology 3 (SH3) domain as well as Ena/VASP homology 1 (EVH1) domains. Overall, the importance of LAP* in Mn-SOD induction provides a unique opportunity to study the molecular mechanisms controlling the function of an intron-specific element along with the role of this not well studied C/EBP β isoform.

Acknowledgments—We thank Dr. Michael Kilberg and the members of the Kilberg laboratory for helpful suggestions and enthusiastic support throughout this work. We also thank Nan Su and all other members of the Nick laboratory for their continuing support.

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