C/EBPα is a major activator for the transcription of rat Cu/Zn superoxide dismutase gene in liver cell

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Abstract The rat Cu/Zn superoxide dismutase (SOD1) is expressed in all tissues. Sequence analysis of the *SOD1* promoter region showed that none of the *cis*-elements of hepatocyte-specific nuclear factors (HNF) were observed. The *cis*-element of C/EBPα in the proximal region of the *SOD1* promoter and the high level of C/EBPα in the liver tissue led us to focus on the transcriptional regulation of the *SOD1* gene by C/EBPα. Cotransfection assays with the plasmid expressing transcription factor C/EBPα showed that C/EBPα transactivated *SOD1* gene by 27 fold. The marked transactivation and direct binding of C/EBPα to the *SOD1* promoter were confirmed by deletion analyses and mobility shift assays. These results suggested that C/EBPα plays a major role in the tissue distribution of SOD1.

Key words: Rat *SOD1* gene; C/EBPα; Tissue distribution; Transcription; Transactivation

1. Introduction

The Cu/Zn superoxide dismutase (SOD1) is a key enzyme in the metabolism of oxygen free radicals. It catalyzes the dismutation of superoxide radicals (O_2^-) to oxygen and hydrogen peroxide [1]. The production and/or removal of superoxides has been observed to play a significant role in a variety of critical homeostatic mechanisms both at the cellular and at the organismic level. It has been reported that *SOD1* can prevent oncogenesis and tumor promotion [2]; reduce the cytotoxic and cardiotoxic effects of anticancer drugs [3]; protect against reperfusion damage of ischemic tissue [4]. Recently, it has been shown that overexpression of SOD1 and catalase can increase the average life span of flies [5].

It is known that SOD1 is expressed in all tissues at varying levels. In particular kidney and liver show the highest Cu/Zn SOD mRNA levels [6]. This distribution is consistent with the relative protein concentration and activity of SOD1 in these tissues [7]. There are four known major factor families expressed in the liver: C/EBP (CCAAT/enhancer-binding protein), HNF-1, HNF-3 and HNF-4 [8]. Members of these protein families are expressed in embryonic liver as well as in the adult. Since sequence analysis of the *SOD1* promoter region showed none of the *cis*-elements of hepatocyte-specific nuclear factors, we performed the deletion experiment to focus the most influential *cis*-element in this promoter. We report here that the transcription factor C/EBPα, which is expressed at high levels in liver and adipose tissue, influenced the expression of *SOD1* gene most.

2. Materials and methods

2.1. Plasmid construction

The 1.7 kb *Bam*HI/*Sma*I fragment (nt −1633 to +85) from the rat *SOD1* gene [9] was inserted into pBLCAT3 [10]. Unidirectional 5′ deletion mutants were produced by cutting 5′ region of the *SOD1* promoter with *Sph*I and *Bam*HI, followed by treatment with exonuclease III [11]. The end points of deletion were confirmed by DNA sequencing with Sequenase kit (US Biochemicals). Plasmid pMSV-C/EBPα was a generous gift from S.L. McKnight. For the construction of pGEX3XC/EBPα, the expression plasmid of the GST-C/EBPα fusion protein, the *Bam*HI fragment of pMSV-C/EBPα was inserted into the *Bam*HI site of pGEX3X (Pharmacia).

2.2. Cell culture and transfection

Human HepG2 hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum/penicillin G sodium at 100 units per ml/streptomycin sulfate at 100 μg per ml/amphotericin B at 250 ng per ml. Cells were seeded into 60 mm plastic culture dishes (30–50% confluence) for 24 h prior to transfection. Equal amounts (3.0 pmol) of the various constructs were transfected to the cells by the calcium phosphate DNA coprecipitation method [12]. 5 μg of pRSVβ-gal plasmid [13] was introduced in all experiments to correct the variations of transfection efficiency. The effector plasmid was cotransfected to test for transacting activities. To determine the effective concentration of effector plasmid, cells were cotransfected with reporter plasmid and various amounts of effector plasmid.

2.3. CAT assay

The CAT assay was performed as described previously [14]. The transfected cells were washed twice with PBS and harvested. The pelleted cells were resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.9) and lysed by three cycles of freezing and thawing. After removal of cell debris by centrifugation, cell extracts were first assayed for β-galactosidase activity [15]. Equal quantities of proteins were assayed for CAT activity on the basis of β-galactosidase activity. Extracts were incubated with 0.025 μCi of [^{14}C]chloramphenicol/0.25 M Tris-HCl pH 7.6/0.4 mM acetyl coenzyme A for 1 h at 37°C. The enzyme assay was terminated by adding ethylacetate. The organic layer was analyzed by thin layer chromatography (TLC) with chloroform/methanol (95:5). After autoradiography, both acetylated and unacetylated forms of [^{14}C]chloramphenicol were scraped from the plate and the conversion of chloramphenicol to the acetylated form was calculated by measuring radioactivity. The relative CAT activities were calculated from the percent conversion. Results are the average of three independent experiments.

2.4. Mobility shift assay

The synthetic oligonucleotides for the transcription factor CP2 binding site were purchased from Genosys. [γ-^{32}P]ATP and [α-^{32}P]dATP were from Amersham. The double-stranded oligonucleotide was labeled with [γ-^{32}P]ATP and polynucleotide kinase [15]. Purified GST-C/EBPα was mixed with CP2 oligonucleotide for 20 min at 20°C in 15 μl solution containing 10 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and 2 μg of

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Abbreviations: SOD1, Cu/Zn superoxide dismutase; *SOD1*, Cu/Zn superoxide dismutase gene; C/EBP, CCAAT/enhancer-binding protein; HNF, hepatocyte nuclear factor; CAT, chloramphenicol acetyl transferase; MSA, mobility shift assay; nt, nucleotide(s); PBS, phosphate-buffered saline

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poly[dI-dC]. The binding reaction mixtures were electrophoresed in 6% acrylamide gels in 0.5×TBE (44 mM Tris, 44 mM boric acid, and 1 mM EDTA). After electrophoresis, gels were dried and exposed to X-ray film. For competition assays, binding reaction was performed with a 50-fold molar excess of cold probe or competitor DNA. Nuclear extracts from HepG2 cells were made as described previously [16].

2.5. Purification of C/EBPα

pGEX3XC/EBPα was introduced in *E. coli* BL21(DE3). To express soluble fusion proteins, overnight cultures of cells were diluted with 10 volumes of induction medium (3.2% bacto-tryptone, 2% yeast extract, 0.1 mM $MgSO_4$, 1 mM $FeCl_3$, 1×M9 salt), cultured for an additional 2 h, and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) at 37°C for 2 h. Cells from 3 ml of culture were harvested and resuspended in 0.5 ml of phosphate-buffered saline (PBS) containing 1% Triton X-100 (PBS-Triton). The lysates were sonicated three times with 15-s pulses, and the debris was pelleted by centrifugation. 10 μl of supernatant was mixed with 50 μl of glutathione-Sepharose (Sigma), equilibrated and suspended in PBS-Triton according to the manufacturer's specification, and incubated at 4°C for 30 min. The Sepharose was collected by centrifugation, washed three times with PBS-Triton, resuspended in 10 μl of sodium dodecyl sulfate (SDS) sample loading buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM dithiothreitol, 0.1% bromophenol blue, 10% glycerol), and assayed by SDS-polyacrylamide gel electrophoresis (PAGE).

3. Results and discussion

3.1. Identification of C/EBPα binding site in SOD1 promoter

As shown in Fig. 1, from the sequence analysis of transcription factor binding sites, the sites for CAAT box binding protein (CP2) and NF-IL6 were located at −73 and −101, respectively. Interestingly, none of the HNF series (HNF-1, 3, and 4) was observed in the promoter region of rat *SOD1* gene. C/EBPα and NF-IL6 (LAP; liver activator protein) are members of a bZIP protein subfamily since they share 71% homology in their basic DNA binding region and leucine zipper region, and they have been shown to form heterodimers [17]. The CP2 site has a CCAAT motif which could also be bound by C/EBPα.

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-412
TGGAGATAGAACATGGGCCTTACACATATTACACCGAGCATCCATCTTGG
-362
CTCACCCCAACTTTCACACAGCAACTGCGGCGCGCTGCAAAGTCAGTCGC
-312
AATCCGCATTTCTAGACAGAGCGGCTTCAGACCTTCCAGGCGCGCACGCA
-262
GGCCTCGCCGAGGTTTCCTCCGCGACTCGGCCGACTTCACAGTTAGAAGA
-212
CAATAGCGACTTTCCCAGCTCTGTCTCGATTCTGGAACTTTCTCAGTCGC
-162
AAGCTCCTGAAGCTGGCGCTCCCCTCAGCCCCGCCCCCAACGTGCCCCGC
-112                                          AP2
GGCCAGGGAACTTCAGGAAGGGTAGGCAGAGACCGCGGCTAGCGATTGGT
                NF-IL6                        CP2
-62
TCCCTGCCAAGGTGGGAGTGGCCAGGCACAGGCATATAAAAGCTCCGCGG
                                          TBP
-12        +1 ↱ tsp
CGCTGGGCCCTCGTTTTGCACCTTCGTTTCCTGCGGCGGCTTCTGTCGTC
+39
TCCTTGCTTTTTGCTCTCCCAGGTTCCGAGGCCGCCGCGCGTCTCCCGGG
+89
GAAGCATG
    MET
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Fig. 1. Sequence analysis of the rat *SOD1* promoter. The sequence is shown from −412 to +96 relative to the transcription start point (tsp). Transcription factor binding sites for AP2, NF-IL6, CP2 and TBP are underlined. The CCAAT box is in a box.

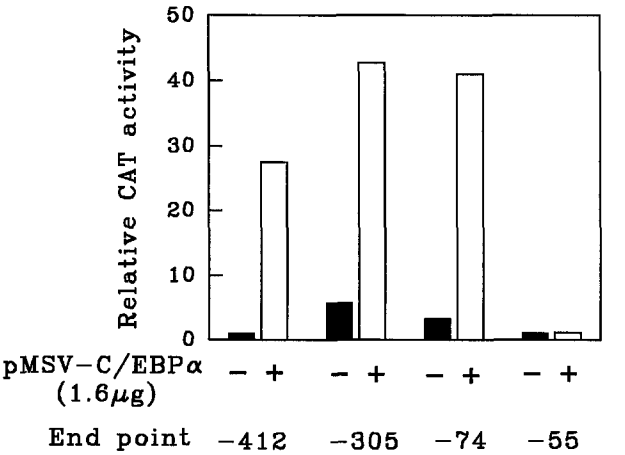

Fig. 2. Activation of expression of the *SOD1* gene by C/EBPα. Deletion mutants were cotransfected with pMSV-C/EBPα, effector plasmid (1.6 μg). When cotransfected with pMSV-C/EBPα, the CAT activities of pRSP-412, pRSP-305 and pRSP-74 were dramatically increased. The solid bar and open bar represent the CAT activity of SOD-CAT fusion plasmid cotransfected without and with effector plasmid, respectively.

A series of 5′ deletion mutants was prepared to determine whether the *SOD1* promoter might harbor *cis*-elements critical for transactivation by C/EBPα. In addition to pRSP-412, deletion mutants terminating at positions −305, −74 and −55 were tested by transient expression experiments (Fig. 2). Each deletion mutant was cotransfected into HepG2 cells with 1.6 μg of pMSV-C/EBPα plasmid. When the expression was examined in the absence of pMSV-C/EBPα, the level of expression of all constructs was very low with a slight difference. As shown in Fig. 2, a substantially higher level of *SOD1* promoter activity was maintained at least −74 bp upstream from the transcription start point (tsp). When the expression was examined in the presence of pMSV-C/EBPα, the level of expression of three constructs was dramatically increased. But the level of expression of pRSP-55 was not changed. The induction fold of pRSP-412 was higher than any other constructs by cotransfection of the pMSV-C/EBPα plasmid.

These results show that C/EBPα can activate the transcription of the *SOD1* promoter when expressed in transiently transfected HepG2 cells. It is known that the *SOD1* promoter contains a canonical NF-IL6 site (−101), and a CP2 site (−73) (Fig. 1). At least these sites may be responsible for the transactivation by C/EBPα. van Ooij et al. [18] reported that C/EBPα and NF-IL6 did appear to have a synergistic effect on the *ADH* gene family. C/EBPα and NF-IL6 heterodimers are more effective than either C/EBP homodimers or NF-IL6 homodimers. In the case of the *SOD1* promoter, the differential responses to C/EBPα may be physiologically significant for the interaction with other factors during liver development and/or adipocyte differentiation.

3.2. C/EBPα activates the transcription of SOD1

To test whether C/EBP might be capable of regulation of the *SOD1* gene, a chimeric SOD-CAT gene was cotransfected into HepG2 cells along with the C/EBP expression vector, pMSV-C/EBPα. Friedman et al. [19] previously reported that the HepG2 cell line appeared to exhibit substantially lower levels of C/EBPα than other liver-derived hepatocytes. The SOD-CAT chimera contained nucleotides −412 to +85

fused to the CAT structural gene. This construct was transfected by the calcium phosphate method into HepG2 together with either pUC19 DNA as a control or increasing amounts of pMSV-C/EBPα DNA. As shown in Fig. 3, the SOD promoter of pRSP-412 was strongly transactivated by C/EBPα. The amount of C/EBPα expression plasmid (1.6 μg) was still in the range of a linear increase of the transcriptional activity of target gene. However, the transcriptional activity was slightly, but reproducibly reduced when cotransfected with a larger amount of C/EBPα expression plasmid (Fig. 3). It has been reported that in at least two different systems, overexpression of a transcriptional activator may cause inhibition of transcription [20]. In this case, overexpressed C/EBPα in HepG2 cell may have a squelching effect on the expression of the *SOD1* promoter.

C/EBPα is expressed at high levels in liver and adipose tissue [21] and is important for the expression of the liver-specific genes as well as several adipose-specific genes. It is possible that C/EBPα plays a role in conferring both the tissue-specific and developmental properties of the *SOD1* gene. Because *SOD1* is expressed at relatively high levels in kidney, the SOD1 level is unlikely to be mediated only by C/EBPα. C/EBPα is one of several factors that regulate *SOD1* gene transcription. The LAP, also known as NF-IL6, site was located at −101 in the *SOD1* promoter. This protein may bind to or

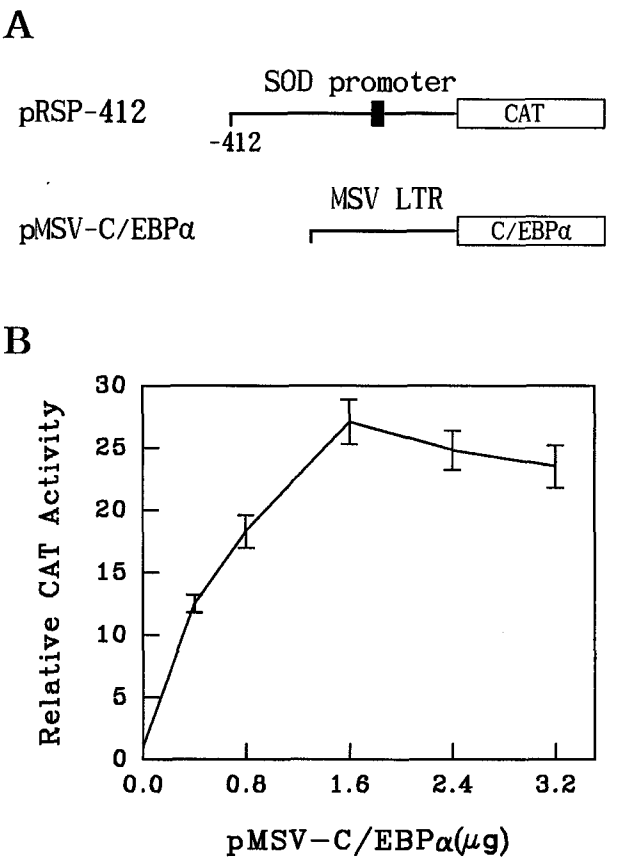

Fig. 3. Determination of an effective concentration of the effector plasmid. A: Schematic diagram of pRSP-412 and pMSV-C/EBPα, the expression vector of the C/EBP gene. B: pMSV-C/EBPα, the effector plasmid was cotransfected with pRSP-412, a *SOD1*-CAT fusion plasmid, in order to check the effective concentration. The maximal CAT activity was shown at the point of about 1.6 μg of effector plasmid.

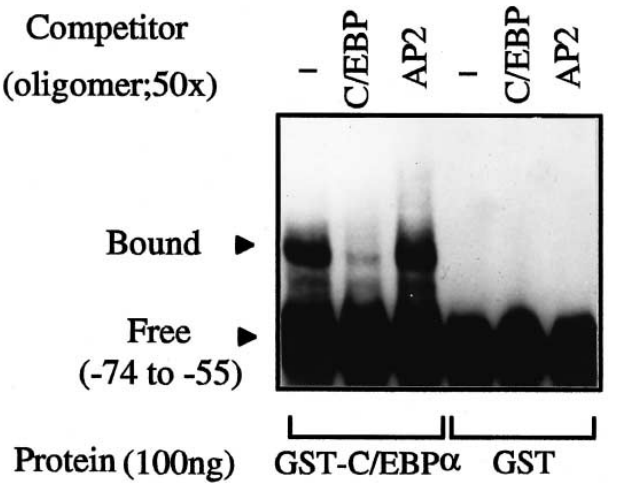

Fig. 4. Direct binding of C/EBPα to the CAAT box sequence of the rat *SOD1* promoter. A ^{32}P-labeled double-stranded oligonucleotide corresponding to the CP2 site (−74 to −55 region in the *SOD1* promoter) was incubated with 100 ng of C/EBPα expressed in *E. coli*. In order to express C/EBPα protein in *E. coli*, a GST-C/EBPα fusion plasmid was constructed as described in Section 2. Purified GST-C/EBPα was subjected to MSA reaction mixture. The C/EBPα consensus oligonucleotide and AP2 consensus oligonucleotide were used as specific and non-specific competitor, respectively. The C/EBPα-DNA complex is indicated by a solid arrowhead.

form a heterodimer with the C/EBPα to stimulate transcription from the *SOD1* promoter.

3.3. Direct binding of C/EBPα to the CP2 site of the SOD1 gene

As shown in Fig. 2, the target sequence for C/EBPα was located in the −74 to −55 region. We therefore tested the direct binding activity of C/EBPα to this site by mobility shift assay (MSA). In order to express C/EBPα protein, GST-C/EBPα fusion plasmid was constructed. The expressed GST-C/EBPα in *E. coli* was purified as described in Section 2. We used double-stranded oligonucleotide corresponding to the CP2 site (−74 to −55 region) in the *SOD1* promoter. 100 ng of GST-C/EBPα protein expressed in *E. coli* was used in the MSA. In Fig. 4, a specific C/EBPα-DNA complex was observed in contrast to the control experiment. The specific DNA-protein complex disappeared when the C/EBPα consensus oligonucleotide was added to the reaction mixture. When the AP2 consensus oligonucleotide was used as a non-specific competitor, the specific C/EBPα-DNA complex was not affected. This result shows that a functional C/EBPα binding site was located in the CP2 site of the *SOD1* promoter and C/EBPα activated the transcription of the rat *SOD1* gene through direct binding.

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