

C/EBP homologous protein (CHOP) up-regulates *IL-6* transcription by trapping negative regulating NF-IL6 isoform

Takayuki Hattori¹, Nobumichi Ohoka, Hidetoshi Hayashi, Kikuo Onozaki*

Department of Molecular Health Sciences, Graduate School of Pharmaceutical Sciences, Nagoya City University, Mizuho, Nagoya 467-8603, Japan

Received 9 December 2002; revised 18 March 2003; accepted 18 March 2003

First published online 26 March 2003

Edited by Ned Mantei

Abstract Interleukin-6 (IL-6) production is up-regulated by several stimuli through the activation of transcription factors. We have previously demonstrated that CCAAT/enhancer binding protein homologous protein (CHOP) positively regulates IL-6 production at the transcriptional level in the human melanoma cell line A375. In this study, we provide evidence that CHOP up-regulates the *IL-6* transcription without binding to the *IL-6* promoter. CHOP dimerized more preferentially with an inhibitory isoform of nuclear factor for IL-6 expression (LIP (liver-enriched inhibitory protein)) than with a positively acting isoform (LAP, liver-enriched activator protein). These results indicate that CHOP plays an important role in IL-6 production without binding to its promoter, probably by trapping protein(s) such as LIP, which would otherwise inhibit *IL-6* transcription. © 2003 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: C/EBP homologous protein;
Nuclear factor for IL-6 expression;
Liver-enriched inhibitory protein; Interleukin-6;
Transcription; Dimer

1. Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine that regulates T cell proliferation [1], B cell differentiation [2], and induction of acute phase proteins [3], and is involved in various autoimmune diseases such as chronic rheumatoid arthritis [4]. The human *IL-6* gene has been isolated [5] and the 350-bp region upstream from the transcription initiation site was found to exhibit strong similarity to the cognate mouse sequence. Several transcriptional regulatory elements were

identified in this region. By using deletion mutants of the *IL-6* promoter region, an IL-1 responsive element was found at position –180 to –122 [6], and a 14-bp palindromic DNA motif (ACATTGCACAATCT) in this region was identified as a binding site for nuclear factor for IL-6 expression (NF-IL6) (C/EBP β), a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors. In this region, between –173 and –151, lies a 23-bp DNA element termed multiple responsible element, which is essential for the induction of *IL-6* transcription by treatment with IL-1, tumor necrosis factor (TNF), forskolin, or phorbol ester. Moreover, an activator protein 1 (AP-1) binding motif upstream of this region and a nuclear factor κ B (NF- κ B) binding motif lying downstream are also involved in IL-6 production by IL-1 or TNF α [4].

NF-IL6 has been initially identified as a factor that binds to the *IL-6* promoter region and enhances expression of IL-6 when hepatocytes are treated with IL-1 [7]. NF-IL6 is a member of the C/EBP family of transcription factors. All C/EBP family members possess a basic leucine zipper domain in their carboxyl-terminal region, with which they form homo- or heterodimers. This dimerization is essential for binding to promoter DNA to activate transcription. Normally, NF-IL6 mRNA is not expressed, but is markedly induced in response to infection or inflammation in almost all tissues [7]. NF-IL6 responsive elements are found in several cytokine genes (IL-1, IL-6, IL-8, TNF α , and granulocyte colony stimulating factor (G-CSF)) and acute phase proteins (haptoglobin, C-reactive protein (CRP), α 1-acid glycoprotein), and NF-IL6 plays multiple roles in various tissues [8].

Three isoforms of NF-IL6 (C/EBP β) are generated from a single mRNA through the use of different translation initiation sites and a leaky ribosomal scanning mechanism [9,10]. All of these isoforms possess a common carboxyl-terminal portion, and therefore they can form dimers and subsequently bind to C/EBP binding elements. Among these isoforms, the translation product initiated at the third AUG (LIP (liver-enriched inhibitory protein)) is devoid of the transcription-activation domain located in the amino-terminal portion of the translation product initiated at the first or second AUG (LAP (liver-enriched activator protein)), and consequently the former product stimulates transcription much less efficiently than the latter two products [9,10].

C/EBP homologous protein (CHOP), a member of the C/EBP family of transcription factors, was originally isolated as the gene induced in response to DNA damage agents [11]; subsequently it has been revealed that CHOP is induced by various extracellular or endoplasmic reticulum stresses [12–

*Corresponding author. Fax: (81)-52-836 3419.

E-mail address: konozaki@phar.nagoya-cu.ac.jp (K. Onozaki).

¹ Present address: Department of Biochemistry 1, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan, and CREST, Japan Science and Technology Corporation, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan.

Abbreviations: AP-1, activator protein 1; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; CHOP, C/EBP homologous protein; CRP, C-reactive protein; G-CSF, granulocyte colony stimulating factor; GST, glutathione *S*-transferase; IL, interleukin; LAP, liver-enriched activator protein; LIP, liver-enriched inhibitory protein; NF-IL6, nuclear factor for IL-6 expression; NF- κ B, nuclear factor κ B; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNF, tumor necrosis factor

17]. By forming heterodimers with other C/EBP family proteins such as NF-IL6, CHOP inhibits their DNA binding activity and transactivational ability [18]. The basic region of CHOP is less conserved than that of other C/EBP family proteins. Actually, at first, CHOP was thought to lack DNA binding activity. Interestingly, however, a CHOP–C/EBP heterodimer has been reported to bind to a unique DNA sequence different from classical C/EBP binding sites and to act as a transactivator [19].

Recently, we demonstrated up-regulation of *IL-6* promoter activity by CHOP in a manner dependent on the leucine zipper domain [20]. However, as described above, CHOP is reported to interfere with the transcriptional activity of NF-IL6 that contributes to *IL-6* transcription. Therefore, these observations are inconsistent. In this study, we demonstrate that CHOP mediates the activation of *IL-6* promoter by a novel mechanism, trapping negative regulating isoform of NF-IL6.

2. Materials and methods

2.1. Reagents

RPMI 1640 and Dulbecco's modified Eagle's medium were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was from JRH Bioscience (Lenexa, KS, USA), anti-Flag monoclonal antibody (M2) from Sigma, and anti-NF-IL6 polyclonal antibody (C-19) from Santa Cruz (Santa Cruz, CA, USA).

2.2. Cell culture

The human melanoma cell line A375, the human embryonic kidney cell line 293, and the human hepatocellular carcinoma cell line HepG2 were cultured as described previously [21].

2.3. Plasmid construction

The plasmid pcDNA3.1-Flag-CHOP, leading to a fusion of the Flag epitope tag to the amino-terminus of human CHOP, was constructed as described previously [21]. The entire coding region of human CHOP was subcloned into pGEX-4T-1 (Amersham Bioscience, Little Chalfont, UK). pCMV-Flag-NFIL6 and pCMV-Flag-NFIL6 Δ Spl were constructed by ligating NF-IL6 or NF-IL6 Δ Spl cDNA from pEF-NFIL6 or pEF-NFIL6 Δ Spl [22], kindly provided by Dr. S. Akira (Osaka University, Japan) with pCMV-Tag2 (Stratagene, La Jolla, CA, USA). pIL6(–1165)-Luc plasmid, an *Asp718*/*Xho*I fragment containing the 1174-bp (–1165 to +9 relative to the transcription initiation site) 5'-upstream sequence of the human *IL-6* gene, was prepared as described previously [20]. For other series of reporter genes with deletion mutants of the 5'-flanking region of the *IL-6* gene, pIL6(–835)-Luc and pIL6(–231)-Luc, each fragment of 5'-upstream region of *IL-6* gene obtained by polymerase chain reaction (PCR) from pIL6(–1165)-Luc was ligated with pGL3-basic (Promega, Madison, WI, USA). To obtain p(C/EBP)₄-Luc, four tandem repeats of C/EBP binding elements in the 5'-upstream region of the *IL-6* gene were amplified by PCR and ligated with pGL3-basic. All constructs were verified by sequencing.

pkB4-Luc was kindly provided by Dr. T. Okamoto (Nagoya City University, Japan).

2.4. Transfection

A375 cells were transfected using Effectene (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 293 and HepG2 cells were transfected by the Chen–Okayama method [23].

2.5. Assay for *IL-6* promoter activity

A375 cells were transiently co-transfected with each pIL6-Luc plasmid, pCMV- β -gal, and plasmids as described in the figure legends in a six-well plate. One day later, the medium was changed. After 24 h, luciferase activity was assayed as described previously [20].

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed according to the method of Masternak et al. with slight modification [24]. 293 cells were transiently transfected with pcDNA3.1-Flag-CHOP or pCMV-Flag-NFIL6. Two days later,

the cells were cross-linked with 1% formaldehyde. Immunoprecipitated DNA was analyzed by PCR using the following primers specific for the *IL-6* promoter: sense 5'-AGTGGTGAAGAGACTCAGTG-3' and antisense 5'-GGCAGAATGAGCCTCAGA-3'. The PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining.

2.7. Promoter pull-down assay

By using PCR with a biotinylated primer and each pIL6-Luc plasmid, the promoter templates were biotinylated at the 5'-end of the lower strand. 293 cells were transiently transfected with pcDNA3.1-Flag-CHOP or pCMV-Flag-NFIL6. After 2 days, cells were lysed in RIPA buffer (0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl at pH 8.0, and protease inhibitors). Cell extracts were supplemented with 0.1 mg/ml poly(dI-dC) and incubated with 2 pmol of biotinylated *IL-6* promoter fragments for 2 h at 4°C. Streptavidin-conjugated magnetic particles (50 μ g; Promega) were then added and incubated for an additional 1 h. The particles were washed four times with TNTE buffer (20 mM Tris–HCl at pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA) and proteins were eluted with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer followed by Western blotting analysis.

2.8. Expression of recombinant CHOP protein and glutathione S-transferase (GST) pull-down assay

GST-tagged CHOP protein was expressed in *Escherichia coli* DH5 α and affinity-purified with glutathione-Sepharose 4B (Amersham Bioscience). The purity and concentration of the recombinant protein were estimated by Coomassie brilliant blue staining.

A375 cells were lysed in RIPA buffer. Aliquots of cell extracts were mixed with GST or GST-CHOP; protein–protein complexes were allowed to form for 1 h at 4°C, and then incubated with glutathione-Sepharose beads for an additional 1 h. The beads were washed four times with TNTE buffer, and proteins were eluted with SDS–PAGE sample buffer followed by Western blotting analysis.

2.9. Immunoprecipitation and Western blotting

Cells were transiently transfected and treated as described in the figure legends. The cells were lysed in RIPA buffer. The lysates were subjected to immunoprecipitation with anti-Flag antibody. One to five percent of lysates and immunoprecipitates were subjected to SDS–12.5% PAGE, transferred onto PVDF membranes and probed with antibody(s) described in the figure legends. The immunoreactive proteins were visualized by ECL Western blotting detection reagents (Amersham Bioscience), and the light emission was quantified with a Lumino image analyzer LAS-1000 (FUJI, Japan).

3. Results

3.1. Determination of CHOP responsive elements in the 5'-flanking region of the *IL-6* gene

We have previously observed that CHOP up-regulates *IL-6* promoter activity without any stimulation or treatment [20]. To determine the 5'-cis-regulatory element(s) involved in the stimulatory effect of CHOP, we generated 5'-deletion mutants of the *IL-6* promoter and ligated them into luciferase reporter plasmids. Each reporter plasmid and the CHOP expression plasmid were transiently co-transfected into human melanoma cell line A375, and then the luciferase activity in the cell lysates was measured. Transfection of CHOP considerably up-regulated the luciferase activity in comparison with mock transfection (Fig. 1A). The luciferase activity was significantly increased when the region from –1165 to –835 bp was deleted, suggesting that a repressor-like factor may affect or a silencer-like element may exist in this region. Even in this case, a stimulatory effect of CHOP on the promoter activity was observed. The luciferase activity was not further changed upon further deletion of the upstream region to –231 bp, even though this deletion removed a putative AP-1 responsive element. In addition, this –231 region, containing NF-IL6

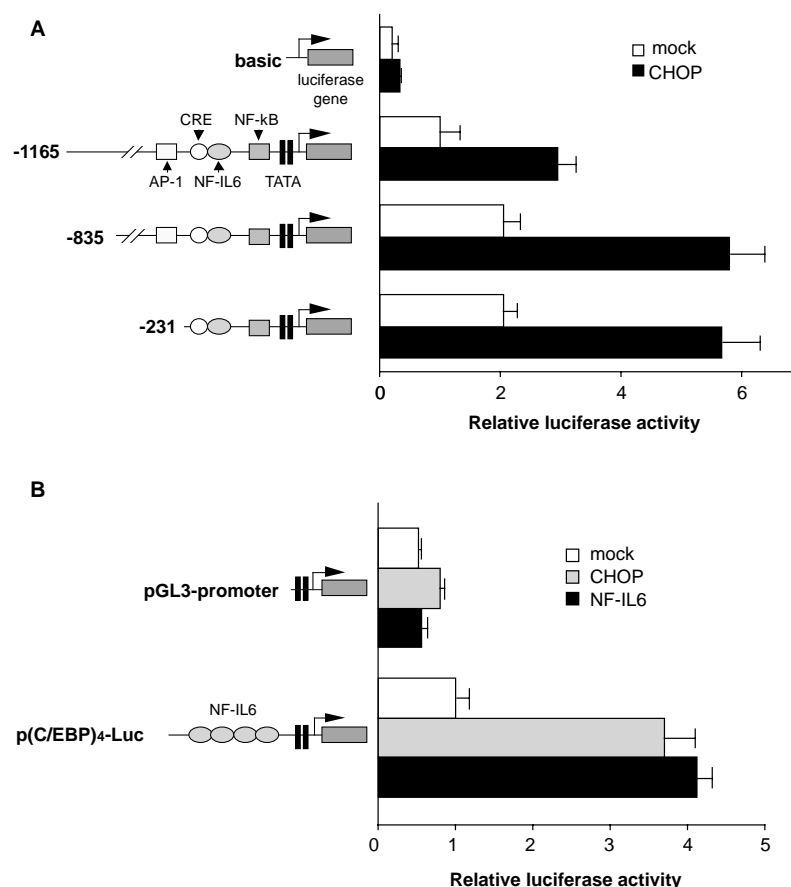


Fig. 1. Deletion analysis of the 5'-flanking region of the human *IL-6* gene in A375 cells. A: Putative consensus sequences in the 5'-upstream region of the human *IL-6* gene fused with luciferase cDNA are illustrated. Numbers indicate distance in base pairs from the transcription start. Each pIL6-Luc reporter plasmid (0.02 µg), β-galactosidase plasmid (0.04 µg) and pcDNA3.1-Hygro (open column) or pcDNA3.1-Myc/hCHOP (closed column) (0.4 µg each) was transiently co-transfected into A375 cells. B: A reporter plasmid containing tandem repeats of a putative C/EBP binding site in the 5'-upstream region of the human *IL-6* gene, fused to luciferase cDNA (0.02 µg), β-galactosidase plasmid (0.04 µg) and pcDNA3.1-Hygro (open column) (0.4 µg), pcDNA3.1-Myc/hCHOP (gray column) (0.4 µg) or pCMV-Flag-NFIL6 (0.04 µg) (closed column) were transiently co-transfected into A375 cells. Plasmid pGL3-promoter was transfected as a control. The relative promoter activity indicates the ratio between the luciferase activity in mock transfectants and those in others after the normalization with β-galactosidase activity expression. Mean ± S.D. based on triplicate cultures is shown.

and NF-κB binding elements, maintained responsiveness to CHOP. Because CHOP readily dimerizes with other C/EBP family members containing NF-IL6 [18], we focused on the NF-IL6 responsive element in this region. To assess whether this element is critical for the CHOP-dependent activation of *IL-6* transcription, we further constructed an NF-IL6-lucifer-

ase reporter gene containing four putative NF-IL6 binding sites derived from the *IL-6* promoter region. As shown in Fig. 1B, overexpression of CHOP produced an increase of luciferase activity comparable to that seen with NF-IL6 in A375 cells. On the other hand, with an NF-κB reporter gene (pκB4-Luc), no activation by CHOP was observed, and

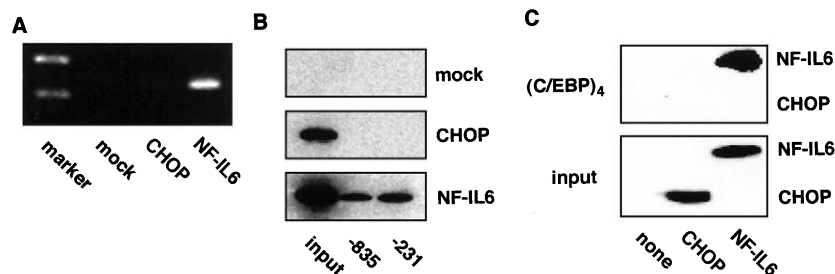


Fig. 2. CHOP does not bind to the promoter region of the *IL-6* gene. A: Cross-linked chromatin isolated from 293 cells transfected with pcDNA3.1-Hyg, pcDNA3.1-Flag-CHOP, or pCMV-Flag-NFIL6 (2 µg each) was immunoprecipitated with antibody against Flag-tag. Immunoprecipitates were analyzed by PCR to confirm the presence of promoter sequence of the human *IL-6* gene. B,C: Recruitment of C/EBP family protein to *IL-6* promoter fragments dependent on NF-IL6 binding element. 293 cells were transiently transfected with pcDNA3.1-Hyg (2 µg), pcDNA3.1-Flag-CHOP (2 µg), or pCMV-Flag-NFIL6 (0.2 µg). Cell extracts (input) were subjected to pull-down assay as described in Section 2. (C/EBP)4 represents four tandem repeats of the putative NF-IL6 binding element (located at position -163 to -150).

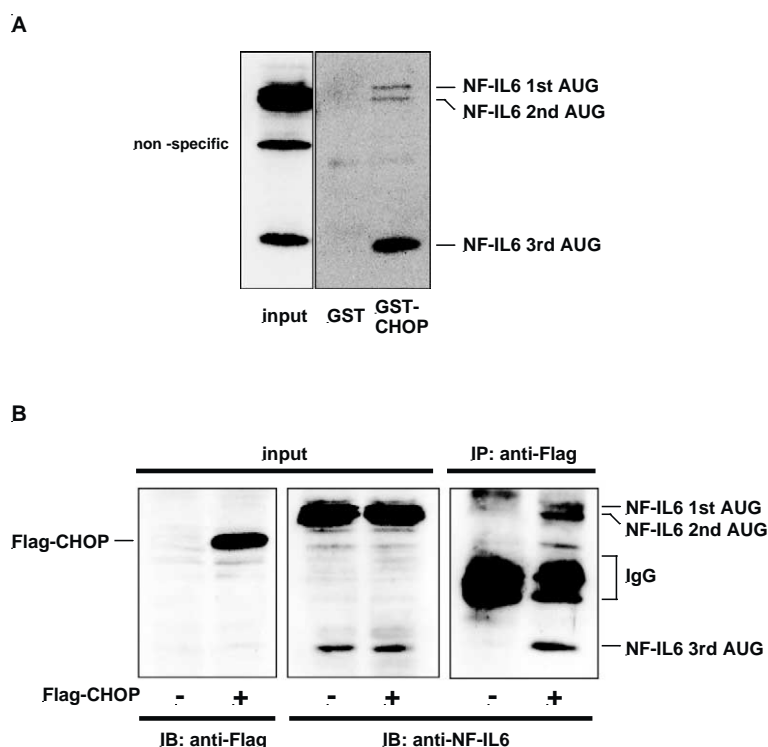


Fig. 3. CHOP preferentially dimerizes with a negatively regulating isoform of NF-IL6, NF-IL6 3rd AUG. A: Cell extracts (input) from A375 cells were subjected to pull-down analysis with recombinant GST-CHOP immobilized on glutathione-Sepharose beads. Proteins associated with GST-CHOP were purified and then analyzed by immunoblotting with anti-NF-IL6 C-terminal antibody. B: HepG2 cells were transiently transfected with pcDNA3.1-Flag-CHOP or empty vector (10 μ g each). After 2 days, cell extracts (input) were subjected to immunoprecipitation (IP) with anti-Flag antibody. Whole extracts and precipitates were analyzed by immunoblotting (IB) using anti-Flag or anti-NF-IL6 C-terminal antibody.

deletion of the NF- κ B binding site from the pIL6(–835)-Luc reporter gene exhibited the same stimulatory response to CHOP as did the wild-type reporter gene (data not shown). These results were also observed in human embryonic kidney cell 293 cells and suggest that CHOP-induced *IL-6* promoter activation could be dependent on the promoter's NF-IL6 response element.

3.2. CHOP is not recruited to the *IL-6* promoter region

By using CHOP mutants, we have previously demonstrated that dimerization ability is important for activation of *IL-6* promoter by CHOP [20]. Thus, it was expected that dimerization of CHOP with other C/EBP family proteins would be required for *IL-6* promoter activation rather than binding to and transactivating the *IL-6* promoter. To determine whether CHOP is physically associated with the *IL-6* promoter in living cells, we next performed ChIP assay. For this experiment it was necessary to use 293 cells, because of their high transfection efficiency. Cross-linked chromatin fragments were isolated from 293 cells transfected with Flag-tagged CHOP or NF-IL6, immunoprecipitated with antibody against Flag-tag, and analyzed by PCR for the presence of specific DNA sequence corresponding to the *IL-6* promoter. As shown in Fig. 2A, *IL-6* promoter sequences were immunoprecipitated with anti-Flag antibody only from Flag-NF-IL6-transfected cells, but not from Flag-CHOP-transfected cells. To confirm the result of the ChIP assay, we examined *in vitro* binding of these C/EBP proteins to *IL-6* promoter sequence by promoter pull-down assay. *IL-6* promoter templates of various lengths were immobilized on magnetic beads and cell extracts from

293 cells transiently transfected with Flag-NF-IL6 or Flag-CHOP were assessed in a 'pull-down' assay by using an anti-Flag antibody. NF-IL6 bound to *IL-6* promoter templates containing the upstream region from –835 to +9 (Fig. 2B). NF-IL6 also bound to promoter template containing the –231-bp upstream region. Furthermore, NF-IL6 bound to the (C/EBP)₄ sequence, which has four tandem repeats of NF-IL6 binding elements as found in the *IL-6* promoter, while CHOP did not bind to any *IL-6* promoter templates at all (Fig. 2C). These findings suggest that NF-IL6 but not CHOP binds to NF-IL6 responsive element in the *IL-6* promoter.

3.3. CHOP preferentially associates with a negatively regulating NF-IL6 isoform, NF-IL6 3rd AUG, rather than with positively regulating isoforms

The results above suggested that CHOP up-regulates *IL-6* transcription without interacting with the *IL-6* promoter region. Accordingly, we hypothesized that CHOP regulates the activity of NF-IL6, which in turn binds to the *IL-6* promoter region and activates *IL-6* transcription. To examine this possibility, cell extracts from A375 cells were subjected to GST pull-down by using GST or GST-CHOP and associated endogenous NF-IL6 isoforms were detected by immunoblotting using an anti-NF-IL6 antibody recognizing its C-terminal region. Compared with the amount of each NF-IL6 isoform in input cell extracts, GST-CHOP bound almost solely the amino-terminal truncated isoform of NF-IL6, NF-IL6 3rd AUG, which has no transcriptional activity (Fig. 3A). These results were also observed in HepG2 and 293 cells (data not shown).

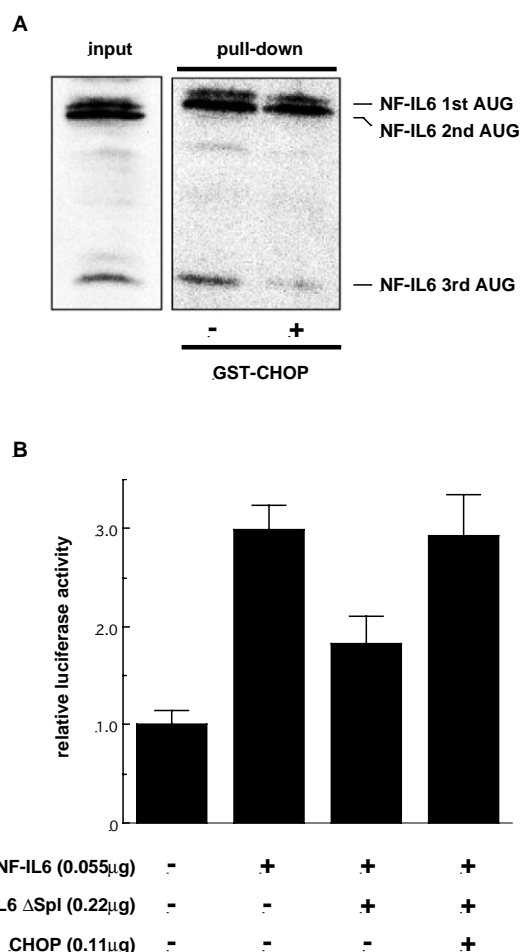


Fig. 4. CHOP activates *IL-6* transcription by removing NF-IL6 3rd AUG from *IL-6* promoter. A: HepG2 cell extracts were subjected to promoter pull-down assay as described in Fig. 2 using a DNA template corresponding to an *IL-6* promoter fragment containing 240 bp (–231 to +9 relative to the transcription initiation site) in the absence or presence of 5 μg GST-CHOP and then analyzed by immunoblotting using anti-NF-IL6 C-terminal antibody. Addition of GST instead of GST-CHOP did not affect the precipitated NF-IL6 isoforms (not shown). B: A375 cells were transiently co-transfected with pIL6(–231)-Luc (0.02 μg), β-galactosidase plasmid (0.04 μg) and the indicated constructs. The relative promoter activity indicates the ratio between the luciferase activity in mock transfectants and that in other conditions after normalization with β-galactosidase activity. Mean ± S.D. based on triplicate cultures is shown.

To examine *in vivo* association of endogenous NF-IL6 isoforms with CHOP, we subjected cell extracts from HepG2 cells, transiently transfected with Flag-CHOP, to immunoprecipitation with an anti-Flag antibody and then analyzed the precipitate by immunoblotting using an anti-NF-IL6 antibody. As in the GST pull-down assay, CHOP preferentially associated with NF-IL6 3rd AUG, rather than with NF-IL6 1st or 2nd AUG (Fig. 3B). These results indicated that CHOP preferentially associates with the transcriptionally inactive NF-IL6 isoform, NF-IL6 3rd AUG, rather than with the transcriptionally active NF-IL6 1st or 2nd AUG isoforms.

3.4. CHOP activates *IL-6* transcription by releasing NF-IL6 3rd AUG from the *IL-6* promoter

Because the CHOP–C/EBP heterodimer could not bind classical a C/EBP binding motif [18], and because CHOP

preferentially associated with NF-IL6 3rd AUG, we next investigated by promoter pull-down assay whether CHOP alters the profile of NF-IL6 isoforms that bind to the *IL-6* promoter region. In the absence of CHOP, all endogenous NF-IL6 isoforms bound to *IL-6* promoter template in the same proportion as in the input extract (Fig. 4A, lanes 1 and 2). In contrast, when recombinant CHOP protein was added to the assay system, NF-IL6 3rd AUG was remarkably removed from the *IL-6* promoter template without any changes in NF-IL6 1st AUG and 2nd AUG binding to the *IL-6* promoter template (Fig. 4A, lane 3). To determine whether CHOP recovers the *IL-6* transcription down-regulated by NF-IL6 3rd AUG, we carried out reporter gene assay using pIL6(–231)-Luc. As shown in Fig. 4B, the luciferase activity was increased by co-transfection with NF-IL6. NF-IL6 ΔSpl, which mimics NF-IL6 3rd AUG [22], inhibited *IL-6* promoter activity up-regulated by NF-IL6. As expected, when CHOP was co-transfected, down-regulation of *IL-6* promoter activity induced by NF-IL6 ΔSpl was recovered to levels comparable to those seen after transfection with NF-IL6 alone. These observations suggest that CHOP up-regulates *IL-6* transcription by eliminating from the *IL-6* promoter dimers comprising NF-IL6 3rd AUG together with NF-IL6 1st or 2nd AUG–NF-IL6.

Finally we examined the dose–response effect of CHOP expression on *IL-6* transcription in A375 cells. As shown in Fig.

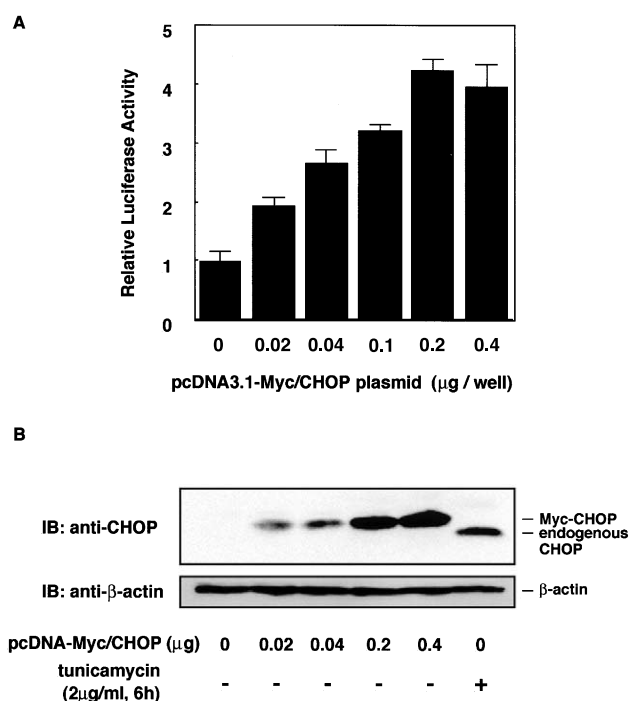


Fig. 5. Dose–response effect of CHOP on the *IL-6* gene transcription. A: A375 cells were transiently co-transfected with pIL6(–231)-Luc (0.02 μg), β-galactosidase plasmid (0.04 μg) and the indicated amounts (0.02–0.4 μg) of pcDNA3.1-Myc/hCHOP. The relative promoter activity indicates the ratio between the luciferase activity in mock transfectants and that in other conditions after normalization with β-galactosidase activity. Mean ± S.D. based on triplicate cultures is shown. B: A375 cells were transiently transfected with the indicated amounts of pcDNA3.1-Myc/CHOP. Cell extracts from each transfectant or from cells treated with 2 μg/ml of tunicamycin for 6 h were analyzed by immunoblotting (IB) using anti-CHOP or anti-β-actin antibody.

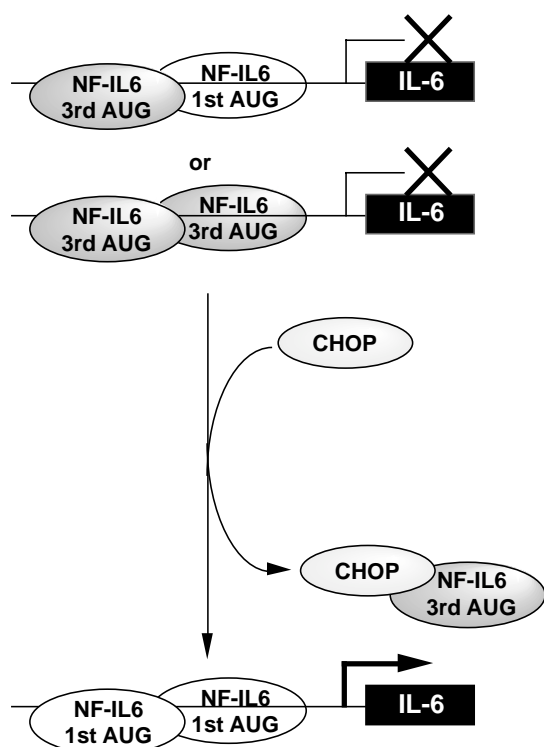


Fig. 6. Proposal mechanism for *IL-6* gene transcription mediated by CHOP. A heterodimer comprising NF-IL6 1st (or 2nd) AUG and NF-IL6 3rd AUG, or an NF-IL6 3rd AUG homodimer, occupies the C/EBP binding element in the *IL-6* promoter and therefore *IL-6* transcription is inactive. CHOP may enhance *IL-6* gene transcription by sequestering the negatively regulating isoform NF-IL6 3rd AUG away from NF-IL6 1st (or 2nd) AUG or from the C/EBP binding element.

5A, CHOP augmented *IL-6* promoter activity in a dose-dependent manner up to 0.2 μ g of plasmid. More than 0.2 μ g of plasmid tended to decrease the transcriptional activity. At high levels of CHOP expression, LIP would be completely sequestered by CHOP, but excess CHOP would then also make LAP unavailable for transactivation. To assess the physiological significance of the phenomenon, we examined the expression level of endogenous CHOP in A375 cells under conditions of stress. The level of CHOP expression in A375 cells treated with tunicamycin is similar to the levels of transfected CHOP expression that enhance *IL-6* expression (equal to transfection of 0.04–0.2 μ g plasmid) (Fig. 5A,B). These results indicated that the endogenous CHOP level in stressed A375 cells is not so high as to exhibit inhibition of *IL-6* transactivation.

4. Discussion

We have previously demonstrated that CHOP up-regulated *IL-6* production at the transcriptional level, and that the leucine zipper domain of CHOP is required for its activity [20]. Because CHOP could not form homodimers [19], it was suggested that heterodimerization of CHOP with other C/EBP family proteins was critical. However, the molecular mechanism for CHOP-induced stimulation of *IL-6* transcription was not well understood.

Originally it had been thought that CHOP acts as an inhibitor for other C/EBP family proteins by forming hetero-

dimers [18]. However, *IL-6* production was up-regulated and an *IL-6* reporter gene was activated when CHOP was expressed [20, Fig. 1]. Therefore, we at first thought that CHOP up-regulated *IL-6* transcription through its own transcriptional activity by binding to a specific motif on the *IL-6* promoter region as reported by Ubeda et al. [19]. Unexpectedly, there was no reported CHOP binding element in the *IL-6* promoter and no binding of CHOP to the *IL-6* promoter could be demonstrated either by ChIP assay or by promoter pull-down assays. In addition, our previous study demonstrated that CHOP could activate the *IL-6* promoter even if the p38 mitogen-activated protein kinase-dependent phosphorylation site or the DNA binding domain was defective [20]. These observations strongly suggest that CHOP activates *IL-6* promoter in a manner independent of its transcriptional activity and interaction with *IL-6* promoter DNA.

As described above, CHOP could activate *IL-6* transcription despite its inhibitory effect on the transcriptional activity of NF-IL6. Of the C/EBP family transcription factors, Ig/EBP (C/EBP γ), ubiquitously expressed in various tissues [25,26], and LIP (NF-IL6 3rd AUG), the translation product initiated at the third AUG on the NF-IL6 mRNA [9,10], have been shown to inhibit the function of C/EBP transcriptional activators by forming heterodimers and have been proposed to act as a buffer against C/EBP-mediated activation. Actually, these inhibitory C/EBPs suppressed activation of *IL-6* promoter induced by NF-IL6 (LAP) even in our model (data not shown, Fig. 4B). These reports and observations suggested to us the following hypothesis accounting for the mechanism of CHOP-mediated *IL-6* promoter activation. At steady state, these inhibitory C/EBP proteins suppress transcriptional activity of NF-IL6 (LAP) by forming heterodimers or occupying the NF-IL6 binding motif in the *IL-6* promoter. However, when CHOP is present, it sequesters the inhibitory C/EBP proteins away from NF-IL6 (LAP) or the *IL-6* promoter by dimerizing with them. Consequently, homodimer formation of NF-IL6 (LAP) and its binding to *IL-6* promoter are augmented, which leads to activation of *IL-6* transcription (Fig. 6). This hypothesis was confirmed in this study. In fact, CHOP dimerized preferentially with NF-IL6 3rd AUG, the inhibitory isoform of NF-IL6, both in vitro and in vivo. Furthermore, CHOP diminished the amount of NF-IL6 3rd AUG bound to an *IL-6* promoter template, whereas NF-IL6 1st and 2nd AUG were not affected. Consistent with these observations, CHOP rescued transcriptional activity of NF-IL6 down-regulated by NF-IL6 Δ Spl, an NF-IL6 derivative that mimics NF-IL6 3rd AUG.

Sylvester et al. reported that during the acute phase response induced by LPS, CHOP seemed to be induced by newly produced NF-IL6 via a C/EBP regulatory site in the promoter region of CHOP in rat liver or human hepatoma cell line [27]. Similar to CHOP induction, acute phase protein induction is temporally delayed relative to NF-IL6 induction. In this case LPS leads to the relatively sustained induction of acute phase proteins compared to the NF-IL6 or CHOP induction. The LIP trapping effect of CHOP might cause this sustained induction.

Recently Hu et al. presented that LIP or NF-IL6 bZIP domain can mediate LPS induction of *IL-6* through NF- κ B activation in murine B lymphoblast P388 cells [28]. In our study, however, LIP overexpression suppressed *IL-6* transactivation and CHOP rescued this effect (Fig. 4B). Moreover,

NF- κ B seemed to contribute little to the CHOP-dependent IL-6 transactivation (data not shown). The expression levels of LIP in A375, 293 and HepG2 cells are relatively low compared to that of LAP. Overexpression of LIP or NF-IL6 bZIP in P388, which probably had no LAP, resulted in a different output. We have to consider the balance of expression levels of LAP/LIP/CHOP in the cells. This balance might be dependent on the cell types and also be changed by various stimulants.

From the dose–response experiments, an optimum dose of CHOP for the IL-6 transcription was observed. At high levels of CHOP expression, NF-IL6 3rd AUG (LIP) would be completely sequestered by CHOP, but excess CHOP would then also make NF-IL6 1st and 2nd AUG (LAP) unavailable for transactivation. CHOP has been reported to be induced by various stresses in cells. Our results transfecting CHOP expression plasmid in A375 cells suggest that the level of endogenous CHOP in stressed cells is enough to trap LIP but not so high as to show the inhibition of IL-6 transactivation. These results indicate that physiologically induced CHOP mostly leads to augmentation of IL-6 transcription by trapping LIP.

This is a novel regulatory mechanism of CHOP on NF-IL6 function. Because NF-IL6 is responsible for activation of many genes including cytokines (TNF α , IL-1, IL-8 and G-CSF) and acute phase proteins (haptoglobin, CRP, α 1-acid glycoprotein) [8], CHOP may also contribute to the regulation of those genes.

Acknowledgements: We thank Dr. S. Akira and Dr. T. Okamoto for providing expression plasmids. This work was supported in part by Grant-in-Aids for Scientific Research (B) from the Japan Society for the Promotion of Science, and Grant-in-Aids for Scientific Research on Priority Areas (C) from The Ministry of Education, Science, Sports and Culture.

References

- [1] Lotz, M., Jirik, F., Kabouridis, R., Tsoukas, C., Hirano, T., Kishimoto, T. and Carson, D.A. (1988) *J. Exp. Med.* 167, 1253–1258.
- [2] Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimizu, K., Nakajima, K., Pyun, K.H. and Kishimoto, T. (1985) *Proc. Natl. Acad. Sci. USA* 82, 5490–5494.
- [3] Gauldie, J., Richards, C., Harnish, D., Lansdorp, P. and Baumann, H. (1987) *Proc. Natl. Acad. Sci. USA* 84, 7251–7255.
- [4] Akira, S., Taga, T. and Kishimoto, T. (1993) *Adv. Immunol.* 54, 1–78.
- [5] Yasukawa, K., Hirano, T., Watanabe, Y., Muratani, K., Matsuda, T., Nakai, S. and Kishimoto, T. (1987) *EMBO J.* 10, 2939–2945.
- [6] Isshiki, H., Akira, S., Tanabe, O., Nakajima, T., Shimamoto, T., Hirano, T. and Kishimoto, T. (1990) *Mol. Cell. Biol.* 10, 2757–2764.
- [7] Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T. and Kishimoto, T. (1990) *EMBO J.* 6, 1897–1906.
- [8] Akira, S. and Kishimoto, T. (1992) *Immunol. Rev.* 127, 25–50.
- [9] Descombes, P. and Schibler, U. (1991) *Cell* 67, 569–579.
- [10] An, M.R., Hsieh, C.C., Reisner, P.D., Rabek, J.P., Scott, S.G., Kuninger, D.T. and Papaconstantinou, J. (1996) *Mol. Cell. Biol.* 16, 2295–2306.
- [11] Fornace, A.J., Neibert, D.W., Hollander, M.C., Luethy, J.D., Papathanasiou, M., Fragoli, J. and Holbrook, N.J. (1989) *Mol. Cell. Biol.* 9, 4196–4203.
- [12] Bartlett, J., Luethy, J., Carlson, S., Sollott, S. and Holbrook, N.J. (1992) *J. Biol. Chem.* 267, 20465–20470.
- [13] Price, B. and Calderwood, S. (1992) *Cancer Res.* 52, 3814–3817.
- [14] Carlson, S.G., Fawcett, T.W., Bartlett, J.D., Bernier, M. and Holbrook, N.J. (1993) *Mol. Cell. Biol.* 13, 4736–4744.
- [15] Marten, N.W., Burke, E.J., Hayden, J.M. and Straus, D.S. (1994) *FASEB J.* 8, 538–544.
- [16] Halleck, M.M., Holbrook, N.J., Skinner, J., Liu, H. and Stevens, J. (1997) *Cell Stress Chaperones* 2, 31–40.
- [17] Zinszner, H., Kuroda, M., Wang, X.-Z., Batchvarova, N., Lightfoot, R.T., Remotti, H., Stevens, J.L. and Ron, D. (1998) *Genes Dev.* 12, 982–995.
- [18] Ron, D. and Habener, J.F. (1992) *Genes Dev.* 6, 439–453.
- [19] Ubeda, M., Wang, X.-Z., Zinszner, H., Wo, I., Habener, J.F. and Ron, D. (1996) *Mol. Cell. Biol.* 16, 1479–1489.
- [20] Hattori, T., Itoh, S., Hayashi, H., Chiba, T., Takii, T., Yoshizaki, K. and Onozaki, K. (2001) *J. Interferon Cytokine Res.* 21, 323–332.
- [21] Hattori, T., Ohoka, N., Inoue, Y., Hayashi, H. and Onozaki, K. (2003) *Oncogene* 22, 1273–1280.
- [22] Nishio, Y., Isshiki, H., Kishimoto, T. and Akira, S. (1993) *Mol. Cell. Biol.* 13, 1854–1862.
- [23] Chen, C. and Okayama, H. (1987) *Mol. Cell. Biol.* 7, 2745–2749.
- [24] Masternak, K., Muhlethaler-Mottet, A., Villard, J., Zufferey, M., Steimle, V. and Reith, W. (2000) *Genes Dev.* 14, 1156–1166.
- [25] Roman, C., Platero, J.S., Shuman, J. and Calame, K. (1990) *Genes Dev.* 4, 1404–1415.
- [26] Cooper, C., Henderson, A., Artandi, S., Avitahl, N. and Calame, K. (1995) *Nucleic Acids Res.* 23, 4371–4377.
- [27] Sylvester, S.L., ap Rhys, C.M.J., Luethy-Martindale, J.D. and Holbrook, N.J. (1994) *J. Biol. Chem.* 269, 20119–20125.
- [28] Hu, H.-M., Tian, Q., Baer, M., Spooner, C.J., Williams, S.C., Johnson, P.F. and Schwartz, R.C. (2000) *J. Biol. Chem.* 275, 16373–16381.