Biochemical and Biophysical Research Communications 442 (2013) 72–78

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of an enhancer region for immune activation in the human GTP cyclohydrolase I gene

Yu Liang [a], Hidehito Inagaki [b], Qinyu Hao [a], Masaru Sakamoto [a], Tamae Ohye [b], Takahiro Suzuki [a,1], Hiroshi Ichinose [a,*]

[a] Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan
[b] Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake 470-1192, Japan

ARTICLE INFO

Article history:
Received 31 October 2013
Available online 9 November 2013

Keywords:
C/EBP
Enhancer
GTP cyclohydrolase I
Lipopolysaccharide
Tetrahydrobiopterin

ABSTRACT

GTP cyclohydrolase I (GCH) catalyzes the first and rate limiting step reaction for the *de novo* synthesis of 5,6,7,8-tetrahydrobiopterin (BH4). The expression of GCH is dramatically elevated by immune activation, while the mechanism remains to be elucidated. In this study, we investigated the transcription mechanism of the GCH gene using lipopolysaccharide (LPS) to stimulate mouse macrophage RAW264.7 cells. With luciferase assay, we found a highly conserved enhancer region spanning approximately 300 bp in intron 1 of GCH gene as a response element to LPS stimulation. The same enhancer region was also responsible for the induction of the GCH gene by IFN-γ and TNF-α in HUVECs. With electrophoresis mobility shift assay (EMSA) and site directed mutation analysis, we identified two key fragments containing C/EBP and Ets binding motifs within the enhancer. Furthermore, C/EBP-β was involved in LPS activated GCH transcription through direct binding to the enhancer shown by supershift, chromatin immunoprecipitation, and RNA interference experiments. In conclusion, our findings uncovered a novel mechanism of GCH transcriptional regulation by immune activation.

1. Introduction

Tetrahydrobiopterin (BH4) is well known as an essential co-factor for three aromatic amino acid hydroxylases (tryptophan hydroxylase, phenylalanine hydroxylase, and tyrosine hydroxylase), nitric oxide synthase (NOS), and alkylglycerol monooxygenase [1]. The physiological role of BH4 has been extensively studied and includes amino acid and catecholamine metabolism, immune activation and endothelial regulation. Deficiency of BH4 has been observed in phenylketonuria, hyperphenylalaninemia, neural disorder, cardiovascular dysfunction, etc., suggesting that regulation of BH4 is tightly coupled to multiple biological processes in physiological and pathological conditions [1–3].

The physiological role of BH4 in the immune system is mainly understood by its key regulatory role in NOS activity, as the dimerization and coupling of NOS controlled by the BH4/BH2 ratio are critical for the synthesis of NO rather than superoxide [4]. Under immune activation, immune cells, such as macrophages, natural killer cells, and monocytes, generate and release NO, with elevated iNOS expression and a concomitant increase of BH4 synthesis. The cellular level of BH4 is mainly regulated by the expression level and catalytic activity of GCH (E.C. 3.5.4.16), the rate-limiting enzyme in the *de novo* biosynthesis of BH4, which converts GTP to 7,8-dihydroneopterin triphosphate. Although the regulatory mechanism of iNOS has been extensively studied, little is known about how GCH expression is regulated during immune activation. In addition, neopterin, another biopterin metabolite, is considered a marker of pro-inflammatory activation and has been widely used in clinical diagnosis as well as the monitoring and prognosis of cell-mediated immunity, such as in cancer, chronic heart failure, and HIV infection [5,6]. However, the physiological role of neopterin during cell-mediated immunity is not fully understood. We believe that studying the transcriptional regulatory mechanism of GCH by immune activation would help to answer these issues.

LPS has been widely used as endotoxin to elicit immune responses. The stimulation of mammalian cells by LPS occurs through binding with a series of partners, including LPS binding protein (LBP), CD14, TLR4 [7,8], and MD-2 [9]. Immune responses are activated via several critical signaling pathways, including p38/MAPK, NF-κB, JNK, and MEK [7]. It has been reported that

Abbreviations: BH4, tetrahydrobiopterin; C/EBP, CCAAT enhancer binding protein; GCH, GTP cyclohydrolase I; HUVEC, human umbilical vein endothelial cell; IFN, interferon; LPS, lipopolysaccharide; NOS, nitric oxide synthase; TNF, tumor necrosis factor.

* Corresponding author. Fax: +81 45 924 5807.
E-mail address: hichinos@bio.titech.ac.jp (H. Ichinose).

[1] Present address: School of Dentistry, Aichi-Gakuin University, Nagoya 464-8650, Japan.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.11.002

GCH mRNA and catalytic activity are enhanced by LPS treatment in the murine neuroblastoma cell line NIE-115 [10]. However, it was unclear how the GCH gene was regulated during immune activation.

In our current research, we studied LPS-activated GCH expression in mouse macrophage RAW264.7 cells by investigating the response elements as well as binding factors. Our findings revealed a key aspect in the transcriptional regulation of GCH by immune activation, that is, the involvement of an essential intronic enhancer and C/EBP-β.

2. Material and methods

2.1. Cell culture and treatment

The mouse macrophage cell line RAW264.7 was cultured in Dulbecco's modified Eagle medium (Wako) with 10% fetal bovine serum (Biowest) at 37 °C, 5% CO_2. LPS treatment was performed by adding 1.0 μg/ml to serum-free medium for 6 h, unless otherwise indicated, with an equivalent amount of PBS as a control.

2.2. Transfection

Transfection was performed with X-tremeGene HP DNA transfection reagent (Roche) according to the manufacturer's instructions. Briefly, cells were cultured in 24-well plates in 500 μL medium one day prior to transfection, which allowed the cells to grow to 70% confluence prior to transfection. DNA plasmid (0.5 μg) was diluted in 50 μL Opti-MEM reduced serum medium (Invitrogen), and 1.5 μL transfection reagent was added and incubated at room temperature for 15 min. In RNA interference experiments, C/EBP-β siRNA mixture or control siRNA (10 pmol, Santa Cruz) together with 0.2 μg reporter plasmids were used. The DNA or DNA/siRNA-reagent complex (50 μL) was added to each well, and the cultures were allowed to grow for 20–24 h, followed by LPS treatment for 6 h.

2.3. Western blot

Cells cultured and treated in 6-well plates were collected and homogenized with NP-40 lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40). The amount of protein in the lysates was quantified by the Bradford method. Lysate (50 μg) was loaded onto an SDS–PAGE gel. After electrophoresis and transfer to PVDF membrane, the amount of GCH protein was detected by incubating with polyclonal GCH antibody (αGCH-N2 164-9-2) [11] at 4 °C overnight. The blots were incubated with secondary antibody (HRP-linked goat anti mouse/rabbit IgG, Amersham Biosciences) for 1 h at room temperature and then washed. The protein bands were visualized with HRP substrate (Millipore).

2.4. GCH activity assay

Cells were collected with a cell scraper, washed twice and then suspended in GCH reaction buffer (100 mM Tris–HCl pH 8.0, 300 mM KCl, 2.5 mM EDTA, 10% glycerol). Cell lysates were obtained by freeze–thaw at −80 °C and centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatants were immediately collected to measure GCH activity as previously described [11].

2.5. Luciferase assay

Dual luciferase assays were performed with a dual-luciferase reporter assay system (Promega). Cells were first transfected with the firefly luciferase insertion plasmid PicaGene Basic Vector 2 (pGV-B2, Toyo Ink) and the *Renilla* luciferase plasmid phRG-TK (Promega), as a control for transfection efficiency, at a 9:1 ratio. The ratio between firefly and *Renilla* luminescence represents the relative transcriptional activity of the reporter plasmid.

2.6. Electrophoretic mobility shift assay (EMSA)

Biotin-labeled and unlabeled DNA oligonucleotides were synthesized as shown in Supplement Table (Operon). Annealing was performed by heating the forward and reverse oligonucleotides at 95 °C followed by slowly cooling down to room temperature. The LightShift Chemiluminescent EMSA kit (Thermo) was used according to the manufacturer's instructions. In each sample, 20 fmol biotin-labeled DNA with or without 100 times the amount of unlabeled DNA (2 pmol) was added to binding buffer (10 mM Tris–HCl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 10 mM EDTA, 50 ng/μL Poly (dI–dC), 2.5% glycerol, 0.05% NP-40, and 1 mM dithiothreitol) containing 8 μg nuclear extract and incubated at room temperature for 20 min. Supershift experiment was conducted by adding 0.8 μg antibody for PU.1 (T-21X, Santa Cruz), Ets-1 (C-20X, Santa Cruz) or C/EBP-β (C-19X, Santa Cruz) or rabbit normal IgG (Santa Cruz) to the binding reaction. Samples were then loaded onto a pre-electrophoresed 6% polyacrylamide gel in 0.5× TBE buffer under constant voltage (100 V) for 50 min. The DNA was transferred to a positively charged nylon membrane by electrical transfer and cross-linked by 312 nm UV light for 15 min. Biotin-labeled DNA was detected with HRP-conjugated avidin by chemiluminescence.

2.7. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed following the protocol of Millipore, with modifications. Briefly, RAW264.7 cells were cultured in 10-cm dishes and treated with 1 μg/mL LPS for 6 h. After crosslinking, the cells were lysed and sonicated to obtain approximately 500-bp DNA fragments. Sonicated samples were pre-cleared with protein G Sepharose beads (preblocked with 0.5 mg/mL BSA and 0.2 mg/mL salmon sperm DNA, GE Healthcare) and an aliquot of the supernatant was retained as the input. The rest of the supernatant was used in immunoprecipitation experiments with 2 μg C/EBP-β antibody (C-19X, Santa Cruz) or rabbit normal IgG at 4 °C overnight under rotation. Immunoprecipitates were captured with protein G Sepharose beads and washed with low salt, high salt, LiCl washing solution, and TE buffer. Immune complexes eluted with 1% SDS in 0.1 M $NaHCO_3$ solution were heated at 65 °C for 4 h to reverse crosslinking, followed by Protease K treatment to release the DNA from the immune complexes. The DNA was finally purified by ethanol precipitation and dissolved in 10 μL TE buffer. Semi-quantitative PCR was performed with primers flanking the C/EBP consensus binding motifs or at the 3′ untranslated region of GCH as a negative control. Primers are listed as a Supplement Table.

3. Results

3.1. Enhanced GCH transcription by LPS requires intron 1

As part of the inflammatory response, the transcription of GCH in immune cells is induced by immunogen like LPS. Therefore we stimulated mouse macrophage RAW264.7 cells with LPS to study immune-activated GCH expression. We first confirmed the induced GCH activity and expression levels in RAW264.7 cells after LPS treatment. Addition of 1 μg/mL LPS to the medium induced GCH activity 5.5-fold after 6 h and 15-fold after 24 h compared with non-treated cells (Fig. 1A). The amount of GCH protein was also

increased markedly in whole cell lysate 6 h after LPS treatment (Fig. 1B).

Next, to examine the transcription mechanism of GCH under LPS stimulation, we transfected RAW264.7 cells with a series of luciferase constructs harboring the 5′ flanking region of the GCH gene and luciferase assays were conducted. Surprisingly, in contrast to the effects of NGF treatment on PC12D cells [12], no elevation in luciferase activity in LPS-treated RAW264.7 cells was observed. As the expression of the GCH gene in RAW264.7 cells was indeed activated, it is highly possible that some extra regulatory elements in addition to the GCH promoter are required for the elevated transcription. Thus, we sought to identify the response elements. We inserted different regions of the human GCH gene into hGCHpro5.2 k, a luciferase construct with a 5.2-kb promoter region of the human GCH gene, and assessed promoter activity by luciferase assay (data not shown). The only construct capable of responding to LPS was hGCHpro5.2 k-int3 k, which has an additional 3-kb fragment in intron 1 (236~504 + 3252) inserted into hGCHpro5.2 k (Fig. 2A and B). These data suggest that the response elements essential for LPS-stimulated GCH transcription were present in the intron 1 fragment.

3.2. Characterization of the enhancer region within intron 1 in response to LPS stimulation

To identify the response elements, we shortened the intronic insertion in the reporter plasmid by deleting the fragment from both the 5′ and 3′ directions, with the aim of identifying a minimal effective region. A series of reporter constructs were prepared (Fig. 2C), and promoter activity was assessed by luciferase assay. A 332-bp fragment, which is highly conserved among species (Supplement Figure), was shown to contain the essential response elements for LPS-induced GCH transcription (Fig. 2C, D).

Interestingly, when this fragment was inverted (hGCHpro5.2 k-int332R), the reporter activity was not affected compared with hGCHpro5.2 k-int332. In contrast, when the hGCHpro5.2 k-int3 k plasmid was truncated by restriction enzyme digestion and re-ligation to remove a 634-bp region (containing the 332-bp fragment), the induced transcription by LPS was almost completely abolished (Fig. 2C, D). These data suggest that this 332-bp region most likely acted as the LPS-response element in a position- and orientation-independent manner.

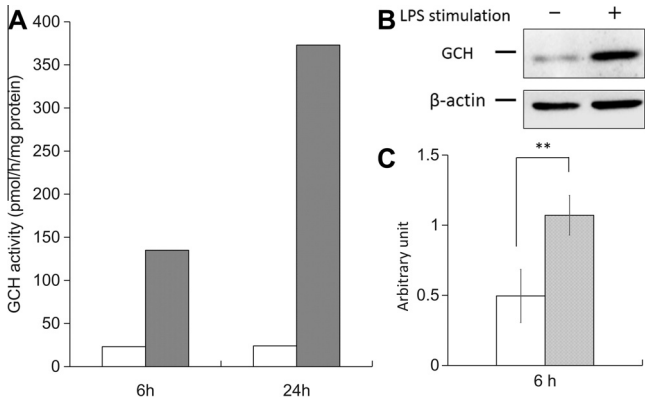

Fig. 1. LPS upregulates GCH activity and expression level. A: GCH activity in RAW264.7 cells was measured with or without LPS treatment (1 μg/mL) for 6 or 24 h. Data are presented as the mean of two independent experiments. B and C: western blot showing whole-cell lysate GCH and β-actin protein levels with or without LPS treatment in RAW264.7 cells. A representative result is shown in B and C shows an average of four independent experiments (intensity ratio of GCH and β-actin). Data are shown as the means ± SEM. Shaded bar, LPS treated; light bar, LPS untreated. ** $p < 0.01$.

As the regulation of GCH expression and activity is an important event in the immune response and is closely related with the coordinated regulation of BH4 synthesis and iNOS expression, the regulatory mechanism uncovered here may be a common fashion for immune-activated GCH expression. It has been shown that GCH expression was also activated by TNF-α/IFN-γ in HUVECs [13]; therefore, we examined the transcription regulatory mechanism in this model with the luciferase assay method used previously in RAW264.7 cells. Similar results were obtained when HUVECs were activated by TNF-α/IFN-γ (Fig. 2E); that is, the reporter plasmid containing only the promoter region was not sufficient for transcription activation, while the same intronic enhancer region acted as the response element. These data suggest that the absolute requirement of the enhancer region in intron 1 acts as a common regulatory element of GCH transcriptional enhancement by immune activation.

3.3. Ets and C/EBP binding sites are critical for LPS-induced GCH transcription

When we tried to further shorten the enhancer region in the reporter constructs, we found that the enhancer region possibly consisted of more than one single element. Deletion of 5′ side from 504 + 1250 to 504 + 1263 or 3′ side from 504 + 1377 to 504 + 1301 caused dramatic reduction in the luciferase activity by LPS stimulation (data not shown). Two conserved fragments (Supplement Figure) in these regions were selected as probes to study the binding with nuclear factors in vitro. EMSA experiments were performed using biotin-labeled fragment 1 and 2 (Fig. 3A) with nuclear extracts from RAW264.7 cells treated with 1 μg/mL LPS for 6 h. There were two major shifted bands observed when nuclear extracts were added to biotin-labeled fragment 1 and 2 (indicated by arrows in Fig. 3B, lane 2 and Fig. 3C, lane 2), which disappeared with the addition of a 100-fold excess amount of cold probe as a competitor (Fig. 3B, lane 3 and Fig. 3C, lane 3).

In order to find out which elements were critical for the complex formation, oligonucleotides that contained only the 5′ half or the 3′ half of each fragment were synthesized and added at a 100-fold excess amount as competitors into the binding reaction to check for a change in the band patterns. We found that neither the 5′ nor the 3′ half of fragment 1 (oligo 1–1 and 1–3; Fig. 3B, lanes 4 and 6) affected the shifted band pattern, while in fragment 2, the 5′ half (oligo 2–1; Fig. 3C, lane 4) did not affected the shifted band pattern, indicating that these regions were not involved in the complex formation composed of nuclear factor(s) and DNA. On the contrary, the 3′ half of fragment 2 (oligo 2–2; Fig. 3C, lane 5) and oligo 1–2 across the middle region of fragment 1 (Fig. 3B, lane 5) almost completely abolished the formation of the complex between nuclear extracts and probes. Further, we noticed that an Ets binding motif and a C/EBP binding motif were included in oligo 1–2 and 2–2, respectively, both of which are highly conserved among species. Therefore, we designed two oligonucleotides with disrupted Ets or C/EBP binding motifs. These mutated oligonucleotides were no longer able to compete with the probes (oligo 1-2 Mut in Fig. 3B, lane 7 and oligo 2-2 Mut in Fig. 3C, lane 6). These data indicated that these two elements (the Ets element in fragment 1 and the C/EBP element in fragment 2) were critical for the nuclear factors to bind to the enhancer region.

Next, we asked whether these two elements were involved for LPS-stimulated GCH transcription by introducing the mutations used in the EMSA experiment into the reporter plasmids. As shown in Fig. 4A, plasmids bearing mutations in either the Ets or C/EBP site showed a significantly reduced response to LPS stimulation, while a double mutation in both sites caused a further reduction in promoter activity, almost to the same level as hGCHpro5.2 k.

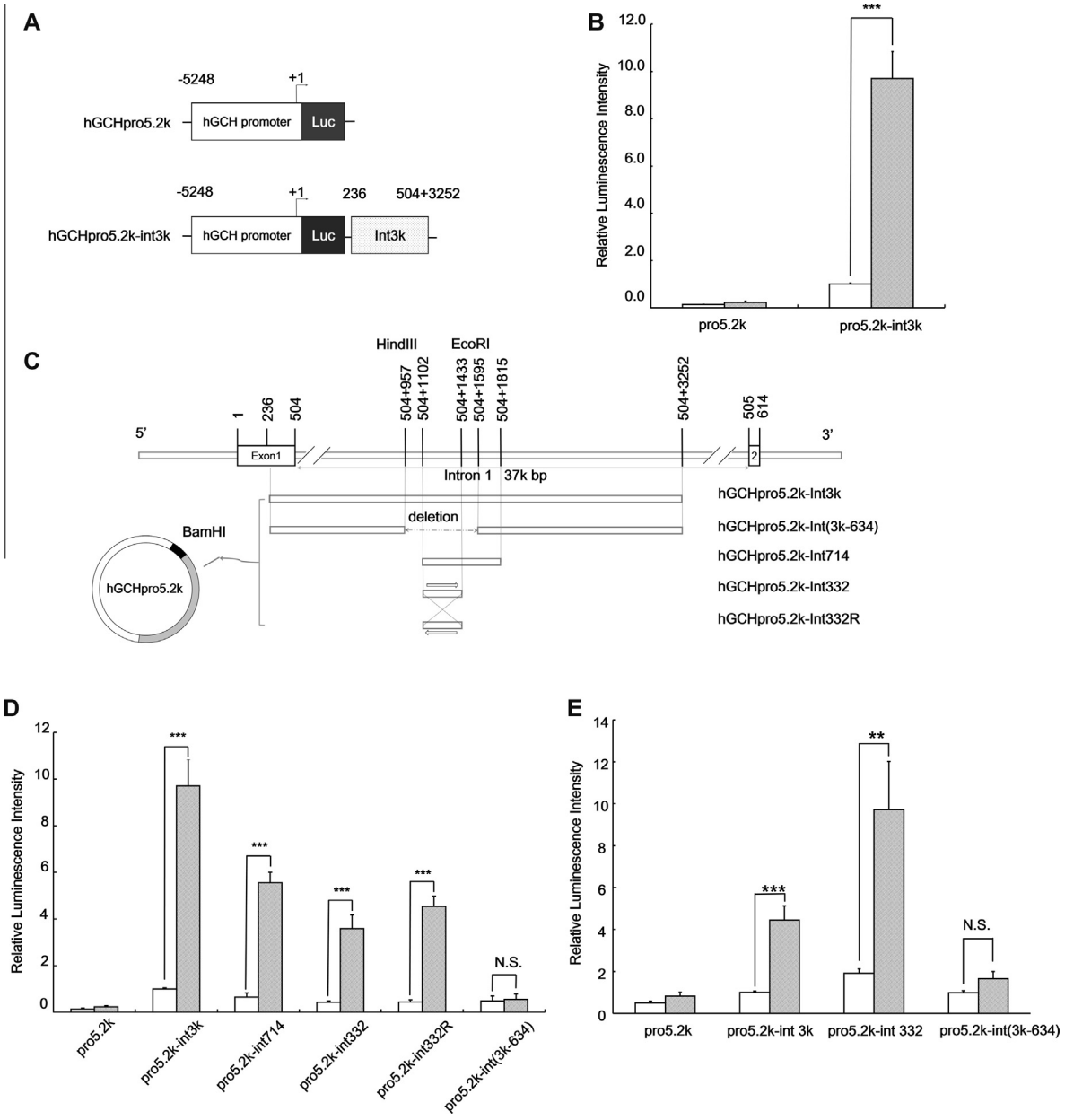

Fig. 2. Identification of an enhancer region within intron 1. (A) Schematic of reporter construct in which the human GCH 5.2-kb promoter and the 3-kb fragment in intron 1 were inserted into pGV-B2. (B) Luciferase activity was measured in LPS-treated RAW264.7. (C) Schematic of luciferase construct with different fragments inserted into hGCHpro5.2k. (D) relative luciferase activity of each construct in RAW264.7 cells was measured after LPS treatment. (E) Relative luciferase activity of reporter constructs was examined in HUVECs following 200 U/mL TNF-α and 200 U/mL IFN-γ treatment for 6 h. At least three independent experiments were conducted. Data are shown as the means ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$. N.S., not significant. Shaded bar, LPS or TNF-α/IFN-γ treated; light bar, untreated.

These results demonstrated that the Ets and C/EBP sites were the critical response elements to LPS stimulation.

3.4. C/EBP-β is recruited to the enhancer region under LPS stimulation

In order to find the specific binding partners to the Ets and C/EBP element, supershift experiments were performed by adding antibodies for Ets and C/EBP family proteins to the binding reaction. Among the C/EBP family members, C/EBP-β is the most extensively studied and well known as a critical transcription factor in regulating multiple target genes related to immune activation, such as TNF-α [14] and IL-6 [15]. Therefore, we first examined whether C/EBP-β bound to the enhancer region. We found that in fragment 2, a C/EBP-β antibody reduced the intensity of the two shifted band while producing a supershift band as shown in lane 8, Fig. 3C, indicating that C/EBP-β was the direct binding partner. Whereas in fragment 1, we tried antibodies against Ets family members, PU.1, and Ets-1, but the patterns of the shifted band were unchanged (lane 10 and 11 in Fig. 3B). The result suggested that they were not likely involved in the complex formation in fragment 1. However, we confirmed that the binding partner was an Ets family member by the fact that an Ets consensus oligonucleotide, but not a mutated one, suppressed the complex formation between fragment 1 and nuclear proteins (lane 8 and 9 in Fig. 3B), although the specific factor was not identified. Next we performed a ChIP assay to examine whether C/EBP-β binds to the enhancer region under LPS stimulation *in vivo* or not. Immunoprecipitates prepared using C/EBP-β antibody or rabbit IgG were assayed for an

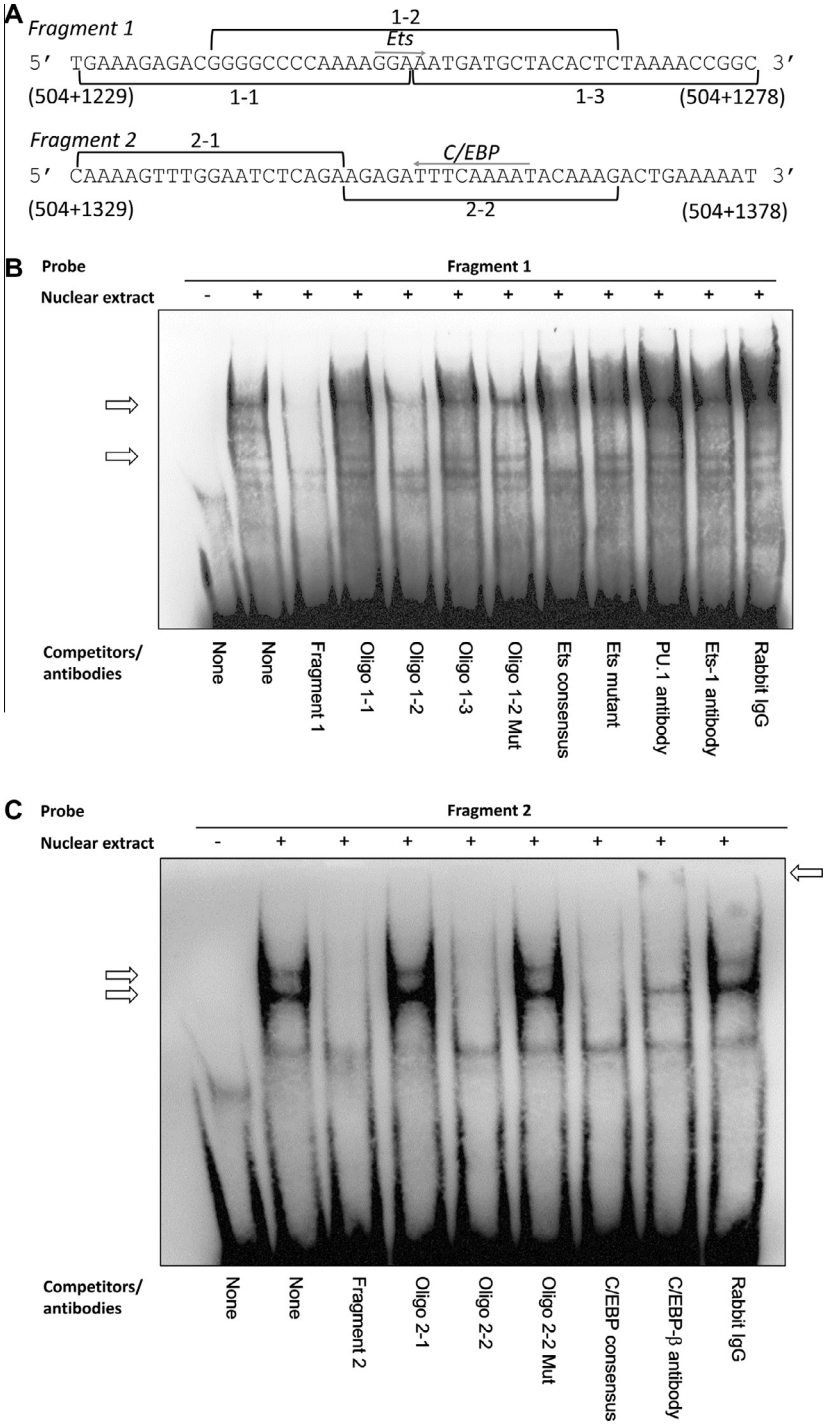

Fig. 3. EMSA experiments demonstrate the binding of C/EBP-β and Ets proteins to the enhancer region. (A) The oligonucleotide sequences used in EMSA experiments. (B) and (C) EMSA experiments using fragment 1 (B) and 2 (C) as probes to bind with nuclear extracts of LPS-stimulated RAW264.7 cells. Specific shifted bands are indicated with arrows to the left of the images. A supershift band formed by C/EBP-β antibody is indicated to the right of the image (C). Unlabeled oligonucleotides at 100-fold as competitors or antibodies were added as indicated under each image.

enrichment of enhancer binding by semi-quantitative PCR, with a set of primers targeting the 3′-untranslated region of the GCH gene as a negative control (Supplement Table). As shown in Fig. 4B, a higher intensity band was detected in the LPS-treated sample compared with the LPS-untreated sample, which suggested that under LPS stimulation, C/EBP-β was recruited to the enhancer region.

The involvement of C/EBP-β in GCH transcriptional activation by LPS was further confirmed by siRNA transfection. When a C/EBP-β siRNA mixture was co-transfected with hGCHpro5.2 k-int332, the promoter activity induced by LPS was significantly reduced compared with control siRNA (Fig. 4C). In addition, we noticed that the basal transcription level of hGCHpro5.2 k-int332 was also attenuated by C/EBP-β siRNA transfection, indicating that C/EBP-β also contributed to the basal transcription of the GCH gene.

4. Discussion

In the current study, we showed for the first time that GCH expression was upregulated by immune activation in a distinct

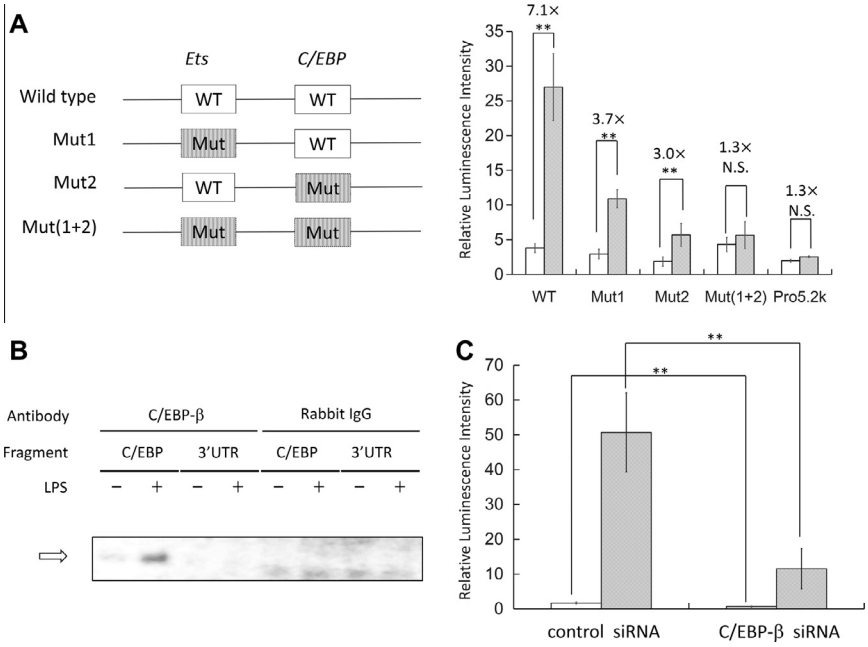

Fig. 4. LPS activated GCH transcription is mediated via the C/EBP and Ets elements and C/EBP-β is one of the factors directly bound to the enhancer region. (A) diagrams of mutated reporter plasmids and luciferase assay measured in LPS-treated RAW264.7 cells. (B) a ChIP assay was performed with C/EBP-β antibody or rabbit normal IgG incubated with sonicated lysate from LPS-treated or untreated RAW264.7 cells. The arrow indicates the PCR product covering the C/EBP binding site. (C) C/EBP-β or control siRNA was co-transfected with hGCHpro5.2k-int332, and relative luciferase activity was measured in RAW264.7 cells. Shaded bar, LPS treated; light bar, LPS untreated. At least three independent experiments were conducted. Data are shown as the means ± SEM. $^{**}p < 0.01$, N.S., not significant.

fashion, employing an enhancer that is located away from the promoter region but within intron 1. To our knowledge, this regulatory mechanism is unique from previous reports of GCH induction by other stimuli, such as cAMP [16] or NGF [12]. The significance of the enhancer during immune-activated GCH transcription was emphasized by the completely non-responsive promoter activity in the absence of the enhancer, while in cAMP- and NGF-activated GCH transcription, only the core promoter region was sufficient. In endothelial or neural cells, synthesis of BH4 is a constitutive and chronically regulated process, whereas the activation of the immune system is a short-term and acute response; therefore, it is understandable that GCH possesses a different regulatory machinery to accommodate the physiological roles of BH4.

Kapatos et al. [17] reported that C/EBP-β and ATF-2 are bound to the GCH core promoter; therefore, it is highly plausible that C/EBP-β bound to the enhancer may bind as a homodimer or heterodimer with C/EBP-β or ATF-2 on the promoter to help form the transcription complex. It has been reported that Ets-1 and C/EBP-α could physically associate via their DNA binding domain to bind to their combined binding sites [18]. In the GCH enhancer reported here, the Ets and C/EBP binding sites are separated by approximately 100 bp; therefore, it would be highly possible that Ets factors and C/EBP-β form a physically interacting complex.

Our data showed that LPS-activated GCH transcription was mediated via the C/EBP and Ets elements cooperatively. However, the binding partners of the Ets element are still unknown, as there are more than 20 members belonging to the Ets family, including Ets-1, Ets-2, Elk, and PU.1, which have identical GGAA/T binding sites [19]. Ets factors have been shown to play important roles in immune regulation [20], including the regulation of enhancer activity in the third intron of TNF-α [21]. The specific Ets factors recruited to the enhancer region largely depends on the expression profile activated by the stimuli, as well as on the interaction preference with other factors. Future studies should investigate which Ets factors bind with the enhancer region.

Among patients with dopa-responsive dystonia, approximately 40% individuals do not carry mutations in exons, the proximal promoter region or splicing junctions known to be related with the disease [22]. Considering that mutated Ets or C/EBP binding sites caused a significantly attenuated response to LPS stimulation, it is plausible that individuals carrying mutations in the enhancer element possibly inappropriately regulate GCH in response to immune system stimulation or other physiological processes. Attention should be paid to the correlation of mutations in the enhancer region with immune disorder, neural deficiency or other BH4-related diseases.

Acknowledgments

We thank the members of our lab for technical help and discussion. This work was supported by Health and Labor Sciences Research Grants for Research on Intractable Diseases from the Ministry of Health, Labor, and Welfare of Japan, and by KAKENHI from MEXT, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.002.

References

[1] B. Thöny, G. Auerbach, N. Blau, Tetrahydrobiopterin biosynthesis, regeneration and functions, Biochem. J. 347 (2000) 1–16.

[2] E. McNeill, K.M. Channon, The role of tetrahydrobiopterin in inflammation and cardiovascular disease, Thromb. Haemost. 108 (2012) 832–839.

[3] R.W. Ernst, N. Blau, B. Thöny, Tetrahydrobiopterin: biochemistry and pathophysiology, Biochem. J. 438 (2011) 397–414.

[4] J. Vásquez-Vivar, P. Martásek, J. Whitsett, J. Joseph, B. Kalyanaraman, The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study, Biochem. J. 362 (2002) 733–739.

[5] C. Murr, B. Widner, B. Wirleitner, D. Fuchs, Neopterin as a marker for immune system activation, Curr. Drug Metab. 3 (2002) 175–187.
[6] A. Berdowska, A.K. Zwirska-Korczala, Neopterin measurement in clinical diagnosis, J. Clin. Pharm. Ther. 26 (2001) 319–329.
[7] B. Beutler, Tlr4: central component of the sole mammalian LPS sensor, Curr. Opin. Immunol. 12 (2000) 20–26.
[8] M. Triantafilou, K. Triantafilou, Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster, Trends Immunol. 23 (2002) 301–304.
[9] Y. Nagai, S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, K. Miyake, Essential role of MD-2 in LPS responsiveness and TLR4 distribution, Nat. Immunol. 3 (2002) 667–672.
[10] A. Ota, S. Yoshida, T. Nomura, S. Matsui, Y. Hagino, K. Umezawa, S. Katoh, T. Nagatsu, Tetrahydrobiopterin biosynthesis enhanced by lipopolysaccharide stimulation in murine neuroblastoma cell line N1E-115, J. Neurochem. 67 (1996) 2540–2548.
[11] T. Suzuki, H. Inagaki, T. Yamakuni, T. Nagatsu, H. Ichinose, Enhanced expression of GTP cyclohydrolase I in V-1-overexpressing PC12D cells, Biochem. Biophys. Res. Commun. 293 (2002) 962–968.
[12] T. Ito, T. Suzuki, H. Ichinose, Nerve growth factor-induced expression of the GTP cyclohydrolase I gene via Ras/MEK pathway in PC12D cells, J. Neurochem. 95 (2005) 563–569.
[13] A. Huang, Y.Y. Zhang, K. Chen, K. Hatakeyama, J.F. Keaney, Cytokine-stimulated GTP cyclohydrolase I expression in endothelial cells requires coordinated activation of nuclear factor-κB and Stat1/Stat3, Circ. Res. 96 (2005) 164–171.
[14] R.M. Pope, A. Leutz, S.A. Ness, C/EBP beta regulation of the tumor necrosis factor alpha gene, J. Clin. Invest. 94 (1994) 1449–1455.
[15] S. Akira, H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, T. Kishimoto, A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family, EMBO J. 9 (1990) 1897–1906.
[16] G. Kapatos, S.L. Stegenga, K. Hirayama, Identification and characterization of basal and cyclic AMP response elements in the promoter of the rat GTP cyclohydrolase I gene, J. Biol. Chem. 275 (2000) 5947–5957.
[17] G. Kapatos, P. Vunnava, Y. Wu, Protein kinase A-dependent recruitment of RNA polymerase II, C/EBPβ and NF-Y to the rat GTP cyclohydrolase I proximal promoter occurs without alterations in histone acetylation, J. Neurochem. 101 (2007) 1119–1133.
[18] K.M. McNagny, M.H. Sieweke, G. Döderlein, T. Graf, C. Nerlov, Regulation of eosinophil-specific gene expression by a C/EBP–Ets complex and GATA-1, EMBO J. 17 (1998) 3669–3680.
[19] B. Wasylyk, S.L. Hahn, A. Giovane, The Ets family of transcription factor, in: P. Christen, E. Hofmann (Eds.), EJB Reviews 1993, Springer, Berlin, Heidelberg, 1994, pp. 7–18.
[20] S. Gallant, G. Gilkeson, ETS transcription factors and regulation of immunity, Arch. Immunol. Ther. Exp. 54 (2006) 149–163.
[21] G.D. Tomaras, D.A. Foster, C.M. Burrer, S.M. Taffet, ETS transcription factors regulate an enhancer activity in the third intron of TNF-alpha, J. Leukocyte Biol. 66 (1999) 183–193.
[22] Y. Furukawa, S.J. Kish, Dopa-responsive dystonia: recent advances and remaining issues to be addressed, Mov. Disord. 14 (1999) 709–715.