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Regulation of the N-Type Calcium Channel Gene by C/EBP

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-ABSTRACT-

Regulation of the N-Type Calcium Channel Gene by C/EBP

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(Directed by Assistant Professor Haeyoung Suh-Kim)

N-type calcium channel ($\alpha_{1B}\,\alpha_2\,\delta\,\beta$) has been known to be specific to nervous tissues and to play important roles in neuronal activities including synaptic transmission and migration of neuronal cells. The α_{1B} promoter gene is a TATA-less gene and has many putative binding sites for several transcription factors including C/EBP (CCAAT/Enhancer Binding Protein), Sp1, and AP1. To further understand regulation of the α_{1B} gene expression in nervous tissues, the investigator determines the role of C/EBP in the α_{1B} gene expression.

In F11 neuroblastoma cell lines, α, β and δ isoforms of C/EBPs enhanced the activity of an α_{1B} reporter gene, pα_{1B}-3992Luc, which contained the 3992 bp fragment of the 5' flanking region of the α_{1B} gene. The α isoform exhibited the highest transactivation of the α_{1B} gene compared to the β and δ isoforms. Deletion analysis showed that C/EBPs enhanced the promoter activity of pα_{1B}-110Luc as well, indicating that C/EBP responsive element locates within –110 bp. To determine the function of CCAAT box-like sequence (CE) at –59 bp as a C/EBP responsive

element, electrophoretic mobility shift assay was carried out. A double-stranded oligonucleotide containing CE could bind specifically C/EBP, which could be also recognized by an anti-C/EBP β antibody. To confirm CE could confer C/EBP-mediated transactivation, pCE1Luc, a reporter gene containing CE, was generated. Expression of the C/EBP isoforms enhanced the luciferase activity of pCE1Luc. C/EBP under the same condition did not increase the pCEm1Luc, a reporter gene that was point-mutated at CCAAT box. Also tested Ras, one of small GTP-binding proteins, which has been reported to enhance the activity of C/EBP. Ras amplified the effect of C/EBP on the α_{1B} gene. Thus cotransfection of C/EBP's and the active form of Ras, RasV12, can synergistically enhanced the pα_{1B}-110Luc or pCE1Luc. Taken with the recent report that the activity of C/EBP β is regulated by NGF or LTP, these result also suggest that C/EBP's may mediate neuronal activity-dependent gene regulation of α_{1B} gene.

Key words : N-type calcium channel, α_{1B}, C/EBP

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I. INTRODUCTION

Calcium Ions and N-Type Calcium Channels

Calcium ions (Ca^{2+}) play critical signaling roles in many biological systems. Extracellular Ca^{2+} entered excitable cells through calcium channels : voltage-dependent calcium channels (VDCC) or ligand-gated calcium channels such as NMDA receptors. In nervous system, especially Ca^{2+} entry via voltage-dependent calcium channel mediates various physiological events such as neurotransmitters release [1], membrane excitability [2], migration of neurons [3] and gene expression [4].

In the brain, VDCC can be classified into two types, high-voltage-dependent calcium channel (HVA) and low-voltage-dependent calcium channel (LVA). HVA has been further classified into several types termed L, N, P, Q, and R, based on differences in current threshold and sensitivity to dihydropyridine (DHP) and ω-conotoxin [5].

The L-type calcium channels, the prototype of VDCC, were purified from

skeletal muscle of rabbit and composed of α_1, α_2, δ, β and γ subunit [6]. The α_1 subunit is a pore-forming unit of the channel [7] and determines the function of the channel, so that as the pore-forming unit of channel, α_1 subunit has been a target of research. The α_2-δ subunits, associated by a disulfide bond, and the cytoplasmic β subunit can regulate functions of channel[8]. The γ subunit is a membrane-spanning protein and its expression is restricted in the skeletal muscle[9].

In neuronal cells, several genes encoding α_1 subunit are cloned and each designated as α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, and α_{1S}. Existence of multiple α_1 subunit genes can indicate that each type of calcium channel is involved in particular calcium-dependent cellular functions. Expression studies have shown that α_{1A} encodes P/Q type channels ; α_{1B} forms N-type calcium channel ; $\alpha_{1C,}$ α_{1D} and α_{1S} define L-type calcium channels ; α_{1E} encodes R-type channel [10].

N type calcium channel has been purified from the brain in use of specific antagonist, ω-conotoxin GVIA [11] and the expression is restricted to nervous tissue[12,13]. N-type calcium channel plays a important role not only in the migration of postmitotic granule cells of mammalian cerebellum by controlling calcium influx [14] but also in the regulation of neurotransmitters release and fast synaptic transmission [15]. N-type calcium channels are composed of $\alpha_{1B,}$ β_3, and α_2 δ [16] .

Pore-forming unit of N-type calcium channel, α_{1B} gene is encoded at distal end of long arm of human chromosome 9 [17]. After initiation of transcription, α_{1B} gene is transcribed into single RNA transcript and the transcript is alternatively spliced and translated into proteins. In the α_{1B} promoter, there is no TATA box but exist many potential transcription factor-binding site such as multiple GC boxes, AP1-, AP2-, and AP4-binding sites, a NRSE-like element, and an inverted CCAAT-box (ATTGG) located at –59 from transcription initiation site [17]. The CCAAT box is one of the most common elements in eukaryotic promoters, found in the reverse or forward orientation. The frequency of CCAAT boxes appears to be higher in TATA-less promoters than TATA-containing promoters, particularly in the reverse

ATTGG orientation. The CCAAT box of TATA-containing promoters is preferentially located in the -80/-100 region (mean position -89) and is not found nearer to the transcription initiation site than -50. In TATA-less promoters the CCAAT box is usually closer to the transcription initiation site (at -66 on average)[18].

Therefore it is possible that C/EBP can bind the CCAAT box of the α_{1B} promoter and regulate the α_{1B} gene expression.

CCAAT/Enhancer Binding Protein (C/EBP)

CCAAT/enhancer binding protein (C/EBP) is a nuclear transcription factor that binds specifically to homologous regulatory elements within the promoters. C/EBPs are critical for normal cellular differentiation and function in broad range of biological responses from the acute phase response to glucose homeostasis. C/EBPs have a basic leucine zipper structure, which is composed of an activation domain, a DNA-binding domain and a dimerization domain[19]. All family members, C/EBP α, β, δ, γ, and ε [20], share the highly conserved dimerization domain, so that they form homo- and heterodimers with other family members and bind to DNA[21,22]. C/EBPs are least conserved in their activation domains and vary from strong activators to dominant negative repressors.

The pleiotropic effects of C/EBPs are also in part that each C/EBP isoforms has different distribution in tissue and function. C/EBPs play essential roles in the adipocyte and the myeloid differentiation program. In the adipocyte differentiation,. C/EBP β is expressed early in clonal expansion, later followed by C/EBP α. C/EBP α has antimitotic function [23] so that its premature expression may block adipocyte differentiation. In the myeloid differentiation program, C/EBP α is a master regulator of myeloid progenitors, C/EBP β plays an important role in the development of macrophage and B-cell, and C/EBP δ is upregulated during myelopoiesis [24]. The formation of alternative combinations of tissue-specific and stage-specific C/EBP dimers may allow differential regulation of target genes in hematopoietic cells and

commitment to distinctive hematopoietic lineages. Recently reported that C/EBP β can regulate neuronal transcription activated by nerve growth factor receptor signaling[25] and that the expression and activities of C/EBP β and δ are intensified by cAMP as well as Ca/Calmodulin kinases activation in hippocampal neurons[26].

In this study, we investigated the effect of C/EBP α, β, and δ on the α1B promoter and further signaling pathways in the regulation of the α_{1B} gene expression.

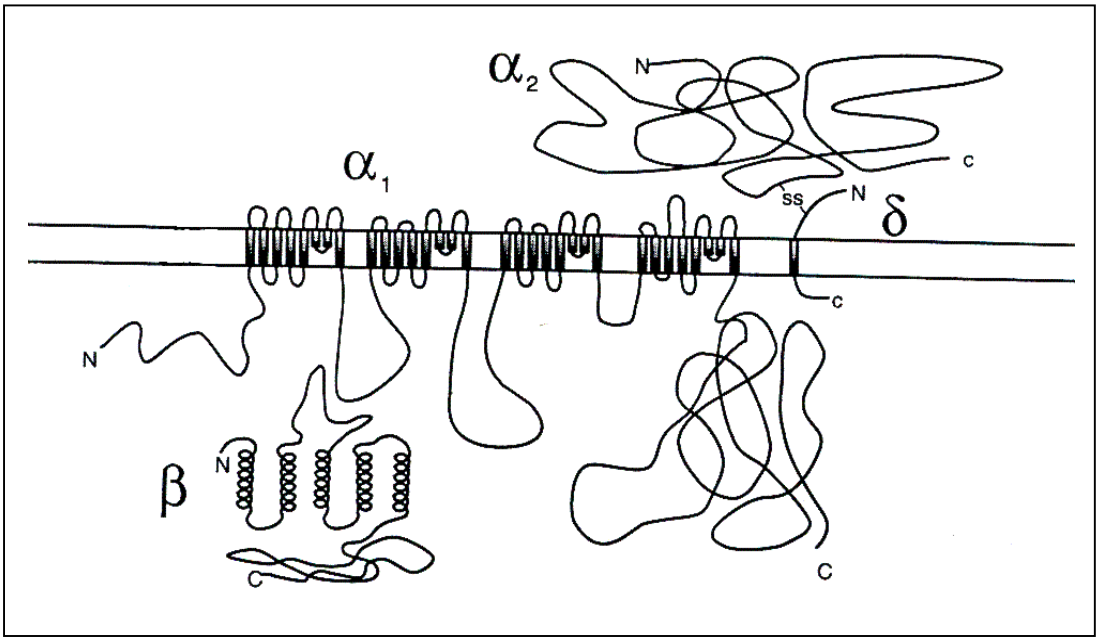


Fig.1 The structure of N-type calcium channel. The N-type calcium channel is composed of α_{1B}, α_2, δ and β subunit. The α_{1B} subunit is the pore-forming unit that determine function of the channel. The α_2 and δ subunit are connected by a disulfide bond and exist as α_2-δ form. The β subunit is cytoplasmic protein that can modulate channel activity (Reviewed by Perez-Reyes E)

II. MATERIALS AND METHODS

A. Materials

Reporter genes for α_{1B} expression, pα_{1B}-3992Luc and –110Luc (Fig. 2) were kindly gifted by Dr. D.S. Kim (Kyungbuk University, Republic of Korea)[1]. Original expression vectors such as pMSV-C/EBP α, pMSV-C/EBP β, pMSV-C/EBP δ, were kindly gifted by S. L. McKnight (Carnegie institution, Boltimore) and modified. The expression vector for constitutive active form of Ras, pSV-RASV12, was kindly gifted by J.H. Kim (Gwangju KAIST, Republic of Korea). PGL3-promoter vector was purchased from Promega (Madison, WI) ; restriction enzymes from Takara (Shuzo, Japan) and Berhinger Manheim (Indianapolis) ; media and supplements for cell culture and transfection from Gibco BRL (Grand Island, N.Y.) ; plastic wares for cell cultures from Falcon (Lincon, New Jersey) ; luciferase assay kit from Promega (Madison, WI) ; C/EBP consensus binding oligonucleotides and anti-C/EBP β antibody from Santacruz Biotechnology (Santacruz, CA) ; α-^{32}P-dATP from Amersham Pharmacia Biotech (U.K.) and Hiperfilm β–Max from Amersham international (Sweden) ; D19 developer and Professional Polymax Fixer from Kodak (Germany) ; other certified materials for study from Sigma (St. Louis, Missouri). F11 cell, a hybridoma cell line of neuroblastoma cells and dorsal root ganglion cells, was provided by Dr. M. Fishman (Harvard University, Cambridge MA). COS cell was purchased from Korean Cell Lines Bank (Republic of Korea).

B. Methods

Cell culture and Transfection

F11 cells and COS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) solution with 1% streptomycin, 1% penicillin and 10% fetal bovine serum (FBS).

At 18 hours before transfection, $2x10^4$ cells of F11 were plated on each well of 6-well plate. The reporter and activator gene were co-transfected by LipofectAMINE. For transfection using LipofectAMINE, 4 μl of LipofectAMINE in 100 μl DMEM supplemented with 0.5% FBS were mixed with 0.4 μg of reporter genes (pα1B-3992Luc and –110Luc, pCE1, and pCEm1) and 0.1 μg of each activator genes. LipofectAMINE-plasmid mistures were transfected into the F11 cells for 3 hours and washed with HBSS. The F11 cells were cultured in DMEM supplemented with 10% FBS at 37 ^oC for 36 hours and harvested for luciferase assay.

Luciferase assay

Thirty six hours after transfection, F11 cells were washed with phosphate-buffered saline, lysated with 1X lysis buffer, and removed the supernatant of cell lysates. The protein amount of the supernatant was determined with standard Bradford assay. For luciferase assay, 30 μl of luciferase substrate were added and incubated for 10 seconds in ice. The luciferase activities were determined with Luminometer by measuring for 5 seconds, three times per one sample. For the average of three values were used and its standard error were tested for student t-test in Sigma Plot 5.0 program. Each transfection was done as duplet.

Electromobility shift assay (EMSA)

COS cells were maintained in DMEM with 10% FBS, 1% streptomycin, and 1% penicillin. For nuclear extract, confluent cells in 100-mm dish were transfected with C/EBP β-expression vector with DEAE/Dextran method and harvested with phosphate-buffered saline (pH 7.4) and centrifuged for 5 min at 14000 rpm. Cell pellet were resuspended with 250 μl of TD buffer (25 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.01% Phenylmethylsulfonyl fluoride (PMSF)) for 5 min at room temperature. Nonidet P-40 was then added to a final concentration of 0.05% and incubated for 2 min at room temperature. After centrifugation at 1,700

× g for 30 min, resulting pellet was suspended in 80 μl of BL buffer (10 mM Tris-HCl (pH 8.0), 400mM LiCl, 0.5 mM DTT, 0.01% PMSF) and kept for 5 min at room temperature. After centrifugation at 12,000 × g for 10 min, the supernatant was harvested as nuclear extract and amount of proteins was determined with Bradford method.

As a probe, a double stranded oligonucleotide containing CCAAT box of α_{1B} promoter, CE1 (5'-GATCCGCGCGGATTGGCCGGGCCCCA-3'), was end-labeled with [α-^{32}P]-dATP using Klenow fragment of DNA polymerase. For EMSA the ^{32}P-labeled probes (40,000 cpm) were added in the reaction containing 20 mM HEPES (pH 7.8) with 7% glycerol, 60 mM KCl, 0.5 mM PMSF, 5 mM $MgCl_2$, 2 mM DTT, and 1 μg of poly(dI-dC) and incubated for 20 min at room temperature. Specific binding was determined by addition of a 30-60 fold excess unlabeled probe. The C/EBP β-probe complex was recognized by addition of an anti-C/EBP β antibody (1.0 μg / lane) to the reaction mixture. Samples were loaded onto 4% polyacrylamide gel (30:1, acrylamide : *m*-bisacrylamide) in 0.5x TBE buffer and subject to electrophoresis at 8 V/cm. The gel was dried with gel-dryer and exposed to Hiperfilm β–Max at –70 ^{o}C for 16 hours. The film was developed with D19 Developer and Professional Polymax Fixer were used.

III. RESULTS

The effects of C/EBP on the α_{1B} gene expression

To determine the mechanism of transcription of α_{1B} gene, reporter genes containing the various length of the α_{1B} promoter were used. pα_{1B}-3992 contained the sequence from –3992 bp to +86 bp of the α_{1B} promoter and pα_{1B}-110 contained the sequence from –110 bp to +86 bp (Fig. 2). F11 cells were transfected with α_{1B}-reporter genes with or without expression vectors for various isoforms of C/EBP, pskMSV-C/EBPα, pskMSV-C/EBPβ, and pskMSV-C/EBPδ. The luciferase activity of the pα_{1B}-3992 was increased by expression of C/EBP α, β, or δ.

Especially, C/EBP α increased most the luciferase activity of the pα_{1B}-3992 as well as pα_{1B}-110 (Fig. 3). The cis-element of C/EBP, CCAAT box, is located in –59 bp upstream sequence from the transcription initiation site of α_{1B} gene. Thus it is possible that C/EBP may bind the CCAAT box of α_{1B} gene resulting in the transactivation of the α_{1B} gene.

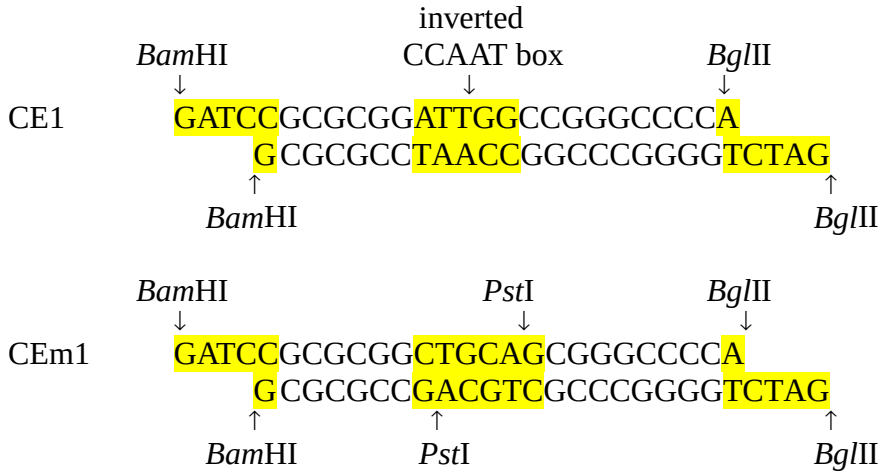
Specific binding between C/EBP and CCAAT box of α_{1B} promoter

To determine whether C/EBP bind specifically to the CCAAT box at –59 bp upstream of the α_{1B} promoter, the electrophoretic mobility shift assays (EMSA) were carried out (Fig. 4). As a source of C/EBP, nuclear extracts were prepared from C/EBP β–transfected COS cells. CE1, a double-stranded oligonucleotide containing CCAAT box was used as a probe. On the gel, there were three bands of DNA-protein complexes thought to be CCAAT box-C/EBP complexes (lane 1). These three bands (arrow 1,2, and 3) were competed with unlabelled CE1 (lane 2 and 3). Among three bands, upper two bands (arrow 1 and 2) were competed with C/EBP consensus binding oligonucleotides, suggesting that the binding of CCAAT box and C/EBP is

specific and CCAAT box is operative (lane 4 and 5). Also upper two bands were recognized by anti-C/EBP β antibody (lane 6).

The construction of pCE1 and pCEm1 *(Fig.5)*

For construction of pCE1, sense and antisense strands of CE1 oligonucleotide were designed so that the 5' and 3' were compatible for *Bam*H1 and *Bgl*II, respectively. The same oligonucleotides were used as a probe and competitors in electrophoretic mobility shift assay (EMSA).



Both single stranded oligonucleotide of CE1 were annealed in boiling water for 3 min and its 5' and 3' ends were phosphorylated by T4 polynucleotide kinase at 37 ^{o}C for 30 min. pGL3 promoter vector were cut by restriction enzyme *Bgl*II at 37 ^{o}C for 1 hour and its 5' and 3' end dephosphorylated by calf intestinal phosphatase at 37 ^{o}C for 30 min. Phosphorylated CE1 and dephosphorylated pGL3 promoter vector were incubated together with T4 DNA ligase at 4 ^{o}C overnight. Ligation mixture was transformed into *Escherisia coli* DH5α and the transformants were cultured in terrific broth for 1 hour at 37 ^{o}C in 225 rpm and plated on the LB plate with 50 μg/ml of ampicillin used as selection antibiotics. About 100 or more colonies grew after incubatrion at 37 ^{o}C for 16 hours and were cultured in 5 ml of terrific broth with 50 μg/ml of ampicillin for 16 hours. From cultured broth, DNA was prepared by

standard alkaline-SDS method. The insertion of CE1 to pGL3 promoter was tested by digesting with *Hind*III and *Xho*I at 37 ^{o}C for 2 hours and confirmed by DNA sequencing using the pGL2 primer.

CCAAT box-mediated transactivation of the α_{1B} promoter by C/EBP

In the Fig 3 and Fig 4, the results showed that C/EBP can enhance the promoter activity of the α_{1B} gene by binding to CCAAT box of the α_{1B} promoter. To determine CCAAT box-mediated transactivation of α_{1B} gene by C/EBP, pCE1 and pCEm1 construct were used in transfection study (Fig. 5).

In the F11 cells, C/EBP α and C/EBP β enhanced the luciferase activity of pCE1 construct (Fig. 6). The promoter activity of pCEm1 did not affected by C/EBP, suggesting C/EBP can recognize CCAAT box of α_{1B} promoter with sequence-specific manner. Together with Fig 3 and Fig 4, the result shown in Fig 6 suggests that C/EBP can bind CCAAT box of α_{1B} promoter specifically and promote the expression of α_{1B} gene (Fig. 8).

The effect of Ras-MAPK Pathway on the C/EBP-mediated transactivation of the α_{1B} promoter

As shown in the Fig. 7 and Fig. 8, constitutive active form of Ras, RasV12, facilitated the C/EBP-mediated transactivation of α_{1B} gene in the F11 cells. These facilitations by RasV12 were shown in the two α_{1B}-luciferase fusion genes and the pCE1 construct but not in the pCEm1, construct containing mutated CCAAT box.

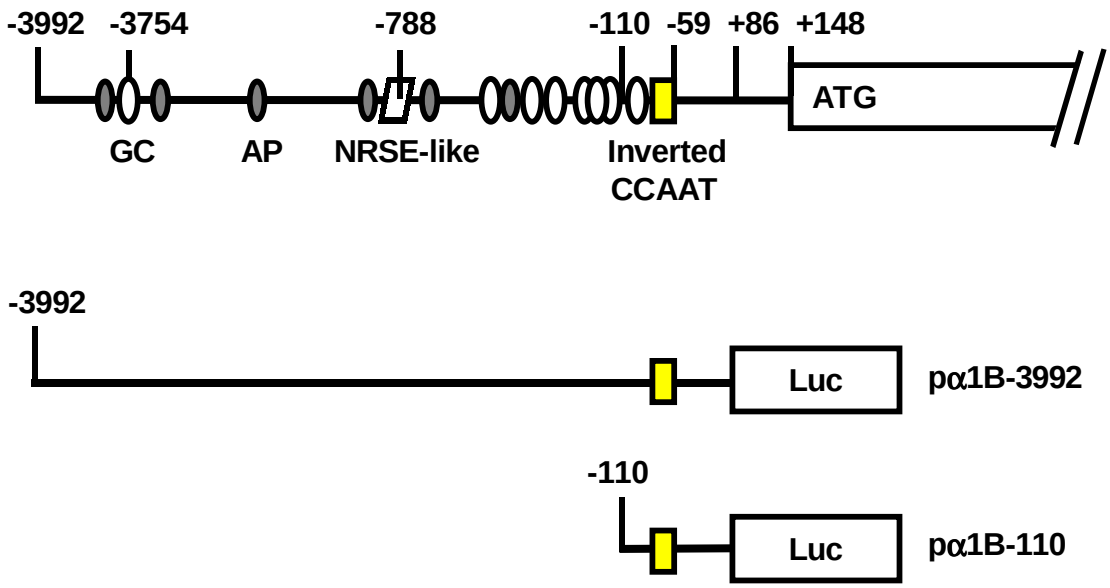


Fig. 2 The structure of the α_{1B} gene and deletion constructs of the α_{1B} gene. The α_{1B} gene has TATA-less promoter with multiple GC box (open circle) , AP1, 2 and 4 binding sites (closed circle), NRSE-like element (open box), and CCAAT box (closed box). To determine the mechanism of transcription of the α_{1B} gene, the α_{1B}-luciferase fusion gene were made by deletion of 5' upstream sequence of α_{1B} promoter (gifted by Dr. D.S. Kim (Kyungbuk University, Republic of Korea). pα_{1B}-3992 contains the sequence from –3992 bp to +86 bp of the α_{1B} promoter and pα_{1B}-110 contains sequence from –110 bp to +86 bp. Both constructs have an inverted CCAAT box.

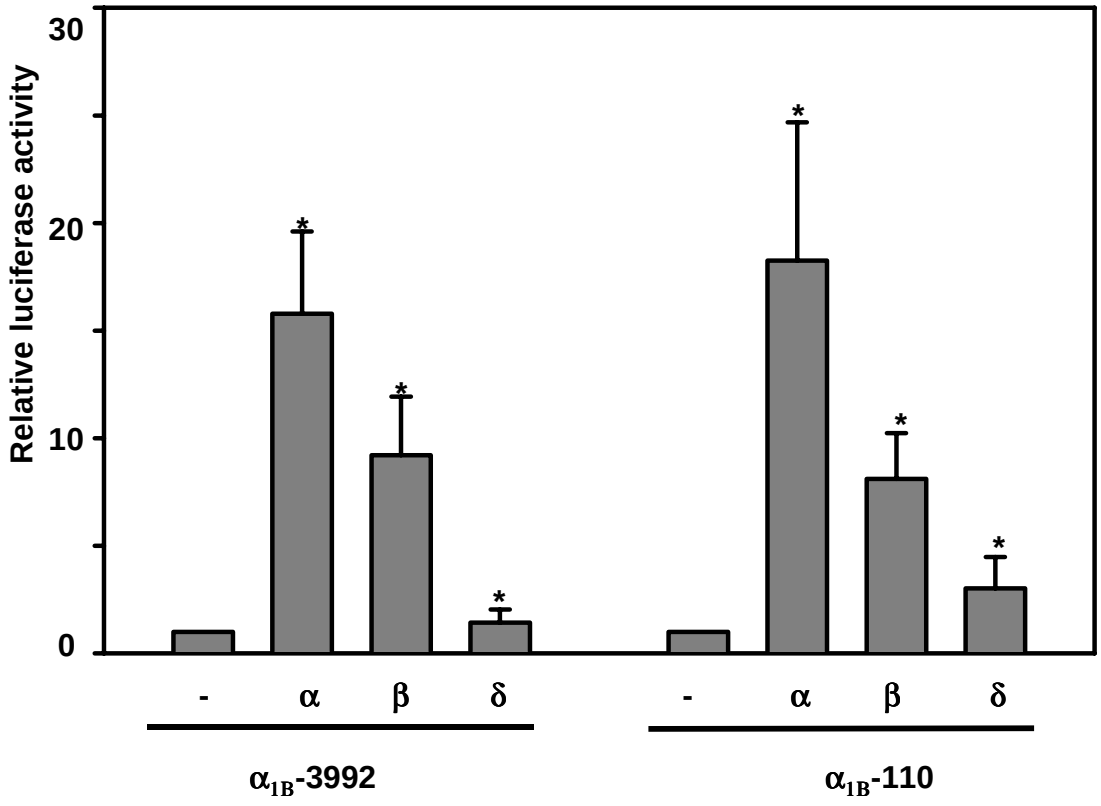


Fig.3 Normalized luciferase activity of C/EBP α, β, and δ on the deletion constructs of α_{1B}. The deletion constructs of α_{1B}, pα_{1B}-3992 and –110, and the expression vector of C/EBP isoforms were co-transfected into F11 cells by LipofectAMINE method. C/EBP α increased most the luciferase activity of pα_{1B}-3992 and –110 but other isoform β and δ of C/EBP at lesser extent (* : $p < 0.05$).

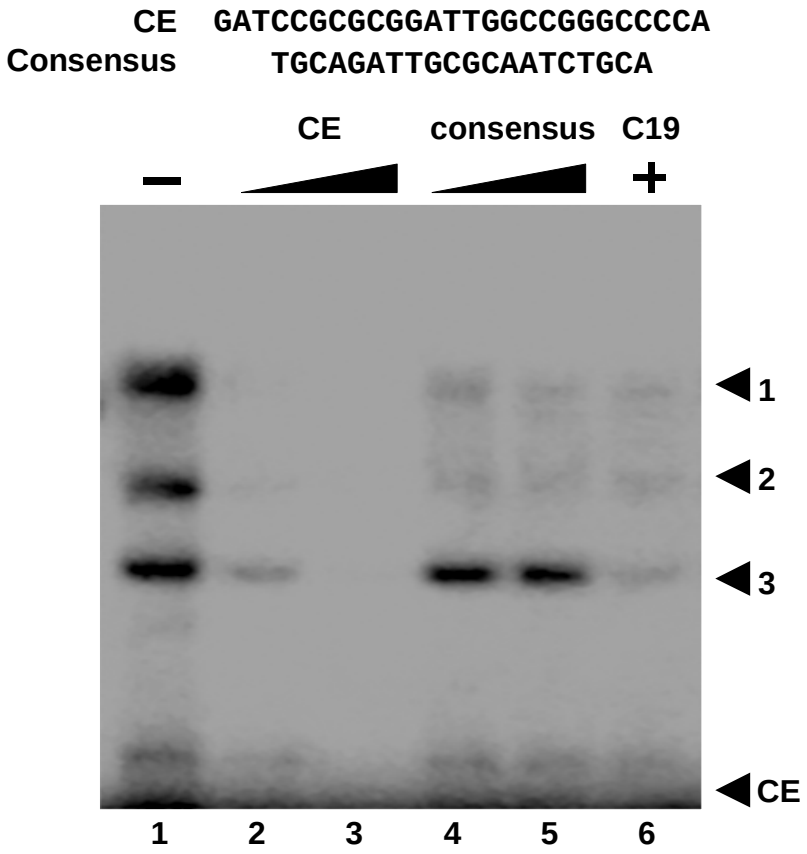


Fig.4 Specific Binding between C/EBP β and CCAAT box of α_{1B} promoter. Nuclear extracts were prepared from C/EBP β−transfected COS cells. Electrophoretic mobility shift assay (EMSA) was carried out with CE1, double stranded oligonucleotides containing CCAAT box. On the gel, there were three bands of DNA-protein complexes thought to be CCAAT box-C/EBP complexes (lane 1). These three bands (arrow 1,2, and 3) were competed with unlabelled CE1 (lane 2 and 3). Among three bands, upper two bands (arrow 1 and 2) were competed with C/EBP consensus binding oligonucleotides, suggesting that the binding of CCAAT box and C/EBP is specific and CCAAT box is operative (lane 4 and 5). Also upper two bands were recognized by anti-C/EBP β antibody (lane 6). Free probes are designated with 'CE' (arrow CE).

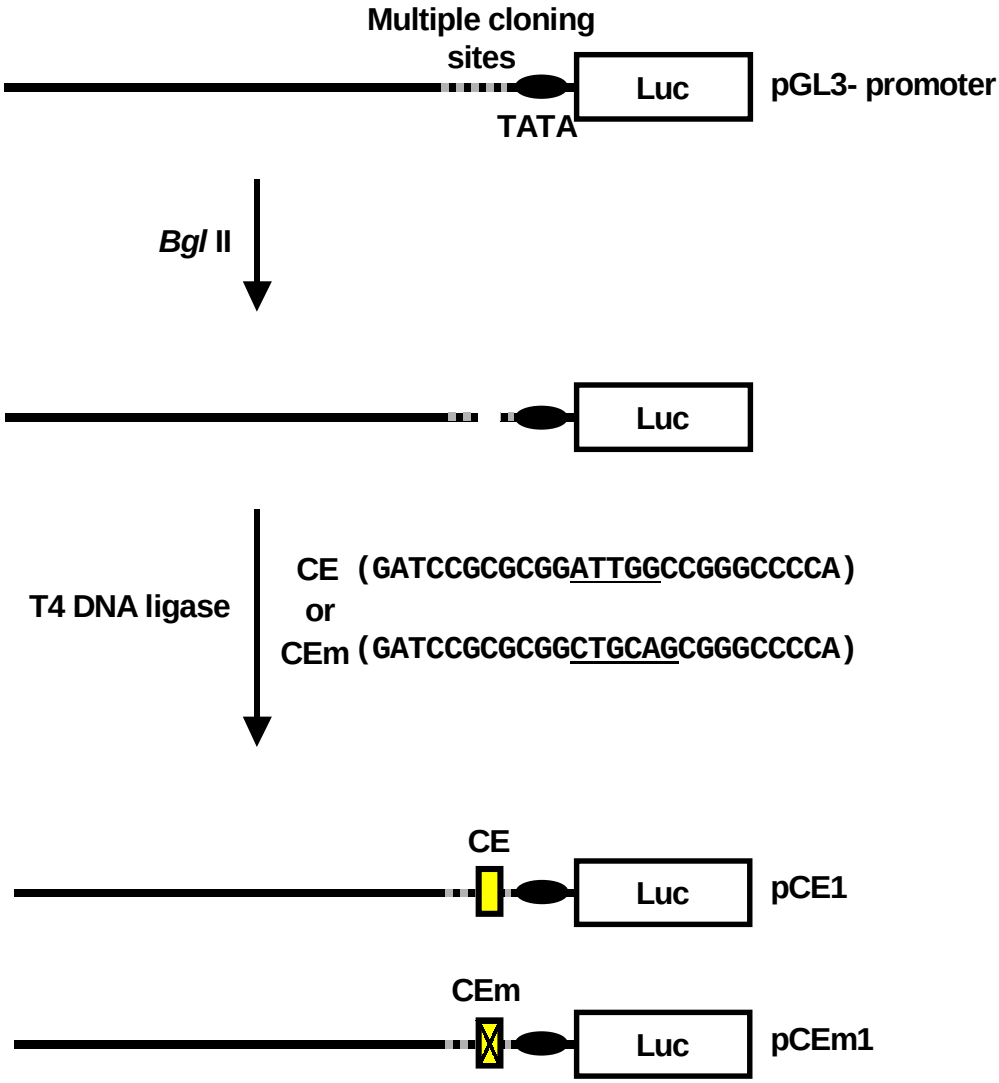


Fig. 5 The construction of pCE1 and pCEm1. pCE1 was constructed by inserion of a CE1, a double-stranded oligonucleotide containing CCAAT box of α_{1B} gene, into pGL3 promoter. pCEm1 was made by insertion of one copy of CEm1 oligonucleotides into pGL3 promoter.

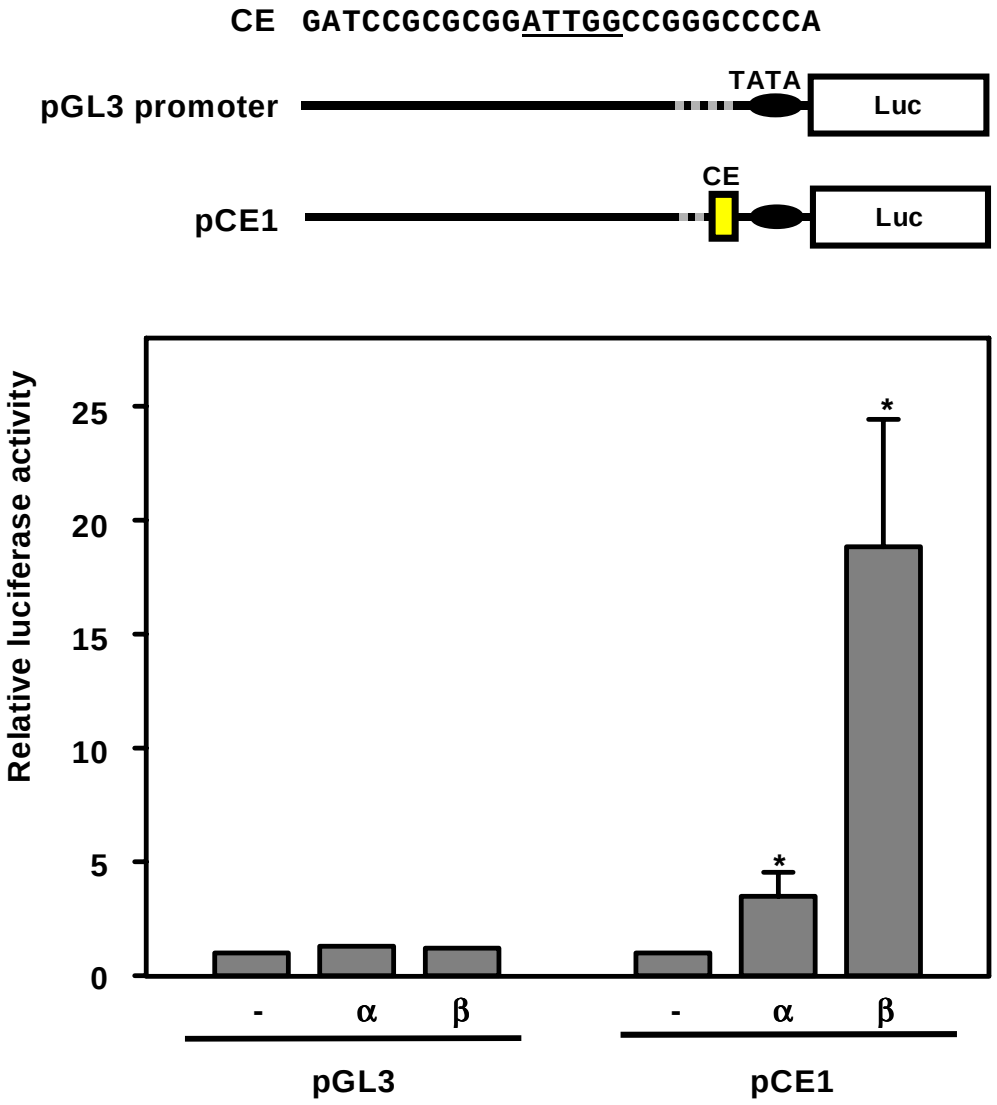


Fig.6 Normalized luciferase activity of C/EBP α and β on the deletion constructs of α_{1B} and pCE1. F11 cells were transfected with pCE1 or empty pGL3 promoter with or without expression vector of C/EBP isoforms by LipofectAMINE method. C/EBP α and β enhanced the luciferase activities of pCE1, the construct inserted with CCAAT box of α_{1B} promoter (* : $p < 0.05$).

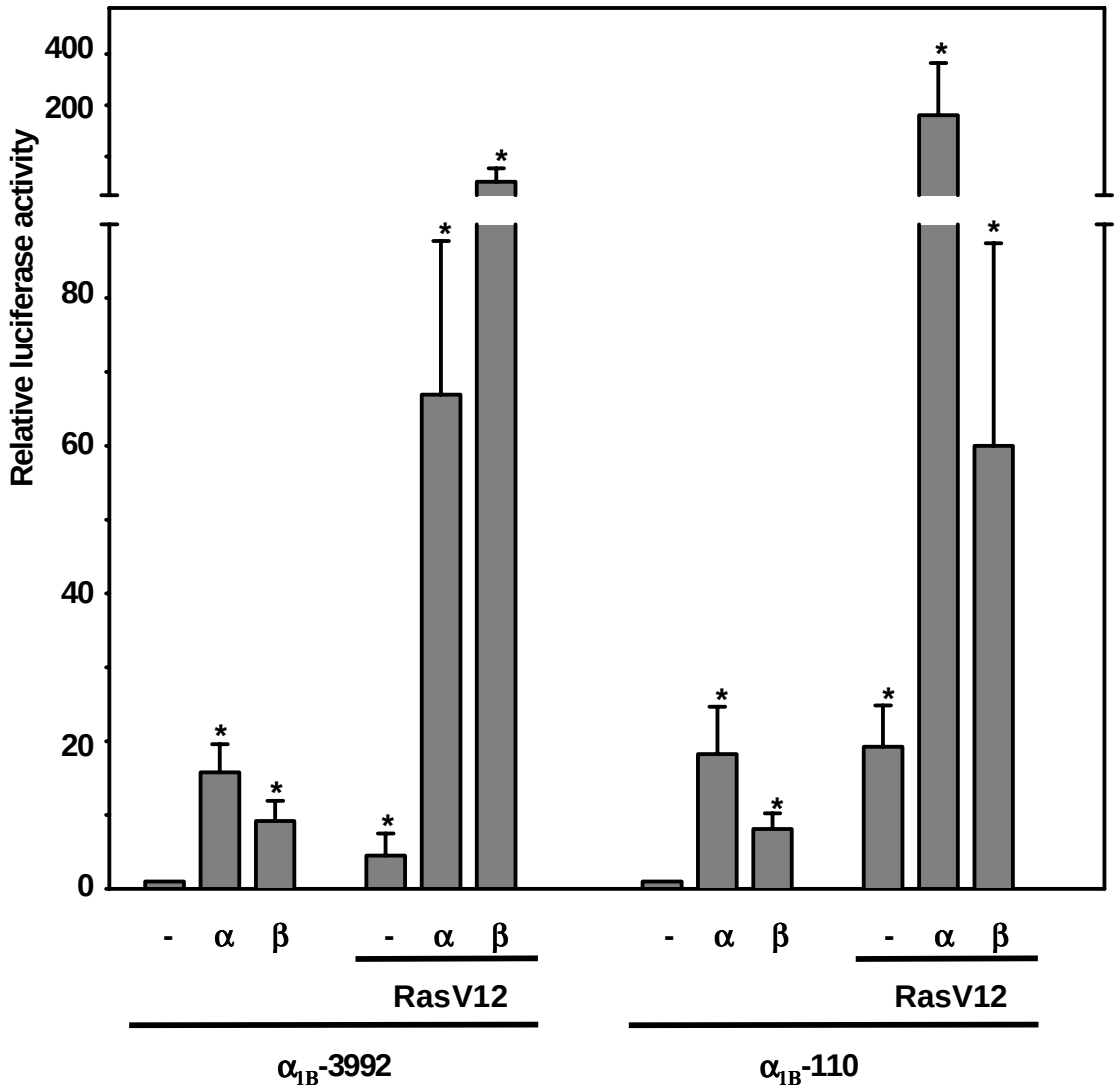


Fig. 7 The constitutive form of Ras, RasV12, facilitated C/EBP–mediated transactivation of the deletion constructs of α_{1B}, pα_{1B}-3992 and –110. F11 cells were transfected with reporter and activator genes by LipofectAMINE method. RasV12 can facilitate not only C/EBP-mediated promoter activity but also the basal activity of deletion constructs of the α_{1B} promoter (* : $p < 0.05$).

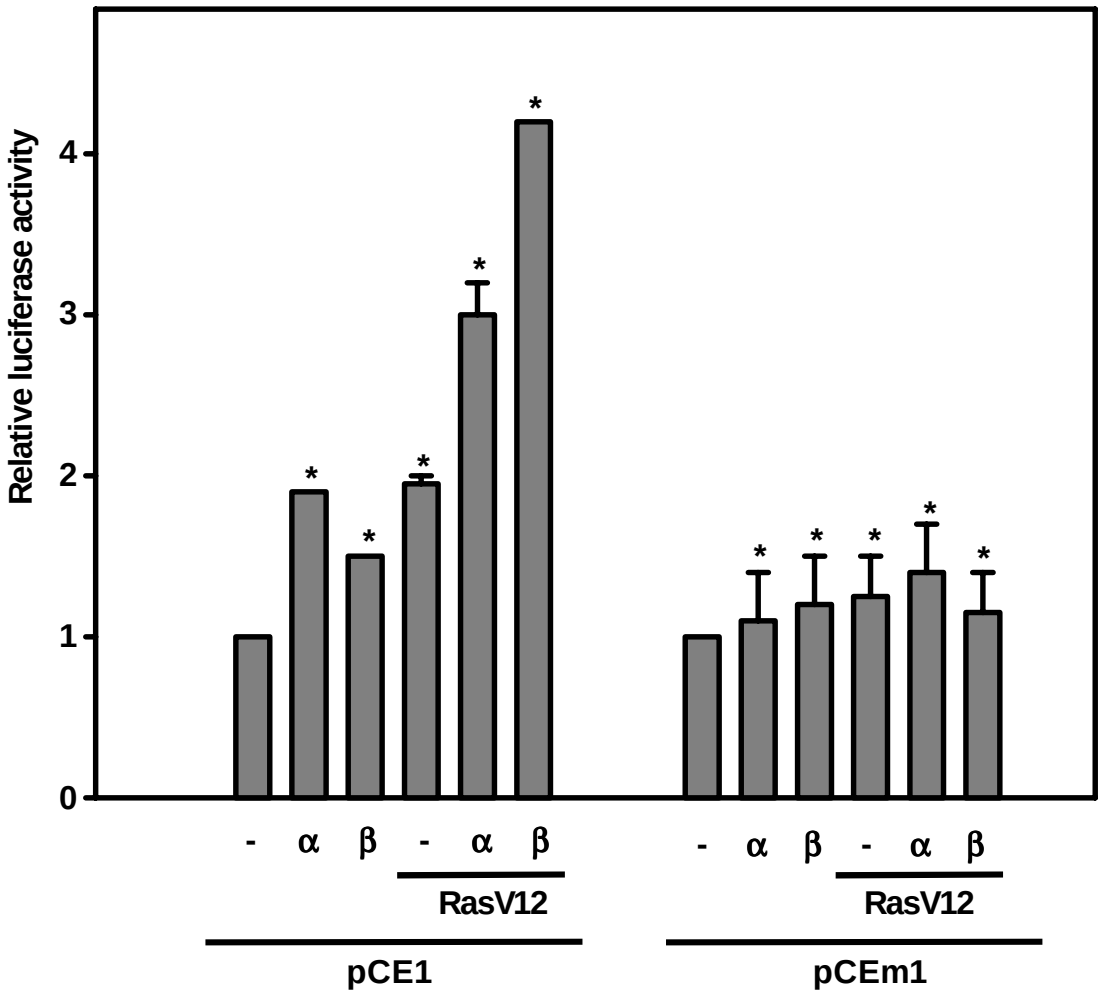


Fig. 8 The promoter activity of pCE1 and pCE1. C/EBP α and β enhanced the luciferase activity of pCE1 but not one of pCEm1, the mutated CCAAT box-inserted pGL3 promoter. These result showed C/EBP can enhance the promoter activity of pCE1 by sequence-specific interaction with CCAAT box. The constitutive form of Ras, RasV12, facilitated the luciferase activity C/EBP-mediated transactivation of pCE1 but not pCEm1. F11 cells were transfected with reporter and activator genes by LipofectAMINE method (* : $p < 0.05$).

Ⅳ. DISCUSSION

From these data, the investigator can conclude that C/EBP can specifically bind to the CCAAT element at -59 bp of the α_{1B} gene and that C/EBP can interact with Ras or can be regulated in the downstream of Ras, resulting the fine regulation of C/EBP-mediated expression of the α_{1B} gene. In the α_{1B} promoter, there are many potential sites for transcription factors including C/EBP, suggesting that transcription factors than C/EBP may regulate the expression of α_{1B} gene. C/EBPs can form homodimers or heterodimers and the partners for dimerization determine the properties of regulation[21,22]. In this study, the interaction of C/EBPs and other transcription factors has not been investigated. Since the α_{1B} gene lacks TATA box[17], therefore importance of CCAAT box is more extended.

All isoforms of C/EBP, α, β, and δ, enhanced the promoter activities of the α1B gene, but the degree of enhancement are different. In the differentiation process of preadipocytes into adipocytes, C/EBP β and δ initiate the differentiation and after expression of C/EBP α result in leading to terminal differentiation into mature adipocytes[23]. In these processes, ratio of C/EBP β and δ with respect to C/EBP α determines the rate and termination of differentiation[23]. The differential effects of C/EBP isoforms on the a1B promoter activity imply that the relative amount of each isoform may be important in the regulating the expression of α_{1B} gene and neuronal differentiation program.

C/EBP β is transcribed a single mRNA but translated into two different forms, 42 and 30 by a leaky ribosomal scanning mechanism. The full length of LAP (42 kDa) has the conserved activation domains found in other C/EBP proteins and the truncated protein whereas LIP (30 kDa) is translated from the third AUG codon in frame and has only the DNA binding and leucine zipper domain. LIP lacks transactivation domain and heteromerization of LAP with LIP attenuates transcriptional activity in substoichiometric amounts

(reviewed by Latchman D.S., Academic press[18]). Since several isoforms of C/EBP exist and they can homo- or hetero-dimerize it is possible the various DNA-protein complex can be detected in EMSA. In the Fig.4 there are 3 complexes specific for the CCAAT box. It is interesting to know why the lowest band was recognized with anti-C/EBP β antibody but not competed with excess amount of consensus oligonucleotides.

C/EBP has been reported that its binding activity and transactivation potentials are modulated by phosphorylation, mainly by protein kinase A, protein kinase C, or calcium/Calmodulin kinase IV[26]. These kinases are known to regulate the gene expression by phosphorylation of C/EBP. This possibility is implied for the α_{1B} gene by that Ras facilitated the C/EBP-mediated transactivation of α_{1B} gene synergistically. Ras has been reported to activate MAPK signaling pathway which leads to phosphorylation of C/EBP. The phosphorylation of C/EBP is major process for long-term potentiation and acute phase response[27] and the α_{1B} subunit plays important roles in synaptic transmission[1]. Therefore, C/EBP-mediated transactivation of the α_{1B} gene may be involved in the constitutive gene expression as well as the inducible expression by Ras-MAPK pathway responsive to specific stimuli. For signal transduction pathway, further investigations are needed.

V. CONCLUSION

From these data, This study shows that C/EBP can specifically bind to the CCAAT element at -59 bp of the α_{1B} gene and that C/EBP may function in the downstream of Ras signaling pathway. These results suggest that the fine regulation of C/EBP-mediated expression of the α_{1B} gene. Further investigation for the possible roles of C/EBP-mediated regulation of α_{1B} gene on the memory formation and further signaling pathway such as PKA, MAPK, or CaMK-mediated pathways for the α_{1B} gene will be needed.

BIBLIOGRAPHY

1. Wheeler DB, Randall A, Tsien RW : Roles of N-type and Q-type calcium channels in supporting hippocampal synaptic transmission. Science 264:107-111, 1994
2. Llinas RR, Sugimori M : Calcium conductances in Prukinje cell dendrites: their role in development and integraion. Prg Brain Res 51:323-334, 1979
3. Komuro H, Rakic P : Selective role of N-type calcium channels in neuronal migration. Science 257:806-809, 1992
4. Bading H, Ginty DD, Greenberg ME : Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. Science 260:181-186, 1993
5. Hofmann F, Biel M, Flockerzi V : Molecular basis for calcium channel diversity. A. Rev. Neurosci 17:399-418, 1994
6. Takahashi M, Seagar MJ, Jones JF, Reber BF, Catterall WA : Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. Proc Natl Acad Sci USA 84:5478-5482, 1987
7. Brust PF, Simerson S, McCue AF, Deal CR, Schoonmaker S, Williams ME, Velicerebi G, Johnson EC, Harfold MM, Ellis SB : Human neuronal voltage-dependent calcium channels : studies on subunit structure and role in channel assembly. Neuropharmacology 32:1089-1102, 1993
8. Isom LL, De Jongh KS, Catterall WA : Auxillary subunits of voltage-gated channels. Neuron 12:1193-1194, 1994
9. Flockerzi V, Oeken HJ, Hofmann F : Purification of a functional receptor for calcium-channel blockers from rabbit skeletal-muscle microsomes. Eur J Biochem 161:217-224, 1986
10. Zhang JF, Randall AD, Ellinor PT, Horne WA, Sather WA, Tanabe T, Schwarz

TL, Tsien RW : Distinctive pharmacology and kinetics of cloned neuronal calcium channel and their possible counterparts in mammalian ENS neurons. Neuropharmacology 32:1075-1088, 1993

11. McEnery MW, Snowman AM, Sharp AH, Adams ME, Snyder SH : Purified ω-conotoxin GVIA receptor of rat brain resemble a dihydropyridine-sensitive L-type calcium channel. Proc Natl Acad Sci USA, 1991
12. Fujita Y : Primary structure and functional expression of the ω-conotoxin-sensitive N-type calcium channel from rabbit brain. Neuron 10:585-598, 1993
13. Dubel SJ, Starr TV, Hell J, Ahlijanian MK, Enyeart JJ, Catterall WA, Snutch TP : Molecular cloning of the α1 subunit of an ω-conotoxin-sensitive calcium channel. Proc Natl Acad Sci USA 89:5098-5062, 1992
14. Komuro H, Rakic P : Selective role of N-type calcium cannels in neuronal migraion : Science 257:806-809,1992
15. Wheeler DB, Randall A, Tsien RW : Roles of N-type and Q-type calcium channels in supporting hippocampal synaptic transmission. Science 264:107-111, 1994
16. Witcher DR, De Waard M, Sakamoto J, Franizini-Armstrong C, Pragnell M, Ka1hl SD, Campbell KP : Subunit identification and reconstitution of the N-type calcium channel complex purified from brain. Science 261:486-489, 1993
17. Kim DS, Jung HH, Park SH, Chin H : Isolation and characterization of the 5' upstream region of the human N-type calcium channel α_{1B} subunit gene. J Biol Chem 272(8):5098-5104, 1997
18. Latchman DS : Eukaryotic transcription factors. 2_{nd} Ed. London, Academic Press, 1995, pp 2-7
19. Landschulz WH, Johnson PF, McKnight SL : The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science 243:1681-1688, 1989
20. Cao Z, Umeck RM, McKnight SL : Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 5:1538-1552,

1991

21. Vinson CR, Sigler PB, McKnight SL : Scissors-grip model of DNA recognition by a family of leucine zipper proteins. Science 246:563-572, 1989
22. Thomassin H, Hamel D, Bernier D, Guertin M, Belanger L : Molecular cloning of two C/EBP-related proteins that bind to the promoter and the enhancer of the α1-fetoprotein gene. Further analysis of C/EBP β and C/EBP γ. Nucleic acids Res 20:3091-3098, 1992
23. Umeck RM, Freidman AD, McKnight SL : CCAAT-Enhancer binding protein : a component of a differentiation switch. Science 251:288-926, 1991
24. Lekstrom-Himes J, Xanthopoulos KG : Biological roles of CCAAT/enhancer binding protein family of transcription factor. J Biol Chem 273(44):28545-28548, 1998)
25. Sterneck E : CCAAT/enhancer binding protein β is a neuronal transcription regulator activated by nerve growth factor receptor signaling. J Neurochem (70):2424-2433, 1998
26. Yukawa K, Tanaka T, Tsuji S, Akira S : Expression of CCAAT/enhancer binding protein β and δ and their activities are intensified by cAMP as well as Ca/Calmodulin kinases activation in hippocampal neurons. J Biol Chem 273(47):31345-31351, 1998
27. Hanlon M, Sealy L : Ras regulates the association of serum response factor and CCAAT/enhancer binding protein β. J Biol Chem 274(20):14224–14228, 1999

- 국문요약 -

Regulation of the N-Type Calcium Channel α_{1B} Gene by C/EBP

N-type calcium channel ($\alpha_{1B}\alpha_2\delta\beta$) 은 신경조직에 특이적으로 발현하여 신경세포연접부위에서의 전달과 신경세포의 이동에 중요한 역할을 한다고 알려져 있다. Pore를 형성하는 부분인 α_{1B} subunit의 프로모터 유전자에는 C/EBP (CCAAT/Enhancer Binding Protein), Sp1등의 여러 전사인자들이 결합할 수 있는 잠재적 결합부위가 존재한다. 신경조직에서 α_{1B} 유전자의 조직특이적 발현을 이해하기 위하여 α_{1B} 프로모터의 5 flanking sequence를 deletion하여 만든 construct를 이용하여 C/EBP의 역할에 대해 조사하였다.

F11 신경모세포종 세포주에서 C/EBP α, β, δ 은 α_{1B} 프로모터의 deletion construct에 대해 프로모터의 활성을 증가시킴을 관찰하였다. 이러한 활성의 증가가 α_{1B} 프로모터의 전사시작부위로부터 –59 bp위치에 있는 CCAAT box에 의한 것임을 확인하기 위하여, electrophoretic mobility shift assay를 수행한 결과 α_{1B} 프로모터의 CCAAT box를 포함한 sequence (CE)와 C/EBP β 단백질이 결합함을 확인하였다. CCAAT box를 경유한 C/EBP의 transactivation을 다시 한 번 확인하기 위하여 α_{1B} promoter의 CCAAT box를 포함한 sequence를 heterologous vector인 pGL3 프로모터 벡터에 연결한 플라스미드 pCE1를 제작하였다. F11 세포주에서 C/EBP α, β isoform은 pCE1의 프로모터의 활성을 증가시킴을 관찰하였다. 위의 결과를 종합해 볼 때 C/EBP는 α_{1B} 유전자의 CCAAT box에 특이적으로 결합하여

프로모터의 활성을 증가시킨다는 결론을 내릴 수 있었다.

따라서 본 연구의 결과들은 C/EBP가 α_{1B}의 발현을 조절하며 또한 조절과정에서 C/EBP가 α_{1B} 프로모터의 CCAAT box에 직접 결합한다는 것을 보여주었으며, 생체 내에서의 그 역할과 신호전달과 관련된 기전 연구는 이후 더 많이 진행되어야 할 것으로 생각된다.

핵심되는 말 : N-type calcium channel, α_{1B}, C/EBP