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Stimulation of corticotropin-releasing factor gene expression by FosB in rat hypothalamic 4B cells

Q1 Kazunori Kageyama[a,b,*], Keiichi Itoi[c], Yasumasa Iwasaki[d], Kanako Niioka[a], Yutaka Watanuki[a], Satoshi Yamagata[a], Yuki Nakada[a], Gopal Das[c], Toshihiro Suda[a,1], Makoto Daimon[a]

[a] Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan
[b] Department of Endocrinology, Metabolism, and Infectious Diseases, Hirosaki University School of Medicine & Hospital, Hirosaki 036-8563, Japan
[c] Laboratory of Information Biology, Graduate School of Information Sciences, Tohoku University, Sendai 980-8579, Japan
[d] Health Care Center, Kochi University, Kochi 780-8520, Japan

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ABSTRACT

The Fos- and Jun family proteins are immediate-early gene products, and the Fos/Jun heterodimer, activator protein-1 (AP-1), may be involved in the regulation of corticotropin-releasing factor (CRF) gene expression. FosB is a member of the Fos family proteins that is expressed in the paraventricular nucleus of the hypothalamus upon stress exposure, but it has not been clear whether FosB participates in the regulation of CRF gene expression. This study aimed to explore the effect of the FosB and cJun proteins on CRF gene expression in rat hypothalamic 4B cells. The levels of FosB mRNA and cJun mRNA increased following treatment with forskolin, phorbol-12-myristate-13-acetate (PMA), or A23187 in the hypothalamic cells. Overexpression of FosB or cJun potently increased CRF mRNA levels. Furthermore, downregulation of FosB or cJun suppressed the CRF gene expression induced by forskolin, PMA, or A23187. In addition, the basal CRF mRNA levels were partially reduced by cJun downregulation. These findings suggest that FosB, together with cJun, may mediate CRF gene expression in the hypothalamic cells.

1. Introduction

Corticotropin-releasing factor (CRF) is a key-player in the hypothalamic-pituitary-adrenal (HPA) stress-axis [27,28]. CRF-expressing neurons are located in the parvocellular division of the paraventricular nucleus of the hypothalamus (PVN) [11], and CRF regulates synthesis and secretion of glucocorticoid from the adrenals *via* adrenocorticotropic hormone (ACTH), while protecting an organism from various stressors [8].

Multiple transcription factors, including the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and activator protein-1 (AP-1), regulate CRF gene promoter activity [25,29]. The cyclic AMP-dependent protein kinase A (PKA) pathway is the major regulatory mechanism for CRF gene expression [16], especially following acute exposure to stressors since phosphorylation of CREB takes place within minutes after cellular stimulation [4]. Protein kinase C (PKC) and p38 mitogen-activated protein (MAP) kinase may also be involved partially in the positive regulation of cAMP-dependent CRF gene expression [16]. However, the functional roles of AP-1 in CRF gene expression remain to be fully understood.

Fos- and Jun family proteins are components of the AP-1 transcription factor and known to be rapidly induced in response to diverse extracellular stimuli as immediate-early gene products [13]. Five Fos family proteins (cFos, Fra-1, Fra-2, FosB, and the naturally truncated form ΔFosB) and three Jun family proteins (cJun, JunB, and JunD) have been identified. The Fos family protein is capable of forming dimers with one of the Jun proteins (Fos/Jun heterodimers), which bind to the regulatory sequences of target genes [18]. The Jun homodimers also bind to the TPA-response element (TRE) of target genes [5,12], although Fos/Jun heterodimers are more stable and have a stronger DNA binding activity than Jun homodimers [24]. cJun, JunB, and JunD were reported to be similar in their properties for DNA binding and interaction with Fos [21], while FosB forms the most stable complex with cJun among the other Fos family proteins[24].

Since cFos is rapidly expressed in neurons following stimulation, it has been utilized as a marker for neuronal activation [18]. In contrast, FosB has been implicated in more delayed or

* Corresponding author at: Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan. Tel.: +81 172 39 5062; fax: +81 172 39 5063.
E-mail address: kkageyama@hkg.odn.ne.jp (K. Kageyama).
[1] Present address: Aomori Rosai Hospital, Hachinohe 031-8551, Japan.

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long-lasing neuronal activation [22]. The functional roles of FosB have been studied most extensively in the nucleus accumbens and dorsal striatum, and the ΔFosB proteins, truncated isoforms of FosB, have been proposed to be involved in the chronic effects of drugs of abuse [22]. Expression of FosB/ΔFosB transcription factors is also induced within the PVN by surgical [7] or restraint stress (our unpublished observation), as well as acute or repeated exposure to drugs of abuse [6]. The FosB/ΔFosB expression is more sustained, which makes contrast to the transient expression of cFos following activation and desensitization by repeated stimuli [3,7,9,20,26].

The immunoreactivity of FosB/ΔFosB is colocalized mostly in CRF-immunoreactive (ir) neurons in the PVN [2,6,7] and vasopressin-ir neurons in the supraoptic nucleus [7] among other hypothalamic neuroendocrine neurons. Although expression of FosB is upregulated in the hypothalamus in response to stress, the role of FosB in CRF gene expression, as well as its molecular mechanisms, has yet to be determined. In the present study, we sought to elucidate whether FosB and cJun proteins mediate CRF gene expression in rat hypothalamic 4B cells. To determine the possible pathways involved, we examined the effects of forskolin (FSK), phorbol-12-myristate-13-acetate (PMA), or A23187 on FosB and cJun mRNA levels in hypothalamic 4B cells. Next, the effects of FosB overexpression and down regulation on stimulated CRF gene expression were also investigated.

2. Materials and methods

2.1. Materials

FSK, PMA, and A23187 were purchased from Calbiochem (San Diego, CA, USA).

2.2. Plasmid construction

cDNAs of mouse FosB and cJun expression vectors were cloned by reverse transcription polymerase chain reaction (RT-PCR) with total RNA extracted from mouse AtT20 corticotroph cells and inserted into a pRc/cytomegalovirus expression vector (BD Clontech, Palo Alto, CA). The specific primers used for PCR were as follows: FosB-forward (F) (5′-TTGCGGCCGC CAGGGAAATG TTTCAAGCTT TTCCC-3′), and FosB-reverse (R), (5′-TTTCTAGAGA GTTTACAGAG CAAGAAGGGA-3′); and cJun-F (5′-CCTCCAAGCT TGCCACCATG ACTGCAAAGA TGGAAACGAC-3′), and cJun-R (5′-CTCGAGCGGC CGCTCAAAAC GTTTGCAACT GCTGCG-3′).

2.3. Cell culture

4B cells were incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 20% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were plated at 1 or 5×10^4 cells/well for one day before transfection for each experiment. One day before each experiment, the cells were washed and then serum-starved overnight in DMEM/F12 supplemented with 0.2% bovine serum albumin (BSA).

2.4. Transfection

The 4B cells, seeded in 12-well plates at a density of 1×10^4 cells/well, were incubated for 24 h. The cells were transiently transfected with X-tremeGENE 9 DNA Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) in accordance with the manufacturer's instructions. A reagent to DNA ratio of 3 μL:1 μg was used. The total amount of transfected plasmid DNA was kept constant by the addition of empty control vectors at 1 μg per well. The cells were harvested 48 h after transfection, and total RNA was extracted.

2.5. RNA interference experiments

FosB (No. SI03116050), cJun (No. SI01526784), and control (No. 1027280) siRNAs were designed and purchased from Qiagen (Hilden, Germany). The 4B cells were transfected with siRNA and HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol. The 4B cells, seeded in 12-well plates at a density of 1×10^4 cells/well, were incubated for 24 h in 1 mL of culture medium containing siRNA for FosB (siFosB), cJun (sicJun), or control (siControl). The reagent to siRNA ratio of 9 μL:75 ng was used per well. After transfection, the cells were incubated with vehicle, 10 μM FSK, 1 μM PMA, or 1 μM A23187. The expression levels of CRF and β2-microglobulin mRNA were examined by quantitative RT-PCR.

2.6. RNA extraction

Total cellular RNA was extracted with an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. cDNAs were then synthesized from total RNA (0.5 μg) with random hexamers as primers with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.7. Real-time RT-PCR

The resulting cDNAs were subjected to real-time RT-PCR to examine changes in mRNA levels. The expression levels of rat CRF mRNA were evaluated with quantitative real-time RT-PCR with the following specific sets of primers and probes: CRF-F (5′-TGGATCTCAC CTTCCACCTT CTG-3′), CRF-R (5′-CCGATAATCT CCATCAGTTT CCTG-3′), and probe (5′-FAM-GCCAGGGCAG AGCAGTTAGC-TAMRA-3′). The expression levels of rat FosB and cJun mRNA were evaluated with quantitative real-time RT-PCR with specific sets of primers and probes (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA, USA). β2-microglobulin was not changed by any treatment and was used as a housekeeping gene to normalize values. Each reaction consisted of 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 1 × Assays-on-Demand Gene Expression Product (Rn04224888 uH for rat FosB, Rn99999045 s1 for rat cJun, and Rn00560865 m1 for rat β2-microglobulin), or a set of CRF primers and probes, and 1 μL of cDNA in a total volume of 25 μL, with the following parameters on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95 °C for 10 min, 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. Data were collected and recorded with ABI PRISM 7000 SDS software (Applied Biosystems) and expressed as a function of the threshold cycle. The amplification efficacies for each gene of interest and the housekeeping gene amplimers were found to be identical when analyzed with diluted samples.

2.8. Statistical analysis

Experiments were performed at least three times. Samples were provided in triplicate for each group of experiments. Each value is expressed as the mean ± standard error of the mean (SEM). Statistical analyses of the data were performed with analysis of variance (ANOVA), followed by the Fisher's protected least-significant difference *post hoc* test. The level of statistical significance was set at $P < 0.05$.

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