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α-Actinin4 nuclear translocation mediates gonadotropin-releasing hormone stimulation of follicle-stimulating hormone β-subunit gene transcription in LβT2 cells

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ABSTRACT

Gonadotropin-releasing hormone (GnRH) regulates the synthesis and secretion of follicle-stimulating hormone (FSH) by stimulating the transcription of *Fshβ* gene. Our iTRAQ quantitative proteomics result showed that the abundance of α-actinin4 (ACTN4) increased in the nuclei of LβT2 cells upon GnRH induction. Using RNA interference, reverse transcription and real-time PCR, luciferase and transient transfection assays, we proved that ACTN4 is involved in the regulation of mouse *Fshβ* gene (*mFshβ*) transcription and its C-terminal calmodulin (CaM)-like domain is crucial for this process. Our study suggests that ACTN4 nuclear translocation mediates GnRH stimulation of *mFshβ* gene transcription.

1. Introduction

Gonadotropin-releasing hormone (GnRH), a hypothalamic decapeptide, serves as a key hormone to regulate reproduction of vertebrates. GnRH is released from hypothalamus and functions through GnRH receptor (GnRHR) binding to induce the activation of multiple downstream signaling pathways, which eventually stimulate the synthesis and secretion of pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [1]. LH and FSH are key regulators of reproductive development and functions. Therefore, elucidating the mechanisms underlying GnRH response to gonadotropins is of great importance to understand the molecular basis of reproduction. The gonadotropins LH and FSH are heterodimeric hormones, each of which contains an identical α-subunit and a unique β-subunit [2]. Since β-subunit confers the physiological specificity for these hormones, studies on the regulation of gonadotropins have mainly focused on their β-subunits.

The molecular mechanisms of the transcriptional regulation of gonadotropins have been studied previously. However, unlike *Lhβ* gene, whose regulatory mechanism has been elucidated and seems to be relatively conserved among the mammalian species (reviewed by [3]), regulations of *Fshβ* gene are still under active investigation and it turns out to be species-specific due to genetic variations in the proximal promoter among several mammalian species [4]. Previous studies on GnRH-induced regulation of *Fshβ* gene have successfully identified factors involved in the regulation of basal transcriptional machinery, which include *cis*-elements and transcription factors (reviewed by [4,5]). Moreover, several GnRH-induced signaling pathways are being revealed, of which the mitogen-activated protein kinase (MAPK) signaling pathways are extensively studied.

To further detect the proteins involved in GnRH-induced regulation of *mFshβ* gene and its signal transduction pathways, we utilized the iTRAQ quantitative proteomics approach to identify differentially expressed proteins in the nucleus of GnRH-treated LβT2 cells. These proteins may be important for hormone-mediated gene regulation. Compared to the former comparative microarray studies [6,7], this proteomics analysis may provide protein information not only limiting to the change in abundance but also the cellular localization, which would greatly increase the opportunity to identify transcription factors that frequently exert their functions through nucleo-cytoplasmic translocation.

Our study identified that α-actinin4 (ACTN4) is one of the up-regulated proteins in the nucleus of LβT2 cells induced by

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GnRH. Functional studies showed that ACTN4 may be a potential positive regulator of *Fshβ* gene transcription, likely through its C-terminal calmodulin (CaM)-like domain.

2. Materials and methods

2.1. Cell culture, transfection and reagents

LβT2 cells (generously provided by Dr. P. Mellon, University of California, San Diego, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% certified fetal calf serum, 10 mM HEPES (pH 7.4), 100 U/ml penicillin and 100 μg/ml streptomycin, which were purchased from Invitrogen (Carlsbad, CA, USA), and were incubated at 37 °C under 5% CO_2. Transfection was carried out at 70–80% confluence using Lipofectamine 2000 (Invitrogen), and the cells were further incubated for 48–72 h before analysis.

Four-plex iTRAQ reagent kit was from AB SCIEX (MA, USA). Polyclonal antisera against ACTN4 was purchased from immunoGlobe (GmbH, Germany). Nucleophosmin (NPM) antibody was purchased from Abcam (Cambridge, UK). Actin antibody was purchased from BD Biosciences (CA, USA). FLAG antibody, GnRH and phosphatase inhibitors, poly-L-lysine, paraformaldehyde, and saponin were purchased from Sigma–Aldrich (MI, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Santa Cruz (CA, USA) and the cocktail proteinase inhibitor was obtained from Roche Diagnostics (GmbH, Germany). All other chemicals are of analytical grade.

2.2. Protein extraction, iTRAQ identification and western blotting

After treatment with 100 nM GnRH or mock, LβT2 cells were rinsed with PBS, and cell pellets were collected by trypsin digestion followed by centrifugation at 2000 rpm for 5 min. For the extraction of total proteins, the cell pellet was lysed in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) followed by centrifugation at 13,000 rpm for 20 min at 4 °C, and the supernatant was used for the analysis. NE-PER kit (Pierce, IL, USA) was used to prepare nuclear and cytoplasmic fractions. The total protein concentration was measured by Bradford protein assay (Bio-Rad, CA, USA), and the protein concentrations of the nuclear and cytoplasmic fractions were determined by RC-DC protein assay (Bio-Rad). iTRAQ identification was carried out as previously described [8]. The nuclear fractions of the mock-treated and GnRH-treated LβT2 cells were labeled with 4-plex iTRAQ reagent kit. Two biological replicates were examined for each mock-treated and GnRH-treated samples. Western blot analysis was performed as described by Luo et al. [9], using polyclonal antisera against ACTN4, NPM, actin and FLAG.

2.3. Confocal microscopy of ACTN4 nuclear translocation and quantitation

LβT2 cells were seeded on poly-L-lysine coated sterilized coverslips in 6-well plates and starved in serum-free medium for 16 h before GnRH treatment. After treated for the indicated times, cells were washed twice with ice-cold Dulbecco's PBS (DPBS; PBS with Ca^{2+} and Mg^{2+}), and then fixed in 3% paraformaldehyde at room temperature for 15 min followed by permeabilization with PBS containing 0.5% saponin at room temperature for 15 min. After permeabilization, the fixed cells were blocked in DPBS containing 10% horse serum plus 1% bovine serum albumin at room temperature for 1 h. Incubation with primary antibodies was done for 1 h at room temperature or overnight at 4 °C. The cells were washed 3 times in DPBS containing 0.1% saponin, and incubated with the secondary antibodies for 1 h at room temperature, followed by 6 times washing in DPBS containing 0.1% saponin. The stained cells were then mounted with 4′,6-diamidino-2-phenylindole (DAPI) containing mounting solution (Santa Cruz), and examined using LSM510 confocal fluorescence microscope (Carl Zeiss, Germany) with 63× Plan-Apo objective lens N.A. = 1.4.

For fluorescence quantitation assays, the unit fluorescence in the nucleus (I_n) and the whole cell (I_w) were calculated as intensity/area. The results were shown as the fold change over mock-treated controls. A minimum of 50 cells (i.e., ±10 microscopic fields) in total were counted. Statistical analyses were performed by two-sample homoscedastic *t*-test.

2.4. Generation of ACTN4 constructs for overexpression and knock-down assays

To generate the ACTN4 overexpression construct ACTN4 OE, the full-length ACTN4 was PCR-amplified from LβT2 cDNA using primers designed to include HindIII and XhoI restriction sites (5′-CCCAAGCTTATGGTGGACTACCACGCAGC-3′ and 5′-CCGCTCGA GTCACAGGTCGCTCTCCCCAT-3′). The digested product was subcloned into pXJ40-FLAG vector. For CaM-deleted ACTN4 mutant, ACTN4 gene from 1–2499 bp was PCR-amplified using the same forward primer for the full-length ACTN4 and the reverse primer, 5′-CCGCTCGAGTCAAATGAAGGCTTGGAAGGTC-3′, digested and inserted into pXJ40-FLAG vector between HindIII and XhoI restriction sites.

For ACTN4 knock-down experiment, two constructs siACTN4$_{976}$ and siACTN4$_{2032}$ containing ACTN4 short hairpin RNA (shRNA) were generated using pSUPER vector (Oligoengine, Seattle, USA). They are designed to target two specific regions of the mouse ACTN4 mRNA. The 19-targeted-oligonucleotides used in siACTN4$_{976}$ is 5′-GCACCT GATGGAAGACTAT-3′ and in siACTN4$_{2032}$ is 5′-GCAATCCAATGAGCA CCTT-3′. The shRNA targeting green fluorescence protein (shGFP) was used as a control, as described by Luo et al. [9].

2.5. RT-PCR and real-time PCR

RNA isolation from LβT2 cells was performed using TRIzol (Invitrogen) according to the manufacturer's protocol, and the total RNA (2 μg) was reverse-transcribed using SuperScript III first-strand synthesis system (Invitrogen). Of the 20 μl cDNA obtained, 1–2 μl was used for a semi-quantitative PCR with specific primers as listed in Supplementary Table 1. Real-time PCR was performed using Power SYBR PCR master mix (Applied Biosystems, CA, USA) with an ABI 7000 sequence detector. Primers for real-time PCR are shown in Supplementary Table 1. The samples were first warmed to 50 °C for 2 min, and then heated to 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative changes in mRNA levels were calculated using ΔΔCt method. Each value is representative of three replicates.

2.6. Luciferase assays

The luciferase reporter construct was generated by fusing the −384/+1 bp of the *mFshβ*-promoter to firefly luciferase gene pGL4.10 (Promega, WI, USA). Firefly luciferase values were normalized to those of *Renilla* luciferase, which was cotransfected as an internal control.

Cells were seeded in 24-well plates. After overnight plating, 500 ng/well of over-expression/shRNA constructs, 500 ng/well of the luciferase reporter constructs and 25 ng/well of simian virus 40 (SV40) were transient–transfected for 48–72 h. After harvest, the cell lysates were split into 96-well plate. Luciferase activity was measured by using the Dual-Glo system (Promega), and a Verita Microplate luminometer (Turner Biosystem, CA, USA). Luciferase activity was normalized to the level of *Renilla* luciferase. The