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Translational level of acetylcholine receptor α mRNA in mouse skeletal muscle is regulated by YB-1 in response to neural activity

Sachiyo Ohashi [1], Masamitsu Moue [1], Toru Tanaka, Shunsuke Kobayashi *

Department of Biochemistry, College of Pharmacy, Nihon University, 7-7-1, Narashinodai, Funabashi, Chiba 274-8555, Japan

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ABSTRACT

Y-box-binding protein 1 (YB-1) binds to mRNAs and affects translation. In this study, we focused on skeletal muscle, in which YB-1 expression is restricted to the early postnatal period, and found that YB-1 binds to acetylcholine receptor α-subunit (AChR α) mRNA. Although transcription of the AChR α gene is known to be regulated by myogenic transcription factors, translational control of the mRNA in response to neuromuscular transmission has not been examined. In undifferentiated C2C12 myoblasts, expression of AChR α remained at a low level. However, translation of the mRNA was increased by knockdown of YB-1. Continued overexpression of YB-1 prevented the cells from differentiating. In myotubes, which show clustering of AChRs, translation of the mRNA was induced within 3 h after treatment with nicotine. The effect of nicotine was inhibited by α-bungarotoxin, and in the presence of cycloheximide the level of AChR α was reduced, even after nicotine treatment. Sucrose gradient analysis revealed that in nicotine-treated myotubes, YB-1-containing polysomes were shifted to the heavier-sedimenting fractions, and showed an apparent decrease in the amount of YB-1 bound to AChR α mRNA. These results suggest that in skeletal muscle cells, neural activity reduces the molar ratio of YB-1 relative to its binding AChR α mRNA, leading to an increase of ribosome binding to the mRNA, and thus activating translation. Furthermore, in postnatal growing mice, as has already been shown, the level of AChR α mRNA declined during the early period with maturation of neuromuscular synapses, but the translation level was found to increase transiently at postnatal day 10, when the level of YB-1 was markedly reduced. It is suggested that although the level of AChR α mRNA is reduced, the translation can be induced by alteration of the ratio of YB-1 protein to the mRNA.

1. Introduction

For gene expression in response to various conditions within cells, as well as transcriptional control, a regulated translation reaction is important. YB-1 is a multifunctional protein that controls gene expression at both the transcriptional and translational levels [1]. In the cytoplasm of normal cells, YB-1 associates with mRNA and alters its translation [2]. We have previously demonstrated that the expression of YB-1 and its association with polysomes are regulated in parallel with growth or aging in a tissue-specific manner [3]. In the brain, the expression of YB-1 is closely associated with development, and YB-1 binds to GluR2 mRNA and CaM1 mRNA to regulate their levels of translation in an activity-dependent manner [4]. However, in other tissues, the mRNA species whose translation is controlled by YB-1 are largely unknown. In skeletal muscle in particular, the period of YB-1 expression in the postnatal period is further limited, being evident for only a few days after birth [3], suggesting that YB-1 is involved in regulation of the translational levels of mRNAs encoding the proteins necessary for development of skeletal muscle in the early postnatal stage.

In the present study, using immunoprecipitation with anti-YB-1 antibody and RT-PCR, we found that YB-1 binds to AChR α mRNA in polysomes of 3-day-old mouse skeletal muscle. AChR α is indispensable for neuromuscular transmission. Therefore, it would be interesting to investigate translational regulation of the mRNA by YB-1 in response to neural activity. In undifferentiated C2C12 myoblasts, YB-1 interacts with the mRNA and exerts an inhibitory effect on its translation. To examine activity-dependent translational regulation of the mRNA, we used differentiated C2C12 myotubes on which AChRs cluster spontaneously [5]. When the myotubes were treated with nicotine, the level of AChR α protein was increased within 3 h after the treatment without any significant change in the level of AChR α mRNA. In the nicotine-treated myotubes, the distribution of YB-1 and S6 ribosomal protein on a sucrose gradient was shifted to heavier-sedimenting polysome fractions, consistent with translational induction. Furthermore, immunoprecipitation analysis and RT-PCR revealed that in the YB-1-associating polysomes, the amount of YB-1 binding to AChR α mRNA was decreased. A transient

* Corresponding author. Fax: +81 47 465 4870.
E-mail address: kobayashi.shunsuke@nihon-u-ac.jp (S. Kobayashi).
[1] These authors contributed equally to this work.

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increase of AChR α protein with decrease of the molar ratio of YB-1 relative to AChR α mRNA was also observed in postnatal growing muscle in which neuromuscular transmission became activated. These observations indicate that in mouse skeletal muscle cells, the translational level of AChR α mRNA is regulated by alteration of the interaction between YB-1 and the mRNA in response to neural activity.

2. Materials and methods

2.1. Cell culture

C2C12 myoblasts were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. For suppression of YB-1 expression, the cells were transfected with YB-1-specific siRNA (Thermo Scientific: M-048134-01) using LipofectAmine 2000 reagent (Invitrogen) in accordance with the manufacturer's protocol. A control siRNA (D-00120-01) was also purchased from Thermo Scientific. For overexpression of YB-1, cells were transfected with a plasmid DNA, pYB-1-GFP, which expresses the YB-1-EGFP fusion protein from the CMV promoter [4]. For formation of myotubes, the cells were cultured in DMEM until approximately 80% confluent, and then the medium was switched to differentiation medium consisting of DMEM with 5% fetal bovine serum for 5 days. To confirm clustering of AChRs, the myotubes were treated with Alexa Fluor 555-conjugated α-bungarotoxin (Invitrogen) and the AChRs was visualized with an Olympus fluorescence microscope linked to a DP-70 imaging system.

2.2. Protein preparation and Western blot analysis

Cells were lysed in TKM buffer, containing 50 mM triethanolamine (pH 7.8), 50 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, protein inhibitors (complete cocktail without EDTA, Roche), 1 mM DTT and ribonuclease inhibitor (0.2 unit/μl, Takara). The lysate was centrifuged at 3000 rpm for 10 min, and the supernatant was used as the postnuclear supernatant (PNS). For preparation of PNS from skeletal muscle of postnatal growing mice, the thigh muscles were homogenized in TKM buffer and centrifuged for 10 min, and then the supernatant was collected. PNS from other tissues were also prepared with TKM buffer. All experimental procedures using animals were approved by the Nihon University Animal Care and Use Committee. Western blot analysis was carried out as described previously [3]. Antibodies used were anti-YB-1 antibody [6], anti-AChR α antibody (BD Transduction Laboratories), anti-β actin antibody (Santa Cruz) and anti-S6 antibody (Cell Signaling Technology).

2.3. Sucrose gradient centrifugation

The PNS was prepared from tissue or cell lysate, with Nonidet P-40 added to 1%. The mixture was centrifuged on a 15–45% sucrose gradient using a SW40Ti rotor (Beckman Coulter). The gradient was fractionated, and used for Western blotting and immunoprecipitation analysis.

2.4. Immunoprecipitation analysis

Immunoprecipitation was performed using Dynabeads M280 sheep anti-rabbit IgG (Invitrogen) in accordance with manufacturer's protocol. Anti-YB-1 antibody (2 μg) was bound to the beads. The PNS or the mixture of polysome fractions was incubated with the beads for 4 h at 4 °C, and then the beads were collected with a magnet. After washing five times with PBS containing 0.1% BSA, the immune complex was eluted with buffer (20 mM Tris–HCl, pH 7.5, 140 mM NaCl, and 2% SDS) and proteins were analyzed by Western blotting.

2.5. Analysis of mRNAs targeted by YB-1

cDNA cloning of YB-1-bound mRNAs was performed using a SMART cDNA Library Construction Kit (Clontech) in accordance with the manufacturer's protocol. To analyze mRNAs in the immune complex, RNA was prepared by phenol–chloroform extraction. After ethanol precipitation, the RNA was dissolved in water and first-strand cDNA was synthesized with a reverse transcriptase MMLV (Takara) using reverse primers specific for each RNA. The primer pairs used for the RT-PCR were as follows: 5′-CTCCACAATGAAAAGACCATCCAGAGATAAACAAG-3′ (forward) and 5′-GATGTAACTCAATGAGCCGACCTGCAAACACAG-3′ (reverse) for AChR α mRNA, 5′-GCGCAAGTACTCAGTGTGGA-3′ (forward) and 5′-CCACAGCACGATTGTCGATT-3′ (reverse) for α-actin mRNA, 5′-TGGGCAAGAGTGAATTTTCC-3′ (forward) and 5′-ACCACCCACTTCA GGTTGAG-3′ (reverse) for dystrophin mRNA, 5′-CTGAGGATGAGCTGGAGGAG-3′ (forward) and 5′-GAATGTTCCGCTC TGCTTTC-3′ (reverse) for ribosomal S6 kinase (RSK) mRNA and 5′-TCCGGTGGATCCGCAGACCGTAACCAT-3′ (forward) and 5′-TCTGCAGTCGACTCGACGCGCATAGGG-3′ (reverse) for YB-1 mRNA.

2.6. Treatment of cells with reagents

C2C12 cells were allowed to differentiate to myotubes in the differentiation medium, and then nicotine was added to the medium at 10 μM. For cycloheximide treatment, the reagent was added 30 min before nicotine treatment. For blocking of the AChRs, α-bungarotoxin was added at 10 μM.

3. Results

3.1. YB-1 binds to AChR α mRNA in skeletal muscle and affects its translation level in C2C12 myoblasts

YB-1 expression in mouse skeletal muscle is restricted to the early postnatal period (Fig. 1A). There were no significant changes in the expression of YB-1 in other tissues between 3-day-old and 10-day-old mice (Fig. 1B). We first attempted to obtain several polysomal mRNAs to which YB-1 binds in hind limb skeletal muscle of 3-day-old mice. The PNS was fractionated on a 15–45% sucrose gradient, and YB-1 and S6 ribosomal protein were detected by Western blotting (Fig. 1C). YB-1 was distributed from non-polysomal to polysomal fractions with the ribosome protein, indicating that YB-1 associates with the translation machinery in skeletal muscle. We collected the heavy-sedimenting polysome fractions (Nos. 1–3) and performed immunoprecipitation with anti-YB-1 antibody. The mRNA was extracted from the immune complex and cDNA cloning was carried out. We identified AChR α mRNA, α-actin mRNA and dystrophin mRNA. The specific binding of YB-1 to the mRNAs was examined by further immunoprecipitation analysis and RT-PCR using specific primers (Fig. 1D). It is known that YB-1 does not bind to ribosomal S6 kinase (RSK) mRNA [7]. The mRNA was not precipitated by anti-YB-1 antibody, indicating that YB-1 binds specifically to AChR α, α-actin, and dystrophin mRNAs of skeletal muscle in postnatal growing mice. In this study, we focused on the participation of YB-1 in neural activity-dependent translational regulation of AChR α mRNA, which is indispensable for neuromuscular transmission. For this purpose, we confirmed that YB-1 binds to AChR α mRNA in C2C12 myoblasts (Fig. 1E). To examine the effects of YB-1 on the translational level of AChR α mRNA in the cells, the expression of YB-1 was decreased by specific siRNA. In the cells, AChR α protein was apparently