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Somatodendritic and excitatory postsynaptic distribution of neuron-type dystrophin isoform, Dp40, in hippocampal neurons

Takahiro Fujimoto, Kyoko Itoh *, Takeshi Yaoi, Shinji Fushiki

Department of Pathology and Applied Neurobiology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

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ABSTRACT

The Duchenne muscular dystrophy (DMD) gene produces multiple dystrophin (Dp) products due to the presence of several promoters. We previously reported the existence of a novel short isoform of Dp, Dp40, in adult mouse brain. However, the exact biochemical expression profile and cytological distribution of the Dp40 protein remain unknown. In this study, we generated a polyclonal antibody against the NH_2-terminal region of the Dp40 and identified the expression profile of Dp40 in the mouse brain. Through an analysis using embryonic and postnatal mouse cerebrums, we found that Dp40 emerged from the early neonatal stages until adulthood, whereas Dp71, an another Dp short isoform, was highly detected in both prenatal and postnatal cerebrums. Intriguingly, relative expressions of Dp40 and Dp71 were prominent in cultured dissociated neurons and non-neuronal cells derived from mouse hippocampus, respectively. Furthermore, the immunocytological distribution of Dp40 was analyzed in dissociated cultured neurons, revealing that Dp40 is detected in the soma and its dendrites, but not in the axon. It is worthy to note that Dp40 is localized along the subplasmalemmal region of the dendritic shafts, as well as at excitatory postsynaptic sites. Thus, Dp40 was identified as a neuron-type Dp possibly involving dendritic and synaptic functions.

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1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked hereditary disease characterized by progressive muscular degeneration [1,2]. About one third of all DMD patients manifest variable degrees of cognitive impairment [3,4]. The *DMD* gene encodes dystrophin (also known as Dp427) and its multiple isoforms, including Dp71 and Dp40, which are produced by alternative promoters and/or alternative splicing [5,6]. Mutations in the *DMD* gene cause an absence or dysfunction of Dp427 localized in the plasma membrane of muscle cells, resulting in DMD [7,8]. Although mutations in all parts of the *DMD* gene can be associated with cognitive impairment [9], mutations in the Dp71 coding region is closely associated with the cognitive impairment observed in DMD patients [10–12]. Dp71 is an abundant product of the *DMD* gene that is expressed in the brain and found in both neurons and glia [5]. Most recently, it was found that the shortest dystrophin isoform, Dp40, the NH_2-terminal partial product of Dp71 produced by alternative splicing [13], is also expressed in the brain [14], although little is known about Dp40.

In this study, as the first step toward understanding the role of Dp40, we generated a polyclonal antibody against the NH_2-terminal region common to both Dp40 and Dp71 and investigated the expression profile of Dp40 in mouse brains. We found that that Dp40 emerged from the early neonatal period to adulthood, with preferential expression in neurons. Furthermore, we dissected the subcellular distribution of Dp40 in cultured hippocampal neurons and showed that Dp40 is somatodendritically localized and detected in the excitatory postsynapses. Thus, Dp40 may play critical roles in dendritic and synaptic functions in the brain.

2. Materials and methods

2.1. Animals

All of the animal experiments in this study were approved by the Institutional Review Board for Biomedical Research using Animals at Kyoto Prefectural University of Medicine, and the animals were handled according to the institutional guidelines and

Abbreviations: DMD, duchenne muscular dystrophy; Dp, dystrophin; DAPI, 4,6-Diamidino-2-phenylindole.

* Corresponding author. Fax: +81 75 251 5849.
E-mail address: kxi14@koto.kpu-m.ac.jp (K. Itoh).

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regulations. The experiments were carried out on ICR mice purchased from a breeder (SHIMIZU Laboratory Supplies Co. Ltd., Kyoto, Japan).

2.2. Antibodies

The antibodies used were as follows: anti-Dp71 (ab15277) from Abcam (Massachusetts, USA), anti-hemagglutinin (HA) (3724) from Cell signaling (Massachusetts, USA), anti-MAP2 (ab17838) from Abcam (Massachusetts, USA), anti-PSD95 (P78352) from UC Davis/NIH NeuroMab Facility (California, USA), anti-synaptophysin (MAB5258) from Millipore (Darmstadt, Germany), and anti-gephyrin (147011) from Synaptic Systems (Gottingen, Germany). The recombinant mouse Dp40 fragment (amino acid residues 1–238) was expressed as a GST fusion protein using the pGEX4T-2 vector (GE Healthcare, England)). The fusion protein was soluble in nondenaturing buffer and was purified with glutathione-Sepharose 4B (GE Healthcare, England). Antiserum was obtained by injecting the recombinant Dp40 protein into a Japanese White rabbit followed by booster injection. The antiserum was purified with an affinity column prepared by cross-linking the recombinant protein to CNBr-activated Sepharose 4B (GE Healthcare, England).

2.3. Immunoprecipitations and Western blotting

Protein extracts from mouse tissues or cultured hippocampal cells were prepared by homogenizing in a lysis buffer (50 mM Tris–HCl, pH7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS). Two milligrams of protein from each tissue or cell was subjected to immunoprecipitations with anti-Dp40 or negative control antibodies. TrueBlot Anti-Rabbit Ig IP Beads (ROCKLAND antibodies & assays, Pennsylvania, USA) was added to the sample and incubated overnight at 4 °C. Then the samples were washed three times in the lysis buffer. Proteins were eluted by SDS sample buffer and separated on a polyacrylamide gel followed by transfer to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween20), the membranes were incubated with anti-Dp40 antibody (dilution 1:1000) overnight at 4 °C. Blots were incubated for 1 h at room temperature (RT) with Rabbit TrueBlot Anti-Rabbit IgG HRP (ROCKLAND antibodies & assays, Pennsylvania, USA). Chemiluminescence was detected using Western BLoT Chemiluminescence HRP Substrate (Takara Bio, Japan) and Hyperfilm™ ECL (GE Healthcare, England).

2.4. Primary culture and transfection

Cultured hippocampal neurons were prepared from ICR mice. Whole brains were isolated from embryonic day 18 mouse embryos, and the hippocampi were dissected out and treated with a papain dissociation system (Worthington Biochemical Co., New Jersey, USA) according to the manufacturer's instructions. The cells were suspended in DMEM (Sigma, Japan) containing 10% horse serum (Life Technologies, Japan) and plated at a density of 7000 cells/cm^2 on poly-D-lysine (Sigma, Japan)-coated 60 mm dishes or glass coverslips (Fisher Scientific, Massachusetts, USA). Four hours after plating, the medium was changed to serum-free Neurobasal medium containing 2% B27 supplement (Life technologies, Japan) and 0.5 mM L-glutamine (Life technologies, Japan), with incubation at 37 °C in a humidified incubator with 5% CO_2/95% air. Cultured hippocampal neurons growing on glass coverslips were subjected to transfection using Lipofectamine LTX reagent (Life Technologies, Japan) at day 4 *in vitro* according to the manufacturer's instructions. After being washed with Hank's Balanced Salt Solution (Life Technologies, Japan), the neurons were grown in Neurobasal medium containing 2% B27 supplement (Life technologies, Japan), 0.5 mM L-glutamine (Life Technologies, Japan) and 5 μM cytosine arabinoside for a further 3 weeks. Hippocampal non-neuronal cells were prepared from embryonic day 18 mouse hippocampi. Dissociated cells were cultured in DMEM containing 10% horse serum and penicillin–streptomycin at 37 °C in a humidified incubator with 5% CO_2/95% air until reaching 100% confluence.

2.5. Construction of the plasmids

In order to generate COOH-terminal HA-tagged fusion protein, the cDNAs encoding full-length mouse *Dp40* was prepared from adult mouse hippocampus by reverse transcription-PCR using a set of primers (5′-GG GGA ATT CCC GCC ACC ATG AGG GAA CAC CTC AAA GGC CAC G-3′ and 5′-GG GGC GGC CGC TCA AGC GTA ATC TGG AAC ATC GTA TGG GTA CGT TTC CAT GTT GTC CCC CTC TA A CAC-3′) and cloned into the pLVSIN-EF1α-IRES-ZsGreen1 vector (Takara Bio, Japan), which co-expresses ZsGreen (reef coral *Zoanthus* sp. green fluorescent protein) via the internal ribosome entry site.

2.6. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at RT, blocked with 5% bovine serum, 0.1% Tx-100, 150 mM NaCl, 50 mM Tris–HCl, pH7.5 for 30 min at RT, and then subjected to double-immunostaining with rabbit polyclonal anti-HA and mouse monoclonal anti-PSD95, anti-MAP, anti-synaptophysin, or anti-gephyrin antibodies. The primary antibodies were visualized with the proper combination of secondary antibodies: goat anti-rabbit IgG conjugated to Alexa Fluor 546 and goat anti-mouse IgG conjugated to Alexa Fluor 647. Nuclear DNA staining was processed by using the ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Japan). Fluorescence images were acquired by a confocal fluorescence microscope (LSM510 Ver. 4.0, Carl Zeiss, Wetzlar, Germany). The fluorescence intensity was quantified using ImageJ software for line scan analysis.

3. Results

3.1. Expression profile of Dp40 protein in the mouse brain

In order to reveal the biochemical expression profile of the Dp40 protein, a polyclonal antibody against the NH_2-terminal region of Dp40 was generated by immunizing a recombinant Dp40 protein in a rabbit. The amino acid sequence used for the antigen is totally included in full-length Dp (also known as Dp427) and a short isoform of Dp, Dp71 (Fig. 1A) [6,14]. To validate the reactivity of the anti-Dp40 antibody, anti-Dp40, anti-Dp71, or control IgG immunoprecipitations were performed using adult mouse cerebrum followed by Western blotting with the anti-Dp40 antibody, showing that the anti-Dp40 antibody was able to immunoprecipitate and detect both Dp40 and Dp71 proteins (Fig. 1B), although the anti-Dp71 antibody immunoprecipitated Dp71, but not Dp40 proteins (Fig. 1B). Next, to examine whether Dp40 would be expressed in the muscle, anti-Dp40 immunoprecipitation was carried out using mouse muscle, revealing that neither Dp40 or Dp71 proteins were detected (Fig. 1C, right half), although other Dp isoforms, including Dp427 were detected (Fig. 1C, right half). Consistent with the results shown in Fig. 1B, Dp40 and Dp71 proteins were obviously detected in the cerebrum (Fig. 1C, left half). These results indicated that the anti-Dp40 antibody we generated in this study

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