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Research article

EF-hand domains are involved in the differential cellular distribution of dystrophin Dp40

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HIGHLIGHTS

- Dystrophin Dp40 mRNA is expressed in PC12 cells.
- Dp40 localizes in the cell periphery/cytoplasm and less extent into the nucleus.
- EF-hand domains are involved in Dp40 cellular distribution.
- Dp40-L93P, Dp40-L170P and Dp40-L93/170P mutants show nuclear localization.
- Dp40 co-localizes with β-dystroglycan.

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ABSTRACT

Dp40 is the shortest DMD gene product that has been reported to date. It is encoded by exons 63–70, a region required for a β-dystroglycan interaction. Its expression has been identified in rat, mouse, and human; however, its function remains unknown. To explore the expression of Dp40 transcript and subcellular localization of epitope-tagged Dp40 proteins, RT-PCR and immunofluorescence assays were performed in PC12 cells. The expression of Dp40 mRNA was found in undifferentiated and nerve growth factor-differentiated PC12 cells. According to immunofluorescence analyses, the recombinant protein Dp40 was mainly localized in the cell periphery/cytoplasm of undifferentiated and differentiated PC12 cells, a small amount of this protein is localized to the nucleus of differentiated cells. With the aim to identify the amino acids involved in the nuclear localization of Dp40, an *in silico* analysis was performed and it predicted that prolines 93 and 170, located within EF1 and EF2-hand domains, are involved in the nuclear localization of this protein. This prediction was confirmed by site-directed mutagenesis, the Dp40-L93P mutant was localized to the nucleus and cell periphery, while Dp40-L170P and Dp40-L93/170P showed mainly a nuclear localization. Dp40 co-localizes with β-dystroglycan and the co-localization score was statistically reduced in Dp40-L93P, Dp40-L170P and Dp40-L93/170P mutants.

1. Introduction

Dystrophin is part of a multimeric complex known as dystrophin-associated proteins (DAP), composed of dystroglycan, sarcospan and sarcoglycans at the transmembrane region and dystrobrevin and syntrophin, which associate with the C-terminus of dystrophin, in the cytoplasm. Full-length dystrophin (Dp427) is composed of four major domains: the *N*-terminal domain contains an actin-binding domain; the largest and central domain consists of 24 triple helical spectrin-like repeats; the third domain, named the cysteine-rich domain, encodes WW, two EF-hands-like (EF1,

Abbreviations: NGF, nerve growth factor; DMD, duchenne muscular dystrophy; NLS, nuclear localization signal; DAP, dystrophin-associated proteins.

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EF2) and ZZ modules; and the *C*-terminal domain is unique to dystrophin, utrophin and dystrobrevins. Internal promoters within the DMD gene and alternative splicing of mRNAs generate multiple *N*-terminal truncated isoforms and variations in the *C*-terminal region [1–3].

The 3′ terminus of the dystrophin mRNA corresponds to exon 79; however, an alternative polyA site is found within intron 70 detected in a rat Schwannoma library. This DMD gene product, named as Apo-dystrophin-3 or Dp40, is transcribed from the Dp71 promoter located within intron 62 and the 3′-UTR derives from intron 70. Therefore, Dp40 mRNA is composed of exons 63–70 and it encodes a predicted protein of 340 amino acids with a corresponding molecular weight of 40 kDa [4].

Dp40 is a dystrophin Dp71 isoform lacking the *C*-terminal end, the seven *N*-terminal amino acids are fused to the cysteine-rich domains (half of the WW domain, the EF1, EF2, and ZZ domains) and the first 48 amino acids of the dystrophin *C*-terminal domain; this region contains the region required for β-dystroglycan interaction. The tissue distribution of the Dp40 transcript is similar to that of Dp71 and it is also expressed in early development and in murine ES cells [4]. Previously, the expression of Dp40 protein in the mouse brain and its interaction with a group of presynaptic proteins was reported [5]. Recently, somatodendritic and nuclear localization of Dp40 wild type, in cultured mouse hippocampal neurons, has been shown and function for this protein in neuronal maturation and postsynaptic processes has been proposed [6]. In order to contribute to the knowledge of Dp40 function, in this work we describe the expression of Dp40 mRNA in PC12 cells and the localization of recombinant Dp40 protein in PC12 and HeLa cells. We found that Dp40 mRNA is expressed in undifferentiated and NGF-differentiated PC12 cells. The Dp40 showed cell peripheral/cytoplasmic localization in undifferentiated PC12 cells, while a small amount of protein was localized to the nucleus of differentiated cells. We identified, by *in silico* analyses, that prolines 93 and 170 are involved in the nuclear localization of Dp40, a finding that was further confirmed by site-directed mutagenesis. The mutant Dp40-L93P was localized to the nucleus and cell periphery, while Dp40-L170P and Dp40-L93/170P were mainly localized to the nucleus. Dp40 can interact with β-dystroglycan as observed by co-localization assays and the co-localization was reduced with Dp40 mutants.

2. Materials and methods

2.1. Cell culture

PC12 cells were grown as previously described [7]. For differentiation, PC12 cells were grown in P100 plates coated with rat collagen in Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA), 1% heat-inactivated horse serum (Gibco) and 50 ng/ml NGF (Gibco) at 4, 8 and 12 days. HeLa cells were grown on P100 plates in DMEM supplemented with 10% fetal calf serum (Gibco), 100 ug/ml penicillin (Gibco), 1 mg/ml streptomycin (Gibco) and 0.25 μg/ml amphotericin B (Gibco).

2.2. RT-PCR and vector construction

Total RNA was obtained using Trizol reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Three micrograms of RNA were retrotranscribed using oligo dT and the Super Script III First-Strand Synthesis System (Invitrogen). PCR reactions were performed with 2.5 μl of cDNA, 200 ng of primers 5′AGTGCTTTCGGCTGCGAGC (5′UTR-Dp71), and 5′AGGAGCTTAGGAGACTGTTTGCAT (3′UTR-Dp40), and 1 unit of Pfu high fidelity DNA polymerase (Invitrogen) and the following parameters: 32 cycles at 94 °C for 30 s and 68 °C for 3 min. The β-actin mRNA was amplified using primers actin-1 and actin-2 [7] at 24 cycles. The amplified products were visualized by electrophoresis on 1.2% Agarose-TBE gels stained with ethidium bromide. The Dp40 products from undifferentiated cells were cloned into an intermediary *p*GEM-T Easy vector (Promega, Madison, WI, USA). For pcDNA4/HisMax-TOPO-Dp40 vector construction, the *p*GEM-T-Dp40 sequence was amplified by PCR using the primers 5′ATGAGGGAACACCTCAAAGGCCACG (rDp71F) and 5′GATCTAGCGGCCGCTCACGTTTCCATGTTGTCCCCCTCTAAC (rDp40R-NotI) and cloned into the expression vector pcDNA4/HisMax-TOPO-TA (Invitrogen) to fuse the Xpress epitope at the *N*-terminus of Dp40. The transformed DH5α cells were identified by PCR and plasmids were sequenced using the Big Dye Terminator V 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

2.3. In vitro *site-directed mutagenesis*

Leucines 93 and 170 in pcDNA4/HisMax-TOPO-Dp40 vector were changed to prolines by site-directed-mutagenesis. For this, primers 5′CTACTATTTATGACCGTCCGGAGCAAGAGCACAACAATTTGG and 5′CCAAATTGTTGTGCTCTTGCTCCGGACGGTCATAAATAGTAG, for amino acid 93, and 5′CGTAGACTGGGTCTTCTTCCGCATGATTCTATTCAAATCC and 5′GGATTTGAATAGAATCATGCGGAAGAAGACCCAGTCTACG, for amino acid 170, (point mutations are underlined) were designed, and the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) was used following the manufacturer's instructions. The double mutant was obtained using the single mutant Xpress-Dp40-L93P as a template. Mutations were confirmed by DNA sequencing.

2.4. Cell transfection

To test the expression of Dp40 proteins, HeLa cells were cultured on P100 plates and transfected for 5 h using Optimem medium (Gibco), 5 μg of plasmid and 20 μl of Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells were harvested and resuspended in 80 μl of extraction buffer (50 mM Tris pH 7.4, 1% Triton X-100, 150 mM NaCl) with COMPLETE protease inhibitors (Roche Applied Sciences, U.S.). Soluble proteins were boiled in Laemmli sample buffer and were analyzed by western blot with the anti-Xpress antibody as previously described [7]. For immunofluorescence assays, PC12 cells were cultured in 6-well plates on cover slips pre-coated with poly-L-lysine and transfected with 2 μg of plasmid and 20 μl of Lipofectamine 2000. The transfection medium was exchanged for growth medium for 24 h or differentiation medium for 72 h. HeLa cells were grown on poly-L-lysine coated cover slips and transfected with 2 μg of plasmid and 5 μl of Lipofectamine 2000.

2.5. Immunofluorescence staining and confocal analyses

Immunofluorescence assays were performed with anti-Xpress (Invitrogen) and LG5 antibodies to detect Xpress-Dp40 and β-dystroglycan proteins as previously described [7]. For each image, 20-30 optical z-sections (0.3 μm thick) were scanned with a 63× oil immersion objective in a Leica Confocal Microscope (TCS-SPE) and the equatorial plane was chosen to show the subcellular localization of proteins. The co-localization was scored from maximal projection of z-sections using the Leica software system (LAS-AF), and at least 10 cells were analyzed for each experiment. The values are expressed as the mean ± standard deviation of three indepen-