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Neuronal differentiation modulates the dystrophin Dp71d binding to the nuclear matrix

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ABSTRACT

The function of dystrophin Dp71 in neuronal cells remains unknown. To approach this issue, we have selected the PC12 neuronal cell line. These cells express both a Dp71f cytoplasmic variant and a Dp71d nuclear isoform. In this study, we demonstrated by electron and confocal microscopy analyses of *in situ* nuclear matrices and Western blotting evaluation of cell extracts that Dp71d associates with the nuclear matrix. Interestingly, this binding is modulated during NGF-induced neuronal differentiation of PC12 cells with a twofold increment in the differentiated cells, compared to control cells. Also, distribution of Dp71d along the periphery of the nuclear matrix observed in the undifferentiated cells is replaced by intense fluorescent foci localized in the center of the nucleoskeletal structure. In summary, we revealed that Dp71d is a dynamic component of nuclear matrix that might participate in the nuclear modeling occurring during neuronal differentiation.

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Duchenne muscular dystrophy gene (DMD) has a complex transcription regulation; it contains at least seven internal promoters that produce different tissue-specific dystrophins, named according with their molecular weights as Dp427, Dp260, Dp116, Dp140, and Dp71 [1]. Dystrophin Dp427 binds to a group of cytoplasmic (syntrophins and dystrobrevins) and membranal proteins (sarcoglycans and dystroglycans) conforming the dystrophin associated protein complex [DAPC] [2,3]. DAPC confers stability to the sarcolemma and participates in the signaling transduction occurring from the extracellular matrix to the actin-cytoskeleton [4,5]. Lost of dystrophin, due to DMD gene mutations, promotes the DAPC disintegration and consequently the development of Duchenne muscular dystrophy [6,7].

Dp71 is transcribed from a housekeeping promoter located between exons 62 and 63, its mRNA suffers alternative splicing on exons 71–74 and 78, which may result in the production of multiple alternative products [8,9]. With the exception of muscle tissue, Dp71 has been found in all tissues tested so far, and represents the principal DMD gene product in the nervous system [9]. We have started a long term study focusing on the role of Dp71 during neuronal differentiation of PC12 cell line. PC12 cells express at least two different Dp71 isoforms, generated by the alternative splicing of exon 78: Dp71d isoform contains the protein domain encoded by exons 78 and 79, while Dp71f variant has an alternative C-terminal domain of 31 amino acids, which are incorporated into Dp71 protein upon the removal of exon 78 from the Dp71 mRNA. Interestingly, the alternative splicing of exon 78 determines the distribution of Dp71 isoforms in differentiated PC12 cells: Dp71f distributes into the cytoplasmic compartment while Dp71d localizes mainly in nuclei. It is likely that function of each Dp71 isoform is related to its specific subcellular localization [10].

Recently, by using an antisense approach, we generated PC12 clones displaying drastic reduction of Dp71 expression. Remarkable, deficiency of Dp71 isoforms expression impairs the NGF-induced neuronal differentiation of PC12 cells [11]; however, the individual contribution of each variant to this phenomenon remains undetermined. Further analysis of Dp71-antisense clones demonstrated that Dp71f variant binds to the β-1 integrin adhesion protein complex modulating the adhesion of PC12 cells to substrates [12,13]. Once the function of the cytoplasmic isoform Dp71f was determined, our immediate interest has been focused on the characterization of the Dp71d nuclear function. Dp71d accumulates in the nucleus of PC12 cells during the late stages of

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PC12 neuronal differentiation [10], suggesting that this protein may mediate nuclear responses during this process.

In this paper, we have established by immunoelectron and immunofluorescence microscopy analyses as well as by Western blot evaluation of cell fractions that Dp71d binds to the nuclear matrix of PC12 cells. Furthermore, we evidenced that the amount and spatial distribution of Dp71d at the nuclear matrix is modulated during PC12 neuronal differentiation.

Materials and methods

Antibodies. Anti-lamin-B1 antibody was purchased from Zymed Laboratories Inc. (South San Francisco, CA, USA), while anti-Sp3, lamin A/C and anti-calnexin antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-dystrophin (2166, [14]) and anti-actin antibodies were kindly provided by Dr. Dereck Blake and Dr. Manuel Hernandez, respectively.

PC12 cell culturing. PC12 cells were grown as previously described [11]. For differentiation studies, cells were treated with 50 ng/ml of NGF (Alomone Laboratories, Jerusalem, Israel) for 1, 3, or 6 days. NGF-containing medium was changed every third day.

Immunoelectron microscopy (IEM). Cells and isolated nuclei were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. Washed cells were gradually dehydrated in increasing concentrations of ethanol and then embedded in LR-White resin (London Resin Co., Ltd., England) which was polymerized under UV at 4 °C overnight. Thin sections were mounted on formvar-covered nickel grids. Immunolabeling was carried out by flotation of the mounted sections on drops of each solution and all steps were conducted at room temperature. To minimize non-specific labeling, grids were incubated with 0.1 M phosphate buffer, pH 7.4, with 150 mM NaCl containing 0.05% skim milk, and 0.05% Tween 20 for 30 min. Sections were incubated with the anti-dystrophin antibody 2166 (dilution 1:50). Next, samples were incubated for 2 h with the corresponding secondary antibody coupled to colloidal gold particles of 20 nm diameter (dilution 1:40 in PBS-0.05% Tween 20) (Ted Pella, INC., CA). Incubation of antibodies was performed in a humid chamber with intervening washes. After washing in PBS and water, sections were contrasted with 2% uranyl acetate and examined in a transmission electron microscope (JEOL 2000EX, Japan) [15,16].

Isolation of cell extracts. Total, cytosolic and nuclear protein extracts were obtained as previously described [17].

Nuclear matrix preparations. To obtain nuclear matrix extracts, pure nuclei were digested with 100 U/ml RNase-free DNAse I (Sigma, Co., St. Louis, MO), following the recommendations of the provider. To remove the chromatin and nuclear soluble proteins, samples were incubated for 15 min on ice with 150 μl of 2× high salt buffer (20 mM Tris–HCl, pH 7.4, 1.3 M $[NH_4]_2$ SO_4) and centrifuged at 10,000 rpm for 10 min at 4 °C. The resulting pellet containing the nuclear matrices was resuspended in 100 μl of RIPA buffer lysis containing complete protease inhibitor mixture (CPIM) (Roche Applied Science, Indianapolis, IN). To obtain *in situ* nuclear matrices, cells plated on coverslips were treated with TM-2 buffer (10 mM Tris–HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM PMSF, and 0.5% Triton X-100) for 20 min at 4 °C. To remove cytosolic soluble proteins, samples were incubated for 20 min at 4 °C, in CSK 1× buffer (10 mM PIPES, pH 6.8, 300 mM Sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, RNase out 5 U/ml (Invitrogen, Carlsbad, CA), and 1% Triton X-100) containing CPIM. Then, chromatin was dissolved by incubating samples with 100 U/ml of RNase-free DNase I for 2 h at 37 °C. To remove DNA and nuclear soluble proteins, preparations were incubated for 20 min at 4 °C with the extraction buffer (CSK 1× buffer containing 650 mM ammonium sulfate). Finally, samples were washed with PBS and then fixed with 4% paraformaldehyde in CSK 1× buffer for 40 min at 4 °C.

Electrophoresis and Western blot analysis. Equal amounts of protein extracts (60 μg) were mixed with Laemmli's sample buffer and boiled for 3 min. Next, proteins were resolved by SDS–PAGE and electrotransferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature in TBS-T (150 mM NaCl, 10 mM Tris–HCl, pH 8, 0.05% Tween 20) containing 6% low-fat dried milk and then, incubated overnight at 4 °C with the corresponding primary antibody. Following three washes in TBS-T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham-Pharmacia, GE Healthcare, Buckinghamshire, UK) and developed using the ECL Western blotting analysis system (Amersham-Pharmacia, GE Healthcare, Buckinghamshire, UK). Quantitative analysis of immunoblots was performed on a digital Science ID system (Kodak).

Immunofluorescence and confocal microscopy analysis. Cells, plated on coverslips were processed for confocal microscopy analysis as described previously. For double labeling, cells were incubated overnight at 4 °C with the anti-Dp71 antibody (2166) and either anti-lamin B1 or anti-Sp3 antibody. The day after, cells were washed three times with PBS and incubated for 1 h at 4 °C with PBS containing both a TRITC-conjugated secondary anti-mouse antibody and a FITC-conjugated secondary anti-goat antibody (Zymed Laboratories Inc., San Francisco, CA). For counterstaining, cells were incubated for 10 min at room temperature with propidium iodide 500 μg/ml (Sigma, St. Louis, MO) or DAPI (Sigma, St. Louis, MO). After washing with PBS, preparations were mounted with VectaShield (Vector Laboratories Inc., Burlingame, CA, USA) and viewed throughout a confocal laser scanning microscope (TCP-SP2, Leica, Heidelberg Germany) using 63× and 100× oil-immersion plan apochromat objectives (NA 1.32 and 1.4, respectively). Single optical sections were selected to analyze the co-localization patterns between two markers.

Statistics. Statistical analyses were performed with the two-tailed Student's *t* test.

Results

Distribution of Dp71d isoform in the PC12 cells by immunoelectron microscopy

To determine the subcellular distribution of Dp71d isoform, Intact PC12 cells and isolated nuclei were processed for immunogold localization using the anti-dystrophin antibody 2166. Immunogold localization of whole PC12 cells indicated that Dp71d is mainly associated with organelles such as the ER and cytoplasmic granules (Supplementary Figure A and B, respectively) but it was not found free in the cytoplasm. Nuclei were intensively decorated by gold particles (Supplementary Figure C), indicating the presence of Dp71d in this organelle. In isolated nuclei, 2166 antibody labeled the nucleolus as well as some electrodense material resembled the nuclear matrix (Fig. 1A–C). Negative controls consisted of sections incubated only with the secondary antibody conjugated to colloidal gold particles or with a non-related rabbit polyclonal antibody, and revealed with the respective secondary antibody conjugated to 20 nm colloidal gold particles, produced only a scarce dispersed gold labeling without any specific association to cells structures (data not shown).

Association of Dp71d to the nuclear matrix in differentiating PC12 cells

To ascertain whether Dp71d associates with the nuclear matrix, *in situ* nuclear matrices and nuclear matrix protein extracts were obtained. The purity of *in situ* nuclear matrices was tested by visu-

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