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Dystrophin Dp71f associates with components of the β1-integrin adhesion complex in PC12 cell neurites

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Abstract

Dystrophin Dp71 has been implicated with cognitive impairment shown by Duchenne muscular dystrophy patients. To study Dp71 neural role, we used PC12 cell line, since these cells differentiate into sympathetic like neurons when stimulated with nerve growth factor. Previously in undifferentiated PC12 cells, it was demonstrated that dystrophin Dp71f is a key component of the β1-integrin adhesion complex that confers proper complex assembly. Since integrin based mediated adhesion is important during neuronal differentiation, it was important to know if dystrophin Dp71f was a structural component of the β1-integrin adhesion complex in neurites of nerve growth factor stimulated PC12 cells. In the present work, by performing immunofluorescence assays, we determined the association of dystrophin Dp71f with some components of the β1-integrin adhesion complex such as β1-integrin subunit, talin, α-actinin and vinculin in neurites of nerve growth factor stimulated PC12 cells seeded onto the extracellular matrix protein laminin. The association was stronger in neural growth cones suggesting that dystrophin Dp71f is important for the function that the β1-integrin complex has during neurite outgrowth.

Key words: Dystrophin Dp71f; neurite outgrowth; β1-integrin; PC12 cells.

Introduction

Duchenne muscular dystrophy (DMD) is caused by an X-linked genetic disorder causing progressive muscle degeneration and death. The DMD gene encodes seven different dystrophins that differ in structure and expression patterns (Ahn *et al.*, 1993). Dystrophin Dp71 is the smallest isoform and shows an alternative splicing of exons 71-74 and 78 (Ceccarini *et al.*, 1997). Although the function of Dp71f is unknown, experimental evidence shows its importance in the nervous system: (a) Dp71 expression occurs in parallel with brain development (Jung *et al.*, 1993); (b) Dp71 is required for the anchorage and/or organization of the dystrophin associated protein complex in the brain (Greenberg *et al.*, 1996); and (c) C-terminal mutations in dystrophin, which would adversely affect Dp71 expression are associated with mental retardation (Moizard *et al.*, 2000). Since dystrophin Dp71 has been associated with cognitive impairment, to study its neuronal function we adopted the noradrenergic clonal line of rat adrenal pheochromocytoma cells (PC12 cells) which respond to nerve growth factor (NGF) and differentiate into sympathetic like neurons (differentiated PC12 cells) (Greene, 1976). PC12 cells express two different dystrophin Dp71 isoforms, one of them is called dystrophin Dp71d. This isoform lacks exon 71 and has nuclear localization. The other is denominated dystrophin Dp71f which lacks exons 71 and 78, showing a cytoplasmic distribution (Marquez *et al.*, 2003). Both Dp71 proteins are upregulated during NGF induced PC12 neuronal differentiation. Dp71f increases 9-fold in total extracts, while Dp71d augments up to 7-fold in nuclear extracts. These results suggest that each Dp71 variant may perform specific tasks in neuronal differentiation (Marquez *et al.*, 2003).

The phenomenon of neuronal differentiation includes a set of separate processes. These are: neuritogenesis, neurite outgrowth, pathfinding, targeting and synaptogenesis (Tojima *et al.*, 2004). Cellular adhesion to extracellular matrix proteins such as laminin collagen and fibronectin is important for neuronal differentiation (Carri *et al.*, 1992). In

undifferentiated PC12 cells, the integrin heterodimer a1b1 mediates adhesion to laminin by the assembly of a multi-protein adhesion complex which contains the proteins talin, α-actinin, FAK, paxillin and actin (Cerna *et al.*, 2006). This complex contains Dp71f as a key component which interacts with most of the complex components and provides an appropriate assembly and stability (Cerna *et al.*, 2006).

In this communication, by performing double immunostaining assays we found that dystrophin Dp71f colocalizes with the β1-integrin subunit, talin, α-actinin and vinculin in the basal portion of neurites of nerve growth factor stimulated PC12 cells seeded onto laminin. We found that such interactions are stronger in neural growth cones. Our results suggest that dystrophin Dp71f is a structural component of the β1-integrin complex in PC12 cell neurites.

Methods and materials

Cell culturing and cell differentiation assay

PC12 cells were grown in RPMI-1640 medium supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum, 100 U/ml (w/v) penicillin and 0.1 mg/ml (w/v) streptomycin, and maintained at 37°C in a humidified incubator with a 5% CO_2 atmosphere. To induce differentiation, 2×10^3 cells were seeded onto coated 18×18 mm coverslips and treated during 5 days with 100 ng/ml nerve growth factor (NGF, Gibco). Medium containing nerve growth factor was changed every third day.

Antibodies

Polyclonal antibodies raised against to β1-integrin subunit, talin, α-actinin and vinculin were purchased from Santa Cruz Biotechnology. The anti-Dystrophin Dp71f monoclonal antibody 5F3 was a donation of Dr. Dominique Mornet (9).

Immunofluorescence and confocal microscopy analysis

Cells, plated onto laminin-coated 18×18 mm coverslips at 2×10^3 cell density, were washed with cytoskeleton buffer [10 mM (w/v) MES, 150 (w/v) mM NaCl, 5 mM (w/v) EGTA, 5 mM (w/v) $MgCl_2$, 5 mM (w/v) glucose], fixed for 5 min at room temperature in PBS containing 4% (v/v) paraformaldehyde and permeabilized for 5 min with PBS containing 0.5% (w/v) Triton X-100. Coverslips were washed three times with PBS, incubated for 1 h at room temperature with the blocking solution (Block ACE, Dainippon Pharmaceuticals) containing 3% (w/v) bovine serum albumin and washed again three times with PBS containing 0.1% (v/v) Tween 20. Cell preparations were double staining by overnight incubation at 4ºC with the following primary antibodies: an anti-Dp71f antibody (5F3) and either anti-β1 integrin, anti-talin, anti α-actinin or anti-vinculin antibody. The day after, cells were washed three times with PBS and incubated for 1 h at 4ºC with PBS containing both a TRITC-conjugated secondary anti-mouse antibody and a FITC-conjugated secondary anti-goat or anti-rabbit antibody (Zymed Laboratories Inc. San Francisco, CA). After washing with PBS, cell preparations were mounted with VectaShield (Vector Laboratories Inc., Burlingame, California, USA) and viewed throughout a confocal laser scanning microscope (TCP-SP2, Leica, Heidelberg Germany) using 63X and 100X oil-immersion plan apochromat objectives (NA 1.32 and 1.4, respectively). Six-eight consecutive single sections were obtained simultaneously for one or two channels throughout the Z-axis of the sample. The resulting stack of images were projected and analyzed onto the two-dimensional plane using a pseudocolor display green (FITC), and red (TRITC) for double labeling experiments. Fluorochromes were excited at 488 nm (for FITC) wavelength for single labeling, and additionally at 560 nm (for TRITC) wavelength in double labeling experiments. Single optical sections of the stacks were selected to analyze the colocalization patterns between two markers.

Results

To test if dystrophin Dp71f interacted with the β1-integrin adhesion complex in neurites of differentiated PC12 cells, we double-stained nerve growth factor stimulated PC12 cells seeded onto laminin by using a mouse anti-dystrophin Dp71f antibody (red channel) and either rabbit polyclonal anti-β1 integrin or goat polyclonal anti-α actinin, anti-talin or anti-vinculin antibodies (green channel). The immunostaining images of the basal portion (contacting the extracellular matrix protein laminin) show that the components of the β1-integrin adhesion complex (β1-integrin subunit, α-actinin, talin and vinculin) are distributed along neurites with a strong accumulation in neural growth cones. By other hand, we found that dystrophin Dp71f shows the same subcellular distribution. By making a merge of signals we observed that dystrophin Dp71f colocalizes with the β1-integrin subunit, talin, α-actinin and vinculin along neurites but importantly a stronger colocalization was observed in growth cones (Fig. 1).

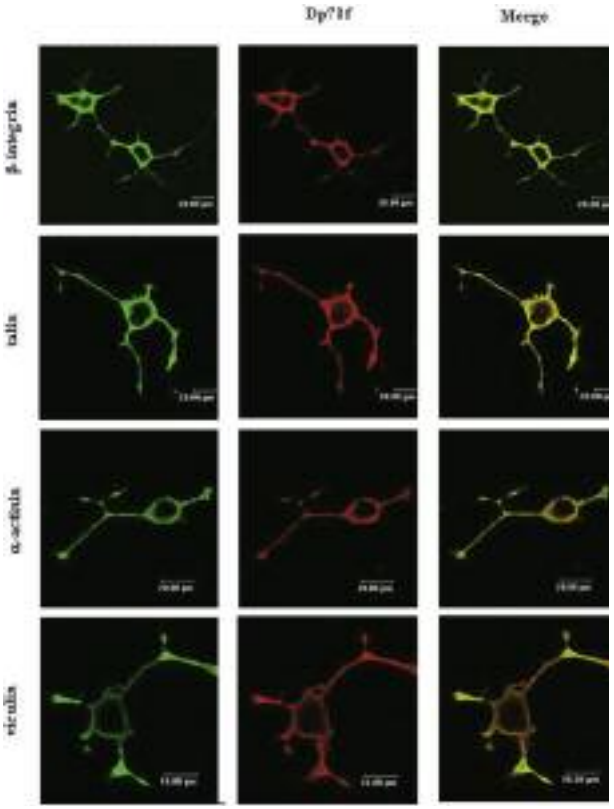

FIG. 1. — Subcellular distribution of dystrophin Dp71f and the adhesion-associated proteins in neurites of nerve growth factor stimulated PC12 cells. Five day nerve growth factor stimulated PC12 cells, plated onto laminin-coated glass coverslips, were double stained with the anti-distrophin Dp71f antibody 5F3 (red channel) and either anti-β1 integrin, anti-vinculin, anti-talin or anti-α actinin antibody (green channel). Images were analyzed by confocal laser microscopy. Focus was adjusted to the basal portion of cells. Merge staining of Dp71f with β1 integrin, talin, α-actinin or vinculin is shown in yellow (right-hand panels).

Discussion

Present work demonstrates that some of the most important components of the β1-integrin complex (β1-integrin subunit, talin, α-actinin and vinculin) are distributed along neurites of nerve growth factor stimulated PC12 cells seeded onto laminin where they colocalize with dystrophin Dp71f. A stronger colocalization with this protein was observed in growth cones.

The importance of Dp71 in the neural function was previously studied by generating a PC12 cell line stably transfected with vectors expressing antisense mRNA against Dp71 mRNA. Antisense-Dp71 PC12 cells, showed impairment in their ability to display neurite outgrowth when induced to differentiate with NGF or dibutyryl cyclic AMP (Acosta *et al.*, 2004). On the other hand there is strong evidence to show the importance of β1-integrin complex in neurons. During central nervous system development, the β1-integrin complex modulates neuronal migration and synaptogenesis (Graus-Porta *et al.*, 2001). Neurons and glia of the β1-integrin knockout mouse die prematurely after birth with severe brain malformations. Cortical hemispheres and cerebellar folia fuse, and cortical laminae are perturbed. These defects result from disorganization of the cortical marginal zone, where beta1-class integrins regulate glial endfeet anchorage, meningeal basement, membrane remodeling, and formation of the Cajal-Retzius cell layer (Graus-Porta *et al.*, 2001). The findings of the present work are supported by previous experiments that revealed the role of Dp71f as a scaffolding protein within the β1-integrin complex (Cerna *et al.*, 2006). It would be interesting to analyze if the mdxcv3 mouse (which does not expresses Dp71) shows a similar phenotype of brain malformations as is observed in the β1-integrin knockout mouse.

This work shows for the first time an association between Dp71f and components of the integrin complex in neurites and growth cones of differentiated PC12 cells, suggesting a common function of Dp71f and the β1-integrin complex during neuritogenesis. A growing tip of neurite, the neuronal growth cone is a highly motile and adhesive form of cytoarchitecture. The growth cone plays a vital role for navigation, elongation and maintenance of neurites (Sobue, 1993). Integrins are heterodimeric receptors composed by the association of one β chain with a α chain. At least 18 distinct α subunits and 8 β subunits have been reported (Lee *et al.*, 2004). By using immunoprecipitation assays, it would be interesting to characterize all the integrin complexes present in PC12 neurites to determine specifically which integrin complexes contain dystrophin Dp71f. Characterization of integrin complexes in neurites of differentiated PC12 cells would require neurite dissection and this process is methodologically out of our hands at this moment as PC12 cell neurites are fine extensions of approximately one micrometer in diameter (Greene *et al.*, 1976). It is probably that a mechanical disruption of neurites without affecting the integrity of PC12 cell body, followed by centrifugation would provide us with pure neurites. This process will be evaluated in future works.

Our results agree with the importance of the integrin based adhesion during neuritogenesis as previously reported (Carri *et al.*, 1992). Although, it had been demonstrated that expression of dystrophin Dp71f increases in nerve growth factor stimulated PC12 cells and that this isoform localize in PC12 cell neurites (Marquez *et al.*, 2003). Association of this

protein with a function during the neuronal differentiation process had not been made.

This report supports hypotheses that dystrophin Dp71f could be a key element of the functions that the β1-integrin adhesion complex has during navigation, elongation and maintenance of neurites.

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