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Journal of Cellular Biochemistry 105:735–745 (2008)

Journal of Cellular Biochemistry

Nuclear and Nuclear Envelope Localization of Dystrophin Dp71 and Dystrophin-Associated Proteins (DAPs) in the C_2C_{12} Muscle Cells: DAPs Nuclear Localization Is Modulated During Myogenesis

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ABSTRACT

Dystrophin and dystrophin-associated proteins (DAPs) form a complex around the sarcolemma, which gives stability to the sarcolemma and leads signal transduction. Recently, the nuclear presence of dystrophin Dp71 and DAPs has been revealed in different non-muscle cell types, opening the possibility that these proteins could also be present in the nucleus of muscle cells. In this study, we analyzed by Immunofluorescence assays and Immunoblotting analysis of cell fractions the subcellular localization of Dp71 and DAPs in the C_2C_{12} muscle cell line. We demonstrated the presence of Dp71, α-sarcoglycan, α-dystrobrevin, β-dystroglycan and α-syntrophin not only in plasma membrane but also in the nucleus of muscle cells. In addition, we found by Immunoprecipitation assays that these proteins form a nuclear complex. Interestingly, myogenesis modulates the presence and/or relative abundance of DAPs in the plasma membrane and nucleus as well as the composition of the nuclear complex. Finally, we demonstrated the presence of Dp71, α-sarcoglycan, β-dystroglycan, α-dystrobrevin and α-syntrophin in the C_2C_{12} nuclear envelope fraction. Interestingly, α-sarcoglycan and β-dystroglycan proteins showed enrichment in the nuclear envelope, compared with the nuclear fraction, suggesting that they could function as inner nuclear membrane proteins underlying the secondary association of Dp71 and the remaining DAPs to the nuclear envelope. Nuclear envelope localization of Dp71 and DAPs might be involved in the nuclear envelope-associated functions, such as nuclear structure and modulation of nuclear processes. J. Cell. Biochem. 105: 735–745, 2008.

KEY WORDS: Dp71; DYSTROPHIN-ASSOCIATED PROTEINS; NUCLEUS; NUCLEAR ENVELOPE; MYOGENESIS

Dystrophin, the protein product of the Duchenne muscular dystrophy gene (DMD) [Hoffman et al., 1987], is a key component of a multi-protein complex known as the dystrophin-associated protein complex (DAPC) [Ervasti et al., 1991]. In skeletal muscle, the DAPC is composed of peripheral and integral membrane proteins; in addition to dystrophin, the basic components of the DAPC include the dystroglycans (α and β) [Ibraghimov-Beskrovnaya et al., 1992] the sarcoglycans (α, β, δ, and γ) [Tome et al., 1994], the syntrophins (α, β1 and β2) [Ahn et al., 1996], dystrobrevin (α and β) [Nawrotzki et al., 1998] and sarcospan [Crosbie et al., 1997]. Dystrophin binds to cytoskeletal actin and to a transmembrane protein, β-dystroglycan; the extracellular domain of β-dystroglycan binds to the peripheral protein α-dystroglycan, which in turns binds to laminin in the basal lamina [Montanaro et al., 1999]. By this way, the DAPC serves as a bridge to connect the extracellular matrix to cytoskeleton, providing structural stability to the sarcolemma during muscle contraction. In addition, several lines of evidence suggest that this protein complex may also play a role in cell signaling. First, the DAPC is associated with several signaling molecules, including Grb2 [Cavaldesi et al., 1999], nNOs [Grozdanovic and Baumgarten, 1999], caveolin 3 [Song et al., 1996], dystrobrevin [Nawrotzki et al., 1998] as well as with regulatory kinases [Cavaldesi et al., 1999]; and second, the DAPC establishes a bidirectional signaling with the integrin adhesion system in cultured L6 myocytes [Yoshida et al., 1998].

Several forms of muscular dystrophy are caused by primary mutations in the genes encoding components of the DAPC [Monaco, 1989; Tsubata et al., 2000]. Primary mutations in the dystrophin

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Received 1 October 2007; Accepted 25 June 2008 • DOI 10.1002/jcb.21870 • 2008 Wiley-Liss, Inc.
Published online 4 August 2008 in Wiley InterScience (www.interscience.wiley.com).

gene cause DMD [Darras et al., 1988], which is characterized by the loss of dystrophin protein and concomitant loss of the entire DAPC. Autosomal recessive limb-girdle muscular dystrophy results from mutations in the sarcoglycan genes, which leads to either partial or complete sarcoglycan deficiency. In addition to muscle tissue, several DAPC-like complexes have been identified in different organs, including brain, lung, retina and kidney [Blake et al., 1999; Cavaldesi et al., 1999; Claudepierre et al., 2000; Loh et al., 2000; Moukhles and Carbonetto, 2001]. In all cases, the DAPCs have been found associated with the cell membrane, therefore, it has been dogmatically accepted that these protein complexes can exist only in this particular cell region. Noticeable, we recently described the existence of a DAPC located in a cell compartment different from cell membrane, the nucleus; we identified a DAPC composed of dystrophin Dp71, β-dystroglycan, α- and β-dystrobrevin and nNOS in the nuclei of HeLa cells [Fuentes-Mera et al., 2006]. Furthermore, we found that nuclear Dp71, β-dystroglycan, nNOS, β-sarcoglycan, α-dystrobrevin and β-dystrobrevin are associated with the nuclear matrix and demonstrated that Dp71, β-dystroglycan and β-dystrobrevin interact with the nuclear matrix proteins lamin B1 and actin. These results indicate that nuclear DAPC may participate in the nuclear architecture of HeLa cells, serving as scaffolding for nuclear processes. The existence of nuclear DAPC raises the question whether this particular localization is restricted to a certain cell line or widely distributed in different cell types. Specifically, it is relevant to ascertain whether a nuclear DAPC might exist in muscle cells, where function deficiency of the cell membrane-associated DAPC has been directly related to muscle pathologies.

In this study we analyzed the subcellular distribution of dystrophin Dp71 and the dystrophin associated proteins (DAPs) in the C_2C_{12} muscle cell line. In addition to cytoplasm and plasma membrane, we demonstrated the presence of Dp71, α-sarcoglycan, α-dystrobrevin β-dystroglycan and α-syntrophin in the nucleus of muscle cells. Furthermore, we observed that myogenic differentiation of C_2C_{12} cells modulates differentially the relative nuclear abundance of DAPs as well as the composition of the nuclear complex, indicating that specific amount of each DAP member is required in the differentiated myotubes nuclei. Furthermore, we found Dp71, β-dystroglycan, α-dystrobrevin and α-syntrophin in the nuclear envelope (NE) fraction of C_2C_{12} muscle cells; however, while the relative protein levels of β-dystroglycan and α-sarcoglycan were found enriched in this nuclear structure, those of the remaining proteins displayed depletion, compared with the nuclear fraction. Our results suggest that β-dystroglycan and α-sarcoglycan might function as inner nuclear membrane proteins in the C_2C_{12} muscle cells, facilitating the further association of the remaining DAPs with the nuclear envelope.

MATERIALS AND METHODS

CELL CULTURE

C_2C_{12} cells were grown in Dulbecco's modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 1 mM sodium pyruvate at 37°C in a water-saturated atmosphere with 5% of CO_2. To induce muscle differentiation, cells at 75% confluence were changed to DMEM supplemented with 2% horse serum instead of 10% fetal bovine serum, and cultured for 5 days.

RNA EXTRACTION AND RT-PCR

Total RNA was isolated from C_2C_{12} cell monolayer with Trizol-reagent (Invitrogen) according to the manufacturer's instructions and dissolved in DEPC-treated H_2O. The reverse transcription reaction was carried out under the following conditions: 5 μg total RNA was mixed with 1X First-Strand buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 10 mM DTT, 0.5 mM of each deoxynucleotide triphosphate, 300 ng random primers and 200 U of M-MLV RT (Invitrogen) and incubated at 37°C for 1 h 30 min followed by inactivation at 96°C for 10 min. The PCR reaction was carried out in a total volume of 50 μl containing 5 μl of cDNA solution, 1× PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 0.2 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 2.5 U of Taq DNA polymerase (Invitrogen) on a Gene Amp PCR system 2400 Thermal Cycler (Perkin Elmer, Boston, MA) for 30 cycles. The PCR conditions included an initial denaturation step at 94°C for 5 min followed by a cyclic reaction consisted of denaturation at 94°C for 45 s, annealing for 30 s and extension for 1 min at 72°C. For each pair of primers, the annealing temperature was chosen 5°C below the lowest primer melting temperature. The sequence of primers for the amplification of the dystrophin-associated proteins was previously reported [Fuentes-Mera et al., 2006].

ANTIBODIES

The following antibodies were used: +78 Dp71, a rabbit polyclonal antibody directed against the last 17 amino acids of the C-terminal domain of dystrophin (antibody synthesized by Genemed Synthesis, Inc. San Francisco, CA and characterized in our laboratory by using mouse muscle and brain protein extracts); a mouse monoclonal antibody against emerin (FL-254, Santa Cruz Biotechnology, Santa Cruz, CA); JAF, a rabbit polyclonal antibody that recognizes the last seven amino acids of the C-terminal of β-dystroglycan [Rivier et al., 1999]; a goat polyclonal antibody raised against a peptide mapping at the C-terminus of β-dystroglycan (C-20, this antibody was used for Immunoprecipitation assays, Santa Cruz Biotechnology); a mouse monoclonal antibody directed to α-dystrobrevin (Transduction Laboratories, Lexington, KY); Sarco3, a rabbit polyclonal antibody against α-sarcoglycan [Rivier et al., 1999]; a rabbit polyclonal antibody directed against amino acids 24–105 of α-sarcoglycan (H-82, Santa Cruz Biotechnology); rabbit polyclonal antibodies directed to calnexin (H-70), lamin A/C (H-110), Sp3 (D-20), myogenin (M-225) and a goat polyclonal antibody against α-syntrophin (N-19, Santa Cruz Biotechnology); a rabbit polyclonal antibody directed against the sequence MDEEEDGAGAEESG-QPRSFTQL of the α1G subunit of the voltage-dependent calcium channel 3.1 (Alomone labs Ltd., Har Hotzvim Hi-Tech Park, Jerusalem, Israel).

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY ANALYSIS

Cells plated on cover slips were washed three times in PBS and fixed with 4% formaldehyde for 10 min at room temperature and permeabilized in PBS containing 0.2% Triton X-100 for 2 min. Next,

cells were blocked during 20 min with gelatin 1% (w/v) and FBS 1.5% in PBS and incubated overnight at 4°C with the appropriate primary antibody Then, cells were washed with PBS and incubated for 1 h at 4°C with fluorescein-conjugated goat anti-mouse IgG or fluorescein-conjugated goat anti-rabbit IgG (1:50 Zymed Laboratories, Inc., San Francisco, CA). For counterstaining, nuclei were dyed with DAPI (Sigma–Aldrich, St. Louis, MO) and actin filaments with TRITC-phalloidin (Sigma–Aldrich). After washing, cover slips were mounted on microscope slides with VectaShield (Vector Laboratories Inc., Burlingame, CA) and cells were examined on a confocal laser scanning microscope (TCP-SP5, Leica, Heidelberg, Germany) using a Plan Neo Fluor 63× (NA = 1.4) oil-immersion objective. For Giemsa staining, cells plated on cover slips were washed three times in PBS, fixed in methanol and stained with 0.5 ml of a 1:25 dilution of Giemsa (Sigma–Aldrich) for 1 h at room temperature. Giemsa was diluted in PBS and the resulting solution was passed through a 0.2 μm filter and centrifuged at 10,000*g* for 2 min to remove particulates. Cells were examined on a Nikon Eclipse 80i microscope using a Plan Neo Fluor 20× (NA = 0.50) objective.

C_2C_{12} CELL FRACTIONATION

To obtain total extracts, 500 μl of lysis buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM PMSF, 1% of NP-40 and 1× complete (Roche Applied Science, Indianapolis, IN)] were added to a cell monolayer and the mixture was incubated in ice during 20 min; after that, cells were scraped and centrifuged at 12,000*g* by 5 min. To isolate cytosolic and nuclear extracts, cells were washed twice with cold PBS, scraped, and centrifuged at 200*g* for 5 min at 4°C. Cell pellet was resuspended in 4.2 ml of cold TM-2 Buffer [10 mM Tris–HCl pH 7.4, 2 mM $MgCl_2$, 0.5 mM PMSF and 1× complete (Roche Applied Science)] and incubated for 10 min in ice; then, 1.8 ml of Triton X-100 5% (v/v) was added and the homogenate was incubated in ice for 10 min. Nuclei were separated from cytosol by ten passes through a 22 gauge needle and centrifugation at 2,000*g* for 5 min at 4°C. The supernatant was saved as a cytosolic fraction, and the pellet was resuspended in 3 ml of buffer A [0.25 M sucrose, 50 mM Tris–HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 1× complete (Roche Applied Science) and 0.5 mM PMSF] at a density of 17×10^6 nuclei/ml, then 6 ml of buffer B [2.3 M sucrose, 50 mM Tris–HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 1× complete (Roche Applied Science) and 0.5 mM PMSF] were added and mixed by inversion. The mixture was then underlayed with 2 ml of buffer B and centrifuged at 141,000*g* for 1 h at 4°C. The nuclei were resuspended in 1 ml of buffer A (at this step, the nuclei can be kept at −75°C) and finally, nuclear proteins were isolated by resuspension of nuclei in lysis buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5 mM PMSF, 1% of NP-40 and 1× complete (Roche Applied Science)] and further sonication. To obtain nuclear envelope extracts, a fractionation procedure described previously was followed [Cronshaw et al., 2002; Matunis, 2006]. Briefly, frozen nuclei were thawed in a 30°C water bath, centrifuged for 5 min at 2,000*g* and resuspended in 1 ml of freshly prepared buffer C [0.1 mM $MgCl_2$, 1 mM DTT, 1× complete (Roche Applied Science), 5 μg/ml DNase I (Sigma–Aldrich), 5 μg/ml RNase A (Sigma–Aldrich) and 0.5 mM PMSF] and incubated at room temperature for 1 h. Next, 4 ml of extraction buffer (8.5) [10% sucrose (w/v), 50 mM Tris–HCl pH 8.5, 0.1 mM $MgCl_2$, 1 mM DTT, 1× complete (Roche Applied Science) and 0.5 mM PMSF] were added drop-wise, while vortexing. The suspension was underlayed with 4 ml of ice-cold sucrose cushion [30% sucrose (w/v), 50 mM Tris–HCl pH 7.5, 0.1 mM $MgCl_2$, 1 mM DTT, 1× complete (Roche Applied Science) and 0.5 mM PMSF] and centrifuged at 4,000*g* for 15 min in a swinging bucket rotor. The pellet was resuspended in 1 ml of ice-cold extraction buffer (pH 7.5) [10% sucrose (w/v), 50 mM Tris–HCl (pH 7.5), 0.1 mM $MgCl_2$, 1 mM DTT, 1× complete (Roche Applied Science) and 0.5 mM PMSF], added with 0.5 ml of extraction buffer (pH 7.5) with 0.3 mg/ml of heparin, and incubated at 4°C for 1 h. The suspension was underlayed with 4 ml of ice-cold sucrose cushion and centrifuged at 4,000*g* for 15 min in a swinging bucket rotor. The resulting pellet corresponding to nuclear envelope fraction was resuspended in lysis buffer and sonicated. Fractionation of cytosol (S100) and total membrane (P100) was carried out according to a protocol previously described [Guillemin et al., 2005], with minor modifications. In brief, cells (8×10^6 cells) were washed and scraped in ice-cold PBS and spun for 5 min at 200*g*. Cells were suspended in 0.5 ml of CLB buffer [10 mM HEPES pH 7.9,10 mM NaCl, 1 mM Kh_2PO_4, 5 mM $NaHCO_3$, 5 mM EDTA pH 8.0, 1 mM $CaCl_2$, 0.5 mM PMSF and 1× complete (Roche Applied Science)]. Homogenization was performed by applying 50 strokes with a Dounce homogenizer. Thereafter, 50 μl of 2.5 M sucrose was added to restore isotonic conditions. Cell homgenates were centrifuged at 6,300*g* for 5 min and postnuclear supernatants were then centrifuged at 107,000*g* for 30 min. The resulting pellet (P100) was suspended in 0.2 ml of lysis buffer, and the supernatant (S100) was supplemented with 0.2 volumes of 5× lysis buffer.

IMMUNOPRECIPITATION ASSAYS

Nuclear extracts (1 mg) were precleared with 20 μl of recombinant protein G-agarose beads (Invitrogen) blocked with 3% of BSA in lysis buffer for 2 h at 4°C. The beads were removed by centrifugation at 16,000*g* for 5 min, and precleared extracts were incubated overnight at 4°C with 2.5 μg of the immunoprecipitating antibody. As negative control, parallel incubations with an irrelevant antibody were performed. Thereafter, 20 μl of protein G-agarose beads blocked with BSA were added and incubated overnight at 4°C. The immune complexes were collected by centrifugation at 16,000*g* for 5 min and washed five times for 10 min with 1 ml of wash buffer (50 mM Tris–Cl pH 8.0, 500 mM NaCl, 1 mM EDTA pH 8.0, 1% v/v Triton X-100 and 0.5 mM PMSF) and then, eluted by boiling in 50 μl of SDS sample buffer (50 mM Tris–Cl pH 6.8, 2% SDS w/v, 10% glycerol v/v, 0.1% 2-mercaptoethanol v/v, 0.001% bromophenol blue w/v). Immunoprecipitated proteins were then analyzed by Immunoblotting.

WESTERN BLOTTING

Protein extract samples (80 μg) were electrophoresed on a 10% SDS–polyacrylamide gel (PAGE) and proteins were then transferred to nitrocellulose (Hybond-N+, Amersham Pharmacia, GE Healthcare, Buckinghamshire, UK) by using a Transblot apparatus (Bio-Rad, Hercules, CA). When rabbit and mouse antibodies were used, the membranes were blocked for 1–3 h in TBST [100 mM

Tris–HCl pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween-20] with 6–15% (w/v) low-fat dried milk and then incubated overnight at 4°C with the appropriate primary antibody. For goat antibodies, the membranes were blocked for 1–3 h in TBST [100 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween-20] with 15% (w/v) low-fat dried milk; next, the membranes were incubated for 3 h in TBS (TBST without Tween-20) with 2.5% of β-mercaptoethanol and then incubated overnight at 4°C with the appropriate primary antibody. Specific proteins were visualized by using the chemiluminescence (ECL) Western blotting system according to the manufacturer's protocol (Amersham Pharmacia).

RESULTS

EXPRESSION OF DYSTROPHIN Dp71 AND DAPs IN THE C_2C_{12} MUSCLE CELLS

To determine the mRNA expression pattern of DAPs in the C_2C_{12} muscle cell line, total RNA extracts obtained from myoblast cultures were subjected to RT-PCR analyses, using different pairs of primers that amplify specifically the cDNA of each DAP [Fuentes-Mera et al., 2006]. The amplification of actin was used as positive control (lane 1), while that of β-dystrobrevin (lane 8), which is not expressed in muscle cells [Blake et al., 1998], was employed as negative control. The expected amplified products for sarcoglycans α, β, γ, and δ (lanes 2–5, respectively) as well as for β-dystroglycan (lane 6), α-dystrobrevin (lane 7), α-syntrophin (lane 9), and β-syntrophin (lane 10) were obtained (Fig. 1). Identity of the PCR products was determined by DNA sequencing (data not shown).

SUBCELLULAR DISTRIBUTION OF Dp71 AND DAPs DURING C_2C_{12} MYOGENESIS

To ascertain whether dystrophin Dp71 and DAPs display nuclear localization in the C_2C_{12} muscle cells, their subcellular distribution were examined in both myoblasts and differentiated myotubes. C_2C_{12} cells were induced to differentiate by lowering horse serum in the culture medium and muscular differentiation was monitored by visualization of Giemsa-stained myotubes and immunodetection of the differentiation protein marker myogenin. Upon differentiation induction for 5 days, C_2C_{12} cells experimented profound morphological changes, they grew tending to align themselves in straight parallel lines forming multinucleated myotubes (Fig. 2A). At the same induction time, the expression of myogenin protein (37 kDa), absent from myoblasts [Cusella-De Angelis et al., 1992], was clearly observed in the protein extracts of myotubes (Fig. 2B). As previously reported, it was observed that Dp71 (71 kDa) and α-sarcoglycan (50 kDa) were present exclusively in myoblasts [de Leon et al., 2005] and myotubes [Wakabayashi-Takai et al., 2001; Delgado-Olguin et al., 2006], respectively (Fig. 2B). Therefore, the subcellular distribution analysis of Dp71 was restricted to myoblasts while that of α-sarcolgycan was approached only in the differentiated myotubes. Immunofluorescence and confocal microscopy analyses were conducted using antibodies directed specifically to each protein and the appropriate fluorescein-conjugated secondary antibodies. Cells were counterstaining with DAPI and TRITC-phalloidin to visualize nuclei and cytoplasmic actin respectively.

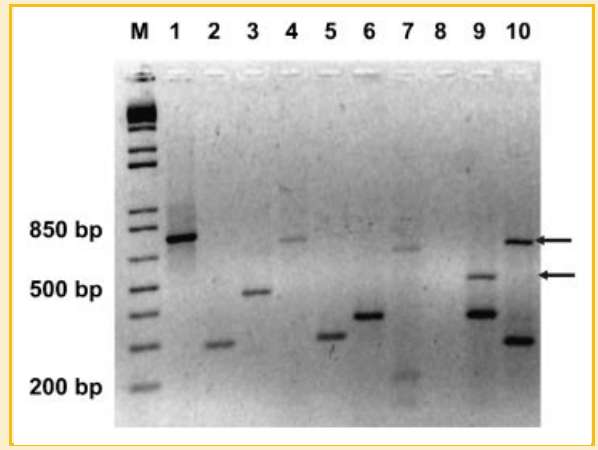

Fig. 1. RT-PCR analysis of DAPs in the C_2C_{12} muscle cells. Total RNA was obtained from C_2C_{12} myoblast cultures to analyze the mRNA expression of DAPs by RT-PCR. The expression of actin and β-dystrobrevin was used as positive and negative controls, respectively. Lanes: M, DNA markers; 1, actin (766 bp); 2, α-sarcoglycan (286 bp); 3, β-sarcoglycan (444 bp); 4, γ-sarcoglycan (700 bp); 5, δ-sarcoglycan (291 bp); 6, β-dystroglycan (348 bp); 7, α-dystrobrevin (569 bp); 8, β-dystrobrevin (185 bp); 9, α-syntrophin (498 bp); and 10, β-syntrophin (702 bp). Arrows point to the specific bands of α- and β-syntrophin. Size of DNA markers is shown on the left.

In C_2C_{12} myoblasts (Fig. 3), the labeling produced by the anti-dystrophin antibody (+78Dp71) seemed to be predominantly cytoplasmic with a diffuse punctuate staining inside the nucleus. It is well established that Dp71 is present in the myoblasts [Howard et al., 1999] and that the full length dystrophin (Dp427) is expressed in the differentiated myotubes [Klamut et al., 1989; Barnea et al., 1990]; therefore, it could be assumed that the anti-dystrophin antibody labeling corresponds to the former protein. On the other hand, α-dystrobrevin, α-syntrophin and β-dystroglycan displayed a strong nuclear Immunostaining, which appeared to be more intensive than their respective labeling generated in the cytoplasm. It is worth to mention that the β-dystroglycan immunolabeling generated a rim-like fluorescence pattern around the nucleus, typical of integral proteins of the inner nuclear membrane. Likewise, it should be noted that α-dystrobrevin nuclear labeling is excluded from nucleoli. In C_2C_{12} differentiated myotubes (Fig. 4), the β-dystroglycan staining was concentrated in the nuclear periphery, showing a less intense signal in the cytoplasm and nuclei of myotubes. On the other hand, the α-dystrobrevin immunolabeling intensity seemed to be similar between nucleus and cytoplasm. Finally, the α-sarcoglycan and α-syntrophin Immunostaining, which was extended throughout the myotube cell dimension, predominated inside the nucleus.

To sustain immunofluorescence results with biochemical evidence, C_2C_{12} myoblasts and myotubes were fractionated into total, plasma membrane, cytoplasmic and nuclear extracts, and the resulting cell preparations were subjected to Western blot analysis using antibodies against Dp71 and each DAP. The analysis of total protein extracts revealed that β-dystroglycan and α-dystrobrevin protein levels increased in the differentiated myotubes (2.2-fold and 1.8-fold, respectively) while those of α-syntrophin diminished 28%,

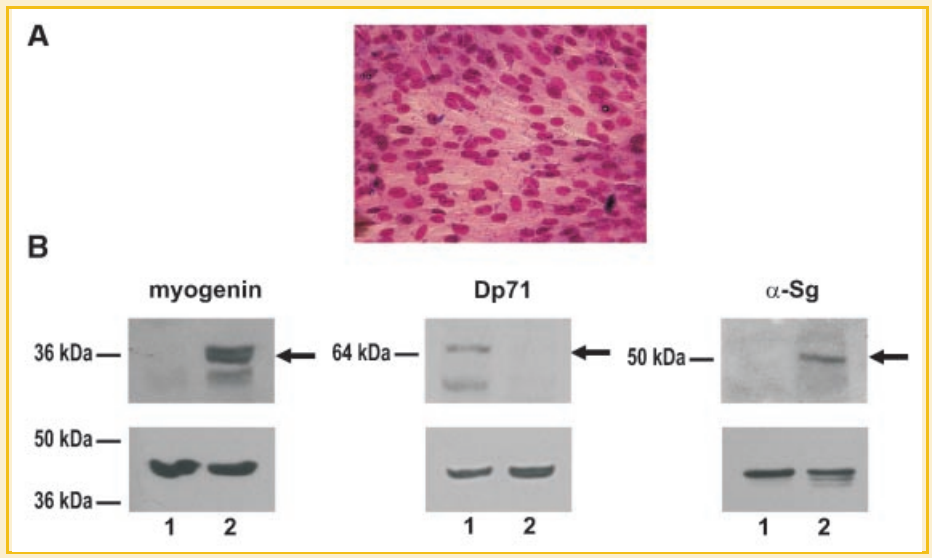

Fig. 2. Expression of Dp71 and α-sarcoglycan during C_2C_{12} myogenesis. A: C_2C_{12} cells were induced to differentiate for 5 days, stained with Giemsa and visualized under the light microscopy. B: Total protein extract, obtained from myoblasts (lane 1) and 5 days differentiated myotubes (lane 2) were analyzed by SDS–PAGE and Immunoblotting, using equal amount of each extract (80 μg) and antibodies against myogenin (a protein marker of differentiated myotubes), Dp71 and α-sarcoglycan (α-Sg). B: Membranes were striped and reprobed with an anti-actin-antibody, as loading control. Arrows at the right denote the specific bands for myogenin and Dp71 and α-sarcoglycan. Migration of protein markers is shown on the left.

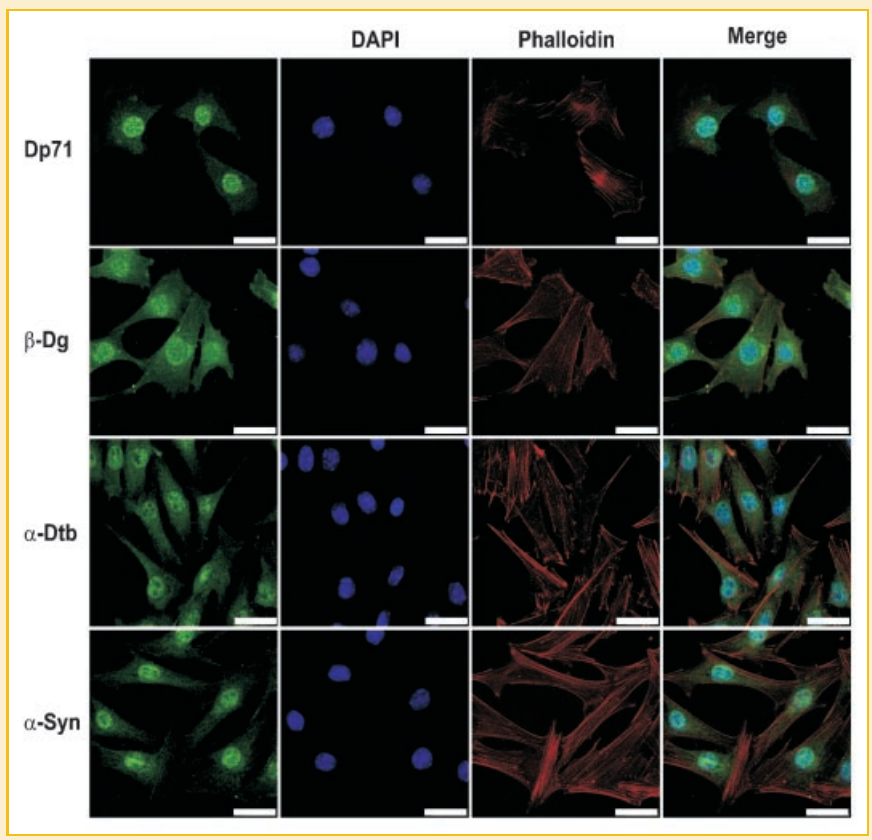

Fig. 3. Immunofluorescence analysis of Dp71 and DAPs in the C_2C_{12} myoblasts. C_2C_{12} myoblasts, cultured on glass cover slips, were immunostained with primary antibodies directed to dystrophin Dp71, β-dystroglycan (β-Dg), α-dystrobrevin (α-Dtb) or α-syntrophin (α-Syn) and the specific signal was developed using a fluorescein-conjugated secondary antibody (green color). Cells were counterstained with DAPI (blue color) and TRITC-phalloidin (red color) to visualize nuclei and cytoplasmic actin, respectively. After triple labeling, cell preparations were subjected to confocal microscopy analysis and single optical Z-sections were selected in each case to show the subcellular distribution of each protein. Merged images are shown on the right panels. Bar = 25 μm.

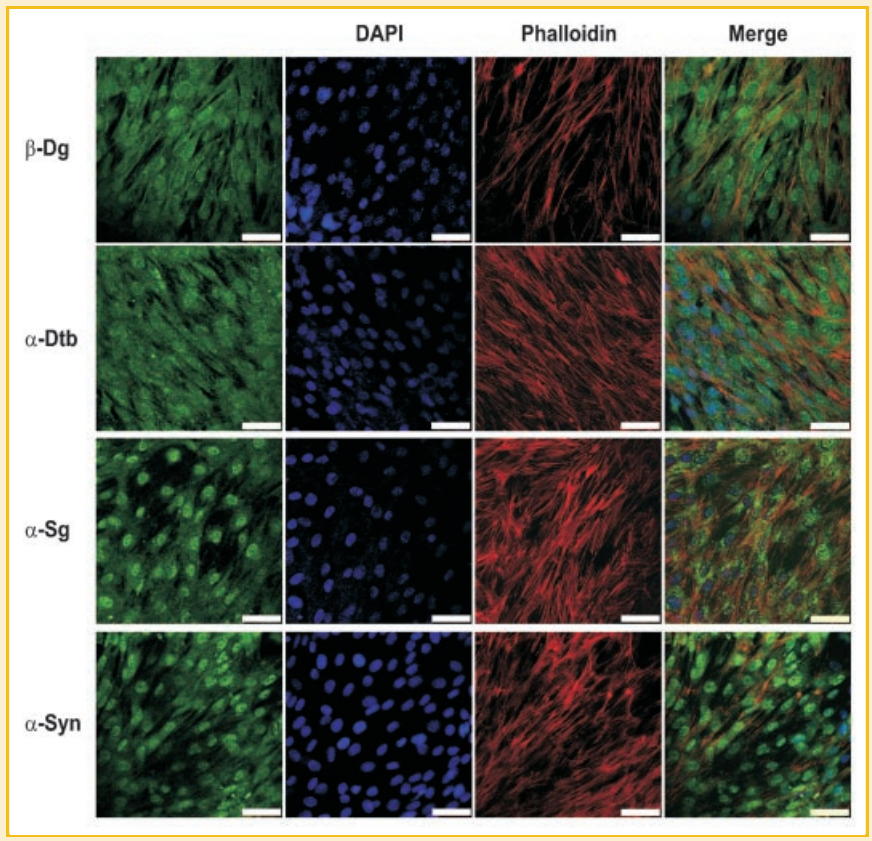

Fig. 4. Immunofluorescence analysis of DAPs in the C_2C_{12} differentiated myotubes. Cells grown on glass cover slips were induced to differentiate by culturing in differentiation medium for 5 days, as described in Materials and Methods Section. Cells were immunostained with antibodies directed against α-dystrobrevin (α-Dtb), α-sarcoglycan (α-Sg), α-syntrophin (α-Syn), or β-dystroglycan (β-Dg), and the appropriate fluorescein-conjugated secondary antibody was employed to develop the specific labeling (green color). For decoration of nuclei (blue color) and cytoplasmic actin, cells were counterstained with DAPI (blue color) and TRITC-phalloidin (red color), respectively. Cell preparations were subjected to confocal microscopy analysis and single optical Z-sections were selected to be shown. Merged images are shown on the right panels. Bar = 50 μm.

compared with myoblasts (data not shown). Figure 5 shows the distribution of Dp71 and DAPs in the C_2C_{12} plasma membrane fraction. First, the purity of the plasma membrane fraction was tested by immunodetection of the α1G subunit of the voltage-dependent calcium channel 3.1, a plasma membrane protein marker. As expected, the α1G subunit was recovered in the plasma membrane fraction obtained from myoblasts and myotubes but not in the corresponding cytosolic extracts. In myoblasts, β-dystroglycan was recuperated in the plasma membrane fraction but not in the cytoplasmic extract; on contrary, Dp71, α-dystrobrevin and α-syntrophin were found only in the cytosolic preparation. Interestingly, in addition to α-sarcoglycan and β-dystroglycan, the myotube plasma membrane fraction contained α-dystrobrevin and α-syntrophin, suggesting that conformation of the dystrophin-associated protein complex at the plasma membrane changes during myogenesis. The nuclear fraction analysis of C_2C_{12} cells is shown in Figure 6. In myoblasts, the Dp71 immunoreactive band appeared to accumulate in the nuclear extract, displaying a relative weaker intensity in the cytoplasm. Two unspecific bands from unknown origin were revealed by the anti-Dp71 antibody in the nuclear extract; the first one migrating above the 64 kDa marker and the second one above the 50 kDa marker. The immunoreactive bands corresponding to β-dystroglycan (43 kDa) and α-dystrobrevin (87 kDa) were found enriched in the nuclear compartment, being lower the cytoplasmic protein levels of the latter protein. Finally, the immunoreactive band of α-syntrophin (58 kDa) was distributed evenly between the two cell compartments. In C_2C_{12} differentiated myotubes, β-dystroglycan and α-sarcoglycan displayed a predominant cytoplasmic localization, while α-syntrophin and α-dystrobrevin were found mostly in the nucleus. Densitometric analysis of the cell fractionation immunoblots revealed that the nucleus/cytoplasm (N/C) ratio of β-dystroglycan decreased in the differentiated myotubes (75%) while that of α-dystrobrevin remained unmodified, as compared with myoblasts. In contrast, the α-systrophin N/C ratio augments 2.3-fold as a consequence of the differentiation process. To confirm the fractionation procedure fidelity of myoblast and myotube cultures, calnexin (an endoplasmic reticulum protein) and lamin A/C (a nuclear matrix-associated protein) were employed as cytoplasmic and nuclear marker, respectively. As expected, calnexin (90 kDa) was absent in the nuclear fraction but present in the cytosolic extract, whereas lamin A/C (69/62 kDa) was found solely in the nuclear fraction (Fig. 6).

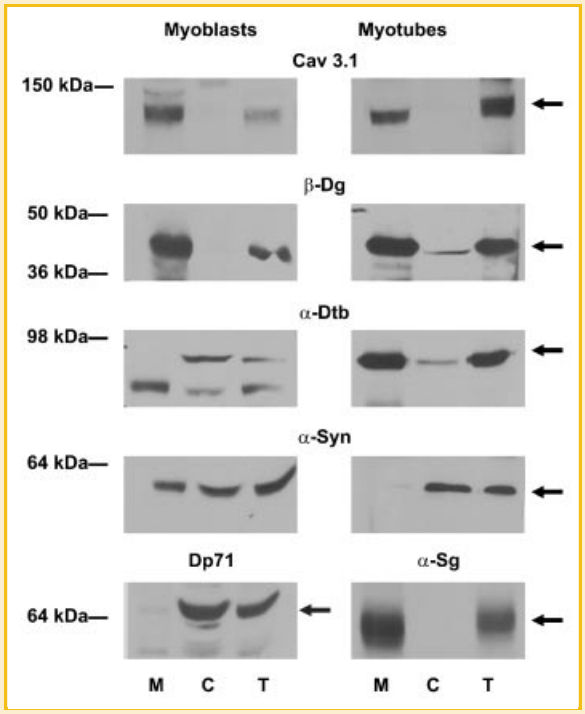

Fig. 5. Distribution of Dp71 and DAPs at the plasma membrane during C_2C_{12} myogenesis. Total (T), plasma membrane (M), and cytosolic (C) protein extracts were prepared from myoblasts or 5 days differentiated myotubes. Cell fractions were resolved by SDS–PAGE and analyzed by Immunoblotting using antibodies against Dp71, β-dystroglycan (β-Dg), α-dystrobrevin (α-Dtb), α-sarcoglycan (α-Sg) or α-syntrophin (α-Syn). Purity of plasma membrane extracts was evaluated by immunodetection of the calcium channel subunit Cav 3.1, a membranal protein marker. Position of protein markers is shown on the left and arrows at the right denote the migration of the expected protein bands.

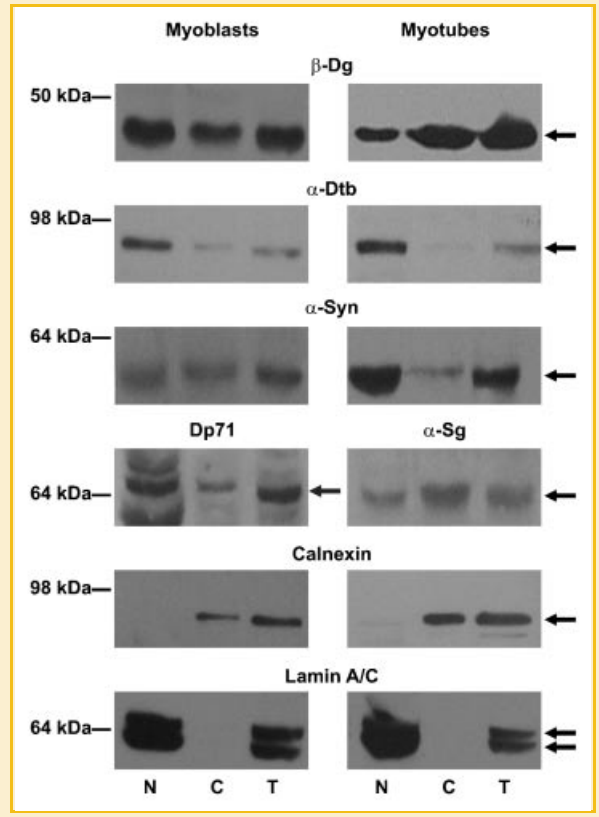

Fig. 6. Nuclear distribution of Dp71 and DAPs during C_2C_{12} myogenesis. Myoblasts or myotubes induced to differentiate for 5 days were fractionated to obtain total (T), nuclear (N) and cytoplasmic (C) protein extracts. A: Equal amount (80 μg) of each protein extract was resolved by SDS–PAGE and subjected to Immunoblot analysis using antibodies directed to Dp71, β-dystroglycan (β-Dg), α-dystrobrevin (α-Dtb), α-sarcoglycan (α-Sg) or α-syntrophin (α-Syn). As loading control, membranes were stripped and reprobed with an anti-actin antibody (data not shown). B: To test purity of cell fractions, immunodetection of nuclear (lamin A/C) and cytoplasmic (calnexin) protein markers was carried out. Position of protein markers is shown on the left. Arrows at the right denote the migration of the expected protein bands.

DAPs FORM A COMPLEX IN THE NUCLEUS OF C_2C_{12} MUSCLE CELLS

To ascertain whether nuclear DAPs associate each one to each other to form a protein complex, nuclear protein extracts from C_2C_{12} cell cultures were immunoprecipitated with the β-dystroglycan antibody C-20 and analyzed by Western blotting with antibodies directed to specific DAPs. Figure 7 show that β-dystroglycan precipitated together with Dp71, α-dystrobrevin and α-syntrophin in myoblasts, and with α-sarcoglycan, α-dystrobrevin and α-syntrophin in myotubes. It should be noted that the amount of α-syntrophin decreased drastically in the complex of myotubes, compared with the myoblast complex, suggesting that most of this protein left the nuclear complex or establish weaker interactions in the differentiated cells that are disrupted during the immunoprecipitation process.

Dp71 AND DAPs ARE COMPONENTS OF THE NUCLEAR ENVELOPE OF MUSCLE CELLS

The cytoplasmic DAPC is organized around the sarcolemma by the interaction of peripheral components (dystrophin, syntrophin, and dystrobrevins) with integral membrane members (sarcoglycans and β-dystroglycan). In analogy with this arrangement, we reasoned that nuclear DAPs might be associated with the inner nuclear membrane of muscle cells, establishing similar type of protein–protein interactions. As a first step to test this hypothesis, we compared the distribution of Dp71 and DAPs in the nuclei and nuclear envelope fractions of C_2C_{12} myoblasts and myotubes, using equal amount of protein sample from both subcellular preparations (Fig. 8). Purity of nuclear envelope extract was verified by Immunodetection of Sp3 (60/100 kDa), a soluble nuclear protein, lamin A/C, a nuclear envelope protein marker, and emerin (34 kDa), a well-characterized inner nuclear membrane protein. As expected, Sp3 was recovered in the nuclear fraction but not in the nuclear envelope preparation, while lamin A/C and emerin were found in both the nuclear and nuclear envelope fractions of myoblasts and myotubes. The immunoreactive bands corresponding to all of the DAPs tested were recovered in the nuclear envelope fraction of myoblasts; however, it should be noted that β-dystroglycan protein levels were clearly enriched in the nuclear envelope fraction, while those of Dp71, α-dystrobrevin and α-syntrophin were depleted,

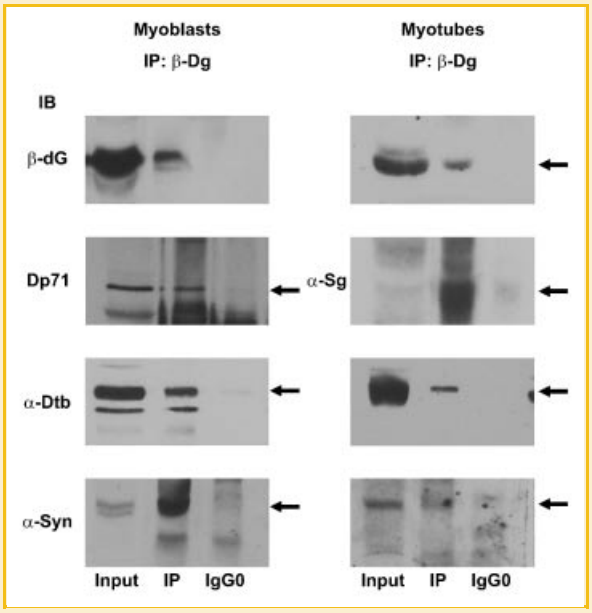

Fig. 7. DAPs establish a protein complex in the nucleus of C_2C_{12} myoblasts and myotubes. Nuclear extracts from C_2C_{12} myoblasts or 5 days differentiated myotubes were immunoprecipitated with the β-dystroglycan antibody C-20. Immunoprecipitated proteins were analyzed by Western blotting with antibodies against β-dystroglycan (β-Dg), Dp71, α-dystrobrevin (α-Dtb), α-syntrophin (α-Syn), and α-sarcoglycan (α-Sg). IP, immunoprecipitation; IB, immunoblotting. IgG0, irrelevant IgG was used as negative control. Input, 5% of the nuclear extract subjected to immunoblotting. Arrows at the right denote the migration of the expected protein bands.

compared with their respective nuclear protein levels. In myotubes, β-dystroglycan and α-sarcoglycan protein bands were found enhanced in the nuclear envelope, whereas those corresponding to α-dystrobrevin and α-syntrophin remained unchanged, compared with their respective nuclear protein bands.

DISCUSSION

Dystrophin and DAPs form a multi-protein complex around the muscle cell membrane to provide sarcolemma stability during contraction and direct cell signaling from the extracellular matrix to the cellular environment [Ehmsen et al., 2002], two relevant functions of the muscle tissue. Interestingly, different studies have provided experimental evidence of the nuclear localization of dystrophin Dp71 and several DAPs in a variety of cell models [Hogan et al., 2001; Kulyte et al., 2002; Marquez et al., 2003], emerging the possibility that these proteins play an alternative function inside nuclei. Recently, we demonstrated the existence of a dystrophin-associated protein complex in the nucleus of HeLa cells [Fuentes-Mera et al., 2006], which is composed of dystrophin Dp71, β-dystroglycan, β-dystrobrevin and nNOs. In addition, we found that Dp71 and DAPC components are present in the nuclear matrix fraction of these cells, suggesting that these proteins may play a role in the nuclear architecture.

Our earlier study encouraged us to analyze whether the nuclear localization of DAPs is extended to muscle cells, where the cell

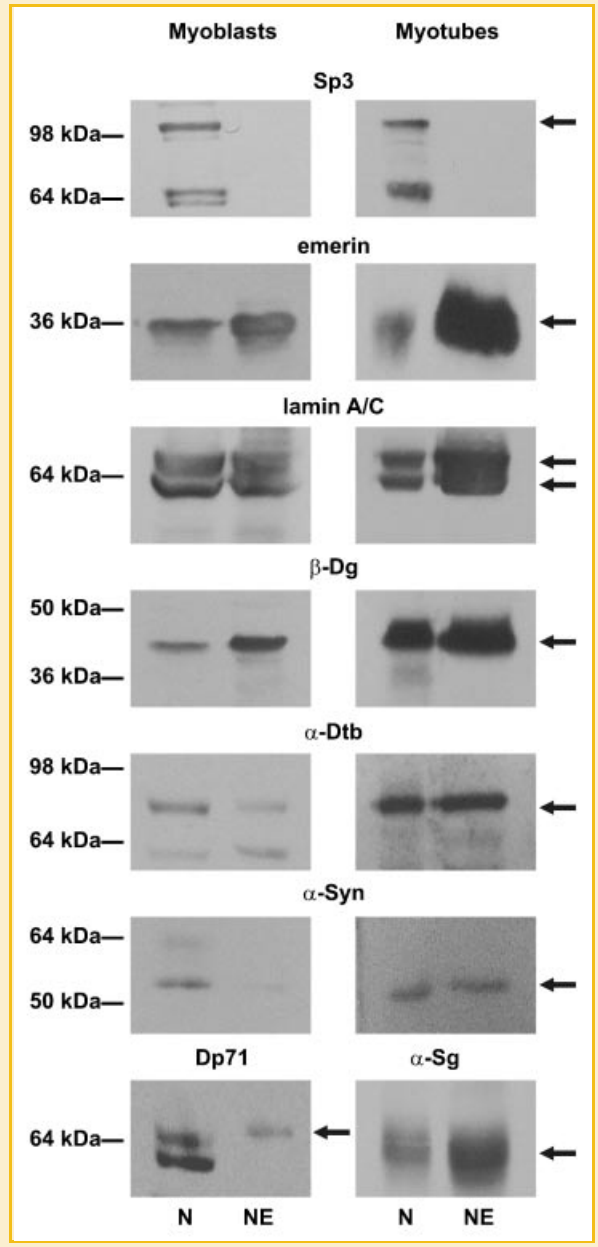

Fig. 8. Presence of Dp71 and DAPs in the nuclear envelope of C_2C_{12} myoblasts and myotubes. Nuclear (N) and nuclear envelope (NE) fractions were obtained from myoblast or 5 days differentiated myotubes, as described in Materials and Methods Section. Equal amount (80 μg) of each protein extract was resolved by SDS–PAGE and subjected to Immunoblot analysis using antibodies against Dp71, a-sarcoglycan (α-(Sg), β-dystroglycan (β-Dg), α-dystrobrevin (α-Dtb) or α-syntrophin (α-Syn). Fidelity of cell fractionation was tested by immunodetection of the nuclear envelope proteins emerin and lamin A/C, and the transcription factor Sp3, a soluble nuclear protein. Position of protein markers is shown on the left. Migration of the expected protein bands is denoted by arrows.

membrane-associated function of these proteins is well characterized. Therefore, we analyzed herein the subcellular distribution of dystrophin Dp71 and DAPs in differentiating C_2C_{12} muscle cells. By using Immunoflurescence assays and Immunoblotting analysis of cell fractions, we demonstrated the presence of Dp71 and DAPs

not only in the cytoplasm and plasma membrane but also in the nucleus of C_2C_{12} cells. Specifically, we found Dp71, β-dystroglycan, α-dystrobrevin and α-syntrophin in the nucleus of myoblasts, and β-dystroglycan, α-dystrobrevin, α-syntrophin and α-sarcoglycan in the nucleus of myotubes. Induction of muscle differentiation had no effect on the DAPs immunoflurescence pattern; but, the relative nuclear abundance of some of these proteins did changed: the nuclear protein levels of β-dystroglycan decreased in differentiated myocytes, whereas those of α-syntrophin increased. In addition, we established by Immunoprecipitation assays that DAPs form a protein complex in the nucleus of C_2C_{12} cells. Interestingly, this complex changes its composition during myogenesis. In myoblasts, the complex is composed of Dp71, β-dystroglycan, α-dystrobrevin and α-syntrophin, while in the differentiated cells the nuclear complex lacks Dp71 but contains α-sarcoglycan, because of the state-specific expression of these two proteins. Furthermore, in spite of being a relatively abundant protein in the nucleus of differentiated cells, a substantial decrease in the amount of α-syntrophin bound to the myotubes complex was observed. It is likely that absence of Dp71 in the myotubes affects the binding of α-syntrophin to the nuclear complex. In contrast, the association of α-dystrobrevin to this complex seemed to be unaltered by the lack of Dp71. It is possible that in absence of Dp71 an unknown intermediary facilitates the engagement of α-dystrobrevin to the myotubes nuclear complex. Myogenic differentiation activates muscle development and maintains muscle phenotype by the concerted action of a family of myogenic transcription factors, such as MyoD, myf-5 and myogenin, which trigger new gene expression profile and activate different signaling pathways [Sartorelli and Caretti, 2005]. Therefore, it seems that muscle differentiation pathway induces DAPs redistribution to assure that the required amount of each of these proteins is present in the nucleus of differentiated myotubes, and ultimately modulates the composition of the DAPs nuclear complex. Identification of the molecular mechanism(s) governing such processes (i.e., modulation of DAPs nuclear import during myogenesis), needs further investigation. Distribution of DAPs at the plasma membrane also changes during myogenesis. While β-dystroglycan was the only DAP present in the myoblasts plasma membrane, α-sarcoglycan, α-dystrobrevin and α-syntrophin were found together with β-dystroglycan in the plasma membrane of myotubes, suggesting that conformation of a complete DAP complex is necessary to provide sarcolemma stability and cell signaling function to differentiated muscle cells.

A necessary step towards the identification of Dp71 and DAPs nuclear function is the definition of their subnuclear localization. On this regards, it is noteworthy that β-dystroglycan and α-sarcoglycan displayed a rim-like immunofluorescence patter that resembles that of inner nuclear membrane proteins. Therefore, we reasoned that these proteins could be inserted in the inner nuclear membrane by using theirs respective transmembrane domains, as they do in the cell membrane [Saito et al., 1999]. To evaluate this hypothesis, we isolated the nuclear envelope (NE) fraction from C_2C_{12} myoblasts and myotubes, using a rigorous biochemical method [Cronshaw et al., 2002; Matunis, 2006]. Components of the NE include the inner nuclear membrane (INM) and outer nuclear membrane (ONM), nuclear pore complexes (NPCs) and the nuclear lamin. The lamin is a fibrous nucleoskeletal structure associated with the INM through interactions with integral membrane proteins [Vlcek et al., 2001]. Noticeable, we found Dp71 and all of DAPs tested (β-dystroglycan, α-dystrobrevin, and α-syntrophin) in the NE fraction of myoblasts; nevertheless, the recovered amount of each protein was different. While β-dystroglycan showed enrichment in the nuclear envelope, the remaining proteins displayed depleted levels, compared with their respective nuclear protein levels. In the C_2C_{12} myotubes, β-dystroglycan as well as α-sarcoglycan displayed enhanced protein levels in the nuclear envelop. These results suggest that β-dystroglycan and α-sarcoglycan are tightly associated with the nuclear envelope, while Dp71 and the remaining DAPs are peripheral proteins that are detached from the nuclear envelope during the purification protocol. Since the inner nuclear membrane protein emerin showed increased levels in the nuclear envelope fraction of C_2C_{12} muscle cells, as β-dystroglycan and α-sarcoglycan did, we consider that the cell fractionation results support the hypothesis that β-dystroglycan and α-sarcoglycan might function as inner nuclear membrane proteins that serve as scaffold to allow the secondary association of Dp71 ant the rest of DAPs with the nuclear envelope. Electron microscopy scanning analysis would be necessary to confirm the subnuclear localization of these proteins. Previously, we demonstrated that Dp71, β-dystroglycan and β-dystrobrevin interact with lamin B1 in the nucleus of HeLa cells [Fuentes-Mera et al., 2006]; therefore, it is likely that additional association could be establish between Dp71 and DAPs with lamin proteins and/or other nuclear proteins of muscle cells.

The nuclear envelope is crucial for muscle tissue function, as has been evidenced by the identification of mutations in the emerin and lamin A/C encoding genes, which cause the X-linked and autosomal-dominant forms of the Emery–Dreifuss muscular dystrophy, respectively [Wilson, 2000]. Two nonexclusive hypotheses have been proposed to explain how deficiency of nuclear envelope proteins causes muscular dystrophy: the mechanical stress hypothesis states that abnormalities in nuclear structure which results from emerin-laminA/C mutations lead to increased susceptibility to cellular damage by mechanical stress. The gene expression hypothesis, which proposes that the nuclear envelope plays a role in tissue-specific gene expression that can be altered by mutations in emerin–laminA/C genes, is based primarily on observed interactions between nuclear envelope and chromatin components [Worman and Courvalin, 2004], including transcription factors an chromatin modifiers [Shaklai et al., 2007]. Given the nuclear envelope localization of Dp71 and DAPs in muscle cells, showed in this study, it is tempting to speculate that these proteins play a role in the nuclear envelope-associated functions, such as nuclear structure integrity and underlying of regulatory nuclear processes. However, due to the essential role of DAPs at the sarcolemma, the design and development of cell and/or mice models with exclusive deficiency of DAPs at nuclear level would be necessary to demonstrate their function in the nucleus of muscle cells.

In summary, we demonstrated that Dp71 and DAPs localize to the nucleus of C_2C_{12} muscle cells where they interact each other to form a protein complex. Furthermore, we found that the nuclear levels of these proteins and the composition of the nuclear complex are modulated during myogenesis. Finally, we offered experimental

evidence showing that Dp71 and DAPs are components of the nuclear envelope and that β-dystroglycan and α-sarcoglycan might function as nuclear membrane proteins.

ACKNOWLEDGMENTS

This work was supported by CONACyT-México Grant No. 54858 (to BC). We thank MS Iván José Galván Mendoza (Unidad de Microscopía Confocal, Centro de Investigación y Estudios Avanzados del IPN) for technical assistance in confocal microscopy.

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