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Dp71ab/DAPs Complex Composition Changes During the Differentiation Process in PC12 Cells

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Abstract PC12 cells express different Dp71 isoforms originated from alternative splicing; one of them, Dp71ab lacks exons 71 and 78. To gain insight into the function of Dp71 isoforms we identified dystrophin associated proteins (DAPs) that associate in vivo with Dp71ab during nerve growth factor (NGF) induced differentiation of PC12 cells. DAPs expression was analyzed by RT–PCR, Western blot and indirect immunofluorescence, showing the presence of each mRNA and protein corresponding to α-, β-, γ-, δ-, and ε-sarcoglycans as well as ζ-sarcoglycan mRNA. Western blot analysis also revealed the expression of β-dystroglycan, α1-syntrophin, α1-, and β-dystrobrevins. We have established that Dp71ab forms a complex with β-dystroglycan, α1-syntrophin, β-dystrobrevin, and α-, β- and γ-sarcoglycans in undifferentiated PC12 cells. In differentiated PC12 cells, the complex composition changes since Dp71ab associates only with β-dystroglycan, α1-syntrophin, β-dystrobrevin, and δ-sarcoglycan. Interestingly, neuronal nitric oxide synthase associates with the Dp71ab/DAPs complex during NGF treatment, raising the possibility that Dp71ab may be involved in signal transduction events during neuronal differentiation. J. Cell. Biochem. 102: 82–97, 2007. © 2007 Wiley-Liss, Inc.

Key words: NGF-induced differentiation; PC12 cells; dystrophin associated proteins (DAPs); dystrophin glycoprotein complex (DGC); dystrophin Dp71ab

Duchenne muscular dystrophy (DMD) is an X-linked genetic disease characterized by progressive muscle degeneration due to the absence of dystrophin [Emery, 1993]. In skeletal muscle, dystrophin associates with different isoforms of syntrophin, dystrobrevin, dystroglycan, sarcoglycan, and sarcospan proteins [Durbeej and Campbell, 2002] which are part of the dystrophin glycoprotein complex (DGC) [Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991]. The large number of signal transduction proteins known to interact with the DGC makes it almost certain that this complex is somehow involved in cell signaling. β-dystroglycan and syntrophin have been shown to bind Grb2 [Yang et al., 1995; Russo et al., 2000; Oak et al., 2001], suggesting a role in signal transduction. Grb2 has also been shown to recruit focal adhesion kinase, FAK 125, to the DGC [Yoshida et al., 1998; Cavaldesi et al., 1999]. Syntrophin also binds neuronal nitric oxide synthase, nNOS [Brenman et al.,

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1996; Hashida-Okumura et al., 1999], muscle and nerve voltage gated Na^+ channels [Gee et al., 1998], SAPK3 [Hasegawa et al., 1999], calmodulin [Madhavan et al., 1992], and diacyl glycerol kinase ζ [Hogan et al., 2001]. In contrast to muscle, much less is known about the molecular mechanisms underlying brain abnormalities in neuromuscular disease in humans [Blake and Kroger, 2000; Mehler, 2000]. The greater diversity of dystrophin and utrophin isoforms present in the brain makes it more difficult to study the central nervous system of dystrophin animals.

Dp71 has been identified as the major DMD gene product in many non-muscle tissues. Dp71 is particularly abundant in brain and its expression increases during the differentiation of the central nervous system [Sarig et al., 1999]. Dp71 associates with dystrophin associated proteins (DAPs) in a similar way to that described for full-length dystrophin. In glial and neuronal cells Dp71 interacts with β-dystroglycan [Austin et al., 1995; Claudepierre et al., 2000b; Haenggi et al., 2004], α- and β-dystrobrevin [Blake et al., 1998; Blake et al., 1999; Claudepierre et al., 2000b; Haenggi et al., 2004], syntrophins [Kramarcy et al., 1994; Blake et al., 1999; Claudepierre et al., 2000b; Haenggi et al., 2004], and δ-sarcoglycan [Claudepierre et al., 2000b]. Besides, Dp71 contains a unique sequence of seven amino acids in the N-terminus that can bind actin [Howard et al., 1998]. All these results suggest that Dp71 is involved in the anchorage and/or organization of specific membrane components.

Dp71 mRNA is alternatively spliced at exons 71–74 and 78 in several tissues, generating multiple Dp71 protein products [Austin et al., 1995, 2000]. Splicing of exons 71–74 does not change the open reading frame (ORF), while splicing of exon 78 eliminates the last 13 amino acids of the original ORF, adding 31 new amino acids known as the founder sequence, whose function is not known [Austin et al., 1995]. The isoform that lacks exon 78 has been named Dp71f, while the isoform containing this exon is named Dp71d. We have previously shown that alternative splicing regulates the nuclear/cytoplasmic distribution of Dp71 isoforms [González et al., 2000; Márquez et al., 2003].

Although, Dp71 is the major DMD gene product expressed in adult human brain tissue [Gorecki and Barnard, 1995], its function is poorly understood. We have adopted the PC12 cell line as an in vitro neuronal model to study Dp71 cellular function [Cisneros et al., 1996; Márquez et al., 2003]. These cells express three Dp71 splicing isoforms: Dp71a (lacking exon 71; GenBank accession number AY326947), Dp71c (lacking exons 71–74; AY326949); both proteins were previously known as Dp71d and Dp71ab (lacking exons 71 and 78; AY326948) previously known as Dp71f [Márquez et al., 2003]. Dp71ab amount increases ninefold in total extracts during nerve growth factor (NGF)-induced PC12 cell differentiation, and localizes in the cell body and neurites of differentiated cells [Márquez et al., 2003].

To gain insight into the function of Dp71 dystrophins, we identified DAPs that interact with Dp71ab during NGF-induced differentiation of PC12 cells. DAPs expression was analyzed by RT–PCR, Western blot, and indirect immunofluorescence coupled to confocal microscopy. Our results show the presence of a Dp71ab/DAPs complex in PC12 cells whose composition changes during the NGF-induced neuronal differentiation process. Interestingly, we identified a protein involved in signal transduction, nNOS, which is incorporated into the Dp71ab/DAPs complex as PC12 cells differentiate, raising the possibility that Dp71ab takes part in signaling events during PC12 cells neuronal differentiation.

MATERIALS AND METHODS

Cell Culture

PC12 cell cultures were grown on collagen-coated P100 plastic tissue culture dishes in a medium containing Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco), 10% heat-inactivated horse serum (Gibco), 5% fetal calf serum (Gibco), 100 U/ml of penicillin (Gibco), 1 mg/ml of streptomycin (Gibco), and 0.25 μg/ml of amphotericin B (Gibco) at 37°C in a water-saturated atmosphere with 5% CO_2. The cultures were exposed to 50 ng/ml of 2.5 S NGF (Invitrogen) for periods of 4, 8, and 12 days for analysis of the expression of sarcoglycans, β-dystroglycan, α1-syntrophin, and dystrobrevins; and for 1–10 and 12 days for the expression of nNOS.

RNA Preparation and RT–PCR

Total RNA extraction was performed using the RNAqueous®-4PCR Kit from Ambion, Inc. Two micrograms of RNA was converted into

complementary DNA (cDNA) using random hexamers as primers and M-MLV reverse transcriptase according to the manufacturer's instructions (Invitrogen). Prior to retro-transcription (RT) reaction, RNA was incubated with RNase-free DNase1 (Invitrogen) to avoid DNA contamination.

Polymerase chain reactions (PCR) took place in a GeneAmp PCR system 2700 thermal cycler with 200 ng of each primer, 0.2 mM of each dNTP, 1.5 units of *Taq*-DNA polymerase in a buffer containing 1.5 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris-HCl (pH 8.4). PCR products were visualized by electrophoresis on 1.5% agarose-Tris-borate buffer gels containing ethidium bromide.

The oligonucleotide sequences, amplified product sizes and PCR conditions are given in Table I. The oligonucleotides were designed according to the rat sarcoglycans sequence reported in GenBank. The PCR sequence was determined using the Dye deoxy terminator cycle sequence kit (Perkin Elmer) and the ABI Prism sequencing apparatus 310 (Perkin Elmer).

Antibodies

The sarco 3, pept 2, pept 1, Nini, and LG7 are polyclonal antibodies (pAb) for α-, β-, γ-, δ-, and ε-sarcoglycans, respectively; LG5 and C4 are pAbs for β-dystroglycan and α1-syntrophin; 5F3 is a monoclonal antibody (mAb) for Dp71ab. All these antibodies have been previously characterized [Rivier et al., 1999; Claudepierre et al., 2000a,b]. α1CT-FP is a pAb for α1-dystrobrevin and β1CT-FP is a pAb which recognizes α1-, α2-, and β-dystrobrevins; these antibodies were kindly provided by Dr. Derek Blake and have been previously characterized [Blake et al., 1998]. The anti-nNOS is a commercial mAb for the nNOS protein (Santa Cruz Biotech).

Immunofluorescence Staining

PC12 cells were grown on cover slips pre-coated with poly-L-lysine. Permeabilization and fixation were performed according to Márquez et al. [2003]. 5F3 and nNOS mAbs were incubated overnight at 4°C and were detected with Alexa 488 secondary anti-mouse IgG antibody (Molecular Probes, Invitrogen), while α-, β-, γ-, δ-, ε-sarcoglycans, and α1-syntrophin were incubated for 1 h at 37°C and were detected with secondary anti-rabbit IgG coupled to Cy3 (Amersham life science products). Mounting was performed in Vectashield mounting medium (Vector laboratories, Inc.) and samples were observed with a 40× oil immersion objective on an epifluorescence Bio-Rad microscope attached to a confocal system (Bio-Rad 1024) using appropriate filters. From each image, 10–15 optical Z-sections (1 μm thick) were scanned. Representative sections were chosen to obtain the distribution of the different proteins in PC12 cells. The images were analyzed using the Image Pro Plus 5.0 program (Media Cybernetics) to obtain the colocalization percentage between Dp71ab and sarcoglycans (α-, β-, γ-, δ-, and ε-), between Dp71ab and α1-syntrophin and between nNOS and α1-syntrophin.

TABLE I. Primer Pair Sequences, Expected Product Size, and PCR Conditions for Sarcoglycans and Actin Reactions

mRNA detected	Primers name	Sequence (5′ to 3′)[a]	Product size	PCR conditions
α-Sarcoglycan	a-Sarco F	TGGCTTCCTCTATGGCACTCC	515 bp	94°C/30 s, 60°C/30 s
	a-Sarco R	GGGGCTCTGGCACTGACTTGT		72°C/30 s 35 cycles
β-Sarcoglycan	b-Sarco F	GGTCTGCTGCGATTCAAGCAAGT	253 bp	94°C/30 s, 60°C/30 s
	b-Sarco R	GAAACTCATGAGTCTCATAGTCT		72°C/30 s 29 cycles
γ-Sarcoglycan	g-Sarco F	CTGAGAACCAGCATGTCTACAAG	304 bp	94°C/30 s, 60°C/30 s
	g-Sarco R	TCTGAGTTGCGAGCATTGACTGT		72°C/30 s 35 cycles
δ-Sarcoglycan	d-Sarco F	GAACAGTACTCACACCACAGGAG	290 bp	94°C/30 s, 60°C/30 s
	d-Sarco R	GCATTACCTGGTCGGGACTTGAT		72°C/30 s 35 cycles
ε-Sarcoglycan	e-Sarco F	GCCGAAAACGTGGGGAAACC	328 bp	94°C/30 s, 60°C/30 s
	e-Sarco R	CGGCACCAACCATGACATAA		72°C/30 s 35 cycles
ζ-Sarcoglycan	z-Sarco F	GAAGTGAGAGCCAGTGAAGAC	346 bp	94°C/30 s, 60°C/30 s
	z-Sarco R	AAGAAGAGAAGGAACCGATTG		72°C/30 s 35 cycles
Actin	Actin 1	TTGTAACCAACTGGGACGATATGG	763 bp	94°C/1 m, 60°C/1 m
	Actin 2	GATCTTGATCTTCATGGTGCTAGG		72°C/1 m 30 cycles

[a]The primer pairs for each sarcoglycan were designed using the rat mRNA sequence found in GenBank (Accession Nos. XM_220884, XM_223355, NM_001006993, XM_340790, AY164274, and XM_344526 for α-, β-, γ-, δ-, ε-, and ζ-sarcoglycans, respectively). Letter F and R of the sarcoglycans name primers denote the forward and reverse orientation for each primer. Actin 1 is the forward primer and Actin 2 is the reverse primer.

Preparation of Cellular Extracts and Western Blots

The PC12 cells were removed from collagen-coated dishes, washed twice with PBS and resuspended in 300 μl of extraction buffer TE (250 mM Tris, 1 mM EDTA pH 8 with 1× complete protease inhibitors cocktail from Roche, Inc.). Cells were sonicated (Soniprep 150, Sanyo) and protein concentrations were determined by Bradford's method. The total extract was resuspended in sample buffer (75 mM Tris-HCl, 15% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 20% glycerol, 0.001 % Bromophenol Blue). 40–100 μg protein was size fractioned on 5–12% gradient or 10% polyacrylamide/SDS gels. Proteins were electrotransferred and the membranes were blocked as previously described [Márquez et al., 2003]. The efficiency of protein transfer was controlled by both Ponceau Red staining of the membrane and Coomassie Blue staining of the remaining gel. Incubation times for primary antibodies ranged from 1 h at room temperature to 24 h at 4°C. All antibody dilutions and washes were carried out in TBS-T buffer. The secondary antibody, mouse anti-IgG or rabbit anti-IgG were peroxidase conjugated and the detection of the immunoreactive bands were carried out using the Western-Blot ECL system (Amersham Pharmacia Biotech).

Coimmunoprecipitation

The coimmunoprecipitations were performed according to Claudepierre et al. [2000b] and Elion [1999]. Briefly, confluent PC12 cells cultures were removed from collagen-coated P100 dishes, washed twice with PBS and resuspended in 700 μl of extraction buffer B (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100 with 1× complete protease inhibitors cocktail from Roche, Inc.), yielding 1–1.2 mg total protein; protein extracts were then incubated on ice for 15 min. Three hundred and fifty microliter of protein extract (0.5–0.6 mg total protein) was incubated with either 5F3 (500 μl), LG5 (20–30 μl), C4 (20–30 μl), or anti-nNOS (10 μg) antibodies. As a negative control, 350 μl of protein extract (0.5–0.6 mg total protein) was used in parallel coimmunoprecipitation assays in which antibodies were not added. Samples were incubated 1.5 h at 4°C under constant agitation, centrifuged for 15 min at 12,000*g*, and the pellet was discarded. Immune complexes were immunoprecipitated for 1 h at 4°C with protein G-Sepharose (Sigma-Aldrich) previously blocked for 1 h at 4°C in 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% BSA, pH 7.4 with 1× complete protease inhibitors cocktail (Roche). Samples were centrifuged 15 min at 12,000*g* and washed three times with extraction buffer B. Precipitate (bead fraction) and supernatant (void fraction) were size fractioned on 3–10% gradient or 10% polyacrylamide/SDS gels and subjected to Western blot.

RESULTS

Sarcoglycan mRNAs Expression During NGF-Induced Neuronal Differentiation of PC12 Cells

Little is known about the expression of sarcoglycans during neuronal differentiation. To gain insight into the role of sarcoglycans in neuronal differentiation, we analyzed sarcoglycan mRNAs expression during the NGF-induced neuronal differentiation of PC12 cells. Total RNA was isolated from undifferentiated PC12 cells as well as from cells treated with NGF for 4, 8, and 12 days. RT–PCR assays were carried out to analyze sarcoglycan mRNAs expression (Fig. 1), the amplified cDNA fragments were visualized in Agarose-TBE gels and stained with ethidium bromide. Actin amplification levels were used as an internal control of cDNA loading in parallel PCR reactions. The identity of each amplified DNA fragment was confirmed by DNA sequencing (data not shown).

Interestingly, all sarcoglycan mRNAs were detected and NGF treatment of PC12 cells differentially affected the expression of some sarcoglycan mRNAs. α-, β-, γ-, and ε-sarcoglycan mRNAs were present during all the differentiation process induced by NGF (Fig. 1A–C,E, respectively), while δ and ζ-sarcoglycan mRNAs were absent in undifferentiated cells and expressed during the differentiation process (Fig. 1D,F); δ-sarcoglycan mRNA appears at time 4 after NGF treatment while ζ-sarcoglycan expression is observed at day 12.

Subcellular Distribution of Sarcoglycans in Undifferentiated and NGF-Differentiated PC12 Cells

We investigated whether NGF treatment affects the subcellular distribution of the sarcoglycan proteins. Indirect immunofluorescence and confocal analysis showed that α-, β-, and

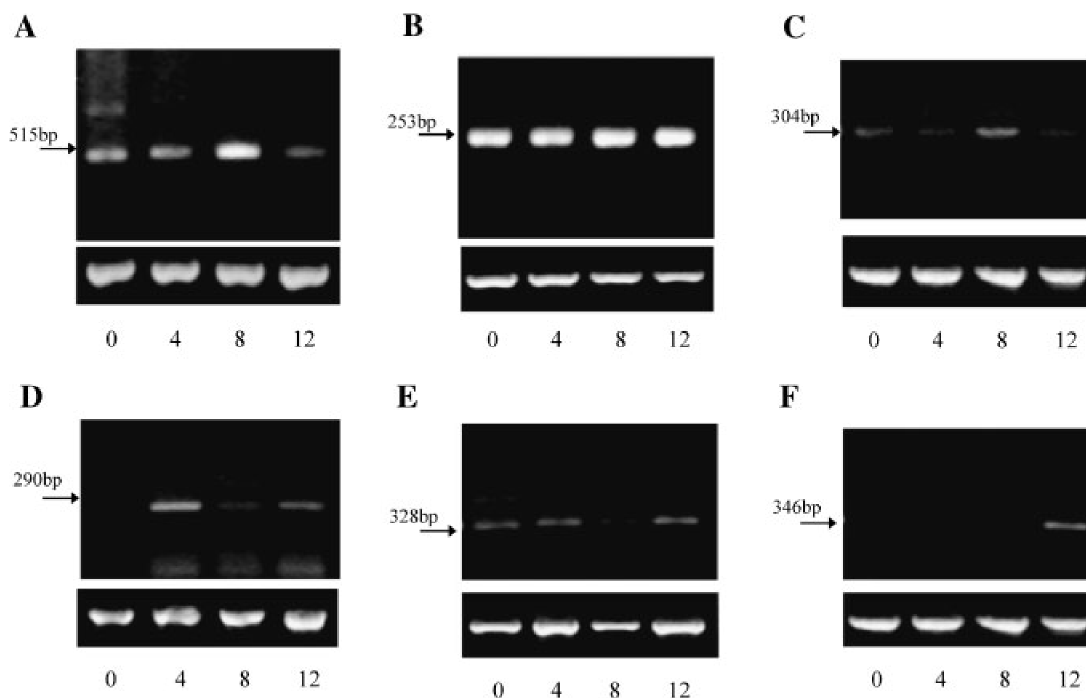

Fig. 1. Expression of sarcoglycan mRNAs during NGF-differentiation of PC12 cells. Total RNA was isolated at different days after NGF treatment (numbers below each lane) and converted into cDNA for the PCR. The oligonucleotides sequence and PCR conditions for each pair of primers are given in Table 1. The PCR products were resolved in a 1.5% agarose gels and stained with ethidium bromide. **Panel A** shows the expression for α-sarcoglycan, **panel B** for β-sarcoglycan, **panel C** for γ- sarcoglycan, **panel D** for δ-sarcoglycan, **panel E** for ε-sarcoglycan, and **panel F** for ζ-sarcoglycan. The lower part of the figures shows the amplification of actin as a loading control. The figures show a representative result.

γ-sarcoglycan displayed a similar punctuated pattern in cytoplasm as well as in the cell periphery. All three proteins also showed a nuclear punctuated distribution albeit much less intense than that in the cytoplasm (Fig. 2B,E,H, respectively). After 10 days of NGF treatment, a very low signal corresponding to α- and γ-sarcoglycans was detected in the cytoplasm and along neurites (Fig. 3B,H, respectively). Similar labeling intensity was detected for β-sarcoglycan protein in both untreated PC12 cells (Fig. 2E) and those treated with NGF for 10 days (Fig. 3E), localizing mainly to the cytoplasm and the cellular periphery, along the neurites, where it was concentrated in the growth cones. A signal was barely detected in the nucleus.

δ-Sarcoglycan mRNA was not detected by RT–PCR in undifferentiated PC12 cells (Fig. 1D); accordingly, no protein was detected by confocal immunofluorescence (Fig. 2K). After 10 days of NGF treatment, δ-sarcoglycan protein was localized mainly in the cytoplasm, in the cell periphery, along neurites and a faint nuclear signal (Fig. 3K).

ε-Sarcoglycan showed a punctuated distribution both in the nucleus and cytoplasm in undifferentiated cells and strong labeling was present at contact sites between cells (Fig. 2N). After 10 days of NGF treatment, ε-sarcoglycan protein showed a punctuated distribution in the cytoplasm, cell periphery, neurites, and growth cones (Fig. 3N). Interestingly, the intensity of the signal at contact sites between the cells in undifferentiated and differentiated cells remained unchanged (data not shown).

Dp71ab Strongly Colocalizes With α, β, and γ-Sarcoglycans in Undifferentiated Cells and With δ-Sarcoglycan in NGF-Differentiated Cells

Several reports have demonstrated the formation of Dp71/DAPs complexes in different tissues, including cells from the central nervous system [Blake et al., 1999; Loh et al., 2000; Claudepierre et al., 2000b; Haenggi et al., 2004; Hernández-González et al., 2005]. However, little is known about the composition of these complexes during the neuronal differentiation process. We investigated whether sarcoglycans

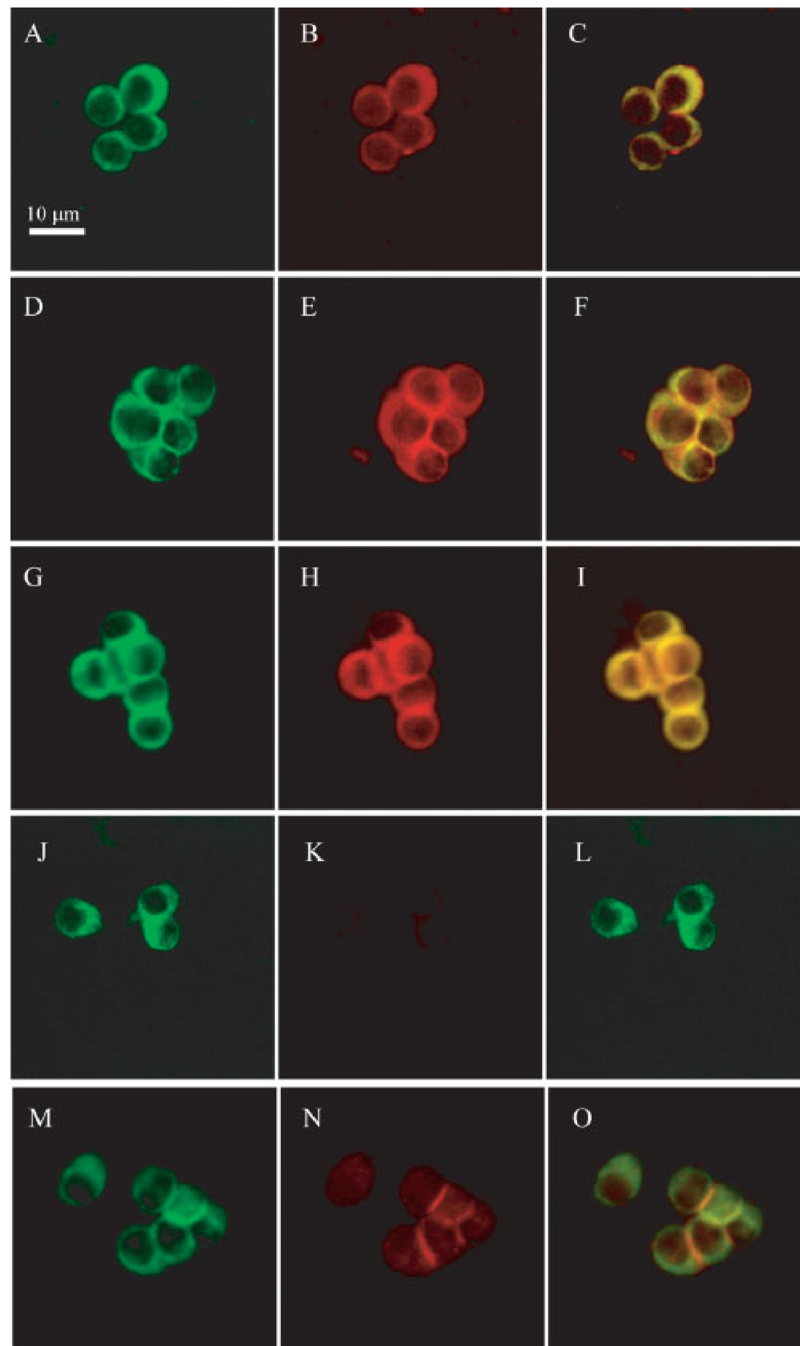

Fig. 2. Subcellular localization of Dp71ab and sarcoglycans in undifferentiated PC12 cells. Undifferentiated PC12 cells were double immunostained with mAb 5F3 against Dp71ab (**A**,**D**,**G**,**J**,**M**) and with the pAbs sarco 3, pept 2, pept 1, Nini, and LG7 against α-, β-, γ-, δ-, and ε-sarcoglycans (**B**,**E**,**H**,**K**,**N**, respectively). Cells were subjected to analysis by confocal laser scanning microscopy and representative single confocal layers were selected in each case to show the subcellular distribution of Dp71ab and sarcoglycans. All the right hand **panels** (**C**,**F**,**I**,**L**,**O**) are merged images of the two preceding images. Size bar corresponds to 10 μm.

colocalized with Dp71ab. As shown in Figure 2, confocal immunofluorescence revealed that Dp71ab has a diffuse punctuated labeling pattern in the cytoplasm and cell periphery in undifferentiated PC12 cells (Fig. 2A,D,G,J,M). In NGF-differentiated PC12 cells, Dp71ab localized to the cell soma cytoplasm, being more abundant at the periphery of the cells, varicosities, and growth cones; a low signal was present in neurites (Fig. 3A,D,G,J,M). These

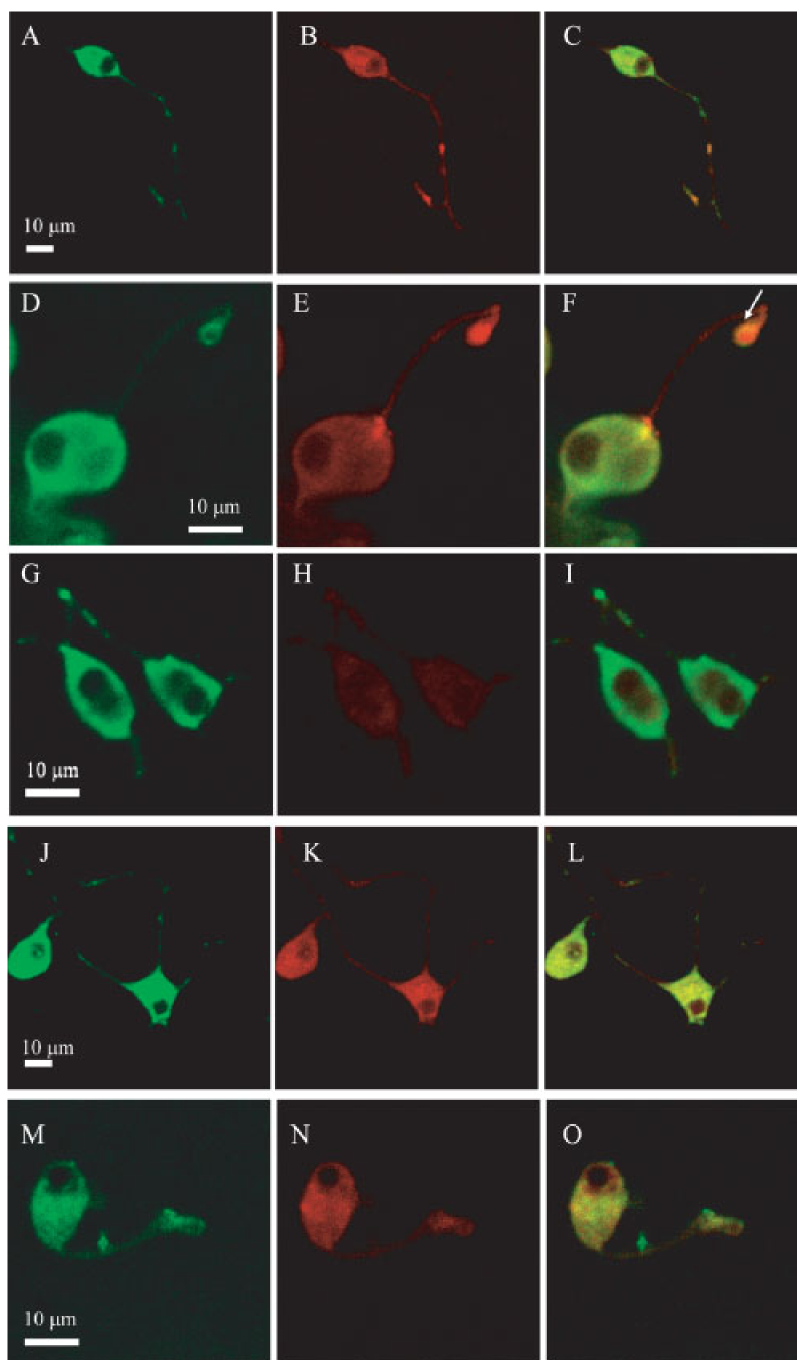

Fig. 3. Subcellular localization of Dp71ab and sarcoglycans in NGF-differentiated PC12 cells. PC12 cells were double immunostained with the mAb 5F3 against Dp71ab (**A**,**D**,**G**,**J**,**M**) and with the pAbs sarco 3, pept 2, pept 1, Nini, and LG7 against α- (**B**), β- (**E**), γ- (**H**), δ- (**K**), and ε- (**N**) sarcoglycans, respectively. Cells were subjected to analysis by confocal laser scanning microscopy and representative single confocal layers were selected in each case to show the subcellular distribution of Dp71ab and sarcoglycans. All the right hand **panels** (**C**,**F**,**I**,**L**,**O**) are merged images of the two preceding images. Arrowhead in panel F indicates colocalization in growth cone. Size bar corresponds to 10 μm.

data are consistent with our previous observations [Márquez et al., 2003]. Double labeling assays revealed that, in undifferentiated PC12 cells, Dp71ab strongly colocalizes with α-, β-, and γ-sarcoglycan in the cytoplasm and at the cellular periphery, where a strong signal is present (yellow Fig. 2C,F,I, respectively). The colocalization averages were $28 \pm 6\%$ between

Dp71ab and α-sarcoglycan (yellow Fig. 2C); 41 ± 14% between Dp71ab and β-sarcoglycan (yellow Fig. 2F), and 44 ± 15% between Dp71ab and γ-sarcoglycan (yellow Fig. 2I). After 10 days of NGF treatment the strong colocalization between Dp71ab and α-, β-, and γ-sarcoglycans observed in undifferentiated cells decreased drastically (9 ± 4%, 8 ± 3%, and 7 ± 3%, respectively; yellow Fig. 3C,F,I, respectively). It is interesting to note that Dp71ab and β-sarcoglycan showed colocalization in the periphery of the growth cone (arrow Fig. 3F).

Dp71ab and δ-sarcoglycan colocalized in the cytoplasm of PC12 cells NGF-treated for 10 days (21 ± 7%), but not in the cellular periphery and neurites (yellow Fig. 3K).

As shown in Figures 2 and 3, colocalization between Dp71ab and ε-sarcoglycan increased slightly as NGF-induced neuronal differentiation progressed. The colocalization average between Dp71ab and ε-sarcoglycan increased from 3 ± 1% in undifferentiated PC12 cells (yellow Fig. 2O) to 10 ± 4% in differentiated cells (Fig. 3O).

Protein Levels of β-Dystroglycan, α1-Syntrophin, α1-, and β-Dystrobrevins Remain Constant During NGF-Induced Neuronal Differentiation of PC12 Cells

Several reports have demonstrated the expression of α-/β-dystroglycan, α1-syntrophin, α-, and β-dystrobrevins in brain or neuronal cells [Culligan et al., 2001; Moukhles and Carbonetto, 2001; Ceccarini et al., 2002]. We analyzed the expression of β-dystroglycan, α1-syntrophin, α-, and β-dystrobrevin proteins during NGF-induced neuronal differentiation of PC12 cells. Total protein was isolated from PC12 cells as well as from cells treated with NGF during 4, 8, and 12 days. Protein levels were determined by Western blot analysis followed by densitometry scanning. Actin protein levels were used as an internal control of protein loading. To compare the relative levels of different proteins (β-dystroglycan, α1-syntrophin, α1-, and β-dystrobrevins), the ratio between the densitometry data for each protein and actin was calculated and plotted as shown in the graphs located next to the Western blot figures (Fig. 4). Our results clearly indicate that the protein levels of β-dystroglycan, α1-syntrophin, α1-, and β-dystrobrevins remained constant during the neuronal differentiation of PC12 cells induced by NGF (Fig. 4).

Dp71ab Associates With β-Dystroglycan, α1-Syntrophin, and β-Dystrobrevin in Undifferentiated and 12 day-NGF-Differentiated PC12 Cells

Márquez et al. [2003], showed that Dp71ab colocalizes with β-dystroglycan, a member of the DAPs, in undifferentiated as well as in NGF-differentiated PC12 cells. This suggests that both polypeptides either interact with each other or are members of the same protein complex along the differentiation process. Therefore, we investigated the interactions between Dp71ab and DAPs; β-dystroglycan, α1-syntrophin, and β-dystrobrevin in undifferentiated and 12 day-NGF-differentiated PC12 cells through coimmunoprecipitation assays. As shown in Figure 5A–C protein complexes containing Dp71ab were immunoprecipitated with mAb 5F3 (which specifically recognizes Dp71ab) from crude membrane extracts prepared from undifferentiated PC12 cell cultures. DAPs were revealed by specific polyclonal antibodies (Fig. 5). In each panel, lane 1 corresponds to control bead fraction (no antibody added) revealing proteins bound non-specifically to Sepharose beads, and lane 2 corresponds to the supernatant fraction of the immunoprecipitation reaction. Members of the DAPs complex that coimmunoprecipitated with Dp71ab are shown in lane 3 of every panel: 43 kDa β-dystroglycan protein (Fig. 5A, lane 3); 59 kDa α1-syntrophin protein (Fig. 5B, lane 3) and 68 kDa β-dystrobrevin (Fig. 5C, lane 3). These data clearly show that Dp71ab is associated with β-dystroglycan, α1-syntrophin, and β-dystrobrevin in undifferentiated PC12 cells.

Coimmunoprecipitation assays, similar to those described above were carried out with protein extracts prepared from 12 day-NGF-differentiated PC12 cells, to analyze the interactions between Dp71ab and DAPs; β-dystroglycan, α1-syntrophin, and β-dystrobrevin. Polyclonal antibodies LG5 and C4 (specifically recognizing β-dystroglycan and α1-syntrophin, respectively) coimmunoprecipitated Dp71ab (Fig. 5D, lane 3 and E, lane 3, respectively). Reciprocally, mAb 5F3 coimmunoprecipitated β-dystrobrevin (Fig. 5F, lane 3). Our results indicate that Dp71ab was associated with β-dystroglycan,

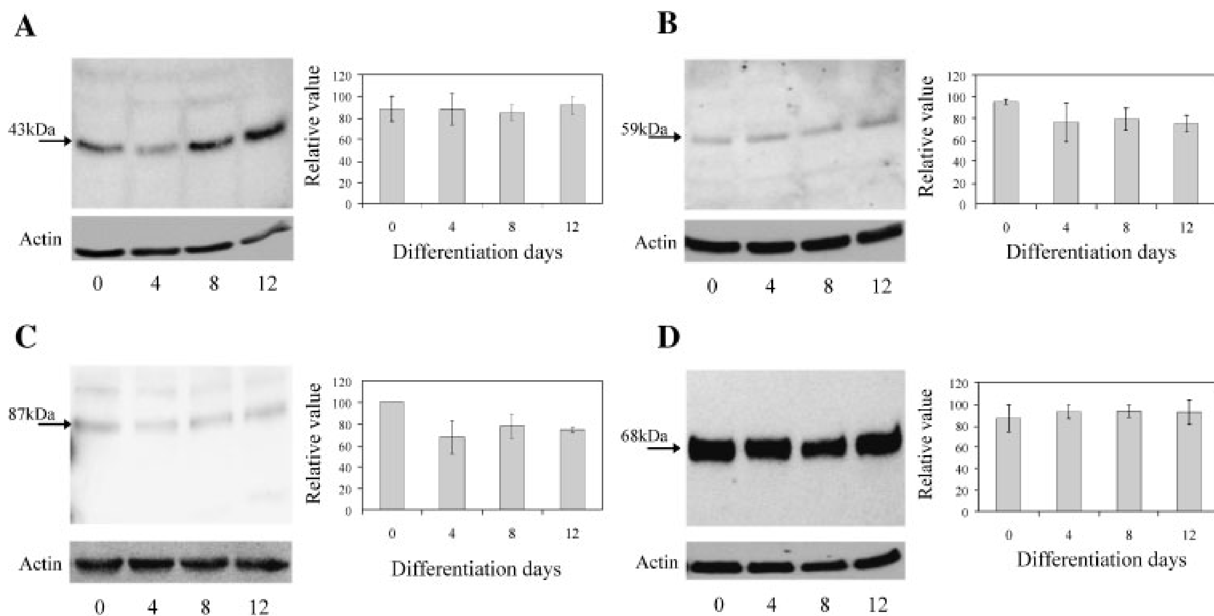

Fig. 4. Expression level of β-dystroglycan, α1-syntrophin, α-, and β-dystrobrevin proteins during NGF-differentiation of PC12 cells. PC12 cells were NGF-differentiated for 0, 4, 8, and 12 days (numbers below each lane). **A**: Immunodetection of β-dystroglycan using LG5 antibody. **B**: Immunodetection of α1-syntrophin using C4 antibody. **C**: Immunodetection of α1-dystrobrevin using α1CT antibody. **D**: Immunodetection of β-dystrobrevin with β1CT antibody. The lower part of the figures shows actin as a loading control, actin was immunodetected using a mAb against actin. The relative expression was quantified by densitometric scanning of the band intensity for β-dystroglycan, α1-syntrophin, α-, and β-dystrobrevin and actin in the Kodak Digital Science 1D program. The ratio between the values for β-dystroglycan, α1-syntrophin, α-, and β-dystrobrevin with actin was graphed (graphics at the right of each panel); standard deviation is indicated. The figures show a representative image of the Western blot and the graphics show the values of at least three independent experiments.

α1-syntrophin, and β-dystrobrevin in PC12 cells treated with NGF for 12 days.

nNOS Protein Is a Member of the Dp71ab/DAPs Complex in Differentiated PC12 Cells

In addition to its structural role, the DGC seems to have signaling functions, due in part to interaction with modular adapter proteins such as syntrophins [Peters et al., 1997]. It has been demonstrated that β-dystroglycan can interact with the adaptor protein Grb2 [Yang et al., 1995; Russo et al., 2000; Oak et al., 2001]. We therefore decided to investigate whether β-dystroglycan interacted with the signaling protein Grb2. Coimmunoprecipitation assays using polyclonal antibody LG5, which specifically recognizes β-dystroglycan, did not coimmunoprecipitate Grb2, indicating that these two proteins were not associated in vivo in PC12 cells (data not shown).

We examined next the relationship between α1-syntrophin and nNOS since it has been reported that α1-syntrophin interacts with the signaling protein nNOS in muscle cells [Brenman et al., 1996] and neurons [Hashida-Okumura et al., 1999]. In PC12 cells, nNOS expression is turned on by NGF [Schonhoff et al., 2001; Kalisch et al., 2002]. In order to study the relationship between nNOS and α1-syntrophin proteins, we first determined the expression pattern of nNOS protein in PC12 cells upon NGF treatment. Our results indicate that nNOS protein is first seen at day 2 after NGF addition and its amount increases along the differentiation process (Fig. 6A,B).

Coimmunoprecipitation assays showed that nNOS and α1-syntrophin proteins were found as an associated-protein complex after 12 days of NGF treatment. A mAb against nNOS protein coimmunoprecipitated α1-syntrophin (Fig. 6C, lane 3). Furthermore, nNOS and Dp71ab associate into a complex since monoclonal antibody 5F3, which specifically recognizes Dp71ab, coimmunoprecipitates nNOS from cultures of PC12 cells at 4 and 10 days of NGF treatment (Fig. 6D, lane 3 and E, lane 3, respectively).

α1-Syntrophin Colocalizes With nNOS and Dp71ab

To determine the subcellular localization of nNOS and α1-syntrophin proteins in undifferentiated as well as in 10 day-NGF-

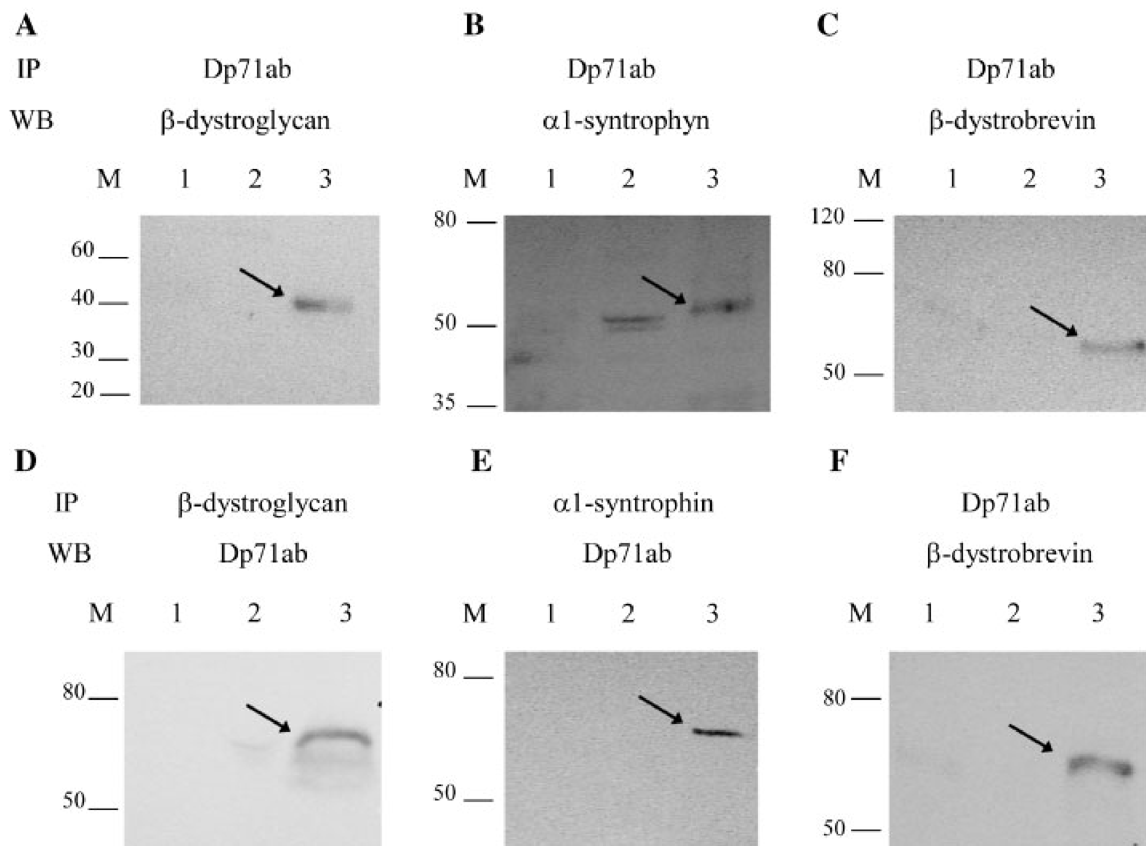

Fig. 5. β-Dystroglycan, α1-syntrophin, and β-dystrobrevin coimmuneprecipitate with Dp71ab in undifferentiated and 12 days NGF-differentiated PC12 cells. Undifferentiated PC12 cells total protein extracts were immunoprecipitated with 5F3 antibody (**A**–**C**). The immune complexes were analyzed by Western blot using LG5 (A), C4 (B), and β-CT (C) antibodies. β-Dystroglycan, α1-syntrophin, and β-dystrobrevin were coimmunoprecipitated with Dp71ab (**lanes 3** in A–C, respectively). Twelve days NGF-differentiated PC12 cells protein extracts were immunoprecipitated with LG5 (**D**) C4 (**E**), or 5F3 (**F**) antibodies. The immune complexes were analyzed by Western blot using 5F3 (D,E) or β-CT (F) antibodies. Dp71ab coimmunoprecipitaed with β-dystroglycan (D, lane 3), α1-syntrophin (E, lane 3), and β-dystrobrevin (F, lane 3) in 12 days NGF-differentiated PC12 cells. **Lanes 1**: control bead fractions. **Lanes 2**: supernatant fractions after immunoprecipitation. **Lanes 3**, proteins immunoprecipitated (bead fraction). Molecular mass markers are indicated on the left.

differentiated PC12 cells, indirect immunofluoresence and confocal analyses were used. As previously shown, nNOS protein is not expressed in undifferentiated PC12 cells (Fig. 7A) while α1-syntrophin shows a punctuated distribution in the cytoplasm (Fig. 7B,H). Ten days after NGF treatment, nNOS (Fig. 7D) and α1-syntrophin (Fig. 7E,K) were mainly localized in the cytoplasm and the cellular periphery, along the neurites and in the growth cones. The colocalization average of $34 \pm 11\%$ (yellow, Fig. 7F) indicates that nNOS and α1-syntrophin proteins were found strongly colocalized in the cytoplasm, neurites, varicosities and growth cones.

Double labeling experiments showed that α1-syntrophin and Dp71ab are colocalized in the cytoplasm of undifferentiated PC12 cells (yellow Fig. 7I; the colocalization average was $23 \pm 8\%$). After 10 days of NGF treatment, α1-syntrophin and Dp71ab were also found strongly colocalized in the cytoplasm and cell periphery and to a minor extent in neurites, varicosities and growth cones (Fig. 7L; the colocalization average was $34 \pm 8\%$). α1-Syntrophin and Dp71ab subcellular colocalization pattern was similar to that of nNOS and α1-syntrophin.

DISCUSSION

The PC12 cell line is a well-established model of neuronal differentiation. We have previously shown the existence of Dp71 isoforms that arise through alternative splicing of the pre-mRNA in this cell line [Márquez et al., 2003].

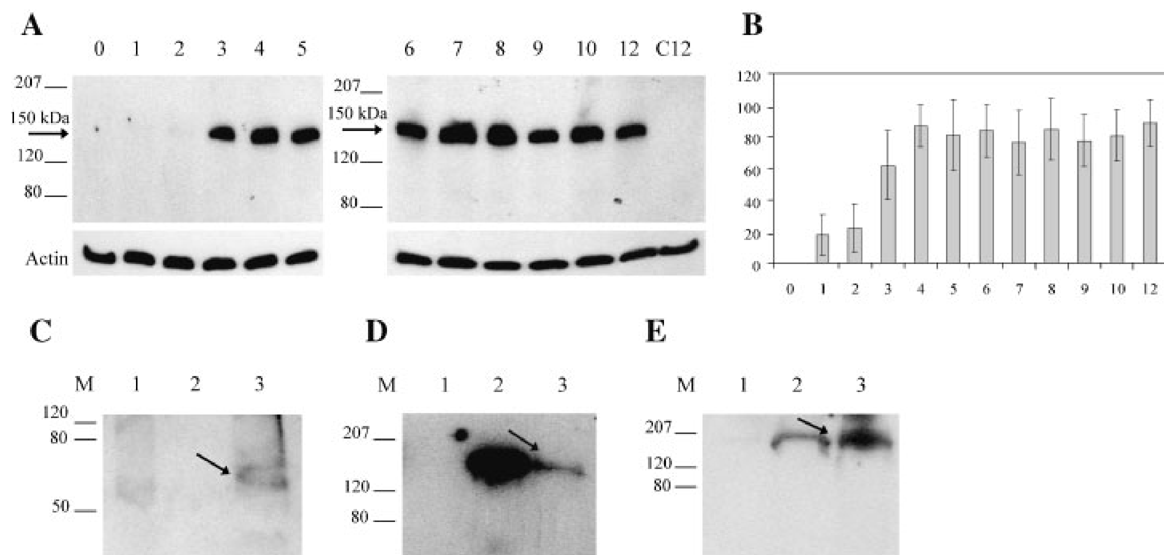

Fig. 6. Coimmunoprecipitation of nNOS with α1-syntrophin and Dp71ab in NGF-induced differentiation of PC12 cells. Western blot experiments were performed to analyze the expression level of nNOS during NGF-differentiation of PC12 cells. PC12 cells were NGF-differentiated for 0–10 and 12 days (numbers below each lane). **A**: Western blot of nNOS shows a 150 kDa immunostaining band. The lower part of the figure shows actin as a loading control, the actin immunodetection was detected with a mAb against actin. The relative expression was quantified by densitometric scanning of the nNOS and actin band intensity in the Kodak Digital Science 1D program. The ratio between the values for nNOS with actin was graphed and the standard deviation is indicated (**B**). The figure show a representative image of the Western blot and the graphic show the values of at least three independent experiments. PC12 cells extracts were immunoprecipitated with anti-nNOS (**C**) or 5F3 (**D**,**E**) antibodies. The immune complexes were analyzed by Western blot using C4 (C) or anti-nNOS (D,E) antibodies. **Lanes 1**: control bead fractions. **Lanes 2**: supernatant fractions after immunoprecipitation. **Lanes 3**: proteins immunoprecipitated (bead fraction). α1-Syntrophin coimmunoprecipitates with nNOS in 10 days NGF-differentiated PC12 cells (C, lane 3). nNOS was coimmunoprecipitaed with Dp71ab in 4 and 10 days after NGF-differentiated PC12 cells (D,E, lanes 3, respectively). Comparison of the void (D,E, lane 2) and bead (D,E, lane 3) fractions showed that although a large portion of nNOS was recovered in the void fraction, a significant portion of nNOS was coimmunoprecipitated with Dp71ab. Molecular mass markers are indicated on the left.

Interestingly, these isoforms show distinct subcellular distribution and their expression at both mRNA and protein level varies during NGF-induced neuronal differentiation [Márquez et al., 2003]. These data strongly suggest that each Dp71 isoform might perform a specific function. Furthermore, Dp71 isoforms seem to play an important role during neuronal differentiation, since ablation of their expression by antisense RNA inhibits NGF-induced neuronal differentiation of PC12 cells [Acosta et al., 2004]. Other cellular components may be important for Dp71 function, for example, Dp71 interacts with trans-membrane and cytoplasmic proteins named DAPs and actin. Although studied, the function of Dp71 isoforms is not yet clear. To gain insight into the function of Dp71 dystrophins, we initiated the identification of DAPs that interact with Dp71ab (which lacks exons 71 and 78) during NGF induced differentiation of PC12 cells.

We have previously demonstrated that β-dystroglycan, a member of the DGC, is expressed during the differentiation process of PC12 cells suggesting the existence of a DGC [Márquez et al., 2003]. In this work, we present evidence that many other proteins of the DGC are also expressed in PC12 cells. Interestingly, we detected mRNA of all known sarcoglycans (α-, β-, γ-, δ-, ε-, and ζ-sarcoglycans). This is relevant because it has been reported that there are alternative types of sarcoglycan complexes, one present in skeletal and cardiac muscle consisting of α-, β-, γ-, and δ-sarcoglycan; and another one, expressed in smooth muscle, containing β-, δ-, ζ-, and ε-sarcoglycan [Straub et al., 1999; Wheeler et al., 2002]. Recently, Anastasi et al. [2005] suggested the existence of a pentameric, or hexameric if considering ζ-sarcoglycan, arrangement of the sarcoglycan subcomplex. The hexameric sarcoglycan subcomplex, along with a differential expression of

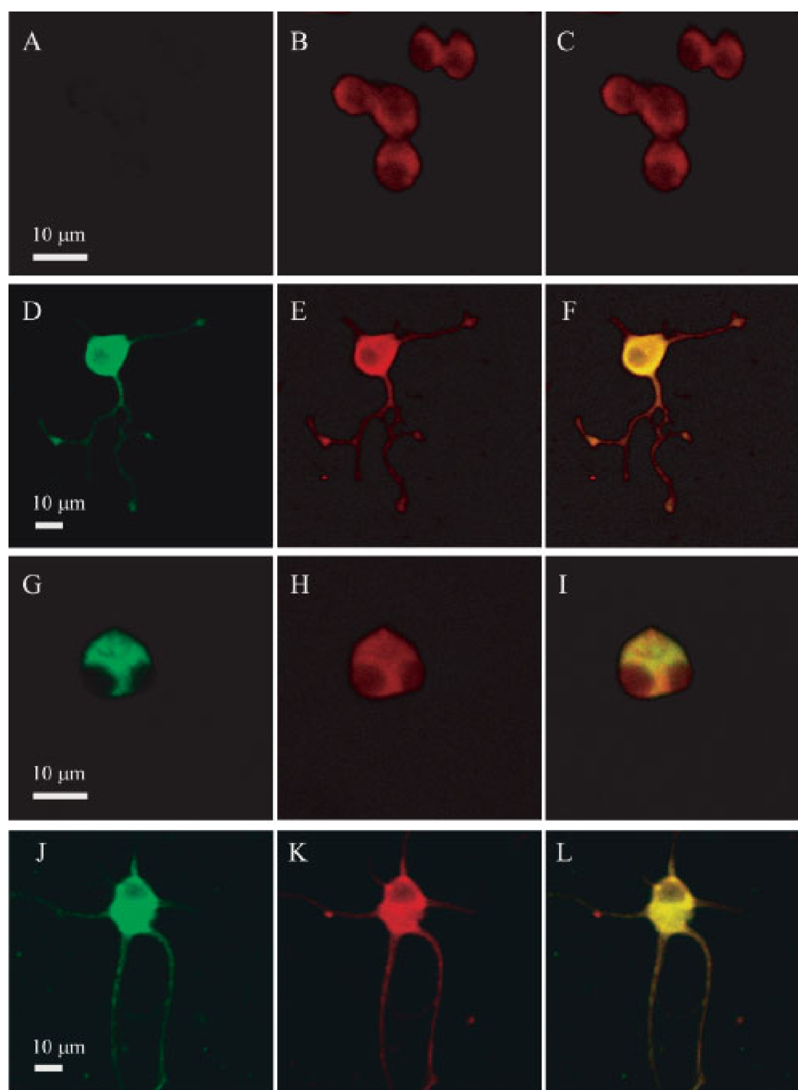

Fig. 7. Subcellular localization of nNOS, α1-syntrophin and Dp71ab in undifferentiated and NGF-differentiated PC12 cells. PC12 cells were double immunostained with the mAb anti-nNOS (**A**,**D**), with the mAb 5F3 against Dp71ab (**G**,**J**) and with the pAb C4 against α1-syntrophin (**B**,**E**,**H**,**K**). Cells were subjected to confocal laser scanning microscopy analysis and representative single confocal layers were selected in each case to show the subcellular distribution of nNOS (A undifferentiated and D 10 days NGF-differentiated), Dp71ab (G undifferentiated and J 10 days NGF-differentiated), and α1-syntrophin (B,H undifferentiated and E,K 10 days NGF-differentiated PC12 cells). All the right hand **panels** (**C**,**F**,**I**,**L**) are merged images of the two preceding images. Size bar corresponds to 10 μm.

single sarcoglycans, may characterize skeletal, cardiac, smooth muscles or other tissues. The existence of a similar sarcoglycan subcomplex in neuronal cells has not been well documented. However, Claudepierre et al. [2000a] reported the expression of δ- and γ-sarcoglycans in Müller glial cells from rat retina. Imamura et al. [2000] reported the expression of β-, δ-, and ε-sarcoglycans in the peripheral nervous system and Royuela et al. [2003] reported the expression of α-, β-, γ-, δ-, and ε-sarcoglycans in sciatic nerve of monkey. Other groups have reported the expression of ε-sarcoglycan in brain [Xiao and LeDoux, 2003; Nishiyama et al., 2004]. However, little is known about the expression of other sarcoglycans in neuronal cells or during neuronal differentiation process.

The expression of α-, β-, γ-, δ-, and ε-sarcoglycan proteins was confirmed in this work by indirect immunofluorescence. Sarcoglycans α-, β-, and γ- showed a cytoplasmic and cellular periphery staining in undifferentiated PC12 cells, which suggest the formation of a sarcoglycan complex in undifferentiated cells.

Colocalization analysis showed that α-, β-, and γ-sarcoglycan could be part of a Dp71ab/DAPs complex in the undifferentiated state (with the colocalization averages of $28 \pm 6\%$; $41 \pm 14\%$ and $44 \pm 15\%$, respectively). Interestingly, this complex may be different in the NGF-differentiated PC12 cells as the colocalization of these proteins varied in this state ($9 \pm 4\%$; $8 \pm 3\%$ and $7 \pm 3\%$ respectively); whereas δ-sarcoglycan colocalized with Dp71ab in the cytoplasm in cells after 10 days of differentiation ($21 \pm 7\%$).

Dickens et al. [2002] identified putative cadherin domains in the ε-sarcoglycan primary sequence. Proteins containing cadherin-domain are adhesion molecules that can modulate a wide variety of processes, including cell polarization and migration [Tepass et al., 2000]. In this work, we found that ε-sarcoglycan showed strong labeling at cell-to-cell contacts. Such localization was not affected by NGF treatment, since after 10 days of NGF treatment; the intensity of the ε-sarcoglycan signal at contact sites between cells was maintained (data not shown). Márquez et al. [2003] showed that Dp71d localizes to cell–cell contacts in PC12 cells. These results suggest that ε-sarcoglycan may be part of a Dp71d-DAPs complex, which may be important to maintain cell–cell contact and/or communication.

Our RT–PCR, indirect immunofluorescence and colocalization evidence suggests that sarcoglycans may play an important role during the neuronal differentiation process induced by NGF, and that PC12 cells may be a suitable system for studying the function of the sarcoglycan complex. The colocalization analysis showed that Dp71ab and sarcoglycans might associate into a multiprotein complex. Indeed, these interactions show a dynamic behavior during the neuronal differentiation of PC12 cells induced by NGF.

Several reports have demonstrated that α/β-dystroglycan, α1-syntrophin, α-, and β-dystrobrevins are expressed in brain [Culligan et al., 2001; Moukhles and Carbonetto, 2001]. Our Western blot results showed that β-dystroglycan, α1-syntrophin, α-, and β-dystrobrevins were constitutively expressed in PC12 cells during all the NGF-differentiation process.

In this work, we provide evidence that almost all the proteins that are part of the DGC are expressed in PC12 cells, being to our knowledge the first evidence that a neuronal cell type can express almost all the proteins of the DGC during the differentiation process.

Several reports have demonstrated or suggested the formation of Dp71/DAPs complexes in different tissues, including cells from the central nervous system [Blake et al., 1999; Loh et al., 2000; Claudepierre et al., 2000b; Haenggi et al., 2004; Hernandez-González et al., 2005]; however, little is still known about the formation of these complexes during the neuronal differentiation process or with Dp71 isoforms. Our coimmunoprecipitation assays showed that β-dystroglycan, α1-syntrophin, and β-dystrobrevin interact with Dp71ab in undifferentiated and NGF-differentiated PC12 cells, allowing us to presume that these proteins may be the core of the Dp71ab/DAPs complex along the differentiation process.

Phung et al. [1999] and Yamazaki et al. [2006] have shown that nNOS protein and NO are crucial for the differentiation and neurite extension of PC12 cells. Although nNOS does not have a transmembrane domain, subcellular fractionation experiments showed that ~60% of the total NOS activity in brain was found in the particulate fraction, suggesting that nNOS is associated with cell membrane, probably through the interaction with membrane associated proteins [Hecker et al., 1994]. Interestingly, a candidate for such an interaction is α1-syntrophin, a dystrophin associated protein that interacts with nNOS through their PDZ domains in muscle cells [Brenman et al., 1996] and neurons [Hashida-Okumura et al., 1999]. Furthermore, expression levels of nNOS protein seem to be tied to the formation of DGC, since antisense oligonucleotides that shut off dystrophin expression also reduce the amount of nNOS in human neurons [Sogos et al., 2003].

Through immunofluoresence and coimmunoprecipitation assays we provide additional evidence pointing out the relationship between nNOS and α1-syntrophin: both proteins strongly colocalized in the cytoplasm, cellular periphery, neurites, varicosities, and growth cones. The colocalization average of Dp71ab/α1-syntrophin ($34 \pm 8\%$) and nNOS/α1-syntrophin ($34 \pm 11\%$) suggest that the nNOS protein interacting with α1-syntrophin also associates with Dp71ab. At the same time nNOS coimmunoprecipitated with α1-syntrophin and Dp71ab. We have previously shown that the amount of Dp71ab protein increases during the

differentiation process [Márquez et al., 2003] while the absence of Dp71 expression results in a marked suppression of neurite outgrowth [Acosta et al., 2004]. Therefore, it will be of interest to determine the importance of the nNOS associated with the Dp71ab/DAPs complex for neurite outgrowth of PC12 cells during NGF-induced neuronal differentiation. Because of that, it is tempting to propose that the Dp71ab/DAPs complex facilitates the migration of nNOS to neurites and growth cones as NGF-induced differentiation progresses.

The differential expression of some of the components of the DGC during neuronal differentiation suggests functional flexibility of the complex in the nervous system. In this scenario, specific associations between different DAPs components may contribute to the organization of distinct DGC-like complexes [Ceccarini et al., 2002]. Interestingly, our coimmunoprecipitation and colocalization results suggest the formation of two different Dp71ab/DAPs complexes in PC12 cells: one consisting of Dp71ab, β-dystroglycan, α1-syntrophin, β-dystrobrevin, α-, β-, and γ-sarcoglycans in undifferentiated PC12 cells (Fig. 8A); and another NGF-induced complex conformed by Dp71ab, β-dytroglycan, α1-syntrophin, β-dystrobrevin, nNOS, and δ-sarcoglycan (Fig. 8B). It has been proposed that the sarcoglycan complex contributes to a more stable association to the DGC in muscle [Ozawa et al., 1998]. Thus, the variation in sarcoglycan composition between both complexes in PC12 cells, as described above, suggest that both complexes fulfill different functions required as the cells differentiate. For example, one of these complexes could facilitate the migration of the Dp71ab/DAPs-nNOS complex to the neurites, growth cones, varicosities, or other structures of differentiated cells. As for Dp71ab, it is possible that this isoform modulates the architectural changes occurring in the cytoskeletal network during

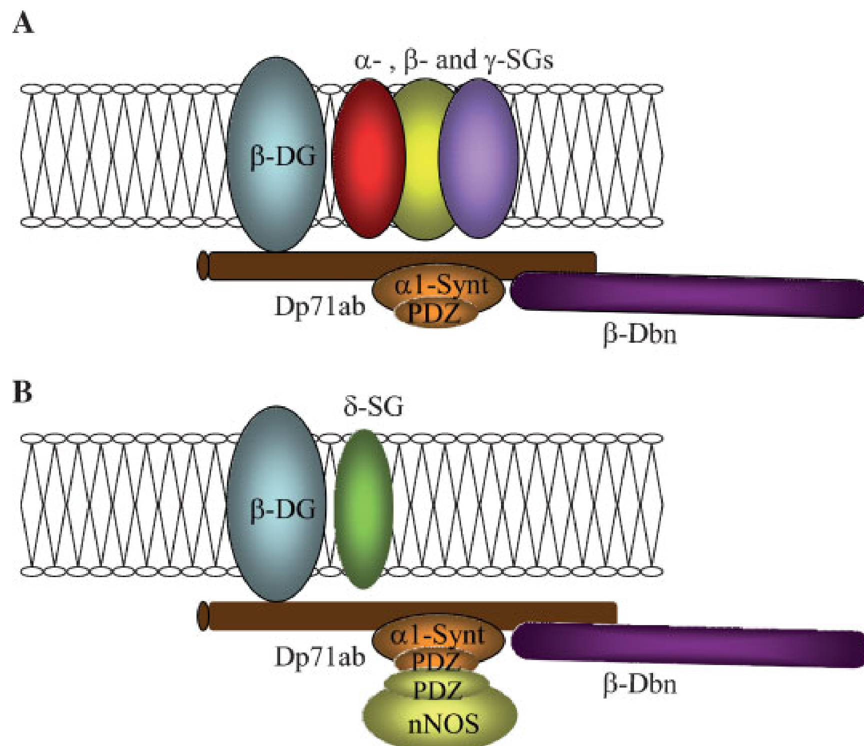

Fig. 8. Model of the Dp71ab/DAPs complex in PC12 cells during NGF-differentiation. In undifferentiated PC12 cells Dp71ab interacts with β-dystroglycan (β-DG), α1-syntrophin (α1-Synt), and β-dystrobrevin (β-Dbn), also α-, β-, and γ-sarcoglycans (-SGs) could be members of this complex (**A**). In NGF-differentiated PC12 cells Dp71ab interacts with β-dystroglycan, α1-syntrophin, and β-dystrobrevin, also δ-sarcoglycan could be a member of this complex, meanwhile α-, β-, and γ-sarcoglycan could be lost from the complex (**B**). Syntrophins are a family of five proteins (α1-, β1-, β2-, γ1-, and γ2-) containing two pleckstrin homology domains and a PDZ domain. The PDZ sequence serves as an adaptor for the recruitment of other proteins such as ion and water channels, receptors, kinases, and neural nitric oxide synthase (nNOS). In differentiated PC12 cells α1-syntrophin interacts with nNOS via its PDZ domains (B).

NGF-induced differentiation through its interaction with β-dystroglycan, α1-syntrophin, β-dystrobrevin, and nNOS.

In summary, our results show the occurrence, during NGF-induced neuronal differentiation of PC12 cells, of dynamic changes at both mRNA and protein levels, as well as in the subcellular distribution of dystrophin Dp71ab and DAPs components. These dynamic changes result in the formation of at least two Dp71ab containing complexes that probably reflect adjustments to changes in the cellular environment produced during the differentiation process: the cytoplasm and cell periphery in undifferentiated cells, and neurites, varicosities, and growth cones in cells treated with neuronal growth factor. Importantly, our work strongly suggests that PC12 cells may be an appropriate model to elucidate the function of Dp71 isoforms.

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