Neuronal nitric oxide synthase localizes through multiple structural motifs to the sarcolemma in mouse myotubes

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Abstract In skeletal muscle, neuronal nitric oxide synthase is localized at the sarcolemma in association with the dystrophin glycoprotein complex (DGC). The nNOS N-terminal 231 amino acids comprise a PDZ domain (residues 1–100) and a β-hairpin finger loop (residues 101–130) which binds α-syntrophin located in the DGC. Endogenous nNOS and GFP-tagged nNOS localize to the sarcolemma in mouse C2C12 myotubes. Expression of GFP-tagged nNOS domains in C2C12 myotubes reveals that the PDZ domain and the β-hairpin finger loop of nNOS are independently capable of localizing to the sarcolemma of C2C12 myotubes. Binding studies indicate that α-syntrophin binds only to the β-hairpin finger loop and not the PDZ domain of nNOS. nNOS may bind to proteins in addition to α-syntrophin at muscle sarcolemma.

Key words: Nitric oxide synthase; Sarcolemmal binding; Skeletal muscle; α-Syntrophin; PDZ domain

1. Introduction

The neuronal isoform of nitric oxide synthase (nNOS) is highly expressed in skeletal muscle [1,2] where it is co-localized to the sarcolemma with the dystrophin glycoprotein complex (DGC) [3]. Association of nNOS with the DGC accounts for its unique sarcolemmal localization in skeletal muscle fibers observed in the immunostaining patterns in cross-sections of skeletal muscle tissues [1,3]. The signaling role of nNOS in skeletal muscle is poorly understood; however in skeletal muscles isolated from mice lacking nNOS, the contraction-stimulated NO production and subsequent cGMP formation signaling pathway is compromised but with no detectable sign of muscle pathology [4].

nNOS is unique among the different nitric oxide synthases in that it possesses an additional 231 amino acid N-terminal extension that interacts with the PDZ domain of α-syntrophin, a 58 kDa protein member of the plasma membrane-associated DGC [5,6]. PDZ domains are characterized by their ability to interact with other proteins to form organized complexes required for diverse signaling pathways and protein interactions take place predominantly through recognition of short consensus carboxyl-terminal peptide motifs, which bind to the consensus peptide binding groove present in PDZ domains. The N-terminal 100 amino acids of nNOS also contain a PDZ domain. The crystal structure of the first 300 amino acids of nNOS bound to the PDZ domain of α-syntrophin depicts a unique β-hairpin finger loop located between residues 100–130 of nNOS which interacts directly with the canonical peptide groove of the PDZ domain of α-syntrophin leaving the peptide binding groove of the PDZ domain of nNOS available for other protein interactions [7].

Disruption of the DGC as in the dystrophin-deficient *mdx* mice results in the absence of nNOS sarcolemmal localization [3,4] in addition to loss of nNOS signaling function [8]. Moreover, in mice lacking α-dystrobrevin ($dbn^{-/-}$) most of the DGC proteins remain intact at the sarcolemma including α-syntrophin [9]. However nNOS and contraction-stimulated cGMP formation are attenuated in skeletal muscles isolated from $dbn^{-/-}$ mice [9]. Despite the essentially intact nature of the DGC observed in $dbn^{-/-}$ mice, they exhibit skeletal muscle and cardiac myopathy. The precise interactions of nNOS with the DGC at the sarcolemma of skeletal muscle are unclear.

In mouse C2C12 myoblasts, dystrophin and many other components of the DGC are not detectable. However as the C2C12 cells fuse to form myotubes, dystrophin is expressed and is localized to myotube tips, Triton X-100-insoluble cytoskeleton and costameres [10]. Other members of the DGC are also expressed and co-localized with dystrophin in differentiated C2C12 myotubes including aciculin [10], α-dystroglycan [11], β-dystroglycan [12] and the members of the sarcoglycan complex [13]. In this study we use differentiated mouse C2C12 myotubes to investigate the nNOS determinants that account for the co-localization of nNOS at the sarcolemma in skeletal muscle. Results suggest multiple binding partners exist for nNOS at the sarcolemma where independent binding exists for both the PDZ domain and the β-hairpin finger loop of nNOS.

2. Materials and methods

Chemicals and biochemicals were purchased from Sigma. Mouse C2C12 cell line was purchased from ATCC. DMEM, cell culture reagents and molecular biology reagents were purchased from GIBCO-BRL, MD, USA. Mouse anti-GFP monoclonal antibody was purchased from Zymed Labs., CA, USA. Rhodamine-red goat anti-rabbit was purchased from Molecular Probes, USA. GST-Sepharose was purchased from Amersham-Pharmacia, UK.

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Abbreviations: nNOS, neuronal nitric oxide synthase; DGC, dystrophin glycoprotein complex; GFP, green fluorescence protein; DMEM, Dulbecco's modified essential medium; HIT media, DMEM containing 2% heat-inactivated horse serum, 10 μg/ml each of insulin and transferrin; PCR, polymerase chain reaction; EGTA, ethyleneglycoltetraacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PSD-95, postsynaptic density protein-95; PDZ, PSD-95 disc-large and ZO-1

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2.1. Cell culture

COS-7 cells were grown and maintained in Dulbecco's modified essential medium (DMEM) and 10% fetal bovine serum in 35 mM culture dishes. Mouse C2C12 myoblast cells were grown on 150 mm dishes in growth media composed of DMEM and 10% fetal bovine serum. All cells were maintained in a humidified incubator at 37°C and 95% air–5% CO_2. Cells were fed every 2 days.

2.2. Differentiation of C2C12 cells

C2C12 myoblasts were differentiated into myotubes using HIT medium (DMEM containing 2% heat-inactivated horse serum, 10 μg/ml transferrin and 10 μg/ml insulin). The media were changed every 48 h.

2.3. Electroporation of C2C12 myoblasts

C2C12 myoblasts 70–80% confluent were harvested by trypsinization and resuspended in 5 ml of growth media and diluted to a concentration of 2×10^7 cells per ml. For transfection, 10^7 cells (0.5 ml) were mixed with 10 μg of plasmid and 120 μg of sheared salmon sperm DNA in a 0.4 cm electroporation cuvette. Electroporation was performed under the following conditions: voltage, 300 V, resistance, R5 and capacitance, 1150 μF. Following electroporation, cells were immediately transferred to 35 mm culture plates containing 4 ml of growth media plus 1% butyrate. Each 35 mm plate had a glass coverslip. Transfected cells were cultured for 24 h after which the media was aspirated and replaced with 4 ml of growth media (control cells) or 4 ml HIT differentiation media (for differentiation of myoblasts into myotubes). Cells were maintained as before and media were changed every 48 h.

2.4. Construction of nNOS-GFP plasmids

Fragments of rat nNOS cDNA [14] were amplified by polymerase chain reaction (PCR) using Expand-PCR (Roche Biochemicals). Reaction mixtures contained the cDNA for the full length rat nNOS (FL-nNOS) as a template and oligonucleotide primer pairs. The following primer pairs (sense/antisense) were synthesized to produce cDNA fragments that encoded nNOS amino acid residues shown in parentheses: 5′-AGAATTCATATGGAAGAGAACACG-3′/5′-CGCACTAGTAGGGCCCCTCAGAATGAG-3′ (1–100), 5′-AGAATTCATATGGAAGAGAACACG-3′/5′-CGCACTAGTGCTTTTCATCTC-3′ (1–231), 5′-TAGAATTCATATGAGGGCTTCACTACA-3′/5′-CGCACTAGTGCTTTTCATCTC-3′ (101–231), 5′-TAGAATTCATATGGGAATCCAGGTGGACAGA-3′/5′-CGCACTAGTGGAGCTGAAAACCTC-3′ (232–1429), 5′-AGAATTCATATGGAAGAGAACACG-3′/5′-CGCACTAGTGGAGCTGAAAACCTC-3′ (1–1429). After incubation at 94°C for 5 min, reactions proceeded with 30 cycles of denaturation, annealing and extension (94°C, 30 s; 55°C 30 s; and 72°C 30 s). Extension times for fragments 232–1429 and 1–1429 were 4 min. PCR fragments were subcloned into the pGreen Lantern Vector (GIBCO-BRL) using *Eco*R1 and *Spe*1 restriction sites. nNOS-GFP fusion constructs (1–100-nNOS-GFP, 1–231-nNOS-GFP, 101–231-nNOS-GFP, 232–1429-nNOS-GFP and FL-nNOS-GFP) were confirmed by automated nucleotide sequencing.

2.5. Transfection of COS-7 cells

COS-7 cells were transfected with nNOS-GFP plasmids when approximately 70–80% confluent using 3 μg plasmid and 4 μl Fugene-6 (Roche Biochemicals) according to the manufacturer's procedure. Cells were harvested 48 h after transfection by scraping into 250 μl of buffer A: 50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, and protease inhibitors: 100 μg/ml phenylmethanesulfonyl fluoride, 20 μg/ml leupeptin, 30 μg/ml aprotinin,1 mM benzamidine, 20 μg/ml pepstatin and stored at −20°C.

2.6. Preparation of C2C12 cell lysates

C2C12 myoblasts or differentiating myotubes were prepared as described above. On days 0, 2, 4, 6, 8, 10 and 12 of differentiation, cells in 60 mm culture dishes were scraped into 250 μl of buffer A as described above for COS-7 cells. Samples were kept frozen at −70°C until used for nNOS expression analysis. In some experiments, lysates from day 12 differentiated C2C12 myotubes were extracted with buffer A supplemented with either 0.3 M NaCl or 1% Triton X-100. These extracts were then centrifuged at $100\,000\times g$ for 30 min at 4°C. The pellets were resuspended in an equivalent volume of SDS–PAGE sample buffer (250 μl) and both supernatant and resuspended pellet fractions were analyzed for distribution of nNOS in C2C12 myotube extracts. Control samples were extracted with buffer A alone and fractionated by centrifugation as described above.

2.7. Expression and purification of α-syntrophin-GST

Human α-syntrophin subcloned into the *Eco*R1 restriction site of pBluescript KSII (Stratagene, CA, USA) was a gift from Louis Kunkel. A 1.9 kb *Eco*R1/*Xho*1 cDNA fragment encoding the full length human α-syntrophin was subcloned into the glutathione reductase (GST) expression plasmid pGEX-2T (Amersham-Pharmacia, UK) using the same restriction sites. Expression of GST-human α-syntrophin fusion protein in DH5α *Escherichia coli* cells was induced using 1 mM IPTG for 3 h at 37°C. Cells were collected ($10\,000\times g$ for 20 min) and extracted by sonication 3×20 s into buffer A (see above). The cell lysate was centrifuged at $10\,000\times g$ for 15 min to remove membranes and particulate material. The clear supernatant fraction was applied to a 2 ml GST-Sepharose column (Amersham-Pharmacia) pre-equilibrated with buffer A. The column resin was washed using 20 ml of buffer A followed by 20 ml buffer A containing 0.5 M NaCl to remove unbound proteins. Purified GST-α-syntrophin protein was eluted from the column with buffer A containing 10 mM glutathione and stored at −20°C.

2.8. Immunolocalization of nNOS in C2C12 myotubes

Coverslips containing myotubes were washed once with 2 ml of 10% PBS and then fixed with 1 ml 4% paraformaldehyde in PBS for 20 min at room temperature. The coverslips were then washed three times with PBS and the cells permeabilized for 15 min using 0.5% Triton X-100 in PBS, and blocked with 2% goat serum (Sigma, USA) in PBS for 1 h. The coverslips were washed once with 2 ml PBS, then overlaid and incubated overnight at 4°C with 500 μl of primary antibody (anti-rabbit nNOS antibody raised against the N-terminal 231 amino acids of rat nNOS). Coverslips were washed four times with PBS, and incubated with 300 μl of secondary antibody, rhodamine goat anti-rabbit 2 mg/ml (Molecular Probes) at 1:200 in PBS for 1 h in the dark at room temperature. Coverslips were washed with water four times and inverted onto a glass slide containing one drop of FluroGuard Antifade Reagent (Bio-Rad, CA, USA). The coverslip was permanently fixed to the glass slide using nail polish.

2.9. Fluorescence imaging

Fluorescence imaging of transfected C2C12 myotubes with different nNOS-GFP constructs was performed using a fluorescence microscope described previously [15]. Fourteen bit fluorescence images were acquired by a cooled CCD camera (Photometrics CCD200, Tucson, AZ, USA) and BDS-Image software (Oncor, Gaithersburg, MD, USA).

2.10. Western blotting and overlaying of nNOS-GFP proteins with GST-α-syntrophin

Protein samples from C2C12 myoblasts, differentiated C2C12 myotubes or transfected COS-7 cell extracts (20 μl) were prepared in SDS–PAGE sample buffer and loaded onto 10% SDS–polyacrylamide gels and resolved for 2 h at 120 V and transferred onto nitrocellulose membrane for immunoblot analysis for expression of nNOS or nNOS-GFP expression using rabbit anti-nNOS (1:1000 dilution), a gift from Bettie-Sue Masters, or mouse anti-GFP monoclonal primary antibody (1:1000 dilution, Zymed Labs., CA, USA) respectively. Antibody binding was visualized using enhanced chemiluminescence (Amersham-Pharmacia, UK). To detect GST-α-syntrophin binding to nNOS-GFP in COS-7 cell lysates, nitrocellulose filters were prepared as before and incubated overnight with GST-α-syntrophin (200 μg/ml) in 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.05% Tween-20 (TBST) containing 5% milk at 4°C. The membranes were washed (3×5 min) with TBST and incubated with mouse anti-GST monoclonal antibody (Sigma, USA) at 1:1000 dilution for 1 h at room temperature. Membranes were washed (3×5 min) and GST-α-syntrophin binding visualized by enhanced chemiluminescence (Amersham-Pharmacia, UK).

3. Results

3.1. Expression of nNOS in differentiating C2C12 myotubes

Immunoblot analysis of extracts from differentiating C2C12 myoblasts shows that expression of nNOS increases significantly during differentiation. In C2C12 myoblasts little if

any nNOS is detected in cell extracts over a period of 10 days in culture (Fig. 1A). In differentiating C2C12 myotubes however, significant nNOS expression is observed by day 4 which continues to increase to day 12 (Fig. 1B). Biochemical analysis of subcellular localization of nNOS in fully differentiated C2C12 myotubes (day 12) shows an approximately equal distribution of nNOS between the soluble and insoluble fractions (Fig. 1). This distribution is similar to our previous findings of nNOS distribution in mouse skeletal muscle extracts [3]. A biochemical characteristic of sarcolemmal bound skeletal muscle nNOS is its solubility in buffers containing high salt and insolubility in buffers containing Triton X-100 [2,3]. In fully differentiated C2C12 myotubes, the insoluble nNOS was completely extracted with buffer containing 1 M NaCl and not with buffer containing 1% Triton X-100 (Fig. 1C, lanes 2 and 3).

3.2. Localization of nNOS to the sarcolemma of C2C12 myotubes

Immunolocalization of endogenous nNOS in C2C12 myotubes differentiated for 12 days shows staining at the sarcolemma (Fig. 2A). Pre-immune serum shows no significant staining, indicating that the fluorescence observed at the sarcolemma of C2C12 myotubes in Fig. 2A is specifically nNOS

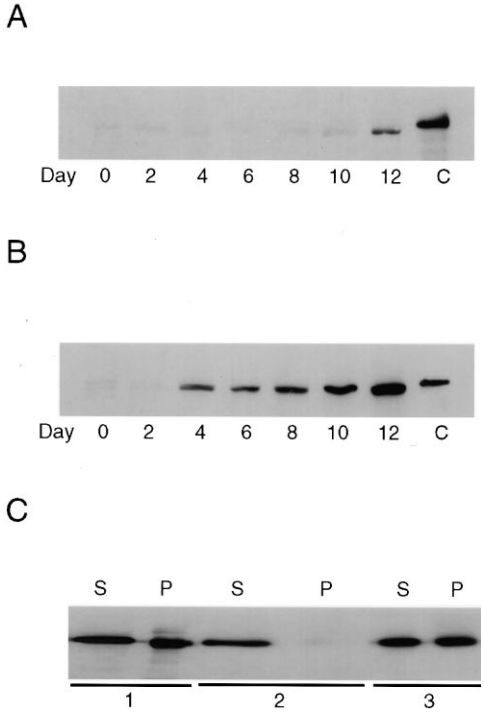

Fig. 1. Expression of endogenous nNOS in differentiating C2C12 myotubes. A: C2C12 myoblasts were grown in DMEM, 20% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. Cells were harvested in buffer A and subjected to SDS–PAGE followed by Western blotting with anti-nNOS Ab with purified nNOS as control (C). B: At day 2, myoblasts maintained in DMEM containing 20% fetal bovine serum were treated with DMEM containing 2% heat-inactivated horse serum, 10 μg/ml insulin, and 10 μg/ml transferrin to induce formation of myotubes. C: C2C12 myotubes were harvested on day 12 and centrifuged at 100 000 × *g* for 30 min to obtain the supernatant (S) and pellet (P) fractions. Lane 1, cells harvested in buffer A; lane 2, in buffer A with 1 M NaCl; and lane 3, in buffer A with 1% Triton X-100.

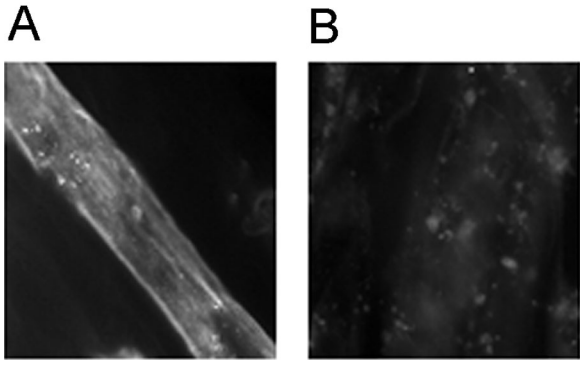

Fig. 2. Immunostaining of C2C12 myoblasts and myotubes with nNOS antibody. Day 12 differentiated C2C12 myotubes (A) and C2C12 myoblasts (B) were stained for endogenous nNOS. Sarcolemmal staining of nNOS is prominent in day 12 differentiated myotubes and not myoblasts.

(Fig. 2B). Localization of endogenous nNOS at the sarcolemma is observed by day 5 of differentiation (not shown). After 12 days of differentiation, the C2C12 myotubes appeared multi-nucleated and twitched spontaneously in culture.

3.3. Full length nNOS-GFP localizes to the sarcolemma of differentiated C2C12 myotubes

In order to determine which domain(s) of nNOS were required for localization to the sarcolemma, GFP-tagged nNOS constructs were prepared and transfected into C2C12 myoblasts. Expression and visualization of GFP-tagged nNOS were monitored as differentiation proceeded. In non-differentiating C2C12 myoblasts, expressed full length nNOS (FL-

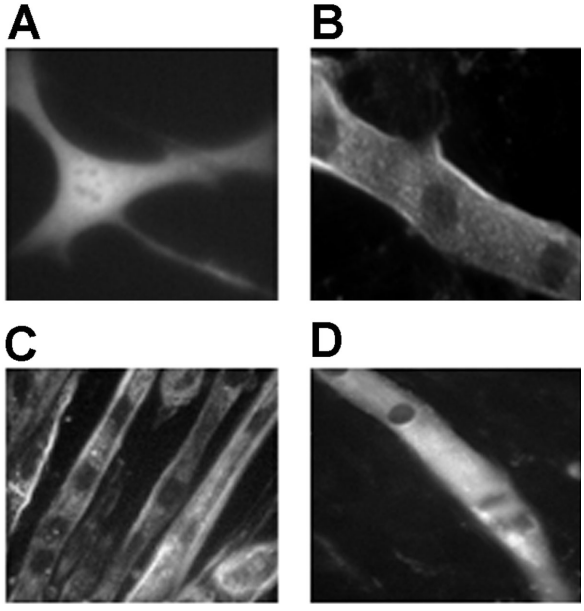

Fig. 3. Fluorescence imaging of C2C12 myotubes and myoblasts transfected with FL-nNOS-GFP. C2C12 myoblasts were transfected with FL-nNOS-GFP (A, B and C) or pGreen Lantern Vector (D) and differentiated into myotubes (B, C and D). Fluorescence images were acquired on day 12 in undifferentiated myoblasts (A), day 6 (B) or day 12 (C and D) of differentiation. FL-nNOS-GFP is at the sarcolemma in day 6 and day 12 differentiated myotubes. GFP alone does not localize to the sarcolemma in day 12 differentiated myotubes (D).

nNOS-GFP) was observed as diffuse fluorescence throughout the cytoplasm of the C2C12 myoblasts (Fig. 3A). However by 6 days fused myoblasts appeared multi-nucleated and sarcolemmal localization of FL-nNOS-GFP was apparent (Fig. 3B). After 12 days of differentiation FL-nNOS-GFP was bound predominantly to the sarcolemma (Fig. 3C). As a control, expression of GFP protein by itself in day 12 differentiated C2C12 myotubes resulted in diffuse fluorescence throughout the myotube with no localization to the sarcolemma (Fig. 3D).

3.4. The N-terminus of nNOS localizes to the sarcolemma in C2C12 myotubes

The fluorescence imaging results obtained in Figs. 2 and 3 showed that endogenous nNOS and FL-nNOS-GFP both localized to the sarcolemma in differentiated C2C12 myotubes consistent with localization of nNOS to the DGC in skeletal muscle tissues. To determine whether or not the N-terminus of nNOS was responsible for the localization, plasmid constructs were made which expressed fragments of the unique N-terminus of nNOS. The GFP-tagged nNOS fragments encoded the whole N-terminal region of nNOS, 1–231-nNOS-GFP; the PDZ domain of nNOS, 1–100-nNOS-GFP; and the region containing the β-hairpin finger loop, 101–231-nNOS-GFP. A C-terminal GFP fusion construct was also prepared that encoded the remaining regions of nNOS, 232–1429-nNOS-GFP. These constructs were transfected into C2C12 myoblasts which were allowed to differentiate for 12 days. When C2C12 myotubes were fully differentiated by day 12, cells transfected with 1–231-nNOS-GFP (the unique N-terminal 231 amino acids) localized to the sarcolemma, (Fig. 4A). In C2C12 myotubes transfected with 232–1429-nNOS-GFP, fluorescence was diffuse and spread throughout the myotube indicating that there are no sarcolemmal localization determinants in the C-terminal of nNOS (amino acids 232–1429; Fig. 4D).

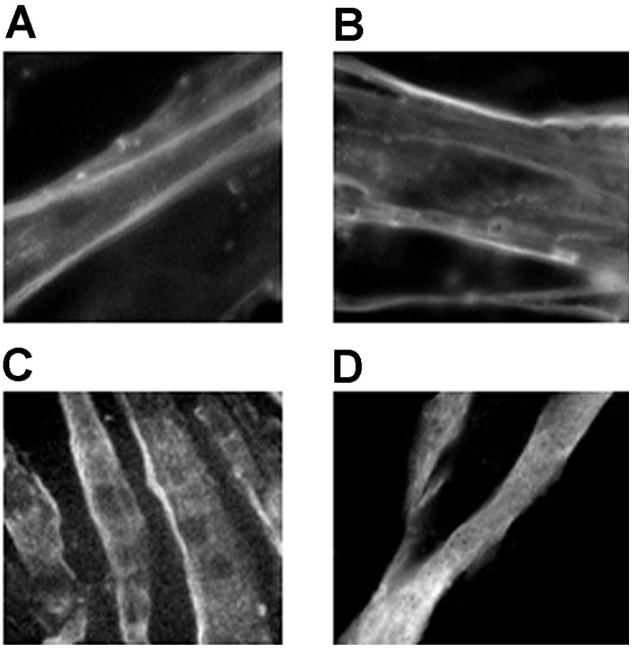

Fig. 4. Fluorescence imaging of C2C12 myotubes transfected with nNOS-GFP fusion constructs. C2C12 myotubes differentiated for 12 days after transfecting myoblasts with 1–231-nNOS-GFP (PDZ domain plus β-hairpin finger loop of nNOS) (A), 1–100-nNOS-GFP (PDZ domain of nNOS) (B), 101–231-nNOS-GFP (β-hairpin finger loop of nNOS) (C), or 232–1429-nNOS-GFP (C-terminal portion of nNOS) (D). Sarcolemmal localization is observed with nNOS-GFP fragments containing the N-terminus (A, B and C) but not with the C-terminal portion of nNOS.

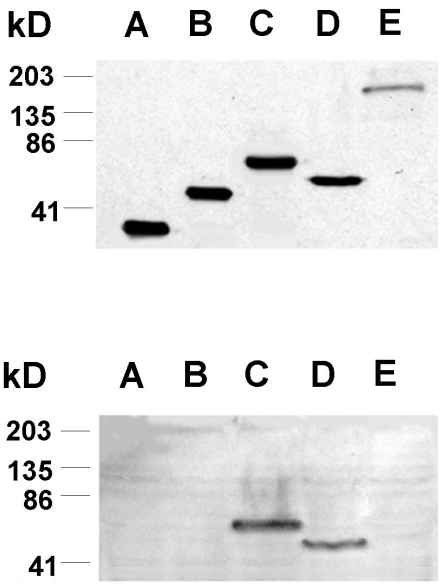

Fig. 5. α-Syntrophin overlay of nNOS-GFP proteins. COS-7 cells were transfected with plasmid constructs of pGreen Lantern Vector (A), 1–100-nNOS-GFP (B), 1–231-nNOS-GFP (C), 101–231-nNOS-GFP (D), and 232–1429-nNOS-GFP (E). Cells were harvested after 48 h, and extracts electrophoresed on 10% SDS–PAGE followed by transfer to nitrocellulose membrane. Nitrocellulose membranes were either immunoblotted with anti-nNOS (upper panel) or overlaid with GST-α-syntrophin and probed with anti-GST (lower panel). GST-α-syntrophin binds only to 1–231-nNOS-GFP (C) and 101–231-nNOS-GFP (D) and not to the 1–100-nNOS-GFP (B) nor the C-terminal nNOS 232–1429-nNOS-GFP.

To determine the structural motifs required for sarcolemmal binding, the PDZ domain and the β-hairpin finger loop were expressed independently as GFP fusion proteins. Cells transfected with the 1–100-nNOS-GFP plasmid encoding the PDZ domain of nNOS localized to the sarcolemma indicating a direct nNOS PDZ domain interaction with the sarcolemma of C2C12 myotubes (Fig. 4B). Cells transfected with the 101–231-nNOS-GFP plasmid which expressed the β-hairpin finger loop also showed fluorescence at the sarcolemma (Fig. 4C). Thus both the PDZ domain (amino acids 1–100) and the region containing the β-hairpin finger loop (amino acids 101–231) are independently sufficient for subcellular localization of nNOS to the sarcolemma in C2C12 myotubes.

3.5. Binding of α-syntrophin to nNOS-GFP fusion proteins

Western immunoblot analysis of extracts of COS-7 cells transfected with nNOS-GFP constructs demonstrated that each construct expressed the expected size of nNOS-GFP fusion protein (Fig. 5A). Identical samples were transferred to a nitrocellulose membrane and subjected to GST-α-syntrophin overlay analysis. Fig. 5B shows that GST-α-syntrophin binds to the 1–231-nNOS-GFP and the 101–231-nNOS-GFP fusion proteins. However, GST-α-syntrophin did not bind to 1–100-nNOS-GFP which contains only the PDZ domain of nNOS. It also did not bind the 232–1429-nNOS-GFP fusion protein.

In control overlay experiments, purified GST did not bind to any of the nNOS-GFP expressed proteins in COS-7 cell lysates (data not shown).

4. Discussion

In the present study, we show that nNOS expression is induced upon C2C12 myotube formation. The nNOS expressed in fully differentiated C2C12 myotubes exhibited similar properties to the nNOS isolated in skeletal muscles in terms of cytosolic and insoluble sarcolemmal distribution [1–3,5]. The major components of the DGC have been shown to be present at the sarcolemma in fully differentiated C2C12 myotubes [10,11,13,14]. FL-nNOS-GFP was also localized to the sarcolemma in fully differentiated C2C12 myotubes and not in C2C12 myoblasts. However, in a recent report where dystrophin and β-dystroglycan were found at the sarcolemma of differentiated C2C12 myotubes, nNOS was not, contrary to the findings of this study [12]. The shorter differentiation period of the C2C12 myoblasts (5 days) in that study may have accounted for the lack of nNOS localization at the sarcolemma. The identical sarcolemmal localization of both endogenous and FL-nNOS-GFP allowed us to address the question as to which region(s) of nNOS was responsible for this localization.

The association of nNOS with the DGC at the sarcolemma is assumed to be mediated by interaction of the unique N-terminal region of nNOS (residues 1–231) with the PDZ domain of α-syntrophin [6,7]. PDZ-mediated interactions of most proteins occur through recognition of short consensus COOH-terminal peptide motifs binding to the peptide binding groove of the PDZ domain [16]. Other types of PDZ-mediated interactions exist which include interactions between PDZ domains of two or more different proteins. These interactions do not involve the recognition of a COOH-terminal sequence by a PDZ domain but instead require the full tertiary structure of the interacting PDZ domains [6]. The PDZ–PDZ interaction between nNOS and α-syntrophin is unique in that it involves the binding of the β-hairpin finger loop found downstream of the nNOS PDZ domain located between residues 101–130 of nNOS to the peptide binding groove in the PDZ domain of α-syntrophin [9]. The result of this interaction is the formation of a heterodimer between nNOS and α-syntrophin. The PDZ domain of nNOS also interacts with PSD-95 in neuronal cells forming a heterodimer [6]. In a recent report, the β-hairpin finger loop without the nNOS PDZ domain was also shown to bind to the PDZ domain PSD-95 [17]. Moreover, the β-hairpin finger loop forms a stable structure by itself. The binding of GST-α-syntrophin to residues 101–231 of nNOS and not residues 1–100 of the PDZ domain of nNOS demonstrates the specific nature of the nNOS/α-syntrophin interaction. These interactions of the β-hairpin finger loop with α-syntrophin PDZ domain leave the peptide binding groove of the nNOS PDZ domain available to interact with additional proteins. The absence of GST-α-syntrophin binding to the PDZ domain of nNOS is similar to the observation that nNOS PDZ domain (residues 1–100) also does not bind to the PDZ domain of the postsynaptic density protein-93 [18].

The biochemical studies of the N-terminal region of nNOS binding to the PDZ domain of α-syntrophin suggest α-syntrophin is required for nNOS to be localized to the sarcolemma [6,7]. This view is supported by the absence of nNOS localization at the sarcolemma in skeletal muscles isolated from mice with ablation of α-syntrophin [19]. In mice lacking α-syntrophin, dystrophin and other components of the DGC are present at the sarcolemma but nNOS is not [19]. In two other reports however, sarcolemmal localization of nNOS was absent despite α-syntrophin at the sarcolemma [9,20]. In one case α-syntrophin was found at the sarcolemma of skeletal muscle from transgenic *mdx* mice expressing only the C-terminal 71 kDa of dystrophin or expressing dystrophin minus the 'rod-like' spectrin motif [20]. However nNOS was not detected at the sarcolemma in skeletal muscles from these mice. In skeletal muscles from dystrobrevin-deficient mice, α-syntrophin was also found at the sarcolemma but nNOS was not [9]. α-Syntrophin therefore may be required but is not sufficient for nNOS localization to the sarcolemma. We show that 1–100-nNOS-GFP also binds to the sarcolemma in differentiated myotubes but does not bind to α-syntrophin. Thus the PDZ domain of nNOS is probably binding to another protein localized at the sarcolemma.

In this study, we show that the nNOS PDZ domain and the β-hairpin finger loop bind to the sarcolemma. In addition, the β-hairpin finger loop binds to α-syntrophin. Therefore it is predicted that either the PDZ domain or the β-hairpin finger loop would confer high-affinity binding of nNOS to the sarcolemma in skeletal muscle. However, nNOS is not localized to the sarcolemma in skeletal muscles from mice lacking α-syntrophin. Conversely, in muscles from mice lacking dystrobrevin, α-syntrophin is present at the sarcolemma but nNOS is not. The difference with our observations in C2C12 myotubes and the results from genetically manipulated mouse models indicate that the situation in the C2C12 myotube system may not be identical to that in skeletal muscles in vivo. Perhaps two different proteins binding to the PDZ and the β-hairpin finger loop respectively are required for nNOS localization to the sarcolemma in skeletal muscle tissues.

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