Selective Loss of Sarcolemmal Nitric Oxide Synthase in Becker Muscular Dystrophy

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Summary

Becker muscular dystrophy is an X-linked disease due to mutations of the dystrophin gene. We now show that neuronal-type nitric oxide synthase (nNOS), an identified enzyme in the dystrophin complex, is uniquely absent from skeletal muscle plasma membrane in many human Becker patients and in mouse models of dystrophinopathy. An NH_2-terminal domain of nNOS directly interacts with α1-syntrophin but not with other proteins in the dystrophin complex analyzed. However, nNOS does not associate with α1-syntrophin on the sarcolemma in transgenic *mdx* mice expressing truncated dystrophin proteins. This suggests a ternary interaction of nNOS, α1-syntrophin, and the central domain of dystrophin in vivo, a conclusion supported by developmental studies in muscle. These data indicate that proper assembly of the dystrophin complex is dependent upon the structure of the central rodlike domain and have implications for the design of dystrophin-containing vectors for gene therapy.

Mutations of the X-linked dystrophin gene are the most common cause of inherited muscular dystrophy and affect ~1:3,500 male births (1). Duchenne muscular dystrophy (DMD)[1], the more common and more severe form of the disease, is associated with mutations that lead to an absence of dystrophin protein in muscle (2, 3). A clinically milder disease, Becker muscular dystrophy (BMD), accounts for ~20% of cases and often involves deletions within the rodlike central domain of dystrophin (4). Muscle dystrophin levels are often nearly normal in BMD, which can preclude diagnosis by immunohistochemical analysis of dystrophin (5).

Dystrophin is a large intracellular protein containing several defined sequence motifs (6). An NH_2-terminal α-actinin–like domain binds to F-actin (7), and is followed by a large rod domain that shares sequence homology with the structural repeats in spectrin. The COOH terminus is unique to dystrophin and related proteins, and this region directly binds to a glycoprotein complex in skeletal muscle (8–10). The structural dystrophin-associated complex includes intracellular proteins, syntrophins (11), as well as integral membranes proteins, the dystroglycans (12) and sarcoglycans; the absence of dystrophin in DMD causes a disruption of this complex (13). These interactions suggest a structural role for dystrophin, physically linking the extracellular matrix to the muscle cytoskeleton (14). In support of this model, genetic mutations in components of the sarcoglycan complex can cause autosomal recessive muscular dystrophy (15–18).

Restoration of a functional dystrophin molecule to muscle represents a primary goal for therapy. To better understand mechanisms for assembly of the dystrophin complex and to identify potential constructs for gene therapy, fragments of dystrophin have been targeted to skeletal muscle of transgenic *mdx* mice, which lack endogenous dystrophin. Replacement with either a full-length dystrophin, a COOH-terminal construct encoding 71 kD of dystrophin, Dp71, or a dystrophin minigene, lacking a large portion of the central spectrinlike repeats, restores the structural dystrophin complex to muscle. Replacement with full-length dystrophin corrects muscular dystrophy in *mdx* mice (19). Despite apparent restoration of the dystrophin complex, *mdx* mice targeted with Dp71, still display severe muscular

[1] *Abbreviations used in this paper:* α-BGT, alpha bungaro-toxin; AChR, acetylcholine receptor; BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; GST, glutathione-S-transferase; nNOS, neural-type nitric oxide synthase; NO, nitric oxide; PH, pleckstrim homology.

dystrophy (8, 9), whereas those containing the minigene, have a very mild disease phenotype (20, 21). These results indicate that all components of the dystrophin membrane cytoskeleton are needed to completely prevent symptoms of muscular dystrophy.

In addition to their cytoskeletal role, dystrophin and associated proteins have been implicated in specific signaling functions of the junctional and extrajunctional sarcolemma. The dystrophin-related protein, utrophin, is concentrated at neuromuscular endplates and is implicated in acetylcholine receptor (AChR) clustering. α-dystroglycan binds with high affinity to agrin and laminin suggesting that the dystrophin-associated complex may serve as a link between the extracellular matrix and intracellular events that help form AChR clusters (22, 23). Signaling by the dystrophin complex may be mediated in part by nitric oxide (NO), a messenger molecule in muscle that can regulate myocyte development (24), AChR function (25), and muscle contractility (26). NO is formed in skeletal muscle by the neuronal-type nitric oxide synthase (nNOS) that is enriched at the sarcolemma of fast twitch muscle fibers in rodents (26) and in both fast and slow twitch fibers in primates (27). Recent studies identify nNOS as a nonstructural component of the dystrophin complex (28). Furthermore, nNOS is absent from skeletal muscle sarcolemma of *mdx* mice and in DMD (28). Biochemical studies in vitro demonstrate that the NH_2 terminus of nNOS, which contains a PDZ protein motif, directly binds to a similar motif in α1-syntrophin (29); furthermore, nNOS and α1-syntrophin coimmunoprecipitate from muscle extracts. Direct binding of nNOS to dystrophin or other associated proteins has not yet been demonstrated.

DMD and *mdx* mice show primary dystrophin deficiency and secondary deficiencies of sarcoglycans, dystroglycans, syntrophins, and nNOS. BMD in humans, due to abnormal dystrophin, generally retains dystrophin-associated proteins, though nNOS has not been evaluated. We now show that nNOS is properly restored to the plasma membrane in transgenic *mdx* mice expressing full-length human dystrophin but is selectively absent from skeletal muscle membranes in *mdx* mice expressing either Dp 71 (DMD phenotype) or a dystrophin minigene lacking many of the spectrinlike repeats (very mild BMD phenotype). Dyslocalization of nNOS in the transgenic mouse models is associated with disruption of the normal nNOS/α1-syntrophin interaction. In human biopsies, we note that loss of sarcolemmal nNOS is commonly observed in BMD. In some patients, lacking as little as three exons in the spectrinlike domain of dystrophin, the absence of sarcolemmal nNOS represents the only identified immunohistochemical abnormality.

Materials and Methods

Antibodies. The following primary antibodies were used: nNOS polyclonal raised against homogenous nNOS protein purified from rat cerebellum (30), nNOS monoclonal (Transduction Labs, Lexington, KY), α1-syntrophin polyclonal, syntrophin monoclonal (31), dystrophin monoclonal (Sigma Chemical Co., St. Louis, MO), β-dystroglycan monoclonal, utrophin monoclonal, and α-sarcoglycan monoclonal (Novacastra Laboratories Ltd., Newcastle upon Tyne, UK).

Immunofluorescence. Unfixed skeletal muscle samples were flash frozen in liquid nitrogen–cooled isopentane, sectioned on a cryostat (10 μm), and melted directly onto glass slides. Sections were then postfixed in 2% paraformaldehyde in PBS or cold acetone. Tissues were "blocked" in PBS containing 1% normal goat serum. Primary antibodies were diluted in blocking reagent and were applied to sections overnight at 4°C. For indirect immunofluorescence, secondary goat anti–rabbit FITC–, or donkey anti–mouse Cy-3–conjugated antibodies were used according to the manufacturer's specifications (The Jackson Laboratory, Bar Harbor, ME; 1:200). Cy-3–conjugated α-BGT (kindly provided by Peter Sargent, University of California, San Francisco) was diluted together with the secondary antibody for double labeling motor endplates.

Tissue Extraction and Western Blot Analysis. Mouse quadriceps skeletal muscle was homogenized in 10 volumes (wt/vol) of buffer A (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF), and heavy microsomes were prepared by a standard protocol with minor modifications. Nuclei were pelleted by centrifugation at 1,000 *g*. The supernatant was then centrifuged at 20,000 *g*, yielding supernatant S_1. The resulting heavy microsomal pellet was resuspended in buffer A containing 500 mM NaCl, incubated for 30 min at 4°C with agitation, and centrifuged at 15,000 *g*, yielding supernatant S_2. This resulting pellet was resuspended in buffer A containing 500 mM NaCl plus 0.5% Triton X–100, incubated for 30 min at 4°C with agitation, and centrifuged at 15,000 *g*, yielding supernatant S_3 and a final pellet P.

Tissue extracts were resolved by SDS-PAGE (7.5% acrylamide) and proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). Membranes were incubated overnight with primary antisera diluted in Tris-HCl buffered saline containing 1% BSA. Immunoreactive bands were visualized by the enhanced chemiluminescence system according to the manufacturer's specifications (Amersham Corp., Arlington Heights, IL).

Immunoprecipitation. Polyclonal antibodies (1 μg) to α1-syntrophin or nonimmune serum were added to 0.5-ml aliquots of solubilized skeletal muscle membranes from wild-type mouse (1 mg/ml) or total solubilized muscle extract from *mdx* mouse (2 mg/ml), and samples were incubated on ice for 1 h. Protein A–Sepharose (50 μl) was used to precipitate antibodies. Protein A pellets were washed three times with buffer containing 100 mM NaCl and 1% Triton X–100. Immunoprecipitated proteins were denatured with loading buffer and resolved by SDS-PAGE.

Fusion Protein Affinity Chromatography. A fusion protein of glutathione-S-transferase (GST) fused to the first 299 amino acids of nNOS was expressed in *Escherichia coli* and purified on glutathione Sepharose beads as described (28). Solubilized skeletal muscle membranes were incubated with control (GST) or GST-nNOS (1-299) beads. Samples were loaded into disposable columns washed with 50 volumes of buffer containing 0.5% Triton X–100 plus 300 mM NaCl, and proteins were eluted with 150 μl of SDS-PAGE loading buffer.

Characterization of Human Tissues. All human muscle biopsies were obtained for diagnostic purposes (dystrophin analysis), and were flash frozen in isopentane cooled in liquid nitrogen. Patients were evaluated for dystrophin expression by immunofluorescence and Western blotting as described (32, 33). Mutation detection in

BMD patients was done by multiplex PCR, as previously described. Mutation detection in α-sarcoglycan (adhalin) was done by RT-PCR and single strand conformation polymorphism, with aberrant conformers sequenced as previously described (34); one patient was homozygous for an Arg77Cys mutation whereas another was a compound heterozygote, Leu31Pro and Arg284Cys. All biopsies used in this study were deemed to be of excellent preservation based on hematoxylin and eosin staining of cryosections.

Results

Previous studies suggest that association of nNOS with the dystrophin complex is mediated by direct binding of the NH_2-terminus of nNOS to the PDZ domain of α1-syntrophin (29). However, during skeletal muscle development, we found a dissociation between nNOS and α1-syntrophin localization in muscle. Throughout postnatal rat development, α1-syntrophin was present at extrajunctional sarcolemma and was particularly enriched at neuromuscular endplates (Fig. 1). By contrast, at postnatal day 3 (P3) and P7, nNOS was observed only at extrajunctional sarcolemma. Enrichment of nNOS at neuromuscular endplates did not become apparent until P12, coincident with accumulation of dystrophin at endplates. Utrophin was enriched at endplates in all stages evaluated.

We compared nNOS and α1-syntrophin expression in

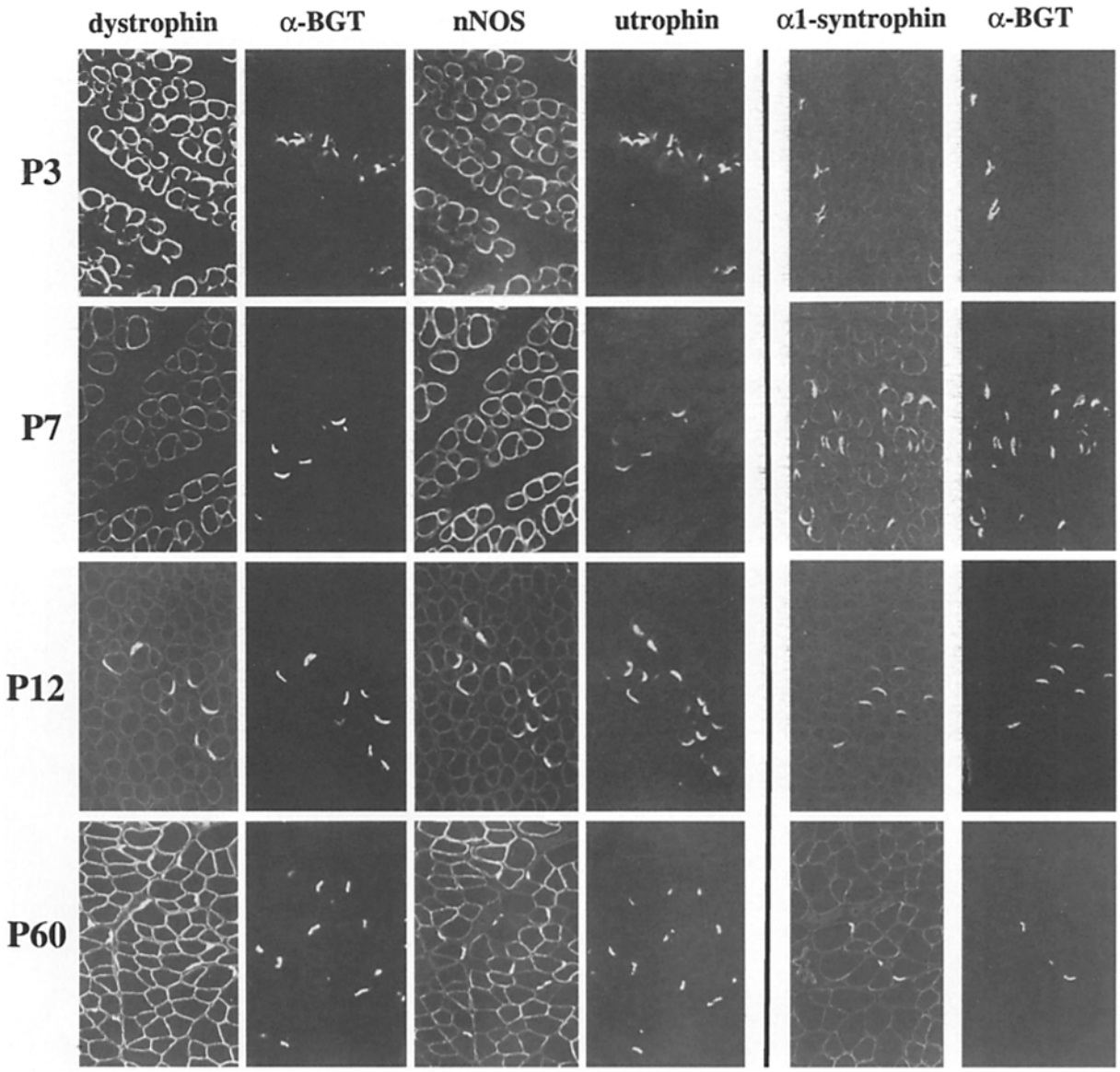

Figure 1. Localization of nNOS and other dystrophin-associated proteins during postnatal development. Adjacent sections of postnatal rat quadriceps muscle were stained for dystrophin, nNOS, α-BGT, and utrophin, and nearby sections were stained for α1-syntrophin and α-BGT. Dystrophin and nNOS stained extrajunctional sarcolemma at P3 and P7 and both became concentrated at neuromuscular endplates at P12 and P60. α1-syntrophin was present at extrajunctional sarcolemma and was enriched at neuromuscular endplates at all ages evaluated. Utrophin staining was restricted to neuromuscular endplates.

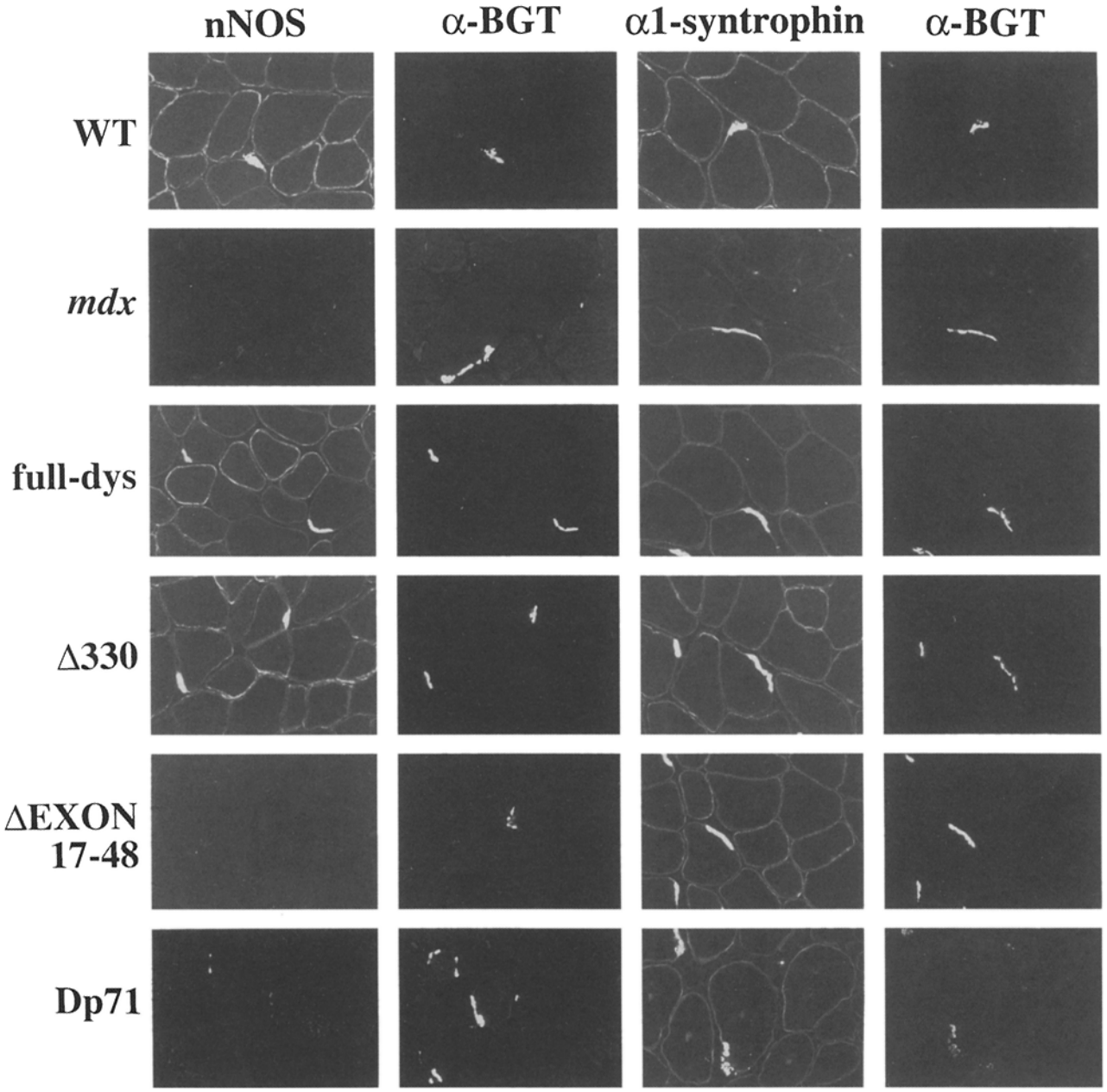

Figure 2. Localization of nNOS and α1-syntrophin in transgenic *mdx* mice. Cryosections from mouse quadriceps were double labeled for either nNOS or α1-syntrophin and α-BGT. Immunofluorescent staining showed that nNOS in wild-type mouse was expressed at extrajunctional sarcolemma of a subset of fibers and was enriched at all neuromuscular endplates. nNOS was absent from junctional and extrajunctional sarcolemma in *mdx* mice. nNOS staining in *mdx* transgenic mice expressing full-length dystrophin (*full-dys*) or truncated dystrophin lacking the COOH-terminal 330 nucleotides (Δ330) resembled that of wild-type mice. *mdx* mice expressing dystrophin-lacking exons 17–48 (*ΔEXON 17-48* mini-dys) or the COOH-terminal 71 kD of dystrophin Dp71 lacked nNOS staining at sarcolemma, similar to nontransgenic *mdx* mouse. α1-syntrophin occurred at extrajunctional sarcolemma and was concentrated at neuromuscular endplates in wild-type mice and was restricted to the endplates in *mdx* mouse. α1-syntrophin expression was restored to sarcolemma in the four transgenic *mdx* mouse lines expressing different portions of the dystrophin gene.

skeletal muscle of wild-type, *mdx*, and various transgenic *mdx* mice (Fig. 2) that express mutant forms of dystrophin (8, 9, 19–21, 35). As previously reported, α1-syntrophin was absent from extrajunctional sarcolemma of *mdx* mouse, but remained at neuromuscular endplates (31, 36). nNOS was absent from both junctional and extrajunctional sarcolemma of *mdx* mouse. Four lines of *mdx* transgenic mice were evaluated. These previously described lines express either full-length dystrophin (line 862CAA [20]), or various truncated dystrophins, lacking either the 330 nucleotides of exons 71–74 near the COOH-terminus (Δ330 [35]), lacking exons 17–48 of the spectrinlike motif (ΔE17-48; CVBA line 12142 [20]), or lacking all but the COOH-terminal 71 kD (Dp71; MCA-1 [8, 9]). As previously re-

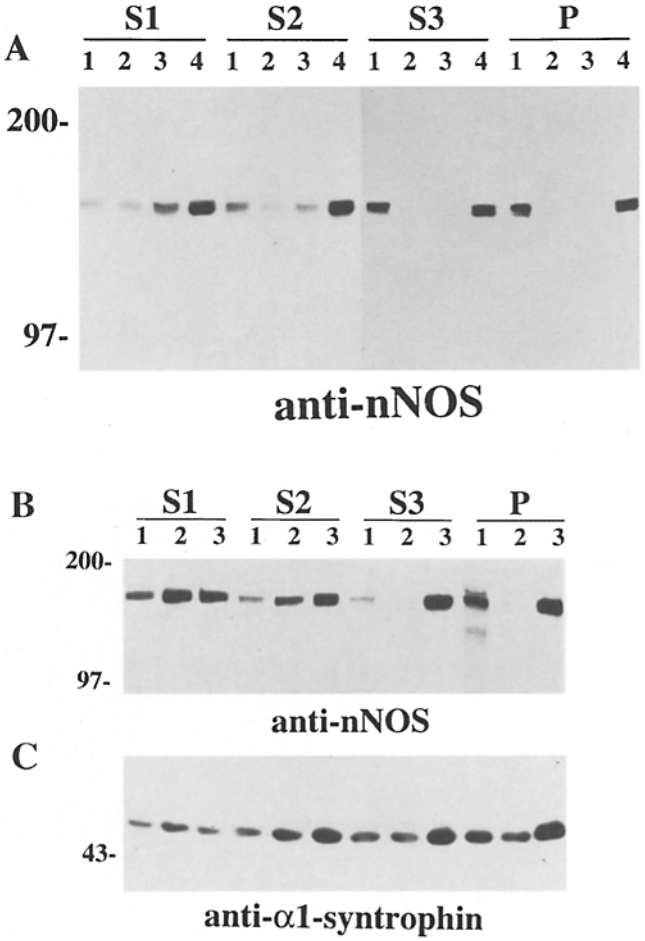

Figure 3. Subcellular distribution of nNOS in transgenic *mdx* mouse. Mouse quadriceps skeletal muscle homogenates were sequentially extracted with buffers containing 100 mM NaCl (*S1*), 500 mM NaCl (*S2*), and 0.5% Triton X–100 (*S3*), leaving an insoluble cytoskeletal pellet (*P*). (*A*) Western blotting indicated that nNOS was enriched in membrane-associated and pellet fractions in wild-type mouse (lanes *1*) and transgenic *mdx* mice expressing full-length dystrophin (lanes *4*). In *mdx* mouse (lanes *2*) and Dp71 transgenic *mdx* mouse (lanes *3*), nNOS was fully extracted by 500 mM NaCl and was absent from membrane-associated and cytoskeletal fractions. (*B* and *C*) A similar fractionation was performed on muscle homogenates from wild-type mouse (lanes *1*), *mdx* mouse expressing dystrophin-lacking exons 17–48 (lanes *2*), or *mdx* mouse expressing full-length dystrophin (lanes *3*). (*B*) nNOS was absent from membrane-associated (*S3*) and cytoskeletal pellet (*P*) in *mdx* mouse expressing the truncated dystrophin. (*C*) Reprobing the blot shows that α1-syntrophin had a generally similar fractionation in muscle from all three mice lines.

ported, α1-syntrophin expression was restored to junctional and extrajunctional sarcolemma in each of the four transgenics. By contrast, nNOS was restored to the sarcolemma only by full-length dystrophin and the Δ330 mutant.

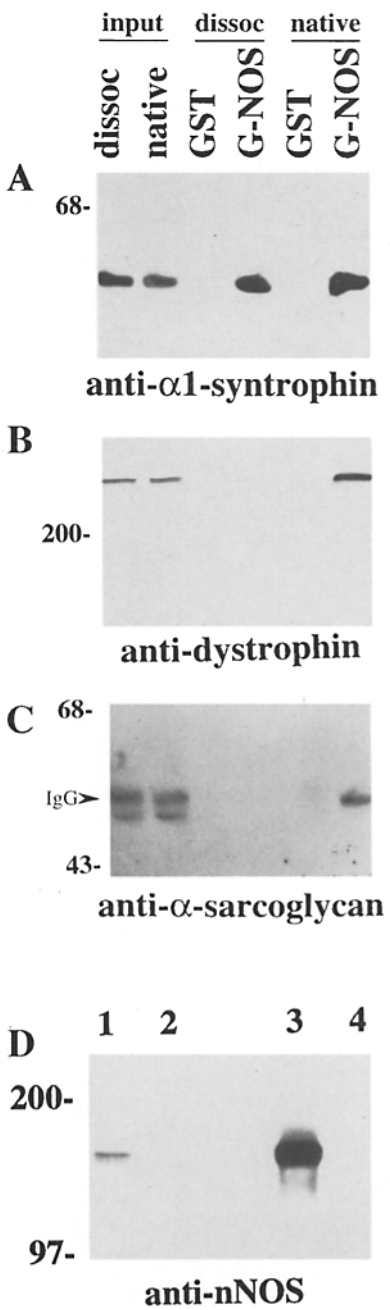

Figure 4. Selective interaction of nNOS and α1-syntrophin. Crude solubilized membranes from mouse quadriceps were titrated with NaOH to pH 11, to dissociate the dystrophin complex, and were neutralized to pH 7.4 with 1 M Tris-HCl. Native and dissociated (*dissoc*) preparations were incubated with agarose beads linked to either GST or GST fused to the first 299 amino acids of nNOS (*G-NOS*). After extensive washing, beads were eluted with loading buffer and proteins were resolved by SDS/PAGE. (*A*) Western blotting showed that α1-syntrophin was selectively retained by G-NOS beads in both native and dissociated preparations. Reprobing the same blot with (*B*) dystrophin or (*C*) α-sarcoglycan revealed that G-NOS beads retained these proteins from native protein preparations. However, after dissociation of the complex, neither dystrophin nor α-sarcoglycan bound to G-NOS. The 55-kD band observed in input lanes from α-sarcoglycan blot appears to be mouse IgG and was reactive with the secondary antibody used for Western blotting. (*D*) Coimmunoprecipitation of nNOS with α1-syntrophin from wild-type and *mdx* mouse skeletal muscle. Total solubilized extract from *mdx* (lanes *1* and *2*) or solubilized membranes from wild-type (lanes *3* and *4*) mouse quadriceps were immunoprecipitated with an antibody to α1-syntrophin (lanes *1* and *3*) or nonimmune serum (lanes *2* and *4*). Western blotting indicates that nNOS was specifically coimmunoprecipitated with α1-syntrophin from *mdx* and wild-type extracts.

Table 1. *Sarcolemmal Expression of nNOS, Dystrophin, and Syntrophin in BMD*

Diagnosis	Exons deleted (mutation)	Age at biopsy	Imunofluorescence at sarcolemma		
			nNOS	Dystrophin	Syntrophin
Normal		53	++++	++++	++++
Normal		41	++++	++++	++++
Mild BMD	45–47	30	0	++++	++++
Mild BMD	52	29	+++	++++	++++
Mild BMD	45–48	8	+	++++	++++
Int. BMD	3–6	13	+	+++	++++
Int. BMD	10–42	24	0	++++	++++
Int. BMD	13–41	12	++	+++	++++
Int. BMD	45	23	0	+	++
Sev. BMD	8	10	0	+	+++
Sev. BMD	3–7	7	0	+	+++
Sev. BMD	45–47	10	0	+++	++++
Sev. BMD	51–52	9	0	+	+++
α-sarcoglycanopathy	(L31P/R284C)	11	++++	++++	++++
α-sarcoglycanopathy	(R77C/R77C)	8	+++	+++	++++

Human muscle biopsies were labeled by immunofluorescence. Sarcolemmal labeling was blindly evaluated by three observers from 0 to ++++. Variation between observers never varied by more than one +, and for those cases, the majority score is reported. *Int.*, intermediate; *Sev.*, severe.

Biochemical studies confirmed that nNOS did not associate with sarcolemma in *mdx* mice or transgenic *mdx* mice expressing either Dp71 or ΔE17-48. In wild-type and *mdx* transgenic mice expressing full-length dystrophin, nNOS was enriched in membrane-associated and cytoskeletal fractions, whereas in *mdx*, Dp71, and ΔE17-48 lines, nNOS was present only in soluble fractions of muscle. As previously reported, α1-syntrophin occurred in sarcolemmal fractions of all four lines of transgenic *mdx* mice evaluated (Fig. 3, and data not shown).

Whereas these studies are consistent with the model that association of nNOS with α1-syntrophin in vivo requires a full-length dystrophin, direct binding of nNOS to the rodlike domain of dystrophin or another dystrophin-associated protein could also explain the data. Previous studies demonstrate that the NH_2-terminal domain of nNOS is necessary and sufficient for interaction with the dystrophin complex (28). We therefore evaluated interaction of dystrophin-associated proteins with a purified fusion protein containing the first 299 amino acids of nNOS. As previously demonstrated, a Sepharose column linked to this fusion protein selectively retained several components of the dystrophin complex from crude skeletal muscle extracts. To determine which components directly interact with nNOS in vitro, we dissociated the dystrophin complex by briefly adjusting the pH of muscle extracts to 11 and then repeating the binding assays immediately after neutralizing the extracts. Previous studies (37) have demonstrated that this procedure reversibly dissociates dystrophin from associated proteins. After dissociation of the complex, α1-syntrophin continued to interact with the nNOS column but dystrophin, α-sarcoglycan, and β-dystroglycan were not retained (Fig. 4, *A–C* and data not shown).

To further verify that nNOS directly interacts with syntrophin in vivo, we conducted immunoprecipitation experiments in *mdx* mouse (Fig. 4 *D*). A polyclonal antibody to α1-syntrophin specifically coimmunoprecipitated a small amount of nNOS from extracts of *mdx* skeletal muscle. nNOS was more efficiently coimmunoprecipitated with α1-syntrophin from solubilized membranes of wild-type mouse as previously shown (29).

We next asked whether mutations in the NH_2-terminal or rodlike domains of dystrophin that cause BMD in humans were associated with altered localization of nNOS. We immunohistochemically evaluated nNOS and α1-syntrophin expression in 12 BMD patients with molecularly defined deletions in the dystrophin gene. Immunohistochemical expression of nNOS, dystrophin, and syntrophin were assessed blindly. Loss of sarcolemmal nNOS, but not α1-syntrophin expression was associated with Becker phenotype (Table 1). Some of the patients, with mild to intermediate disease, showed reduced but detectable nNOS

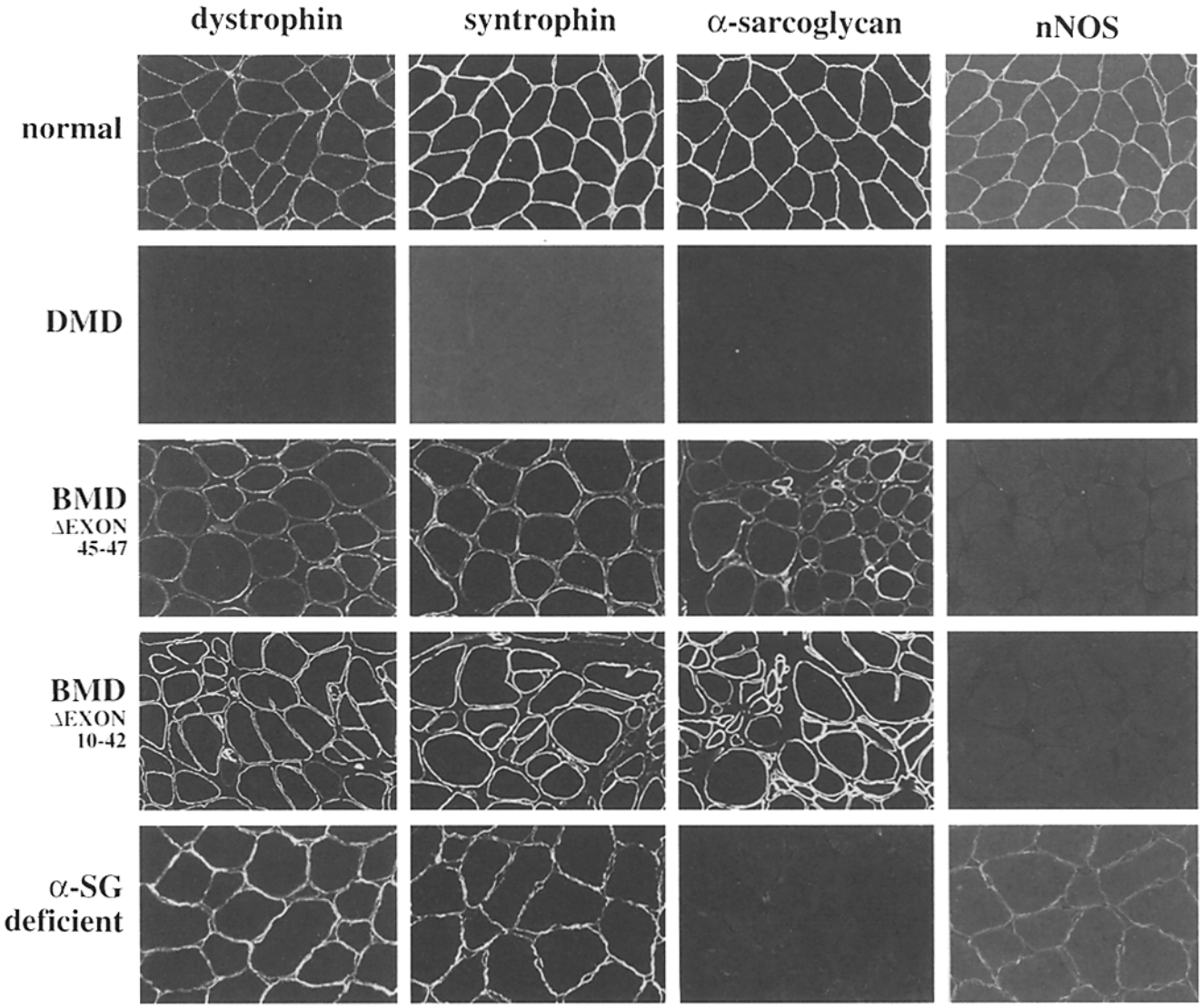

Figure 5. nNOS is absent from skeletal muscle sarcolemma in certain patients with BMD. Skeletal muscle cryosections from human biopsies were immunostained with monoclonal antibodies to dystrophin, syntrophin, α-sarcoglycan, or polyclonal antibody nNOS. All four antibodies showed sarcolemmal staining in normal patients and essentially no sarcolemmal labeling in patients with DMD. In two patients with BMD, due to loss of exons 45–47 or 10–42 of dystrophin, immunofluorescent labeling for dystrophin, syntrophin, and α-sarcoglycan was detected at the membrane. By contrast, nNOS sarcolemmal staining was undetectable in these two BMD patients. nNOS labeling was present in a patient with α-sarcoglycan deficiency.

staining of sarcolemma. In several patients, loss of sarcolemmal nNOS occurred despite apparently normal assembly of other components of the dystrophin-associated glycoprotein complex (Fig. 5). By contrast, we found that nNOS expression was intact in two patients with primary α-sarcoglycan deficiency. This is consistent with the normal status of dystrophin and syntrophins in this disorder.

Discussion

A principal finding of this work is that assembly of nNOS into the dystrophin complex is dependent upon the normal structure of the rodlike domains of dystrophin. Previous analyses of specific protein contacts involved in maintenance of the dystrophin complex have focused on protein interactions at the NH_2- and COOH-terminal domains of dystrophin. These studies identify a functional F-actin binding site near the NH_2-terminus (7) and binding sites for β-dystroglycan and syntrophins in the COOH-terminal domain of dystrophin (10). Understanding the mechanism for nNOS association with the dystrophin complex is important because nNOS is uniquely absent from sarcolemma in certain animal models of muscular dystrophy and in certain patients with BMD. Absence of sarcolemmal nNOS in *mdx* mouse expressing a dystrophin minigene indicates a role for the rodlike domain of dystrophin for binding of nNOS. Studies of nNOS expression in BMD patients demonstrate that distinct deletions in the NH_2-terminal or central domain of dystrophin disrupt recruitment of nNOS to the sarcolemma. These results indicate that a

unique nNOS interaction domain may not be present in dystrophin, but that proper conformation is required for assembly of nNOS into the dystrophin complex.

Previous studies suggest that direct interaction of nNOS with α1-syntrophin accounts for association of nNOS with the dystrophin complex (29). Three syntrophin genes have been identified and each contains two pleckstrin homology (PH) domains. The first PH domain is split by a PDZ motif, and the second PH domain is followed by a COOH-terminal region unique to the syntrophins (38). Interaction of nNOS with α1-syntrophin is mediated by direct association of PDZ protein–binding interfaces near the NH_2-terminus of nNOS and α1-syntrophin. Studies here are consistent with this model and demonstrate that α1-syntrophin, but not dystrophin, β-dystroglycan or α-sarcoglycan binds to the PDZ-containing domain of nNOS. NOS isoforms lacking a PDZ motif do not associate with the dystrophin complex, further suggesting that the PDZ domain of nNOS represents the relevant domain for interaction (29).

The precise binding site(s) for syntrophins within the dystrophin complex is uncertain. In vitro studies show that the COOH-terminal region of syntrophin directly interacts with a splice-prone COOH-terminal domain of dystrophin (39–41). However, syntrophins are present in dystrophin complexes of the Δ330 transgenic mouse that lacks the identified syntrophin interaction domain, suggesting additional bindings sites for syntrophins (35). It is not clear which domain of syntrophins might interact with these additional sarcolemmal binding sites. PH domains are known to interact with specific membrane proteins and phospholipids (42), and these regions of syntrophins represent candidate interaction domains. Because the first PH domain of syntrophin is split by the PDZ domain (38), it is possible that simultaneous occupation of the PH1 and PDZ sites is sterically prohibited. Therefore, membrane association of syntrophin mediated by its PH domains could preclude binding of nNOS to the PDZ domain. This may explain the observed sarcolemmal expression of syntrophin and absence of nNOS in certain disease states.

nNOS appears not to interact with utrophin-containing complexes. During early postnatal muscle development, nNOS is not concentrated with complexes of α1-syntrophin and utrophin at neuromuscular endplates. Similarly, utrophin complexes at neuromuscular endplates of *mdx* mouse lack nNOS. Taken together with biochemical studies showing direct interaction of nNOS with α1-syntrophin in vitro, we propose that sarcolemmal localization of nNOS requires both syntrophin and dystrophin. It is alternatively possible that nNOS primarily binds directly to dystrophin in vivo. We have, however, been unable to detect direct interaction between nNOS and dystrophin in vitro and would disfavor this model. Future studies of nNOS expression in mice lacking syntrophin isoforms may be necessary to definitively clarify this issue.

Loss of sarcolemmal nNOS does not appear to be a generic consequence of muscle disease. nNOS expression occurs normally at the sarcolemma in a variety of inflammatory, neuropathic, and idiopathic muscle disorders (Chao, D.S., and D.S. Bredt, unpublished observations), and in *dy* mutant mice that have muscular dystrophy associated with loss of extracellular M-laminin (merosin; 28). Three patients with similar deletions of exons 45–47 of dystrophin all showed loss of sarcolemmal nNOS but exhibited a wide spectrum of clinical variability. This domain of dystrophin is apparently critical for assembly of nNOS, and the clinical differences could be caused by environmental and/or epigenetic factors. nNOS was expressed normally in two patients with autosomal recessive muscular dystrophy due to mutations in α-sarcoglycan (adhalin). Abnormality of nNOS expression, therefore, appears specific for dystrophin-related disease, and immunohistochemical analysis for nNOS may provide a supplemental diagnostic test.

Abnormal expression of nNOS may play a role in the pathophysiology of BMD. Decreased expression of nNOS alone is not sufficient to produce muscular dystrophy, as we have not detected muscle pathology in $nNOS^{\Delta/\Delta}$ mice that have a targeted disruption of nNOS (Chao, D.S., and D.S. Bredt, unpublished observations). However, endogenous NO does play a role in regulation of skeletal muscle development and contractility (24, 26). Disruption of these NO signaling pathways may contribute to abnormal muscle function and incomplete myofiber regeneration seen in muscular dystrophy. In *mdx* mice expressing Dp71 or dystrophin minigene, nNOS is the only known dystrophin-associated protein absent from the sarcolemma. Transgenic *mdx* mice expressing the dystrophin minigene have an extremely mild muscular dystrophy characterized by only a modest increase in central nuclei and serum pyruvate kinase activity (20, 21). A human patient with an identical mutation had a mild dystrophy and was ambulatory with the aid of a stick at age 61 (43). In contrast to the mild clinical phenotype, this minigene mutation in a 25-year-old patient was associated with severe histopathological muscle fiber atrophy, extensive replacement by fat and fibrous connective tissue, and few surviving fibers of normal diameter (43).

These findings may be relevant in designing therapies for DMD. We find that nNOS does not associate with utrophin-containing complexes, so that strategies for upregulation of utrophin in DMD would not be expected to restore sarcolemmal nNOS. Because of the large size of the dystrophin protein, vectors for gene therapy may need to encode truncation mutants. Expression of a minigene lacking exons 17–48 of dystrophin fails to recruit nNOS to sarcolemma of *mdx* mouse and is associated with a very mild Becker phenotype. Complete rescue of muscle function may require replacement with dystrophin constructs that properly recruit nNOS to skeletal muscle membranes.

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