α1-Syntrophin Gene Disruption Results in the Absence of Neuronal-type Nitric-oxide Synthase at the Sarcolemma but Does Not Induce Muscle Degeneration*

(Received for publication, June 29, 1998, and in revised form, October 13, 1998)

Shuhei Kameya‡, Yuko Miyagoe‡, Ikuya Nonaka§, Takaaki Ikemoto¶, Makoto Endo¶, Kazunori Hanaoka, Yo-ichi Nabeshima***, and Shin’ichi Takeda‡ ***

From the Departments of 3Molecular Genetics and 5Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, the 6Department of Pharmacology, Saitama Medical School, Moroyama-machi, Saitama 350-04, the 7Department of Biosciences, School of Science, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, and the **Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565, Japan

α1-Syntrophin is a member of the family of dystrophin-associated proteins and is strongly expressed in the sarcolemma and the neuromuscular junctions. All three syntrophin isoforms have a PDZ domain that appears to participate in protein-protein interactions at the plasma membrane. α1-Syntrophin has additionally been shown to associate with neuronal nitric-oxide synthase (nNOS) through PDZ domains in vitro. These observations suggest that α1-syntrophin may work as a modular adaptor protein that can link nNOS or other signaling enzyme to the sarcolemmal dystrophin complex. In the sarcolemma, nNOS regulates the homeostasis of reactive free radical species and may contribute to the oxidative damage to muscle protein in muscle disease such as Duchenne muscular dystrophy. In this study, we generated α1-syntrophin knock-out mice to clarify the interaction between α1-syntrophin and nNOS in the skeletal muscle. We observed that nNOS, normally expressed in the sarcolemma, was largely absent from the sarcolemma, but considerably remained in the cytosol of the knock-out mice. Even though the distribution of nNOS was altered, the knock-out mice displayed no gross histological changes in the skeletal muscle. We also discovered that muscle contractile properties have not been influenced in the knock-out mice.

Syntrophins are intracellular peripheral membrane proteins weighing 58 kDa, originally identified in the postsynaptic membrane of Torpedo (1). In mammalian skeletal muscle, syntrophins co-purify with dystrophin, the protein products of the Duchenne muscular dystrophy (DMD) gene (2, 3), and complex with several other dystrophin-associated proteins. The dystrophin complex appears to link the cytoskeleton to the extracellular matrix in skeletal muscle and stabilize the sarcolemma (4–6).

In human and mouse, three highly conserved but distinct syntrophin isoforms, α1-, β1-, and β2-syntrophin, are encoded by different genes (7–9). The β1- and β2-syntrophin transcripts are expressed in wide variety of tissues, whereas α1-syntrophin transcript is predominantly expressed in skeletal and cardiac muscle (7, 8). An immunohistochemical study using specific antibodies revealed that three syntrophins are concentrated at the neuromuscular junction, but α1-syntrophin is also present at the extrasynaptic sarcolemma (9, 10). The expression of β1-syntrophin at the sarcolemma, however, is reported to be restricted to fast twitch muscle fibers (9).

All three syntrophins have four protein domains (7); two pleckstrin homology domains, a PDZ domain, and a carboxy-terminal syntrophin unique domain. Pleckstrin homology domain is a small domain containing less than 100 residues and originally identified as an internally duplicated motif in pleckstrin (11). It was also revealed in a number of intracellular signaling and membrane-associated cytoskeletal proteins. The 57 carboxy-terminal amino acids of three isoforms, the syntrophin unique domain (7), are highly conserved and may serve in a specific interaction with dystrophin and its relatives, dystrobrevin (8, 12). Finally, the PDZ domain is composed of 90 amino acids and was originally identified in postsynaptic density-95, disc large, and ZO-1 (13). The PDZ domain is present in diverse families of enzymes and structural proteins, all of which are concentrated at specialized cell-cell junctions, such as neuronal synapses, epithelial zona occludens, and septate junctions. These observations suggest that PDZ domain may participate in protein-protein interactions at the plasma membrane (14). Indeed, in skeletal muscle, the association of neuronal nitric-oxide synthase (nNOS) and α1-syntrophin has been demonstrated using a yeast two-hybrid system (15). α1-Syntrophin may work as a modular adaptor protein that can link a signaling enzyme and nNOS to the sarcolemmal dystrophin complex.

In DMD and its experimental mouse model, mdx, which lacks dystrophin expression, nNOS is absent from the sarcolemma and partially accumulates in the cytosol (16). The altered regulation of nNOS in the dystrophic muscle may augment the toxicity of NO or superoxide and therefore contribute to myofiber necrosis (17); the derangement of NO metabolism is responsible for tissue damage in certain diseases (18, 19).

Endogenous NO produced near the sarcolemma has been reported to depress contractile function (20), although the mechanism of NO action in muscle contraction is poorly understood. Galler et al. (21) showed NO-depressed muscle contrac-
tion in regard to both mechanical properties and myofibrillar ATPase activity using the skinned fibers method. Aghdashi et al. (22) hypothesized that oxidants and NO interact directly with Ca\(^{2+}\) release channel (ryanodine receptor 1) in the T-system. The overall magnitude of cGMP-dependent changes appears to be small (20).

To investigate the role of a1-syntrophin in skeletal muscle and the interaction between a1-syntrophin and nNOS in vivo, we generated a1-syntrophin knock-out mice. The deficiency of a1-syntrophin in these mice causes the decrease of nNOS expression at the sarcolemma, indicating the direct interaction of a1-syntrophin and nNOS in vivo. Very interestingly, the absence of nNOS at the sarcolemma did not result in an overt degeneration of skeletal muscle in this mutant mouse. Therefore, the absence of nNOS at the sarcolemma may not be the major causative factor of muscle degeneration in DMD or in mdx mice. In addition, the force-frequency relationship between wild-type and a1-syntrophin knock-out mice showed no significant difference in muscle contractile properties. We also examined the effects of the NOS inhibitors, 7-nitroindazole (7-NI) and nitro-l-arginine methyl ester (L-NAME), and the exogenous NO donor, sodium nitroprusside (NP), on muscle contraction. Treatment by NOS inhibitors caused the shift of stimulation-tension curves in the mutant mice, suggesting that muscle contractile properties of a1-syntrophin knock-out mice are regulated by the nNOS activity in the cytosol.

### MATERIALS AND METHODS

#### Targeting Vector Construction and Transfection of ES Cells—The mouse a1-syntrophin gene was isolated from a mouse genomic library prepared from 129/SvJ mice DNA (Stratagene). It was probed with a 164-bp pair RT-PCR product from exon 2 (7). The targeting vector, as shown in Fig. 1a, consisted of the 6.0-kb genomic DNA containing the 1.2-kb neomycin resistance gene (BbsI site of exon 2) derived from pMC1neo(polyA) (24). A 1.8-kb herpes simplex virus thymidine kinase gene construct was attached at the 5'-end of the a1-syntrophin-neomycin construct. Finally, the targeting vector was linearized using the restriction enzyme, Ncol, before transfection by electroporation into E14 embryonic stem cells obtained from the inbred mouse strain 129/SvJ (23). After deleting the ES cells (10\(^3\)) from their plates using trypsin, they were suspended in 3.2 ml of cold Hanks' solution and then mixed with the targeting vector DNA at a concentration of 25 \(\mu\)g/ml in an electroporation cuvette. The electroporation was carried out at 200 V and 500 microfarads using a Bio-Pulsar (Bio-Rad) apparatus. Two days following the electroporation, we added 300 \(\mu\)g/ml Neomycin (G418) to the culture, and electroporated myoblasts were allowed to fuse and form myotubes in the cultures to a concentration of 2 \(\mu\)g to isolate the cells containing a targeted disruption by positive-negative selection. Then surviving colonies were cloned after a 10-day selection period. Homologous recombinants were screened by PCR using the primers described below. The forward primer (P1), designed from neomycin resistance gene, was ATTCGCAATGTCAAGAC (24), and the reverse primer (P2), outside the targeting vector, was CCTCTATTACATCTGTGTCATC. PCR was carried out for 40 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min by a DNA thermal cycler (Perkin-Elmer).

**Generation of Chimeric Mice with Germline Transmission**—We generated chimeric mice using the blastocyst injection (23) and aggregation (25) methods. Blastocysts were recovered from a mating of B6C3Fl (F1 of C57BL6 and C3H/HeJ) and C57BL/6 mice. ES-injected blastocysts were transplanted into uteri of pseudopregnant ICR mice. The resulting chimeric mice were mated with BALB/c mice, and homozygous mutant mice were generated by inter-crossing of heterozygotes. The aggregation method was performed as described previously, with some modifications to suit our experimental conditions (25). Chimeric mice were mated with C57BL/6 mice, and homozygous mutant mice were generated by inter-crossing of heterozygotes.

**Genomic Southern Blot Analysis**—We isolated genomic DNA from ES cells or mouse tails by phenol extraction and ethanol precipitation. The genomic DNA was digested by EcoR1, fractionated using electrophoresis through 0.8% agarose gels, and transferred to Hybond N+ nylon membrane (Amersham Pharmacia Biotech). The blots were hybridized to the 32P-labeled Nael-Spel fragment of genomic DNA produced using a Random primer DNA labeling kit (Takara).

**Northern Blot Analysis and RT-PCR—**Total RNA was isolated by using Rnazo1 B (Tel-Test, Inc.). We purified the poly(A)+ RNA using the mRNA Purification Kit (Amersham Pharmacia Biotech). Poly(A)+ RNA was electrophoresed in a 1.0% denaturing agarose-formaldehyde gels and transferred to Hybond N+ nylon membrane (Amersham Pharmacia Biotech). The hybridization with the 32P-labeled DNA probes constructed by employing a random primer DNA labeling kit (Takara). The density of the detected band were calculated using BAS-2000 (Fuji). Mouse a1-syntrophin cDNA (nucleotides 1180–1355) (26) and mouse nNOS cDNA (nucleotides 1547–2105) (27) were used to detect the mRNA of a1-syntrophin and nNOS. For RT-PCR, first strand cDNA was synthesized using random hexamers. The forward primer (P1) and (P2), from exon 1 of a1-syntrophin gene (7) was AGCTGCCAGAAGCGCTGCTG and GCTGGGCATCAGCATCAAGG, respectively. The reverse primer (R1), from exon 3 of the a1-syntrophin gene (7) was GAGGTGAGTTGCCAGCAACGC.

**Western Blot Analysis for a1-Syntrophin**—The total cellular protein was extracted from mouse hind-limb muscle for Western blot analysis. We used Bradford method and Coomassie Brilliant Blue G-250 (Bio-Rad) to determine the protein concentrations. The protein fractions were extracted with a reducing sample buffer (10%) SDS, 70 mM Tris-HCl, 5% β-mercaptoethanol, 10 mM EDTA) 15 \(\mu\)g protein/lane were separated on a SDS-polyacrylamide gel (8%). The resulting gel was subsequently transferred to a polyvinylidene difluoride membrane (Millipore) employing an amperage of 242 mA for 2 h. The transferred membrane was blocked with anti-a1-syntrophin antibody. a1-Syntrophin polyclonal antibody was prepared by injecting purified synthetic peptides into rabbits according to standard protocols. Anti-a1-syntrophin antibody was raised against the peptides CRQPSSPGPQPRNLSEA (amino acids 191–206) (26) of a1-syntrophin plus an amino-terminal cysteine. The antibody was used at a 1:40 dilution, and its signal was detected using the enhanced chemiluminescence method (Amersham Pharmacia Biotech) and a 1:5000 dilution of the anti-rabbit secondary antibody (Tago Immunologics).

**Histological Analysis, Immunohistochemistry, and NDP Activity Assay—**The skeletal muscle of mice were excised postmortem and then rapidly frozen in liquid nitrogen-cooled isopentane. 6-μm frozen sections were cut and placed on poly-l-lysine-coated slides. The slides were brought to room temperature, air dried, and acetone-fixed for 10 min. For histological analysis, the fixed sections were stained with hematoxylin and eosin. For immunostaining, the sections were incubated with anti-a1-syntrophin antibody at a dilution of 1:40 and then stained using fluorescein isothiocyanate-conjugated anti-rabbit goat antibody (Tago Immunologics). An NDF activity assay was performed as described previously (28). The sections were postfixed in 2% paraformaldehyde in phosphate-buffered saline, pH 7.4, at 2 h. After rinsing in phosphate-buffered saline, the sections were incubated in 0.2% Triton X-100 for 10 min at 37 °C. The subsequent reaction was carried out for 1 h in a dark, humidified chamber at 37 °C in 0.2% Triton X-100, 0.1 mM NADPH, and 0.16 mg/ml nitro blue tetrazolium. The reaction was terminated by washing with water.

**Extraction and Western Blot Analysis for nNOS—**The extraction of nNOS was performed according to the method described by Brennan et al. (16). To isolate nNOS, the quadriceps muscle was homogenized in 10 volumes (w/v) of buffer A (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA). The nuclei of the muscle were pelleted by centrifugation at 100,000 \(\times\) g for 30 min at 4 °C with 40% ammonium sulfate and centrifuged for 1 h at 15,000 \(\times\) g. The supernatant was then centrifuged at 20,000 \(\times\) g to yield the supernatant S1. The remaining high microsomal pellet was resuspended in buffer B (500 mM NaCl added to buffer A), incubated for 30 min at 4 °C with agitation, and centrifuged at 15,000 \(\times\) g, yielding supernatant S2. The pellet from this last centrifugation was resuspended in buffer B containing 0.5% Triton X-100, incubated for 30 min at 4 °C with agitation, and centrifuged at 15,000 \(\times\) g to create supernatant S3 and the final pellet, P. The fractions were resolved using the sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (6% polyacrylamide). The proteins were transferred to a polyvinylidene difluoride membrane (Millipore), which was later incubated with anti-nNOS antibody (Transduction Laboratories).

**NOS Catalytic Assays—**NOS catalytic assay was carried out according to the method described by Brennan et al. (16). The quadriceps muscle from wild-type, mdx, and homozygous mutant mdx were homogenized in 10 volumes of buffer containing 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 0.1 mM NaCl. The homogenate was centrifuged at 20,000 \(\times\) g to reveal the soluble fraction. The pellet was extracted in the same buffer containing 0.5 mM NaCl and centrifuged at 20,000 \(\times\) g to create a particulate fraction. To perform the catalytic assay, aliquots from these fractions were assayed in 125-μl reactions.
containing 1.8 × 10^3 cpm of [3H]arginine (53.0 Ci/mmol), 1 mM NADPH, 640 µM CaCl_2, 1 µM calmodulin, 3 µM each of tetrahydrobiopterin, FAD, and FMN. After incubation for 25 min at 22 °C, the assays were terminated with 2 ml of 20 m M HEPES, pH 5.5, 2 m M EDTA. The samples were then applied to 1-ml columns of Dowex AG50WX-8 (Na\(^{+}\) form), which were eluted with 2 ml of water. [3H]citrulline was quantified by liquid scintillation spectroscopy of the 4-ml flow-through. Differences of activity of soluble or particulate fractions between wild-type and knock-out or mdx mice were calculated using Student’s t test.

Analysis of Contractile Properties of Muscles—We dissected the muscle fiber bundles from the diaphragms and extensor digitorum longus of mice and tied them at both ends with silk filaments. The bundles were then mounted in a small bath between a pair of stainless steel rods, one of which was attached to a force transducer. We then immersed the bundles in physiological salt solution containing: 150 mM NaCl, 4 mM KCl, 2 mM CaCl_2, 1 mM MgCl_2, 5 mM HEPES, 5.6 mM glucose, pH 7.4. Electrical stimulation was applied through a pair of platinum wires placed on both sides of the bundles. The stimulus lasted 0.5 ms and measured a voltage of 20 V. The relative force was calculated by counting the force recorded at a stimulus frequency of 125 Hz as 100%. In the experiments using bundles from diaphragm, to assess the effects of NOS inhibitor and NO donor on contraction, 1 mM 7-NI, 5 mM l-NAME, or 1 µM NP was added to the physiological salt solution.

RESULTS

Generation of α1-Syntrophin Knock-out Mice—We cloned and mapped the mouse α1-syntrophin gene and used that information to disrupt it in the E14 ES cell line, a line derived from the 129/SvJ mouse strain. Homologous recombination between the targeting vector and the α1-syntrophin gene resulted in the replacement of the second exon of the gene with the neomycin resistance gene, thus knocking out the α1-syntrophin gene (Fig. 1a). Five positive clones were identified from 288 G418- and GANC-resistant clones. Southern blot analysis using a probe that recognizes an 7.0-kb EcoRI fragment from the endogenous α1-syntrophin allele and to a 3.6-kb EcoRI fragment from the disrupted gene. b, Southern blot analysis of EcoRI-digested genomic DNA from mouse tails.

Fig. 1. a, restriction map of the endogenous α1-syntrophin gene, the targeting vector, and the properly disrupted α1-syntrophin gene. The herpes simplex virus thymidine kinase gene (TK) and the neomycin resistance gene (NEO) are also illustrated. Homologous recombination results in the insertion of the neomycin resistance gene into the second exon of the α1-syntrophin gene. The location of the probe used for Southern blot analysis is shown. The probe hybridizes to a 7.0-kb EcoRI fragment from the endogenous α1-syntrophin allele and to a 3.6-kb EcoRI fragment from the disrupted gene. b, Southern blot analysis of EcoRI-digested genomic DNA from mouse tails.

-7.0 kb
-3.6 kb

Two germline-transmitting mice from the two independently derived E14 ES cell clones were obtained and further studied. Heterozygous animals identified by Southern blot analysis were bred with each other to obtain homozygous animals. Heterozygous and homozygous mice are indistinguishable from wild-type mice and develop normally in nearly all respects.

α1-Syntrophin Expression Is Absent in Homozygous Mutant Mice—Our Northern blot analysis revealed the expression of α1-syntrophin mRNA in the skeletal muscle of wild-type mice through strong signals at 2.2 and 2.6 kb (Fig. 2a). This result is consistent with a previous report regarding α1-syntrophin expression (7). The size of α1-syntrophin mRNA of heterozygous mutant mice was identical to that of wild-type mice, but the...
amount of α1-syntrophin mRNA was about 70% of that in wild-type mice. The mRNA of homozygous mutant mice included a 1.9-kb band and light smears in the vicinity of 1.5 kb. The amounts were 20% that of wild-type mice.

Our RT-PCR analysis of the skeletal muscle mRNA of wild-type and homozygous mutant mice employed pairs of primers that spanned exon 1 and 3 of the α1-syntrophin gene. Wild-type mice revealed a PCR product with an expected size, but homozygous mutant mice had a shorter than expected product (Fig. 2c). We cloned the PCR product from homozygous mutant mice and found that the entire exon 2 of the α1-syntrophin gene was missing.

To investigate the expression of α1-syntrophin at the protein level, we prepared an anti-α1-syntrophin antibody. The polyclonal antibody was raised against synthetic peptide corresponding to α1-syntrophin (amino acids 191–206). Using this antibody, we carried out Western blot analysis of the skeletal muscle lysates of wild-type, heterozygous mutant, and homozygous mutant mice (Fig. 2d). Wild-type mice revealed a 59-kDa band that corresponds to α1-syntrophin, whereas homozygous mutant mice showed no detectable α1-syntrophin. In addition, we could not detect any truncated fragments that could have been derived from the exon 2-lacking transcript. Heterozygous mutant mice produced nearly half the amount of α1-syntrophin compared with wild-type mice.

Histological and Immunohistochemical Analysis of α1-Syntrophin Knock-out Mice—We examined frozen sections of the tibialis anterior and soleus muscles of wild-type and homozygous mutant mice using hematoxylin and eosin staining (Fig. 3, a–d). There were no striking morphologic changes in these skeletal muscles. We could detect neither degeneration and regeneration of muscle fibers nor cellular infiltrations and proliferation of connective tissues in the α1-syntrophin knock-out mice. Histochemical analysis using Myosin-ATPase staining indicated that the differentiation of muscle fiber types has not been affected.

Using anti-α1-syntrophin antibody, we examined adult skeletal muscle immunohistochemically (Fig. 3, e and f). In wild-type mice, the expression of α1-syntrophin was detected at the sarcolemma and was particularly enriched at the neuromuscular junction, as already reported (10). In homozygous mutant mice, the expression of α1-syntrophin was completely absent in both the sarcolemma and the neuromuscular junction. The labeling pattern for the α1-syntrophin of heterozygous mutant...
mice was indistinguishable from that of wild-type mice.

To investigate the expression of dystrophin-glycoprotein complex in α1-syntrophin knock-out mice, we performed immunohistochemical analysis of adult skeletal muscles using anti-dystrophin and anti-α-sarcoglycan antibody. The expression of dystrophin and α-sarcoglycan appeared to be not altered in homozygous mutant skeletal muscle (data not shown).

nNOS Expression in α1-Syntrophin Knock-out Mice—Recent research has suggested that α1-syntrophin anchors nNOS to the sarcolemma (14). We investigated the influence of the loss of α1-syntrophin on the expression of nNOS in the homozygous mutant muscle. We did this by performing a Northern blot analysis of adult skeletal muscle (Fig. 4a). In wild-type mice, a single 10-kb band corresponding to nNOS mRNA was detected. Homozygous mutant mice produced an equal amount of nNOS mRNA. In mdx skeletal muscle, the amount of nNOS mRNA was decreased to 50% in accord with the previous report (29). In DMD and mdx mice, nNOS is absent from the sarcolemma but expressed in the cytosol although in small amounts. Like nNOS, NDP activity in skeletal muscle fibers is also concentrated in the sarcolemma and colocalizes with nNOS (28) (Fig. 4c). In α1-syntrophin knock-out mice, however, NDP activity was completely absent in the sarcolemma (Fig. 4d).

We also investigated the subcellular distribution of nNOS in the quadriceps muscle of α1-syntrophin knock-out mice (Fig. 4, e and f). In wild-type mice, significant amounts of nNOS remained in the insoluble pellet (P) even after the sequential S1, S2, and S3 extractions of skeletal muscle homogenates. By contrast, nNOS was largely removed with 100 mM NaCl (S1) and did not remain in the pellet (P) fraction in α1-syntrophin knock-out mice.

Our study of nNOS catalytic activity demonstrated that this activity for nNOS was strongest in the particulate fractions of skeletal muscle homogenates in wild-type mice in accord with previous report (15) (Table I). In mdx mice, some nNOS activity remained in the soluble fractions, whereas activity in the particulate fractions was decreased. Homozygous mutant has decreased nNOS activity in the particulate fraction but maintained the activity in the soluble fractions, compared with wild-type mice. In addition, the total nNOS activity for homozgyous mutant mice was 59% that of wild-type mice; the activity for mdx mice was only 27% when calculated using the mean value.

Contractile Properties of Skeletal Muscles in α1-Syntrophin Knock-out Mice—To examine the influence of intracellular distribution of nNOS in knock-out mice on muscle contraction, we measured contractile properties of the diaphragm and the extensor digitorum longus muscles (Fig. 5). We first measured the relative forces exerted during submaximal contractions to determine the steady state force-frequency relationship in the diaphragm of the wild-type and α1-syntrophin knock-out mice. No significant difference was observed between wild-type and...
a1-syntrophin knock-out mice at any stimulus frequency measured. Extensor digitorum longus muscle was also investigated but again did not produce any differences between wild-type and knock-out mice (data not shown). In diaphragm, we further investigated the influence of NOS inhibitors and NO donor on muscle contractions (Fig. 5). Exposure to the NOS inhibitor 7-NI shifted the force-frequency relationship up and to the left at both wild-type and a1-syntrophin knock-out mice, and the relative forces showed no significant difference between the mice. Exposure to the NOS inhibitor, l-NAME also shifted the force-frequency relationship up and to the left at both wild-type and a1-syntrophin knock-out mice. However, l-NAME had a relatively weak effect on contraction in knock-out mice compared with the effect in wild-type mice. Treatment by the NO donor NP did not result in any significant changes from the steady state.

**DISCUSSION**

Our a1-syntrophin knock-out mice produce a truncated transcript that lacks 186 base pairs of exon 2 of the a1-syntrophin gene. This missing segment could cause an in-frame deletion and produce a truncated a1-syntrophin lacking the PDZ domain. However, Western blot analysis using an anti-nNOS antibody indicated that significant amounts of nNOS remained in the insoluble pellet (P) even after sequential extraction of skeletal muscle homogenates with 100 mM NaCl (S1), 500 mM NaCl (S2), and 0.5% Triton X-100 (S3) in wild-type mice (e, upper panel). In a1-syntrophin knock-out mice, nNOS was largely extracted with 100 mM NaCl (S1) and was not detected in a pellet (P) fraction (f, upper panel). Control immunoglobulin bands were shown (e and f, lower panels).

| Fraction | Soluble | Significance | Particulate | Significance |
|----------|---------|--------------|-------------|-------------|
| +/-      | 3.77 ± 0.44 | 7.80 ± 1.34 | 7.60 ± 1.34 | NS          |
| mdx      | 4.42 ± 1.35 | 2.28 ± 0.82 | 2.45 ± 0.82 | p < 0.02    |
| mdx      | 2.33 ± 0.58 | 0.74 ± 0.40 | 0.74 ± 0.40 | p < 0.005   |

In a1-syntrophin knock-out mice, there is no NDP activity in the sarcolemma and a considerable amount of nNOS remains at the soluble fraction. The interaction of a1-syntrophin and nNOS has been demonstrated in vitro by using the yeast two-hybrid system, pull-down assay, and protein overlay assay (15). Although there is an altered distribution of nNOS in DMD and mdx mice, it is not known which protein among the dystrophin-
associated proteins is responsible for the disappearance of nNOS from the sarcolemma. Our data strongly suggest that α1-syntrophin interacts with nNOS in vivo via the PDZ domain and anchors nNOS to the submembranous dystrophin complex.

The role of NO in skeletal muscle is not clear. Altering the regulation of nNOS in dystrophic muscle can augment the toxic interaction of NO and superoxide with the cell and contribute to myofiber necrosis (17). In DMD and mdx mice, the preferential degeneration of fast twitch muscle fibers is explained by the selective enrichment of nNOS in fast twitch muscle fibers (20). Our knock-out mice have no obvious degeneration of their muscle fibers despite the disappearance of α1-syntrophin and nNOS from the sarcolemma. This suggests that the degeneration process does not arise merely from the altered distribution of nNOS.

NO produced by nNOS near the sarcolemma of fast twitch muscle fibers appears to regulate the muscle contraction by opposing the generation of contractile force (20). Our functional analysis of the contractile properties of wild-type and α1-syntrophin knock-out mice revealed no significant changes when tested at steady state. This observation shows that the muscles of the knock-out mice still retain enough NO to regulate muscle contraction by depressing the contractile force. This was also supported by the findings that treatment with a NOS inhibitor, 7-NI, shifted the force-frequency relationship to the left not only in wild-type mice but also in α1-syntrophin knock-out mice (20).

**Fig. 5.** Force-frequency relationship of the skeletal muscles of wild-type (a) and α1-syntrophin knock-out mice (b). The relative force was calculated by counting the force recorded at a stimulus frequency of 125 Hz as 100%. Means ± S.E. has been shown using symbols and bars. 7-NI caused a significant shift to the left of the force-frequency relationship in wild-type mice. L-NAME caused a moderate shift to the left of the force-frequency relationship in wild-type muscle. The contractile properties of normal, 7-NI-treated and NP-treated α1-syntrophin knock-out mice were not different from wild-type mice. L-NAME had relatively weak effect on contraction in α1-syntrophin knock-out mice compared with the effect in wild-type mice. c, the relative force at a stimulus frequency of 60 Hz in wild-type (+/+) and α1-syntrophin knock-out mice (−/−). Treating with 7-NI and L-NAME showed statistically significant effect on contraction both wild-type and knock-out mice when compared with steady state contraction. Differences of relative force between NOS inhibitors or donor-treated and steady state were calculated by t test.
mice. However, another NOS inhibitor, L-NAME, had a comparatively weak effect on muscle contraction of α1-syntrophin knock-out mice but not the effect on that of wild-type mice. We cannot exclude the possibility that the modest response to L-NAME reflects reduced expression of nNOS and/or the altered distribution of nNOS in α1-syntrophin knock-out mice. Although Kobzik et al. (20) showed that the exogenous NO donor, sodium nitroprusside, could depress the development of tension, our findings reveal that sodium nitroprusside has no significant effect on contraction. Because the effect of the exogenous NO donor was relatively modest compared with the NOS inhibitor in the previous study, the exogenous NO donor might have a weak effect on contraction in our particular assay.

The biochemical nNOS catalytic activity assay showed that α1-syntrophin knock-out mice maintained 59% of nNOS activity compared with wild-type mice and that certain activities were still retained at the cytosol fraction. We believe that the remaining nNOS activity in the cytosol regulates the contractile properties of α1-syntrophin knock-out mice. Although the target of NO in skeletal muscle contraction has not yet been assigned to either myofibrillar proteins, the Ca²⁺ release channel, or any other factors, our results suggest that cytosolic nNOS could compensate for the lost activity of membrane-associated nNOS and provide enough NO to depress the excessive contraction.

α1-Syntrophin transcript is also expressed in tissues other than skeletal and cardiac muscles. The amount of mRNA in tissues other than striated muscle is very low, and its expression in other tissues has not yet been fully verified and therefore must be investigated. Although we have demonstrated an important role for α1-syntrophin in the function of nNOS within the sarcolemma, further study will be needed to gain a deeper understanding of the function of α1-syntrophin in the contraction and maintenance of the structure of skeletal muscle.

Acknowledgments—We thank Dr. Hideo Sugita and Daniel North for review of this manuscript.
α1-Syntrophin Gene Disruption Results in the Absence of Neuronal-type Nitric-oxide Synthase at the Sarcolemma but Does Not Induce Muscle Degeneration
Shuhei Kameya, Yuko Miyagoe, Ikuya Nonaka, Takaaki Ikemoto, Makoto Endo, Kazunori Hanaoka, Yo-ichi Nabeshima and Shin’ichi Takeda

J. Biol. Chem. 1999, 274:2193-2200.
doi: 10.1074/jbc.274.4.2193

Access the most updated version of this article at http://www.jbc.org/content/274/4/2193

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 12 of which can be accessed free at http://www.jbc.org/content/274/4/2193.full.html#ref-list-1