Deficiency of Dystrophin-associated Proteins in Duchenne Muscular Dystrophy Patients Lacking COOH-terminal Domains of Dystrophin

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Abstract

Dystrophin, the protein product of the Duchenne muscular dystrophy (DMD) gene, is a cytoskeletal protein tightly associated with a large oligomeric complex of sarcosomal glycoproteins including dystroglycan, which provides a linkage to the extracellular matrix component, laminin. In DMD, the absence of dystrophin leads to a drastic reduction in all of the dystrophin-associated proteins, causing the disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix which, in turn, may render muscle cells susceptible to necrosis. The COOH-terminal domains (cysteine-rich and carboxyl-terminal) of dystrophin have been suggested to interact with the sarcosomal glycoprotein complex. However, truncated dystrophin lacking these domains was reported to be localized to the sarclemma in four DMD patients recently. Here we report that all of the dystrophin-associated proteins are drastically reduced in the sarclemma of three DMD patients in whom dystrophin lacking the COOH-terminal domains was properly localized to the sarclemma. Our results indicate that the COOH-terminal domains of dystrophin are required for the proper interaction of dystrophin with the dystrophin-associated proteins and also support our hypothesis that the loss of the dystrophin-associated proteins in the sarclemma leads to severe muscular dystrophy even when truncated dystrophin is present in the subsarcolemmal cytoskeleton. (J. Clin. Invest. 1993. 92:866–871.) Key words: Duchenne muscular dystrophy • dystrophin • dystrophin-glycoprotein complex • dystrophin-associated proteins • dystroglycan

Introduction

Duchenne muscular dystrophy (DMD) is caused by the absence of dystrophin, a large membrane cytoskeletal protein (1).

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1. Abbreviations used in this paper: BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; 35DAG, 35-kD dystrophin-associated glycoprotein; 43DAG, 43-kD dystroglycan; 50DAG, 50-kD dystrophin-associated glycoprotein; DRP, dystrophin-related protein; 156DAG, 156-kD dystroglycan; 59DAP, 59-kD dystrophin-associated protein; SCARMID, severe childhood autosomal recessive muscular dystrophy.

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2. Dystrophin is associated with a large oligomeric complex of sarcosomal glycoproteins, including dystroglycan, which binds the extracellular matrix component, laminin (3–10). Dystrophin also interacts with F-actin (11, 12). These findings indicate that the dystrophin–glycoprotein complex spans the sarclemma to provide a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix. In DMD, the absence of dystrophin leads to a great reduction in all of the dystrophin-associated proteins, causing the disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix. This disruption of the linkage may, in turn, render muscle cells susceptible to necrosis (4, 10, 13–15). The same sarcosomal defect also exists in a mosaic of dystrophin-deficient muscle fibers in asymptomatic DMD carrier and is presumed to be responsible for the muscle fiber degeneration in this condition (16). The significant role of the loss of the dystrophin-associated proteins in the molecular pathogenesis of DMD is supported by our recent findings that the specific deficiency of the 50-kD dystrophin-associated glycoprotein (S0DAG) alone causes severe childhood autosomal recessive muscular dystrophy with DMD-like phenotype (SCARMID) (15).

In our proposed model of the dystrophin–glycoprotein complex, dystrophin is presumed to be localized to the subsarcolemmal region due to its dual interaction with the glycoprotein complex in the sarclemma and the subsarcolemmal F-actin cytoskeleton (9). Recently the NH2-terminal domain of dystrophin was expressed as a fusion protein and shown to interact with F-actin (11, 12). The exact domain of dystrophin which interacts with the dystrophin-associated proteins remains unknown. However, the COOH-terminal domains (cysteine-rich and carboxyl-terminal) of dystrophin have been suggested to be involved in the interaction with the dystrophin-associated proteins because of the following observations (9): (a) the lack of significant homology between the carboxyl-terminal domain of dystrophin and proteins of known function except for dystrophin-related protein (DRP), an autosomal homologue of dystrophin (2, 17), (b) the conservation of the cysteine-rich and carboxyl-terminal domains among different species (18), (c) the clinical observation that the phenotype of the patients with deletions in the cysteine-rich and carboxyl-terminal domains is severe (19), and (d) the results of immuno-gold labeling studies (20, 21). The results of limited calpain digestion of the purified dystrophin-glycoprotein complex and the demonstration of the association of DRP with the dystrophin-associated proteins in mdx muscle are consistent with this hypothesis (22–24).

Recently, truncated dystrophin lacking the COOH-terminal domains was reported to be properly localized to the sarclemma in four unique patients with DMD, leading to an alter-
native hypothesis that another domain in dystrophin may inter-
tact with the sarcolemmal glycoprotein complex (25–29). A
modified model of the dystrophin–glycoprotein complex was
proposed (26) and the NH₂-terminal domain of dystrophin
was hypothesized to interact with the dystrophin-associated
proteins (26, 28). However, the possibility that truncated dys-
rophin with the intact NH₂-terminal domain may be properly
targeted to the sarcolemma by associating with other subsarco-
lemmal cytoskeletal component(s) even though it is not asso-
ciated with the dystrophin-associated proteins in the sarco-
lemma was not addressed. Furthermore, the molecular mech-
anism underlying the severe phenotype of these patients in spite
of the expression and proper localization of truncated dystro-
phin is unknown (25–29). To address these issues, we investi-
gated the status of the dystrophin-associated proteins in the
DMO patients lacking the COOH-terminal domains of dys-
trophin.

Methods

Case reports

Patient 1. This 18-mo-old patient was fully described elsewhere (26).
Briefly, the patient suffers from a combination of muscular dystrophy,
glycerol kinase deficiency, congenital adrenal hypoplasia, and poor
psychomotor development (26). DNA analysis of the dystrophin gene
revealed a large deletion removing the distal part of the gene beyond
exon 49 which encodes the distal portion of the rod domain, the cy-
teine-rich domain, and the carboxyl-terminal domain, and extending
beyond the glycerol kinase and congenital adrenal hypoplasia genes
(26). A 270-kD band was detected by the antibody against the NH₂-
terminus or central domain of dystrophin but was not detected by the
antibody against the COOH terminus in the immunoblot analysis
(26). The biopsy specimen investigated in the present study was ob-
tained from the deltoid muscle at the age of 18 mo.

Patient 2. This 2-yr-old boy has positive family history with one
maternal uncle with DMD. He has a history of progressive weakness of
his legs starting at 18 mo of age. Neurological examination showed
proximal weakness of the extremities, hypertrophy of the calves, and
tightness of the heel cords. Serum creatine kinase level was elevated to
15,000 IU (normal 50–200 IU). Histology of the biopsied quadriceps
muscle was consistent with severe myopathy. DNA analysis of the dys-
trophin gene was not performed.

Patient 3. This 4-yr-old boy, the elder brother of patient 2, was
developmentally delayed: he crawled at 18 mo, walked at 2 yr, said his
first word at 3 yr, and was not toilet trained at 4 yr of age. First signs
of weakness manifested at the age of 3 yr. Neurological examination
revealed proximal weakness of the extremities, positive Gowers sign, and
hypertrophy of the calves. Serum creatine kinase level was elevated to
35,760 IU. Histology of the biopsied quadriceps muscle was consistent
with severe myopathy. DNA analysis of the dystrophin gene was not per-
formed.

Antibodies

Affinity-purified rabbit antibodies against the first 15 amino acids of
the NH₂-terminus and the last 10 amino acids of the COOH-terminus
dystrophin were characterized previously (6–8, 13). A cDNA clone
corresponding to repeat units 21 and 22 of the rod domain of human
dystrophin (residues 2263 to 2557) was isolated from a rabbit skeletal
muscle cdNA library, subcloned into pGEX vector (Pharmacia LKB
Biotechnology, Piscatway, NJ) and expressed as a GST-fusion protein
by IPTG induction (2, 10). Sheep antibody against this fusion protein
was affinity purified as described (13, 14). Monoclonal antibody
IVD₃, against the 50-kD dystrophin-associated glycoprotein (50DAG)
was characterized previously (4, 6). Specific antibodies against the
156-kD dystroglycan (156DAG), 59-kD dystrophin-associated pro-
tein (59DAP), 50DAG, 43-kD dystroglycan (43DAG), and 35-kD
dystrophin-associated glycoprotein (35DAG) were affinity purified as
described (10, 13–15).

Immunohistochemistry

Indirect immunofluorescence microscopy of 7-µm thick cryosections
from skeletal muscle biopsy specimens was performed as described
previously (6, 13–15). Blocking was performed by a 30-min incubation
with 5% BSA in PBS (50 mM sodium phosphate, pH 7.4, 0.9% NaCl).
Incubation with primary antibodies was performed for 1 h. In
the case of rabbit or mouse primary antibodies, cryosections were
incubated with 1:200 diluted fluorescein-labeled goat anti-rabbit IgG or
anti-mouse IgG (Boehringer-Mannheim Corp., Indianapolis, IN) for 1
h. In the case of sheep primary antibodies, cryosections were incubated
for 30 min with 1:500 diluted biotinylated rabbit anti-sheep IgG (Vec-
tor Laboratories, Inc., Burlingame, CA) followed by incubation for 30
min with 1:1,000 diluted fluorescein-conjugated streptavidin (Jackson
Immunoresearch Laboratories, Inc., West Grove, PA). Incubation with
all antibodies was performed at room temperature. Each incubation
was followed by extensive washing with PBS. Final specimens were
examined under a Zeiss Axioplan fluorescence microscope. For reli-
able comparison, cryosections from different patients were placed
on the same microscopy slide and processed identically. In addition, pho-
tographs were taken under identical conditions with the same exposure
time.

Results and Discussion

Antibodies against the NH₂ terminus, rod domain, and COOH
terminus of dystrophin and antibodies against the dystrophin-
associated proteins all stained the sarcolemma in the skeletal
muscle from normal humans as described previously (4, 10,
14, 15). (Figs. 1 and 2). In the skeletal muscle from typical
DMO patients, sarcolemma was not stained by the antibodies
against the NH₂ terminus, rod domain or COOH terminus of
dystrophin, and the staining for all of the dystrophin-associated
proteins was greatly reduced in the sarcolemma (Figs. 1 and 2)
(4, 10, 14, 15, 24).

In the skeletal muscle from the three DMO patients de-
scribed in Case Reports, antibodies against the NH₂ terminus
and rod domain of dystrophin stained the sarcolemma but an
antibody against the COOH terminus of dystrophin did not
stain the sarcolemma (Figs. 1 and 2). The staining for all of the
dystrophin-associated proteins was greatly reduced in the sar-
colemma (Figs. 1 and 2). The staining pattern of the dystro-
phin-associated proteins was indistinguishable from that of typi-
cal DMO patients (Figs. 1 and 2).

Here we have investigated the status of all the components
of the dystrophin-glycoprotein complex in three DMO patients
with peculiar expression of dystrophin. Our patients included a
patient reported previously (patient 1) (26). DNA analysis of the
dystrophin gene in this patient indicates that truncated dys-
trophin in this patient lacks the distal portion of the rod
domain and the entire cysteine-rich and carboxyl-terminal
domains. Information about the defect of the dystrophin gene was
not available for the other two patients (patients 2 and 3).
However, the results of immunohistochemistry using antibod-
ies against the three distinct domains of dystrophin indicate
that the defect involves a portion of dystrophin distal to the
21st and/or 22nd repeat units of the rod domain in these two
brothers.

Immunohistochemistry using antibodies against the three
distinct domains of dystrophin demonstrated that dystrophin
lacking the COOH-terminal portions was expressed and proper-
ly localized to the sarcolemmal region in all three patients
reported here. These results are identical to those reported pre-
Figure 1. Immunohistochemical analysis of dystrophin and the dystrophin-associated proteins in the skeletal muscle from a normal human (Normal), patient 1, and a typical DMD patient (DMD). Transverse cryosections from biopsied skeletal muscle were immunostained with antibody against the NH\textsubscript{2} terminus of dystrophin (N-Ter), antibody against the rod domain of dystrophin (Rod), antibody against the COOH terminus of dystrophin (C-Ter), and antibodies against the 156DAG, 59DAP, 50DAG, 43DAG, and 35DAG. In patient 1, antibody against the NH\textsubscript{2} terminus or the rod domain of dystrophin stained the sarcolemma but no staining was observed using an antibody against the COOH terminus of dystrophin. Staining for the dystrophin-associated proteins was greatly reduced as in a typical DMD patient. Bar, 100 \mu m.
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Figure 2. Immunohistochemical analysis of dystrophin and the dystrophin-associated proteins in the skeletal muscle from a normal human (*Normal*), patient 2, patient 3, and a typical DMD patient (*DMD*). Transverse cryosections from biopsied skeletal muscle were immunostained with antibody against the NH₂ terminus of dystrophin (*N-Ter*), antibody against the rod domain of dystrophin (*Rod*), antibody against the COOH terminus of dystrophin (*C-Ter*), and antibodies against the 156DAG, 59DAP, 50DAG, 43DAG, and 35DAG. In patients 2 and 3, antibody against the NH₂ terminus or the rod domain of dystrophin stained the sarcolemma but no staining was observed using an antibody against the COOH terminus of dystrophin. Staining for the dystrophin-associated proteins was greatly reduced as in a typical DMD patient. Bar, 100 μm.
viously (25–29). However, the present study is the first to demonstrate the loss of all the dystrophin-associated proteins in the sarcolemma of these patients, a phenomenon characteristic of DMD sarcolemma. The loss in all of the dystrophin-associated proteins including the laminin-binding dystroglycan indicates that the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix is disrupted in spite of the presence of truncated dystrophin in these patients. This is presumed to be the cause of severe muscular dystrophy. This hypothesis is also consistent with our observations that the dystrophin-associated proteins were fairly well preserved in the sarcolemma of the Becker muscular dystrophy (BMD) patients having in-frame deletions in the rod domain of dystrophin (repeat units 3–20) (30).

Our results suggest that the domain of dystrophin which interacts with the glycoprotein complex is missing in the three patients reported here and thus support the hypothesis that the COOH-terminal portions of dystrophin are required for proper stabilization of the glycoprotein complex. This is consistent with the recent biochemical findings that the cysteine-rich domain and the first half of the carboxyl-terminal domain remained bound to the dystrophin-associated proteins after limited calpain digestion (22) and with the recent demonstration that DRP, which has the cysteine-rich and carboxyl-terminal domains highly homologous to those of dystrophin, is associated with the dystrophin-associated proteins in mdx muscle (17, 24).

In our proposed model of the dystrophin-glycoprotein complex, the 59DAP was hypothesized to be associated with the other dystrophin-associated proteins on the COOH-terminal domains of dystrophin but the possibility that the 59DAP may be associated with the other domains of dystrophin, apart from the other dystrophin-associated proteins, was not excluded (9). Thus, the present result demonstrating the loss of the 59DAP in the DMD patients with truncated dystrophin suggests that the 59DAP interacts with the COOH-terminal domains of dystrophin.

The mechanism by which dystrophin lacking the COOH-terminal domains was properly localized to the sarcolemma in these patients is unclear. Presumably, truncated dystrophin with the intact NH2-terminal domain can interact properly with other subsarcolemmal cytoskeletal component(s). For instance, the actin-binding site in the NH2-terminal domain may associate with subsarcolemmal cytoskeletal proteins such as γ-actin which could target truncated dystrophin to the subsarcolemmal region.

The present study has significant implications not only for the differential diagnosis between DMD and BMD, but also for the elucidation of the molecular mechanism leading to muscle degeneration in these diseases. Our results suggest that the status of the dystrophin-associated proteins in the sarcolemma may be more directly correlated with the phenotype of the patients than the status of the expression of dystrophin itself. The recent finding that the specific deficiency of the 50DAG alone causes SCARMD also supports this hypothesis (15). The phenotype of DMD is presumed to be caused by the loss of the dystrophin-associated proteins in the sarcolemma which, in turn, can be due to either the complete absence of dystrophin or the specific absence or dysfunction of the domain of dystrophin which interacts with the dystrophin-associated proteins (Fig. 3). The former is the case in the typical DMD patients and the latter is the case in the DMD patients lacking the COOH-terminal domains of dystrophin as in our patients. Our hypothesis is also consistent with the observation of Koenig et al. (19), who correlated the phenotype with dystrophin alterations at the genomic level and reported severe phenotype for the patients with deletions involving the exons which correspond to the cysteine-rich and the first half of the carboxyl-terminal domains. Indeed our findings provide a functional explanation for their observation.

Our hypothesis predicts that the in-frame mutations of the dystrophin gene having no effects on the domain which interacts with the glycoprotein complex will not result in the loss of the dystrophin-associated proteins and thus, lead to only a mild disease phenotype. One exception could be the mutations which affect the domain of dystrophin which interacts with F-actin. Indeed mutations involving the NH2-terminal domain of dystrophin lead to a phenotype of severe BMD (19, 31, 32).
Thus, it is important to investigate the status of the dystrophin-associated proteins in BMD patients having a variety of mutations to clarify the molecular mechanism leading to muscle degeneration in BMD.

Finally, our results indicate that caution is required in the interpretation of the immunohistochemical and immunoblot analyses of dystrophin in biopsied skeletal muscle from DMD/BMD patients: analysis using only antibodies against dystrophin can be quite misleading as exemplified in the case of the DMD patients lacking the COOH-terminal domains of dystrophin. Furthermore, we have shown that secondary loss of dystrophin due to the deficiency of the 50DAG can occur in the advanced stages of SCARMID, making the differential diagnosis between DMD, BMD, and SCARMID difficult by the immunohistochemical analysis of dystrophin alone (15). Thus, immunohistochemical analysis of the dystrophin-associated proteins, in addition to dystrophin, will help the accurate diagnosis of muscular dystrophies.

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References