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Restoration of dystrophin-associated proteins in skeletal muscle of mdx mice transgenic for dystrophin gene

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Duchenne muscular dystrophy (DMD) patients and mdx mice are characterized by the absence of dystrophin, a membrane cytoskeletal protein. Dystrophin is associated with a large oligomeric complex of sarcolemmal glycoproteins, including dystroglycan which provides a linkage to the extarcellular matrix component, laminin. The finding that all of the dystrophin-associated proteins (DAPs) are drastically reduced in DMD and mdx skeletal muscle supports the primary function of dystrophin as an anchor of the sarcolemmal glycoprotein complex to the subsarcolemmal cytoskeleton. These findings indicate that the efficacy of dystrophin gene therapy will depend not only on replacing dystrophin but also on restoring all of the DAPs in the sarcolemma. Here we have investigated the status of the DAPs in the skeletal muscle of mdx mice transgenic for the dystrophin gene. Our results demonstrate that transfer of dystrophin gene restores all of the DAPs together with dystrophin, suggesting that dystrophin gene therapy should be effective in restoring the entire dystrophin–glycoprotein complex.

Duchenne muscular dystrophy; Mdx mouse; Transgenic mouse; Dystrophin; Dystrophin–glycoprotein complex; Dystrophin-associated protein

1. INTRODUCTION

The absence of dystrophin, a membrane cytoskeletal protein, is the primary cause of Duchenne muscular dystrophy (DMD) and mdx mice [1,2]. Dystrophin is associated with a large oligomeric complex of sarcolemmal glycoproteins [3–6], including dystroglycan which binds laminin, a major protein component of the extracellular matrix [7]. Although dystrophin constitutes only 0.002% of the total skeletal muscle [1], it constitutes 2% of total sarcolemmal proteins and 5% of sarcolemmal cytoskeletal proteins [8,9]. Dystrophin also interacts with F-actin [10–12]. Thses findings indicate that the dystrophin–glycoprotein complex (DGC) is a major trans-sarcolemmal structure which provides a linkage between the subsarcolemmal cytoskeleton and the extracellular matrix.

In DMD patients and mdx mice, the absence of dystrophin leads to a drastic reduction in all of the dystrophin-associated proteins (DAPs) in the sarcolemma, thus causing the disruption of the linkage between the subsarcolemmal cytoskeleton and the extracellular matrix [4,7,13,14]. This is presumed to cause the sarcolemmal instability, which, in turn, may render muscle fibers susceptible to necrosis [13,14]. The significant role of the dysfunction/disruption of the DGC in the molecular pathogenesis of muscular dystrophies is exemplified by our recent finding that the specific deficiency of the 50 kDa dystrophin-associated glycoprotein (50DAG) alone causes a severe autosomal recessive DMD-like muscular dystrophy [15]. These findings indicate that the efficacy of dystrophin gene therapy will depend not only on replacing dystrophin but also on restoring all of the DAPs in the sarcolemma of DMD patients.

In the present study, we investigated the status of the DAPs in the skeletal muscle of mdx mice transgenic for the dystrophin gene, in order to know if the DAPs are restored properly in the sarcolemma when dystrophin is expressed. We performed the immunohistochemical and immunoblot analyses of these proteins in the large-caliber skeletal muscles of transgenic mdx mice, since the status of the DGC is well characterized in these muscles of mdx mice [13]. The analysis of the phenotype of the transgenic mdx mice, including the histological study of various muscles, and the analysis of dystrophin expression in various tissues are reported in detail elsewhere (C.C. Lee et al., submitted for publication).

2. MATERIALS AND METHODS

A vector based on the muscle creatine kinase (MCK) promoter [16] was generated to drive the full-length dystrophin cDNA in transgenic mice (C.C. Lee et al., submitted for publication). The MCK promoter construct (pCCLMCK-II) was constructed to contain the E1 and E2 enhancers, and the 14 kb dystrophin cDNA was cloned into this vector to generate pCCLMCK-DMD. Using the restriction enzymes *Sal*I

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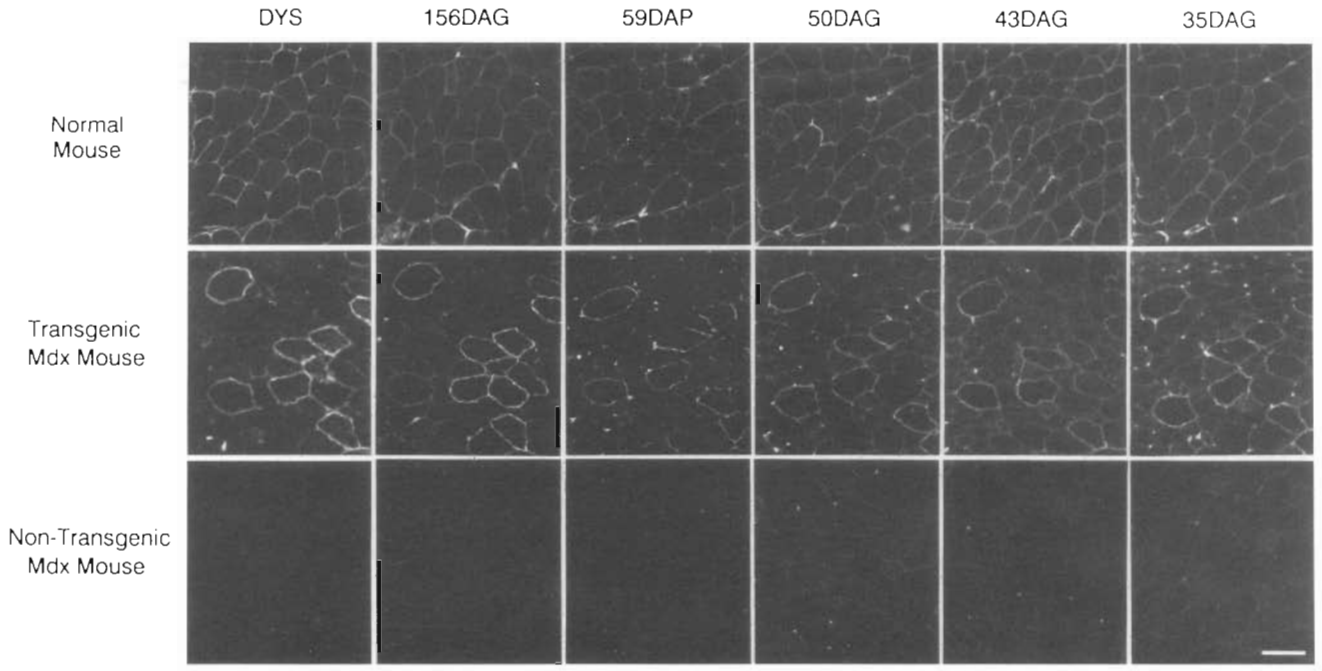

Fig. 1. Immunohistochemical analysis of dystrophin and the DAPs in the quadriceps muscle from a 15-week-old normal mouse, mdx mouse transgenic for the dystrophin gene and non-transgenic mdx sibling. Bar, 100 μm.

and *Not*I, the MCK-DMD construct was excised from the plasmid sequence, gel electrophoresis separated and purified for microinjection into mouse embryos. Microinjection of FVB mouse embryos with MCK-DMD construct DNA generated a male transgenic mouse with this construct as determined by Southern blotting. Mdx transgenic mice were generated by breeding this male transgenic animal with mdx females to produce F1 transgenic and non-transgenic mdx male progeny. Sibling mdx male progeny of transgenic and non-transgenic phenotype derived from this breeding scheme were analyzed in this study.

Immunohistochemistry of the skeletal muscle (quadriceps and gastrocnemius) was performed as described previously [8,13,14,17]. Serial transverse cryosections (7 μm) were immunostained with affinity-purified rabbit polyclonal antibody against the last 10 amino acids of the C-terminus of dystrophin, affinity-purified rabbit polyclonal antibody against the last 12 amino acids of the C-terminus of dystrophin-related protein and affinity-purified sheep polyclonal antibodies against the 156 kDa dystroglycan (156DAG), the 59 kDa dystrophin-associated protein (59DAP), the 50DAG, the 43 kDa dystroglycan (43DAG) and the 35 kDa dystrophin-associated glycoprotein (35DAG) [4,5,7,8,13,14,15,17].

Skeletal muscle membranes prepared from back and limb muscles and/or SDS extracts of skeletal muscle cryosections were separated on 3–12% SDS-PAGE and immunoblotted as described previously [13,15]. Immunoblots were stained with affinity-purified rabbit polyclonal antibody against the last 10 amino acids of the C-terminus of dystrophin, monoclonal antibody against the 156DAG (IIH6), and a cocktail of affinity-purified sheep polyclonal antibodies against the 59DAP, 50DAG, 43DAG and 35DAG as described previously [13,15].

For both immunohistochemical and immunoblot analyses, muscles from transgenic mdx mice were always compared with those from age-matched normal mice and non-transgenic mdx siblings simultaneously.

3. RESULTS AND DISCUSSION

Southern analysis of the founder transgenic mouse has revealed the presence of a single copy transgene integration since a unique restriction enzyme band was observed with several enzymes on Southern blotting when probed with the 3′ MCK-DMD DNA fragment (not shown). The size of restriction enzyme bands from several of these enzymes was incompatible with a tail to tail in tandem integration. These results indicated that

Table I

Restoration of dystrophin and the DAPs in the skeletal muscle of mdx mice transgenic for the dystrophin gene.

	Immunohistochemistry	Immunoblotting
Age (Weeks)	Muscle Fibers Positive for Dystrophin and DAPs (%)[a]	Dystrophin (%)[b] /156DAG (%)[c]
3	25	15/40
15	14	12/35
30	Not Determined	11/37
40	92	100/96
45	47	44/58

[a]The prevalence of muscle fibers with positive staining for dystrophin and the DAPs. A total of 500 to 1,000 muscle fibers were counted for each mouse.

[b] Percentage of the amount of dystrophin compared with normal mouse (100%) as determined by the densitometry of the autoradiograms of the immunoblots of the SDS extracts of the skeletal muscle (3-week- and 15-week-old mice) or the skeletal muscle membranes (other mice). Dystrophin was not detected in the non-transgenic mdx siblings.

[c] Percentage of the amount of the 156DAG compared with normal mouse (100%) as determined by the densitometry of the autoradiograms of the immunoblots of the SDS extracts of the skeletal muscle (3-week- and 15-week-old mice) or the skeletal muscle membranes (other mice). Non-transgenic mdx siblings had the 156DAG of 10–20% of the normal level.

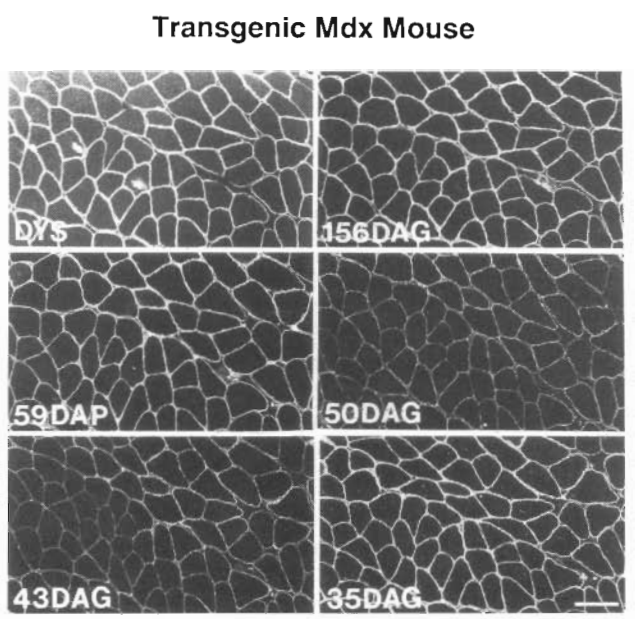

Fig. 2. (A) Immunohistochemical analysis of dystrophin and the DAPs in the quadriceps muscle from a 40-week-old mdx mouse transgenic for the dystrophin gene. Bar, 100 μm.

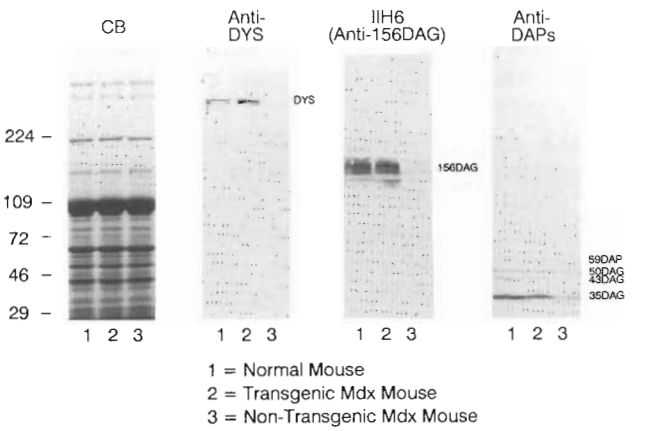

Fig. 2. (B) Immunoblot analysis of dystrophin and the DAPs in the skeletal muscle membranes from 40-week-old normal mouse (lane 1), mdx mouse transgenic for the dystrophin gene (lane 2) and non-transgenic mdx sibling (lane 3). One hundred and 400 μg of membranes were loaded for Coomassie-blue staining of the gel and immunoblotting, respectively. Molecular weight standards ($\times 10^{-3}$) are shown on the left.

the transgenic animal had a unique integration site with a single copy gene.

Immunostaining for dystrophin expression in these F1 progeny has revealed significant variation of dystrophin expression in different progeny derived from this one founder (Table I). Variation is also observed within different muscle fibers from different regions of the animal (Table I, Figs. 1 and 2a). It is highly possible that this variation in expression is inherent to the pCCLMCK-II construct since it may lack certain cis-acting elements for ubiquitous expression as demonstrated for the globin locus [18]. The variation of transgene expression in various progeny derived from a single founder may be due to the outbred genetic background of the progeny from breeding FVB founder mouse with C57 black mdx female mouse. Variation in progeny genetic background from such a breeding strategy may contribute to the differences observed in transgene expression since a strain-specific modifier [19] has been described to regulate transgene expression.

In order to investigate the status of the components of the DGC in the skeletal muscle of mdx mice transgenic for the dystrophin gene, we performed the immunohistochemical analysis of dystrophin and the DAPs. As controls, we investigated both age-matched normal mice and non-transgenic mdx siblings simultaneously. As described previously [13], dystrophin and all of the DAPs were co-localized to the sarcolemma in normal mice, while dystrophin was absent and all of the DAPs were drastically diminished in the sarcolemma of non-transgenic mdx siblings (Fig. 1). In order to show the specificity of the restoration of the DAPs in mdx mice transgenic for the dystrophin gene, we investigated transgenic mice expressing dystrophin at various levels (range 14–92%, Table 1). As shown in Figs. 1 and 2a, muscle fibers expressing dystrophin along the sarcolemma were found in transgenic mice. Examination of the serial transverse cryosections revealed that all of the DAPs were not only restored but also co-distributed with dystrophin in the sarcolemma of dystrophin-positive fibers (Figs. 1 and 2a). Dystrophin-related protein (DRP), an autosomal homologue of dystrophin [17,20], did not co-localize with dystrophin and the DAPs in these fibers (not shown).

Restoration of dystrophin and the DAPs in the skeletal muscle of transgenic mdx mice was confirmed by the immunoblot analysis of the skeletal muscle membranes (Fig. 2b) and/or the SDS extracts of the skeletal muscle (not shown) (Table I). While dystrophin was absent and the DAPs were drastically reduced in the skeletal muscle of non-transgenic mdx siblings as reported previously [13], dystrophin and the DAPs were restored to various degrees in the skeletal muscle of transgenic mdx mice (Fig. 2b, Table I). The level of restoration of these proteins on immunoblot analysis showed a good correlation with that on immunohistochemistry (Fig. 2, Table I).

In mdx mice, a very small percentage of muscle fibers (less than 1 to 2%) are reported to show dystrophin staining along the sarcolemma [21]. Somatic reversion or suppression of mdx mutation in vivo was proposed as an explanation for these rare fibers [21]. However, a possibility that the present findings represent this phenomenon rather than the effect of transfection of the dystrophin gene is negated based on the following: (1) in non-transgenic mdx siblings, dystrophin-positive muscle fibers were quite rare (less than 1%) on immu-

nohistochemistry and dystrophin was not detected on immunoblot analysis; (2) the prevalence of dystrophin-positive muscle fibers in transgenic mdx mice was high (Table I).

Thus, we have demonstrated the co-restoration of dystrophin and all of the DAPs in the sarcolemma of mdx mice transfected with the dystrophin gene. Most importantly, dystroglycan (156DAG/43DAG) which provides a linkage to the extracellular matrix and the 50DAG whose deficiency alone causes severe childhood autosomal recessive muscular dystrophy with DMD-like phenotype (SCARMD) were restored together with dystrophin, 59DAP and 35DAG.

Previously, we have demonstrated that the absence of dystrophin leads to the deficiency in all of the other components of the DGC in DMD and mdx skeletal muscle. In SCARMD, the deficiency of the 50DAG is presumed to lead to the secondary reduction of the 35DAG. There are a few genetic diseases in which a deficiency of one component in a protein complex can result in the loss of the other components. For instance, hereditary elliptocytosis, characterized by a defect in spectrin, is also associated with deficiencies in protein-4.1 and minor sialoglycoproteins [22]. Skeletal muscle phosphorylase kinase deficiency is characterized by the combined loss of all four subunits of this enzyme [23]. However, the present study is the first to demonstrate that transfer of the causative gene restores not only its protein product but also the other components of the complex.

Recently, we have identified a single gene encoding both the 156DAG and 43DAG, and have demonstrated that the level of expression of dystroglycan (156DAG/43DAG) mRNA is indistinguishable between normal and mdx mice [7]. These findings suggested that these DAPs were produced at the normal level, but were not properly assembled and/or integrated into the sarcolemma or were degraded, in the absence of dystrophin in mdx mice [7]. Thus, it is most likely that dystrophin facilitates the assembly of the DAPs into the sarcolemma and/or stabilizes them in the sarcolemma in transgenic mdx mice.

Currently, research is in progress on dystrophin gene therapy. However, recent discoveries on the role of the DAPs in the molecular pathogenesis of DMD raise serious questions concerning the efficacy of dystrophin gene therapy [24–27]. In order for this therapy to be successful, the entire DGC will have to be restored in DMD muscle. Thus, the goal of dystrophin gene therapy must be the replacement of dystrophin and the restoration and stabilization of the DAPs, in order to restore the normal linkage between the subsarcolemmal cytoskeleton and the extracellular matrix. The observation of dystrophin along the sarcolemma after such therapies cannot indicate the restoration of all of the DAPs in the sarcolemma, because dystrophin could be properly localized to the sarcolemmal region due to its association with other subsarcolemmal cytoskeletal components such as g-actin even when it is not associated with the DAPs. Indeed we have demonstrated recently that dystrophin is properly localized to the SCARMD sarcolemma in which the deficiency of the 50DAG causes a reduction of the 35DAG [15]. We have also found that truncated dystrophin lacking the C-terminal domains is properly localized to the sarcolemma even though it is not associated with the DAPs (K. Matsumura, personal communication) and that dystrophin is localized to the sarcolemma in Fukuyama-type congenital muscular dystrophy in which the DAPs are reduced in the sarcolemma [28]. Thus, our results demonstrating the co-restoration of dystrophin and all of the DAPs in the sarcolemma of a substantial percentage of muscle fibers in transgenic mdx mice answer this crucial question and indicate that dystrophin gene therapy could be effective.

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