Exogenous Dp71 restores the levels of dystrophin associated proteins but does not alleviate muscle damage in *mdx* mice

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Dp71 is a non-muscle product of the Duchenne muscular dystrophy gene. It consists of the cysteine-rich and C-terminal domains of dystrophin. We have generated transgenic *mdx* mice which do not have dystrophin but express Dp71 in their muscle. In these mice, Dp71 was localized to the plasma membrane and restored normal levels of dystrophin associated protiens (DAPs), indicating that Dp71 is capable of interacting with the DAPs in a similar manner to dystrophin. However, the presence of Dp71 and DAPs in the muscle fibres of *mdx* mice was not sufficient to alleviate symptoms of muscle degeneration.

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Duchenne muscular dystrophy (DMD) is a lethal X-linked degenerative disorder of muscle (reviewed in ref. 1). The 427 kDa protein which is deficient in DMD, dystrophin, is expressed primarily in muscle and the nervous system (reviewed in ref. 2). It contains four distinct domains: an N-terminal actin binding domain, a large region of 24 spectrin-like triple helix repeats, a cysteine-rich domain containing two potential Ca^{++} binding sites and a C-terminal region. In muscle, dystrophin is found under the sarcolemma and has been shown to bind to a complex of membrane proteins (known as the dystrophin-associated proteins or DAPs) some of which are glycosylated (dystrophin-associated glycoproteins or DAGs[3–5]). The large dystrophin-DAP complex is believed to link the intracellular cytoskeleton, the sarcolemma and the extracellular matrix. DMD patients and *mdx* mice (a mouse strain lacking dystrophin), have reduced levels of DAPs in skeletal muscle[3,6]. This may be due to a destabilization of the complex in the absence of dystrophin. It has recently been shown that the absence of one of the DAPs, (50 DAG or adhalin), is associated with a rare form of severe childhood autosomal recessive muscular dystrophy (SCARMD), very similar to DMD[7]. The affected children have, however, normal levels of dystrophin associated with the sarcolema. This raised the possibility that the pathology of DMD is due to the reduced levels of DAPs in the sarcolema of DMD patients.

Dystrophins are encoded by an extremely large and complex gene, spanning more than 2.5 megabases (Mb). Five promoters, which regulate the expression of at least three isoforms of dystrophin and two other smaller proteins have been identified so far[2,8]. One of these smaller products is Dp71, a 71 kDa protein which contains the cysteine-rich and C-terminal domains of dystrophin and a short unique amino terminus[9–13]. The large domain of spectrin-like repeats and the N-terminus actin-binding domain are not present in Dp71. This protein is of special interest since it is found in most or all non-muscle tissues

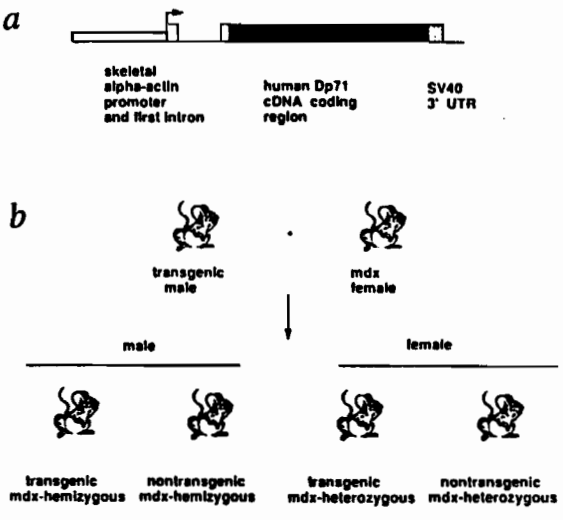

Fig. 1 *a*, Schematic representation of the DNA construct introduced into fertilized oocytes. The thin empty box represents the skeletal actin promoter region; thick boxes, exons; and line, skeletal actin first intron. The black box represents the Dp71 cDNA and the hatched box, the SV40 3' region. *b*, Scheme of the four genotypes of animals generated and used in this study. Because of the X-linked nature of the gene, only the male hemizygous mice had a *mdx* phenotype. One half of all offspring were transgenic.

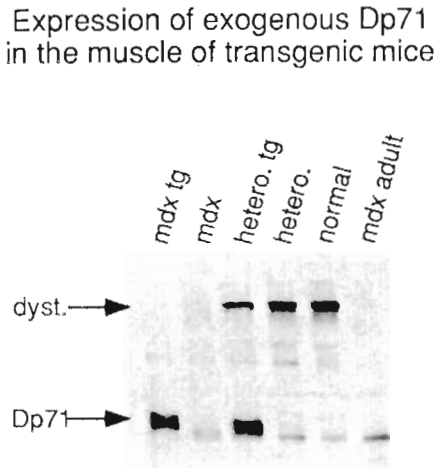

Fig. 2 Western blot analysis of Dp71 and dystrophin in skeletal muscle of 4–8-week-old transgenic mice. Whole muscle extracts were size fractionated, blotted and stained with mAb MANDRA1. mdx tg, *mdx* transgenic mice; *mdx*, male mice with the *mdx* phenotype; hetero, females heterozygous for the *mdx* phenotype; and normal, CD1 control. The position of dystrophin (dyst) and Dp71 fractions are shown.

and is the most abundant known DMD gene product in the brain. It is detectable during early mouse development and in undifferentiated embryonic stem (ES) cells[14]. Like dystrophin, Dp71 has been shown to be associated with the plasma membrane[15]. Dp71 is absent or barely detectable in skeletal muscle[10,13]. Its function is unknown and it is unclear whether it binds to the DAPs, most of which are also widely expressed in many non-muscle tissues and cell types.

To elucidate the molecular interactions of Dp71 *in vivo*, and to test whether Dp71 can functionally replace dystrophin in muscle, we generated transgenic *mdx* mice in which Dp71 is expressed in the skeletal muscle. We found that in these mice Dp71 is associated with the plasma membrane and is capable of restoring normal levels of the DAPs, indicating that Dp71 can associate with the cell membrane in a manner similar to dystrophin. However, Dp71 expression and the restoration of normal levels of the DAPs is not sufficient to alleviate the pathology in the muscle of these mice.

Ectopic expression of Dp71 in transgenic mice

A chimaeric gene construct containing the coding region of human Dp71 (ref. 10) under the control of a skeletal muscle actin promoter, was microinjected into fertilized mouse oocytes (Fig. 1*a*). One hundred offspring were analysed and one was found to contain 2–3 copies of the integrated transgene. With other genes we normally find the transgene in about 15–25% of mice born from injected fertilized oocytes. The reason for the low rate of transgenics obtained with the Dp71 transgene is unknown. It is possible that overexpression of the exogenous Dp71 has a deleterious effect on embryo development. Transgenic male offspring were crossed with *mdx* females to produce *mdx* mice carrying the transgene (Fig. 1*b*).

The expression of the transgene in skeletal muscle tissue was analysed by western blotting using the monoclonal antibody MANDRA1, that recognizes the C-terminal region of dystrophin[16] and therefore also reacts with Dp71. Results showed that exogenous Dp71 is expressed in skeletal muscle; the level of Dp71 being aproximatily equal to that of dystrophin in the transgenic heterozygous females (Fig. 2).

Exogenous Dp71 associates with the sarcolemma

To examine the localization of the exogenous Dp71, frozen skeletal muscle tissue from transgenic *mdx* mice was sectioned and stained with MANDRA1 and a rhodamine labelled second antibody. Dp71 was found to be localized to the plasma membrane (data not shown), confirming our previous results that showed that the cysteine-rich and C-terminal domains are sufficient for membrane localization in muscle and non-muscle cells[15]. Identical results were obtained using polyclonal antibodies against the C-terminus of dystrophin (Fig. 3, C-DYS). It is important to note that unlike dystrophin, the exogenous Dp71 was not found in the microsomal fraction, suggesting a looser association of this protein with the membrane than that of endogenous dystrophin (data not shown).

Restoration of normal levels of DAPs

Since the levels of the DAPs which complex with dystrophin are reduced significantly in muscle lacking dystrophin, we examined the effect of the presence of exogenous Dp71 on the levels of DAPs in the skeletal muscle of *mdx* mice. Immunostaining of muscle sections with antibodies directed against three of the dystrophin associated proteins — 59 DAP, 50 DAG and 43 DAG (α dystroglycan) — showed that, as reported, non-transgenic *mdx* mice had greatly reduced levels of these proteins in the membrane. In contrast, transgenic *mdx* mice which contain Dp71 in the muscle, had normal levels of the three DAPs tested

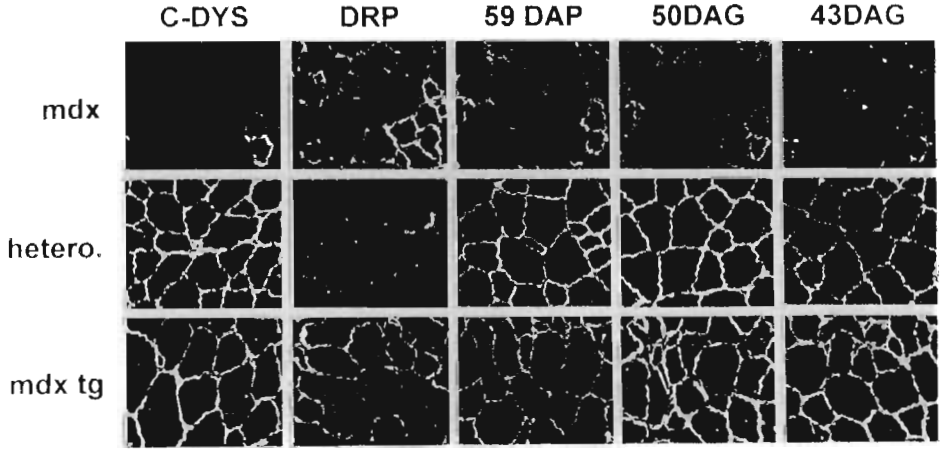

Fig. 3 Immunolocalization of dystrophin/Dp71 (C-DYS), DRP and DAPs in *mdx* (mdx), heterozygous (hetero.) and transgenic *mdx* (mdx tg) skeletal muscle. Transverse skeletal muscle sections were labelled by indirect immunofluoresence with affinity purified antibodies against the C-terminus of dystrophin (C-DYS), DRP, 59 DAP, 50 DAG and 43 DAG.

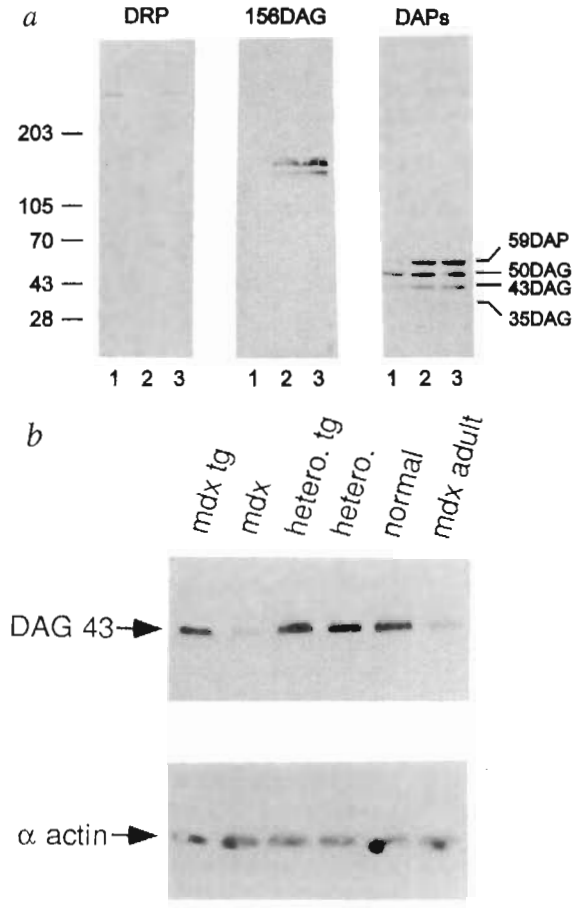

Fig. 4 *a*, Western blot analysis of DRP and DAPs in the skeletal muscle of six-week-old mice. Membrane enriched fractions were immunoblotted and identical blots were stained with rabbit polyclonal antibody against DRP, a monoclonal antibody IIH6 against 156 DAG or a mixture of anti-dystrophin-associated protein antibodies.1, male mice with the *mdx* phenotype; 2, female heterozygous for the *mdx* phenotype; 3, transgenic *mdx* mice. *b*, western blot analysis of 43 DAG in the skeletal muscle of 4–8-week-old or six-month-old (adult) mice. Muscle samples were prepared and fractionated as described for Fig. 2 and in the Methodology. The blot was stained with the mAb α43, and stripped and reprobed with an anti skeletal muscle actin mAb as a loading control. Lanes are as in Fig. 2. The positions of DAG 43 and α actin are shown.

(Fig. 3). Western blot analysis of the microsomal fraction using the same antibodies, and an antibody against 156 DAG (β dystroglycan) (Fig. 4*a*) and western blot analysis of total muscle protein (Fig. 4*b*) using an anti 43-DAG monoclonal antibody confirmed these results. These results suggest that in the muscles of the transgenic animals, Dp71 interacts with the DAPs in the plasma membrane and that this association is sufficient to restore normal levels of all the DAPs, probably through a stabilization of the proteins when bound in a complex with Dp71.

It has been shown that the amount of dystrophin related protein (DRP) is elevated in the muscle of DMD patients and *mdx* mice[17]. Interestingly, the expression of Dp71 in the muscles of transgenic mice did not cause a reduction in the level of DRP to that seen in normal mice and in heterozygous females(Figs 3 and 4*a*).

DAPs do not alleviate muscle damage

In order to investigate whether the restoration of the DAPs to normal levels in the transgenic *mdx* mice was sufficient to alleviate the symptoms of muscle degeneration we analysed serum creatine kinase (CK) levels in these mice. *Mdx* mice have very elevated CK serum levels, similar to those seen in DMD patients. Figure 5 shows that the CK levels in the transgenic *mdx* mice are nearly the same as in their non-transgenic *mdx* littermates. The CK level in the serum of the heterozygous females was not significantly higher than that of non-transgenic mice. Histological examination of the diaphragms and thigh muscles of *mdx* transgenic mice showed areas of extensive degeneration and regeneration similar to or even greater than that seen in nontransgenic *mdx* mice. The significance of the apparent enhanced muscle damage in transgenic *mdx* mice needs further investigation. Finally, many nuclei in the muscle of the transgenic mice were in a central, rather than in the normal peripheral, localization (Fig. 6) a sign of extensive muscle fibre regeneration which is seen in the fibres of DMD patients and *mdx* mice.

Discussion

Dystrophin is the main product of the DMD gene in muscle cells. Several non-muscle products of the DMD gene have also been identified. However, while the function of the muscle dystrophin isoform is at least partially understood the role of the other DMD gene products is completely unknown.

Dp71 is a 71 kDa protein containing the cysteine rich and C-terminal domains of dystrophin[10]. It is expressed in a wide range of non-muscle tissues including brain and liver and also in heart and smooth muscle. It shares with dystrophin the region which has been mapped as the region important for binding to the DAP complex[18]. However whether Dp71 can interact with the intact DAP complex was not known although it has been shown[19] that Dp71 interacts with a 58 kDa protein (syntrophin) which may be identical with 59 DAP. In this communication we have shown that exogenous Dp71 is able to restore normal levels of all of the DAPs in the muscles of transgenic *mdx* mice, indicating that Dp71 is capable of binding to and stabilizing the DAP-complex. Since Dp71 and some of the DAPs are expressed in many cell types and tissues it is conceivable that Dp71-DAP association occurs normally in non-muscle cells. The function of this complex in those cell types is as yet unknown.

It was previously shown that the cloned Dp71 cDNA lacks the dystrophin exons 71 and 78 and that, due to a frame shift caused by alternative splicing, the last C-terminal 13 amino acids of dystrophin are replaced by a unique 31 amino acid sequence which is not found in muscle dystrophin[10]. Cox *et al.*[20] have generated transgenic mice containing an exogenous Dp71 like transgene with a construct derived from dystrophin cDNA and therefore containing exons 71 and 78 encoding the 13 amino acid C-terminus of dystrophin. They obtained results similar to ours with regards to the association of Dp71 to the sarcolemma and restoration of normal levels of DAPs, indicating that the alternative splicing variations of the C-terminal do not affect the ability of the protein to associate with the DAPs.

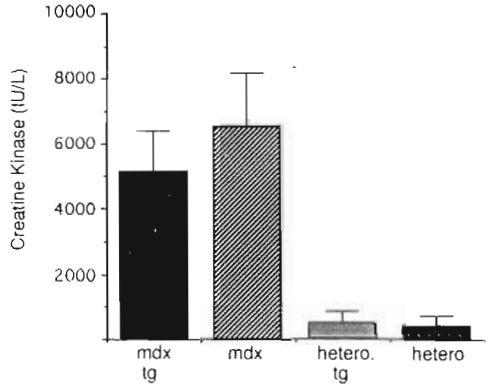

Fig. 5 Creatine kinase levels in transgenic mice. Blood samples were drawn from the tails of 4–8-week-old mice and serum prepared. Each point represents the mean CK activity of samples from six mice. Column abbreviations are as given in Fig. 2.

The restoration of normal levels of the DAPs in transgenic *mdx* mice expressing Dp71 in muscle did not alleviate the pathology of the disease. It therefore seems that an intact DAP complex is required but not sufficient for normal muscle function. Muscle damage does occur even in the presence of normal levels of DAPs. It would seem that in addition to the DAP complex a functional dystrophin protein containing the actin binding domain and/or rod domain is also crucial for muscle integrity.

Since it has been shown that deletion of a large part of the rod domain of dystrophin in human patients may only cause a very mild dystrophic phenotype[21], it is likely that the actin binding domain is crucial for function of the protein. This is consistent with the identification of DMD patients which have in frame deletions resulting in the absence of the actin binding region. These patients have severe Duchenne muscular dystrophy even though dystrophin was localized to the membrane and there is an intact C-terminal and cystein rich region[22–24]. Recently a case was described where a single amino acid substitution in the actin binding domain resulted in a DMD phenotype[25].

It has been shown that the extracellular protein, merosin (muscle laminin) binds to the dystrophin-DAPs complex in muscle[26] and recently it was shown that merosin is absent in *dy/dy* mice[27]. These mice display a severe dystrophic syndrome with some similarities to DMD even though levels of dystrophin and the DAPs in these mice are normal. In SCARMD, the absence of 50 DAG is associated with muscle degeneration despite the presence of normal levels of dystrophin. The work described here shows that the absence of the actin binding domain and/or the spectrin like domain of dystrophin causes muscle degeneration even in the presence of normal levels of DAPs. It therefore seems that the absence or misfunction of any component of the normal anchorage between the cytoskelaton and the extracellular matrix, (that is, dystrophin-DAPs-merosin) can destabilize the sarcolemma and induce severe muscle damage.

Methodology

Plasmid constructs and microinjection. Plasmid constructs were made as described in Sambrook *et al.*[28]. A 1.8 kb fragment of the rat skeletal muscle actin gene containing 1.2 kb of promoter, the first exon, first intron and 25 bp of the second exon was placed upstream to a 2.2 kb human cDNA clone containing the entire coding region of the human Dp71 cDNA and 190 bp of the 3' untranslated region. Three prime to the Dp71 cDNA a 339 bp fragment containing the SV40 3'UTR was added. Vector sequences were removed and the insert was microinjected into (BALB\cj × C57BL\6) F1 fertilized oocytes as described in Hogan *et al.*[29]. Oocytes were transferred into CD1 females and positive transgenic animals were identified by Southern blotting of DNA from tail biopsies, as described[28].

Preparation of muscle extracts and western blot analysis. Total muscle extracts were prepared by homogenization of 250 mg of tissue in 5 ml of electrophoresis sample buffer. Microsomal fractions were prepared from skeletal muscle as described[4,6,30]. Western blot analysis of total muscle extracts was done using 3–

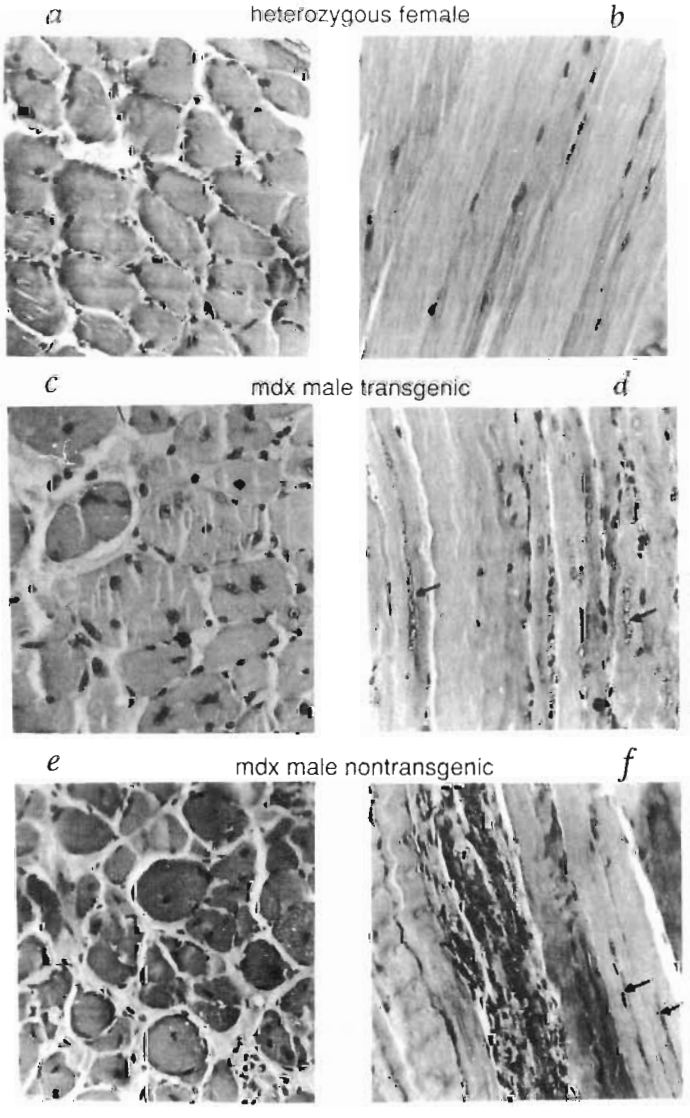

Fig. 6 Histological sections of six-week-old mice. Tissues were fixed and stained with eosin and heamotoxylin. *a*, *c* and *e*, Cross sections of diaphragm. *b*, *d* and *f*, Transverse sections of thigh muscles. Arrows in *d* and *f* mark the position of rows of centrally located nuclei. Magnification, 250×.

10% polyacrylamide/SDS gradient gels as described by Pons *et al.*[17]. Western blot analysis of microsomal fractions was done with 800 µg of total microsomes separated on a 3–12% polyacrylamide/SDS gel in the presence of 1% 2-mercaptoetanol and transferred to nitrocellulose. Detection of the immunoreactive bands was done using an ECL kit (Amersham).

Antibodies. MANDRA1 is a monoclonal antibody which recognizes the C-terminal domain of human dystrophin[16]. α43 is a monoclonal antibody against 43DAG . Monoclonal antibody IIH6 against α-dystroglycan (156DAG) was characterized previously[4]. Specific antibodies against the dystrophin-glycoprotein complex were raised in sheep using the purified dystrophin-glycoprotein complex, and antibodies against individual components of the complex were affinity-purified as described[29]. Affinity-purified rabbit antibodies against the C-terminal of DRP, 50 DAG (adhalin) and affinity purified sheep polyclonal antibody specific for the dystrophin c-terminus (C-DYS), were characterized previously[31,32]. Anti-α-sarcomeric actin is a monoclonal antibody specific for skeletal muscle actin(Sigma).

Immunocytochemistry and histology analysis. Skeletal muscle tissues were immersed in Tissue-tek (Miles) and frozen in liquid nitrogen cooled isopentane. Indirect immunofluoresence microscopy of 7µm-thick transverse cryosections of skeletal muscle was performed as described[33]. Sections were immunostained with affinity-purified sheep polyclonal antibodies specific for dystrophin C-terminal domain, 59 DAP, β-dystroglycan (43 DAG)[4] or rabbit polyclonal antibodies against DRP or adhalin (50DAG).

Creatine kinase assay. Creatine kinase was analysed from blood from tail bleeds of four-week-old mice. Serum was prepared and the CK activity determined using a Trace-CK-activity kit according to the manufacturers instructions (Trace Scientific, Australia).

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