Independent localization of dystrophin N- and C-terminal regions to the sarcolemma of *mdx* mouse myofibres in vivo

Matthew G. Dunkley1, Kim E. Wells2, Tony A. Piper1, Dominic J. Wells3 and George Dickson1,*

1Department of Experimental Pathology, UMDS, Guy’s Hospital, London SE1 9RT, UK
2Transgenic Research Unit, Rayne Institute, St Thomas’s Hospital, London SE1 7EH, UK
3Department of Veterinary Basic Sciences, The Royal Veterinary College, London NW1 0TU, UK

*Author for correspondence

SUMMARY

Dystrophin has been proposed to associate with the skeletal muscle membrane by way of a glycoprotein complex that interacts with its C-terminal domains. Transfection of *mdx* mouse myotubes in culture or myofibres in vivo with recombinant genes encoding human dystrophin deletion mutants shows, however, that not only the C terminus of dystrophin but also its N-terminal actin-binding domain can locate independently to the muscle sarcolemma. This observation suggests that lack of sarcolemma-associated dystrophin in Duchenne muscular dystrophy (DMD) muscle may result from enhanced degradation of truncated mutation products rather than their inability per se to associate with the sarcolemma.

Key words: dystrophin, sarcolemma, *mdx* mouse, gene transfer

INTRODUCTION

The structural and cellular biochemistry of skeletal muscle dystrophin has been extensively studied in recent years (Anderson and Kunkel, 1992; Ahn and Kunkel, 1993), and this molecule has been clearly established as one component of the so-called costamere lattice that forms part of the cortical cytoskeleton of the mature muscle fibre (Ervasti and Campbell, 1993a; Porter et al., 1992; Straub et al., 1992). Structural predictions based on amino acid sequence (Koenig et al., 1988; Davison and Critchley, 1988; Koenig and Kunkel, 1990; Cross et al., 1990), and biochemical investigations with recombinant and proteolytic polypeptide fragments (Hemmings et al., 1992; Way et al., 1992; Suzuki et al., 1992; Levine et al., 1992) indicate that the dystrophin molecule has four distinct domains: an N-terminal domain that can bind actin filaments; a series of α-helical repeats forming the central rod domain; and two globular C-terminal domains involved in interaction with a membrane-associated glycoprotein complex (DGC; Ervasti and Campbell, 1993b).

Early hypotheses that the C-terminal domains of dystrophin were uniquely involved in its association with the plasma membrane arose initially from clinical observations of the absence of dystrophin expression in the sarcolemma of DMD patients with deletions eliminating this region (Hoffman et al., 1988; Malhotra et al., 1988; Wessel, 1990). Furthermore, direct biochemical evidence has clearly demonstrated that at least one wheat germ agglutinin (WGA) binding component of the DGC interacts with the cysteine-rich C-terminal region of dystrophin (Suzuki et al., 1992). However, more recent clinical reports have described examples of mutant dystrophin molecules lacking C-terminal domains yet retaining the ability to localize to the myofibre membrane (Hoffman et al., 1991; Helliwell et al., 1992; Récan et al., 1992). It therefore appears likely that variable metabolic instability of truncated polypeptides or mRNAs may be a major contributing factor in whether these mutant dystrophins are observed at the sarcolemma or not. Furthermore, it is feasible that by interaction with specific β- or α-actin isoforms in the cortical cytoskeleton underlying the myofibre plasma membrane (Ervasti and Campbell, 1993a), the actin-binding domain at the dystrophin N terminus may itself be able to interact directly with the sarcolemma.

In order to begin to address these possibilities we have directly expressed recombinant polypeptide fragments corresponding to N- and C-terminal regions of human dystrophin in *mdx* mouse myotubes in culture, and in mature myofibres in vivo. We report here that high level expression of these gene constructs results in truncated N- and C-terminal products that can both localize independently to the muscle sarcolemma.

MATERIALS AND METHODS

Gene constructions

The construction of cDNAs encoding full-length normal human dystrophin (pDMD1), and also a much smaller centrally-deleted BMD-based molecule (pBN1) have been described elsewhere (Dickson et al., 1991; Acsadi et al., 1991; Dunckley et al., 1992). To prepare a cDNA corresponding to the C-terminal region of the deleted BMD molecule, a BstEII (blunted)-SalI fragment of plasmid pMINI-1B (a gift from Dr Don Love and Prof. Kay Davies) was cloned into the SnaBl-SalI sites of the pBabeNeo vector (Morgenstern and Land, 1990a) to yield plasmid pBN-CT3. In this case, the first initiation
codon encountered in the cDNA is the in-frame methionine codon at amino acid 2509 in the published dystrophin sequence (Koenig et al., 1988), and corresponds to exon 51, which encodes the 3rd hinge region of the dystrophin molecule (Roberts et al., 1993). For expression of the N-terminal region of the dystrophin molecule, the EcoRI insert of pCf27 (Smith et al., 1987; Dickson et al., 1991) was cloned into the EcoRI site of pBabeNeo to yield plasmid pBN-NT1. This construct encodes amino acids 1-713 of dystrophin corresponding to exons 1-17 of the gene terminating within the 2nd hinge region of the protein. In the pBabeNeo or pRSV (full-length cDNA) vectors, inserted cDNA genes are transcribed from the Moloney murine leukemia virus promoter and polyadenylated by a polypurine tract provided by the 3′LTR or SV40 sequences, respectively. For in vitro and in vivo transfection studies all plasmids were prepared in predominantly closed circular form by two sequential purifications on CsCl2 density gradients. Where appropriate, transfection and microinjection procedures were positively-controlled using either plasmids pCH110 (Pharmacia) or pRSV-LacZ (Wolff et al., 1991) encoding E. coli β-galactosidase transcribed from the SV40 or Rous sarcoma virus (RSV) promoters, respectively.

**COS-7 cell transfections**

COS-7 cells were grown in DME medium containing 10% (v/v) fetal calf serum (FCS) and transfected with supercoiled plasmid DNA constructs pBN1, pBN-NT1 and pBN-CT3 described above. After 48 hours, transfected cultures were fixed for immunocytochemistry or harvested for western blot analyses as described (Barton et al., 1988; Dickson et al., 1991).

**Microinjection of cultured mdx myotubes**

Satellite cells from mdx skeletal muscle were prepared by enzymic dissociation and placed in culture on laminin-coated culture surfaces as previously described (Dunckley et al., 1992; Dickson et al., 1992). At 80% confluence myoblast cultures were transferred to DME containing 5% heat-inactivated horse serum and allowed to differentiate and fuse into myotubes, many of which showed cross-striated appearance and spontaneous contractile activity 7 days later. For expression of cDNA constructs, 10- to 14-day-old cultures were rinsed in serum-free DME medium lacking Ca2+ and Mg2+, and multiple nuclei within selected myotubes (at least 300 nuclei/culture) microinjected with approximately 0.3 pl of 200 µg/ml solutions of test cDNAs dissolved in injection buffer (137 mM KCl, 2.6 mM NaCl, 7.8 mM Na2HPO4, 2H2O, 1.4 mM KH2PO4) (Minaschek et al., 1989). An electronically regulated microinjection system (Eppendorf Micromanipulator 5171) set at an injection pressure of 200 HpA over 0.2 seconds (resistance pressure 20 HpA) was used in conjunction with a Zeiss Axiosvert 135 microscope with phase-contrast optics. After microinjection, cultures were returned to standard culture conditions for 24 hours and then processed for immunostaining or β-galactosidase histochemistry.

**Intramuscular plasmid injections**

Plasmids isolated by double CsCl2 gradient purification and estimated as over 90% supercoiled by agarose gel electrophoresis were used for transfection of mdx skeletal myofibres in vivo by direct injection of concentrated plasmid DNA (Acsadi et al., 1991; Wells and Goldspink, 1992). DNAs for injection were prepared in Dulbecco’s PBS (Ca2+- and Mg2+-free) at a concentration of 3-4 mg/ml and 100 µl injected into the left quadriceps muscles of 6-week-old male mdx mice. After 7-14 days muscle tissues were collected, including the contralateral muscle as a control, and frozen in liquid N2-cooled isopentane for cryosectioning and immunostaining, or β-galactosidase histochemistry.

**Western blotting, immunostaining analyses and β-galactosidase histochemistry**

COS-7 cell cultures (5 × 105 cells/60 cm Petri dish) were harvested and samples subjected to discontinuous SDS-PAGE on 6% gels, and western blot analyses as previously described (Wells et al., 1992). For western blotting, anti-dystrophin monoclonal antibodies 12B2, 6D3...
and 6C5, to N-terminal, central rod and C-terminal epitopes, respectively, were used (a kind gift from Dr Louise Nicholson) at dilutions of 1/50 hybridoma supernatant. For immunofluorescence staining analyses, cell cultures or 10 µm cryosections were fixed in methanol (−20°C) for 5 minutes and incubated with rabbit antibodies to either the C-terminal or N-terminal dystrophin synthetic peptides (G6 and G8, respectively; Dickson et al., 1992; Wells et al., 1992) in combination with biotinylated monoclonal antibody MANDYS-1 (Morris et al., 1993) as previously described (Dunckley et al., 1992, 1993). Dual immunodetection of rabbit and mouse antibodies was then performed with FITC sheep anti-rabbit Ig, in combination with streptavidin-Texas Red. Samples were post-fixed again in cold methanol, mounted in Citifluor (Amersham) and viewed on a Zeiss Axiophot microscope with phase-contrast and epifluorescence optics. Histochemistry for the detection of β-galactosidase was performed essentially as described (Turner and Cepko, 1987).

RESULTS

Expression of dystrophin cDNAs in COS cells

A series of dystrophin cDNA gene constructs were assembled in expression vector pJ4Ω (Morgenstern and Land, 1990b) and retroviral vector pBabeNeo (Morgenstern and Land, 1990a) utilizing the MoMuLV promoter for the transcription of inserted recombinant cDNA sequences. As shown in Fig. 1, recombinant dystrophin cDNAs were prepared encoding a BMD-based minidystrophin (construct pBN1) lacking residues 665-2299 from part of the central rod domain (England et al., 1990), as well as N-terminal (residues 1-713) and C-terminal (residues 2509-3685) constructs (pBN-NT1 and pBN-CT3, respectively). Hence, these latter two constructs closely correspond to two parts of the Becker molecule either side of the deletion breakpoint. Transient transfection into COS-7 cells resulted in expression of appropriate recombinant products of 229 and 139 kDa corresponding to the Becker-type and C-terminal truncated dystrophins, respectively (Fig. 2A). Cross-reactivity of C- and N-terminal monoclonal antibodies with (probably) myosin heavy chain at ~200 kDa is likely to be due to the extremely large amounts of this protein in muscle relative to dystrophin (0.002% of muscle protein). A faint cross-reactive band at ~145 kDa is present in all COS cell samples, including untransfected controls, but the band at 139 kDa (Fig. 2A, track 7) corresponding to the C-terminal dystrophin polypeptide is nevertheless clearly specific. In the case of the N-terminal construct, no specific product was detected in transfected COS-7 cells with antibodies 12B2 or G6, suggesting that this type of product may undergo rapid turnover as has been suggested to be the case in the mdx mouse, where a point mutation gives rise to a stop codon instead of amino acid 993 (Sicinski et al., 1989).

Expression of recombinant dystrophin domains at the sarcolemma of cultured mdx myotubes

In order to assess expression in the correct cellular environment of the differentiated muscle cell two experimental approaches were adopted, one in vitro and one in vivo. Firstly, primary cultures of mdx mouse myoblast were prepared and allowed to differentiate into fully contracting cross-striated myotubes. Multiple nuclei (~300 in total) of these dystrophin-deficient myotubes were then injected with approximately 0.1 pg of the cDNA constructs. This quantity corresponds to injection of about 1000 copies per nucleus (Minaschek et al., 1989). 24 hours later, cultures were fixed and stained for dystrophin using combinations of rabbit polyclonal antibody to either the N- or C-terminal epitopes of dystrophin (antibodies G6 and G8, respectively) in conjunction with mouse monoclonal antibody (MANDYS-1) to an epitope in the central rod domain, deleted in each of the constructs.
domain of the native molecule but absent in the various deletion constructs.

In control mdx cultures injected with a control plasmid construct encoding β-galactosidase (RSV-Lac Z; Acsadi et al., 1991) myotubes expressing β-galactosidase were observed, but none were immunoreactive for dystrophin (data not shown). However, with all four dystrophin cDNAs including the N- and C-terminal deletion constructs, expression of dystrophin immunoreactivity was clearly observed in the majority of injected myotubes with an apparent membrane-associated distribution pattern (Fig. 3).

**Expression of recombinant dystrophin domains in mdx muscle in vivo**

To further confirm the membrane expression pattern of recombinant dystrophin polypeptides observed in vitro, mdx muscle fibres were also transfected in vivo by direct intramuscular injection of supercoiled plasmid DNA as described previously (Wolff et al., 1991). Muscles were removed for analyses 7-14 days after administration of the DNA and examined for recombinant dystrophin expression by dual-label immunostaining as for the cultured myotubes with the exception that a directly-conjugated mouse antibody to dystrophin (biotinylated MANDYS-1; Morris et al., 1993) was used to eliminate conjugate background cross-reactivity. In line with the in vitro studies, all four cDNA constructs expressed the recombinant dystrophin polypeptides in vivo which localized to the myofibre sarcolemma (Fig. 4). In the cases of the three deletion constructs, transfected myofibres were clearly distinguished from the small endogenous population of 'revertants'
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(Hoffman et al., 1990) by the dual-label analyses and lack of reactivity with antibodies to the deleted rod-domain (Fig. 4I and J, arrowed).

**DISCUSSION**

Immunofluorescence localization, subcellular fractionation studies and biochemical analyses of dystrophin clearly suggest that this molecule belongs to a class of proteins involved in linkage of the cytoskeleton to membrane components that in turn allow transmembrane association to the extracellular matrix (Dickson et al., 1992; Ervasti and Campbell, 1993a). In this context, the C-terminal domains of dystrophin are thought to mediate association with a large oligomeric integral membrane-protein complex containing six sub-unit components, four of which are glycoproteins (the dystrophan-glycoprotein complex, DGC; Ervasti and Campbell, 1991; Suzuki et al., 1992). In contrast, the N-terminal domain of dystrophin is thought to contain probably two actin-binding sites (Levine et al., 1992). Thus, chimaeric molecules comprising the first 233 amino acids of dystrophin fused to the last 645 of a-actinin localize to actin-containing microfilamentous structures in transfected COS cells (Hemmings et al., 1992) and bacterially-expressed fusion proteins containing this region of the dystrophin molecule cosediment from solution with purified F-actin (Way et al., 1992), as does the whole DGC (Ervasti and Campbell, 1993a). In the present study, we have expressed the N- and C-terminal domains of human dystrophin as recombinant polypeptides in non-muscle cells and muscle cells of the dystrophic mdx mouse, and have shown clearly that both sub-structures can independently associate with the sarcolemma both in cultured myotubes and in myofibres in vivo.

With the C-terminal construct utilised here, the structure of the expressed product corresponds closely to that of naturally-occurring truncated dystrophin isoforms (Dp116, Byers et al., 1992). In contrast, the N-terminal domain of dystrophin is thought to contain probably two actin-binding sites (Levine et al., 1992). Thus, chimaeric molecules comprising the first 233 amino acids of dystrophin fused to the last 645 of a-actinin localize to actin-containing microfilamentous structures in transfected COS cells (Hemmings et al., 1992) and bacterially-expressed fusion proteins containing this region of the dystrophin molecule cosediment from solution with purified F-actin (Way et al., 1992), as does the whole DGC (Ervasti and Campbell, 1993a). In the present study, we have expressed the N- and C-terminal domains of human dystrophin as recombinant polypeptides in non-muscle cells and muscle cells of the dystrophic mdx mouse, and have shown clearly that both sub-structures can independently associate with the sarcolemma both in cultured myotubes and in myofibres in vivo.

**Fig. 4.** Expression of recombinant dystrophin cDNAs in mdx mouse muscle in vivo. At 7-14 days after direct in vivo injection of plasmid DNA constructs into quadriceps muscles, methanol-fixed frozen sections of control normal C57Bl/10 (A,B) and injected mdx mouse muscle (C-J) were immunostained using antibodies to the C-terminus, antibody G8 (A,C,G,I), N-terminus, antibody G6 (E), and mid-rod domain of dystrophin, biotinylated MANDYS 1 antibody (B,D,F,H,J). In control muscle, dystrophin immunoreactivity for the C-terminal (A), mid-rod (B), and N-terminal domains (not shown) was clearly observed as continuous bright fluorescent staining at the sarcolemma. However, mdx muscles injected with the Becker minidystrophin construct, pBN1, expressed the C terminus (C) but not the mid-rod domain of dystrophin (D) at the sarcolemma of transfected myofibres. Similarly, myofibres in muscles injected with the C-terminal dystrophin construct, pBN-CT3, expressed a polypeptide specifically immunoreactive to the dystrophin C-terminal antibody, G8 (G), and not the mid-rod domain antibody, MANDYS 1 (H). Furthermore, consistent with the results in vitro (Fig. 3G and H), mdx muscles injected with the N-terminal dystrophin construct, pBN-NT1, specifically expressed the N terminus (E) and not the mid-rod domain of dystrophin (F) localised to the sarcolemma. Rare, dystrophin-positive (revertant) myofibres in mdx muscles (I,J arrowed) were distinguished from fibres expressing recombinant molecules by immunoreactivity to the mid-rod (J) as well as N- (not shown) and C-terminal (I) domains of dystrophin. Bar, 20 µm.
In conclusion, this study shows that sarcolemmal association is a property of both the N- and C-terminal domains of the dystrophin molecule. Not only does this provide experimental data to match recent observations from DMD/BMD patients, but also demonstrates a strategy that may be applied to defining further the exact structural features of these dystrophin domains responsible for membrane association. In addition, colocalization and immunoprecipitation studies using recombinant dystrophin polypeptides such as those described here will allow interactions with other components of the sarcolemmal membrane and cytoskeleton to be determined. In this way a structure/function map of the dystrophin molecule, largely defined at present by primary and secondary structure predictions from sequence data and genotype-phenotype correlations of DMD/BMD patients, could be confirmed and more clearly established.

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REFERENCES


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