Evolutionary conservation of the dystrophin central rod domain

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Dystrophin cDNA fragments encoding the C-terminal repeats of the central rod region have been expressed as fusion proteins. The polyclonal antisera raised to the purified fusion proteins have been characterized and neither antiserum cross-reacted with dystrophin-related protein. Antisera detected dystrophin with molecular mass close to that of the human in all terrestrial vertebrates and amphibia studied. Experiments with antisera to the N-terminal region of the dystrophin rod confirmed that epitopes to the rod region were conserved during this evolutionary period and the length of this domain remained unaltered.

INTRODUCTION

Dystrophin is a sarcolemmal protein which is defective in both Duchenne muscular dystrophy and the milder allelic disorder, Becker muscular dystrophy. The dystrophin gene is located on the short arm of the X-chromosome (Xp21 region) and encompasses at least 2.3 Mb, making it one of the largest genes presently identified. The dystrophin cDNA predicts a 3685-amino acid sequence (molecular mass 427 kDa), which can be divided into four distinct regions (Koenig *et al.*, 1988): (i) an N-terminal α -actinin-like region; (ii) a central rod domain similar to that of spectrin, with 24 triple-helical repeat structures and four potential hinge regions (Koenig & Kunkel, 1990); the domain contains approx. 2800 amino acids (76% of the entire protein sequence); (iii) a cysteine-rich domain with potential Ca^{2+} -binding sites (EF hands); (iv) a C-terminal region that has no homology to previously sequenced proteins, although a related autosomal gene has subsequently been identified by low-stringency hybridization procedures (Love *et al.*, 1989).

Two deletion hot spots have been identified in the region of the gene which encodes the central rod domain (Leichti-Gallati *et al.*, 1989). Most deletions in Becker muscular dystrophy have breakpoints in the hot spots and are predicted not to disrupt the translational reading frame (Koenig *et al.*, 1989). Truncated dystrophin molecules which lack fragments of the central rod domain as the result of an in-frame deletion have been detected in Becker muscular dystrophy (England *et al.*, 1990; Bulman *et al.*, 1991); however, the loss of fragments of the rod domain does not appear to affect the ability of the truncated protein to localize to the sarcolemma (Arahata *et al.*, 1991). This observation, together with the identification of a patient with mild Becker muscular dystrophy with a deletion of 60% of the rod domain (England *et al.*, 1990), implied a minor functional role for the domain. Variation in both the quantity of dystrophin and the severity of clinical phenotype was subsequently demonstrated in patients with deletions of identical fragments of the rod domain (Beggs *et al.*, 1991). This suggests that factors other than the deletion may contribute to the clinical phenotype, complicating the unambiguous determination of the rod domain's function.

Contradictory to the idea that the central rod domain plays only a minor role in the function of dystrophin are studies examining the evolutionary conservation of this region. At the cDNA level, the predicted amino acid sequence of the central rod

domain shows 75% conservation between human and chicken (Lemaire *et al.*, 1988). A preliminary study using cDNA probes has examined the structural organization of the dystrophin gene among mammals and birds and has found a size and complexity similar to that seen in the human gene (Meng *et al.*, 1990). Individual studies with antibodies directed toward dystrophin fragments located in the N-terminal half of the rod domain have indicated conservation of epitopes between human, mouse, rat and *Torpedo sp.* (Hoffman *et al.*, 1987; Chang *et al.*, 1989; Jasmin *et al.*, 1990). Although these antibody studies imply that the size of the rod domain may be widely conserved, this hypothesis has not been rigorously tested.

We have produced novel polyclonal antibodies (denoted P6 and H12) to the C-terminal region of the dystrophin rod domain. The H12 antibody has been used, together with the 60 kDa antibody to the N-terminal region of the rod domain (Hoffman *et al.*, 1987), to study the evolution of the whole rod domain within the dystrophin molecule. We find that, not only is dystrophin detectable in all terrestrial vertebrates and amphibia studied, but that the size of the rod domain remains highly conserved. Preliminary results describing other uses of the P6 antibody have already appeared in abstract form (Strong *et al.*, 1990; Gangopadhyay *et al.*, 1990).

MATERIALS AND METHODS

Plasmid constructs

Human dystrophin cDNA clone 63-1 was digested with restriction enzymes *Hind*III and *Pst*I. After agarose-gel electrophoresis, a 1.2 kb *Hind*III fragment and a 0.6 kb *Pst*I fragment were purified using a Gene Clean kit (Bio 101, La Jolla, CA, U.S.A.). The *Hind*III fragment, blunt-ended with Klenow polymerase, and the *Pst*I fragment were ligated into the expression vector pRIT2T (Pharmacia) at the *Sma*I and *Pst*I sites respectively. After transformation of *Escherichia coli* N4830-1 (Pharmacia), recombinants were selected by hybridization and the correct insert orientation was determined by restriction analysis. *E. coli* N4830-1 were freshly transformed with the appropriate *Pst*I and *Hind*III recombinants and fusion proteins were induced according to established protocols (Pharmacia). Growth medium was chilled on ice, centrifuged and the cell pellet resuspended in 30 ml of ice-cold TST buffer (50 mM-Tris/HCl, pH 7.6, 150 mM-NaCl, 0.05% Tween 20). The cell suspension was sonicated on ice (4 μ m amplitude, 8 $\times$ 30 s) and centrifuged

Abbreviation used: PBS, phosphate-buffered saline.

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(20000 *g*, 20 min, 4 °C) and fusion proteins were identified in the supernatant by Western blotting (see below), with whole sheep serum as the primary antibody.

Fusion protein isolation

Fusion proteins were purified on a 2 ml IgG-Sepharose 6FF column (Pharmacia) at 4 °C. The cell extract was recycled twice through the column and the column was then washed with TST buffer (20 ml), followed by 5 M-ammonium acetate, pH 5 (4 ml). Fusion proteins were subsequently eluted with 0.5 M-acetic acid, pH 3.4 (10 ml); 1 ml samples were collected and immediately neutralized with 5 M-NaOH. The affinity eluates were pooled and dialysed against phosphate-buffered saline (PBS; Dulbecco 'A'; Unipath, Basingstoke, U.K.) (16 h, 4 °C) and concentrated to 1.5 ml with Aquacide IIA (Calbiochem). Each concentrate was run on an SDS/polyacrylamide gel. The fusion protein was located by silver staining a portion of the gel (Morrissey, 1981), and the corresponding (unstained) fusion protein bands were cut from the remaining gel and electroeluted into 2 mM-Tris base/150 mM-glycine, pH 8.3 (5 mA, 18 h, 10 °C). Eluates were dialysed against 10 mM-Tris base/1 mM-EDTA, pH 7.4 (16 h, 4 °C) and concentrated as before.

Antiserum production

Rabbits were injected subcutaneously with 50 µg of purified fusion protein, emulsified in complete Freund's adjuvant, followed by two 50 µg boosters (incomplete adjuvant) at 3-week intervals. Then 1 week after the final boost, serum was collected and frozen in liquid N₂. Antisera raised to the fusion proteins encoded by the *Pst*I and *Hind*III recombinants were denoted P6 and H12 respectively.

Antibody purification

The fusion protein (100 µg) used to raise antiserum P6 was coupled to 1 ml of tressyl-activated Sepharose 4B (Pharmacia) in 0.1 M-NaHCO₃/0.5 M-NaCl, pH 8.0, for 4 h at 25 °C. Residual active sites on the column were blocked with 0.1 M-Tris/HCl, pH 8.0, for 4 h at 4 °C. IgG-binding sites on Protein A were blocked with 4 ml of human serum/PBS (1:1), which was then recirculated four times. The column was washed with PBS (5 ml) and then 2 ml of P6 antiserum/PBS (1:1) was recirculated four times. The column was washed with 10 ml of PBS, then eluted with 0.1 M-glycine/HCl, pH 2.5; 0.5 ml portions were neutralized on collection with 2 M-Tris base, pooled and dialysed against PBS (16 h, 4 °C). Antibodies directed against protein A were removed by passing the eluate over a 1 ml protein A-Sepharose affinity column (Porton Products Ltd., Maidenhead, Berks., U.K.) which was blocked before use with human serum as described above. The eluate from the fusion protein column was recirculated ten times to ensure complete removal of all protein A antibodies.

Sample preparation

Skeletal muscle was removed from the various species immediately after death and a normal human skeletal-muscle biopsy was obtained as previously described (Heckmatt *et al.*, 1984). All samples were immediately frozen in liquid N₂. Approx. 20 mg of frozen tissue was placed between sheets of aluminium foil cooled in liquid N₂ and crushed between steel weights. The powdered tissue was dissolved in 30 µl of solubilization buffer [1 % (w/v) SDS, 10 mM-iodoacetamide, 5 mM-EGTA, 10 mM-benzemethonium chloride, 10 mg of phenylmethanesulphonyl

fluoride/ml, 2.5 mg of pepstatin A/ml]. Samples were centrifuged (14000 *g*, 5 min) and the pellets discarded. A 5 µl sample of the supernatant was assayed for protein (Lowry *et al.*, 1951), with BSA as standard. The remaining supernatant was snap-frozen in liquid N₂ until further use. Some 60 µg of total protein from each supernatant was initially analysed by SDS/PAGE (see below), stained with Coomassie Blue R250 and scanned with a densitometer (Joyce-Loebel) in order to determine the relative density of the myosin band in each sample. The volume of each solubilized muscle sample was then adjusted to provide a constant myosin loading and then the samples were re-run on Western blots (see below).

SDS/PAGE and Western blotting

Samples were diluted with an equal volume of Laemmli buffer, heated to 100 °C for 3 min and then electrophoresed on 4–20 % linear gradient gels (Laemmli, 1970) for 18 h at 20 mA (10 °C). Molecular masses were estimated by using rainbow markers (Amersham). Gels were washed for 5 min in transfer buffer (2 mM-Tris base/150 mM-glycine) and the proteins were electrophoretically transferred in the same buffer on to nitrocellulose paper for 24 h at 250 mA (10 °C). Blots were blocked for 1 h with 3 % (w/v) milk powder in PBS and then incubated with dystrophin antisera diluted with PBS/0.05 % Tween 20 as follows: P6 and H12 antibodies, 1:3000; 60 kDa antibody, 1:1000. Whole sheep serum was diluted 1:300. Bound antibody was visualized using an appropriate biotinylated secondary antibody and streptavidin/biotinylated horseradish peroxidase (Dako), followed by diaminobenzidine as previously described (Patel *et al.*, 1988).

RESULTS

Dystrophin cDNA fragments were expressed as fusion proteins by cloning a *Pst*I fragment (nucleotides 8652–9291) and a *Hind*III fragment (nucleotides 8012–9281) into the pRIT2T expression

Fig. 1. Expression and purification of fusion proteins

(a) SDS/PAGE of *E. coli* lysates (50 µg of protein per lane). Gel stained with Coomassie Blue. Lane 1, molecular-mass markers (kDa); lane 2, transformation with the *Hind*III recombinant; lane 3, transformation with the *Pst*I recombinant. (b) Western blot of *E. coli* lysates (50 µg of protein per lane). Lane 1, transformation with the *Hind*III recombinant; lane 2, transformation with the *Pst*I recombinant; lane 3, transformation with pRIT2T; lane 4, untransformed *E. coli*. (c) SDS/PAGE of proteins affinity-purified from lysates of *E. coli* transformed with the *Hind*III recombinant (lane 1) and the *Pst*I recombinant (lane 2). Gel stained with Coomassie Blue; 10 µg of protein per lane. (d) Western blot of the SDS/polyacrylamide gel shown in (c). (e) Western blot of electroeluted fusion proteins. Lane 1, protein of molecular mass 75 kDa from the *Hind*III recombinant, lane 2, protein of molecular mass 50 kDa from the *Pst*I recombinant.

Fig. 2. Schematic representation of the dystrophin protein (Koenig & Kunkel, 1990) showing the predicted positions of the epitopes for the 60 kDa, H12 and P6 antibodies (arrows)

Dystrophin domains are as follows: ■, N-terminal α -actinin-like region; ▨, putative hinge regions; ▩, proteinase-sensitive site; □-□, rod domain repeats; ■, cysteine-rich region; □, C-terminal region, unique to dystrophin family.

Fig. 3. Characterization of antisera against Western blots

Lane 1, human skeletal muscle probed with preimmune rabbit serum diluted 1:3000; lane 2, human skeletal muscle probed with H12 antiserum; lane 3, human skeletal muscle probed with P6 antiserum; lane 4, human skeletal muscle probed with P6 affinity preparation (not quantified); lane 5, *mdx* mouse skeletal muscle probed with H12 antiserum. Migration of 97 and 200 kDa markers is indicated, and migration of dystrophin is indicated by arrow. The dilutions used to obtain qualitatively similar results suggest that many high-affinity antibodies bind irreversibly to the fusion protein affinity matrix and cannot be recovered. Although 1:100000 dilutions of P6 and H12 antisera were able to detect dystrophin, the best signal-to-noise ratio was obtained with antiserum dilutions of approx. 1:3000, where other specificities bound to Western blots were near their limit of detection.

vector. The restriction fragments encode 214 and 423 amino acids respectively from the C-terminus of the rod domain. These rod-domain fragments are flanked on both sides by Protein A sequences when expressed as pRIT2T fusion proteins. Although the fusion proteins were not overexpressed in the *E. coli* lysate (Fig. 1a), they could easily be detected on Western blots probed with whole serum (Fig. 1b, lanes 1 and 2), since the IgG-binding domain of protein A is maintained in the 21 kDa fragment fused to the N-terminus of the dystrophin sequence. Expression of the 26 kDa truncated form of Protein A encoded by non-recombinant pRIT2T can be seen in Fig. 1(b) (lane 3). Subsequent affinity chromatography of the cell lysates on an IgG affinity column resulted in a partial purification of the fusion proteins (Figs. 1c and 1d). Final purification to homogeneity was achieved when the 75 kDa and 50 kDa fusion proteins were cut out and electroeluted from a preparative SDS/polyacrylamide gel of material partially purified after the IgG affinity-column step (Fig. 1e). Approx. 30 μ g of pure fusion proteins were isolated from 1 litre of induced *E. coli* ($A_{600} = 1.0$ at induction).

The rabbit whole antisera collected after immunization with the purified 75 kDa and 50 kDa fusion proteins were denoted H12 and P6 respectively. The 60 kDa antibody (Hoffman *et al.*, 1987) also used in this study was whole antiserum raised to a fusion protein containing a dystrophin fragment encoded by mouse cDNA. The fusion proteins from which the H12 and

Fig. 4. Western-blot analysis of dystrophin in skeletal muscle from several animal classes

(a) 60 kDa antiserum; (b) H12 antiserum. Lane 1, mouse (C57/BL10); lane 2, rat (Wistar); lane 3, crow (*Corvus corone*); lane 4, lizard (*Lacerta* sp.); lane 5, toad (*Bufo* sp.); lane 6, aquatic toad (*Xenopus laevis*); lane 7, frog (*Rana temporalis*); lane 8, human; lane 9, rainbow trout (*Salmo gairdneri*); lane 10, locust (*Locusta* sp.); lane 11, housefly (*Musca domestica*); lane 12, lobster (*Homarus gammarus*); lane 13, crab (*Cancer pagurus*). The position of myosin (200 kDa marker) is shown, and migration of dystrophin is indicated by an arrow.

60 kDa antibodies are derived each encompass approximately four repeats at opposite ends of the rod domain (Fig. 2). Epitopes of the P6 antibody are contained within the region recognized by the H12 antibody. Three bands in the region of 400 kDa were detected when Western blots of normal human skeletal muscle were probed with either H12 or P6 antibodies (Fig. 3, lanes 2 and 3 respectively). Preimmune rabbit serum produced no significant labelling (Fig. 3, lane 1). Since the staining pattern was indistinguishable when blots were probed with either affinity-purified P6 antibody (Fig. 3, lane 4) or whole diluted antiserum, the latter was used routinely. Antibodies against Protein A did not cross-react with proteins in skeletal muscle, as indicated by the lack of signal when a blot of skeletal muscle from the dystrophin-negative *mdx* mouse was probed with H12 antibody (Fig. 3, lane 5). This also demonstrates that H12 antibody does not cross-react with the recently identified dystrophin-related protein (Khurana *et al.*, 1990; Love *et al.*, 1991).

Dystrophin (approx. 400 kDa) was detected in all the terrestrial vertebrates and amphibia with both the 60 kDa and H12 antibodies (Figs. 4a and 4b respectively). Dystrophin appeared as multiple bands in each case. A high-molecular-mass doublet

was consistently detected with both antisera, although in the amphibia, the lower band was clearly visible only with increased protein concentrations (results not shown). Despite the difficulty in determining small variations in molecular mass in the 400 kDa range with this system, slight shifts of the top dystrophin band were observed between species. In four separate experiments, no detectable molecular-mass differences were observed between rat and human (Fig. 4, lanes 2 and 8). However, a slight decrease in dystrophin molecular mass was observed with mouse, crow, lizard and amphibian skeletal muscle (Fig. 4, lanes 1, 3, 4–7). The same pattern was observed with both antibodies. These qualitative differences are not sufficient to allow absolute determination of the dystrophin molecular mass in these vertebrates, but it is apparent that the size differs slightly from that of human. The molecular-mass difference between human and amphibian dystrophin was approx. 10 kDa, which is about the limit of high molecular-mass resolution for this system (results not shown).

A significant decrease in antibody signal intensity was observed in the classes of vertebrate more distantly related to the mammalia. This decrease in signal intensity was most pronounced with antibody H12. In the case of mammalia (Figs. 4a and 4b, lanes 1, 2 and 8), antibodies bound more efficiently than in crow and lizard (Fig. 4, lanes 3 and 4). A further decrease in signal intensity was observed for the amphibia (Fig. 4, lanes 5, 6 and 7).

Muscle samples from the trout and several species of terrestrial and aquatic invertebrate exhibited no bands common to 60 kDa and H12 antibodies (Fig. 4, lanes 9–13). Staining with the 60 kDa antibody was non-specific, as this was observed on an identical blot probed with non-immune sheep serum (results not shown). The quantity and quality of muscle protein was determined on a gel stained for total protein. The transfer efficiency was constant for all muscle samples presented, as indicated by the 200 kDa myosin band, which stained non-specifically by the secondary antibody (results not shown).

DISCUSSION

Detection of dystrophin in the 400 kDa region with antisera to both ends of the central rod domain indicated that the length of the domain was not significantly different in terrestrial vertebrates and amphibia (Figs. 4a and 4b, lanes 1–8). Although the molecular-mass differences (approx. 10 kDa) between human/rat and the remaining vertebrates could be within experimental error, any real discrepancy is unlikely to be due to differences in the size of the rod domain, given that the rod domain in chicken (Lemaire *et al.*, 1988) is only seven amino acids shorter than in human. The intramolecular distance between the putative hinge regions (Koenig & Kunkel, 1990) may impose a functional constraint on the length of the rod domain.

The origin of the multiple immunoreactive dystrophin bands is unknown, but the pattern is in accordance with that detected by other dystrophin antibodies (Hoffman *et al.*, 1988; Nicholson *et al.*, 1989; Bulman *et al.*, 1991). Three alternatively spliced forms of dystrophin mRNA have been demonstrated in human skeletal muscle (Feener *et al.*, 1989). The predicted molecular-mass difference of the encoded proteins was, however, too small to correlate with the separation of the high-molecular-mass doublet observed here (approx. 20 kDa). Post-translational modification may be responsible, but to date there is no evidence. A third possible explanation is that the bands represent enzymic cleavage of the high-molecular-mass form. The reproducibility of the high-molecular-mass doublet between species is evidence for the evolutionary conservation of the mechanism responsible for the multiple bands.

The decrease in band intensities with evolutionary time cannot

be accounted for in terms of shorter rod domains. The H12 and 60 kDa antibodies encompass approximately four of the triple-helical repeats each (approx. 110 amino acids per repeat) and so the observed molecular-mass shifts are insufficient to affect antibody binding. Comparison of human and chicken dystrophin (Lemaire *et al.*, 1988) showed amino acid substitution over approx. 25% of the sequence recognized by the 60 kDa and the H12 antibodies. Presumably a comparable loss of sequence similarity occurs between human and crow. The decrease in band intensity between crow and amphibia, without significant decrease in molecular mass implies a further loss of sequence similarity. Both antisera will therefore bind to epitopes with greater than 25% sequence variation. This amino acid substitution could be partially responsible for the apparent molecular-mass variation between species.

The detection of dystrophin of 400 kDa in cartilaginous fish with the 60 kDa antibody (Chang *et al.*, 1989) suggests that both the length of the rod domain and to a degree the sequence are preserved in more primitive species than amphibia. Muscle samples from several other classes, from which the amphibia diverged around 350 million years ago, were, however, negative for 60 kDa and H12 antibodies. The rod domain repeats are likely to have evolved as the result of exon duplication (Koenig *et al.*, 1988) and so the lack of any significantly truncated molecule is noteworthy, considering that the two antibodies will recognize epitopes in the rod domain with greater than 25% sequence divergence and, these epitopes encompass 30% of the rod domain.

We conclude that the length of the rod domain was established early in vertebrate evolution and has been preserved, from the period when amphibia and reptiles diverged (around 300 million years ago). It is therefore tempting to speculate that the constant size of this domain has functional significance. Furthermore, the lack of an immunoreactive dystrophin band on invertebrate blots suggests that, if invertebrate dystrophin exists, its structure is likely to differ significantly from the mammalian form.

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