Characterization and localization of a 77 kDa protein related to the dystrophin gene family

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The Duchenne muscular dystrophy gene gives rise to transcripts of several lengths. These mRNAs differ in their coding content and tissue distribution. The 14 kb mRNA encodes dystrophin, a 427 kDa protein found in muscle and brain, and the short transcripts described encode DP71, a 77 kDa protein found in various organs. These short transcripts have many features common to the deduced primary structure of dystrophin, especially in the cysteine-rich specific C-terminal domains. The dystrophin C-terminal domain could be involved in membrane anchorage via the glycoprotein complex, but such a functional role for these short transcript products has yet to be demon-

strated. Here we report the first isolation of a short transcript product from saponin-solubilized cardiac muscle membranes using alkaline buffer and affinity chromatography procedures. This molecule was found to be glycosylated and could be easily dissociated from cardiac muscle and other non-muscle tissues such as brain and liver. DP71-specific monoclonal antibody helped to identify this molecule as being related to the dystrophin gene family. Immunofluorescence analysis of bovine or chicken cardiac muscle showed a periodic distribution of DP71 in transverse T tubules and this protein was co-localized with the dystrophin glycoprotein complex in the Z-disk area.

INTRODUCTION

One of the most common muscular dystrophies is related to the Xp21 locus which is spanned by the largest known gene: the Duchenne muscular dystrophy (DMD) gene (*DMD*). The transcription product of this gene in muscle is a 14 kb mRNA encoding a 427 kDa protein called dystrophin [1], which is associated with the muscle cell sarcolemma and absent or altered in Duchenne and Becker muscular dystrophies respectively [2–4]. The amino acid sequence and sequence similarity deductions suggest that this protein consists of four domains: an N-terminal actin-binding region [5–8], a rod structure with repetitive sequences arranged in a triple helix that may confer associative properties [9–12], a cysteine-rich domain which shares sequence similarity with the calcium-binding domain of α -actinin [13,14] and a C-terminal extremity which interacts with the membrane via a glycoprotein complex [15–19]. Dystrophin belongs to an expanding protein family. Indeed, different 400 kDa forms have been identified and produced either by alternative splicing of the 3' part of the dystrophin gene [20] or by alternative promoter utilization in the 5' part of the gene, thus conferring cell-type specificity [21]. A dystrophin-related protein (DRP) encoded on the autosomal chromosome 6 [22], the cDNA of which was recently sequenced [23], shows sequence similarities with dystrophin mainly in its N- and C-terminal parts. Moreover, short transcripts related to the *DMD* gene have been detected [24–26]. They give rise to a 77 kDa protein named dystrophin product 71 (DP71) which was first identified in liver, hepatoma cells and also in cardiac muscle [27]. This protein is the major *DMD* gene product in many non-muscle tissues, including brain, neurons and glial cell cultures [28]. It has been partially modified by alternative exon utilization. Moreover, it lacks the entire large domain of spectrin-like repeats and the actin-binding domain, which are replaced by seven new amino acids contained within one exon not present in dystrophin mRNA. This exon and the DP71 promoter are located between exons 62 and 63 of the

dystrophin mRNA [28]. The C-terminal part of DP71 is also modified as the last 13 amino acids contained in dystrophin are replaced by 31 new ones obtained by frameshifting in the splicing [24]. Although a role in maintaining membrane integrity and/or a regulator role in calcium channels have been proposed for dystrophin, nothing is known concerning DP71 function. Using various biochemical procedures such as subcellular fractionation and immunostaining analysis, DP71 was found to be associated with the plasma membrane in HeLa cells. Transfection experiments, with a vector expressing DP71, revealed an association of this protein with the plasma membrane in cultured myogenic cells from *mdx* mouse cells [29].

In this study, we isolated, characterized and localized a 77 kDa protein related to the dystrophin gene obtained from chicken cardiac muscle and other non-muscle tissues such as brain and liver. The localization of this protein was also analysed by immunofluorescence studies on chicken and bovine cardiac muscles.

MATERIALS AND METHODS

Preparation of crude extracts

Total membrane extracts were prepared from fresh tissues (1 g) homogenized in 5 vol. of extraction buffer containing 50 mM Tris/HCl (pH 9.0) in the presence of 0.01% saponin and a protease inhibitor cocktail. Homogenates were centrifuged for 10 min at 2000 g and the supernatants were mixed with equivalent volumes of SDS buffer and denatured for 5 min at 100 °C. Samples of each extract were separated on 3–9% SDS/polyacrylamide gels.

Preparation of chicken cardiac muscle to obtain pure 77 kDa protein

The first steps were performed as previously described [12] but with slight modifications. Briefly, chicken cardiac muscles were

cut into very small pieces immediately after killing. Extraction buffer was freshly made by the addition of protease inhibitors as follows: 0.1 M Tris/KOH (pH 12.5), 1 mg/ml soybean trypsin inhibitor, 1 mg/ml leupeptin, 10 mM iodoacetamide and 1 mM phenylmethanesulphonyl fluoride in the presence of 0.01 % saponin. After homogenization in 10 vol. of this buffer, the solution was clarified by 30 min centrifugation at 8000 *g* and then the supernatant was precipitated with solid $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 40 % (w/v). The pellet was resuspended in extraction buffer without saponin. This solution, obtained by minimum buffer addition, was gently vortex-stirred. Complete clarification of the solution was obtained by ultracentrifugation for 1 h at 140 000 *g*. The resulting soluble fraction enriched in 77 kDa protein was passed through a hydroxyapatite column, equilibrated with 10 mM phosphate buffer (pH 7) to separate the different proteins present in this solution. High-molecular-mass and pigmented proteins were completely absorbed on the hydroxyapatite column and the eluted proteins were concentrated with an Amicon cell using a PM10 membrane under nitrogen pressure. After dialysis against 0.1 M Tris/HCl (pH 8) for 2 h, the resulting concentrated proteins were repeatedly passed through an S-Sepharose fast-flow column. Pure 77 kDa protein was specifically eluted over a 0–500 mM NaCl gradient as the first elution peak.

Monoclonal antibody production

Monoclonal antibody 9C11 was obtained after injection of a saponin extract of chicken cardiac muscle which was used as antigen. Screening was performed using the same antigen by Western-blot and e.l.i.s.a. techniques to select monoclonal antibodies raised against a 77 kDa protein. This antibody was characterized using different recombinant proteins, named H, H₁ and H₂, expressing different C-terminal parts of dystrophin. H contains the last 303 residues of the chicken C-terminal dystrophin part (amino acids 3357–3660), H₁ contains 127 residues (amino acids 3357–3484) and H₂ contains 258 residues (amino acids 3357–3615). These dystrophin parts were fused to β -galactosidase. We also produced a DRP fusion protein corresponding to the b3 fragment described by Khurana [30]. A detailed cloning procedure of these constructions is described elsewhere [31].

Monoclonal antibody 5F3 was obtained after injections of a fusion protein expressing the previously defined last 31 residues of DP71 [24]. The construction was performed in pEX3 vector dephosphorylated at the *Sma*I site and verified by sequencing. The insert was obtained after a PCR reaction using an M13mp18 construction as template (containing the last 93 coding nucleotides of DP71 cDNA and part of a non-coding sequence obtained from D. Yaffe's laboratory) and *Eco*RI digestion followed by Klenow treatment.

The primer's sequences were sense 5'-CGGAATTCAAGAGGACACAATGTAGGAAGT 3' and antisense 5'-GCGCATGCATAGACGTGTAACCTGC-3'. The construction was verified by sequencing.

Monoclonal antibody dys2 was obtained from Novocastra, and was able to detect the 400 kDa dystrophin band in all tissues tested, but failed to detect the 77 kDa short transcript isolated from chicken cardiac muscle.

Polyclonal antibody C-4-22, raised against the central part of the molecule (residues 1173–1728), was previously characterized as dystrophin-specific [32].

Expression of the recombinant protein and Western-blot analyses were performed as previously reported [31].

Glycoprotein detection

Detection of the 77 kDa protein was performed using a Glyco-DIG kit (Amersham) according to the manufacturer's recommended procedure.

Deglycosylation treatment was performed using recombinant N-glycosidase F enzyme (Biolabs) for 1 h at 37 °C.

Immunofluorescence studies

Longitudinal cryosections of cardiac muscles (10–12 μm) were treated with monoclonal antibody 5F3 which was then detected with a fluorescein-labelled second antibody as previously described [33].

RESULTS

DP71 presence in chicken and rat crude extracts

In previous experiments using RNAase-protection assays, PCR amplification and Western-blot techniques, dystrophin, the DP71 small transcript and DP71 were detected and their tissue distributions characterized [24–28]. We analysed crude extracts from different chicken or rat tissues with dystrophin monoclonal antibody H'5A3 which we previously characterized and raised against the last 303 residues of the chicken skeletal dystrophin [31]. The presence of DP71 is shown in Figure 1. The C-terminal distal product of the *DMD* gene was detected and found to have a molecular mass of about 77 kDa, which is in full agreement with previous studies in brain, liver and cardiac muscle from rat and chicken. Surprisingly, DP71 was detected in chicken skeletal muscle, whereas it was reportedly undetectable in similar extracts from rat (as noted here also).

Monoclonal antibody characterization

To purify a short transcript product from the *DMD* gene, we selected the cardiac muscle in which this molecule was previously found to be rather abundant. In a second step, chicken cardiac saponin extract was used as antigen for monoclonal antibody

Figure 1 Comparative immunoblot analysis of rat and chicken extracts from various tissues

The tissue distribution of 400 kDa dystrophin and the 77 kDa short transcript product were analysed using a monoclonal anti-dystrophin antibody H'5A3 directed against the C-terminal domain of chicken skeletal dystrophin as previously reported [30]. Various tissues were analysed. In lanes 1, 2, 3 and 4 are samples from brain, cardiac muscle, liver and skeletal muscle from rat and chicken respectively. The molecular mass standard (kDa) is indicated on the left.

Figure 2 Characterization of monoclonal antibody 9C11 detecting dystrophin fusion protein

The constructions used are shown after Coomassie Blue staining and revelation by immunoblot analysis with 9C11 monoclonal antibody. Neither the β -galactosidase part nor the H_1 dystrophin segment could be detected, while both H and H_2 recombinant proteins gave positive results. Proteins analysed in lanes 1, 2 and 3 are H , H_2 and H_1 respectively. The molecular mass standard (kDa) is indicated on the left.

Figure 3 Elution pattern of the 77 kDa protein identified as a short transcript product related to the *DMD* gene

The elution pattern of the first peak is presented after Coomassie Blue staining or after transfer to nitrocellulose and immunoblot analysis using the previously characterized monoclonal antibody 9C11. Lane 1 corresponds to a sample from a 77 kDa protein-enriched preparation and lanes 2, 3, 4 and 5 correspond to the 3, 4, 5 and 6 fractions in which the protein was eluted. Each lane contained 40 μ g of protein. The molecular mass standard (kDa) is indicated on the left.

production. Specific monoclonal antibodies were characterized as suitable to identify selectively the presence of the protein throughout the purification procedure. These new monoclonal antibodies were first screened according to their reactivity towards proteins with molecular masses around 80 kDa. Twenty-one antibodies were obtained and among them 10 monoclonal antibodies detected such 80 kDa protein bands in total protein extracts from chicken brain, liver and cardiac tissues. Only three of them, 9C11, 14F10 and 9G11, also detected similar proteins in corresponding mouse, rat and rabbit extracts.

To increase the performance of such monoclonal antibodies, a second screening was carried out to determine their ability to detect different dystrophin fusion proteins containing overlapping fragments of the dystrophin C-terminal part. The results presented in Figure 2 showed that the most reactive antibody, monoclonal antibody 9C11, detected only the H and H_2 fusion proteins but never the β -galactosidase part or the dystrophin

Figure 4 Characterization of the 77 kDa protein

Shown are immunoblots revealed with monoclonal antibodies 5F3, 9C11 and anti-glycoprotein antibody. In the upper blots, lanes 1, 2 and 3 are samples from brain, heart and liver respectively. An equal volume of sample was loaded in each lane to show the respective purification yield of the 77 kDa protein in each organ. Lower blots show samples of the cardiac 77 kDa protein before and after deglycosylation treatment in lanes 1 and 2 respectively. The molecular mass standard (kDa) is indicated on the left.

part contained in the H_1 fusion protein. The dystrophin segment detected corresponded to the overlapping sequences between H and H_2 which are not contained in H_1 . Moreover, this monoclonal antibody was able to detect a recombinant fragment encompassing residues 3161–3388 of the human *DRP* sequence, which also have homologous domains with the central region of H fusion protein (results not shown). We then used the specificity of such monoclonal antibodies to isolate a short transcript product related to the *DMD* gene family from cardiac muscle.

Purification of a 77 kDa protein

The strategy to obtain pure 77 kDa protein involved first extracting an enriched 77 kDa protein preparation from chicken cardiac muscle. Two main conditions were essential for extraction: a 50 mM Tris/KOH buffer (pH 12.5) and the presence of 0.01% saponin as detergent. We followed a three-step procedure for this protein purification. First, the preparation underwent $(NH_4)_2SO_4$ fractionation followed by high-speed

Figure 5 Immunohistochemical images of the 77 kDa protein in bovine and chicken cardiac muscles using monoclonal antibody 5F3

Chicken cardiac muscle is shown after haematoxylin-eosin coloration (panel A) and treatment with the second antibody alone (panel B). Periodic striation labelling is shown in cardiac muscle from chicken (panel D) and bovine heart (panel C) using monoclonal antibody 5F3. The arrows show visible fluorescent bands that bridged the distance between two consecutive transverse striations. Higher magnification is shown in panel E. Panels A, B, C and D ($\times 300$); panel E ($\times 1200$).

centrifugation. The hydroxyapatite column allowed us to eliminate other high-molecular-mass proteins and pigmented components. At this step, no other glycoproteins were found to be associated with the 77 kDa protein according to this purification procedure, as controlled using the Glyco-DIG detection kit (results not shown). The enriched 77 kDa protein sample was then submitted to S-Sepharose fast-flow chromatography. The first elution peak for each fraction, after washing the column without any ionic strength buffer and starting the NaCl gradient, is presented in Figure 3. The presence of the protein was identified after analysis on SDS/PAGE, either stained by Coomassie Blue or revealed on nitrocellulose sheets using the monoclonal antibody 9C11 characterized above. In contrast with reported dystrophin properties, this purified protein was not retained on either the wheat-germ-agglutinin-lectin or concanavalin affinity columns (results not shown).

Following the same procedure, similar 77 kDa molecular-mass proteins were purified from chicken liver and brain extracts. Although we agreed with previous results [28] showing the brain to be the most abundant source of DP71, our experimental conditions gave us a poor extraction yield. This was mainly due to the fact that no attempts were made to optimize extraction conditions for this protein in each tissue. Our experimental conditions were kept identical for comparison of the different tissues. We thus found that DP71 was easier to purify from liver and heart than from brain.

Characterization of the 77 kDa protein

Using monoclonal antibody 9C11, the 77 kDa protein was found to contain the C-terminal part of dystrophin. Moreover, the commercially available dystrophin-specific monoclonal antibody

Figure 6 Comparative analysis of bovine cardiac muscle by indirect cytochemical fluorescence labelling

Single or double labelling of 10–12 μm -thick cryostat sections of unfixed tissues from bovine heart were treated for epifluorescence. Double labelling of muscle tissue sections was carried out in sequential steps as follows: first, section incubation with the anti-dystrophin polyclonal antibody C-4-22 together with specific DP71 monoclonal antibody, followed by incubation with rhodamine-conjugated anti-(mouse IgG) (1/100, TEBU) together with fluorescein-conjugated anti-(rabbit IgG) (1/50, Jackson Immuno Research Laboratories). The white arrows showed an identical periodic striation in the transverse T tubules. The scale marker (white bar) corresponds to 20 μm .

dys2 was unable to detect this 77 kDa protein, revealing that it could not be a proteolytic product of the dystrophin C-terminal part. To investigate whether the 77 kDa protein corresponded to DP71, we also produced another specific monoclonal antibody (5F3) directed against the previously described last 31 amino acids of DP71 [24]. The purified 77 kDa proteins obtained from chicken brain, cardiac muscle and liver were positively detected with the specific antibody 5F3 (Figure 4). Monoclonal antibodies 5F3 and 9C11 thus clearly revealed that the purified 77 kDa proteins were short transcript products related to the *DMD* gene.

Unexpectedly, the use of a glycoprotein detection kit gave positive detection on the purified 77 kDa protein from cardiac muscle, indicating that this protein was glycosylated (Figure 4). This result was supported by the fact that when an N-deglycosidase enzyme (PGNaseF) was used, the molecular mass shifted from 77 kDa to 71 kDa (Figure 4).

Localization of DP71

Immunofluorescence studies were performed on bovine and chicken cardiac muscle sections using the monoclonal antibody 5F3 characterized above (Figure 5). Regular transverse periodic striations were observed. Many non-fluorescent areas were also detected in the sections examined. In some places (as shown by the arrows) visible fluorescent bands bridged the distance between consecutive periodic striations of the same myofibril at the sarcolemma. Another dystrophin-specific antibody (C-4-22) was used to corroborate this observation with previous identification of the cardiac muscle structure and to give clearer interpretation to our results. Thus we comparatively assessed fluorescent immunostaining obtained with monoclonal antibodies 5F3

(DP71-specific) and C-4-22. The 5F3 monoclonal antibody was detected with fluorescein-coupled anti-(mouse IgG) second antibody, while the C-4-22 polyclonal antibody was revealed with rhodamine-coupled anti-(rabbit IgG) secondary antibody. As expected, dystrophin was found at the periphery of the sarcolemma membrane. With both antibodies, the periodic staining was similar and revealed co-distribution of both DP71 and dystrophin molecules (Figure 6). However, on the ultrathin section of cardiac muscle, the thickness of the section obtained in this investigation was not homogenous and an adjustment was required to be able to focus on both antibodies. These data, along with the fact that the dystrophin–glycoprotein complex was previously localized at the level of the transverse T tubules [34], indicated that in heart DP71 is co-distributed within the Z-disk in the transverse T tubule structures. This distribution was also similar to those previously reported for skeleton, a muscle protein of the intermediate filament [35].

DISCUSSION

DP71 expression is stringently controlled during the development stages and is down-regulated during myogenesis *in vitro* [6]. Here we observed that DP71 is expressed in chicken skeletal muscle, but not in rat skeletal muscle. These two different patterns might be explained by different regulation pathways in these two species. It would be of interest to investigate whether there is any functional significance to this difference.

We purified DP71 from different sources using an identical procedure for each tissue. The conditions were not modified to increase the extraction yield in each tissue and thus allowed us to compare the performance of the method within organs. In

contrast with our expectations, the most dystrophin-rich organ, the brain, provided a less abundant extraction than liver or heart. Purification of the 77 kDa protein and the production of specific monoclonal antibodies were the first steps in studying the biochemical properties of DP71 and in defining its functional role. Saponin was used during this extraction as previously described in cardiac muscle to obtain cell skinning [36]. The use of saponin as detergent led to detachment of the 77 kDa protein from the muscle membrane, an adaptation to a previously described single-step rapid procedure to prepare cardiac dystrophin [12]. The use of different protease inhibitors with reduced preparation time was critical for isolating intact 77 kDa protein. A relationship between saponin and the ring-like micellar structure is characteristically favoured by cholesterol in the plasma membrane. This detergent effect, its perturbation (increase membrane permeability to sodium) and the easy extraction of the 77 kDa protein by such treatment suggest formation of a complex between this 77 kDa protein and different membranous proteins. Extraction of the 77 kDa protein was performed at pH 12.5. In such conditions, using digitonin-solubilized rabbit skeletal muscle membrane, the 156 and 59 kDa dystrophin-associated proteins were co-extracted, while the 50, 43 and 35 kDa proteins remained within the membrane pellet [16]. These results also suggest that the 77 kDa protein is not an integral membrane protein and is not linked to the 50, 43 and 35 kDa components of the dystrophin complex, as they were not present in our extract as expected. However, characterization of this 77 kDa protein as a glycosylated protein indicates that it could be an easily extractable membrane protein when using alkaline treatments, suggesting localization in the plasma membrane as is the case for the 156 kDa glycoprotein. Such a localization is in full accordance with recent reports from Nudel's group who found that DP71 was associated with the cell membrane [29]. This post-translational event does not occur in dystrophin, as previously reported [15]. N-deglycosidase treatment confirmed that this protein was glycosylated, and the new 71 kDa molecular mass is in full agreement with the calculated molecular mass from DP71 cDNA sequences.

Expression of the DP71 small transcript and the corresponding protein was mainly obtained using RNAase-protection assays, PCR amplification and Western-blot techniques. Although RNAase-protection experiments are useful in detecting the tissue distribution of this transcript since specific probes can be used, studies at the protein level using immunochemical detection cannot be so specific as the monoclonal antibodies used until now detect both dystrophin and DP71. We show that two new monoclonal antibodies are specific to the 77 kDa protein. Monoclonal antibody 5F3 specifically detected this protein. Its epitope was raised against the C-terminal part of the protein the sequence of which is absent in dystrophin. Monoclonal antibody 9C11 detected the 77 kDa protein and also the dystrophin fusion protein of the C-terminal part of dystrophin, but did not detect dystrophin in our extracts. This could be explained by the fact that we produced an antibody against a native form of the protein. It is thus possible that in dystrophin, the corresponding epitope is hidden by post-translational events and not in DP71. Taken together, these results support the identification of this 77 kDa protein as belonging to the dystrophin gene family and indicate that the molecule must have some specific properties that are not common to dystrophin, even though similar sequences are present.

Previous reports have localized dystrophin and the glycoprotein complex in transverse T tubules [34]. Here we localized DP71 in the Z-disk area within the same structure as dystrophin, where numerous other proteins are also present. Future work

should focus on analysing potential interactions of the 77 kDa protein with different purified components of the Z-disk. Moreover, immunocytochemistry techniques should be used to compare normal cardiac muscles with those of DMD patients, where there is a deletion in the region corresponding to DP71, and also in *mdx* mice which are dystrophin deficient but in which DP71 is still expressed.

These results also pointed out the need for studies at the protein level, as post-translational modifications may be important for protein function and should be considered before carrying out any efficient gene therapy studies. It would be of major interest to determine, in Fukayama-type congenital muscular dystrophy [37] and severe childhood autosomal recessive muscular dystrophy [38], events responsible for the absence of the 43 kDa and 50 kDa glycoproteins respectively, although the results are controversial [39]. A deficiency in their primary sequence or absence of a glycosylation pathway might make these proteins more prone to degradation.

Production of the new monoclonal antibodies used here will help in various fields: (i) to avoid misinterpretation in diagnosis of DMD or Becker muscular dystrophy and (ii) to investigate specifically the exact role and localization of proteins related to the dystrophin gene family in different muscle and non-muscle tissues.

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