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Dp71, the nonmuscle product of the Duchenne muscular dystrophy gene is associated with the cell membrane

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The 70.8 kDa protein, Dp71, is the major Duchenne muscular dystrophy (DMD) gene product in many nonmuscle tissues including the brain. Dp71 shares most of the C-terminal and cysteine-rich domains with the dystrophins but lacks the entire large rod shaped domain of spectrin-like repeats, and the N-terminal actin-binding domain. The function of Dp71 is unknown. Using subcellular fractionation and immunostaining we show that Dp71 is associated with the plasma membrane. Dp71 is also associated with the plasma membrane in *mdx* myogenic cells transfected with a vector expressing Dp71.

Duchenne muscular dystrophy; Dp71; Dystrophin; Immunostaining; Plasma membrane

1. INTRODUCTION

The main product of the DMD gene in the muscle is a 427 kDa protein called dystrophin. It consists of four domains: an N-terminal actin binding domain; spectrin-like repeats; a cysteine-rich region, sharing homology with the Ca^{2+} binding domain of *Dictyostelium* α-actinin; and a C-terminal domain with no similarity to any other known protein [1], except for the dystrophin related protein (DRP) [2]. Electron microscopy and cytoimmunostaining studies have demonstrated that in the muscle, dystrophin is associated with the cell membrane [3,4]. This association is mediated by a number of glycoproteins which complex with dystrophin [5,6].

A very similar isoform of dystrophin is expressed in the brain. Its mRNA is transcribed from the same gene but it is regulated by a different promoter located 75–300 kb 5′ to the promoter of the muscle type dystrophin [7–14].

Recently, we and others have described an additional product of the same gene having a mass of only 70.8 kDa (Dp71) [15–19]. This protein is expressed in many nonmuscle cell types and it is the major product of the DMD gene in brain, and in neuron and glia cell cultures [15–17]. It is relatively abundant in embryonic stem cells where it is the only detectable DMD gene product [20]. Dp71 does not contain the actin binding domain or the large region of spectrin-like repeats which confer a rod shape to dystrophin [15,16]. It does contain the cysteine-rich and the C-terminal domains of dystrophin. However, due to alternative splicing, 13 amino acids are deleted in the C-terminal domain and the last 13 amino acids are replaced by 31 amino acids, which are not found in skeletal muscle dystrophin. This substitution increases the calculated hydrophobicity of the C-terminus of Dp71. In addition, the sequence encoding the first 7 amino acids at the N-terminus of the protein, and the 5′ untranslated region are located in a unique exon not present in dystrophin mRNA. This exon and the promoter of Dp71 are located in the intron between exon 62 and 63 of dystrophin mRNA ([17,18] and Lederfein et al., in preparation).

The occurrence of Dp71 in a wide range of cells and its regulated expression during early embryogenesis suggest that it has an important biological function, which is as yet unknown. To approach this question we investigated the cellular localization of Dp71. The results presented here show that at least a significant fraction of Dp71 is associated with the cell membrane. These results also indicate that the cysteine-rich and the C-terminal domains are sufficient for the association with the plasma membrane.

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Abbreviations. DMD, Duchenne muscular dystrophy; kDa, kilodalton; DRP, dystrophin related protein; kb, kilobase(s); PM, plasma membrane; ER, endoplasmic reticulum; Ab(s) antibody(ies); mAb, monoclonal antibody; pAb, polyclonal antibody.

2. EXPERIMENTAL

2.1. *Cell cultures*

Human hepatoma HepG2 cells and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum. For immunofluorescence staining the cells were grown on cover slips pre-coated with gelatin.

2 2. *Establishment of a myogenic cell line from* mdx *mice and transfection*

The MDXL myogenic cell line was established from thigh muscle of 3-week-old *mdx* male mouse (Yaffe and Saxel, unpublished) using the culture conditions, and differential plating, serial cell passaging and cloning methods as described previously [21,22] The cells were grown and induced to differentiate as described in Yaffe and Saxel [22] The cells proliferated and differentiated in a manner similar to other myogenic cell lines, but did not express dystrophin. Cultures of proliferating mononucleated MDXL cells were cotransfected with a plasmid containing the entire coding region of the Dp71 cDNA under the control of the skeletal muscle actin gene promoter, and the plasmid pTM which confers G418 resistance. G418 resistant clones expressing Dp71 after differentiation were isolated and used for immunofluorescence staining.

2.3. *Preparation of subcellular fractions*

Monolayer cultures of HepG2 cells were rinsed with PBS and the cells were collected using a rubber policeman and centrifuged for 10 min at 1,500 rpm The pellet was resuspended in a hypotonic solution (10 mM HEPES, pH 7 4, 10 mM KCl, 0.1 mM EDTA, 0 1 mM EGTA, 1 mM DTT, 0 5 mM PMSF, 1 μg/ml leupeptine, 1 μg/ml pepstatine, 1 μg/ml aprotenine) and homogenized with a B-pestle Dounce homogeneizer (20 strokes). Nuclei were pelleted by 10 min of centrifugation at 1.000 rpm. The supernatant fraction was centrifuged at 25,000 $\times g$ for 15 min. The resulting supernatant contained mainly soluble proteins and enzymes The pellet was resuspended in a buffer containing 0.3 M sucrose, 10 mM tricine, pH 8, 1 mM K-EDTA, 1 mM PMSF, 0.1 mM PABA, 1 μg/ml leupeptin, 1 μg/ml pepstatin and layered on the top of a centrifuge tube containing several layers of sucrose in the above-mentioned buffer (20% and 35% sucrose). After spinning for 90 min at 25,000 rpm (Beckman rotor SW41), two fractions were collected. These were the interphase between the 20% and 35% sucrose layers, that contained the plasma membrane (PM); and the pellet, that mostly contained mitochondria and endoplasmic reticulum (ER). The fractionation was done at 4°C. All the fractions were assayed for protein concentration (Bradford assay) and stored at −70°C. For Western blot analysis, the sample buffer components were added to a final concentration of: 67.5 mM Tris-HCl (pH 6.8), 20% glycerol, 15% SDS, 5% 2-mercaptoethanol, 0 001% Bromophenol blue.

The preparation of subfractions from rat liver was done according to Hirschberg et al. [23].

2 4. *Western blot analysis*

Western blot analysis was done using 3–10% polyacrylamide/SDS gradient gels as described by Laemmli [24] and modified by Pons et al. [25]. Detection of the immunoreactive bands was done with the ECL kit (Amersham).

2.5. *Immunofluorescence staining*

For indirect immunofluorescence staining, the cells were rinsed briefly with PBS and then fixed for 5 min in cold methanol (−20°C). Fixed cells were then permeabilized with PBS, 0.2% Triton X-100 for 15 min and blocked with PBS, 5% milk, 0.2% Triton X-100 for another 30 min. Incubations of the antibodies (Abs) were done in PBS, 0.2% Triton X-100, 5% milk at room temperature. The primary and secondary Abs were incubated for 3 and 1 h, respectively, followed by several washes with PBS. Mounting was done in 60% glycerol, 2% *n*-propyl gallate and 0.2 M Tris-HCl (pH 8.1)

2.6. *Antibodies*

MANDRA1 and MANDRA6 are monoclonal antibodies (mAb) against two different epitopes in the C-terminal domain of human dystrophin. The two Abs do not react with DRP [26] NCl Dys-1 is a mAb against the mid rod domain with epitopes of human dystrophin (Novocastra Laboratories) R-156 is a polyclonal antibody (pAb) raised against *N*-cadherin [27]. For immunofluorescence the secondary Ab used was Rhodamine anti-mouse TRITC from Jackson Immuno Laboratories Photographs were taken with a Axioskop Zeiss immunofluorescence microscopy using Kodak T-max ASA 3200 film

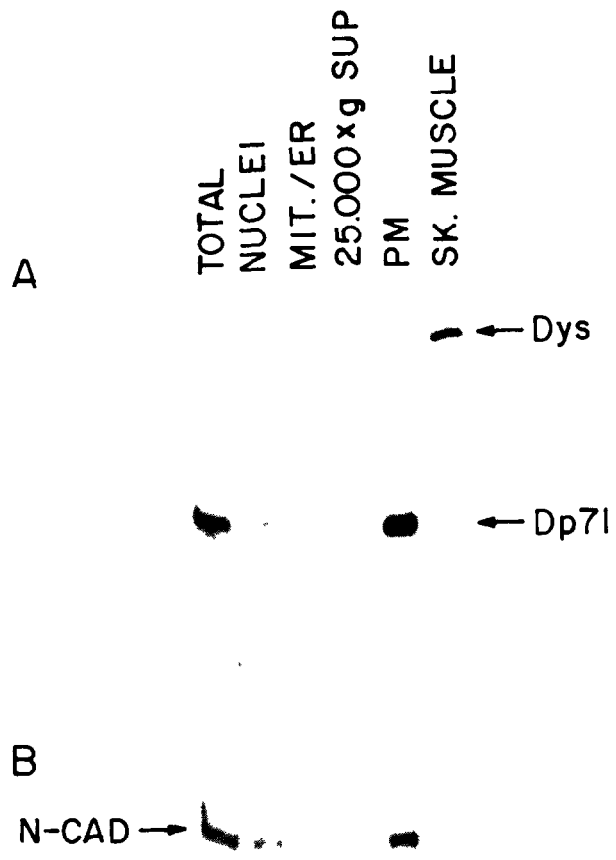

Fig 1. Distribution of Dp71 in subcellular fractions of HepG2 cells. Protein samples (15 μg) were sized-fractionated on a 3–10% polyacrylamide/SDS gel, blotted onto a nitrocellulose sheet, and immunostained with the mAb MANDRA1 (A) and pAb R-156 (B) The positions of dystrophin (Dys), Dp71 and *N*-cadherin (N-CAD) are indicated. The samples are: total extract of HepG2; nuclei; mitochondria and endoplasmic reticulum (MIT./ER); 25,000 $\times g$ supernatant (sup); plasma membrane (PM) and total skeletal muscle extract (sk. muscle)

3. RESULTS

3.1. *Subcellular fractionation*

To determine the cellular localization of Dp71 we prepared subcellular fractions from human hepatoma HepG2 cells. The relative concentration of Dp71 in the various fractions was determined by Western blot analysis using the mAb MANDRA1, which recognizes both dystrophin and Dp71 [16,26]. Dp71 is highly enriched in the sucrose-layer purified PM fraction. However, it is also present in other fractions (Fig. 1A). Recovery calculations indicated that 5–20% of total Dp71 was found in the purified PM fraction. As can be seen in Fig. 1B, the subcellular distribution of *N*-cadherin, a known membrane protein which is associated with cell–cell ad-

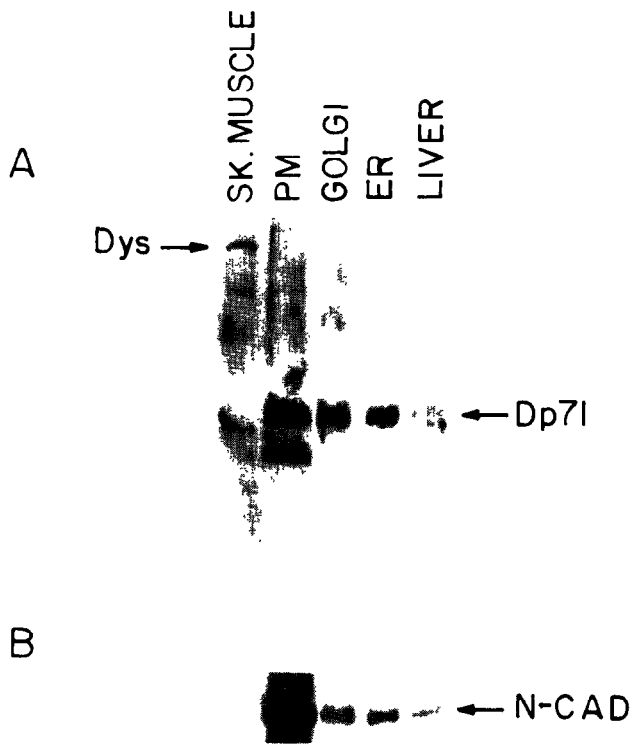

Fig. 2. Distribution of Dp71 in subcellular fractions of liver cells. Protein samples (4 μg) were sized-fractionated on a 3–10% polyacrylamide/SDS gel, blotted onto a nitrocellulose sheet, and immunostained with the mAb MANDRA1 (A) and pAb R-156 (B). The positions of dystrophin (Dys), Dp71 and *N*-cadherin (N-CAD) are indicated. The samples are ER, heavy Golgi subfraction (Golgi), PM and total skeletal muscle and liver extracts. The lower band in the PM fraction may be a degradation product of Dp71.

hesion [27], is similar to that of Dp71. It is therefore likely that all the fractions are contaminated to some extent with PM.

In another experiment we compared the level of Dp71 in PM, ER fractions and a heavy Golgi fraction obtained from normal rat liver [23]. The purity of each fraction and cross-contamination was tested by assaying the activity of enzyme markers for PM (alkaline phosphodiesterase), ER (glucose-6-phosphatase) or Golgi (galactosyltransferase). The PM was essentially free of ER (1.7% contamination). However, the ER fraction was contaminated with significant amounts of PM (33%) and the heavy Golgi subfraction was contaminated with 13% of PM. As can be seen in Fig. 2A, Dp71 is enriched in the highly purified PM fraction but is also present in the other fractions which are contaminated with PM. The distribution of Dp71 in liver fractions is similar to that of *N*-cadherin (Fig. 2B) as observed in the HepG2 subcellular fractions.

These experiments show that Dp71 is associated with the PM. We cannot exclude the possibility that some Dp71 is also normally present in other cellular compartments. However, the similarity of the distribution of Dp71 and *N*-cadherin suggests that the presence of Dp71 in other fractions is mostly due to contamination with PM.

3.2. *Immunofluorescence localization of Dp71 in cells*

To further examine the localization of Dp71, we used the mAb MANDRA1 for indirect immunofluorescence staining of cell types which do not express dystrophin. Fixed and permeabilized HepG2 and HeLa cells were first stained with MANDRA1 followed by staining with rhodamine conjugated rabbit anti-mouse antibody. As can be seen in Fig. 3A, the immunofluorescence staining was associated with the cell membrane. No staining was observed with mAb NCL Dys-1 which recognizes an epitope in the spectrin-like repeat domain of dystrophin, which is absent in Dp71 (Fig. 3C). No membrane associated staining was observed when mAb MANDRA1 was omitted from the staining reaction (second Ab only; not shown).

Membrane staining of HepG2 cells was also obtained with the mAb MANDRA6, which is directed against a different epitope in the C-terminal region of the protein (not shown).

It should be pointed out that in the HepG2 cells the membrane staining was observed in regions of cell aggregates density with extensive cell-to-cell contact. We do not know whether this pattern of staining represents clonal diversity or a density-dependent stimulation of Dp71 expression. Further investigations are required in order to evaluate these possibilities.

As can be seen in Fig. 4, the immunostaining of HeLa cells, with the mAb MANDRA1 is also associated with the PM.

Due to the low abundancy of Dp71 in liver tissues, we could not detect significant specific staining of Dp71 in frozen liver sections.

3.3. *Localization of exogenous Dp71 expressed in* mdx *myogenic cells*

As described elsewhere, Dp71 is not detectable in differentiated muscle fibers [16,17]. It was of interest to test whether Dp71 expressed from a transfected gene is capable of binding to the muscle fiber sarcolemma.

Due to a mutation resulting in a stop codon (nucleotide 3185 of dystrophin mRNA), *mdx* mice do not express dystrophin [28]. The mutation does not affect the expression of Dp71 which is transcribed from the 3′ region of the gene in the relevant tissues. However, as in normal mice, Dp71 is undetectable in skeletal muscle of *mdx* mice ([16] and Fig. 5).

A myogenic cell line established in our laboratory from *mdx* mice, MDXL, was transfected with a plasmid containing a cloned Dp71 cDNA under the control of the rat skeletal muscle actin gene promoter. Some of the transfected clones expressed Dp71 in differentiated cultures at a level comparable to the levels of dystrophin in muscle cell lines from normal mice.

Specific staining of the muscle fiber membrane was

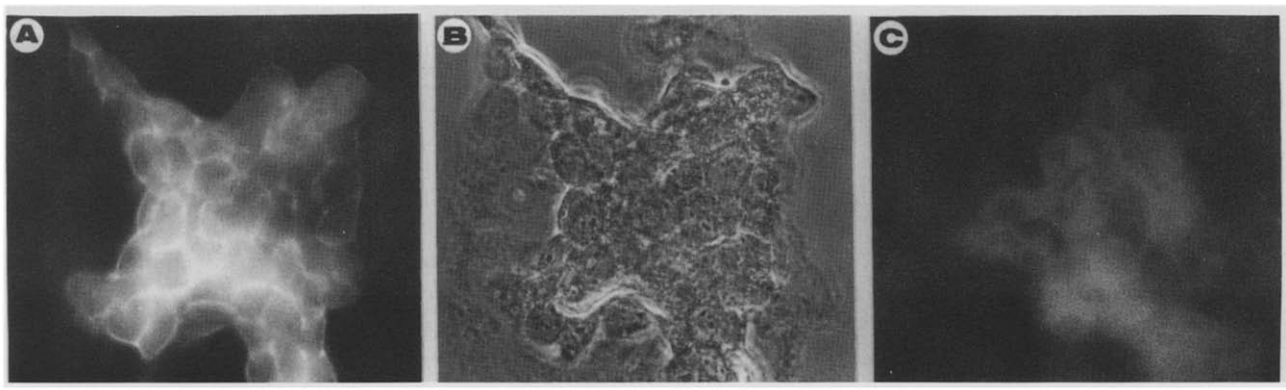

Fig. 3. Immunofluorescence staining of Dp71 in HepG2 cells. (A) Immunofluorescence staining with mAb MANDRA1. (B) Phase-contrast of the field shown in (A). (C) Immunofluorescence staining with mAb NCL Dys-1. Magnification: 350 ×.

observed only in differentiated MDXL myogenic cultures that expressed exogenous Dp71, indicating that at least a significant fraction of Dp71 expressed from the cloned cDNA became associated with the sarcolemma (Fig. 5). Since the only difference between the transfected and nontransfected cells is the expression of Dp71, this experiment also provides evidence that the membrane staining is not due to crossreaction of the mAb with other proteins. The intensity and distribution of the membrane staining was similar to that observed in L185 cells, a rat myogenic cell line that expresses dystrophin (not shown).

4. DISCUSSION

Both the immunostaining and the subcellular fractionation of HepG2 cells clearly demonstrate that in these cells Dp71 is primarily associated with the PM; however, the presence of some Dp71 in other cellular compartments cannot be excluded. Dp71 is also enriched in a purified PM fraction in a normal liver tissue, and its presence in other subcellular fractions is probably mainly due to contamination of these fractions with PM.

Membrane staining with a mAb against an epitope in the C-terminal domain shared by dystrophin and Dp71 was also observed in HeLa cells. These cells contain Dp71 mRNA and small amounts of dystrophin mRNA. However, only the Dp71 protein is detectable in extracts from these cells when analysed by Western blot (not shown). The specific membrane staining which is visible only with anti-C-terminal Abs indicates that Dp71 is also associated with the PM in HeLa cells.

The interaction of dystrophin with the sarcolemma is mediated through binding to several glycoproteins [29,30]. It was first suggested [5] and then demonstrated [6] that the C-terminal and cysteine-rich domains of dystrophin are involved in this association. On the other hand, evidence coming from studies of DMD patients with truncated dystrophins that do not contain the C-terminal domain of the protein, suggested that the N-terminal domain and/or the spectrin-like repeats may be sufficient for the association of the protein with the membrane [31–33].

As Dp71 contains only the C-terminal and cysteine-rich domains of dystrophin, our results indicate that these two domains of the protein are sufficient for membrane association in muscle and nonmuscle cells. It re-

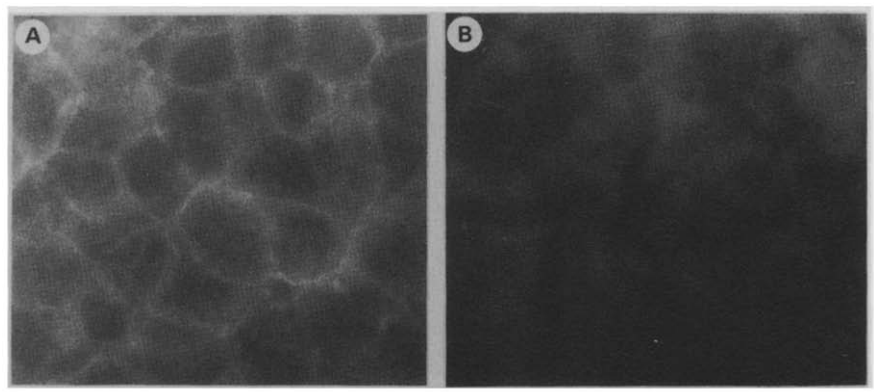

Fig. 4 Immunofluorescence staining of Dp71 in HeLa cells. Immunofluorescence staining with mAb MANDRA1 (A) and mAb NCL Dys-1 (B). Magnification. 350 ×.

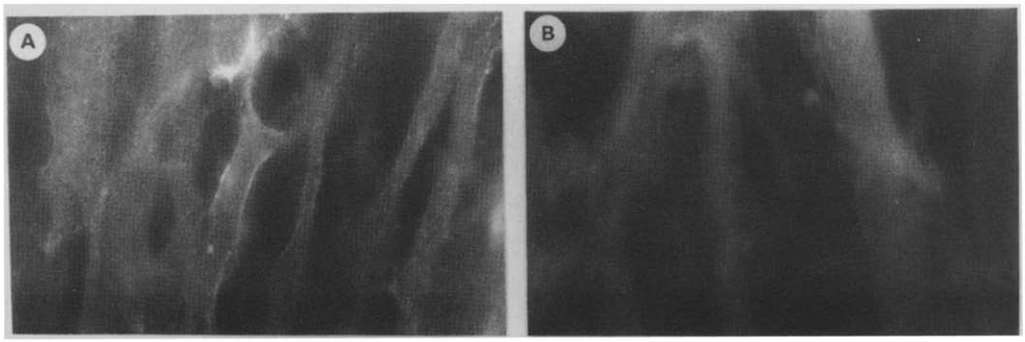

Fig. 5. Immunofluorescence staining of exogenous Dp71 in *mdx* myogenic cells: (A) Differentiated, stable transfected MDXL cultures expressing exogenous Dp71. (B) Control untransfected differentiated MDXL cultures. Stained with mAb MANDRA1. Magnification. 400 ×.

mains to be determined whether Dp71 binds to the same glycoproteins that bind dystrophin, other glycoproteins, or whether it is associated with the cell membrane by a different mechanism.

On the basis of the rod-shaped structure of dystrophin, its localization to the membrane, and the damage of the sarcolemma in DMD patients, it was speculated that dystrophin is a cytoskeletal protein which provides mechanical support to the membrane in skeletal muscle. Other studies have shown some linkage between dystrophin and the calcium leak channels (reviewed in [34]). Dp71 is a much smaller, nonfibrilar, membrane-associated protein, containing potential Ca^{2+} binding sites, and is expressed in many nonmuscle cell types. As Dp71 does not contain the spectrin-like repeats and the actin binding domain of dystrophin it is unlikely that this protein functions as a mechanical support to the cell membrane. In view of its localization, relative abundancy in embryonic stem cells and early embryos, the possibility that Dp71 is involved in interactions between cells should be investigated.

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