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Rapid Communication

Xp21 Dystrophin and 6q Dystrophin-related Protein

Comparative Immunolocalization Using Multiple Antibodies

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A protein of M_r 400 K and slightly lower M_r than Xp21 dystrophin was detected in skeletal muscle from patients with Duchenne muscular dystrophy by three antibodies raised angainst the midrod and C-terminal portions of chicken dystrophin, and by antibodies to dystrophin-related protein. Immunocytochemistry showed continuous sarcolemmal staining of Duchenne muscle with these antibodies. Subcellular localization to the inner face of the plasma membrane of Duchenne muscle was demonstrated by immunoelectron microscopy using the model of a Duchenne patient deleted for most of the dystrophin gene. Other antibodies were specific for Xp21 dystrophin. In conclusion, a dystrophin homologue that may be identical to the previously described dystrophin-related protein (DRP)[1] is expressed in Duchenne muscle with intracellular distribution similar to Xp21 dystrophin in normal muscle. (Am J Pathol 1991, 139:969–976)

Dystrophin is the protein product of the Duchenne muscular dystrophy (DMD) gene locus on chromosome Xp21.[2] Extensive sequence homologies to spectrins and alpha-actinins have led to the suggestion that these molecules may be members of a family of large structural proteins expressed in muscle.[3,4,5] An autosomal homologue to dystrophin was discovered when Love et al. screened a fetal muscle cDNA library with cDNA clones encoding the final C-terminal domain of Xp21 dystrophin and found a closely related sequence with 83% sequence homology to dystrophin at the predicted amino-acid level over a stretch of 490 amino acids.[6] This homologous sequence identified a 13 kb mRNA and mapped to chromosome 6.[6] No further data are as yet available on the nucleotide sequence of this chromosome 6-encoded gene. Subsequent studies using pAbs to a recombinant protein containing a 656 bp-region of the homologous cDNA identified a 400 K protein, called dystrophin-related protein (DRP), which was expressed in normal and DMD skeletal muscle.[1] Unlike dystrophin, this 6q-DRP was also found to be expressed at high levels in many nonmuscle tissues such as kidney, spleen, liver, and placenta.[1,7]

In previous studies, we have made use of the cross-reaction of polyclonal antibodies raised against different fragments of chicken dystrophin to identify a protein with molecular mass (M_r) of 400 K on Western blots which is expressed at the neuromuscular junctions in skeletal muscle of normal and DMD patients, and of normal and dystrophin-deficient mdx mice.[8] Similar immunolocalization to the neuromuscular and myotendinous junctions in skeletal muscle was shown for 6q-DRP.[9] The study defined the immunolocalization of this dystrophin homologue in DMD skeletal muscle by the use of newly generated monoclonal antibodies to human and chicken dystrophin, and compared structural similarities between Xp21 dystrophin and 6q-DRP at the protein level. All results were compared with those obtained for antibodies raised against a DRP fusion protein.

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Materials and Methods

Patients

Normal muscle was obtained from orthopedic surgery for nonmuscle-related reasons. Muscle from DMD patients was obtained from diagnostic needle biopsies and had previously been shown to lack dystrophin completely by immunofluorescence and Western blotting.[10] For immunolocalization of DRP at the light and electron microscopic level, the muscle of the DMD patient M.A. which was known to be deleted exons 1 to 52 of the dystrophin gene as well as for the muscle and brain promotor regions and the genomic probe 754,[8,10] was used thereby excluding crossreaction of antibodies to dystrophin or to any alternative splicing products of the dystrophin gene in this sample. Immunoelectron microscopy of DMD muscle was exclusively performed from this specimen, but for light microscopy and Western blotting the results were further compared with those of two DMD patients and with those of four normal controls. The DMD patients aged 3 months and 5 years lacked dystrophin in their muscle specimens but had no detectable deletions of the dystrophin gene.[10]

Antibodies

The polyclonal antibodies generated from rabbits immunized with a fragment of the central rod domain of chicken dystrophin (pAbs C4/6: residues 1173–1728) were described previously.[8] Furthermore, four new mAbs were recently obtained by lymphocyte hybridizations from three different mice immunized with recombinant dystrophin fragments spanning three different regions of human and chicken skeletal muscle dystrophin.[8,11]

- mAbs Do.9C2 and Do.3F6 were obtained using a fragment of the human dystrophin midrod domain (residues 1840–2266) as immunogen.
- mAb C.4G10 was obtained using a fragment of the chicken dystrophin midrod domain (residues 1173–1728) as immunogen.
- mAb H.5A3 was obtained using the C-terminal domain of chicken dystrophin (residues 3357–3660) as immunogen.

All of these pAbs and mAbs detected their own antigen in ELISA. They all stained the sarcolemma of normal human skeletal muscle on immunofluorescence and detected a protein of M_r 400 K on Western blots. The mAb H.5A3 also reacted with the DRP fusion protein in ELISA and on Western blots. In addition, mAb dys 2 raised against a synthetic peptide consisting of the last 17 amino acids at the C-terminus of human dystrophin was given by Dr. L. V. B. Nicholson, Newcastle-upon-Tyne, UK.[12] The pAbs raised against a DRP fusion protein that were used in this study were a gift from Drs. T. S. Khurana and L. M. Kunkel, Boston, and have been characterized previously.[1]

Immunofluorescence and Western Blotting

Immunofluorescence and Western blotting were carried out as described.[10,13] Briefly, for immunofluorescence serial 5-μm cryosections were incubated with the primary antibody (undiluted culture supernatant for mAbs Do.3E6, C.4G10, and H.5A3, mAb Do.9C2 was diluted 1:10, pAbs C4/6 were diluted 1:50, anti-DRP diluted 1:100, and mAb dys 2 diluted 1: 50) in PBS for 2 hours. This was followed by the respective secondary Abs (biotinylated sheep–anti-mouse or biotinylated donkey–anti-rabbit from Amersham) diluted 1: 200 for 30 minutes and by streptavidin-Texas red (Amersham) diluted 1: 200 in phosphate-buffered saline (PBS) for 1 hour. Sections were viewed and photographed in a Zeiss III RS photomicroscope equipped for epifluorescence. Electrophoresis was carried out by separating muscle homogenates on 4–20% sodium dolecyl sulphate (SDS) gradient gels with a 3.5% stacking gel and applying 100 μg of protein per lane. Transfer and immunostaining were carried out as described previously.[10]

Electron Microscopy

To preserve antigenicity we developed a two-step fixation procedure for cryomicrotomy modifying a protocol by Tokuyasu.[14] Fresh muscle tissue was immersed in 0.3 M ethylacetimidate (Sigma) in PBS for 30 minutes and then transferred into 8% paraformaldehyde in PBS for 20 minutes. The specimens were subsequently dehydrated in 2.3 M sucrose for 4 hours and then snapfrozen onto a silver pin in liquid N_2. Sections of 100 nm thickness were cut in a RMC MT 6000 ultracut at −100°C, mounted on carbon–formvar-coated nickel grids, immunostained as for immunofluorescence using 10 nm protein A-gold for pAbs from rabbit and 10 nm anti-mouse IgG from goat (Amersham) for mAbs as secondary antibodies. Thin sections were embedded in 0.6% (w/v) methylcellulose containing 2% uranyl acetate and viewed in a Philips CM 10 electron microscope.

Results

Immunofluorescence

The pAbs C4/6 and the mAb C.4G10 directed against the rod portions of chicken dystrophin as well as mAb

H.5A3 (against the C-terminus of chicken dystrophin) labelled the sarcolemma of both normal and DMD muscle (Figure 1 a–f). For all these antibodies sarcolemmal staining of DMD muscle was less intense than for normal muscle. In addition, the staining pattern observed in DMD muscle showed variable intensity of surface staining between different fibers. All antibodies that labelled the sarcolemma of DMD muscle also showed intense staining of intramuscular nerves (not shown) and blood vessels in normal and DMD muscle (Figure 1 f, i). If the first antibody was omitted, none of the secondary antibodies showed any staining on muscle sections.

The mAbs Do.9C2 and Do.3E6 (against the rod portion of human dystrophin) as well as mAb dys 2 showed sarcolemmal staining of normal muscle but complete lack of staining in DMD muscle (shown for mAb Do.9C2 in Figure 1 g, h). There was no prominent staining of intramuscular nerves or vessels in normal or DMD muscle for any of these antibodies.

Staining of the sarcolemma in normal and DMD muscle was also found with pAbs to DRP if they were used at a dilution of 1:100 (Figure 1 i, j). The staining characteristics observed for anti-DRP in DMD muscle were similar to those found for pAbs C4/6 and for mAbs C.4G10 and H.5A3.

Electron Microscopy

To determine the precise intracellular localization of the sarcolemmal staining observed at the light microscopic level, thin sections of DMD muscle that had large deletion of the dystrophin gene, from the DMD patient, were stained with pAbs C4/6 and with mAb H.5A3. Both antibodies showed predominant labelling on the cytoplasmic face of the plasma membrane (Figure 2 a, b). Occasional labelling in the plasma membrane or on the outer surface was also observed. No other intracellular structures of the muscle fiber were stained. Again, the same results were obtained with antibodies to DRP (Figure 2c). Thin sections of normal muscle showed stronger label of the same distribution when stained with pAbs C4/6, anti-DRP, or mAb H.5A3 than the DMD muscle of the patient (not shown).

Western Blotting

Muscle homogenates from the three DMD patients lacked dystrophin completely on immunoblots labelled with mAb dys 2 (Figure 3, blot A). The same result was obtained for mAbs Do.9C2 and Do.3E6, which detected dystrophin as a band of M_r 400 K in normal muscle and lacked binding to the sarcolemma of DMD muscle by immunofluorescence.

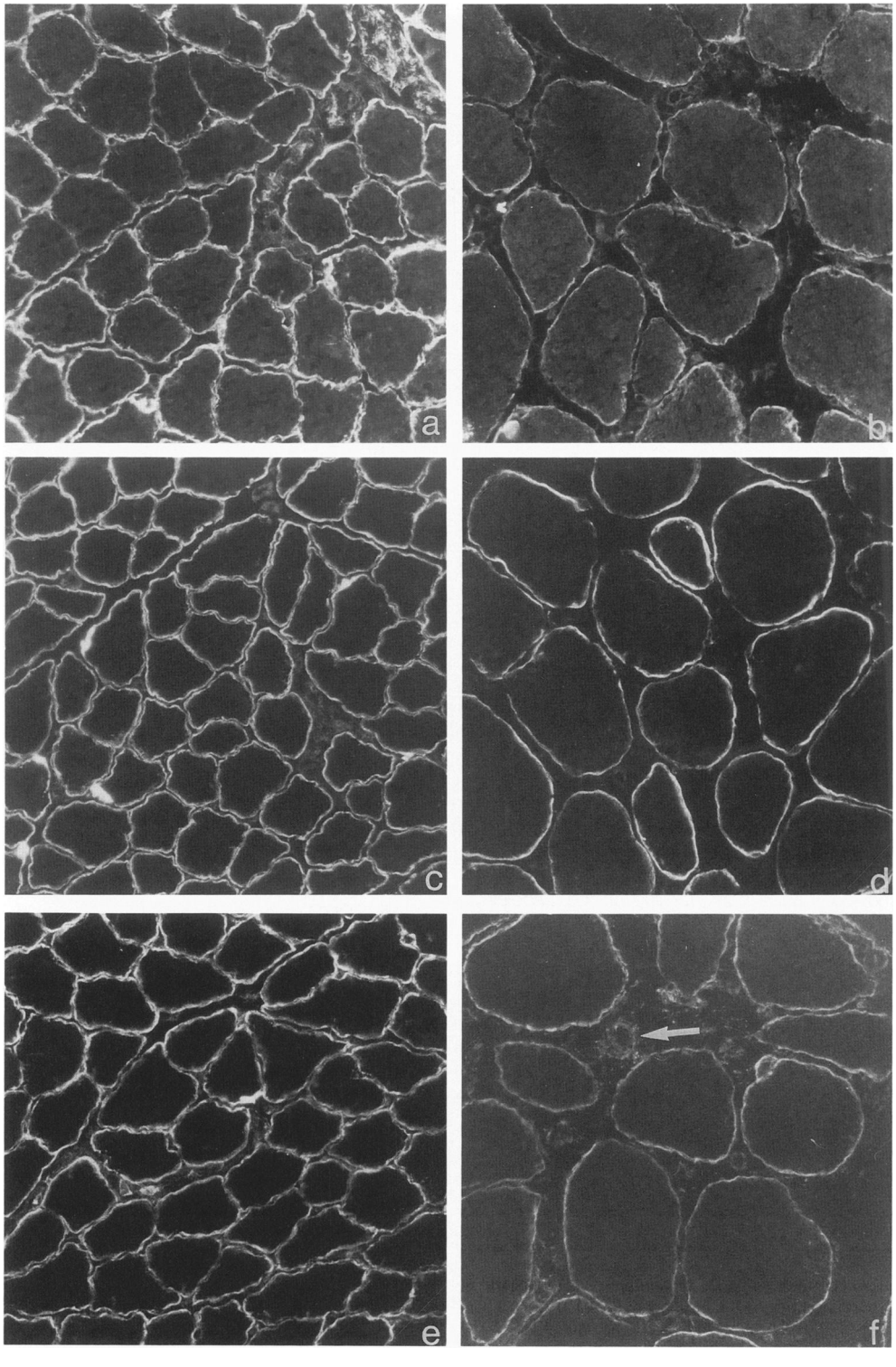
In contrast, the pAbs C4/6 as well as mAbs C.4G10 and H.5A3 showed strong labelling of dystrophin in normal muscle plus binding to a protein of slightly smaller M_r and reduced abundance if compared with dystrophin in DMD muscle (shown for mAbs C.4G10 and H.5A3 in Figure 3, blots B, C). A protein of identical M_r was detected in DMD muscle by anti-DRP (Figure 3, blot D). The staining of the corresponding band in normal muscle by anti-DRP was more intense than in DMD muscle suggesting crossreaction of anti-DRP to Xp21 dystrophin.

For all antibodies that detected a band of M_r 400 K in DMD muscle the difference in M_r to Xp21 dystrophin was minimal and could only be clearly seen on adjacent lanes of a blot. Because all the anti-dystrophin antibodies stained a strong band in normal muscle it was impossible to distinguish between a Xp21 dystrophin-band and a 6q-DRP-band in normal muscle.

Interestingly, the antibodies raised against C-terminal portions of dystrophin recognized Xp21 dystrophin as a single band on Western blots (Figure 3, blots A, C). One of these mAbs, which showed crossreaction to a 400 K protein in DMD muscle also labelled this protein as a single band (Figure 3, blot C). In contrast, antibodies directed against the rod-portion of dystrophin labelled Xp21 dystrophin as a doublet and also sometimes detected a doublet of slightly smaller M_r in DMD muscle (Figure 3, blot B, lane 4). In addition, mAb C.4G10 also consistently labelled a faint cross-reactive band just above myosin in DMD muscle only (Figure 3, blot B). Similar to what was found for mAb H.5A3 directed against the C-terminus of dystrophin, anti-DRP which was raised against a recombinant protein containing parts of the C-terminal DRP sequence also only labelled a single band in DMD muscle (Figure 3, blot D).

Discussion

A dystrophin homologue with M_r 400 K was previously shown to be expressed at the neuromuscular junctions of normal individuals and DMD patients.[8] This protein was also detected in vascular and nerve tissue of normal and mdx mice.[15] The precise immunolocalization of this dystrophin homologue in DMD muscle was further characterized by newly generated pAbs and mAbs raised against various portions of human and chicken dystrophin. Three different antibodies, two against the rod portion and one against the C-terminus of dystrophin showed crossreaction to a 400 K dystrophin homologue expressed at the sarcolemma of DMD muscle. This dystrophin homologue must be transcribed from a gene other than Xp21 dystrophin because it was consistently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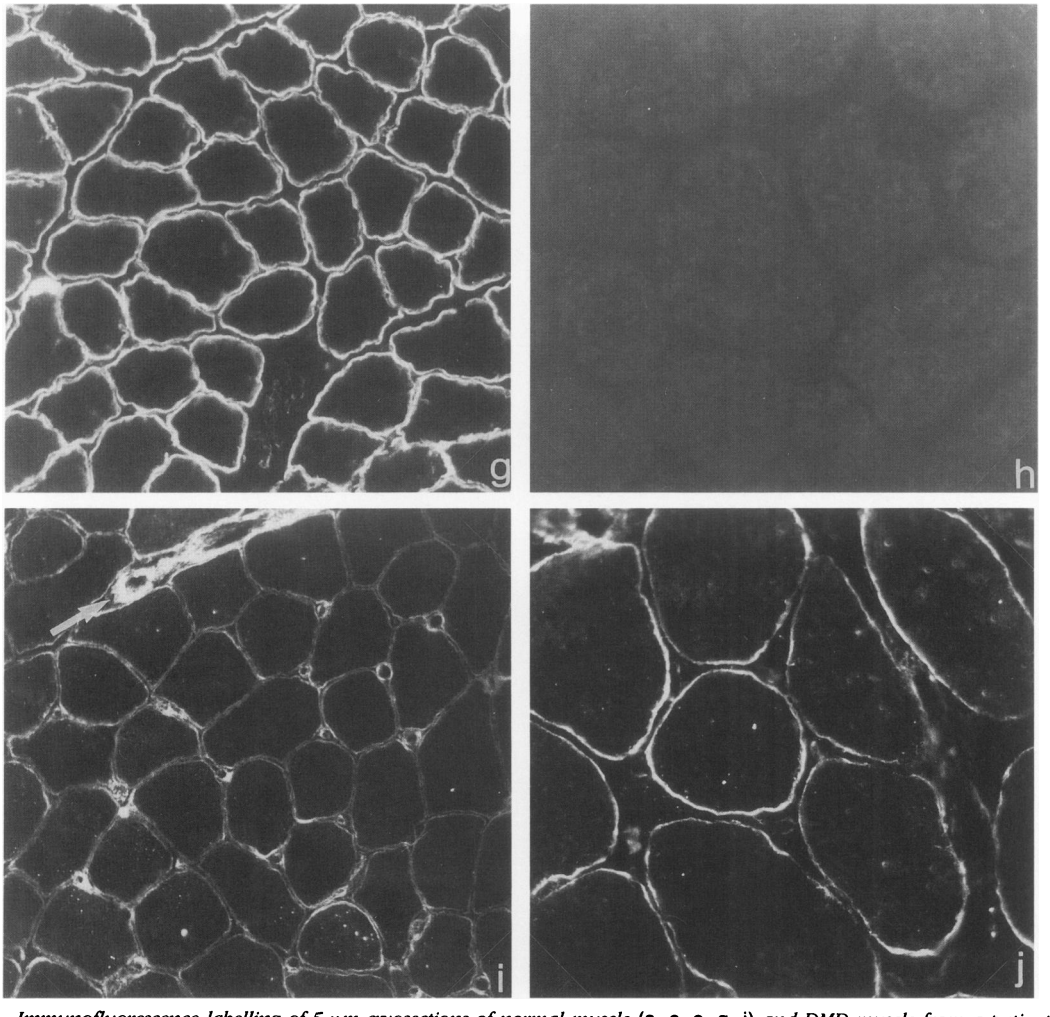

Figure 1. *Immunofluorescence labelling of 5-μm cryosections of normal muscle* (**a, c, e, g, i**) *and DMD muscle from a patient with a deletion of exons 1 to 52 of the dystrophin gene* (**b, d, f, h, j**) *using various antibodies to dystrophin and DRP, ×180.* **a,b**: *pAbs C4/6 raised against the rod portion of chicken dystrophin stain the sarcolemma of normal muscle fibers* (**a**) *but also show continuous but less intense labelling of the sarcolemma in DMD muscle* (**b**). **c,d**: *mAb C.4G10 raised against the rod portion of chicken dystrophin strongly label the sarcolemma of normal muscle* (**c**) *but also stain DMD muscle surface membrane with reduced intensity* (**d**). **e,f**: *mAb H.5A3 raised against the C-terminus of chicken dystrophin stain the sarcolemma of normal muscle* (**e**) *but also delineate the sarcolemma of DMD muscle at reduced intensity* (**f**). *Intramuscular blood vessels are also stained* (**f**, *arrow*). **g,h**: *mAb Do.9C2 raised against the rod portion of human dystrophin label the sarcolemma of normal muscle intensely* (**g**) *but show complete lack of staining in DMD muscle* (**h**). **i,j**: *pAbs to DRP label the sarcolemma of normal muscle continuously* (**i**) *but also exhibit continuous labelling of DMD muscle sarcolemma* (**j**). *Intramuscular blood vessels are brightly stained* (**i**, *arrow*).

detected in muscle from a DMD patient lacking exons 1 to 52 of the dystrophin gene.

Sarcolemmal staining in the DMD muscle specimens was also observed with pAbs to DRP, which had previously been shown to label the neuromuscular and myotendinous junctions of skeletal muscle.[9] Because the mAb H.5A3 also reacted with the DRP fusion protein, the similar immunolocalization of the protein detected by these various antibodies argues in favor of the fact that they all recognize 6q-DRP. The staining pattern of positively labelled intramuscular nerves and vessels observed for pAbs C4/6 and mAbs C.4G10 and H.5A3 as well as for anti-DRP further supports this assumption.

The detection of 6q-DRP at the sarcolemma of DMD muscle in addition ot the previously described expression of this protein at the neuromuscular junction can be explained by the higher affinity for 6q-DRP of the newly generated mAbs as well as the higher sensitivity of the streptavidin–Texas-red amplification method if compared with directly fluorescein-conjugated secondary antibodies as used in the previous study ([8] and T. Voit, unpublished results).

Muscle–fiber-surface staining had previously been noted in mdx mice when polyclonal antibodies raised against the C-terminus of dystrophin were used, and these pAbs also detected a 400 K band.[16] Identity of this protein with 6q-DRP was postulated and is likely in view of our identical results.

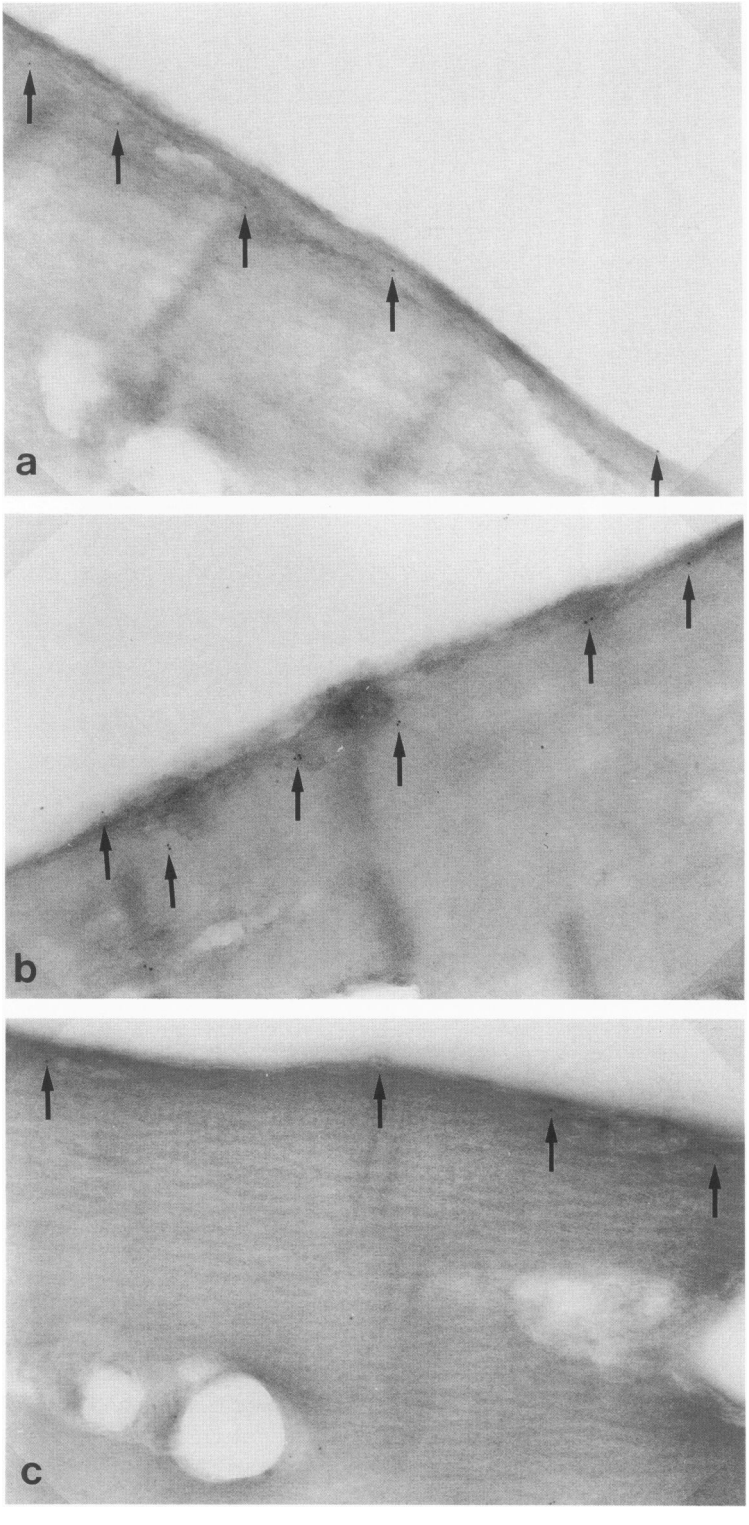

Figure 2. *Immunogold labelling of thin cryosections of DMD skeletal muscle from a patient who carries a deletion of exons 1 to 52 of the dystrophin gene using antibodies to different portions of the dystrophin molecule* (**a**, **b**) *and to DRP* (**c**). **a**: *pAbs C4/6 directed against the rod portion of chicken dystrophin detect a homologous protein at the cytoplasmic face of the plasma membrane in DMD muscle (arrows),* ×*30,000.* **b**: *mAb H.5A3 directed against the C-terminus of chicken dystrophin label a homologous protein at the cytoplasmic face of the plasma membrane (arrows),* ×*45,000.* **c**: *pAbs to DRP show labelling at the cytoplasmic face of the plasma membrane in DMD muscle (arrows),* ×*31,500.*

The identity of the 400 K dystrophin homologue to 6q-DRP was further supported by the subcellular localization of this protein at the cytoplasmic face of the plasma membrane in DMD muscle by pAbs C4/6 and by mAb H.5A3 as well as by anti-DRP. This localization is similar to that of Xp21 dystrophin in normal muscle.[17,18]

On Western blots, the protein detected by pAbs C4/6 and by mAbs C.4G10 and H.5A3 in DMD muscle was of slightly lower M_r than the protein(s) detected in normal muscle. In previous studies 6q-DRP was found to comigrate with dystrophin on Western blots.[9] The difference in M_r detected in our system, however, was not significant

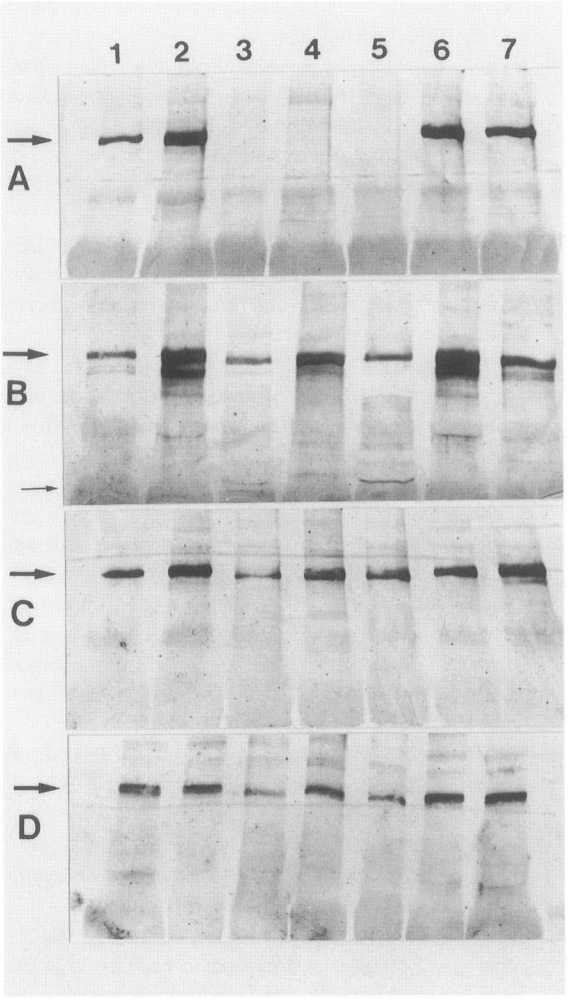

Figure 3. *Four parallel Western blots of skeletal muscle homogenate with identical loading* (**A, B, C, D**), *probed with various antibodies to different portions of Xp21 dystrophin, and with pAbs against 6q-DRP. Lanes were loaded as follows: 1, 2, 6, 7 = normal controls; 3 = DMD muscle from a 3-month-old patient; 4 = muscle from DMD patient MA with a deletion of exons 1 to 52 of the dystrophin gene; 5 = muscle from a 5-year-old DMD patient. Only the upper-half of the blots is shown.* **A**: *Xp21 dystrophin is detected as a single band of M_r 400 K in normal muscle (lanes 1, 2, 6, 7) but lacking completely in DMD muscle (lanes 3–5) when probed with the Xp21 dystrophin-specific mAb dys 2 (blot A, arrow).* **B**: *The mAb C.4G10 directed against the rod portion of chicken dystrophin shows a dystrophin doublet in normal muscle (blot B, arrow). A protein of slighty lower M_r and reduced abundance is labelled as a single (lanes 3, 5) or double band (lane 4) in DMD muscle. An additional band of M_r 220 K is found in DMD muscle only (small arrow).* **C**: *The mAb H.5A3 detects dystrophin as a single band of M_r 400 K in normal muscle and also labels a protein of slightly lower M_r in DMD muscle as a single band. The protein detected in DMD muscle is less abundant than the corresponding band in normal muscle.* **D**: *Polyclonal Abs to DRP label a single band of M_r 400 K in normal skeletal muscle (lanes 1, 2, 6, 7) and also detect a protein of slightly lower M_r and reduced abundance in DMD muscle (lanes 3–5).*

enough to rely on it for diagnostic purposes because the two proteins were not clearly separated. The slightly higher M_r of the protein(s) detected in normal muscle can be explained by the reaction of the different anti-dystrophin antibodies with Xp21 dystrophin as well as by crossreaction of anti-DRP to Xp21 dystrophin. Because the resolution of this difference in molecular weight is incomplete by the gel system used, the bands of Xp21 dystrophin and 6q-DRP merged into one band with resulting higher abundance and slightly higher M_r in normal muscle whereas only DRP with lower M_r was detected in DMD muscle.

Although all antibodies raised against C-terminal portions of either Xp21 dystrophin or 6q-DRP identified Xp21 dystrophin in normal muscle and 6q-DRP in DMD muscle as a single band on Western blots, mAb C.4G10 labelled Xp21 dystrophin as a doublet and also sometimes detected 6q-DRP in DMD muscle as a doublet. The detection of a Xp21 dystrophin doublet in normal muscle by antibodies raised against the rod portion of dystrophin is a well-known feature[2,10] and has been linked to the existence of dystrophin isoforms[13,19] as well as to protein degradation.[20]

In addition, a crossreactive band of M_r 220 K was detected by mAb C.4G10 in DMD muscle only. This additional band most likely represents a degradation fragment of 6q-DRP, possibly comparable to the Xp21 dystrophin fragment of similar M_r detected by antibodies to the N-terminus or rod portion.[20] This Xp21 dystrophin fragment is frequently observed in BMD muscle[21] and can be produced experimentally by limited proteolysis.[20] The detection of a similar fragment of 6q-DRP in DMD but not in normal muscle suggests the presence of a similar site sensitive to endogenous proteolysis in diseased muscle. The presence of secondary degradation of 6q-DRP in DMD muscle might also play a role in the unequal expression of sarcolemmal staining in DMD muscle, which was observed for all antibodies with (cross-) reaction to this protein. Increased expression in regenerating fibers may be an alternative or complementary explanation.[9]

The sequence homologies of 6q-DRP to Xp21 dystrophin over the C-terminal part of the molecule have been firmly established[6] but nothing is known yet about the more N-terminal sequence. The finding that the pAbs C4/6 and the mAb C.4G10, both raised against the rod portion of chicken dystrophin, showed crossreaction to 6q-DRP strongly suggests the presence of at least one further epitope localized on the rod portion of Xp21 dystrophin and common to both proteins. In addition, a 400 K protein was detected in DMD muscle and believed to correspond to DRP by pAbs (called 1-2a) raised against N-terminal sequences of Xp21 dystrophin.[20,22] Taken together with our results, these findings support the concept of a chromosome 6-encoded homologue to dystrophin, which shares common epitopes with Xp21 dystrophin over the C-terminus, rod portion, and N-terminus, and may be presumed to represent a rod-shaped cytoskeletal protein possibly derived from a common ancestral gene. However, differences in epitopes can also be predicted from the Xp21 dystrophin-specific labelling

found for mAbs Do.9C2 and Do.3E6 raised against the rod portion of human dystrophin.

As a practical consequence, antibodies to dystrophin used for diagnostic purposes should be carefully characterized and crossreaction to 6q-DRP and possibly other homologous proteins be excluded before any conclusions can be drawn from the results. Serious diagnostic errors could result from the use of anti-dystrophin antibodies with unidentified crossreaction to 6q-DRP, which would yield positive sarcolemmal staining of DMD muscle and label a 400 K band in DMD muscle homogenates on Western blots.

From a more theoretical point of view, the question if 6q-DRP is also expressed at the sarcolemma of normal muscle must be left open until specific antibodies without crossreaction to Xp21 dystrophin become available. At present, overexpression of 6q-DRP in DMD muscle or lack of downregulation of the protein which is strongly expressed in fetal muscle[1] could account for the sarcolemmal expression of the dystrophin homologue in DMD muscle.

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