Are Cysteine-rich and COOH-Terminal Domains of Dystrophin Critical for Sarcolemmal Localization?

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Abstract

It has been hypothesized that the tight localization of dystrophin at the muscle membrane is carried out by its cysteine-rich and/or carboxyl domains. We report the results of biochemical and immunocytochemical investigations of dystrophin in muscle from a 1-yr-old patient with a large deletion that removes the distal part of the dystrophin gene, thus spanning the exons coding for the cysteine-rich and the carboxy-terminal domains, and extends beyond the glycerol kinase and congenital adrenal hypoplasia genes. Immunological analysis of muscle dystrophin shows that the deletion results in the production of a truncated, but stable, polypeptide correctly localized at the sarcolemma. These data indicate that neither the cysteine-rich domain, nor the carboxyl domain, are necessary for the appearance of normal dystrophin sarcolemmal localization. (*J. Clin. Invest.* 1992. 89:712–716.) Key words: Duchenne/Becker muscular dystrophy • gene deletion • muscle membrane

Introduction

Dystrophin, the 427 kD protein produced by the Duchenne muscular dystrophy/Becker muscular dystrophy (DMD/BMD) gene, shares many features with the cytoskeletal proteins spectrin and alpha-actinin. It has an extended spectrin-like central domain, and more globular amino- and carboxyl-terminal domains (1). Its location in muscle at the inner surface of the plasma membrane (2, 3), despite the lack of any transmembrane domain, suggests that dystrophin is associated with membrane proteins. Very likely candidates are the integral membrane sarcolemmal glycoproteins, characterized by Campbell et al. (4, 5). However, the precise domain(s) of interaction remains speculative. It has been hypothesized that the tight membrane association is mediated by the cystein-rich and/or COOH-terminal domains of dystrophin. This assumption was first based on indirect arguments, such as the very high degree of conservation at nucleotide and amino-acid levels of these domains (6), the correlation between the disease severity (Duchenne versus Becker), and dystrophin–domain alteration (7). It was further assessed by ultrastructural studies using a COOH-terminal antibody (25).

Here, we report the case of a 1-yr-old boy carrying a large deletion removing the distal part of the dystrophin gene beyond exon 49, and encompassing the glycerol kinase and congenital adrenal hypoplasia genes. In skeletal muscle we found a truncated dystrophin, that, although lacking the cysteine-rich and COOH-terminal domains, seems to be stable and to retain normal sarcolemmal localization.

Methods

Case. The proband A.B. is a male patient with muscular dystrophy, glycerol kinase deficiency, congenital adrenal hypoplasia, and poor psychomotor development. Diagnosis was made at birth because of adrenal failure. The histological and histochemical study of a muscle biopsy specimen taken at the age of 9 mo showed the characteristic features of muscular dystrophy: (*a*) numerous necrotic muscle fibers; (*b*) foci of regenerating fibers; (*c*) discrete type 1 fiber predominance; and (*d*) marked increase in collagen endomysial tissue. Full familial, clinical, and molecular details will be given elsewhere. PCR–multiplex analysis of peripheral blood DNA, to explore the muscle promoter, and 17 exons of the dystrophin gene (8, 9), showed that exons 50, 51, 52, and 60 were missing. The size of the deletion was further documented by Southern analysis that showed a terminal deletion of the dystrophin gene with a proximal breakpoint between exons 49 and 50. A junction fragment was detected on EcoRI and XmnI digests (Fig. 1). The distal breakpoint of the deletion mapped between C7 and L1 probes (data not shown).

Immunoblotting. Western blot analysis of muscle biopsy specimens from normal and patient A.B. was performed according to Nicholson et al. (10), using polyclonal (60 kd) (11) and monoclonal (Dy4/6D3) (10) antibodies directed against the central part of dystrophin, a monoclonal antibody (Dy10/12B2) directed against an epitope in the NH_2-terminal part of the protein within exons 10 to 12 (12), and a monoclonal antibody Dy8/6C5 directed against the 17 last amino acids (12).

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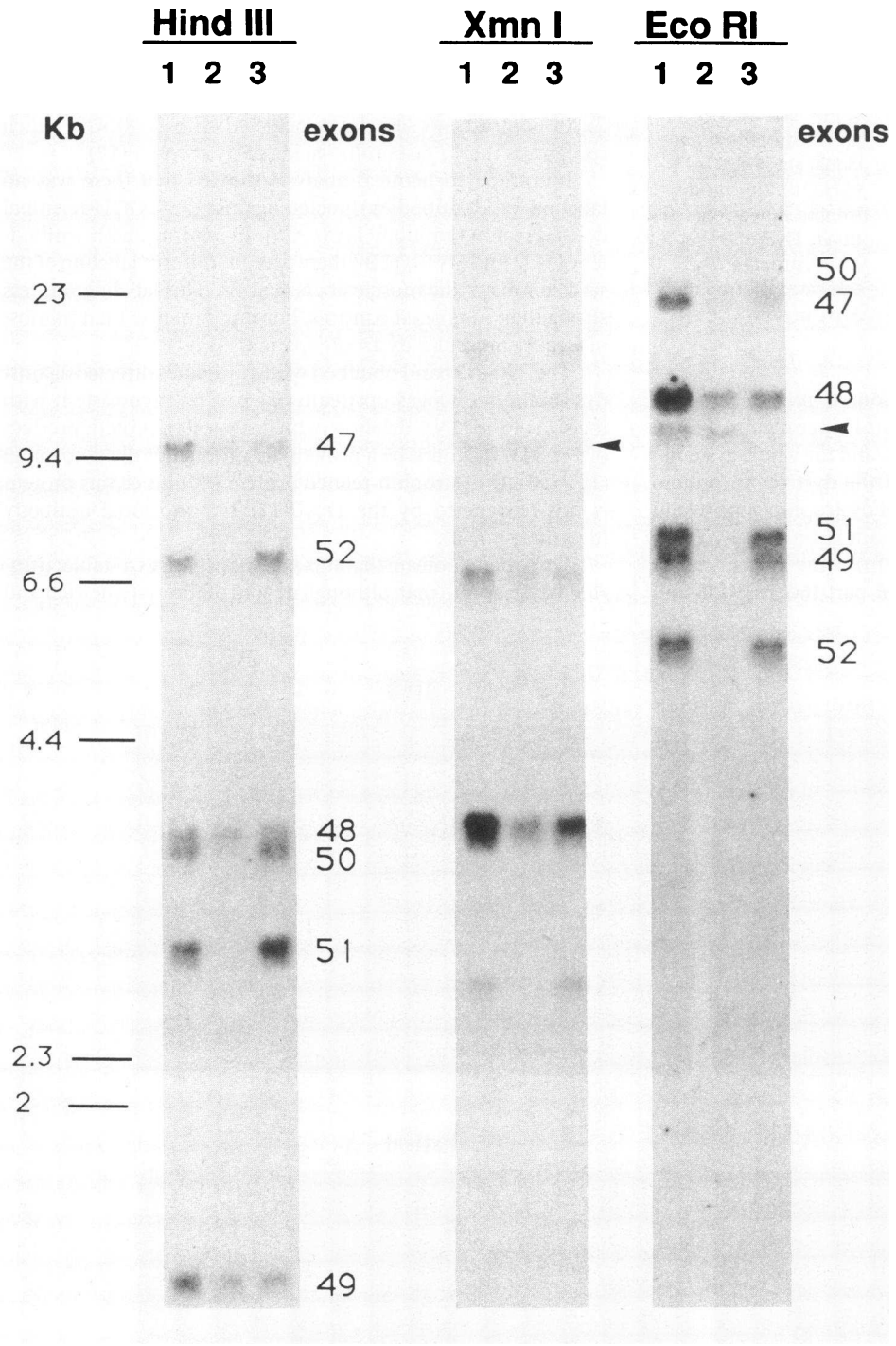

Figure 1. Southern blot of genomic DNA digested by HindIII, XmnI, and EcoRI, and hybridized with the cDNA probe 8 (4). (Lane *1*) patient A.B.'s mother; (Lane *2*) patient A.B.; (lane *3*) normal male control DNA. Hybridization pattern on patient A.B.'s DNA (lane *2*) shows the absence of fragments containing exons 50–52 on HindIII digest, and a junction fragment corresponding to exon 49 on XmnI and EcoRI digests (*arrow*). Gene dosage and junction band on DNA of his mother (lane *1*) indicate her carrier status.

Immunocytochemistry. The muscle biopsy specimens were immediately frozen in isopentane, cooled in liquid nitrogen, and stored at −80°C. Histochemical studies using conventional techniques and immunocytochemical characterization of dystrophin were performed in transverse cryostat sections. The immunodetection was performed on 4 to 6-μm sections by indirect immunofluorescence, using the above mentioned polyclonal and monoclonal antidystrophin antibodies, and revealed with corresponding fluorescein isothyocyanate-conjugated IgG (13). Sections were examined with a Leitz photomicroscope equipped with epifluorescence illumination.

Results and Discussion

From the physical map of the Xp21 region constructed by pulse field gel electrophoresis (14–16), we estimate that the deletion in patient A.B. encompasses at least 4,000 kb of genomic DNA, starting between exons 49 and 50, and ending between C7 and L1. The position of the distal breakpoint confirms the proximal position of C7 (16, 17), and indicates that the deletion eliminates the congenital adrenal hypoplasia and

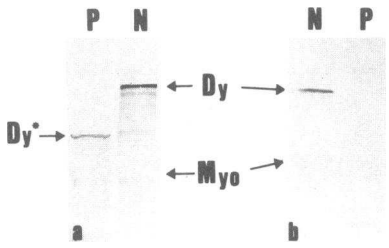

Figure 2. Western blot of control human normal muscle (*N*) and patient muscle specimen (*P*) probed with (*a*) anti-dystrophin monoclonal antibody Dy4/6D3 (10); (*b*) anti-dystrophin monoclonal antibody Dy8/6C5 (12). *Dy,* dystrophin; *Dy*,* truncated dystrophin; *Myo,* myosin.

glycerol kinase genes (17). In addition, it removes the sequences corresponding to part of the third hinge region, the five last repeats of the spectrin-like domain, and the whole cysteine-rich and COOH-terminal domains of the dystrophin protein (18). We thus expected that the deleted dystrophin gene would produce an unstable truncated dystrophin. Surprisingly, Western blot analysis of a muscle biopsy from the patient, using antibodies directed against the central part (60 kd) (11) and (Dy4/6D3) (10), and against the NH_2-terminal part (Dy10/12B2) of dystrophin, showed the presence of a truncated dystrophin of about 270 kD, which was only slightly diminished in labeling intensity (about 60% of the normal dystrophin) (Fig. 2). In contrast, no signal was detected with Dy8/6C5, which recognizes the 17 last amino-acids of dystrophin (12).

Immunocytochemical analysis showed that there was no labeling with antibodies directed against the COOH-terminal deleted region (antibody Dy8/6C5). In contrast, with antibodies 6O kD and Dy10/12B2 there was an uniform labeling of the sarcolemma of the muscle fibers (Fig. 3). This labeling was less strong than in normal controls, but more intense than in most of Becker muscular dystrophy patients (Fig. 3).

The 270-kD band obtained with antibodies directed against dystrophin sequences upstream of exon 50 is consistent with the size of the DNA deletion, which suggests a protein product of 280 kD. It cannot be a proteolytic product of the autosomal (19) 420-kD dystrophin-related protein (20), since this protein is not recognized by the Dy10/12B2 monoclonal antibody (data not shown).

These data indicate that this patient produces a stable, truncated dystrophin, that, although devoid of the cysteine-rich and

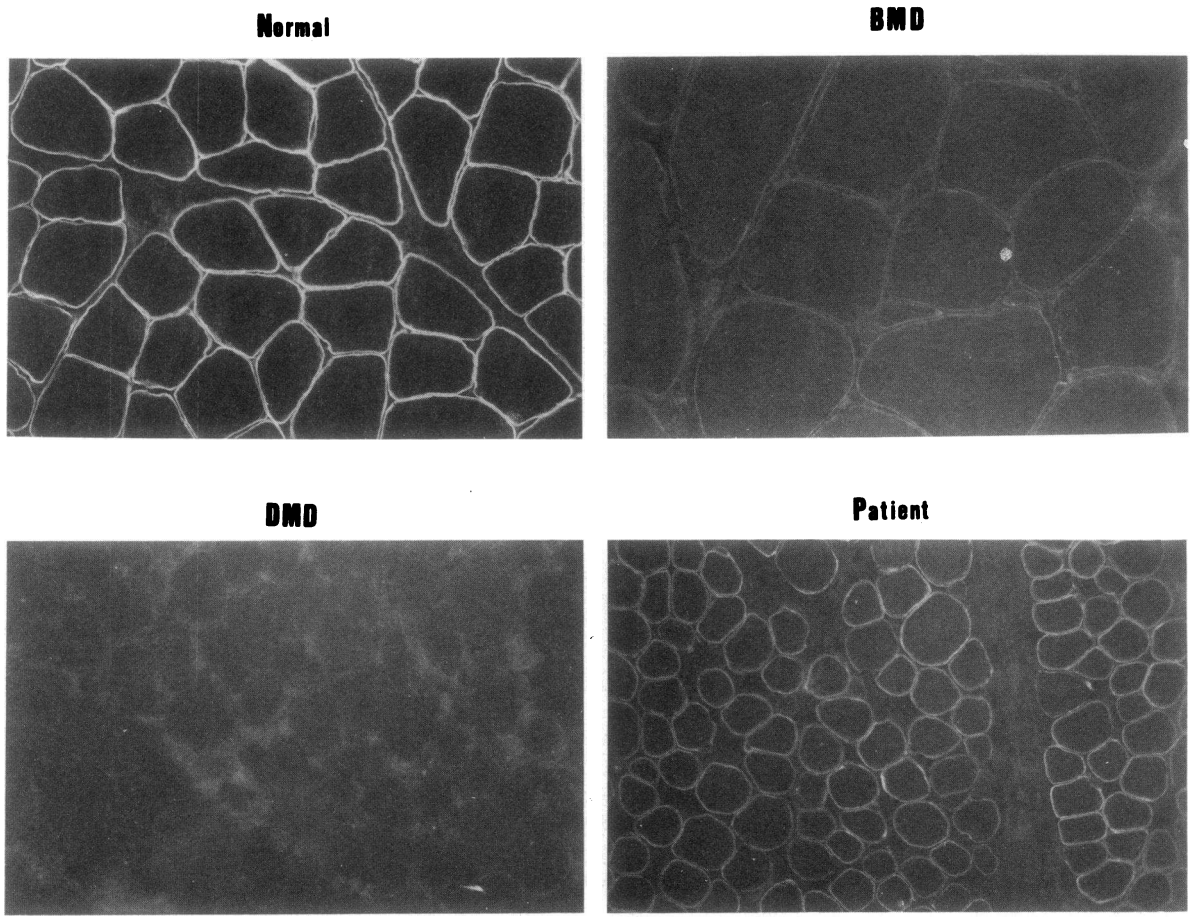

Figure 3. Indirect immunofluorescence localization of dystrophin using anti-dystrophin monoclonal antibody DY10/12B2 (12) in unfixed cryostat transverse section of muscle fibers. (*Normal*) even distribution of dystrophin in the sarcolemma of all fibers. (*BMD*) marked diminution of the labeling of the sarcolemma in a Becker muscular dystrophy patient aged 29. (*DMD*) absence of dystrophin in a 6-yr-old Duchenne muscular dystrophy patient. (*Patient*) the labeling is strictly sarcolemmal and of intermediate intensity between normal and Becker muscular dystrophy. Magnification 175.

COOH-terminal domains, appears to be correctly located at the muscle cell sarcolemma. This unexpected result suggests that these domains are not essential for the sarcolemmal localization of dystrophin, and that interactions between other regions of the dystrophin molecule and proteins closely associated with the membrane must occur, as previously suggested by Cross et al. (21). Interestingly, the truncated dystrophin (deletion of exons 17 to 48) of the Becker patient reported by England et al. (22) is normally localized at the sarcolemma. In addition, deletions removing various parts of the dystrophin gene, somewhere between exon 1 and exon 53, and producing a Becker phenotype, have been reported (7, 23). Although these reports do not mention immunohistochemical analysis, one may infer that the truncated dystrophin detectable on Western blots (23) is attached in vivo to sarcolemma. Altogether, these results, combined with the data observed in patient A.B., suggest that the COOH-terminal domain, which seems to be attached to the membrane (24, 25), is not critical in membrane connection. Other more proximal domains of dystrophin might be involved, thus allowing some membrane binding if one of them is missing. In Fig. 4, we propose a speculative model, derived from the one recently presented by Ervasti et al. (24).

Another possibility is that the truncated dystrophin of patient A.B. has a new COOH-terminal end, deriving from the translation of an unknown sequence downstream of the deletion breakpoint. However, the size of the truncated dystrophin is not in favor of this hypothesis. The fact that the amount of truncated dystrophin is only slightly diminished suggests that the mRNA is stable, although it lacks the 3-kb-long, highly conserved untranslated sequence. The transcript is being currently analyzed in order to define precisely the position of the stop codon, and the sequence beyond it.

The propositus is now 18 mo old, and it is difficult to anticipate what will be the clinical evolution of the muscle disease, i.e., towards a Duchenne or a Becker phenotype. Although traces of truncated dystrophin may be present in Duchenne patients (26), the presence of a significant amount of dystrophin is, in principle, indicative of a Becker-type disease (25). Indeed, we recently found substantial amounts of dystrophin in muscle samples taken in infants who were diagnosed at birth several years ago, on the basis of high plasma creatine-kinase activity, and who had ultimately developed a Becker phenotype (unpublished data). One could thus anticipate that the patient reported here should have a mild phenotype. However, it remains possible that its truncated but stable dystrophin is nonfunctional, and that a more severe disease will develop. No correlation can be made with previously reported cases associating myopathy, glycerol kinase deficiency, adrenal insufficiency, and a deletion starting in the distal portion of the dystrophin gene (28, 29), since the status and localization of dystrophin in these patients have not yet been reported.

The distal part of dystrophin is highly conserved, both at the amino acid and at the nucleotide level (3). This is thought to reflect stringent requirements for attachment to the membrane through interacting proteins. This explanation may now be questioned, since it appears that membrane binding can be ensured by other domains of dystrophin. One can hypothesize that the high degree of conservation of this portion of the gene is due rather to its involvement in the production of other essential proteins, such as the 80-kD protein (Chelly et al., manuscript in preparation) corresponding to the recently described 6.5-kb mRNA that is transcribed by the distal part of the dystrophin gene (30).

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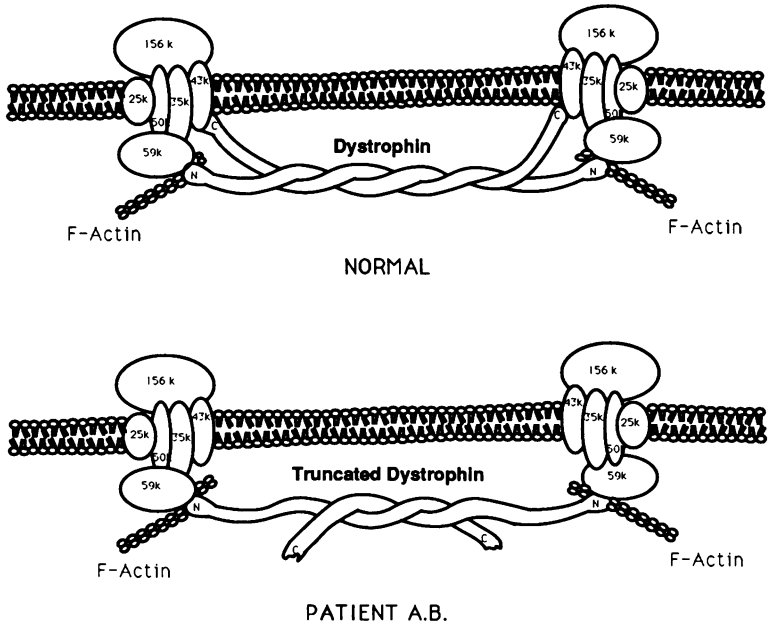

Figure 4. Speculative model of dystrophin, combining the model of Ervasti et al. (24) and our findings, showing the truncated dystrophin still attached to the membrane by its NH_2-terminal end in patient A.B. In this model, additional possible interaction between internal parts, such as hinge regions and membrane, is not represented.

Note added in proof. After submission of our manuscript, a paper by Eric P. Hoffman et al. (Is the carboxyl-terminus of dystrophin required for membrane assocation? A novel, severe case of Duchenne muscular dystrophy) describing similar findings appeared in *Annals of Neurology* (1991. 30:605–610).

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