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Monoclonal antibodies against defined regions of the muscular dystrophy protein, dystrophin

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Nineteen monoclonal antibodies which bind to native dystrophin in the plasma membrane of frozen muscle sections were obtained using a recombinant fusion protein as immunogen. On Western blots of normal mouse muscle extracts, the antibodies bind specifically to a 400000 M_r protein which is absent from dystrophic mouse (*mdx*) muscle. At least four distinct epitopes have been identified by cleavage mapping methods. Although the fusion protein contained 25% of the human dystrophin sequence (Cys^{816}-Asp^{1747}; M_r 108000), most of the monoclonal antibodies (15 out of 19) recognize a single fragment of M_r 27500.

Dystrophin; Muscular dystrophy; Monoclonal antibody; Epitope mapping; Fusion protein; Nitrothiocyanolsenzoic acid

1. INTRODUCTION

Dystrophin is a muscle membrane protein which is absent, reduced or altered as a result of mutation in the Duchenne muscular dystrophy (DMD) gene or its homologue in the mouse [1]. The large size of dystrophin (427 kDa) and its low abundance (<0.01% of the total muscle protein) are hindrances to the isolation of intact, native protein for structure/function studies.

Monoclonal antibodies against defined regions of dystrophin provide a means of studying its structure and function, interactions with other proteins and the nature of the partial gene products produced in some patients carrying deletions in the dystrophin gene [2]. Predictions from the sequence suggest a structural protein on the inner face of the membrane, consisting of a 25-repeat, rod-like, triple-helical domain separating an N-terminal actin-binding domain from two C-terminal domains, one of which is rich in cysteine [3]. Polyclonal antisera have been prepared against two helical regions using fusion protein immunogens [1] and a monoclonal antibody against the beginning of the helical region has been prepared from a synthetic peptide [4]. We now describe the production of monoclonal antibodies which can identify at least four distinct regions of dystrophin in the proposed helical region.

2. METHODS

2.1. *Preparation of immunogen and monoclonal antibodies*

A 4.3 kb dystrophin cDNA fragment designated Cf23 [5,6] was cloned into the *Eco*RI site of pEX2 (Genofit Ltd), an expression vector containing the lacZ gene [7], and the recombinant plasmid (pEX2:Cf23) was digested with *Pst*I or *Bam*HI plus *Bgl*II to produce deletion derivatives pEX2:Cf23b and pEX2:Cf23c respectively (fig.1a). The two plasmids thus derived were introduced into *E. coli* POP2136 to enable expression of the corresponding β-galactosidase fusion proteins, designated LacZ-108kD and LacZ-42kD respectively (fig.1c,e). POP2136 (pEX2:Cf23b) cells were grown at 30°C to log phase and then incubated for 1.5 h at 44°C to induce expression of the fusion protein. Inclusion bodies were released from the cell pellet by sonication at 4°C in TNE buffer (50 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0) and collected by centrifugation at 10000 × *g* for 10 min. The pellet was boiled in 2 ml of SDS buffer (2% SDS, 50 mM Tris-HCl, pH 6.8) with 5% 2-mercaptoethanol (2-ME). After centrifugation at 100000 × *g* for 20 min, the supernatant was subjected to gel filtration (85 × 1.2 cm; Ultrogel AcA34, LKB) in SDS buffer with 1 mM 2-ME. Fractions containing lacZ-108kD without major contaminants were identified by SDS-PAGE and pooled. The protein was precipitated with ethanol (50% v/v), redissolved in 0.3 ml 8 M urea in PBS, diluted to 3 M urea and incubated at 37°C for 30 min. No attempt was made to remove DNA or residual traces of SDS. An emulsion in complete Freund's adjuvant was used to immunise Balb/c mice (ca. 100 μg), with boosts 1 month later in incomplete adjuvant and twice in PBS, 2,3 or 4 days before fusion, after a rest period of 1-2 months. Sera were tested 10 days after the second injection. Fusions were performed [8] with myeloma lines Sp2/0, NS0 and NS1 and screened by ELISA [9], using microtiter plates coated with either lacZ-108kD or pEX2 β-galactosidase alone. Hybridoma culture supernatants were further tested by immunofluorescence microscopy before cloning to homogeneity (at least twice) by limiting dilution.

2.2. *Immunofluorescence microscopy*

Sections (7 μm) of human or mouse muscle frozen in isopentane were mounted on glass slides and stored at −70°C. Bound antibody was detected with FITC-labelled rabbit anti-mouse Ig (DAKO), using a Leitz Dialux microscope with epifluorescence optics and Ilford HP5 film was exposed for 30 s [10].

2.3. *Western blotting*

To minimise degradation, fresh minced muscle was dropped into 4 vols of boiling extraction buffer (10% SDS, 10% EDTA, 5% 2-ME, 10% glycerol, 50 mM Tris-HCl pH 6.7) [4], homogenised immediately in a Silverson blender, boiled again for 3 min and centrifuged (100

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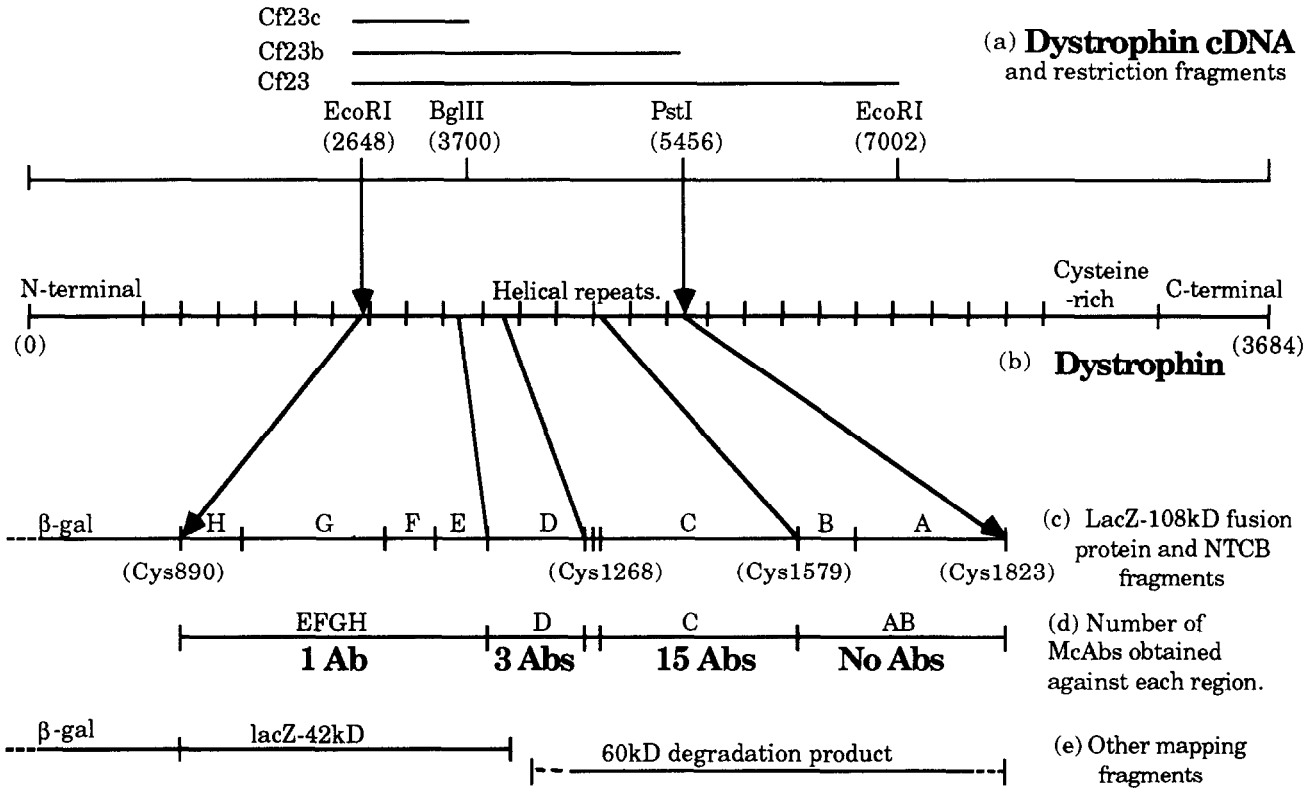

Fig.1. Production of recombinant fusion proteins and identification of monoclonal antibody binding sites by NTCB cleavage at Cys residues.

K, 20 min). After SDS-PAGE on 4–12.5% gradient gels [1] (10 × 12 cm wide), protein bands were transferred electrophoretically (BioRad Transblot) to nitrocellulose at 100 mA overnight in 25 mM Tris, 192 mM glycine. Blots were developed with monoclonal antibody culture supernatants (1/100) as described previously [8].

3. RESULTS

Fig.1. shows (a) dystrophin cDNA and the restriction fragments derived from it, (b) the domain structure of dystrophin predicted from its primary sequence [3] and (c) the β-galactosidase fusion protein (lacZ-108kD) which we prepared from cDNA encoding part of the rod-like helical repeat region. Mice were immunised with partially purified, solubilised lacZ-108kD for monoclonal antibody production. From one mouse, 136 out of 587 hybridoma-containing wells (23% of the total) produced antibody recognising lacZ-108kD, but not lacZ alone, in ELISA. Those which bound most strongly and specifically to the plasma membrane of frozen sections of adult mouse or human muscle (fig.2a) were selected for cloning. Sixteen were obtained from this mouse and three from a second. All of the antibodies identified the same 400 kDa protein on Western blots of normal mouse muscle (fig.2b). None of the antibodies reacted with any protein in *mdx* dystrophic mouse muscle, either in frozen sections or Western blots (fig.2).

For epitope mapping, we used a combination of nitrothiocyanobenzoic acid (NTCB) cleavage at Cys residues [11,12], proteolytic cleavage and DNA deletion analysis. Incomplete digestion by NTCB produces a ladder of partial cleavage fragments (A, AB, ABC, etc.) from which each antibody selects a characteristic 'fingerprint'. Fig.3 shows predicted NTCB fragments (up to 60 kDa) for antibodies binding to fragments A–D (predictions for E–H or above 60 kDa are complicated by the β-galactosidase contribution) alongside actual

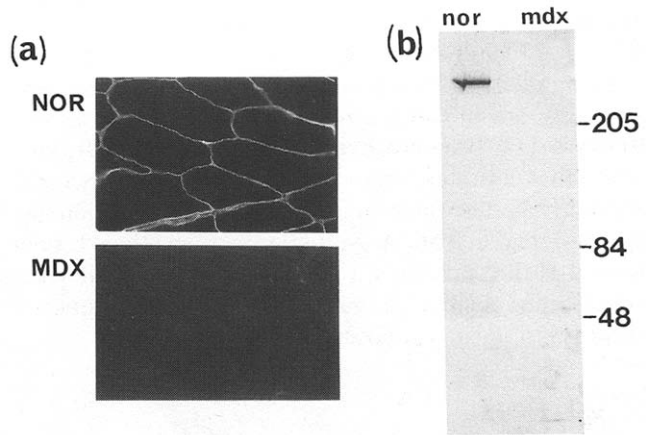

Fig.2. Monoclonal antibody characterization. (a) Monoclonal antibody binding to the plasma membrane of muscle from normal (NOR), but not dystrophic (MDX), mouse. (b) The 400kDa protein present in normal mouse muscle (nor) is absent from dystrophic muscle (mdx). M_r markers: myosin (205 kDa), phosphofructokinase (84 kDa), fumarase (48 kDa). The antibodies shown belong to the 'C' epitope class (fig.1d), but all 19 antibodies gave similar results.

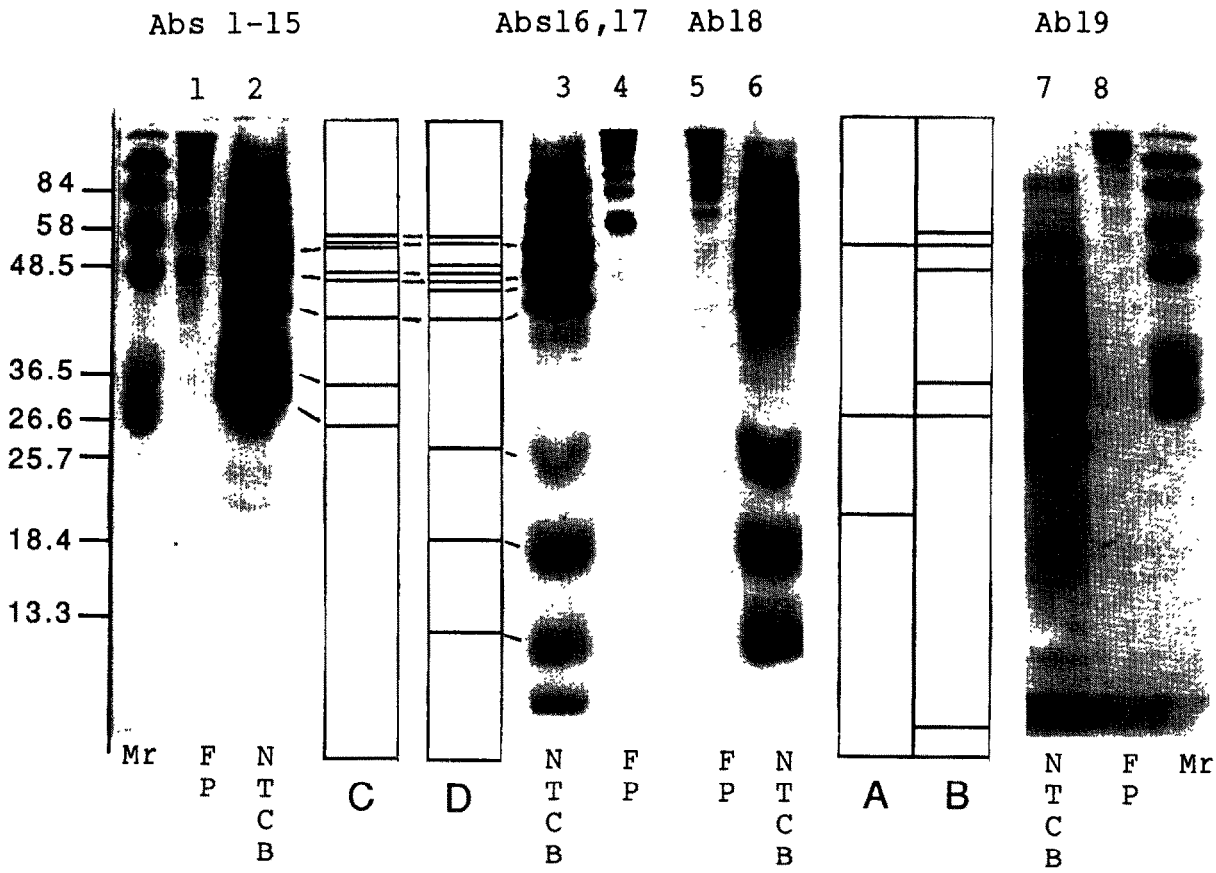

Fig.3. Epitope mapping of monoclonal antibodies by Western blotting. The crude pellet of lacZ-108kD inclusion bodies was boiled in SDS buffer (FP; lanes 1,4,5,8) or cleaved with nitrothiocyanobenzoic acid (Sigma) in 5 M guanidine-HCl as described [11] (NTCB; lanes 2,3,6,7), separated on a SDS-15% polyacrylamide gel (10 × 12 cm) and blotted by diffusion [8]. The antibodies used to develop the blots were MANDYS1,16,18 and 19, representing the 3 classes in fig.1d. Predicted NTCB fragment sizes for A, B, C and D (fig.1c) are shown alongside the actual cleavage products and prestained molecular weight markers (M_r; up to 25.7 kDa, Gibco-BRL; 26.6–84 kDa, Sigma). The 60 kDa degradation product (lanes 1 and 4) and the smallest NTCB fragment (lane 3), which may be derived from it, are indicated by closed and open circles respectively between lanes 4 and 5.

Western blots for the 3 classes of antibody. Attention should be paid to the pattern of fragments in the 10–40 kDa region, rather than to the precise position of any one fragment, since the relationship between molecular weight and migration position in SDS gels often shows deviations of 2–3 kDa or more. The lane 2 pattern (15 antibodies) corresponds quite closely to that predicted for fragment C and lanes 3 and 6 (3 antibodies) to a fragment D pattern, notably in the smallest predicted fragments (C, 27 500; D, 12 600) and the smallest fragment common to both (CD, 40 000). There is no resemblance to the predicted A and B fragment patterns in this 10–40 kDa region. MANDYS19 is the only antibody which binds in ELISA to a shorter fusion protein (lacZ-42kD) containing E–H and part of D (fig.1e; results not shown) and its NTCB fingerprint differs from any predicted (lane 7). This demonstrates an epitope within EFGH and supports an ABCD allocation for the other 18. All 15 'C' antibodies bind to a major degradation product in lacZ-108kD (lane 1) but the 'EFGH' antibody (MANDYS19; lane 8) and one of the 3 'D' antibodies (MANDYS18; lane 5) do not, demonstrating a proteolytic cleavage site within 'D'. Its size (60 kDa) indicates a fragment containing all, or nearly all, of ABC (55 kDa) and part of D (fig.1e). The unpredicted, smallest fragment in lane 3 may also arise from this proteolytic cleavage within 'D' since only those 'D' antibodies which recognise the 60 kDa fragment bind to it. Thus, the results of three different mapping techniques are internally consistent.

4. DISCUSSION

We have shown that monoclonal antibodies which detect dystrophin on both Western blots and frozen tissue sections bind to at least four different dystrophin sequences. Such antibodies could be used in a rapid test for specific dystrophin epitopes in those MD patients with an altered from of dystrophin [2].

The high proportion of antibodies against fragment C could be explained by our selection of antibodies which bind both to native, authentic dystrophin *in situ* and to partially denatured recombinant dystrophin on ELISA plates. This might select antibodies which bind

to accessible and denaturation-resistant epitopes. Alternatively, high immunogenicity might be an intrinsic property of this dystrophin region. Hoffmann et al. [1] found that a smaller pATH fusion protein (trpE-30kD), containing an insert which includes part of fragment C, was much more immunogenic than one containing 60 kDa from a different helical region. The presence of stable repeating structures could explain higher immunogenicity (cf. collagen repeats [14]), since there is no evidence of any locally high density of amino-acid differences between human and mouse dystrophin to account for it [13] and all the antibodies cross-react with mouse dystrophin. The 30 kDa insert (Glu^{1181}–Phe^{1388}) of Hoffmann et al. [1] and fragment C (Cys^{1268}–Cys^{1579}) each contain three triple-helical repeats and they overlap by one complete repeat [3].

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