Monoclonal antibodies for dystrophin analysis

Epitope mapping and improved binding to SDS-treated muscle sections

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A group of 44 monoclonal antibodies (mAbs) raised against the central helical rod (25 mAbs) and C-terminal (19 mAbs) regions of dystrophin were prepared using *trpE* recombinant fusion proteins as immunogens. Some mAbs cross-react with the structurally related proteins, α -actinin and utrophin. Epitope mapping revealed uneven distribution of mAb-binding sites, no mAbs being produced against the C-terminal end of the helical fragment or the cysteine-rich region of the C-terminal dystrophin fragment. The failure of these large regions of the recombinant immunogens to elicit anti-dystrophin antibodies may be because of their inability to fold into the correct dystrophin-like conformation. The mAbs were selected for their ability to recognize 427 kDa dystrophin on Western blots after SDS/PAGE, and/or for immunostaining of the membrane in frozen muscle sections. Although some mAbs obtained by Western-blot screening failed to bind native dystrophin in frozen muscle sections, successful binding could be obtained after SDS or urea treatment of the tissue section to expose the epitopes. This increases the range of mAbs available for detection of dystrophin deletions in muscular dystrophy and evaluation of myoblast therapy.

INTRODUCTION

Dystrophin, a structural protein on the inner face of muscle membranes, is the 427 kDa protein product of a gene on the human X chromosome which is altered by mutation in both Duchenne and Becker muscular dystrophies [1]. Predictions from the amino-acid sequence suggest a rod-shaped triple-helical region separating an N-terminal actin-binding region from a cysteine-rich and a C-terminal region [2]. In the milder Becker form, the genetic deletions are often expressed as in-frame deletions in the dystrophin mRNA, the protein products of which are detectably smaller but usually have an intact C-terminus [3]. In the more severe Duchenne form, dystrophin is either undetectable [4] or lacks the C-terminal region [5,6]. A high proportion of known deletions begin at the 'P20 breakpoint' between exons 44 and 45 in the central helical-rod region [7].

In addition to the study of the structure and function of normal dystrophin, monoclonal antibodies (mAbs) against different epitopes along the dystrophin molecule are clearly of potential value in studies of altered dystrophins in muscular dystrophy patients [5,8–11], alternative transcripts of the dystrophin gene [12–14], relationships in sequence and structure between dystrophin and other proteins, notably α -actinin [15,16] and the autosomal dystrophin-related protein (DRP), utrophin [17,18], and for distinguishing successful myoblast transplants from 'revertant' fibres in muscular dystrophy patients [19].

The preparation and epitope mapping of 22 mAbs raised against one section of the central rod region (amino acids 815–1749) have already been described [20,21]. We have now used an adjacent fragment of the central rod (amino acids 1749–2248, including the P20 region) and a C-terminal fragment to prepare, characterize and map 44 additional mAbs. Previously, we have briefly described the cross-reaction of one of the rod mAbs, MANDYS141, with α -actinin [15] and the diagnostic application of one of the C-terminal mAbs, MANDRA1 [22]. One advantage

of large panels of mAbs is illustrated by the identification of apodystrophin-1, a novel 70–80 kDa protein product of an alternative C-terminal transcript of the dystrophin gene [23–25], for which only a few mAbs are suitable. The C-terminal mAbs may prove useful in the analysis of dystrophin isoforms produced by alternative splicing of mRNA in this region [13].

EXPERIMENTAL

Antibody production

For dystrophin rod antibodies, a dystrophin cDNA fragment encoding amino acids 1749–2248 was obtained by digestion with *Pst*I and *Hind*III and cloned into the expression vector pATH2. A subfragment encoding amino acids 2102–2248, obtained by digestion with *Bgl*II, was cloned into the pEX1 expression vector in the correct reading frame. For C-terminal antibodies, a *Dra*I fragment of dystrophin cDNA encoding amino acids 3200–3684 (end) was cloned into the *Sma*I site of pATH2. Two subfragments, obtained with *Pst*I and *Sac*I respectively, encoded N-terminal (amino acids 3200–3304) and C-terminal (amino acids 3558–3684) parts of the *Dra*I fragment and they were cloned in-frame into pEX2 and pEX3 respectively.

Fusion proteins with the *Escherichia coli trpE* gene product were induced by resuspending log-phase cells in growth medium lacking tryptophan and with 20 μ g of indoleacrylic acid/ml and incubating at 37 °C for 2–4 h [26]. lacZ fusion proteins from pEX vectors were induced by incubation at 42 °C as described previously [20]. Purification of the 90 kDa *trpE* fusion proteins by extraction of inclusion bodies with 2% (w/v) SDS and gel filtration on Ultragel AcA34 (LKB) were performed as described previously [20]. mAbs were produced by immunization of Balb/c mice and fusion of spleen cells with Sp2/0 myeloma cells as described previously [20]. To distinguish between anti-dystrophin and anti-*trpE* antibodies, hybridoma culture supernatants were screened twice by e.l.i.s.a. [20] using microtitre plates coated with

Abbreviations used: mAb; monoclonal antibody; NTCB, nitrothiocyanobenzoic acid; DRP, dystrophin-related protein; PBS, phosphate-buffered saline.

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the two different fusion proteins (binding to the non-specific fusion protein reveals anti-trpE antibodies). They were further selected for binding either to the 427 kDa dystrophin band on Western blots of mouse muscle extracts, or to the sarcolemma in frozen, human muscle sections. All hybridoma cell lines were subcloned twice by limiting dilution. The immunoglobulin subtype of each antibody was determined with an isotyping kit (Serotec, Oxford, U.K.).

Western blotting

Freshly dissected mouse tissues were weighed, dropped into 4 ml/g of boiling SDS extraction buffer [10% (w/v) SDS, 10% (w/v) EDTA, 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol, 50 mM-Tris/HCl, pH 6.7], homogenized in a Silverson blender, boiled for 2 min and centrifuged at 100 000 g for 20 min. *E. coli* cells were washed with phosphate-buffered saline (PBS), sonicated in 10 vol. of Tris/HCl, pH 6.8, containing 2% (w/v) SDS and 5% (w/v) 2-mercaptoethanol, boiled for 2 min and centrifuged at 100 000 g for 20 min.

Extracts (250 µl) were loaded as a strip on to 4–12.5% gradient gels with a 4% stacking gel. After electrophoresis, proteins were transferred electrophoretically (Bio-Rad Transblot) to nitrocellulose sheets (Schleicher & Schull, Dassel, Germany; BA85) at 100 mA for 16 h in 25 mM-Tris/192 mM-glycine/0.003% SDS. Blots were blocked for 1 h in 3% (w/v) skimmed-milk powder dissolved in incubation buffer [0.05% Triton X-100 in PBS (25 mM-sodium phosphate, pH 7.2, 0.9% NaCl)]. After two 5-min-long washes in PBS, a 'miniblotter' apparatus (Immunetics, Cambridge, MA, U.S.A.) was used to apply up to 28 different mAb-culture supernatants (1:50 dilution in PBS) as vertical lanes across all the protein bands on the blot. After incubation for 1 h at 20 °C and three washes (for 5 min) with PBS, blots were incubated either with biotinylated anti-(mouse Ig) antibody and a peroxidase-avidin detection reagent (Vectastain ABC kit), according to the manufacturer's instructions (Vector Laboratories, Peterborough, U.K.) for tissue extracts, or with peroxidase-labelled rabbit anti-(mouse Ig) antibody (DAKOpatts, High Wycombe, Bucks., U.K.) for *E. coli* extracts. After four washes (each for 5 min) with PBS, substrate was added [0.4 mg of diaminobenzidine (Sigma)/ml in 25 mM-phosphate-citrate buffer, pH 5.0, with 0.012% H₂O₂].

Immunohistochemistry

Frozen sections (7 µm) of human muscle were allowed to attach to untreated glass slides and were stored at –80 °C. After incubation for 30 min at 20 °C with mAb-culture supernatants, diluted 1:3 with PBS, and three 5-min-long washes with PBS, sections were incubated with the second antibody for 30 min at 20 °C. The second antibody [DAKOpatts Ltd; 1:40 dilution in PBS containing 1% (v/v) horse serum/1% (v/v) foetal calf serum/0.3% BSA] was anti-(mouse Ig) antibody labelled with fluorescein. After three further washes (5 min each) in PBS, slides were mounted in 70% (w/v) glycerol in PBS, examined with a Leitz (Leica) epifluorescence photomicroscope with appropriate filters, and photographed for a fixed time (60 s) using Kodak Tri-X Pan film.

RESULTS

Antibody production

Fig. 1 shows the separation of a trpE fusion protein containing amino acids 1749–2248 of dystrophin from the majority of *E. coli* proteins by gel filtration in the presence of 2% (w/v) SDS. The fractions chosen for immunization (Pool in Fig. 1) contain, in addition to the main 95 kDa band of fusion protein, a doublet of

higher molecular mass as the principal *E. coli* contaminant and some lower molecular mass proteins. Twenty-five mAbs raised against the dystrophin rod were obtained from a single fusion of Sp2/O myeloma cells with spleen cells of a mouse immunized with this fusion protein. Out of 768 wells 669 showed hybridoma growth (87%). Of these, 247 (37% of hybridomas) recognized the fusion protein in e.l.i.s.a., although 100 of these also recognized a fusion-protein preparation without dystrophin sequences obtained from the pATH2 vector alone. Of the remaining 147, 75 were screened further by immunofluorescence microscopy of frozen human muscle sections and 72 on Western blots of mouse muscle extracts. After two rounds of cloning, we obtained 14 hybridoma lines (MANDYS 101–111 and 141–143) by the first screening method, and 11 (MANDYS 121–131) by the second.

The C-terminal antibodies were obtained from two Sp2/O myeloma fusions with spleen cells from mice immunized with a trpE fusion protein containing the last 485 amino acids of dystrophin. In the first fusion, although nearly all of 768 wells had hybridoma growth, only three were strongly positive in e.l.i.s.a., and two which recognized dystrophin on both frozen sections and Western blots were cloned twice (MANDRA1 and 2). In a second fusion, 62 were e.l.i.s.a.-positive, although 16 of these also recognized the control fusion protein from pATH2 without a cDNA insert. All 17 clones (MANDRA3–19) selected from the remaining 46 were dystrophin-positive, first on human frozen muscle sections and also on subsequent Western blots of mouse muscle extracts.

Antibody characterization

Antibodies were numbered according to characteristics which emerged during screening (Table 1). Thus, MANDYS101–111 mAbs bind native human dystrophin (immunofluorescence microscopy) but not denatured mouse dystrophin (Western blots after SDS/PAGE). Five are human-specific since they do bind human dystrophin on Western blots, but at least two, MANDYS105 and 110, are specific for native dystrophin since they recognize both human and mouse dystrophin but only on frozen sections (Table 1). These antibodies can nevertheless be shown to be dystrophin-specific by the absence of membrane staining in Duchenne patients and *mdx* mice (results not shown). In contrast, MANDYS121–131 recognize denatured dystrophin on Western blots only, and stain the sarcolemma only very weakly in frozen muscle sections (Fig. 2a). However, brief pretreatment of the muscle section with 1% (w/v) SDS after formalin fixation results in excellent sarcolemmal staining by

Fig. 1. Purification of trpE-fusion-protein immunogen by gel filtration

Fractions (2 ml) were collected from the Ultrogel AcA34 (LKB) column and 10 µl of each was loaded on to a 7% acrylamide gel in the SDS/PAGE system of Laemmli [34] after addition of 2-mercaptoethanol (5% v/v) and sucrose (5% w/v). One lane contained molecular mass markers (Sigma). After electrophoresis, the gel was stained with Coomassie Blue R250. The position of the 95 kDa fusion protein is indicated (FP) and fractions 3–5 were pooled for use as immunogen.

Table 1. Characterization of 44 mAbs raised against dystrophin

IMF(Hu): staining of sarcolemma in immunofluorescence microscopy of frozen human muscle sections [reaction with mouse (mo+) or chicken (ch+) sections shown for MANDYS mAbs only and relative intensity indicated for MANDRA mAbs only]. Blot(Mo): reaction with 427 kDa dystrophin band on Western blots of mouse muscle extracts [reaction with human 427 kDa band (hu+) indicated for MANDYS101–111 only and relative intensity indicated for MANDRA mAbs only]. Blot (fish): reaction with 427 kDa band in skate (*Raja clavata*) muscle extracts. Cross-react: reaction with actinin or mouse utrophin (DRP) [23] on Western blots or internal staining of muscle fibres in immunohistochemistry are indicated. Some react only with recombinant utrophin (recomb). NTCB group (MANDYS mAbs only): mAbs are divided into three groups according to the pattern of cysteine cleavage fragments which they recognize (see Fig. 6). *SacI–DraI* (MANDRA mAbs only): some mAbs recognize this recombinant subfragment of the immunogen containing the last 128 amino acids of dystrophin (see Fig. 4); results from Fig. 5 are summarized. Abbreviations: nd, not determined; w, weak; IMF, immunofluorescence; hu, human; mo, mouse; ch, chicken.

mAb	Ig subtype	IMF (Hu)	Blot (MO)	Blot (fish)	Cross-react	NTCB group
MANDYS101	G2b	+	–	–		
MANDYS102	G2a	+	–(hu+)	–		3
MANDYS103	G2a	+	–(hu+)	–		2
MANDYS104	G2a	+	–(hu+)	–		2
MANDYS105	G1	+(mo+ ch+)	–	–		
MANDYS106	G2a	+	–(hu+)	–		2
MANDYS107	G2b	+	–	–		
MANDYS108	G2a	+	–(hu+)	–		2
MANDYS109	G1	+	–	–		2
MANDYS110	G1	+(mo+)	–	–		
MANDYS111	G2a	+	–	–	Fibres	
MANDYS121	G1	–	+	+		1
MANDYS122	G1	–	+	–		2
MANDYS123	G1	–	+	–		1
MANDYS124	G1	–	+	–		2
MANDYS125	G2b	–	+	–		1
MANDYS126	G1	–	+	–		1
MANDYS127	G1	–	+	–		1
MANDYS128	G1	–	+	–		2
MANDYS129	G1	–	+	–		1
MANDYS130	G1	–	+	–		1
MANDYS131	G1	–	+	–		2
MANDYS141	G2b	–	+	+	Actinin	
MANDYS142	G2a	–	+	+	Actinin	
MANDYS143	G2b	+	–	nd	Fast fibres	
						<i>SacI–DraI</i>
MANDRA1	G1	++	++	++		+
MANDRA2	G1	++	++	+		–
MANDRA3	G1	++	+	+	DRP	+
MANDRA4	G1	w	+	+		+
MANDRA5	G2a	+	++	+		+
MANDRA6	G1	+++	++	++		–
MANDRA7	G1	++	+	+		–
MANDRA8	G1	w	+	–		–
MANDRA9	G1	++	+	–		–
MANDRA10	G1	w	w	–		–
MANDRA11	G1	w	++	+		–
MANDRA12	M	w	++	++		–
MANDRA13	M	w	++	++		–
MANDRA14	G1	w	+	nd		–
MANDRA15	G2b	w	+	+	200 kDa	–
MANDRA16	G1	++	++	++	DRP	+
MANDRA17	G1	++	++	+	DRP	–
MANDRA18	G1	++	++	++	DRP (recomb)	+
MANDRA19	G1	+	++	++	DRP (recomb)	+

these antibodies (Fig. 2*d*). Fig. 2(*b*) shows that formalin fixation alone has no effect. Use of 8 M-urea, instead of SDS, also increases sarcolemmal staining to a lesser degree (Fig. 2*c*), which suggests that exposure of the epitopes by dystrophin unfolding *in situ* is responsible. MANDRA1–19 mAbs bind both native and denatured dystrophin on frozen sections and Western blots, though some of them show preference for one or the other in terms of strength of binding (weak to ++ in Table 1).

Cross-reaction with dystrophin in fish skeletal muscle (Table 1) reflects sequence conservation during evolution; thus, the C-terminal domain of dystrophin is known to be the most highly conserved [27], and 15 out of 18 MANDRA antibodies tested

recognized skate (*Raja clavata*) muscle dystrophin, while only three out of 24 rod antibodies tested did so. Similarly, all 19 C-terminal antibodies recognized the more closely related mouse dystrophin, but only 15 out of 25 rod antibodies did so (Table 1).

Although most mAbs appear to be dystrophin-specific, some cross-reactions with non-dystrophin proteins were observed. Utrophin [23], a DRP encoded by the autosomal *DMDL* gene, is similar in size to dystrophin and shares sequence similarities, especially at the conserved C-terminal end [17,18]. Four of the C-terminal MANDRA antibodies clearly recognize a lacZ recombinant fusion protein containing the last 329 amino acids of human utrophin as well as the trpE-fusion-protein immunogen

Fig. 2. Improved immunohistochemical detection of dystrophin in the muscle membrane after SDS treatment of frozen human muscle sections

(a) Untreated sections; (b) sections fixed with 15% (w/v) HCHO in PBS (adjusted to pH 7.0 with NaOH) for 10 min and blocked with 1 M-glycine in PBS for 10 min. (c) Treatment as in (b) was followed by exposure to 8 M-urea in PBS for 30 s. (d) Treatment as in (b) was followed by treatment with 1% (w/v) SDS in PBS for 30 s. After washing with PBS, subsequent antibody steps were performed as described in the Experimental section.

Fig. 3. Cross-reaction of C-terminal mAbs with the autosomal homologue of dystrophin, DRP

An extract of *mdx* mouse lung was loaded as a strip on to a 4–12.5% gradient gel. Lanes 1–19 were developed with dystrophin C-terminal mAbs in the following order: MANDRA 5, 14, 15, 11, 6, 12, 18, 10, 7, 8, 3, 4, 19, 17, 16, 9, 13, 1, 2. The bands that run across all lanes and the gaps between lanes are non-specific cross-reactions of the second antibody detection system.

Fig. 4. Summary of epitope-mapping results by restriction enzyme deletion analysis

Restriction enzymes used to generate subfragments for mapping are shown in the first line. The second line shows approximate positions of dystrophin ‘domains’ [2,28] and the P20 ‘breakpoint’ [7]. The expanded form shows the number of mAbs which bind to each subfragment.

Fig. 5. Binding on Western blots of 19 C-terminal mAbs to (a) the *DraI* trpE-fusion-protein immunogen and (b) the *SacI* subfragment in a lacZ fusion protein

Crude fusion proteins (*Escherichia coli* inclusion body pellet) were boiled in a 2% (w/v) SDS sample buffer and loaded on to 7% acrylamide gels [34]. Lanes 1–19 were developed with MANDRA3–19 inclusive (plus two controls) in the following order: MANDRA 15, 9, 8, 19, 14, 4, 6, 10, control culture supernatant, 16, 3, 18, 5, 13, control culture supernatant, 17, 7, 12, 11. Lanes 20–28 are, in order: PBS, a mouse antiserum against the *DraI*-fusion-protein immunogen, PBS, mAb against trpE, culture medium control, MANDRA1, mouse antiserum raised against the C-terminal region of human utrophin [18], MANDRA2, culture medium control. The seven mAbs and two antisera which react with the smaller subfragment are shown below (b).

with the last 500 amino acids of human dystrophin. Only two of these four recognized authentic 400 kDa utrophin on Western blots of *mdx* mouse lung, a tissue rich in this protein [18] (Fig. 3). This may be a reflection of sequence differences between human and mouse DRPs. A cross-reaction with a 400 kDa band in *mdx* tissues was also observed with MANDRA3 (Fig. 3) and two rod antibodies, MANDYS121 and 127 (Table 1); this is likely to be utrophin also. MANDRA15 cross-reacts with an approx. 200 kDa protein in *mdx* lung which has not yet been identified (Fig. 3). MANDYS141 and 142 cross-react with α -actinin in both its native and denatured form, but recognize only denatured dystrophin as described in detail elsewhere [15]. MANDYS143 and 111 both show clear sarcolemmal staining of native dystrophin but they also show internal sarcoplasmic staining, either of all muscle fibres (MANDYS111) or of fast muscle fibres only (MANDYS143; fibre typing by histochemical ATPase staining). We cannot yet say whether α -actinin or some other sarcoplasmic protein is responsible in these cases since these two antibodies do not bind to any SDS-denatured protein on Western blots, not even to dystrophin itself.

Epitope mapping

Antibodies were mapped by deletion analysis using the available restriction-enzyme subfragments shown in Fig. 4. Mapping by Western blotting is illustrated in Fig. 5. All C-terminal mAbs recognized the complete 95 kDa fusion protein used as immunogen (Fig. 5a), but only seven out of 19 recognized the last 126 amino acids (amino acids 3558–3684) in a 130 kDa lacZ fusion protein obtained by *SacI* digestion (Fig. 5b). Predictably, a mAb raised against trpE (lane 23) binds only in Fig. 5(a), while an antiserum against a cross-reactive region of utrophin (lane 26) binds to both fusion proteins in Fig. 5. None of the mAbs recognized the fusion protein containing amino acids 3200–3304 (results not shown).

None of the 25 rod antibodies recognized a trpE fusion protein

Fig. 6. Binding of a selection of dystrophin rod mAbs to cysteine cleavage fragments of the trpE-fusion-protein immunogen

Crude fusion protein (*E. coli* inclusion body pellet) was cleaved with NTCB as described elsewhere [33] and the products were applied as a strip to a 14% acrylamide gel [34]. Lanes 1–24 were developed with mAbs in the following order: MANDYS 127, 125, 141, 123, 121, 130, 124, 142, 131, 143, 102, 129, 122, 128, 142(rpt), culture medium control, 126, 126(rpt), culture medium control, 111, control, 104, control, 102. The band in the last lane, which distinguishes Group 3 from Group 2 mAbs, is shown by an arrow and groups are shown below.

obtained from the *Bg*III–*Hind*III subfragment, suggesting that all epitopes lie between amino acids 1749 and 2102 (Fig. 4). Further division of the rod antibodies into epitope groups was possible using cysteine cleavage of the fusion protein with nitrothiocyanobenzoic acid (NTCB). Three different patterns of cleavage fragments were revealed on Western blots developed with the rod antibodies (Fig. 6), allowing their classification into three epitope groups (Table 1). Group 3 mAbs differ from those in Group 2 in binding to one additional NTCB band (illustrated by the arrow in Fig. 6). Since there are only two Cys residues within the 1749–2102 sequence (Cys-1891 and Cys-2023), one would not expect more than three different patterns. Although it is sometimes possible to correlate bands on the Western blot with specific amino-acid sequences [20], partial NTCB cleavages and Cys residues in the trpE part of the fusion protein make it difficult in this case. However, the fact that all three human-specific mAbs belong to NTCB groups 2 and 3 suggests that these may bind to the last two predicted fragments (Cys-1892–Cys-2024 and Cys-2024–Ser-2102) where nearly all significant sequence differences between human and mouse are found (Fig. 7). Together with specificity differences shown in Table 1, the results show that at least 10 different epitopes are represented among the 25 rod antibodies. Similarly, at least seven different epitopes are recognized by the 19 C-terminal mAbs.

DISCUSSION

To prepare site-specific mAbs against dystrophin, we have followed the strategy used for the preparation of polyclonal antisera by Kunkel and co-workers [1,28]. This consists of using as immunogens large recombinant fragments (approx. 500 amino acids) produced as trpE fusion proteins from dystrophin cDNA cloned into pATH2 expression vectors. Large fragments are more likely to contain at least some areas which have refolded into a dystrophin-like conformation, though smaller fragments, if immunogenic, would require less epitope mapping effort. The average exon in the dystrophin gene encodes 50–60 amino acids [2,7], so production of exon-specific mAbs requires mapping to this accuracy, at least. Synthetic peptides composed of 15–50 amino acids have been used effectively as immunogens, though mainly for the N-terminal and C-terminal globular domains of dystrophin and related proteins rather than the more highly structured helical-rod regions [29,30]. Use of boiling 2% (w/v) SDS at an early stage in immunogen preparation has the advantage of minimizing solubility problems as well as enabling one-step purification by simple gel filtration. However, SDS is difficult to remove from proteins and, although some proteins can refold well after SDS treatment [31], refolding may be limited to local folding of secondary structures in other proteins. On the other hand, mAbs raised against truly native protein epitopes are often unable to bind the denatured protein on Western blots after SDS/PAGE and this can cause characterization problems as well as limiting their potential applications.

In view of these considerations, it is not surprising that half of the dystrophin rod mAbs recognized dystrophin on Western blots but not native dystrophin on frozen sections. This might have been a grave disadvantage since immunolocalization is a major application of mAbs, but we have shown that it can be easily overcome by a simple SDS pretreatment of tissue sections (Fig. 2). At least two mAbs, MANDYS105 and 110, are specific for native dystrophin which suggests that some refolding did occur after partial removal of SDS before immunization. Selection during screening for mAbs which bind both native and denatured dystrophin is possible and was followed for the C-terminal mAbs. Dystrophin regions which are incapable of eliciting such antibodies for structural reasons would, however, be missed by this procedure. If, for example, the cysteine-rich domain should require disulphide-bridge formation for correct refolding after denaturation, then this might explain why no mAbs were obtained against this part of the C-terminal fusion protein (Fig. 4).

Epitope mapping has shown that no mAbs were obtained against the C-terminal end of the dystrophin helical-rod fusion protein (Fig. 4). A similar result was obtained with pEX2 fusion protein containing the preceding dystrophin sequence (amino

Fig. 7. Sequence differences between human and mouse dystrophins in the rod-mAb-binding region

The full sequence of human dystrophin [2] between Cys-1749 and Ser-2102 is shown with differences in the corresponding mouse sequence (EMBL Nucleic Acid Database) below (after alignment). The two additional Cys residues for NTCB cleavage are numbered.

acids 815–1748), which failed to elicit antibodies against the last 243 amino acids [20]. This could be coincidental or could be a consequence of terminating the sequence at an inappropriate point and disrupting the preceding protein structure over some distance. There are two problems which this could cause. First, the incorrectly folded end might elicit antibodies which fail to recognize the intact dystrophin molecule. Alternatively, or additionally, this region might be more sensitive to proteolysis and would consequently be under-represented in the final immunogen. Proteolysis of the 95 kDa protein undoubtedly occurs, though in Fig. 5(a), at least, it does not appear to be extensive. The problem may not arise with the C-terminal fusion protein which terminates normally. In this case, no mAbs were produced against the cysteine-rich area, but in the remaining C-terminal domain numbers of mAbs are distributed approx. according to fragment size (Fig. 4).

More detailed mapping is required to determine whether the 44 mAbs described here recognize 44 different epitopes, though results so far suggest that a minimum of 17 epitopes are involved. It is not uncommon to obtain mAbs against epitopes which lie close together on the antigen and are therefore difficult to separate by fragmentation techniques [21]. Differences between such antibodies are often revealed by species-specificity differences resulting from single amino-acid changes in the antigen [32,33].

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