# Monoclonal antibody evidence for structural similarities between the central rod regions of actinin and dystrophin

Nguyen thi Man[1], J.M. Ellis[1], I.B. Ginjaar[2], M.M.B. van Paassen[2], G.-J.B. van Ommen[2], A.F.M. Moorman[3], A.J. Cartwright[1] and G.E. Morris[1]

[1] *Research Division, NE Wales Institute, Deeside, Clwyd CH4 5BR, UK,* [2] *Department of Human Genetics, Sylvius Laboratories, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands* and [3] *Anatomisch-Embryologisch Laboratorium, Academisch-Medisch Centrum, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands*

Received 30 July 1990

A monoclonal antibody, MANDYS141, binds to both dystrophin and actinin on Western blots (SDS-denatured), but only to actinin in frozen sections of human muscle (native conformation). It differs from a polyclonal cross-reacting antiserum in that it binds to several muscle isoforms of actinin (smooth, fast and slow) from man, mouse and chicken and recognises a quite different part of the proposed triple-helical region of dystrophin (amino acids 1750–2248). The results suggest that structural homologies between actinin and dystrophin occur more than once in their central helical regions and provide experimental support for an actinin-like central rod model for dystrophin.

Dystrophin; Actinin; Monoclonal antibody; Muscular dystrophy; Recombinant fusion protein; pATH vector

## 1. INTRODUCTION

Dystrophin is a 427 kDa muscle membrane protein which is absent, reduced or altered as a result of mutation in the Duchenne muscular dystrophy (DMD) gene or its homologue (mdx) in the mouse [1]. Predictions from the sequence suggest a structural protein on the inner face of the membrane, consisting of a rod-shaped triple-helical domain separating an N-terminal actin-binding domain from two C-terminal domains, one of which is rich in cysteine [2]. A polyclonal rabbit antiserum raised against part of the proposed helical region (amino acids 1181–1388 in a recombinant fusion protein) of mouse cardiac dystrophin was found to cross-react with a fast skeletal muscle isoform of actinin, even after affinity purification [1,3]. This cross-reaction was only obtained when native protein was used as immunogen. More recently, an antiserum against dystrophin which reacts with the fast skeletal muscle isoform of C-protein has been described [4].

We now describe a monoclonal antibody, MANDYS141, raised against a quite different part of the central helical region of human skeletal muscle dystrophin, which cross-reacts with actinin from fast, slow and smooth muscle.

## 2. MATERIALS AND METHODS

### 2.1. *Preparation of immunogen and monoclonal antibodies*

A dystrophin cDNA fragment coding for amino acids 1750–2248 was obtained by digestion with *Pst*I and *Hin*dIII and cloned into pATH2, an expression vector containing part of the *E. coli* trpE gene inducible with indoleacrylic acid and tryptophan starvation [5].

Inclusion bodies were released from transformed and induced *E. coli* by sonication at 4°C in TNE buffer (50 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0) and the 90 kDa fusion protein was partially purified by gel filtration in 2% SDS buffer as described in [6]. Immunization, fusion and hybridoma screening were performed as in [6]. MANDYS141 belongs to the $IgG_{2b}$ subclass.

### 2.2. *Immunofluorescence microscopy*

7 $\mu$m sections 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.

### 2.3. *Cysteine cleavage*

Cleavage of actinin by nitrothiocyanobenzoic acid in 5 M guanidine-HCl/0.2 M Tris-acetate, pH 9.0, and subsequent analysis on 10% polyacrylamide gels was performed as previously described [6,7].

### 2.4. *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) [8], homogenised immediately in a Silverson blender, boiled again for 3 min and centrifuged (100 K, 20 min). After SDS-PAGE on 4–12.5% gradient gels [1], protein bands were transferred to nitrocellulose overnight in 25 mM Tris, 192 mM glycine either electrophoretically (BioRad Transblot; 100 mA) or by diffusion. Blots were developed with monoclonal antibody culture supernatants as described previously [9].

## 3. RESULTS

The monoclonal antibody, MANDYS141, binds to two major bands on Western blots of normal mouse

*Correspondence address:* G.E. Morris, Research Division, NE Wales Institute, Deeside, Clwyd CH4 5BR, UK

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/90/$3.50 

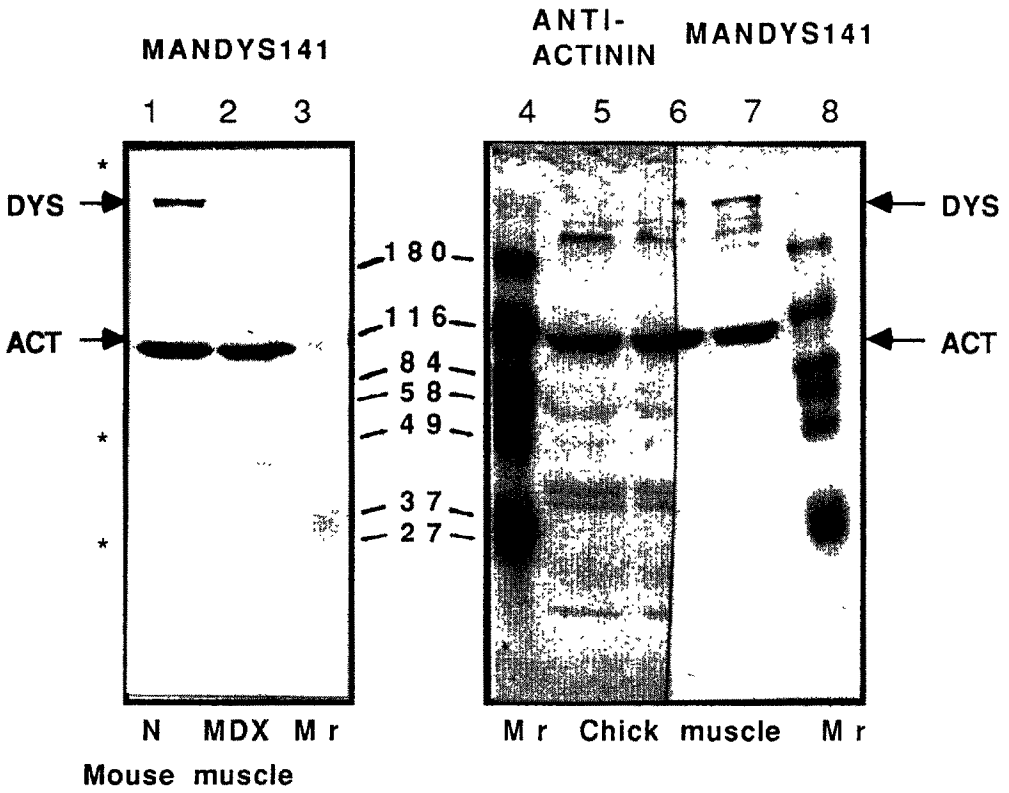


Fig. 1. Specificity of MANDYS141 antibody for dystrophin (DYS) and actinin (ACT). Additional faint bands indicated by * are non-specific reactions visible even when MANDYS141 is omitted (the smaller ones are mouse Ig chains reacting with the second antibody). Another band (- - -) may be actinin dimer. The second blot was produced by diffusion, rather than electrophoretically, so the high molecular weight dystrophin was transferred less efficiently. It was cut down the middle of lane 6 for accurate alignment of protein bands and developed with rabbit anti-actinin serum (left half) or MANDYS141 (right half).

muscle extracts at about 100 kDa and 400 kDa (Fig. 1, lane 1). The absence of the larger protein from extracts of dystrophic mouse (mdx) muscle (lane 2) confirms its identity as dystrophin. The 100 kDa protein, present in both normal and mdx mouse muscle, comigrates with the major protein in chicken breast muscle recognised by an antiserum raised against chicken gizzard actinin (Sigma; lanes 5, 6, 7).

Immunolocalization in longitudinal sections of mdx mouse muscle supports the identification of the 100 kDa protein as actinin; both MANDYS141 (Fig. 2a) and the anti-actinin serum (Fig. 2b) give the same pattern of narrow cross-striations, consistent with the Z-line location of actinin and similar to that observed by Hoffman et al. [3] using a polyclonal anti-dystrophin/actinin serum. Monoclonality of the hybridoma line was ensured by two rounds of limiting dilution cloning at 0.5 cells per well or less. Several (4–8) hybridoma supernatants were tested after each cloning and all stained both proteins on Western blots. This rules out any possibility of there being two different antibodies present in the culture supernatant.

Although MANDYS141 recognises both 'native' actinin in frozen sections and 'denatured' actinin on Western blots after SDS-PAGE, it does not recognise native dystrophin in frozen sections. Fig. 2c shows a transverse section of normal human muscle in which the antibody binds to all fibres internally without binding to dystrophin in the membrane, unlike the polyclonal antiserum of Hoffman et al. which stains only fast fibres internally [3]. (Fig. 2d shows typical dystrophin staining by a different monoclonal antibody, MANDYS1 [6], which stains the same 400 kDa protein on Western blots.) A mosaic pattern of internal staining by MANDYS141 is sometimes observed, with some fibres more intensely stained than others, as in Fig. 2a for example. These quantitative, rather than qualitative, differences have not been sufficiently consistent to determine whether fast and slow fibres are involved. All sections in Fig. 2 did contain both fast and slow fibres as determined with an antibody against fast myosin and by differential ATPase staining [14].

We attempted to localise the epitope on actinin recognised by MANDYS141 by chemical cleavage of chicken gizzard actinin (Sigma) at Cys residues with nitrothiocyanobenzoic acid [7]. The antibody binds to actinin itself and only to one major fragment of about 41 kDa (Fig. 3), although stained gels and blots (not shown) show that many other fragments, both larger and smaller, are available as predicted from actinin

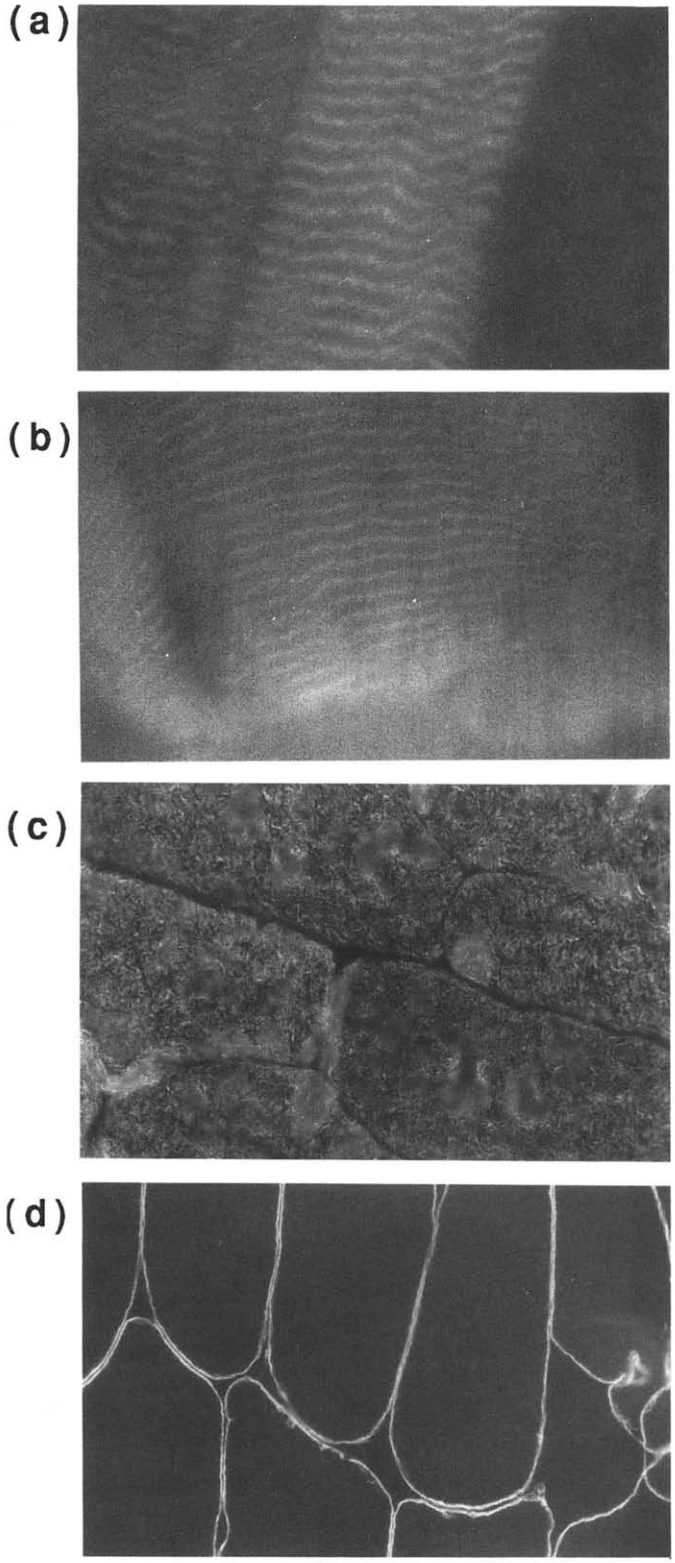


Fig. 2. Immunostaining of human and mouse muscle by monoclonal antibodies. Frozen longitudinal sections from mdx mouse thigh muscle (a, b) or transverse sections from human muscle biopsies (c, d) were stained using monoclonal antibodies MANDYS141 (a, b, c) or the dystrophin-specific MANDYS1 (d). Magnifications: a, b: 1000 ×; c, d: 400 ×.

amino acid sequences [10,11]. Predictably, a polyclonal antiserum binds to many more fragments, though it also fails to bind to any fragment smaller than 41 kDa (Fig. 3). Attempts to identify the 41 kDa fragment by N-terminal microsequencing (not shown) were unsuccessful, possibly because of amino-group modification after chemical cleavage.

## 4. DISCUSSION

Sequence similarities between dystrophin and actinin have been reported for both actin-binding and cysteine-rich regions, but the sequence similarity of their helical domains is no greater than for many other proteins with a coiled-coil helical structure, such as spectrin and myosin. There are no identical linear stretches greater than three or four amino acids, though, on a higher level, there are significant homologies in their helical repeat structures [12]. Indeed, epitopes on triple-helical structures may consist of side-chains exposed on one face of a helix or they may even be shared between the surfaces of adjacent helices. Assembly of epitopes common to dystrophin and actinin by protein folding in this way might explain why they are not revealed by linear

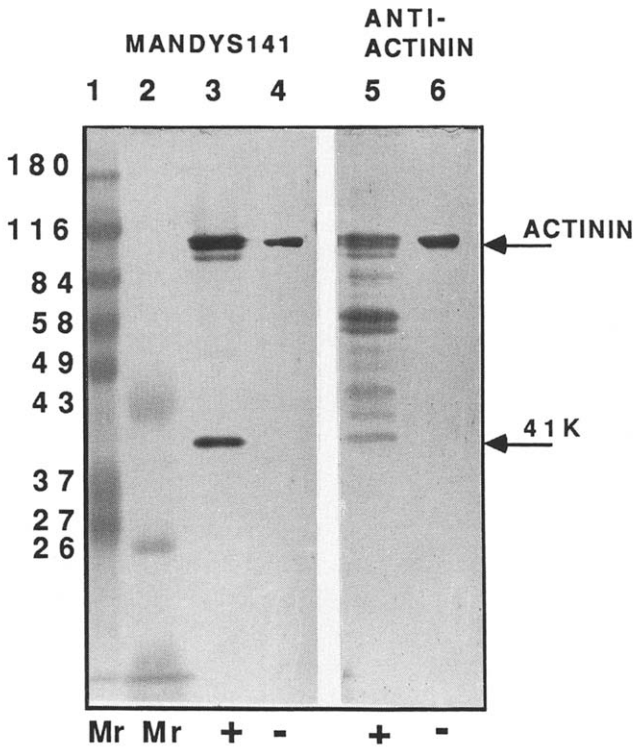


Fig. 3. Binding of MANDYS141 to chicken smooth muscle (gizzard) actinin and to a 41 kDa cleavage product. Gizzard actinin (Sigma) before (−) and after (+) partial cleavage with nitrothiocyanobenzoic acid was subjected to SDS-PAGE on 10% gels (with prestained blue $M_r$ markers) and transferred to nitrocellulose by diffusion. Identical mirror-blots from the same gel were developed with MANDYS141 (lanes 3, 4) or rabbit anti-actinin serum (lanes 5, 6; Sigma) as in Fig. 1.

sequence comparisons. Reaction on Western blots does not exclude an epitope assembled from distant amino acids in the linear sequence, since many antigens are capable of significant (though usually not complete) refolding during electrophoresis in 0.1% SDS [7,13].

MANDYS141 binds to native, myofibrillar actinin in frozen muscle sections but not to native dystrophin in the membrane. The opposite effect was observed with the Hoffman antiserum; good membrane and myofibrillar staining was obtained on muscle sections, but the antibody reacted weakly with dystrophin on Western blots relative to actinin [3]. One explanation is that the MANDYS141 epitope is obscured on native dystrophin, either by protein folding or by interaction with other proteins in the cell, while the epitope on actinin is accessible in both conformations. Alternatively, MANDYS141 could have a high affinity for partially-denatured protein (on blots) but a low affinity for native protein (in frozen sections) and perhaps detect only the high abundance protein, actinin, in the latter case. In contrast, the Hoffman antiserum might have a lower affinity for denatured dystrophin and actinin than for the corresponding native proteins; this antiserum was obtained only when a soluble native form of immunogen was used [1,3], while MANDYS141 was raised from an SDS-treated immunogen.

Chemical cleavage results (Fig. 3) support an epitope assembled by protein folding rather than one formed by local folding of a linear amino acid sequence, since we might expect the latter to be present on a continuous 'ladder' of partial digestion fragments [6,7]. It is not possible to identify the 41 kDa actinin fragment unequivocally from the positions of Cys residues in the sequence (although only part of chicken gizzard actinin has been sequenced directly, it is thought to be the product of the same gene as fibroblast actinin [10]). The largest predicted fragment after complete digestion would be Cys-480–Cys-768 (sequence of [10]), or about 33 kDa. This predicted fragment covers the major part of the helical region of actinin (residues 280–750) where the homology with our helical dystrophin fragment is expected to lie, but is too small to be the 41 kDa fragment in Fig. 3. Refolding of the MANDYS141 epitope may require an intact chain at one or more of the Cys residues.

*Acknowledgements:* We thank the Muscular Dystrophy Group of Great Britain and Northern Ireland for grant support (to G.E.M.), Tim Helliwell (Royal Liverpool Hospital) for frozen sections and Graham Bulfield (AFRC, East Lothian) for mdx mice.

## REFERENCES

[1] Hoffman, E.P., Brown, R.H. and Kunkel, L.M. (1987) Cell 51, 919–928.
[2] Koenig, M., Monaco, A.P. and Kunkel, L.M. (1988) Cell 53, 219–228.
[3] Hoffman, E.P., Watkins, S.C., Slayter, H.S. and Kunkel, L.M. (1989) J. Cell Biol. 108, 503–510.
[4] Yamaguchi, M., Ishiura, S., Takanoohmuro, H., Tsukahara, T., Arahata, K., Obinata, T., Tamiya, T., Tsuchiya, T. and Sugita, H. (1990) Biochem. Biophys. Res. Commun. 169, 57–63.
[5] Dieckmann, C.L. and Tzagoloff, A. (1985) J. Biol. Chem. 260, 1513–1520.
[6] Nguyen thi Man, Cartwright, A.J., Morris, G.E., Love, D.R., Bloomfield, J.F. and Davies, K.E. (1990) FEBS Lett. 262, 237–240.
[7] Morris, G.E. (1989) Biochem. J. 257, 461–469.
[8] Shimizu, T., Matsumura, K., Hashimoto, K., Mannen, T., Ishiguro, T., Eguchi, C., Nonaka, I., Yoshida, M. and Ozawa, E. (1988) Proc. Jap. Acad. (Ser. B) 64, 205–208.
[9] Morris, G.E. and Head, L.P. (1982) FEBS Lett. 145, 163–168.
[10] Baron, M.D., Davison, M.D., Jones, P. and Critchley, D.R. (1987) J. Biol. Chem. 262, 17623–17629.
[11] Arimura, C., Suzuki, T., Yanagisawa, M., Imamura, M., Hamada, Y. and Masaki, T. (1988) Eur. J. Biochem. 177, 649–655.
[12] Davison, M.D. and Critchley, D.R. (1988) Cell 52, 159–160.
[13] Simmerman, H.K.B., Lovelace, D.E. and Jones, L.R. (1989) Biochim. Biophys. Acta 997, 322–329.
[14] Brooke, M.H. and Kaiser, K.K. (1970) Arch. Neurol. 23, 369–379.