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Full-length and short forms of utrophin, the dystrophin-related protein

Nguyen thi Man[a], T.R. Helliwell[b], C. Simmons[a], S.J. Winder[c], J. Kendrick-Jones[c], K.E. Davies[d], G.E. Morris[a,*]

[a]*MRIC Biotechnology Group, N.E. Wales Institute, Deeside, Clwyd, CH5 4BR, UK*
[b]*Department of Pathology, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, UK*
[c]*MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK*
[d]*Molecular Genetics Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DU, UK*

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Abstract All previous studies of the localization of utrophin (the dystrophin-related protein) in muscle and other tissues have been performed only with antibodies against the C-terminal region of the protein. Since several short forms of dystrophin, the apo-dystrophins, are produced from the 3′ end of the dystrophin gene, there is a possibility that similar short forms of utrophin exist and that these could be responsible for some of the many different localizations of 'utrophin' in muscle. We have produced a new panel of 15 mAbs against the N-terminal region of utrophin and we have used it together with mAbs against the C-terminal region to show that full-length utrophin is present at neuromuscular junctions, in nerves, blood vessels and capillaries in normal muscle and in the sarcolemma of patients with muscular dystrophy and dermatomyositis. However, two of the 15 mAbs also recognised rat/mouse utrophin and both of these detected an additional 62 kDa protein on Western blots of rat C6 glioma cells. This potential 62 kDa 'apo-utrophin' was not detected in human cerebral cortex, in rat Schwannoma cells nor in any of the non-nerve cells and tissues tested.

Key words: Dystrophin; Muscular dystrophy; Dermatomyositis; Monoclonal antibody; Actin binding; Neuromuscular junction

1. Introduction

Dystrophin is a large, membrane-associated cytoskeletal protein encoded by a gene on the X-chromosome and found mainly in muscle and brain [1,2]. The absence or malfunction of this protein is responsible for the Xp21-linked muscular dystrophies (Duchenne and Becker types) [1,2]. Utrophin is a very similar protein encoded by an autosomal gene and found in nearly all tissues and cell types [3–5]. Both proteins have long rod-like, central helical regions separating C-terminal domains, which interact with transmembrane glycoproteins, from an N-terminal domain which interacts with actin, suggesting that they act as links between the cytoskeleton and the membrane [6,7]. In skeletal muscle, dystrophin is restricted to the sarcolemma but utrophin is found at neuromuscular junctions, in nerve fibres and in blood vessels and capillaries [4,8]. The dystrophin-associated glycoproteins in the sarcolemma interact extracellularly with the basement membrane protein, merosin (laminin-M) [9], whereas utrophin-associated glycoproteins at neuromuscular junctions interact with agrin, a protein involved in synapse formation [10,11]. Several short forms of dystrophin, the apo-dystrophins, have been shown to exist [12–17], though only the 71 kDa apo-dystrophin-1 has a wide cell and tissue distribution. The apo-dystrophins may be particularly important for brain cell function [13] and all of the apo-dystrophins identified so far contain the cysteine-rich domain and the first half of the C-terminal domain of dystrophin, regions which are known to be important for membrane interaction [18,19]. Corresponding short forms of utrophin have not yet been identified; lower M_r bands on Western blots have certainly been detected using antibodies against the utrophin C-terminal domain [5], but these could be degradation products of the full-length utrophin which is found in all tissues (the identification of apo-dystrophins was facilitated by the absence of full-length dystrophin from many cell lines and tissues containing apo-dystrophins [5]).

Initially, only a part of the 3′ end of utrophin cDNA was cloned and sequenced [3] and consequently all antibodies so far produced against utrophin have been directed against the C-terminal domain [4,8,20]. This leaves open the possibility that some of the minor localizations of utrophin identified with these antibodies, such as nerves or capillaries, could be due to short C-terminal forms, analogous to the apo-dystrophins. Even the appearance of utrophin in the sarcolemma of patients with Xp21-linked muscular dystrophies or with inflammatory myopathies [21,22] could arguably be due to short forms. Increases in the 400 kDa utrophin band have been reported on Western blots of Duchenne muscle [22], but some of this increase might be due to a higher proportion of blood vessels and other utrophin-containing components in dystrophic muscle. The recent cloning of the complete human utrophin cDNA [7] has enabled production of recombinant protein fragments of the N-terminal domain of utrophin. We have prepared a new panel of monoclonal antibodies (mAbs) against amino acids 113–371 of utrophin and have used them to confirm that full-length, or near full-length, utrophin is found at all reported localities in normal muscle and in the sarcolemma of dystrophic muscle. A possible 62 kDa short form of utrophin was found using the N-terminal mAbs, but only in glioma cells.

2. Materials and methods

2.1. mAb production

Recombinant utrophin fragments (residues 1–261 and 113–371) were purified as described previously [23] and the latter was used to immunise BALB/c mice. Two injections with adjuvant of 50 μg, 4 weeks apart, were followed after 5–6 weeks by 150 μg intraperitoneally 2 and 3 days before fusion of spleen cells with Sp2/O myeloma cells. Two separate fusions produced 7 and 8 cloned hybridoma lines respectively. Culture supernatants were screened against the recombinant antigen by ELISA and positive wells were screened further against full-length

*Corresponding author. Fax: (44) (244) 814 305.
E-mail: morrisge@newi.ac.uk

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utrophin on Western blots of human lung extracts. A further selection was then applied by immunofluorescence microscopy on normal human muscle frozen sections with screening for binding to blood vessels (a site of utrophin localization; see Fig. 1) but not to the sarcolemma (the site of dystrophin localization).

2.2. Western blotting

Tissue samples or cell pellets were sonicated and boiled in 4 vols. of SDS sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 50 mM Tris-HCl, pH 6.8). After centrifugation at 100,000 × *g* for 20 min., extracts were subjected to SDS-PAGE. Electrophoretic blotting onto nitrocellulose sheets in the presence of 0.003% SDS and development with mAbs and the Vectastain Elite second antibody system has been described elsewhere [4]. Diffusion blotting is performed by immersing the gel/blot assembly vertically in 50 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl pH 7.0 for 18–36 h with stirring [24]. Prestained M_r markers were from Sigma.

2.3. Immunohistochemistry and ELISA

Immunohistochemistry of frozen muscle sections using a biotin–avidin method with horseradish peroxidase has been described elsewhere [21]. The enzyme-linked immunosorbent assay (ELISA), using antigen-coated 96-well microtitre plates and peroxidase-labelled anti-(mouse Ig) as second antibody has been described elsewhere [25].

3. Results

Table 1 shows 15 mAbs prepared against amino-acids 113–371 of utrophin. The mAbs were selected during screening for lack of cross-reaction with dystrophin. Only two of the 15 mAbs cross-reacted with rat/mouse utrophin (Table 1). Only three of the 15 mAbs bound to utrophin residues 1–261 in an ELISA assay. Since residues 1–261 comprise nearly all of the N-terminal domain, it is likely that most of the other 12 mAbs require, or are directly solely against, the start of the rod domain. There are 4 mAbs in Table 1 which do not recognise utrophin on Western blots, though they do recognise UTR113–371 in ELISA; it may be that they recognise some conformation of the recombinant antigen which is not shared by utrophin itself.

Table 1
15 mAbs against the utrophin N-terminal region (aminoacids 113–371)

Antibody	Clone No.	Ig subclass	Binding to		
			Utrophin (blots)	Rat Utophin (blots)	UTR1-261 (ELISA)
MANNUT1	3B6	G1	++	++	–
MANNUT2	5C9	G2b	++	++	–
MANNUT3	4E3	G2b	+++	–	++
MANNUT4	6H4	G2a	+	–	++
MANNUT5	8C11	G1	+	–	–
MANNUT6	10D4	G2a	++	–	–
MANNUT7	6A10	G1	–	–	–
MANNUT8	10A7	G2a	+++	–	–
MANNUT9	7C11	G1	–	–	–
MANNUT10	3C10	n.d.	+++	–	–
MANNUT11	3B3	G1	+++	–	–
MANNUT12	4B12	G2a	++	–	–
MANNUT13	2A5	G2a	–	–	–
MANNUT14	8A3	G2b	+	–	+
MANNUT15	5E10	G2b	–	–	–

All mAbs bound to UTR113-371 in ELISA and none of the mAbs bound to dystrophin on Western blots. Binding on blots was determined using extracts of human lung and, for rat utrophin, C6 glioma cells. Ig subclass was determined using a mouse isotyping kit (Serotec).

Fig. 1a shows that both N-terminal and C-terminal utrophin mAbs give the same immunohistochemical labelling of nerve fibres, blood vessels (arteries and veins) and capillaries in a normal human muscle section and that they do not label the sarcolemma. Fig. 1b shows that both mAbs label a neuromuscular junction. This suggests that full-length utrophin is present at all these sites, rather than any short form. Similarly, Fig. 2 shows that the utrophin found in the sarcolemma in Duchenne

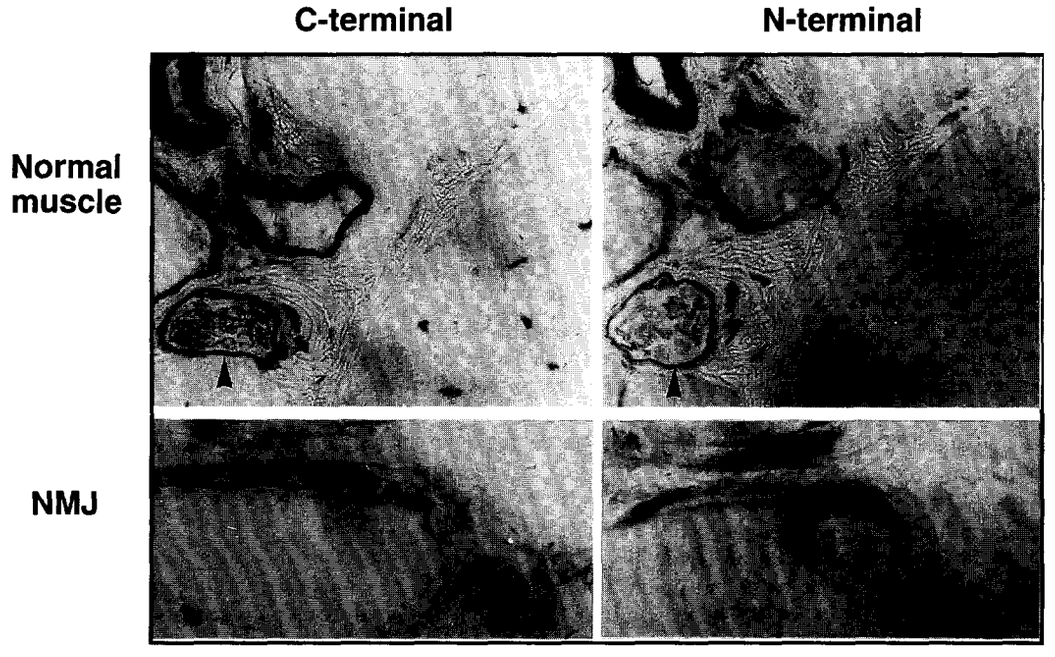

Fig. 1. N-terminal and C-terminal utrophin mAbs recognize the same structures in normal muscle sections. In the upper figure (magnification 700×), major blood vessels are labelled (upper left) together with a nerve fibre (arrow, lower left) and capillaries (right); the sarcolemma of the muscle fibres is not labelled (right). In the lower figure (magnification 1000×), a neuromuscular junction (NMJ) is labelled by both mAbs.

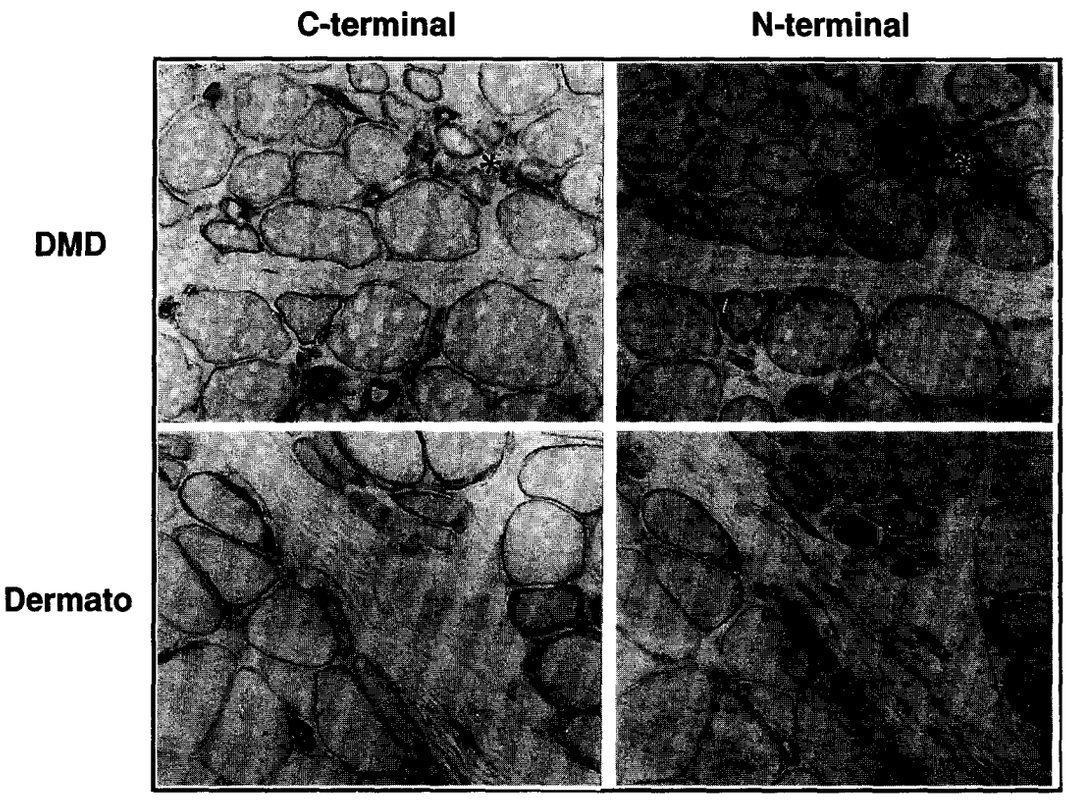

Fig. 2. In Duchenne muscular dystrophy (DMD) and dermatomyositis (Dermato), the sarcolemma is labelled by both N-terminal and C-terminal utrophin mAbs. Membrane labelling is somewhat weaker in an area of regeneration (*) (magnification 500×).

muscular dystrophy (DMD; Fig. 2a) and in dermatomyositis (Fig. 2b) is also full-length, or near full-length. In DMD, all fibres are labelled, though smaller, regenerating fibres are weaker, while in dermatomyositis, sarcolemmal utrophin labelling is more variable, most large fibres and some small fibres being utrophin-positive in Fig. 2b [21].

Protein bands of lower Mr than utrophin are often detected with C-terminal utrophin mAbs on Western blots [5]. It is impossible to say, however, whether these smaller proteins are the products of different mRNA transcripts of the utrophin gene, analogous to the apo-dystrophins, or whether they are simply degradation products of utrophin. Using our new panel of mAbs on Western blots, we found a 62 kDa protein in rat C6 glioma cells but not in other cell lines or in human tissues (Fig. 3). We feel this is unlikely to be a degradation product of utrophin because preliminary studies have shown that a corresponding mRNA of appropriate size also exists in C6 glioma cells (Zuellig and Davies, unpublished data). The specificity of the protein for C6 glioma cells also argues against its being a degradation product. In human brain extracts (Fig. 3b), we could detect only full-length utrophin (the two lower M_r bands being cross-reactions of the second antibody detection system). We found that the 62 kDa protein is best transferred by diffusion blotting (Fig. 3a) though the higher M_r utrophin is not transferred under these conditions. The 62 kDa protein is still detectable after electrophoretic blotting, however, together with utrophin itself (Fig. 3c). The same 62 kDa protein is detected by two different mAbs, so it is unlikely to be a cross-reactive antigen unrelated to utrophin.

4. Discussion

We have prepared a panel of 15 mAbs against part of the N-terminal region of utrophin (amino acids 113–371). Residues 113–371 of utrophin comprise the C-terminal half of the actin binding domain and the beginning of the spectrin-like coiled coil repeats. Utrophin 113–371 does bind actin (K_d 275 μM; [23]) but rather weakly compared to the complete actin binding domain, residues 1–261 (K_d 19 μM; [23]). It is likely, as with dystrophin, that more than one region in the linear sequence of utrophin contributes to actin binding [23].

Utrophin at the neuromuscular junction and in nerves, blood vessels and capillaries, as well as in the sarcolemma of patients with certain myopathies, is the full-length protein, since mAbs directed against the N- and C-termini give the same labelling pattern. This does not, however, exclude the possibility of a contribution to the labelling from apo-utrophins. The detection of a possible 62 kDa N-terminal short form of utrophin in a rat glioma cell line shows that the problem of short forms may well be a real one, though, unlike the well-characterized apo-dystrophins, they have not been shown to be widely present in human tissues.

The observation that utrophin appears in the extra-junctional sarcolemma only in Duchenne/Becker muscular dystrophies (where dystrophin is absent or reduced) and in inflammatory myopathies (where dystrophin levels are normal) suggests a possible role for the inflammatory response in inducing utrophin. Mast cells are present in muscle in Duchenne MD and inflammatory myopathies, but they may be localised to areas

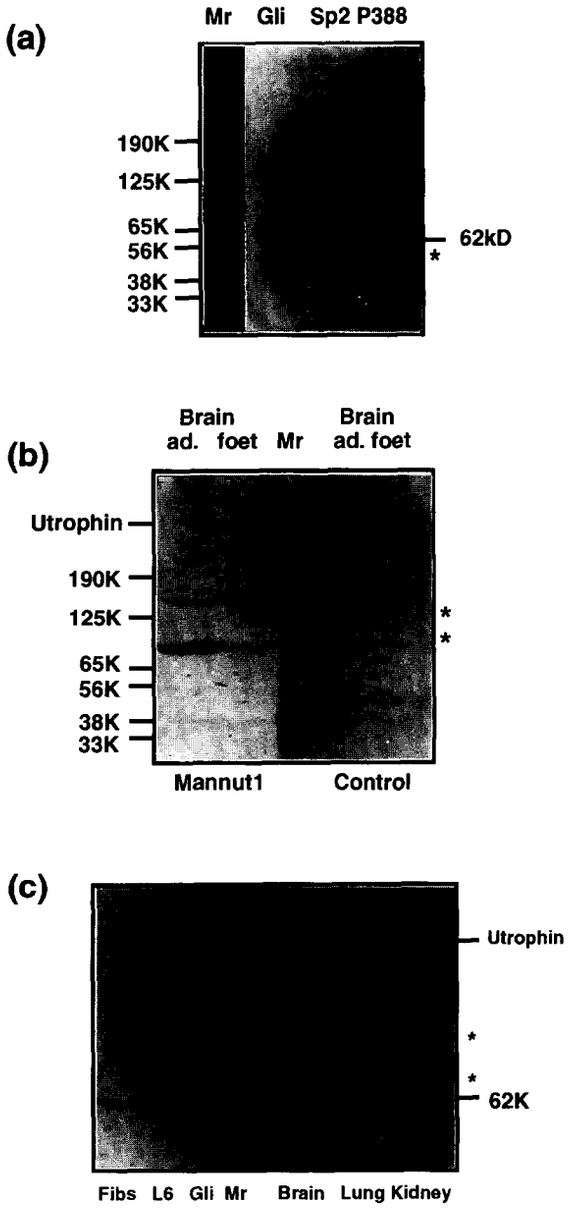

Fig. 3. A 62 kDa protein band is detected by N-terminal utrophin mAbs in rat C6 glioma cell extracts (Gli) but not in in other human tissues or cell lines. Diffusion blotting was used in (a) to enhance transfer of low M_r proteins and utrophin is not transferred for this reason. Non-specific reactions of the second antibody detection system are shown by asterisks and can be identified by control experiments in which the primary anti-utrophin mAb (Mannut1) is omitted (b). The cell extracts in (c) are from human skin fibroblasts (Fibs), rat L6 myoblasts (L6) and C6 glioma cells (Gli) and the tissue extracts are adult human. Utrophin (395 kDa) is present in all extracts. The 62 kDa protein was also undetectable in HeLa and SWA Schwannoma cells (not shown).

of necrosis and regeneration [26,27], so they are unlikely to account for the uniform effect on all muscle fibres in Duchenne and Becker MDs [21]. The possibility, however, of a common mediator of utrophin induction, such as elevated calcium, in the two types of muscle disease cannot be ruled out at this stage.

The molecular mechanism by which utrophin interacts with the dystrophic sarcolemma is not yet clearly established. Dystrophin at the sarcolemma interacts with extracellular laminin via a dystroglycan complex (extracellular 156DAG and transmembrane 43DAG) [9] and a similar utrophin complex interacts with extracellular agrin at neuromuscular junctions [10]. It is not yet known whether the same or another complex of extracellar and transmembrane proteins is involved in mediating the appearance of utrophin in the myopathic sarcolemma; there is some correlation between the distributions and levels of 43DAG and utrophin labelling, but it is not always clear-cut [28].

This panel of mAbs will be useful for future screening for new utrophin isoforms and products of mutations in the utrophin gene.

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