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Calmodulin-binding profiles for nebulin and dystrophin in human skeletal muscle

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Nebulin and dystrophin are two high-molecular-mass skeletal muscle proteins that have both been associated with the defective gene in Duchenne muscular dystrophy, although the function of neither protein is known. Other high-molecular-mass, calmodulin-binding proteins have recently been implicated in regulating calcium release from skeletal muscle. Western blots of human skeletal muscle biopsy samples were probed with biotinylated calmodulin; nebulin was identified as a prominent high-molecular-mass calmodulin-binding protein but dystrophin did not bind detectable amounts of biotinylated calmodulin. Dystrophin was absent in a Duchenne muscle biopsy.

Muscle protein; Duchenne muscular dystrophy; Nebulin; Dystrophin; Calmodulin; SDS-PAGE

1. INTRODUCTION

The structure and function of high-molecular-mass proteins in junctional sarcoplasmic reticulum and the cytoskeletal matrix have recently received much attention. Skeletal muscle contraction is regulated by release of calcium from specialized regions in the junctional reticulum, known as junctional triads or feet, which in the electron microscope are seen as a four-leafed clover structure, spanning the membrane between the transverse tubular system and the sarcoplasmic reticulum. The calcium-release channel protein is one of a number of these high-molecular-mass proteins which both bind calmodulin and are phosphorylated by a variety of endogenous protein kinases [1,2]. Dystrophin, the protein associated with the defective gene product in Duchenne muscular dystrophy [3], has recently been shown to fractionate with junctional feet membrane preparations, although it is distinct (both immunologically and on the basis of size) from the calcium-release channel protein [4]. The N-terminal amino acid sequence of dystrophin shows considerable similarities to the highly conserved actin-filament-binding domain of chicken cytoskeletal α-actinin [5]. This has led to the suggestion that dystrophin might form an anchor for the triads to the myofibrillar cytoskeleton, through the binding of actin filaments [4]. Nebulin is another high-molecular-mass protein of the myofibrillar matrix that has been linked with the defective gene in Duchenne muscular dystrophy [6]. Nebulin coexists with thick and thin filaments and, speculatively, provides a scaffold for these filamentous structures [7]. Calmodulin is involved in skeletal muscle contraction at many levels, e.g. the regulation of calcium release from the sarcoplasmic reticulum and the interaction with cytoskeletal calcium regulatory proteins [8,9]. Little is known about the functional roles of either nebulin or dystrophin; as one approach to this pro-

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Abbreviations: PBS, phosphate-buffered saline; TP buffer, PBS containing 0.05% Tween 20; TCAP buffer, TP buffer containing 1 μM Ca^{2+}; PMSF, phenylmethylsulphonyl fluoride

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blem, we have examined whether either of these two proteins are involved in calcium regulation, by examining their ability to interact with calmodulin.

2. EXPERIMENTAL

2.1. *Materials*

Samples of fresh human muscle were obtained by needle biopsy of the vastus lateralis, from patients attending the muscle clinic. Patient diagnosis was assessed by clinical, histochemical, electron microscopical, serum enzyme and electromyographical criteria [10]. Control muscle samples were from biopsies which showed no clinical abnormalities. Sheep antibodies to the 60 and 90 kDa dystrophin fusion proteins were the generous gift of Drs E. Hoffman and L. Kunkel (Boston, USA). Goat antibody to nebulin was a generous gift from Drs J. Whardale and J. Trinnick (Bristol, England). Streptavidin-biotinylated peroxidase was obtained from DAKO, streptavidin-biotinylated alkaline phosphatase from Vector Labs and biotinylated calmodulin from Penninsular Labs. Biotinylated IgG (donkey, anti-sheep/goat) was obtained from Amersham and biotinylated IgG (rabbit, anti-goat) from Tissue Culture Services. Human serum was obtained from the North London Blood Centre. Nitrocellulose blotting matrices were purchased from Schleicher and Schuell. Electrophoresis reagents were obtained from BDH; protease inhibitors and diaminobenzidine were obtained from Sigma.

2.2. *Electrophoresis*

Human muscle biopsy samples (approx. 2 mg) were homogenised in 1% SDS (w/v) containing 1 mg/ml PMSF, 1 mM iodoacetamide, 1 mM benzemethonium chloride, 5 mM EGTA, 0.25 mg/ml pepstatin A. After centrifugation at 12000 $\times g$ for 1 min, the supernatant was assayed for protein and a volume corresponding to 50 μg protein was electrophoresed on a 4–20% linear gradient SDS polyacrylamide gel [11]. Gels were then fixed with 20% trichloroacetic acid and stained with 0.5% Coomassie brilliant blue R250 in 45% methanol/10% acetic acid. Alternatively, after electrophoresis, gels were gently agitated in blotting buffer (20 mM Tris base, 150 mM glycine) for 30 min before being electrophoretically transferred onto nitrocellulose paper (500 mA, 15 h, 15°C), conditions which have been optimised in our laboratory for the transfer of high-molecular-mass proteins [12]. Proteins remaining on the polyacrylamide gel were stained with silver [13]. Blot transfers were probed as indicated below.

2.3. *Calmodulin binding*

Blots were preincubated with TCaP buffer to block non-specific binding [14] before incubation for 1 h with biotinylated calmodulin (1 μl/ml) containing 1 μM Ca^{2+}. Blots were then incubated for 1 h with a preformed streptavidin-biotinylated peroxidase complex (each reagent diluted 1:5000 in 1 μM Ca^{2+}/PBS and mixed for 30 min) before being finally exposed to freshly prepared diaminobenzidine (0.025%)/hydrogen peroxide (0.005%) for less than 1 min. Blots were washed (3-times) between each step, with TCaP buffer. Calcium-independent calmodulin binding was determined in the absence of added Ca^{2+} and in the presence of 5 mM EGTA.

2.4. *Dystrophin detection*

Non-specific binding was blocked with TP buffer; the buffer was also used to wash the blots (3 times) between each processing stage. Blots were initially probed with antibody to a 90 kDa fusion protein derived from a fragment of a dystrophin cDNA clone isolated from mouse cardiac muscle [3]. Blots were then labelled with biotinylated donkey anti-sheep/goat IgG (1:400 in human serum/TP buffer, 1:1) for 60 min before being finally probed with a streptavidin-peroxidase complex as described above. As an alternative to peroxidase staining, the blot was finally probed with a streptavidin-alkaline phosphatase complex (prepared as for the peroxidase complex, using 1:250 dilutions of each reagent), followed by visualisation with Fast Red solution [10 mg naphthol phosphate in 1 ml dimethylformamide/50 mg Fast Red in 49 ml of 0.1 M Tris buffer (pH 8.2) and filtered before use].

2.5. *Nebulin detection*

TP buffer was used, both to block non-specific binding sites and to wash the blots, as described above. Blots were initially probed with anti-nebulin antibody (1:1000 in TP buffer) for 1 h. The blots were then incubated with biotinylated rabbit anti-goat IgG (1:200 in human serum/TP buffer, 1:1) for 1 h and processed with the streptavidin-peroxidase complex as described above.

3. RESULTS

We found that nebulin was very susceptible to degradation and could only be reproducibly observed on SDS gels with a size of approx. 500 kDa (fig.1) if a cocktail of protease inhibitors was included in the muscle homogenisation medium, in agreement with previous results [7]. The natural abundance of dystrophin was too low to be detected in whole muscle by direct protein staining.

Biotinylated calmodulin has recently been shown to be a convenient and sensitive probe for detecting calmodulin-binding proteins of membrane preparations after gel electrophoresis [15]. With this particular probe, nebulin appears to be a major calmodulin-binding protein in human skeletal muscle. Fig.2 shows the results from a normal muscle biopsy sample run out on an SDS gel and probed with (A) anti-nebulin antibody, (B) biotinylated calmodulin and (C) anti-dystrophin antibody (to 90 kDa fusion protein). Evidence of the susceptibility of nebulin to degradation is also shown in the number of immunologically reactive lower molecular mass bands seen in fig.2A. The presence of dystrophin in the biopsied muscle can readily be seen on the blot probed with the dystrophin antibody, as an immunoreactive doublet (fig.2C). We have no indication as to the

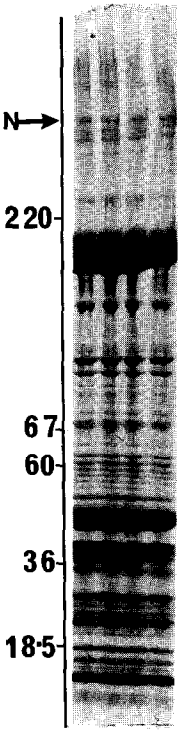

Fig.1. SDS-PAGE of identical samples of a normal human muscle biopsy, solubilised with a cocktail of protease inhibitors and stained with Coomassie blue; N, nebulin. Molecular mass markers indicated (in kDa).

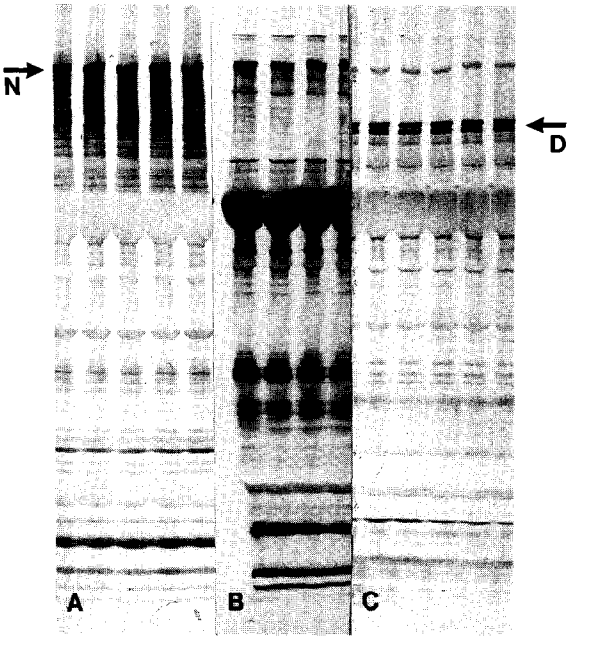

Fig.2. Calmodulin-binding profiles of nebulin and dystrophin. SDS-PAGE of identical, normal human muscle biopsy samples, electrophoretically transferred to nitrocellulose and probed with (A) anti-nebulin antibody, (B) biotinylated calmodulin and (C) anti-dystrophin antibody. N, nebulin; D, dystrophin.

origins of this doublet at present but it is a regular feature from blots of all muscle samples containing dystrophin that we have examined. In comparison to nebulin, dystrophin appears to be an extremely stable protein and can be detected with the antibody probe, irrespective of whether protease inhibitors are included during muscle homogenisation (not shown). Similar results were obtained with antibody to the 60 kDa dystrophin fusion protein (not shown). There is no evidence from fig.2B to suggest that dystrophin is a major calmodulin-binding protein although we cannot rule out the possibility that binding occurs below the levels of our detection. The efficiency of transfer of these high-molecular-mass proteins to the blotting membrane was verified by silver staining the post-blotted polyacrylamide gel (not shown). Control experiments, omitting either anti-dystrophin or anti-nebulin antibodies, showed no staining with second antibody.

The binding of calmodulin to nebulin (as well as to all the other calmodulin-binding proteins detected in fig.2B) was calcium-dependent. Fig.3 shows SDS gel profiles of five different muscle biopsy samples (four normal, one Duchenne) probed with biotinylated calmodulin/1 μM Ca^{2+} (A), biotinylated calmodulin/5 mM EGTA (B) and anti-dystrophin antibody (against 90 kDa fusion protein) (C). Similar (but more intensely stained) binding patterns were seen in the presence of 1 mM Ca (not shown). None of the proteins identified in fig.3A were stained in the presence of the calcium-chelating agent, EGTA (fig.3B). Apart from nebulin, many other calmodulin-binding proteins were labelled (fig.3A) and those tentatively identified include myosin heavy chain (200 kDa), calmodulin-dependent sarcolemmal Ca^{2+}-ATPase (approx. 150 kDa), myosin light chain kinase (95 kDa), calsequestrin (63 kDa), and troponin I (23 kDa). The Duchenne muscle sample run out on lane e clearly contains nebulin (as shown by calmodulin binding, fig.3A), but totally lacks dystrophin (fig.3C).

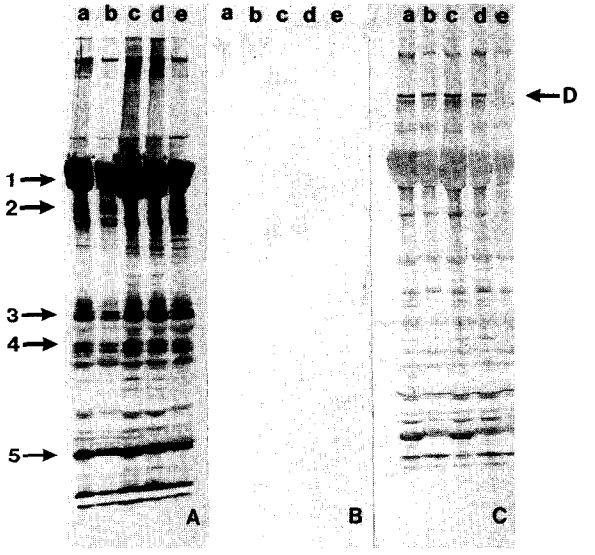

Fig.3. Calcium dependence of calmodulin binding. SDS-PAGE of four different, normal human muscle biopsy samples (lanes a–d) and a Duchenne dystrophy muscle biopsy sample (lane e), electrophoretically transferred to nitrocellulose and probed with (A) biotinylated calmodulin, (B) biotinylated calmodulin in the presence of 1 mM EGTA and (C) anti-dystrophin antibody. 1, myosin heavy chain (200 kDa); 2, calmodulin-dependent sarcolemmal Ca^{2+}-ATPase (approx. 150 kDa); 3, myosin light chain kinase (95 kDa); 4, calsequestrin (63 kDa); 5, troponin I (23 kDa).

4. DISCUSSION

Nebulin is one of the major myofibrillar proteins of skeletal muscle [7]. Until recently it was believed to play a purely structural role in stabilising the myofilament lattice. However direct evidence from radiation inactivation experiments suggests that both nebulin and titin (another high-molecular-mass myofibrillar protein) are associated with the maintenance of resting tension within the muscle fibre [16]. Titin and nebulin can be phosphorylated, although only 15% of total titin phosphates exchanged with cytosolic ATP within 3 days; it has been suggested that the majority of phosphorylation sites are therefore more likely to have a structural, rather than a regulatory role [17]. However, the fact that nebulin is much more intensively labelled than titin [17], coupled with our observations that nebulin is a major calmodulin-binding protein, indicate that some of these phosphorylation sites may indeed play a regulatory role in the assembly and turnover of the myofibril, at least as far as nebulin is concerned.

Until dystrophin was identified, nebulin had been postulated to be the defective gene product of Duchenne dystrophy [6]. The identification of nebulin in the Duchenne muscle biopsy studied here (illustrative of many other Duchenne biopsies examined) provides further support against this argument. Nebulin expression has recently been shown to be normal in other Duchenne muscle biopsies, using a combination of immunocytochemical and immunoblotting techniques [18]. Dystrophin appears to be a rather stable protein, unlike many other high-molecular-mass muscle proteins such as nebulin, titin and junctional feet proteins, which are extremely sensitive to degradation by proteases. The origins of the immunoreactive doublet invariably observed when muscle samples are probed with anti-dystrophin antibody are intriguing; whether this represents different gene products or a form of post-translational modification is unclear at present.

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