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Storage of phosphorylated desmin in a familial myopathy

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The quantity and the electrophoretic characteristics of desmin were analyzed in a familial skeletal muscle disorder, characterized by the intra-sarcoplasmic accumulation of an electron-dense granulo-filamentous material facing the Z-lines and reacting strongly with polyclonal anti-desmin antibodies. The analysis was performed on biopsies from the deltoid muscles of 4 patients, members of 2 families. In the 4 biopsies, an increase in the relative amount of desmin compared to that of actin or insoluble proteins (3 fold) and in the number of isovariants (6 instead of 3) was observed. The isovariants of desmin were similar to those described in Purkinje fibres of the heart as a phosphorylated form of the protein [(1987) Eur. J. Cell Biol. 44, 68–78]. Therefore, post-translational events could affect both the polymerization and the amount of desmin filaments in this autosomal dominant familial myopathy.

Familial cardiomyopathy; Desmin immunoquantitation; Desmin isovariant; (Skeletal muscle)

1. INTRODUCTION

A familial muscular disorder characterized by an intra-sarcoplasmic accumulation of an electron-dense granulo-filamentous material was described in 1978 by Fardeau et al. [1]. Recently these authors revealed by immunohistochemical labelling that this material reacts specifically with anti-desmin antibodies [2]. In adult skeletal muscle desmin is the constitutive protein of the intermediate filaments (IFs) that link individual myofibrils laterally at their Z disks and to the sarcolemma [3]. An accumulation of IFs has also been described in other muscular diseases by electron microscopy [4—6], but it was not clear if it was the result of a relative decrease in the amount of myofibrillar proteins and/or in an increase in IFs with altered capacities of polymerization.

The regulatory mechanisms that govern IF assembly-disassembly are not known. It was found recently that vimentin [7] and desmin [8] when phosphorylated in vitro by protein kinase A lose their ability to form normal IFs. In vivo phosphorylation of desmin results in an increased number of isovariants [9].

Therefore in order to better characterize the familial disorder described by Fardeau et al. [1] we have used immunological methods to analyse both the electrophoretic characteristics and the quantity of desmin in the skeletal muscle biopsies presenting an ultrastructural accumulation of electron-dense granulo-filamentous material. Since the resistance of IFs to extraction has been well documented [10] a one step partial purification of desmin was performed prior to the quantitative and qualitative analysis. A simple and sensitive method for evaluating the amount of desmin as a function of the protein and/or actin content in the extract was adapted using a dot-blot immunolabelling technique. Actin was chosen as an internal marker since the protein is known to be strongly associated with desmin throughout the purification processes [11] and since we observed that its relative amount in the protein extracts did not vary significantly among biopsies from controls and patients. The present experiments show that in the 4 patients' biopsies analysed, there is an increase in both the

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amount (2–3.8-fold) and the number of isovariants (up to 6) of desmin.

2. MATERIALS AND METHODS

Biopsies were obtained from deltoid muscles of 4 adult patients, 3 of them belonging to the same family (fig.1). The diagnosis was based on clinical symptoms and characteristic morphological changes [1]. Clinically it is characterised by a hypertrophic cardiomyopathy with a diffuse, symmetrical, non-selective involvement of skeletal muscles predominantly in distal and velopharyngeal muscles. Muscle biopsies, from patients displaying no clinically definite muscle disease and which were considered to be morphologically normal, were used as controls.

Biopsy material, previously frozen in liquid nitrogen and stored at −80°C, was homogenized at 4°C in 20 vols of 5 mM Hepes (pH 7.5), 25 mM sucrose, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) with protease inhibitor (1 μg/ml leupeptine, 0.5 mM benzamidine, 5 μg/ml of M-tosyl phenylalanine chloromethylketone (TPCK), using a polytron PT 10-35 homogenizer (1 time 5 s, plot 2 and 2 times 5 s, plot 3). 0.5% Triton X-100 was added and the samples were gently mixed for 15 min at 4°C and centrifuged (15 min at 10000 rpm (rotor Sorvall SS-34). The pellet was solubilized in 50 mM Tris-HCl (pH 7.7), 5 mM $MgCl_2$, 0.01% DNase, 0.005% RNase, 0.1 mM PMSF, 8 M urea, 0.5% SDS, 1% 2-mercaptoethanol, and protease inhibitors in the same amount as above. Aliquots were stored frozen at −20°C. Protein content was determined by Bradford's procedure [12].

The anti-actin antibodies were obtained from Miles Laboratory. Immunoquantitation and immunoblotting of desmin were performed using polyclonal immune serum raised in rabbit immunized with electrophoretically eluted chicken gizzard desmin extracted according to Geisler and Weber [12]. The antidesmin antiserum was characterized by the Ouchterlony test, immunofluorescence labelling of cardiac cells (not shown) and immunotransfer (see fig.2).

Mono- and two-dimensional gel electrophoresis (mini-apparatus, Biorad) were performed with slightly modified conditions from Laemmli [13] and O'Farrell [14], adapted for cytoskeletal proteins: electrofocusing gels (6.6 cm) containing 1.6% ampholine pH 4–6.5, and 0.4% ampholine 2D pH 3–10 (Pharmacia) were run for 1250 V·h. Equilibration was performed just prior to the run of the second dimension. Equal amounts of protein (30 μg) were applied on each gel. Proteins electrophoretically transferred to nitrocellulose sheets according to the method of Towbin et al. [15] were either stained with ink [16] or immunolabelled.

Immunoquantitation of desmin was an adaptation to dot-blot (SRC 96 apparatus from Schleicher and Schull) of Burnette's method [17]. Briefly, aliquots (80 μl) of increasing amounts of proteins from insoluble extracts were blotted onto nitrocellulose sheets previously soaked in 25 mM Tris, 192 mM glycine and 20% methanol. After preincubation with 5% bovine serum albumin (BSA) and washing in 10 mM Tris-HCl (pH 8.3), 0.9% NaCl, 0.05% NP-40, proteins were probed for reactivity with desmin anti-serum (1/250) or actin antibodies (1/100). The amount of either desmin or actin on each dot was revealed by incubation of the nitrocellulose sheet with iodinated protein A (2.5×10^5 cpm/ml for 60 min). After washing the extent of ^{125}I-protein A binding was visualized by exposure of the nitrocellulose sheet to an X-ray film, then the dots were excised and the amount of radioactivity determined. The latter was plotted as a function of the protein loaded on each dot.

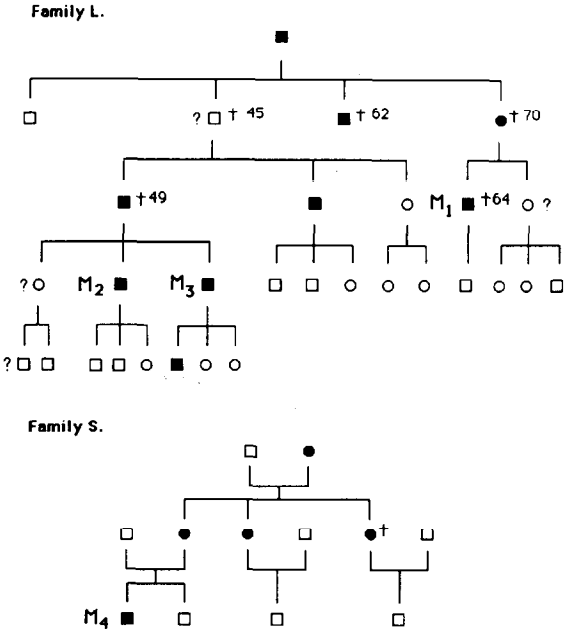

Fig.1. Family trees of the patients (M_1–M_4) whose biopsies presented ultrastructural alterations and accumulation of IFs.

3. RESULTS AND DISCUSSION

Fig.2 shows that after one step partial purification desmin and actin extracted from muscle biopsies were concentrated in the pellet rich in myofibrillar proteins. The desmin antiserum and anti-actin antibodies reacted only against desmin and actin, respectively.

Electrophoretic characteristics of desmin were analysed by 2D gel electrophoresis (fig.3). Both ink-staining and immunolabelling revealed that in all the controls desmin had the classical pattern of 2–3 isovariants, the more basic with the isoelectrofocusing characteristics of α-actin being the more important. In biopsies from patients desmin appeared to be composed of up to 6 isovariants of similar size, 3 of which were similar to those of the controls and the other three being more acidic. In fact an identical electrophoretic pattern of desmin has been observed in normal cardiac Purkinje

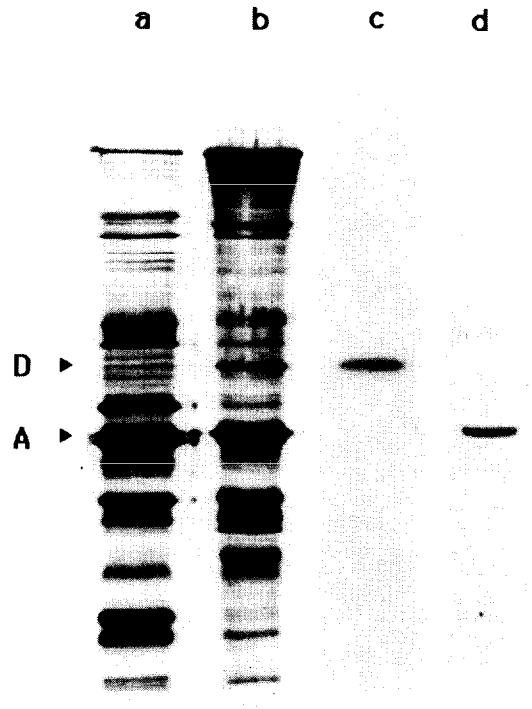

Fig.2. Gel electrophoresis of normal human muscle extracts. Soluble (lane a) and pellet extracts (lanes b–d) were stained with ink (lane a,b), with desmin antiserum (lane c) or with anti-actin antibodies (lane d). Actin and desmin are respectively marked as A and D.

fibres [18] where it was described as a highly phosphorylated form of the protein [9].

Furthermore it appeared from the ink staining of the nitrocellulose sheet that the intensity of staining of the desmin spots was clearly increased in patient's biopsies whilst that of actin spots did not vary among biopsies from controls and patients. Therefore, this apparent increase in the amount of desmin in the biopsies was further evaluated by immunoquantitation. Fig.4 is a typical curve of the immunoquantitation of desmin following dot-blot immunolabelling with desmin antiserum and ^{125}I-Protein A of increasing amounts of protein extracts. The amount of desmin and actin (not shown) in each muscle extract were evaluated in the linear portion of the curves as cpm of bound ^{125}I-Protein A per μg of loaded protein. The data summarized in table 1, show that in biopsies from 3 of the 4 patients (M_2, M_3, M_4) the amount of desmin was greater than in controls whilst that of actin remained constant. As a consequence the desmin/actin ratio (D/A) was markedly increased in these biopsies. Interestingly in the M_1 biopsy, the amount of desmin appeared to be similar to that of control muscles and only the actin quantitation enables us to reveal an increase in the D/A ratio as in the other biopsies.

In conclusion we have shown that the accumulation of intra-sarcoplasmic electron-dense granulo filaments of desmin, previously reported to characterize a familial muscle disorder [1] is accompanied by a large increase (up to 3.8 fold) in the amount of a desmin that has the typical electrophoretic pattern of the phosphorylated form of the protein [9].

The question that arises is whether any relationship exists between the alterations in the distribution and polymerisation state of IFs and the increase in the concentration and phosphorylation state of their constitutive protein. In vitro both kinase A and kinase C are able to phosphorylate the IF protein desmin [8] and vimentin [9] but only kinase A has been shown to induce a transition towards the depolymerized state of the IFs [7,8]. IFs with highly phosphorylated forms of desmin appeared either as filaments normally localised at the level of Z discs in cardiac Purkinje fibres [9] or as a granulo-filamentous material in pathological skeletal muscle ([1] and this paper). This suggests that in vivo the phosphorylation sites of desmin may be differently implicated in impaired filament formations. In vivo the modulation of the IF structure and organisation was shown to depend at least partially on a kinase A dependent single site-specific phosphorylation [19].

According to studies on neurofilaments, phosphorylation might be a factor implicated in stabilizing compacted forms of filaments [20]. It could be postulated that similarly phosphorylation of desmin increases IF stability and thus favors the relative accumulation of the protein observed in both cardiac Purkinje fibres [18] and in the pathological skeletal muscle (this paper).

Numerous ultrastructural studies have described abnormalities in the intermediate filaments distribution in different muscular diseases [4–6] and recent in vitro analyses have focused on the role of phosphorylation of vimentin and desmin on the IFs state of polymerization [7,8]. This study is the

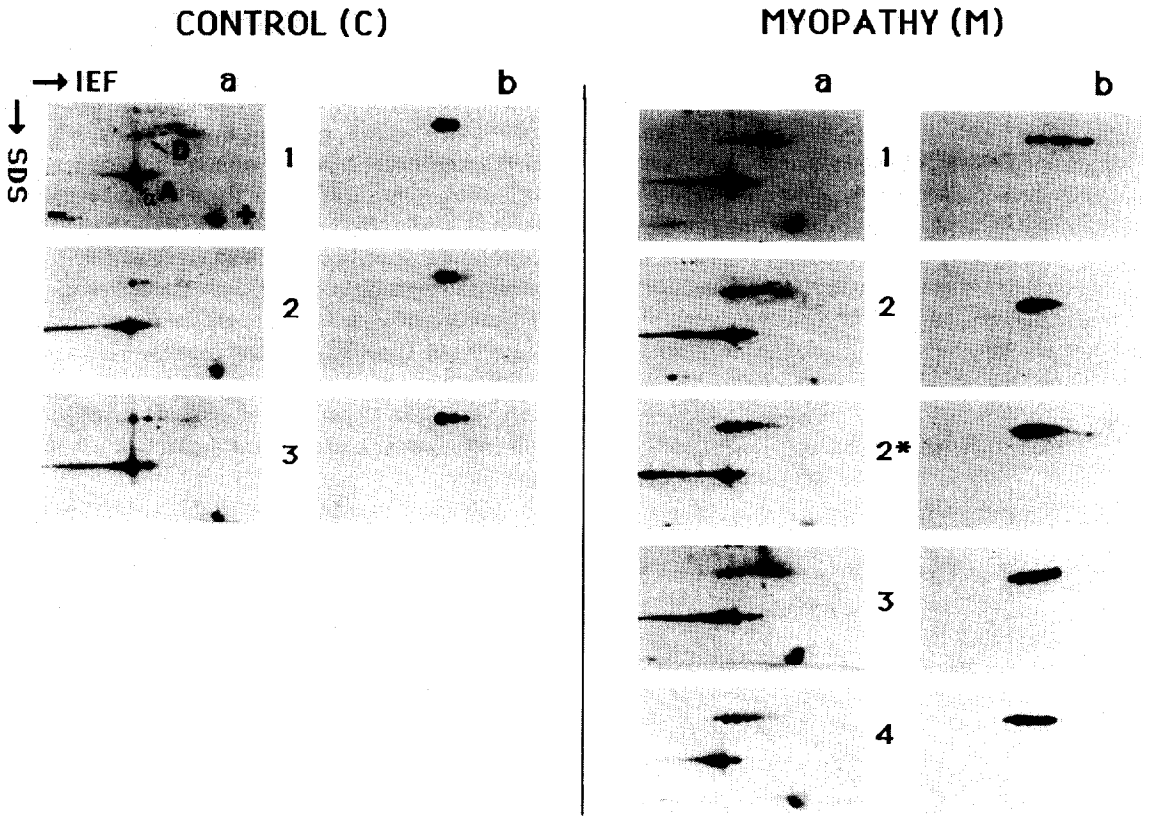

Fig.3. Increase in the number of isovariants of desmin in the biopsies from members of the two families showing intra-sarcoplasmic accumulation of IFs. Equal amounts of protein of the pellet extracts were submitted to 2D-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose sheet stained either with ink (a) or antidesmin labelled with ^{125}I-protein A (b). M_2 and M_2* are two biopsies taken at different time intervals from the same patient.

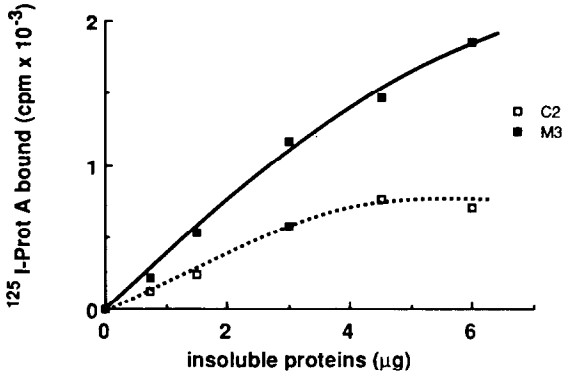

Fig.4. Typical curve of the immunoquantitation of desmin in a pellet extract from control (C_2) and patient's biopsies (M_3). The amount of ^{125}I-protein A bound on each dot was expressed as a function of the amount of protein applied. The relative amount of desmin in each biopsy extract was evaluated in the linear portion of the curve.

Table 1

Storage of desmin in myopathy

	Desmin (D) (cpm/µg insol. prot.)	Actin (A) (cpm/µg insol. prot.)	D/A
Control (n = 7)	247 ± 31*	383 ± 28*	0.66 ± 0.08*
Myopathy			
Family L.			
M_1	280 (× 1.13)	199 (× 0.52)	1.41 (× 2.1)
M_2	1076 (× 4.36)	429 (× 1.12)	2.51 (× 3.8)
M_3	800 (× 3.24)	450 (× 1.17)	1.78 (× 2.7)
Family S.			
M_4	916 (× 3.71)	401 (× 1.05)	2.28 (× 3.4)

Data were calculated according to the immunoquantitation method described in section 2. n, number of samples.
*, mean ± SE

first demonstration of an in vivo alteration of desmin characteristics which are associated with changes in the IF organization. These results are in favor of an impairment at the level of the specific enzymatic control of phosphorylation-dephosphorylation events that may be involved in the physiological modulation of intermediate filament integrity and structure. The nature of the gene responsible for this desmin disorder and the relative importance of this impairment in the development of the clinical syndrome observed in this familial muscle disease remained to be elucidated.

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