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The fast-twitch muscle calsequestrin isoform predominates in rabbit slow-twitch soleus muscle

Larry Fliegel*+, Ekkehard Leberer*, N. Michael Green+ and David H. MacLennan*

*Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, 112 College Street, Toronto, Ontario M5G 1L6, Canada and +National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, England

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The major form of calsequestrin in rabbit slow-twitch soleus muscle is shown to be identical to that isolated and cloned from rabbit fast-twitch muscle on the following bases: identity of cDNAs cloned from mRNAs from the two muscle sources; equivalent hybridization of a fast-twitch calsequestrin cDNA probe to mRNAs isolated from fast-twitch and slow-twitch muscles; identity of the 23 amino-terminal amino acids; strong binding of $^{45}Ca^{2+}$ in a gel overlay of slow muscle sarcoplasmic reticulum protein to a band at the level of the fast-twitch calsequestrin isoform and only weak binding at the level of the cardiac isoform. No evidence was obtained for developmentally regulated alternative splicing of the calsequestrin transcript in mature slow or fast-twitch muscle.

Calsequestrin; Slow-twitch muscle; cDNA cloning

1. INTRODUCTION

Calsequestrin [1] is a low-affinity, high-capacity Ca^{2+}-binding protein found in the terminal cisternae of the sarcoplasmic reticulum. The protein plays an important role in sequestering Ca^{2+} within the sarcoplasmic reticulum and concentrating it at the junctional face of the terminal cisternae, near the sites of Ca^{2+} release [2]. The sequence of the protein has been determined by peptide sequencing and by cDNA cloning of both a fast-twitch skeletal muscle isoform [3] and a cardiac muscle isoform [4]. Studies of the Ca^{2+} ATPase of sarcoplasmic reticulum have demonstrated the expression of two isoforms, both of which are alternatively spliced. One isoform is expressed in fast-twitch skeletal muscle [5] and alternative splicing is developmentally regulated [6]. The other is expressed in slow-twitch and cardiac muscles [6,7] and an alternatively spliced form is expressed in smooth muscle (Lytton, J., Zarain-Herzberg, A., Periasamy, M. and MacLennan, D.H., in preparation) and in nonmuscle cells [8,9]. By analogy, it was of interest to determine whether different calsequestrin isoforms are expressed in different skeletal muscles and whether there is developmentally regulated alternative splicing of the calsequestrin transcript.

Correspondence address: D.H. MacLennan, Banting and Best Department of Medical Research, Charles H. Best Institute, University of Toronto, Toronto, Ontario M5G 1L6, Canada

+ *Present address:* Department of Pediatrics, University of Alberta, Edmonton, Alberta T6G 2R7, Canada

2. MATERIALS AND METHODS

The isolation of rabbit neonatal fast-twitch muscle calsequestrin cDNA was described earlier [3]. The preparation of the adult fast-twitch cDNA library was described by Brandl et al. [6]. Calsequestrin cDNAs were isolated using nick-translated restriction endonuclease fragments of neonatal calsequestrin cDNA [3] as hybridization probes. Double-stranded cDNA was synthesized from adult rabbit slow-twitch soleus muscle mRNA and inserted into the expression vector λgt 11 [10]. The resulting library of 10^6 recombinant clones was screened [10] with affinity-purified polyclonal antibodies which were raised in a goat against rabbit fast-twitch skeletal muscle calsequestrin as described previously for other sarcoplasmic reticulum proteins [11]. Rabbit anti-goat IgG linked to horseradish peroxidase

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(Jackson Immunoresearch Laboratories) was used as the second antibody. Overlapping restriction endonuclease fragments were subcloned into the pTZ vector (Pharmacia) and single-stranded cDNAs made in this vector were sequenced by the dideoxy chain termination method of Sanger et al. [12]. RNA blot analysis was performed as described earlier [5].

Calsequestrin was purified from adult slow-twitch muscle by the method of K.P. Campbell (see [13]). The purified protein was sequenced on an Applied Biosystems 470 A gas-phase peptide sequencer according to Morrice et al. [14]. Ca^{2+} binding to proteins was detected using the Ca^{2+} overlay technique of Maruyama et al. [15].

SDS-polyacrylamide gel (10%) electrophoresis was carried out following the procedure of Laemmli [16], and immunoblotting was performed according to Towbin et al. [17]. Rabbit skeletal muscle sarcoplasmic reticulum membranes were isolated as described by Campbell and MacLennan [18]. Rabbit cardiac sarcoplasmic reticulum was isolated as described earlier [19] and was a gift from Dr M. Michalak. Rabbit soleus muscle sarcoplasmic reticulum was isolated by homogenizing tissue in 5 vols of a solution containing 0.3 M sucrose, 0.1 M KCl, 20 mM imidazole, pH 7.5, and 0.1 mM PMSF, centrifuging the homogenate twice at 10000 × *g* for 30 min to remove nuclei and mitochondria, and centrifuging the supernatant at 100000 × *g* for 1 h to obtain a microsomal pellet.

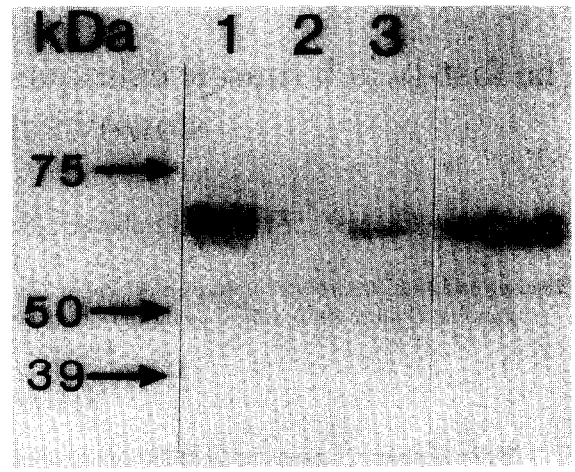

Fig.1. Immunoblot staining of sarcoplasmic reticulum from fast-twitch (lane 1), cardiac (lane 2) and slow-twitch muscle (lane 3) with affinity-purified polyclonal antibodies directed against the fast-twitch isoform of calsequestrin (CaS). Protein amounts applied to each lane were 15 μg. The positions of marker proteins are indicated (kDa). The antibody used in this study identifies a fast-twitch isoform of calsequestrin in both fast- and slow-twitch muscles, but does not react with cardiac calsequestrin.

3. RESULTS AND DISCUSSION

The similarity among calsequestrins from rabbit slow-twitch soleus muscle, rabbit fast-twitch skeletal muscle and rabbit cardiac muscle was tested immunologically. Polyclonal antibodies against fast-twitch muscle calsequestrin reacted with one band of the same apparent molecular mass in both fast- and slow-twitch muscle sarcoplasmic reticulum (fig.1), confirming the previous findings of Leberer and Pette [20]. The antibodies did not react with any protein in cardiac muscle sarcoplasmic reticulum (fig.1). This suggested that a form of calsequestrin of similar size and with an epitope identical to that in fast-twitch muscle calsequestrin was present in slow-twitch muscle but not in cardiac muscle.

The similarity between the skeletal muscle forms of calsequestrin was explored further through the isolation and characterization of calsequestrin cDNA clones from the neonatal muscle cDNA library and a mature slow-twitch muscle cDNA library. Restriction maps of clones isolated from these sources were identical (fig.2). The identity between these cDNA clones was confirmed by nucleotide sequence analysis (not shown).

It was of interest to determine whether there was developmentally regulated alternative splicing of the fast-twitch calsequestrin in slow- or fast-twitch muscle tissues because we had observed a discrepancy between the carboxyl-terminal amino acid sequence of calsequestrin from adult rabbit fast-twitch muscle and the sequence deduced from a neonatal cDNA in an earlier study [3]. Alternative splicing at the 3′-end was considered unlikely in the light of our finding that the carboxyl-terminus of the protein is encoded at the center of a very large exon [21]. The results illustrated in fig.2 show that all cDNAs had an identical 3′-sequence. Thus there is no 3′-alternative splice between the neonatal transcript and mature forms of either the fast- or slow-twitch muscle calsequestrin transcripts. The discrepancy between the amino acid sequence and the sequence deduced from a cDNA [3] must have another explanation such as posttranslational modification or an error in peptide sequencing caused by the difficulty in analysing a string of aspartate residues.

The use of a fast-twitch calsequestrin probe to isolate cDNA from the soleus muscle library left open the possibility that the clone represented only a minor proportion of the calsequestrin cDNAs present in the library. In order to determine

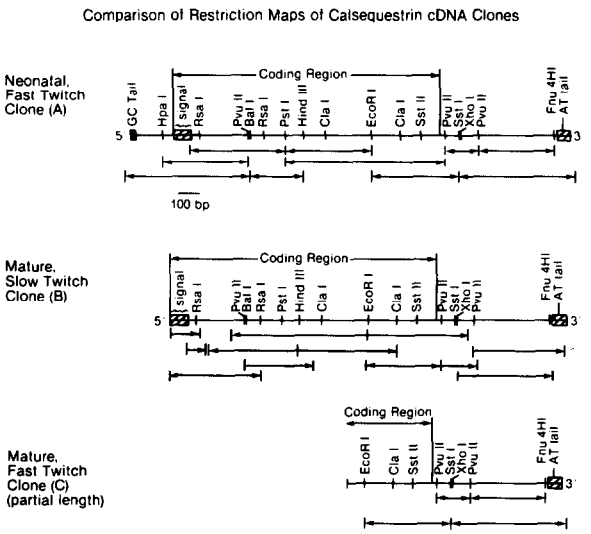

Fig.2. Restriction map and sequencing strategy of cDNA clones encoding calsequestrin. Clone A was isolated from a neonatal skeletal muscle cDNA library [3], clone B from a mature slow-twitch cDNA library, and clone C from a mature fast-twitch cDNA library. The restriction map displays five- and six-base sites used in sequencing. Each of these clones was sequenced and the arrows indicate the direction and extent of each sequencing run. The sequences obtained were identical in every case.

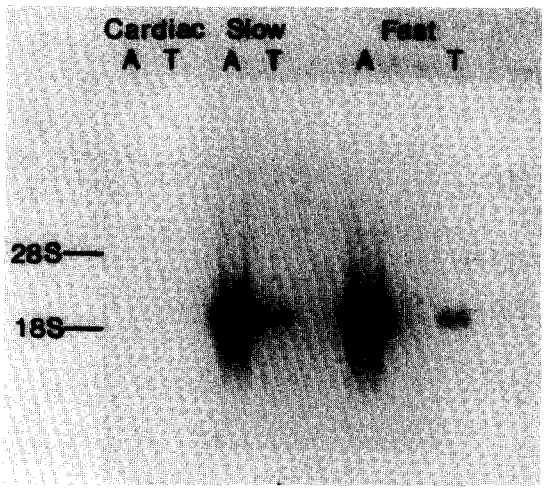

Fig.3. RNA blot analysis of total (T) and poly(A)$^+$ (A) RNA from adult rabbit cardiac, slow-twitch and fast-twitch muscles. Residues 790–1115 of neonatal fast-twitch calsequestrin [3] were used as a probe. The same results were obtained with a probe composed of residues 411–789 from the same cDNA clone.

whether the fast-twitch transcripts represented a major or a minor component in the soleus muscle mRNA, we carried out Northern blot hybridization on mRNAs isolated from fast-twitch, slow-twitch and cardiac muscles. We found that a fast-twitch calsequestrin probe hybridized to mRNAs in both fast- and slow-twitch preparations (fig.3). The size of the transcript was identical in both cases and the proportion of the calsequestrin transcript in fast- and slow-twitch mRNA populations was very similar. We did not observe any hybridization to transcripts in cardiac muscle mRNAs.

These observations demonstrate that the fast-twitch calsequestrin transcript is absent from cardiac muscle but is a major form in both fast- and slow-twitch skeletal muscle. Scott et al. [4] have carried out a comparable experiment with a cardiac calsequestrin probe. They found that cardiac calsequestrin was absent from fast-twitch muscle mRNA, gave a weak signal in slow-twitch muscle mRNA and a strong signal in cardiac muscle mRNA. In considering all of these observations, we conclude that the fast-twitch transcript is the sole transcript in fast-twitch muscle, it is absent from cardiac muscle and it is the predominant transcript in slow-twitch muscle. By contrast, the cardiac transcript is the sole transcript in cardiac muscle, it is absent from fast-twitch muscle and it is a minor component in slow-twitch muscle.

In order to confirm that the amount of calsequestrin mRNA in slow muscle is reflected at the protein level, we examined the form of calsequestrin present in slow-twitch muscle by taking advantage of its Ca^{2+}-binding properties in a gel overlay system [15]. Fig.4 illustrates that rabbit skeletal and cardiac calsequestrins can be distinguished as a result of their different mobilities in SDS-polyacrylamide gels [22]. In this experiment fast-twitch muscle can be seen to contain a Ca^{2+}-binding protein migrating only with the mobility of fast-twitch calsequestrin, while slow-twitch muscle contains a major labelled band comigrating with the fast-twitch isoform and a minor labelled component comigrating with the cardiac isoform of calsequestrin.

As further confirmation of the presence of fast-twitch calsequestrin in soleus muscle, we carried out amino-terminal sequence analysis on the protein isolated from this tissue. The preparation gave

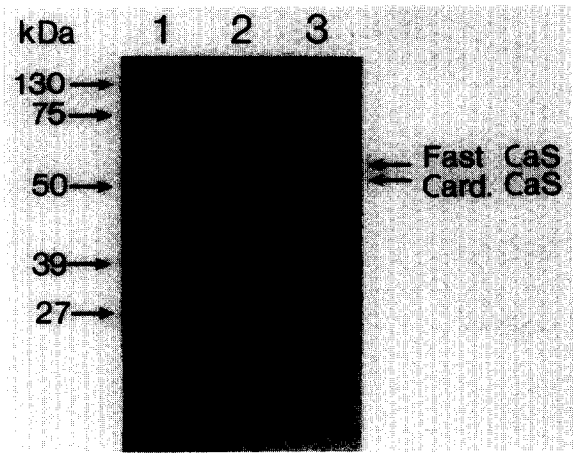

Fig.4. Identification of different isoforms of calsequestrin (CaS) after separation in SDS-polyacrylamide gels [17] and overlay with $^{45}Ca^{2+}$ [16]. Lanes: 1, rabbit fast-twitch muscle sarcoplasmic reticulum (5 μg protein); 2, purified rabbit cardiac calsequestrin (2.5 μg); 3, soleus muscle sarcoplasmic reticulum (15 μg protein). Soleus muscle contains a major Ca^{2+}-binding band with the same mobility as fast-twitch muscle calsequestrin (Fast CaS) and a minor Ca^{2+}-binding band of the same mobility as cardiac muscle calsequestrin (Card. CaS). The arrows indicate the positions of marker proteins (kDa).

a clear sequence as follows: EEGLDFP EYDGVDRVINVNAKNY. No minor sequence was observed. This is the precise fast-twitch calsequestrin amino-terminal amino acid sequence, confirming the vast predominance of the fast-twitch isoform of calsequestrin in slow-twitch muscle.

The predominance of fast-twitch calsequestrin in soleus muscle points to the probability that sarcoplasmic reticulum in slow-twitch muscle has a hybrid character. The cardiac isoform of the Ca^{2+} ATPase [5,6] and the cardiac form of phospholamban [23] are expressed in soleus muscle. These proteins must function together with a fast-twitch isoform of calsequestrin, in contrast to cardiac sarcoplasmic reticulum which has the cardiac isoforms of all 3 proteins.

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