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Structural diversity of triadin in skeletal muscle and evidence of its existence in heart

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Abstract

Triadin has been characterized as an abundant protein co-localized with the calcium release channel on the terminal cisternae of the sarcoplasmic reticulum of the skeletal muscle. Its localization to terminal cisternae of the sarcoplasmic reticulum and functional studies suggest that it has an important role in excitation-contraction coupling. In this study we identify three triadin isoforms in rabbit skeletal muscle and by Northern blot analysis demonstrate that triadin also exists in the heart.

Key words: Calcium release channel; Sarcoplasmic reticulum; Calcium; E-C coupling; Triad

1. Introduction

The exact mechanism of excitation–contraction (E-C) coupling in skeletal muscle is incompletely understood. The dihydropyridine (DHP) receptor which is on the α_1 subunit of the L-type voltage-dependent calcium channel and the ryanodine receptor are the two main components that are thought to mediate E-C coupling [1]. The DHP receptor may be the voltage sensor on the T-tubule which 'detects' depolarization [2,3]. The signal is thought to be transduced to the ryanodine receptor through a second messenger or by some mechanical linkage [1]. In cardiac muscle Ca^{2+} is believed to be the second messenger and calcium-induced calcium release is considered to be the major mechanism for E-C coupling [4–6]. In skeletal muscle, data support a mechanism for a linkage between the DHP receptor and the ryanodine receptor [1,7]. However, no direct interaction between the two molecules has been demonstrated.

Triadin is a 95 kDa junction-specific protein which co-localizes with the ryanodine receptor on the terminal cisternae of the sarcoplasmic reticulum (SR) in skeletal muscle [8–10]. It is present in a roughly 1:1 molar ratio with the ryanodine receptor [9]. The functional role of triadin in E-C coupling is controversial; it has been suggested by Caswell et al. [8,9], that it may serve as a linkage protein which mediates the mechanical coupling between the DHP receptor and the calcium release channel (ryanodine receptor). Recently, Knudson et al. [11], proposed that triadin may be a skeletal muscle-specific protein serving to anchor calsequestrin to the SR junction. To further elucidate the role of triadin in E-C coupling, we utilized molecular biological techniques to obtain the primary structure of triadin in rabbit skeletal muscle. Three isoforms were found to exist, and Northern blot analysis showed that triadin exists in heart as well as in skeletal muscle.

2. Experimental procedures

2.1. cDNA cloning and sequencing

An oligo(dT)-primed size-selected (> 1.5 kb) cDNA library was constructed in λgt11 [12] and λZAPII vector (Stratagene) using young adult rabbit back muscle. 10^6 recombinants were screened using a *picoblue* immunoscreening kit from Stratagene from each library, with the monoclonal anti-triadin antibody GE4.90. Nine positive clones from the λgt11 library and 13 positive clones from the λZAPII library were found. Two clones (95-1 and 95-7) from the λgt11 library and one clone (95-5) from the λZAPII library were sequenced from both orientations using the dideoxy chain termination method (13). Since the 5′ end was missing from all the clones, a triadin-specific cDNA library was constructed to λZAPII vector using one-month-old rabbit back skeletal muscle with primer 1870 (GCAGTTGATGACTCTC). The library was constructed by using the SuperScript Choice System for cDNA Synthesis kit from BRL and was screened with the 5′ end of 95-1, a 900 bp *Eco*RI/*Xba*I fragment. Out of 40 positive clones five positive clones (141, 30, 121, 12, 71) were sequenced as before, and analyzed using a DNA Analysis program [14].

2.2. Internal protein sequencing

The purified triadin [9] was reduced and fluorescently labeled with TBP and ABD-F [15] and precipitated with acetone. The precipitate was dissolved in 8 M urea and digested with endoproteinase Lys-C for 24 h at 37°C in 50 mM NH_4HCO_3 and 2 M urea. The digest was directly injected onto a 2.1 mm Vydac C_{18} reverse phase HPLC column equilibrated in 0.1% trifluoroacetic acid in water and eluted with a gradient of acetonitrile. Peak 10 eluting at 15% acetonitrile was collected for sequence analysis. The sequence analysis was carried out on a Porton

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Abbreviations: E-C coupling, excitation–contraction coupling; DHP receptor, dihydropyridine receptor; SR, sarcoplasmic reticulum; RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TBP, tributyl phosphine; ABD-F, 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole; APRT, adenosine phosphoribosyl transferase.

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2090 E sequencer in the Protein Sequencing Core facility of the University of Cincinnati College of Medicine.

2.3. Reverse transcription and polymerase chain reaction (RT-PCR) to amplify the isoforms from RNA

Total RNA was isolated from rabbit skeletal muscle as described [16]. The reverse transcription (RT) was done in 20 μl containing 1 μg of skeletal muscle total RNA, 2.5 unit/μl reverse transcriptase, 1 unit/μl RNase inhibitor, 0.75 μM downstream primer, 1 mM dATP, 1 mM dCTP, 1 mM dTTP, 1 mM dGTP, 6 mM $MgCl_2$ and 1 × PCR buffer II (Perkin Elmer Cetus). The RT reaction was carried out at 42°C for 15 min, the enzyme was inactivated at 99°C for 5 min and the sample incubated on ice for 5 min. PCR was carried out in 100 μl containing the RT mix (above) and 80 μl of PCR master mix containing 2 mM $MgCl_2$, 1 × PCR buffer II, 0.15 μM upstream primer and 2 units of thermal stable Taq (Perkin Elmer Cetus). The reaction was performed under the following conditions: initial denaturation at 95°C for 2 min, 45 cycles at 58°C for 1 min then 72°C for 30 s and 94°C for 1 min. The product was run on a 3% agarose gel. Specific bands were cut out and purified through a prep-A-Gene kit (Bio-Rad). The DNA recovered from the gel was sequenced directly with a Circum Vent thermal cycle dideoxy DNA sequencing kit using Vent (exo-) DNA polymerase (Bio-Rad) from both orientations. The primers for sequencing were ^{32}P-labeled at the 5′ end with T4 polynucleotide kinase.

2.4. Northern blot analysis

Total RNA was isolated from one-month-old rabbit back skeletal muscle, heart, cerebellum, kidney and stomach by a modified guanidinum thiocyanate/phenol procedure [16]. Poly(A)$^+$ RNA was isolated using an oligo(dT)-cellulose column [17]. The mRNA (2 μg) was electrophoresed through 1% agarose/0.8% formaldehyde denaturing gel and transferred to a nylon filter. The filter was hybridized with ^{32}P random prime labeled probes under 6 × SSPE (20 × SSPE = 175.3 g NaCl, 27.6 g NaH_2PO_4 and 7.4 g EDTA in 1 liter of H_2O, pH 7.4), 0.5% SDS, 5 × Denhardt's Sol, 50% formamide and 100 μg/ml of salmon sperm DNA at 42°C for 16 h [17]. The filter was washed under 2 × SSPE, 0.1% SDS at room temperature for 15 min, 1 × SSPE, 0.1% SDS at 67°C for 15 min and 0.1 × SSPE, 1% SDS at 67°C for 20 min. Autoradiography was carried out for 16 h at −70°C.

3. Results and discussion

Eight overlapping clones were identified by immunoscreening two rabbit skeletal muscle cDNA expression libraries with the anti-triadin monoclonal antibody GE4.90 and rescreening the triadin specific library with the *EcoRI/XbaI* fragment of clone 95-1 (Fig. 1). Six of the overlapping clones (95-1, 95-5, 95-7, 12, 30, 112) revealed a 4525 nucleotide sequence that contains a 2118-nucleotide open-reading frame (triadin 1). This sequence shows 99.9% homology with the 94 kDa glycoprotein (triadin) sequence published recently by Knudson et al. [11]. The amino acid sequence of an endoproteinase Lys-C proteolytic fragment of the purified triadin ((K)XALHGKPEE) is also consistent with the deduced protein sequence of triadin (amino acids 540–549 according to triadin 1 sequence) (Fig. 2).

Clone 141 showed two deletions in the sequence of triadin 1. The first deletion, D1, is located at nucleotides 1247–1273 (27 bp), and the second, D2, is between nucleotides 1754 and 1774 (21 bp). The second deletion was also observed in clone 71; the amino acid sequences for these deletions are shown in Fig. 2. Both deletions are located in the highly positive charged region of the protein. The existence of these deletions was confirmed by RT-PCR. With an upstream primer at bp 1159 and a downstream primer at bp 1455, two bands (296 bp and 269 bp) were amplified (Fig. 3a). Two additional bands (227 bp and 206 bp) were amplified using an upstream primer at 1687 and a downstream primer at 1914

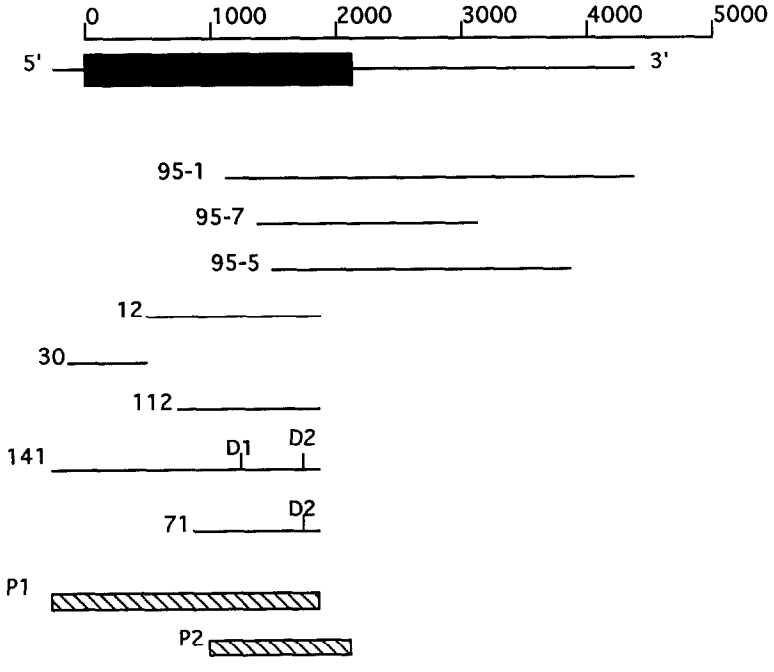

Fig. 1. Positive cDNA clones isolated from the rabbit skeletal muscle cDNA library. Overlapping clones 95-1, 95-7, 95-1, 121, 12 and 30 encode the sequence of triadin 1. Clone 141 encodes for the triadin 2 isoform which is characterized by two deletions: D1 and D2. Clone 71 encodes the triadin 3 isoform which has a single (D2) deletion. P1 and P2 is the 5′ probe and 3′ probe using for Northern blot analysis.

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                401                                                 450
Triadin 1       EKHEEPAKSTKKEHAAPSEKQAKAKIERKEEVSAASTKKAVPAKKEEKTT
Triadin 2       **************E---------**************************
Triadin 3       **************************************************

                451                                                 500
Triadin 1       KTVEQETRKEKPGKISSVLKDKELTKEKEVKVPASLKEKGSETKKDEKTS
Triadin 2       **************************************************
Triadin 3       **************************************************

                501                                                 550
Triadin 1       KPEPQIKKEEKPGKEVKPKPPQPQIKKEEKPEQDIMKPEKTALHGKPEEK
Triadin 2       **************************************************
Triadin 3       **************************************************

                551                                                 600
Triadin 1       VLKQVKAVTTEKHVKPKPAKKAEHQEKEPPSIKTDKPKSTSKGMPEVTES
Triadin 2       **********************************-------E********
Triadin 3       **********************************-------E********

                601             620
Triadin 1       GKKKIEKSEKEIKVPARRES
TRIADIN 2       ********************
TRIADIN 3       ********************
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Fig. 2. Comparison of deduced amino acid sequences of triadin 1, triadin 2 and triadin 3. The stars indicate the amino acid identity. The dashed lines indicate the deletion regions. The underlined amino acids are the sequence obtained from the endoproteinase Lys-C fragment derived from the purified triadin.

(Fig. 3b). The PCR products were sequenced from both orientations. The 269 bp DNA product encodes a 27 bp deletion which is identical to D1, and the 206 bp DNA product encodes a 21 bp deletion identical to D2. Therefore, we conclude that triadin exists in at least three isoforms in rabbit skeletal muscle.

Tissue-specific distribution of triadin transcripts were determined by Northern blot analysis of rabbit tissues using the cDNA probe derived from nucleotides −180 to 1870 of the rabbit skeletal muscle triadin 2 (probe 1). Three major mRNA transcript sizes, 4.6 kb, 2.5 kb and 2.0 kb, were found in skeletal muscle (Fig. 4a). The 4.6 kb band at least codes for triadin 1 isoform which is 4525 bp in length characterized by cDNA cloning and sequence. Only a 48 bp and a 21 bp deletion have been identified in triadin 2 and triadin 3, respectively. Both deletions are not large enough to shift the mRNA from 4.6 kb to 2.5 kb or 2.0 kb. Therefore, it is suspected that triadin 2 and triadin 3 isoforms may have other divergent regions comparing to triadin 1. Also, it cannot be ex-

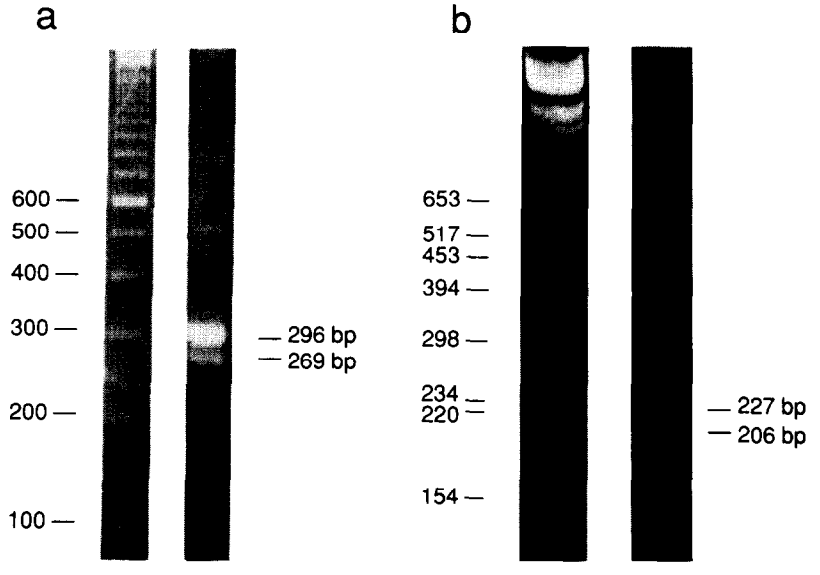

Fig. 3. Reverse transcription and polymerase chain reaction (RT-PCR) to amplify the three isoforms from RNA. (a) With an upstream primer at bp 1159 (CCTGCAGAAGAACAGCCCAAG) and a downstream primer at bp 1455 (GGAAGCTGGAACCTTCAC) of triadin 1, two bands (296 bp and 269 bp) were amplified. (b) With an upstream primer at bp 1687 (CATGTCAAGCCAAAACCAGC) and a downstream primer at bp 1914 (TTCTTTTGATCCTCTTGCAGG) of triadin 1, two bands (227 bp and 206 bp) were amplified.

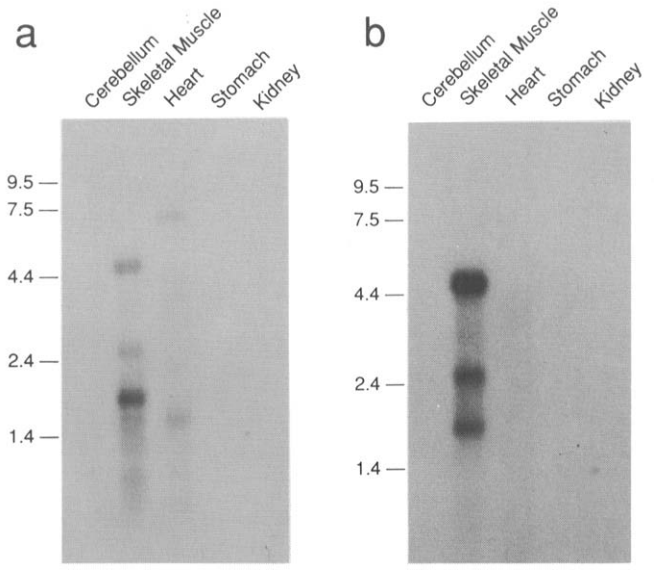

Fig. 4. Northern blot analysis of mRNA from different rabbit tissues. 2 μg of skeletal muscle, heart, cerebellum, stomach and kidney mRNA were used. (a) The mRNA was hybridized with the 5′ end probe (probe 1) including the sequence from −180 to 1870 of triadin 2. (b) The mRNA was hybridized with the 3′ end probe (probe 2) including the sequence from 1100 to 2030 of triadin 1.

cluded that other isoforms, which have yet to be identified, correspond to the 2.5 kb and 2.0 kb mRNAs. Two transcript sizes, 6.7 kb and 1.6 kb, were found in heart (Fig. 4a). A second probe covering base pairs 1100–2030 (probe 2) was used and three transcripts (4.6 kb, 2.5 kb and 2.0 kb) were again detected in skeletal muscle but not in heart. Both experiments were repeated twice, and the blots were hybridized with a probe to the housekeeping APRT gene to ensure that none of the RNA prepared from different tissues was degraded. These results provide evidence that triadin exists in both skeletal muscle and in heart. The heart isoform is different from all of the three isoforms cloned in skeletal muscle. The N-terminal portion of the heart triadin (amino acids 1–366) has significant homology to the skeletal muscle triadin. However, the C-terminal portion (amino acids 367–706) shows little homology. Knudson et al. [11] used a probe similar to our probe 2 and failed to detect any bands in the heart, and therefore proposed that triadin is unique to skeletal muscle, possibly explaining the differences in E-C coupling between skeletal muscle and heart. Our results clearly show that triadin does indeed exist in the heart, a finding which is consistent with the detection in the heart of a homologous protein which binds to the triadin monoclonal antibody, GE4.90, and which has similar molecular weight and subcellular distribution to the skeletal muscle triadin [18]. In the heart, antibody GE4.90 also detected a 60 kDa band [18]. This band may be a protease product or another isoform of heart triadin corresponding to the 1.6 kb mRNA transcript. By using our skeletal muscle triadin cDNA probes, triadin was not found in the stomach, kidney and cerebellum of the rabbit.

The C-terminal portion of triadin may be responsible for the interaction with the skeletal muscle ryanodine receptor (RyaR1) (unpublished data). Therefore, our result that the C-terminus portion of the heart triadin(s) differs from that of the skeletal muscle is not surprising because of the significant differences in the heart and the skeletal muscle ryanodine receptors. Since the C-terminal half of the skeletal muscle triadin isoforms are very rich in charges residues and these amino acids may be missing in the heart triadin, we speculate that they may be involved in communication between the DHP receptor and the ryanodine receptor in skeletal muscle E-C coupling.

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