Biochemical Characterization and Molecular Cloning of Cardiac Triadin*

(Received for publication, August 3, 1995, and in revised form, September 22, 1995)

Wei Guo†, Annelise O. Jorgensen§, Larry R. Jones¶, and Kevin P. Campbell||

From the Howard Hughes Medical Institute and the Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, the §Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario MSS 1A8, Canada, and the ¶Kranert Institute of Cardiology and the Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202.

Triadin is an intrinsic membrane protein first identified in the skeletal muscle junctional sarcoplasmic reticulum and is considered to play an important role in excitation-contraction coupling. Using polyclonal antibodies to skeletal muscle triadin, we have identified and characterized three isoforms in rabbit cardiac muscle. The cDNAs encoding these three isoforms of triadin have been isolated by reverse transcription-polymerase chain reaction and cDNA library screening. The deduced amino acid sequences show that these proteins are identical in their N-terminal sequences, whereas the C-terminal sequences are distinct from each other and from that of skeletal muscle triadin. Based upon both the amino acid sequences and biochemical analysis, all three triadin isoforms share similar membrane topology with skeletal muscle triadin. Immunofluorescence staining of rabbit cardiac muscle with antibodies purified from the homologous region of triadin shows that cardiac triadin is primarily confined to the I-band region of cardiac myocytes, where the junctional and corbular sarcoplasmic reticulum is located. Furthermore, we demonstrate that the conserved region of the luminal domain of triadin is able to bind both the ryanodine receptor and calsequestrin in cardiac muscle. These results suggest that triadin colocalizes with and binds to the ryanodine receptor and calsequestrin in cardiac muscle. These results suggest that triadin colocalizes with and binds to the ryanodine receptor and calsequestrin and carries out a function in the lumen of the junctional sarcoplasmic reticulum that is important for both skeletal and cardiac muscle excitation-contraction coupling.

Ca2+ release from the sarcoplasmic reticulum of skeletal and cardiac muscle is regulated by similar but distinct mechanisms (1–3). In cardiac muscle, depolarization leads to the opening of voltage-gated Ca2+ channels. Ca2+ influx through L-type Ca2+ channels (the dihydropyridine receptor) triggers the opening of the ryanodine receptor/Ca2+ release channel in the sarcoplasmic reticulum. However, in skeletal muscle, entry of extracellular calcium is not needed for this signal transduction process. The skeletal muscle dihydropyridine receptor interacts either directly or indirectly with the ryanodine receptor, thereby activating the Ca2+ release channel without a requirement for extracellular calcium. Despite this difference, many factors that modulate the channel properties of the skeletal muscle ryanodine receptor also affect the cardiac ryanodine receptor in a similar fashion (4, 5). Identification of protein components in the junctional sarcoplasmic reticulum is fundamental to our understanding of the mechanisms of Ca2+ storage and release in muscle cells. So far, the major components of the excitation-contraction coupling, such as the ryanodine receptor, the dihydropyridine receptor, and calsequestrin, have been extensively studied in skeletal muscle, and their counterparts have later been identified and characterized in cardiac muscle.

Triadin is an intrinsic membrane protein originally identified in skeletal muscle (6–8). It is specifically enriched in the junctional sarcoplasmic reticulum, where it colocalizes with the ryanodine receptor/Ca2+ release channel (6, 8). In addition, triadin probably forms multimers in the lumen of the sarcoplasmic reticulum through disulfide bonds (6–8). Triadin was previously proposed to bind the ryanodine receptor and the dihydropyridine receptor and to serve as the “linking protein” that mediates the signal transduction process between these two Ca2+ channels in skeletal muscle (8). Recently, triadin has been found to interact with both the ryanodine receptor and calsequestrin in the lumen of the skeletal muscle sarcoplasmic reticulum (9). These results (9) along with membrane topology analysis (7, 23) suggest that triadin anchors calsequestrin to the junctional face membrane near the sarcoplasmic reticulum “foot” ryanodine receptor and is probably involved in the functional coupling between the Ca2+ release channel and the intraluminal calcium-binding protein calsequestrin (10–13).

Recent reports have described the possible existence of triadin in cardiac muscle (14–17). To gain further insight into the function of triadin and to examine the molecular differences between skeletal and cardiac muscle triadin, we have identified and biochemically characterized three cardiac triadin isoforms and subsequently cloned the cDNAs encoding these proteins. Immunofluorescence staining indicates that these cardiac triadin isoforms are primarily confined to the junctional and corbular sarcoplasmic reticulum. In addition, using an affinity binding assay, we found that the conserved region of the luminal domain of triadin is able to interact with both the ryanodine receptor and calsequestrin in the lumen of the cardiac muscle junctional sarcoplasmic reticulum. These results suggest that triadin does not carry out a skeletal muscle-specific function. Instead, it probably plays an important role in both skeletal and cardiac muscle excitation-contraction coupling.
EXPERIMENTAL PROCEDURES

Generation of Anti-triadin Polyclonal Antibodies—Skeletal muscle triadin luminal domain-GST fusion protein (L-triadin) (9) was used to generate polyclonal antibodies in sheep (Sh33). Also, full-length skeletal muscle triadin was purified from SDS-polyacrylamide gel slices and was used to generate polyclonal antibodies in guinea pigs (GP57 and GP58).

Isolation of Cardiac Microsomes—Fresh whole adult rabbit hearts were rapidly frozen using liquid nitrogen. Cardiac muscle microsomes were prepared from the frozen hearts as described previously (7). Protein samples were analyzed by SDS-PAGE using the buffer system of Laemmli (18) and transferred to nitrocellulose according to Towbin et al. (19). The polyclonal antibodies were used for immunoblot assay as described previously (6).

Vesicle Protection Assay of Rabbit Cardiac Muscle Triadin—Trypsin was used at a 1:50 (w/w) ratio to digest rabbit cardiac muscle microsomes in the presence or absence of 0.3% CHAPS for 15 min at 37 °C. The reactions were quenched with 2 mM phenylmethylsulfonyl fluoride and 3% SDS in Laemmli sample buffer (18). The protein samples were separated using 3–15% gradient SDS-PAGE and transferred to nitrocellulose. Polyclonal antibody GP58 was used at a 1:1000 dilution for the immunoblot analysis.

Isolation of cDNAs Encoding Three Isoforms of Cardiac Triadin—Rabbit cardiac muscle total RNA was isolated by homogenization in RNAzol according to the protocol of Cinna/Biotecx followed by chloroform extraction. cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). A DNA forward primer corresponding to skeletal muscle triadin nucleotides 1–23 (F1) and reverse primers corresponding to nucleotides 818–845 (R2) and 2002–2124 (R3) were used to amplify cardiac triadin sequence from the reverse-transcribed cDNA using polymerase chain reaction (RT-PCR). A DNA fragment of 845 bp was generated by primers F1 and R2. This RT-PCR product was subcloned into pBluescript SK(+) vector for sequencing. The sequence was analyzed using PCGENE software from IntelliGenetics, Inc. (Mountain View, CA).

Affinity Purification of Polyclonal Antibodies—The DNA fragment corresponding to the conserved region of the luminal domain of triadin (amino acids 69–264) was subcloned into pGEX-2T vector by the EcoRI site and expressed as a GST fusion protein (H-triadin). Purified H-triadin was used to purify antibodies GP58 and Sh33 as described previously (24). After purification, anti-GST antibodies were removed with GST fusion protein-nitrocellulose strips.

Immunofluorescence Labeling of Papillary Muscle Cardiocytes—Dissection, cryofixation, and cryosectioning (6–8 μm) of atrial and ventricular muscle tissues of anesthetized adult rabbit hearts were performed as described previously (24, 26). Indirect immunofluorescence labeling of cardiac myocytes was carried out as described previously (24). After purification, anti-GST antibodies were removed with GST fusion protein-nitrocellulose strips.

Affinity Purification of Polyclonal Antibodies—The DNA fragment corresponding to the conserved region of the luminal domain of triadin (amino acids 69–264) was subcloned into pGEX-2T vector by the EcoRI site and expressed as a GST fusion protein (H-triadin). Purified H-triadin was used to purify antibodies GP58 and Sh33 as described previously (24). After purification, anti-GST antibodies were removed with GST fusion protein-nitrocellulose strips.

Immunofluorescence Labeling of Papillary Muscle Cardiocytes—Dissection, cryofixation, and cryosectioning (6–8 μm) of atrial and ventricular muscle tissues of anesthetized adult rabbit hearts were performed as described previously (24, 26). Indirect immunofluorescence labeling of cardiac myocytes was carried out as described previously (24). After purification, anti-GST antibodies were removed with GST fusion protein-nitrocellulose strips.

RESULTS

Identification of Three Triadin Isoforms in Cardiac Muscle—Polyclonal antibodies generated from skeletal muscle L-triadin-GST fusion protein (Sh33) and polyclonal antibodies generated from native full-length 95-kDa skeletal muscle triadin (GP57 and GP58) all recognized three proteins in rabbit cardiac muscle microsomes (Fig. 1). One of the three proteins has a molecular mass of 92 kDa. The other two have molecular masses of 35 and 40 kDa, respectively. Monoclonal antibody 11G12, which was previously used to characterize skeletal muscle triadin (7), does not recognize any protein in cardiac muscle microsomes (Fig. 1).

Confocal microscopy was carried out with a photomicroscope (Nikon Optiphot-2, Tokyo, Japan). Antibodies were applied to cardiac microsomes in the presence of 0.1% CHAPS and 5 mM β-mercaptoethanol. This is a major difference between the two different tissues. Brandt et al. (16) reported earlier that reducing reagents did not alter the mobility of the large cardiac triadin isoform. Our result is consist-

1The abbreviations used are: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); L-triadin, luminal domain triadin fusion protein; H-triadin, homologous region of the luminal domain triadin fusion protein; C-triadin, cytoplasmic domain of triadin fusion protein.
Interestingly, the nucleotide sequence of this fragment is the same as skeletal muscle sequence 1–845. The amplified fragment corresponds to the C-terminal sequence of skeletal muscle triadin, poly(A) RNA. However, using primers F1 and R1, which correspond to the skeletal muscle triadin sequence, but differs from CT1 and CT2. This sequence probably represents part of the 92-kDa cardiac triadin (CT3) sequence. A forward primer corresponding to nucleotides 795–815 was synthesized (TCAGTATGCATTCTGTCGATA). Using this primer together with pBluescript primer KS, we were able to amplify the 3’-sequence from the zAPII library to obtain the full-length open reading frame of CT3. Fig. 5C shows the nucleotide sequence of CT3 and its translation. In comparison with skeletal muscle triadin (Fig. 6), except for the deletion of GCA from positions 991 to 993, CT3 has the same sequence from nucleotides 1 to 1932. The rest of the 3’-sequence is totally different. The translated protein therefore has a unique C-terminal sequence of 23 amino acids. Fig. 6 shows the alignment of the three cardiac triadin isoforms together with the skeletal muscle sequence. Since the conserved regions of these triadin sequences are the same at the nucleotide level, these isoforms are probably the result of alternative splicing of the same gene. Hydrophobicity analysis of the proteins was conducted according to the method of Kyte and Doolittle (20) using PC/GENE software (Fig. 7). The hydrophobicity plots suggest that all three proteins share similar membrane topology with their skeletal muscle counterpart, although their luminal segments have different lengths.

Cellular and Subcellular Distribution of Triadin in Ventricular Tissue—Since each of the three triadin isoforms in cardiac muscle contains a common homologous luminal domain (H-triadin; amino acids 69–264), the cellular distribution of triadin was probed using two affinity-purified antibodies against H-triadin (Sh33 and GP58). Confocal imaging showed that...
specific labeling for H-triadin was densely distributed in rabbit atrial (Fig. 8, a) and ventricular (Fig. 8, b and c) myofibers, where it was localized to discrete foci. In contrast, the intensity of labeling of vascular smooth muscle cells (sm; Fig. 8, a), endothelial cells (E; Fig. 8a), and fibroblasts (not shown) present in the same sections was only marginally higher than that of the background. Since the marginal increase in the labeling intensity was diffusely distributed throughout these cells, it seems unlikely that it represents specific labeling for triadin in these subcellular organelles. Furthermore, as expected, specific labeling of atrial and ventricular myocytes as well as of non-muscle cells was not detected with monoclonal antibody IIG12 (6, 7), which is specific for the C terminus of skeletal muscle triadin (not shown).

Regarding the subcellular distribution of triadin, confocal imaging of longitudinal cryosections from rabbit papillary muscle shows that specific labeling was mainly confined to parallel strands oriented transversely to the longitudinal axis of the fibers (Fig. 8, b and c). These strands were frequently resolved into discrete foci (Fig. 8, c, white arrows). Phase-contrast imaging of the same field (Fig. 8, d) shows that the discrete fluorescent foci were primarily localized to the center of the I-band region (Fig. 8, d, white arrows), where most of the internal junctional and corbular sarcoplasmic reticulum is localized in mammalian myofibers.
rose was incubated with the radiolabeled material, and the amount of bound receptor was determined by counting the [3H]ryanodine on the Sepharose. As shown in Fig. 9B, both L-triadin and H-triadin bound the labeled receptor, whereas C-triadin did not bind to the receptor. As a positive control, the ryanodine receptor polyclonal sheep antibody (6) Sepharose was capable of binding to the solubilized ryanodine receptor.

**DISCUSSION**

We have previously cloned skeletal muscle triadin and found that triadin was present only in skeletal muscle by Northern and Western blot analyses (7). This suggested that triadin carries out a function that is specific to skeletal muscle excitation-contraction coupling. Recently, however, several reports have suggested the existence of triadin in cardiac muscle (14–17). Peng et al. (15) were able to detect several messages of triadin in cardiac muscle using Northern blot analysis. Notably, the messages could be identified using a probe corresponding to the 5'-fragment (but not the 3'-fragment) of skeletal muscle triadin. This result suggests that skeletal and cardiac muscle triadin isoforms share similar sequences at the N-terminal region, but differ at their C termini.

Using polyclonal antibodies, we demonstrated the existence of three isoforms of triadin in cardiac muscle microsomes (Fig. 1). One migrates at a molecular mass of 92 kDa, slightly smaller than the skeletal muscle counterpart of 95 kDa. The other two form doublets of 35 and 40 kDa, respectively. Interestingly, none of the proteins could be detected by monoclonal antibody IIG12, which was used to isolate the skeletal muscle triadin cDNA (7). In fact, none of the monoclonal antibodies that were used by Knudson et al. (6, 7) to characterize skeletal muscle triadin were able to recognize the cardiac muscle triadin isoforms by either Western blot analysis or immunocytochemical staining (data not shown). The antigenic epitopes for these monoclonal antibodies have now been mapped to the C-terminal region of skeletal muscle triadin (data not shown), further supporting the prediction that the triadin isoforms differ at their C termini.

By RT-PCR and library screening, we have obtained cardiac muscle triadin sequences (Fig. 5). The alignment of triadin sequences is shown in Fig. 6. These sequences are the same until nucleotide 793. For the long cardiac triadin isoform CT3, except for the deletion of the trinucleotide GCA at positions 991–993, the nucleotide sequence is identical to that of skeletal muscle triadin from nucleotides 1 to 1932. The rest of the sequence is different. As a result of the GCA deletion, an alanine residue at position 331 is missing. The translated protein is therefore basically homologous to skeletal muscle triadin amino acids 1–665, but has a unique C terminus of 23 amino acids. The cardiac triadin isoform CT3 is 39 amino acids shorter than its skeletal counterpart, which explains why it has a slightly smaller molecular mass than skeletal muscle triadin as detected by Western blot analysis.

In skeletal muscle triadin, there are two cysteine residues at positions 270 and 671 in the proposed luminal segment. These two residues are believed to be responsible for the disulfide-linked homomultimeric structure of triadin (7). Interestingly, for CT3, there is only one cysteine residue (position 270) in the luminal segment. Fig. 3 demonstrates that CT3 only migrates...
as a single band of ~92 kDa in the presence of N-ethylmaleimide. Therefore, CT3 does not form multimeric complexes through disulfide bonds. The lack of a cysteine residue might affect the formation of multimeric structures of cardiac triadin.

Based on hydrophobicity analysis by the method of Kyte and Doolittle (20), each of the three cardiac triadin isoforms contains a single transmembrane domain that separates it into cytoplasmic and luminal segments (Fig. 7). Since cardiac triadin isoforms are homologous to skeletal muscle triadin at their N-terminal portions, previous analysis of this portion of the sequences (7) applies to these cardiac proteins. Like their skeletal muscle counterpart, only the N-terminal 47 amino acids are cytoplasmic, with the bulk of the proteins located in the lumen of the sarcoplasmic reticulum. This prediction is consistent with the results of the vesicle protection assay (Fig. 2).

The cellular and subcellular distribution of triadin in cardiac muscle was also examined by immunofluorescence staining (Fig. 8). Specific staining was primarily detected in cardiac myocytes where the labeling of triadin corresponds very closely to that of the ryanodine receptor and calsequestrin, which were previously demonstrated to be localized to the junctional and corbular sarcoplasmic reticulum in cardiac myocytes (24, 25, 27, 28). The subcellular distribution of triadin in cardiac muscle in this study is very similar to that reported by Brandt et al.
and Carl et al. (17) using a monoclonal antibody that recognizes both skeletal and cardiac muscle triadin. We also examined the possible interaction of triadin with the ryanodine receptor and calsequestrin in cardiac muscle. As shown in Fig. 9, the homologous region of the triadin luminal segment (H-triadin) was able to bind [3H]ryanodine-labeled cardiac receptor and cardiac calsequestrin.

Triadin was originally found only in skeletal muscle (6, 7). It was therefore proposed that triadin carries out a function that is specific to skeletal muscle excitation-contraction coupling (6, 7). Recently, we found that triadin interacts with the ryanodine receptor and calsequestrin in the lumen of the skeletal muscle sarcoplasmic reticulum (9). Triadin may be the transmembrane protein that anchors calsequestrin to the junctional sarcoplasmic reticulum near the "sarcoplasmic reticulum foot" ryanodine receptor and thus may be involved in the functional coupling between these two proteins (10–13). In cardiac muscle, calsequestrin and the ryanodine receptor are localized to the junctional and corbular sarcoplasmic reticulum membrane. If triadin is the anchoring protein for calsequestrin in skeletal muscle, there should be triadin homologs in cardiac muscle. In this study, we report the identification, molecular cloning, immunofluorescent localization, and biochemical characterization of three isoforms of triadin in rabbit cardiac muscle. We find that cardiac triadin isoforms share a large region of sequence identity with their skeletal muscle counterpart. In addition, their similar membrane topologies and patterns of molecular interactions suggest that triadin plays a role that is important to both skeletal and cardiac muscle excitation-contraction coupling.

Acknowledgment—We thank Wayne Arnold for expert technical assistance.
REFERENCES

22. Deleted in proof