Identification of Triadin 1 as the Predominant Triadin Isoform Expressed in Mammalian Myocardium*

(Received for publication, July 8, 1999, and in revised form, July 19, 1999)

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Triadin is an integral membrane protein of sarcoplasmic reticulum shown to interact with the ryanodine receptor/Ca\(^{2+}\) release channel, junctin, and calsequestrin. Several triadin isoforms have been postulated to exist in cardiac muscle, but to date none has been conclusively identified. Here, we show that only triadin 1 is significantly expressed. We cloned and sequenced cDNAs encoding canine cardiac triadin 1 and 3 but found no evidence for triadin 2. From deduced primary structures, antibodies against domains common to all triadins and an antibody against the unique C terminus of triadin 1 were raised. All antibodies detected two prominent proteins of molecular masses 35 and 40 kDa on immunoblots from cardiac microsomes, including the antibody that recognizes only triadin 1. The 40-kDa mobility form was shown to correspond to the glycosylated form of triadin 1, not a distinct triadin 2 isoform as previously hypothesized. Confirming this, overexpression of triadin 1 in transgenic mouse hearts produced both the 35-kDa deglycosylated and the 40-kDa glycosylated mobility forms. The glycosylation site of triadin 1 was localized to asparagine residue 75, and its bitopic arrangement in the membrane was confirmed. Although a 92-kDa immunoreactive protein could be tentatively identified in myocardium as triadin 3, its expression level was insignificant (≤5%) compared with that of triadin 1. We conclude that triadin 1 is the triadin isoform most likely to play a role in Ca\(^{2+}\) release in heart.

The junctional SR\(^1\) is the site of Ca\(^{2+}\) release in cardiac and skeletal muscle (1). Ca\(^{2+}\) release occurs here through the Ca\(^{2+}\) release channel or the RyR, which resides in the junctional SR membrane in association with a complex of proteins including triadin, junctin, and calsequestrin (2–4). Calsequestrin is a high capacity, moderate affinity Ca\(^{2+}\)-binding protein localized in the lumen of the junctional SR in cardiac and skeletal muscle which stores the Ca\(^{2+}\) required for Ca\(^{2+}\) release (5, 6). Triadin (4, 8) and junctin (9) are structurally similar junctional SR proteins that bind directly to calsequestrin and the RyR (2–4). Both triadin and junctin are integral membrane proteins that may serve to tether calsequestrin to the Ca\(^{2+}\) release channel, thus facilitating the transfer of Ca\(^{2+}\) across the junctional membrane during coupling of excitation to contraction (2–4). Recent reports suggest that triadin inhibits the activity of the RyR in skeletal muscle by decreasing the open state probability of the channel (10–12). From this type of evidence, triadin is proposed to have an important regulatory role in the Ca\(^{2+}\) release process as it occurs in both cardiac (2, 4) and skeletal muscle (3, 13).

Triadin was first identified by Caswell and co-workers (14, 15) as a major 95-kDa membrane protein endogenous to skeletal muscle junctional SR membranes. Subsequently, the primary structure of skeletal muscle SR membranes was deduced from its cDNA sequence by Knudson et al. (8). Only a single triadin isoform was identified in skeletal muscle, which was predicted to be an intrinsic membrane protein containing a single membrane-spanning domain (8, 16). The protein contains a short N terminus located in the cytoplasm, and a large, highly charged C-terminal domain proposed to reside entirely within the lumen of the SR (8, 16). A glutathione S-transferase fusion protein containing the luminal domain of triadin was shown to bind directly to calsequestrin and the RyR, providing direct evidence for a physical association between all three proteins (3).

Conclusive identification of triadin protein(s) in cardiac tissue has proven to be problematic. By using \(^{125}\)I-labeled calsequestrin in overlay assays, Mitchell et al. (7) identified three major calsequestrin-binding proteins in cardiac junctional SR vesicles of apparent molecular weights of 28,000, 35,000, and 40,000. The 26-kDa calsequestrin-binding protein was recently purified, sequenced, and cloned and named junctin (9). Identical junctins were expressed in cardiac and skeletal muscle (9), and like triadin, junctin was demonstrated to bind to both calsequestrin and the RyR (2). Although the 35- and 40-kDa calsequestrin-binding proteins have been consistently observed in canine cardiac microsomes and excluded from being junctin isoforms (2, 7, 9), their exact relationship to triadin or triadin isoforms has remained ambiguous.

Recent cloning results utilizing a rabbit heart cDNA library predicted three unique triadin protein isoforms expressed in cardiac muscle, named cardiac triadins 1, 2, and 3 by Guo et al. (4). The deduced amino acid sequences of all three cardiac triadins (and skeletal muscle triadin) were identical between amino acid residues 1–264. This shared region included the N-terminal cytoplasmic domains, the transmembrane segments, and the C-terminal, intralumenal domains shown to bind calsequestrin and the RyR. The sequences of the cardiac triadins diverged after amino acid 264 by the inclusion of unique C-terminal tails, giving predicted molecular weights of approximately 32,000, 35,000, and 75,000 for rabbit cardiac
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triadins 1, 2, and 3, respectively (4). On immunoblots of rabbit cardiac SR vesicles using generic triadin antibodies that did not discriminate between isoforms, three prominent triadin mobility forms were detected with apparent molecular weights of 35,000, 40,000, and 92,000, suggesting that these three immunoreactive bands represented cardiac triadins 1, 2, and 3, respectively, predicted by cDNA cloning (4). Consistent with this, the apparent molecular weights of the 35-kDa and 40-kDa calceustrin-binding proteins in canine cardiac junctional SR vesicles (2, 7, 9) also matched very closely with the predicted molecular weights for rabbit cardiac triadins 1 and 2 (4). Moreover, purification and partial amino acid sequencing of the 35-kDa calceustrin-binding protein from dog heart junctional SR vesicles gave several peptide fragments of identical amino acid sequences as regions of the common domains shared by all of the rabbit cardiac triadin isoforms predicted from cDNA cloning (4). Based upon all of these observations, it was proposed that three major triadin isoforms indeed existed in heart (4). However, direct identification of the predicted protein isoforms and determination of their relative expression levels still has not been performed.

Here, we directly addressed the issue of the identities of the different triadin isoforms expressed in heart. Remarkably, we find that only triadin 1 is expressed to any significant extent in myocardium. We could find no evidence for the existence of triadin 2, and expression of triadin 3, although detected provisionally at the protein level, appears marginal compared with the level demonstrated for triadin 1 in all species examined.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from New England Biolabs. Growth media constituents for plasmid propagation in Escherichia coli were purchased from Fisher and Sigma. Library isolates and constructs were sequenced using SequenaseTM (Amersham Pharmacia Biotech), and sequence was analyzed with MacVectorTM (Oxford Molecular). All oligonucleotides were purchased from Life Technologies, Inc. Radioactive nucleotides were obtained from NEN Life Science Products.

Cloning of Canine Triadin Isoforms—Oligonucleotides flanking the cDNA sequence encoding the common lumenal region shared by the rabbit triadin isoforms (residues 69–264) were used to PCR-amplify a 588-bp [588-bp]PdCCTP- and [588-bp]AdATP-labeled cDNA probe using rabbit cardiac triadin 1 cDNA as template (4). The probe was used to screen a canine cardiac λgt10 cDNA library (5, 17), and positive isolates were subcloned into the EcoRI site of pBluescript II SK (Stratagene). All of these isolates displayed a 5’ partial open reading frame encoding triadin 1 and triadin 2, and 3, respectively, which were used to first amplify a mutant product. A second PCR reaction used primers 1 and 2 with 2 µl of the first PCR product mix to amplify the full-length triadin 1 encoding the N75A mutation. The final PCR product carrying the full-length protein coding region for N75A-triadin 1 was ligated into the EcoRI-BgII sites of baculovirus transfer plasmid pVL1393 and pAcGS2 (Pharmingen), respectively.

To compare the canine cardiac triadin isoforms to their skeletal muscle counterpart, a UniZap canine skeletal muscle cDNA library (Stratagene) was screened using the common 588-bp cDNA probe described above. Positive clones were isolated according to manufacturers’ specifications. Clone 2d (Fig. 1) contained the full-length canine skeletal muscle triadin protein-coding region.

Glycosylation Site Mutant of Triadin 1—Asparagine residue 75, the potential glycosylation site conserved in all triadin isoforms, was mutated to alanine in cardiac triadin 1 to produce an N75A-triadin 1 mutant. The mutated cDNA was generated by PCR of the assembled full-length triadin 1 clone as follows. An N75A sense primer encoding an alanine mutation at asparagine residue 75 and primer 2 (Fig. 1B) were used to first amplify a mutant product. A second PCR reaction used primers 1 and 2 with 2 µl of the first PCR product mix to amplify the full-length triadin 1 encoding the N75A mutation. The final PCR product carrying the full-length protein coding region for N75A-triadin 1 was ligated into the EcoRI-BgII sites of a baculovirus transfer plasmid and expressed in insect cells.

Recombiant Triadin Expression in Sf21 Insect Cells—Canine cardiac triadin 1 and N75A-triadin 1 cDNA protein-coding regions were subcloned into the EcoRI-BgII restriction enzyme sites of the baculovirus transfer plasmids pVL1393 and pAcGS2 (Pharmingen), respectively. Transfer vectors were cotransfected with BaculoGoldTM-linearized baculovirus DNA (Pharmingen) into Sf21 insect cells (Invitrogen) according to manufacturers’ specifications. Recombinant virus isolation and amplification were performed as described in baculovirus protocols (18).

Purification of Recombinant Cardiac Triadin—Insect cells infected with recombinant baculovirus encoding canine cardiac triadin 1 or N75A-triadin 1 were extracted at pH 11.4 with sodium carbonate to obtain carbonate pellets highly enriched in the recombinant proteins (19). Triadins were then solubilized from the carbonate pellets by use of Triton X-100 and purified by phosphocellulos chromatography as described previously (9).

Antibodies to Triadin and Epitope Mapping—Site-specific antibodies were raised in rabbits against synthetic peptides (Genosys Biotechnologies) corresponding to the following regions: the common N terminus (cytoplasmic domain) of cardiac and skeletal muscle triadins of all known species isoforms (residues 2–18, C-TEITAEGNASTTTTVI) (N-terminal antibody); the common lumenal domain of mouse cardiac and skeletal muscle triadins (residues 146–160, C-QEKAEEKEEFEKKIQ) (lumenal antibody); and the unique C-terminal tail of canine cardiac triadin 1 (residues 262–278, C-EVEAGSSKRTLGGKQIQ) (T1-specific antibody). Peptides with N-terminal cysteines were coupled to ovalbumin using Inject™ Conjugation Kit (Pierce), and conjugated samples were used as immunogen for rabbits. The resulting antiserum was affinity purified following the method of Olmsted (2, 9). Polyclonal antibodies produced to each peptide were affinity purified from rabbit antiserum using the corresponding peptides cross-linked to Sulfolink™ gel columns (Pierce). In addition, a generic triadin antiserum was raised in rabbits against purified recombinant triadin 1, and antibodies were affinity purified following the method of Olmsted (22).

Epitopes recognized by the triadin antibodies were mapped to high resolution using cellulose-bound PepSpots™ (Jerini Bio Tools). The PepSpots™ sheet contained a series of immobilized 13-mer synthetic peptides, each overlapping by 11 residues, that moved down the length of the primary structure of canine triadin 1. Immunoblotting on PepSpots™ was done as described below.

Preparation of Cardiac Microsomes—Procedure I cardiac microsomes (21) were isolated from the ventricles of dogs, rabbits, mice, rats, humans, and guinea pigs. Subfractionation of Procedure I microsomes from dogs into free and junctional SR vesicles was conducted as described (21). Skeletal muscle microsomes were isolated from canine hind limb muscle.

Vesicle Protection Assay—A protease protection assay was used to determine the topology of cardiac triadin 1 in the SR membrane as described previously for junctin (9). 50 µg of intact or 0.2% Triton X-100-permeabilized canine cardiac microsomes were treated with 1 µg of trypsin for 30 min at room temperature. SDS-PAGE of microsomes was then conducted, and membrane proteins were transferred to nitrocellulose for incubation with the N-term or T1-specific antibodies. Calsequestrin, an entirely intracellular protein, was also probed with a polyclonal antibody to canine cardiac calsequestrin (2), as a control for vesicle leakiness.

SDS-PAGE and Immunoblotting—40–50 µg of microsomal protein were dissolved in SDS sample buffer containing 7% SDS plus 80 mT

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Fig. 1. Canine triadin cDNA clones. A, shows restriction maps for assembled full-length cDNA clones encoding canine cardiac triadin 1, canine cardiac triadin 3, and canine skeletal muscle triadin. Boxes denote open reading frames with start (ATG) and stop (TGA) codons indicated. Open boxes designate regions of identical cDNA sequence. Regions encoding unique C-terminal tails are dotted (triadin 1), shaded (triadin 3), or solid black (skeletal muscle triadin). The region of sequence overlap between triadin 3 and skeletal muscle triadin is striped. In this region, the tether indicates a part of the sequence that is missing from skeletal triadin. The thin lines indicate the untranslated regions, which were identical at the 5' ends but different at the 3' ends for all of the clones. B, shows maps of partial cDNA clones isolated from canine cardiac libraries used to assemble the full-length clones. Primers 1, 2, and 3 were used to confirm the full-length cDNA sequences by RT-PCR are indicated by the arrows.

RESULTS

Cloning Canine Cardiac and Skeletal Muscle Triadin cDNAs—By using a rabbit triadin (4)-based cDNA probe, Agt10 (5, 17) and Lambda Zap Express canine cardiac cDNA libraries were screened. The Agt10 library yielded 50 positive clones, each of which carried a triadin 5' partial open reading frame. Isolate 4c contained a 720-bp EcoRI fragment, the longest clone, incorporating the 5'-untranslated region, the ATG start codon, and part of the 5' protein-coding region of triadin through amino acid residues 1–174 (Fig. 1B). The Lambda Zap Express library yielded 15 more positive clones, none of which carried a full-length protein-coding region. However, 13 of these clones contained partial open reading frames encoding the unique C-terminal end of triadin 1. Of these, clone 5z was longest and had the most overlap with clone 4c (Fig. 1B). Only two cDNA clones were found encoding a unique C terminus similar to that previously reported for rabbit triadin 3 (4). Of these, clone 3b was the longest with the most overlap with clone 4c (Fig. 1B). The full-length assembled sequences of triadins 1 and 3 depicted in Fig. 1A were confirmed in heart by RT-PCR of canine left ventricle total RNA using canine-specific primers to yield the predicted size bp products for triadins 1 and 3 (data not shown). The cDNA for canine triadin 1 predicts a 278-amino acid protein with a calculated molecular weight of 30,757. The cDNA for canine triadin 3 predicts a 597-amino acid protein with a calculated molecular weight of 64,823 (Fig. 2). To compare the primary structures of canine cardiac triadins 1 and 3 to their skeletal muscle equivalent, a UniZap canine skeletal muscle cDNA library was screened using the same probe. Clone 2d (Fig. 1A) contained the full-length protein-coding region for canine skeletal muscle triadin. The de-
duced primary structure for canine skeletal muscle triadin predicts a 701-amino acid protein with a calculated molecular weight of 78,274 (Fig. 2).

Thus, our cloning results identifying canine cardiac triadins 1 and 3 and canine skeletal muscle triadin are very similar to cloning results first reported for these triadin isoforms in the rabbit (4). Similar to rabbit triadin isoforms, all canine triadins are predicted to be identical from amino acid residues 1–257 and then each protein diverges with its own unique C-terminal sequence, canine cardiac triadin 1 containing unique amino acid residues 258–278, canine cardiac triadin 3 containing residues 258–597, and canine skeletal muscle triadin containing residues 258–701 (Fig. 2). Cardiac triadin 3 and skeletal muscle triadin are identical over most of the region extending from residues 258–541 of triadin 3, and then each protein diverges with its own unique C terminus (Fig. 2). Residues 1–257 (the common regions) of the canine and rabbit (4, 8) triadins are highly conserved and share 92% amino acid identity. The unique C termini of canine and rabbit cardiac triad (4) triadins are less conserved and share 86% homology for triadin 1 and 57% homology for triadin 3. The unique C termini of canine and rabbit skeletal muscle triadins share 77% homology.

In contrast to results previously reported using a rabbit cDNA library (4), we could find no evidence for the existence of a canine cardiac triadin 2 cDNA clone encoding a third cardiac triadin with its own unique C-terminus, either by screening the two different canine cardiac cDNA libraries or by use of RT-PCR of canine cardiac total RNA.

Triadin Protein Mobility Forms in Canine Cardiac Microsomes Recognized by Site-specific Antibodies—Site-specific antibodies were raised in rabbits to identify the predicted triadin isoforms expressed in heart. Three site-specific antibodies to triadin were generated as follows: one to residues 2–18 of the common cytoplasmic domain (N-term); one to residues 146–160 of the common lumenal domain (Lumenal); and one to residues 262–278 of the unique C terminus predicted only for cardiac triadin 1 (T1-specific). These antibodies were affinity purified from rabbit antiserum, and their antigenic epitopes were mapped at high resolution using a series of peptide spots encompassing the entire amino acid sequence of canine cardiac triadin 1 (Fig. 3). The epitope for the N-term antibody was mapped to triadin residues 10–18 (ASTTTTVID); the epitope for the Lumenal antibody to residues 147–158 (EKAEKEEKPERK); and two T1-specific antibody epitopes were found, one to residues 262–270 (EEVAGGSKR) and one to residues 266–278 (GGSKRTLGKKQIQ) (Fig. 3). Microsomes isolated from canine ventricle and skeletal muscle were next analyzed by immunoblotting with the three site-specific antibodies (Fig. 3). The N-term and Lumenal antibodies detected two prominent proteins in cardiac microsomes of molecular masses 35 and 40 kDa (T1), and a very minor protein of 92-kDa (asterisk), which accounted for less than 5% of the total immunoreactivity (Fig. 3). The T1-specific antibody...
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Fig. 3. Characterization of site-specific triadin antibodies. A, depicts three autoradiograms mapping the N-term, Lumenal, and T₁-specific antibody epitopes using the PepSpots™ sheet. 133 overlapping peptides (Pep. #), encompassing the amino acid sequence of triadin 1, were immobilized on the nitrocellulose sheet and scanned by immunoblotting. Amino acid residues of triadin 1 scanned along each row are listed on the right (T₁ Res.). B, shows immunoblots of canine cardiac (C) and skeletal muscle (S) microsomes incubated with the N-term, Lumenal, and T₁-specific antibodies. The 35- and 40-kDa molecular mass forms of triadin 1 (T₁) are bracketed. The asterisk depicts the 92-kDa molecular mass protein identified in cardiac microsomes. Molecular mass standards (x 10⁻³) are shown on the right.

Fig. 4. Triadin in cardiac membrane subfractions. The upper panel shows an immunoblot of Procedure I canine cardiac membrane vesicles (MV) and derived subfractions A–E obtained by Ca²⁺-oxalate loading followed by sucrose density gradient centrifugation. 40 μg of membrane protein were analyzed per gel lane. The immunoblot was developed with a generic antibody raised to recombinant canine cardiac triadin 1 (T₁). The faintly reacting 92-kDa protein in subfraction D is indicated by the asterisk. The lower panel depicts results when the same subfractions were processed and probed with an antibody to calsequestrin (CSQ).

To confirm the localization of the cardiac triadin isoforms to the junctional SR in heart, Procedure I microsomes were subfractionated into membranes enriched in free SR (subfraction E) and junctional SR (subfraction D) by calcium-oxalate loading followed by sucrose density gradient centrifugation (21), and immunoblotting was conducted with a generic triadin antibody raised to purified recombinant triadin 1 expressed and purified from insect cells. Use of the generic antibody revealed that both the 35- and 40-kDa mobility forms of triadin 1 were highly enriched in the junctional SR subfraction D, along with the minor 92-kDa protein that reacts with non-selective triadin antibodies (Fig. 4, upper panel). Identical results were obtained with the Lumenal antibody; however, when the T₁-specific antibody was used, only the 35- and 40-kDa triadin mobility forms were detected in subfraction D (data not shown). Immunoblotting of the same subfractions with a calsequestrin antibody (23) showed that calsequestrin copurified with triadin in the junctional SR subfraction D (Fig. 4, lower panel), along with junctin and the RyR as demonstrated in our previous study (9).

Cardiac Triadin 1 Is Glycosylated—The doublet detected for triadin 1 (the 35- and 40-kDa mobility bands described above) on immunoblots could result from partial glycosylation of the protein, since all the triadins share a consensus N-linked glycosylation site at asparagine residue 75 (Fig. 2). It is known that skeletal muscle triadin is glycosylated (13, 16). To test if triadin 1 is partially glycosylated, membranes were treated with endo H, which hydrolyzes N-glycans of the high mannose type, and then subjected to SDS-PAGE followed by immunoblotting using the generic triadin antibody made to recombinant triadin 1. Endo H treatment of canine cardiac microsomes completely converted the 40-kDa triadin band into the 35-kDa mobility form (Fig. 5, left panel). Thus, the 40-kDa triadin protein recognized by triadin antibodies appears to be the glycosylated form of triadin 1, not a unique triadin 2 isoform as previously suggested (4). PhosphorImager analysis of the 35- and 40-kDa mobility forms indicates that the intensity of the

raised to the unique C terminus of canine cardiac triadin 1 detected the same two prominent protein bands at 35 and 40 kDa, suggesting that they both arose from triadin 1. This T₁-specific antibody did not cross-react with the minor 92-kDa cardiac microsomal protein (the putative triadin 3 isoform, see below) nor did it cross-react with any triadin isoform present in skeletal muscle microsomes (Fig. 2B), corresponding to the skeletal muscle isoform of triadin. (Resolution of rabbit skeletal muscle triadin into these two anomalous mobility forms of 95 and 60 kDa has been reported previously (15, 16, 24).) Thus, results with all three antibodies suggest that triadin 1 is the only major triadin isoform expressed in microsomes isolated from canine ventri-
35-kDa band is doubled upon endo H treatment of the microsomes, suggesting that canine cardiac triadin 1 is approximately 50% glycosylated.

The generic triadin antibody also detected the faint 92-kDa molecular mass protein when the same immunoblot was exposed 13 times longer, and like triadin 1, the mobility of the 92-kDa protein was increased by approximately 4 kDa by endo H treatment (Fig. 5, right panel). This suggests that the 92-kDa triadin isoform present in cardiac microsomes is also glycosylated.

To test if triadin 1 is the major triadin isoform present in cardiac microsomes from different mammalian species, cardiac microsomes prepared from rabbits, mice, rats, humans, and guinea pigs were incubated in the presence and absence of endo H and then immunoblots were probed with the Lumenal antibody. For all species tested, the same two major 35- and 40-kDa molecular mass proteins were detected, and in all cases, treatment of cardiac microsomes with endo H completely converted the upper 40-kDa mobility form of triadin 1 into the lower 35-kDa mobility form (Fig. 6A). Identical results were obtained when blots were probed with the specific triadin antibody raised to recombinant canine cardiac triadin 1. Use of the T1-specific antibody recognizing the distinct C terminus of canine cardiac triadin 1 confirmed the identity of triadin 1 in microsomes from the same species (Fig. 6B). However, the cross-reactivity of this antibody with triadin 1 in the different species was poor, especially for cardiac microsomes isolated from humans, rats, and mice. Five times more concentrated antiserum and much longer autoradiographic exposure times were required for detection of triadin 1 in microsomes from these latter species, and especially for mouse microsomes, the signal was at the limit of detectability. Poor cross-reactivity was most likely due to the less stringent conservation of amino acids at the distinct C terminus of triadin 1. For example, the epitopes recognized by the T1-specific antibody in canine cardiac triadin 1, EEAAGGSK and GGSKRTLGKKQIQ, are EEAAGCFK and GCFKRTLGKK-QMQ in rabbit cardiac triadin 1 (4).

Similar to results obtained with dog cardiac microsomes, we could detect very faint 92-kDa molecular mass proteins in cardiac microsomes from all species tested when blots were probed with generic triadin antibodies, and exposure times for the autoradiograms were long. The mobilities of the 92-kDa proteins recognized by generic triadin antibodies were also increased by approximately 4-kDa with endo H treatment (data not shown). However, the expression levels for these 92-kDa triadins were minor in comparison to that of triadin 1 for all species tested. No evidence for a triadin 2-immunoreactive protein was found in microsomes from any mammalian species.

Overexpression of Canine Cardiac Triadin 1 in Transgenic Mouse Hearts—To confirm the post-translational modification of triadin 1, we overexpressed the canine cardiac isoform of the protein in transgenic mouse hearts using the α-myosin heavy chain promoter to drive protein expression (23). Microsomes were prepared from control and transgenic mouse hearts and probed with the T1-specific antibody, which recognizes canine cardiac triadin 1 strongly but mouse cardiac triadin 1 only very weakly, and the Lumenal antibody, which recognizes both triadins equally well (Fig. 6). Use of the T1-specific antibody revealed that overexpression of canine cardiac triadin 1 in transgenic mouse hearts produced two protein mobility forms of molecular masses 35 and 40 kDa, and as expected, endo H treatment completely converted the 40-kDa molecular mass form into the 35-kDa mobility form (Fig. 7, 1st panel). This demonstrates that canine cardiac triadin 1 is also partially glycosylated when expressed in the mouse cardiac background. The T1-specific antibody to the unique C terminus of canine cardiac triadin 1 did not detect mouse triadin 1 in control microsomes under the conditions used (2nd panel). However, the Lumenal antibody did detect both species forms of triadin 1, at the same time showing that canine triadin 1 was substantially overexpressed in microsomes from transgenic animals (3rd and 4th panels). Importantly, the canine and murine triadins exhibited superimposable electrophoretic mobilities on SDS-PAGE; proteins originating from either species migrated as two mobility forms and, in both cases, the upper 40-kDa form was completely converted to the lower 35-kDa form by endo H treatment. These results directly demonstrate the gly-

**Fig. 5.** Endo H effect on triadin mobility in canine cardiac microsomes. Canine cardiac microsomes were incubated in the presence and absence of endo H (+/−) and then immunoblotted using the generic antibody raised to recombinant canine cardiac triadin 1. The autoradiogram was exposed for one (left) or 13 (right) h. ψ denotes the glycosylated form of triadin 1 (T1) of apparent molecular mass of 40 kDa. The 92-kDa antibody-binding protein is designated by the asterisk.

**Fig. 6.** Triadins in cardiac microsomes from different animal species. 50 µg of cardiac microsomal protein from dog, rabbit, guinea pig, mouse, rat, and human species were incubated in the presence and absence of endo H (+/−) and immunoblotted using the Lumenal antibody (A) or the T1-specific antibody (B). Samples in A were incubated with the Lumenal antibody at a 1:250 dilution, and the exposure time on all autoradiograms was 1.5 h. Dilutions of the T1-specific antibody used and autoradiographic exposure times for this antibody are indicated below B. The protein of molecular mass greater than 45 kDa in the guinea pig lanes of B reacted nonspecifically with the antibody. ψ denotes the glycosylated form of triadin 1 (T1) visible in all species.
cosylation of triadin 1 in myocardium and, furthermore, emphasize that triadin 1 is the only major triadin isoform expressed in mouse heart as well as in dog heart.

Glycosylation Site of Cardiac Triadin 1—Cardiac triadin 1 contains a single consensus site for N-linked glycosylation at asparagine residue 75 (Fig. 2). To test if this putative glycosylation site is utilized, we expressed and purified native triadin 1 and N75A-triadin 1 from SF21 insect cells. Immunoblotting with the T1-specific antibody revealed that native triadin 1 expressed in insect cells migrates as a major 35-kDa mobility form along with two slower mobility forms of 38 and 42 kDa (Fig. 8). Endo H treatment of the native protein converted the mobilities of the 38- and 42-kDa molecular mass bands into that of the 35-kDa band, suggesting that canine cardiac triadin 1 is also partially glycosylated when expressed in insect cells. Confirming this, expression of N75A-triadin 1 in insect cells gave rise only to the high mobility form migrating at 35 kDa, which corresponds to the mobility of the native protein in its deglycosylated form. These experiments localize the site of glycosylation of cardiac triadin 1 to asparagine residue 75.

Membrane Topology of Cardiac Triadin 1—The topology of the triadins in the SR membrane is controversial; Caswell and co-workers (13) have proposed that skeletal muscle triadin contains four transmembrane segments, whereas most groups have concluded that skeletal muscle triadin contains only one membrane-spanning segment, and these latter groups suggest that skeletal muscle triadin has a short N terminus projecting into the cytoplasm and that the C-terminal end of the molecule is accessible and localized on the outer surface of the SR vesicle membrane. Here we show that only one triadin isoform, cardiac triadin 1, the smallest of the known triadins, is expressed to a significant extent in mammalian myocardium. The discrete localization of triadin 1 to the junctional sarcoplasmic reticulum in heart (25), along with its direct association with the ryanodine receptor, calsequestrin, and junctin (2, 4), suggests that triadin 1 plays an important role in Ca$^{2+}$ release. On SDS-PAGE, triadin 1 runs as doublet of protein bands of apparent molecular weights 35,000 and 40,000. Originally it was proposed that the upper band of this doublet is contributed by a different unique cardiac triadin isoform, cardiac triadin 2 (4), but the results presented here clearly demonstrate that this is not the case. The upper band of the doublet is the glycosylated form of triadin 1; both bands react equally well with an antibody that recognizes only the unique C terminus of triadin 1; deglycosylation with endo H completely converts the upper band of the doublet into the lower band; and triadin 1, expressed either in approximately 4-kDa (middle panel). Since the T1-specific antibody recognizes the C-terminal end of the triadin 1 molecule, the results indicate that the C terminus of triadin is protected by its localization in the SR lumen.

Confirming this, expression of N75A-triadin 1 in insect cells gave rise only to the high mobility form migrating at 35 kDa, which corresponds to the mobility of the native protein in its deglycosylated form. These experiments localize the site of glycosylation of cardiac triadin 1 to asparagine residue 75.

Membrane Topology of Cardiac Triadin 1—The topology of the triadins in the SR membrane is controversial; Caswell and co-workers (13) have proposed that skeletal muscle triadin contains four transmembrane segments, whereas most groups conclude that skeletal muscle triadin contains only one membrane-spanning segment, and these latter groups suggest that skeletal muscle triadin has a short N terminus projecting into the cytoplasm and that the C-terminal end of the molecule is accessible and localized on the outer surface of the SR vesicle membrane. Here we show that only one triadin isoform, cardiac triadin 1, the smallest of the known triadins, is expressed to a significant extent in mammalian myocardium. The discrete localization of triadin 1 to the junctional sarcoplasmic reticulum in heart (25), along with its direct association with the ryanodine receptor, calsequestrin, and junctin (2, 4), suggests that triadin 1 plays an important role in Ca$^{2+}$ release. On SDS-PAGE, triadin 1 runs as doublet of protein bands of apparent molecular weights 35,000 and 40,000. Originally it was proposed that the upper band of this doublet is contributed by a different unique cardiac triadin isoform, cardiac triadin 2 (4), but the results presented here clearly demonstrate that this is not the case. The upper band of the doublet is the glycosylated form of triadin 1; both bands react equally well with an antibody that recognizes only the unique C terminus of triadin 1; deglycosylation with endo H completely converts the upper band of the doublet into the lower band; and triadin 1, expressed either in approximately 4-kDa (middle panel). Since the T1-specific antibody recognizes the C-terminal end of the triadin 1 molecule, the results indicate that the C terminus of triadin is protected by its localization in the SR lumen. The gain in electrophoretic mobility of triadin 1 produced by trypsin treatment, along with the loss of the N-terminal epitope, is consistent with cleavage of triadin 1 by trypsin at lysines 30 or 33, or arginine 34, when the protein resides in the intact SR vesicle membrane. When SR vesicles were proteolyzed in the presence of 0.2% Triton X-100, all triadin immunoreactivity was lost (Fig. 9), due to complete digestion of the protein after loss of protection from the membrane. As an internal control, we observed that digestion of cardiac calsequestrin, an entirely intralumenal protein (23), occurred only in the presence of Triton X-100 (right panel). Thus, our results confirm the membrane topology for cardiac triadin previously proposed by Guo et al. (4), in which the protein contains only one transmembrane domain, a short N-terminal segment facing the cytoplasm, and a large C-terminal domain localized entirely in the lumen of the SR.

**DISCUSSION**

Here we show that only one triadin isoform, cardiac triadin 1, the smallest of the known triadins, is expressed to a significant extent in mammalian myocardium. The discrete localization of triadin 1 to the junctional sarcoplasmic reticulum in heart (25), along with its direct association with the ryanodine receptor, calsequestrin, and junctin (2, 4), suggests that triadin 1 plays an important role in Ca$^{2+}$ release. On SDS-PAGE, triadin 1 runs as doublet of protein bands of apparent molecular weights 35,000 and 40,000. Originally it was proposed that the upper band of this doublet is contributed by a different unique cardiac triadin isoform, cardiac triadin 2 (4), but the results presented here clearly demonstrate that this is not the case. The upper band of the doublet is the glycosylated form of triadin 1; both bands react equally well with an antibody that recognizes only the unique C terminus of triadin 1; deglycosylation with endo H completely converts the upper band of the doublet into the lower band; and triadin 1, expressed either in approximately 4-kDa (middle panel). Since the T1-specific antibody recognizes the C-terminal end of the triadin 1 molecule, the results indicate that the C terminus of triadin is protected by its localization in the SR lumen. The gain in electrophoretic mobility of triadin 1 produced by trypsin treatment, along with the loss of the N-terminal epitope, is consistent with cleavage of triadin 1 by trypsin at lysines 30 or 33, or arginine 34, when the protein resides in the intact SR vesicle membrane. When SR vesicles were proteolyzed in the presence of 0.2% Triton X-100, all triadin immunoreactivity was lost (Fig. 9), due to complete digestion of the protein after loss of protection from the membrane. As an internal control, we observed that digestion of cardiac calsequestrin, an entirely intralumenal protein (23), occurred only in the presence of Triton X-100 (right panel). Thus, our results confirm the membrane topology for cardiac triadin previously proposed by Guo et al. (4), in which the protein contains only one transmembrane domain, a short N-terminal segment facing the cytoplasm, and a large C-terminal domain localized entirely in the lumen of the SR.
the transgenic mouse heart or in Sf21 insect cells, produces both mobility forms of triadin 1.

Our cloning results of the canine isoforms of triadin support the conclusion that triadin 1 is the only major triadin isoform expressed in heart. In canine cardiac cDNA libraries, of 15 triadin isoform-specific clones identified, 13 encoded amino acid sequence corresponding to triadin 1, only 2 encoded sequence for triadin 3, and no clone could be found encoding the putative triadin 2 isoform. RT-PCR of canine cardiac total RNA also failed to reveal any triadin 2 isoform. During screening of canine cDNA libraries, we encountered many partial and corrupt clones that had unrelated sequences ligated adjacent to correct triadin protein-coding sequence. This was most likely due to false priming at internal sites during construction of the libraries. The triadin luminal domain is particularly enriched in lysine residues, which are encoded mainly by the base adenosine. To identify bona fide triadin isoform-specific clones, the validity of each triadin clone isolated from the cDNA library had to be confirmed by RT-PCR of canine cardiac total RNA. By this approach, several false clones were eliminated. Although we cannot be certain, it seems possible that the rabbit cardiac triadin 2 cDNA clone previously characterized (4) may have arisen from a cloning artifact. Regardless, we can conclude from our antibody results that there is no significant expression of triadin 2 in myocardium in any of the mammalian species investigated here.

In agreement with Guo et al. (4), who analyzed a rabbit cardiac cDNA library, we were successful in isolating cDNA clones from canine libraries that encoded a putative large cardiac triadin 3 isoform. The predicted sequence of triadin 3 overlapped with skeletal muscle triadin throughout most of its sequence (4). On immunoblot analysis, we detected a trace protein of apparent molecular weight 92,000 in cardiac microsomes that was recognized by our generic triadin antibodies. This 92-kDa trace protein colocalized with triadin 1 and calsequestrin to the junctional SR fraction and also appeared to be glycosylated. A similar 95-kDa molecular mass protein in cardiac microsomes that cross-reacted with a monoclonal antibody to skeletal muscle triadin was reported earlier by Carl et al. (26). Therefore, it appears that the 92-kDa triadin-immunoreactive protein detected presently and in previous studies (4, 26) may correspond to the predicted cardiac triadin 3 isoform. Although the apparent molecular weight of triadin 3 on SDS-PAGE is much greater than the molecular weight calculated from the amino acid sequence (approximately 65,000), the anomalous mobilities of the large triadins on SDS-PAGE is well known (8, 13, 24). In an attempt to conclusively identify this 92-kDa protein in cardiac microsomes as the cardiac triadin 3 isoform, we tried to raise an antibody to the unique C terminus predicted for triadin 3, but unfortunately we have been unsuccessful in producing a usable antisemur to date. Therefore, the identity of this 92-kDa protein as triadin 3 remains tentative at present. It should be emphasized, however, that the level of expression of the 92-kDa triadin-immunoreactive protein in cardiac SR vesicles is very low (<5% of triadin 1), suggesting that it may subserve only a minor role in Ca\(^{2+}\) release in relation to triadin 1. We also analyzed whole ventricular homogenates and atrial microsomes with our antibodies, and again we found triadin 1 to be the major triadin isoform detected in all fractions examined (data not shown). Thus, we could find no evidence for selective localization of a given triadin isoform to atrium or ventricle or to one subcellular compartment or another.

In this study, we localized the glycosylation site of triadin 1 to asparagine residue 75. In eukaryotic cells, N-linked core glycosylation occurs at Asn residues within the sequon, Asn-X-(Ser/Thr)-Y, where X and Y are any amino acid, and X≠Pro. Efficiency of this glycosylation depends on the amino acid present at the X and Y positions, the accessibility of the sequon to oligosaccharyltransferase during protein synthesis, and the length of the polypeptide (27, 28). The functional sequon we identified for triadin 1, NFSA, is conserved among all the triadins (4, 8). This suggests that skeletal muscle triadin, a known glycoprotein (16), is also glycosylated at asparagine residue 75, although Fan et al. (13) have proposed that a different amino acid is glycosylated. Based on this sequon, the N-linked core glycosylation should be relatively efficient. However, the proportion of triadin 1 glycosylated varied greatly between species (Fig. 6). It could be that inefficient glycosylation at asparagine 75 is due to the closeness of the sequon to the membrane segment. In a recent study it was reported that for efficient glycosylation to occur, the receptor site must be spaced at least 12–14 residues from the transmembrane segment (29); asparagine residue 75 of triadin 1 is only 7 amino acids removed from the predicted membrane segment. Heterologous expression of triadin 1 in insect cells also showed that triadin 1 is partially glycosylated. SF cells are capable of complex glycosylation of mammalian glycoproteins, although the processing is slightly different (18). This may explain the heterogeneity in electrophoretic mobilities of the glycosylated forms of triadin 1 in insect cells versus heart.

By using our site-specific antibodies, we confirmed the membrane topology of cardiac triadin 1 previously proposed by Guo et al. (4). Both the glycosylated and deglycosylated forms of the protein contain a very short N-terminal segment that is positioned in the cytoplasm, a single transmembrane segment, and a highly charged C-terminal domain located entirely in the lumen of the SR. This membrane topology of triadin 1 is identical to that recently demonstrated for junctin, the related junctional SR protein (9). Both proteins have homologous amino acid sequences around the transmembrane regions and both are missing methionine residue 1. Neither junctin nor triadin have signal peptides. It may be that their structurally similar transmembrane domains aid in proper vectorial insertion of the proteins into the membrane.

The functional role of triadin 1 in the heart remains to be determined. In preliminary experiments, we have observed that our transgenic mice overexpressing triadin 1 exhibit cardiac hypertrophy, contractility changes, and down-regulation of the associated junctional SR proteins junctin and the RyR.3 Experiments are currently in progress to determine if triadin 1 alters Ca\(^{2+}\) release directly.

Acknowledgments—We thank Wei Guo and Kevin Campbell for supplying us with the rabbit cardiac triadin cDNA clone. The excellent technical assistance of Glen Schmeisser in production and characterization of antibodies is greatly appreciated.

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